สารยับยั้งแอลฟากลูโคซิเดสจากใบและเมล็ดมะรุม *Moringa oleifera*

นางสาววรรณวิศา สุดมาตร

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α-GLUCOSIDASE INHIBITORS FROM LEAVES AND SEEDS OF MORINGA *Moringa oleifera*

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Field of Study	Biotechnology
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วรรณวิศา สุดมาตร: สารยับยั้งแอลฟากลูโคซิเดสจากใบและเมล็ดมะรุม *Moringa oleifera*. (α-GLUCOSIDASE INHIBITORS FROM LEAVES AND SEEDS OF MORINGA *Moringa oleifera*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.ปรีชา ภูวไพรศิริศาล, 58 หน้า.

มะรุมเป็นพืชรับประทานได้ ที่ใช้รักษาเบาหวานมาเป็นเวลานาน ในประเทศไทยจำหน่ายใบและเมล็ดมะรุมเพื่อลดระดับน้ำตาลในเลือดหลากหลายรูปแบบ เช่น ชาชง และแคปซูล ในงานวิจัยนี้ได้แสดงให้เห็นถึงสารออกฤทธิ์ทางชีวภาพที่มีส่วนช่วยลดระดับน้ำตาลในเลือดจากมะรุมด้วย bioassay-guided โดยการแยกส่วนสกัดน้ำ, ส่วนสกัดเมทานอลจากใบมะรุม และส่วนสกัดเมทานอลจากเมล็ดมะรุม ได้สารบริสุทธิ์ 9 ชนิด คือ *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside, keampferol, kaempferol-3-*O*-β-glucopyranoside, 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside, 4-methoxybenzene-1,3-diol, hydroxybenzoic acid, *N,N*-Bis(4-hydroxybenzyl) urea, uridine และ สารใหม่ คือ ME51-4 ซึ่งเป็นอนุพันธ์ของเอไมด์ ผลการทดสอบฤทธิ์ยับยั้ง α-glucosidase ของสารบริสุทธิ์พบว่า มีเพียง keampferol และ kaempferol-3-*O*-β-glucopyranoside ซึ่งแยกได้จากส่วนสกัดน้ำจากใบมีฤทธิ์ยับยั้ง α-glucosidase ทั้งจากเบเกอร์ยีสต์และลำไส้หนูได้ดีกว่าสารในกลุ่มอื่น ๆ อย่างไรก็ตาม keampferol มีฤทธิ์ยังยั้ง α-glucosidase ดีกว่า kaempferol-3-*O*-β-glucopyranoside เพียงเล็กน้อย จากผลการยับยั้ง α-glucosidase ที่มีประสิทธิภาพของ keampferol และ kaempferol-3-*O*-β-glucopyranoside สามารถสรุปได้ว่าการใช้ใบมะรุมในรูปแบบของชาชงน่าจะมีส่วนช่วยควบคุมระดับน้ำตาลในเลือดและรักษาโรคเบาหวานได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

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KEYWORDS: α-GLUCOSIDASE INHIBITORS / *Moringa oleifera* / Diabetes

WANVISA SUDMART: α-GLUCOSIDASE INHIBITORS FROM LEAVES AND SEEDS OF MORINGA *Moringa oleifera*. ADVISOR: ASST. PROF. PREECHA PHUWAPRAISAN, Ph.D., 58 pp.

Moringa oleifera is an edible plant that has long been used for diabetes therapy. In Thailand, leaves and seeds of *M. oleifera* are sold in many forms such as herbal infusion and capsule, and used to suppress blood glucose level. To proof this claim, the components responsible for this activity were identified using bioassay-guided isolation. The isolation of aqueous and methanolic extracts of leaves together with methanolic extract of seeds yielded nine compounds named *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside, keampferol, kaempferol-3-*O*-β-glucopyranoside, 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside, 4-methoxybenzene-1,3-diol, *p*-hydroxybenzoic acid, *N*,*N*-Bis(4-hydroxybenzyl) urea, uridine as well as new amide derivative named ME51-4. All the isolated compounds were tested for inhibitory activity against α-glucosidase. Only keampferol and kaempferol-3-*O*-β-glucopyranoside, which were isolated from leaves aqueous extract, broadly inhibited α-glucosidases intestinal from baker's yeast and rat intestine. However, kaempferol inhibited α-glucosidase with more slightly improved inhibition than kaempferol-3-*O*-β-glucopyranoside. Due to highly potent inhibition of keampferol and kaempferol-3-*O*-β-glucopyranoside, the consumption of *M. oleifera* leaves in form of herbal infusion would result in higher effective control of blood glucose level and diabetes therapy.

Field of Study : Biotechnology Student's Signature .....

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
HCl	hydrochloric acid
KCl	potassium chloride
KOH	potassium hydroxide
L	liter
μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
nm	nanometer
TLC	thin-layer liquid chromatography
UV	ultraviolet
V	voltage
v/v	volume by volume
w/w	weight by weight

CHAPTER I

Introduction

Diabetes mellitus (DM) is a group of chronic disease, which a person has high blood sugar (glucose) levels that results from defects in insulin secretion, or its action, or both. Normally, blood glucose levels are tightly controlled by insulin, a hormone produced by the pancreas. When the blood glucose elevates, after eating food, insulin is released from the pancreas to normalize the glucose level. In patients with diabetes, the absence or insufficient production of insulin causes hyperglycemia. Diabetes is a chronic medical condition, meaning that although it can be controlled, it lasts a lifetime. The effects of diabetes mellitus include long term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss.

According to the 2006 World Health Organization (WHO) report, the estimated diabetes prevalence for 2000 is 171 million and is expected to affect 366 million people by 2030, which amount to an increase of 144% over the next 30 years (Wild, 2004). Diabetes and its numerous complications are extremely burdensome on the health and economies of countries worldwide. In high income countries, for instance, treatment of diabetic foot complications accounts for 15-25% of total healthcare resources for diabetes. This is an enormous waste not only of scarce public health resources but also of healthy lives. It is estimated that with basic diabetes management and care, up to 80% of all diabetic foot amputations can be prevented. Data from the 2011 Center for Disease Control and Prevention (CDC) shows the number of diabetic patient in the United States study found diabetes affects 25.8 million people (children and adults) 8.3% of the United States populations. A total cost of diagnosed diabetes in the United States in 2007 is $US 174 billion.

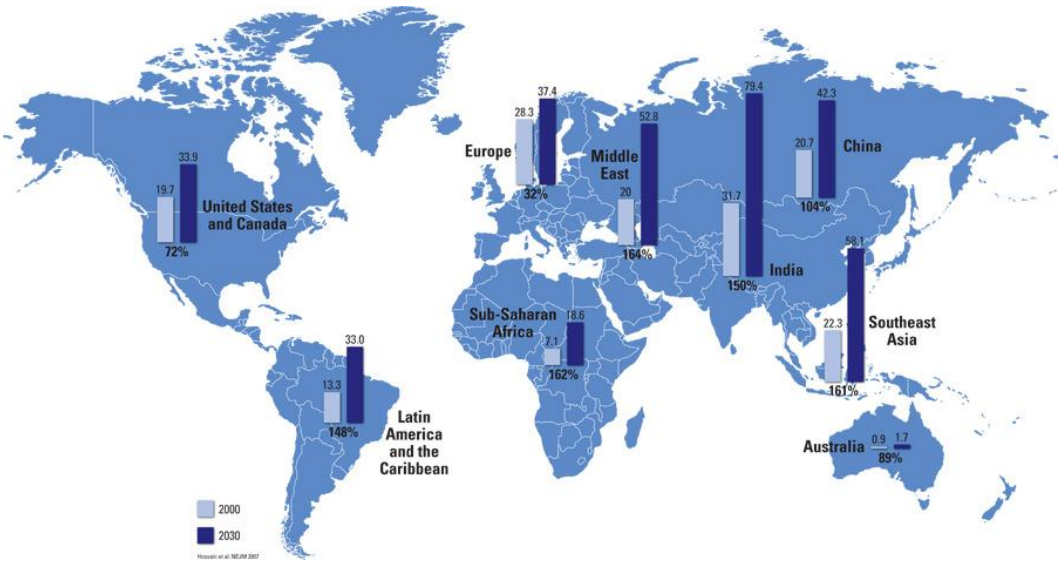


Figure 1.1 Worldwide prevalence of diabetes in 2000-2030 (www.diabetesmapsandgraphs.com/worldpreva.htm)

1.1 Classifications, causes and complication of diabetes mellitus

In 1980, the WHO expert committee classification of diabetes mellitus into two types are currently known; type I or insulin-dependent diabetes mellitus (IDDM) and type II or non-insulin-dependent diabetes mellitus (NIDDM) (Figure 1.2). The classification was widely accepted and is used internationally.

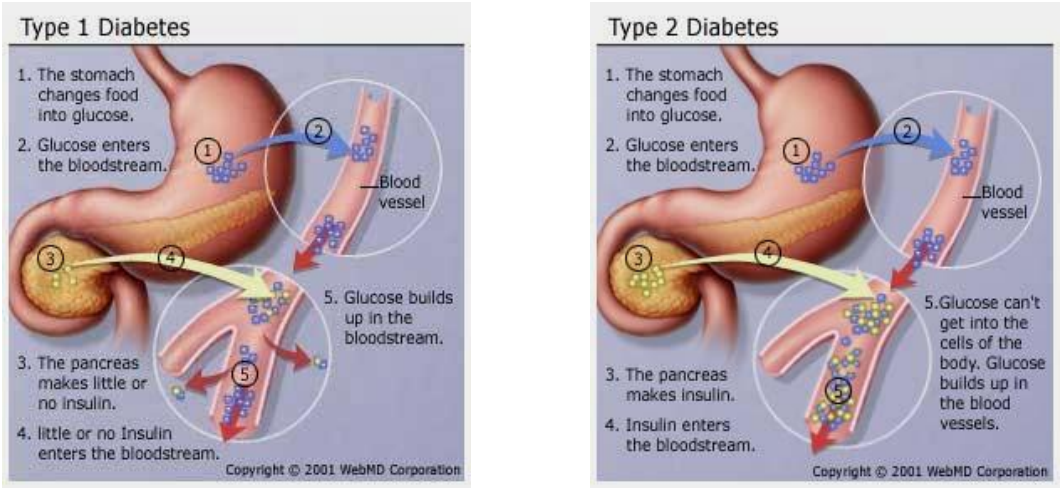


Figure 1.2 Regulation of glucose enters bloodstream in type I and II diabetes (www.uscf.mightyminnow.com/images/charts).

