ANALYSIS OF VOLATILE CONSTITUENTS FROM POST-HARVESTING HANDLING AND OPTIMIZATION OF EXTRACTIVE SOLVENT ON THE QUALITY OF THE LEAVES OF *PREMNA SERRATIFOLIA* L.

By
Miss Khin Su YEE

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES (INTERNATIONAL PROGRAM)
Graduate School, Silpakorn University
Academic Year 2021

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โดย
MissKhin Su YEE

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Title	Analysis of volatile constituents from post-harvesting handling and optimization of extractive solvent on the quality of the leaves of *Premna serratifolia* L.
By	Miss Khin Su YEE
Field of Study	PHARMACEUTICAL SCIENCES (INTERNATIONAL PROGRAM)
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MISS KHIN SU YEE : ANALYSIS OF VOLATILE CONSTITUENTS FROM POST-HARVESTING HANDLING AND OPTIMIZATION OF EXTRACTIVE SOLVENT ON THE QUALITY OF THE LEAVES OF *PREMNA SERRATIFOLIA* L. THESIS ADVISOR : ASSOCIATE PROFESSOR UTHAI SOTANAPHUN, Ph.D.

The objective of this study was to compare the chemical composition of volatile oils, phenolic content and antioxidant activity of the fresh, dried, and fermented leaves of *Premna serratifolia*, and to find a suitable extractive solvent to prepare a good quality extract from its leaves. Volatile oils from the fresh, dried and fermented leaves were analyzed by gas chromatography–mass spectrometry. The phenolic content and antioxidant activity were determined by DPPH and Folin-Ciocalteu methods. A total of 77, 82 and 90 compounds were detected in the fresh, dried and fermented leaf volatile oils, respectively. The main compounds of the fresh leaf volatile oils were amyl vinyl carbinol (15.8–32.6%), linalool (11.1–15.1%), phytol (7.7–12.5%), salicylic acid methyl ester (3.9–7.2%) and (*E*)-caryophyllene (3.1–6.6%). After drying and fermentation, amyl vinyl carbinol was decreased to 6.3–13.8% and 6.9–11.5%, respectively. Likewise, linalool and phytol decreased to 6.3–7.5% and 7.3–9.0% after drying, and decreased to 5.3–7.9% and 2.0–3.4% after fermentation. After drying, (*E*)-caryophyllene increased significantly to 6.6–12.2%, becoming the most prevalent compound, and palmitic acid ethyl ester (1.17%) dramatically hydrolysed to palmitic acid (5.0%). The fermentation method resulted in a significant increase in phenolic compounds, particularly *p*-vinylanisole (2.4–41.1%), which became the predominant compound, as well as a significant decrease in phytol and the finding of acorenone B (4.4%) as a new compound. Among the 80% ethanolic extracts of fresh, dried and fermented leaves, fresh leaf extract possessed the highest phenolic content of 6.08%GAE and 67.24% of antioxidant activity at the concentration of 100 µg/mL. Premnaodoroside A and the mixture of glucose and fructose were isolated from the dried leaves. A TLC-densitometric method was developed using premnaodoroside A as a standard marker. The method demonstrated satisfactory specificity, precision and accuracy with good linearity ($R^2 > 0.99$) in the range of 0.11-0.87 µg/spot, with a limit of quantitation and a limit of detection of 0.04 and 0.13 µg/spot, respectively. The analysis of 0, 20, 40, 60, 80 and 100% ethanolic extracts of three samples revealed that 100% ethanolic extract exhibited the highest content of premnaodoroside A (3.23–5.25%), total phenolic content (7.56-8.24% GAE) and antioxidants (4.92–6.66% AAE), but its extractive yields (13.57-19.57%) were the lowest. On the contrary, solvents with a lower percent of ethanol gave a higher percent of extractive yield. 80% Ethanol gave the optimization of both the yield of the extract and the content of the interesting compounds.

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Miss Khin Su YEE

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

KI	- Kovats Retention Index
TLC	- Thin Layer Chromatography
R_f	- Retardation factor, Relative front, Rate of flow
HPTLC	- High Performance Thin Layer Chromatography
GC-MS	- Gas Chromatography Mass Spectrophotometry
DPPH	- 1,1-diphenyl-2-picrylhydrazyl
IC_{50}	- % Inhibitory Concentration
AAE	- Ascorbic acid equivalent
GAE	- Gallic acid equivalent
mm	- Millimeter
mL	- Milliliter
cm	- Centimeter
cm^{-1}	- Per centimeter
pg	- Picogram
mg	- Milligram (s)
g	- Gram (s)
%	- Percentage
℃	- Degree centigrade
SD	- Standard Deviation
UV	- Ultraviolet
UV/Vis	- Ultraviolet- Visible Spectroscopy
ESI-MS	- Electrospray Ionization Mass Spectrophotometry
MS	- Mass Spectrophotometry
eV	- Electron-Volt
DB-5	- 5% phenyl and 95% dimethylpolysiloxane polymer
PEG	- Polyethylene glycol
ID	- Internal diameter
MeOH	- Methanol
CH_2Cl_2	- Dichloromethane
H_2O	- Water

EtOH	- Ethanol
FT-IR	- Fourier Transform Infra-red Spectroscopy
KBr	- Potassium bromide
MW	- Molecular weight
[M]+	- Molecular ion
m/z	- Mass to charge ratio
MHz	- Mega hertz
TMS	- Tetramethylsilane
MeOD	- Deuterated methanol
ppm	- Part per million
ν_{max}	- Wavenumber at maximum absorption
min	- Minutes
h	- (Hour) s
α	- Alpha
β	- Beta
δ	- Chemical shift
DEPT	- Distortionless Enhancement by Polarization Transfer
^{1}H-NMR	- Proton Nuclear Magnetic Resonance Spectroscopy
^{13}C- NMR	- Carbon Nuclear Magnetic Resonance Spectroscopy
s	- Singlet
d	- Doublets
dd	- Doublets of doublets
t	- Triplet
m	- Multiplet
J	- Coupling constant
µg	- Microgram (s)
µL	- Microliter

CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the problem

Myanmar has a rich source of medicinal plants and a strong system of traditional medicine. Myanmar people are profoundly relied on the Myanmar traditional medicine and medicinal plants. *Premna serratifoila* L. (synonym: *P. integrifolia* L.) (family Lamiaceae) is a well-known traditionally reputed plant. This plant is naturally growing in most parts of Myanmar and abundantly found in Mandalay Division, Myanmar. Similarly, the plant is abundantly found in India, Laos, Cambodia, Thailand, Malaysia, Indonesia, Australia, Vietnam (1). Its Thai name is Sam Pra Nga. The leaves *of P. serratifolia* are used as food additives to reduce fishy smell in Celebes (2), as a meal after boiling in Peninsula, Malaysia and Indonesia (3). In Myanmar, its leaves are very popular and used for the relieve and curing of a cancer and variety of diseases such as antibacterial, anti-inflammatory, antidiabetic, and pain (personal communications).

P. serratifolia have properties of antibacterial, anti-inflammatory, antioxidant, antidiabetic and antitumor activities (4, 5). Furthermore, *P. serratifolia* contains phenolic compounds, iridoids (6), glycosides, flavonoids, alkaloids, terpenoids, tannins and saponins (7). A number of iridoid glycosides have been isolated from the leaves of this plant (8). Iridoid glycosides have numerous biological activities and plays an important position in the fields of natural product chemistry and biology (8). HPLC/MS, HPLC-DAD-ESI/MS, and HPTLC have been reported for the quantitation of some iridoid glycosides from *Veronica* sp. (9), *Globularia punctate* (10) and, *Gmelina aroborea* (11). In practice, the leaves of *P. serratifolia* are easily fermented, turned to black color, and caused changes in its chemical constituents. To prepare herbal extracts in the phytomedicine industry, drying the leaves immediately after collection was important to keep their quality for long-term use. Therefore, dried leaves have the advantage of reducing quality degradation, slowing microorganism formation, preventing biochemical reactions (12) and being convenient to use as raw material.

The leaf of *P. serratifolia* is widely used by Myanmar people for the treatment of liver cancer. Some people take a preparation of fresh leaves as a medicine and others use the dry leaves to make herbal preparation. However, when the fresh leaves are kept at room temperature or dried in the shade under unsuitable conditions, some of the leaves will be fermented and turn a dark brown color. Generally, a variety of processing methods have been developed. In Traditional Chinese Medicine, including cleaning, cutting, peeling, boiling, steaming, and stir-frying (13) are widely used for the purposes of relieving or decreasing toxicity, changing medicinal properties, and improving therapeutic efficiency. Therefore, post-harvest processing plays an essential role in the production of herbal drugs, and it may impact on the organoleptic and chemical properties, as well as the clinical efficacy and safety of the produced herbal drugs (13). Many medicinal plants have been reported for chemical variation between their fresh and dried leaves such as *Tapinanthus bangwensis* (14), *Artemisia afra* (15), *Ocimum sanctum* (16), *Cymbopogon citratus* (17). Concerned with *P. serratifolia*, its leaves have very distinct characteristics. The odor change of leaves caused by dried and fermented leaves could be due to a change in chemical reactions. These might be affected by the volatile and chemical constituents in the leaves. This kind of scientific research has not been retrieved, and therefore, it was of keen interest to investigate the different volatile constituents by using gas chromatography coupled with mass spectrometry (GC-MS) and phenolic content and antioxidant activity among fresh, dried, and fermented leaves of *P. serratifolia* by the Folin-Ciocalteu reagent and DPPH assay method, respectively.

Herbal extracts are common raw material in the production of modern herbal medicinal products because unwanted materials are removed, their chemical constituents are concentrated, and they can be further processed into several herbal dosage forms (18). To obtain a good herbal extract in both quality and quantity aspects, an appropriate percentage of ethanolic solvent needs to be optimized. However, there were no validated analytical methods for standardization and optimization of extractive solvent on the quality of the leaves of *P. serratifolia*. Therefore, this study aimed to gain some basic information on the effect of solvent on the extraction of this plant. Optimization of solvent concentration is crucial in ensuring the complete extraction of a compound of interest from the leaves of *P.*

serratifolia in order to develop a good quality herbal raw material. Various percentages of ethanol were used for the extraction of the leaves of *P. serratifolia* and the quality of the extraction was examined based on the extractive yield, the contents of the interesting analytical markers such as major a iridoid glycosides and total phenolic compounds, and its antioxidant activity. The major iridoid glycoside was isolated from the leaves of this plant and identified by using spectroscopic methods. Its content in the extract was quantified by a reliably developed TLC-densitometric method. The appropriate percentage of ethanolic solvent was optimized to obtain a good herbal extract of the leaves of *P. serratifolia* in both quantity and quality aspects.

1.2 Aims and objectives

1. To compare quality of fresh, dried and fermented leaves of *P. serratifolia*.
2. To isolate and identify marker compound from dried leaves of *P. serratifolia*.
3. To develop and validate method for quantification of the compound by TLC-densitometer.
4. To find suitable extractive solvent to prepare the extract from dried leaves of *P. serratifolia*.

CHAPTER 2
LITERATURE REVIEW

Premna was previously classified as a member of the Verbenaceae family, but has now been reclassified as a member of the Lamiaceae family. This genus has 200 species that are predominantly found in tropical and subtropical Asia, Africa, Australia, and the Pacific Islands. The word *Premna* comes from the Greek word 'premnon,' which relates to the short and twisted trunks of *P. serratifolia* L., the genus's first collected species. This species is commonly used in tropical Asia and East Africa to treat neuralgia and headaches, stomachic, fevers, colds, and coughs, as well as liver and cardiac disorders. The leaves, roots, or inner bark are used by the local people in Myanmar, Thailand, the Malay Peninsula, and Indonesia to relieve stomach ache discomfort, as a diuretic, or to treat diarrhea. In India, four species of *Premna* (*P. herbacea*, *P. integrifolia*, *P. latifolia*, and *P. tomentosa*) are used in Ayurvedic medicine, either alone or together with other plants, and are still available as an over-the-counter medicine for local people (19).

2.1 Plant morphological characters

P. serratifolia is a shrub or tree that is 1-8 m tall. Leaves are opposite and the leaf blade is glabrous and broadly ovate. The margin is entire, and slightly undulate. The apex is acute to rarely acuminate or obtuse. The base is broadly cuneate, and the petiole is 0.5-7mm long and glabrous. Flowers are inflorescences and the calyx is cup-shaped, 2-lipped, and the lower lip is subentire to shortly 3-dentate. The upper lip is longer than the lower lip and has 2-dentates. The corolla is yellowish-green and villous in the throat. The ovary is glabrous, and the style is 3.5–4 mm. The fruit is globose with 4 seeds (20, 21).

2.2 The bioactivity of *P. serratifolia*

The leaf extracts of *P. serratifolia* possess cytotoxic, antioxidant, anti-diabetic, hepatoprotective, antibacterial, and analgesic activities. The bioactivity of *P. serratifolia* can be concluded and shown in Table 1.

Table 1 Bioactivity of *P. serratifolia*

Plant part	Activity	Design of Study	References
Leaves	Cytotoxic	*In vitro*, MTT assay, MCF-7 (breast cancer) cell lines	(22)
		In vitro, SRB assay MCF7 (breast cancer), HepG2 (liver cancer) and A549 (lung cancer) cell lines	(23)
		In vitro, trypan blue exclusion method, EAC cell lines	(24)
	Antioxidant	DPPH assay	(25)
	Hepatoprotective	Carbon tetrachloride induced hepato-toxicity in rats	(24)
	Antidiabetic	α-Glucosidase inhibition assay	(25)
	Antibacterial	Disc diffusion method against *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Shigella dysenteriae*, *Shigella sonnei* and *Escherichia coli*	(26)
	Analgesic	Acetic acid induced writhing model in mice	
Flower	Antioxidant	Nitric oxide scavenging activity	(4)

Table 1 Bioactivity of *P. serratifolia* (continued)

Plant part	Activity	Design of Study	References
Flower	Anti-inflammatory	*In vitro*, Human Red Blood cell (HRBC) membrane stabilization	(4)
Stem bark and wood	Antioxidant	DPPH, FRAP, reducing power and nitric oxide scavenging assays	(27, 28)
	Cardiac stimulant	Isolated frog heart perfusion	(29)
Roots	Anti-inflammatory Immunomodulatory	cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) enzyme-based assays, lymphocyte proliferation assay, pro-and anti-inflammatory cytokines measurement	(1)
	Antimicrobial	*Staphylococcus aureus, Coagulase Negative Staphylococca, Escherichia coli Klebsiella pneumoniae, Pseudomonas aeuroginosa, Salmonella typhi, Salmonella typhi A, Salmonella typhi B, Vibrio chlorea, Entero cocci, Candida albicans, Aspergillus niger, Pencillium hrysogenum, Microsporum gypseum and Epidermatophyton flocossum*	(30)

2.3 Phytochemical constituents of *P. serratifolia*

The phytochemical screening of the leaf extract of *P. serratifolia* demonstrated the presence of glycosides, flavonoids, alkaloids, tannins and terpenoids. Chemical constituents of *P. serrtifolia* are shown in Table 2.

Table 2 Phytochemical constituents of *P. serratifolia*

Plant part	Class	Compound name	References
Leaves	Iridoid glycosides	6-*O*-α-L-(2″-*O*-*cis*-*p*-coumaroyl) rhamnopyranosylcatpol	(31)
		6-*O*-α-L-(2″-*O*-*trans*-*p*-coumaroyl) rhamnopyranosylcatpol	(31)
		Premcoryoside	(31)
		6-*O*-α-L-(2″-caffeoyl) rhamnopyranosylcatpol	(31)
		6-*O*-(3″-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosylcatalpol (Premnacorymboside B)	(32)
		Scutellarioside II	(32)
		Premnaodoroside A	(32)
		10-*O*-*trans*-*p*-methoxycinnamoylcatalpol	(8, 32)
	Flavonoids	Acacetin	(22)
		Acacetin-7-*O*-glycoside	(22)
		Quercetin-3-D-xyloside	(33)
		Kaempferol-3,7-*O*-bis-α-L-rhamnoside	(33)
		Isorhamnetin-3-*O*-glucoside	(33)
		Luteolin-3',7-di-*O*-glucoside	(33)
		Eriodictyol-7-*O*-glucoside	(33)

Table 2 Phytochemical constituents of *P. serratifolia* (continued)

Plant part	**Class**	**Compound name**	**References**
Leaves	Flavonoids	Syringetin-3-*O*-galactoside	(33)
		Petunidin-3-*O*-β-glucopyranoside	(34)
		Vitexin-2″-*O*-rhamnoside	(34)
		Neohesperidin	(34)
		Apigenin-7-*O*-glucoside	(34)
		Catechin hydrate	(34)
		Cyanidin chloride	(34)
		Quercetin-3-galactoside	(34)
		Diosmin	(34)
		Genistein	(34)
		Malvin chloride	(34)
		Myricitrin	(34)
		Poncirin	(34)
		Vitexin	(34)
		Tilirosid	(34)
	Triterpenoids	Oleanolic acid	(35)
	Steroids		
		Stigmasterol	(35)
	Volatile oils	Phytol	(36)
		α-Humulene	(36)
		Eugenol	(36)
		Spathulenol	(36)
		1-octen-3-ol	(36)
		2,5-Furandione, 3-methyl	(5)

Table 2 Phytochemical constituents of *P. serratifolia* (continued)

Plant part	**Class**	**Compound name**	**References**
Leaves	Volatile oils	2,3-dihydrobenzofuran	(5)
		2-Hydroxy-3-methylbenzaldehyde	(5)
		Dodecanoic acid	(5)
		2-Propenoic acid, 3-(4-methoxyphenyl)-	(5)
		4-(3-hydroxy-1-propenyl)-2-methoxyphenol	(5)
		2-Propenoic acid, 3-(4-methoxyphenyl)-, ethyl ester	(5)
		1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	(5)
		n-Hexadecanoic acid	(5)
		Octadecanoic acid, ethyl ester	(5)
		1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyloctasiloxane	(5)
Flower buds	Volatile oils	1-octen-3-ol	(37)
		(*Z*)-n-hexanol	(37)
		2-phenyl ethyl alcohol	(37)
		(*E, Z*)-2,4-nonadienal	(37)
		Linalool	(37)

Table 2 Phytochemical constituents of *P. serratifolia* (continued)

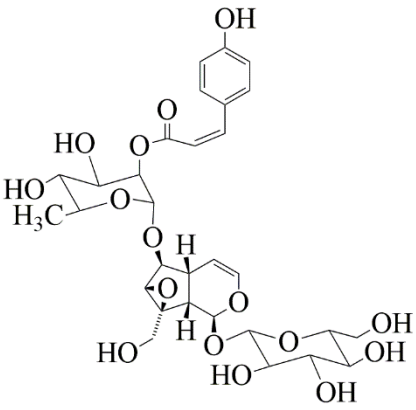
Plant part	**Class**	**Compound name**	**References**
Wood	Lignans	1α-Hydroxyl-6-epipinoresinol	(38)
		Xanthoxyol	(38)
Stem	Lignans	4β-hydromxyasarinin-1-*O*-β-glycopyranoside	(27)
		4-epi-gummadiol-4-*O*-β-glycopyranoside	(27)
		Plucheoside D1	(27)
Stem bark	Lignans	Premnadimer	(27)
Flower	Iridoid glycosides	10-*O*-trans-*p*-coumaroylcatapol	(35)
		4″-hydroxy-*E*-globularinin	(35)
		Premnosidic acid	(35)
		6-*O*-α-L-(10-*O*-trans-*p*-coumaroyl)-rhamnopyrnosylcatapol	(35)
		6-*O*-(3″-*O*-acetyl-2″-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosylcatalpol (Premnacorymboside A)	(27)
Root bark	Diterpenoids	1β,3α,8β-trihydroxy-pimara-15-ene	(39)
		6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene	(39)
		2α, 19-dihydroxy-pimara-7,15-diene	(39)
		11,12.16-trihydroxy-2-oxo-5-methyl-10-demethyl-abieta-1-[10]6,8,11,13-pentene	(40)
		6-hydroxy salvinolone	(1)

Table 2 Phytochemical constituents of *P. serratifolia* (continued)

Plant part	**Class**	**Compound name**	**References**
Root and twigs	Diterpenoids	Isopimara-7,15-dien-1β, 3β-diol	(41)
		Isopimara-7,15-dien-1β, 19-diol	(41)
		13-epi-5,15-rosadien-3a,11β-diol	(41)
		Abietatrien-1β-ol	(41)
		Abietatrien-1β,12-diol	(41)
		6α,11,12-Trihydroxy-7β,20-epoxy-8,11,13-abietatriene	(41)
		5α,11,12-Trihydroxy-6-oxa-abieta-8,11,13-trien-7-one	(41)
		Obtusinone A	(41)
		Obtusinone B	(41)
		Obtusinone C	(41)
	Phenylethanoid glycoside	Acteoside (verbacoside)	(41)
	Volatile oils	2,5-Furandione, 3-methyl	(5)
		5-(hydroxymethyl)-furan-2-carbaldehyde	(5)
		Benzofuran, 2,3-dihydro-	(5)
		2-Hydroxy-3-methylbenzaldehyde	(5)
		Seychellene	(5)

Table 2 Phytochemical constituents of *P. serratifolia* (continued)

Plant part	**Class**	**Compound name**	**References**
Root and twigs	Volatile oils	Dodecanoic acid	(5)
		1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7 trimethyl-4-methylene-, [1ar(1aα,4aα,7β,7aβ,7bα)]	(5)
		3-(4-methoxyphenyl)-2-propenoic acid	(5)
		3,7,11,15-Tetramethyl-2-hexadecen-1-ol	(5)
		Phytol	(5)
		n-Hexadecanoic acid	(5)
		Octadecanoic acid, ethyl ester	(5)
		2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-Octahydro 4b,8,8-trimethyl-1-(1-methylethyl)-, (4bs-trans)-	(5)



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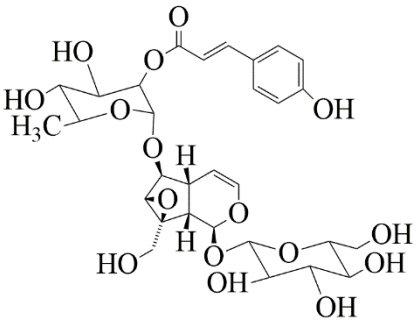

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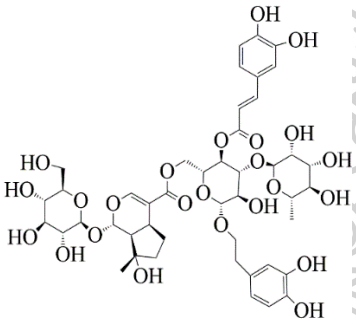
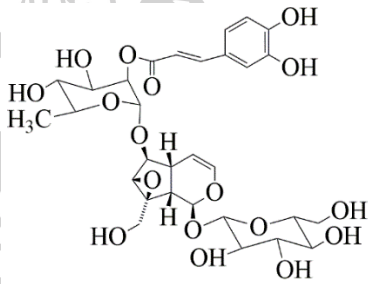
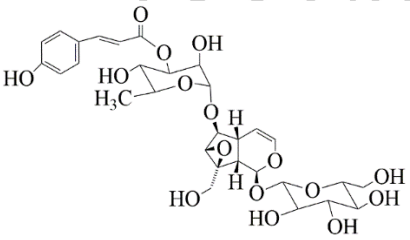


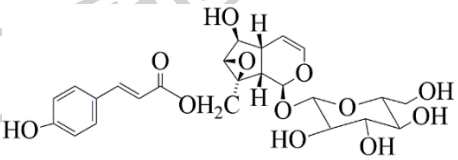
Premcoryoside



6-*O*-α-L-(2″-caffeoyl) rhamnopyranosylcatalpol

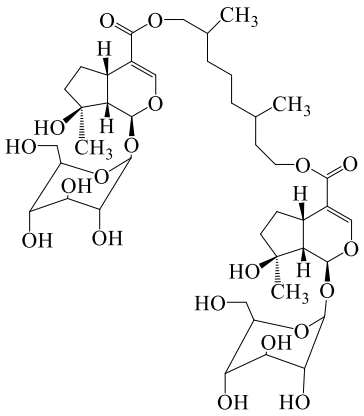


6-*O*-(3″-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosylcatpol (Premnacorymboside B)

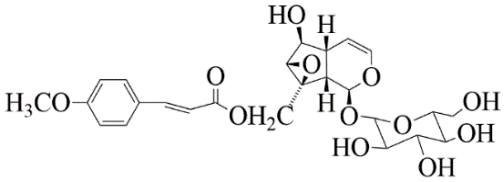


Scutellarioside II

Figure 1 Iridoid glycosides from *P. serratifolia* leaves

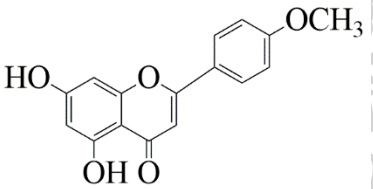


Premnaodoroside A

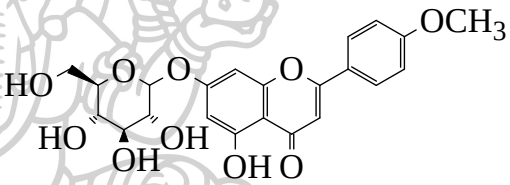


10-*O*-*trans*-*p*-methoxycinnamoylcatalpol

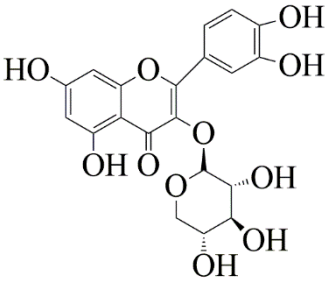
Figure 1 Iridoid glycosides from *P. serratifolia* leaves (continued)



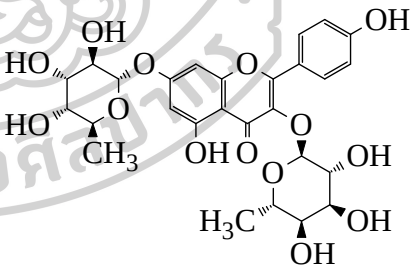
Acacetin



Acacetin-7-*O*-glycoside

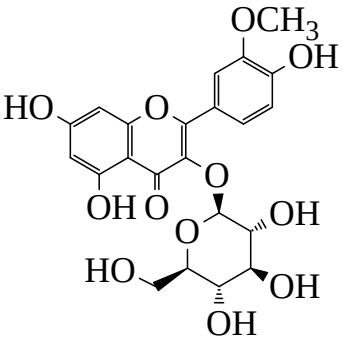


Quercetin-3-D-xyloside

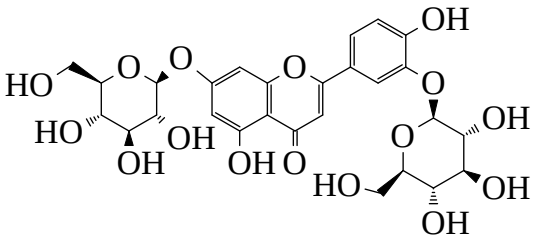


Kaempferol-3,7-*O*-bis-α-L-rhamnoside

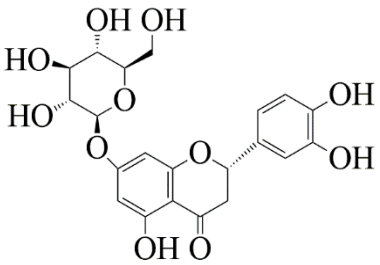
Figure 2 Flavonoids from *P. serratifolia* leaves



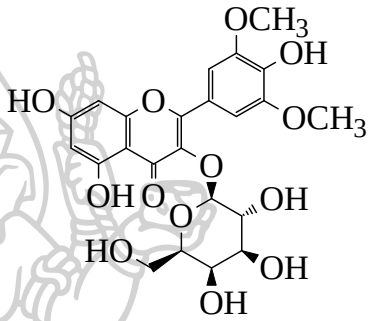
Isorhamnetin-3-*O*-glucoside



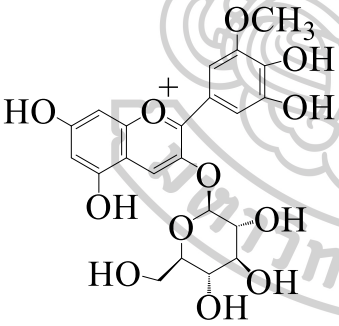
Luteolin-3'-7-di-*O*-glucoside



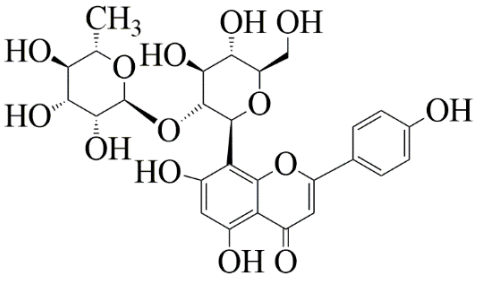
Eriodictyol-7-*O*-glucoside



Syringetin-3-*O*-galactoside

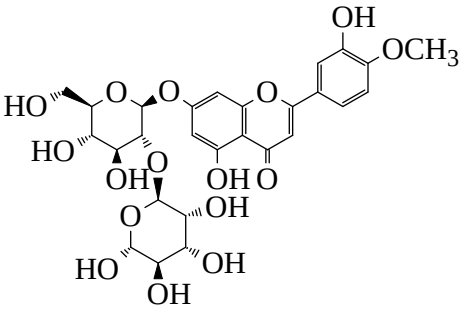


Petunidin-3-*O*-β-glucopyranoside

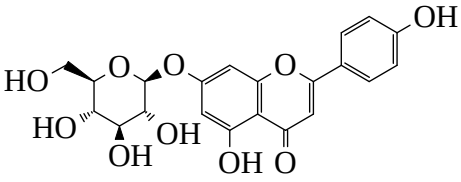


Vitexin-2''-*O*-rhamnoside

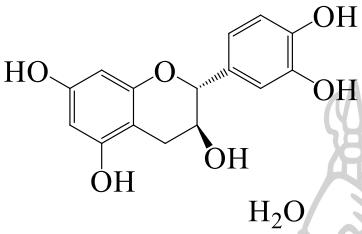
Figure 2 Flavonoids from *P. serratifolia* leaves (continued)



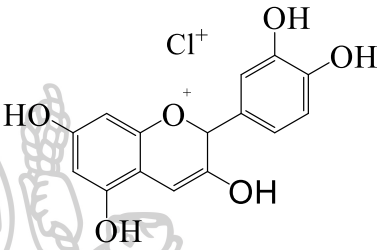
Neohesperidin



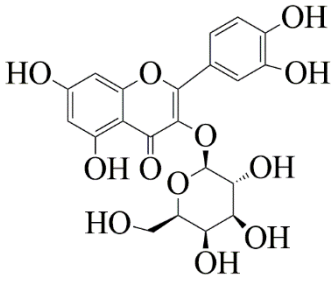
Apigenin-7-*O*-glucoside



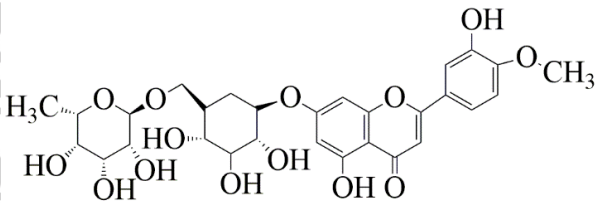
Catechin hydrate



Cyanidin chloride

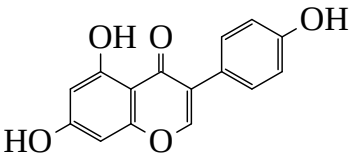


Quercetin-3-galactoside

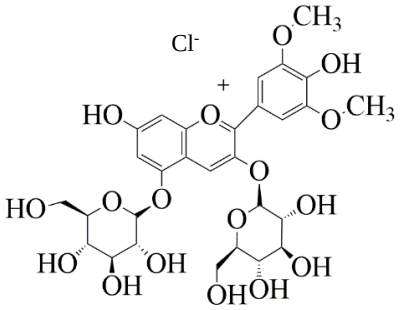


Diosmin

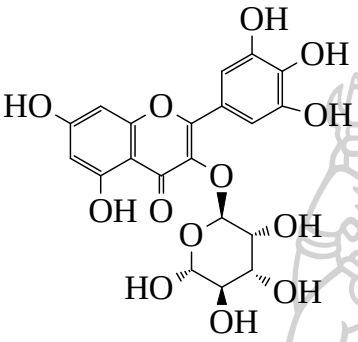
Figure 2 Flavonoids from *P. serratifolia* leaves (continued)



