สารยับยั้งแอลฟากลูโคซิเดสจากเมล็ดกระเจี๊ยบเขียว *Abelmoschus esculentus* และใบมะตูมซาอุ *Schinus terebinthifolius*

นางสาววรรณิศา ธนะโกสัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2555

α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS AND *Schinus terebinthifolius* LEAVES

Miss Wannisa Thanakosai

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS AND *Schinus terebinthifolius* LEAVES
By	Miss Wannisa Thanakosai
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Preecha Phuwapraisirisan, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr. rer. nat)

THESIS COMMITTEE

……...Chairman
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

…….....Thesis Advisor
(Assistant Professor Preecha Phuwapraisirisan, Ph.D.)

…… Examiner
(Associate Professor Chanpen Chanchao, Ph.D.)

…….. External Examiner
(Assistant Professor Wimolpun Rungprom, Ph.D.)

วรรณิศา ธนะโกสัย: สารยับยั้งแอลฟากลูโคซิเดสจากเมล็ดกระเจี๊ยบเขียว *Abelmoschus esculentus* และใบมะตูมซาอุ *Schinus terebinthifolius* (α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS AND *Schinus terebinthifolius* LEAVES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ปรีชา ภูวไพรศิริศาล, 59 หน้า

การค้นพบสารยับยั้ง α-glucosidase จะสามารถนำไปสู่การพัฒนาการรักษาโรคเบาหวานชนิดที่ 2 ได้ โดยสารสกัดจากเมล็ดกระเจี๊ยบเขียว และใบมะตูมซาอุซึ่งจัดเป็นพืชกินได้นั้นได้ถูกเลือกมาศึกษาฤทธิ์ในการยับยั้ง α-glucosidase การแยกส่วนสกัดเมทานอลจากเมล็ดกระเจี๊ยบเขียวได้สารกลุ่ม flavonoid glycosides 2 ชนิดคือ quercetin-3-*O*-β-glucoside หรือ isoquercetin และ quercetin-3-*O*-β-glucosyl (1‴→6″) glucoside โดยสารสกัดที่แยกได้ ไม่สามารถ ยังยั้ง α-glucosidase จากเบเกอร์ยีสต์ ในขณะที่ isoquercetin สามารถยับยั้ง มอลเทสและซูเครสได้มากกว่า diglucoside ถึง 6-10 เท่า ชี้ให้เห็นว่าการที่สารมีขั้วมากขึ้นซึ่งเป็นผลมาจากปริมาณของน้ำตาลที่เพิ่มขึ้นในโครงสร้างจะส่งผลให้ฤทธิ์ในการยับยั้งเอนไซม์ลดน้อยลงซึ่งส่วนสำคัญที่ทำหน้าที่ในการยับยั้งเอนไซม์คือ aglycone quercetin โดย quercetin ที่เตรียมได้จาก rutin ได้ถูกนำมาทดสอบการยับยั้ง α-glucosidase ด้วย ซึ่งสามารถยับยั้ง α –glucosidase จากเบเกอร์ยีสต์, มอลเทส และ ซูเครสที่ IC_{50} 62.8, 12.32 และ 31.69 ไมโครโมล ตามลำดับ การศึกษากลไกการยับยั้ง α-glucosidase โดยการวิเคราะห์ทางไคเนติกส์ของ quercetin พบว่า เป็นการยับยั้ง แบบ competitive inhibition ในซูเครส ในขณะที่เป็นการ ยับยั้งแบบ mixed type inhibition ในมอลเทส

การแยกส่วนสกัดเมทานอลจากใบมะตูมซาอุสามารถแยกสารกลุ่ม hydrolysable tannin ได้ 3 ชนิดคือ methyl gallate, β-glucogallin, 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside หรือ PGG และ inositol ซึ่ง PGG สามารถยับยั้งมอลเทสและซูเครสได้สูงที่สุดที่ IC_{50} 27.21 และ 37.53 ไมโครโมล ตามลำดับ การศึกษากลไกการยับยั้งทางไคเนติกส์ของ PGG พบว่าเป็นแบบ mixed type inhibition ทั้งในมอลเทสและซูเครส นอกจากนี้ PGG ยังเสริมฤทธิ์ร่วมกับ acarbose (synergistic effect)ในการยับยั้งมอลเทสทำให้มีเปอร์เซ็นต์การยับยั้งเพิ่มขึ้นถึง 7.1 และ 7.9 % โดยการศึกษาทางไคเนติกส์และ synergistic effect ของ PGG จากงานวิจัยนี้ยังไม่มีการรายงานมาก่อน

สาขาวิชา… เทคโนโลยีชีวภาพ…...ลายมือชื่อนิสิต……..……..……

ปีการศึกษา……2555...……ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์..…….....

##5372464023: MAJOR BIOTECHNOLOGY
KEYWORDS: α-GLUCOSIDASE INHIBITORS / *Abelmoschus esculentus*/*Schinus terebinthifolius*/ DIABETES MELLITUS

WANNISA THANAKOSAI: α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS AND *Schinus terebinthifolius* LEAVES. ADVISOR: ASST. PROF. PREECHA PHUWAPRAISAN, 59 pp.

Discovery of α-glucosidase inhibitors has been actively pursued with the aim of developing therapeutics for the treatment of type 2 diabetes mellitus. In examining inhibitory effect of edible plants, extracts of *Abelmoschus esculentus* seeds and *Schinus terebinthifolius* leaves revealed promising results, and they thus were selected for further investigation.The methanol crude extracts from seeds of *A. esculentus* afforded two flavonoid glycosides named, quercetin-3-*O*-β-glucoside or isoquercetin and quercetin-3-*O*-β-glucosyl(1′′→6′′) glucoside. The isolated compounds selectively inhibited α-glucosidase whereas their inhibition against α-glucosdase from baker's yeast was not observed. Isoquercetin inhibited maltase and sucrase 6-10 times more potent than its related diglucoside, thus indicating that increased hydrophilicity by glucose moiety reduced inhibitory effect and aglycone quercetin is critical for enzyme inhibition. Quercetin prepaed from commercially available rutin was also examined for α-glucosidase inhibition. It showed board inhibition against α-glucosidase from baker's yeast, maltase and sucrose with IC_{50} value of 62.8, 12.32 and 31.69 μM, respectively. The kinetic study of quercetin showed competitive inhibition in sucrase whereas mixed type inhibition against maltase was observed.

The methanol crude extracts from *Schinus terebinthifolius* leaves afford 3 hydrolysable tannins named methyl gallate, β-glucogallin, 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside (PGG) and inositol. PGG revealed highest inhibition against maltase and sucrose with IC_{50} values of 27.21 and 37.53 μM, respectively. The kinetic analysis of PGG showed mixed type inhibition against maltase and sucrase. In addition, PGG showed dominant synergistic effect in rat intestinal maltase, thus leading to 7.1 and 7.9 % higher inhibition. Notably, the kinetic analysis and synergistic effect of PGG is first report herein.

Field of Study: ……Biotechnology…… Student's Signature ……..
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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
CD_3OD	Deuterated methanol
DMSO	Dimethyl sulfoxide
Cm	centimeter
^{13}C NMR	Carbon-13 nuclear magnetic resonance
COSY	Correlated spectroscopy
2D NMR	Two dimensional nuclear magnetic resonance
DM	Diabetes mellitus
d	Doublet (NMR)
dd	Doublet of doublet (NMR)
EC	Enzyme Commission
ESIMS	electrospray ionization mass spectrometry
HCl	hydrochloric acid
HMBC	Heteronuclear multiple bond correlation experiment
Hz	Hertz
h	Hour
IC_{50}	Concentration that required for 50% inhibition in *vitro*
J	Coupling constant
kg	kilogram
K_m	Michaelis constant
l	liter
µg	microgram
µl	microliter
M	mole per liter (molar)
MW	molecular weight
µM	micromolar
m	meter
mg	milligram
min	minute

ml	milliliter
mM	millimolar
m/*z*	Mass per charge
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NMR	Nuclear magnetic resonance
nm	nanometer
OD	optical density
S	singlet
TLC	thin-layer liquid chromatography
U	unit
UV	ultraviolet
V_{max}	maximum velocity
δ	Chemical shift

CHAPTER I

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose levels (prosprandial hyperglycemia) that result from defects of insulin, inadequate pancreatic insulin secretion or absent insulin. Diabetes mellitus was first identified as a disease associated with "sweet urine". Elevated levels of blood glucose (hyperglycemia) lead to spillage of glucose into the urine. Normally, the last process of carbohydrate hydrolyzing is small intestine which breaks down carbohydrates to monosaccharide (glucose). Glucose is absorbed through the cells of the intestine into bloodstream. High level of glucose in blood stream will promote the β-cell of pancreas to release more insulin into bloodstream for help transporting glucose into the cells, leading to the decline in the glucose levels (Fig1.1). However, if pancreas cannot produce insulin or if do not have enough insulin, this will result in diabetes.

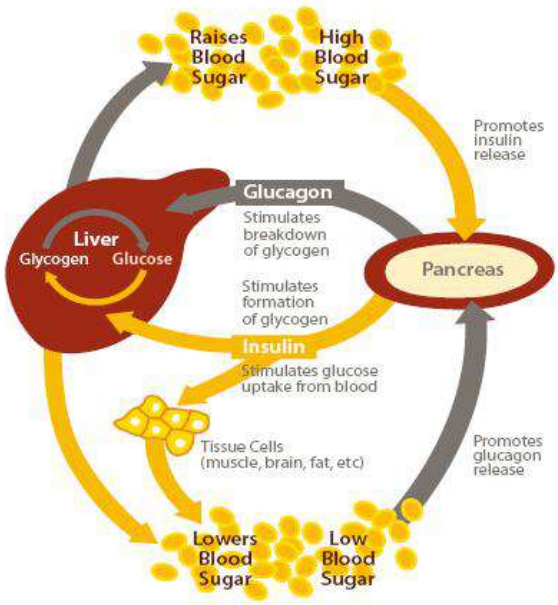


Figure 1.1 Blood sugar regulation

Insulin can regulate high glucose level by using insulin receptors of most body's cell. Insulin receptors bind to insulin, which is in the circulation. When a cell has insulin attached to its surface, the cell activates glucose transporter designed to absorb glucose from the bloodstream into the cell (Fig 1.2). Currently, the outbreak of

diabetes mellitus can be found worldwide and diabetic patients is increasing. According to Word Health Organization (WHO) projection, around 150 million people worldwide have diabetes, and this will 300 million by 2025 (Yi Hung *et al*., 2012). In addition, the postprandial hyperglycaemia leads to the development of chronic complications such as retinopathy, nephropathy, neuropathy and other (Heydari *et al.*, 2010), therefore elevating the annual health care budgets.

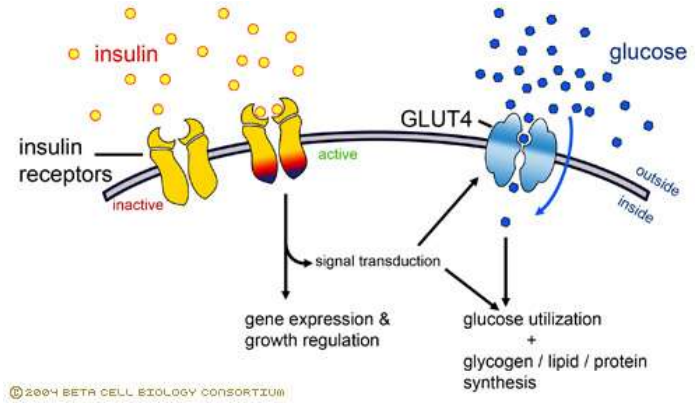


Figure 1.2 Glucose transport activated by insulin
(http://www.betacell.org/content/articlepanelview/article_id/1/panel_id/3)

1.1 Diabetes mellitus: classifications, causes and its complication

Diabetes mellitus was classified into two major classes (WHO, 1999); it was referred to insulin-dependent (type I diabetes) and non-insulin-dependent (type II diabetes).

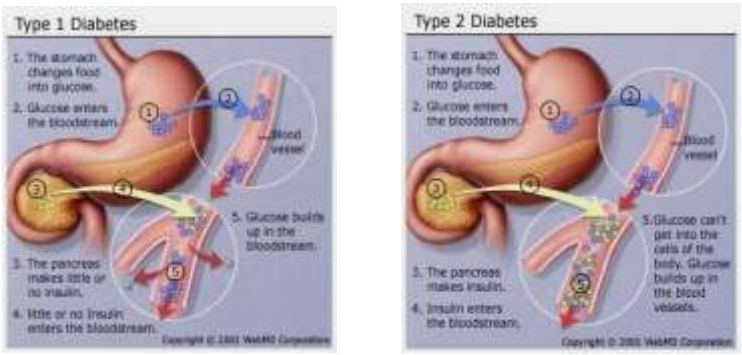


Figure 1.3 Regulation of glucose enters bloodstream in type I and II diabetes
(http://www.mindthesciencegap.org/2012/01/30/type-1-diabetes-on-the-rise/)

1.1.1 Type I diabetes mellitus

According to the report of World Health Organization (WHO) in 1999, type I diabetes, or juvenile–onset diabetes, results from autoimmune mediated impairment of the β- cells of the pancreas lead to unable to produce any insulin. Type I usually appears before the age of 40 and this type is the least common of the two main types approximately for 10%. Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease, which subsequently develop to coma and death. Since pancreas cannot produce insulin, therefore the patients necessarily to receive insulin injection for therapy.

1.1.2 Type II diabetes mellitus

Type II diabetes or adult - onset diabetes (> 40 years old) is the most common encountered diabetes and is characterized by disorders of insulin secretion and relative insulin deficiency. Once the insulin becomes less effective in suppressing blood glucose, the resulting blood glucose will increase. This physiological is recognized as insulin resistance condition. Mostly, insulin resistance is an early event due to environmental factors such as obesity, and gradually functions of β-cell (Silke *et al*., 2011). Type II diabetes makes up about 90% of the diabetic population and highly associated with a family history of diabetes (Maciej, 2005). The development of type II diabetes resulted from a combination of lifestyle, genetic factor and the prevalence of diabetes increases with age while is highest in those older than 60 years (Ali, 2009).

Insulin secretion from the pancreas normally attenuates glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid released from fat tissue. The decline in insulin secretion will reduce insulin signaling in its target tissues. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycemia of diabetes. The diagnosis of diabetes could be conducted by fasting blood glucose test. Normal fasting plasma glucose levels are less than 100 milligrams per deciliter (mg/dl). Fasting plasma glucose levels of more than 126 mg/dl on two or more tests on different days indicate diabetes. A random blood glucose test can also be used to

diagnose diabetes; a blood glucose level of 200 mg/dl or higher indicates diabetes. Postprandial hyperglycemia, following a rapid increase in blood glucose, is one of the earliest risk factors associated with the development of type II DM such as coronary artery disease, microvascular damage, eventual failure of the eyes, kidneys and nerves (Alain, 2001; Marcelo *et al.*, 2003). In its early stages, many people with type II DM can control their blood glucose levels by losing weight, eating properly and exercising. Many patients with type 2 diabetes require oral medication, and eventually need insulin shots to control their diabetes.

1.2 Oral antihyperglycemic therapy for type 2 diabetes mellitus (α-glucosidase inhibitors)

Diabetes treatment largely depends on the type and severity of the diabetes. Type I diabetes is treated with insulin, exercise, and a diabetic diet. Type II diabetes is first treated with weight reduction, a diabetic diet, and exercise. When these treatments fail to control the elevated blood sugars, oral medications are used. If oral medications are still insufficient, insulin medications and other injectable medications are considered. Oral medications are dividing into three groups, namely insulin secreatagogue, insulin sensitizer and α-glucosidase inhibitor (Andrew, 2005).

Of oral antihyperglycemic drugs currently used, α-glucosidase inhibitors including acarbose (Precose® or Glucobay®), voglibose (Basen®) and miglitol (Glyset®) are the first choices the therapy. They inhibit intestinal α-glucosidases (sucrase, maltase and isomaltase), thus retarding the rate of carbohydrate digestion. This approach can delay the postprandial hyperglycemia (Guk Hwang *et al*., 2011). The α-glucosidase inhibitors competitively inhibit the activity of α-glucosidases in the brush border of enterocytes lining the intestinal villi (Fig 1.4). High affinity of inhibitor and enzyme binding could prevent the cleavage of disaccharide and oligosaccharide into monosaccharide, therefore delaying absorption of glucose in gastrointestinal tract. The use of α-glucosidase inhibitors should be taken with meals containing digestable carbohydrate led to high efficiency to inhibit glucose absorption (Andrew *et al*., 2005). However, α-glucosidase inhibitors have side effects such as

bloating, flatulence and diarrhea. Therefore, it is of interest to search for drugs from new sources, including Thai herbs that can minimize the side effects.

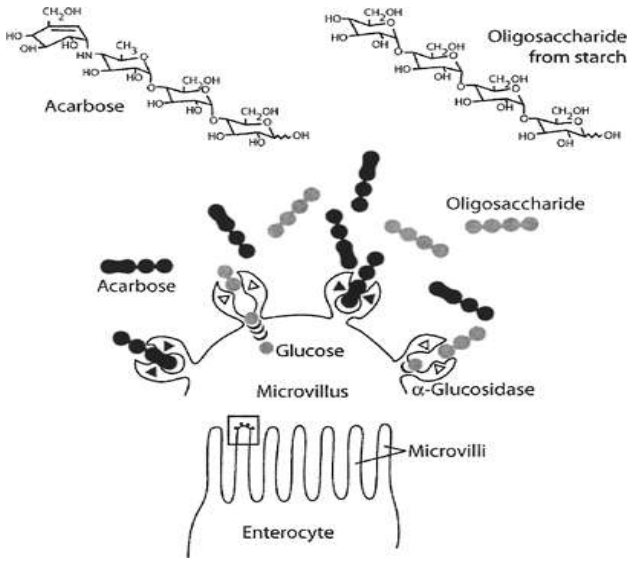


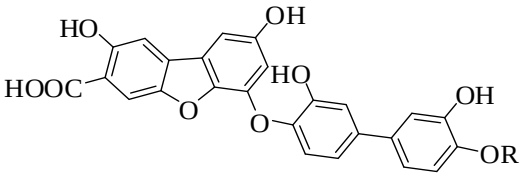
Figure 1.4 α-Glucosidase inhibitors competitively inhibit the activity of α-glucosidases in the brush border of small intestinal

1.3 Antidiabetes drugs from medicinal plants

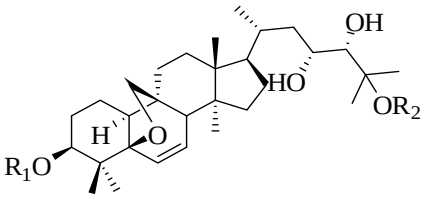
Since oral diabetic drugs have many undesirable side effects, herbal medicine, medicinal plants or know as traditional medicine was interested to help reduce side effect from normal oral diabetic drugs and use as alternative way for type II DM therapy. It is an accepted fact that herbal medicine is playing an important role in care diabetes includes cardiovascular diseases, musculo-skeletal disorders, cancer and mental disorders. In addition, it also can be incorporated into lifestyles and low cost. Around 80% of the population continues to use traditional medicine in Africa, Asia and Latin America (Payyappallimana, 2009).

