EXTRACTION OF SPENT GREEN TEA LEAVES AS BIOACTIVE-COMPOUND SOURCE

Miss Monsichar Yannarat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
Common Course
Faculty of Science
Chulalongkorn University
Academic Year 2018

การสกัดกากใบชาเขียวเพื่อเป็นแหล่งของสารออกฤทธิ์ทางชีวภาพ

น.ส.มนสิชา ญาณรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	EXTRACTION OF SPENT GREEN TEA LEAVES AS BIOACTIVE-COMPOUND SOURCE
By	Miss Monsichar Yannarat
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Science
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor PREECHA PHUWAPRAISIRISAN, Ph.D.)

..... Thesis Advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

..... Examiner
(Assistant Professor Varapha Kongpensook, Ph.D.)

..... External Examiner
(Wachiraporn Phoonan, Ph.D.)

มนสิชา ญาณรัตน์ : **การสกัดกากใบชาเขียวเพื่อเป็นแหล่งของสารออกฤทธิ์ทางชีวภาพ**. (EXTRACTION OF SPENT GREEN TEA LEAVES AS BIOACTIVE-COMPOUND SOURCE) **อ.ที่ปรึกษาหลัก** : **ผศ. ดร.วรินทร ชวศิริ**

กากใบชาเขียวเป็นปัจจัยหลักที่ทำให้ต้นทุนการผลิตชาเขียวพร้อมดื่มสูง เนื่องจากมีปริมาณของเหลือทิ้งมากและมีค่าใช้จ่ายในการกำจัดสูง งานวิจัยนี้ศึกษาตัวทำละลายและเวลาในการสกัดกากใบชาเขียวที่ให้ผลผลิตของสารประกอบที่ดี และเพื่อแยกสารออกฤทธิ์ทางชีวภาพออกมาในรูปของส่วนที่มีปริมาณของสารสำคัญสูง และสารบริสุทธิ์ ส่วนที่แยกได้ถูกทดสอบฤทธิ์ต้านออกซิเดชั่น ฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส ฤทธิ์ยับยั้งเอนไซม์คลอไลน์เอสเทอเรส และฤทธิ์กระตุ้นการเจริญเติบโตของพืช สารกาเฟอีนถูกพบในทุกสิ่งสกัด สิ่งสกัดจากตัวทำละลายทั้งห้าชนิด สิ่งสกัดด้วยไดคลอโรมีเทนให้ปริมาณกาเฟอีนสูงที่สุด (92.8%) ในขณะที่สิ่งสกัดเมทานอลมีปริมาณสารในกลุ่มคาเทชินสูงสุด (64.5%) ถูกแยกต่อโดยวิธีสกัดด้วยของเหลวและของเหลว ส่วนของน้ำถูกสกัดด้วยเอทิลเอทิลอะซิเตต ได้สิ่งสกัดที่มีปริมาณของสารในกลุ่มคาเทชินสูง (EGC, C, EC, EGCG, ECG) และปริมาณกาเฟอีนต่ำ ส่วนที่มีปริมาณสารในกลุ่มคาเทชินสูงถูกแยกได้เป็นสี่ส่วนย่อย สิ่งสกัดที่มีปริมาณกาเฟอีนสูงและสิ่งสกัดที่มีปริมาณสารในกลุ่มคาเทชินสูงมีประสิทธิภาพในการออกฤทธิ์ทางชีวภาพค่อนข้างน่าสนใจ กาเฟอีน, กรดแกลลิก และ ECG เป็นสารออกฤทธิ์ทางชีวภาพที่มีอยู่ในกากใบชาเขียว สารออกฤทธิ์ทางชีวภาพถูกแยกจากกากใบชาเขียวแสดงฤทธิ์ทางชีวภาพ ได้แก่ ฤทธิ์ต้านออกซิเดชั่น, ฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส, ฤทธิ์ยับยั้งเอนไซม์คลอไลน์เอสเทอเรส และ ฤทธิ์ควบคุมการเจริญเติบโตของพืช กากใบชาเขียวเป็นแหล่งที่มาของสารออกฤทธิ์ทางชีวภาพที่ยอดเยี่ยม และสิ่งสกัดที่มีปริมาณของสารที่สนใจสูงเป็นส่วนที่น่าสนใจสำหรับใช้ในการพัฒนาสูตรอาหารและยาสมัยใหม่

สาขาวิชา	**เทคโนโลยีชีวภาพ**	**ลายมือชื่อนิสิต** .....
ปีการศึกษา	2561	**ลายมือชื่อ อ.ที่ปรึกษาหลัก** .....

5972135123 : MAJOR BIOTECHNOLOGY

KEYWORD: Spent green tea leaves extract, Enrich-caffeine fraction, Enrich-catechin family fraction

Monsichar Yannarat : EXTRACTION OF SPENT GREEN TEA LEAVES AS BIOACTIVE-COMPOUND SOURCE. Advisor: Asst. Prof. Warinthorn Chavasiri, Ph.D.

Spent green tea leaves (SGTL) are a major contributor to the high cost of commercial tea products because of the large amount of leftover and the cost of disposal. This research investigates on the solvent and time for the extraction of SGTL that provides good yield of potential compound and to separate bioactive compounds as enrich-fractions and pure compounds. The obtained fractions were evaluated for antioxidant, antityrosinase, anticholinesterase and plant growth regulator activities. Caffeine was found in all samples. Among five extracts, the dichloromethane extract revealed high caffeine content (92.8 %peak area), while the methanol extract showed the highest five-catechin content (64.5 %peak area). Further separation of the latter by liquid-liquid extraction, the aqueous phase was extracted with ethyl acetate to gain high catechin family content (EGC, C, EC, EGCG, ECG) fraction with low caffeine. Four subfractions were separated from enrich-catechin family fraction. The enrich-caffeine and enrich-catechin family fractions displayed remarkable biological effects. Caffeine, gallic acid and ECG were bioactive compounds responsible for the biological activity in SGTL. Bioactive compound were isolated from SGTL showed bilogical activity including antioxidant, antityrosinase, anticholinesterase and plant growth regulation. SGTL was proved to be an excellent source of bioactive compounds. The enrich fractions served as prospective materials for the exploration of new multi-functional food and drug formulations.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, the author would like to express her sincere gratitude to Assistant Professor Dr. Warinthorn Chavasiri, Center of Excellence in Natural Product Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University for his generous guiding, kindly helpful suggestions, valuable assistance and encouraging throughout the entire period of this research.

Sincere thanks are extended to Associate Professor Preecha Phuwapraisirisan, Assistant Professor Dr. Varapha Kongpensook, Dr. Wachiraporn Phoonan serving as the chairman and members of thesis committee, for their valuable comments, discussion and suggestions. Many thanks to Oishi group public company limited for providing plant material (Spent green tea leaves) used in this study.

The author would like to thank Ms. Asshaima Paramita Devi, Ms. Suekanya Jaruinthusophon and all member in Center of Excellence in Natural Product Chemistry and Program in Biotechnology, Faculty of Science, Chulalongkorn University for the advice and scientific techniques during research. Thanks, are also extended to my friends and sister for their friendliness, helpful advice, cheerful attitude and encouragement.

Moreover, the author would like to express her deepest appreciation and gratefulness to her parents including Mr. Anawat Yannarat and Mrs. Soontaree Yannarat and my family member for their love, understanding, inspiration, great support and encouragement throughout the entire study. Without them, the author would never have been able to achieve this goal.

Monsichar Yannarat

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

page

LIST OF SCHEMES

page

LIST OF ABBREVIATIONS

µL	microliter
µm	micrometer
°C	degree Celsius
^{13}C-NMR	carbon-13 nuclear magnetic resonance
^{1}H-NMR	proton nuclear magnetic resonance
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ACh	acetylcholine
AChE	Acetylcholinesterase enzyme
AD	Alzheimer's disease
BChE	Butyrylcholinesterase enzyme
BHT	Butylated hydroxytoluene
C	catechin
$CDCl_3$	deuterate chloroform
CF	caffeine
CH_2Cl_2	dichloromethane
Cm	centimeter
CQE	mg catechin equivalent
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMSO-d_6	deuterate dimethylsulfoxide
Dopa	3,4-dihydroxyphenylalanine

DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC	epicatechin
EC_{50}	Half maximal effective concentration
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EN	enrich fraction
EtOAc	ethyl acetate
EtOH	ethanol
g	gram
GA	gallic acid
GAE	mg gallic acid equivalent
GTL	green tea leaves
H_2O	water
HBTA	5-hydroxy-1,4-benzothiazinylalanine
HPLC	high performance liquid chromatography
IC_{50}	Half maximal inhibition concentration
ICAQ	indole-2-carboxylic acid-5,6-quinone
IQ	indole-5,6-quinone
L-DOPA	L-3,4-dihydroxyphenyl alanine
MeOH	methanol
mg	milligram

mL	milliliter
mm	millimeter
MS	Microsoft
nm	nanometer
OH	hydroxide
ORAC	Oxygen radical absorbance capacity
PGRs	plant growth regulators
ppm	part per million
R	antioxidant radical
R:H	antioxidant radical scavenger
R^2	regression
SD	standard deviation
SGTL	spent green tea leaves
TFC	total flavonoid content
TPC	total phenolic content
TRP	tyrosinase related protein
TYR	tyrosinase
UV	ultraviolet
w/v	weight per volume

CHAPTER I

INTRODUCTION

Tea (*Camellia sinensis*; Ericales; Theaceae) is the most widely consumed beverage aside from water in many countries around the world (Primavesi, Piantanida, and Pravettoni, 2014). This consumption is accompanied by the discarding of large amounts of tea waste into the environment (G. News, 2014). World tea production and consumption are forecasted to significantly increase during the period 2014–2020 (Transparency, Market, and Research, 2015). Three major kinds of tea, namely, black tea (fully fermented), oolong tea (partially fermented) and green tea (unfermented) are produced based on their manufacturing processes. Recently, green tea is receiving considerable attention for specific health benefits due to the presence of large amount of caffeine and catechins. (Wiseman, Balentine, and Frei, 1997) Thus, green tea is the most popular among the others.

Green tea has become popular beverage in Thailand for many years. In 2016–2017, the marketing value of green tea drink was 3,187 million Bahts, a decrease of 12% from 2015. As a result, the entrepreneurs want to stimulate the marketing and decreasing of production costs. Spent green tea leaves (SGTL) are another factor contributing to higher production costs because of high waste disposal cost. There also have caused growing environmental problems, as those contain substantial levels of organic substances which require costly treatment upon their disposal. Many researchers have been interested in utilizing SGLT for particle board production (M. A. Batiancela, M. N. Acda, and R. J. Cabangon, 2014) and hydrogen production (Ayas and Esen, 2016). The purpose of this research is to examine type of solvent and extraction time of SGTL and to separate the extract into enrich fraction and compound, and to further study on their chemical and biological properties. The study also has the aim to increase value of SGTL from green tea drink industry.

1.1 General characteristics of green tea

Green tea (**Figure 1.1**) was non-fermented tea and was a famous herbal plant as an antioxidant with abundant health benefits and considered as one of the most popular beverages in the world. There is increasing evidence that specific substances found in the tea leaves can enhance health benefit. Recently, researchers suggest that some compounds including caffeine and catechins in tea leaves play an important role to prevent cardiovascular disease and some cancers. There are many catechins in green tea leaves, and principal catechins are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), and (+)-catechin (C). The chemical structures of five catechins and caffeine are shown in **Figure 1.2**

Figure 1.1 Characteristics of green tea leaves

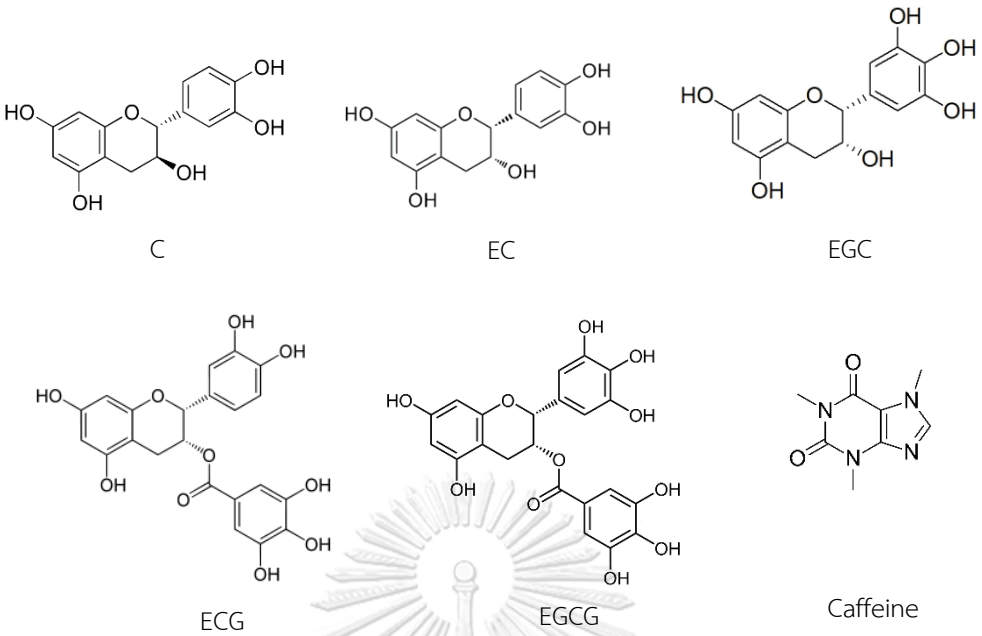


Figure 1.2 Chemical structures of catechins and caffeine

1.1.1 Chemical constituents in green tea

1.1.1.1 Catechin

Catechin is present in many dietary products, plants, fruits (such as apples, blueberries, gooseberries, grape seeds, kiwi, strawberries), green tea, red wine, beer, cacao liquor, chocolate, cocoa, *etc.* (Anand, Gill, and Mahdi, 2014) Catechins (flavan-3-ols) belong to the group of polyphenols. Catechin (C), (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC) (**Figure 1.2**) were the major polyphenols in green tea. (McKay and Blumberg, 2002) Green tea and its catechin components are known to stimulate antioxidant activity by scavenging free radicals, inhibiting pro-oxidant enzymes, and stimulating antioxidant enzymes. (Leelayuwat, 2017)

1.1.1.2 Caffeine

Caffeine is the most abundant alkaloid in green tea, being its amount in fresh leaves around 2–5% mass of the dry weight. (Park, Im, and Kim, 2012; Perva-Uzunalić *et al.*, 2006) The effects of caffeine as stimulant of the central nervous system are well known. Some adverse effects are derived from its consumption, including sleep

deprivation, tachycardia, abortion and miscarriages. Similarly to caffeine, theophylline and theobromine stimulate the central nervous system, but its amount in green tea is lower than 0.5% mass of the dry weight (Engelhardt, 2010; Zhao *et al.*, 2011).

1.2 Biological activity

1.2.1 Antioxidant activity (N. Liang and Kitts, 2014)

Antioxidants are those substances that can donate a hydrogen atom or an electron to a radical, and thereby disrupt its ability to continue the free radical chain propagation process. By donating a hydrogen atom or an electron, the oxidized antioxidant becomes a radical species. It is important that the antioxidant radical that is formed be sufficiently stable (low in energy) to be unable to continue the free radical propagation. Evaluation of the antioxidant capacity of sample therefore has been the focus for many studies that have used distinct *in vitro* chemical and enzymatic assays; some of which employ stable radicals as probes to measure free radical scavenging activity. In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses absorption once the odd electron is paired off by a hydrogen or electron-donating antioxidant. (**Figure 1.3**)

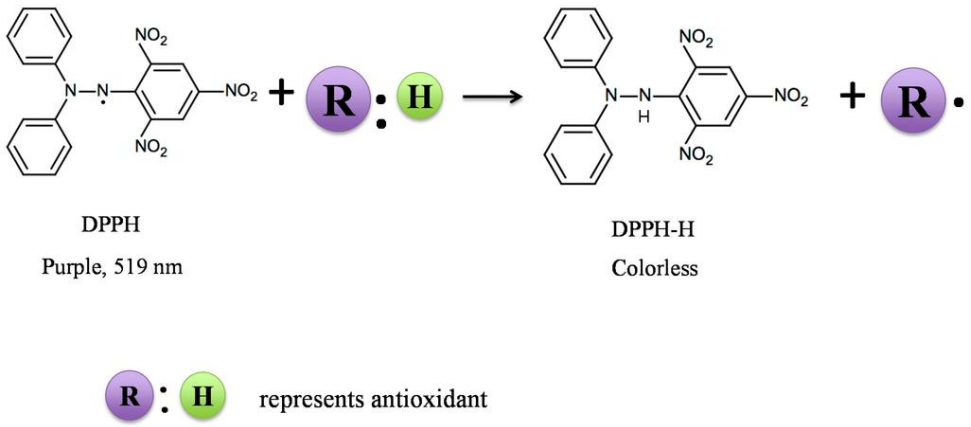


Figure 1.3 Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. R:H = antioxidant radical scavenger; R = antioxidant radical

1.2.2 Anti-tyrosinase activity (Chang, 2009)

Tyrosinase (EC 1.14.18.1) has copper structure on the active site that accelerates the hydroxylation reaction. The hydroxylation of L-tyrosine was changed to L-DOPA (L-3,4-dihydroxyphenyl alanine) and the oxidation of L-DOPA was as dopamine (L-dopaquinone) until the pathway of two pigments were produced. The first type was brown or black pigment (eumelanin) that found in dark skin. Then, red or yellow pigments (pheomelanin) was found in paleface. The melanocytes occurred at the epidermis. Melanin was produced to protect ultraviolet radiation. If the melanin was damaged, it could cause skin disorders such as dark circles, freckles and spots. The melanin production process could be controlled by inhibition of the enzyme activity. (**Figure 1.4**)

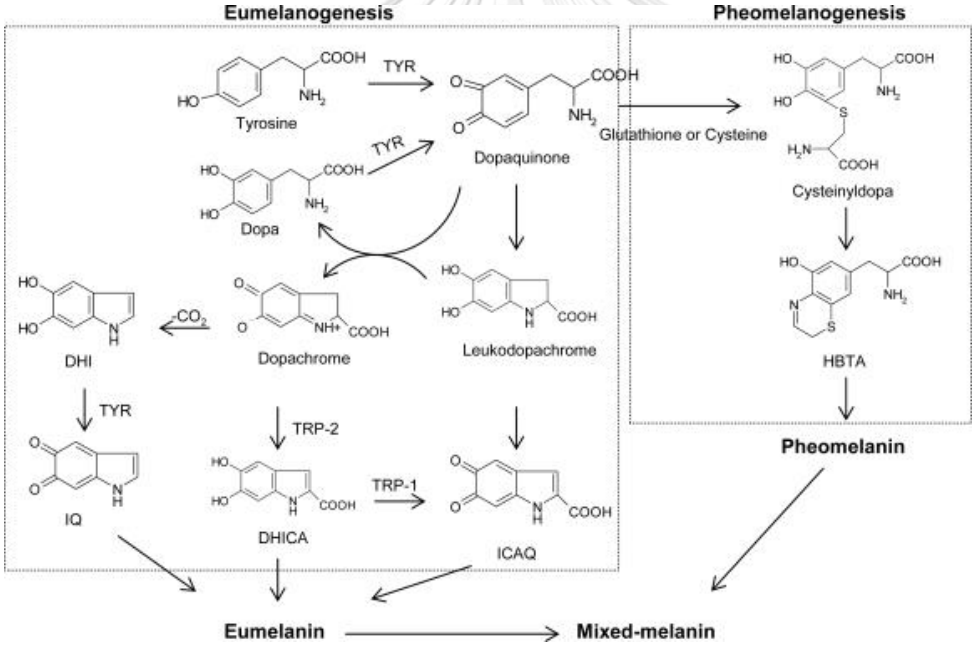


Figure 1.4 Biosynthetic pathway of melanin

(TYR, tyrosinase; TRP; tyrosinase related protein; dopa, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5,6-quinone; IQ, indole-5,6-quinone; HBTA, 5-hydroxy-1,4-benzothiazinylalanine)

1.2.3 Cholinesterase inhibition (Pohanka, 2014)

Inhibitors of AChE (EC 3.1.1.7.) and BChE (EC 3.1.1.8) are neurotoxic compounds capable of causing central, peripheral or both central and peripheral cholinergic crises. A number of these compounds have also found application as drugs developed for the treatment of Alzheimer's disease (AD) and myasthenia gravis. These are based on the premise that increasing the availability of acetylcholine (ACh) at acetylcholine receptors in the brain, results in better neuron to neuron transport that will improve cognitive function. Cholinergic nerves, however, can be found in both the central (CNS) and peripheral (PNS) nervous systems and disparate body tissues. The pathway of cholinesterase enzyme is shown in **Figure 1.5**. ACh was a CNS messenger important for learning and memory. ACh was degraded by AChE. In AD, ACh level was low due to excessive degradation by AChE. Cholinesterase inhibitors corrected the deficit of ACh by blocking the action of AChE and thereby increasing the amount of Ach, that remained in the synaptic cleft. Cholinesterase inhibitors as disease progress, the brain gradually produced less acetylcholine and cholinesterase inhibitors may lost their effect.

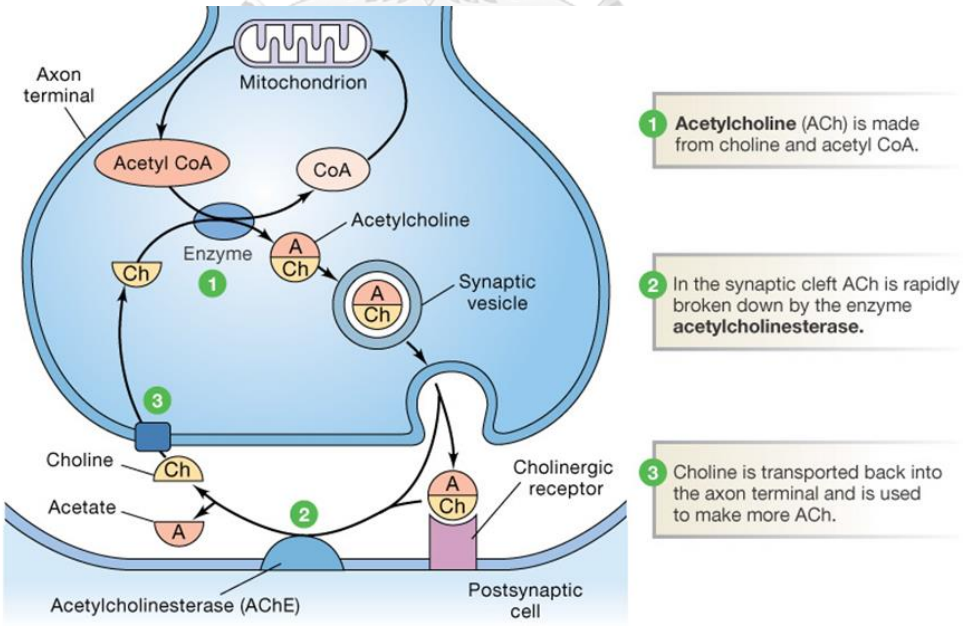


Figure 1.5 Principle of the anticholinesterase pathway

1.2.4 Plant growth regulation (Gray, 2004)

Plant growth regulators (PGRs) possessed similar characteristic as plant hormone. PGRs were chemicals applied by a horticulturist to regulate plant growth. However, the difference between PGRs and plant hormone was PGRs were no made within the plant and do not meet all the criteria of plant hormones listed above. Many PGRs were synthetic chemicals and include herbicides, defoliants, rooting compounds, and compounds used in tissue culture. Hormones and PGRs were broadly grouped as ethylene, auxins, cytokinin, jasmonic acid, brassinosteroids, gibberellins and abscisic acid. (**Figure 1.6**)

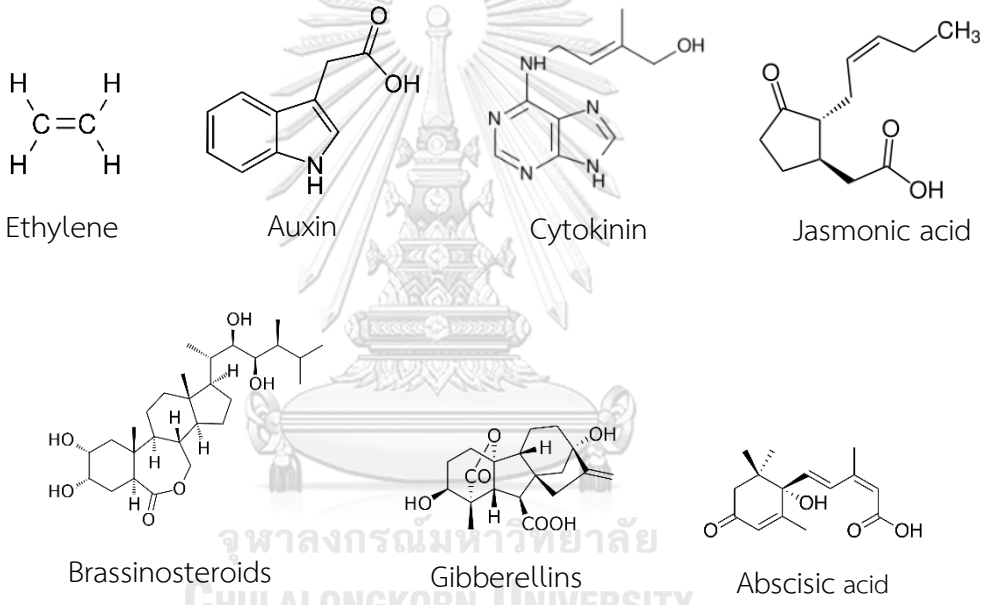


Figure 1.6 Chemical structures of plant hormone and plant growth regulators

1.3 Literature review

The chemical composition of green tea leaves has been widely investigated and reported to contain catechin and its glycosides, chlorogenic acid, coumarylquinic acid and theogallin (3-galloylquinic acid), caffeine, theobromine and theophylline. The major chemical composition of green tea leaves was catechins 30-42% (Graham, 1992). There are many reports to affirm that green tea was health beneficial due to the presence of large amount of caffeine and catechins. Caffeine content was high in green

tea leaf extract and was studied for toxicity and insecticidal activity in coffee arabica extracts and caffeine were examined. The dichloromethane extract gave the best results for EC_{50} of 4380 ppm on insect repellent. (Phankaen *et al.*, 2017) Caffeine showed plant growth regulator of seeds and stem cutting. (Puengyen, 2005)

Green tea has the highest catechin and caffeine content comparing with other tea types.(Carloni *et al.*, 2013) The antioxidant activity was conducted in several assays such as DPPH, ABTS and ORAC. The activity was in well corresponded with the amount of catechin content. (Senanayake, 2013) The antioxidant potential of green tea can lower cholesterol and diabetes (Spadiene *et al.*, 2014) and showed anticancer such as lung cancer, oral cancer, esophageal cancer, stomach cancer, liver cancer, prostate cancer, *etc*. (Hamilton-Miller, 2001) Green tea constituents were bioactive with beneficial effects on the body such as the prevention of Alzheimer's, anti-inflammatory, antimicrobial activity *etc*. (Cabrera, Artacho, and Giménez, 2006). As good biological activity in cosmetic, green tea constituents also revealed high levels of collagenase inhibitory and antityrosinase activity. (Makimura *et al.*, 1993)

Green tea is mainly obtained from the leaves of *Camellia sinensis* L. Spent green tea leaves (SGTL) remained after the preparation of green tea beverage and resulted in as solid waste product. These were not only polluting the environment but also represented a loss of valuable resource. To tackle this problem, during the past few decades, some researchers investigated the possibility of exploiting SGTL as a feedstuff in the diet of ruminant (Kondo, Kita, and Yokota, 2006) and lambs (Nasehi *et al*. 2014), pest management of formicine ant (Dieng, Zawawi, Yusof, Ahmad, and Abang, 2016), particle board production (M. A. Batiancela, M. N. Acda, and R. J. J. J. o. C. M. Cabangon, 2014), hydrogen production (Ayas and Esen, 2016), adsorbents for synthetic dyes and toxic metals (Lin *et al*. 2007, Wan *et al*. 2014, Chandrashekhar *et al*. 2019), polymer composites (Mattos *et al.*, 2014; Xia *et al.*, 2015), bio-film (Duan *et al.*, 2016) and bio-energy (Naruephat and Patcharee 2015). In comparison, the SGTL showed higher polyphenol and antioxidant activity than oolong tea and black tea. EC, EGC and GCG were major catechins present in SGTL (Nadiah and Uthumporn, 2015). SGTL can be used as source of protein at 5-10% (Theeraphaksirinont, Chanpongsang, Chaiyabutr,

and Topanurak, 2009). In the study reported by Tsubaki and Azuma (Tsubaki and Azuma, 2013), green tea residue was totally fractioned by a combination of microwave-assisted alkaline pretreatment and enzymatic hydrolysis. Alkaline pretreatment (Nadiah and Uthumporn, 2015) improved solubilization of green tea residue up to 74%. Alkali-soluble fraction contained polysaccharides, protein, phenolics and aliphatic compounds. Subsequent enzymatic hydrolysis mainly converted cellulose into glucose. The final residue was predominantly composed of aliphatic cuticular compounds which is a potential alternative feedstock of oil plants. In addition, SGTL showed antibacterial activity against *Pectobacterium* spp. causing soft rot on carrot. (Melvin Joe, Chanbhasa, John Henry, Benson, and 2017)

1.4 The goal of research

1.4.1 To study solvent and extraction time of spent green tea leaves and separation into enrich-fraction and as pure compound.

1.4.2 To determine the biological activity such as antioxidant, anti-tyrosinase, Cholinesterase inhibition and plant growth regulator of enrich fraction and compound.

CHAPTER II

MATERIAL AND METHODS

2.1 Plant material

Dried spent green tea leaves (SGTL) were obtained from Oishi group public company limited, Bangkok, Thailand in September 2017.

Germination tests were performed using seedlings of *Helianthus annuus* L. (sunflower), *Capsicum flutescens* Linn. (chilli), *Cuourtita moschao* Decen (pumpkin), *Zea mays* Linn. (corn), *Cucumis melo* L. (melon), *Dracaena goldieana* (queen of Dracaenas) and *Cymbopogon citratus* Stapf. (lemon glass) were selected as plant model for root growth promotion test. Stock plants were maintained under outdoor conditions so that succulent new shoots were available for cutting material throughout the testing period.

2.2 Chemicals

All solvents (acetone, dichloromethane, methanol, ethanol, hexane, ethyl acetate) from RCI Labscan Limited were commercial grade. Solvents for HPLC analysis were acetonitrile and methanol. The chemicals used were as follows: Folin-Ciocaltue reagent, gallic acid, sodium nitrite, aluminium trichloride, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, tris-HCl buffer from Sigma Aldrich chemical company.

2.3 Enzymes

Tyrosinase enzyme from mushroom, acetylcholinesterase enzyme and butyrylcholinesterase enzyme were purchased from Sigma chemical company.

2.4 Equipment and instruments

Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 F_{254}). Column chromatography was performed on silica gel (Merck Kieselgel 60 G).

High performance liquid chromatography (HPLC) (Waters Corporation, Anaheim, California, USA) was conducted using cosmosil C18 AR-II column (250 mm length, 10 mm i.d.; AllTech Associates, Waters) and UV detector. UV-visible adsorption was recorded on microplate reader spectrometer (BioTek instrument, headquartered in Winooski, VT, USA).

2.5 The extraction of spent green tea leaves

Dried SGTL were extracted using reflux method for 5, 15, 30, 60, 120 and 240 minutes with various solvents (acetone, dichloromethane, methanol, water and 50%ethanol) in ratio 1:10. The extract was filtered off and evaporated by rotatory evaporator to dryness to yield the crude extract.

2.6 Determination of total phenolic content (TPC) and total flavonoid content (TFC)

TPC was determined according to Folin-Ciocaltue assay method (Nadiah and Uthumporn, 2015). Briefly, 20 µL of sample, in triplicate, were introduced into 96 well plate, followed by 100 µL 10% Folin-Ciocaltue reagent and 80 µL of sodium carbonate solution (7.5% w/v). The solutions were well mixed and placed in dark for 30 minutes at room temperature. The absorbance was measured at 765 nm using UV-visible spectrophotometer. Gallic acid was used for constructing the standard curve. TPC was expressed as mg gallic acid equivalent (GAE), per g dry weight material.

TFC was determined according to Nadiah's method using aluminium trichloride (Nadiah and Uthumporn, 2015). Briefly, 25 µl of the extract solution was added to 96 well plate with 100 µl of distilled water, subsequently, about 50 µl of sodium nitrite solution (5% w/v) was added to the mixture and kept for 5 minutes, followed by adding 15 µl of 0.75 M aluminium trichloride. After incubation for 6 minutes, 50 µl of 1 M sodium hydroxide was added to the solution. The mixture was further diluted with 50 µl of distilled water and shaken vigorously. The absorbance of the mixture was measured at 510 nm using UV-visible spectrophotometer and TFC was expressed as mg catechin per g dry weight material.

2.7 Separation of spent green tea leaves extract

2.7.1 Separation of spent green tea leaves extracts to enrich fraction

Dried SGTL were extracted with dichloromethane using 1:10 ratio by refluxing method for 4 hours. The extract of SGTL was filtered off and evaporated by rotatory evaporator to dryness to get "enrich-caffeine" fraction. Then, the residue was extracted with methanol. Crude methanol was dissolved with distilled water and extracted with dichloromethane for 3-5 times. Two phases were collected. The aqueous was extracted with ethyl acetate to get "enrich-catechin family" fraction.

2.7.2 Separation of "enrich-catechin family" fraction

The "enrich-catechin family" fraction (ethyl acetate fraction) was chromatographed on silica gel column eluting with ethyl acetate: dichloromethane: hexane: acetic acid (3:1:2:0.5), followed by methanol. All eluted fractions (15 mL/fraction) were collected and the chemical profile of each fraction was determined by TLC. Due to the similar pattern of chemical compounds on TLC, fractions were pooled and evaporated. The fractions were further purified by HPLC (ReproSil-Pur 120 C18 AQ, 5 µm, 250x10 mm) using linear gradient of A (acetonitrile:water:acetic acid (193:6:1)) and B (acetonitrile:water:acetic acid (139:60:1)) .The separation furnished two compounds, namely gallic acid and epicatechin gallate (ECG). Their structures were elucidated by means of ^{1}H and ^{13}C NMR spectroscopy.