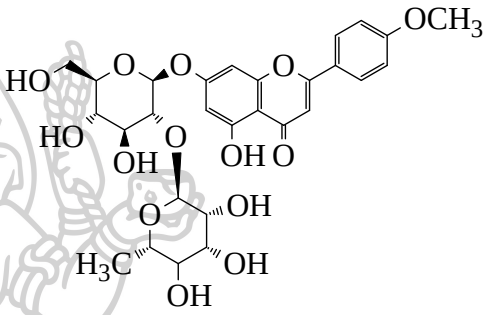
Genistein



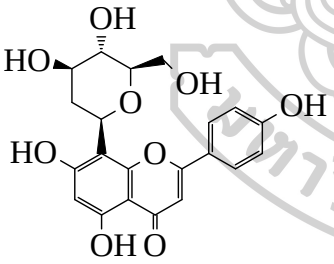
Malvin chloride



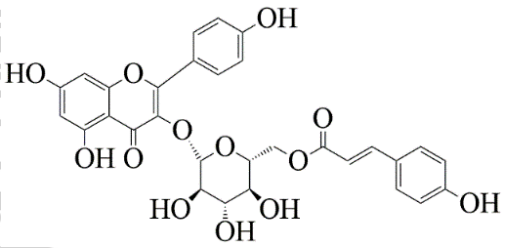
Myricitrin



Poncirin

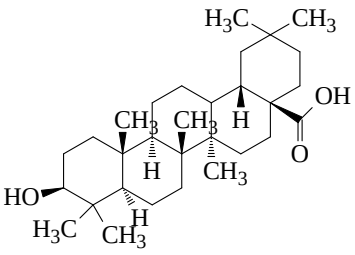


Vitexin

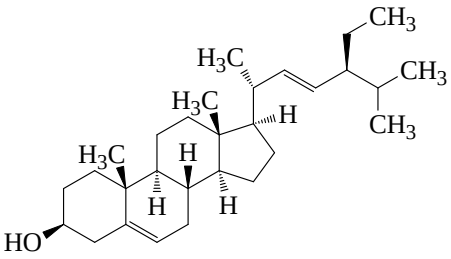


Tiliroside

Figure 2 Flavonoids from *P. serratifolia* leaves (continued)

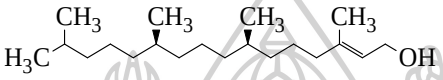


Oleanolic acid

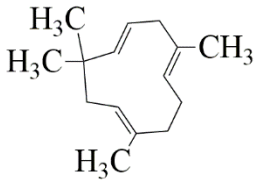


Stigmasterol

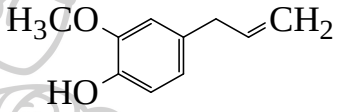
Figure 3 Triterpenoids and steroids from *P. serratifolia* leaves



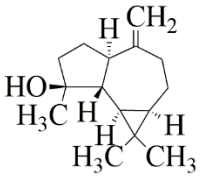
Phytol



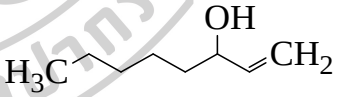
α-Humulene



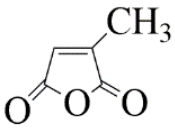
Eugenol



Spathulenol

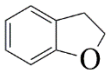


1-Octen-3-ol

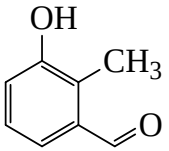


2,5-Furandione, 3-methyl

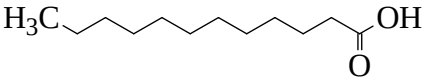
Figure 4 Volatile oils from *P. serratifolia* leaves and flower buds



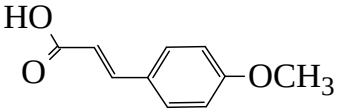
2,3-dihydrobenzofuran



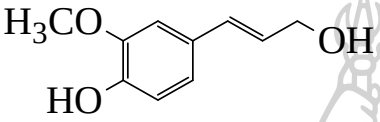
2-Hydroxy-3-methylbenzaldehyde



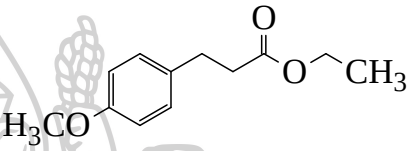
Dodecanoic acid



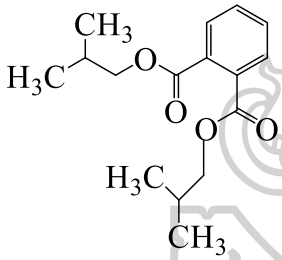
2-Propenoic acid, 3-(4- methoxyphenyl)-



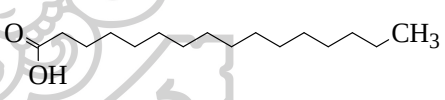
4-(3-hydroxy-1-propenyl)-2methoxyphenol



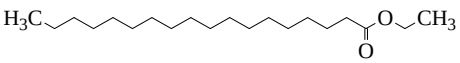
2-Propenoic acid, 3-(4-methoxyphenyl), ethyl ester



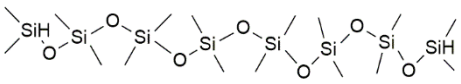
1,2-Benzenedicarboxylic acid, bis(2methylpropyl) ester



n-Hexadecanoic acid

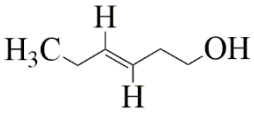


Octadecanoic acid, ethyl ester

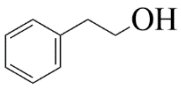


1,1,3,3,5,5,7,7,9,9,11, 11,13,13,15, 15-hexadecamethyloctasiloxane

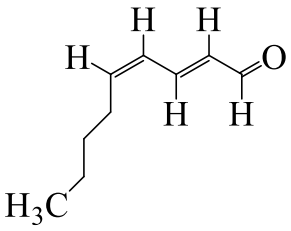
Figure 4 Volatile oils from *P. serratifolia* leaves and flower buds (continued)



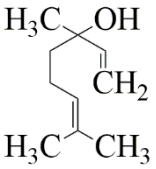
(*Z*)-n-Hexanol



2-Phenyl ethyl alcohol

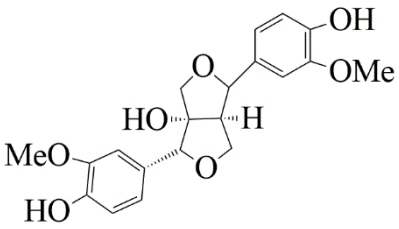


(*E, Z*)-2,4-Nonadienal

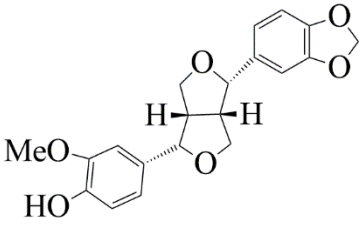


Linalool

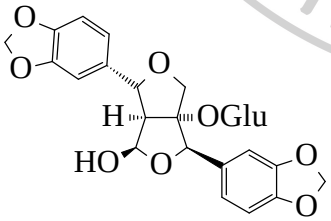
Figure 4 Volatile oils from *P. serratifolia* leaves and flower buds (continued).



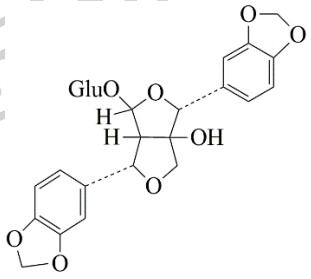
1α-Hydroxyl-6-epipinoresinol



Xanthoxyol

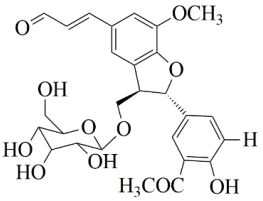


4β-hydroxyasarinin-1-*O*-β-glucopyranoside

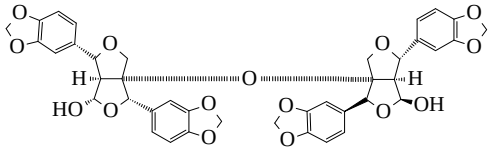


4-epi-gummadiol-4-*O*-β-glucopyranoside

Figure 5 Lignans from *P. serratifolia* wood and stem bark

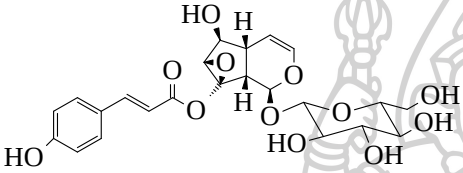


Plucheoside D1

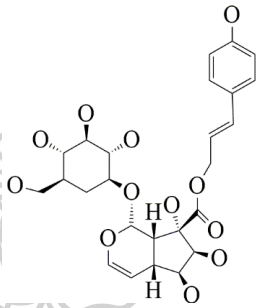


Premnadimer

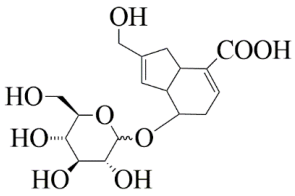
Figure 5 Lignans from *P. serratifolia* wood and stem bark (continued).



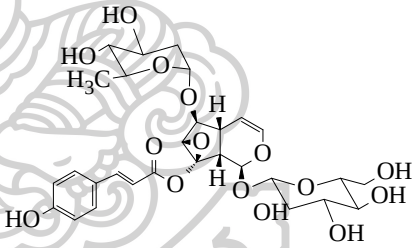
10-*O*-*trans*-*p*-coumaroylcatapol



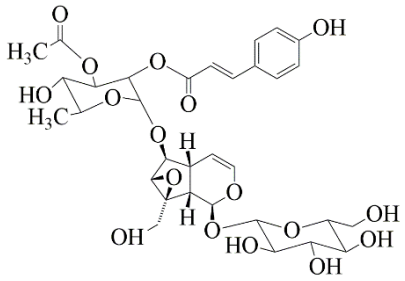
4″-hydroxy-*E*-globularinin



Premnosidic acid



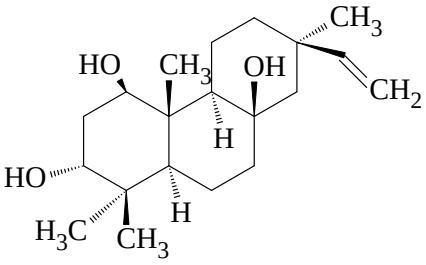
6-*O*-α-L-(10-*O*-*trans*-*p*-coumaroyl)-rhamnopyranosylcatapol



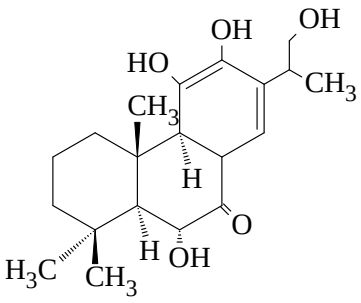
6-*O*-(3″-*O*-acetyl-2″-*O*-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosylcatpol

Premnacorymboside A

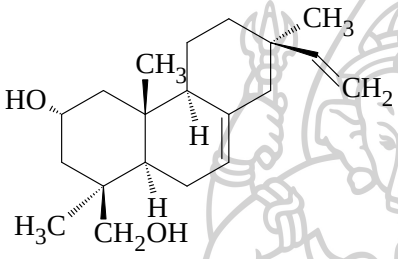
Figure 6 Iridoids glycosides from *P. serratifolia* stem and flower



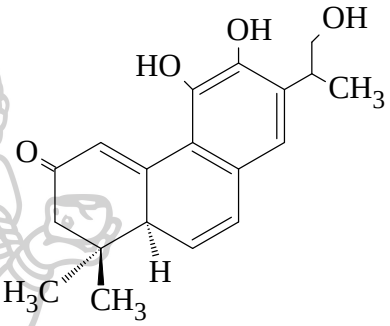
1β,3α,8β-trihydroxy-pimara-15-ene



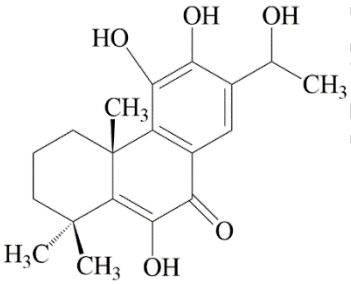
6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene



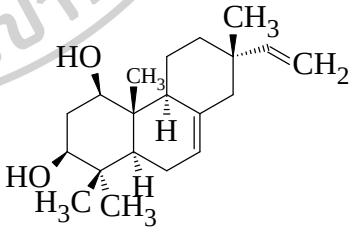
2α, 19-dihydroxy-pimara-7, 15-diene



11,12,16-trihydroxy-2-oxo-5-methyl-10- demethyl-abieta-1-[10]6,8,11,13-pentene

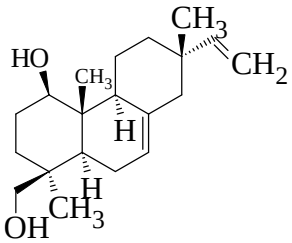


6-Hydroxy salvinolone

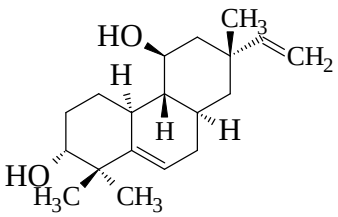


Isopimara-7,15-diene-1β, 3β-diol

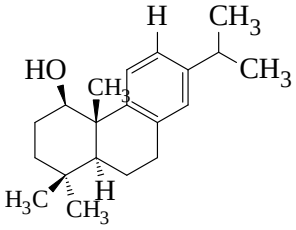
Figure 7 Diterpenoids from *P. serratifolia* root bark and twigs



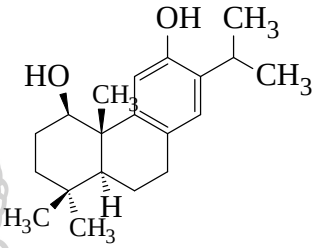
Isopimara-7,15-diene-1β,19-diol



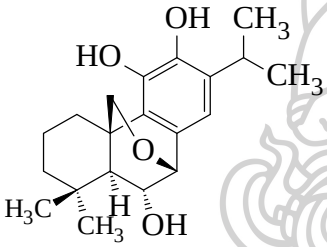
13-epi-5,15-rosadien-3α,11β-diol



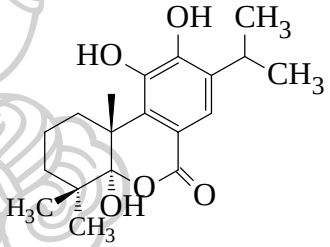
Abietatrien-1β-ol



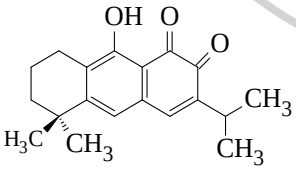
Abietatrien-1β,12-diol



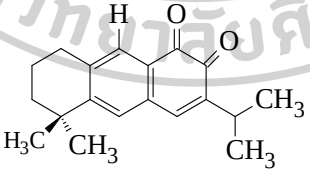
6α,11,12-Trihydroxy-7β,20-epoxy-8,11,13-abietatriene



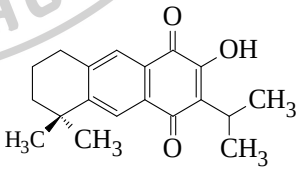
5α,11,12-Trihydroxy-6oxa-abieta-8,11,13-trien-7-one



Obtusinone A

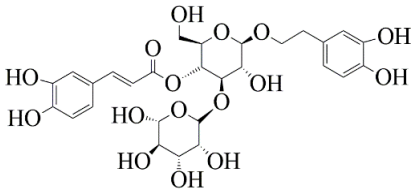


Obtusinone B

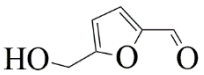


Obtusinone C

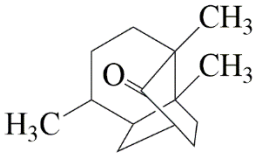
Figure 7 Diterpenoids from *P. serratifolia* root bark and twigs (continued)



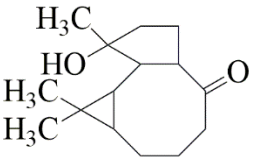
Acteoside (verbacoside)



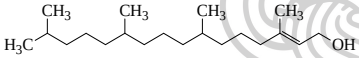
5-(hydroxymethyl)-furan-2-carbaldehyde



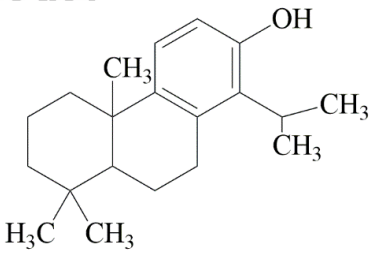
Seychellene



1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7 trimethyl-4-ethylene[1ar(1aα,4aα,7β,7aβ,7bα)]



3,7,11,15-Tetramethyl-2-hexadecen-1-ol

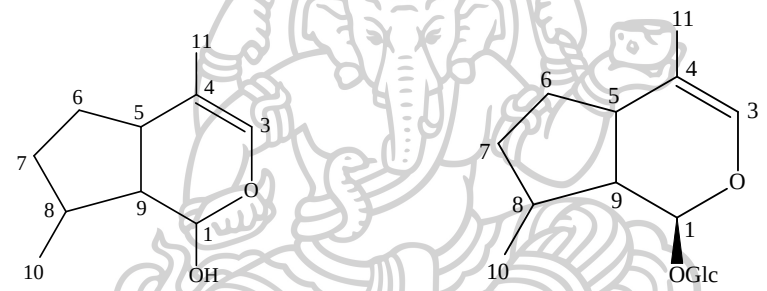


2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-Octahydro 4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans)-

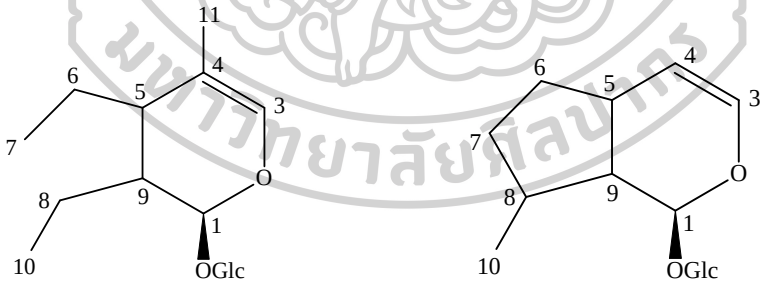
Figure 8 Phenylethanoid glycosides and volatile oils from *P. serratifolia* root and twigs

2.4 Iridoids and biosynthesis of iridoids

Iridoids are also known as monoterpene lactones which usually found in plants as glycosides. They can be occurred in dicotyledon angiosperms within the superorders Corniflorae, Gentianiflorae, Lamiiflorae and Loasiflorae. Their structures are based on cyclopentan[c]pyran skeleton represented as iridane (*cis*-2-oxabicyclo[4.3.0]nonane). Iridoids are broadly classified into two groups: iridoid glycosides and secoiridoid glycosides (Figure 9). Iridoid glycoside has sugar unit attached at carbon 1 position of iridoids, and secoiridoids are formed by the cleavage of cyclopentane ring at the C-7–C-8 bond. Iridoid glycosides have another subgroup: nine carbon basic skeleton (one carbon loss in carbon 4 position) called 4-demethyl iridoids glycosides (42).



Iridoid Iridoid glycosides



Secoiridoid glycosides 4-demethyl iridoid glycosides

Figure 9 Structures of iridoids basic skeletons

The biosynthesis pathway of terpenoids involve some essential metabolic intermediates, such as mevalonic acid (Figure 10). Despite the fact that acetyl-CoA is the initial primary metabolite, the 5-carbon isoprene units in the form of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) define the basic

structure of all terpenoids. The geranyl pyrophosphate (GPP), which is made up of two isoprene units, is the precursor of all terpenoids in the subsequent phases of the process. Sesquiterpenes (15 carbons), diterpenes (20 carbons), and triterpenes (30 carbons) are well-known terpenoids that result from the condensation of these isoprene units in a series of enzyme-catalyzed processes. The GPP produces a variety of biologically important cyclic and acyclic monoterpenes, while the 8-hydroxygeraniol pathway produces iridoids and their derivatives (43).

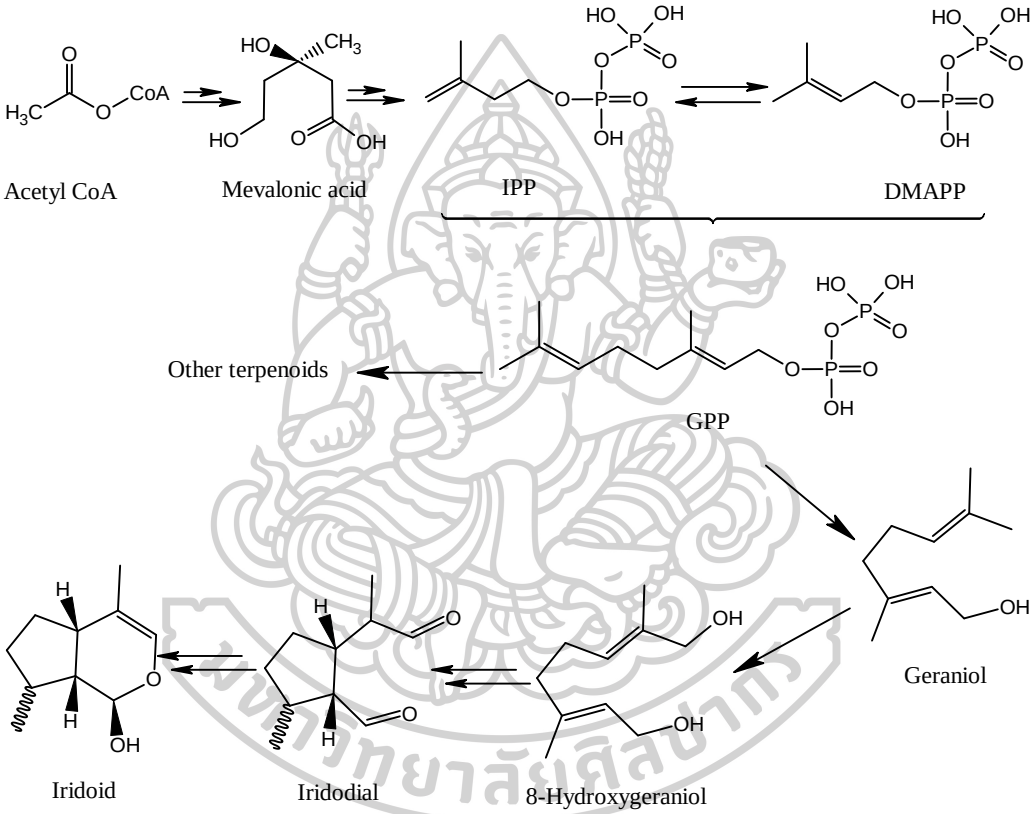


Figure 10 An overview of biosynthesis pathway of iridoids (43)

2.5 Chromatography

Chromatography is a technique for separating compounds from complex mixtures. It can be used for both qualitative and quantitative analyses of phytochemical research. Adsorption, partition, ion-exchange, affinity, and size-exclusion chromatography are five types of interactions in a chromatographic system. The chromatographic method has two phases: the stationary phase, which can be solid

or liquid, and the mobile phase, which can be liquid or gas. A selected mobile phase is primarily used for the separation of compounds (44).

2.6 Gas chromatography

The gas chromatography method is used for separating mixture components of vaporizable volatile compounds and is commonly used for qualitative and quantitative analyses of samples. When the sample is injected into the column (stationary phase), the sample is passed through the column by the flow of the inert gaseous mobile phase. The analyte goes through the column and the detector detects the time between the sample injection and the analyte eluted from the end of the column. Elution, retention time, theoretical plate, height equivalent to a theoretical plate (HEPT), capacity factor, selectivity, and resolution are all important aspects of GC chromatography (44).

2.6.1 Elution

Elution is the continuous addition of a mobile phase to transport the sample through the column. The duration an analyte spends in the mobile phase determines the average rate at which it flows through the column.

2.6.2 Retention time (tR)

The retention time is the time between sample injection and an analyte peak reaching a detector at the end of the column. Each analyte in a sample have a retention time and the time taken for the mobile phase to pass through the column is known as tM.

2.6.3 Capacity factor

The retention factor, often known as the capacity factor, is a term used to describe the migration rate of an analyte of a column. The retention factor for analyte A is defined as

$$k'A = (tR - tM)/tM$$

The chromatogram can be used to determine tR and tM. When the retention factor of an analyte is less than one, elution is very fast, and when it is larger

than 20, elution takes a long time. For an analyte, the retention factor should be between 1 and 5.

2.6.4 Height equivalent to a theoretical plate (HEPT)

Theoretical plate is a large number of separate layers in the chromatographic column. In these plates the sample is equilibrated separately between the stationary and mobile phase. The analyte travels down the column by transfer of equilibrated mobile phase from one plate to the next.

2.6.5 Resolution

Separation of band center is referred to as resolution. To get high resolution, an increase in N, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening.

2.6.6 Mass spectrometry

The molecular ion is fragmented in mass spectrometer into small fragments with different mass to charge ratio and can be used for identification of compound. Mass spectrometer contains five components of sample inlet, ion source, mass analyzer, detector and data system (44).

2.6.7 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is gas chromatography coupled with a mass spectrometer and is widely used in medicinal chemistry, pharmaceutical analysis, and pharmaceutical biotechnology. The function of GC-MS is that when the sample molecule comes out of the GC column, it enters the ionization chamber of mass spectrometry. In the ionization chamber, a sample molecule is converted into a molecular ion by ionization energy. A non-ionized molecule is pulled off a vacuum pump, which is joined to an ionization chamber. Some molecules are transferred with a negative ion, and they are absorbed by repeller plates. The ionized molecule passes through a mass analyzer in which molecular ions break down into small fragments according to their mass to charge ratio (*m/z*). When an electron collides with the surface of an electron multiplier, it is possible that more electrons will be released, resulting in a higher signal. The recorder captures the detector's signal and generates a mass spectrum (44).

2.7 Planar chromatography

Planar chromatography is a type of chromatography in which the stationary phase is dispersed on a flat planar surface. TLC is a common planar chromatographic technique that is extensively used as a low-cost approach for analyzing simple mixtures quickly. TLC requires small amount of sample and can analyze multiple samples simultaneously. Sample accessibility in planar format offers greater flexibility and simplicity in sample evaluation, post-chromatographic derivatization for identification and quantification, and separation archives for later evaluation. It can be used to analyze samples with minimal pre-purification and where the analyte lacks a suitable chromophore, making detection by other approaches challenging. Therefore, the TLC method is widely used for separation, detection, and qualitative and quantitative determination of biomolecules belonging to different chemical classes (45).

2.7.1 High Performance Thin Layer Chromatography (HPTLC)

HPTLC is advanced form of TLC and applicable for both qualitative and quantitative methods. HPTLC has the advantages of being able to handle a large number of samples at once and can be compared to a standard for identification. Moreover, absorption or fluorescence of the substance on the plate can be measured by TLC densitometry scanner. HPTLC has frequently been found to be superior to HPLC in term of cost, time needed for pharmaceutical analyses in comparative studies (45). Comparative factors of TLC and HPTLC, and HPTLC and HPLC are shown in Tables 3 and 4 respectively.

Table 3 Comparison between HPTLC and TLC (45)

Parameters	HPTLC	TLC
Technique	automated/instrumental	manual
Mean particle size	5–6 µm	10–12 µm
Layer thickness	100 µm	250 µm
Plate height	12 µm	30 µm
Efficiency	high due to smaller particle size	generated less
Separations	3–5 cm	10–15 cm
Analysis time	shorter migration distance and the analysis time is greatly reduced	slower
Sample volume	0.1–0.5 µL	1–5 µL
Starting spot's diameter	1–1.5 mm	3–6 mm
Separated spot's diameter	2–5 mm	6–15 mm
Sample tracks per plate	≤ 36	≤ 10
Separation time	3–20 min	20–200 min
Detection limit (absorption)	100–500 pg	1–5 ng
Detection limits (fluorescence)	5–10 pg	50–100 pg

Table 4 Comparison of HPTLC and HPLC
(44)

Parameters	HPTLC	HPLC
Stationary phase	solid	solid
Mobile phase	liquid	liquid
Sample	non-volatile	non-volatile
System	open system	closed system
Separating medium	plate	tubular column
Sample analysed at a time	up to 22	1 sample
Derivatization	easy	very difficult
Maintenance	low	high
Running cost	low	high
Analysis time	1 to 5 min/sample	5-60 min/sample

2.8 Densitometry

Densitometric method is available for qualitative and quantitative analysis using TLC/HPTLC chromatogram. The retardation factor (R_f) value and the color of the spot after reaction with detection reagent are two simple characteristics that can be employed for qualitative analysis by TLC/HPTLC method. The R_f can be defined as the ratio of the distance of the spot migration from the start to the distance of the mobile phase migration from the start. UV 254/366 nm (if the compounds have a UV absorbing and fluorescencing functional group) can also be used to visualize the analyte spot. By using scanning densitometry, the TLC/HPTLC chromatogram can be transformed into a densitogram, in which all spots are visible as peaks. Moreover, TLC/HPTLC chromatogram is the most frequently approached for quantitative analysis using densitometry. Both external and internal standardization approaches can be used for calibration. Therefore, densitometry is an accurate, and reliable way to quantify the results of a TLC/HPTLC chromatogram and has become the most popular technique for determining the concentration of chromatographic zones on a TLC/HPTLC layer.

The instrument is an integral computer-controlled device that leads to highly reproducible and accurate results (1% standard deviation). The technology is based on a pre-set wavelength of electromagnetic radiation (typically UV/visible from 190–800 nm) that either moves at a pre-determined rate through the chromatographic zones or, whilst the beam remains stationary, the TLC/HPTLC layer is moved under the control of a motorised base plate. The technique of scanning the plate under these conditions is called spectro-densitometry. The scanning process is relatively fast (up to 100 mm s^{-1}) with a spatial resolution in steps of 25–200 mm. The instrument can be pre-programmed to scan at a variety of wavelengths and scan automatically all of the chromatographic tracks on the developed layer. Chromatograms resulting from scanning the developed tracks are very similar to those obtained in HPLC, normally displaying a series of peaks with baseline resolution where the zones are well separated. The purity of chromatographic zone can also be evaluated by taking full spectra at the beginning, apex, and end of the peak. If the spectra are identical, then the peak is considered to be of high purity (45).

2.9 Method validation

Analysis method must to be validated before using to prove that the method is suitable for its intended use and provide reliable, accurate, and reproducible results. Method validation parameters include specificity, calibration curve, precision, accuracy, limit of detection and limit of quantitation, robustness (46).

2.9.1 Calibration curve and range

Linearity is known as the ability of an analytical process to produce test results that are directly proportional to the concentration (amount) of an analyte in the sample (within a specific range). Visual evaluation of a plot of signals as a function of analyte concentration or content should be used to determine linearity. If there is a linear relationship, test findings should be examined using proper statistical methods, such as the least squares approach to calculating a regression line. Data from the regression line itself may be useful in estimating the degree of linearity mathematically. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares are necessary to submit. At least, 5-6 concentrations are needed to construct a calibration curve. Linearity should be within the minimum and

maximum concentration ranges. The minimum specified ranges for the assay of a drug substance or a finished (drug) product are 80 to 120 percent of the test concentration.

Regression line, $y = ax + b$

a is the slope of regression line

b is the y – intercept

x is the analyte concentration

y is the signal responses

2.9.2 Accuracy

Accuracy is the assessment of how close of the result value to the true value. Assessment of accuracy should be done after establish of specificity/selectivity, determination of the linear range, and determination of the precision of the method.

2.9.3 Specificity

Specificity refers to the ability to accurately and precisely measure the analyte of interest in the presence of other components such as contaminants, degradation products, and matrix components that could be present in the sample matrix.

2.9.4 Precision

Precision illustrates the closeness of replicated values within the same day (intra-day) and different continuous days (inter-day) under similar specified conditions. There are three levels of precision: repeatability, intermediate precision, and reproducibility. The precision under the same operational conditions over a short interval of time is referred to as repeatability (intra-day precision). Intermediate precision (inter-day precision) is present within-laboratory variation, different days, different analysts, different equipment. A minimum of nine determinations covering the specified range for the procedure should be used to assess repeatability (i.e., three concentrations and three replicates of each concentration, or using a minimum of six determinations at 100% of the test concentrations). The precision of an analytical procedure is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements. Reproducibility refers to the precision of results

among laboratories (collaborative studies, usually applied to standardization of methodology). An inter-laboratory trial is frequently used to demonstrate reproducibility.

2.9.5 Limit of detection and limit of quantification

The lowest amount of analyte in a sample that can be reliably detected and classified, but not necessarily quantified under the defined experimental conditions of the analytical method is known as the limit of detection (LOD). Limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantitatively measured with suitable accuracy and precision under experimental conditions established for the analytical method.

2.9.6 Robustness

Robustness is the evaluation of the performance of the method that can vary the chromatographic parameter.

CHAPTER 3
MATERIALS AND METHODS

3.1 Determination of post-harvest effects on quality of fresh, dried and fermented leaves of *P. serratifolia*

3.1.1 Plant materials

The plant materials were collected from Mandalay Division, Myanmar in October 2018. The taxonomic species was identified by comparing its flower and leaf characters with the references (20, 21). The voucher specimen (Ps172018) was deposited with the herbarium of the Department of Pharmacognosy, Silpakorn University, Thailand.

3.1.2 Preparation of plant materials

The collected leaves were prepared into three parts. The first part was the fresh leaves, the second part was dried in the shade, and the third part was fermented by packing in a plastic bag and placed at room temperature for 10 days in the shade until the leaves turned to black color.

3.1.3 Preparation of the volatile oils

The fresh leaf sample (490 g), the dried leaf sample (244 g) and the fermented leaf sample (201 g) were weighed. They were separately chopped into small pieces and placed in round bottle flask. Distilled water was added into half of the bottle and volatile oils were extracted by hydrodistillation process using Clevenger apparatus for 5 h. The collected oils were dried over anhydrous sodium sulfate and store at 4ºC in air-tight glass bottles. The yields of the volatile oils of the fresh, dried and fermented leaves were 0.008, 0.139 and 0.139%, respectively, calculated based on fresh, dry and fermented weight of the plant materials.