For type II DM therapy, there have been several reports indicating that many herbs can reduce blood glucose level such as extracted of *Momordica charantia* (Grover *et al*., 2004), *Psidium guajava* (Wang *et al.*, 2010), *Camellia sinensis* (Kamiyama *et al*., 2010) and other. In 2012, the review of Hung and coworker summarized the discovery of novel anti-diabetic natural product extracts such as a

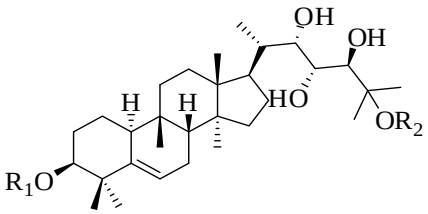
vanillic acid derivative (**1**) and its sulfate adduct (**2**) isolated from green algae, *Cladophora socialis* (Chlorophyceae). They showed potent inhibition of protein tyrosine phosphatase 1B (PTP1B), an important enzyme in regulating the insulin receptor, with IC_{50} values of 3.7 and 1.7 μM, respectively. Karaviloside XI (**3**) and four cucurbitane glycosides, momordicosides Q, R, S, T, (**4**, **5**, **6**and**7**) isolated from bitter melon (*Momordica charantia*) as well as their aglycons stimulated glucose transporter 4 (GLUT4) translocation to the cell membrane. In 2010, Kamiyama and coworker reported that catechin gallates inhibited against rat intestine α-glucosidase and glycogen phosphorylase *in vitro* experiment. The result showed that the polyphenol components, catechin 3-gallate (CG), gallocatechin3-gallate (GCG), epicatechin 3-gallate (ECG) and epigallocatechin 3-gallate (EGCG) (Fig 1.5) revealed good inhibition toward rat intestinal maltase and rabbit glycogen phosphorylase (GP) b.



1 R=H
2 R=SO_3H

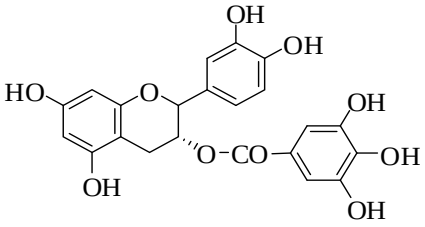


3 R_1=All', R_2=H
4 R_1=Glc', R_2=H
5 R_1=All', R_2=Glc''

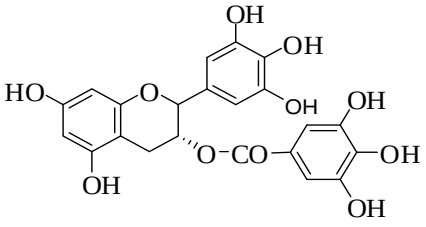


6 R_1=Glc''(1→6)-Glc', R_2=Glc'''
7 R_1=Xyl''(1→4)-[Glc''(1→6)]-Glc', R_2=Glc''''

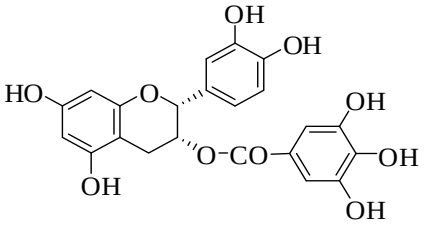
Glc=b-D-glucopyranosyl
All=b-D-allopyranosyl
Xyl=b-D-xylopyranosyl



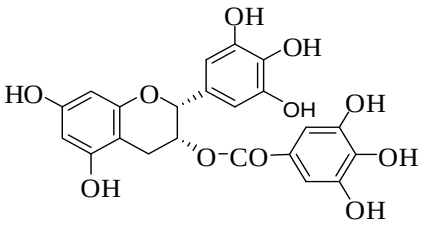
Catechin gallate (CG)



Gallocatechin gallate (GCG)



Epicatechin gallate (ECG)



Epigallocatechin gallate (EGCG)

Figure 1.5 Structures of selected natural products having antidiabetic properties.

CHAPTER II

α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS

Figure 2.1 Botanical aspects of *Abelmoschus esculentus*: (a) flower, (b) pod and (c) seed.

2.1 Introduction

2.1.1 Botanical aspect and distribution

Abelmoschus esculentus or okra is an herb, known as Lady's finger. The plant is also called gombo in Africa and bhindi in India (Camciuc *et al.*, 1998). In Thailand, it is known as "krachiap mon". It is edible plant in family of Molvaceae and other related species include cotton. The origin of okra is in Africa and it can grow in many other areas such as Turkey, Japan, India, Philippine and Thailand (Arapitsas, 2008). It can growing to 2 m tall. The fruit is a capsule containing numerous seeds**.** The leaves are 10–20 cm long and broad, palmately lobed with 5–7 lobes. Characteristics of okra pods are greenish and rich in mucilage**.**

2.1.2 Phytochemical and pharmacological investigation

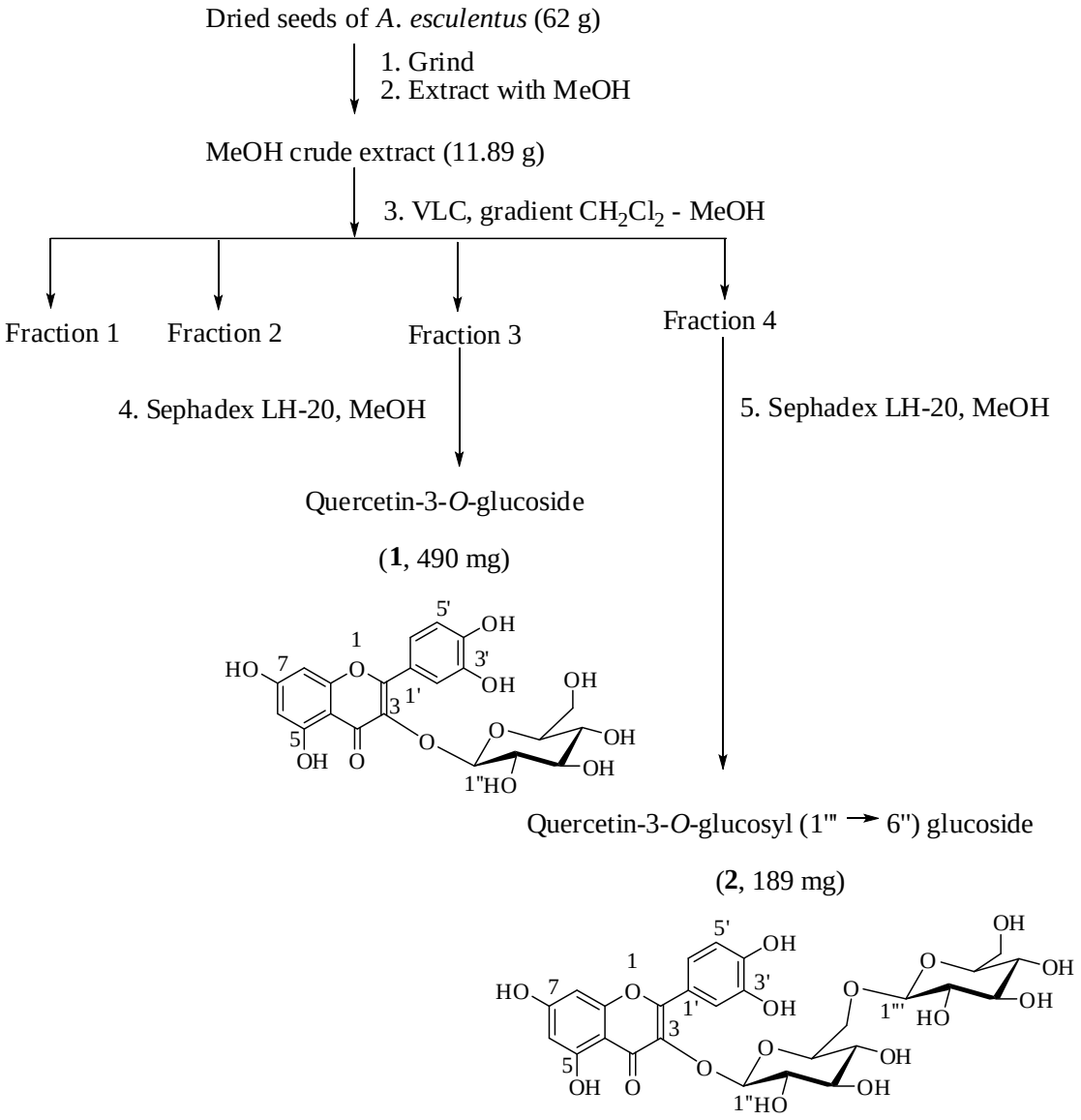
Okra is rich in mucilage (dietary fiber) and volatile compounds including polyphenolic compounds. It possesses several biological activities such as antioxidant. The major antioxidant compounds were quercetin derivatives and epigallocatechin (Shui *et al.*, 2004). The abundant volatile compounds in okra seed coat are used as a

scent by the local population in Angola (Camciuc *et al*., 1998). Mucilage from leaves and pods were used as alternative natural excipient in the modified drug delivery systems and used as a mucilaginous food against gastric irritative and inflammative diseases (Arapitsas, 2008; Ameena *et al.*, 2010). The mucilage from pods also had inhibitory activity against disacharidase (maltase, sucrase and lactase) for type II diabetes therapy (Hassan, 2009). In previous report, okra seeds could decrease blood glucose level in diabetic rat by hot water (435 mg/kg), cold water (230 mg/ml) and 80% ethanolic extracts (210 mg/kg) when compared with antidiabetic drug tolbutamide (100 mg/kg). In addition, roasted and ground seeds of okra have been used as traditional medicine in Turkey for postprandial hypoglycemia (Aslan *et al*., 2003). However, the active components responsible for this effect have not been identified. Therefore, it is of interest to isolate, separate and identify active components using α-glucosidase inhibition guided isolation.

2.2 Results and discussion

2.2.1 Isolation

Dried seeds of okra (62 g) were ground and extracted with methanol. The metanolic extract was evaporated in rotary evaporator and isolated by quick column chromatography to give four main fractions. Fractions 3 and 4 were purified by Sephadex LH-20 and eluted with 100% MeOH, leading to two known flavonoid glycosides named, quercetin-3-*O*-glucoside (**1**) and quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**) (Scheme 2.1). The chemical structures of isolated compounds were identified by NMR and mass spectroscopy.



Scheme 2.1 Isolation procedure of isolated compounds from *Abelmoschus esculentus* seeds

2.2.2 Structure elucidation of quercetin-3-*O*-glucoside (1)

Quercetin-3-*O*-glucoside (**1**) was obtained as yellow solid. The positive ESIMS spectrum of **1** exhibited $[M+ Na]^+$ ion at *m/z* 487 that corresponded with molecular formula of $C_{21}H_{20}O_{12}$. The ^1H and ^{13}C NMR spectra of **1** showed characteristic signals of flavonoid glycoside, which was identifiled as quercetin-3-*O*-glucoside. The signal of aromatic ring B was appear at δ_H 7.71 (1H, *J* = 2.0 Hz), 7.59 (1H, *J* = 8.4, 2.0 Hz) and 6.87 (1H, *J* = 8.4 Hz) for H-2′, H-6′ and H-5′, respectively. The two meta coupled protons in aromatic ring A resonated at δ_H 6.40 and 6.20 for H-8and H-6, respectively. Six proton signals of a sugar moiety resonated at δ_H 3.20-3.72 for H-2″, H-3″, H-4″, H5″ and H-6″, respectively. In addition, the signal of methyl group was not appear in spectum, suggesting that compound **1** comprised a glucose unit. The presence of anomeric proton at δ_H 5.26 with a coupling constant of 7.2 Hz indicated a *β*-configutation of glucose while HMBC correlation between H-1″ and C-3 confirmed glucosidic linkage to quercetin. The complete structure and key HMBC correlations for **1** are shown in Figure 2.2. In addition, the NMR data of **1** were consistent with previous report (Caldwell *et al*., 2006).

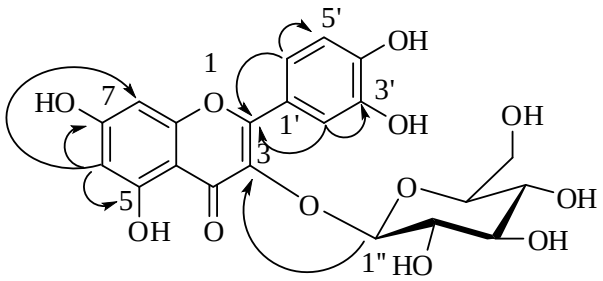


Figure 2.2 Chemical structure of quercetin-3-*O*-glucoside and selected HMBC correlations (arrow curves)

2.2.3 Structure elucidation of quercetin-3-*O*-glucosyl *(1′′′→6′′)* glucoside (2)

Quercetin-3-*O*-glucosyl (1′′′→6′′) glucoside (**2**) was obtained as dark yellow solid. The positive ESIMS spectrum of **1** exhibited $[M+ Na]^+$ ion at *m/z* 649 that corresponded with molecular formula of $C_{27}H_{30}O_{17}$**.** The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** except for the presence of two anomeric proton at δ_H 5.20 (1H, *J* = 7.6 Hz) and 4.15 (1H, *J* = 7.6 Hz) for H-1′′ and H-1′′′. The HMBC correlation observed between the anomeric proton at δ_H 4.15 (H-1′′′) and C-6′′ (δ_C 68.1) indicated that terminal glucose was connected to the C-6′′ position of another glucose (Figure 2.3). In addition, the NMR data of **2** were consistent with previous report (Hirayama *et al*., 2008).

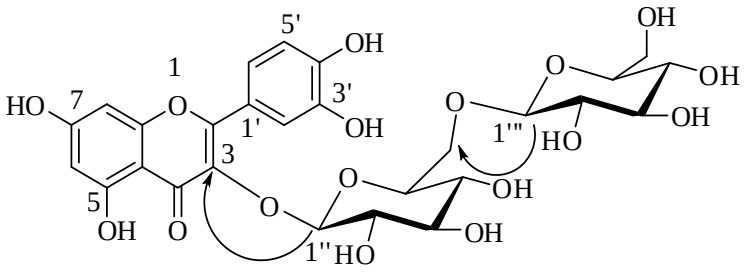


Figure 2.3 Chemical structure of quercetin-3-*O*-glucosyl *(1′′′→6′′)* glucoside and selected HMBC correlations (arrow curves)

2.2.4 Structure elucidation of quercetin (3)

To investigate α-glucosidase inhibitory effect of aglycone, quercetin **(3)** was prepared. Rutin, other commercial available form of quercitin glycoside, was used as starting material instead of **1** and **2.** Quercetin **(3)** from acid hydrolysis of rutin was obtained as yellow solid. The ^1H and ^{13}C NMR spectra of **3** were similar to those of **1**, but signals of sugar disappeared.

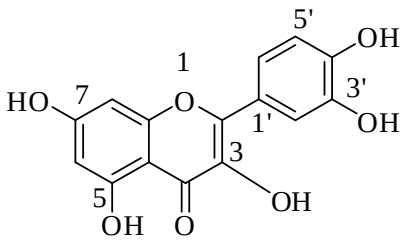


Figure 2.4 Chemical structures of quercetin

2.2.5 α-Glucosidase inhibitory activity of compounds 1-3

Baker's yeast and rat intestine were used as sources of α-glucosidase. Inhibitory activity of the isolated compounds (**1** and **2**) was determined comparison with quercetin (**3**) and type II diabetes drugs (acarbose) by colorimetric method. The results are shown in Table 2.1.

Table 2.1 α-Glucosidase inhibitory activity of **1-3**

compounds	IC_{50} [a] (μM)		
	α-glucosidase		
	baker's yeast	rat intestinal	
		maltase	sucrase
1	NI [c]	64.14 [b]	42.52
2	NI	646.99	265.74
3	62.80	12.32	31.69
Acarbose	215.35	54.67	47.08

[a] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of enzyme activity

[b] Values were performed in duplicate

[c] No inhibition, less than 50% inhibition at 10 mg/mL

The three flavonoids have different numbers of sugar linked to aromatic ring. Increase in number of sugar led to differentα-glucosidase inhibition. **1** and **2** have one and two sugar moieties in structures, respectively. Percentage inhibition of **1** and **2** against baker's yeast were less than 50% but selective against rat intestinal glucosidase. As the result from table 2.1 was found that, increased hydrophilic by glucose moiety of isolated compounds (**1** and **2**) led to reduced inhibition ten time *vs* maltase and six time *vs* sucrase. We assume that the flavonoid aglycone is critical for inhibite enzyme. To confirm this result, aglycone guercetin (**3**) was assayed compare with isolated compounds (**1** and **2**). Compound **3** which obtained from hydrolysis of rutin for remove glucose moiety therefore sugar moiety in **3** disappears. The result

showed that, **3** boardly inhibited α-glucosidase more potent than acarbose. In maltase, **3** showed increased inhibition more than **1** five times and one time *vs* sucrase therefore confirm that aglycon quercetin (**3**) is critical part for against α-glucosidase (Jo S-H *et al.,* 2010).

In addition, quercetin could be effective in controlling fasting and postprandial blood glucose levels in diabetic animal models. The research of Ji-Hye Kim *et al.,* 2011 showed that oral administration of quercetin (100 mg/kg) or acarbose (40 mg/kg) to diabetic rats significantly decreased plasma glucose levels 30-180 min after a single oral dose of starch *in vitro*. Small intestinal maltase activities were significantly reduced by consumption of quercetin or acarbose.

The kinetic analysis of quercetin (**3**) was estimated in previously report. In 2006, the report of Kenjiro *et al.,* quercetin showed mixed type inhibition close to noncompettitive inhibitionin baker's yeast. This result similar to report of Yan Qin *et al.*, in 2009. However, there is still no report about kinetic analysis in rat intestine therefore it was first study for investigated kinetic of quercetin **(3)** in rat intestine. The kinetic analysis in rat intestine (sucrase and maltase) was carries out using Lineweaver-Burkplots. It was showed competitive inhibitionin sucrase, consider from intersection of each quercetin concentration line showed in y axis (Fig 2.5). Owing to type of inhibition is competitive therefore it has K_i value only. K_i value was investigate though secondary replot of slope vs. [I] from a primary Lineweaver–Burk plot (Fig 2.6) which indicates the affinity of inhibitor to free enzyme (**E**). The analysis showed that K_m increased with unchanged V_{max} values follow by Table 2.2 therefore confirm that quercetin possesses competitive inhibition in sucrase.

On the contrary in maltase, it was showed mixed type inhibition (Fig 2.7) because intersection of each quercetin concentration line showed in second quadrant. The data of K_m and V_{max} analysis consistent with Table 2.2 that K_m increased with decreas V_{max} values (data not show) therefore confirm that quercetin possesses mix type inhibition. Mix type inhibition has both K_i and K_i' values. The K_i' show affinity of inhibitor to enzyme-substrate complex (**ES**) and obtained by plotting between secondary replot of intercept *vs*. [I] from a primary Lineweaver-Burk plot (Fig 2.9). Dissociation constants of K_i and K_i' values were compared, it was found that the K_i

value of quercetin less than K_i' value 5 times (Table 2.3), suggesting that binding affinity of quercetin to free enzyme (**E**) stronger than enzyme-substrate complex (**ES**). The data implied that quercetin is dominant in competitive inhibition**.**

Table 2.2 Inhibition mechanism

Type of inhibition	K_m	V_{max}	**Intersection**
Competitive	increase	unchanged	Y axis, Y>0
Non-competitive	unchange	decrease	X axis, X<0
Uncompetitive	decrease	decrease	no intersection
Mixed	increase	decrease	second quadrant

Table 2.3 The values from kinetic analyses of quercetin (**3**)

dissociation constant	**sucrase**	**maltase**
K_i	0.2 mM	0.4 mM
K_i'	-	2.1 mM

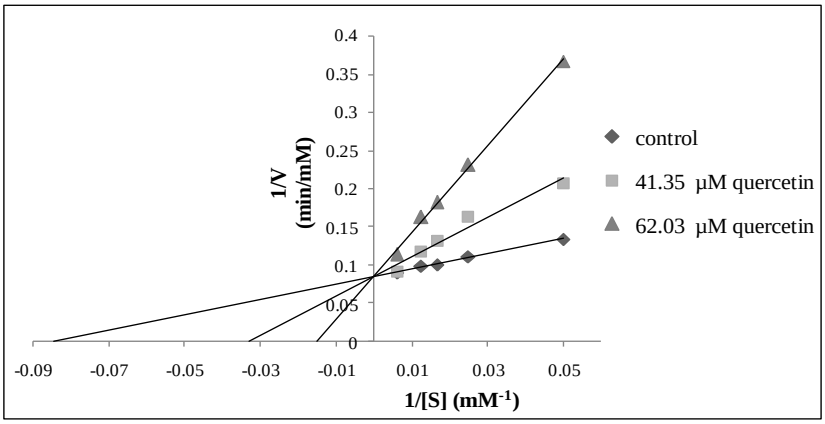


Figure 2.5 Lineweaver-Burk plot for inhibitory activity of quercetin (**3**) on the intestinal sucrase.