Characteristics of type 1 diabetes mellitus are mostly in children and adults up to 40 years of age, the effects of the disease will carry throughout their life. Type 1 diabetes mellitus is a chronic immunoinflammatory disease resulting from the destruction of insulin-producing pancreatic beta cells mediated by autoreactive T lymphocytes, natural killer (NK) cells and macrophages (Mangano, 2012). This type of diabetes is the least common of the two main types and accounts for between 5 - 10% of all people with diabetes. There is no cure for Type 1 diabetes, but improvements are being made each year in treatments, blood sugar checking, and insulin delivery.

Characteristics of type 2 diabetes mellitus are mostly in adults after 40 years of age. Type 2 diabetes mellitus is the most common form of diabetes accounts for approximately 90 - 95% of all cases of diabetes. Normally, the body produced insulin from the beta-cell of pancreas. Insulin secretion from the pancreas normally reduces glucose output by the liver, skeletal muscle, and fat tissue. Type 2 diabetes develops when the beta-cell of pancreas can still secret some insulin, but not enough, or when the insulin that is produced does not work properly (known as insulin resistance) as show in Figure 1.2.

Pathogenesis of type 2 diabetes has various factors affect both insulin secretion and insulin action. Insulin is a hormone secreted by the beta cell of the pancreases as the islets of langerhans, which to move glucose from the bloodstream into the body's cells to produce energy. The small intestine breaks down dietary carbohydrates into glucose, as the body's main source of fuel. When glucose is absorbed through the intestine into bloodstream, a blood glucose level is rise. This causes beta cells in the pancreas to produce and secrete insulin in to bloodstream.

All of cells in the body have insulin receptors in their plasma membrane. The stimulus for insulin secretion is high blood glucose (which occurs within moments of consuming a carbohydrate-containing meal) results in an increased number of insulin receptors bound to insulin. Insulin binding to the extracellular domain of the insulin receptor and the intracellular tyrosine kinase domain of the receptor is activated. The results of these activated signaling pathways depend on the cell type. For example, in muscle and adipose cells, vesicles containing the insulin-regulated glucose transporter

(Glut4) fuse with the plasma membrane, which remove glucose from the blood by carrying Glut4 across the cell membrane to the inside of the cell (Figure 1.3).

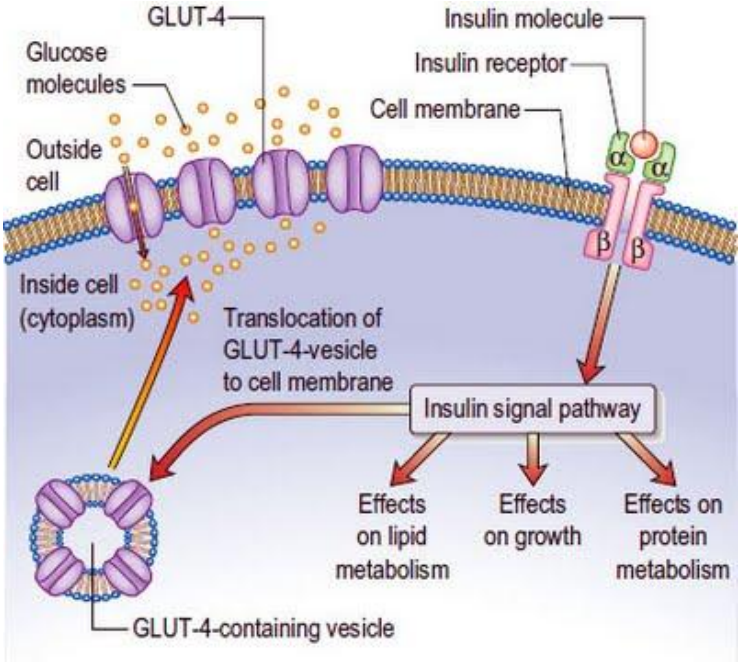


Figure 1.3 The mechanism of insulin on blood glucose level control (http://www.namrata.co/insulin-biosynthesis-secretion-and-action/)

Diabetes occurs when there are insulin problems. When the digestive system breaks down dietary carbohydrate to produce glucose and the body's blood glucose level rises while the body fails to use the insulin effectively because the cells do not respond to the presence of insulin. As a result, more insulin is required to decrease the blood glucose level, causing the pancreas to work harder to maintain with the demand. Eventually, the pancreas cannot keep up with the demands and begins to fail known as insulin resistance, causing the blood glucose level to rise exponentially and become to type 2 diabetic mellitus (Figure 1.4).

The symptoms and complications of diabetic mellitus usually appear more gradually, which include polyuria, polydipsia and weight loss. High blood sugar levels contribute to microorganism (bacteria and yeast) infections process. Diabetes patient are especially prone to foot infections, yeast infections, urinary tract infections and surgical site infections. Foot infections are the most common problems in persons

with diabetes. In addition, yeast cells (*Candida albicans*) that occur naturally in the mucous membranes (such as mouth, vagina and nose) can simply enter the blood system. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. People with diabetes are often found hypertension and abnormalities of lipoprotein metabolism.

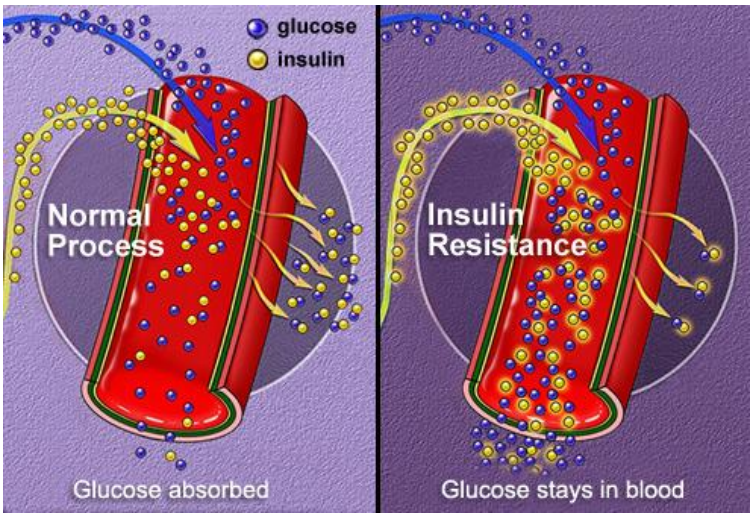


Figure 1.4 Pathogenesis of insulin resistance in type 2 diabetes (http://wp.stockton.edu/gfb1/tag/type-2-diabetes/)

1.2 Diagnostic criteria for diabetes mellitus

The diagnostic criteria for diabetes mellitus have been greatly simplified (Table 1.1). A normal fasting plasma glucose level (FPG) is less than 100 mg/dL and normal 2-hour oral glucose tolerance test (OGTT) levels are less than 140 mg/dL. Blood glucose levels above the normal level but below the criterion established for diabetes mellitus indicate impaired glucose homeostasis or pre-diabetes. Persons with fasting plasma glucose levels ranging from 100 to 126 mg/dL are said to have impaired fasting glucose, while those with a 2-hour oral glucose tolerance test (OGTT) level between 140 mg/dL and 200 mg/dL are said to have impaired glucose tolerance.

Both impaired fasting glucose and impaired glucose tolerance are associated with an increased risk of developing type 2 diabetes mellitus. Lifestyle changes, such as weight loss and exercise, are treated in pre-diabetic patients. Persons with fasting plasma glucose levels more than 126 mg/dl and a 2-hour oral glucose tolerance test (OGTT) level more than 200 mg/dL are said to have diabetes mellitus.

Table 1.1 Criteria for the diagnosis of diabetes mellitus

Stage	Test	
	Fasting Plasma Glucose (FPG)	2-Hour Oral Glucose Tolerance Test (OGTT)
Diabetes	≥126 mg/dl	≥200 mg/dl
Pre-diabetes	≥100 and <126 mg/dl	≥140 and <200 mg/dl
Normal	<100 mg/dl	<140 mg/dl

1.3 Oral antidiabetic drugs

Type 2 diabetes mellitus is a complex disorder that is difficult to treat effectively in the long term. People with diabetes are overweight or obese at diagnosis and will be unable to achieve or sustain near normoglycaemia without oral antidiabetic agents; a sizeable proportion of patients will eventually require insulin therapy to maintain long-term glycaemic control, either as monotherapy or in conjunction with oral antidiabetic therapy. Today's clinicians are usually presented oral antidiabetic drugs for type 2 diabetes. These diabetes medicines are usually prescribed to people with type 2 diabetes along with recommendations for making specific dietary changes and getting regular exercise.

The aim of oral therapy in type 2 diabetes is to reach normoglycemia to prevent later complications. The main classes of oral antidiabetic drugs include agents that stimulate insulin secretion (sulphonylureas and rapid-acting secretagogues),

reduce hepatic glucose production (biguanides), improve insulin action (thiazolidinediones) and delay digestion and absorption of intestinal carbohydrate (α-glucosidase inhibitors). The major effects of oral antidiabetic agents are rapidly blood glucose-lowering, α-glucosidase inhibitors being rather less effective. For person with diabetes, α -glucosidase inhibitors are safety agents since they can be used either as monotherapy or in combination with other oral hypoglycemic agents or insulin. The α-glucosidase inhibitors for treating diabetes are to suppress the post-prandial hyperglycemia. This can be performed by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolysing enzymes α-glucosidase (sucrase, maltase and isomaltase) in the small intestine (Figure 1.5). Inhibitors of intestinal α-glucosidase enzymes retard the rate of carbohydrate digestion, thereby providing an alternative means to suppress postprandial hyperglycaemia.

The α-glucosidase inhibitors bind to the active site of α-glucosidase enzymes in the brush border of enterocytes lining the intestinal villi. The α-glucosidase inhibitors will bind to α-glucosidase enzymes to prevent the cleavage of disaccharide and oligosaccharide substrates into monomeric glucose prior to absorption. The theoretical result of taking an α-glucosidase dose with a meal is therefore to delay, but not stop, carbohydrate absorption. The inhibition of these enzymes thus reduces the rate of formation of "absorbable sugars" and thus delays the rise in blood glucose concentration following meals (postprandial).