3.1.4 GC-MS analysis

The volatile oils were analyzed by Agilent 6890 gas chromatography equipped with Agilent technology, 5973N mass selective spectrometric detector (EIMS, electron energy, 70 eV, scanning from 40 to 500 *m/z*) with a quadrupole analyzer and an Agilent Chem Station data system (Agilent Technologies, U.S.A.). Two columns, fused silica capillary column (5%-phenyl)-methylpolysiloxane DB-5

(30 m x 0.32 mm ID x 0.25 µm film thickness) and Carbowax 20M polyethylene glycol (PEG) (60 m x 0.25 mm ID x 0.25 µm film thickness) were used. Ultra-high purity helium gas (99.999%) was used as a carrier gas at a flow rate of 1 mL/min. The sample (1.0 µL) was injected with a splitless mode. The solvent delay for the detector was 3 minutes. The ion source temperature was 230°C and quadrupole temperature was programmed at 150°C. For the DB-5 column, the initial oven temperature was 80°C and increased to 130°C at the 5°C/min, then increased to 280ºC at the 10ºC/min and hold for 5 min. For Carbowax 20M column, the initial oven temperature was 60ºC and increased to 220ºC at the 2ºC/min.

3.1.5 Identification of chemical compounds of the volatile oils

Identification of compounds was performed by comparing their RI relative to *n*-alkanes (C8-C26) with Adams (47), NIST Chemistry WebBook (48), SRD 69, Babushok et al., (49) and Leffingwell et al., (50). Their mass spectra were also compared with libraries databases of Wiley7n.l and NIST 05.

3.1.6 Determination of total phenolic content and antioxidant activity

3.1.6.1 Extraction

The samples of fresh, dried and fermented leaves (11 g) were separately macerated with 200 mL of 80% methanol at room temperature for 48 h. After that, they were filtered and evaporate to dryness on a water bath. The extracts were weighed and calculated for %yield.

3.1.6.2 Determination of total phenolic content

Folin-Ciocalteu method was used to determine the total phenolic content by some modification from the method in reference (25). Sample solution (10 mg/10 mL in methanol) was prepared and 0.5 mL of this solution was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent (Fluka; Buchs, SG, Schweiz). The mixture solution was allowed to stand for 5 min. Then, 2 mL of 5% sodium carbonate was added, mixed and kept for 180 min. The absorbance was measured at 765 nm using a spectrophotometer (Hitachi U-2000 spectrophotometer, Tokyo, Japan). A calibration curve of gallic acid (Fluka; Buchs, SG, Schweiz) (10-70 µg/mL) which used as reference was constructed between the final concentration and absorbance

value. The total phenolic content of the extract was expressed as gallic acid equivalent (%GAE). The experiment was done in triplicate.

3.1.6.3 Determination of antioxidant activity

Radical scavenging activity was used to determine the antioxidant activity. The procedure was done with some modification from the method in reference (Timotius, et.al. 2018) (25). The sample solutions (2 mg/mL) were serially diluted to the concentrations of 200, 100, 20, 10, and 2 µg/mL, respectively. Then, DPPH solution (Fluka; Buchs, SG, Schweiz) (75 µg/mL) was prepared. After preparing the solution, 4 mL of each concentration of sample was mixed with 4 mL of DPPH solution in a test tube and kept in a dark place for 60 minutes. After incubation for 60 min, the absorbance of the sample was measured at 517 nm using a spectrophotometer (Hitachi U-2000 spectrophotometer, Tokyo, Japan). The sample solutions without DPPH solution was used as a negative control, and methanol was used as a blank. The percentage of DPPH scavenging activity was calculated as follows:

$$\% \text{ scavenging activity} = [(A_{DPPH} - (A_{sample+\ DPPH} - A_{sample}) / A_{DPPH}] \times 100$$

A_{DPPH} is the absorbance of a DPPH solution without extract. $A_{sample\ +\ DPPH}$ is the absorbance of a sample in DPPH solutions. A_{sample} is the absorbance of a sample in methanol solution. Ascorbic acid (Fluka; Buchs, SG, Schweiz) was employed as a positive control and it was prepared in the same way as the tested sample. The regression was constructed between % scavenging activity and concentration of the sample, and were used to calculate IC_{50}. The 50% inhibitory concentration (IC_{50}) was reported as the amount of antioxidant necessary to reduce the DPPH concentration by 50%.

3.2 Isolation and identification of chemical constituents of the leaves of *P. serratifolia*

3.2.1 Preparation of plant materials

The collection and identification of plant materials were described in section 3.1.1. The collected leaves were thoroughly washed with water, and then they were dried in the shade. The dried leaves were processed into coarse powdered using blender.

3.2.2 Extraction

The dried coarse leaf powder (480 g) were extracted by a Soxhlet apparatus with hexane for 16 h. The extract solution was evaporated under reduced pressure to give the crude extract (13.81 g, 2.87 % yield). The marc was re-extracted with dichloromethane and methanol, respectively for 16 h. Each extract was concentrated under reduced pressure (Rotarvapor, Buchi R-134, Flawil, Switzerland) and evaporated on water bath to obtain crude extracts of dichloromethane (8.16 g, 1.7 %yield) and methanol (112.79 g, 23.49 %yield) (Figure 11).

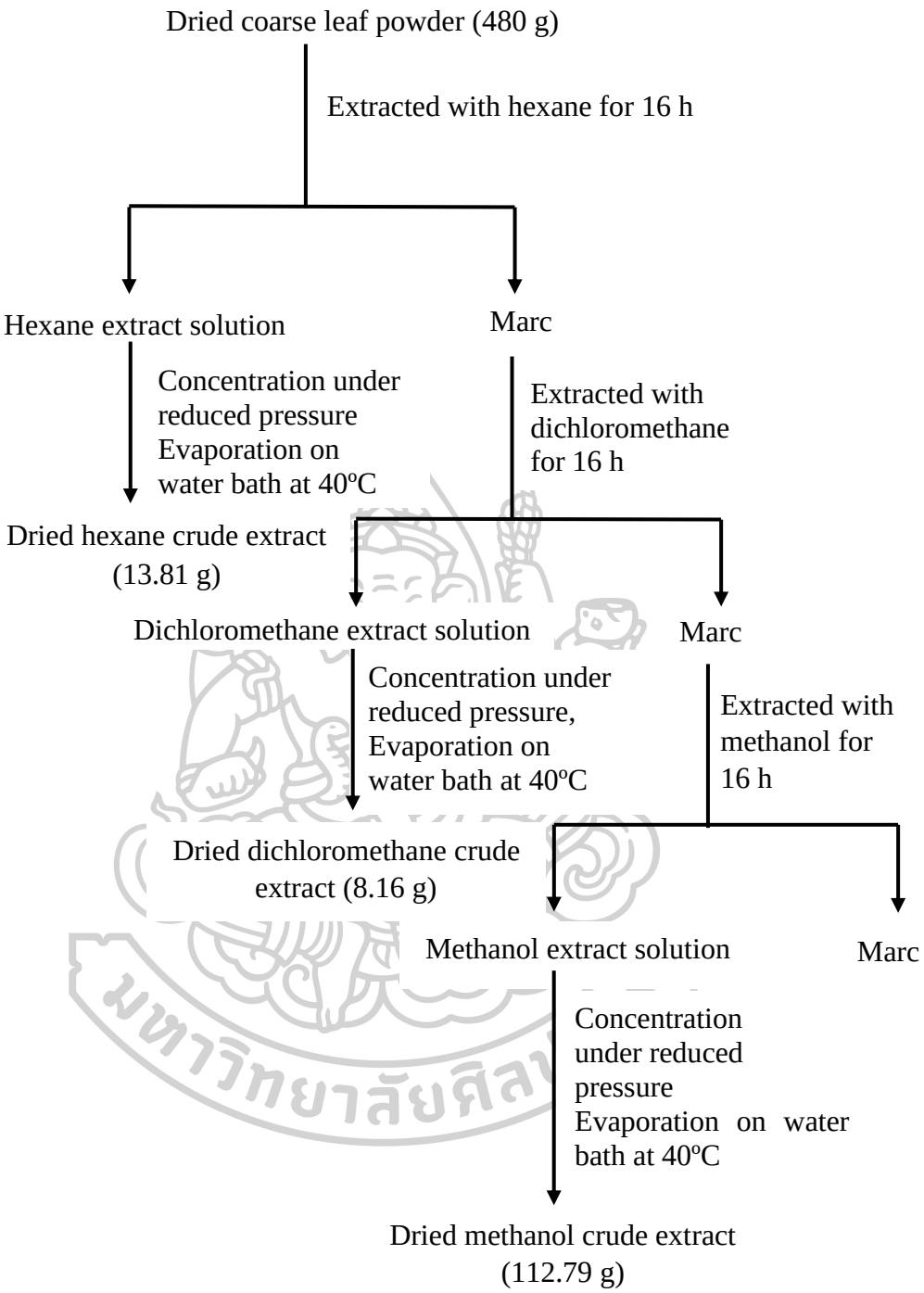


Figure 11 Flow diagram of extraction of *P. serratifolia* leaves

3.2.3 Phytochemical screening tests

The hexane, dichloromethane and methanol extracts were investigated for the presence of phenolic, saponins, alkaloids, steroids, flavonoids, and iridoids. The phytochemical screening test was described as follows (51):

3.2.3.1 Test for phenolics

A small amount of each extract was dissolved in 2 mL of methanol. One drop of 9% ferric chloride solution was added into each extract solution and checked the color. The dark blue or green color indicated the presence of phenolics.

3.2.3.2 Test for saponins

A small amount of each extract was diluted with 10 mL of water. It was shaken vigorously and allowed to stand for 15 min. The formation of a layer of foam indicated the presence of saponins.

3.2.3.3 Test for alkaloids

A small amount of each extract was placed in a porcelain spotting plate and dissolved with two drops of 10% sulfuric acid. Then, three drops of Drangendroff's reagent were added to the spot plate. The formation of reddish-brown precipitates indicated the presence of alkaloids.

3.2.3.4 Test for steroids and triterpenoids

A small amount of each dried extract was placed in a porcelain spotting plate and dissolved with three drops of acetic anhydride. Then, one drop of concentrated sulfuric acid was added gently. The occurrence of green or purple color indicated the presence of steroids or triterpenoids, respectively.

3.2.3.5 Molisch's test

A small amount of each extract was dissolved in 1 mL of methanol in a test tube. Two drops of 5 % α–napthol in ethanol were added and they were mixed well. Following that, gentle drop of concentrated sulfuric was added into the side of test tube until two phases appeared. The appearance of the purplish red color in the interphases indicated the presence of sugar.

3.2.3.6 Test for iridoids

A small amount of each extract was dissolved in 1 mL of methanol in a test tube. Then, 1 mL of Trim-Hill reagent was added and heated with alcohol burner. The appearance of green in color indicated the presence of iridoids.

3.2.4 Chromatographic procedure

3.2.4.1 Analytical Thin Layer Chromatography (TLC)

Technique	: One dimension, ascending
Adsorbent	: Silica gel 60 F254 (0.040-0.063 mm, Merck, Germany) precoated on aluminium sheet
Layer thickness	: 0.2 mm
Distance	: 6 cm
Temperature	: Laboratory temperature (25-30 °C)
Solvent	: Methanol-Dichloromethane-Water (4:1:0.1)
Detection	: 254 and 366 nm
	: Spraying with 1% vanillin-sulfuric acid solution and heating at 110 °C for 5 min

3.2.4.2 Gel filtration chromatography

Column	: Glass column (2.5 cm in diameter)
Gel Filter	: Sephadex LH-20 (Amersham Biosciences ®, No.51-2180-00-DB)
Solvent	: Methanol
Packing method	: Wet method
Sample loading	: The sample was dissolved in 10 mL of eluent and it was introduced gently into the top of the column with pasture pipette
Detection	: Collected fractions were detected by TLC technique

3.2.4.3 Column chromatography

Column	: Glass column (2.5 cm in diameter)
Adsorbent	: Silica gel 60 (0.040-0.063 mm, 64271 Darmstadt, Germany)
Solvent	: Methanol-Dichloromethane-Water (4:1:0.1)
Packing method	: Wet method
Sample loading	: The sample was dissolved in 10 mL of eluent and it was subjected gently into the top of the column with pasture pipette
Detection	: Collected fractions were detected by TLC technique

3.2.5 Isolation of premnaodoroside A

The methanolic extract (18 g) was dissolved in methanol, and the undissolved mass (0.62 g) was discarded. The solution was applied over a Sephadex LH-20 chromatography and eluted with methanol. Each fraction was collected according to their color band and checked with TLC by using methanol-dichloromethane-water (4:1:0.1). This process was repeated nine times, each time using 2 g of crude methanol extract. Fractions with similar patterns were combined to afford 8 fractions. They were PSM 1 (1.19 g), PSM 2 (0.10 g), PSM 3 (0.35 g), PSM 4 (3.11, giving a major purple color band on TLC), PSM 5 (0.48 g), PSM 6 (4.01 g, giving a major black color band on TLC), PSM 7 (2.39 g), and PSM 8 (0.74 g) (Table 5). Fraction PSM 4 was subjected to a silica gel 60 column chromatography and eluted with methanol-dichloromethane-water (4:1:0.1). Eighty-nine fractions (30 mL each) were collected and fractions with similar pattern on the TLC plate were combined together to give 12 fractions (PSP 1 to PSP 12) (Table 6). Fraction 10 (0.10 g) was purified by recrystallization in methanol and acetone, providing a yellowish white amorphous powder of the pure compound premnaodoroside A (54.1 mg). The process of separation and isolation is shown in Figure 12.

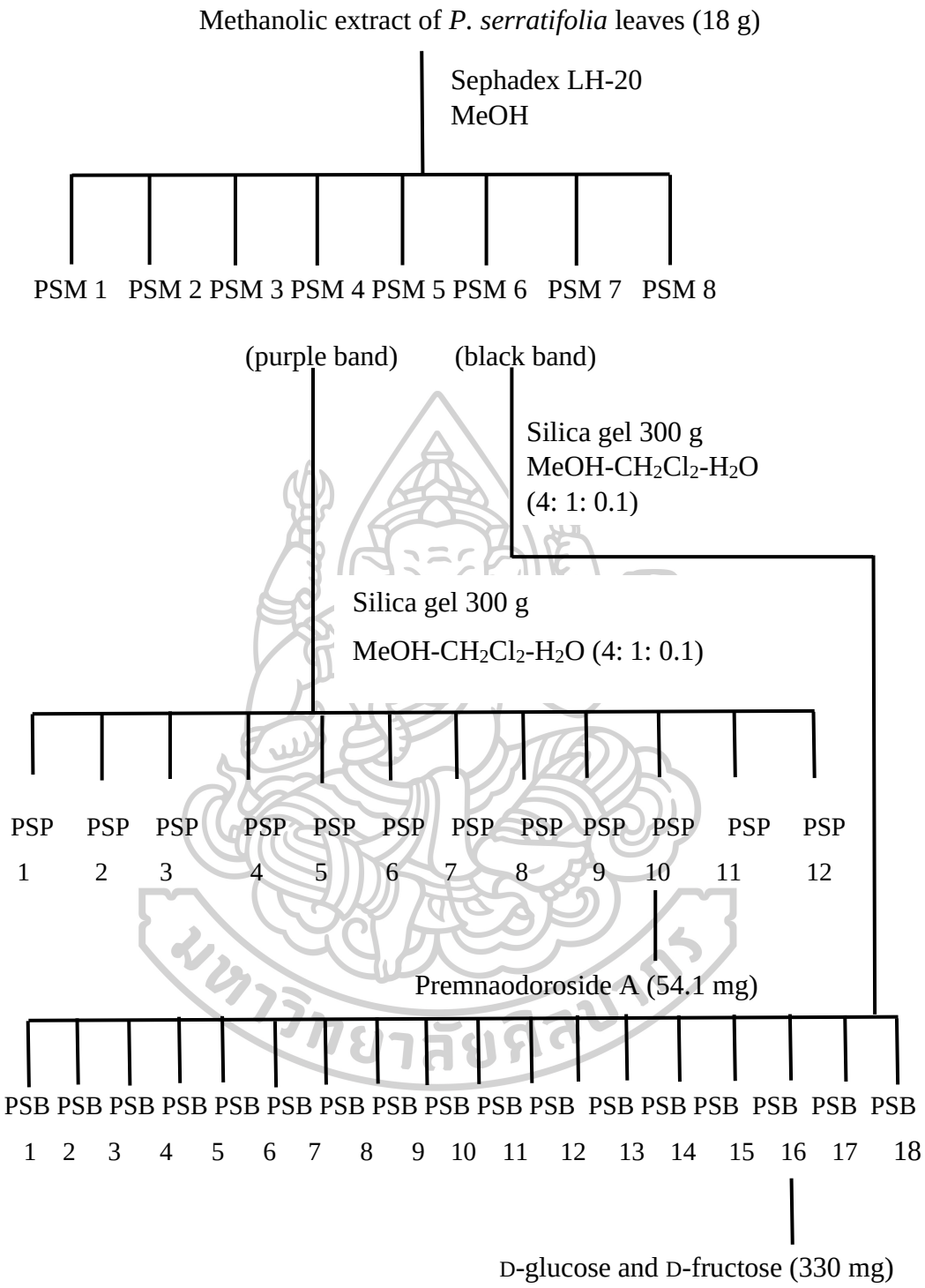


Figure 12 Flow diagram of isolation of the compounds from the methanolic extract

Table 5 Combined fractions of the Sephadex LH20 column of methanolic extract

Fraction code	No. of eluate	Weight (g)
PSM 1	1	1.19
PSM 2	2	0.10
PSM 3	3	0.35
PSM 4	4-5	3.11
PSM5	6	0.48
PSM 6	7-8	4.01
PSM 7	9-12	2.39
PSM 8	13-18	0.74

Table 6 Combined fractions of the silica gel column chromatography of the fraction PSM 4

Fraction code	No. of eluate	Weight (g)
PSP 1	1-6	0.01
PSP 2	7-9	0.06
PSP 3	10-11	0.04
PSP 4	12-15	0.12
PSP 5	16-23	0.11
PSP 6	24-34	0.20
PSP 7	35-43	0.11
PSP 8	44-54	0.11
PSP 9	55-61	0.14
PSP 10	62-71	0.10
PSP 11	72-80	0.70
PSP 12	81-89	0.08
PSP 13	Washing column with MeOH	1.32

3.2.6 Isolation of D-glucose and D-fructose

Fraction PSM 6 (4.01 g) was introduced into a silica gel 60 column chromatography and eluted with methanol-dichloromethane-water (4:1:0.1) to obtain 120 fractions. Fractions with similar patterns on TLC plate were combined to give 18 fractions (PSB 1 to PSB 18) (Table 7). Fraction 16 afforded the mixture of D-glucose and D-fructose (330 mg) as powdered.

Table 7 Combined fractions of the silica gel column chromatography of the fraction PSM 6.

Fraction code	No. of eluate	Weight (g)
PSB 1	1-12	0.01
PSB 2	13-16	0.04
PSB 3	17-20	0.12
PSB 4	21-25	0.13
PSB 5	26-31	0.1
PSB 6	32-40	0.12
PSB 7	41-45	0.12
PSB 8	46-51	0.08
PSB 9	52-57	0.05
PSB 10	58-60	0.02
PSB 11	61-66	0.06
PSB 12	67-74	0.14
PSB 13	75-76	0.04
PSB 14	77-80	0.13
PSB 15	81-91	0.33
PSB 16	92-99	0.33
PSB 17	100-111	0.35
PSB 18	112-120	0.16
PSB 19	Wash column with MeOH	1.68

3.2.7 Identification of the isolated compounds

3.2.7.1 Spectroscopic techniques

3.2.7.1.1 UV-Vis spectrophotometry

The UV spectra were measured with a UV-Vis spectrophotometer (Hitachi U-2000 spectrophotometer, Tokyo, Japan) at the Faculty of Pharmacy, Silpakorn University.

3.2.7.1.2 Mass spectrometry

Mass spectra were recorded on Liquid Chromatography/Mass Spectrometry (1100 series/LC-MSD-Trap-SL) with an ESI Source (G2440DA) (USA) at the Faculty of Pharmacy, Silpakorn University.

3.2.7.1.3 Infrared absorption spectrometry

IR spectra were recorded on an FTIR spectrometer (Thermo Nicolet 4700, Madison, WI, USA) operated with Thermo Electron's OMNIC software (Version 7.2a, Thermo Nicolet, Madison, WI, USA) at the Faculty of Pharmacy, Silpakorn University. The sample was placed as thin-film in the middle of a dry KBr window and the FTIR spectrum was recorded in the wave number range of 4000-400 cm^{-1}.

3.2.7.1.4 Nuclear magnetic resonance spectrometry

The ^{1}H-NMR (300 MHz), and ^{13}C-NMR (75 MHz) spectra were recorded on a Bruker Bio 300 MHz NMR Spectrometer at the Faculty Science, Silpakorn University). Deuterated methanol (MeOD) and deuterated water (D_2O) were used as solvents. The position of signals was measured on a ppm scale, and tetramethylsilane (TMS) was used as a reference.

3.2.8 Characterization of the isolated compounds

3.2.8.1 Premnaodoroside A

Appearance - Yellowish white amorphous powder (solvent use to crystallize)

Solubility - Methanol

ESI-MS - *m/z*: 888.5 $[M-H]^-$ ($C_{42}H_{66}O_{20}$, M=890);

^{1}H-NMR - δppm, 300 Hz, in MeOD;

5.45 (2H, *d*, *J*=4.5 Hz, H-1a, H-1b), 7.39, (H, *s*, H-3a), 7.41 (H, *s*, H-3b), 3.2~3.3 (proton overlapped by the solvent, H-5), 1.65 (H, *m*, H-6), 1.75 (5H, *m*, H-6), 1.78, (H, *m*, H-7), 2.22 (2H, *dd*, *J*= 9.3, 4.2 Hz, H-9), 1.32 (3H, *s*, H-10), 4.17 (*t*, *J*=5.9 Hz, H-1″), 1.59 (*m*, H-2″, 1.74, *m*, H-2″), 1.4-1.5 (*m*, H-3″), 1.19~1.37 (proton overlapped by the water, H-4″, H-5″, H-6″), 1.82 (*m*, H-7″), 3.92 (H, *m*, H-8″), 0.91 (3H, *d*, *J*=6.3 Hz, H-9″), 0.96 (3H, d, *J*=7.2 Hz, H-10″), 4.68 (H, *d*, *J*=7.8 Hz, H-1′), 3.2~3.3 (proton overlapped by the solvent, H-2′, H-3′, H-4′), 3.37 (2H, *t*, H-5′), 3.90 (2H, *dd*, *J*=11.9, 6.3 Hz, H-6′);

^{13}C-NMR δppm, 75 Hz, in MeOD;

95.61, 95.61 (C-1), 152.07, 152.07 (C-3), 113.71, 113.75 (C-4), 32.23, 32.23 (C-5), 31.09, 31.06 (C-6), 40.80, 40.84 (C-7), 80.69, 80.72 (C-8), 52.42, 52.42 (C-9), 24.82, 24.82 (C-10), 169.19, 169.25 (C-11), 63.65 (C-1″), 36.91 (C-2″), 31.15 (C-3″), 38.24 (C-4″), 25.34 (C-5″), 34.85 (C-6″), 34.06 (C-7″), 70.09 (C-8″), 20.03 (C-9″), 17.56 (C-10″), 99.86, 99.98 (C-1′a, 1′b), 74.76, 74.89 (C-2′a, 2′b), 78.40, 78.53 (C-3′a, 3′b), 71.75, 71.87 (C-4′a, 4′b), 78.02, 78.13 (C-5′a, 5′b), 62.98, 63.1(C-6′a, 6′b).

3.2.8.2 D-glucose and D-fructose

Appearance - Yellowish amorphous powder

Solubility - Water

IR - ν_{max}cm^{-1} (KBr): 3360.8 and 1055.8 cm^{-1}

ESI-MS - *m/z*: 203 $[M+Na]^+$ ($C_6H_{12}O_6$, M=180);

^{13}C-NMR - δ ppm, 75 Hz, in D_2O;

- α-D-Glucopyranose: 92.04 (C-1), 71.43 (C-2), 72.35 (C-3), 71.38 (C-4), 69.59 (C-5), 60.53 (C-6)

- β-D-Glucopyranose: 95.86 (C-1), 74.39 (C-2), 75.70 (C-3), 71.38 (C-4), 69.59 (C-5), 60.70 (C-6)

- β-D-Fructopyranose: 63.83 (C-1), 98.02 (C-2), 67.49 (C-3), 69.54 (C-4), 69.15 (C-5), 63.31 (C-6)

- α-D-Fructofuranose: 62.85 (C-1), 104.39 (C-2), 81.91 (C-3), 75.31 (C-4), 81.24 (C-5), 62.85 (C-6).

- β-D-Fructofuranose: 62.59 (C-1), 101.45 (C-2), 75.89 (C-3), 74.08 (C-4), 80.62 (C-5), 62.33 (C-6).

3.2.9 Purity of premnaodoroside A

The stock solution of premnaodoroside A (5 mg/mL) was diluted with ethanol to yield a working solution with a concentration of 0.436 mg/mL. Two microliters of working solution were spotted on the TLC plate to obtain the concentration of 0.872 µg/spot. The chromatogram was developed (see details in section 3.3.3) and the plate was scanned and analyzed (see details in section 3.3.4). Then, CAMAG densitometer (CAMAG CATS 1.4.9.210184 software) was used to scan the TLC chromatogram in reflectance mode and detect the absorption at wavelength 254 nm. The purity was the percent proportion of the area of the standard (the main peak) to total peak area.

3.3 Development and validation of TLC-densitometric method

3.3.1 Preparation of standard stock solution and calibration curve

The standard stock solution (5 mg/mL) of premnaodoroside A was prepared in ethanol. The working solution (0.055-0.436 mg/mL) was prepared by appropriate dilution of the standard solution with ethanol. Two microliters of each solution were spotted on a TLC plate in triplicate. The chromatogram was developed (see details in section 3.3.3) and the plate was scanned and analyzed (see details in section 3.3.4). The calibration curve was constructed between peak areas and amounts of the compound of 0.11-0.872 µg/spot by least square linear regression.

3.3.2 Sample preparation

Prepare the sample extracts as described in section 3.4. 0% ethanolic extract (423.4 mg/10 mL), 20% ethanolic extract (152.6 mg/10 mL), 40% ethanolic extract (151.6 mg/10 mL), 60% ethanolic extract (94.2 mg/10 mL), 80% ethanolic extract (95.3 mg/10 mL), 100% ethanolic extract (51.2 mg/10 mL) were prepared and sonicated for 30 min in ultrasonic bath (Elma® transonic 890/H: Singen, Germany).

3.3.3 Chromatographic conditions

Technique	: One dimension, ascending
Adsorbent	: Silica gel 60 F254 (0.040-0.063 mm, Merck, Germany) precoated on aluminium sheet 20 x 10 cm
Layer thickness	: 0.2 mm
Applied volume	: 2 µL for sample and standard solutions
Distance	: 8 cm
Temperature	: Laboratory temperature (25-30 °C)
Solvent	: Ethyl acetate-Methanol-Ammonium hydroxide solution (50 : 35 : 15), saturated for 180 min
Detection	: Ultraviolet and Visible light (200 and 800 nm)
	: Dipping with 1% vanillin-sulfuric acid solution in tank and heating at 110 °C for 5 min

3.3.4 Densitometric parameter

The developed TLC chromatogram was scanned at 5 min after derivatization by using CAMAG TLC Scanner II operated by CAMAG CATS 1.4.9.210184 software in the reflectance-absorbance mode at the wavelength of 520 nm. The slit dimension of 12.00 x 0.90 mm, macro, and 20 mm/s scanning speed were employed for densitometric parameters.

3.3.5 Method validation

3.3.5.1 Determination of identity and peak purity

The standard (0.34 μg/spot) and 0% ethanol extract (84.68 μg/spot), 20% ethanol extract (30.52 μg/spot), 40% ethanol extract (30.32 μg/spot), 60% ethanol extract (18.84 μg/spot), 80% ethanol extract (19.06 μg/spot), 100% ethanol extract (10.24 μg/spot) were spotted on TLC plate. The chromatogram was developed (see detail in section 3.3.3) and the plate was scanned and analyzed (see detail in section 3.3.3). Spectra in the range of 450-700 nm of the peak of premnaodoroside A in the extracts were compared with standard for peak identity, and those of peak start, peak maximum and peak end positions of each peak were compared for peak purity.

3.3.5.2 Precision and accuracy

The repeatability (intra-day precision) and intermediate (inter-day precision) were performed to analyze the five replications of three concentrations standard 0.11, 0.44 and 0.87 μg/spot. The experiment was carried out over three days in order to determine intermediate precision. Precision was measured in percent relative standard deviation (%RSD), where %RSD = (Mean/SD) x100. Accuracy was described as percentage (detected concentration x 100/ loading concentration).

3.3.5.3 Recovery

The sample was dissolved in ethanol to the concentration of (15 mg/mL), it was spiked with three different concentrations of standard compound (0.22, 0.35 0.44 μg/spot). The spiked samples were analyzed in triplicate. AUC of spike samples was then determined and calculated for the amount of the standard by

comparing with the calibration curve. The recovery was expressed as percent recovery (detected amount x 100/added amount).

3.3.5.4 Limit of detection and limit of quantitation

LOD and LOQ were calculated based on the standard deviation (SD) of the y-intercept and the slope of (S) of the calibration curve. 3.3 x SD/S and 10 x SD/S were used for LOD and LOQ, respectively.

3.3.5.5 Robustness

Minor changes in chromatographic parameters, such as mobile phase composition, plate treatment time, delay between spotting and plate development, and plate heating time, could all affect the efficiency of the method. This study was interested in the effect of time varying from derivatization to TLC scanning by densitometer due to the spot color readily fading after derivatization. The standard was used in five concentrations (0.11-0.87 µg/spot) for robustness. To determine the AUC of each concentration, the time gap was fixed at 5 to 25 min after derivatization.

3.4 Optimizing solvent extraction of dried leaves of *P. serratifolia*

3.4.1 Plant materials

Three samples of the leaves of *P. serratifolia* were collected from Aungmyethazan (Sample 1), Chanayethazan (Sample 2) and Pathingyi (Sample 3), Mandalay, Myanmar, in December, 2018. Identification of the plant was done by comparing with references as mention in 3.1.1. The voucher specimen is deposited in the herbarium of the Department of Pharmacognosy, Silpakorn University, Thailand. The leaves were air-dried, ground into powder and passed through a 0.85 mm sieve.

3.4.2 Sample preparation

The dried leaves powdered (30 g) were extracted with 0% ethanol (distilled water), 20, 40, 60, 80 and 100% ethanol by heating at 100 °C for 2 h. Before heating, weigh of sample (30 g) and solvent (600 mL) were recorded. After place on water bath for 2 h, they were cooled, weigh again and adjust the volume to reach original weight. The extraction solutions were filtered. Extract solution 100 mL were taken from that solution, evaporated to dryness and calculated for %yield. The

remaining extract solution were also evaporated to dryness and combined with the first portion. The extraction of sample number 2 and 3 were done as the same procedure.

3.4.3 Determination of the content of premnaodoroside A

The determination of premnaodoroside A in samples were done by TLC method that developed with method as described in section 3.3.

3.4.4 Determination of total phenolic content

The extract (200 mg/10 mL) was diluted with ethanol to obtain working solution (10 mg/10 mL) and the determination method was described in section 3.1.6.2. The calibration curve was constructed using gallic acid (10-70 µg/mL) as standard.

3.4.5 Determination of antioxidant activity

The extract (200 mg/10 mL) was diluted with ethanol to obtain working solution (200 µg/mL) and the determination method was described in section 3.1.6.3. The calibration curve was constructed using ascorbic acid (2-16 µg/mL) as standard. Radical scavenging activity of the sample was calculated as ascorbic acid equivalent (%AAE).

3.4.6 Statistical analysis

All data were demonstrated as mean ± standard deviation, and analysis of variance was performed using the Excel. A statistically significant *p*-value was estimated to be less than 0.05.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Determination of post-harvest effects on quality of fresh, dried and fermented leaves of *P. serratifolia*

4.1.1 Identification of chemical constituents of fresh, dried and fermented leaf volatile oils

The volatile oils of fresh, dried, and fermented leaves of *P. serratifolia* were prepared by Clevenger's apparatus. The yields of the volatile oils of the fresh, dried, and fermented leaves were 0.008, 0.139 and 0.139%, respectively, calculated based on the fresh, dry and fermented weight of the plant material. They were clear yellowish color. Their chemical constituents were analyzed by GC-MS using DB-5 and Carbowax 20M columns which are suitable for determination of nonpolar and polar compound, respectively. The lists of identified compounds and compound categories in three types of leaf volatile oils are shown in Tables 8, 9.