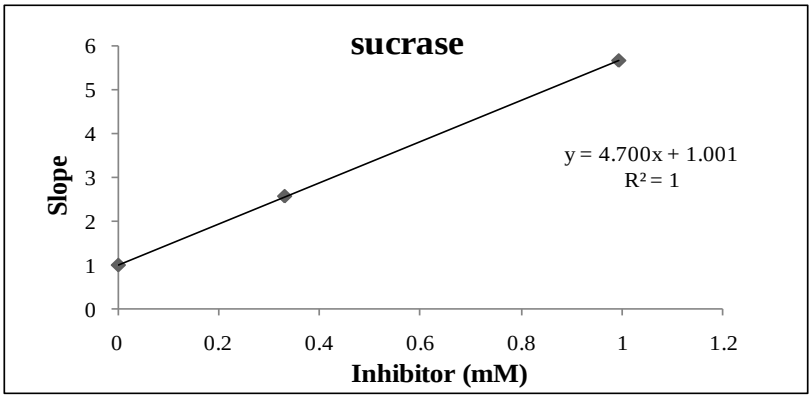


Figure 2.6 Secondary replot of slope *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i

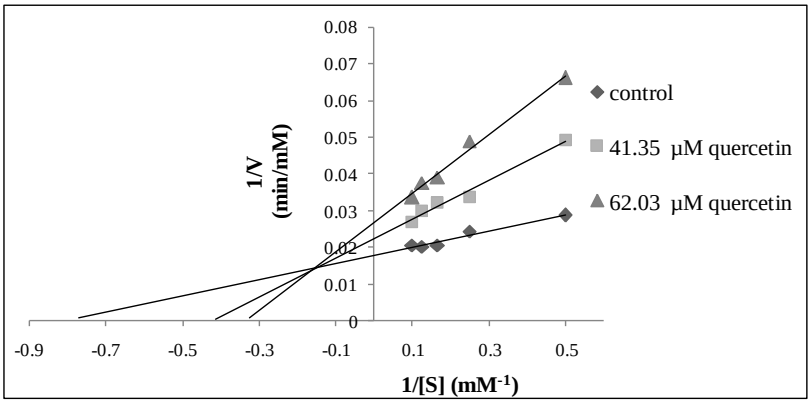


Figure 2.7 Lineweaver-Burk plot for inhibitory activity of quercetin (**3**) on the intestinal maltase.

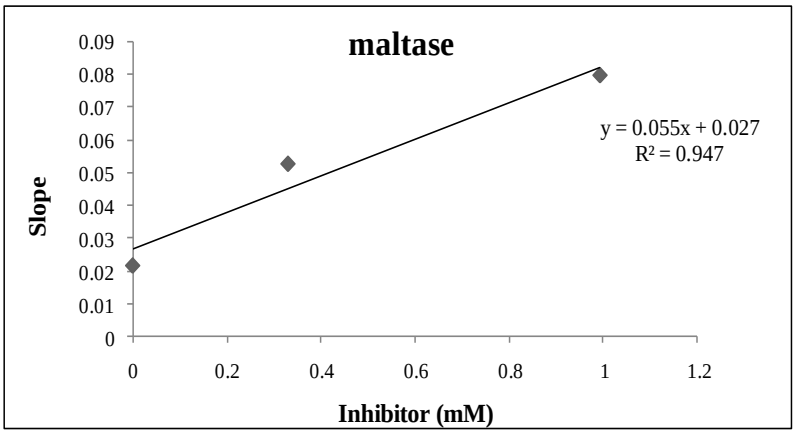


Figure 2.8 Secondary replot of slope *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i

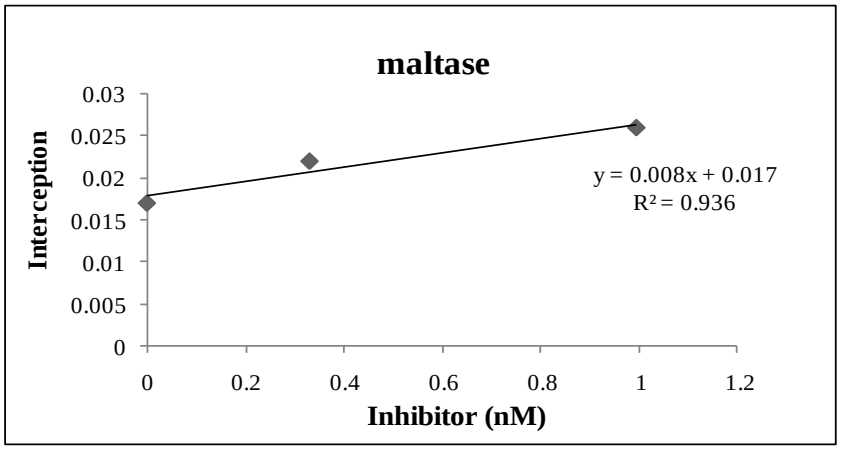


Figure 2.9 Secondary replot of intercept *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i'

2.3 Experiment section

2.3.1 General experimental procedures

The ^{1}H and ^{13}C NMR spectra (CD_3OD) were recorded with a Varian Mercury+ 400 NMR spectrometer. The chemical shift in δ (ppm) was assigned with reference to the signals of residual protons in deuterated solvents. ESIMS was obtained from microTOF 72 MS spectrometer. TLC was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF254). Gel filtration chromatography was performed on Sephadex LH-20.

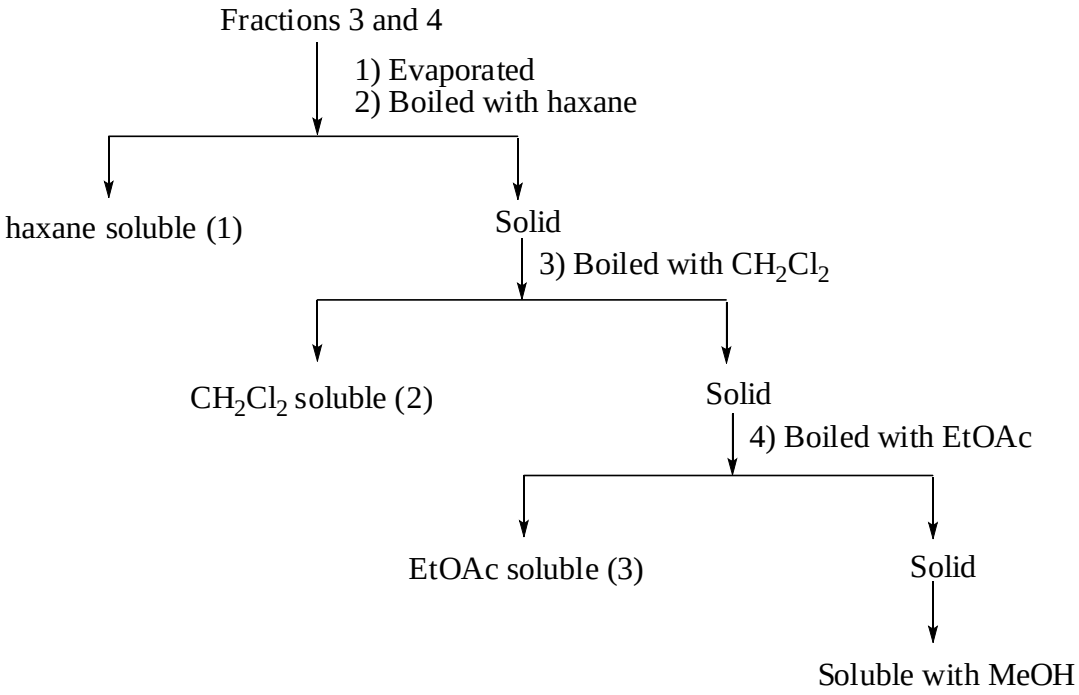
2.3.2 Plant material

Okra pods were collected from Huai Monthong Nakhon Pathom, Thailand in November 2010. Plant authentication was performed by Mrs. Parinyanoot Klinratana, and the specimen (BCU013433) is deposited at the Botanical Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University.

2.3.3 Extraction and isolation

Okra seeds were separated from pods and air-dried (62 g). Ground seeds were extracted with methanol (2 × 250 mL) for two days at room temperature, and this

procedure was repeated three times. Methanolic extract was evaporated in rotary evaporator, yielding brown sticky paste. The methanol extract (11.89 g) was isolated by quick column chromatography and then eluted with CH_2Cl_2 containing increasing volume of MeOH (100:0, 90:10, 80:20 and 50:50 v/v, 400 mL) to give four main fractions. Each fraction was then evaporated under reduced pressure at 40 °C to dryness before biological evaluation against α-glucosidase. The fractions showing high inhibitory activity were further purified by column chromatograpy. To remove fat and oil from the solid compound, fractions 3 and 4 were boiled with hexane, CH_2Cl_2 and EtOAC, respectively (Scheme 2.2) for purified solid compound. The residues after being boiled with EtOAC were purified by Sephadex LH-20 and eluted with MeOH (100%), leading to two known flavonoid glycosides named quercetin-3-*O*-glucoside (**1**, 490 mg) and quercetin-3-*O*-glucosyl(1‴→6″) glucoside (**2**, 189 mg).



Scheme 2.2 The process in removing fat and oil from fractions **3** and **4**

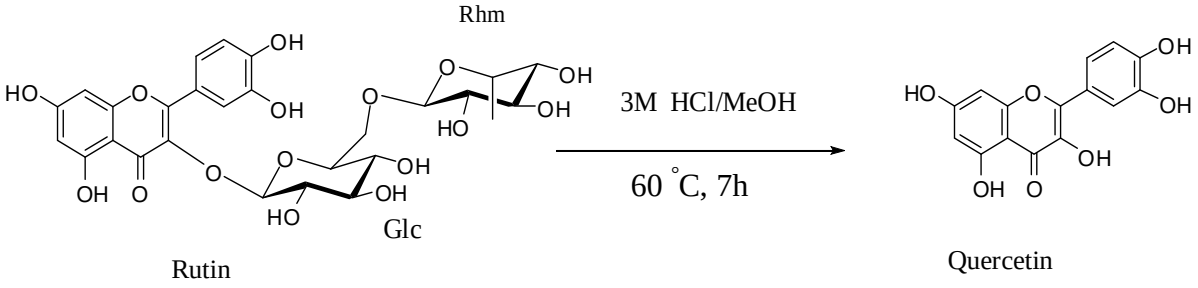
Quercetin-3-*O*-glucoside (**1**); yellow solid; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.71 (d, J = 2.0 Hz, 1H, H-2′), 7.59 (dd, J = 8.4, 2.0 Hz, 1H, H-6′), 6.87 (d, J = 8.4 Hz, 1H, H-5′), 6.40 (s, 1H, H-8), 6.20 (s, 1H, H-6), 5.26 (d, J = 7.2 Hz, 1H, H-1″), 3.72

(dd, *J* = 12.0, 2.0 Hz, 1H, H-5″), 3.58 (dd, *J* = 12.0, 5.2 Hz, 2H, H-6″), 3.20-3.51 (m, 3H, H-2″, H-3″, H-4″) ^{13}C NMR (CD_3OD, 100 MHz) δ 177.7, 165.0, 162.0, 158.0, 158.0, 148.0, 144.0, 134.0, 121.8, 121.4, 116.1, 114.6, 104.0, 102.8, 98.5, 93.3, 77.0, 76.6, 74.3, 69.7, 61.1; ESIMS, m/z 487 $[M+Na]^+$

Quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**); dark yellow solid; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.70 (d, *J* = 2.0 Hz, 1H, H-2′), 7.66 (dd, *J* = 8.4, 2.0 Hz, 1H, H-6′), 6.86 (d, *J* = 8.4 Hz, 1H, H-5′), 6.40 (d, *J* = 2.0 Hz, 1H, H-6), 6.19 (d, *J* = 2.0 Hz, 1H, H-8), 5.20 (d, *J* = 7.6 Hz, 1H, H-1″), 4.15 (d, *J* = 7.6 Hz, 1H, H-1‴), 3.16-3.99 (m, 10H, H-2″, 3″, 4″, 5″, 6″, 2‴, 3‴, 4‴, 5‴ and 6‴) ^{13}C NMR (CD_3OD, 100 MHz) δ 177.9, 164.6, 161.4, 157.6, 157.0, 148.4, 144.4, 134.3, 122.2, 121.6, 116.1, 114.7, 104.3, 103.0, 102.8, 98.6, 93.5, 76.4, 76.3, 76.2, 76.1, 74.1, 73.6, 69.8, 68.1, 60.9, 56.6; ESIMS, m/z 649 $[M+Na]^+$

2.3.4 Hydrolysis of rutin

To obtain quercetin (**3**) for α-glucosidase inhibition assay compared with isolated compounds from *Abelmoschus esculentus* seeds, hydrolysis of rutin was carried out via the general procedure of Kajjout *et al.*, (2011). Briefly, rutin (100 mg) was dissolved in 3M ethanolic HCl (3 mL) and the mixture was stirred vigorously at 60 °C for7 h. The reaction mixture was evaporated to dryness and washed with H_2O to afford quercetin (**3**, 45.8 mg). The structure of quercetin was then confirmed by NMR.



Scheme 2.3 Hydrolysis of rutin

Quercetin **(3)**; yellowsolid; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.64 (d, J = 2.0 Hz, 1H, H-2′), 7.54 (dd, J = 8.52, 2.0 Hz, 1H, H-6′), 6.79 (d, J = 8.4 Hz, 1H, H-5′), 6.29 (d, J = 2.0 Hz, 1H, H-8), 6.08 (d, J = 2.0 Hz, 1H, H-6)**.**

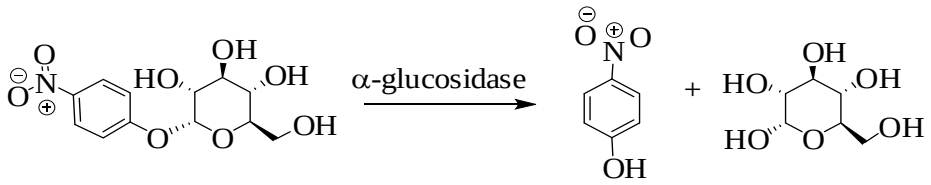
2.3.5 α-Glucosidase inhibitory activity

2.3.5.1 Chemical and equipment

The α-glucosidase (EC 3.2.1.20) from Baker's yeast and *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as a synthetic substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The *p*NPG solution was prepared in 0.1 M phosphate buffer, adjusted to pH 6.9, whereas yeast glucosidase was dissolved in 0.1 M phosphate buffer, pH 6.9, to yield 6 U/mL stock-solution, and further diluted with 0.1 M phosphate buffer to get 0.1 U/mL. Acarbose (Glucobay® 50 N 1; Bayer Vital, Leverkusen, Germany) was used as a synthetic inhibitor of α-glucosidase. Bio-Rad microplate reader model 3550 UV was used to measure the absorbance at 405 and 503 nm of enzyme reaction. Rat intestinal glucosidase and substrate (maltose and sucrose) were supplied by Sigma Aldrich Co. (USA). Glu-kit was purchased from Human Gesellschaft für Biochemica und Diagnostica mbH (Germany). The crude enzyme solution prepared from rat intestinal acetone powder was used as a source of maltase (0.45 U/mg proteins) and sucrase (0.09 U/mg proteins).Rat intestinal acetone powder (1.0 g) was resuspended in 30 mL of 0.9 % NaCl solution. After centrifugation (12,000*g*, 30 min), supernatant was used for the assay.

2.3.5.2 α-Glucosidase inhibitory activity from baker's yeast

Assay for inhibitory effect against α-glucosidase from baker's yeast was performed using colorimetric method. The enzymatic hydrolysis of the *p*NPG was monitored based on the amount of *p*-nitrophenol released from the reaction mixture by Bio-Rad microplate reader using a 405 nm absorbance (Scheme 2.4)



p-nitrophenyl- α -D-glucopyranoside (colorless) | *p*-nitrophenol (yellow) | glucose

Scheme2.4 Hydrolysis of *p*NPG by α-glucosidase from baker's yeast

Procedure

Assay was performed according to a slightly modified method described by Guk Hwangand coworker (2011). In the 96-well plate, 10 μL of sample dissolved in DMSO (0.1, 1 and 10 mg/mL) was added to a solution of 0.1 M phosphate buffer (pH 6.9, 50 μL) and α-glucosidase (0.1 U/mL, 40 μL). After preincubation at 37 °C for10 min, 50 μL of1 mM *p*NPG prepared in the same buffer was added. The reaction mixture was incubated for additional 20 min, and terminated by adding100 μL of 1 M Na_2CO_3. *p*-Nitrophenol released from the reaction of *p*NPG was quantified by measuring absorbanceat 405 nm. The percentage inhibition of activity was calculated as follows

$$\% \text{ inhibition} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance without the sample

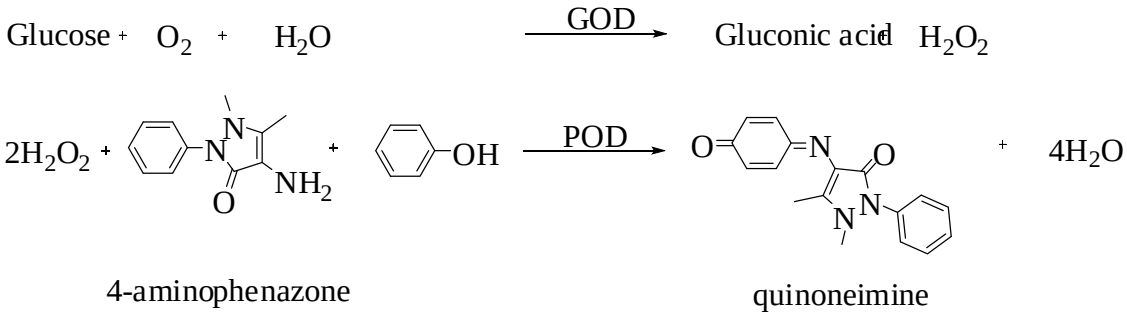
A_1 is the absorbance with the sample

The IC_{50} value was determined from a plot of % inhibition and final concentration of reaction. Experiment was run in duplicate.