2.8 Biological activity tests of enrich fractions and isolated compounds

2.8.1 Antioxidant activity

The free radical scavenging activity of each sample was measured from the reduction of DPPH radical (Marinova, Batchvarov, and 2011)using eight different concentrations of sample in methanol. For each concentration, 50 µL was mixed with 100 µL of 0.05 g/L DPPH, incubated at room temperature for 30 minutes in the dark and then the absorbance was measured at 517 nm using microplate reader. Ascorbic acid was used as standard reference. Percent of DPPH inhibition was calculated from the following formula (2.1) and half maximal inhibitory concentration (IC_{50}) at 50% was determined for each sample.

$$\%\ DPPH\ inhibition = \frac{\Delta A\ control - \Delta A\ sample}{\Delta A\ control} x\ 100 \qquad (2.1)$$

where "$\Delta A\ control$" was the absorbance of control sample (including all reagents except the extract), "$\Delta A\ sample$" was the absorbance of solution while adding the sample extract.

2.8.2 Anti-tyrosinase activity

Tyrosinase inhibition assays were performed with L-tyrosine as substrate (Larik, Saeed, Channar, and Muqadar, 2017). The reaction mixture in 96 well plate contained 50 μL of mushroom tyrosinase (250 U/mL), 50 μL of sample solution in buffer and 50 μL of 5 mM of substrate. After the addition of substrate, the reaction was immediately monitored at 490 nm for dopachrome formation in the reaction mixture. Kojic acid was used as positive control. The concentration range of extract used for the mushroom tyrosinase inhibition assay was 0–1 mg/mL. Percent of tyrosinase inhibition was calculated from the following formula (2.2) and IC_{50} was determined for each sample.

$$\%\ Tyrosinase\ inhibition = \frac{\Delta Acontrol - \Delta A\ sample}{\Delta A\ control} x\ 100 \qquad (2.2)$$

Where "$\Delta Acontrol$" was the change of absorbance at 490 nm without test sample, "$\Delta Asample$" was the change with test sample.

2.8.3 Cholinesterase inhibition

The cholinesterase inhibition activity was measured using Ellman's reagent (Barbosa Filho *et al.*, 2006). Briefly 25 μL of 1.5 mM substrate (ATCI and BTCI), 50 μL of 50 mM tris–HCl buffer (pH 8.0), 125 μL of 3 mM DTNB, and 25 μL of sample dissolved in 50 mM tris–HCl buffer (pH 8.0) were added to the wells, incubated 10 minutes followed by 25 μL of 0.3 U/ml enzyme (AChE and BChE). The reaction was measured at 405 nm, using microplate reader, in triplicate experiments. Enzyme activity was calculated as percentage of the velocities of sample compared to the

negative control. Percent of cholinesterase inhibition was calculated from the following formula (2.3) and IC_{50} was determined for each sample. Galantamine was used as a positive control.

$$\%\ EA = \frac{\Delta E - \Delta S}{\Delta E} x\ 100 \qquad (2.3)$$

Where *"E"* was the activity of the enzymatic without test sample, *"S"* was the activity of enzymatic with test sample.

2.8.4 Plant growth regulation

Plant growth regulation test was measured on seed germination and root growth promotion to verify the presence of bioactive compounds. (Puengyen, 2005)

2.8.4.1 Primary screening on germination of selected plant seeds

Tested solutions of enrich fractions and isolated compounds were prepared at the concentration of 1, 10, 100, 1000 ppm. 10 mL of tested solutions were added into a glass vial. A controlled vial contained an equal amount of the solvent used to prepare the tested solutions. Nine seeds of selected plants were soaked into glass vial containing tested and controlled solutions for 3 hours, allowed to dry in air for 5 minutes. Then the seeds were placed into petri dish (25 mm diameter) containing filter paper disc wetted with 10 mL of distilled water and covered by the lid and sealed with parafilm to prevent the evaporation of water. These petri-dishes were kept under daylight for 7 days. Growth was quantified by measuring dry weight of roots and shoots both from treated and controlled plants. All tested and controlled experiments were run in 4 replications (10 seeds of each plant per test per replication) and averaged. Percent of growth promotion was calculated from the following formula:

$$\%\ Growth\ promotion = \left[\left(\frac{T}{C}\right) x\ 100\right] - 100\% \qquad (2.4)$$

Where "*T*" was the mean roots dry weight of treated plant, "*C*" was the mean roots dry weight of controlled plant.

2.8.4.2 Root growth promotion

Enrich fractions and isolated compounds were dissolved in proper solvent and diluted with water to make the concentration of 1, 10, 100, 1000 ppm. Distilled water was used as control. 50 mL of prepared solution was added into plastic bottle. A controlled bottle contained an equal amount of the solvent used to prepare the tested solutions. Stem-cutting of *Dracaena goldieana* and *Cymbopogon citratus* were dipped into the solution for 30 minutes and allowed to dry in air for 5 minutes. The treated plants were individually dipped in the rooting medium, containing distilled water in plastic bottle. Then the plants were under outdoor conditions. After 7-14 days, the treated plants were removed from the medium. Growth was quantified by measuring dry weight of roots both treated and controlled plants. All tested and controlled experiments were run in 4 replications (16 samples per test per replication) and averaged. Percent of growth promotion was calculated from the following formula (see (2.4)).

2.9 Statistical Analysis

The results are presented as mean ± standard deviation (SD) of three replicates. Factorial ANOVA was used to test for significant differences in the percentages of inhibition and growth promotion. Least-significant different (LSD) ($p < 0.05$) were applied for the pairwise multiple comparisons. The statistical analyses were performed using IBM SPSS statistics version 22 for windows.

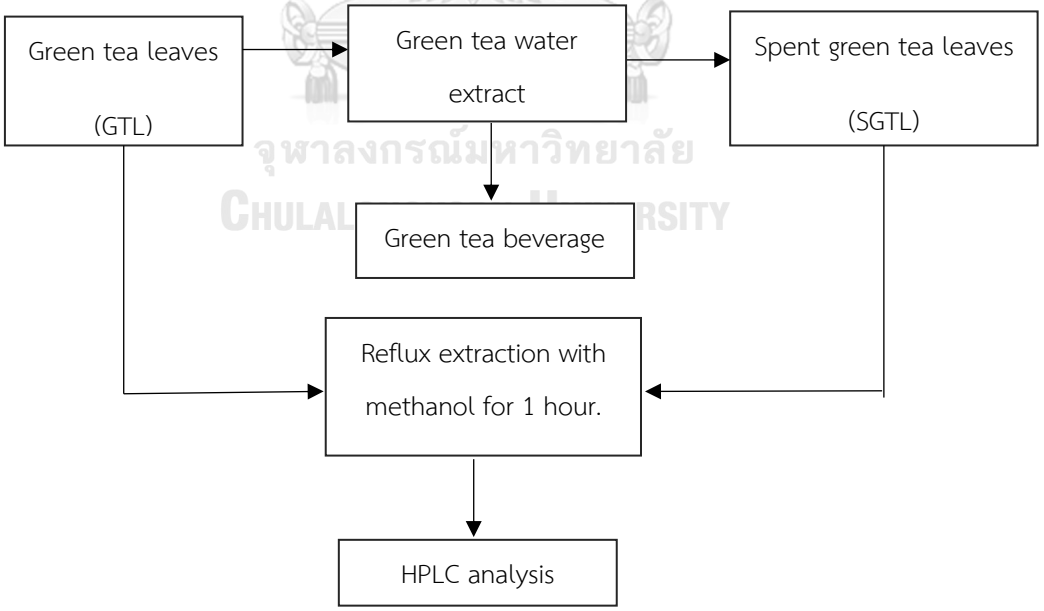
CHAPTER III

RESULTS AND DISCUSSION

The main aim of this research was to utilize SGTL as bioactive sources in form of enrich fraction or as pure compounds. The optimization for the extraction of SGTL using reflux method was justified by determination of percentage yield, total phenolic content (TPC) and total flavonoid content (TFC). The extract was separated in form of enrich fraction or as pure compounds. The preliminary screening tests including antioxidant, anti-tyrosinase, cholinesterase inhibition and plant growth regulator of enrich fraction and pure compounds were carried out. The outcome from this study would lead to new options for the utilization of SGTL.

3.1 Preliminary study of green tea and SGTL methanol extracts

SGTL is the leftover from green tea beverage production process. The preliminary study on chemical constituents in original green tea leaf (GTL) compared with SGTL is shown in **Scheme 3.1.**



Scheme 3.1 The preliminary study on original green tea leaf (GTL) and SGTL

The percentage yield and constituent distribution of both extracts are shown in **Figure 3.1** and **Table 3.1.**

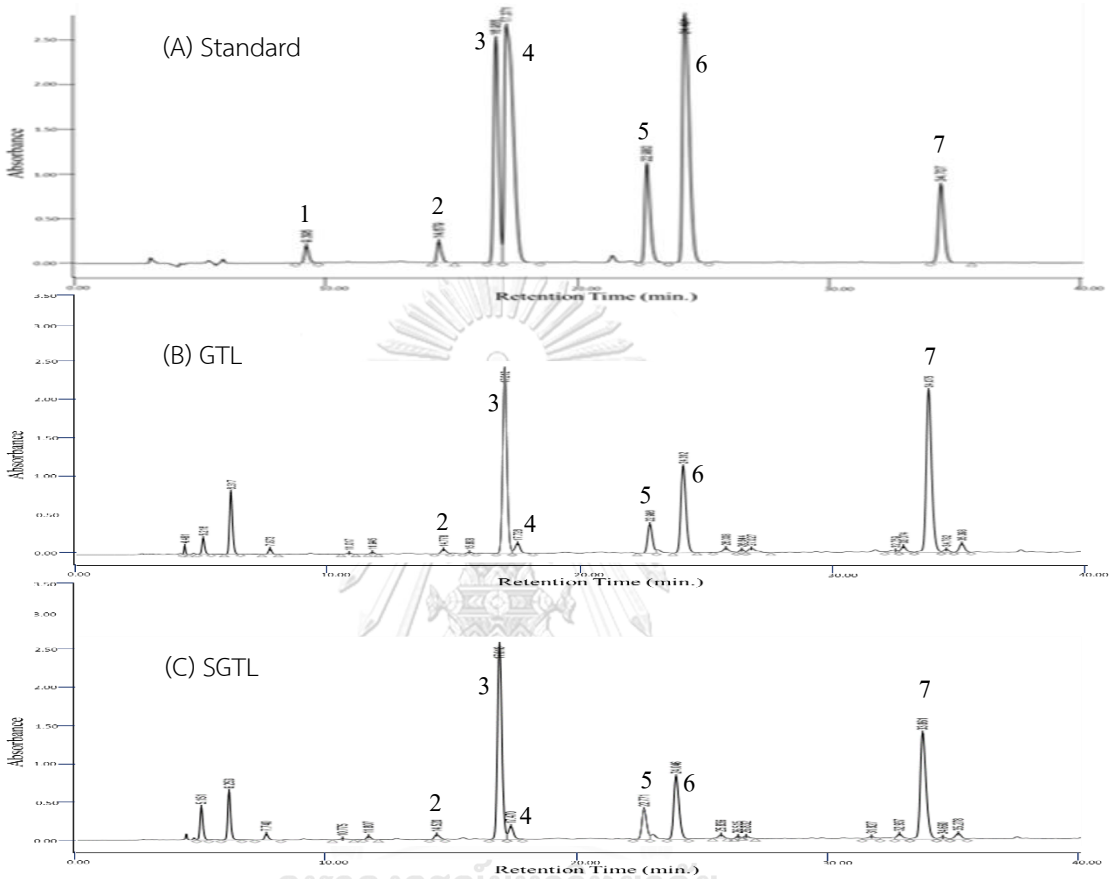


Figure 3.1 HPLC chromatograms of green tea and SGTL methanol extracts

1: GC (gallocatechin), 2: EGC (epigallocatechin), 3: CF (caffeine), 4: C (catechin), 5: EC (epicatechin), 6: EGCG (epigallocatechin gallate), 7: ECG (epicatechin gallate)

Table 3.1 The chemical composition of GTL and SGTL methanol extracts using HPLC

Sample	%yield	Constituent distribution (%peak area)						
		EGC	CF	C	EC	EGCG	ECG	Unknown
GTL	21.5	0.8	27.9	2.1	4.9	15.7	31.3	17.3
SGTL	10.5	1.1	34.7	2.8	6.0	13.0	24.7	17.7

* EGC: epigallocatechin, CF: caffeine, C: catechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate

The industrial process to extract GTL has been limited with the amount of caffeine containing in green tea beverage, which is regulated not to harm to consumers. Adults should not get caffeine exceed 250 mg per day. The legal of caffeine in green tea beverage should indicate caffeine content per volume of package on label attached packaging (Khan and Mukhtar, 2013). Green tea beverages in the market have caffeine content of 21-51 milligrams per 250 milliliters (T. News, 2012). Therefore, green tea beverages are extracted bioactive compounds not completely from green tea leaves. In the present study, methanol was used as extraction solvent. For comparison, GTL and SGTL were performed. The highest percent yield compared to SGTL extract (approximately 22 and 11%, respectively). The chemical compositions of all five catechins and caffeine in GTL and SGTL methanol extracts are shown in **Table 3.1**. Caffeine content in SGTL (34.7 %peak area) was higher than GTL (27.9 % peak area) methanol extracts. While five-catechin content of SGTL (47.6 %peak area) was lower than GTL (54.8 %peak area) methanol extracts that can be calculated as percent recovery approximately 15%. This might be due to the process during the green tea beverage manufacturing that reduced the levels of catechin.

3.2 Extraction of SGTL

The extraction yields normally depend on solvent, pH, temperature, extraction time, and composition of the sample. In this work, SGTL was extracted by diverse solvents including acetone, dichloromethane, methanol, water and 50%ethanol with various times (5, 15, 30, 60, 120 and 240 minutes). The percentage yields of SGTL extracts with various solvents and time shows in **Figure 3.2**.

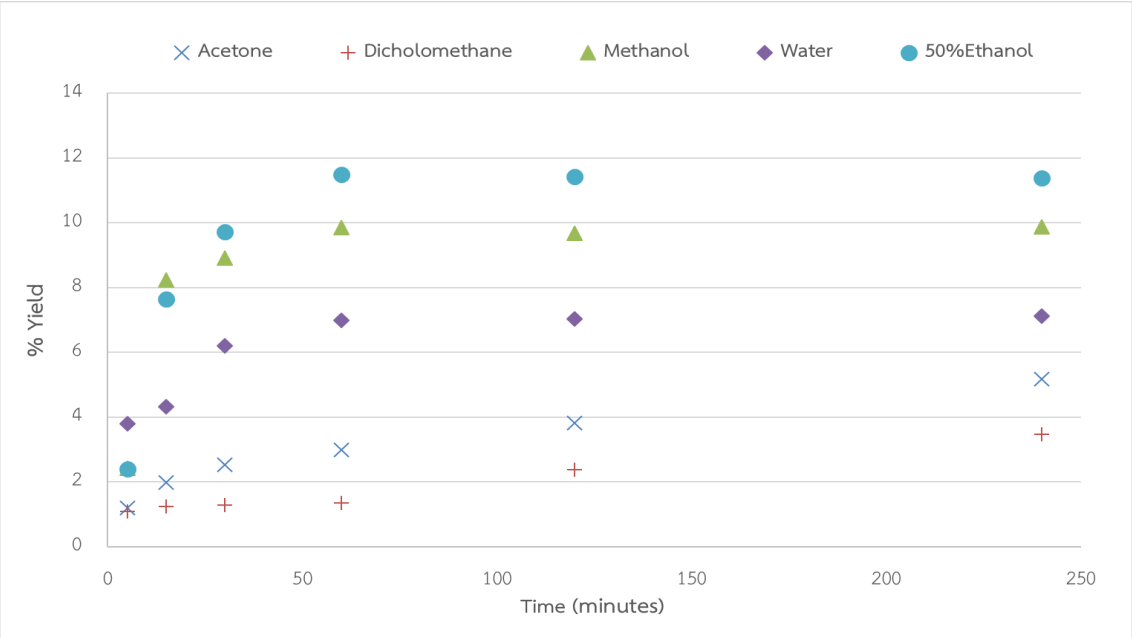


Figure 3.2 Percentage yield of SGTL extracts with various solvents and time

The duration of extraction is one of the major factors which influence the yield (Vuong, Golding, Stathopoulos, Nguyen, and Roach, 2011). The optimized time for the extraction with 50% ethanol, water and methanol was 1 hour. The SGTL extraction with acetone and dichloromethane reached its maximum after 4 hours; nevertheless, it was plausible that the yield could be further increased with longer extraction time. The highest yield of extraction by various solvents were in the following order: 50% ethanol > methanol > water > acetone > dichloromethane. The extract of each solvent was selected to determine TPC and TFC.

TPC of the extracts was calculated based on the calibration curve of gallic acid (R^2 = 0.993) whereas TFC was calculated from that of catechin (R^2 = 0.990) (**Appendix 1**). Phenolic compounds in plants have been known to display certain antioxidant behavior. (Škerget *et al.*, 2005). Their free radical scavenging ability is facilitated by their hydroxyl groups, the TPC could be used as basis for rapid screening of antioxidant activity. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of both *in vitro* and *in vivo* depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun, 2005). The results of TPC and TFC are presented in **Figure 3.3**.

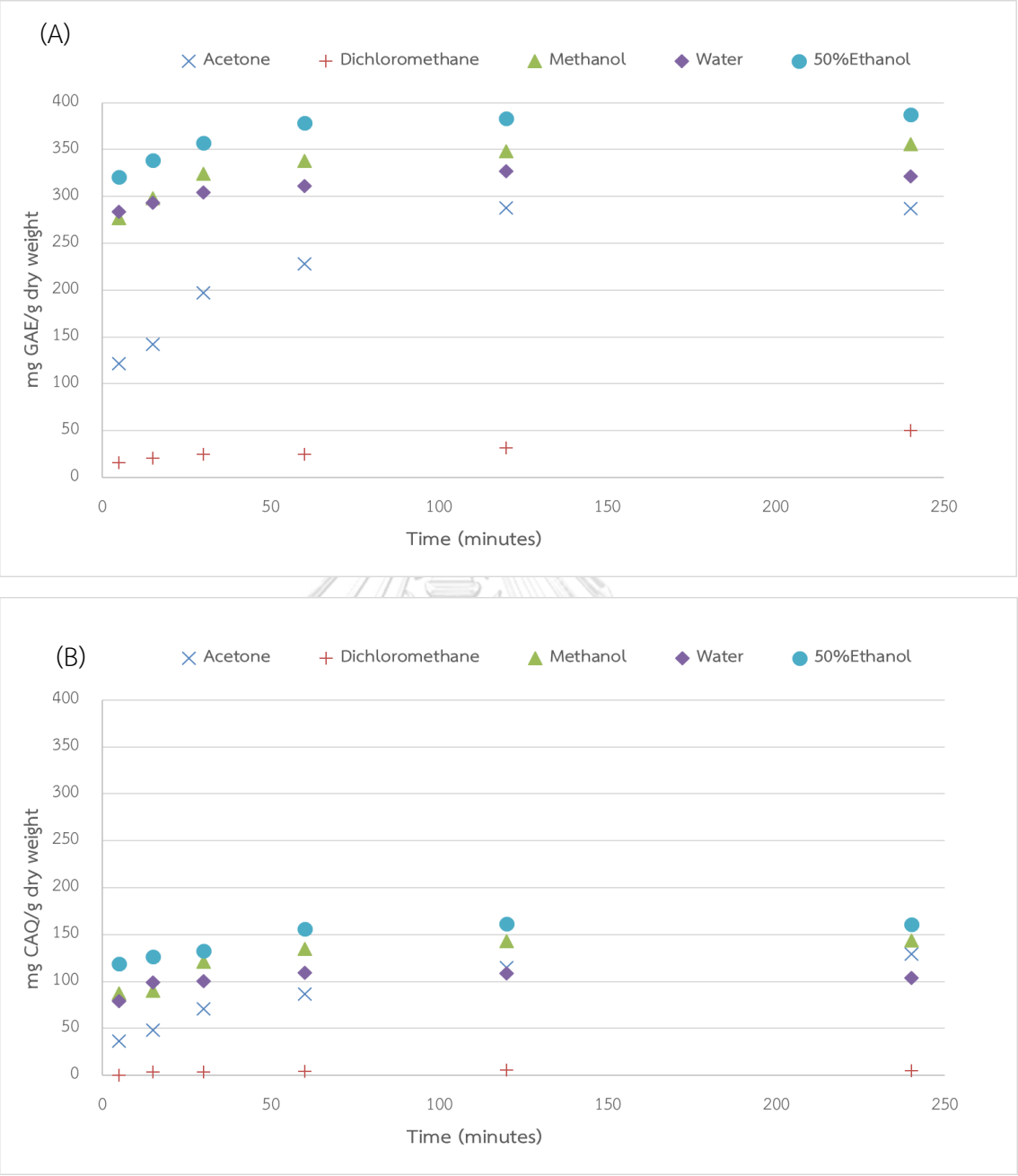


Figure 3.3 (A) TPC and (B) TFC of SGTL extracts

As the result, SGTL extract with 50% ethanol showed the highest TPC and TFC following by that with methanol while the dichloromethane extract showed the lowest. According to percentage yield, TPC and TFC, the appropriate extraction times of each solvent are summarized in **Table 3.2.**

Table 3.2 The percentage yield, TPC and TFC of SGTL extracts

Solvent	Time (minute)	%yield	TPC (mg GAE/g)	TFC (mg CEQ/g)
Acetone	240	5.2	288.87 ± 2.65	129.44 ± 0.68
Dichloromethane	240	3.5	50.06 ± 1.01	5.42 ± 0.07
Methanol	60	9.9	337.66 ± 1.73	134.99 ± 1.78
Water	60	7.0	311.05 ± 8.55	109.56 ± 1.46
50% Ethanol	60	11.5	378.62 ± 0.43	156.06 ± 3.70

GAE = gallic acid equivalent; CEQ = catechin equivalent.

Green tea is a rich source of phenolics, so the extraction efficiency of these compounds is highly dependent on the time of extraction and the solvents used (Rusak, Komes, Likić, Horžić, and Kovač, 2008). The main classes of polyphenols present in green tea are flavanols and phenolic acids. These compounds constitute 16–30% of dry weight of fresh leaf (Graham, 1992). Flavonoids are the most common, important, and widely distributed group of phenols that are present in plants with highly effective antioxidants (Panche, Diwan, and Chandra, 2016; Yanishlieva-Maslarova, 2001). **Table 3.2** shows the TPC and TFC of SGTL extracts measured using Folin-Ciocalteu's colorimetric method and aluminium trichloride. The SGTL extract with 50% ethanol for 1 hour showed the highest TPC and TFC (378.62 ± 0.43 mg GAE/g dry weight and 156.06 ± 13.70 mg CEQ/g dry weight, respectively) followed by that with methanol and water (337.66 ± 1.75, 311.05 ± 8.55 mg GAE/g dry weight and 134.99 ± 1.78, 109.56 ± 1.46 mg CEQ/g dry weight, respectively). Then, these samples were determined for the chemical composition by HPLC. (**Figure 3.4, Table 3.3**).

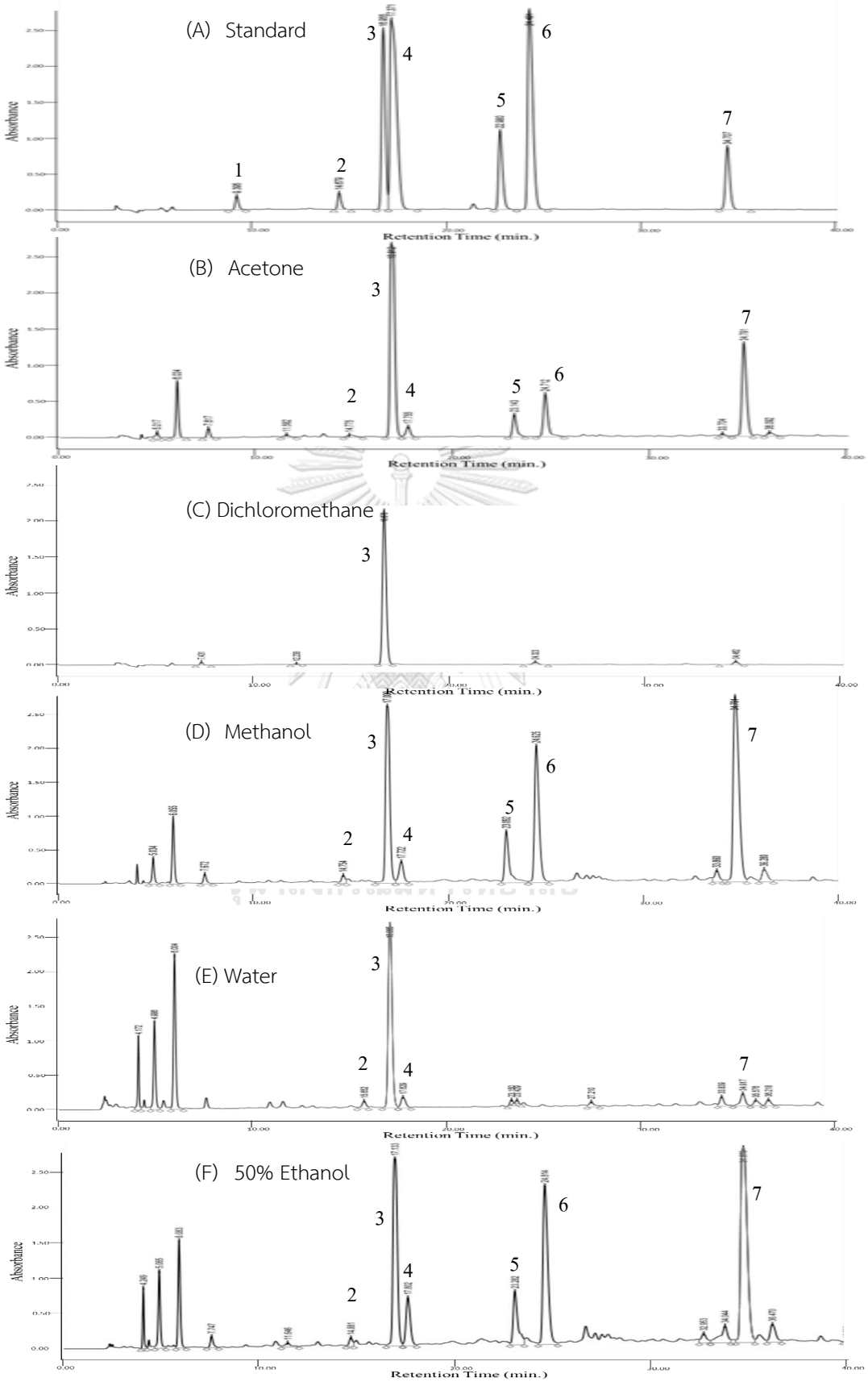


Figure 3.4 HPLC chromatograms of SGTL extract

1: GC (gallocatechin), 2: EGC (epigallocatechin), 3: CF (caffeine), 4: C (catechin), 5: EC (epicatechin), 6: EGCG (epigallocatechin gallate), 7: ECG (epicatechin gallate)

Table 3.3 The chemical composition of SGTL extract using HPLC

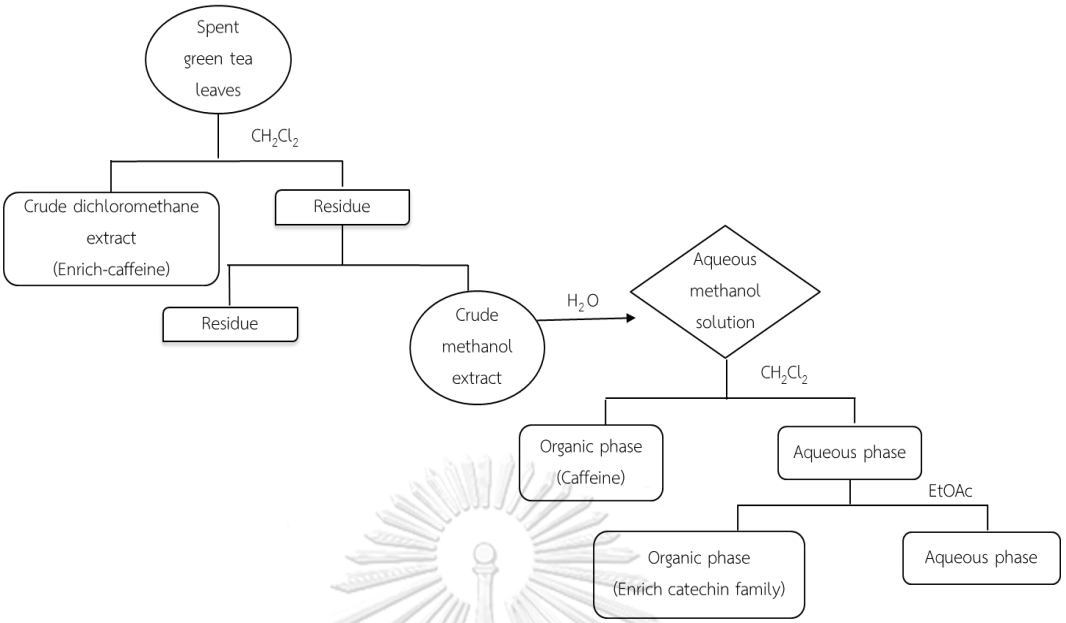
Solvent	Constituent distribution (%peak area)						
	EGC	CF	C	EC	EGCG	ECG	Unknown
Acetone	1.0	47.8	2.5	4.6	9.9	21.7	12.5
Dichloromethane	-	92.8	-	-	2.4	2.6	2.2
Methanol	1.0	23.8	3.2	7.8	19.2	33.3	11.7
Water	1.9	43.6	3.2	-	-	3.7	47.6
50% Ethanol	0.8	19.7	5.0	5.9	18.5	30.3	19.8

* EGC: epigallocatechin, CF: caffeine, C: catechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate

Leaves of green tea are rich in bioactive compounds, particularly phenolic compounds with antioxidant activity. The elevated proportion of catechins is related to biological functionality (Carloni *et al.*, 2013). The contents of caffeine (CF) and five catechins (EGC, C, EC, EGCG, ECG) in SGTL extract of each solvent are shown in **Figure 3.4** and **Table 3.3**. Caffeine was found in all samples. Among five extracts, the dichloromethane extract showed high caffeine content (92.8 %peak area, **Figure 3.4C**). Five catechins (EGC, C, EC, EGCG, ECG) were detected in acetone, methanol and 50%ethanol extracts (**Figures 3.4 B, D, F**). This result is supported by Zuo, Chen, and Deng, 2002, which stated that large amount of polyphenols and caffeine still remained in SGTL. The polarity of methanol has influenced for the extractability of polyphenols in SGTL. The methanol extract showed the highest five-catechin content with 64.5%peak area, whereas the water extract showed the highest unknown content, presumably hydrolyzable and condensed tannin (Graham, 1992).

3.3 Separation for enrich fraction

The solvent extraction was used to separate for enrich fraction. First, SGTL was extracted with dichloromethane furnishing enrich caffeine fraction. Then the residue was further extracted with methanol. The separation procedure of SGTL is shown in **Scheme 3.2.**



Scheme 3.2 Separation procedure for enrich-caffeine and enrich-catechin family fractions

From HPLC analysis (**Figure 3.4**), it could be concluded that the "enrich-caffeine" fraction was derived from the dichloromethane extract while five catechins were gained from the methanol extract. Therefore, methanol was used to extract five catechins after "enrich-caffeine" fraction was obtained. Decaffeination of the methanol extract was performed by liquid-liquid extraction with dichloromethane. After that "enrich catechin family" fraction was further extracted into ethyl acetate phase.

3.3.1 Examination of enrich caffeine fraction

SGTL (300 g) was refluxed with dichloromethane for 4 and 24 hours, respectively. The solvent was evaporated by rotatory evaporator. The percentage yield and constituent distribution of both extracts are shown in **Figure 3.5** and **Table 3.4.**

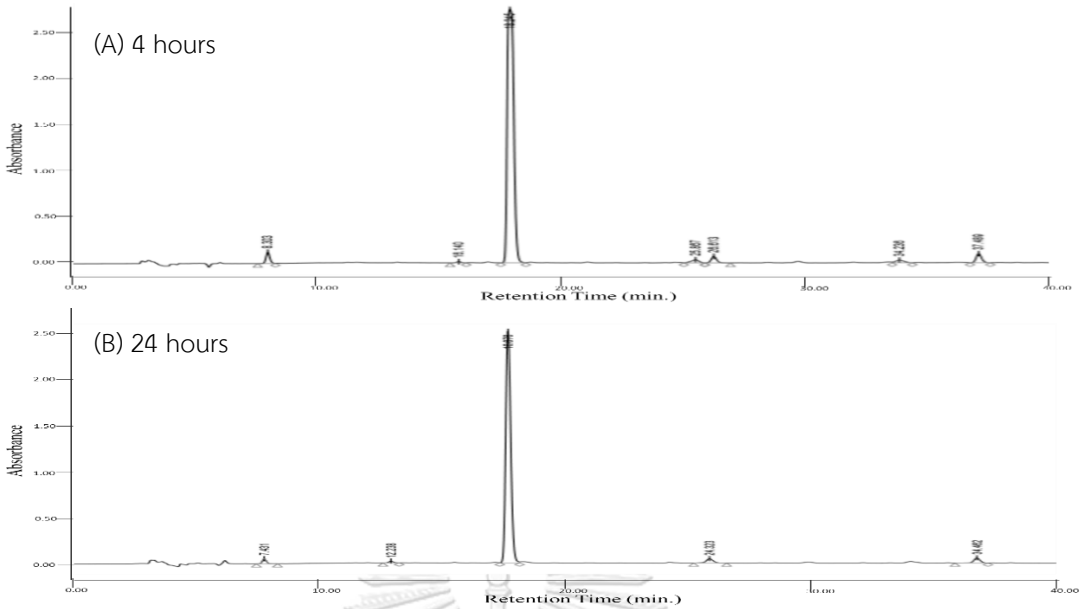


Figure 3.5 HPLC chromatograms of the dichloromethane extracts at (A) 4 and (B) 24 hours

Table 3.4 Percentage yield and constituent distribution of the dichloromethane extracts at 4 and 24 hours

Time (hours)	%Yield	Constituent distribution (%peak area)						
		EGC	CF	C	EC	EGCG	ECG	unknown
4	3.46	-	92.8	-	-	2.4	2.6	2.2
24	3.67	-	95.9	-	-	0.4	0.5	3.1

* EGC: epigallocatechin, CF: caffeine, C: catechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate

Both extracts did not show any different in percentage yield and caffeine content. The dichloromethane extracts at 4 and 24 hours showed the caffeine content of 92.8 and 95.9 %peak area, respectively. Thus, the extraction with dichloromethane for 4 hours was used for extraction of “enrich-caffeine” fraction. This fraction was further washed with acetone three times to furnish **compound 1** which was identified as caffeine. The ^{1}H and ^{13}C NMR spectra of compound **1** are shown in **Figure 3.6** and its spectral assignment is presented in **Table 3.5**.