Currently, the α-glucosidase inhibitors such as acarbose (Precose® or Glucobay®), miglitol (Glyset®) and voglibose (Basen®) are known to reduce postprandial hyperglycemia primarily by interfering with the carbohydrate digestive enzymes and by delaying glucose absorption (Hsieh, 2009). The most common problems with α-glucosidase inhibitors are gastrointestinal adverse effects. If the dosage is too high, undigested oligosaccharides pass into the large bowel and fermented by the flora cause flatulence, abdominal discomfort and sometimes diarrhea.

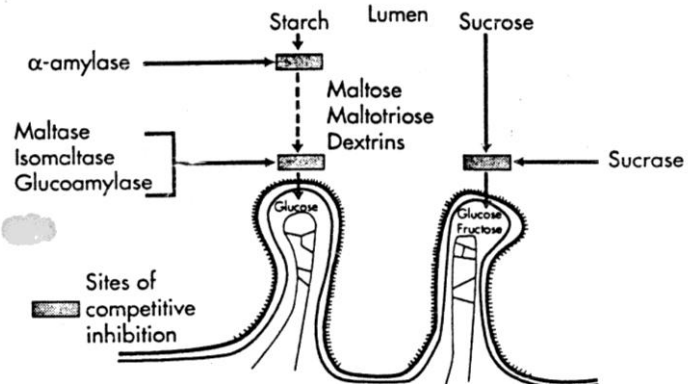


Figure 1.5 In normal digestion, oligosaccharides hydrolyzed by α-glucosidase located in the intestinal brush border to monosaccharide, which are then absorbed.

1.5 Antidiabetes drugs from medicinal plants

Current scientific evidence demonstrates that much of the morbidity and mortality of diabetes can be eliminated by aggressive treatment with diet, exercise, and new pharmacological approaches to achieve better control of blood glucose level. Furthermore, the possibility of preventing the onset of diabetes using dietary supplements and/or herbal medicines has attracted increasing attention. Herbal medicines are prescribed widely because of their effectiveness, fewer side effect and relatively low cost. To this end, research has begun to embrace traditional medicines from various cultures, as scientists search for clues to discover new therapeutic drugs for diabetes (Li, 2004). Traditional Indian and Chinese medicine have long used plant and herbal extracts as anti-diabetic agents (Chen, 2001; Grover, 2002). Therefore, investigation on such agents from traditional medicinal plants has become more important and researches are competing to find the new effective and safe therapeutic agent for the treatment of diabetes.

In 1976 nojirimycin (Figure 1.6) was discovered as the 1-deoxynojirimycin (DNJ) was later isolated from the roots of mulberry trees and called molanoline (Yagi, 1976). DNJ is also produced by many strains in Bacillus and Streptomyces, which shown to be a potent inhibitor of both α- and β-glucosidases (Schmidt, 1979). Despite the excellent α-glucosidase inhibitory activity in vitro, its efficacy in vivo was only

moderate Therefore, a large number of DNJ derivatives were prepared in the hope of increasing the in vivo activity. The *N*-alkyl derivatives were most effective and this led to the development of *N*-hydroxyethyl deoxynojirimycin (known as Miglitol or Glyset®) as an oral treatment of the type 2 diabetes with fewer gastrointestinal side effects (Melo, 2006).

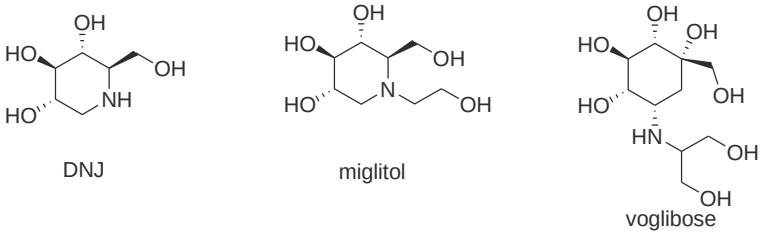


Figure 1.6 structures of 1-deoxynojirimycin (DNJ), miglitol and voglibose

Volglibose (Basen®), an N-substituted derivative of valiolamine isolated from the fermentation broth of *Streptomyces hygroscopicus subsp. limoneus*, is a potent and structurally novel inhibitor of the intestinal disaccharidases (Luo, 2001). Voglibose can be regarded as derivative of 1-deoxynojirimycin (DNJ), which also has a potent inhibitory activity against sucrase and maltase. It has been employed in Japan for the treatment of diabetes since 1994. Volglibose was studies based on α-glucosidase inhibitory activity; it was shown to be 20 to 30 times more potent than acarbose, thus increasing glucose tolerance by inhibiting its digestion and absorption in the small intestine, especially after meals (Yasuda, 2003). The most common adverse effect of voglibose is hepatotoxicity and gastrointestinal disturbance induced by fermentation of unabsorbed carbohydrate in the bowel and increments of gastrointestinal motility (Nakamura, 1993). Additionally, the use of volglibose led to less adverse effects including flatulency and abdominal distention, as shown in a random comparative study (Melo, 2006).

In late 1990, salacinol (Figure 1.7) was isolated from water-soluble extract of the roots and stems *Salacia reticulate* as a potent α-glucosidases inhibitor, which has been traditionally used in India and Sri Lanka for the treatment of diabetes. *Salacia reticulata* Wight, known as kothalahimbutu distributed in Sri Lanka and Indian forests, has been used as a supplementary food in Japan to prevent obesity and

diabetes. The mode of action of salacinol was also proved to be the competitive inhibition against α-glucosidase, and *Ki* values against maltase, sucrase, and isomaltase were revealed as 3.2, 0.84, and 0.59 mg/ml, respectively. The inhibitory activities toward maltase and sucrase are nearly equal to those of acarbose and that toward isomaltase is much more potent than that of acarbose (Yoshikawa 1997). Kotalanol, a derivative of 1,2,3-trihydroxy-propyl-salacinol showed more potent inhibitory activity against sucrase than salacinol and acarbose, which was developed to diabetic drug that used generally in name Diabosol® (Yoshikawa, 1998).

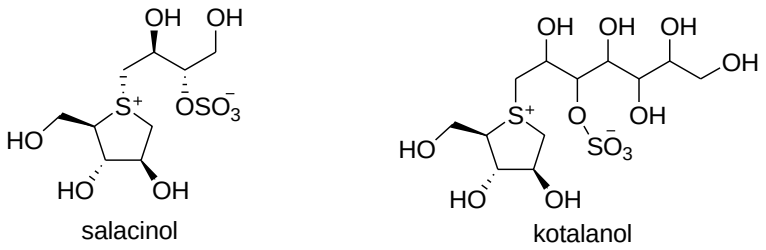


Figure 1.7 structures of salacinol and kotalanol

Since antiquity, diabetes has been treated with herb medicines. In Thailand, approximately 200 plants species are employed as antidiabetes agents. Thai Traditional Medicine was long-term mainstream medical system, which has benefit of the healthcare system. In addition, there are many reports showed increasing use of medicinal plants as antidiabetic drugs, which studied both in subject and animal models. Therefore, Thai medicinal plants have potentially antidiabetic. In this research, an attempt plant was performed, using the following criterion.

1. Reducing blood sugar and no toxic in long-term administration
2. Commercially or readily available and use less time in production
3. No report on the use as α-glucosidase inhibitors

CHAPTER II

α-GLUCOSIDASE INHIBITORS FROM *Moringa oleifera* LEAVES AND SEEDS

2.1. Introduction

2.1.1 Botanical aspect and distribution of *Moringa oleifera*

Moringa oleifera Lam. (Syn. *Moringa pterygosperma* Gaerth) belong to family Moringaceae. It is native to the sub-Himalayan tract of Northern India, which is commonly known as drumstick tree, horseradish tree, benzolive tree, kelor, marango, molnge, moonga, mulangay, saijhan (in India) and in Thai as MaRum. This is rapid growing tree is perennially softwood tree with timber of low quality, but which for centuries has been advocated for traditional, medicinal and industrial uses (Banerji, 2009). The tree ranges in height from 5 to 10 m and sometimes even 15 m. The leaves, flowers, fruits (which are called "pods"), and roots of the tree are used as vegetables and the trunk is used in the paper industry. The pods are usually 25-45 cm long; pods contain ~ 20 seeds, which are globular, ~ 1 cm in diameter. Fully mature, dried seeds (on average weigh ~ 0.3 g) are round or triangular shaped, the kernel being surrounded by a lightly wooded shell with three papery wings (Tsaknis, 1999). The bark is grey and thick and looks like cork, peeling in patches. Roots have the taste of horseradish. Leaves are longitudinally cracked leaves, 30-75 cm long main axis and its branch jointed, glandular at joints, leaflets are glabrous and entire. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with red-tinged mid-veins, with entire (not toothed) margins, and are rounded or blunt-pointed at the apex and short-pointed at the base. The twigs are finely hairy and green (Mishra, 2011).

M. oleifera loses its leaves from December to January and new growth starts in February to March. It produces cream colored flowers when it is 8 months old and the flowering season begins in January and continues through to March. The fruit ripens from April to June (Fahey, 2005).

Figure 2.1 *Moringa oleifera*

2.1.2 Pharmacological and phytochemical investigation of *Moringa oleifera*

M. oleifera is an important food commodity which has enormous attention as the "natural nutrition of the tropics". The leaves, fruit, flowers and immature pods of this tree are used as a highly nutritive vegetable in many countries, particularly in India, Pakistan, Philippines, Hawaii and many parts of Africa (Anwar, 2007). The leaves of this plant are highly nutritious, which contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, more potassium than bananas and more protein than milk and eggs. *M. oleifera* is rich in various phytochemicals like carotenoids, vitamins, minerals, amino acids, sterols, glycosides, alkaloids, flavonoids, moringine, moringinine, phytoestrogens caffeoylquinic acids and phenolics in flowers, leaves, roots, fruits and seeds (Sreelatha, 2011).

Pharmacological properties and medicinal uses, *Moringa* is very important for its medicinal value. Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological, hepatorenal disorders, cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, hepatoprotective, antioxidant, antihypertensive, cholesterol lowering and antidiabetic (Anwar, 2007).