2.3.5.3 α-Glucosidase inhibitory activity from rat intestine

The α-glucosidases from rat intestine was used as a source of maltase and sucrase. The enzymatic hydrolysis of the maltose and sucrose were monitored based on the amount of glucose. The concentration of glucose released from the reaction mixture was determined by the glucose oxidase (GOD) method using a glu-kit

(Human, Germany). Enzymatic activity was quantified by measuring absorbance of quinoneimine at 503 nm (Scheme 2.5).



4-aminophenazone quinoneimine

Scheme 2.5 Enzymatic oxidation of glucose by GOD

Procedure

Assay was performed according to a method described by Jo and coworkers (2010) with slight modification. In the 96-well plate, 10 µL of sample (0.1, 1 and 10 mg/mL in DMSO) was added with 30 µL of the 0.1 M phosphate buffer (pH 6.9), 20 µL of the substrate solution (maltose: 10 mM; sucrose: 100 mM) in 0.1 M phosphate buffer, 80 µL of glucose kit and 20 µL of the crude enzyme solution. The reaction mixture was then incubated at 37 °C for 10 min (for maltose) and 40 min (for sucrose). The concentration of glucose released from the reaction mixture was determined by the glucose oxidase method using a glu-kit (Human, Germany). Voglibose was used as positive control in this assay. Enzymatic activity was quantified by measuring absorbance at 503 nm. The percentage inhibition and IC_{50} value were calculated the same as baker's yeast.

2.3.6 Measurement of kinetic constant

To characterize the pattern of quercetin inhibition, kinetic analyses of quercetin on rat intestinal (maltase and sucrase) was observed at different quercetin concentrations. The concentrations of quercetin were used at 0, 41.35 and 62.03 µM, respectively. Enzyme and quercetin were incubated with increasing concentration of maltose 0.5-20 mM for maltase inhibition and sucrose 5-200 mM for sucrase inhibition and other compounds were used same α-glucosidase inhibition assay

describe in above. The type of inhibition was determined by Lineweaver-Burk plot for calculation of K_i constants, secondary replot of slope *vs*. [I] from a primary Lineweaver–Burk plot and Secondary replot of intercept *vs*. [I] from a primary Lineweaver–Burk plot for the determinationof K_i'.

Supporting information

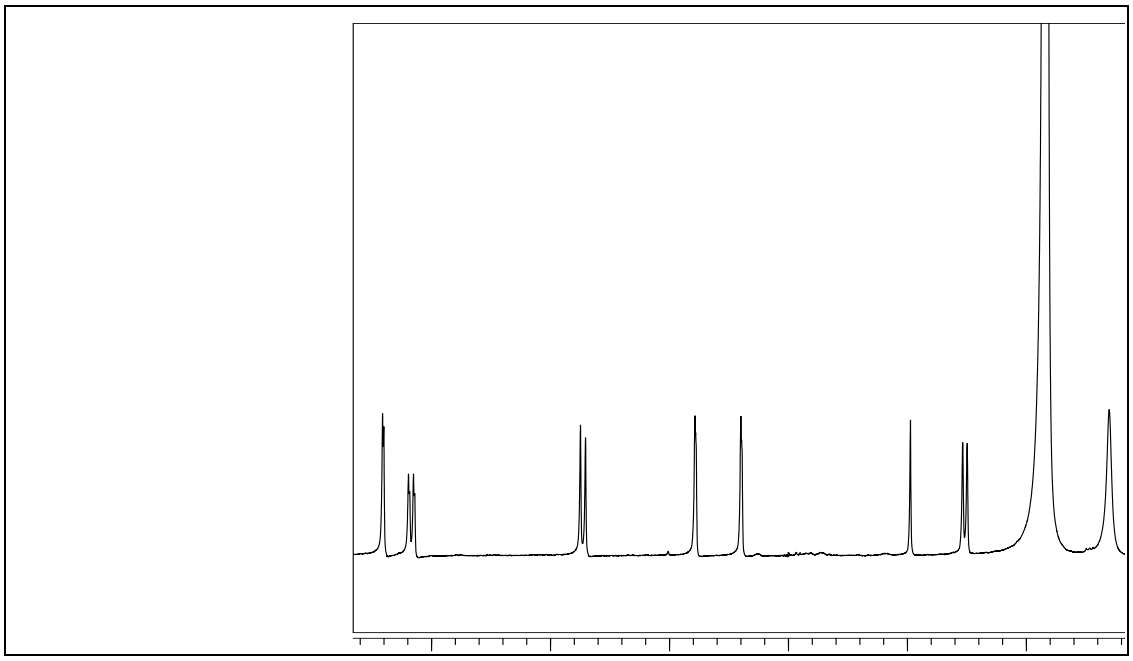


Figure 2.10 ^{1}H NMR (CD_3OD) spectrum of quercetin-3-*O*-glucoside (**1**)

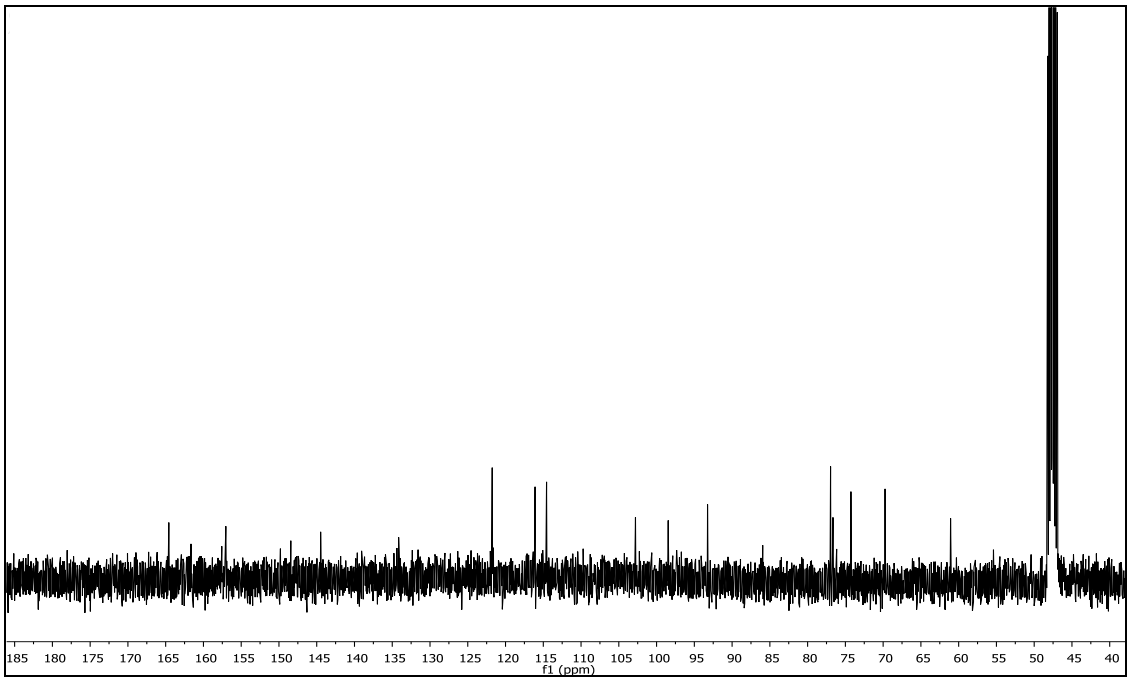


Figure 2.11^{13}C NMR (CD_3OD) spectrum of quercetin-3-*O*-glucoside (**1**)

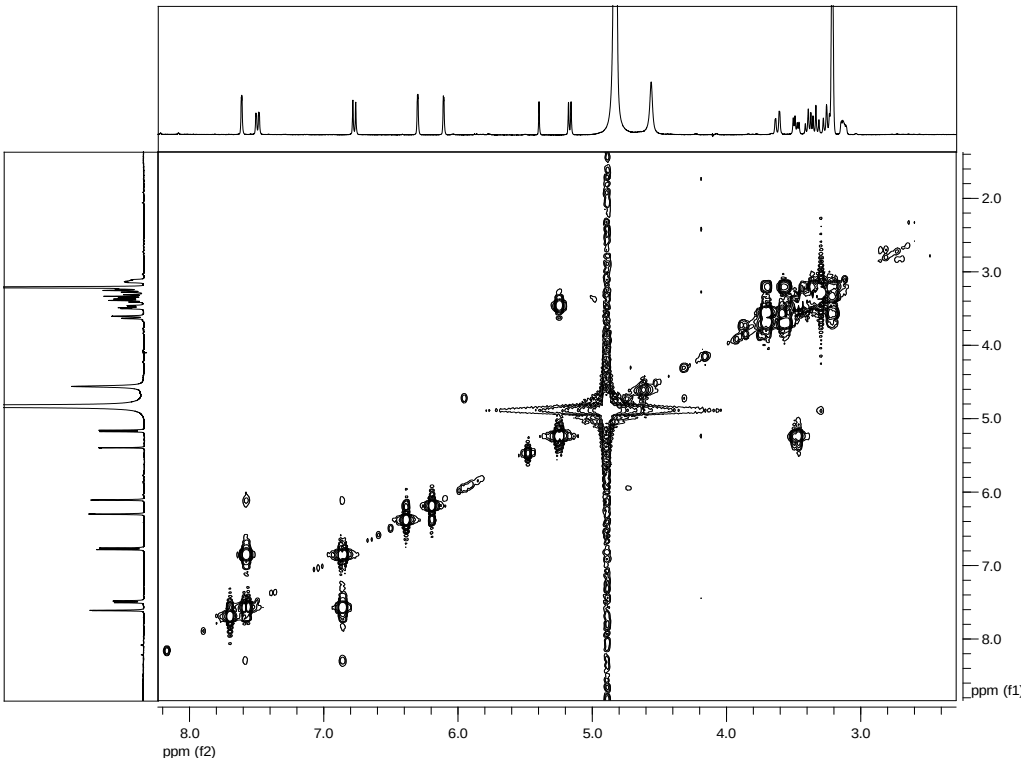


Figure 2.12 COSY spectrum of quercetin-3-*O*-glucoside (**1**)

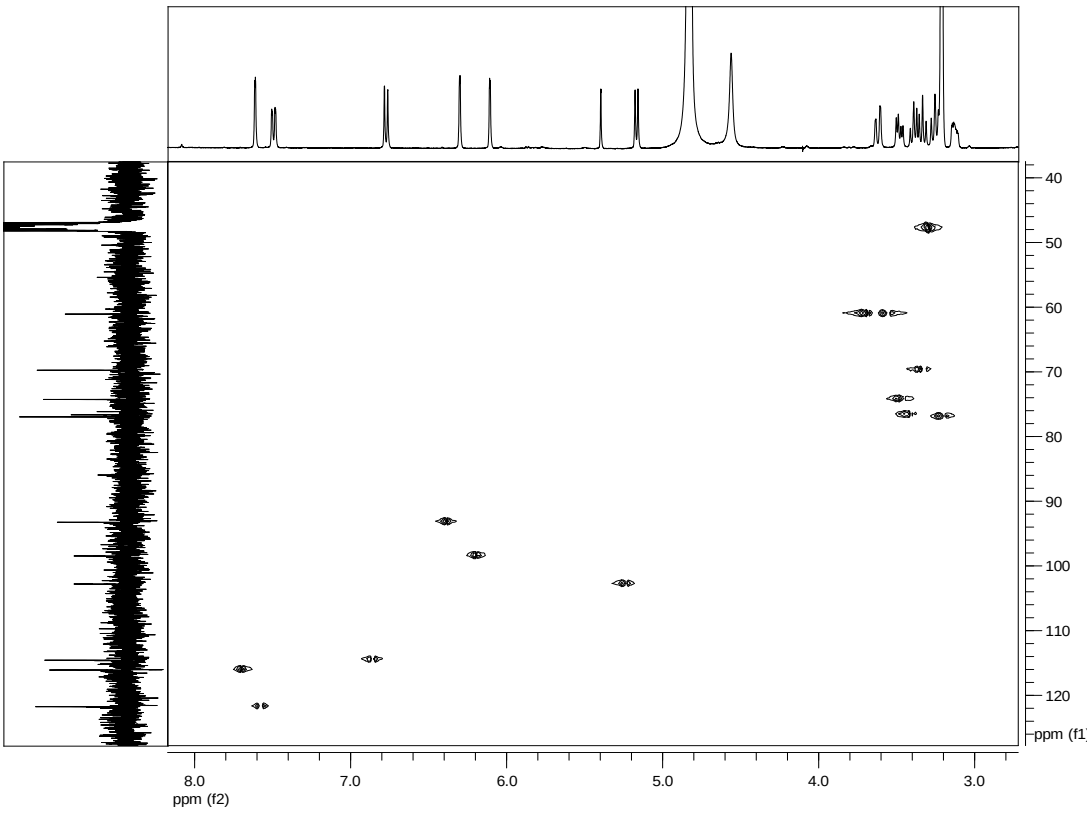


Figure 2.13 HMQC spectrum of quercetin-3-*O*-glucoside (**1**)

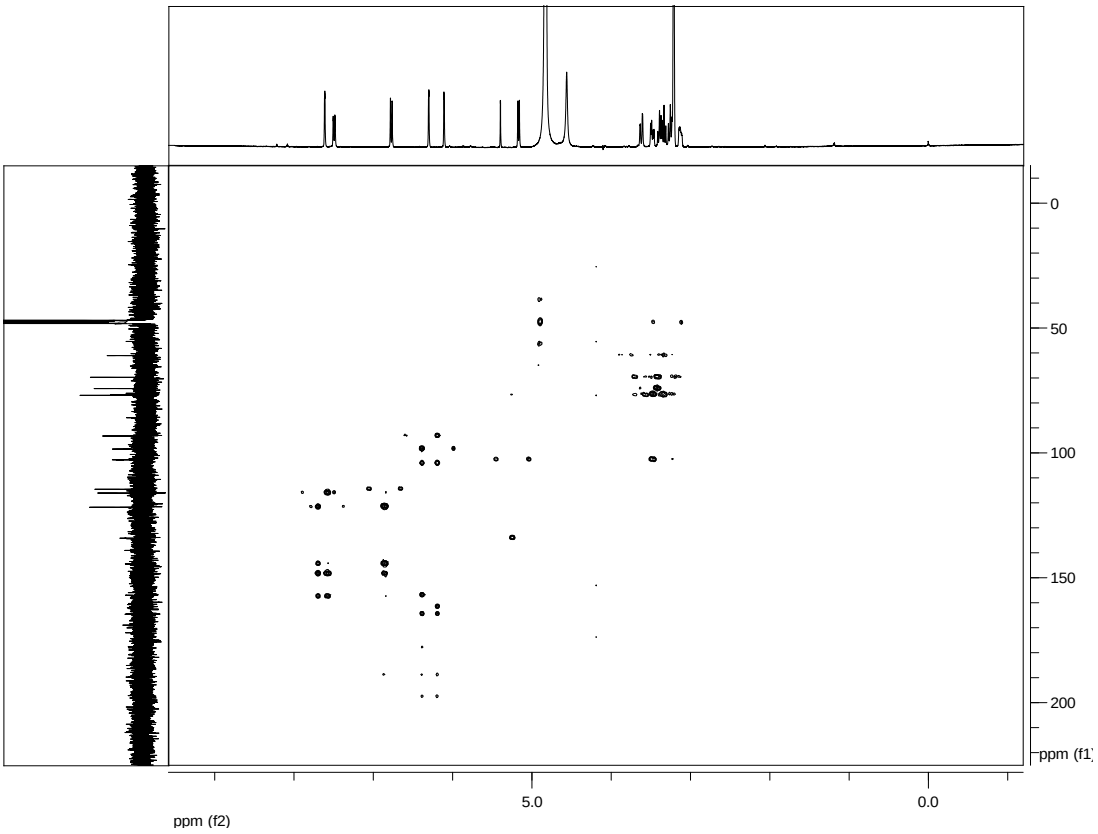


Figure 2.14 HMBC spectrum of quercetin-3-*O*-glucoside (**1**)

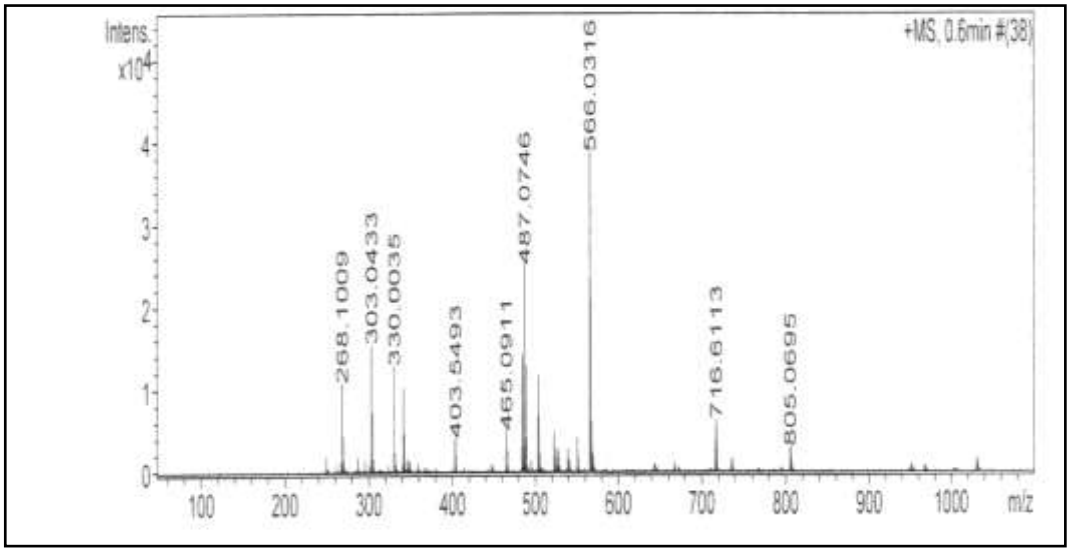


Figure 2.15 Mass spectrum of quercetin-3-*O*-glucoside (**1**)

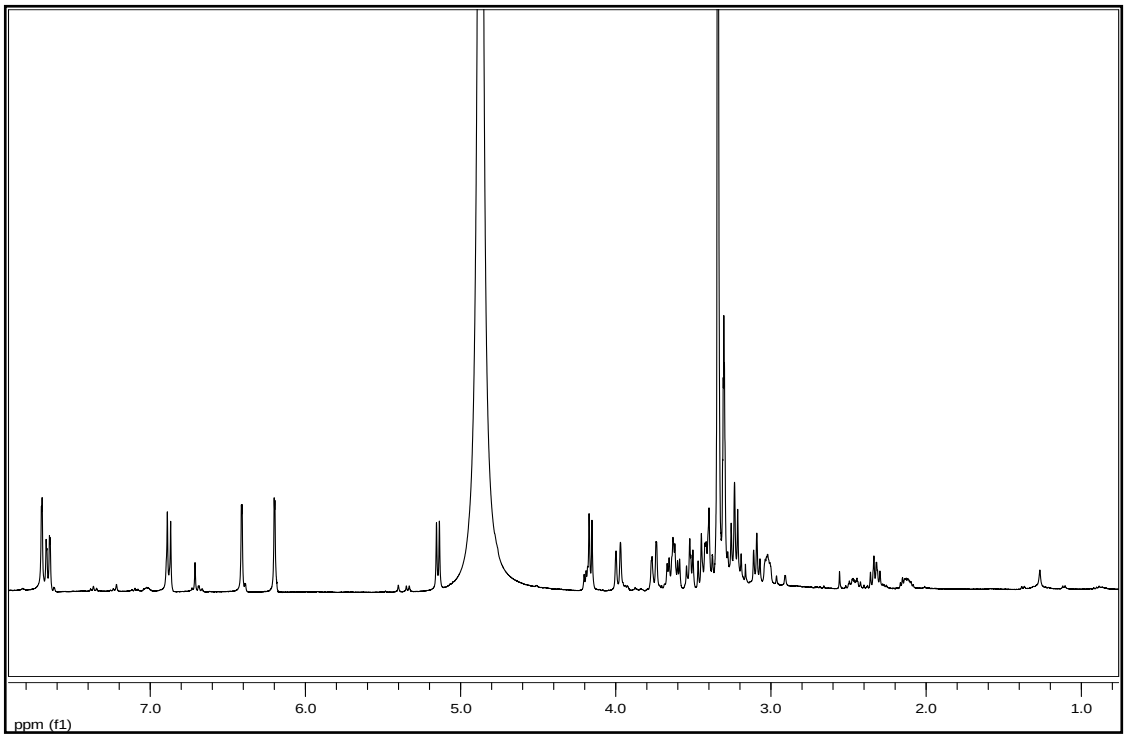


Figure 2.16 ^{1}H NMR (CD_3OD) spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