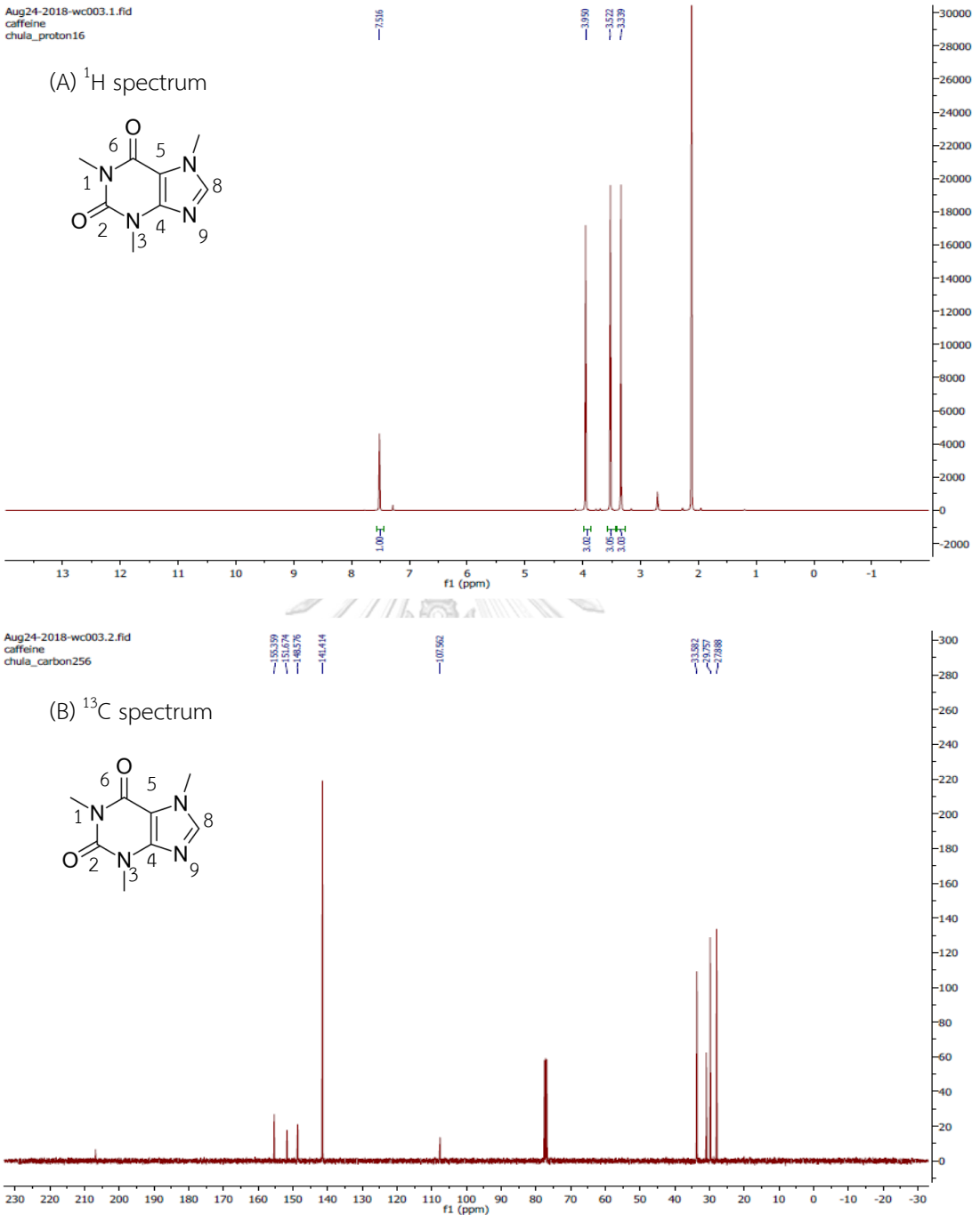


Figure 3.6 ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of **compound 1.**

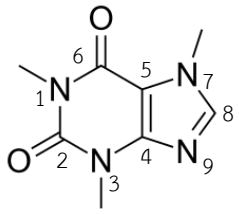


Table 3.5 The ^{1}H and ^{13}C spectral assignment of **compound 1**

Position	**Chemical shift (ppm)**			
	Compound 1		**Caffeine (Sitkowski *et al.*, 1995)**	
	^{1}H	^{13}C	^{1}H	^{13}C
1-CH_3	3.31	27.89	3.4	27.5
2	-	151.67	-	151.3
3-CH_3	3.50	29.76	3.6	29.3
4	-	148.58	-	148.3
5	-	107.56	-	107.1
6	-	155.36	-	154.9
7-CH_3	3.93	33.58	4.0	33.2
8	7.49	141.41	7.5	141.2

3.3.2 Enrich catechin family fraction

The residue of SGTL after extraction with dichloromethane was further refluxed with methanol for 1 hour. The percentage yield and constituent distribution are shown in **Figure 3.7B** and **Table 3.6**. The decaffeination was carried out (**Scheme 3.1**) by dissolving the methanol extract with distilled water and partitioned by liquid-liquid extraction. Then, aqueous phase was extracted with ethyl acetate to yield high catechin family content (C, EC, EGCG, ECG) and low caffeine content. (**Figure 3.7C, Table 3.6)** Thus, this fraction was designated as “enrich catechin family fraction”. (**Scheme 3.2**).

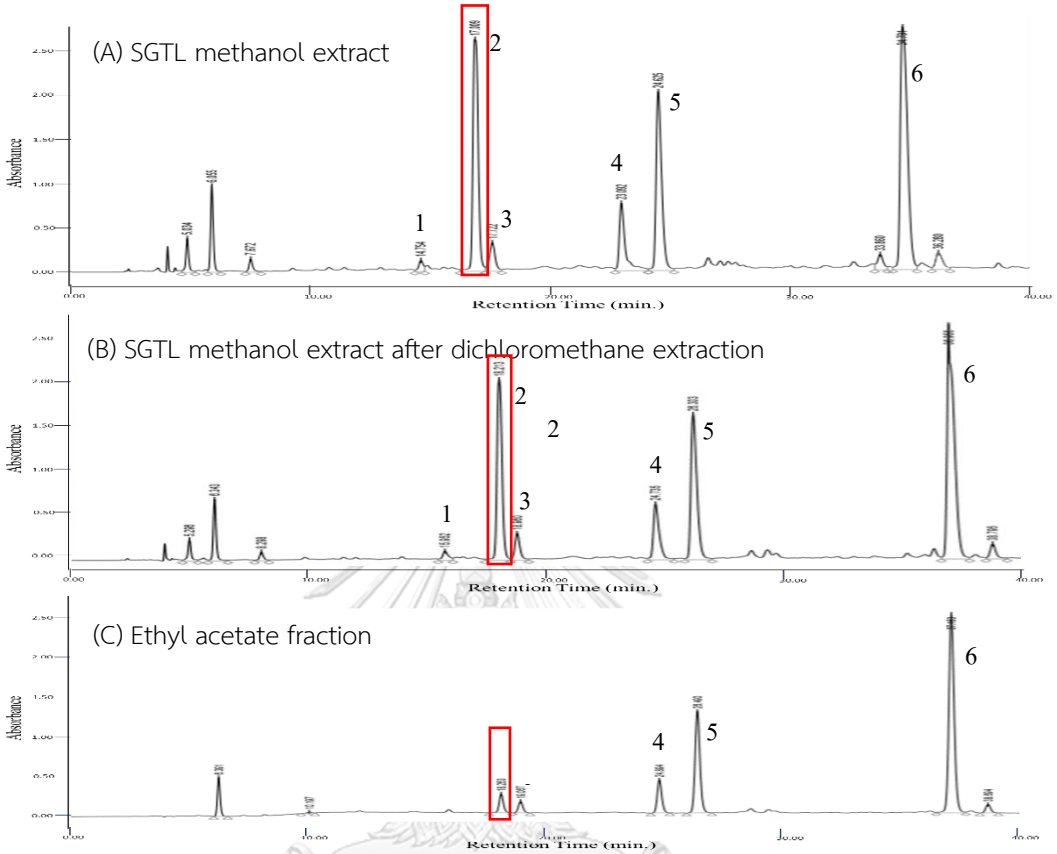


Figure 3.7 HPLC chromatograms of (A) SGTL methanol extract, (B) SGTL methanol after dichloromethane extraction and (C) ethyl acetate fraction

1: EGC (epigallocatechin), 2: CF (caffeine), 3: C (catechin), 4: EC (epicatechin), 5: EGCG (epigallocatechin gallate), 6: ECG (epicatechin gallate)

Table 3.6 Percentage yield and chemical composition of SGTL methanol extract, SGTL methanol after dichloromethane extraction and ethyl acetate fraction

Fraction	%Yield	Constituent distribution (%peak area)						
		EGC	CF	C	EC	EGCG	ECG	unknown
SGTL methanol extract	9.9	1.0	23.8	3.2	7.8	19.2	33.3	11.7
SGTL methanol after dichloromethane extraction	11.8	1.3	21.2	3.3	7.7	20.2	37.2	9.1
Ethyl acetate fraction	54.9	-	4.0	2.6	7.5	25.0	52.8	8.04

* EGC: epigallocatechin, CF: caffeine, C: catechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate

The comparison of the SGTL methanol extract and the SGTL methanol extract after dichloromethane extraction (**Figure 3.7A**) showed the reduction of caffeine contents as 23.8 and 21.2 %peak area. The HPLC chromatogram of the ethyl acetate fraction showed 87.9% peak area of catechins (**Figure 3.7C** and **Table 3.6**). As the result, it could conclude that "enrich-catechin family" was gained in ethyl acetate fraction.

3.4 Primary biological activity of enrich fractions

The "enrich-caffeine" and "enrich-catechin family" fractions were subjected to biological activity including antioxidant, anti-tyrosinase, cholinesterase inhibition and plant growth regulation. The results are presented in **Tables 3.7, 3.8 and 3.9.**

Table 3.7 Antioxidant, antityrosinase and anticholinesterase of enrich-caffeine and catechin family fractions at 100 ppm

Fractions	%Inhibition			
	Antioxidant	Antityrosinase	Anticholinesterase	
			AChE*	BChE*
Enrich-caffeine	19.5±4.85	6.16±5.18	71.2±2.41	22.7±7.70
Enrich-catechin family	93.1±1.19	83.7±1.74	65.3±1.71	59.4±2.40

*AChE: Acetylcholinesterase enzyme, BChE: Butyrylcholinesterase enzyme

"Enrich-catechin family" showed high activity of antioxidant and antityrosinase (93.1±1.19% DPPH inhibition and 85.7±1.89% tyrosinase inhibition) at 100 ppm. The catechin family was reported to contribute for antioxidant property of green tea infusions (Peluso and Serafini, 2017; Šilarová, Česlová, and Meloun, 2017). The relative activities of catechin family in scavenging DPPH and ABTS were EGCG ≈ ECG > EGC > EC, indicating green tea which contained large relative amount of these catechins having higher antioxidant activity (Higdon and Frei, 2003). The tyrosinase inhibitory activity might depend on hydroxyl groups of phenolic compounds such as gallic acid, ellagic acid, kojic acid, resveratrol, and several catechins that could form hydrogen bonding to enzyme, leading to lower enzymatic activity (Jae *et al.*, 1999). Enrich-caffeine showed high activity on AChE (71.2±2.41% inhibition) but not showed activity on BChE at 100 ppm. Caffeine is an alkaloid with a stimulant effect in the body. It can interfere in transmissions based on acetylcholine, epinephrine, norepinephrine, serotonin, dopamine and glutamate that can act as a non-competitive inhibitor of AChE in the body (Pohanka and Dobes, 2013). Anticholinesterase activity of enrich-catechin family showed 65.3±1.71 and 59.4±2.40 %inhibition on AChE and BChE, respectively. As the result, enrich-catechin family fraction showed high activity on all biological tests (more than 50% inhibition) while enrich-caffeine fraction showed high anticholinesterase on

AChE. Enrich-catechin family fraction was further fractionated. Further study was performed by these biological activities of each fraction at concentration of 100 ppm.

Table 3.8 The effect of enrich-caffeine and catechin family fractions on seed germination

Plant	%seed germination							
	Enrich-caffeine fraction (ppm)				Enrich-catechin fraction (ppm)			
	1000	100	10	1	1000	100	10	1
Helianthus annuus L. (sunflower)	1.00 ±7.92	2.45 ±6.67	9.81* ±5.65	0.94 ±4.71	-0.38[b] ±7.89	1.33[a]* ±3.40	0.18[b] ±0.59	-1.32 ±0.54
Triticum aestivum L. (wheat)	-0.29 ±5.64	1.26 ±2.06	5.84* ±1.07	-3.00 ±3.31	1.98* ±3.21	-0.75 ±3.78	-2.50 ±4.14	-2.98 ±1.50

[a,b] The mean difference significant at 0.05 level (LSD's test).

*Highest seed germination

Table 3.9 The effect of enrich-caffeine and catechin family fractions on stem cutting

Plant	%Plant growth regulator							
	Enrich-caffeine fraction				Enrich-catechin family fraction			
	1000	100	10	1	1000	100	10	1
Dracaena braunii **Engl.** (belgain evergreen)	14.13 [b] ±4.04	21.15 ±8.88	50.93[a]* ±5.22	11.39[b] ±4.10	21.79* ±8.54	14.73 ±13.08	12.91 ±9.36	10.22 ±7.18
Cymbopogon citratus **Stapf.** (lemon Grass)	39.20 ±8.16	45.44 ±11.51	56.41* ±9.61	45.69 ±7.42	20.65 ±14.14	43.21* ±6.71	28.49 ±6.84	24.11 ±6.34

[a,b] The mean difference significant at 0.05 level (LSD's test).

*Highest seed germination

As the results, the percentage of seed germination and plant growth promotion were not significant difference ($P > 0.05$). Enrich-caffeine fraction showed high

promotion of seed germination (sunflower: 9.81, wheat: 5.84 %seed germination) and stem cutting (Belgain evergreen: 50.93±5.22, lemon grass: 56.41±9.61 % plant growth regulator) more than enrich-catechin family fraction at concentration of 10 ppm.

To sum up, enrich caffeine fraction should contain plant growth regulator. Further studies on seed germination and stem cutting of the enrich caffeine fraction and pure caffeine were investigated. The results are presented in **Tables 3.10 and 3.11.**

Table 3.10 The effect of enrich-caffeine fraction and pure caffeine on seed germination

Plant	%seed germination							
	Enrich-caffeine fraction (ppm)				Caffeine (ppm)			
	1000	100	10	1	1000	100	10	1
Helianthus annuus L. (sunflower)	1.00 ±7.92	2.45 ±6.67	9.81* ±5.65	0.94 ±4.71	1.23[b] ±4.86	3.77 ±0.77	8.24[a]* ±2.28	0.51[b] ±2.33
Triticum aestivum L. (wheat)	-0.29 ±5.64	1.26 ±2.06	5.84* ±1.07	-3.00 ±3.31	0.12[b] ±5.69	1.93 ±3.48	5.46[a]* ±3.86	0.23[b] ±1.77
Cucurbita moschata **Decne.** (pumpkin)	13.23[a]* ±3.44	3.33[b] ±2.88	-2.86[b] ±4.84	-3.35[b] ±2.85	2.24[b] ±3.60	12.09[a]* ±4.39	2.46[b] ±2.87	-0.95[b] ±6.01
Cucumis melo **L.** (melon)	3.95[a]* ±3.47	-0.40[b] ±2.90	-1.32[b] ±1.63	-1.60[b] ±2.90	-0.55[b] ±1.75	3.10[a]* ±1.81	1.01 ±0.01	-0.70[b] ±0.02
Capsicum flutescens **Linn.** (chilli)	-0.79[b] ±2.54	4.39 ±2.18	8.42[a]* ±2.95	0.39[b] ±3.72	-0.52[c] ±2.34	6.31[b] ±1.76	12.8[a]* ±2.81	-1.96[c] ±0.95
Zea mays **Linn.** (corn)	1.06[b] ±1.40	1.80[b] ±1.40	7.75[a]* ±2.65	-1.50[b] ±4.41	1.18[b] ±2.83	3.53[b] ±2.03	7.98[a]* ±1.79	2.89[b] ±1.84

[a,b,c] The mean difference significant at 0.05 level (LSD's test).

*Highest seed germination

Table 3.11 The effect of enrich-caffeine fraction and pure caffeine on stem cutting.

Plant	%Plant growth regulator							
	Enrich-caffeine fraction				Caffeine			
	1000	100	10	1	1000	100	10	1
***Dracaena braunii* Engl.** (belgain evergreen)	14.13[b] ±4.04	21.15 ±8.88	50.93[a]* ±5.22	11.39[b] ±4.10	9.47[b] ±8.14	23.68 ±10.5	52.35[a]* ±10.8	10.46[b] ±3.29
***Cymbopogon citratus* Stapf.** (lemon Grass)	39.20 ±8.16	45.44 ±11.5	56.41* ±9.61	45.69 ±7.42	35.96[b] ±7.24	41.75 ±5.05	59.50[a]* ±12.0	46.45 ±4.02

[a,b] The mean difference significant at 0.05 level (LSD's test).

*Highest seed germination

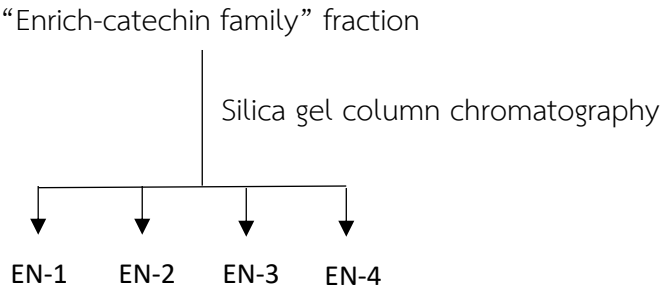
The growth promotion of plant seeds for caffeine at different levels of concentration was significant difference ($P < 0.05$). At 10 ppm of caffeine, the high level of growth promotion of *Helianthus annuus* L., *Triticum aestivum* L., *Capsicum flutescens* Linn. and *Zea mays* Linn could be observed whereas for *Cucurbita moschata* Decne and *Cucumis melo* L., the concentration of caffeine needed was 100 ppm.

The percentage growth promotion against *Dracaena braunii* Engl. and *Cymbopogon citratus* Stapf. of caffeine at concentration of 1000, 100, 10 and 1 ppm are shown in **Table 3.11**. 10 ppm of caffeine exposed very good activity to root growth promotion approximately 52 and 60% respectively that was significant difference ($P < 0.05$). The function of caffeine to stimulate rooting and elongation of roots was found to be like auxin and cytokinin. Each plant species has different threshold for auxin; if this threshold is exceeded, then the plant growth is inhibited by the application of additional auxin (Laplaze, Benkova, Casimiro, and Maes, 2007).

3.5 Purification of "enrich-catechin family" fraction

"Enrich-catechin family" fraction showed high antioxidant and anti-tyrosinase activity. This fraction was separated by silica gel column eluting with ethyl acetate:

dichloromethane: hexane: acetic acid (3: 1: 2: 0.5). Each subfraction was monitored by TLC and those which revealed similar components were combined to yield four fractions as presented in **Scheme 3.3**.



Scheme 3.3 The separation of "enrich-catechin family" fraction

All four subfractions of enrich-catechin family were subjected to biological activity tests. The results of antioxidant, anti-tyrosinase and anticholinesterase at 100 ppm are shown in **Table 3.12.**

Table 3.12 Antioxidant, antityrosinase and anticholinesterase activity of subfractions of "enrich-catechin family" fraction at 100 µg/mL

Fractions	%Inhibition			
	Antioxidant	Antityrosinase	Anticholinesterase	
			AChE	BChE
EN-1	92.6±2.00	89.9±2.63	55.1±2.00	61.3±2.05
EN-2	90.7±0.44	96.9±1.37	0	22.3±5.53
EN-3	91.6±0.72	89.4±1.00	14.1±5.20	32.3±2.74
EN-4	92.4±0.23	94.9±0.44	34.1±1.08	39.9±2.50

*AChE: Acetylcholinesterase enzyme, BChE: Butyrylcholinesterase enzyme

As the results attained, **EN1-EN-4** showed high antioxidant activity more than 90 %inhibition and antityrosinase more than 85 %inhibition. **EN-1** showed the highest anticholinesterase activity of approximately 61 %inhibition while **EN2-EN4** revealed lower than 50%. Catechins from plant sources are considered to exert protective effects against cancer, inflammatory and cardiovascular diseases through antioxidant activities (Dreosti, 1996; Kumar and Rajapaksha, 2005; Lakenbrink, Lapczynski, Maiwald,

and Engelhardt, 2000). Green tea contains 15–20% of catechins and is an important source of natural antioxidant. (H. Liang, Liang, Dong, and Lu, 2007; Y. R. Liang *et al.*, 2008). EGCG, EGC, and GA have significant inhibitory activity against melanin synthesis (Sato and Toriyama, 2009). No, Kim *et al*. 1999 indicated that flavan-3-ols containing gallic acid moiety at 3 position showed strong anti-tyrosinase activity. Four subfractions (**EN1-EN4**) were further analyzed for their chemical composition by HPLC. (**Figure 3.8, Table 3.13**).

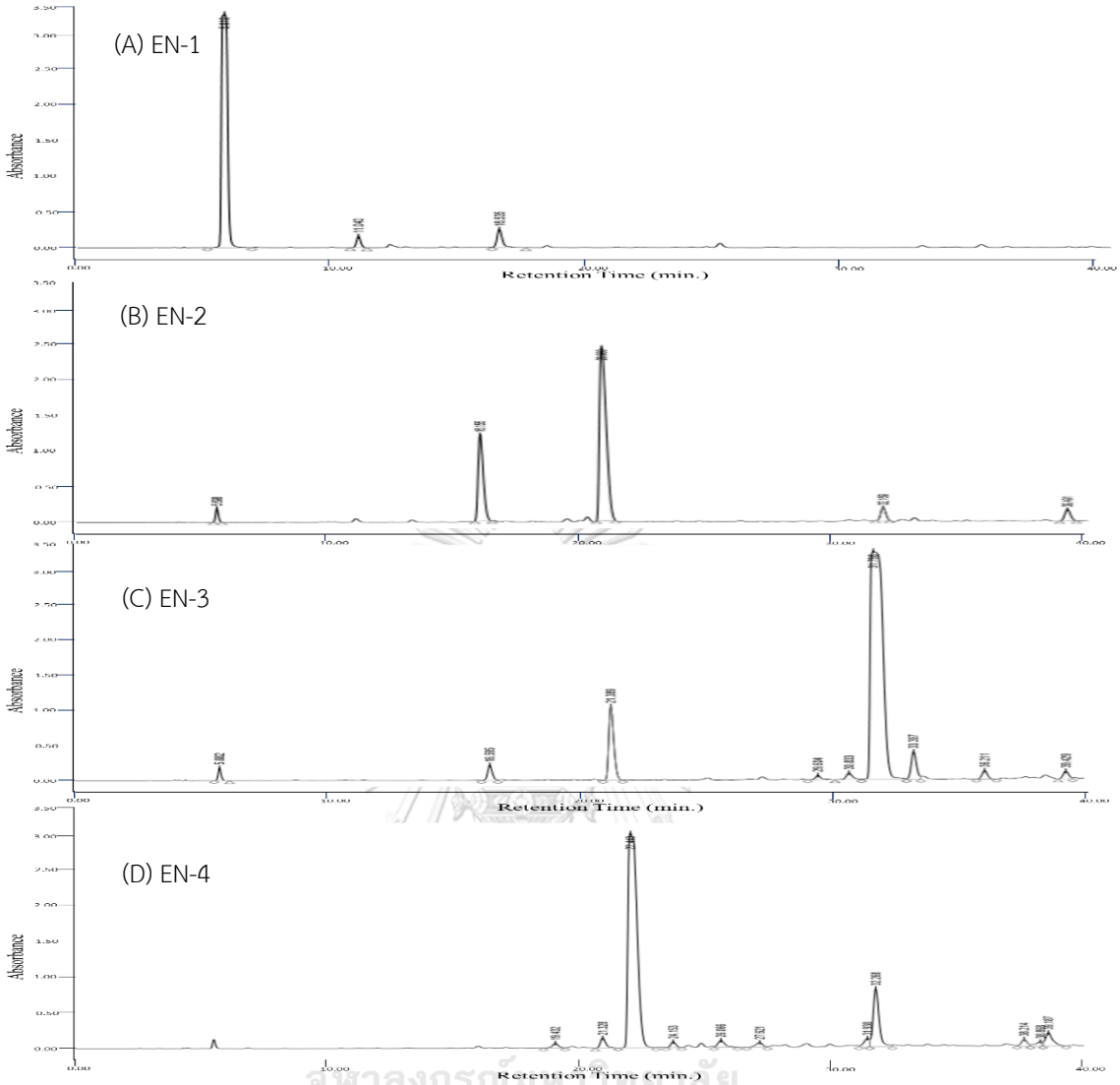


Figure 3.8 HPLC chromatograms of **EN1-4**.

Table 3.13 The chemical composition of EN1-EN4 fraction

Fraction	Weight (g)	Product distribution (%peak area)					
		EGC	C	EC	EGCG	ECG	unknown
EN-1	0.315	-	6.1	-	-	-	93.9
EN-2	0.137	-	25.0	65.8	-	-	9.2
EN-3	0.756	-	2.0	10.4	-	78.1	9.5
EN-4	0.573	-	-	2.3	46.2	36.8	14.7

* EGC: epigallocatechin, C: catechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate

From the result of HPLC analysis (**Figure 3.8**), **EN-1** showed high content of unknown 1 (approximately 89 %peak area), **EN-2** displayed high content of EC (approximately 66 %peak area) while **EN-3** revealed high content of ECG (approximately 78 %peak area) and **EN-4** showed high content of EGCG and ECG (approximately 46 and 37 %peak area, respectively). **EN-1** showed high antioxidant, antityrosinase and anticholinesterase activity and was consecutively separated by HPLC. The HPLC conditions were as follows: injection volume, 100 µL; column, SB-C18 5µm, 4.6 mm x 150 mm (Agilent Technology, USA); mobile phase A, acetonitrile: acetic acid: water (6: 1: 193); mobile phase B, acetonitrile: acetic acid: water (60: 1: 139); gradient elution, mobile phase B increased from 30% to 85% by linear gradient within 35 minutes and further holding at 85% for 5 minutes; flow rate 2 ml/minutes; detecting wavelength 280 nm. **Compound 2** was obtained as white solid (80.5% yield).

The ^{1}H and ^{13}C NMR spectra of **compound 2** are shown in **Figure 3.9** and **Table 3.14.**

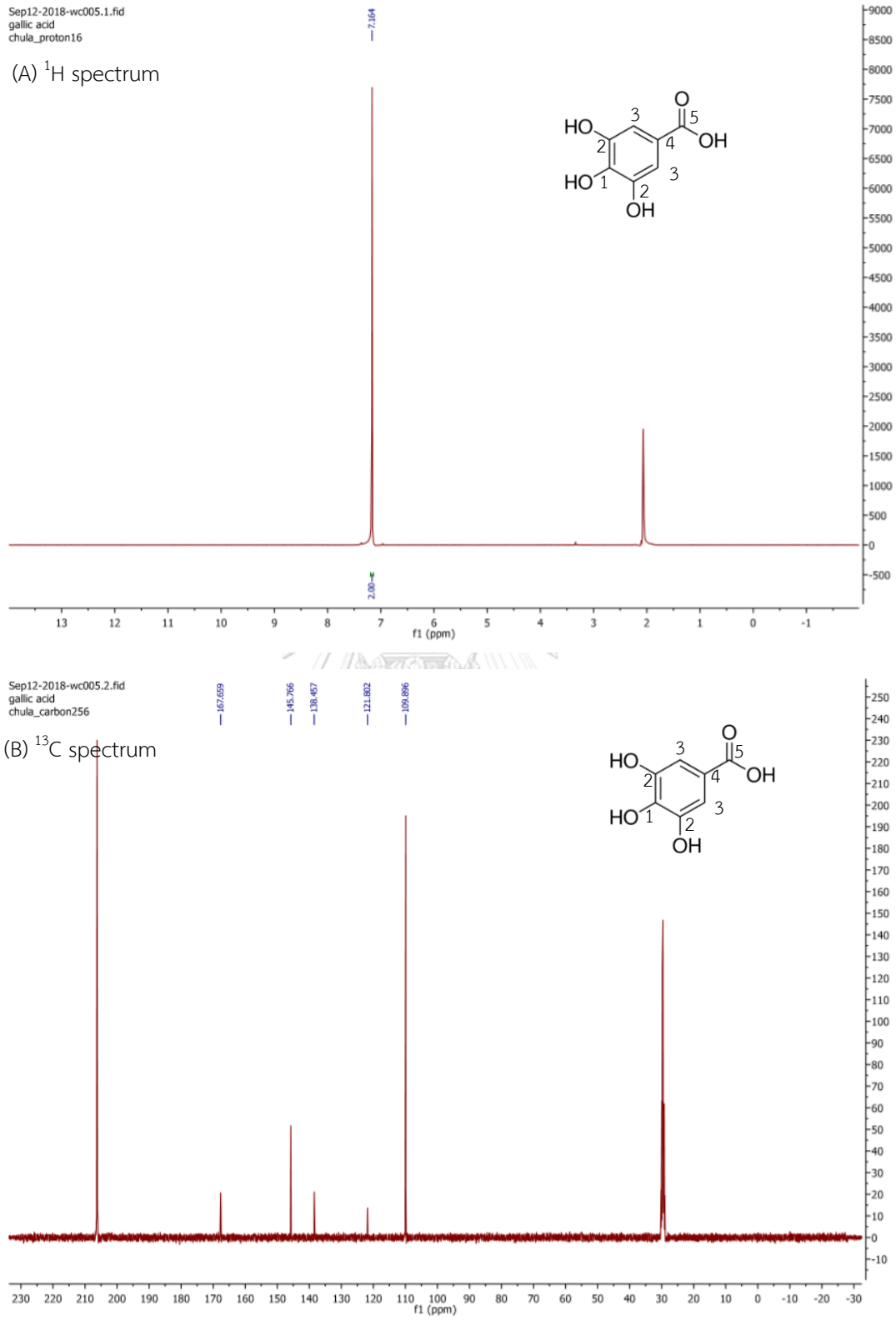


Figure 3.9 ^{1}H and ^{13}C NMR spectra (acetone-D_6) of **compound 2**

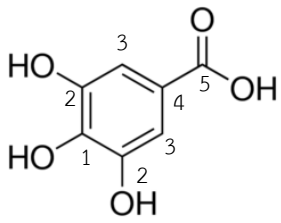


Table 3.14 The ^{1}H and ^{13}C spectral assignment of **compound 2.**

Position	**Chemical shift (ppm)**			
	Compound 2		**Gallic acid (Abri and Maleki, 2016)**	
	^{1}H	^{13}C	^{1}H	^{13}C
1-OH	-	138.70	8.8	138.1
2-OH	-	146.01	9.2	145.5
3	7.16	110.14	6.9	108.8
4	-	122.05	-	120.5
5	-	167.90	-	167.6

Gallic acid is an organic acid, also known as 3, 4, 5-trihydroxybenzoic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. Gallic acid is found both free and as part of tannins. Biological studies show that gallic acid has various properties, including anti-fungal, anti-viral, antioxidant and anti-cancer activities. Gallic acid is also employed as a source material for inks and paints, and as an antioxidant in food, in cosmetics and in the pharmaceutical industry (Ow and Stupans, 2003)

The chemical composition of enrich catechin-family fraction (**Figure 3.8, Table 3.5**) showed high content of ECG in **EN-3**. Thus, this compound was separated from **EN-3** by HPLC and designated as **compound 3**. Pure red brown of compound 3 was 60.5% yield. The ^{1}H and ^{13}C NMR spectra of **compound 3** are presented in **Figure 3.10** and **Table 3.15.**