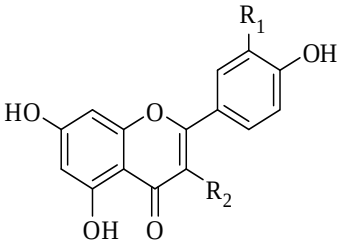
Some researchers reported *M. oliefera* hypoglycemic potential (Kar, 2003). Moreover, anti-diabetic property of *M. oleifera* in animal model was reported.

Francis and coworker investigated insulin secretagogues from *M. oleifera*, in addition to cyclooxygenase enzyme and lipid peroxidation inhibitory activities. They found that phenolic rhamnosides named 1-*O*-phenyl-α-L-rhamnopyranoside and methyl *N*-{4-[(α-L-rhamnopyranosyl)benzyl]}carbamate significantly stimulated insulin release (ca. 30 mg/mg protein) in rat pancreatic β-cells. This study indicated antidiabetic potential of *M. oleifera* as sulfonylurea, an insulin-releasing stimulator (Francis, 2004).

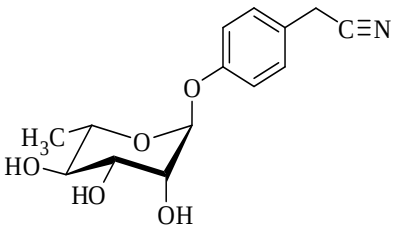
In 2007, Ndong and coworker reported effects of oral administration of *M. oliefera* on glucose tolerance in Wistar rats and Goto-Kakizaki rats, modeled type 2 diabetes. The results indicated that the major polyphenols were quercetin glycosides, rutin, kaempferol glycosides and chlorogenic acid has an ameliorating effect for glucose intolerance (Ndong, 2007).

In 2009, Jaiswal and coworker studied the effect of leave aqueous extract on blood glucose levels of normal and hyperglycemic rats. The extract reduced the blood glucose level in normal rat and normalized high blood glucose levels in sub, mild and severely diabetic rats within 3 h. In addition, it improved glucose tolerance both in normal and hyperglycemic rat more effectively than reference drug Glipizide (Jaiswal, 2009).

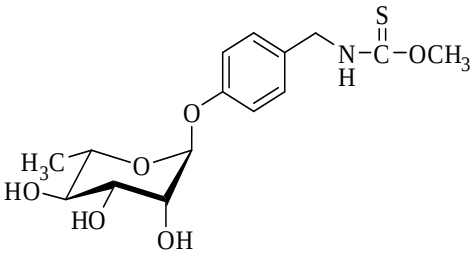
Phytochemistry of leaves, containing two major compounds, which is phenolic glycoside (almost sugar moiety as rhamnose) and flavonoid. The major compounds were found to be flavonoid group such as kaempferol and quercetin (Siddhuraju and Becker, 2003). However, the structure of selected phytochemicals from *M. oleifera* leaves was shown in Figure 2.2 and reviewed by Anwar, 2007; Bennett, 2003 and Sahakitpichan, 2011.



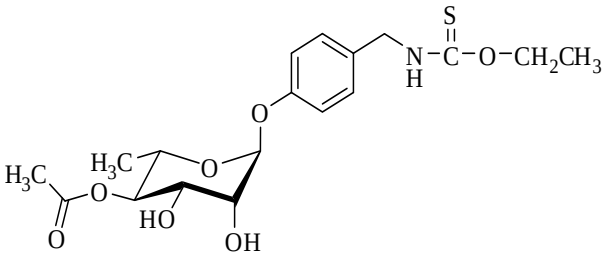
	R1	R2
kaempferol 3-O-glucoside	H	Glc
kaempferol 3-O-(6''-malonylglucoside)	H	Glc-6"-O-CO-CH_2-COOH
quercetin 3-O-glucoside	OH	Glc
quercetin 3-O-(6"-malonylglucoside)	OH	Glc-6"-O-CO-CH_2-COOH
quercetin 3-O-rhamnosylglucoside (Rutin)	OH	Glc-Rham



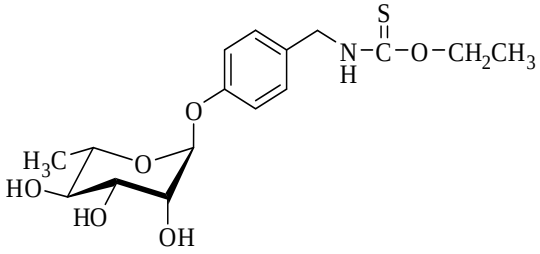
niazirin



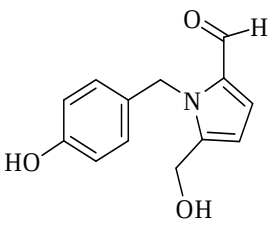
niazinin



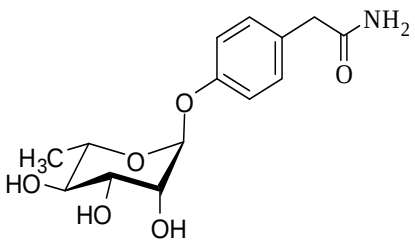
niaziminin



niazimicin



pyrrolemarumine



4'-hydroxyphenylethanamide-α-L-rhamnopyranoside

Figure 2.2 Structures of selected phytochemicals from *Moringa oleifera*

As aforementioned reports, hypoglycemic activity of *M. oleifera* in animal models inspired us to identify the compounds that responsible for above activity. In Thailand, *M. oleifera* leaves were sold either as herbal infusion or as capsule whereas its seeds were consumed fresh or dry. In this research, we designed three different extraction methods to emulate consumption behavior:

1) Leaves were extracted with hot water in order to identify active components possibly found in herbal infusion.

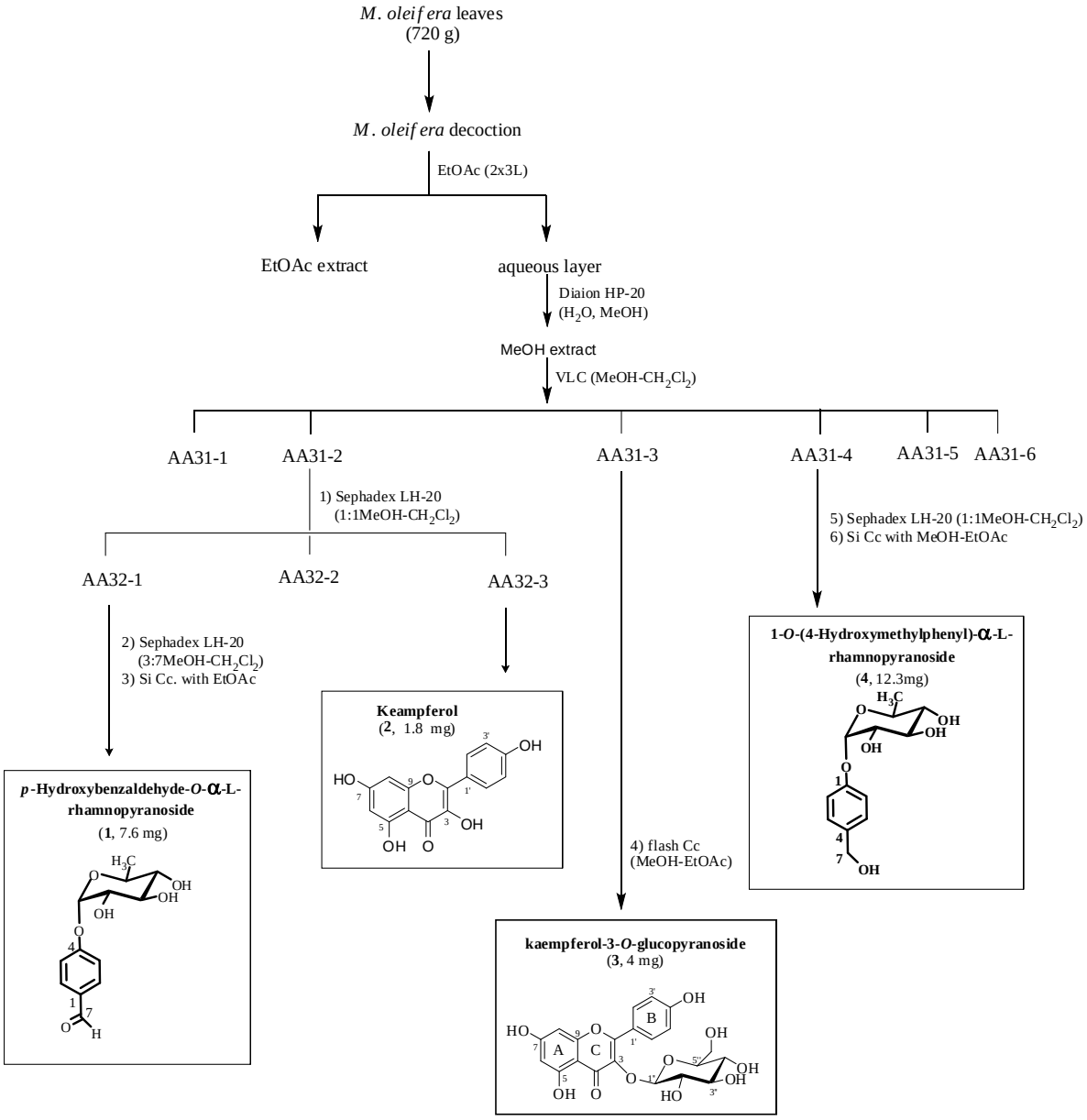
2) Leaves were extracted with methanol in order to identify active components possibly found in herbal capsule.

3) Seeds were extracted with methanol in order to characterize active components found if they were eaten fresh or dry.

2.2 α-Glucosidase inhibitors from aqueous extract *Moringa oleifera* Lam. leaves

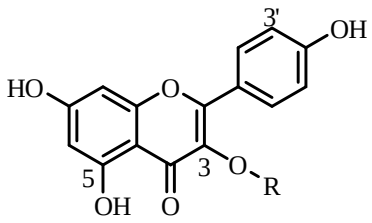
2.2.1 Extraction and isolation

The air-dried leaves of *Moringa oleifera* (720 g) were boiled with H_2O at 80-90 °C for 1 h. The aqueous extract was partitioned with ethyl acetate. The aqueous soluble fraction was subjected to diaion HP-20 column and eluted with H_2O and methanol. The fraction eluted with methanol was subjected to vacuum liquid column chromatography (VLC) over silica gel, which eluted using gradient solvent system methanol/dichloromethane, to provide six fractions (AA31-1 to AA31-6). Fraction AA31-2 was applied over sephadex LH20 to yield three subfractions. Subfraction AA32-1 was purified by sephadex LH-20 column, after which adsorbed onto silica gel to afford *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**). Subfraction AA32-3 afforded keampferol (**2**). Fraction AA31-3 was purified by flash column chromatography using MeOH-EtOAc to afford flavoniod glycosides named kaempferol-3-*O*-β-glucopyranoside (**3**). Fraction AA32-4 was purified by sephadex LH-20 column, after which adsorbed onto silica gel, to afford 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**).