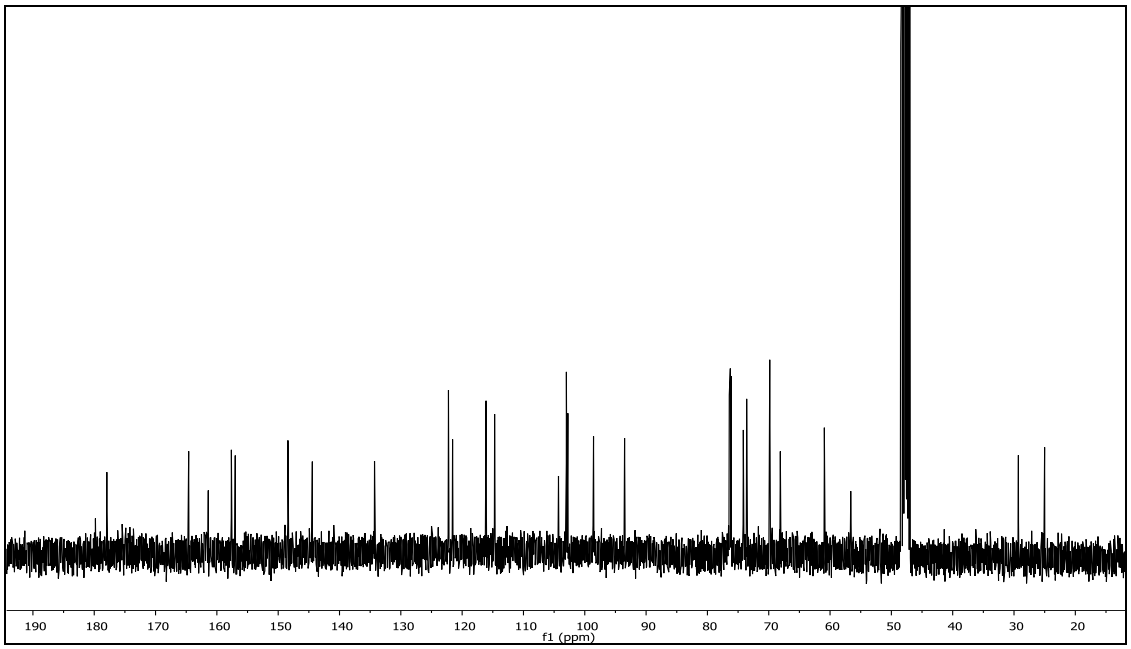


Figure 2.17^{13}C NMR (CD_3OD) spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

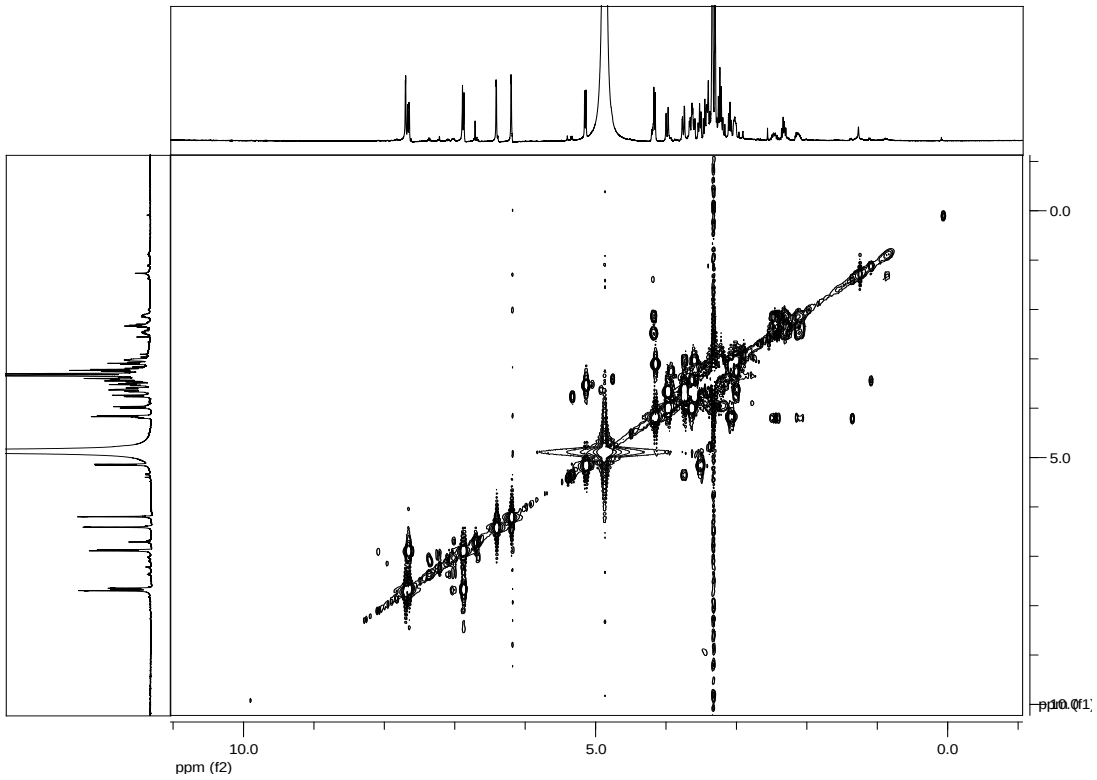


Figure 2.18 COSY spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

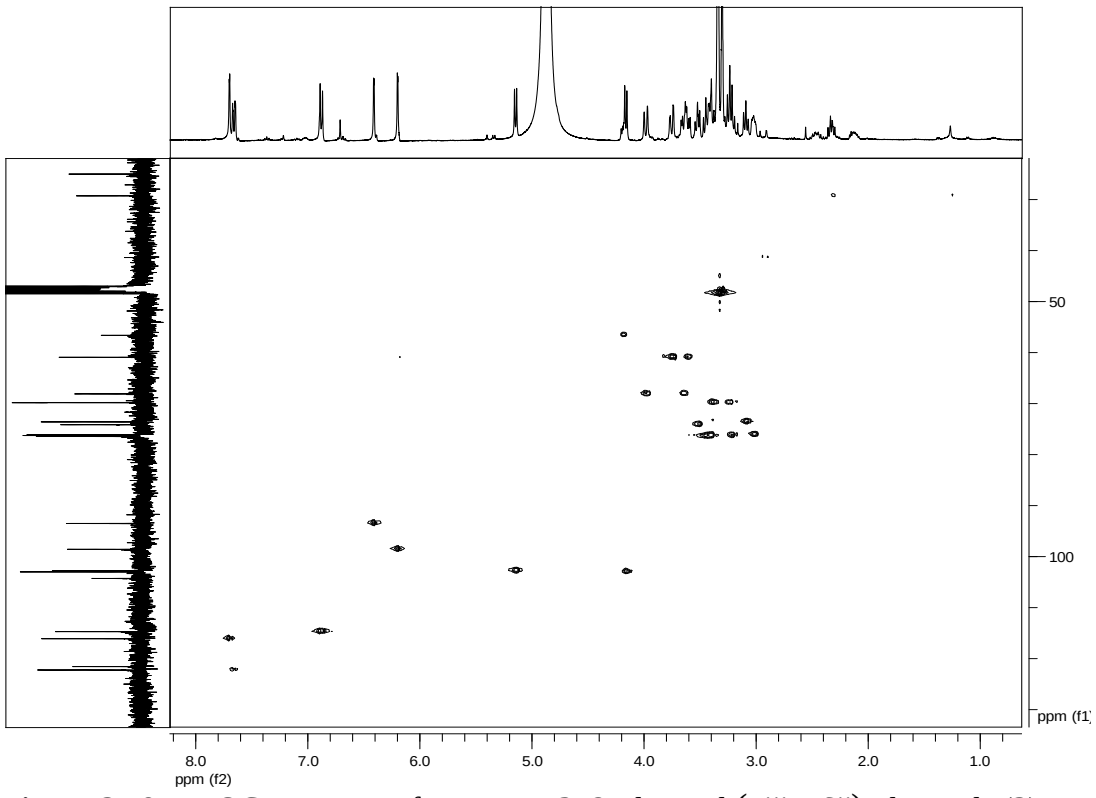


Figure 2.19 HMQC spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

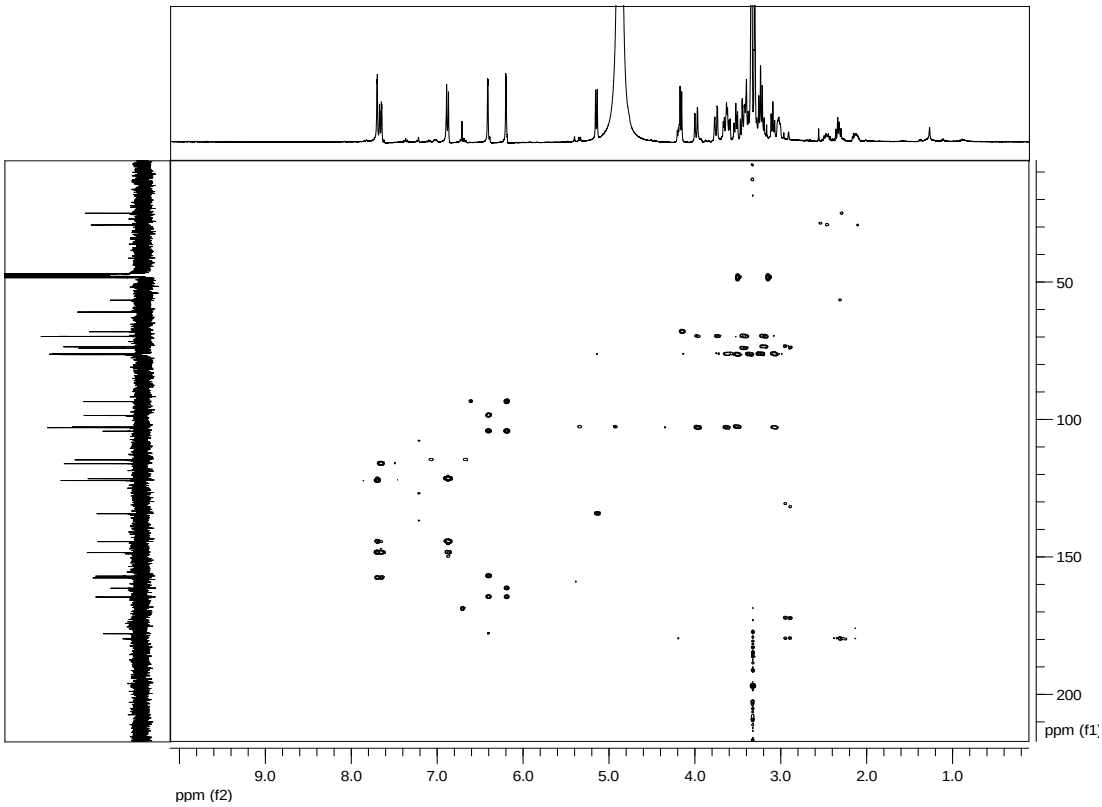


Figure 2.20 HMBC spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

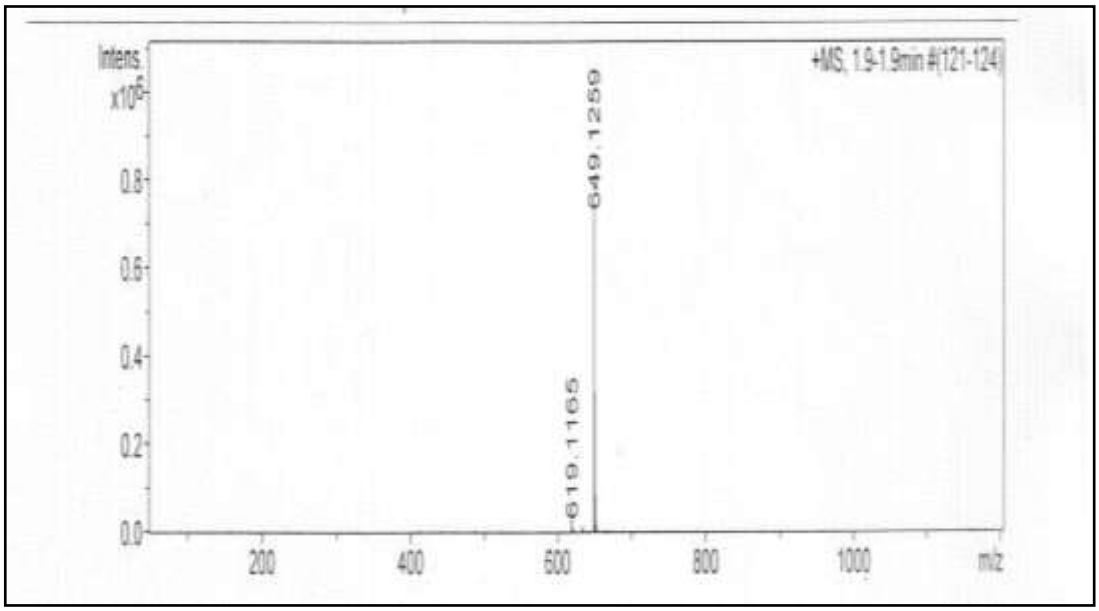


Figure 2.21 Mass spectrum of quercetin-3-*O*-glucosyl (1‴→6″) glucoside (**2**)

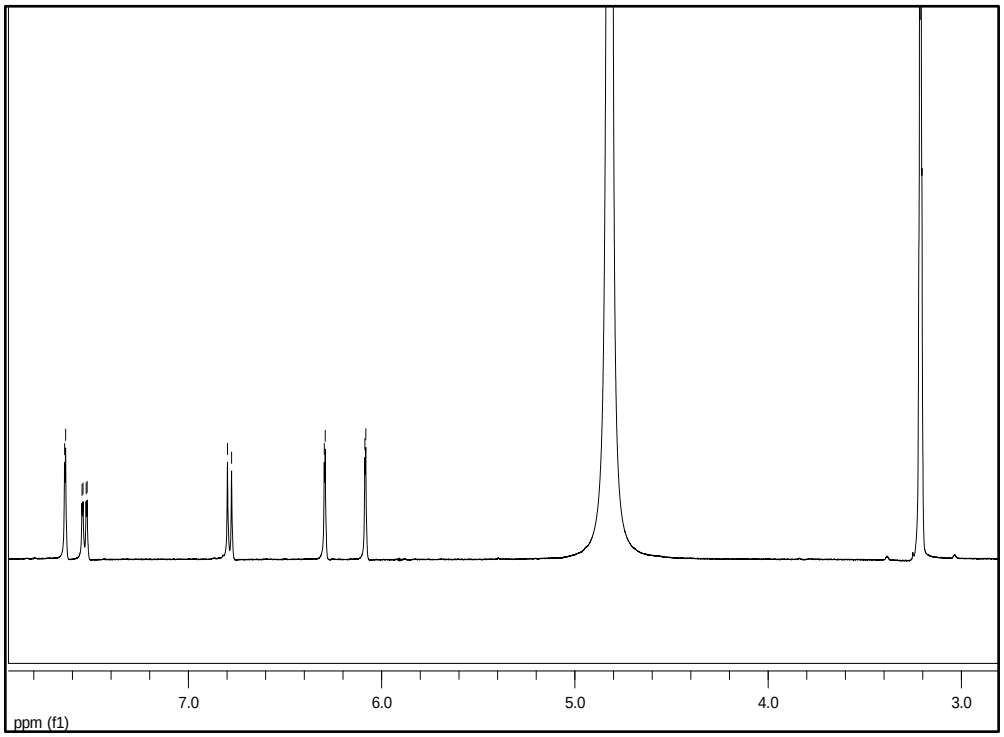


Figure 2.22 ^{1}H NMR (CD_3OD) spectrum of quercetin (**3**)

CHAPTER III

α-GLUCOSIDASE INHIBITORS FROM THE LEAVES OF *Schinus terebinthifolius*

Figure 3.1 *Schinus terebinthifolius*

3.1 Introduction

3.1.1 Botanical aspect and distribution of *Schinus terebinthifolius*

Schinus terebinthifolius or Brazilian pepper tree is a tree belongs to the family Anacardiaceae. Brazilian pepper tree is a perennial tree native to Argentina, Brazil, and Paraguay (Fernando *et al*., 2009). It has been distributed widely such as parts of Central America, North Africa, South Africa and Southern Asia, including Thailand. It is also known as Chrismas berry, pimiento de Brazil and chichita. In Thailand, it was called "Matum-sau". Brazilian pepper tree is a multiple-stemmed evergreen shrub, usually 2 to 6 m tall. The panicles contain many small white flowers. The fruits are small bright-red spherical (Langel and Cherry, 2008).