Table 8 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using DB-5 column

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
alpha-Pinene	-	-	1015	0.7	-	-	939[47]
Amyl vinyl carbinol	1031	15.8	1030	6.3	1031	6.9	979[47]
Amyl ethyl ketone	-	-	-	-	1034	1.2	984[47]
beta-Myrcene	-	-	1037	8.6	1037	6.7	991[47]
Amyl ethyl carbinol	1049	6.4	-	-	1040	5.7	1002[48]
Unknown	-	-	-	-	1048	0.7	-
m-cymene	1059	0.2	-	-	-	-	1026[48]
Limonene	1069	0.3	1065	0.3	1066	0.4	1029[47]
Benzeneacetaldehyde	1079	1.5	-	-	1076	1.0	1042[47]
Unknown	-	-	-	-	1115	1.2	-
Linalool	1126	15.1	1122	6.3	1123	7.9	1097[47]
(*Z*)-beta-Terpineol	-	-	-	-	1125	0.6	1144[47]
Unknown	-	-	-	-	1144	0.4	-
p-Vinyl Anisole	1172	4.5	1172	8.9	1184	1.4	1160(48)

Table 8 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using DB-5 column (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
neo-Menthol	-	-	1190	0.6	-	-	1166[47]
Unknown	-	-	-	-	1190	1.1	-
Naphthalene	1204	0.6	-	-	-	-	1181[47]
Unknown	-	-	-	-	1205	0.5	-
alpha-Terpineol	-	-	-	-	1208	0.7	1189[47]
Unknown	-	-	1208	5.9	-	-	-
Salicylic acid methyl ester	1216	7.2	-	-	-	-	1193[49]
beta-Cyclocitral	1236	0.7	-	-	-	-	1218[49]
Nerol	1239	0.5	1237	0.3	-	-	1230[47]
5-(1′-1′-Dimethylethyl) bicycle[3,10] hexan-2-one	-	-	-	-	1256	1.7	-
p-Anisaldehyde	-	-	-	-	1260	1.9	1270[48]
Unknown	-	-	1270	1.0	-	-	-
alpha-Benzeneacetaldehyde ethylidene-	-	-	1282	0.1	1279	0.8	1279[48]
Salicylic acid ethyl ester	1282	1.1	-	-	-	-	1311[48]
Carbamic acid	-	-	1307	0.6	-	-	-
3,4,4a,5,6,8a-Hexahydro-2,5,5,8a-tetramethyl- (2 .alpha.,4a.alpha.,8a.alph)-2H-1-benzopyran	1307	0.5	-	-	-	-	1318[48]
p-Vinylgyaiacol	1323	1.1	1322	0.1	1322	0.6	1324[48]
Benzene,4-ethyl-1,2-dimethoxy-	-	-	-	-	1329	0.6	-
Unknown	-	-	-	-	1332	0.7	-
Acetanisole	-	-	1361	0.1	1362	0.8	1352[47]
Eugenol	1366	1.1	1365	0.7	1366	1.7	1359[47]
Unknown	1369	1.3	1368	0.6	-	-	-
3,4-Dimethoxystyrene	-	-	-	-	1371	0.6	1368[48]
Unknown	-	-	-	-	1384	1.4	-
(*E*)-beta-Damascenone	1392	1.3	1392	0.7	-	-	1385[47]
beta-Elemene	1400	0.3	1400	0.4	1400	0.6	1391[47]
Methyleugenol	-	-	1407	0.4	1407	0.9	1404[47]
Unknown	-	-	1413	0.4	-	-	-
Isocaryophyllene	-	-	-	-	1420	1.1	1423[48]

Table 8 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using DB-5 column (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
Unknown	-	-	1421	0.7	-	-	-
(*E*)-Caryophyllene	1436	6.6	1438	12.2	1437	12.7	1433[48]
Dihydro-beta-ionone	-	-	1447	0.4	-	-	1444[48]
Neryl acetone	-	-	1456	1.0	1456	0.6	1456[48]
(*E*)-beta-Farnesene	1459	0.3	1460	0.5	1460	0.6	1457[47]
alpha-Humulene	1467	1.6	-	-	-	-	1463[48]
1,1,4,8-Tetramethyl-(*Z, Z, Z*)-4,7,10-cycloundecatriene-	-	-	1468	2.4	1468	3.0	-
(*E*)-beta-Ionone	1491	0.9	1493	2.9	1493	1.7	1489[47]
Unknown	-	-	-	-	1499	1.1	-
alpha-Farnesene	1509	0.2	-	-	-	-	1506[48]
beta-Bisabolene	1514	3.0	1515	3.8	1515	4.8	1512[48]
Unknown	-	-	1529	0.8	1526	1.4	-
beta-Cadinene	-	-	-	-	1533	1.1	1539[47]
Unknown	-	-	1534	1.4	-	-	-
Nerolidol	1566	0.8	1566	1.3	1567	1.3	1563[47]
Caryophyllene oxide	1597	1.8	1598	2.6	1598	2.9	1583[47]
Humulene oxide	-	-	-	-	1628	0.4	1602[49]
3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde	1629	0.3	-	-	-	-	-
beta-Tumerone	-	-	1674	1.0	-	-	1669[47]
Unknown	-	-	-	-	1678	2.4	-
Unknown	-	-	-	-	1681	2.7	-
Acorenone B	-	-	-	-	1708	4.4	1698[47]
Myristatic acid ethyl ester	1794	0.3	-	-	-	-	1794[48]
Isopropyl myristate	1826	0.3	-	-	-	-	1824[48]
Hexahydrofarnesyl acetone	1848	0.3	1844	3.6	1849	1.5	1844[48]
Isobutyl phthalate	1875	0.2	-	-	1875	0.3	1874[48]
Palmitoleic acid methyl ester	-	-	1912	0.9	1911	0.3	1912[48]
Farnesyl acetone C	1925	0.4	1926	2.0	1925	0.7	1920[49]
Isophytol	-	-	-	-	1951	0.1	1948[47]
(*Z*)-11-Hexadecenoic acid	-	-	1958	1.8	-	-	1953[48]

Table 8 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using DB-5 column (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
Unknown	1964	1.3	1963	1.0	1963	0.8	-
Palmitic acid	-	-	1978	4.9	-	-	1968[49]
(*E*)-11-Hexadecenoic acid ethyl ester	1980	0.8	-	-	1979	0.4	1974[48]
Palmitic acid ethyl ester	1990	1.2	-	-	-	-	1990[48]
Geranyl linalool isomer1	-	-	2038	0.2	-	-	-
Linoleic acid methyl ester	-	-	2098	0.4	-	-	2097[48]
Linolenic acid methyl ester	-	-	2106	1.2	2105	0.3	2108[48]
Phytol	2128	12.5	2124	7.3	2120	3.4	2122[48]
(*E*)-9-Octadecenoic acid	-	-	2146	0.4	-	-	2133[49]
Linoleic acid ethyl ester	2167	0.6	-	-	2165	0.1	2164[48]
Linolenic acid ethyl ester	2175	2.3	2174	0.4	2173	0.6	2170[48]
Geranyl linalool isomer2	-	-	2180	0.4	-	-	-
15-Methyl-heptadecanoic acid ethyl ester	2194	0.4	-	-	-	-	-
n-Docosane	-	-	-	-	2199	0.1	2200[50]
n-Tricosane	2299	0.2	2299	0.1	2299	0.1	2300[50]
n-Tetracosane	2399	0.04	2399	0.1	2399	0.1	2400[50]
n-Pentacosane	2499	0.2	2499	0.1	2499	0.2	2500[50]
Bis-(2-ethylhexyl) phthalate	2554	0.3	2552	0.1	-	-	2556[50]
n-Hexacosane	2599	0.1	2598	0.1	2598	0.2	2600[50]
n-Heptacosane	2700	0.4	2699	0.4	2698	0.3	2700[50]
Squalene	2826	0.2	-	-	2825	0.1	2847[50]
n-Nonacosane	2897	3.0	2898	0.4	2898	0.4	2900[50]

Table 9 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using Carbowax 20M column

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
(*E*)-2-Hexenal	1271	0.6	1278	0.7	1279	0.3	1216[48]
(*E*)-beta-Ocimene	-	-	1282	0.2	1283	0.2	1250[48]
Amyl ethyl ketone	1299	7.8	1297	0.2	1298	0.7	1264[48]
p-Cymene	-	-	1307	0.2	1300	0.2	1270[49]
alpha-Terpinolen	-	-	1308	0.1	-	-	1282[49]
Amyl vinyl ketone	1325	0.3	1326	1.4	1326	0.2	1301[49]
6-Methyl-5-hepten-2-one	-	-	1355	0.1	1355	0.1	1345[48]
Amyl carbinol	1379	5.4	-	-	-	-	1371[48]
(Z)-3-Hexen-1-ol	1401	2.2	-	-	-	-	1373[49]
Amyl ethyl carbinol	1423	8.1	1414	2.1	1416	6.2	1392[49]
3,5,5-Trimethyl-3-cyclohexen-1-one	-	-	1425	0.1	-	-	1420[48]
Amyl vinyl carbinol	1474	32.6	1460	13.8	1461	11.5	1444[49]
(*E*)-Linalool oxide	1476	0.2	-	-	-	-	1454[49]
(*Z*)-Linalool oxide	1491	0.2	1486	0.2	1486	0.3	1474[49]
(*E, E*)-2,4-Heptadienal	1502	0.1	1498	0.2	1499	0.2	1491[49]
3,4,4a,5,6,8a-Hexahydro-2,5,5,8a-tetramethyl-(2alpha.,4a.alpha.,8a.alpha) 2H-1-benzopyran	1527	0.2	-	-	-	-	-
Camphor	-	-	1531	0.1	-	-	1515[49]
(*E*)-2-Nonenal	-	-	1538	0.2	1540	0.2	1536[49]
Linalool	1554	11.1	1549	7.5	1549	5.3	1543[49]
1-Methyl-4-(1-methylethyl)-*trans*-2-cyclohexen-1-ol	-	-	-	-	1577	0.3	1571[48]
beta-Elemene	-	-	1578	0.2	-	-	1574[48]
(*E, Z*)-2,6-Nonadienal	-	-	1583	0.4	1585	0.6	1582[49]
(*E*)-Caryophyllene	1594	3.1	1596	14.6	1597	8.4	1599[49]
Unknown	-	-	-	-	1603	0.6	-
2-Acetylthiazole	1632	0.1	-	-	-	-	1634[48]
Acetophenone	1645	2.0	-	-	-	-	1648[48]
(*E*)-beta-Farenesene	-	-	-		1651	0.3	1664[49]
p-Vinyl anisole	1659	2.4	1660	13.1	1667	41.1	1670[48]
Unknown	1667	0.2	-	-	1671	0.6	-

Table 9 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using Carbowax 20M column (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
4-Oxoisophorone	-	-	1694	0.2	1696	0.2	1690[48]
(*E, E*)-2,4 Nonadienal	-	-	-	-	1698	0.1	1696[49]
beta-Bisabolene	1706	1.5	1707	6.0	1706	2.7	1699[48]
Naphthalene	1717	0.2	1717	0.1	-	-	1709[48]
alpha-Farnesene	-	-	1722	0.4	1722	0.2	1744[49]
Unknown	1722	0.2	-	-	-	-	-
delta-Cadinene	-	-	1737	0.2	-	-	1756[49]
Salicylic acid methyl ester	1755	3.9	1751	0.5	1752	0.3	1768[49]
Salicylic acid ethyl ester	1788	0.4	-	-	-	-	1798[48]
Nerol	-	-	1802	0.3	1801	0.1	1795[49]
Unknown	1802	0.4	-	-	-	-	-
(*E*)-beta-Damascenone	1814	0.5	1813	0.9	1813	0.2	1821[49]
Dihydro-beta-Ionone	-	-	1827	0.3	-	-	1825[48]
Unknown	1830	0.2	-	-	-	-	-
Neryl acetone	1843	0.1	1844	1.2	1844	0.4	1835[48]
Geraniol	1852	0.7	1851	0.7	1851	0.4	1851[48]
alpha-Ionone	-	-	1853	0.6	-	-	1857[48]
4-Ethyl-1,2-dimethoxy-benzene	-	-	-	-	1870	0.4	1875[48]
Unknown	1877	0.6	1876	0.7	1877	1.1	-
(*E ,E,E*)-2,4,6-Nona-trienal	-	-	1889	0.2	1889	0.4	-
Unknown	1913	0.2	-	-	-	-	-
Unknown	1923	0.2	-	-	1923	0.5	-
(E)-beta-Ionone	1943	0.2	1945	2.2	1944	0.4	1936[49]
Unknown	1978	0.2	-	-	-	-	-
Caryophyllene oxide	1991	0.7	1992	2.5	1991	1.0	1986[49]
Methyleugenol	-	-	2002	0.7	2002	0.5	2006[49]
4-(2,2,6-Trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-3-buten-2-one	2002	0.1	-	-	-	-	2002[48]
p-Anisaldehyde	2020	0.4	2020	1.7	2021	1.5	2011[49]
Nerolidol	2029	0.4	2029	1.4	2029	0.4	2036[49]
Myristatic acid ethyl ester	2037	0.1	-	-	-	-	2045[48]

Table 9 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using Carbowax 20M column (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
Zingiberenol	-	-	-	-	2114	0.1	2109[48]
Hexahydro farnesyl acetone	2121	0.1	2124	4.1	2122	0.8	2129[48]
Unknown	-	-	-	-	2100	0.2	-
Acetanisole	2144	0.1	2144	0.4	2145	0.7	2148[48]
Eugenol	2151	0.6	2151	1.5	2151	1.0	2150[48]
Longiborneol	-	-	2160	0.4	2159	0.2	2157[49]
2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene	2175	0.6	2174	1.1	2174	0.4	-
p-Vinylguaiacol	2184	0.6	-	-	2184	0.4	2180[48]
alpha-Cadinol	2191	0.1	2191	0.3	-	-	2191[48]
Unknown	-	-	2202	1.7	2202	0.5	-
Acorenone B	-	-	-	-	2217	2.4	-
Palmitoleic acid methyl ester	2226	0.1	2227	0.8	-	-	2225[48]
Palmitic acid ethyl ester	2245	0.4	-	-	2243	0.2	2235[48]
beta-Tumerone	-	-	2246	0.5	-	-	-
9-Hexadecenoic ethyl ester	2268	0.3	-	-	2266	0.2	2269[48]
Unknown	-	-	-	-	2281	1.7	-
Isophytol	-	-	2284	0.3	-	-	2293[49]
2,4-Bis(1,1-dimethylethyl)-phenol	2291	0.04	2291	0.6	2291	0.2	2316[48]
3,7,11-Trimethyl-(*E*, *E*)-2,6,10-dodecatrien-1-ol	-	-	-	-	2357	0.1	2366[49]
Farnesyl acetone C	2362	0.1	2363	1.2	2362	0.4	2377[49]
Ketole (1H-Indole)	-	-	2425	0.2	2425	0.2	2420[48]
Octadecanoic acid ethyl ester	2453	0.1	-	-	-	-	2450[48]
Olealic acid ethyl ester	2468	0.2	-	-	-	-	2461[48]
Linoleic acid methyl ester	-	-	2478	0.3	-	-	2480[48]

Table 9 Chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves analysed by GC-MS using Carbowax 20M column. (continued)

Compound	Fresh leaves		Dried leaves		Fermented leaves		
	RI	%Relative amount	RI	%Relative amount	RI	%Relative amount	Literature RI
Linoleic acid ethyl ester	2515	0.2	-	-	-	-	2519[48]
Linolenic acid methyl ester	-	-	2547	1.1	2546	0.2	2550[48]
Linolenic acid ethyl ester	2584	0.7	-	-	2582	0.4	2594[48]
Phytol	2625	7.7	2620	9.0	2617	2.0	2613[49]
Dibutyl phthalate	-	-	2704	0.5	-	-	2705[48]
3-(4-methoxyphenyl),2-propenoic acid ethyl ester	2641	0.5	-	-	-	-	-

Tables 8 and 9 displayed that a total of 77 compounds were detected in fresh leaf volatile oils and identified compounds were categorized into hydrocarbons (26.1% in DB-5 and 57.1% in Carbowax 20M), terpenoids (42.8% in DB-5 and 26.0% in Carbowax 20M) and phenolics (15.0% in DB-5 and 10.9% in Carbowax 20M) compound categories. Some fatty acids, apocarotenoids, and miscellaneous compounds were also detected (Table 10). The major compounds were amyl vinyl carbinol (15.8% in DB-5 and 32.6% in Carbowax 20M), linalool (15.1% in DB-5 and 11.1% in Carbowax 20M), phytol (12.5% in DB-5, and 7.7% in Carbowax 20M), salicylic acid methyl ester (7.2% in DB-5 and 3.9% in Carbowax 20M) and (*E*)-caryophyllene (6.6% in DB-5 and 3.1% in Carbowax 20M).

Dried leaf volatile oils contained a total of 82 compounds and the identified compounds were categorized into hydrocarbons (7.6% in DB-5 and 19.2% in Carbowax 20M), terpenoids (47.6% in DB-5 and 44.8% in Carbowax 20M) and phenolics (10.2% in DB-5 and 18.4% in Carbowax 20M) compound categories. The major compounds were (*E*)-caryophyllene (12.2% in DB-5 and 14.6% in Carbowax 20M), linalool (6.3% in DB-5 and 7.5% in Carbowax 20M), phytol (7.3% in DB-5 and 9.0% in Carbowax 20M), amyl vinyl carbinol (6.3% in DB-5 and 13.8% in

Carbowax 20M) and caryophyllene oxide (2.6% in DB-5 and 2.5% in Carbowax 20M).

Fermented leaf volatile oils contained a total of 92 compounds, and the identified compounds were categorized into hydrocarbons (16.7% in DB-5 and 20.3% in Carbowax 20M), terpenoids (51.3% in DB-5 and 24.5% in Carbowax 20M) and phenolics (7.9% in DB-5 and 46.2% in Carbowax 20M) compound categories. The major compounds were *p*-vinyl anisole (1.4% in DB-5 and 41.1% in Carbowax 20M), (*E*)-caryophyllene (12.7% in DB-5 and 8.4% in Carbowax 20M), linalool (7.9% in DB-5 and 5.3% in Carbowax 20M), phytol (3.4% in DB-5 and 2.0% in Carbowax 20M), and amyl vinyl carbinol (6.9% in DB-5 and 11.5% in Carbowax 20M).

Table 10 Category of chemical constituents of the volatile oils of the fresh, dried and fermented *P. serratifolia* leaves

Compound category	**Compound sub-category**	**% Relative amount**					
		Fresh leaves		**Dried leaves**		**Fermented leaves**	
		DB-5	**Carbowax 20M**	**DB-5**	**Carbowax 20M**	**DB-5**	**Carbowax 20M**
Terpenoids	Monoterpene hydrocarbons	0.5	-	9.7	0.4	7.1	0.4
	Monoterpene alcohols	15.6	11.8	7.1	8.5	9.2	6.1
	Monoterpene ketones	-	-	-	0.1	-	-
	Miscellaneous oxygenated monoterpenes	-	0.4	-	0.2	-	0.3
	Sesquiterpene hydrocarbons	12.1	4.7	19.3	21.4	23.8	11.5
	Sesquiterpenes alcohols	-	0.1	-	0.7	-	0.3
	Sesqutiterpene ketones	-	-	1.0	0.5	4.4	2.4
	Miscellaneous oxygenated sesquiterpenes	1.8	0.7	2.6	2.5	3.2	1.0
	Diterpenoid hydrocarbons	-	0.6	-	1.1	-	0.4
	Diterpenoid alcohols	12.5	7.7	7.9	9.3	3.5	2.0
	Triterpenoids	0.2	-	-	-	0.1	-
Phenolics		15.0	10.9	10.2	18.4	7.9	46.2
Fatty acids	Fatty acids	-	-	7.2	-	-	-
	Fatty acids esters	5.7	2.1	2.9	2.2	1.7	1.0
Hydrocarbons	Hydrocarbon alcohols	22.2	48.2	6.3	15.9	12.5	17.8
	Hydrocarbon aldehydes	-	0.7	-	1. 7	-	1.7
	Hydrocarbon ketones	-	8.1	-	1.6	2.9	0.9
	Long chain hydrocarbons	3.9	-	1.2	-	1.2	-
Apocarotenoids	Apocarotenoids	4.9	1.7	11.9	12.1	5.7	2.8
Miscellaneous		2.8	0.2	0.9	0.9	2.7	0.2
Unknowns		2.6	2.1	11.8	2.3	13.9	5.2

In terms of the relative amounts of different compound categories, fresh leaf volatile oils contained the highest amounts of hydrocarbons (26.1% in DB-5 and 57.1% in Carbowax 20M), while dried leaf volatile oils contained the least (7.6% in DB-5 and 19.2% in Carbowax 20M). However, dried leaf volatile oils contained the highest amounts of terpenoids (47.6% in DB-5, 44.7% in Carbowax 20M), but fermented leaf volatile oils contained the lowest amounts of apocarotenoids and fatty acids in both columns. On the other hand, the highest amounts of phenolic acids (46.2% in Carbowax 20M) occurred in fermented leaf volatile oils and the lowest amount in fresh leaf volatile oils (10.9% in Carbowax 20M). The total % of relative amount of compound categories are shown in Figure 13.

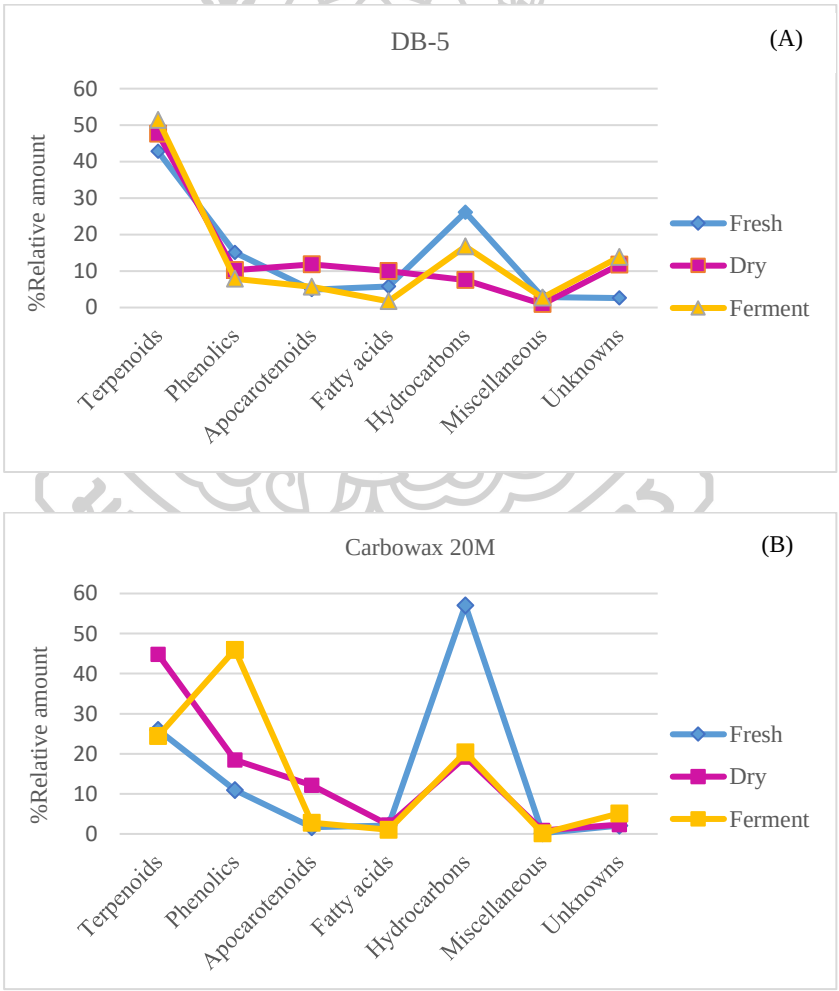


Figure 13 Total %relative amount of compound categories in fresh, dried and fermented leaf volatile oils in DB-5 (A) and Carbowax 20M (B) columns

Furthermore, the results mentioned above indicated that the major compounds of fresh, dried, and fermented leaf volatile oils were amyl vinyl carbinol, linalool, (*E*) caryophyllene, and *p*-vinyl anisole. In the literature, they reported that four compounds were found in *P. serratifolia* fresh leaf volatile oils (column type was not mentioned in the literature): eugenol (47.9%), eugenyl acetate (9.1%), and *cis*-2-oxabicyclo, 4.4.0-decane (12.4%) (2). In our study, fresh leaf volatile oils showed eugenol (1.2%) and eugenyl acetate, *cis*-2-oxabicyclo, 4.4.0-decane were not detected in our study. Another study reported that air-dried leaf volatile oils of *P. intergrifolia* (synonym of *P. serratifolia*) by VF-5 capillary column contained main components of phytol (27.3%), α-humulene (14.2%), spathulenol (12.1%), 1-octen-3-ol (8.2%), eugenol (6.7%), phenylethyl alcohol (5.8%) and caryophyllene oxide (2.6%) (36). However, our study revealed that the dried leaf volatile oils contained phytol (7.3%) and a small amount of eugenol (0.7%), but not much different in the amount of 1-octen-3-ol (6.3%) and caryophyllene oxide (2.6%). This might be due to the fact that the composition of volatile oils can vary depending on the extraction methods and columns used, and a variety of extraction methods and columns should be evaluated in order to comprehensively identify the composition of an volatile oils (52). In addition, quantitative and qualitative volatile oils could be varied by different locations and climates of the plant's origin (53).

4.1.2 Comparison of fresh, dry, and fermented leaf volatile oils by using DB-5 and Carbowax 20M columns

A total number of 95 compounds and 90 compounds were identified by DB-5 and Carbowax 20M columns, respectively (Tables 8 and 9). A number of compounds detected in the fresh, dried, and fermented leaf volatile oils were 47, 52, and 62 for DB-5 column; and 54, 54, and 56 for Carbowax 20M column. Consider on AUC, the results demonstrated that the amounts of fresh, dried, and fermented leaf volatile oils detected in DB-5, both DB-5 and Carbowax 20M and Carbowax 20M were 3.1, 78.0, 18.8%; 15.5, 74.0, 10.5%; and 9.9, 81.3, 8.8%, respectively (Figure 14).

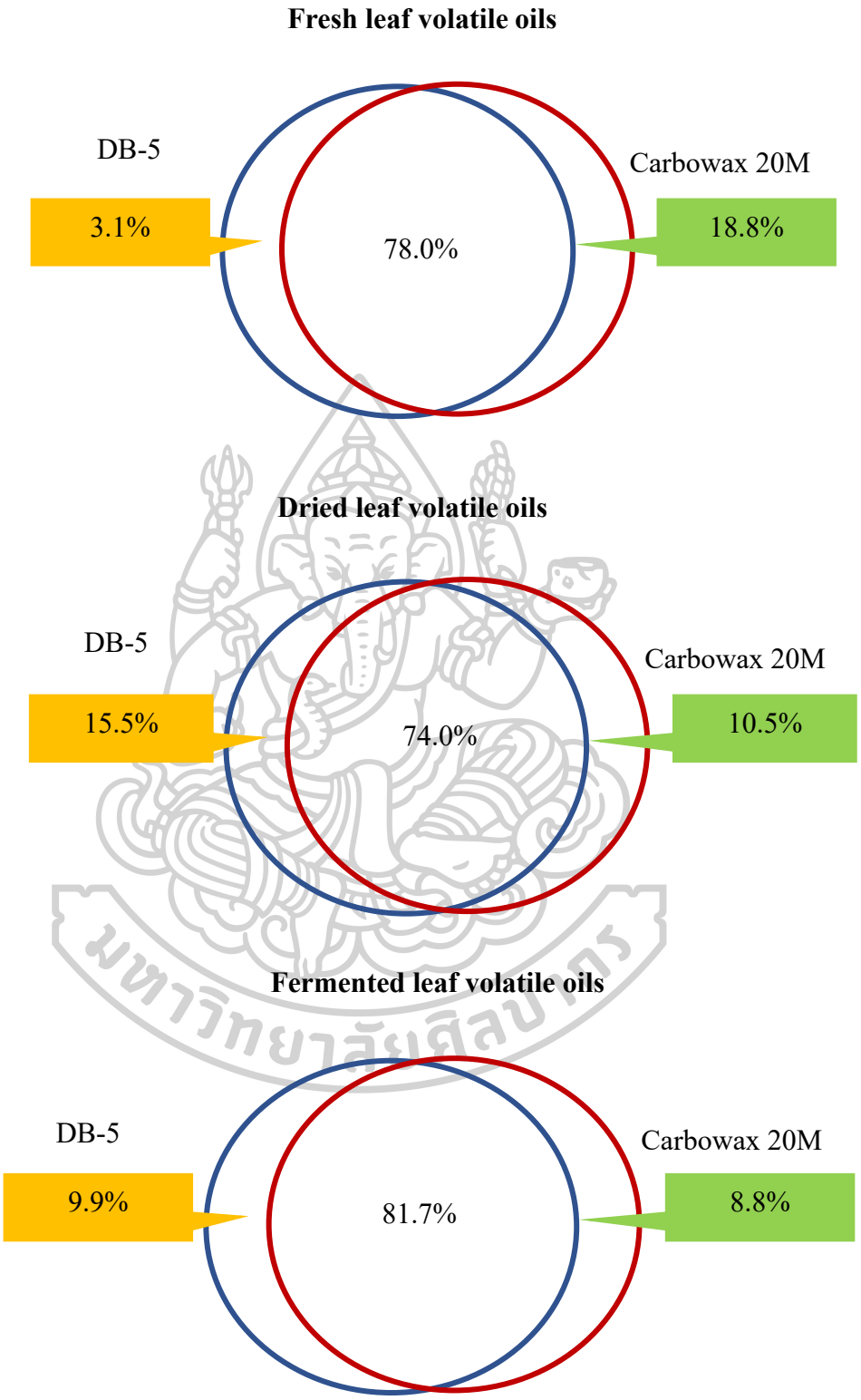


Figure 14 Compound distribution of fresh, dry and fermented leaf volatile oils in DB-5, and Carbowax 20M Columns

According to the results, the varied percentage amounts of nonpolar, moderate polar, and polar compounds in fresh, dried, and fermented volatile oils were detected in DB-5, and Carbowax 20M Columns. Data from DB-5 column implied that some of the polar and moderate polar compounds in the fresh leaves were decreased and some of non-polar compounds were increased by the drying or fermentation processes.

Moreover, it was interesting to note that the relative amounts of some compounds in fresh leaf volatile oils were significantly changed after drying and fermentation. The compounds that decreased in fresh leaf volatile oils were: linalool (11.1-15.1% to 6.3-7.5% and 5.3-7.9%), amyl ethyl carbinol (6.4-8.1% to 0-2.1% and 5.7-6.2%), amyl vinyl carbinol (15.8-32.6% to 6.4-13.8% and 6.9-11.5%), salicylic acid methyl ester (3.9-7.22% to 0-0.5% and 0-0.3%), palmitic acid ethyl ester (0.4-1.17% to 0-0.2%) and linoleic acid ethyl ester (0.2-0.6% to 0-0.1%). After fermentation, phytol was dramatically decreased from 7.7-12.5% to 2.0-3.4%. After drying, (*E*)-caryophyllene, beta-bisabolene and caryophyllene oxide were increased from (3.2-6.6%, 1.5-3.1 and 0.7-1.8%, respectively), to (12.2-14.6%, 3.8-6.0% and 2.5-2.6%), respectively and they were also increased to (8.4-12.7%, 2.7-4.8% and 1.0-2.9%), respectively, after fermentation. From data of Carbowax 20M, *p*-vinyl anisole was dramatically increased to 2.4-41.1% after fermentation (Figure 15).

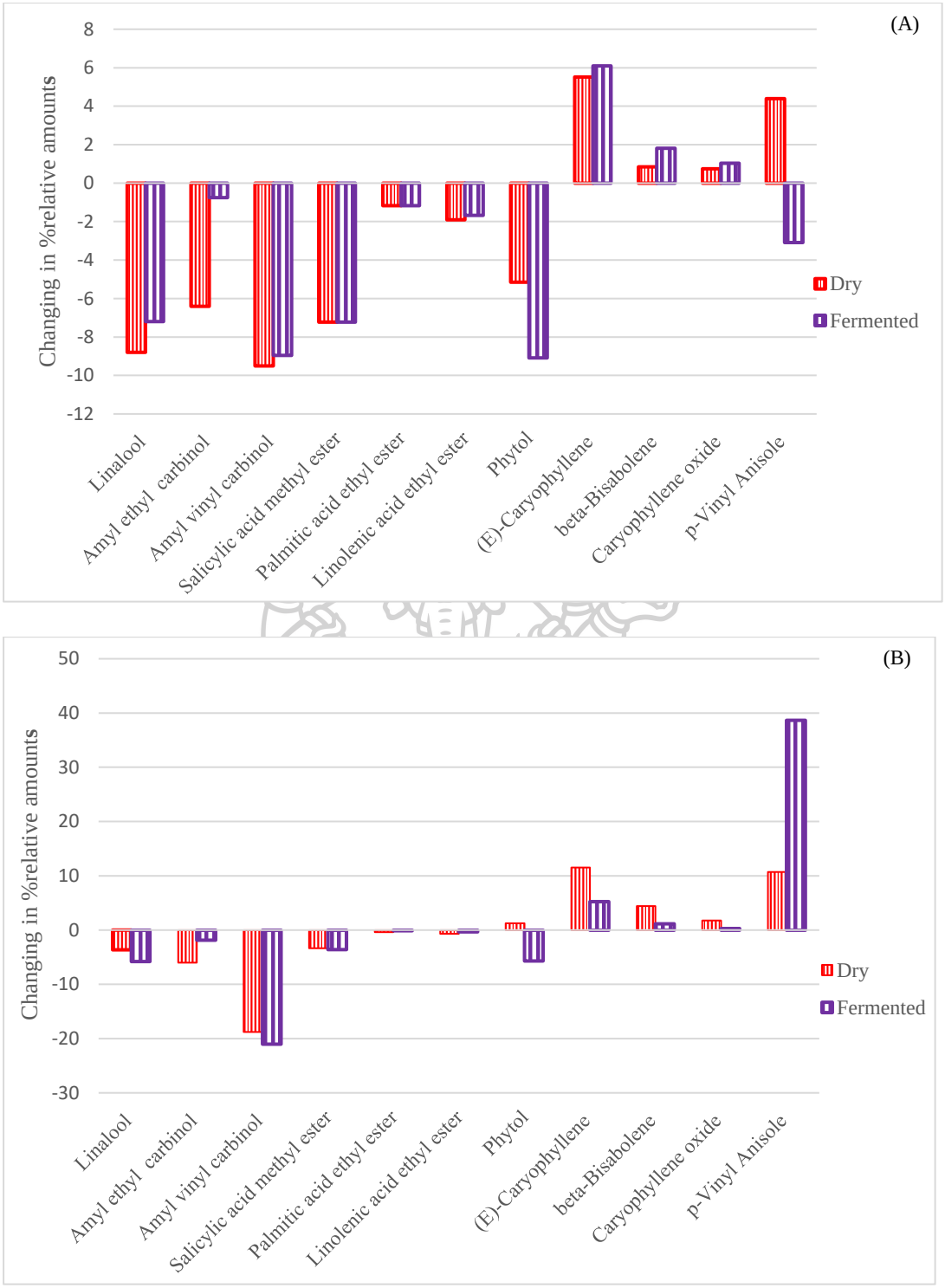


Figure 15 Changing in %relative amounts of some constituents of the volatile oils of the leaves of *P. serratifolia* after drying and fermentation, analysed by DB-5 (A) and Carbowax 20M (B) columns

It was significant to mention that some compounds were disappeared and some new compounds were detected in dried and fermented leaf volatile oils (Figure 16). beta-Myrcene was a new detected compound in dried and fermented leaf volatile oils. It possesses the same chemical skeleton of acyclic monoterpenoids as linalool and might be the dehydrated product of linalool. There has been reported that linalool could be conversed to beta-myrcene by microbial biotransformation using linalool dehydratase isomerase (54). Palmitic acid, 4.9% in DB-5 was detected only in dried leaf volatile oils. This might be due to hydrolysis of palmitic acid ethyl ester to palmitic acid. It was observed that some fatty acid esters might be hydrolysed and cause the formation of fatty acids due to endogenous or microbial enzymes (55). In addition, a disappearing compound was found from fresh leaf volatile oils. It was noteworthy that alpha-humulene disappeared after drying and fermentation, whereas increased relative amounts of (*E*)-caryophyllene and caryophyllene oxide were observed. That reason might be alpha-humulene and (*E*)-caryophyllene possess a similar sesquiterpenoid skeleton that contain humulyl cation as the same biosynthetic precursor (56). Some unproven factors might affect their biosynthesis expression, and (*E*)-caryophyllene could be further oxidized to caryophyllene oxide (57) (Figure 16).