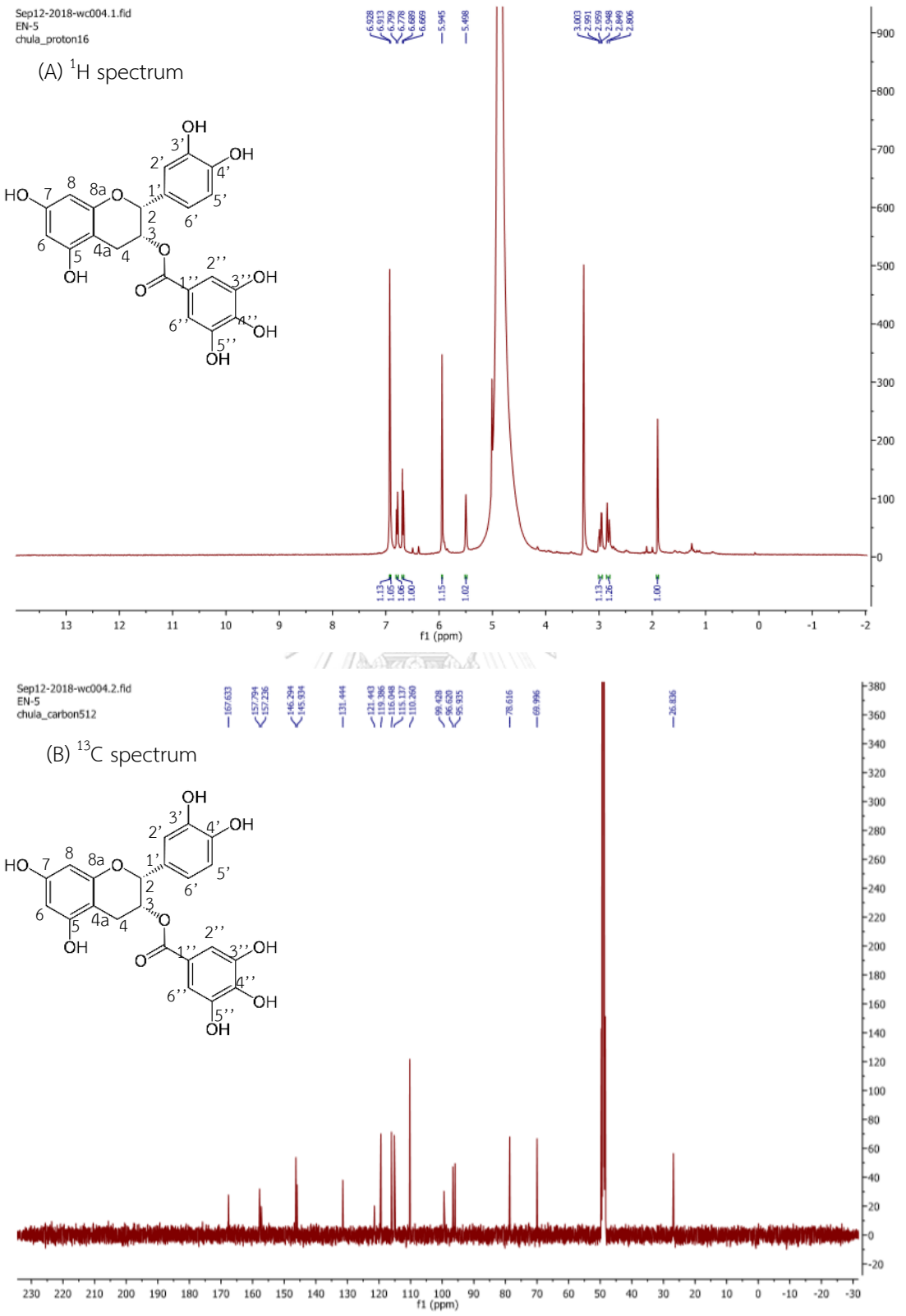


Figure 3.10 ^{1}H and ^{13}C NMR spectra (MeOH-D_4) of **compound 3**

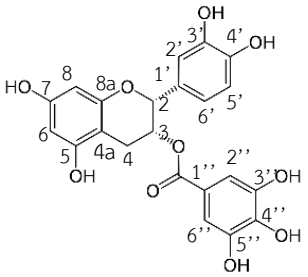


Table 3.15 The ^{1}H and ^{13}C spectral assignment of **compound 3**

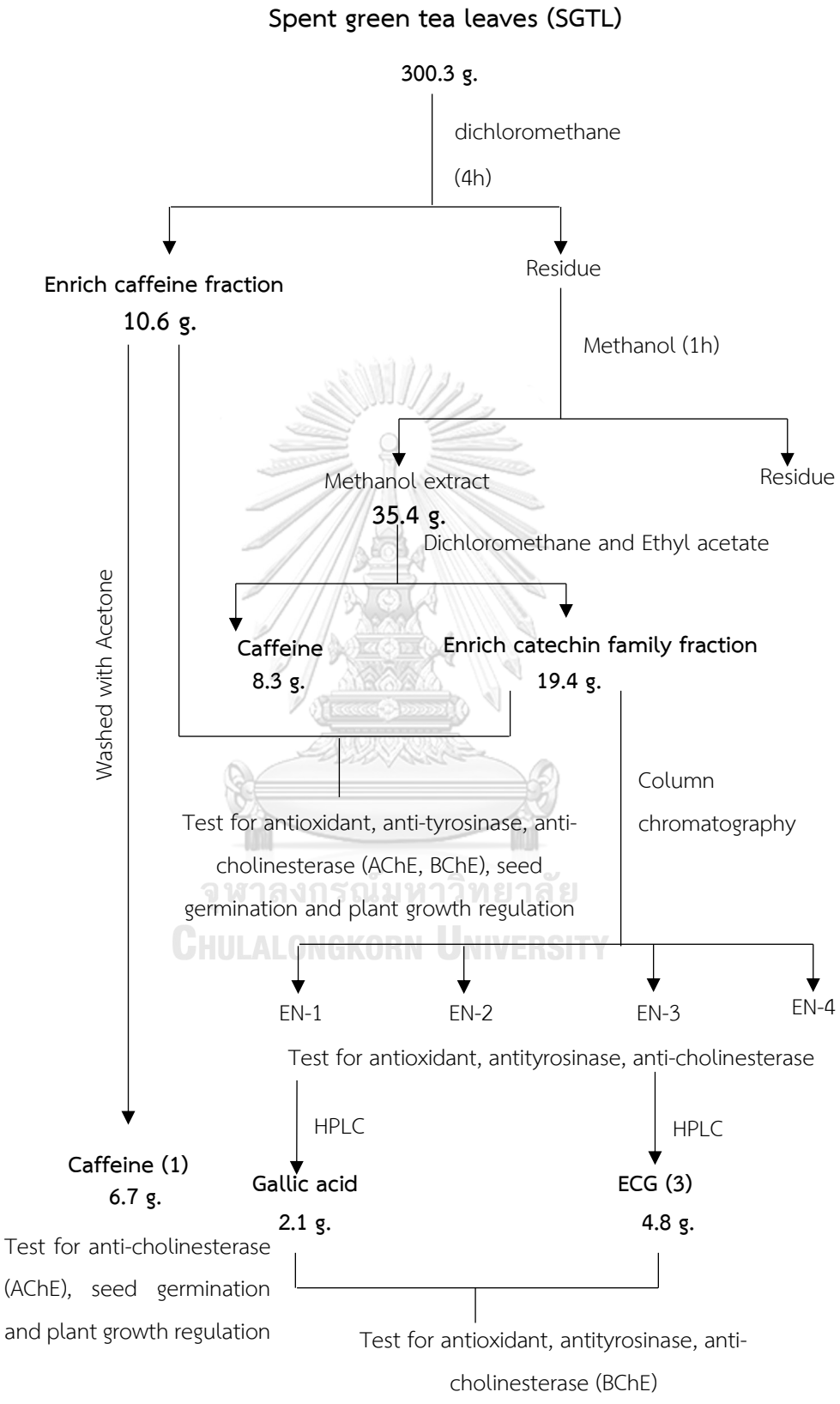
Position	Chemical shift (ppm)			
	Compound 3		ECG (Davis, Cai, Davies, Lewis, and 1996)	
	^{1}H	^{13}C	^{1}H	^{13}C
2	5.01	78.62	5.1	78.1
3	5.50	70.00	5.5	69.3
4α	2.83	26.84	3.0	26.6
4β	2.98	26.84	2.9	26.6
4a	-	99.43	-	99.03
5	-	157.80	-	157.5
6	5.94	96.62	6.0	96.5
7	-	157.80	-	157.8
8	5.94	95.94	6.0	95.8
8a	-	157.24	-	157.1
1’	-	131.45	-	131.4
2’	6.93	115.14	7.0	114.9
3’	-	146.30	-	145.6
4’	-	146.30	-	145.5
5’	6.68	116.05	6.7	115.6
6’	6.79	119.39	6.8	119.2
1”	-	121.45	-	121.9
2”	6.93	110.26	7.0	110.0

Position	Chemical shift (ppm)			
	Compound 3		ECG (Davis *et al.*, 1996)	
	^{1}H	^{13}C	^{1}H	^{13}C
3”	-	145.93	-	145.9
4”	-	131.45	-	138.8
5”	-	131.45	-	138.8
6”	6.93	131.45	7.0	138.8
C=O	-	167.64	-	166.0

Epicatechin gallate was isolated from tea and numerous other plant species including rhubarb and grapes. It differs from epicatechin in its gallate moiety positioned at carbon 3 of the C-ring (Yilmaz, 2006). Epicatechin gallate revealed anticancer effects could inhibit β-catenin signaling and cyclin D1 expression (Lim *et al.*, 2006).

3.6 Biological activity of enrich-caffeine fraction, enrich-catechin family fraction, EN1-4, caffeine, gallic acid and ECG

The summary of the separation of SGTL extract and tested procedures for antioxidant, antityrosinase, anticholinesterase and plant growth regulation are depicted as shown in **Scheme 3.4**.



Scheme 3.4 Separation procedure and biological test of SGTL extract

The half maximal inhibitory concentration (IC_{50}) of enrich-caffeine, enrich-catechin family, **EN-1**, **EN-2**, **EN-3**, **EN-4**, gallic acid and ECG were determined to compare the potency of substance in inhibiting a specific biological activity. The results are showed in **Table 3.16**.

Table 3.16 IC_{50} of antioxidant, antityrosinase and anticholinesterase on enrich fraction, subfractions of enrich-catechin family fraction and pure compounds

Sample	IC_{50} (ug/mL)			
	Antioxidant	Antityrosinase	Anticholinesterase	
			AChE	BchE
Enrich-caffeine	-	-	49.3	-
Enrich-catechin family	5.48	23.7	94.0	75.3
EN-1	5.18	16.7	127.0	48.8
EN-2	11.04	7.8	-	-
EN-3	5.25	19.0	-	-
EN-4	7.11	13.7	-	-
Caffeine	-	-	47.9	-
Gallic acid	2.94	20.6	106.3	48.2
Epicatechin gallate	3.68	33.3	-	-
Ascorbic acid	8.18	-	-	-
Kojic acid	-	2.32	-	-
Galanthamine	-	-	4.3	9.4

*AChE: Acetylcholinesterase enzyme, BChE: Butyrylcholinesterase enzyme

IC_{50} of antioxidant of enrich-catechin family, **EN-1**, **EN-3, EN-4**, gallic acid and ECG exhibited lower value than ascorbic acid as a positive control. The most potent DPPH radical scavengers (IC_{50}) were gallic acid followed by ECG, **EN-1**, **EN-3** (2.94, 3.68, 5.18 and 5.25 ug/mL, respectively). **EN-1** showed higher DPPH scavenger than other subfractions because it contained high content of gallic acid (approximately 89 %peak area). From the result, it can suggest that gallic acid displayed high DPPH scavenging activity in SGTL extract. ECG and **EN-3** showed high antioxidant activity. Previous

reported revealed that gallic acid was better antioxidant than other polyphenols such as quercetin, rutin and protocatechuic acid. (Fu, Chen, Li, Zheng, and Li, 2013; López-Martínez, Navarro-Lozano, and Psychology, 2015). The DPPH radical-scavenging ability of the catechins was EGCG > ECG > EGC > EC and theaflavins. ECG also showed higher DPPH radical- and superoxide-scavenging abilities than carnosol, carnosic acid, and BHT. (Chen and Ho, 1995)

The enzyme inhibitory activity of samples was tested against tyrosinase, AChE and BChE using 96-well plate. The results are given in **Table 3.15**. The lowest IC_{50} of antityrosinase was kojic acid (positive control) at 2.32 ug/mL. **EN-2** showed lower IC_{50} than other fractions. From the result of HPLC (**Figure 3.7, Table 3.12**), **EN-2** mainly contained mainly of EC and C (approximately 66% and 25%peak area, respectively). The subfractions (**EN-1-EN-4**) and gallic acid showed IC_{50} lower than enrich-catechin family fraction. Gallic acid having hydroxyl groups, showed tyrosinase inhibitory effect. (No & Kim, 2004) Enrich-caffeine fraction and caffeine showed high efficiency to AChE inhibition (IC_{50} = 49.3 and 47.9 ug/mL, respectively). While galanthamine (positive control) has an excellent inhibitor effect on both AChE and BChE. The observed differences could be explained by the differences of active site in the enzymes. Similar case was reported by several researchers (Şenol, Yilmaz, Şener, Koyuncu, and Orhan, 2010; Tundis, Bonesi, Menichini, and Loizzo, 2016). Caffeine can act as a non-competitive inhibitor against AChE in the body and had lower binding affinity to BChE (Pohanka and Dobes, 2013).

From the results, it can be concluded that SGTL can be used as bioactive compounds source. The enrich-caffeine and catechin fraction showed biological activity like pure compound. The enrich fractions displayed better alternative than pure compounds.

CHAPTER IV
CONCLUSION

Many researchers investigated on the possibility to utilize SGTL such as feedstuff, pesticide, particle board production, adsorbents for synthetic dyes and toxic metals *etc*. In this research, SGTL were investigated as bioactive compounds source. Enrich-caffeine fraction (3.53 %yield) was derived from the extraction of SGTL with dichloromethane, while the enrich-catechin family fraction (EGC, C, EC, EGCG, ECG) (11.78 %yield) was achieved from the separation of the methanol extract. Enrich-caffeine fractions and caffeine (2.76 %yield) revealed, anticholinesterase and plant growth promotion activities. Whereas enrich-catechin family fractions, gallic acid (0.69 %yield) and ECG (1.59 %yield) displayed promising antioxidant and antityrosinase activity.

Proposal for future work

The results of all tested biological activities revealed that enrich fractions from SGTL extract were potent. The possible future work related to this research would be the development of the separation technique to get high yield of enrich fraction from SGTL extract. In addition, encapsulation of enrich fraction should be studied to serve as prospective material for the exploration of new multi-functional food and drug formulations.

REFERENCES

Abri, A., and Maleki (2016). Isolation and identification of gallic acid from the elaeagnus angustifolia leaves and determination of total phenolic, flavonoids contents and investigation of antioxidant activity. *Iranian Chemical Communication, 4*, 146-154.

Anand, R., Gill, K. D., and Mahdi, A. A. (2014). Therapeutics of Alzheimer's disease: Past, present and future. *Neuropharmacology, 76*, 27-50.

Ayas, N., and Esen, T. (2016). Hydrogen production from tea waste. *International Journal of Hydrogen Energy, 41*(19), 8067-8072.

Barbosa Filho, J., Paula Medeiros, K., Fátima F.M. Diniz, M., Batista, L., Athayde-Filho, P., Silva, M., Cunha, E., Almeida, J. R., and Quintans-Júnior, L. (2006). Natural products inhibitors of the enzyme acetylcholinesterase. *Revista Brasileira de Farmacognosia, 16*(2), 258-285.

Batiancela, M. A., Acda, M. N., and Cabangon, R. J. (2014). Particleboard from waste tea leaves and wood particles. *Journal of Composite Materials, 48*(8), 911-916.

Cabrera, C., Artacho, R., and Giménez, R. (2006). Beneficial effects of green tea—a review. *Journal of the American College of Nutrition, 25*(2), 79-99.

Carloni, P., Tiano, L., Padella, L., Bacchetti, T., Customu, C., Kay, A., and Damiani, E. (2013). Antioxidant activity of white, green and black tea obtained from the same tea cultivar. *Food Research International, 53*(2), 900-908.

Chang, T. S. (2009). An updated review of tyrosinase inhibitors. *International Journal of Molecular Sciences, 10*(6), 2440-2475.

Chen, C. W., and Ho, C. T. (1995). Antioxidant properties of polyphenols extracted from green and black teas. *Journal of Food Lipids, 2*(1), 35-46.

Davis, A. L., Cai, Y., Davies, A. P., Lewis, J. R., and (1996). ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. *Magnetic Resonance in Chemistry, 34*(11), 887-890.

Dieng, H., Zawawi, R. B. M., Yusof, N. I. S. B. M., Ahmad, A. H., and Abang, F. (2016). Green tea and its waste attract workers of formicine ants and kill their workers—implications for pest management. *Industrial Crops and Products, 89*, 157-166.

Dreosti, I. E. (1996). Bioactive ingredients: antioxidants and polyphenols in tea. *Nutrition Reviews, 54*(11), 51-58.

Duan, J., Reddy, K. O., Ashok, B., Cai, J., Zhang, L., and Rajulu, A. V. (2016). Effects of spent tea leaf powder on the properties and functions of cellulose green composite films. *Journal of Environmental Chemical Engineering, 4*(1), 440-448.

Engelhardt, U. H. (2010). Chemistry of tea. In *Comprehensive Natural Products II: Chemistry and Biology* (Vol. 3, pp. 999-1032).

Fu, Y., Chen, J., Li, Y. J., Zheng, Y. F., and Li, P. (2013). Antioxidant and anti-inflammatory activities of six flavonoids separated from licorice. *Food chemistry, 141*(2), 1063-1071.

Graham, H. N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Preventive Medicine, 21*(3), 334-350.

Gray, W. M. (2004). Hormonal regulation of plant growth and development. *PLoS biology, 2*(9), E311-E311.

Hamilton-Miller. (2001). Anti-cariogenic properties of tea (*Camellia sinensis*). *Journal of medical microbiology, 50*(4), 299-302.

Higdon, J. V., and Frei, B. (2003). Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Critical Reviews in Food Science and Nutrition, 43*(1), 89-143.

Jae, K. N., Do, Y. S., You, J. K., Kyung, H. S., Yeong, S. J., Sook, H. R., Yokozawa, T., and Hae, Y. C. (1999). Inhibition of tyrosinase by green tea components. *Life Sciences, 65*(21), 241-246.

Khan, N., and Mukhtar, H. (2013). Tea and health: studies in humans. *Current Pharmaceutical Design, 19*(34), 6141-6147.

Kondo, M., Kita, K., and Yokota, H. O. (2006). Evaluation of fermentation characteristics and nutritive value of green tea waste ensiled with byproducts mixture for ruminants. *Asian Australasian Journal of Animal sciences, 19*(4), 533.

Kumar, N. S., and Rajapaksha, M. (2005). Separation of catechin constituents from five tea cultivars using high-speed counter-current chromatography. *Journal of Chromatography A, 1083*(1-2), 223-228.

Lakenbrink, C., Lapczynski, S., Maiwald, B., and Engelhardt, U. H. (2000). Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *Journal of Agricultural Food Chemistry, 48*(7), 2848-2852.

Laplaze, L., Benkova, E., Casimiro, I., and Maes, L. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. *The Plant cell, 19*(12), 3889-3900.

Larik, F. A., Saeed, A., Channar, P. A., and Muqadar, U. (2017). Design, synthesis, kinetic mechanism and molecular docking studies of novel 1-pentanoyl-3-arylthioureas as inhibitors of mushroom tyrosinase and free radical scavengers. *European Journal of Medicinal Chemistry, 141*, 273-281.

Leelayuwat, N. (2017). Update of Nutritional Antioxidants and Antinociceptives on Improving Exercise-Induced Muscle Soreness. In *Physical Activity and the Aging Brain* (pp. 199-208): Elsevier.

Liang, H., Liang, Y., Dong, J., and Lu, J. (2007). Tea extraction methods in relation to control of epimerization of tea catechins. *Journal of the Science of Food Agriculture, 87*(9), 1748-1752.

Liang, N., and Kitts, D. D. (2014). Antioxidant property of coffee components: assessment of methods that define mechanisms of action. *Molecules, 19*(11), 19180-19208.

Liang, Y. R., Ye, Q., Jin, J., Liang, H., Lu, J. L., Du, Y. Y., and Dong, J. J. (2008). Chemical and instrumental assessment of green tea sensory preference. *International Journal of Food Properties, 11*(2), 258-272.

Lim, Y. C., Lee, S.-H., Song, M. H., Yamaguchi, K., Yoon, J.-H., Choi, E. C., and Baek, S. J. (2006). Growth inhibition and apoptosis by (−)-epicatechin gallate are mediated by cyclin D1 suppression in head and neck squamous carcinoma cells. *European Journal ofCcancer (Oxford, England : 1990), 42*(18), 3260-3266.

López-Martínez, O., Navarro-Lozano, J. J. E. J. o. E., and Psychology. (2015). Influencia de una metodología creativa en el aula de primaria. *European Journal of Education and Psychology, 3*(1). 89-102

Makimura, M., Hirasawa, M., Kobayashi, K., Indo, J., Sakanaka, S., Taguchi, T., and Otake, S. (1993). Inhibitory effect of tea catechins on collagenase activity. *Journal of Periodontology, 64*(7), 630-636.

Marinova, G., Batchvarov, V., and (2011). Evaluation of the methods for determination of the free radical scavenging activity by DPPH. *Bulgarian Journal of Agricultural Science, 17*(1), 11-24.

Mattos, B. D., Misso, A. L., de Cademartori, P. H. G., de Lima, E. A., Magalhães, W. L. E., and Gatto, D. A. (2014). Properties of polypropylene composites filled with a mixture of household waste of mate-tea and wood particles. *Construction Building Materials, 61*, 60-68.

McKay, D. L., and Blumberg, J. B. (2002). The Role of Tea in Human Health: An Update. *Journal of the American College of Nutrition, 21*(1), 1-13.

Melvin Joe, M., Chanbhasa, M., John Henry, A., Benson, A., and (2017). Treatment of carrots with spent green tea extract reduces the soft rot of *Pectobacterium spp.* *Archives of Phytopathology Plant Protection, 50*(9-10), 491-503.

Nadiah, N. I., and Uthumporn, U. (2015). Determination of phenolic and antioxidant properties in tea and spent tea under various extraction method and determination of catechins, caffeine and gallic acid by HPLC. *International Journal on Advanced Science, Engineering Information Technology, 5*(3), 158-164.

News, G. (2014). Teabags targeted for new compost scheme. [Online][Available: hhttp://www.theguardian.com/environment/2012/may/23/tea-targeted-new-compost-scheme, access Sep. 10, 2018]

News, T. (2012). ตะลึงพบ'คาเฟอีน'ในชาขวดเพียบ ปริมาณเท่าเครื่องดื่มผสมคาเฟอีน จี้อย.เตือนผู้ผลิตหวั่นเด็กอันตราย.[Online][Available: https://www.tcijthai.com/news/2012/02/scoop/1392, access Oct. 29, 2018]

Ow, Y. Y., and Stupans, I. (2003). Gallic acid and gallic acid derivatives: Effects on drug metabolizing enzymes. *Current Drug Metabolism, 4*(3), 241-248.

Panche, A. N., Diwan, A. D., and Chandra, S. R. (2016). Flavonoids: an overview. *Journal of Nutritional Science, 5*, 47-47.

Park, H. S., Im, N. G., and Kim, K. H. (2012). Extraction behaviors of caffeine and chlorophylls in supercritical decaffeination of green tea leaves. *LWT-Food Science Technology, 45*(1), 73-78.

Peluso, I., and Serafini, M. (2017). Antioxidants from black and green tea: from dietary modulation of oxidative stress to pharmacological mechanisms. *British Journal of Pharmacology, 174*(11), 1195-1208.

Perva-Uzunalić, A., Škerget, M., Knez, Ž., Weinreich, B., Otto, F., and Grüner, S. (2006). Extraction of active ingredients from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. *Food Chemistry, 96*(4), 597-605.

Phankaen, Y., Manaprasertsak, A., Pluempanupat, W., Koul, O., Kainoh, Y., and Bullangpoti, V. (2017). Toxicity and repellent action of *Coffea arabica* against *Tribolium castaneum* (Herbst) adults under laboratory conditions. *Journal of Stored Products Research, 71*, 112-118.

Pohanka, M. (2014). Alzheimer s disease and oxidative stress: a review. *Current Medicinal Chemistry, 21*(3), 356-364.

Pohanka, M., and Dobes, P. (2013). Caffeine inhibits acetylcholinesterase, but not butyrylcholinesterase. *International Journal of Molecular Sciences, 14*(5), 9873-9882.

Primavesi, L., Piantanida, M., and Pravettoni, V. (2014). Chapter 66 - Studying tea polyphenols and their protective effects on skin. In R. R. Watson, V. R. Preedy, & S. Zibadi (Eds.), *Polyphenols in Human Health and Disease* (pp. 849-859). San Diego: Academic Press.

Puengyen, N. (2005). *Promotion of adventitious root growth by coffee extracts.* Chulalongkorn University, [Online][Available: http://cuir.car.chula.ac.th/handle/123456789/7066 , access Sep. 10, 2018]

Rusak, G., Komes, D., Likić, S., Horžić, D., and Kovač, M. (2008). Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. *Food Chemistry, 110*(4), 852-858.

Sato, K., and Toriyama, M. (2009). Effect of pyrroloquinoline quinone (PQQ) on melanogenic protein expression in murine B16 melanoma. *Journal of Dermatological Science, 53*(2), 140-145.

Senanayake, S. N. (2013). Green tea extract: Chemistry, antioxidant properties and food applications–A review. *Journal of Functional Foods, 5*(4), 1529-1541.

Şenol, F. S., Yilmaz, G., Şener, B., Koyuncu, M., and Orhan, I. (2010). Preliminary screening of acetylcholinesterase inhibitory and antioxidant activities of Anatolian Heptaptera species. *Pharmaceutical Biology, 48*(3), 337-341.

Šilarová, P., Česlová, L., and Meloun, M. (2017). Fast gradient HPLC/MS separation of phenolics in green tea to monitor their degradation. *Food Chemistry, 237*, 471-480.

Sitkowski, J., Stefaniak, L., Nicol, L., Martin, M. L., Martin, G. J., and Webb, G. A. (1995). Complete assignments of the ^{1}H, ^{13}C and ^{15}N NMR spectra of caffeine. *Spectrochimica Acta Part A: Molecular Biomolecular Spectroscopy, 51*(5), 839-841.

Škerget, M., Kotnik, P., Hadolin, M., Hraš, A. R., Simonič, M., and Knez, Z. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry, 89*(2), 191-198.

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

Spadiene, A., Savickiene, N., Ivanauskas, L., Jakstas, V., Skesters, A., Silova, A., and Rodovicius, H. (2014). Antioxidant effects of *Camellia sinensis* L. extract in patients with type 2 diabetes. *Journal of Food Drug Analysis, 22*(4), 505-511.

Theeraphaksirinont, T., Chanpongsang, S., Chaiyabutr, N., and Topanurak, S. (2009). *Effects of green tea waste in total mixed ration on productive performances in cross-bred lactating cows.* Paper presented at the Proceedings of the 47th Kasetsart University Annual Conference, Kasetsart, 17-20 March, 2009. Subject: Animals.

Transparency, Market, and Research. (2015). Tea Market – Global industry analysis, trend, size, share and forecast 2014–2020. [Online][Available: http://www.reportbuyer.com/product/2841844/tea-market-global-industry-analysis-trend-size-share-and-forecast-2014-2020.html, access Sep. 10, 2018]

Tsubaki, S., and Azuma, J.-i. (2013). Total fractionation of green tea residue by microwave-assisted alkaline pretreatment and enzymatic hydrolysis. *Bioresource Technology, 131*, 485-491.

Tundis, R., Bonesi, M., Menichini, F., and Loizzo, M. R. (2016). Recent knowledge on medicinal plants as source of cholinesterase inhibitors for the treatment of dementia. *Mini Reviews in Medicinal Chemistry, 16*(8), 605-618.

Vuong, Q. V., Golding, J. B., Stathopoulos, C. E., Nguyen, M. H., and Roach, P. D. (2011). Optimizing conditions for the extraction of catechins from green tea using hot water. *Journal of Separation Science, 34*(21), 3099-3106.

Wiseman, S. A., Balentine, D. A., and Frei, B. (1997). Antioxidants in tea. *Critical Reviews in Food Science and Nutrition, 37*(8), 705-718.

Xia, G., Reddy, K. O., Maheswari, C. U., Jayaramudu, J., Zhang, J., Zhang, J., and Rajulu, A. V. (2015). Preparation and properties of biodegradable spent tea leaf powder/poly (propylene carbonate) composite films. *International Journal of Polymer Analysis Characterization, 20*(4), 377-387.

Yilmaz, Y. (2006). Novel uses of catechins in foods. *Trends in Food Science Technology, 17*(2), 64-71.

Zhao, Y., Chen, P., Lin, L., Harnly, J. M., Yu, L., and Li, Z. (2011). Tentative identification, quantitation, and principal component analysis of green pu-erh, green, and white teas using UPLC/DAD/MS. *Food Chemistry, 126*(3), 1269-1277.

Zuo, Y., Chen, H., and Deng, Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. *Talanta, 57*(2), 307-316.

APPENDIX

Appendix A

Preparation of solutions

A1. Preparation of chemical for determined biological activity

A1.1 Preparation of 0.05 g/L 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in MeOH

the formula of DPPH = $C_{18}H_{12}N_5O_6$ (MW = 394.32)

1) Calculated amount of DPPH radical solution to use in that time (30 sample x triplicate x 100 µL per well = 9000 µL= 9 mL)

2) 0.05 mg DPPH in 1 mL MeOH. This study needs to prepare 10 mL of DPPH solution. (0.05 mg x 10 mL = 0.5 mg of DPPH)

∴Weigh 0.5 mg DPPH and dissolve in 10 ml MeOH to provide 0.05 g/L DPPH radical solution

A1.2 Preparation of 2.5 mM L-Tyrosine

the formula of L-Tyrosine = $C_9H_{11}NO_3$ (MW= 181.19)

1) Weigh 14.79 mg L-Tyrosine and dissolve in 30 mL of ammonium buffer pH 6.8 to provide 2.5 mM L-Tyrosine

A1.3 Preparation of 1.5 mM Acetylthiocholine iodide (ATCI)

1) Weigh 2.68 mg acetylthiocholine iodide and dissolve in 50 mL of milli Q water to provide 1.5 mM acetylthiocholine iodide

A1.4 Preparation of 1.5 mM Butyrylthiocholine iodide (BTCI)

1) Weigh 2.68 mg butyrylthiocholine iodide and dissolve in 50 mL of milli Q water to provide 1.5 butyrylthiocholine iodide

A2. Preparation of buffer

A2.1 Preparation of sodium phosphate buffer (pH 6.8)

1) A: Weigh $Na_2HPO_4 \bullet 2H_2O$ (MW= 177.99 g/mole) 0.44 g was dissolved in 50 mL of DI water
2) B: Weigh $NaH_2PO_4 \bullet 2H_2O$ (MW= 156.006 g/mol) 0.39 g was dissolved in 50 mL of DI water
3) Then, the solution A and solution B were mixed together in volumetric flask 250 mL to provide sodium phosphate buffer pH 6.8.