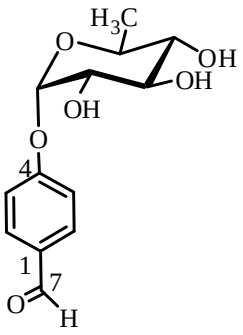


Scheme 2.1 Isolation procedure of aqueous extract *Moringa oleifera* leaves.

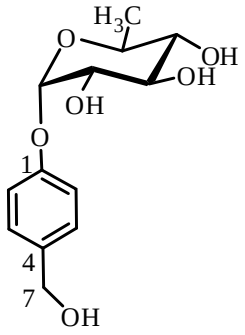
2.2.2 Structure elucidation of isolated compounds 1-4



2 R = H
3 R = Rhamnose



1



4

Figure 2.3 The chemical structures of compounds **1-4** isolated from aqueous extract *Moringa oleifera* leaves

2.2.2.1 *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (1)

Compound **1** was obtained as yellow oil. The structure was deduced by the results from ^{1}H, ^{13}C and confirmed with 2D NMR spectroscopic methods. The ^{1}H NMR showed characteristic signal of para-disubstituted aromatic ring [δ_H 7.78 (2H, d, *J* = 8.0 Hz, H-2 and H-6) and 7.14 (2H, d, *J* = 8.0 Hz, H-3 and H-5)]. The singlet proton at δ_H 9.79 was deduced as the aldehyde group (H-7). The sugar unit was suggested to be a α-L-rhamnopyranosyl moiety apparently from anomaric and secondary methyl protons at δ_H 5.53 and 1.07 (3H, d, *J* = 4.0 Hz), respectively. The ^{13}C NMR indicated 1, 4-disubstituted benzene at δ_C 156.0 and 124.4. Moreover, NMR data showed that the sugar exist in only one anomeric from as the ^{13}C NMR spectrum

has only one anomeric carbon signal (98.2). Therefore, the structure of compound **1** was therefore identified as *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (Leuck, 1998).

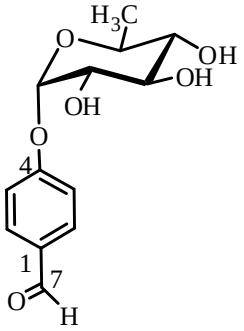


Figure 2.4 *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**).

2.2.2.2 Keampferol (2)

Compound **2** was obtained as a yellow solid. The structure was deduced mainly by the results from ^{1}H NMR. The ^{1}H NMR spectral showed a typical pattern of a kaempferol; two doublet signals at δ_H 8.09 (H-2' and H-6') and 6.90 (H-3' and H-5') with coupling constants of 8.8 Hz. together with two doublet signals of H-6 and H-8 at δ_H 6.18 and 6.40 (J = 2.0 Hz). Therefore, the structure of compound **2** was deduced as kaempferol (Lin, 2009).

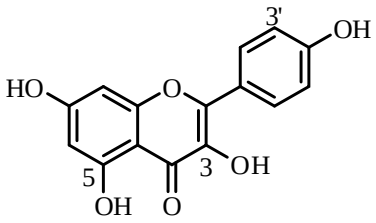


Figure 2.5 keampferol (**2**)

2.2.2.3 Keampferol-3-*O*-β-glucopyranoside (3)

Compound **3** was obtained as a yellow solid. The structure was deduced mainly by the results from ^{1}H NMR. The ^{1}H spectrum of compound **3** showed characteristic signals of kaempferol and sugar moiety. The aglycone moiety was identified as kaempferol from two methine signals at δ_H 6.12 and 6.32 (J = 2.0 Hz), which were assigned to H-6 and H-8 for aromatic ring A, and two doublets for aromatic ring B at δ_H 7.96 (H-2' and H-6') and 6.79 (H-3' and H-5'). These proton signals displayed two pairs of the ortho couple doublet with J = 8.8 Hz. The ^{1}H NMR spectra of compound **3** showed signals similar to those of compound **2**, except the presence of anomaric proton at δ_H 5.17 and other oxygenated OH, indicating that compound **3** comprised the one glucose unit. The identity of **3** was eventually confirmed by co-TLC with authentic sample of kaempferol-3-*O*-β-glucopyranoside deposited in our laboratory. Therefore, the structure of compound **3** was identified as kaempferol-3-*O*-β-glucopyranoside (Lin, 2009).

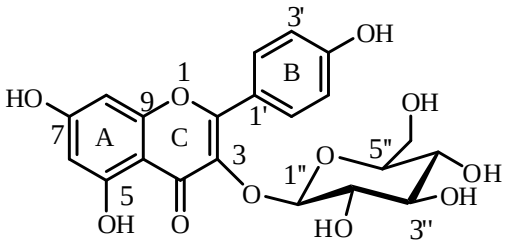


Figure 2.6 kaempferol-3-*O*-β-glucopyranoside (**3)**

2.2.2.4 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (4)

Compound **4** was obtained as yellow oil. The structure was deduced by the results from ^{1}H, ^{13}C and confirmed with 2D NMR spectroscopic methods. The ^{1}H NMR showed characteristic signal of para-disubstituted aromatic ring [δ_H 7.16 (2H, d, J = 8.0 Hz, H-2 and H-6), 6.90 (2H, d, J = 8.0 Hz, H-3 and H-5)]. The sugar unit was suggested to be an α-L-rhamnopyranosyl moiety apparently from anomaric protons at

δ_H 5.53 (1H, s). The ^{13}C NMR indicated 1, 4-disubstituted benzene at δ_C 156.5 and 136.9. In addition, ^{13}C NMR data showed characteristic signal of only one anomeric carbon at δ_C 99.5. Therefore, the structure of compound **4** was identified 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (Grond, 2002).

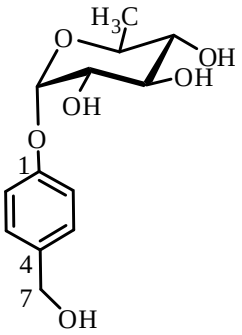


Figure 2.7 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**)

2.2.3 Experiment section

2.2.3.1 General experiment procedures

The ^1H and ^{13}C NMR spectra (in $CDCl_3$, CD_3OD and acetone-d_6) were recorded with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal standard in some cases. ESIMS was obtained from Mass Spectrometer Model VG TRIO 2000. Sephadex LH-20 and silica gel 60 Merck cat. No. 7734 and 7729 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.2.3.2 Plant material

The leaves of *M. oleifera* (voucher's specimen number: BCU 013507) were collected in Lampang, Thailand in January 2010.

2.2.3.3 Extraction and isolation

The air-dried leaves of *Moringa oleifera* (720 g) were boiled with H_2O at 80-90 °C for 1 h and cooled down to room temperature. The aqueous extract was partitioned with ethyl acetate (2×3L). The aqueous soluble fraction was subjected to diaion HP-20 column and eluted with H_2O and methanol. The fraction eluted with methanol was further purified by chromatographic techniques. The fraction eluted with methanol was subjected to vacuum liquid column chromatography (VLC) over silica gel, which eluted into 500 ml fractions using solvent systems of dichloromethane, methanol/dichloromethane (10:90), methanol/dichloromethane (20:80), methanol/ dichloromethane (40:60) and methanol, respectively, to provide six fractions (AA31-1 to AA31-6). Fraction AA31-2 was applied over sephadex LH20 using 1:1 methanol/dichloromethane to yield three subfractions. Subfraction AA32-1 was purified by sephadex LH-20 column using 3:7 MeOH-CH_2Cl_2, after which adsorbed onto silica gel and eluted with EtOAc to afford *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**, 7.6 mg). Subfraction AA32-3 afforded keampferol (**2**, 1.8 mg). Fraction AA31-3 was purified by flash column chromatography using 1:9 MeOH-EtOAc to afford flavoniod glycosides named kaempferol-3-*O*-β-glucopyranoside (**3**, 4 mg). Fraction AA32-4 was purified by sephadex LH-20 column using 1:1 MeOH-CH_2Cl_2, after which adsorbed onto silica gel and eluted with 1:9 MeOH-CH_2Cl_2, to afford 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**,7.1 mg).

***p*-Hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (1)** ^{1}H NMR (400 MHz, acetone-d_6) δ 9.79 (1H, s, H-7), 7.78 (2H, d, *J* = 8.0 Hz, H-2 and H-6), 7.14 (2H, d, *J* = 8.0 Hz, H-3 and H-5), 5.53 (1H, s, H-1′), 3.64-3.12 (sugar moiety); ^{13}C NMR (100 MHz, acetone-d_6) δ 190.9, 131.6, 129.3, 116.9, 116.6, 98.2, 72.3-70.4 (sugar moiety).

Keampferol (2) as a yellow solid ; ^{1}H NMR (400 MHz, methanol-d_4) δ 8.09 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 6.90 (2H, d, *J* = 8.8 Hz, H-3' and H-5'), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 6.18 (1H, d, *J* = 2.0 Hz, H-6).