3.1.2 Phytochemical and pharmacological investigation of *Schinus terebinthifolius*

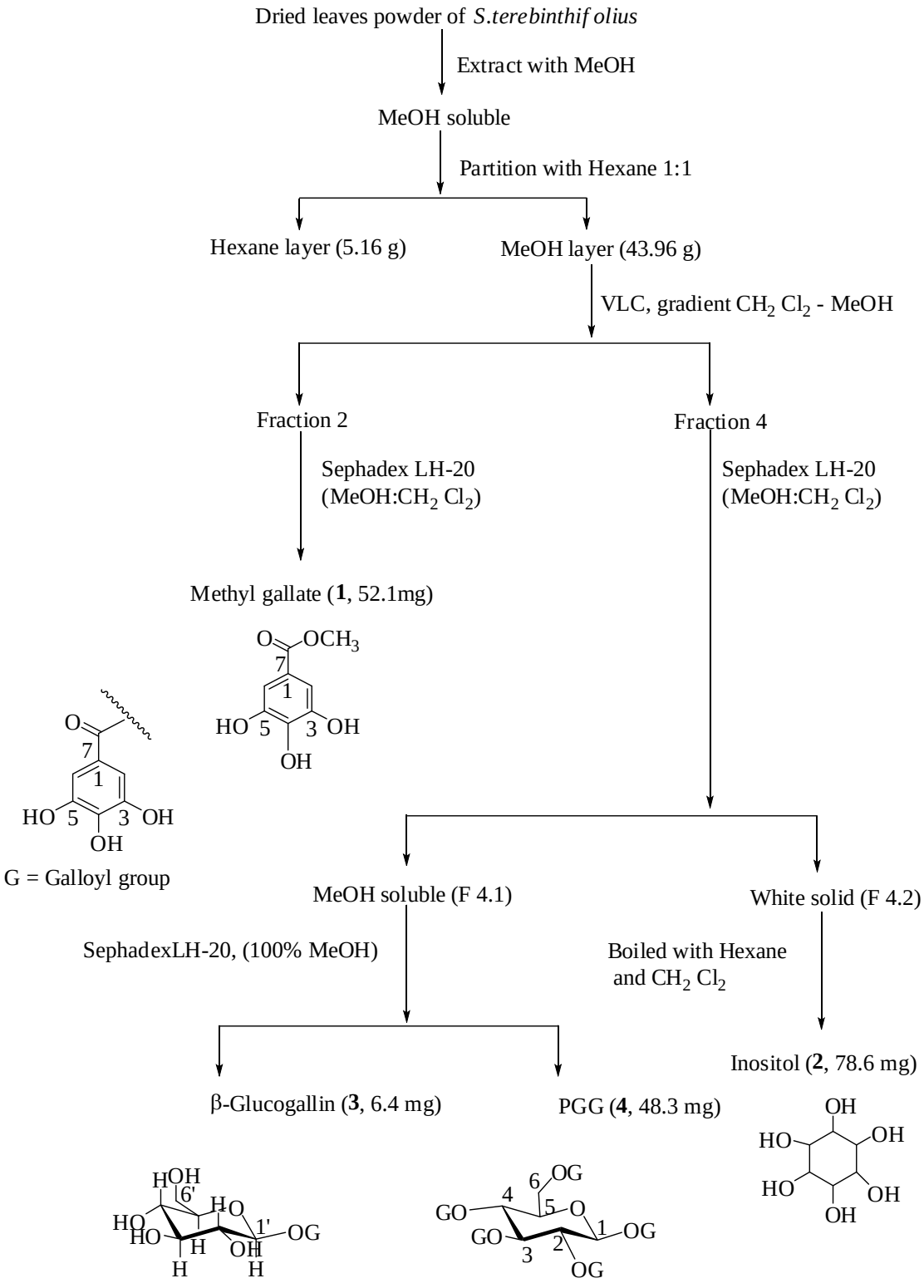
The previously phytochemical analysis of Brazilian pepper tree showed that it contains tannins, alkaloids, flavonoids, terpenes, sterols and abundant of essential oil. In folk medicine, it is used as rheumatism, cadiotonic, hypotensive (Amal *et al.*, 2007), anti-inflammatory, febrifuge, analgesic, to treat illnesses of the urogenital

system (Cavalher-Machado *et al*., 2008) and other. The essential oil was also showed biological activity such as antibacterial, antifungal and antioxidants (Gundidza *et al.*, 2009). For type II diabetes, the bioassay guide isolation of this plant showed high percentage α-glucosdase inhibition when compared with other plants. It is of interest to isolate α-glucosidase inhibitiors from this plant, including identified chemical structure of isolated compounds by NMR spectroscopy technique.

3.2 Results and discussion

3.2.1 Isolation

The air dried leaves of *Schinus terebinthifolius* were ground to coarse powder by blender before extracted with MeOH. The methanolic extract was partition with hexane in a ratio of 1:1. The methanolic layer was evaporated to dry and separated by quick column chromatography eluted with the gradient system to obtain four main fractions. Fraction 2 was purified by sephadex LH-20 to afford methyl gallate (**1**). Fraction 4 was divided in to two parts, F 4.1 was purified by Sephadex LH-20 leading to two compounds named 1-*O*-galloyl-β-D-glucopyranoside (**3**) and 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside (**4**). F 4.2 was further purified by defatting with hexane and CH_2Cl_2, yielding white solid of inositol (**2**) (Scheme2.1)**.**



Scheme 3.1 Isolation procedure of isolated compounds from *Schinus terebinthifolius* leaves

3.2.2 Structure elucidation of methyl gallate (1)

Methyl gallate (**1**) was obtained as white solid. The ^{1}H NMR spectrum of **1** showed two singlet protons. Galloyl proton appeared at δ_H 7.07 (2H, s, H-2 and H-6) and OMe resonated at 3.83 (3H, OCH_3). The ^{13}C NMR spectra showed six carbons. Characteristic signal of carboxyl group at δ_C 169.1(C-7) and carbon signal of methyl group at 52.3 (C-8) indicated the presence of methyl carboxylate. The NMR data of **1** were consistent with previous report of Cynthia *et al*., 1988.

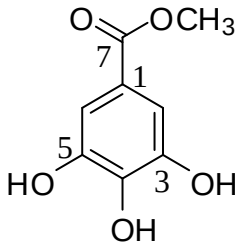


Figure 3.2 Chemical structure of methyl gallate

3.2.3 Structure elucidation of inositol (2)

Inositol (**2**) was obtained as white solid. This compound cannot to dissolve in MeOH but highest dissolve in H_2O. The ^{1}H NMR spectra of **2** showed range of signal at δ_H 3.15-3.94 but anomeric proton signal is disappear in spectrum, therefore it's not sugar. In addition, ^{13}C NMR spectra showed four carbon signals at δ_C 74.4, 72.4, 72.2 and 71.2 respectively. This data indicated that compound **2** was inositol but, configuration is not determined. The NMR data of **1** were consistent with previous report of Cerdan *et al*., 1986.

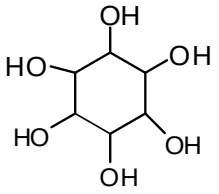


Figure 3.3 Chemical structures of inositol

3.2.4 Structure elucidation of 1-*O*-galloyl-β-D-glucopyranoside or β-glucogallin (3)

1-*O*-Galloyl-β-D-glucopyranoside or β-glucogallin (**3**) was obtained as white powder. The ^{1}H and ^{13}C NMR spectra were similar to those of compound **1** for galloyl proton and carbon but signal of OMe disappeared. Six proton signals at δ_H 3.38-3.85 (6H, H-2′, 3′, 4′, 5′ and 6′) indicated the presence of a sugar moiety. A doublet signal of anomeric proton appearing at δ_H 5.65 (1H, H-1′) with a coupling constant of 8.0 Hz indicated a β-linked anomeric proton. In addition, carbon signals of glucose were also observed at δ_C 96.0, 78.9, 78.2, 74.2, 71.2 and 62.4 for C-1, 2, 3, 4, 5 and 6, respectively. The NMR data of **3** were consistent with those previously reported by Muhammed *et al*., 2009.

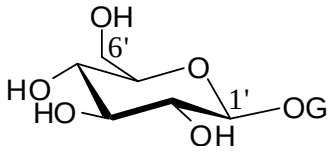


Figure 3.4 Chemical structure of β-glucogallin

3.2.5 Structure elucidation of 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside or PGG (4)

1,2,3,4,6-Pentagalloyl-*O*-β-D-glucopyranoside or PGG (4) was obtained as brown film. The ^{1}H NMR spectra of **4** showed signals in two distinct regions, aromatic and unusual downfied of sugar moiety. PGG had five galloyl groups conjugated to glucose core. Each proton of galloyl group showed singlet signal at δ_H 6.89, 6.95, 6.97, 7.05 and 7.11. Signal of glucose appear at δ_H 5.90 (1H, H-3), 5.59 (2H, H-2, H-4), 4.51 (1H, H-5) and 4.39 (1H, H-6). A doublet signal resonated at δ_H 6.23 (1H, H-1) with a coupling constant of 8.0 Hz, indicating a β-linked anomeric proton. Further ^{13}C NMR was analysis showed thirty-one carbon signals typical of galloyl groups and glucose moiety. Anomeric carbon was observed at δ_C 93.9 in the ^{13}C NMR spectrum. The NMR data of **4** were consistent with previous report of Nicola and coworkers (1994).

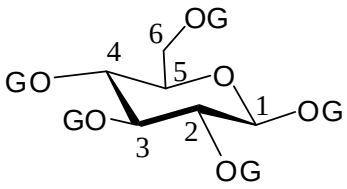


Figure 3.5 Chemical structure of PGG

3.2.6 α-Glucosidase inhibitory activity of compounds 1-4

Inhibitory activity of the isolated compounds (**1-4**) was determined comparison with type II diabetes drug (acarbose) by colorimetric method. The results are shown in Table 3.1

Table 3.1 α-Glucosidase inhibitory activity of **1-4**

compounds	IC_{50} [a] (μM)		
	α-glucosidase		
	baker's yeast	rat intestinal	
		maltase	sucrase
1	NI [c]	191.74[b]	152.09
2	303.16	NI	NI
3	96.32	273.32	135.16
4	NI	27.21	37.53
Acarbose	215.35	54.67	47.08

[a] The IC_{50} value is defined as the inhibitor concentration to inhibit 50%of enzyme activity

[b] Values are performed in duplicate

[c] No inhibition, less than 50% inhibition at 10 mg/mL

Compounds **1**, **3** and **4** from *S. terebinthifolius* leaves were classified in hydrolysable tannin. **3** and **4** showed different number of galloyl group on the glucose core. They have one and five galloyl groups, respectively while **1** have methyl group instead of glucose core. From the Table 3.1, compound **2** and **3** showed inhibitory

effect against α-glucosidase from baker's yeast, whereas inhibitory activityof **1**and **4** were less than 50%. Compound **4** showed highest α-glucosidase inhibitory activity toward rat intestine (maltase and sucrase) followed by acarbose. On the other hand, **1** and **3** showed inhibition nearly identical, whereas inhibition of **2** against rat intestine was less than 50%. Pentagalloyl glucose (PGG, **4**) was much more active than β-glucogallin (**3**) or methyl gallate (**1**). It was indicated that increasing number of galloyl units in the molecule led to increase inhibitory activity in rat intestine (maltase and sucrase). This hypothesis was supported by the reports of Miou *et al*., 2000 and Shruti *et al*., 2011. The presence of five galloyl groups could interact more effectively with the enzyme than gallic acid or β-glucogallin (Miou *et al*., 2001). In 2006 Yulin and coworkers also reported that, **4** could activate the insulin receptor, stimulate glucose transport in adipocytes, and reduce blood glucose in diabetic animals**.** In addition, it showed multiple biological activities such as anti-oxidative effect, cardiovascular protective, and hypo-cholestrolemic affect, which is a great potential in therapy and prevention of several major human diseases includingcancer and diabetes (Jinhui *et al*., 2009). Since **4** displayed an intense inhibitory activity in rat intestine, therefore mechanism underlying the inhibition of **4** was determined by Lineweaver– Burk plot analysis type as well as synergistic effect of **4**.

3.2.7 The kinetic inhibition of PGG on rat intestinal glucosidase

Herein, it is first time for kinetic study of PGG (**4**). The kinetic assay was performed using Lineweaver-Burkplots (Adisakwattana *et al*., 2009).The inhibitory activity against the intestinal maltase and sucrase showed the same trend. They were showed mixed type inhibition. The inhibitory mechanisms of sucrase by PGG are shown in Fig. 3.5.The intersections of each PGG concentration line showed in second quadrant and the analysis of K_m increased with unchanged V_{max} values same in Table 2.2 descript in chapter II indicate that mix-type inhibition in sucrase. In addition, the affinity of inhibitor to free enzyme (**E**) was demonstrate in K_i value (Fig 3.6) while the affinity of inhibitor to enzyme-substrate complex (**ES**) was demonstrate in K_i' value (Fig 3.7). The possible binding mode of this inhibitor was assumed that one inhibitor can bind either to active site of free enzyme (**E**) or to the enzyme–substrate complex (**ES**). According to analysis of K_i value was lower than K_i' value at sixteen

time (Table 3.2) indicate that, binding affinity of PGG to free enzyme (**E**) stronger than enzyme–substrate complex (**ES**). The data implied that PGG is dominant in competitive inhibition.

Similar in sucrase the inhibitory mechanisms of maltase shown mixed type inhibition (Fig 3.8) while value of K_i (**E**) less than K_i' (**ES**) at five time, respectively (table 3.2).It was indicated that, binding affinity of PGG to free enzyme (**E**) stronger than enzyme–substrate complex (**ES**). The data implied that PGG is dominant in competitive inhibition.

Table 3.2 The values from kinetic analyses of PGG (**4**)

dissociation constant	sucrase	maltase
K_i	0.12 mM	0.20 mM
K_i'	2.02 mM	1 mM

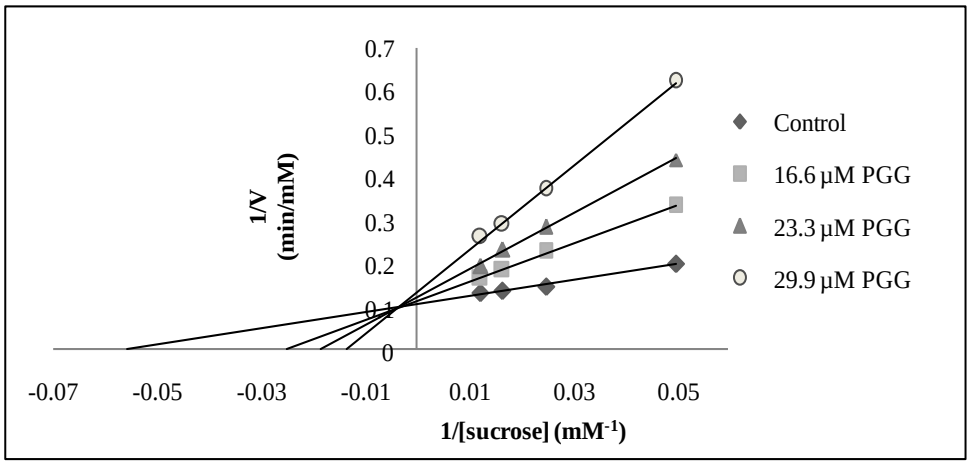


Figure 3.6 Lineweaver-Burk plot for inhibitory activity of PGG (**4**) on the intestinal sucrase.

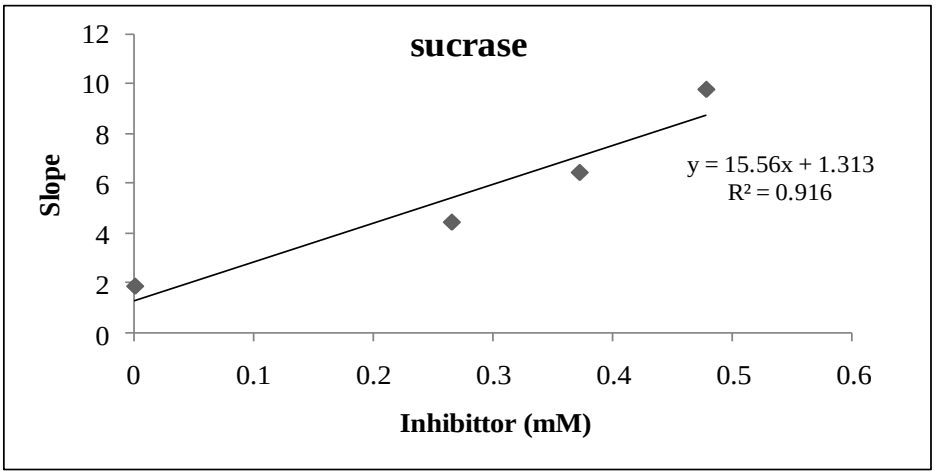


Figure 3.7 Secondary replot of slope *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i

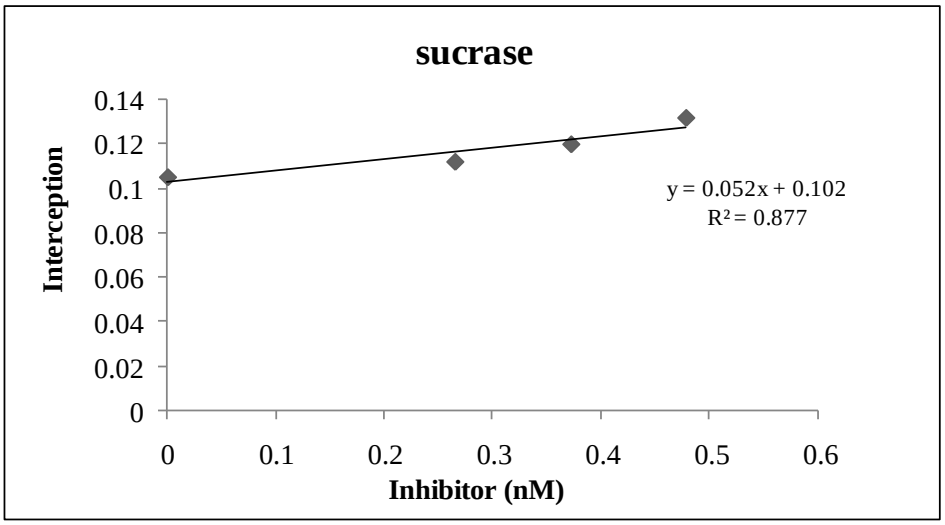


Figure 3.8 Secondary replot of intercept *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i'

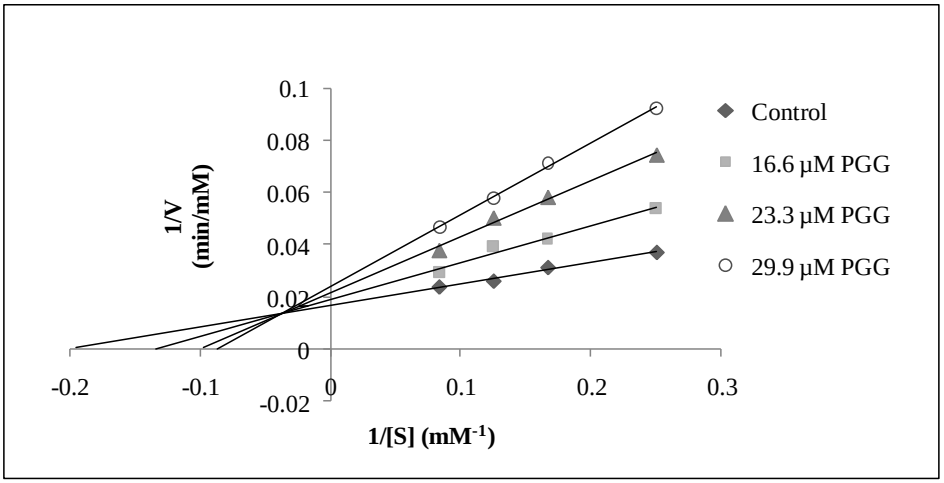


Figure 3.9 Lineweaver-Burk plot for inhibitory activity of PGG (**4**) on the intestinal maltase

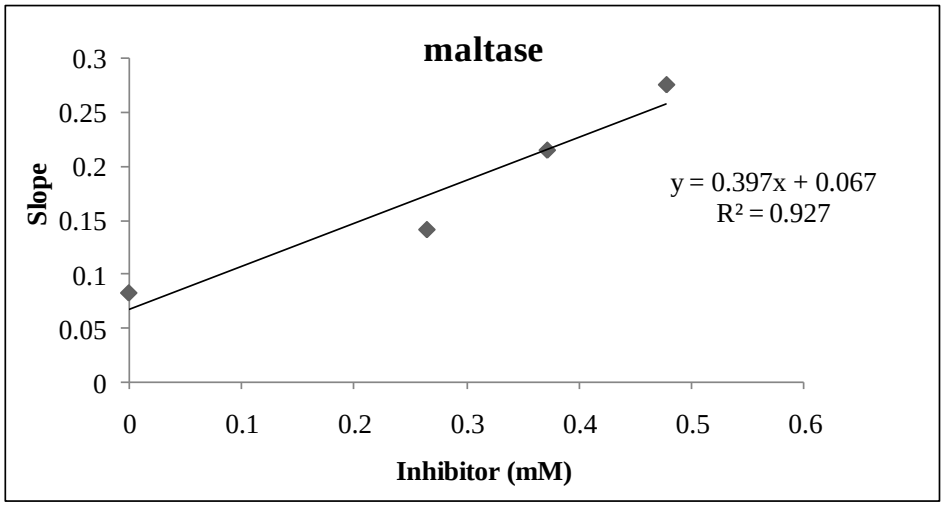


Figure 3.10 Secondary replot of slope *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i