A2.2 Preparation of 50 mM Tris-HCl buffer (pH 8.0)- Buffer A

1) Weigh Tris-HCl (MW= 121.14 g/mole) 3.94 g was dissolved in 430 mL of milli Q water

A2.3 Preparation of 50 mM Tris-HCl buffer with 0.1%BSA (pH 8.0)- Buffer B

1) Weigh Tris-HCl (MW= 121.14 g/mole) 3.94 g was dissolved in 50 mL of milli Q water
2) Weigh BSA (Bovine Serum Albumin) 0.5 g was dissolved in 50 mL of milli Q water
3) Then, the solution A and solution B were mixed together in volumetric flask 500 mL to provide Buffer B

A2.4 Preparation of 50 mM Tris-HCl buffer with 0.1 M NaCl and 0.02 M $MgCl_2 \bullet 6H_20$ (pH 8.0)- Buffer C

1) Weigh Tris-HCl (MW= 121.14 g/mole) 3.94 g was dissolved in 50 mL of milli Q water
2) Weigh NaCl (MW= 58.44 g/mole) 2.92 g was dissolved in 50 mL of milli Q water
3) Weigh $MgCl_2 \bullet 6H_20$ (MW= 203.295 g/mole) 2.03 g was dissolved in 50 mL of milli Q water

4) Then, the solution A, solution B and solution C were mixed together in volumetric flask 500 mL to provide Buffer B

A3. Preparation of enzyme

A3.1 Preparation of 250 units/ml of mushroom tyrosinase

Mushroom tyrosinase enzyme (labeled activity 2687 U/mg)

1) preparation stock of 5000 units/ml of mushroom tyrosinase

18.62 mg was dissolved in 10 mL of 80 mM potassium phosphate buffer pH 6.8

2) Keep in the dark at -20°C until use
3) Dilute to 250 units/ml of mushroom tyrosinase from stock at 5000 units/ml

$$C_1V_1 = C_2V_2$$

$$5000 \text{ units/ml} \times V_1 = 250 \text{ units/ml} \times 5 \text{ mL}$$

$$V_1 = 0.25 \text{ mL}$$

$\therefore$ 0.25 ml of 5000 units/ml of mushroom tyrosinase is mixed with 4.75 ml of 80 mM potassium phosphate buffer pH 6.8 to provide 250 units/ml of mushroom tyrosinase

A3.2 Preparation of 0.3 units/ml of acetylcholinesterase enzyme

Acetylcholinesterase enzyme (labeled activity 2687 U/mg)

1) preparation stock of 100 units/ml of acetylcholinesterase enzyme
2) 18.62 mg was dissolved in 10 mL of buffer B (Keep in the dark at -20°C until use)
3) Dilute to 0.3 units/ml of acetylcholinesterase enzyme from stock at 100 units/ml

$$C_1V_1 = C_2V_2$$

$$100 \text{ units/ml} \times V_1 = 0.3 \text{ units/ml} \times 10 \text{ mL}$$

$$V_1 = 0.03 \text{ mL}$$

∴ 30 µL of 100 units/ml of acetylcholinesterase enzyme is mixed with 4.75 ml of buffer B to provide 0.3 units/ml of acetylcholinesterase enzyme

Appendix B

Regression analysis of TPC, TFC, antioxidant, antityrosinase and anticholinesterase with correlation coefficient (r^2)

B.1 Standard curve of total phenolic content and total flavonoid content

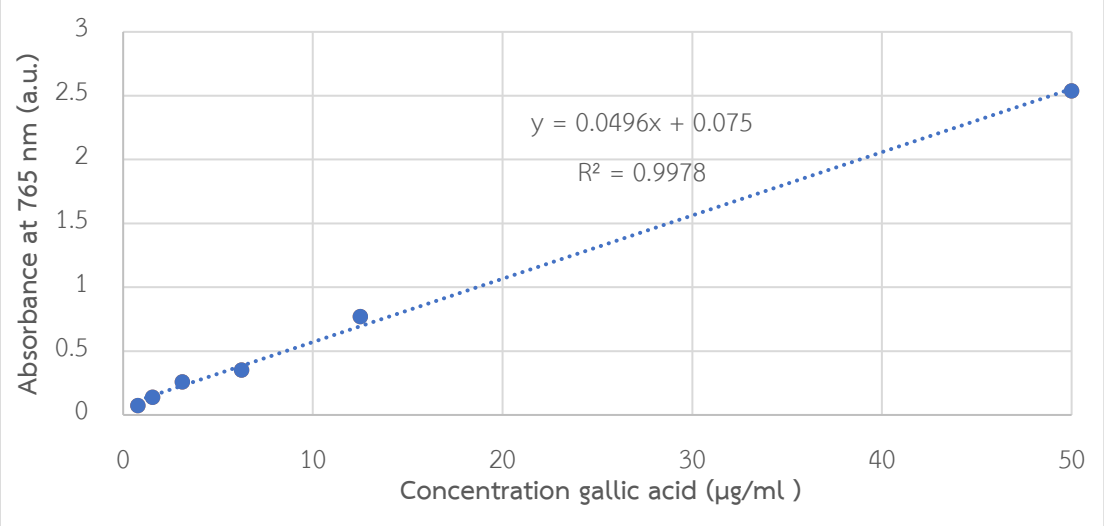


Figure B1.1 Standard curve of gallic acid

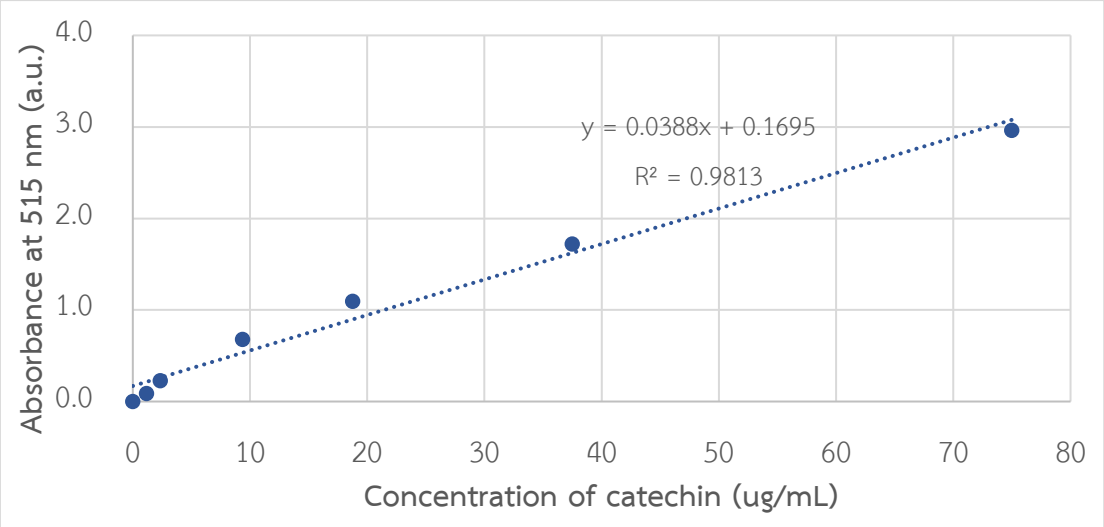


Figure B1.2 Standard curve of catechin

B.2 Regression analysis of antioxidant activity with correlation coefficient (r^2)

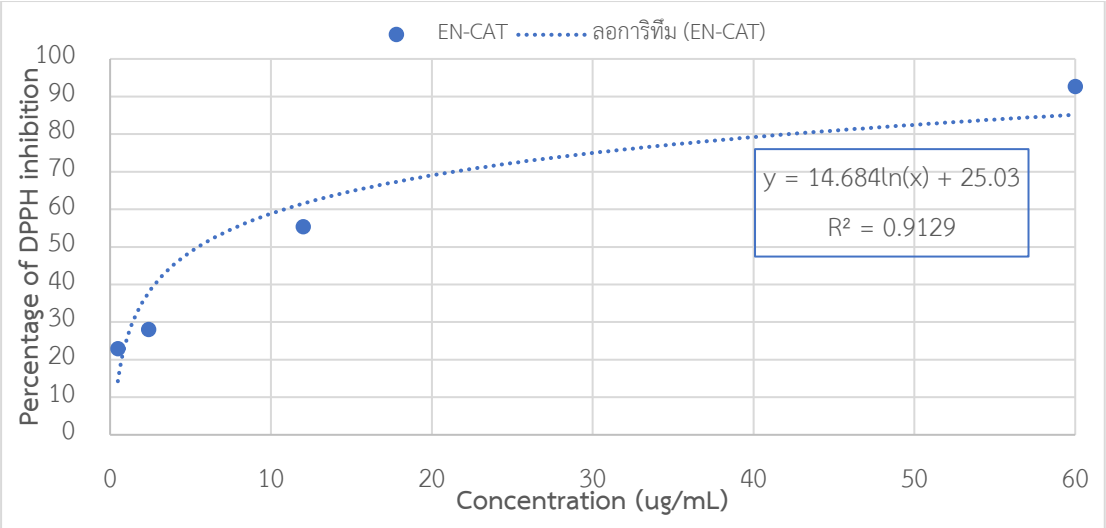


Figure B2.1 Antioxidant activity of EN-Catechin family

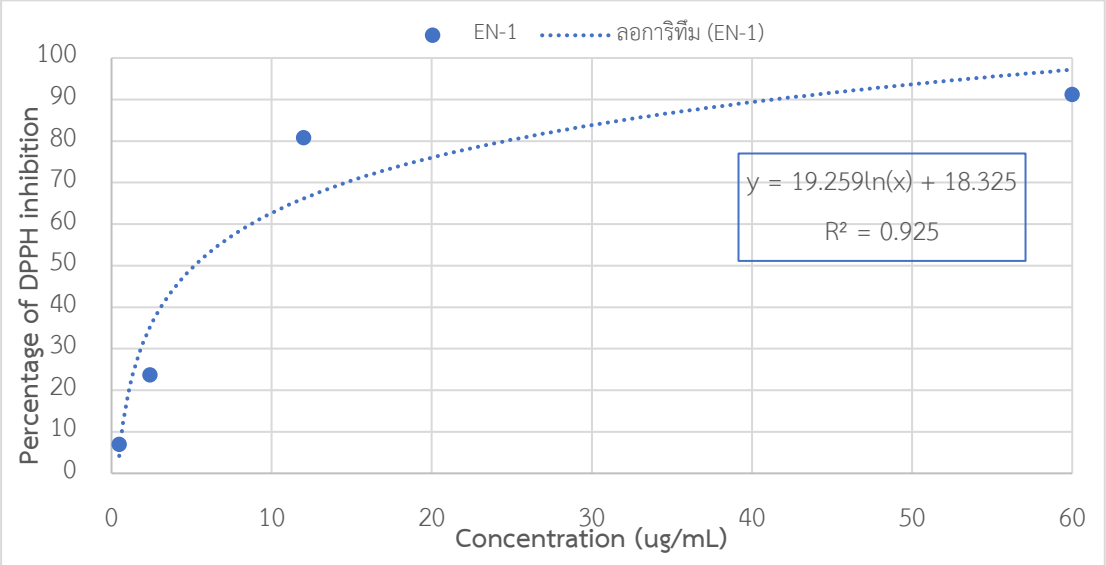


Figure B2.2 Antioxidant activity of EN-1

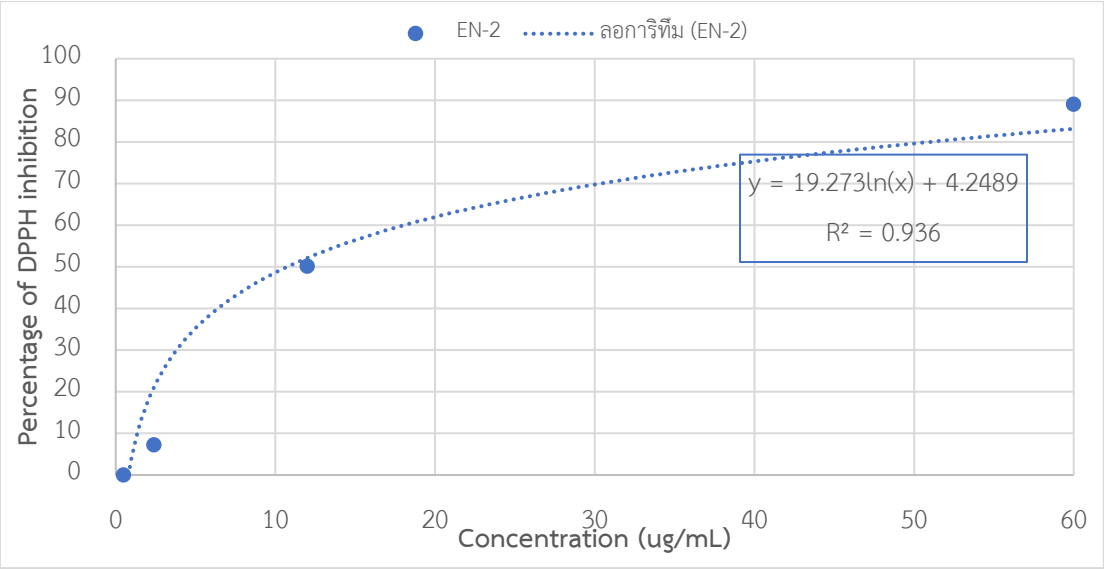


Figure B2.3 Antioxidant activity of EN-2

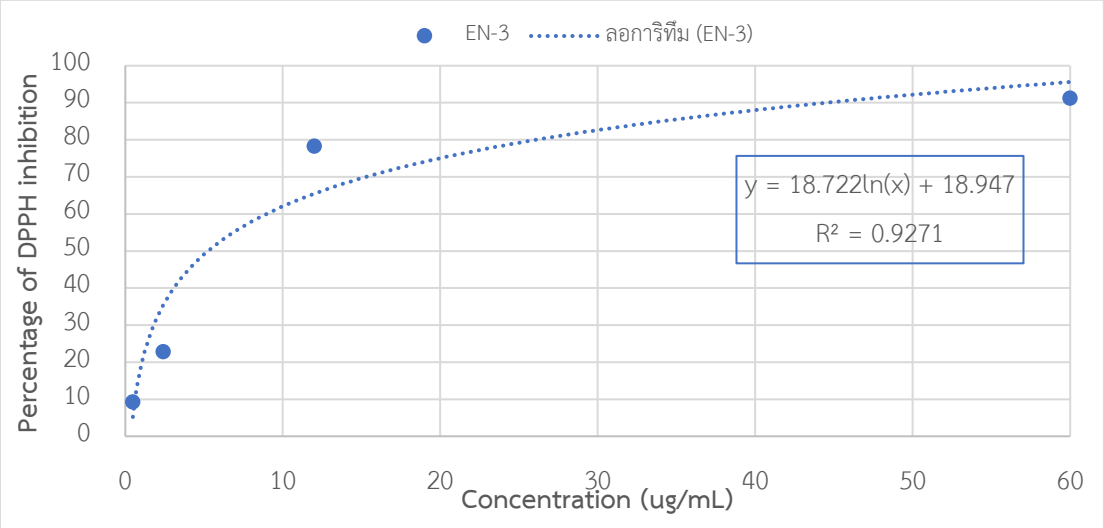


Figure B2.4 Antioxidant activity of EN-3

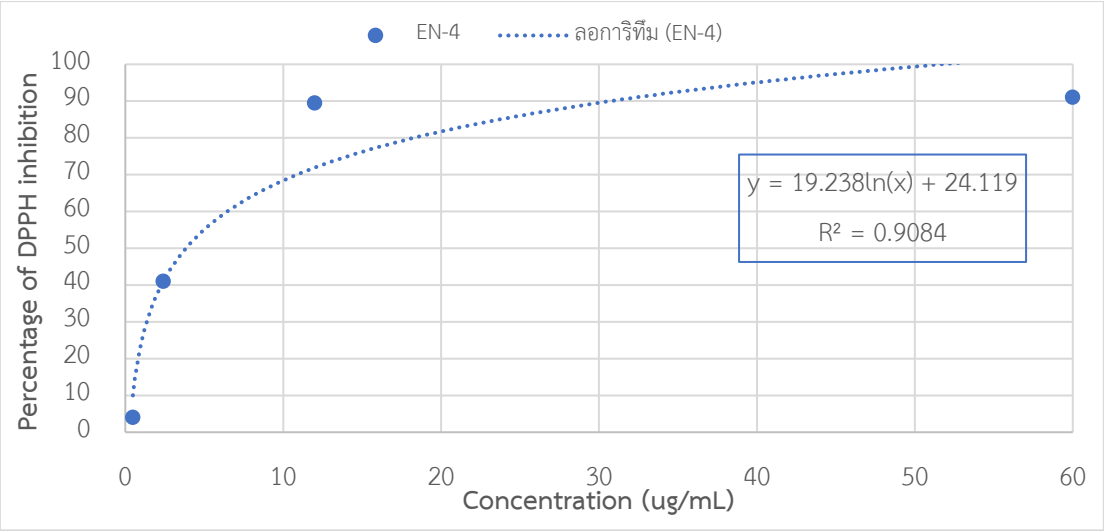


Figure B2.5 Antioxidant activity of EN-4

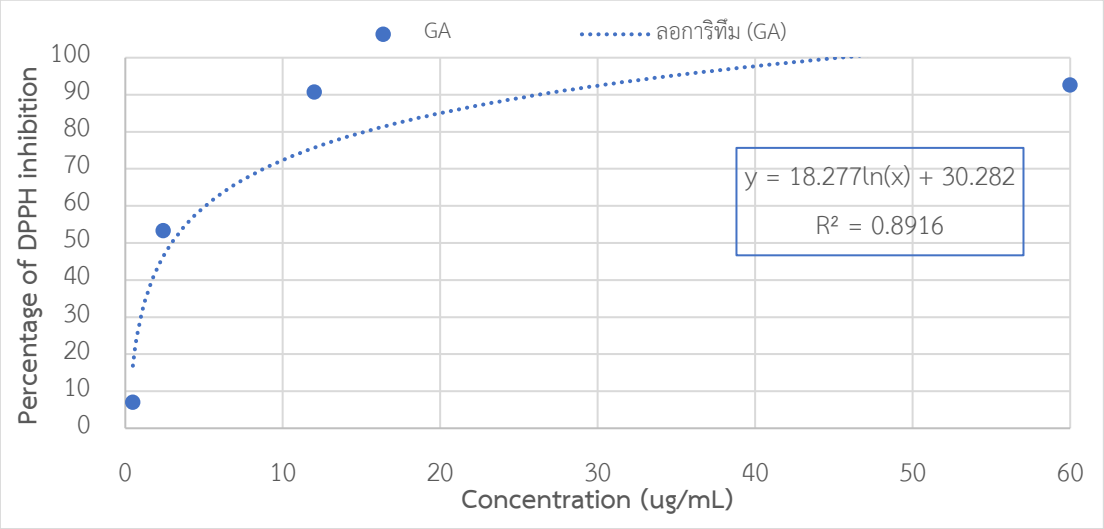


Figure B2.6 Antioxidant activity of gallic acid

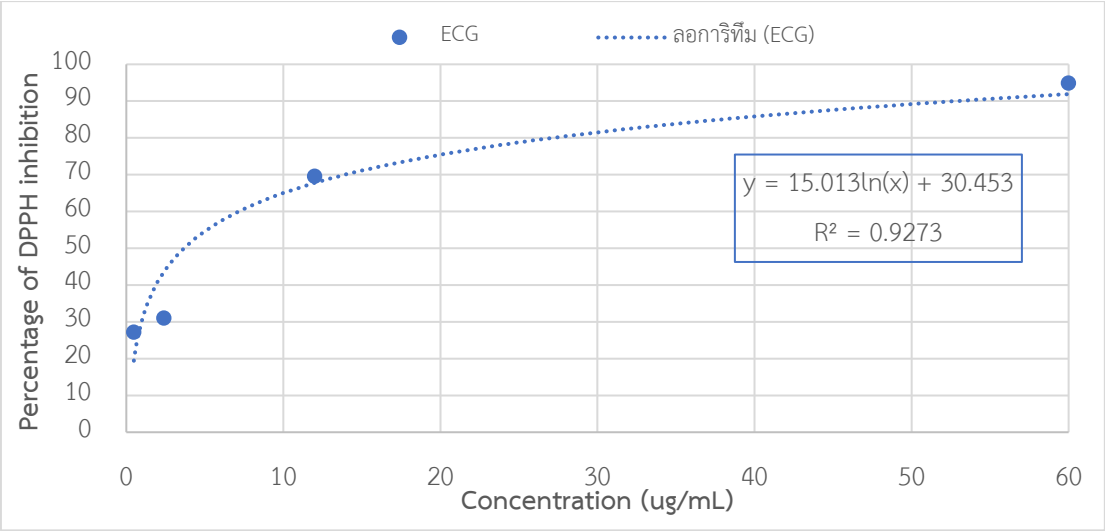


Figure B2.7 Antioxidant activity of ECG

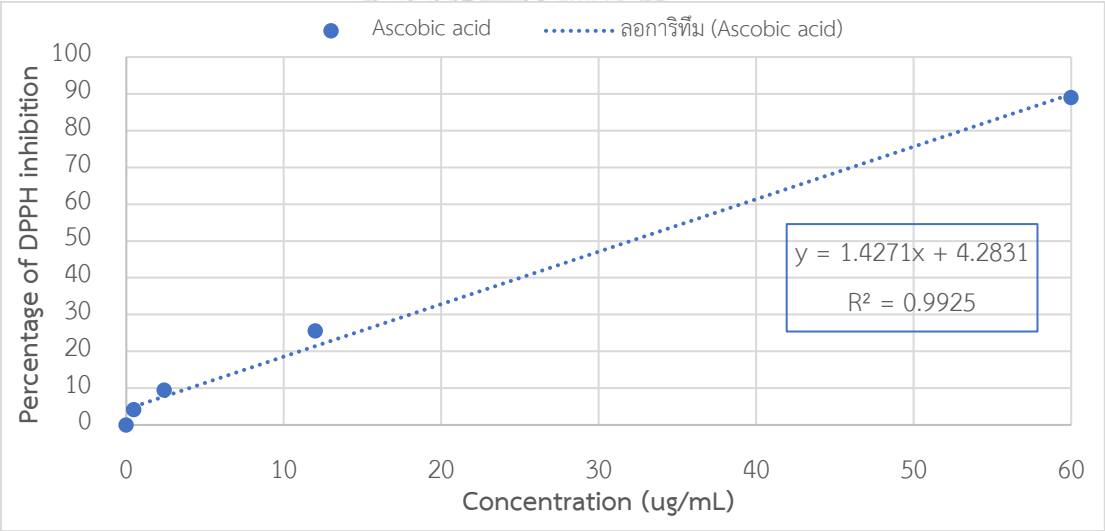


Figure B2.8 Antioxidant activity of ascorbic acid (positive control)

B.3 Regression analysis of antityrosinase activity with correlation coefficient (r^2)

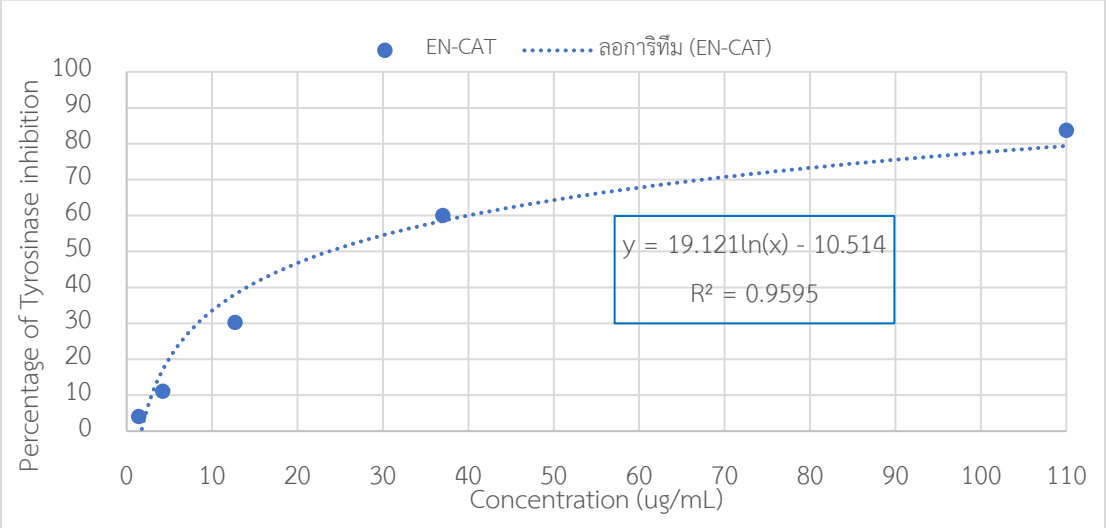


Figure B3.1 Antityrosinase activity of EN-Catechin family

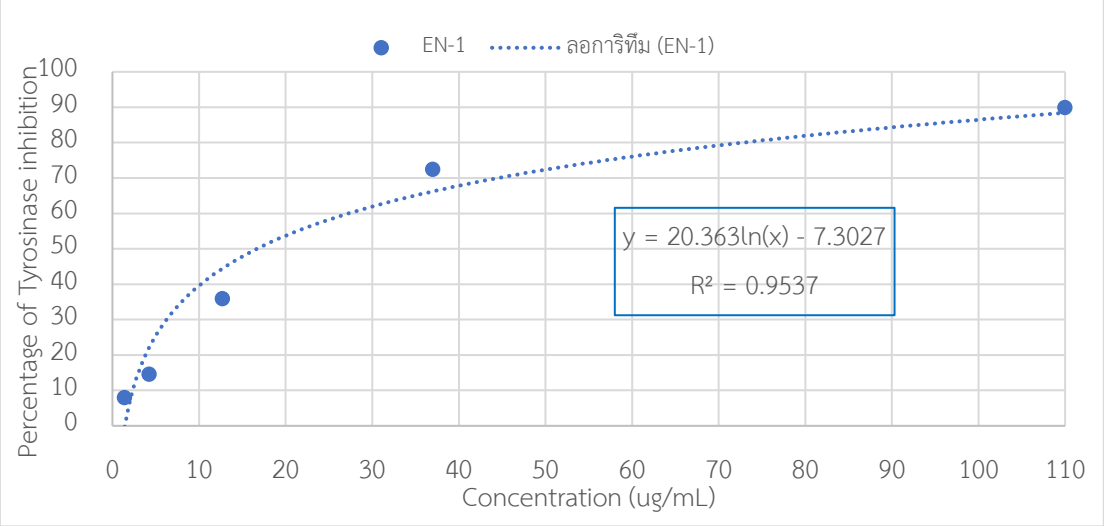


Figure B3.2 Antityrosinase activity of EN-1

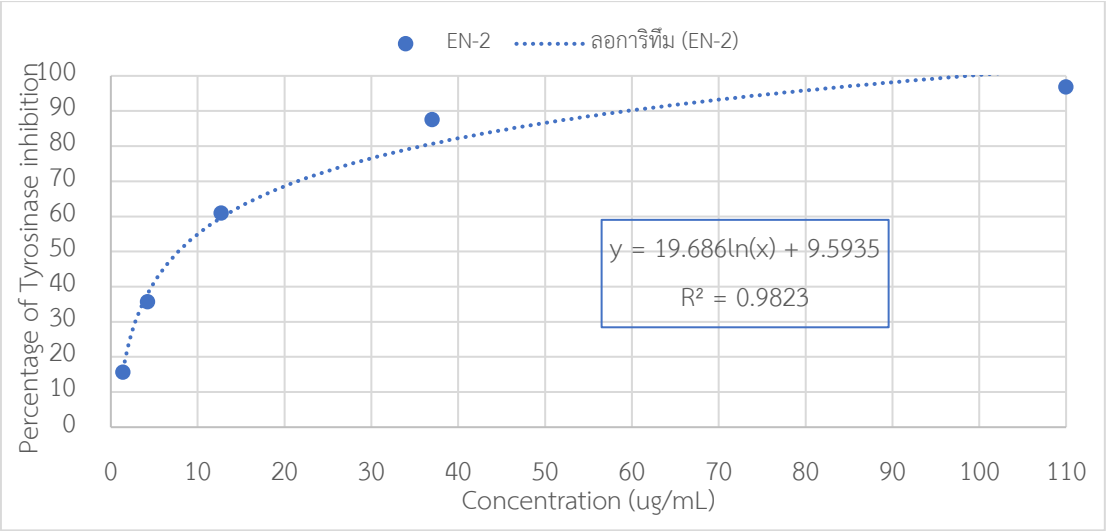


Figure B3.3 Antityrosinase activity of EN-2

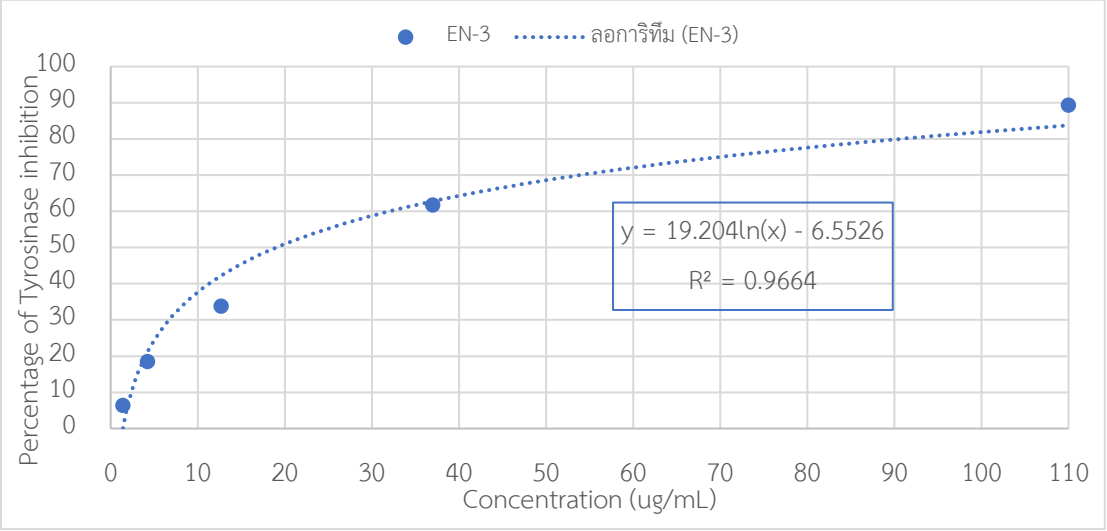


Figure B3.4 Antityrosinase activity of EN-3

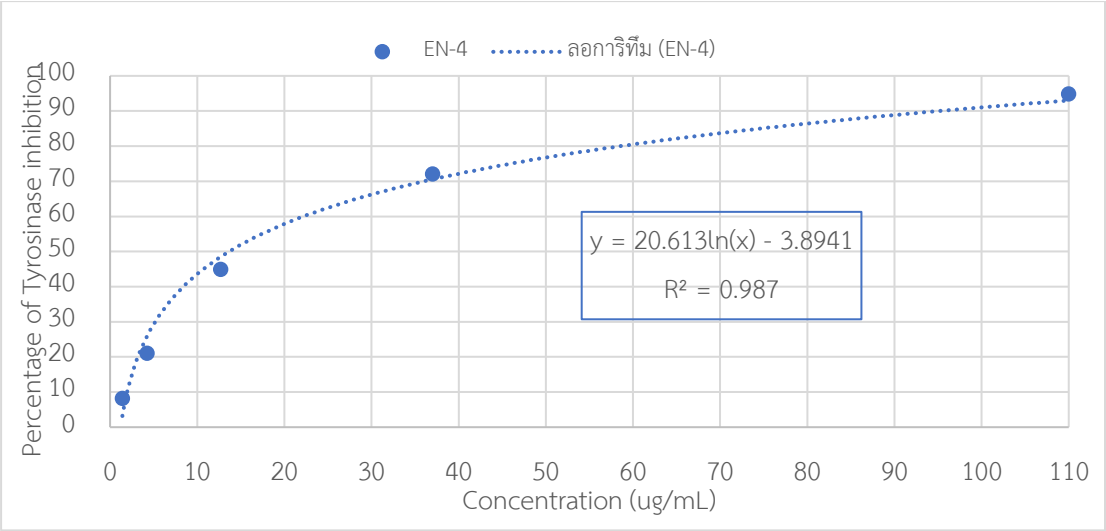


Figure B3.5 Antityrosinase activity of EN-4

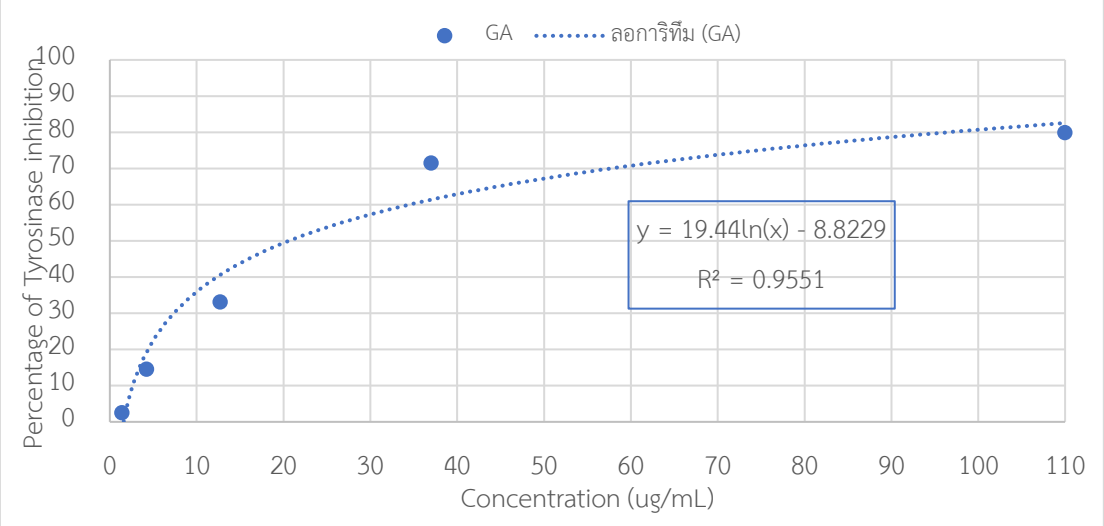


Figure B3.6 Antityrosinase activity of gallic acid

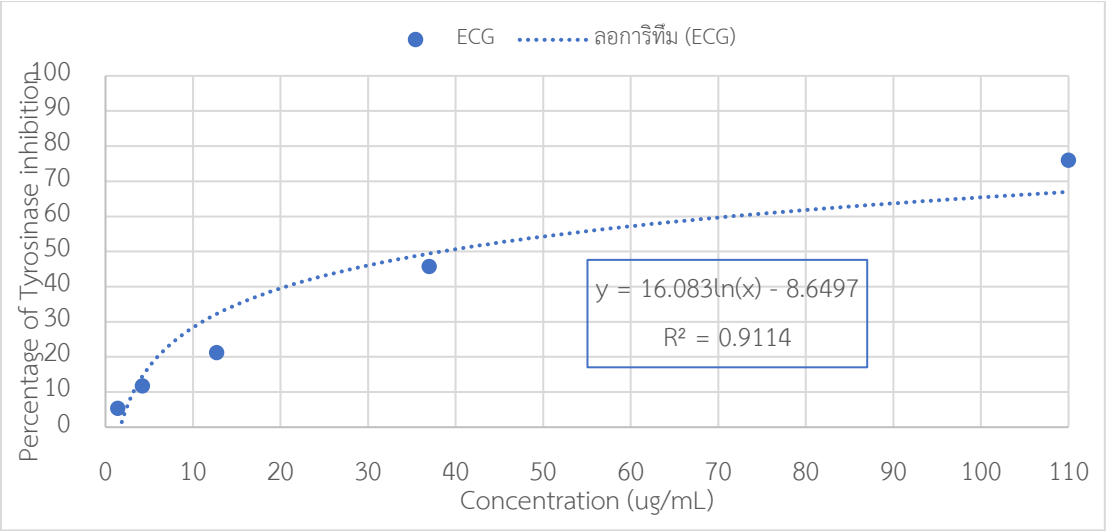


Figure B3.7 Antityrosinase activity of ECG

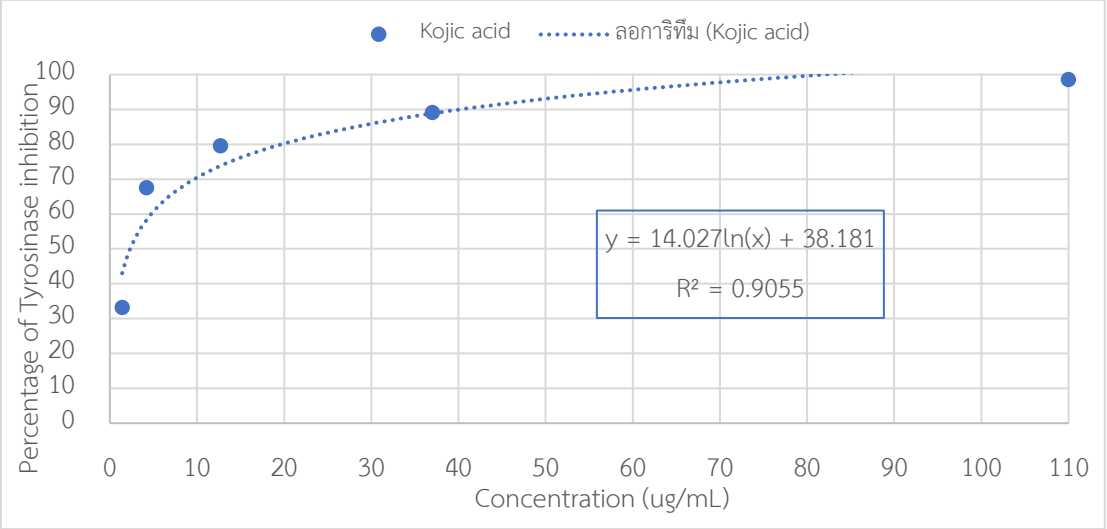


Figure B3.8 Antityrosinase activity of kojic acid (positive control)