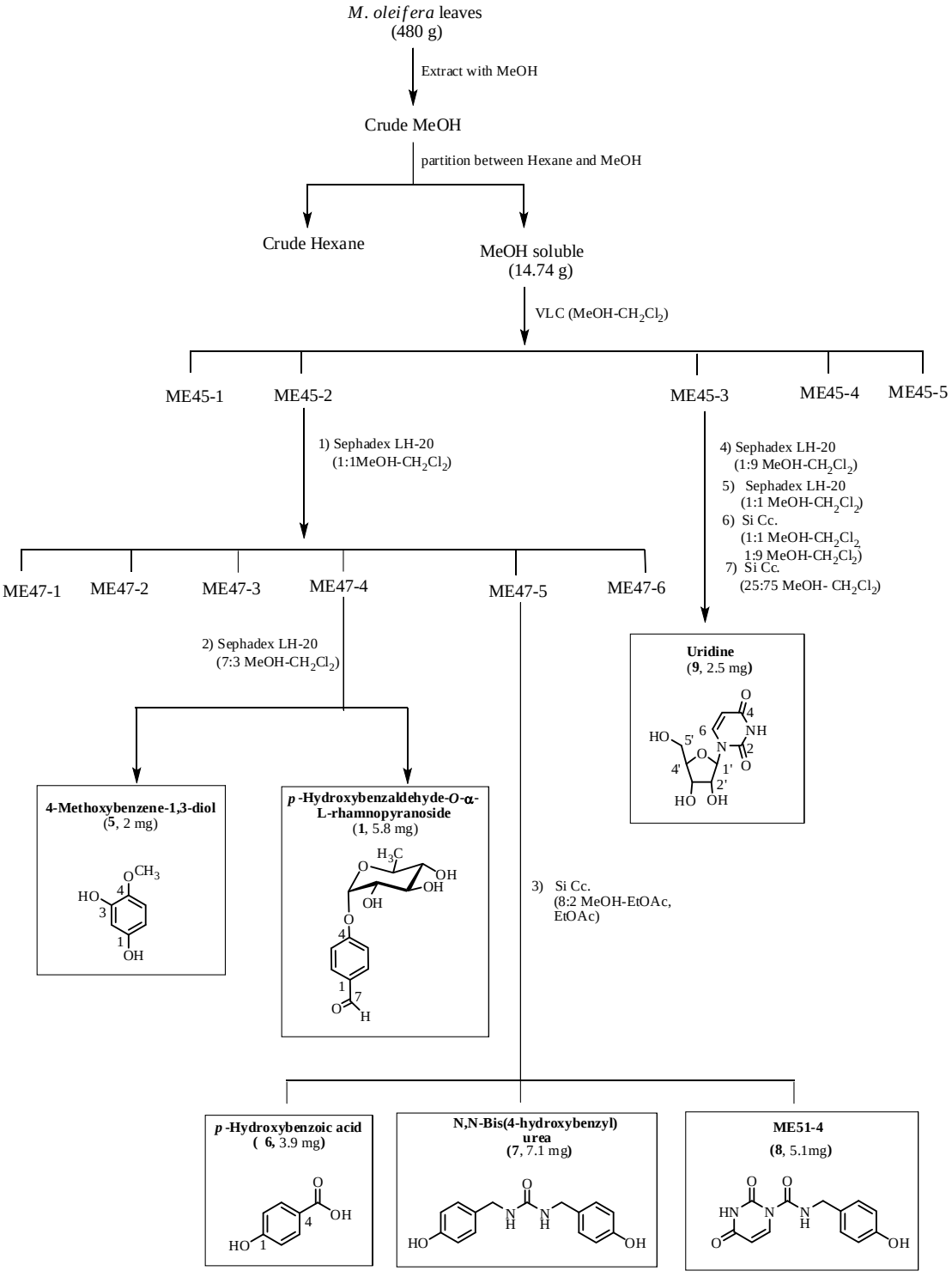
Kaempferol-3-*O*-β-glucopyranoside (**3**) as a yellow solid ; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.96 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 6.79 (2H, d, *J* = 8.8 Hz, H-3' and H-5'), 6.31 (1H, d, *J* = 2.0 Hz, H-8), 6.11 (1H, d, *J* = 2.0 Hz, H-6), 5.17 (1H, d, *J* = 2.0 Hz, H-1"), 3.61-3.10 (sugar moiety).

1-*O*-(4-Hydroxymethylphenyl)-α-L-rhamnopyranoside (4) ^{1}H NMR (400 MHz, acetone-d_6) δ 7.16 (2H, d, *J* = 8.0 Hz, H-2 and H-6), 6.90 (2H, d, *J* = 8.0 Hz, H-3 and H-5), 5.33 (1H, s, H-1′), 4.42-4.40 (2H, m, H-7), 3.90-3.36 (sugar moiety); ^{13}C NMR (100 MHz, acetone-d_6) δ 156.5, 128. 9, 117.2, 136.9, 117.2, 128.9, 64.3, 99.5-18.2 (sugar moiety).

2.3 α-Glucosidase inhibitors from methanolic extract *Moringa oleifera* Lam. leaves

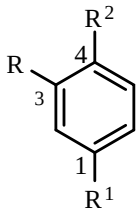
2.3.1 Extraction and isolation

The air dried leaves of *Moringa oleifera* were extracted with MeOH. The MeOH extracts were partitioned between MeOH and hexane. The methanolic layer was separated through vacuum liquid column chromatography (VLC) eluted with a gradient system to obtain five main fractions (ME45-1 to ME45-5). Fraction ME45-2 was purified by Sephadex LH-20 column using 1:9 MeOH-CH_2Cl_2 to obtain six main subfractions (ME47-1 to ME47-6). Subfraction ME47-4 was purified by silica gel column using 7:3 MeOH-CH_2Cl_2 to obtain 4-Methoxybenzene-1,3-diol (**5**) and *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**2**). Subfraction ME47-5 was purified by silica gel column using 8:2 EtOAc/Hexane and EtOAc to obtain *p*-Hydroxybenzoic acid (**6**), *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**) and ME51-4 (**8**). Fraction ME45-3 was purified by Sephadex LH-20 column and silica gel column chromatography, affording uridine (**9**).

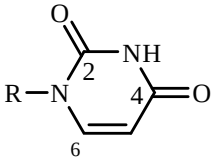


Scheme 2.2 Isolation procedure of methanolic extract from *Moringa oleifera* leaves.

2.3.2 Structure elucidation of isolated compounds 5-9



	R	R^1	R^2
5	—OH	—OH	$-OCH_3$
6	—H	—OH	$-C(=O)-OH$
7	—H	—OH	$-CH_2NH-C(=O)-NH-CH_2-C_6H_4-OH$



	R
8	$-C(=O)-NH-CH_2-C_6H_4-OH$
9	—Ribose

Figure 2.8 The chemical structures of compounds **5-9** isolated from methanolic extract *Moringa oleifera* leaves

2.3.2.1 Aromatic as 5, 6 and 7

Compound 5 4-Methoxybenzene-1,3-diol was obtained as brown oil. The structure was deduced by the results from ^{1}H, ^{13}C and 2D NMR spectroscopic methods. The ^{1}H spectrum of compound **5** showed aromatic signals at δ_H 7.55 (m, 2H), 6.83 (d, J = 8.0 Hz, 1 H), which were assigned to H-2, H-6 and H-5 for aromatic ring, respectively. On the other hand, singlet signals at δ_H 3.89 was characterized to be methoxyl proton. The ^{13}C NMR spectrum exhibited signals for seven carbon atoms of the molecule as three sp^2 CH, three sp^2 quaternary carbons and one methoxyl carbons. A quaternary carbon at δC 152.6, 121.0 and 148.7 was assign as C-1, C-3and C-4, respectively. The HMBC correlations between the methoxyl proton at δ_H 3.89 and C-4 (δ_C 148.7). Therefore, the structure of compound **5** was deduced 4-methoxybenzene-1,3-diol.

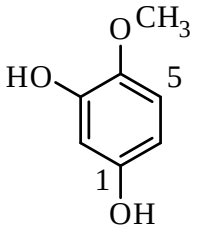


Figure 2.9 4-Methoxybenzene-1,3-diol (**5**)

Compound 6 *p*-hydroxybenzoic acid was obtained as white solid. The structure was deduced by the results from ^{1}H, ^{13}C NMR spectroscopic methods. The ^{1}H NMR spectrum exhibited two signals for *para*-disubstituted aromatic ring of the moleculs. The ^{1}H NMR showed characteristic signal of *para*-disubstituted aromatic ring [δ_H 7.87 (2H, d, J = 8 Hz, H-2 and H-6) and 6.81 (2H, d, J = 8.0 Hz, H-3 and H-5)]. This structure was confirmed by the ^{13}C NMR chemical shifts with signal of carboxyl group at δ_C 170.2. The ^{13}C NMR dedicated 1, 4-disubstituted benzene at δ_C 163.3 and 122.9. Therefore, the structure of compound **6** was identified as *p*-hydroxybenzoic acid (Peungvicha, 1998).

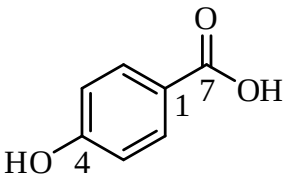


Figure 2.10 *p*-hydroxybenzoic acid **(6)**

Compound 7 ***N,N*-Bis(4-hydroxybenzyl) urea** was obtained as white solid. Compound **7** has a molecular formula of $C_{15}H_{16}N_2O_3$, as deduced from the ESIMS ion at *m*/*z* [M - H]$^-$ 272.12 and from ^{1}H and ^{13}C NMR data analysis. The ^{1}H NMR spectrum exhibited signals for three proton atom of the molecule as signal of para-disubstituted aromatic ring and methylene proton. The ^{1}H NMR showed characteristic signal of para-disubstituted aromatic ring [δ_H 7.09 (4H, d, *J* = 8.0 Hz, H-2 and H-6) and 6.72 (4H, d, *J* = 8.0 Hz, H-3 and H-5)]. The ^{1}H NMR showed characteristic signal of methylene proton at δ_H 4.20 (4H, d, *J* = 8.0 Hz, H-7). The ^{13}C NMR dedicated 1, 4-disubstituted benzene at δ_C 157.5 and 132.0. The ^{13}C NMR showed characteristic signal of methylene and carbonyl carbon at δ_C 44.5 and 161.1, respectively. The mass spectrum data was subsequently confirmed its symmetrical structure. Therefore, the structure of compound **7** was identified as same the structure of *N,N*-Bis(4-hydroxybenzyl) urea (Tomita, 1992)

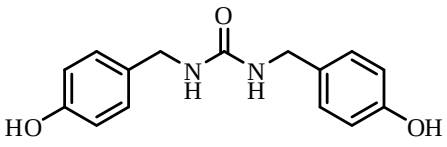


Figure 2.11 *N,N*-Bis(4-hydroxybenzyl) urea (**7**)

2.3.2.2 Uracil 8 and 9

Compound 8 ME51-4 was obtained as white solid. The structure was deduced by the results from ^{1}H, ^{13}C and 2D NMR spectroscopic methods. Interpretation of NMR data resulted in the construction of two separated aromatic systems, which were connected through amide linkage. In the ^{1}H NMR spectrum, the two two-proton mutually coupled doublet at δ_H 7.10 (d, *J* = 8.0 Hz, 2H) and 6.72 (d, *J* = 8.0 Hz, 2H) were assigned to *p*-disubstituted benzene, which was accommodated by hydroxyl group. The singlet signal at δ_H 4.17 was methane protons. In addition, the ^{1}H NMR spectrum showed two one-proton doublets at δ_H 7.39 (d, *J* = 8.0 Hz, 1H) and 5.61 (d, *J* = 8.0 Hz, 1H) due to CH proton of uracil unit. A *p*-disubstituted benzene and uracil were connected through amide moiety (–CONH-) based on HMBC correlation from H-2′ (7.10, d, *J* = 8.0 Hz) to C-7′ and H-7′ (4.17, s) to C-7 (table 2.1). The tentative structure and key HMBC correlations for **8** were shown in Figure 2.12. To our knowledge, the structure of compound **8** has been reported herein for the first time.