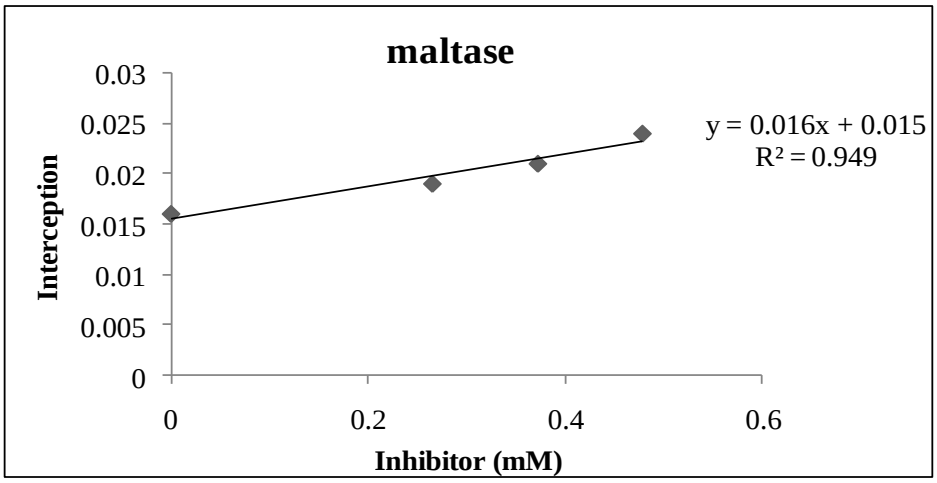


Figure 3.11 Secondary replot of intercept *vs.* [I] from a primary Lineweaver–Burk plot for the determination of K_i

3.2.8 Synergistic effect of PGG (4) with acarbose on rat intestinal α-glucosidase inhibition

From the highest inhibition of PGG against rat intestine, it is of interest to studies combination of PGG with acarbose might interact synergistically on α-glucosidase. Synergistic effect of PGG was carried out for the first time; low concentration of PGG (0.01 mg/mL) which had 5-10% inhibition. It was combined with different concentration of acarbose (0.01-50 mg/mL). The combination of PGG and acarbose were compared with acabose alone. As the result, PGG showed dominant synergistic effect on rat intestine (maltase) adverse in sucrase, the result show in Fig 3.11. Inhibitory activity of PGG combined with acarbose significantly more than acarbose alone. It was showed increase inhibitory activity in range of acarbose 1 and 10 mg/mL (7.1% and 7.9%) whereas other acarbose concentrations have not different percentage inhibition between acarbose alone and it's combination. The combination of PGG with acarbose was no increase inhibitory activityin sucrase, indicating that PGG no produced synergistic inhibition in sucrase (Fig 3.12). However, this effect should be further studies in *in vivo* for combination between PGG and acabose on lowering blood glucose level of type 2 diabetes.

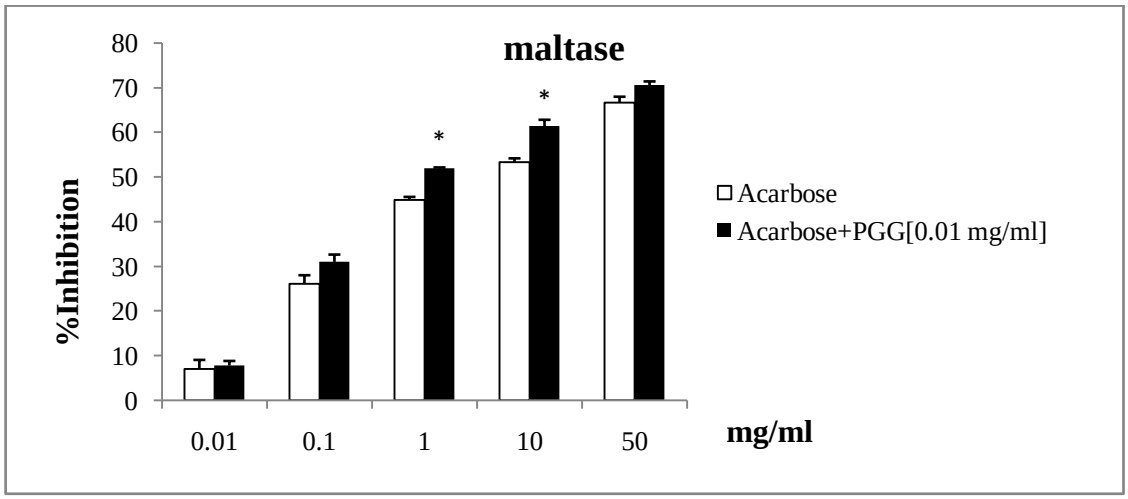


Figure 3.12 The percentage intestinal inhibition of acarbose and its combination with PGG (0.01mg/ml) against rat intestinal maltase

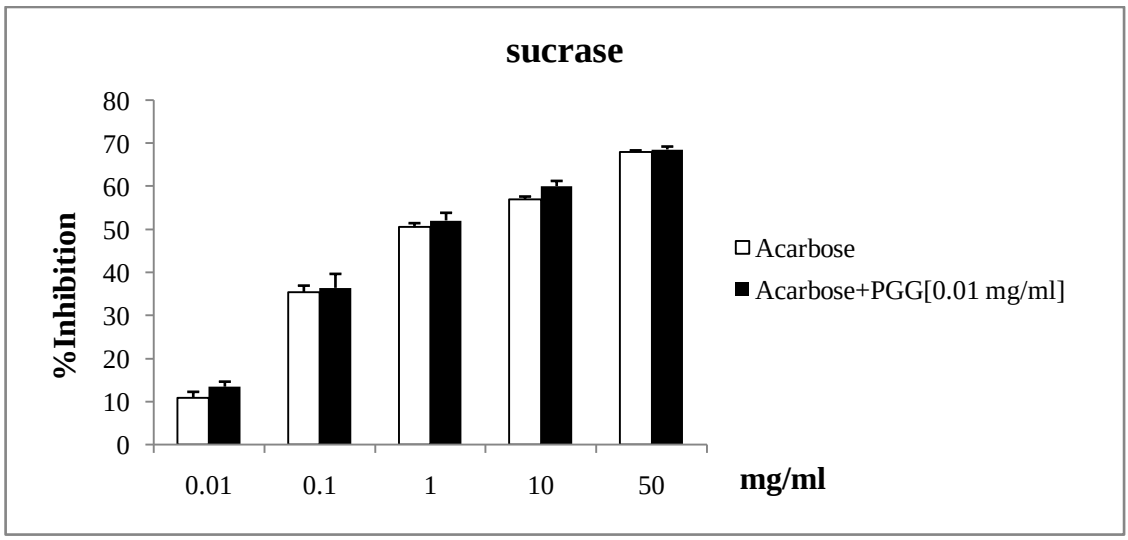


Figure 3.13 The percentage intestinal inhibition of acarbose and its combination with PGG (0.01mg/ml) against rat intestinal sucrase

3.3 Experiment section

3.3.1 General experimental procedures

The ^{1}H and ^{13}C-NMR spectra (CD_3OD) were recorded with a Varian Mercury+ 400 NMR spectrometer. The chemical shift in δ (ppm) was assigned with reference to the signals of residual protons in deuterated solvents. Sephadex LH-20,

silica gel 60 Merck cat. No. 7734 and 7731 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 PF254 plates.

3.3.2 Plant material

The leaves of *Schinus terebinthifolius* were collected from Nakhon Ratchasima, Thailand in December 2011. Plant authentication was performed by Mrs. Parinyanoot Klinratana, and the specimen (BCU013526) was deposited at the Botanical Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University.

3.3.3 Extraction and isolation

The air dried leaves of *Schinus terebinthifolius* were blended until powder before extracted with MeOH (5×700mL) at room temperature.The MeOH extract was partitioned between 1:1 MeOH and hexane. The methanol layer was evaporated to dry (43.96 g) and separated by quick column chromatography eluted with the gradient system (stepwise 0:100, 10:90, 20:80 and 100:0 MeOH- CH_2Cl_2) to obtain four main fractions.Each fraction was evaluation for α-glucosidase inhibitory. The fractions showing high inhibitory activity were further purified by column chromatograpy. Fraction 2 was purified by sephadex LH-20 (10% MeOH- CH_2Cl_2) to afford a known compound, named methyl gallate (**1**, 52.1mg). Fraction 4 was divided two part (F 4.1 and F 4.2). F 4.1 can soluble in MeOH and was purified by sephadex LH-20 (100 % MeOH) leading to two known compounds named 1-*O*-Galloyl-β-D-glucopyranoside or β-glucogallin (**3**, 6.4 mg) and 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside or PGG (**4**, 48.3 mg). F 4.2 is a white solid from quick column chromatography (20% MeOH- CH_2Cl_2), which can soluble in water. It was boiled in hexane and CH_2Cl_2 for washed the solid. Named of white solid is inositol (**2**,78.6 mg). The structure of known compounds was identified by comparison of their ^{1}H and ^{13}C NMR data with those in the previous reports.

Methyl gallate (**1**): white solid; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.07 (2H, s, H-2, H-6), 3.83 (3H, OCH_3) ^{13}C NMR (CD_3OD, 100 MHz) δ 169.1, 146.5, 139.8, 121.5, 110.2, 52.3

Inositol (**2**): white solid: configuration not determined; ^{1}H NMR (D_2O, 400 MHz) δ 3.94 (1H, t, *J*=2.8 Hz), 3.50 (2H, t, *J*= 9.6 Hz), 3.41 (2H, dd, *J*= 10.0, 3.3 Hz), 3.15 (1H, t, *J*=9.2 Hz) ^{13}C NMR (D_2O, 100 MHz) δ 74.4, 72.4, 72.2, 71.2

1-*O*-Galloyl-β-D-glucopyranoside or β-glucogallin (**3**): white powder ;^{1}H NMR (CD_3OD, 400 MHz) galloyl, δ 7.12 (2H, s, H-2, H-6); glucose, δ 5.65 (1H, d, *J* = 8.0 Hz), 3.85 (1H, d, *J*=12 Hz), 3.69 (1H, dd, *J*= 4.4, 4.4 Hz), 3.38-3.48 (4H, m)^{13}C NMR (CD_3OD, 100 MHz) δ 167.2, 146.5, 140.4, 120.8, 110.6, 96.0, 78.9, 78.2, 74.2, 71.2, 62.4

1,2,3,4,6-Pentagalloyl-*O*-β-D-glucopyranoside or PGG (**4**): brown film ; ^{1}H NMR (CD_3OD, 400 MHz); galloyl group δ 6.89, 6.95, 6.97, 7.05,7.11 (5×2H, s); glucose δ 6.23(1H, d, *J*=8.0 Hz, H-1), 5.90 (1H, t, *J*=10.0 Hz, H-3), 5.59 (2H, q, *J*=10.0 Hz, H-2, H-4), 4.51(1H, d, *J*=8.0 Hz, H-5), 4.39 (1H, t, *J*=10.0 Hz, H-6) ^{13}C NMR (CD_3OD, 100 MHz) δ 168.0, 167.3, 167.0, 167.0, 166.3, 146.9, 146.8, 146.5, 146.4, 146.3, 140.8, 140.4, 140.3, 140.2, 140.0, 121.1, 120.4, 120.3, 120.3, 119.8, 110.7, 110.5, 110.5, 110.5, 110.4, 93.9, 74.5, 74.2, 72.3, 70.0, 63.2

3.3.4 α-Glucosidase inhibitory assay

The α-glucosidase inhibitory effect of isolated compounds was evaluated using the same procedure described in Chapter II.

3.3.5 Measurement of kinetic constant

To characterize the pattern of PGG inhibition, kinetic analyses of PGG on rat intestinal (maltase and sucrase) was observed at different PGG concentrations. The concentrations of PGG at 0, 16.6, 23.3 and 29.9 μM respectively were evaluated using the same procedure described in Chapter II.

3.3.6 Synergistic effect of PGG with acarbose on rat intestinal α-glucosidase inhibition

Acording to rat intestinal inhibition of PGG showed highest inhibitory effect therefore, we interest to studies synergistic effect of PGG combined with acarbose. Assay was performed according to a slightly modified method of Adisakwattana *et al.*, 2009. The reaction was performed according to α -glucosidase inhibitory activity from rat intestine described in Chapter II. The various concentrations of acarbose (0.01 -50 mg/mL) were combined with low concentration of PGG (0.01mg/mL). Results were expressed as a percentage inhibition of the corresponding control values (percentage inhibition of acarbose only). Data was expressed as mean ±S.E.M. (n = 3). Statistical analysis was performed by Student's *t*-test ($P<0.01$).

Supporting information

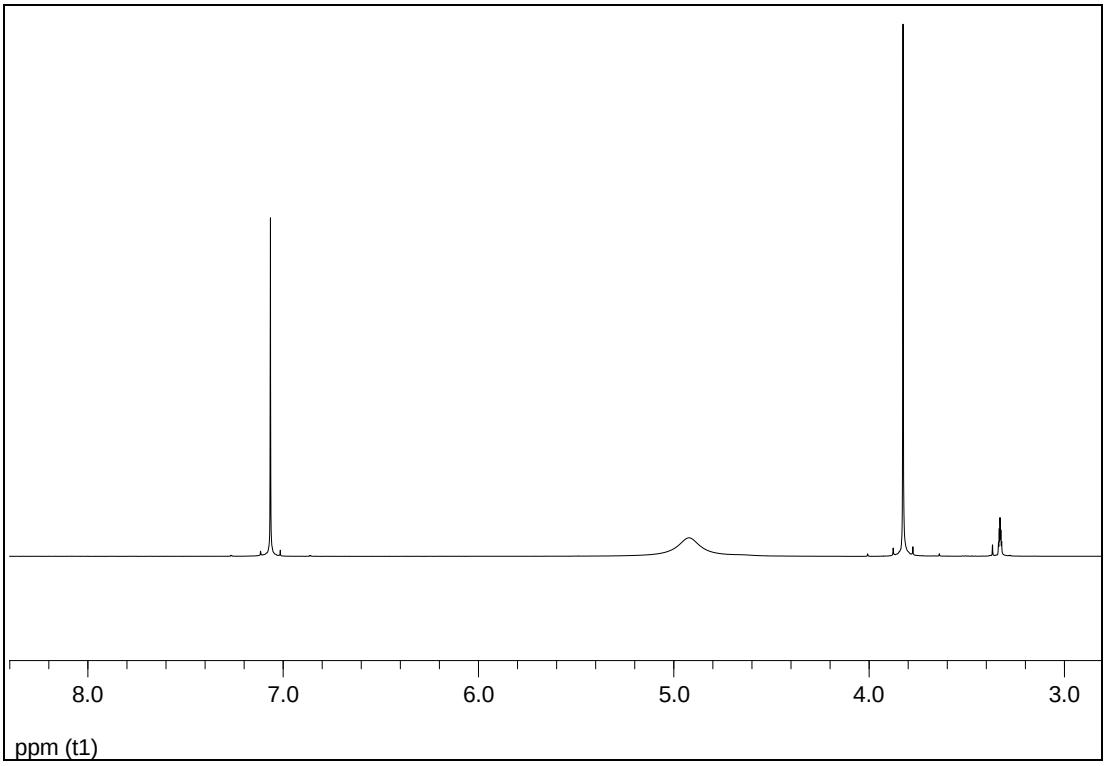


Figure3.14 ^{1}H NMR (CD_3OD) spectrum of methyl gallate (**1**)

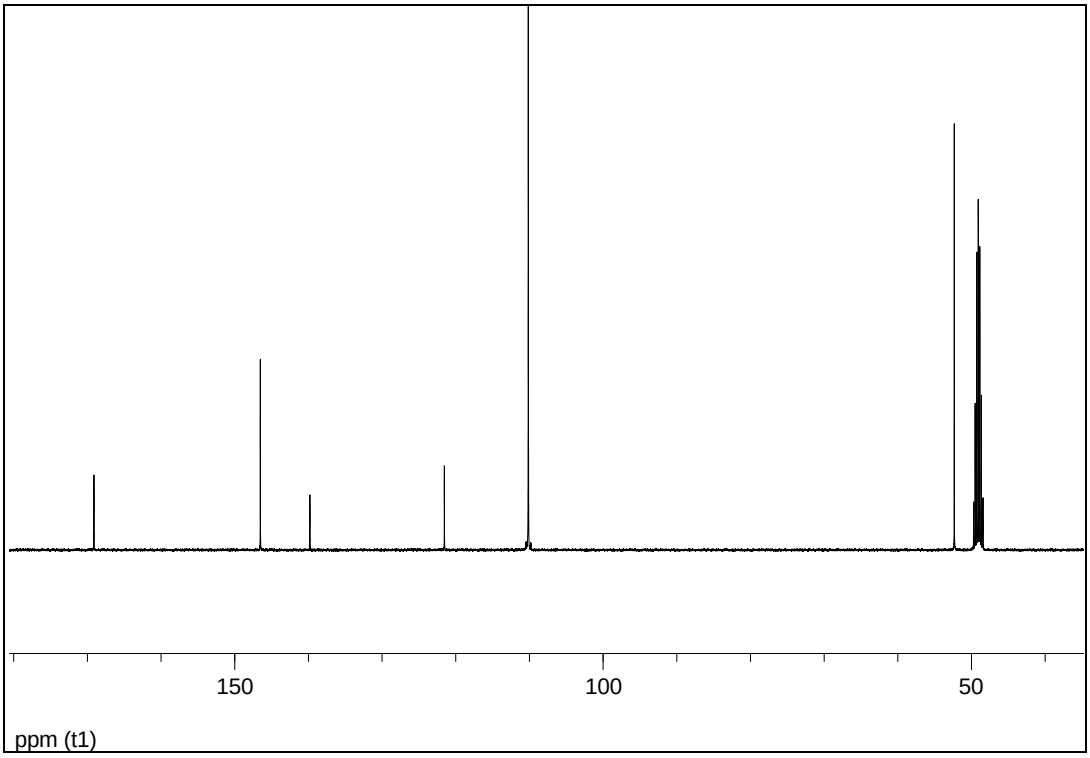


Figure 3.15 ^{13}C NMR (CD_3OD) spectrum of methyl gallate (**1**)