B.4 Regression analysis of anticholinesterase activity (AChE and BChE) with correlation coefficient (r^2)

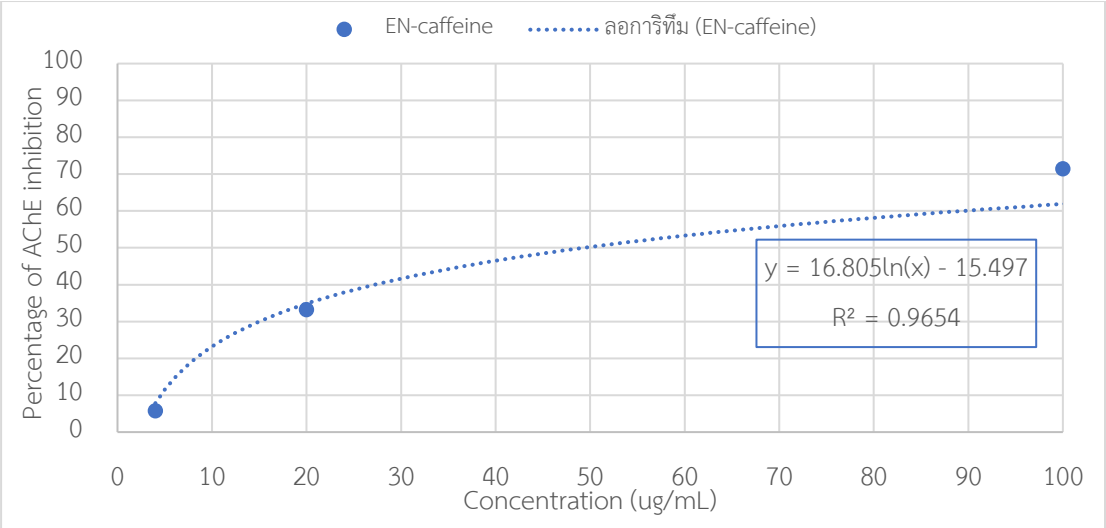


Figure B4.1 Anticholinesterase activity (AChE) of enrich-caffeine fraction

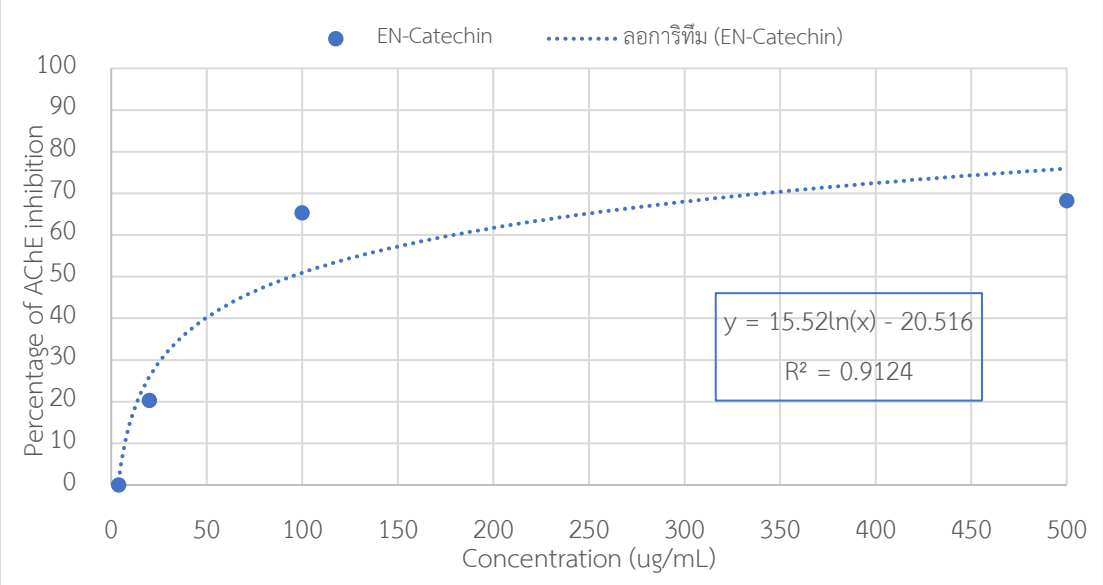


Figure B4.2 Anticholinesterase activity (AChE) of enrich-catechin family fraction

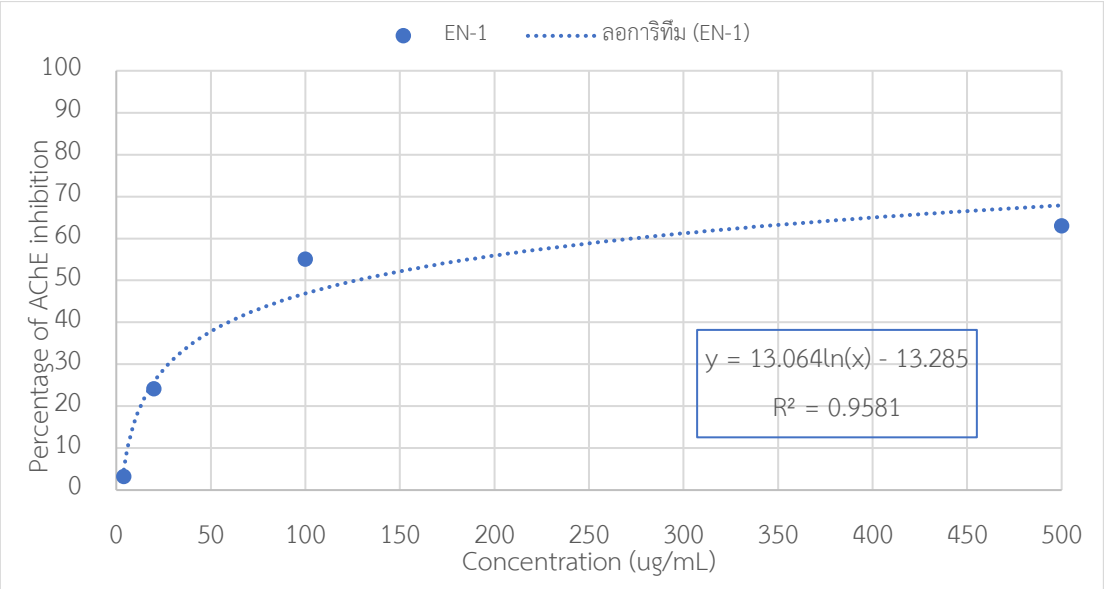


Figure B4.3 Anticholinesterase activity (AChE) of EN-1

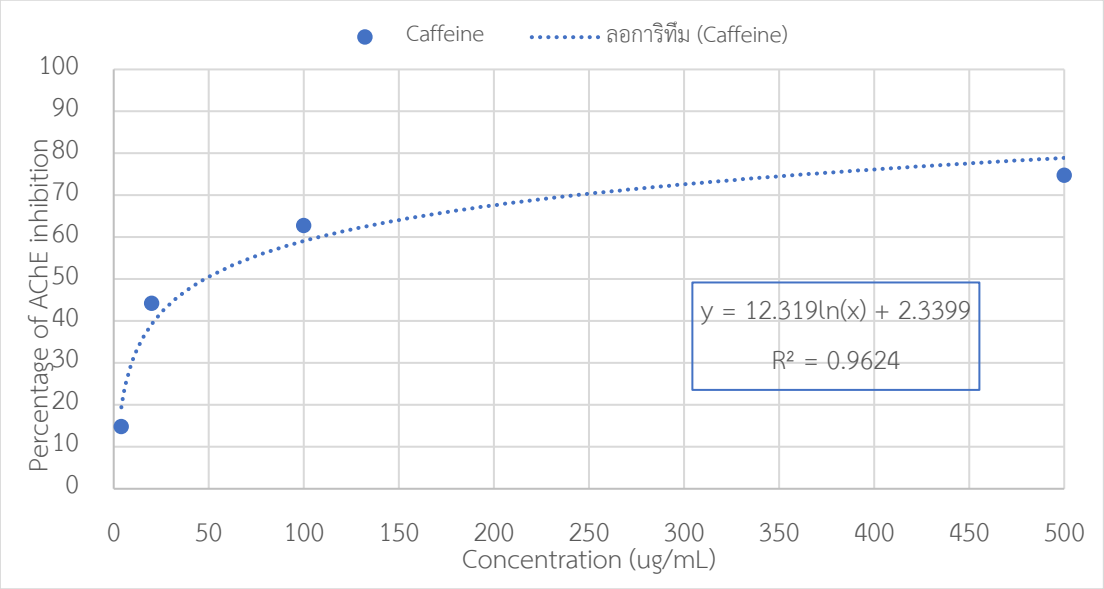


Figure B4.4 Anticholinesterase activity (AChE) of caffeine

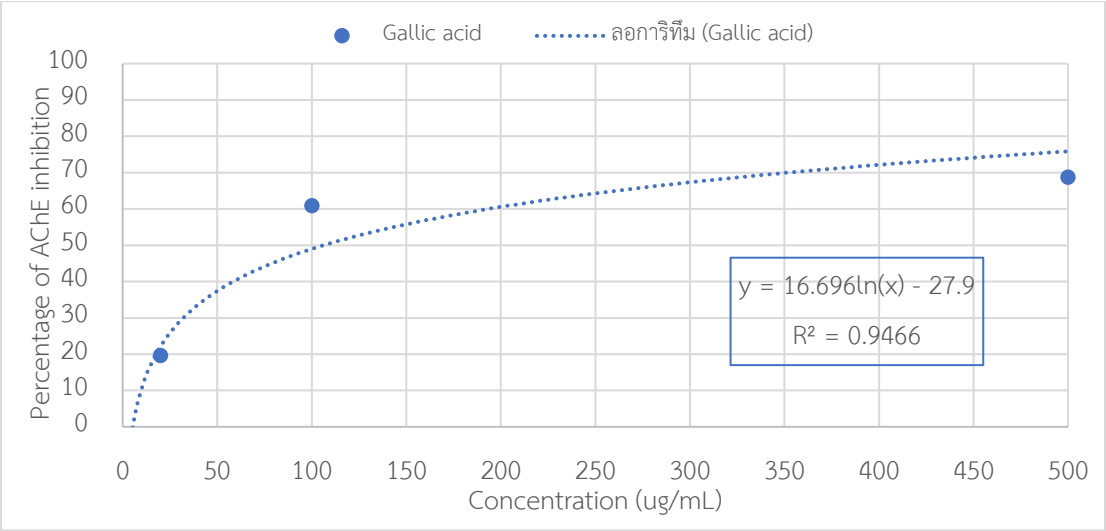


Figure B4.5 Anticholinesterase activity (AChE) of gallic acid

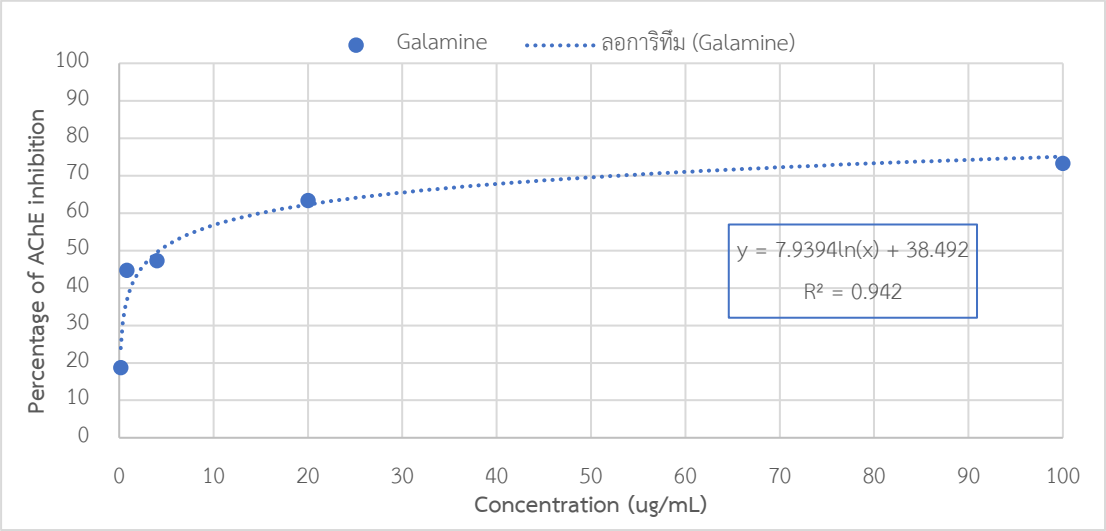


Figure B4.6 Anticholinesterase activity (AChE) of galantamine (positive control)

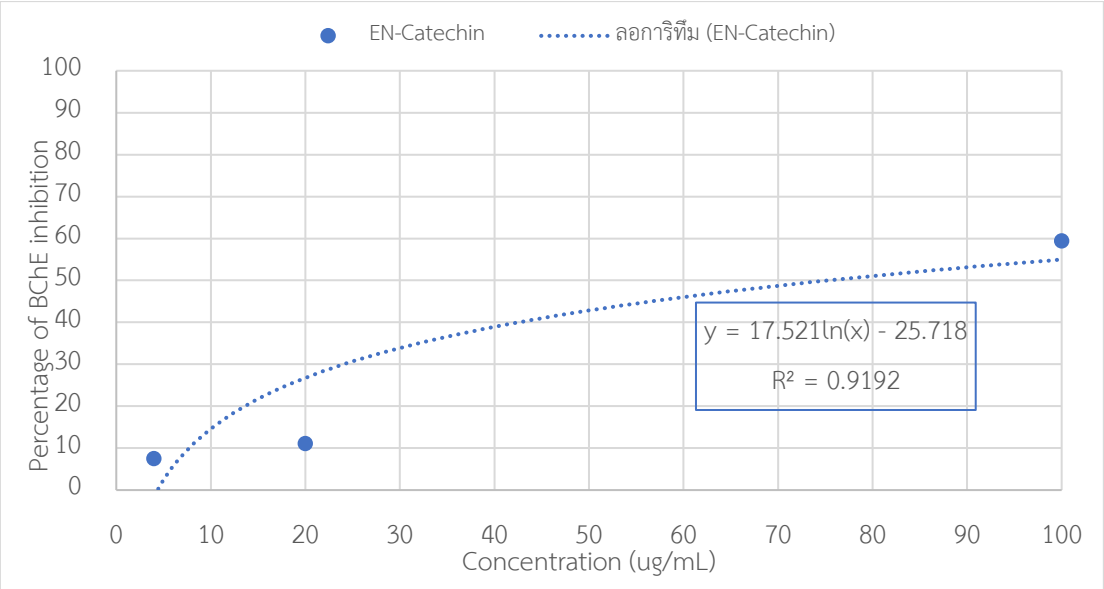


Figure B4.7 Anticholinesterase activity (BChE) of enrich-catechin family

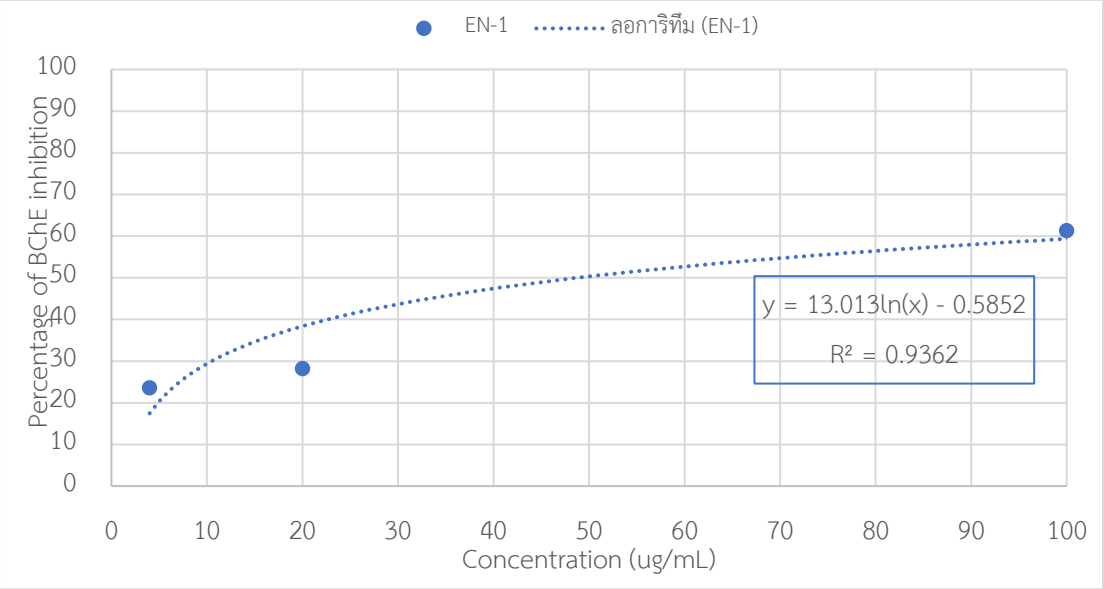


Figure B4.8 Anticholinesterase activity (BChE) of EN-1

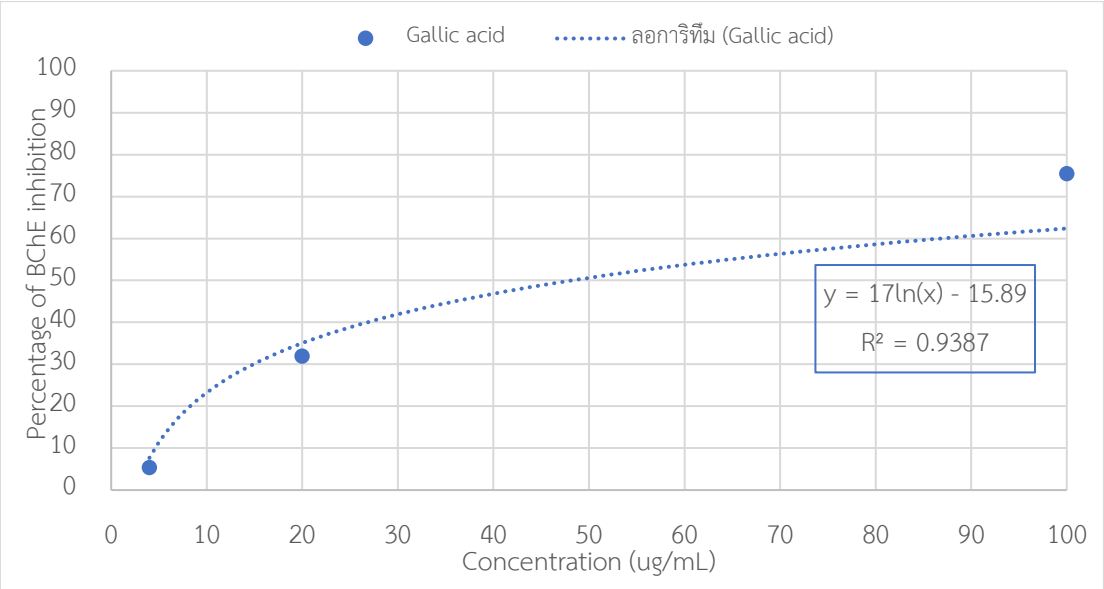


Figure B4.9 Anticholinesterase activity (BChE) of gallic acid

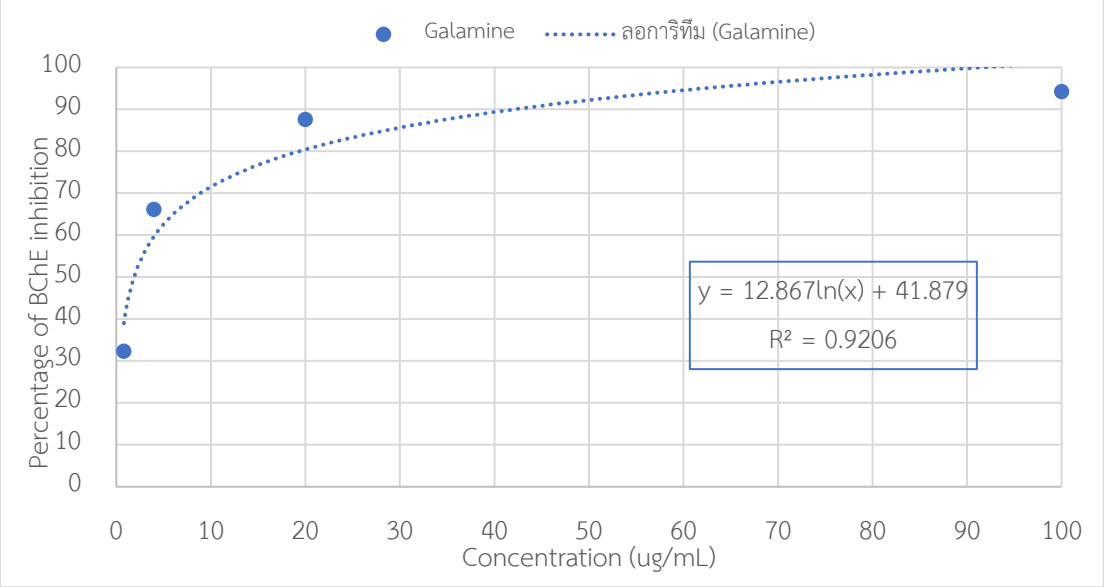


Figure B4.10 Anticholinesterase activity (BChE) of galamine (positive control)

Appendix C

NMR Spectral and chromatogram of HPLC

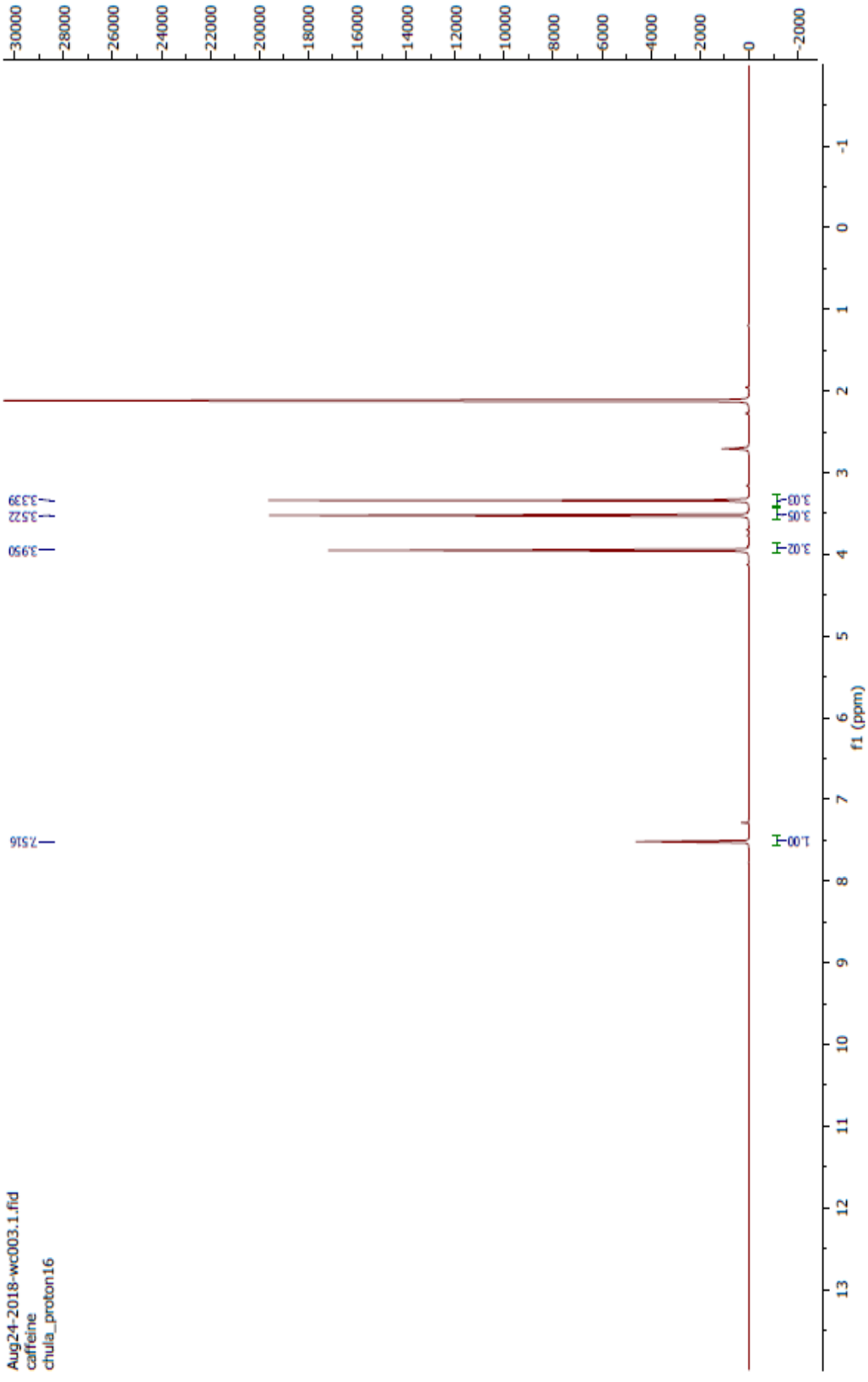


Figure C1.1 The ^{1}H NMR spectrum of caffeine

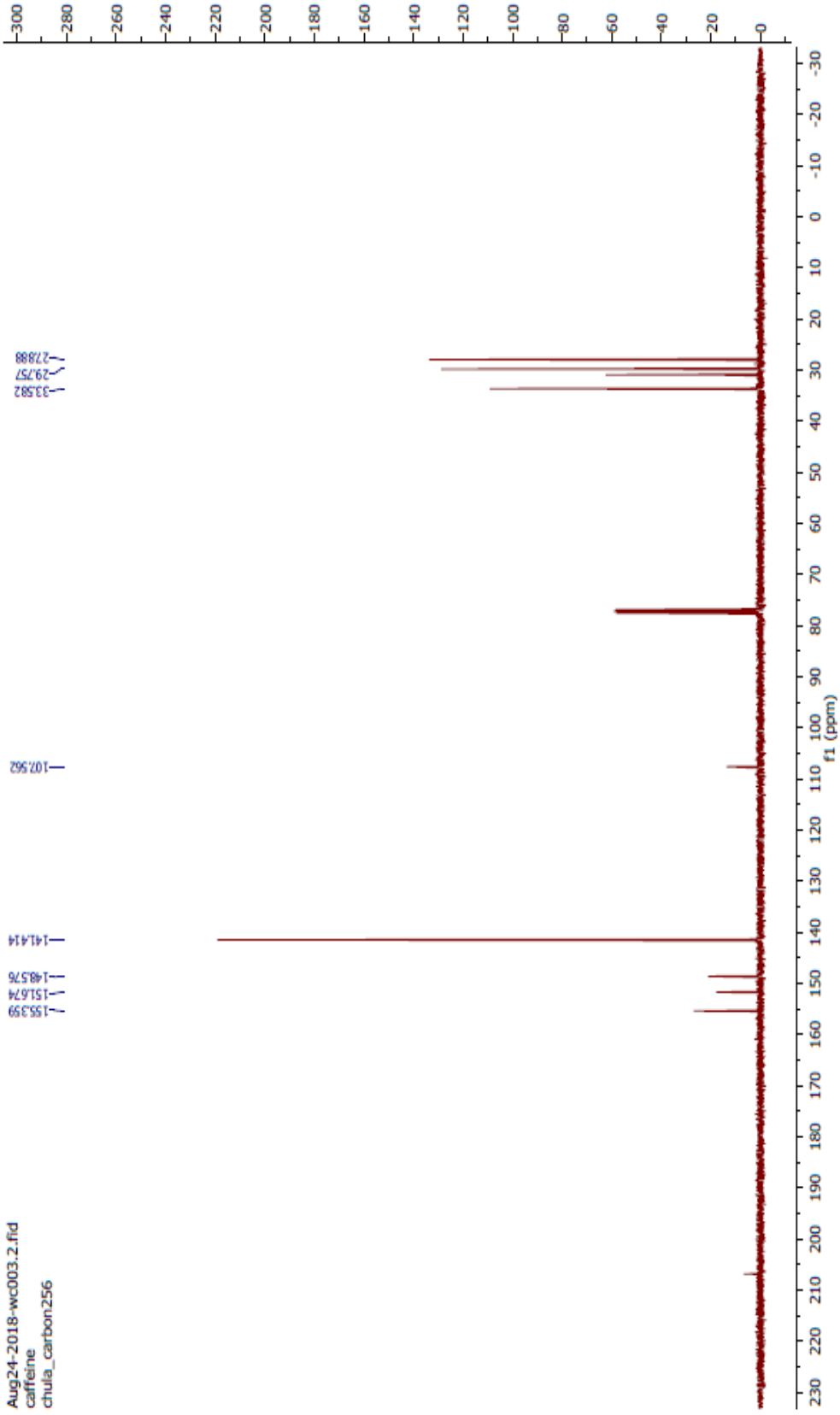


Figure C1.2 The ^{13}C NMR spectrum of caffeine

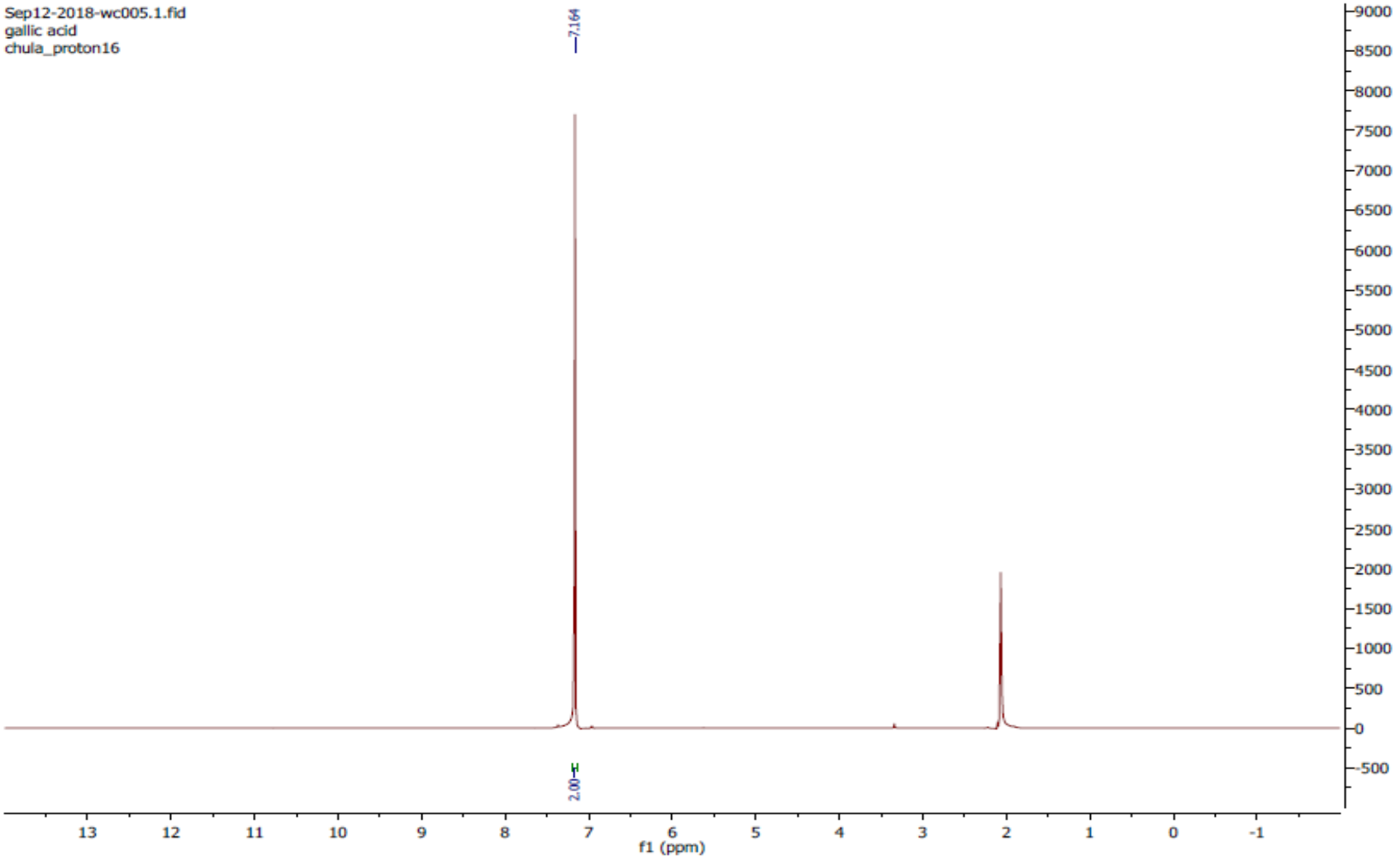


Figure C1.3 The ^{1}H NMR spectrum of gallic acid.