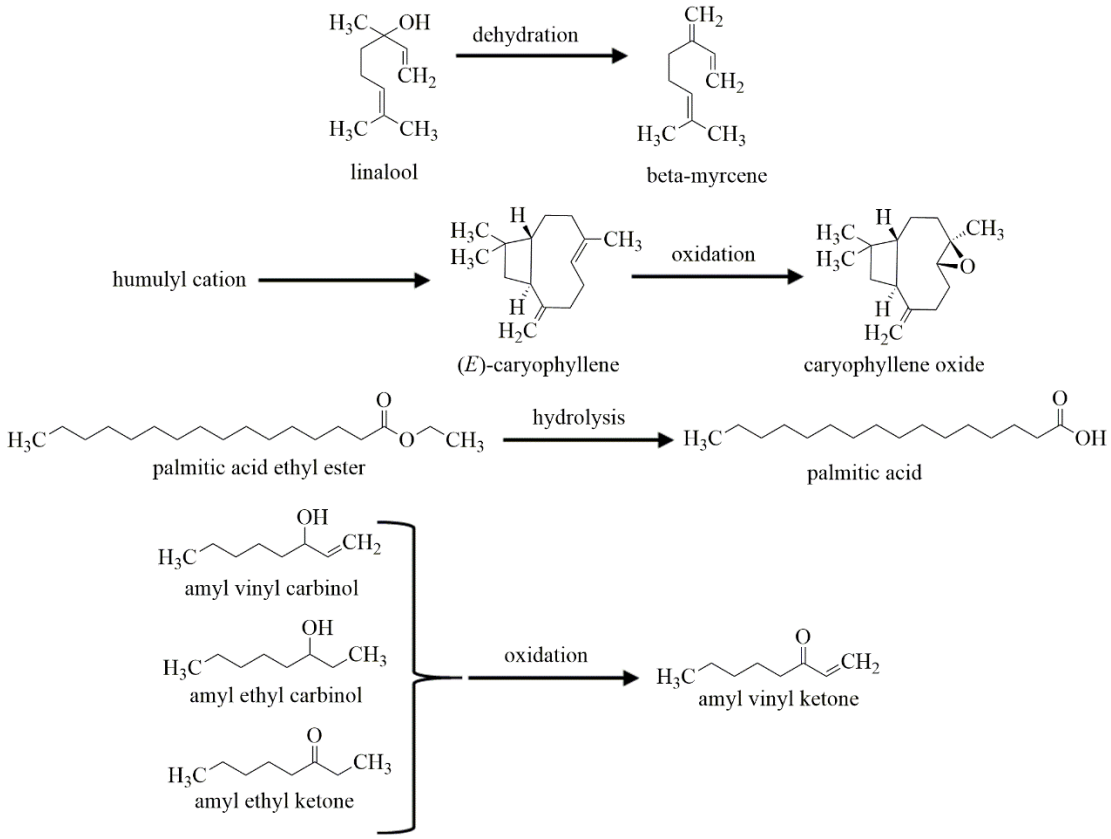


Figure 16 Proposed transformation mechanisms of some volatile compounds of the leaves of *P. serratifolia* after drying and fermentation

Apocarotenoids were detected in dried and fermented leaf volatile oils. Apocarotenoids are the known degradative products derived from carotenoids by enzymatic and non-enzymatic oxidation. Many plant materials, such as *Morus alba*, *M. nigra* (58), black tea, and oolong tea (59), showed an increase in their amounts after drying or fermentation. The result of the present study was also in the same manner. After drying and fermentation, an increase in their proportion was observed. The relative amounts of apocarotenoids in the fresh leaf volatile oils were (4.9% in DB-5 and 1.7% in Carbowax 20M). After drying and fermentation, the amount of apocarotenoids was increased, and it was much higher in the dried leaf volatile oils (11.9% in DB-5 and 12.1% in Carbowax 20M) than in the fermented leaf volatile oils (5.7% in DB-5 and 2.8% in Carbowax 20M). Additionally, three significantly increasing compounds, beta-ionone, hexahydrofarnesyl acetone and farnesyl acetone C, were detected in dried and fermented leaf volatile oils. Degradation mechanisms of

carotenoids to form these three compounds have already been reported (58) (60) According to the results, the post-harvesting effects of drying and fermentation could change the chemical composition and relative amounts of volatile oils of leaves of *P. serratifolia* and could induce the transformation reactions of some compounds by dehydration, hydrolysis, and oxidation.

4.1.3 Determination of total phenolic content and antioxidant activity

4.1.3.1 Determination of total phenolic content

The total phenolic content of fresh, dried, and fermented leaf extracts was determined, and the results are shown in (Table 11). The three extracts contained phenolic content ranging from 3.6 to 6.1%GAE. Fresh leaf extract had the highest total phenolic content (6.1% GAE), followed by dried and fermented leaf extracts (3.9 and 3.6%GAE, respectively) (Figure 17). The results were agreement with those of Dewi et al. (2019) (61), who found that fresh leaves of *Rubus fraxinifolius* had higher phenolic and flavonoid content than air-dried and oven dried (61). However, Garcìa, L.M., discovered that freeze and oven drying of *Urtica dioica* leaves had higher phenolic content and antioxidant activity than fresh leaf extracts (62). All things considered, the variability of the results could be due to changes in the relative quantities of the antioxidant chemical constituents in the samples (63), as well as the processing method utilized on the samples, which affect phenolic content and antioxidant activity (64).

4.1.3.2 Determination of antioxidant activity

The fresh, dried, and fermented leaf extracts were determined for antioxidant activity using DPPH assays. The reaction with the antioxidant compounds changed the color of DPPH from deep violet to light purple, light red, yellow and the degree of discoloration reveals the scavenging potency of the extract. In the present study, the three extracts at various concentrations (1-100 µg/mL) were determined for their antioxidant activity. The result showed that the percentage of antioxidant activity increased with the concentration of extracts (Table 12). At a concentration of 100 µg/mL, the antioxidant activity of the fresh, dried and fermented leaf extracts was 67.24%, 23.68% and 18.70% respectively (Figure 18), that were weaker than that of ascorbic acid 96.77%. It was indicated that fresh leaf extracts had

higher antioxidant activity than dried and fermented leaf extracts. That might be due to the volatile oils of the fresh leaves of *P. serratifolia* contained the highest relative amount of linalool (11.1-15.1%), phytol (7.7-12.5%), and methyl salicylate (3.9-7.2%), which could be attributed to the electron donating ability of more bioactive compounds in the fresh leaf extract. Previous research found that linalool has antimicrobial, anti-inflammatory, anticancer, and antioxidant properties (65). (*E*)-Caryophyllene, palmitic acid, phytol, apocarotenoids and amyl vinyl carbinol had anticancer, and antioxidant activities (66-70). *p*-Vinyl anisole and methyl salicylate were observed to have antioxidant activity (71, 72). Furthermore, several phenolic compounds have been reported from the leaves of this plant, for example, *p*-anisaldehyde, methyl salicylate, *p*-vinyl anisole, acacetin, acacetin-7-*O*-glycoside, quercetin, kaempferol, and luteolin (22, 33). Phenolic compounds are usually related to antioxidative activity and have a therapeutic effect on inflammation, oxidative stress and other metabolic diseases. The leaves of this plant have also been reported to have antioxidant activity (25, 73-76). Therefore, in this experiment, total phenolic content and antioxidant activity might be considered as the quality markers of the extracts of this plant.

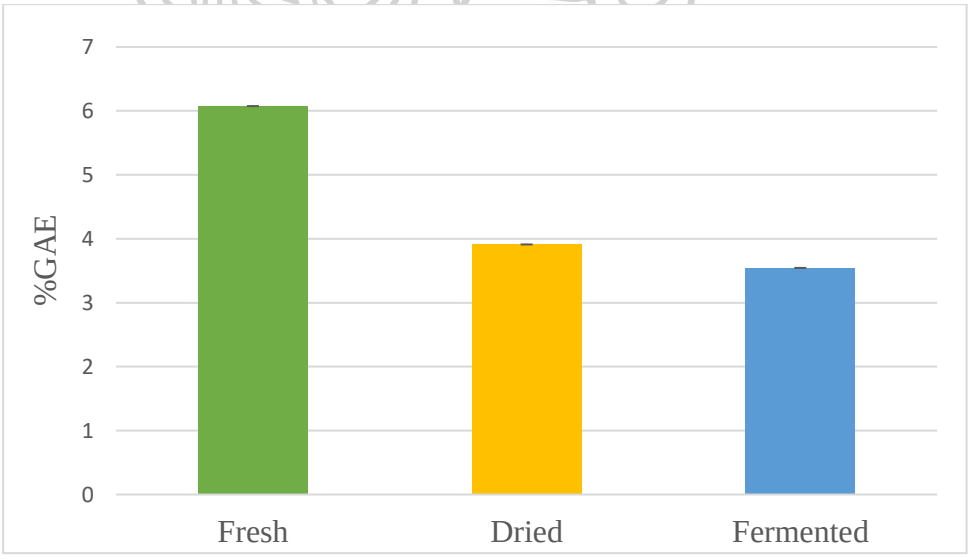


Figure 17 %GAE of 80% ethanol of fresh, dried and fermented leaf (n = 3)

Table 11 %GAE of fresh, dried, fermented leaf extracts (n = 3)

Sample extracts	% GAE
Fresh	6.08 ± 0.005
Dried	3.92 ± 0.009
Fermented	3.55 ± 0.006

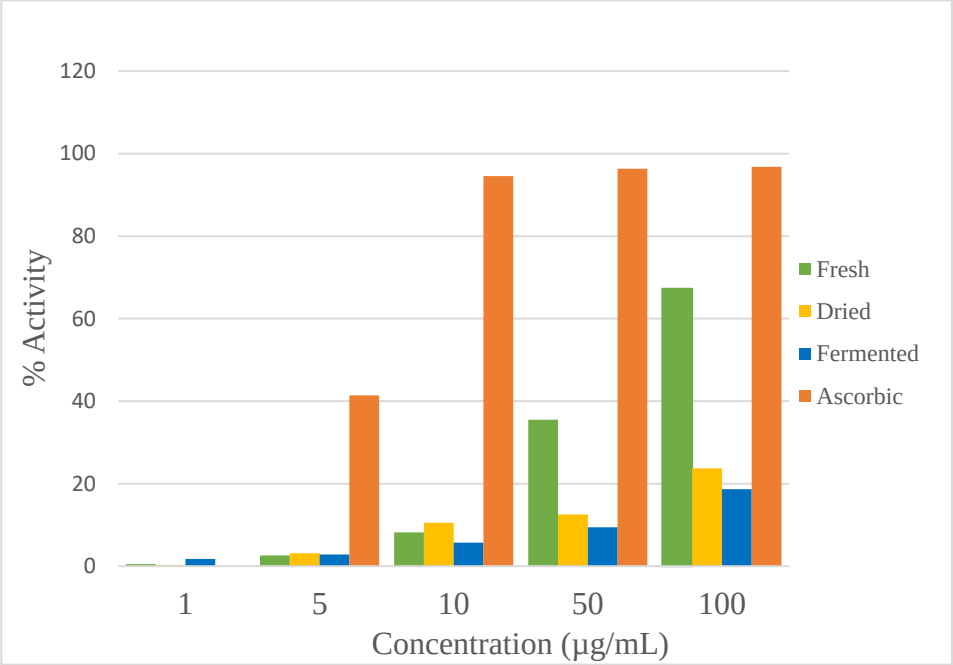


Figure 18 %Radical scavenging activity of fresh, dried, fermented leaf extracts and ascorbic acid at different concentrations (n = 3)

Table 12 %Radical scavenging activity of fresh, dried, fermented leaf extracts and ascorbic acid at different concentrations (n = 3)

Concentration (µg/mL)	% of activity			
	Fresh leaves	Dried leaves	Fermented leaves	Ascorbic acid
1	0.51	0.31	1.79	0.002
5	2.63	3.12	2.81	41.4
10	8.23	10.54	5.71	94.53
50	35.50	12.51	9.47	96.37
100	67.24	23.68	18.70	96.77
	Concentration (µg/mL)			
IC_{50}	73.43	283.65	318.44	5.45

4.2 Isolation and identification of chemical constituents of the leaves of *P. serratifolia*

4.2.1 Phytochemical screening tests

The hexane, dichloromethane and methanol extracts were investigated for the presence of phenolics, saponins, alkaloids, steroids, flavonoids, and iridoids. The results of their phytochemical screening were exhibited in Table 13.

Table 13 Phytochemical screening of hexane, dichloromethane and methanol extracts of *P. serratifolia* leaves

Test	Hexane extract	Dichloromethane extract	Methanol extract
Phenolic ($FeCl_3$) test	- Yellow color	- Yellow color	+++ Dark brown color
Saponin test	- No foam	- No foam	+ Foam
Alkaloids test (Drangendroff's test)	- Yellow color	- Yellow color	+++ Reddish brown precipitate
Steroids test	+++ Green color	+++ Green color	+++ Green color
Molisch's test	-	-	+++
Iridoid test (Trim-Hill) test	- Yellow color	+ Green color	+++ Dark green

4.2.2 Identification of premnaodoroside A

Premnaodoroside A (54.1 mg) was isolated from methanolic dried extract (18 g) of the leaves of *P. serratifolia*. Premnaodoroside A was yellowish white amorphous powder. The compound gave positive result to Trim-Hill reagent, it was possible to be iridoids. The molecular formula, $C_{42}H_{66}O_{20}$, was determined by the ESIMS *m/z* 888.5 $[M-H]^-$ (Figure 32). The UV spectrum showed a maximum absorption band at 225 nm. Comparison of its ^{1}H-NMR and ^{13}C-NMR data with the previous literature (Tables 14, 15), this compound was identified as premnaodoroside A (Figure 19). From the ^{1}H-NMR and ^{13}C-NMR data, (Figures 33 and 37), there were twenty methine, twelve methylene, six quaternary and four methyl groups. The downfield shifts of proton at δ 7.41, 7.39 ppm (Figure 36) and the downfield shifts of carbon at δ 152.07, 152.07 ppm (Figure 41) were the characteristic of iridoid skeleton with an olefinic functional group at position 3/4. The doublet of doublet signal of a

methine proton at δ 2.22 was the characteristic of H-9 (Figure 34). A downfield carbon signals at δ 169.19, 169.25 ppm were the signals of two ester carboxyl carbon (C-11) (Figure 41). The downfield shifts of CH_3-10 at δ 24.82, and C-8 at δ 80.69, 80.72 suggested that hydroxyl groups were attached at position 8 (Figures 38, 40). The appearance of a doublet proton signal at δ 5.45 was the oxygenated methine group at position 1 (H-1) (Figure 36). Their carbon signals presented in the region of 95.61 ppm (Figure 41). The signal of anomeric protons was observed at δ 4.68 which indicated the presence of glucose units (Figure 36). The anomeric proton at δ 4.68 (H-1') had a coupling constant of 7.8 Hz, indicating that the glucose moiety was linked to the iridoid aglycone as β linkage. The downfield signal of anomeric carbon at δ 99.98 (C-1') (Figure 41) revealed that glucose was connected to the aglycone with an *O*-linkage. The ^{13}C-NMR spectrum displayed ten signals which were two oxygenate methylene carbon signals at δ 63.65 (C-1″), δ 70.09 (C-8″) (Figure 40), two methine carbon signals at δ 31.15 (C-3″), δ 34.06 (C-7″) (Figure 39), two methyl carbon signals at δ 20.03 (C-9″), δ 17.56 (C-10″) (Figure 38) and four methylene carbon signals at δ 36.91 (C-2″), δ 38.24 (C-4″), δ 25.34 (C-5″), δ 34.85 (C-6″) (Figure 39), indicating that it was an acyclic monoterpene derivative. All NMR data were compared with those in reference (8, 73) and confirmed that the compound was identified as premnaodoroside A.

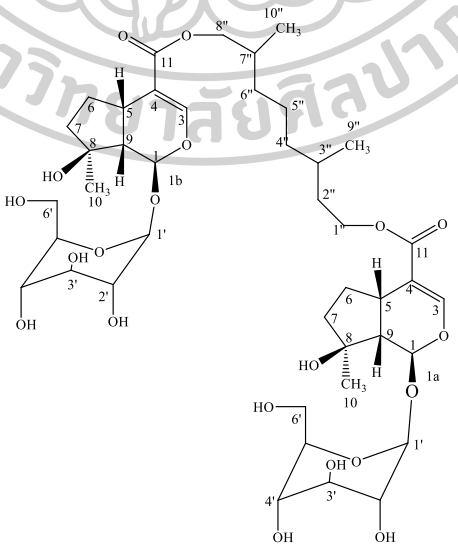


Figure 19 Structure of premnaodoroside A

Table 14 The ^{1}H-NMR spectral data of premnaodoroside A (300 Hz, MeOD)

Position	Premnaodoroside A	Reference (77)	Reference (8)
Iridoid aglycone			
1	5.45 (*d*, 4.5)	5.45 (2H, *d*, 4.3)	5.47 (H, *d*, 4.5)
2	-	-	-
3	7.39 (H, *s*)	7.39 (H, *s*)	7.41 (H, *d*, 1.0)
	7.41 (H, *s*)	7.40 (H, *s*)	
4	-	-	-
5	3.2~3.3 (proton overlapped by the solvent)	3.2~3.3 (proton overlapped by the solvent signal)	3.20 (H, *m*)
6	1.6-1.7 (*m*)	1.57 (H, *m*)	1.62 (2H, *m*)
		1.72 (5H, *m*)	1.74 (2H, *m*)
7	1.78 (*m*)	1.81 (H, *m*)	1.77 (2H, *m*)
8	-	-	-
9	2.22 (2H, *dd*, 9.3, 4.2)	2.22 (2H, *dd*, 4.3, 9.2)	2.24 (2H, *dd*,1.0, 6.0)
10	1.32 (3H, *s*)	1.32 (3H, *s*)	1.34 (3H, *s*)

Table 14 The ^{1}H-NMR spectral data of premnaodoroside A (300 Hz, MeOD) (continued)

Position	Premnaodoroside A	Reference (77)	Reference (8)
Monoterpene Portion			
1″	4.17(*t*, 5.9)	-	4.18 (*t*, 6.5)
2″	1.59 (*m*)	-	1.50 (*m*)
	1.74 (*m*)		1.73 (*m*)
3″	1.4-1.5 (*m*)	-	1.45 (*m*)
4″	1.19~1.37 (proton overlapped by the water)	1.19 (2H, *m*)	1.22 (*m*)
			1.34 (*m*)
5″	1.19~1.37 (proton overlapped by the water)	-	1.33(*m*)
6″	1.19~1.37 (proton overlapped by the water)	-	1.22 (*m*)
			1.40 (*m*)
7″	1.82 (*m*)	-	1.85 (*m*)
8″	3.92 (*m*)	3.92(H, *dd*, 6.70)	3.92 (*m*)
			4.00 (*m*)
9″	0.91 (3H, *d*, 6.3)	0.92 (3H, *d*, 6.70)	0.92 (*d*, 6.5)
10″	0.96 (3H, *d*, 7.2)	0.96 (3H, *d*, 6.7)	0.98 (*d*, 6.5)

Table 14 The ^{1}H-NMR spectral data of premnaodoroside A (300 Hz, MeOD) (continued)

Position	Premnaodoroside A	Reference (77)	Reference (8)
Glucose			
1′	4.68 (H, *d*, 7.8)	4.68 (H, *d*, 7.9)	4.69 (H, *d*, 7.5)
2′	3.2~3.3 (proton overlapped by the solvent)	-	3.23 (*dd*, 7.5, 9.0)
3′	3.2~3.3 (proton overlapped by the solvent)	-	3.23 (*m*)
4′	3.2~3.3 (proton overlapped by the solvent)	-	3.26 (*t*, 9.0)
5′	3.37 (*t*)	-	3.36 (*t*, 9.0)
6′	3.64 (2H, *dd*, 6.3, 11.9)	3.64 (2H, *dd*, 6.1, 6.6)	3.66 (*dd*, 6.0, 12.0) 3.92 (*d*, 12.0)

Table 15 The ^{13}C-NMR spectral data of premnaodoroside A (75 Hz, MeOD)

Position	Premnaodoroside A		Reference (77)		Reference (8)	
Iridoid aglycone	1a	1b	1a	1b	1a	1b
1	95.61	95.61	95.43	95.47	95.43	95.47
3	152.07	152.07	151.91	151.94	151.91	151.90
4	113.71	113.75	113.59	113.62	113.57	113.60
5	32.23	32.23	32.07	32.11	32.06	31.10
6	31.09	31.06	30.93	31.01	30.91	31.00
7	40.80	40.84	40.68	40.71	40.65	40.95
8	80.69	80.72	80.55	80.58	80.54	80.57
9	52.42	52.42	52.30	52.29	52.59	52.59
10	24.82	24.82	24.70	24.67	24.67	24.67
11	169.19	169.25	169.03	169.07	169.04	169.09
Monoterpene Portion						
1″	63.65		63.5		63.50	
2″	36.91		36.8		36.77	
3″	31.15		31.0		30.95	
4″	38.24		38.2		38.15	
5″	25.34		25.3		25.22	
6″	34.85		34.8		34.71	
7″	34.06		34.0		33.96	

Table 15 The ^{13}C-NMR spectral data of premnaodoroside A (75 Hz, MeOD) (continued)

Position	Premnaodoroside A	Reference (77)	Reference (8)
Monoterpene Portion			
8″	70.09	69.9	69.89
9″	20.03	20.0	19.93
10″	17.56	17.5	17.49
Glucose			
1′a, 1′b	99.98	99.86	99.84
2′a, 2′b	74.89	74.76	74.75
3′a, 3′b	78.53	78.40	78.37
4′a, 4′b	71.87	71.75	71.73
5′a, 5′b	78.13	78.02	77.99
6′a, 6′b	63.10	62.98	62.96

4.2.3 Identification of D-glucose and D-fructose

The mixture of D-glucose and D-fructose (330 mg) was isolated from methanolic extract (18 g) of the leaves of *P. serratifolia*. It was yellowish amorphous powder. The compound gave positive result to Molisch's test, it was possible to be sugar. The molecular formula of monosaccharide, $C_6H_{12}O_6$, was suggested by the molecular ion at *m/z* 203 $[M+Na]^+$ in ESIMS (Figure 43). The TLC chromatogram of the compound was compared with standard D-glucose and D-fructose. TLC system was developed using the mixture of chloroform-methanol-water (30 : 15 : 3) as the mobile phase. After development, the TLC plate was sprayed with vanillin-sulfuric reagent and heated on the plate. The TLC chromatogram showed a pale green spot at the same R_f 0.32 as the standard glucose and a dark green spot at the same R_f 0.33 as the standard fructose (Figure 20). Therefore, this compound was possibly the mixture of glucose and fructose. Hydroxy functional groups of glucose and fructose were confirmed by IR bands at 3360.8 and 1055.8 cm^{-1} of O-H stretching and C-O stretching, respectively (Figure 42). The ^{13}C-NMR and DEPT-135 spectra showed a

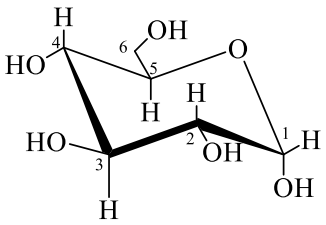
total of thirty carbons (Figures 46 and 47). The ^{1}H-NMR spectrum of the mixture of D-glucose and D-fructose was showed in Figure 44 and the two doublet proton signals at δ 5.23 and 4.64 ppm, which corresponded to the anomeric protons (C-1) of the glucose was shown in Figure 45. Their coupling constants were 3.8 and 7.9 Hz, that indicated the α and β forms of D-glucose, respectively. The overall spectrum of ^{1}H-NMR was complicated and could not be assigned, but ^{13}C-NMR spectra produced the distinctive peaks for the identification of the compound (Figure 48). The chemical shifts of carbons were compared with the previous literatures (Tables 16, 17). The ^{13}C-NMR spectra showed the chemical shift of the carbon signals at δ 92.04 and 95.86 ppm which revealed the presence of the anomeric carbons of α-D-glucose and β-D-glucose, respectively. Moreover, the chemical shift of carbons signals at δ 98.02, 104.39 and 101.45 ppm (Figure 51) revealed the presence of the anomeric carbons (C-2) of β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose, respectively. The carbon signals at δ 71.43, 74.39 (C-2), 72.35, 75.70 (C-3), 71.38, 71.38 (C-4), 69.59, 69.59 (C-5) and 60.53, 60.70 (C-6) were observed for α-D-glucose and β-D-glucose, respectively. The carbon resonance of chemical shifts of the β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose were at δ 63.83, 62.85, 62.59 for C-1, δ 67.49, 81.91, 75.89 for C-3, δ 69.54, 75.31, 74.08 for C-4, δ 69.15, 81.24, 80.62 for C-5 and δ 63.31, 62.85, 62.33 for C-6, respectively (Figures 49 and 50). All chemical shifts were similar to those of the literatures (78, 79). The carbon signals of β-D-fructopyranose and β-D-fructofuranose were more intense than those of α-D-fructofuranose and ^{13}C of glucose were not much less intense than fructose. Barclay T, et.al., 2012 (80) and Maple SR, et.al., 1987 (81) reported determination of the D-fructose and D-glucose tautomeric compositions in D_2O of ^{13}C-NMR. They described that fructose had totally five tautomers. β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose were the major tautomers, whereas α-D-frucotpyranose and the linear keto form of fructose were the minor tautomers. The equilibrium tautomeric composition at 30°C for β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose was 64.8, 25.25 and 6.5% (80). Likewise, the major tautomers of glucose at 27 °C were α-D-glucopyranose, β-D-glucopyranose, whereas the minor tautomers were α-D-glucofuranose, and β-D-glucofuranose. The tautomeric composition of α-D-glucofuranose, β-D-glucofuranose were very low

(0.14-0.69%) (81). In the present study, the major tautomers of D-fructose and D-glucose were consistent with the literature. Therefore, the compound was identified as a mixture of D-glucose and D-fructose.

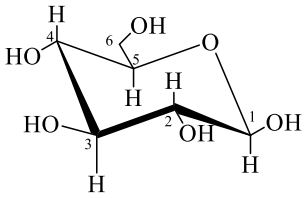
Figure 20 TLC chromatogram of the mixture of D-glucose and D-fructose

Chloroform-methanol-water (30 : 15 : 3)) as mobile phase, detection under UV 366 nm, and detection under visible under light after spraying with 1% vanillin-sulfuric acid

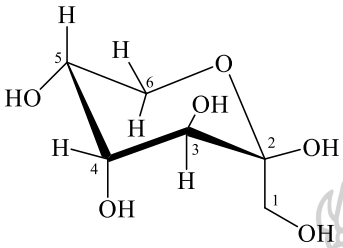
1 = isolated mixture of D-glucose and D-fructose in methanol (5 µL), 2 = standard glucose (5 µL), 3 = standard fructose (5 µL)



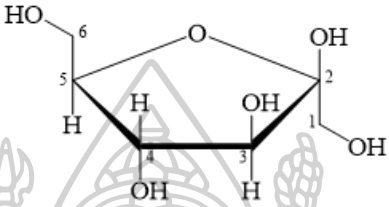
α-D-glucopyranose



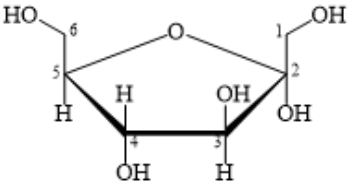
β-D-glucopyranose



β-D-fructopyranose



β-D-fructofuranose



α-D-fructofuranose

Figure 21 Structures of D-glucose and D-fructose.

Table 16 ^{13}C-NMR spectral data of D-glucose (75 Hz, D_2O)

Position ^{13}C	D-glucose		D-glucose (78)	
	α	β	α	β
1	92.04	95.86	91.4	95.9
2	71.43	74.39	71.8	74.1
3	72.35	75.70	72.4	75.8
4	71.38	71.38	71.2	71.2
5	69.59	69.59	69.8	69.8
6	60.53*	60.70*	60.3	60.3

*May be interchanged.

Table 17 ^{13}C-NMR spectral data of D-fructose (75 Hz, D_2O)

Position ^{13}C	D-fructose			D-fructose (79)		
	β-Pyranose	α-Furanose	β-Furanose	β-Pyranose	α-Furanose	β-Furanose
1	63.83	62.85	62.59	64.91	63.94	63.71
2	98.02	104.39	101.45	98.89	105.23	102.31
3	67.49	81.91	75.89	68.57	82.96	76.37
4	69.54	75.31	74.08	70.68	77.02	75.41
5	69.15	81.24	80.62	70.16	82.16	81.51
6	63.31	62.85	62.33	64.24	62.08	63.34

4.2.4 Purity of premnaodoroside A

Premnaodoroside A was the main iridoid glycoside, then it was selected as a standard for further experiment. Its purity was determined by the densitometric method. The mobile phase systems of ethyl acetate-methanol-ammonium hydroxide solution (50 : 35 : 15) was used to give good separation and provide good peak shape resolution. The purity of premnaodoroside A was found to be 87. 2 ± 11.17%. The results are shown in Figure 22 and Table 18.

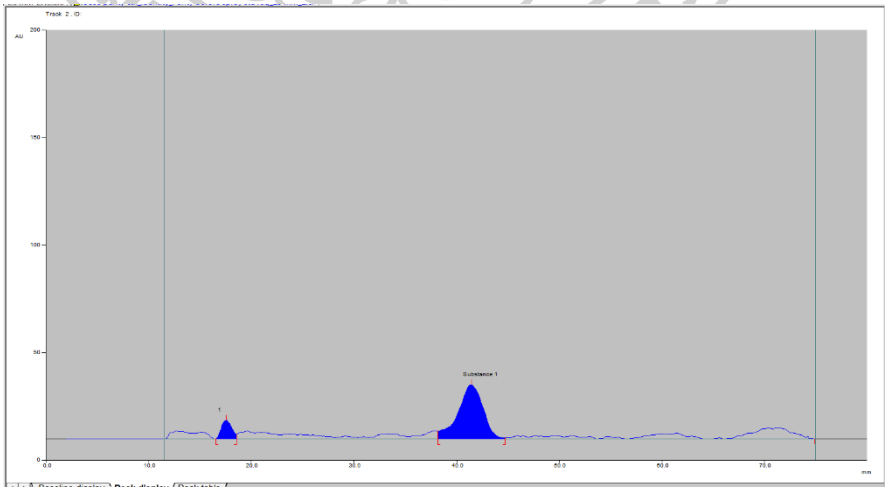


Figure 22 TLC-densitogram of the standard premnaodoroside A

Table 18 Purity of the standard premnaodoroside A (n = 3)

Compounds	AUC	Total AUC	Purity (%)
Premnaodoroside A	839.8	964.5	87.1
Other compounds	124.7		

4.3 Development and validation of TLC-densitometric method

4.3.1 Calibration curve

The calibration curve was constructed between concentration range (0.11-0.87 µg/spot) and area under the curve (AUC) of premnaodoroside A. The calibration curve was shown in (Figure 23). The calibration lines were represented by linear equation: y =5993.7x+234.07, y = 2139.9x+128.29 and y = 3912.7x+174.66, where y is the AUC and x is the concentration (µg/spot). For these equations the correlation coefficient R^2, were 0.9921, 0.9908, and 0.9836, which demonstrated a good linearity (R^2 > 0.99) within the concentration range of 0.11-0.87 µg/spot.