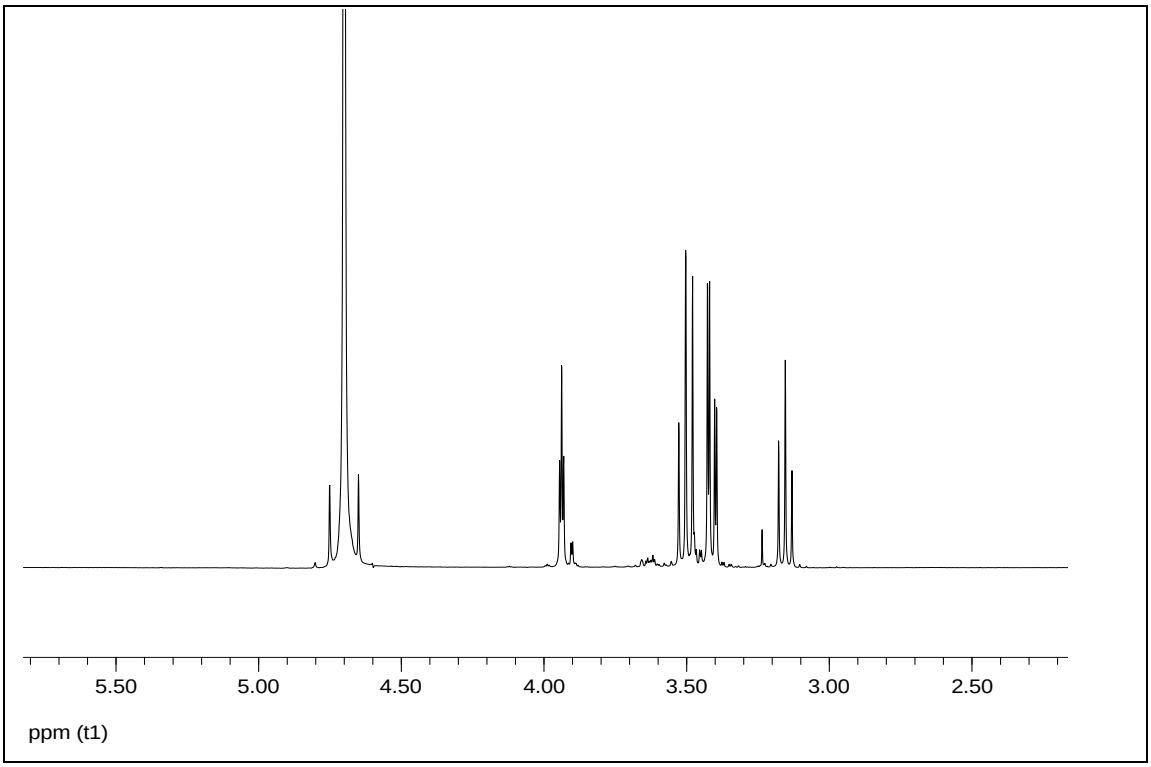


Figure3.16 ^{1}H NMR (CD_3OD) spectrum of inositol (**2**)

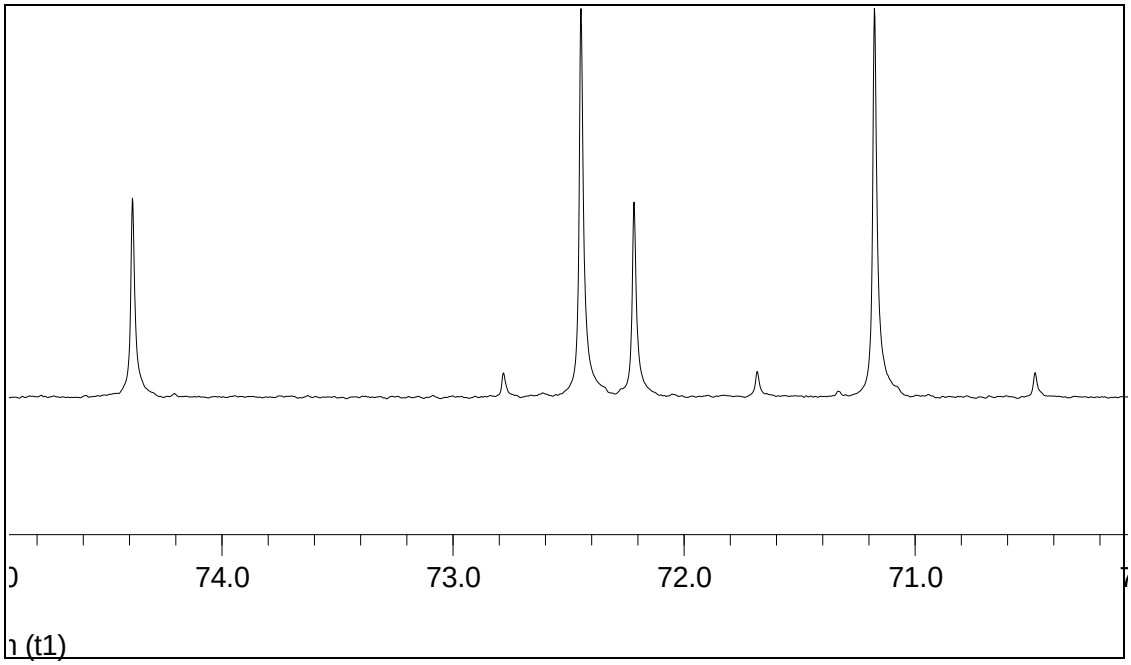


Figure 3.17 ^{13}C NMR (CD_3OD) spectrum of inositol (**2**)

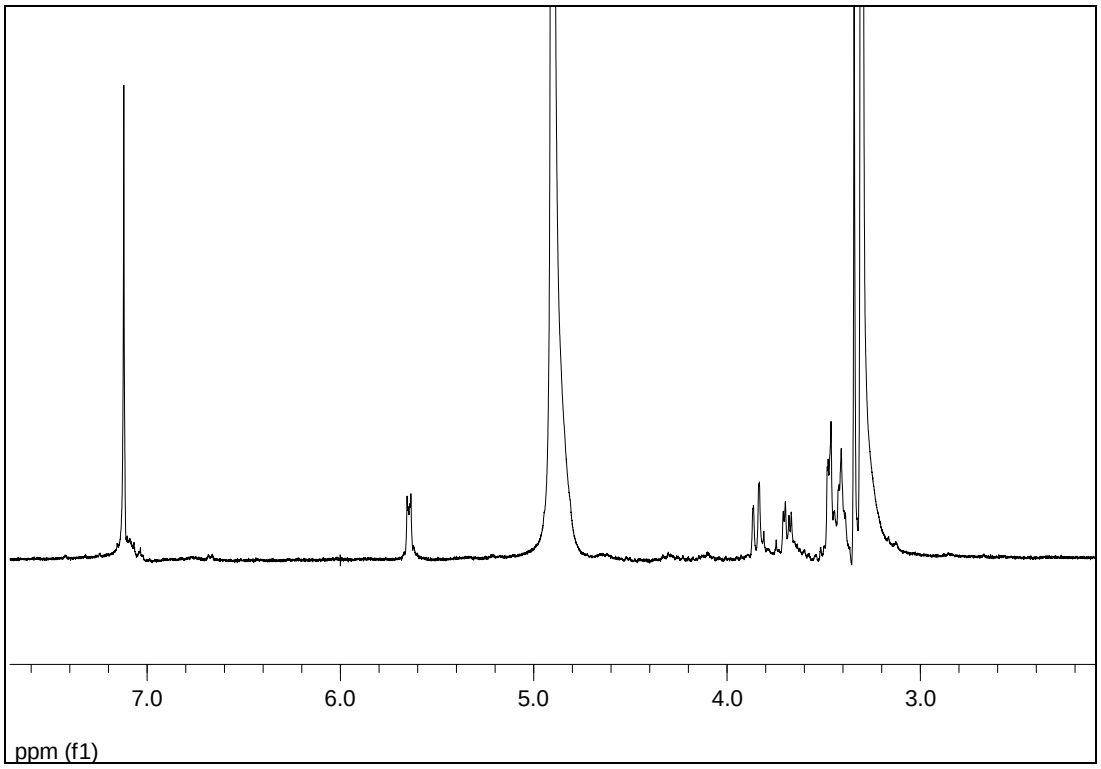


Figure 3.18 ^{1}H NMR (CD_3OD) spectrum of 1-*O*-galloyl-β-D-glucopyranoside (**3**)

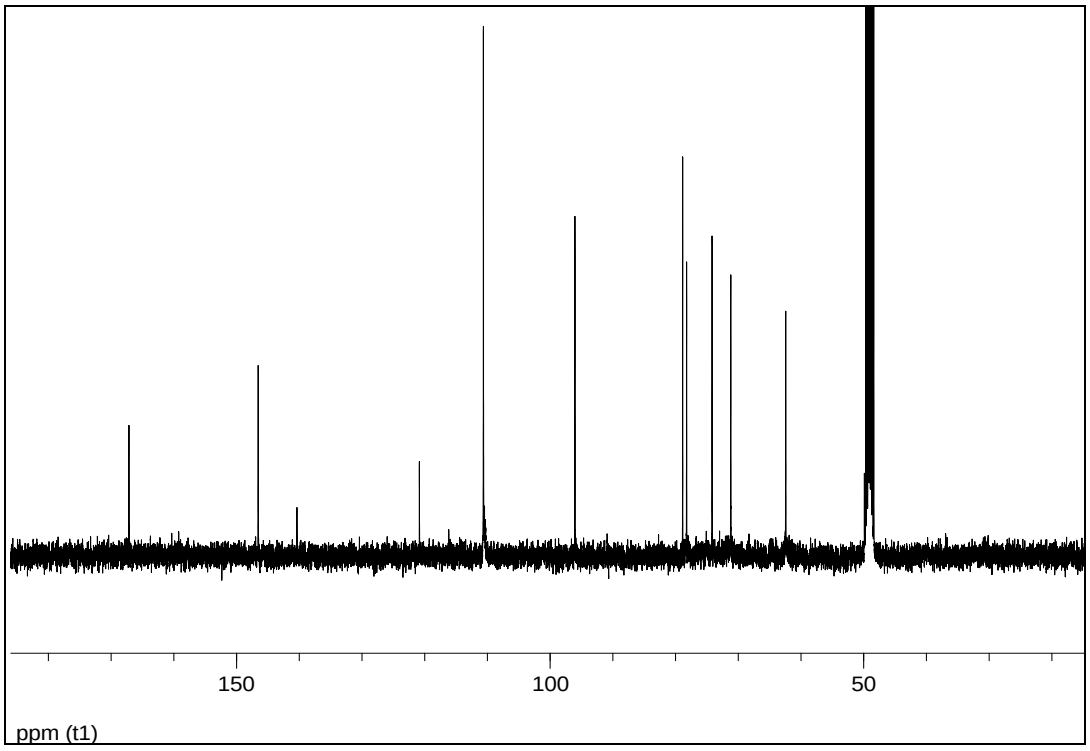


Figure 3.19^{13}C NMR (CD_3OD) spectrum of 1-*O*-galloyl-β-D-glucopyranoside (**3**)

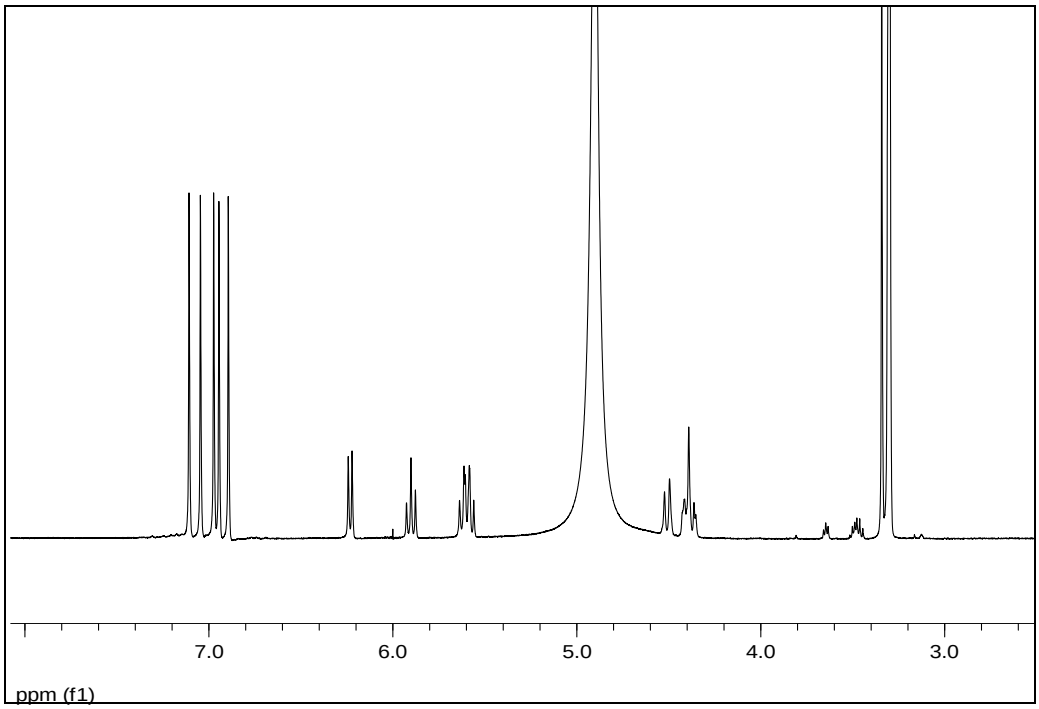


Figure 3.20 ^{1}H NMR (CD_3OD) spectrum of 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside (**4**)

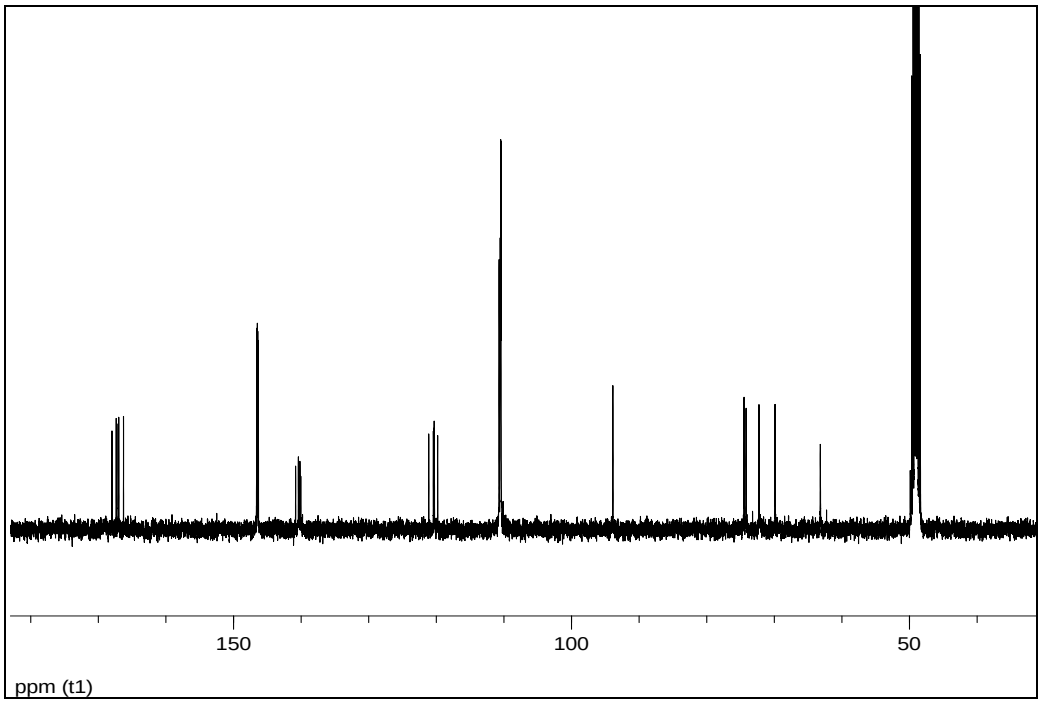


Figure 3.21^{13}C NMR (CD_3OD) spectrum of1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside (**4**)

CHAPTER IV

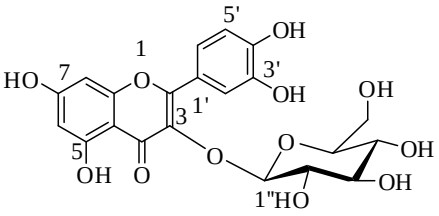
CONCLUSION

The α-glucosidase inhibitors from *Abelmoschus esculentus* seeds and leaves of *Schinus terebinthifolius* were identified. The MeOH extract from the seeds of *Abelmoschus esculentus* afforded two major compounds, which consisted of flavonoid glycosides named, quercetin-3-*O*-glucoside (**1**) and quercetin-3-*O*-glucosyl (1′′′→6′′) glucoside (**2**). The isolation of MeOH extract from leaves of *Schinus terebinthifolius* yielded methyl gallate (**1**), inositol (**2**), 1-*O*-galloyl-β-D-glucopyranoside or β-glucogallin (**3**) and 1,2,3,4,6-pentagalloyl-*O*-β-D-glucopyranoside or PGG (**4**). The structure of all isolated substances from seeds of *Abelmoschus esculentus* and leaves of *Schinus terebinthifolius* were summarized in Figures 5.1 and 5.2, respectively.

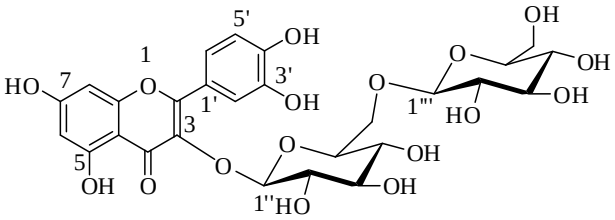
The inhibitory activity against α-glucosidases from baker's yeast and rat intestine (maltase and sucrase) of compounds isolated from both plants were evaluated using colorimetric method. The isolated compounds from *Abelmoschus esculentus* (**1** and **2**) were assayed compared with flavonoide aglycone (quercetin (**3**)) which obtained from hydrolysis of rutin. As a result show, **1** and **2** showed weak inhibition against baker's yeast α-glucosidase (less than 50%). In rat intestine, **3** showed highest against α-glucosidase inhibitory activity followed by **1** and **2**, respectively. Therefore, the kinetic of **3** was carries out using Lineweaver-Burkplots. It showed competitive inhibition a gainst sucrase whereas mixed type inhibition of competitive and noncompetitive was found in maltase.

On the other hand, the isolated compounds from *Schinus terebinthifolius* (**1**, **2**, **3** and **4**) generally demonstrated broad inhibition against α-glucosidases from baker's yeast and rat intestine (maltase and sucrose). Compounds **2** and **3** showed inhibitory effect against α-glucosidase from baker's yeast, whereas inhibitory activity of **1** and **4** were less than 50%. Compound **4** showed highest inhibitory activity against rat intestine (maltase and sucrase). Therefore, the kinetic analysis of **4** was investigated for the first time. It showed mixed type inhibition of competitive and noncompetitive against both maltase and sucrase. In addition, **4** was also analyzed for synergistic

effect for the first time. PGG (**4**) showed dominant synergistic effect in rat intestine maltase but produced no such effect against sucrase.

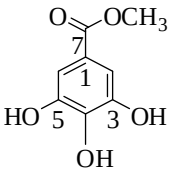


Quercetin-3-*O*-glucoside (**1**)

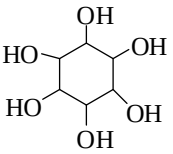


Quercetin-3-*O*-glucosyl (1''' → 6'') glucoside (**2**)

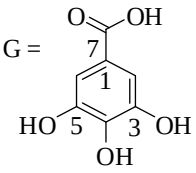
Figure 5.1 The chemical structures of isolated compounds from *Abelmoschus esculentus* seeds

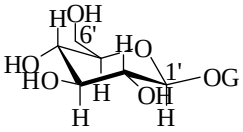


Methyl gallate (**1**)

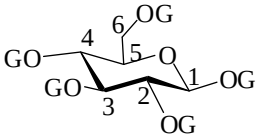


Inositol (**2**)





β-Glucogallin (**3**)



PGG (**4**)

Figure 5.2 The chemical structures of isolated compounds from *Schinus terebinthifolius* leaves

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VITA

Miss Wannisa Thanakosai was born on June 29, 1987 in Bangkok, Thailand. She graduated with Bachelor' s Degree of Science in Biotechnology from Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, in 2009. During studying in Master Degree in Biotechnology program, she was attended conference proceedings of Pure and Applied Chemistry International Conferences (PACCON 2012) in the topic of α-GLUCOSIDASE INHIBITORS FROM *Abelmoschus esculentus* SEEDS page 998-1000.