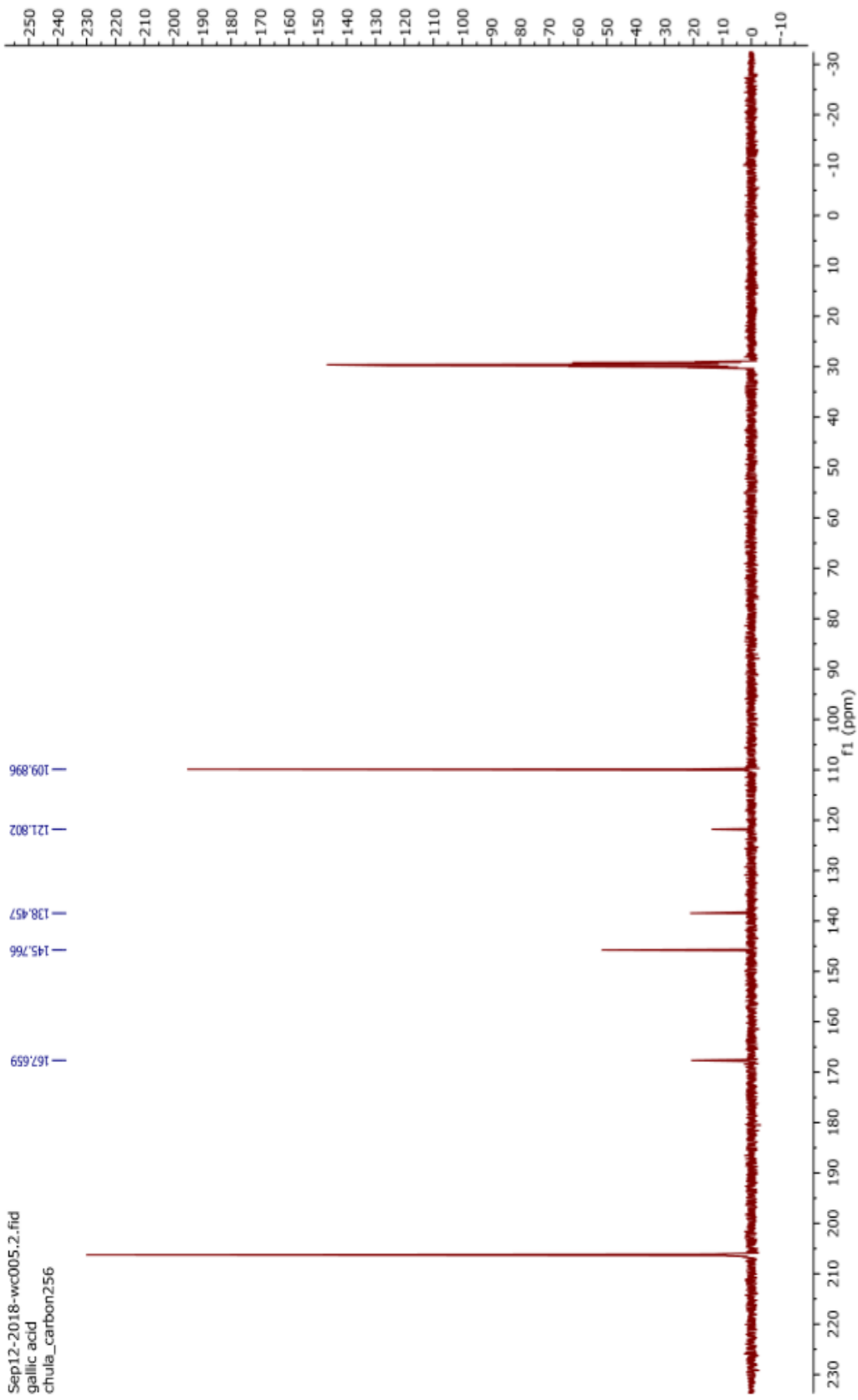


Figure C1.4 The ^{13}C NMR spectrum of gallic acid

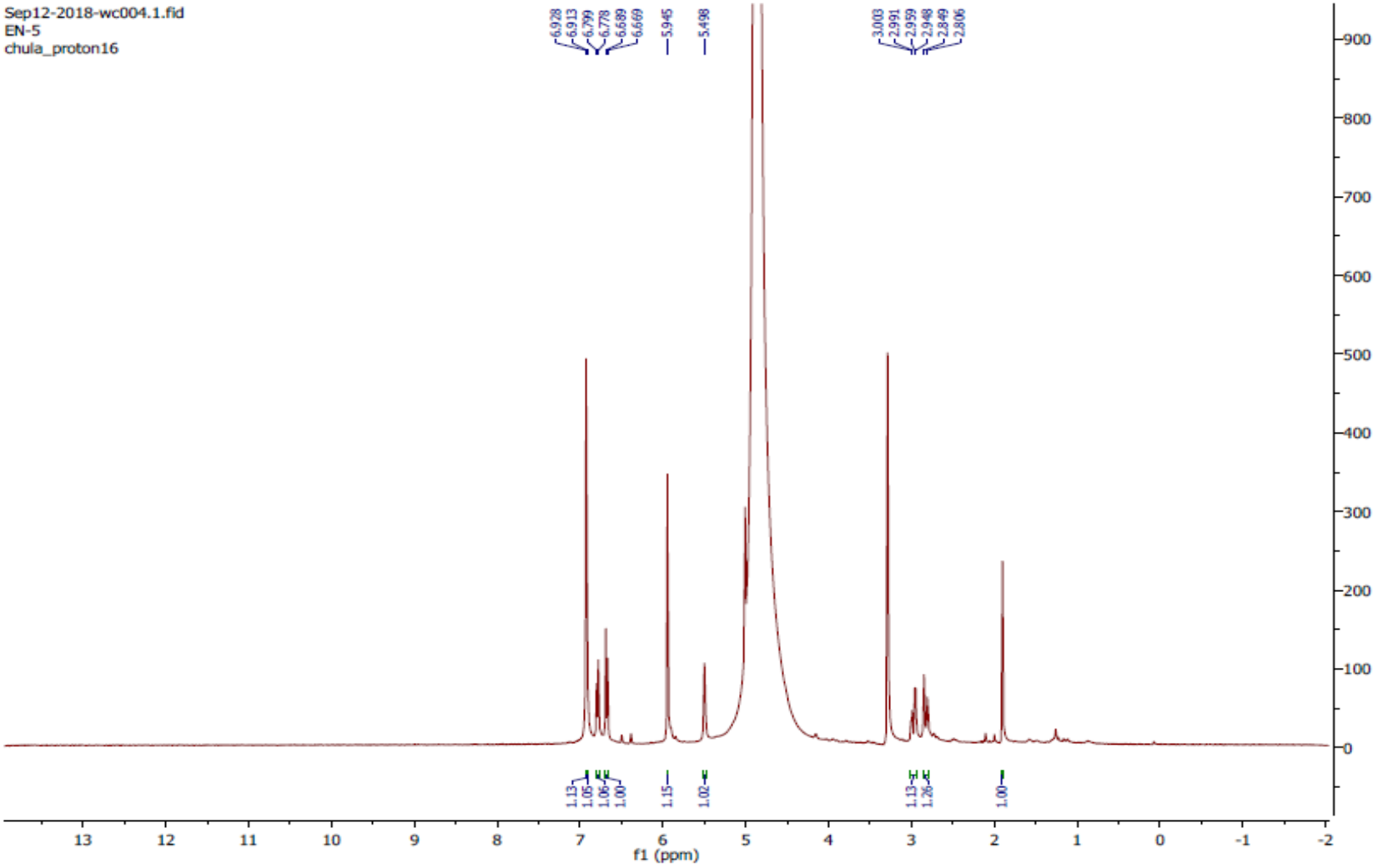


Figure C1.5 The ^{1}H NMR spectrum of ECG

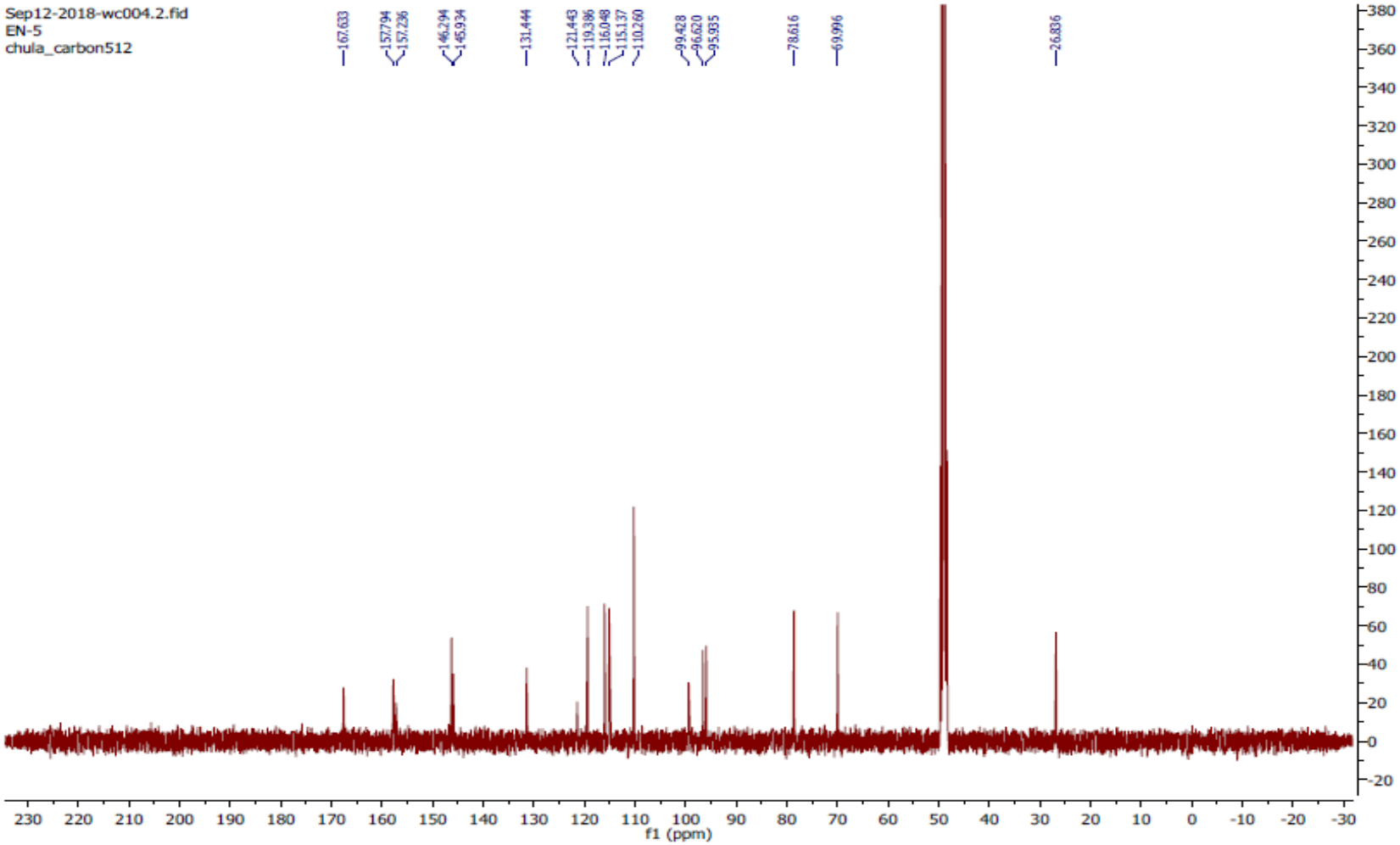


Figure C1.6 The ^{13}C NMR spectrum of ECG

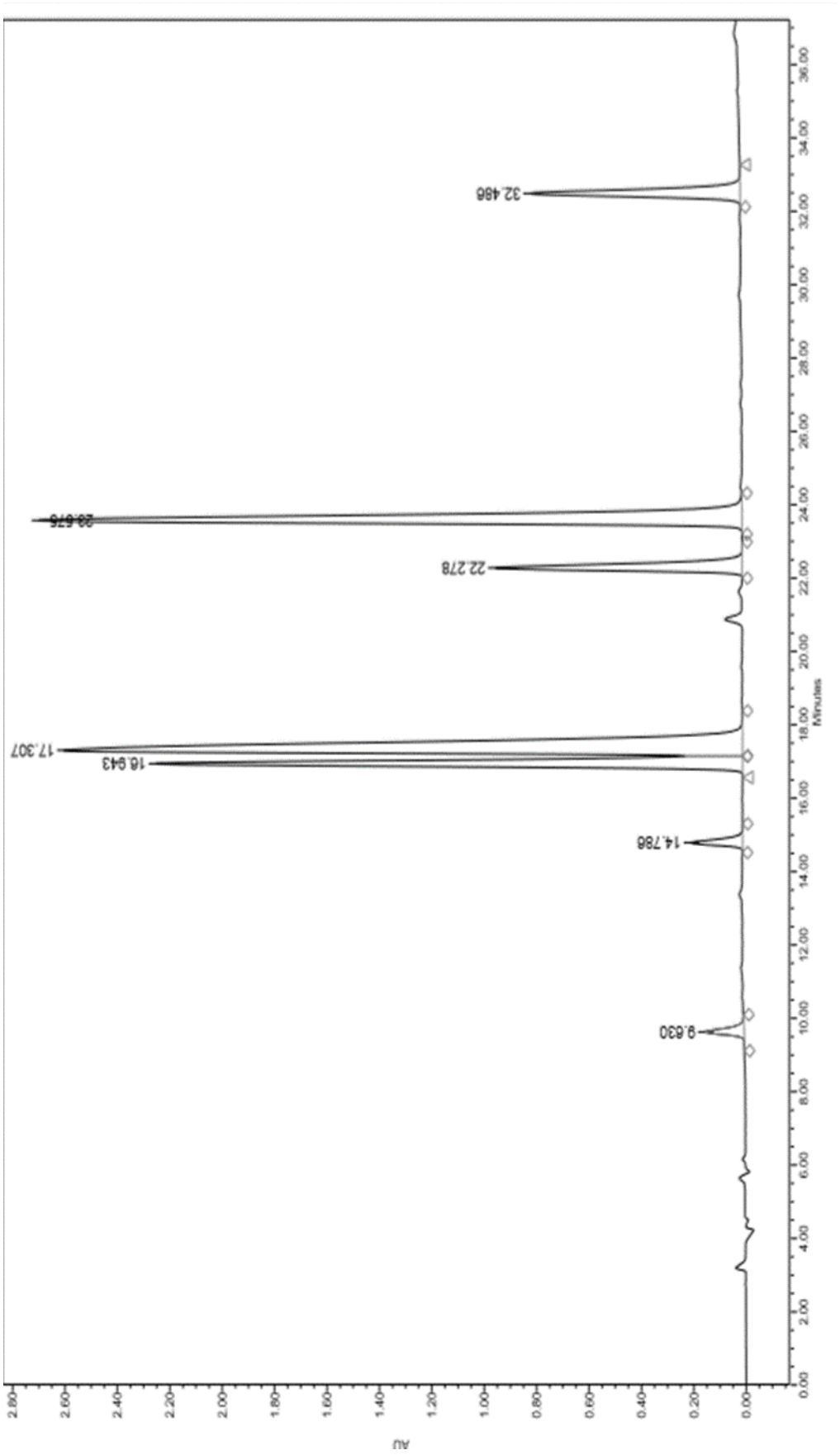


Figure C1.7 The HPLC chromatogram of standard

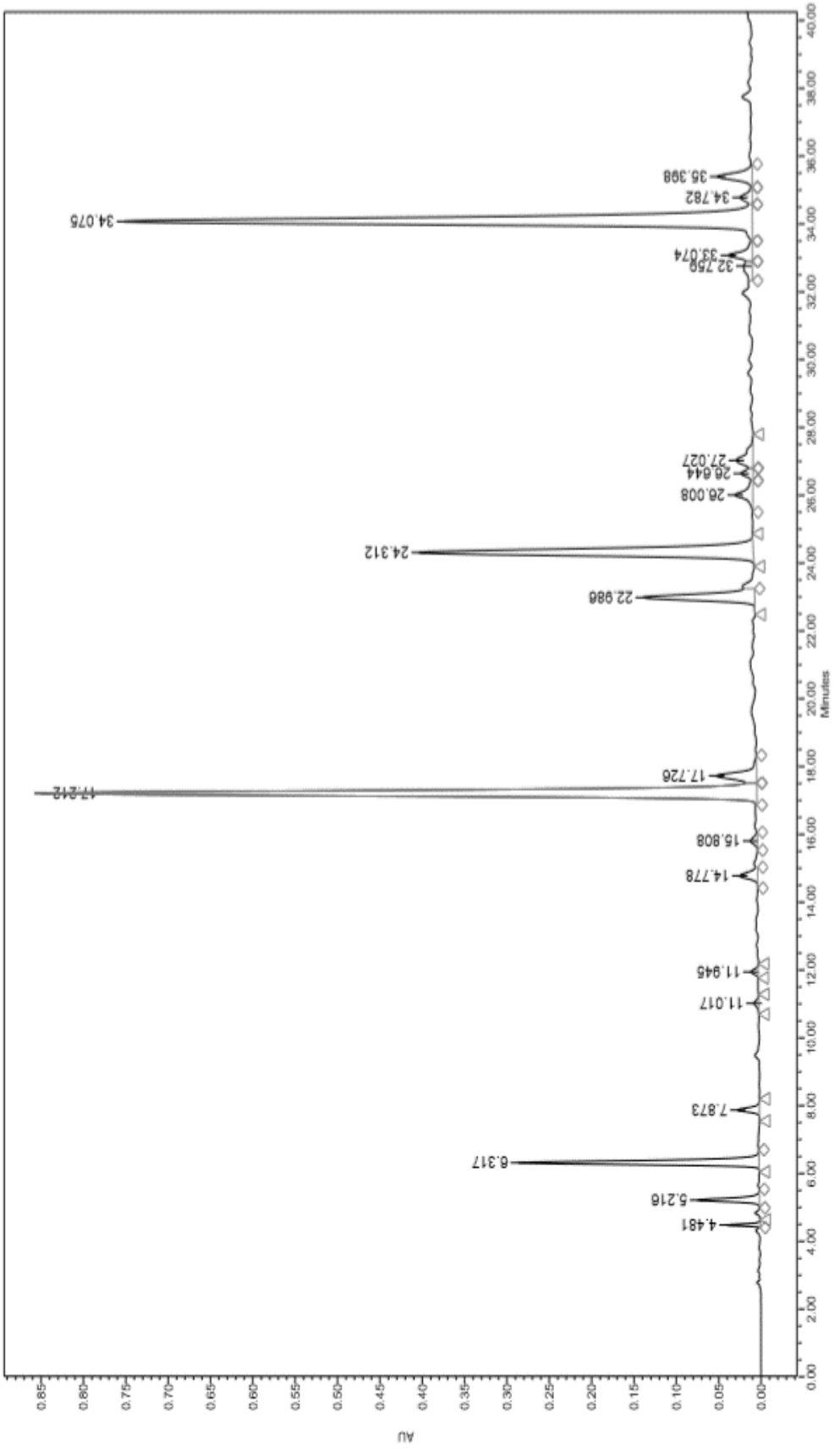


Figure C1.8 The HPLC chromatogram of GTL methanol extract.

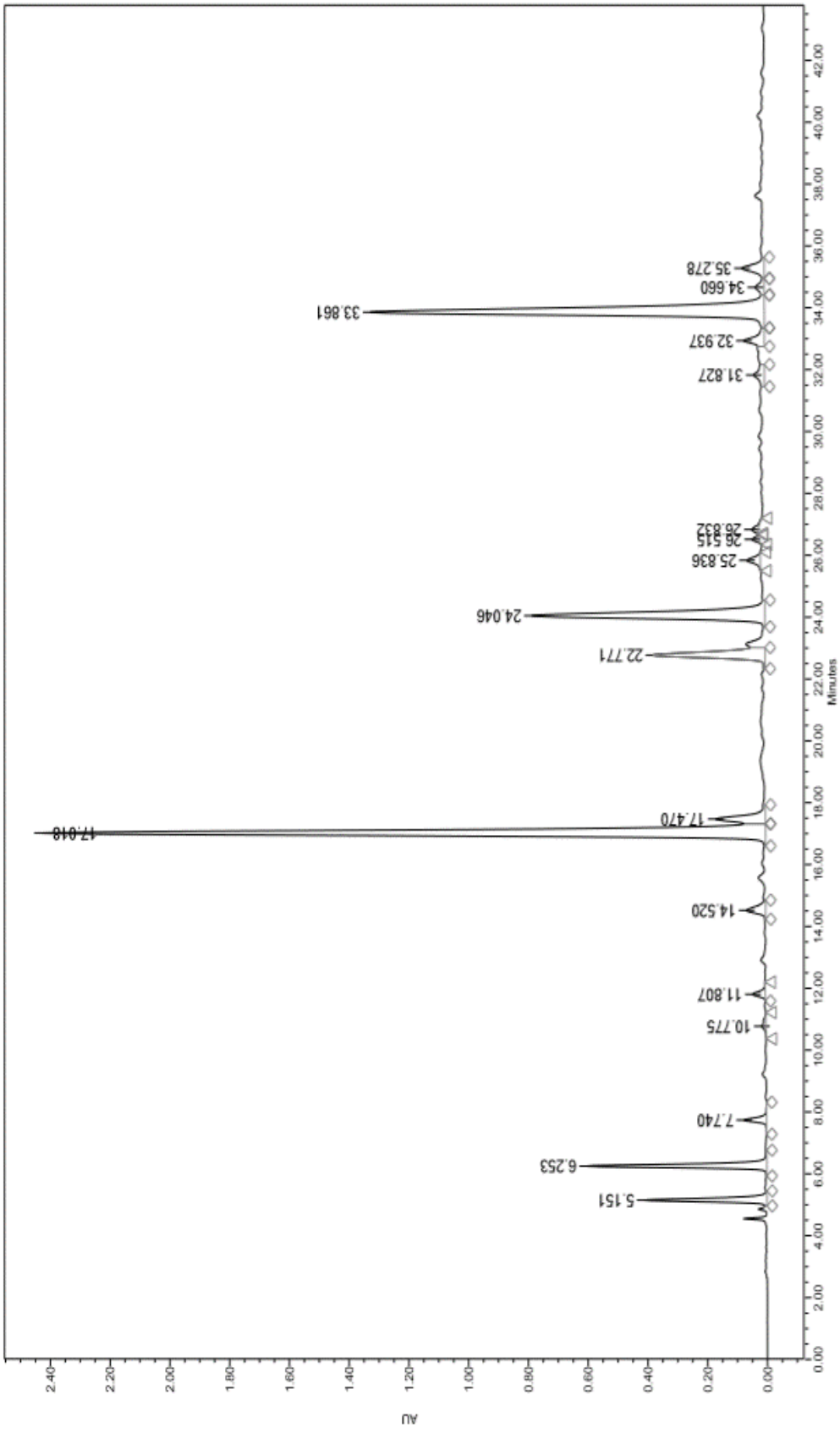


Figure C1.9 The HPLC chromatogram of SGTL methanol extract.