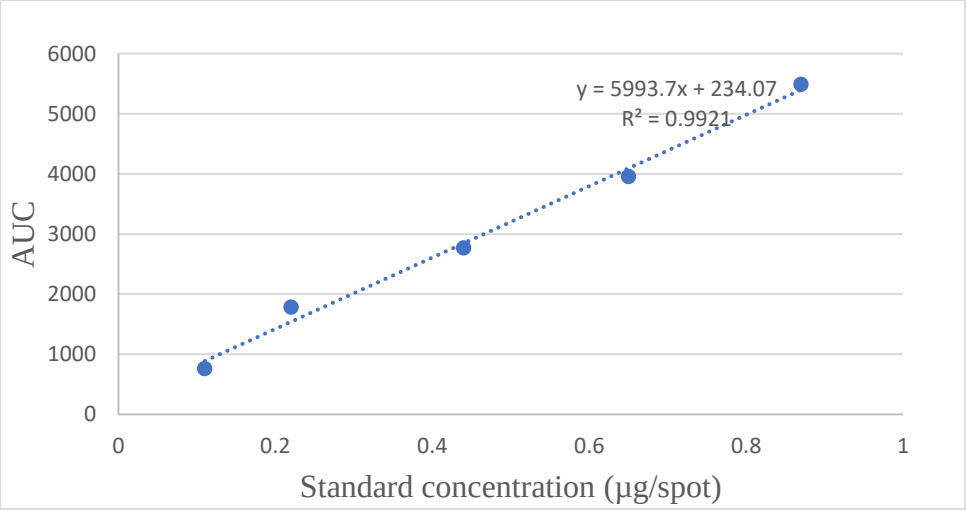
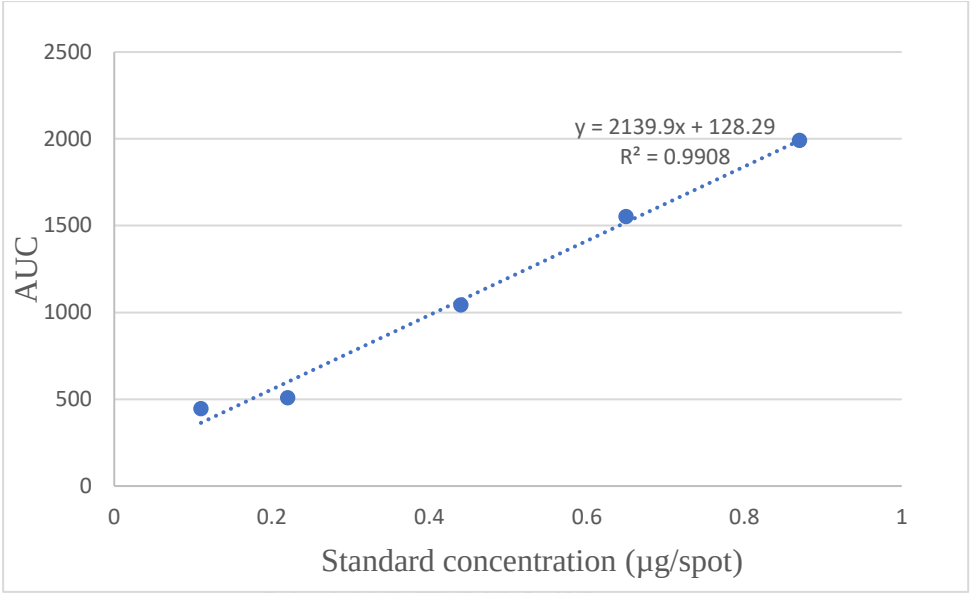


Figure 23 Calibration curve of standard premnaodoroside A



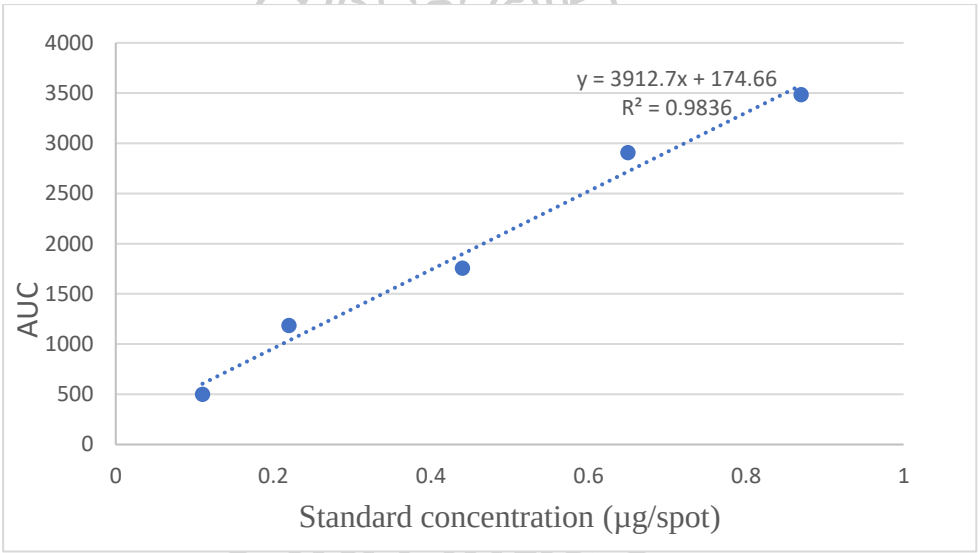


Figure 23 Calibration curve of standard premnaodoroside A (continued)

4.3.2 Peak identity and peak purity

The specificity of the developed method was tested by quantifying peak identity and peak purity. Peak identity between peaks of premnaodoroside A in the extracts and standard was determined by comparing the spectra in the range of 450-700 nm (Figure 52) and they gave a good correlation (Table 19). The spectrum of premnaodoroside A of standard with those of in extracts, which showed quite good correlation that more than 0.96. This suggested that peak identity of all extract was quite good. Peak purity of premnaodoroside A in the extracts was determined by

comparing the spectra in the range of 450-700 nm of peak start, peak maximum and peak end positions of the band, (Figure 53). The correlation of respected spectra of the peak start (S) and peak maximum (M), (r (S, M)) and the correlation of respected spectra of the peak maximum (M) and peak end (E) (r (M, E)) were calculated by CAMAG CATS software. The correlation r (S, M) and r (M, E) of standard and all extracts were more than 0.99. There was no interfering peak observed in densitogram of the standard and the extracts, which indicated the specificity of the result (Figure 24 and Table 19).

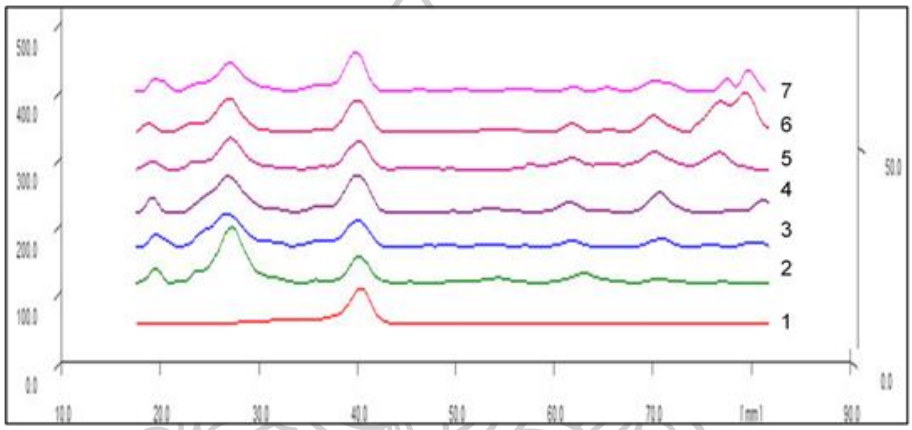


Figure 24 Densitogram of standard premnaodoroside A and the ethanolic extracts.
1 = standard (0.34 µg/spot), 2 = 0% ethanol extract (84.68 µg/spot), 3 = 20% ethanol extract (30.52 µg/spot), 4 = 40% ethanol extract (30.32 µg/spot), 5 = 60% ethanol extract (18.84 µg/spot), 6 = 80% ethanol extract (19.06 µg/spot), 7 = 100% ethanol extract (10.24 µg/spot)

Table 19 Peak identity and peak purity

Standard and Sample	Identity	Purity	
	r (S, A)*	r (S, M)**	r (M, E)***
Premnaodoroside A	0.0000	0.9997	0.9984
Sample 0%	0.9618	0.9995	0.9989
Sample 20%	0.9734	0.9991	0.9971
Sample 40%	0.9980	0.9992	0.9959
Sample 60%	0.9988	0.9961	0.9942
Sample 80%	0.9989	0.9983	0.9965
Sample 100%	0.9998	0.9997	0.9985

*r (S, A) = Correlation of standard analysis track

**r (S, M) = Correlation of peak start to peak maximum

***r (M, E) = Correlation of peak maximum to peak end

4.3.3 Precision and accuracy

The repeatability (intra-day) and intermediate precision (inter-day) were performed by determining the amount of premnaodoroside A at three concentrations of 0.11, 0.44 and 0.87 µg/spot in 5 replications. The results of %RSD of intra-day and inter-day precision were lower than 3%. The accuracy of the method was in the range of 99-100% The results are shown in Table 20.

Table 20 Precision and accuracy

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Loading (µg/spot)	Day	Amount detected (µg/spot) (n=5)	Repeatability (n=5)	Intermediate precision	%Accuracy
0.11	1	0.11± 0.0008	0.77		100.07
	2	0.11 ± 0.0001	0.13	0.09	99.99
	3	0.11 ± 0.0005	0.47		99.86
0.44	1	0.44 ± 0.002	0.04		100.04
	2	0.44 ± 0.002	0.41	0.28	99.72
	3	0.44 ± 0.009	2.16		100.27
0.87	1	0.87 ± 0.003	0.41		99.92
	2	0.87 ± 0.007	0.83	0.11	100.18
	3	0.87 ± 0.009	1.11		100.04

4.3.4 Recovery

The average recovery of three concentrations of spiked standard compound (0.22, 0.35, and 0.44 µg/spot, n=3) was 97.08-101.16%, indicating the good accuracy of the method. The results are shown in Table 21.

Table 21 Recovery

Amount added (µg/spot)	Amount detected (µg/spot) (n =3)	Recovery (%)	Average recovery (%)
0.22	0.22 ± 0.01	101.16 ± 5.45	
0.35	0.34 ± 0.01	97.32 ± 3.78	98.52 ± 2.29
0.44	0.43 ± 0.01	97.08 ± 3.16	

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

LOD and LOQ were calculated based on the standard deviation (SD) of the Y-intercept and the slope of (S) of the calibration curve. 3.3 x SD/S and 10 x SD/S were used for calculating LOD and LOQ, respectively. LOD and LOQ were 0.04 and 0.13 µg/spot, respectively (Table 22).

Table 22 LOD and LOQ data

Parameter	Mean	SD
Slope	4015.433	
Intercept	179.01	
R^2	0.9915	0.001
Limit of detection (LOD) (µg/spot)	0.04	
Limit of quantitation (LOQ) (µg/spot)	0.13	

Calibration curve	slope	intercept
1	5993.7	234.07
2	2139.9	128.29
3	3912.7	174.66
Mean	4015.43	179.01
SD	1928.95	53.02

LOD = 3.3xSD of intercept/mean of slope

LOQ = 10xSD of intercept/mean of slope

4.3.6 Robustness

The time effected the intensity of color after derivatization of compounds on TLC plate. The effect of time before scanning TLC plate by densitometer was investigated for robustness. Robustness testing was performed at five concentrations (0.11-0.87 µg/spot). The result indicated that peak area decreased with time after derivatization from 5 to 25 min. Therefore, TLC scanning started after derivatization for 5 min and continued to measure every 5 min interval till 25 min for all concentrations. The results are shown in Table 23. From data implied that the robustness of this method depended on detection time of chromatogram after derivatization. Then the fixed measuring time after derivatization was important for

accuracy and precision of premnaodoroside A quantitation, and it was fixed at 5 minutes for all analyses.

Table 23 Robustness of the method (peak area after derivatization)

Standard concentration (µg/spot)	Area of peak measure at time after derivatization (min)					Mean	SD	% RSD
	5	10	15	20	25			
0.87	4479.2	3821.0	3447.9	3278.9	3117.8	3628.9	542.3	14.9
0.65	4385.7	3807.8	3523.6	3365.4	3247.4	3665.9	453.9	12.4
0.44	2236.1	1977.9	1822.3	1822.3	1676.5	1891.3	223.1	11.8
0.22	1254.9	1100.8	1035.8	1035.8	953.3	1066.5	118.9	11.2
0.11	538.6	501.4	473.7	473.7	455.3	485.3	35.0	7.2

The TLC-densitometric method has been generally used for the quantitation of iridoids in several previous publications (6, 82, 83). It used less chemicals, low cost, short time analysis and ecofriendly (6). In this study, the developed TLC-densitometric method was validated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Humans: Validation of Analytical Procedure Guideline (ICH guideline) (46). After validation in compliance with ICH guidelines, the method was found to be reliable and suitable for the quantitation of premnaodoroside A. However, the color of the compound on TLC faded with time after derivatization, and it should be much concerned.

4.4 Optimizing solvent extraction of dried leaves of *P. serratifolia*

4.4.1 Yield of the extracts

The leaves of *P. serratifolia* were extracted using ethanol in the concentrations of 0, 20, 40, 60, 80, and 100%. Three samples from different locations were used to confirm the result. Table 24 showed the yields of the extracts. The lowest %yield of all samples was 100% ethanol, while the highest %yield varied

among the samples. The 80% ethanol gave the highest yield for sample 1, but it was the 40% ethanol for the other two samples.

Table 24 Yield of the ethanolic extracts

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Solvent (%Ethanol)	% Yield		
	Sample 1	Sample 2	Sample 3
0	38.36	38.96	31.57
20	40.53	44.89	34.96
40	41.74	45.96	36.37
60	42.35	41.65	34.16
80	47.53	38.54	27.74
100	18.37	19.57	13.57

4.4.2 Determination of the contents of premnaodoroside A

The quantitative determination of premnaodoroside A in three ethanolic extracts was performed by the TLC-densitometric method. The results pattern of the three samples were in the same manner as shown in Figure 25. Different percentages of ethanol gave a significant difference in the concentration of this compound ($p < 0.05$). Its content increased with the percentage of ethanol. The 0% ethanol extract had the lowest concentration (0.53-0.89%), and the highest concentration was detected in 100% ethanol extract (3.23-5.25%) (Table 25). The sample from Chanayethazan (Sample 2) contained the highest amount of premnaodoroside A in all percentage of ethanolic extracts, while that of Patheingyi (Sample 3) contained the lowest.

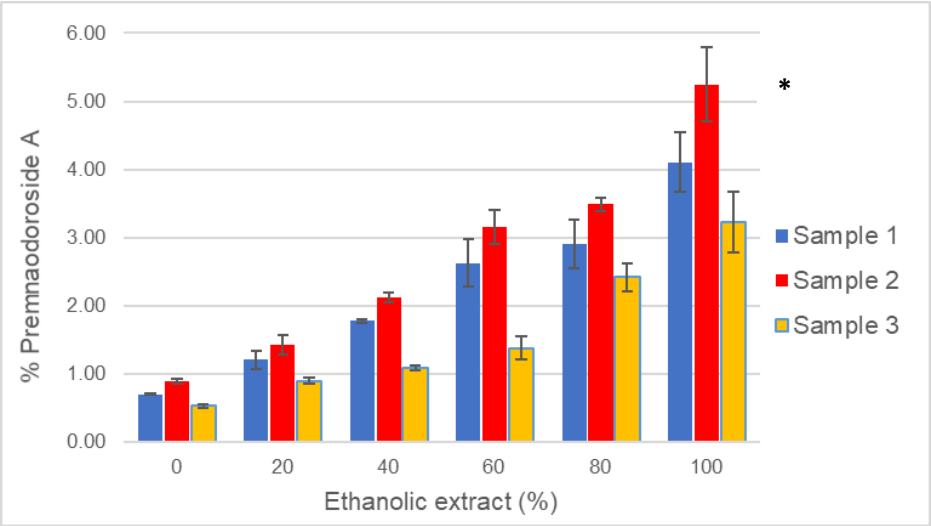

Figure 25 Contents of premnaodoroside A in the extracts

Table 25 Contents of premnaodoroside A in the extracts

Ethanolic extract (%)	% Premnaodoroside A		
	Sample 1	Sample 2	Sample 3
0	0.70 ± 0.01	0.89 ± 0.04	0.53 ± 0.02
20	1.21 ± 0.14	1.43 ± 0.15	0.90 ± 0.04
40	1.78 ± 0.03	2.12 ± 0.07	1.02 ± 0.04
60	2.63 ± 0.34	3.16 ± 0.25	1.38 ± 0.17
80	2.91 ± 0.36	3.49 ± 0.10	2.42 ± 0.20
100	4.11 ± 0.44	5.25 ± 0.54	3.23 ± 0.44

4.4.3 Determination of total phenolic content and antioxidant activity

Total phenolic content (%GAE) and radical scavenging activity (%AAE) of the extracts were determined by Folin-Cioalteu and DPPH assay method, respectively. The results of all samples were in the same manner (Figures 26, 27). Different percentage of ethanol of each sample gave a significantly different result ($p < 0.05$). %GAE and %AAE increased with the percentages of ethanol. 100% ethanol extracts exhibited the highest %GAE (7.56-8.24%) and %AAE (4.92-6.66%) (Tables 26, 27).

The contents of premnaodoroside A in the 0% ethanol extract were the lowest and would increase with increasing percent of ethanol solvent. That might be due to several highly inert polar substances, such as protein, pectin, mucilage and inorganic salts in *P. serratifolia* leaves, dissolved in water and diluted the content of the interesting marker compounds. This result suggested that solubility of premnaodoroside A in ethanol was better than in water. This finding contradicted the traditional preparing method of this plant like infusion and decoction in water for antidiabetic food and drink additive. Some investigators described that infusion and decoction of *P. serratifolia* leaves have antidiabetic and antioxidant activity (25), and could be consumed as food and drink additives for antidiabetic. Infusion and decoction might give medicinal recipes with gentle potency, and they were convenient method according to the traditional style. However, this study indicated that to develop a highly quality extract, the concentration of the premnaodoroside A was highest in 100% ethanolic extract but its yield was the lowest (Table 24). Its extract yield was 2.5 times lower than of the highest yield of 80% ethanol of sample 1, and 40% ethanol extracts of samples 2 and 3. Therefore, quality and quantity should be considered together for the production of herbal extracts.

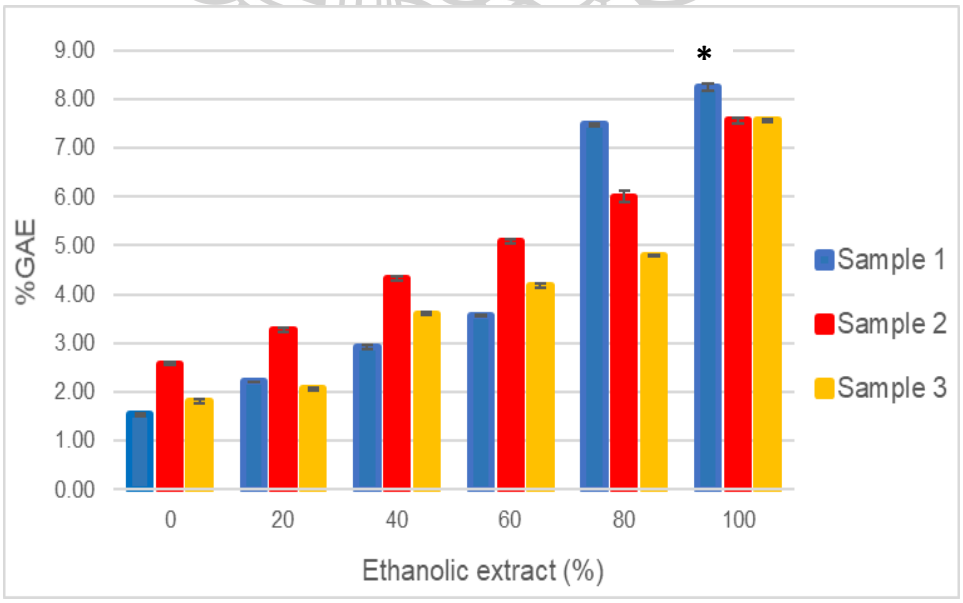

Figure 26 Total phenolic content of the extracts

Table 26 Total phenolic content of the extracts

Ethanolic extract (%)	%GAE		
	Sample 1	Sample 2	Sample 3
0	1.54 ± 0.02	2.58 ± 0.02	1.80 ± 0.04
20	2.21 ± 0.01	3.27 ± 0.05	2.06 ± 0.03
40	2.92 ± 0.03	4.33 ± 0.04	3.60 ± 0.03
60	3.57 ± 0.02	5.08 ± 0.04	4.18 ± 0.04
80	7.48 ± 0.03	6.00 ± 0.12	4.80 ± 0.02
100	8.24± 0.08	7.56 ± 0.03	7.56± 0.03

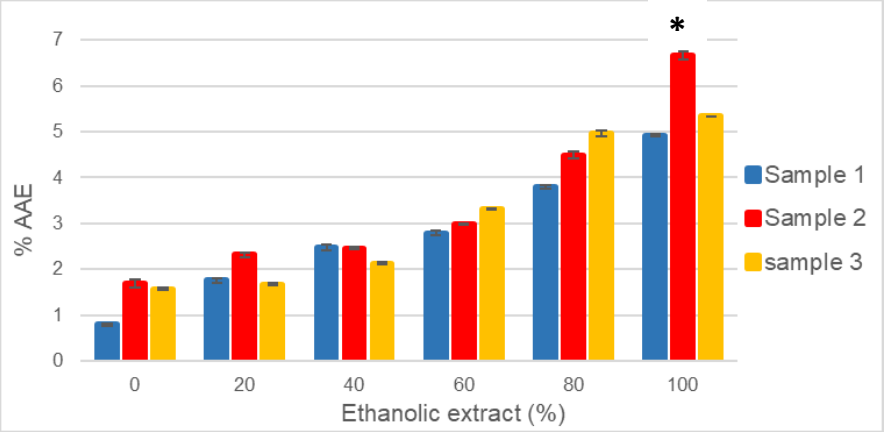

Figure 27 DPPH radical scavenging activity of the extracts

Table 27 DPPH radical scavenging activity of the extracts

Ethanolic extract (%)	%AAE		
	Sample 1	Sample 2	Sample 3
0	0.79 ± 0.02	1.68 ± 0.09	1.57 ± 0.02
20	1.76 ± 0.05	2.32 ± 0.05	1.67 ± 0.02
40	2.48 ± 0.06	2.46 ± 0.02	2.13 ± 0.03
60	2.79 ± 0.05	2.98 ± 0.02	3.32 ± 0.01
80	3.79 ± 0.03	4.49 ± 0.07	4.96 ± 0.07
100	4.92 ± 0.03	6.66 ± 0.09	5.33 ± 0.01

To optimize the percentage of the using ethanol solvents, extractive amounts of premnaodoroside A, total phenolic compounds and antioxidants from plant material were calculated from yields of the extract and the contents of these compounds in the extracts (Figure 28). The percentage of extractive amount was calculated as (Extractive amount (%) of premnaodoroside A = % Yield x % premnaodoroside A /100). The extractive amount of premnaodoroside A, total phenolic content and antioxidant from plant material of each % ethanol extractant are shown in Figure 28 and Tables 28, 29, 30.

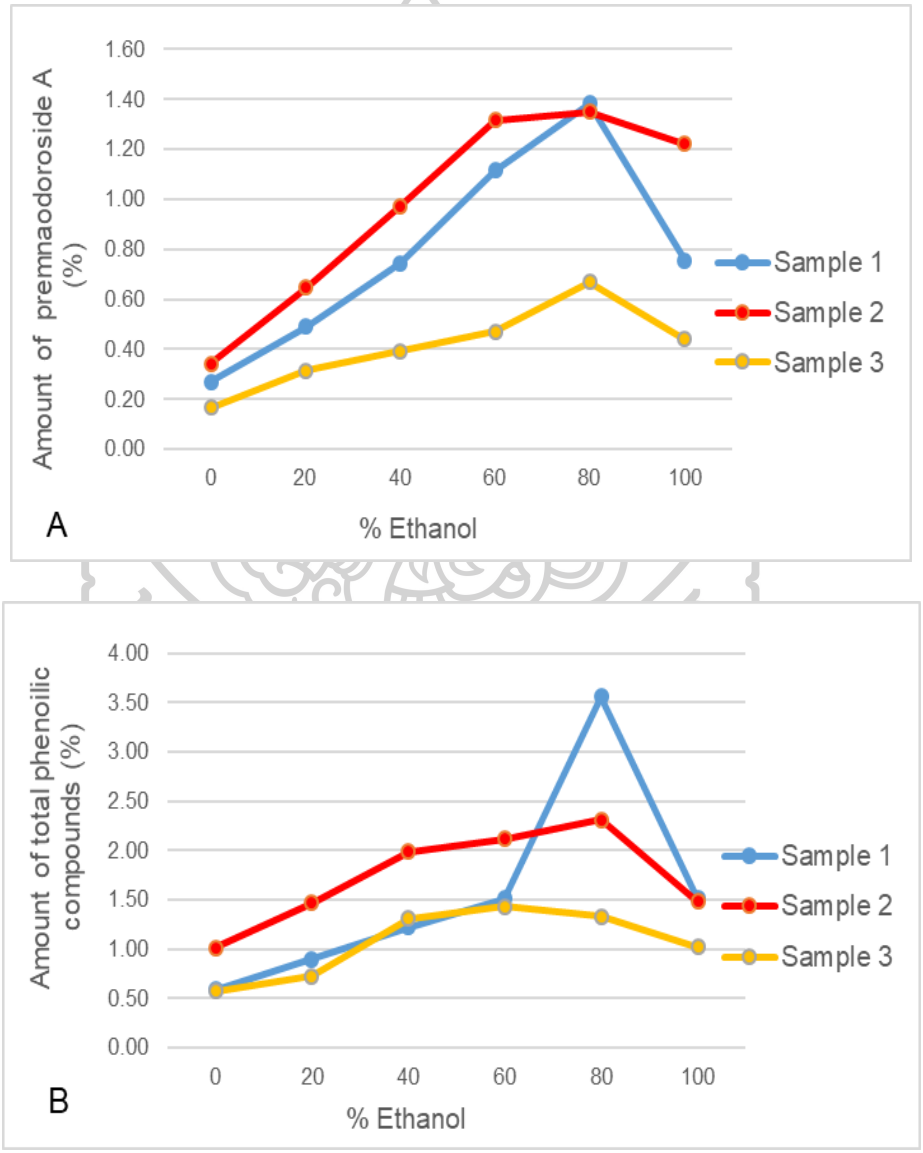

Figure 28 Extractive amount (%) of premnaodoroside A, (B) total phenolic compounds and (C) antioxidants from *P. serratifolia* leaves by using various percent of ethanol as extraction solvent

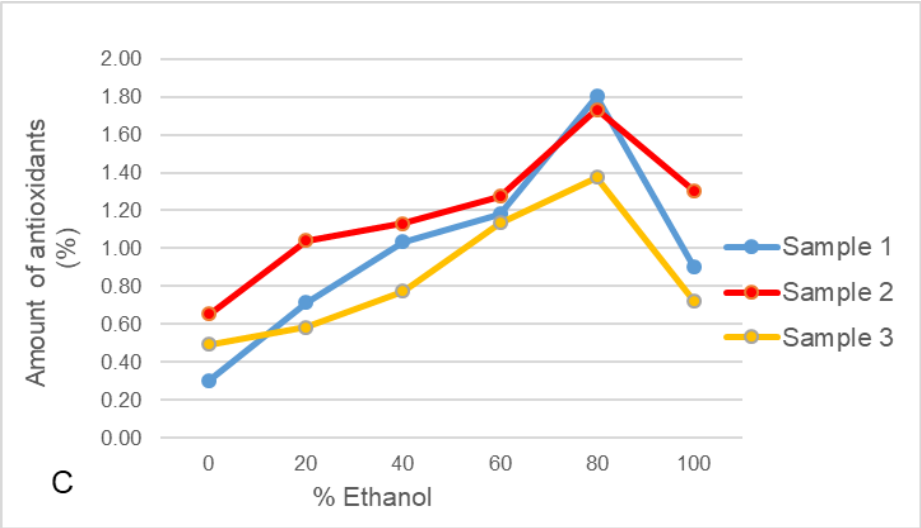

Figure 28 Extractive amount (%) of premnaodoroside A, (B) total phenolic compounds and (C) antioxidants from *P. serratifolia* leaves by using various percent of ethanol as solvent (continued)

Table 28 Extractive amount (%) of premnaodoroside A from *P. serratifolia* leaves by using various percent of ethanol as solvent

% Ethanol	Sample 1	Sample 2	Sample 3
0	0.27	0.34	0.17
20	0.49	0.65	0.32
40	0.74	0.97	0.39
60	1.12	1.32	0.47
80	1.38	1.35	0.67
100	0.75	1.22	0.44

Table 29 Extractive amount (%) of total phenolic compounds from *P. serratifolia* leaves by using various percent of ethanol as solvent

% Ethanol	Sample 1	Sample 2	Sample 3
0	0.59	1.01	0.57
20	0.90	1.47	0.72
40	1.22	1.99	1.31
60	1.51	2.11	1.43
80	3.56	2.31	1.33
100	1.51	1.48	1.02

Table 30 Extractive amount (%) of antioxidants from *P. serratifolia* leaves by using various percent of ethanol as solvent

% Ethanol	Sample 1	Sample 2	Sample 3
0	0.30	0.65	0.50
20	0.71	1.04	0.58
40	1.04	1.13	0.77
60	1.18	1.27	1.13
80	1.80	1.73	1.38
100	0.90	1.30	0.72

According to the calculation from Tables 28, 29, 30, the results of premnaodoroside A content, total phenolic and antioxidant activity of all samples were in the same manner. 80% Ethanol could extract the highest amounts of premnodoroside A, phenolic content and antioxidant from plant material, except for sample 3, where the highest amounts of phenolic compounds were obtained from 60% ethanol (but not much different from 80%). Then 80% ethanol was considered to possess the highest extractive power for the extraction of these bioactive compounds from *P. serratifolia* leaves.

Overall, results revealed that quality and quantity of the extract should be considered to facilitate the pharmaceutical production of the process. In term of the quality of the extract, 100% ethanol provided the highest amount of premnaodoroside A, total phenolic content, and antioxidant activity, but it had the lowest amount of extractive yield. Although its productivity was limited by the lowest extractive yield, 100% ethanol extract might be useful in practice for administration at lower doses with high potency. In term of the quantity of extract, 80% ethanol extract provided the higher extractive yield and % extractive amount. A higher extraction yield could result in a greater number of therapeutic dosages in the production process.

CHAPTER 5
CONCLUSION

The volatile oils obtained from the fresh, dried, and fermented leaves of *P. serratifolia* were determined by gas chromatography-mass spectrometry (GC-MS) using DB-5 and Carbowax 20M columns. The results of the analysis found that some compounds in the volatile oils of fresh leaves were changed when the leaves were dried or fermented. Amyl vinyl carbinol, linalool, and phytol were the major changed compounds. The relative amounts of these compounds were reduced after drying and fermentation. However, the relative amounts of (*E*)-caryophyllene and *p*-vinylanisole were increased after drying and fermentation. In dried and fermented leaves volatile oils, some compounds (alpha-humulene) disappeared and other compounds appeared (beta-myrcene). This was suggested to be a transformation reaction of certain chemicals through hydrolysis, dehydration, and oxidation, and could be attributable to the effect of the post-harvesting procedure on the quality of the plant. Folin-Ciocalteu and DPPH methods were used to measure the phenolic content and antioxidant activity of post-harvesting impacts of the leaves. The phenolic content and antioxidant activity of 80% ethanolic extract of the dried and fermented leaves were lower than those of the fresh leaf extract. The fresh leaf extract showed higher amounts of phenolic content (6.1%GAE) and antioxidant activity (IC_{50}=73.43%) However, The IC_{50} value of the fresh leaf extract was higher than that of ascorbic acid. Therefore, fresh leaf extract had less antioxidant activity than ascorbic acid.

The dried leaf is more suitable than the fresh and the fermented leaves for use as raw material in herbal extract industry. Phytochemical study showed that dried leaves contained premnaodoroside A and a mixture of glucose and fructose as the major chemical constituents. The identification of these compounds was based on their spectroscopic data comparing with previous literatures. Premnaodoroside A which is an iridoid glucoside, was selected as the analytical marker for the quality analysis.