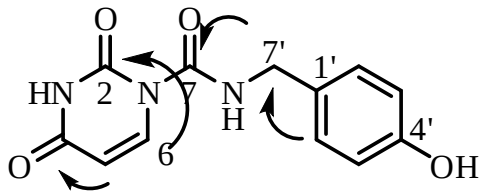


Figure 2.12 Selected HMBC correlations of ME51-4 (**8**)

Table 2.1 ^{1}H, ^{13}C and HMBC NMR data of ME51-4 (**8**) in methanol-d_4

position	δ_C	δ_H (mult, *J* in Hz)	HMBC correlations
1			
2	152.0		
3			
4	167.4		
5	101.8	5.61, d, 8.0	C-4, C-6
6	143.5	7.39, d, 8.0	C-2, C-4, C-5
7	162.0		
1′	132.4		
2′,6′	129.7	7.10, d, 8.0	C-7′, C-3′, C-4′
3′,5′	116.3	6.72, d, 8.0	C-2′, C-4′
4′	157.2		
7′	44.4	4.17, s	C-7,C-1′, C-2′

Compound 9 uridine was obtained as colorless needles. The structure was deduced by the results from ^{1}H, ^{13}C NMR and 2D spectroscopic methods. The ^{1}H NMR dedicated characteristic signal of nitrogen-containing heterocyclic ring as base uracil [δ_H 7.92 (1H, d, *J* = 8 Hz, H-6), (5.61 (1H, d, *J* = 8 Hz, H-5)]. In addition, the sugar moiety, suggest to be a ribose moiety were revealed anomaric protons at δ_H 5.80 (1H, d, *J* = 4 Hz, H-1′). The ^{13}C NMR showed characteristic two carbonyl carbon on base uracil at δ_C 165.1(C-4) and 151.2 (C-2). The carbon chemical shift at δ_C 89.2 (H-1') was assigned as anomaric carbon. Thus, the structure of compound **9** was the same structure of uridine.

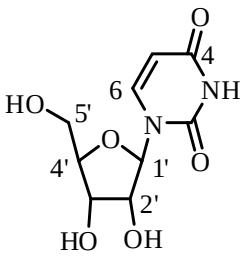


Figure 2.13 Uridine (**9**)

2.3.3 Experiment section

2.3.3.1 General experiment procedures

The ^{1}H and ^{13}C NMR spectra (in $CDCl_3$, CD_3OD and acetone-d_6) were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal standard in some cases. ESIMS was obtained from Mass Spectrometer Model VG TRIO 2000. Sephadex LH-20 and silica gel 60 Merck cat. No. 7734 and 7729 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.3.3.2 Plant material

The leaves of *M. oleifera* (voucher's specimen number: BCU 013507) were collected in Lampang, Thailand in January 2010

2.3.3.3 Extraction and isolation

The air dried leaves of *Moringa oleifera* were extracted with MeOH. The MeOH extracts were partitioned between MeOH and hexane. The methanolic layer was separated through vacuum column chromatography eluted with a gradient system (stepwise 0:100, 5:95, 10:90, 20:80 and 100:0 MeOH-CH_2Cl_2) to obtain five main fractions (ME45-1 to ME45-5). Fraction ME45-2 was purified by Sephadex LH-20 column using 1:9 MeOH-CH_2Cl_2 to obtain six main subfractions (ME47-1 to ME47-6). Subfraction ME47-4 was purified by silica gel column using 7:3 MeOH-CH_2Cl_2 to obtain ME49-1 (**5**, 2 mg) and *p*-Hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**2**, 5.8 mg). Subfraction ME47-5 was purified by silica gel column using 8:2 EtOAc/Hexane and EtOAc to obtain *p*-Hydroxybenzoic acid (**6**, 3.9 mg), *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**, 7.1 mg) and ME51-4 (**8**, 5.1 mg). Fraction ME45-3 was purified by Sephadex LH-20 column and silica gel column chromatography, affording a uridine (**9**, 2.5 mg).

Compound 5 4-Methoxybenzene-1,3-diol as white solid; (400 MHz, methanol-d_4) δ 7.54 (2H, m, H-2 and H-6), 6.83 (1H, d, J = 8 Hz, H-5); ^{13}C NMR (100 MHz, methanol-d_4) δ 152.6 (C-1), 148.7 (C-4) 121.0 (C-3), 125.3 (C-5), 115.9 (C-2), 113.9 (C-6), 56.5 (OCH_3).

Compound 6 *p*-hydroxybenzoic acid as white solid; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.87 (2H, d, J = 8.0 Hz, H-2 and H-6), 6.81 (2H, d, J = 8.0 Hz, H-3 and H-5); ^{13}C NMR (100 MHz, methanol-d_4) δ 170.2 (C-7), 163.3 (C-4), 133.0 (C-2) and (C-6), 122.9 (C-1),116.0 (C-3) and (C-5).

Compound 7 *N,N*-Bis(4-hydroxybenzyl) urea as white solid (400 MHz, methanol-d_4) δ 7.02 (4H, d, J = 8.0 Hz, H-2 and H-6), 6.72 (4H, d, J = 8.0 Hz, H-3 and H-5); ^{13}C NMR (100 MHz, methanol-d_4) δ 161.1(C-7), 157.5 (C-1), 132.0 (C-4), 129.6 (C-3) and (C-5), 116.5 (C-2) and (C-6)**.**

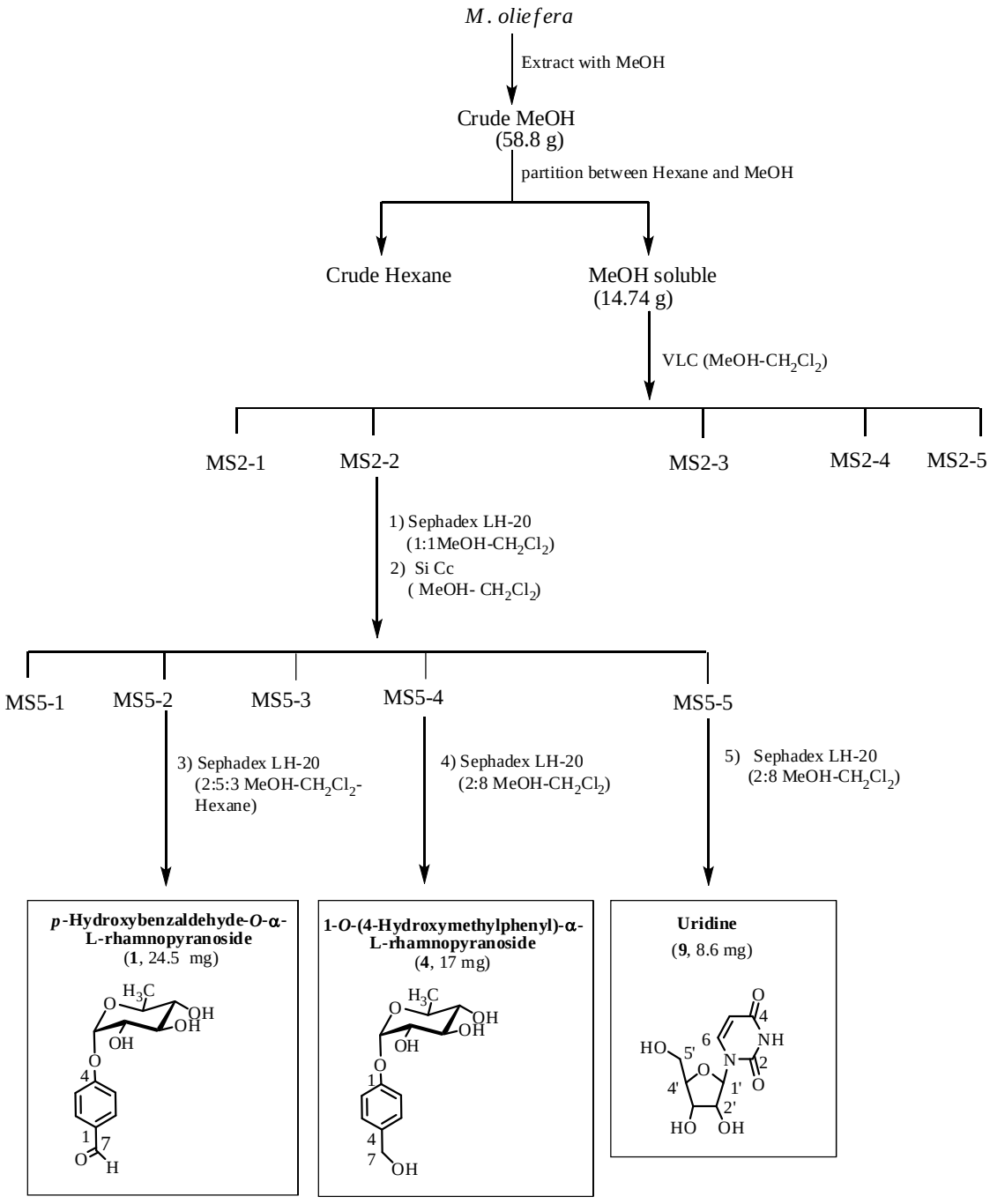
Compound 8 ME51-4 as white solid (400 MHz, methanol-d_4) 7.39 (1H, d, J = 8.0 Hz, H-4), 7.10 (2H, d, J = 8.0 Hz, H-2′ and H-6′), 6.72 (2H, d, J = 8.0 Hz, H-3′ and H-5′), 5.61 (1H, d, J = 8.0 Hz, H-3), 4.17(2H, s, H-7′); ^{13}C NMR (100 MHz, methanol-d_4) 167.4 (C-2), 162.0 (C-5), 157.2 (C-4′), 152.0 (C-1), 143.5 (C-4), 132.4 (C-1′), 129.7 (C-2′and C-6′), 116.3 (C-3′and C-5′), 101.8 (C-3), 44.4 (C-7′).