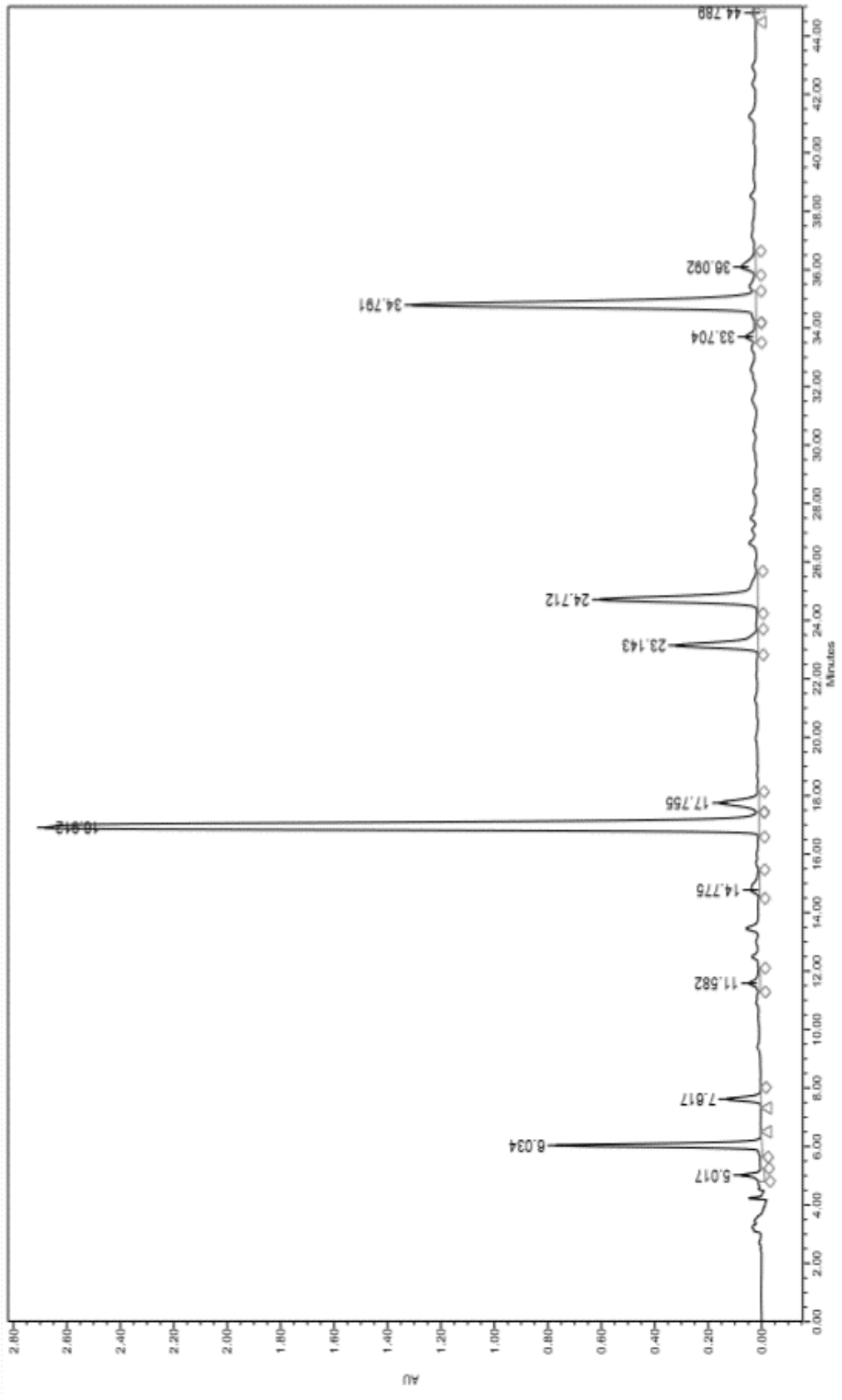


Figure C1.10 The HPLC chromatogram of SGTL acetone extract for 1 hour

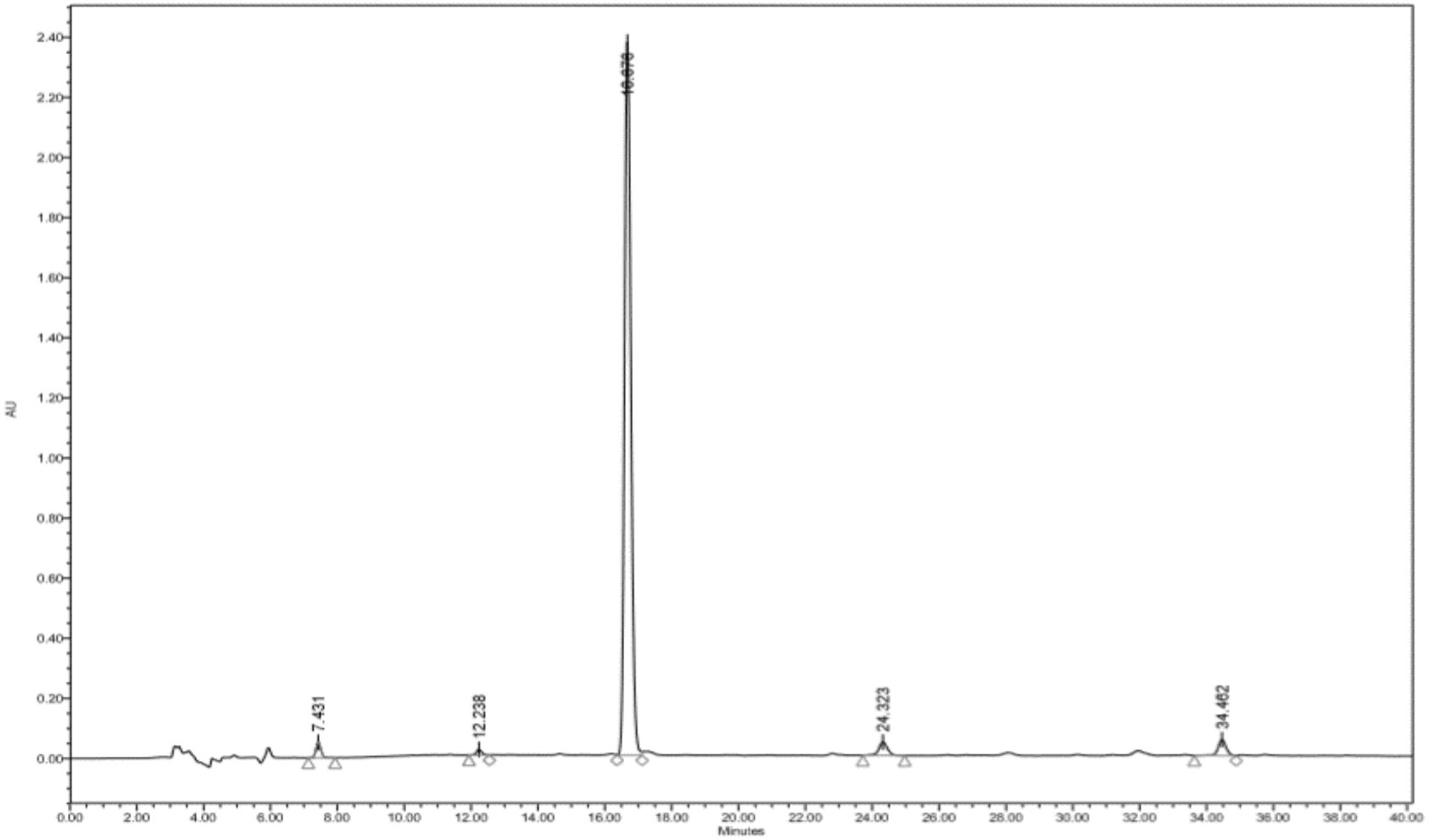


Figure C1.11 The HPLC chromatogram of SGTL dichloromethane extract for 1 hour

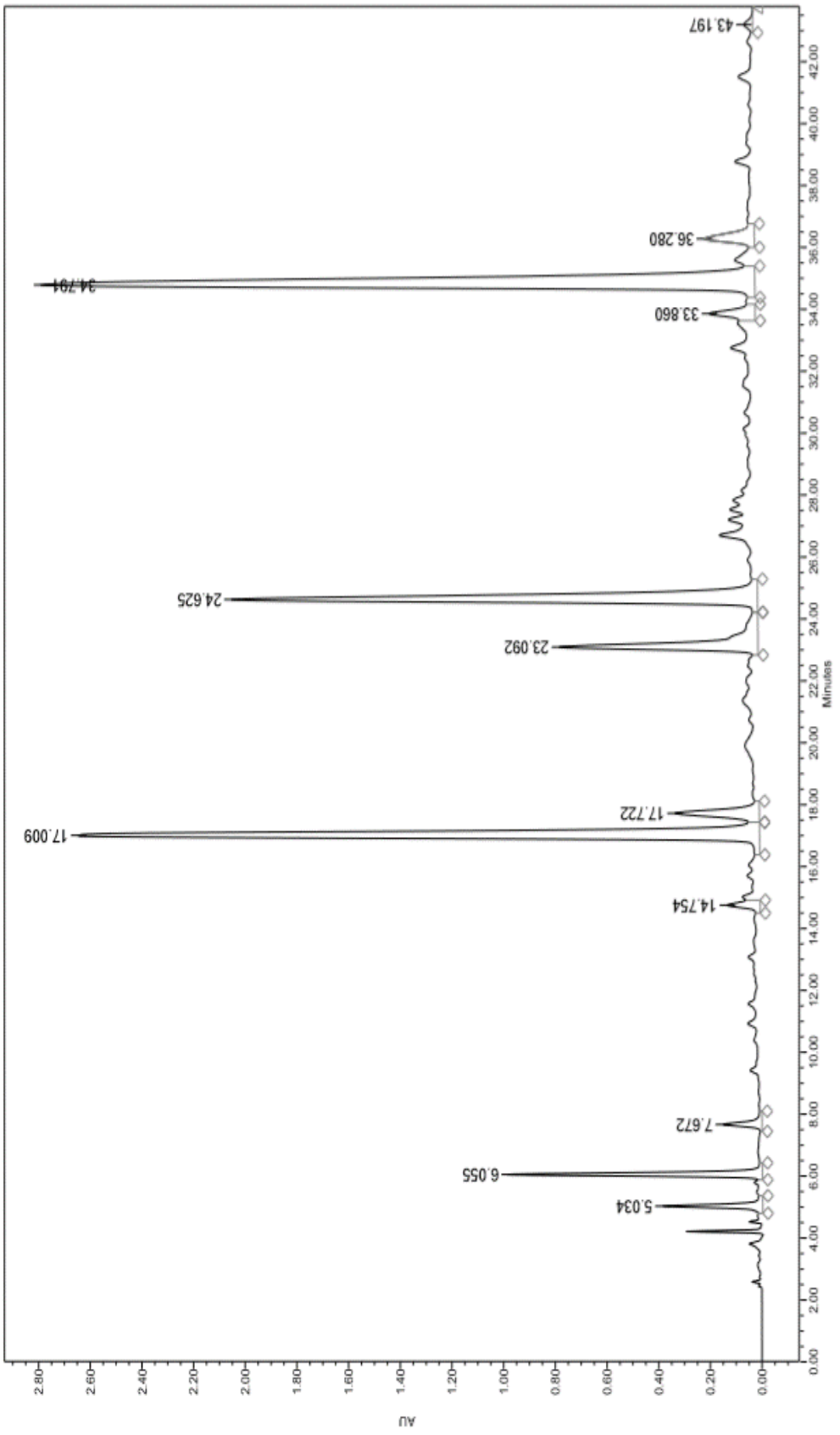


Figure C1.12 The HPLC chromatogram of SGTL methanol extract for 1 hour

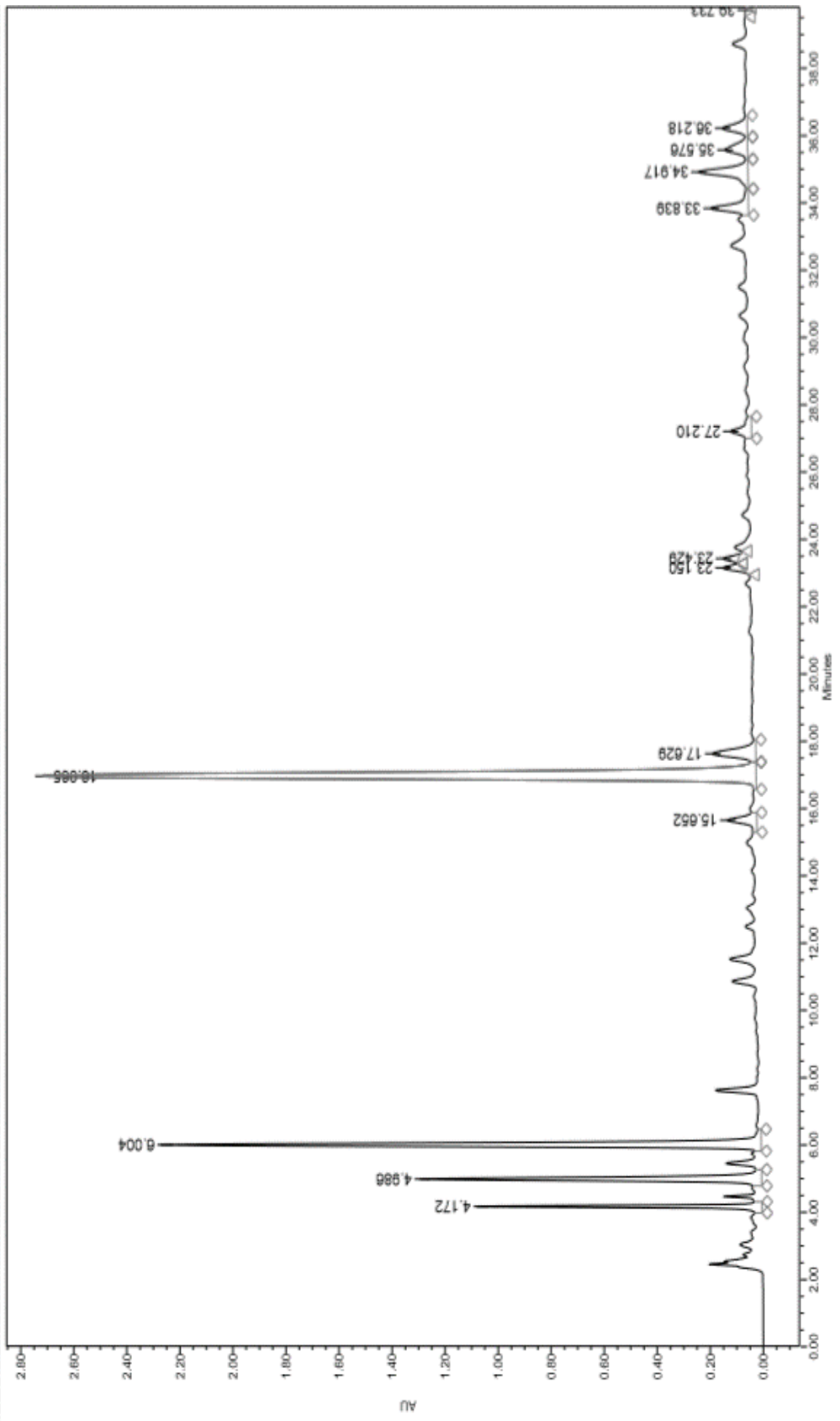


Figure C1.13 The HPLC chromatogram of SGTL water extract for 1 hour

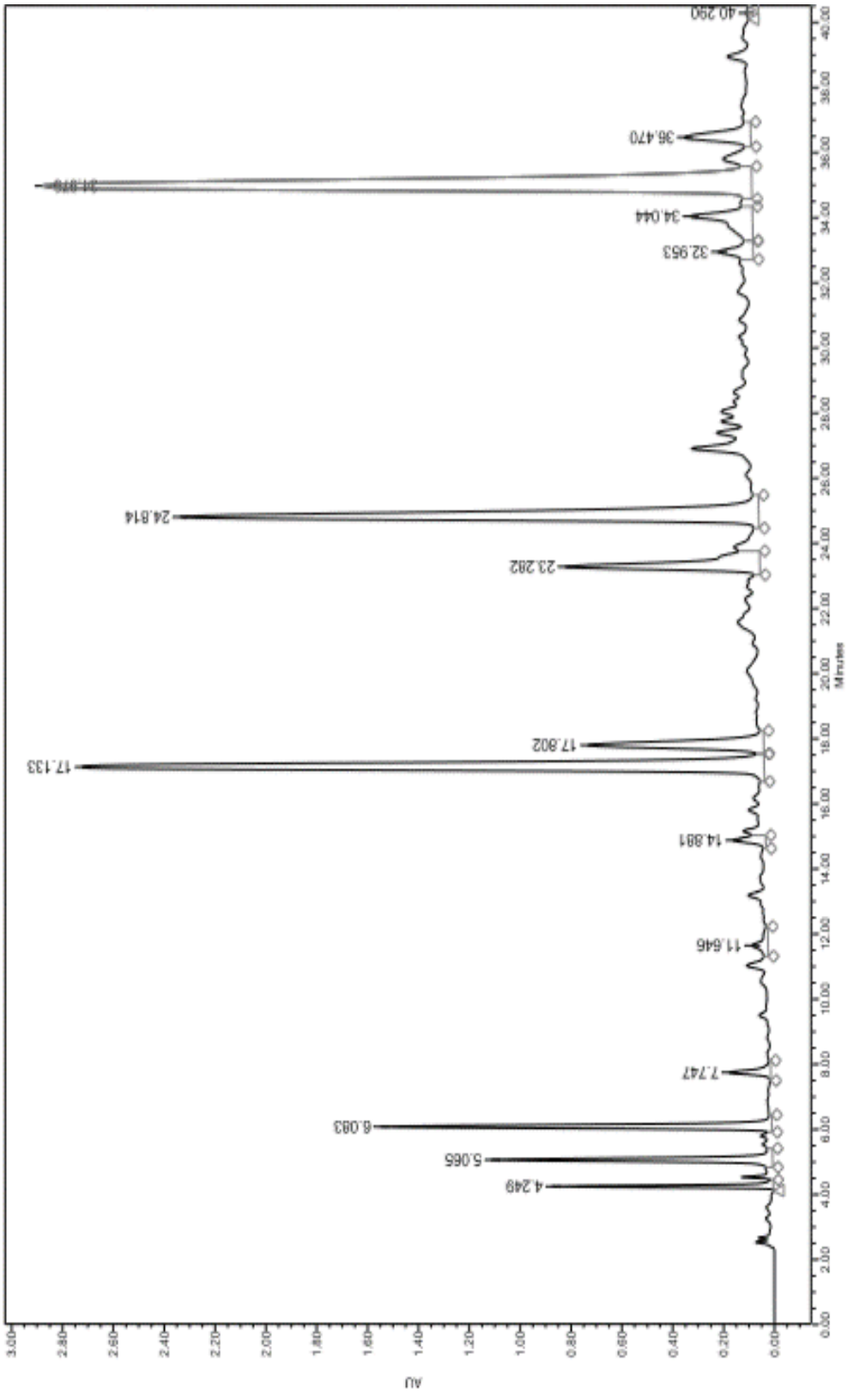


Figure C1.14 The HPLC chromatogram of SGTL 50%ethanol extract for 1 hour

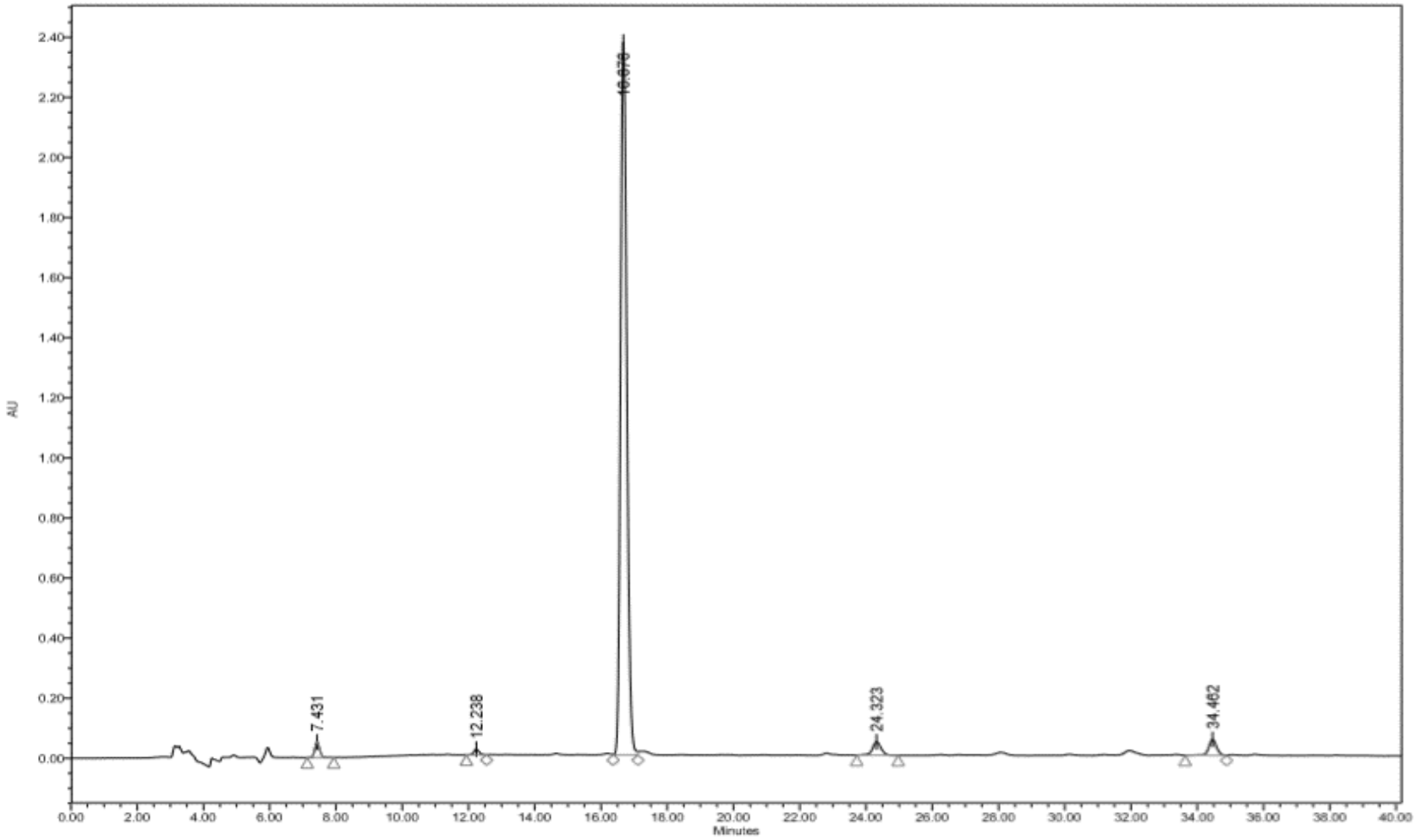


Figure C1.15 The HPLC chromatogram of SGTL dichloromethane extract for 4 hours

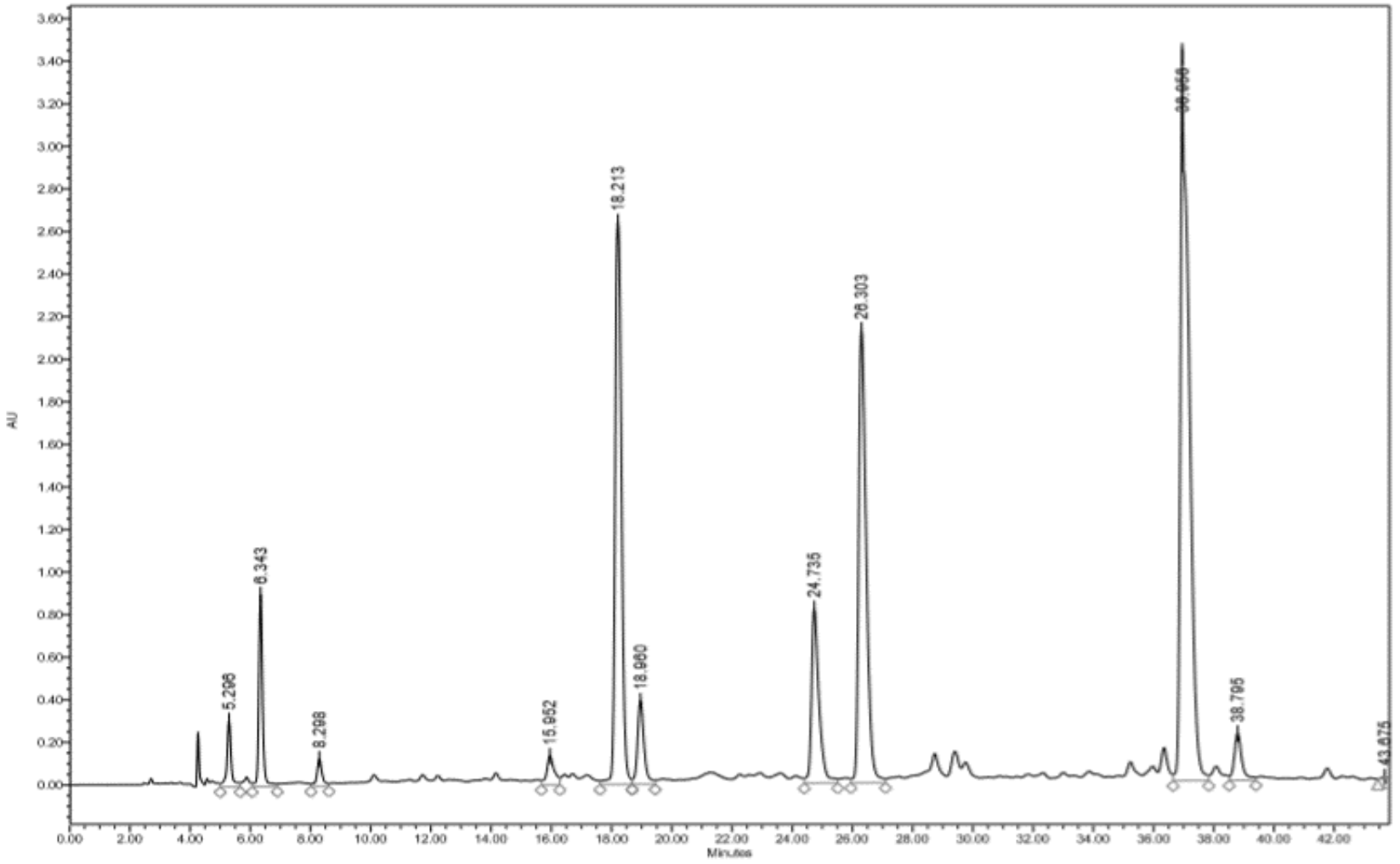


Figure C1.16 The HPLC chromatogram of SGTL methanol extract after dichloromethane extraction

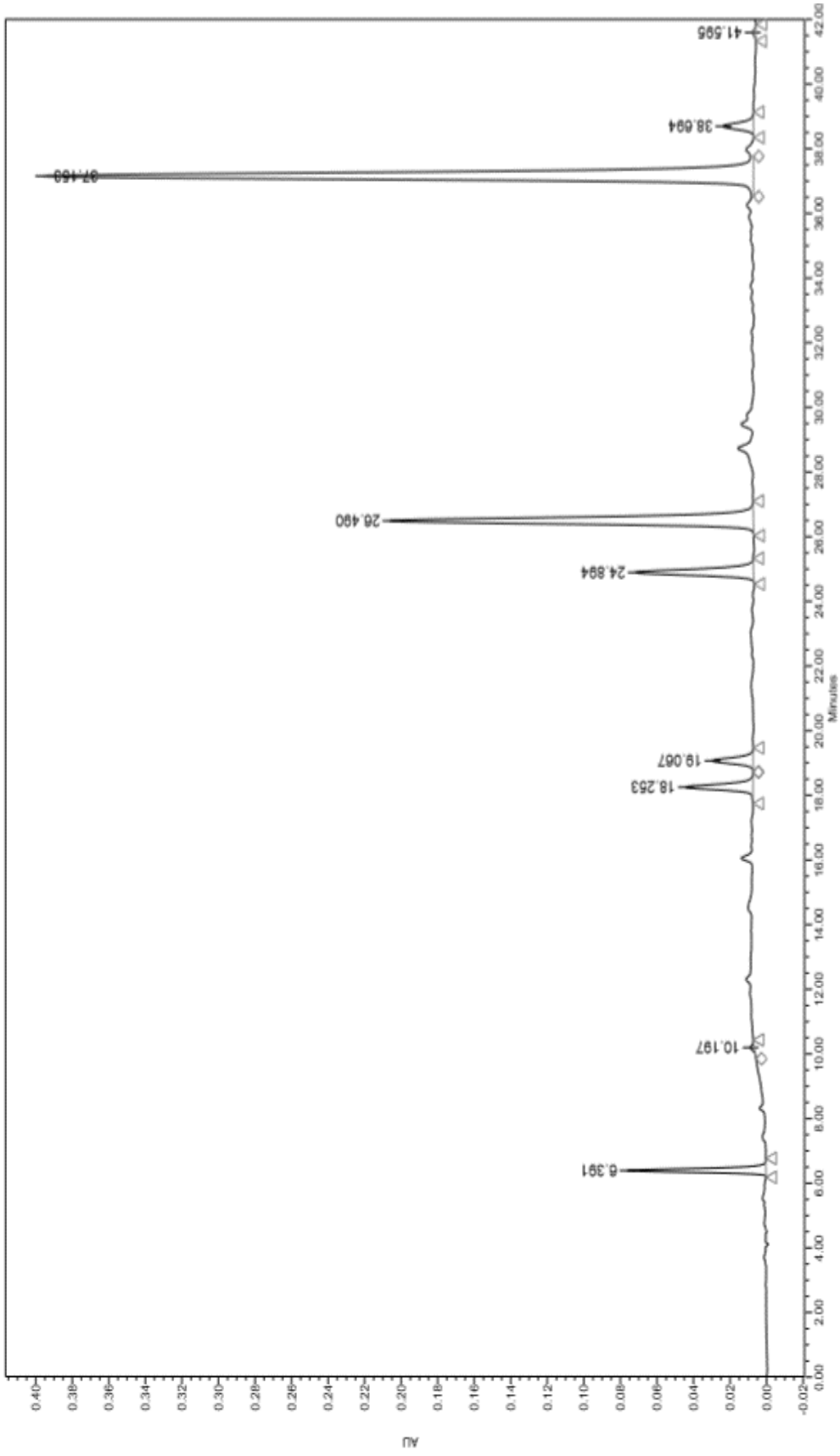


Figure C1.17 The HPLC chromatogram of ethyl acetate fraction

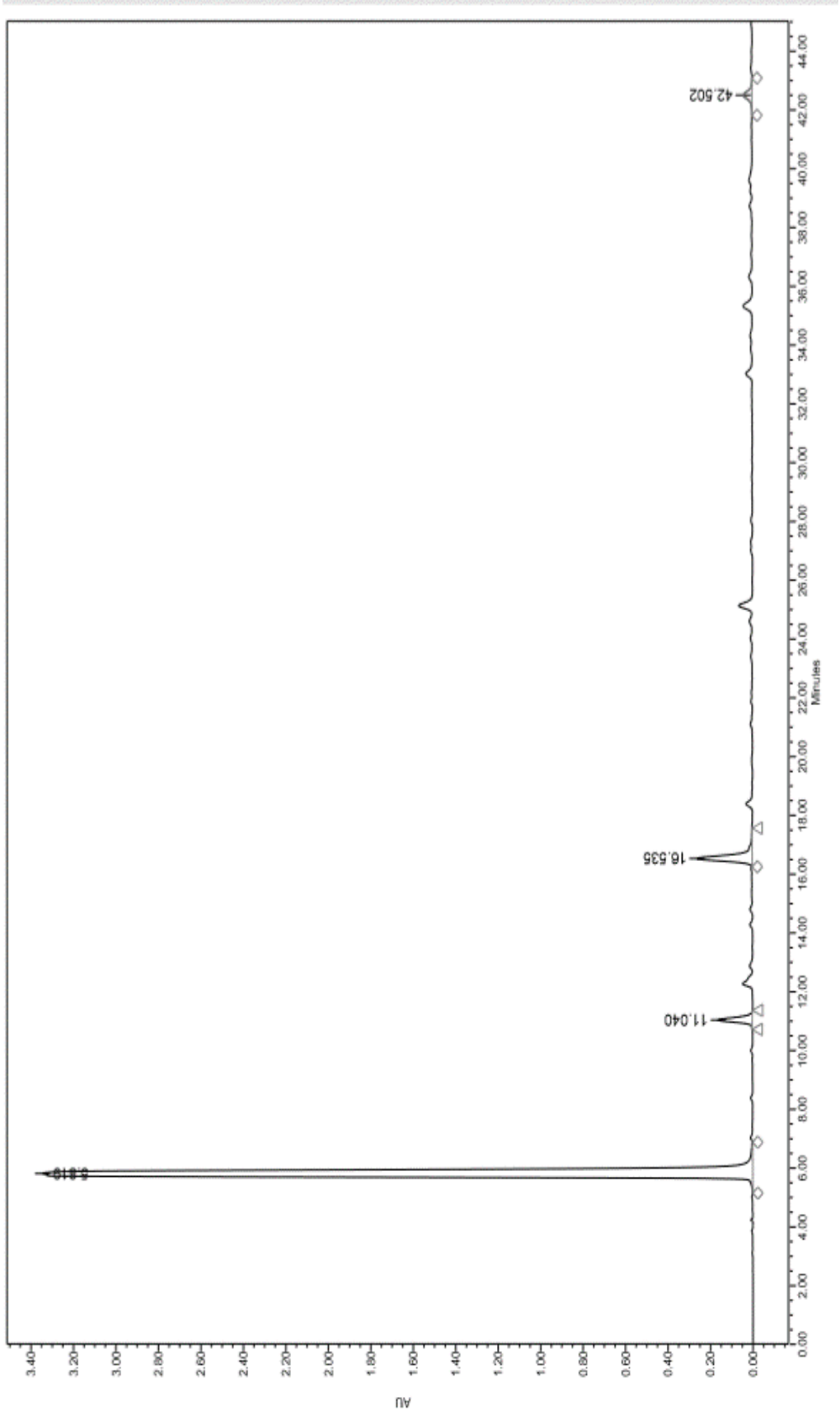


Figure C1.18 The HPLC chromatogram of **EN-1**

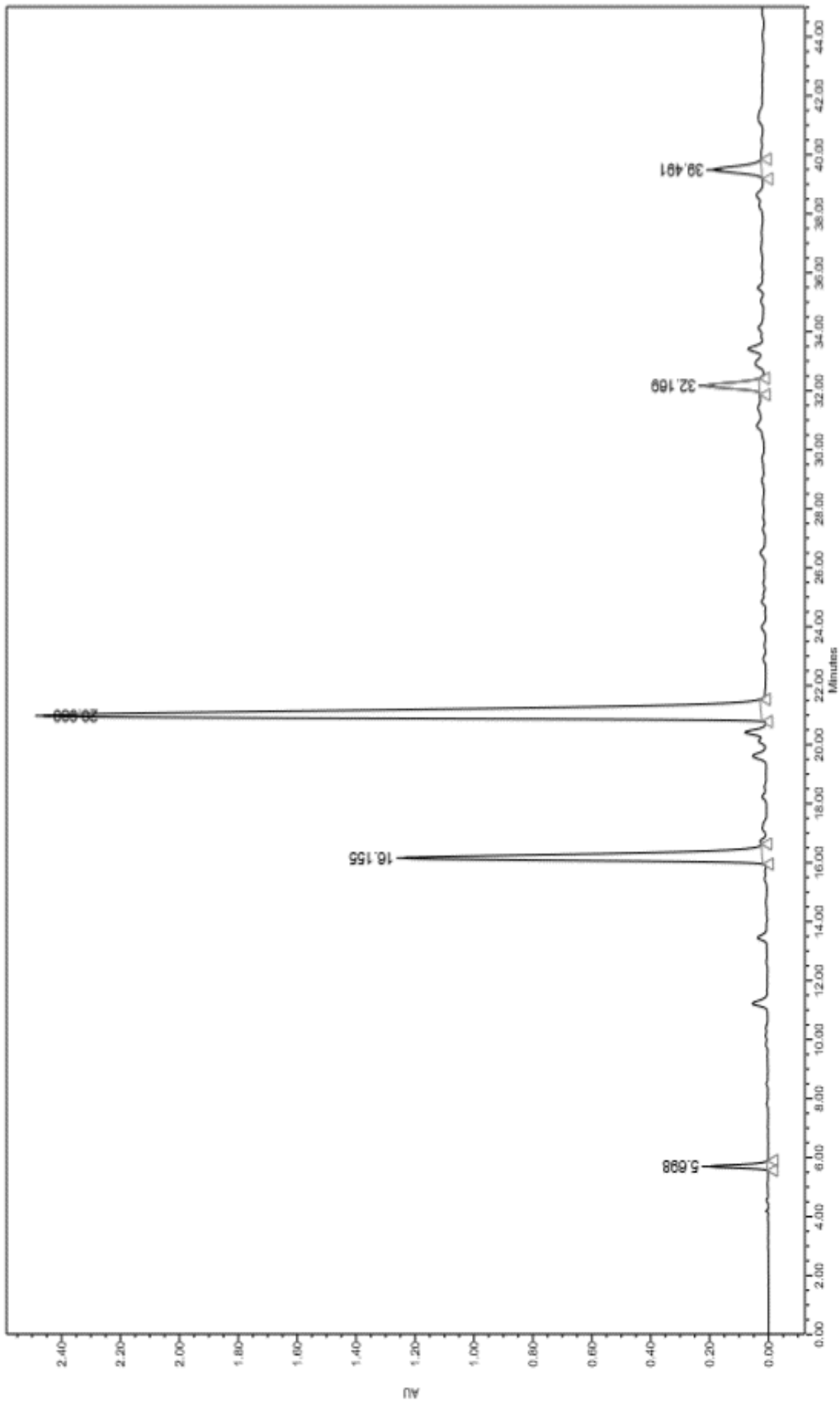


Figure C1.19 The HPLC chromatogram of **EN-2**

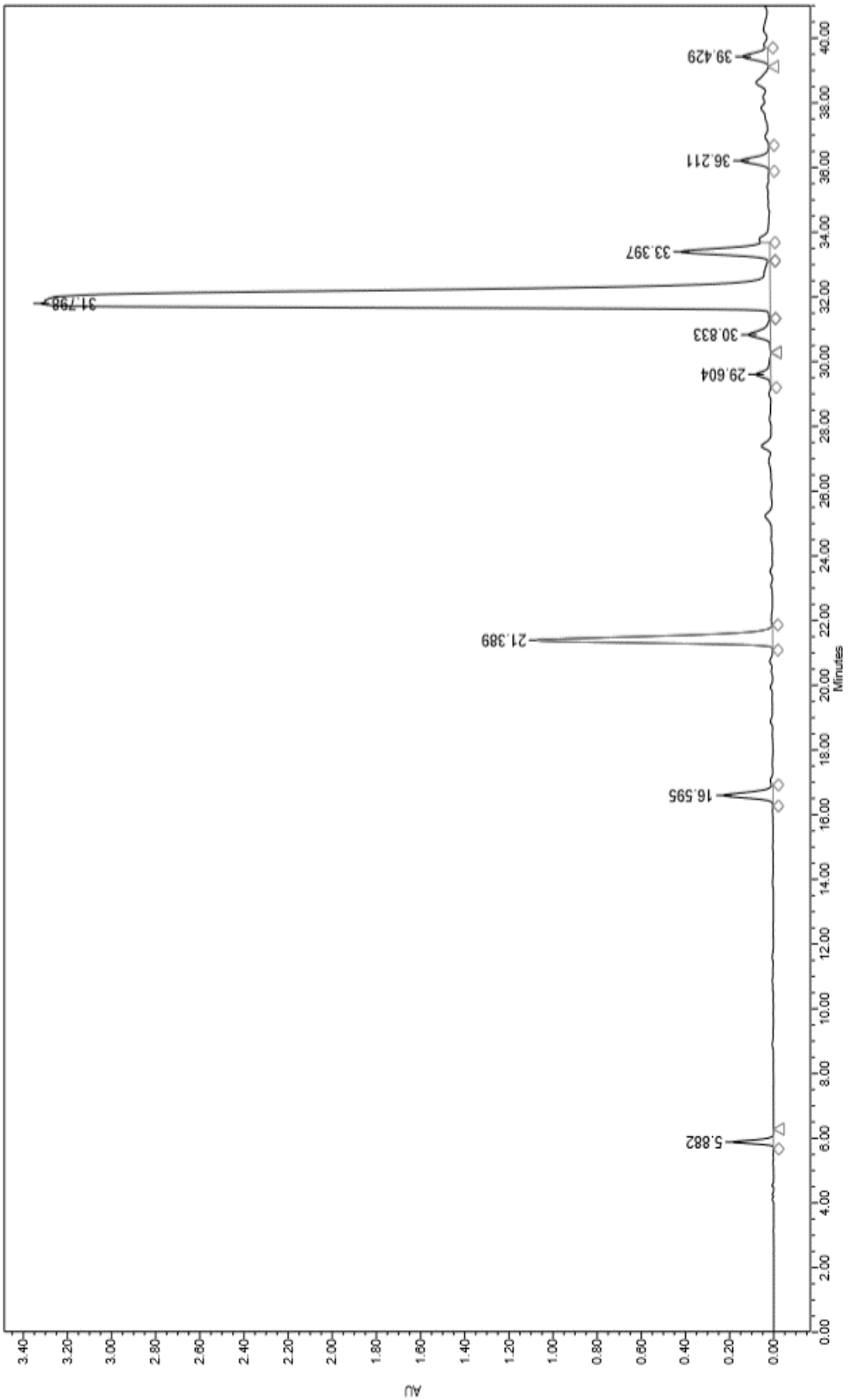


Figure C1.20 The HPLC chromatogram of **EN-3**

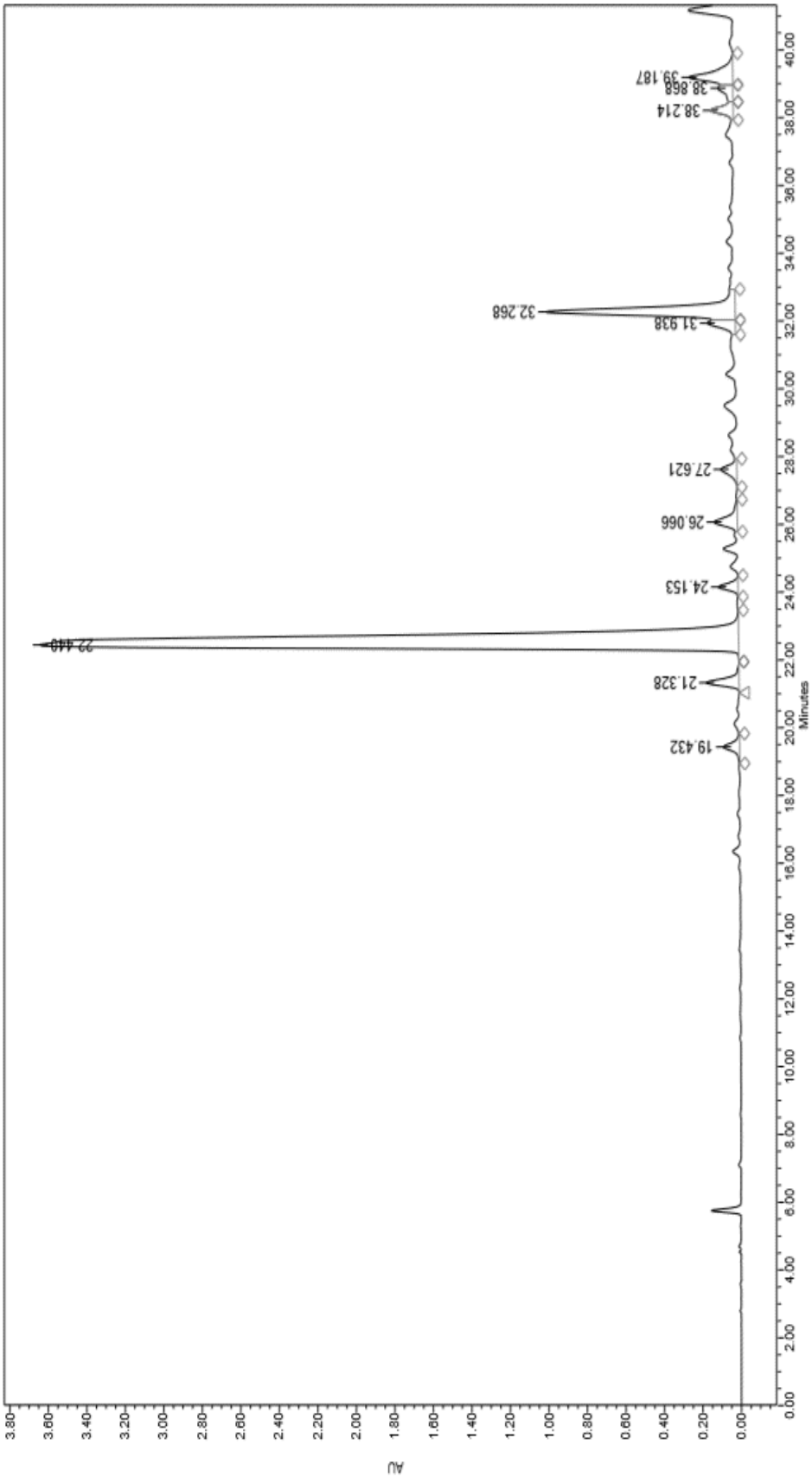


Figure C1.21 The HPLC chromatogram of **EN-4**

VITA

NAME	Ms. Monsichar Yannarat
DATE OF BIRTH	5 April 1994
PLACE OF BIRTH	Nongkhai province, Thailand
INSTITUTIONS ATTENDED	Degree of Bachelor (Biotechnology) from Faculty of Agro-Industry, Chiangmai University in 2015
HOME ADDRESS	Chiang Mai province, Thailand
PUBLICATION	ANRES 2018