A TLC-densitometric method for the analysis of the content of premnaodoroside A was developed and was validated according to ICH guidelines. The calibration curve in the concentration range of (0.87-0.11 µg/spot) demonstrated

a good linearity ($R^2 > 0.99$). The specificity of the method was ensured by analyzing of peak purity and peak identity. Correlations (r^2) among the spectra of peak start, peak maximum and peak end of the band were more than 0.99. The spectra of the sample and standard peaks were identical with the correlations (r^2) closed to 0.99. Moreover, the absence of the any interfering peak revealed that the method is specific. Repeatability and intermediate precisions were carried out by using the three different concentrations of standard at 0.11, 0.44 and 0.87 µg/spot with five replications. The % RSD value of all results was lower than 3%. Accuracy was performed by standard addition method with three standard concentrations (0.22, 0.35and 0.44 µg/spot). The recovery was in the range of 97.08- 101.16%. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from calibration curve. The LOD and LOQ were 0.13 and 0.04 µg/spot, respectively. The robustness method was determined by changes in scanning time after derivatization (5-25 minutes) using five different standard concentrations of 0.11-0.87 µg/spot. Changes in peak area occurred in time variation. According to the results, the proposed method gave satisfactory linearity, specificity, precision and accuracy, and was available for intended use, only scanning time after derivatization must be fixed to minimize error. TLC-densitometric method is simple, low cost, and less time consuming. It could be used for the routine analysis of premnaodoroside A in the extracts of dried leaves of *P. serratifolia*. Extracts of dried leaves were prepared with different percentages of ethanol (0, 20, 40, 60, 80 and 100%) to compare for their yield and quality. The developed TLC-densitometry method was used for the determination of the content of premnaodoroside A. Their antioxidant activity, the total phenolic content was examined by Folin-Cioclateus and DPPH method, respectively. 100% of ethanol extract had the highest content of premnaodoroside A, %GAE and %AAE, although its extractive yield was the lowest. As a result, the findings of this study provided the basic information on solvent concentrations that had a significant effect on extractive yield, the contents of iridoid glycosides (premnaodorside A), total phenolics, and antioxidants in *P. serratifolia* leaf extracts. The benefits and drawbacks of each percent of extract had to be considered in terms of how it would be used in the future.

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APPENDICES

APPENDIX 1

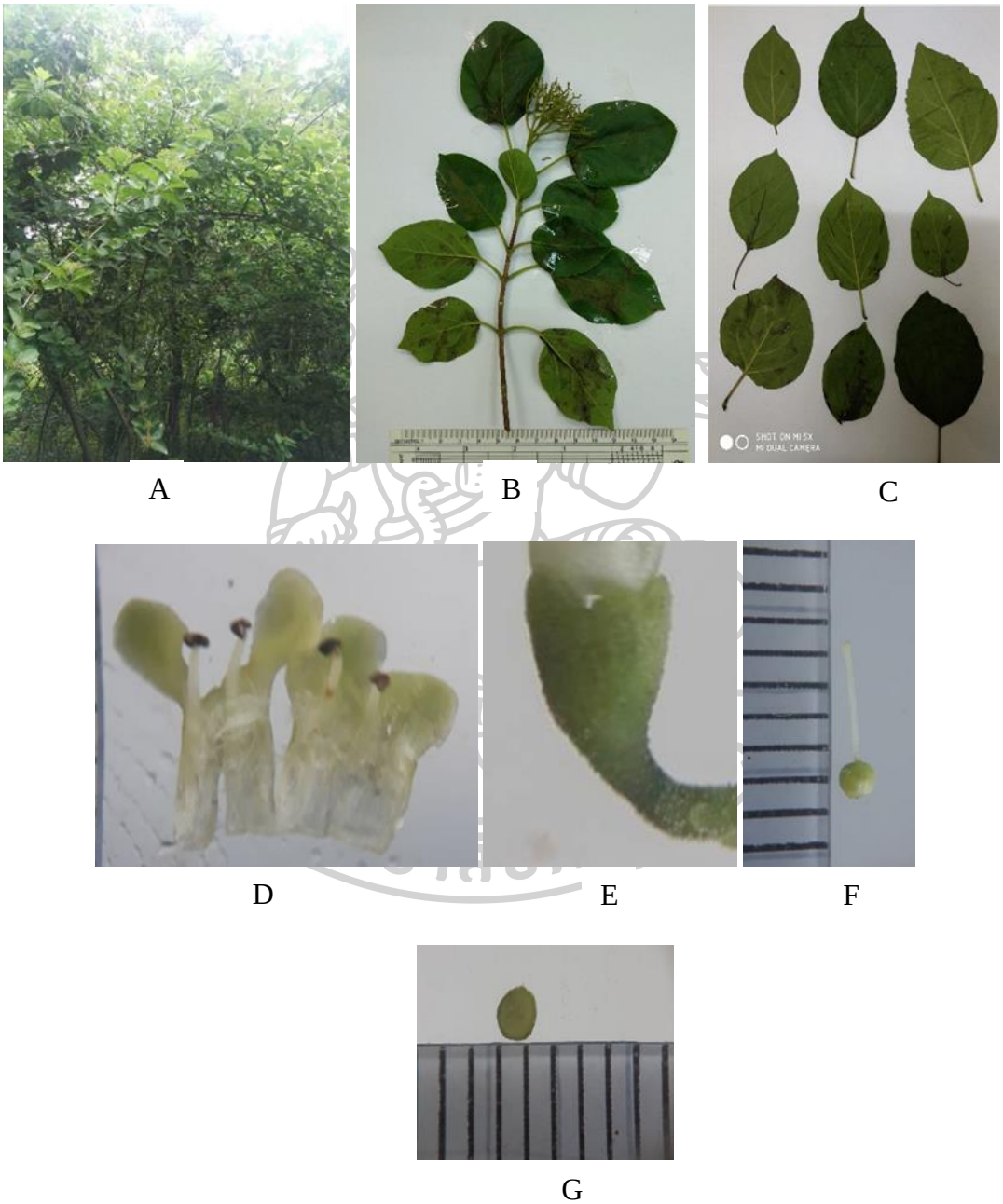

Figure 29 *P. serratifolia* (A) Habit of the plant (B) Flowering branch (C) Fresh leaves with lower surface (D) Vertical cut of corolla (E) Calyx (cup shape) (F) Pistil (G) Cross section of ovary.

APPENDIX 2

Drangendorff's reagent

Two solutions were prepared.

Solution (a) – 0.85 g of bismuth (III) nitrate was dissolved in 10 mL of glacial acetic acid and 40 mL of water.

Solution (b) – 8g of potassium iodide was dissolved in 20 mL of water.

Solution (a) and solution (b) were mixed in equal parts.

Trim-Hill reagent

10 mL of glacial acetic acid were mixed with 1 mL of 0.2% copper sulfated solution and 0.5 mL of concentrated hydrochloric acid. Then, they were mixed well.

Vanillin-sulfuric acid solution

Solution (a) – Dissolve 0.1g of vanillin in 10 mL of ethanol

Solution (b) – 1 mL of sulfuric acid in 10 mL of ethanol

Solution (a) and (b) were mixed together.

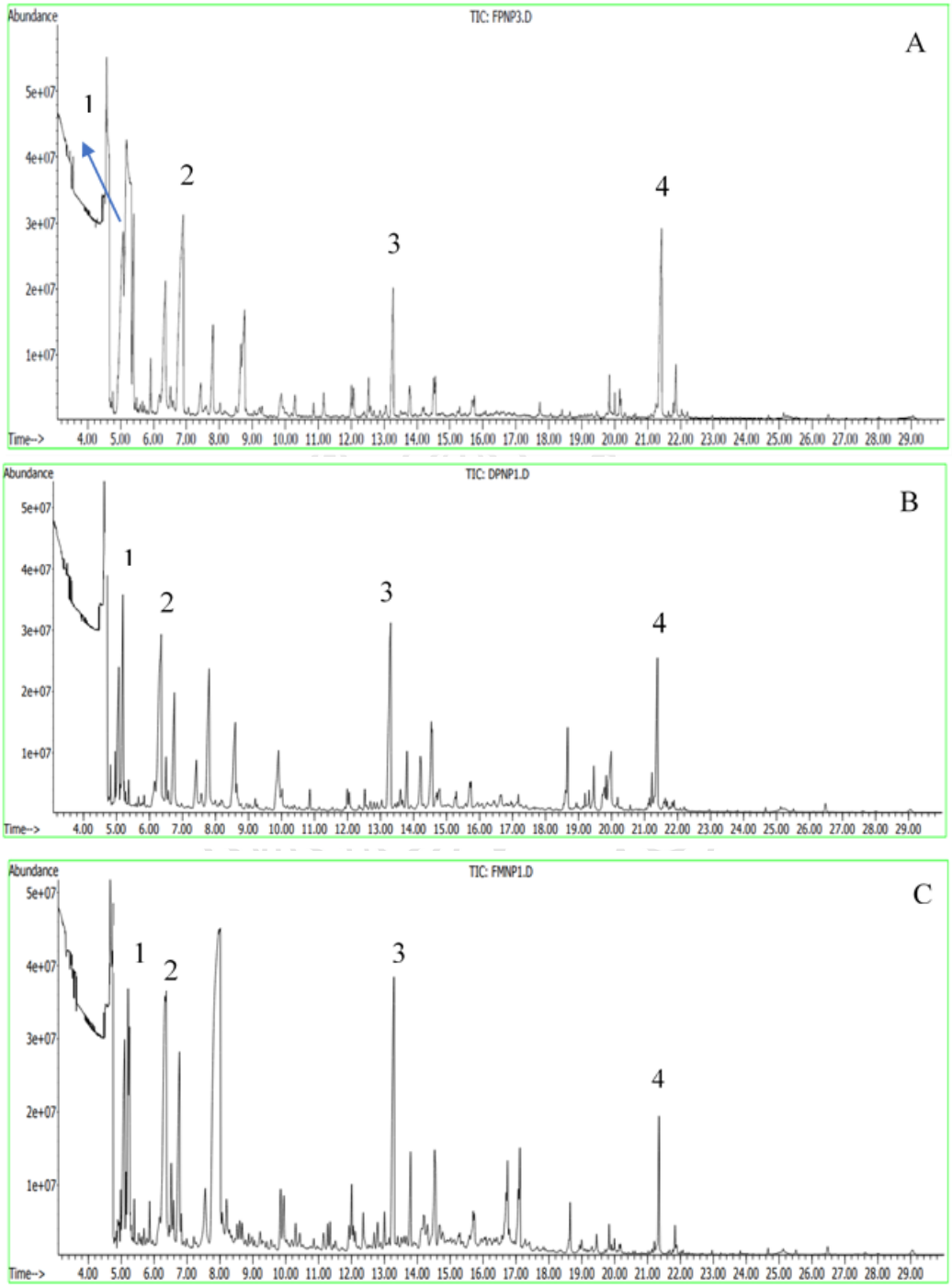

Figure 30 GC chromatograms and their major component of volatile oils (A) fresh, (B) dried and (C) fermented leaf analyzed by DB-5 column

Peak 1. Amyl vinyl carbinol, Peak 2. Linalool, Peak 3. (*E*) Carophyllene, Peak 4. Phytol.

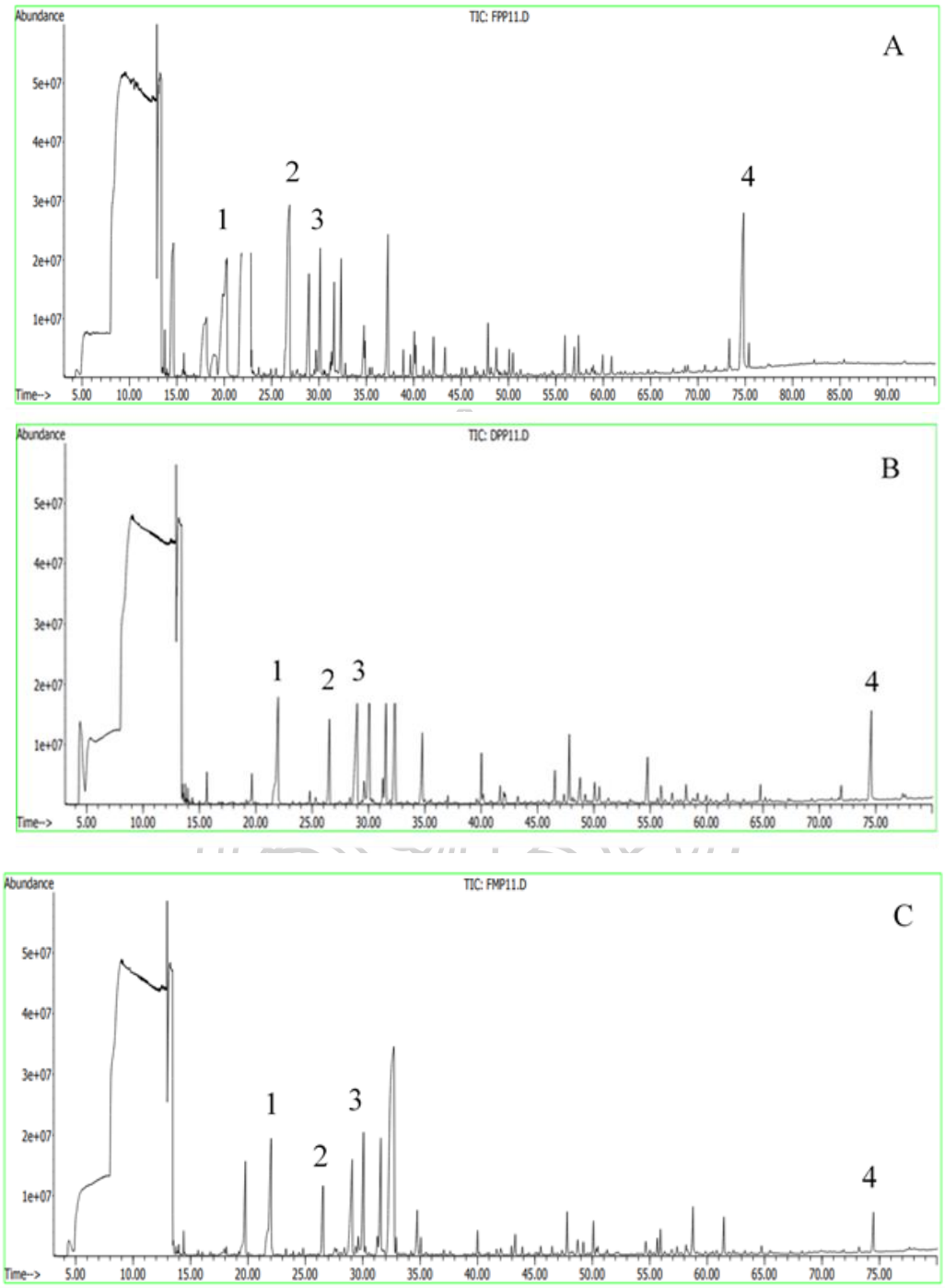

Figure 31 GC chromatograms and their major component of volatile oils (A) fresh, (B) dried and (C) fermented leaf analyzed by Carbowax 20M

Peak 1. amyl vinyl carbinol, Peak 2. linalool, Peak 3. (*E*) carophyllene, Peak 4. Pytol.

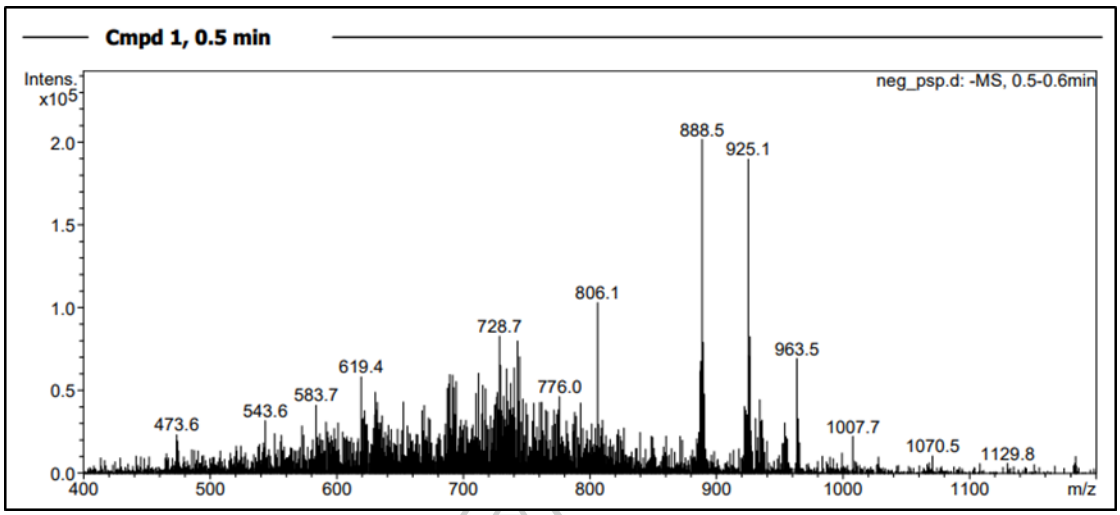

Figure 32 Mass spectrum of premnaodoroside A.

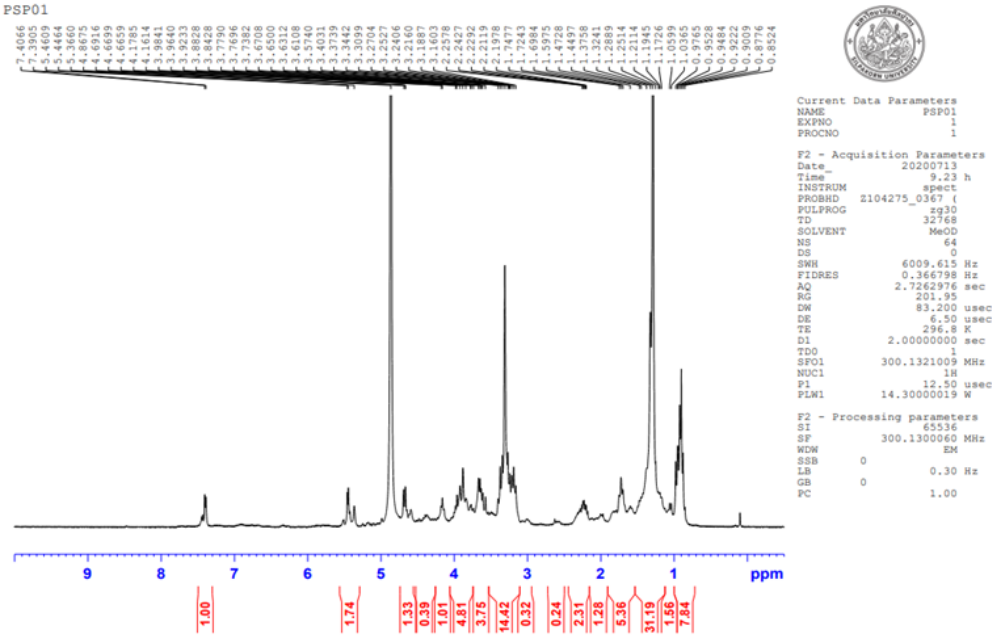

Figure 33 The ^{1}H-NMR (300 MHz) spectrum of premnaodoroside A (in MeOD)

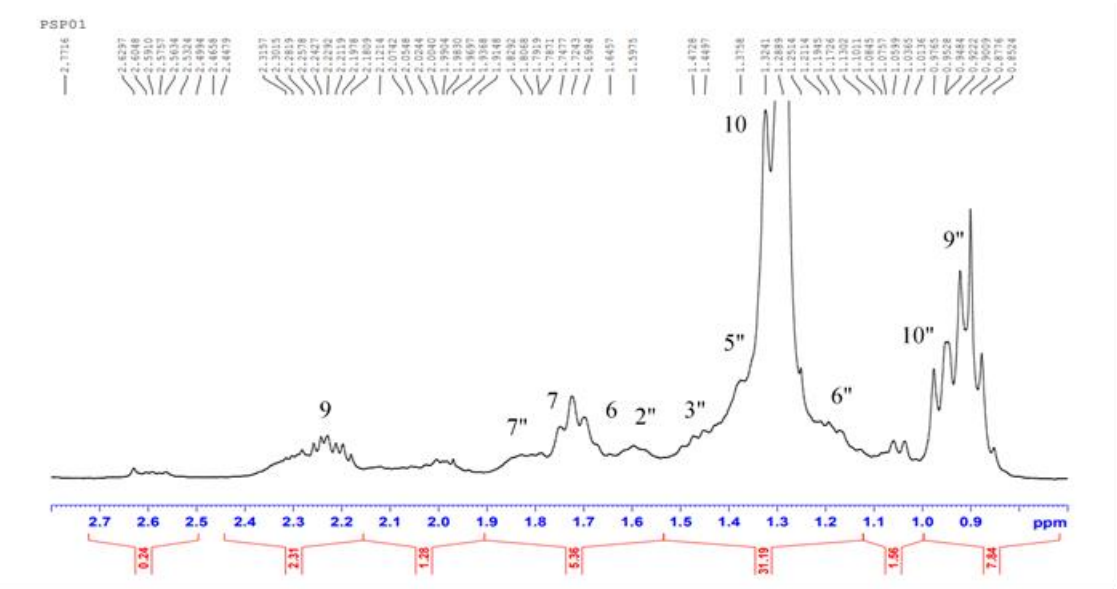

Figure 34 Expanded ^{1}H-NMR (300 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 2.7-0.9 ppm.

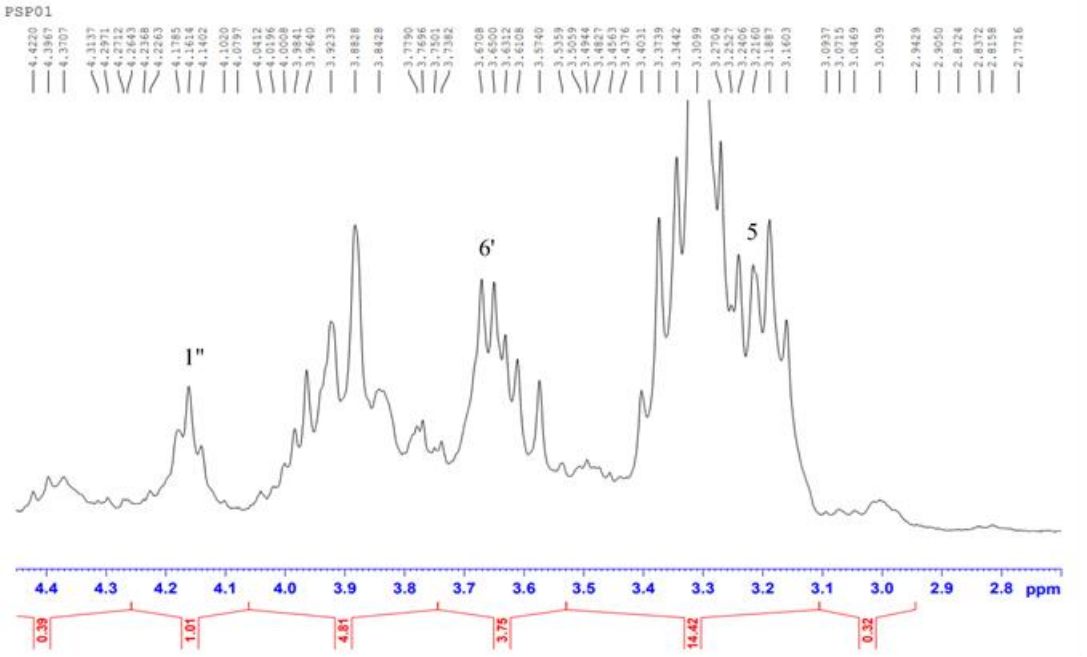

Figure 35 Expanded ^{1}H-NMR (300 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 4.4-2.8 ppm.

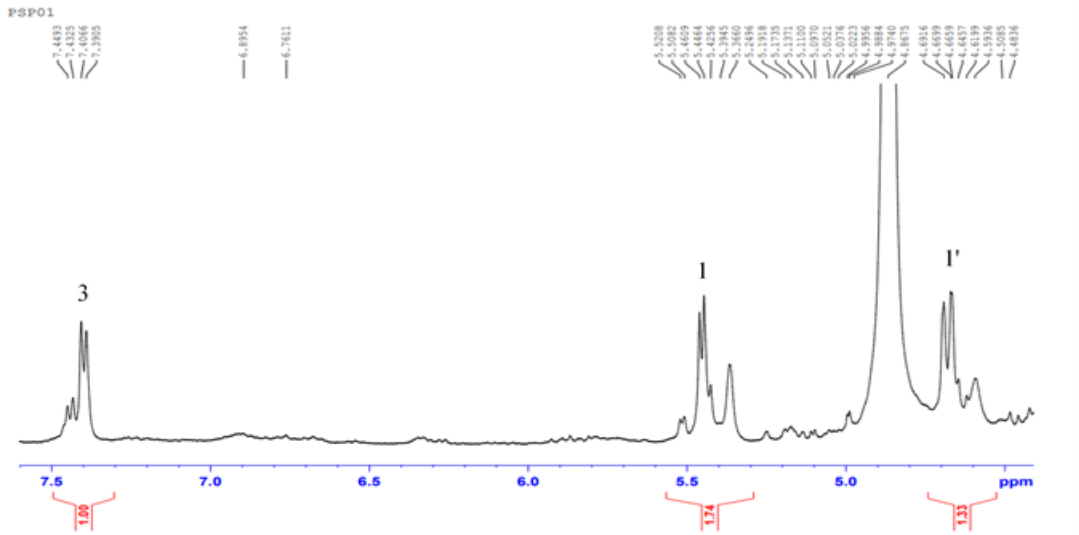

Figure 36 Expanded ^{1}H-NMR (300 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 7.5-5.0 ppm.

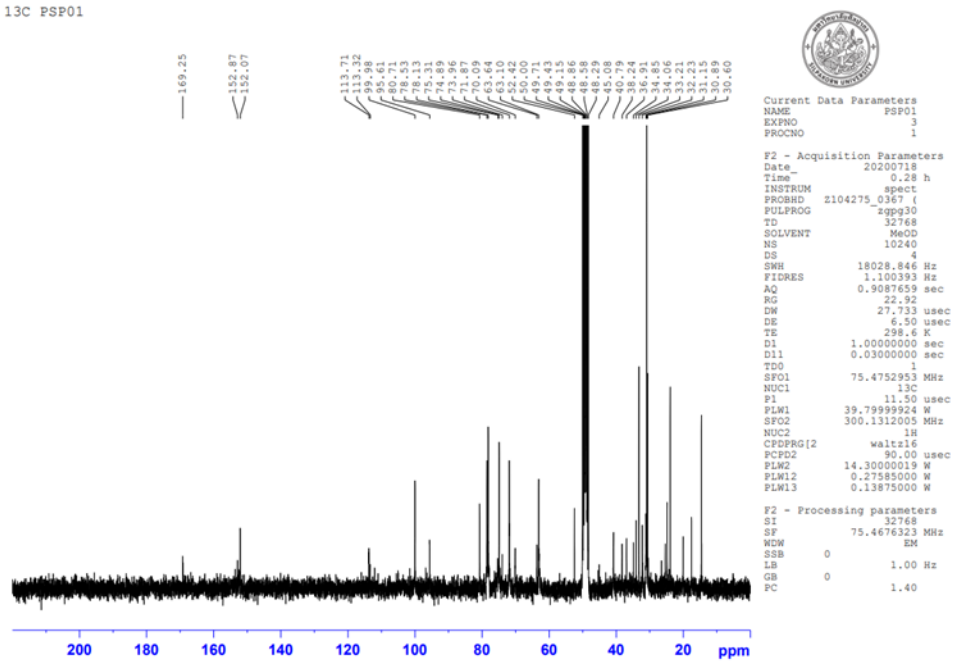

Figure 37 The ^{13}C-NMR (75 MHz) spectrum of premnaodoroside A (in MeOD)

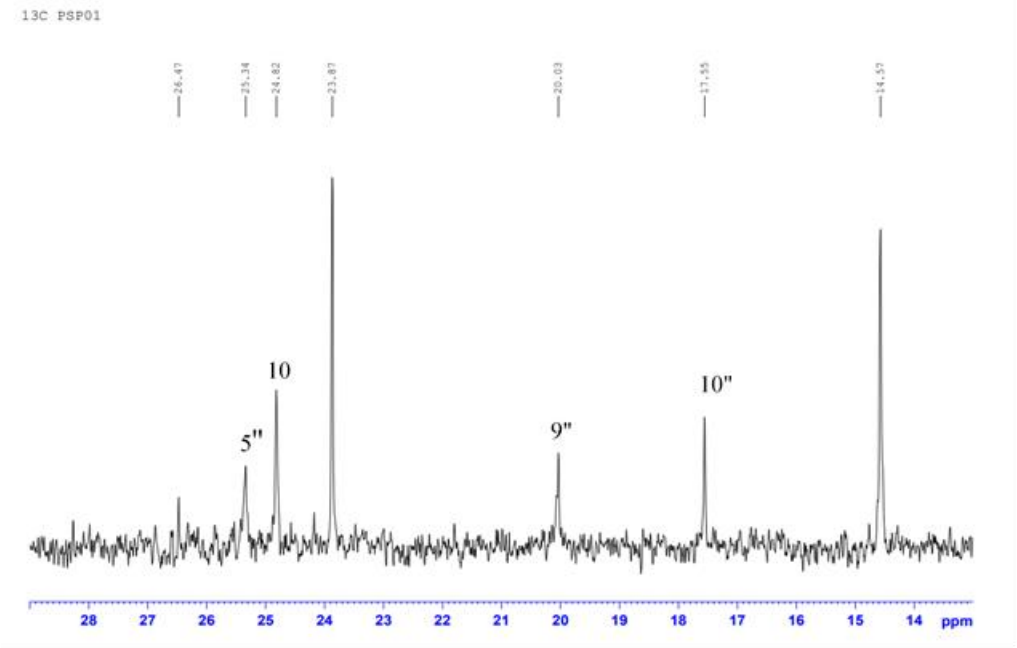

Figure 38 Expanded ^{13}C-NMR (75 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 28.0-14.0 ppm

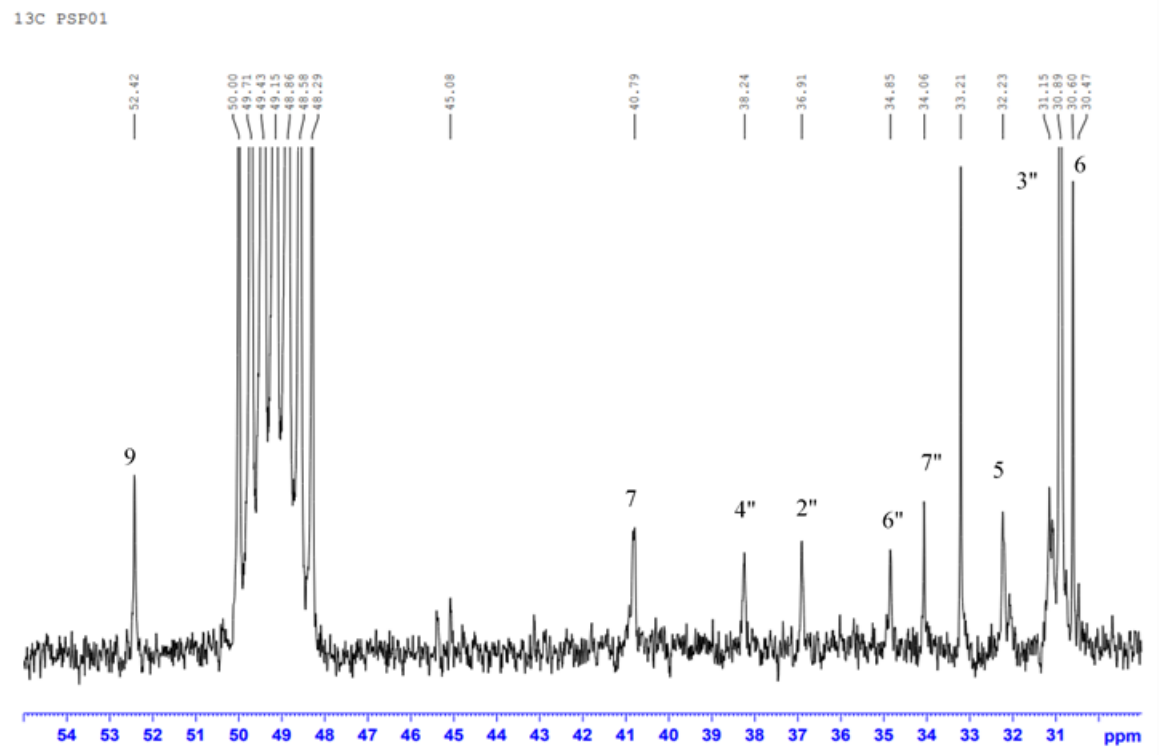

Figure 39 Expanded ^{13}C-NMR (75 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 54.0-31.0 ppm

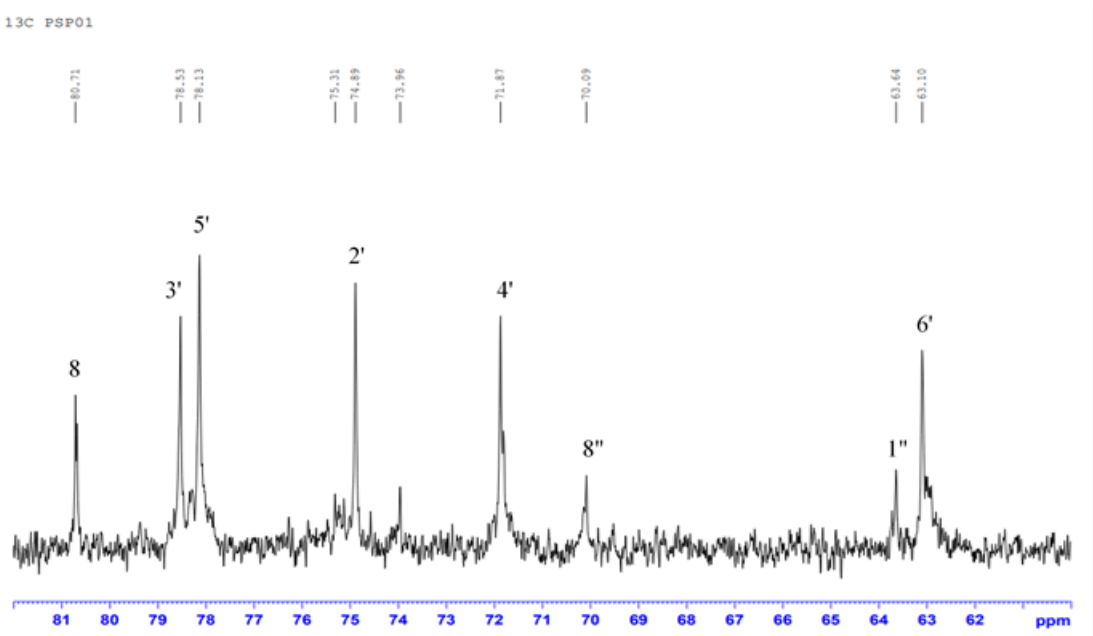

Figure 40 Expanded ^{13}C-NMR (75 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 81.0-62.0 ppm

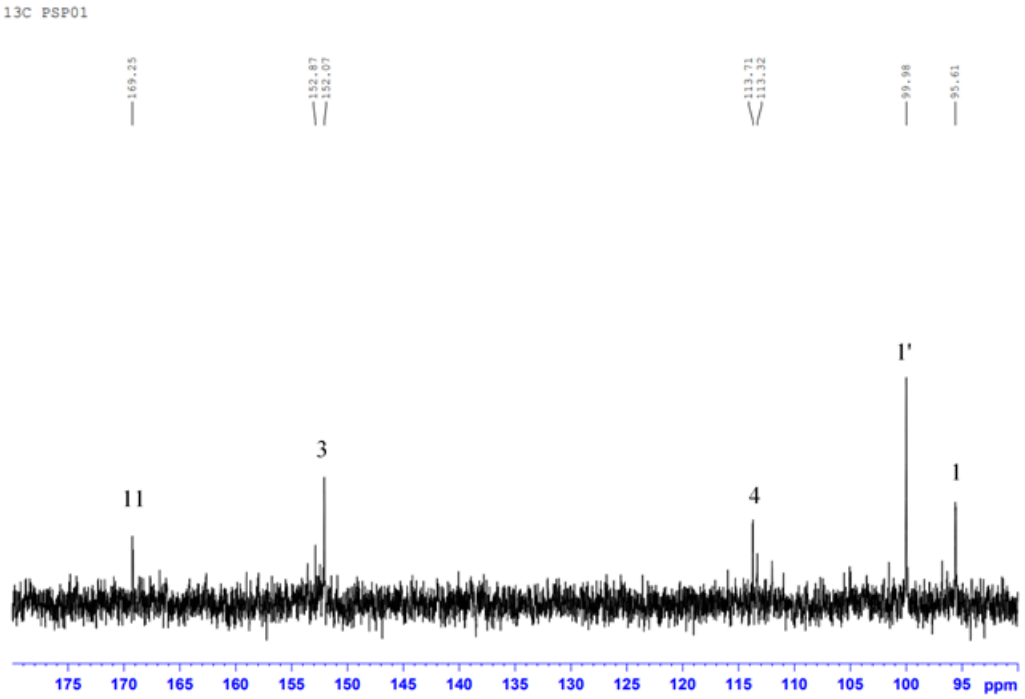

Figure 41 Expanded ^{13}C-NMR (75 MHz) spectrum of premnaodoroside A (in MeOD) in the range of δ 175.0-95.0 ppm

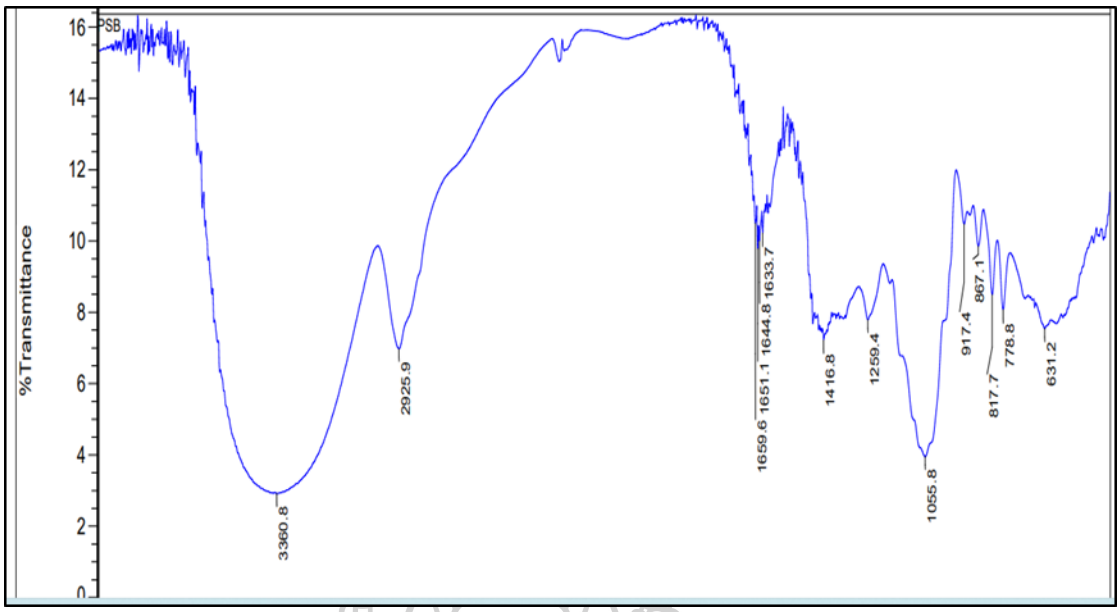

Figure 42 FTIR spectrum of the mixture of D-glucose and D-fructose.

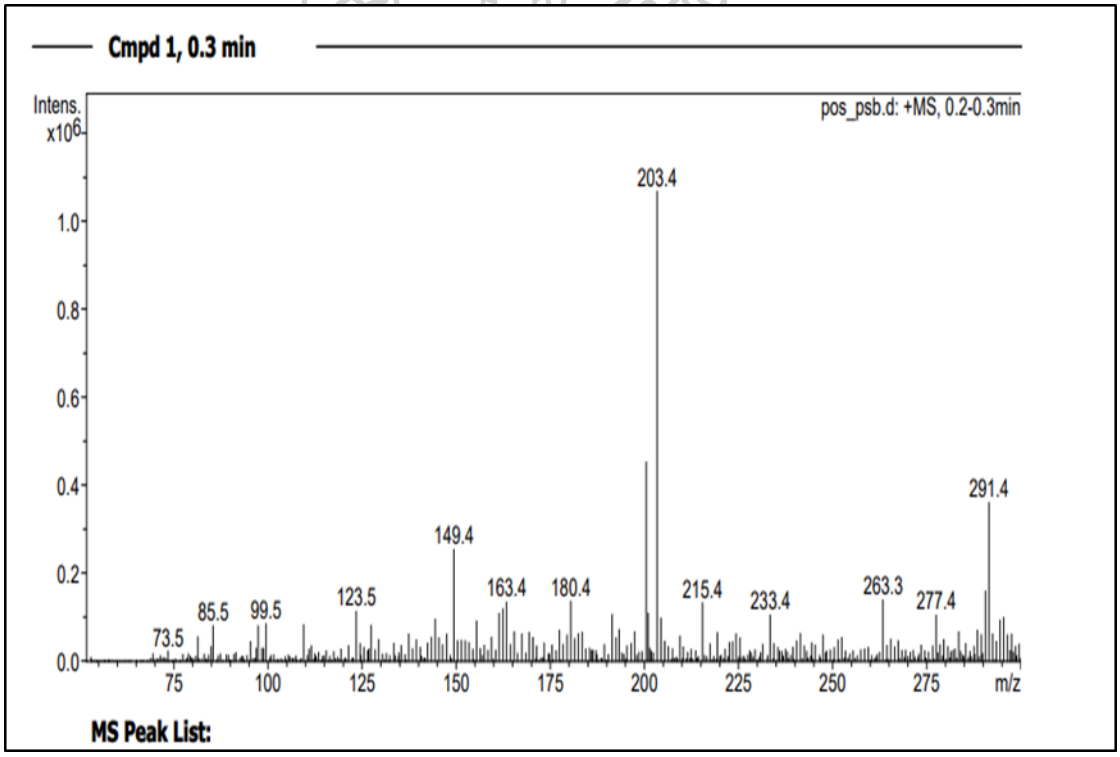

Figure 43 Mass spectrum of the mixture of D-glucose and D-fructose.

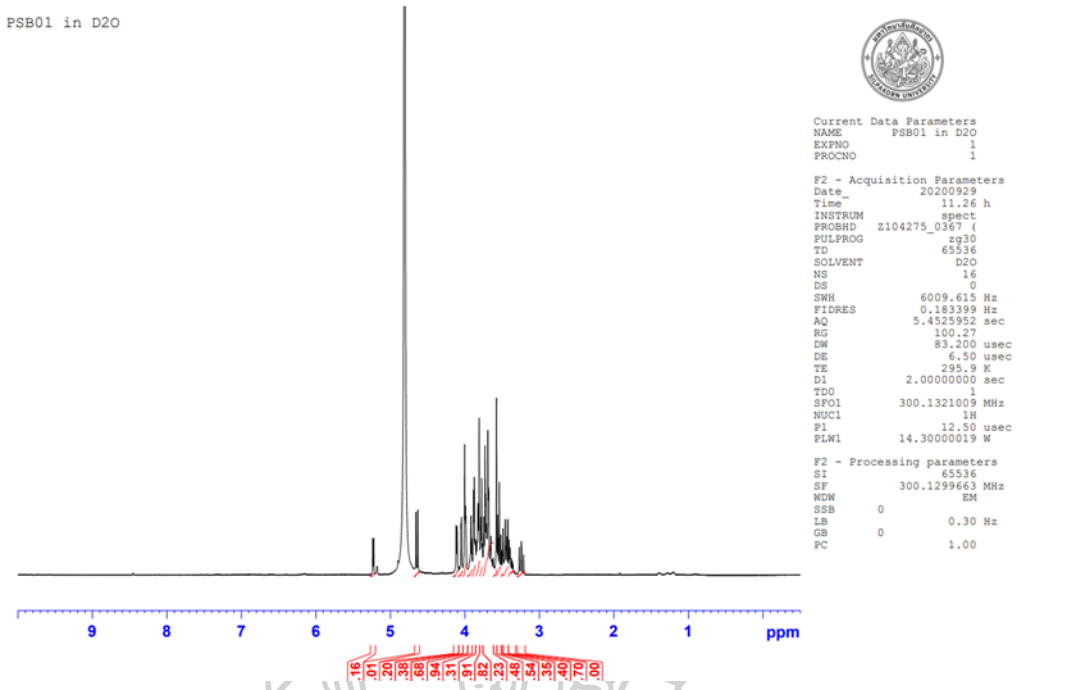

Figure 44 The ^{1}H-NMR (300 MHz) spectrum of the mixture of D-glucose and D-fructose (in D_2O).

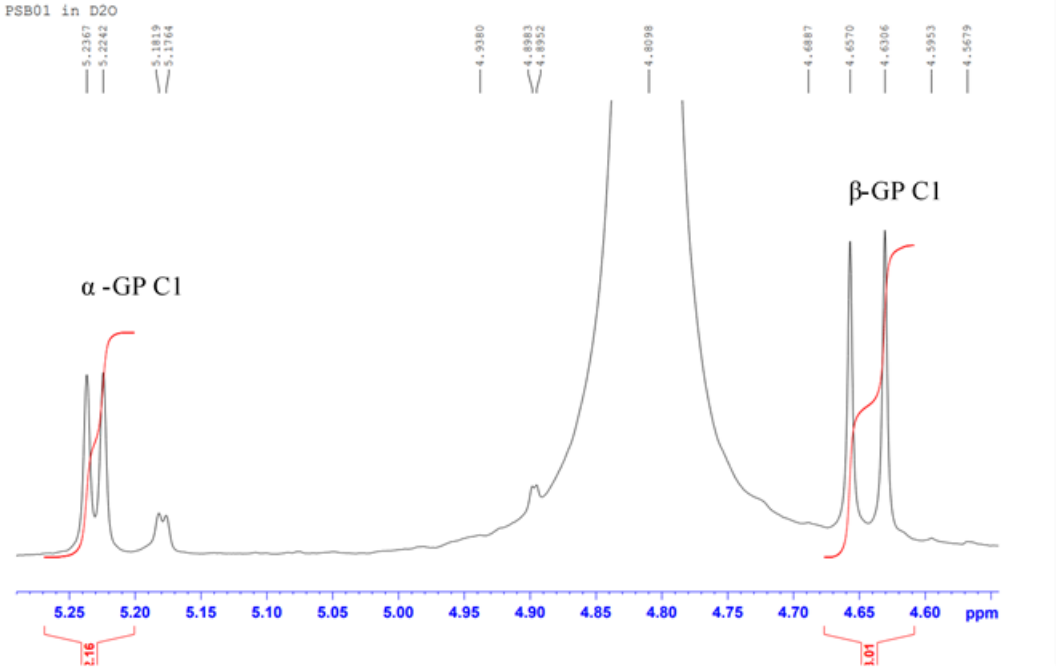

Figure 45 Expanded ^{1}H-NMR (300 MHz) spectrum the mixture of D-glucose and D-fructose (in D_2O) in the range of δ 5.25-4.60 ppm.

(α-GP = α-D-glucopyranose, β-GP = β-D-glucopyranose)

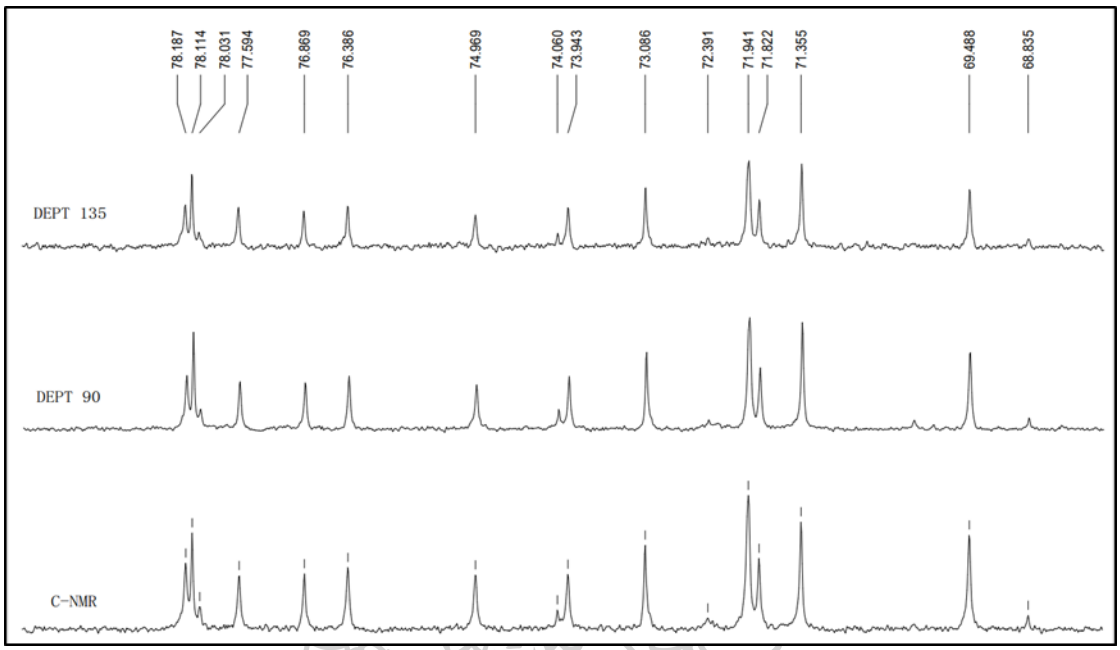

Figure 46 Expanded ^{13}C-NMR and DEPT 135 (75 MHz) spectrum of the mixture of D-glucose and D-fructose (in MeOD) in the range of δ 78.2-68.8ppm

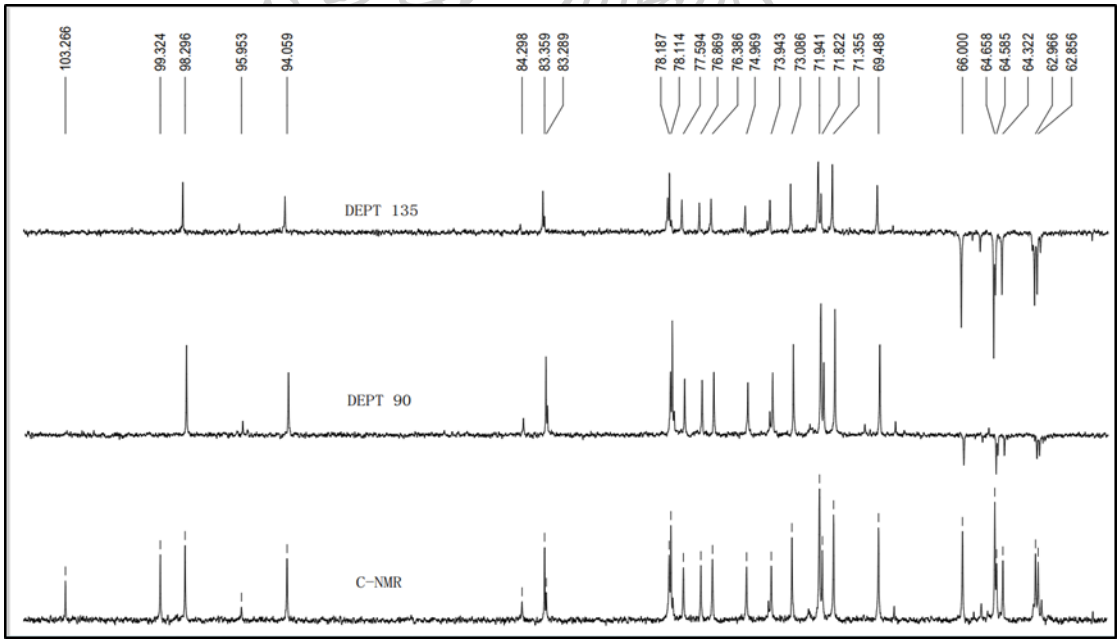

Figure 47 Expanded ^{13}C-NMR and DEPT 135 (75 MHz) spectrum of the mixture of D-glucose and D-fructose (in MeOD) in the range of δ 103.3-62.9ppm

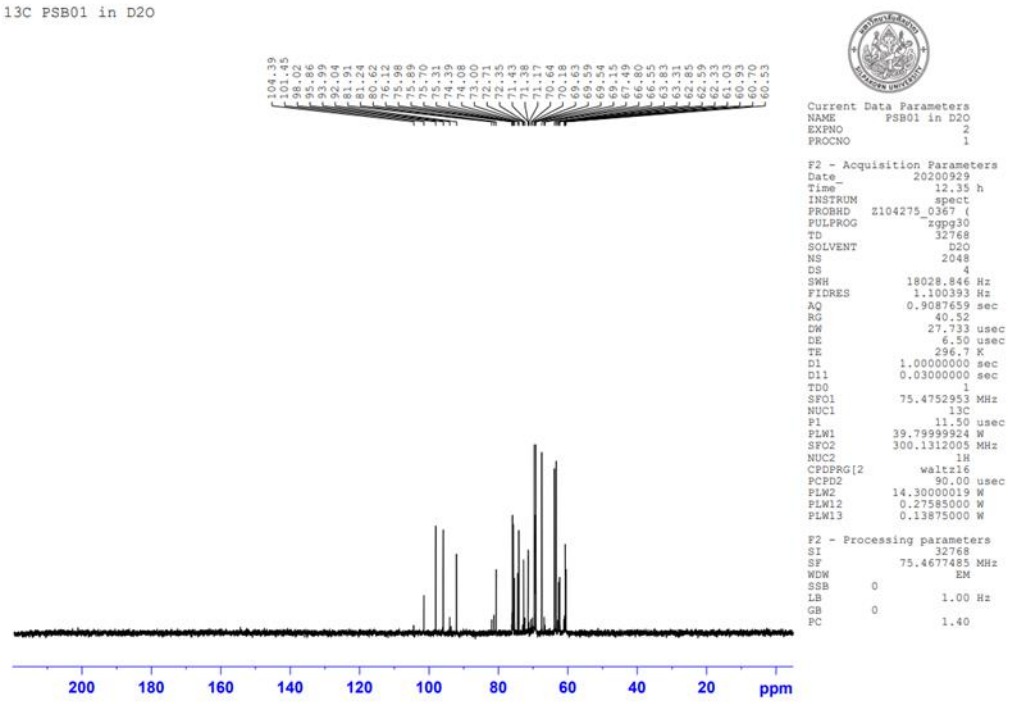

Figure 48 The ^{13}C-NMR (75 MHz) spectrum of the mixture of D-glucose and D-fructose (in D_2O)

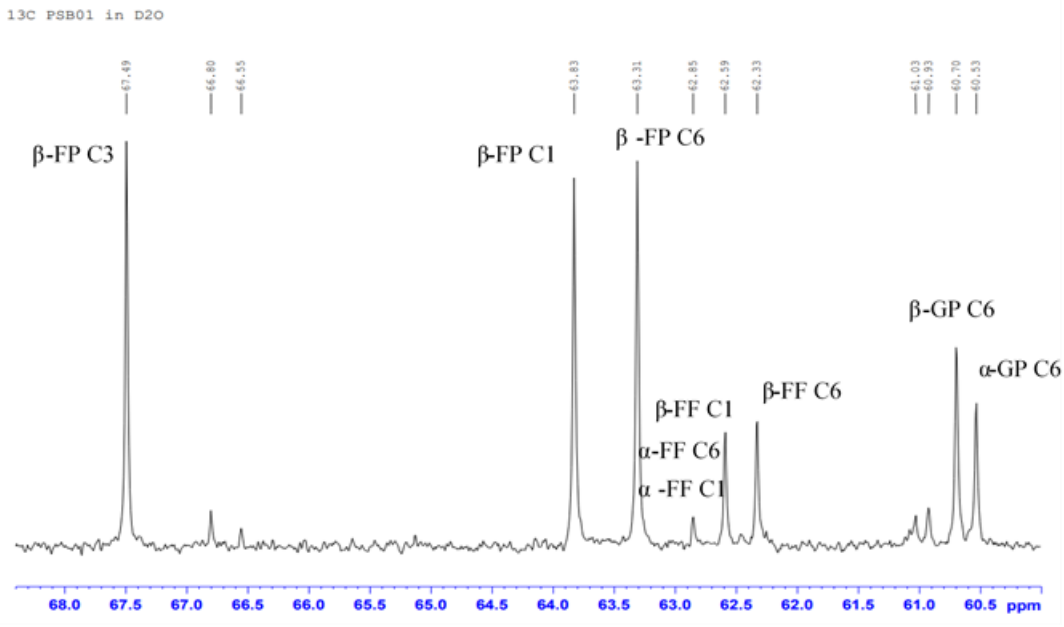

Figure 49 Expanded ^{13}C-NMR (75 MHz) spectrum of the mixture of D-glucose and D-fructose (in D_2O) in the range of δ 68.0-60.5ppm

(β-FP = β-D-fructopyranose, β-FF = β-D-fructofuranose, α-FF = α-D-fructofuranose, β-GP = β-D-glucopyranose, α-GP = α-D-glucopyranose)

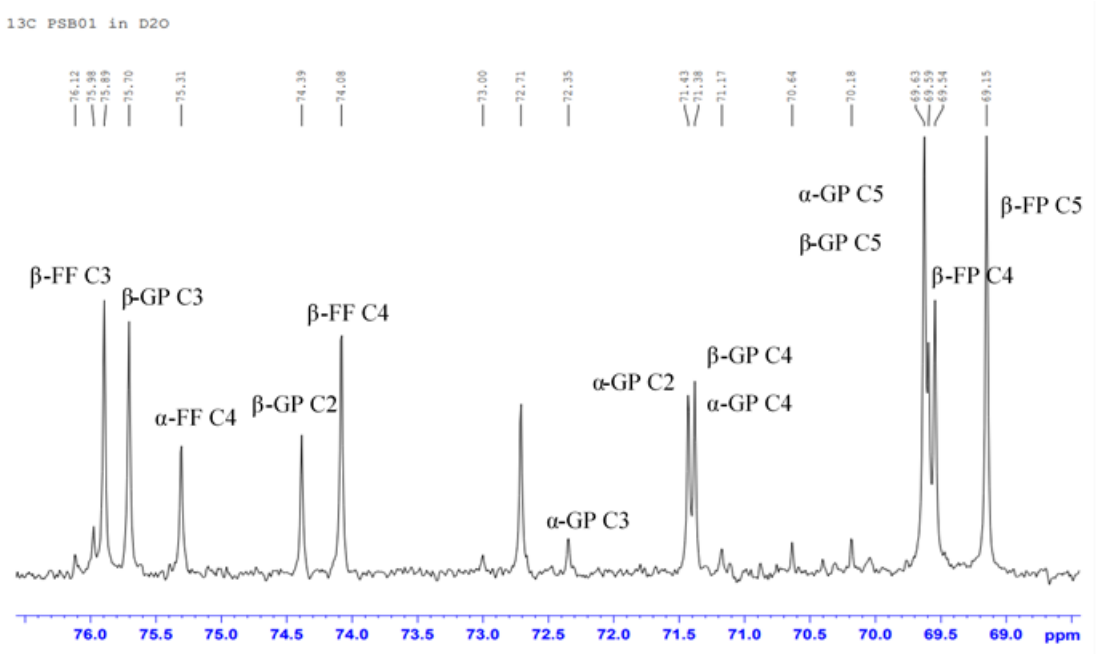

Figure 50 Expanded ^{13}C-NMR (75 MHz) spectrum of the mixture of D-glucose and D-fructose in D_2O) in the range of δ 76.0-69.0ppm

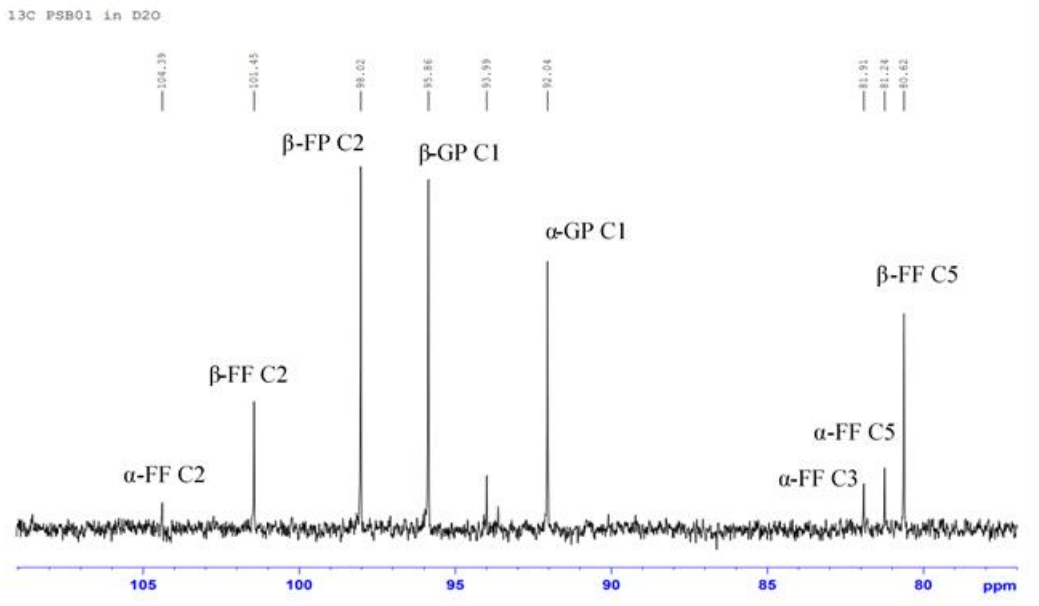

Figure 51 Expanded ^{13}C-NMR (75 MHz) spectrum of the mixture of D-glucose and D-fructose (in D_2O) in the range of δ 105.0-80.0ppm

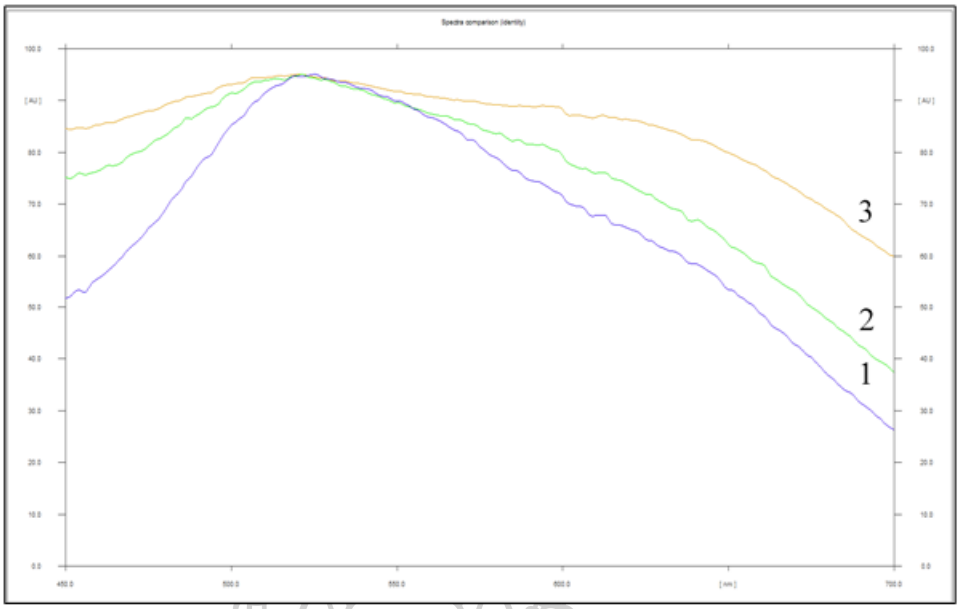

Figure 52 Overlay spectra at 450-700 nm of standard premnaodoroside A and sample: standard (1), 60% ethanolic extract (2), 100% ethanolic extract (3)

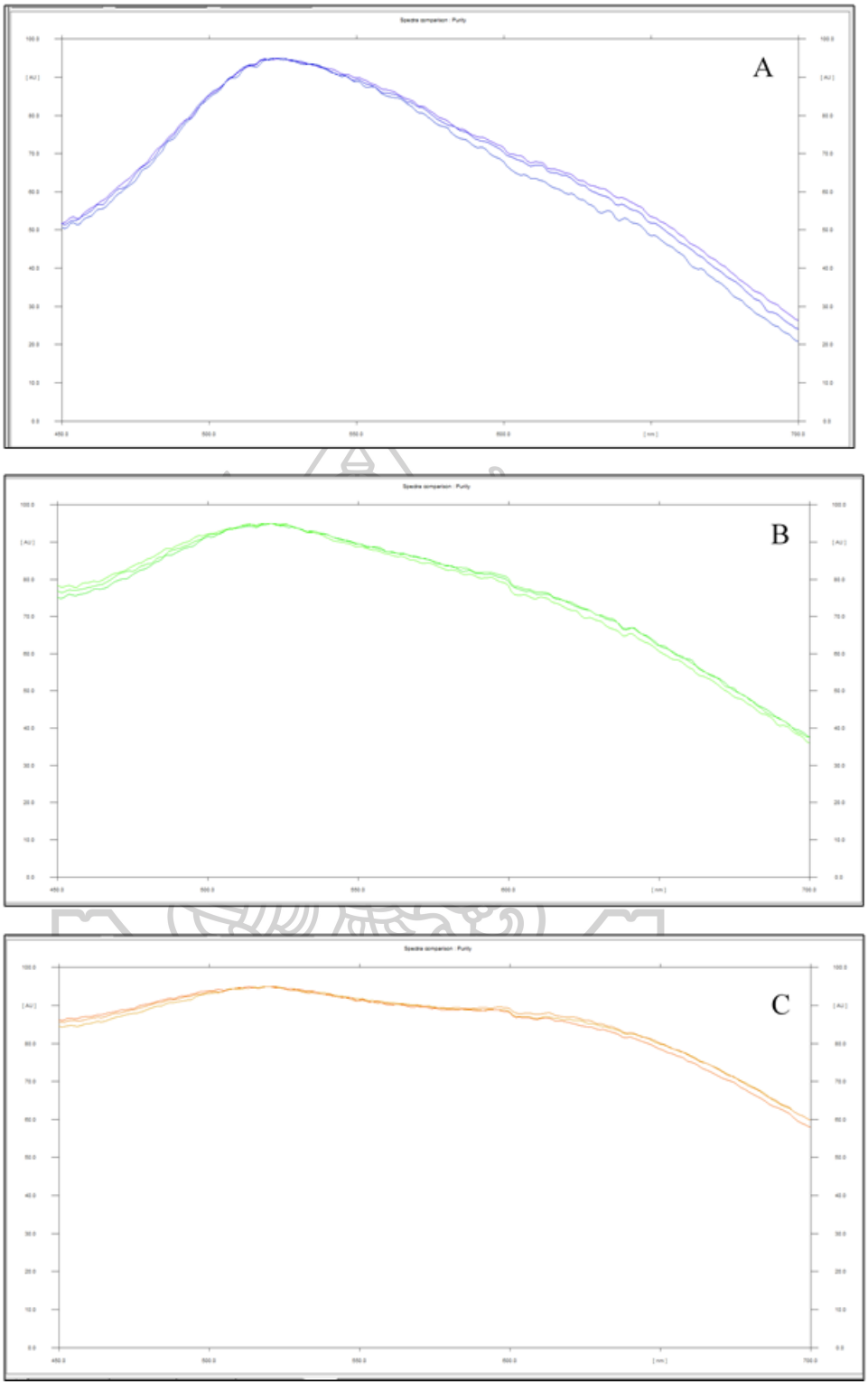

Figure 53 Overlay spectra of peak start, peak maximum and peak end at 450-700 nm of standard premnaodoroside A and sample: standard (A), 60% ethanolic extract (B), 100% ethanolic extract (C)

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