Compound 9 Uridine as ^{1}H NMR (400 MHz, methanol-d_4) δ 7.92 (1H, d, J = 8.0 Hz, H-6), 5.80 (1H, d, J = 4.0 Hz, H-1′), 5.61 (1H, d, J = 8.0 Hz, H-5), 4.09-3.73 (sugar moiety); ^{13}C NMR (100 MHz, methanol-d_4) δ 165.1 (C-4), 151.2 (C-2), 141.3(C-6), 101.2(C-5), 89.2 (C-1′), 84.9(C-4′), 74.3(C-3′), 69.9(C-2′), 60.8 (C-5′).

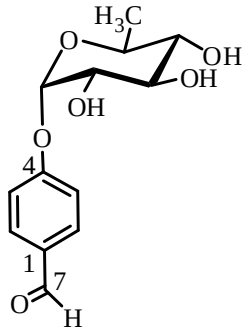
2.4 α-Glucosidase inhibitors from methanolic extract of *Moringa oleifera* seeds.

2.4.1 Extraction and isolation

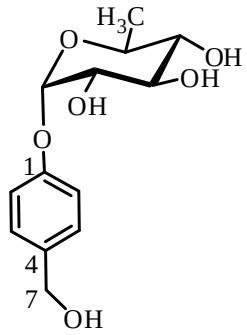
The fully ripe seeds (1 kg) were crushed and extracted with methanol (3×4L). The methanol extract was partitioned with hexane to remove lipid. The methanol extract was subjected to vacuum liquid column chromatography (VLC) over silica gel, eluted with gradient systems of MeOH-CH_2Cl_2, to provide 5 fractions (MS2-1 to MS2-5). Fraction MS2-2 was applied over sephadex LH20 and eluted with methanol, after which adsorbed onto silica gel and eluted with MeOH-CH_2Cl_2 system to afford 5 subfractions (MS5-1 to MS5-5). The subfraction MS5-2 was applied over sephadex LH20, and eluted with MeOH-CH_2Cl_2-hexane 2:5:3 to give a *p*-hydroxy benzaldehyde-*O*-α-L-rhamnopyranoside (**1**). The subfraction MS5-4 was applied over sephadex LH20, and eluted with MeOH-CH_2Cl_2 (20:80) to give a 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**). The subfraction MS5-5 was applied over sephadex LH20, eluting with MeOH-CH_2Cl_2 (20:80) to give uridine (**9**).



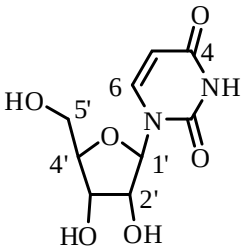
Scheme 2.3 Isolation procedure of methanolic extract from *Moringa oleifera* seeds



1



4



9

Figure 2.14 The chemical structures of compounds **1**, **4** and **9** isolated from methanolic extract *Moringa oleifera* seeds

2.4.2 Structure elucidation of isolated compounds 1, 4 and 9

Structure elucidation of isolated compounds **1**, **4** and **9** was performed the same as previously described.

2.4.3 Experiment section

2.4.3.1 General experiment procedures

The ^{1}H and ^{13}C NMR spectra (in $CDCl_3$, CD_3OD and acetone-d_6) were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal

standard in some cases. ESIMS was obtained from Mass Spectrometer Model VG TRIO 2000. Sephadex LH-20 and silica gel 60 Merck cat. No. 7734 and 7729 were used for open column chromatography. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer).

2.4.3.2 Plant material

The seeds of *M. oleifera* (voucher's specimen number: BCU 013507) were collected in Suphanburi, Thailand in April 2011

2.4.3.3 Extraction and isolation

The fully ripe seeds (1 kg) were crushed and extracted with methanol (3×4L). The methanol extract was partitioned with hexane to remove lipid. The methanol extract was subjected to column chromatography over silica gel, which eluted into 500 ml fractions using solvent systems of CH_2Cl_2, MeOH-CH_2Cl_2 (10:90), MeOH-CH_2Cl_2 (20:80), MeOH-CH_2Cl_2 (40:60) and MeOH, respectively, to provide 5 fractions (MS2-1 to MS2-5). Fraction MS2-2 was applied over sephadex LH20 and eluted with methanol, after which adsorbed onto silica gel and eluted with MeOH-CH_2Cl_2 system to afford 5 subfractions (MS5-1 to MS5-5). The subfraction MS5-2 was applied over sephadex LH20, and eluted with MeOH-CH_2Cl_2-hexane 2:5:3 to give a *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**, 24.5 mg). The subfraction MS5-4 was applied over sephadex LH20, and eluted with MeOH-CH_2Cl_2 (20:80) to give a 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**, 17 mg). The subfraction MS5-5 was applied over sephadex LH20, eluting with MeOH-CH_2Cl_2 (20:80) to give uridine (**9**, 8.6 mg).

Supporting information

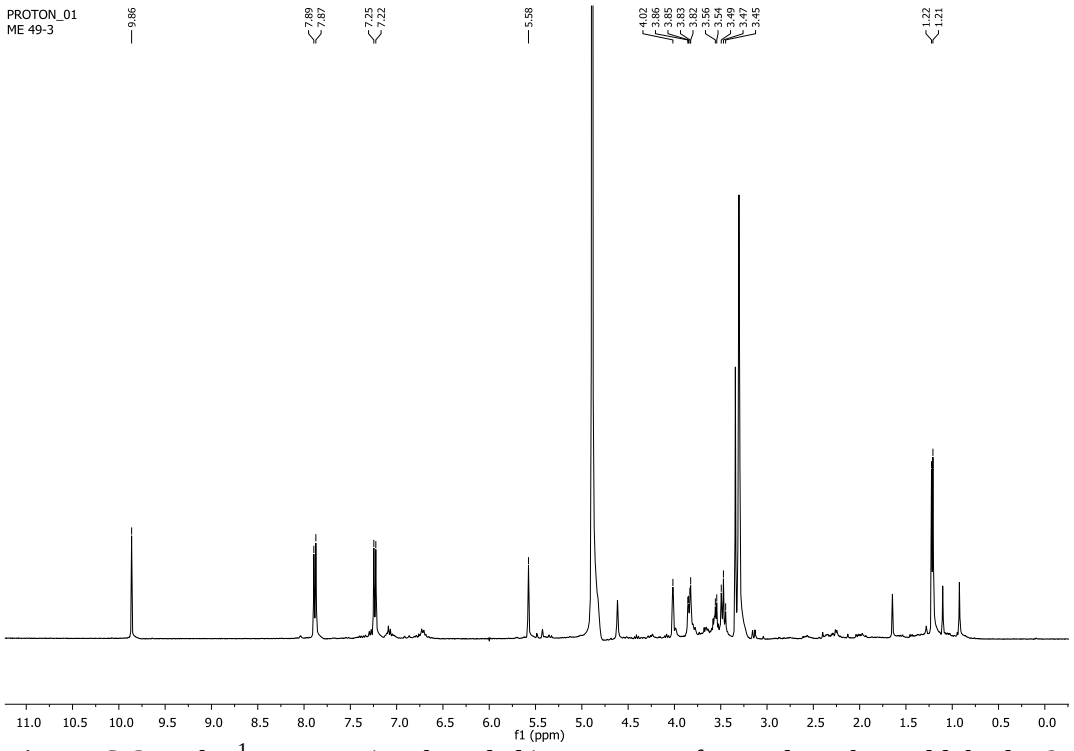


Figure S-2.1 The ^{1}H NMR (methanol-d_4) spectrum of *p*-Hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**).

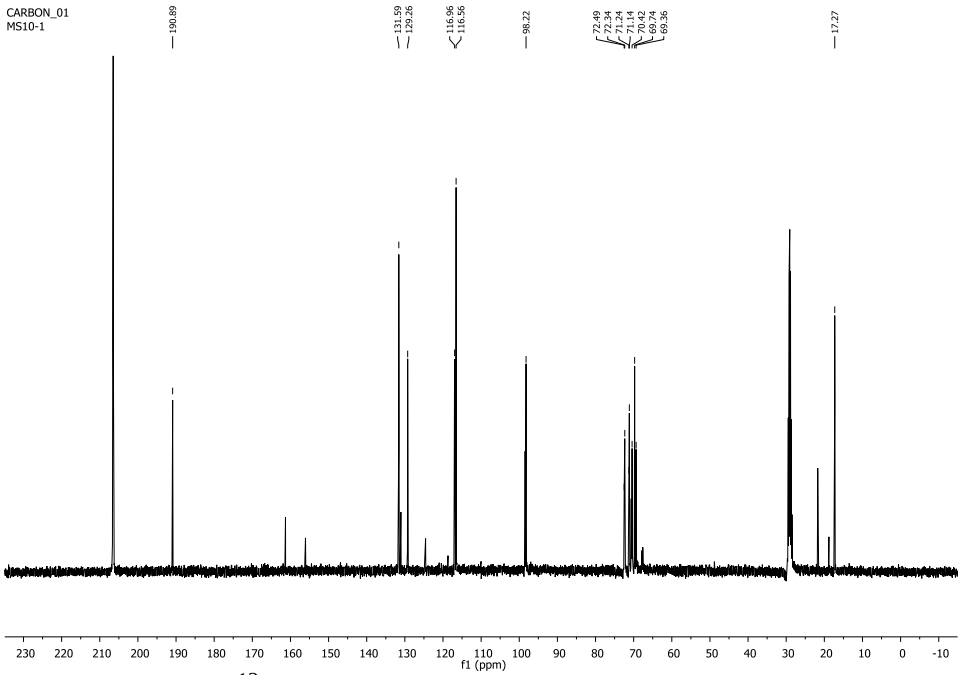


Figure S-2.2 The ^{13}C NMR (acetone-d_6) spectrum of *p*-Hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**).

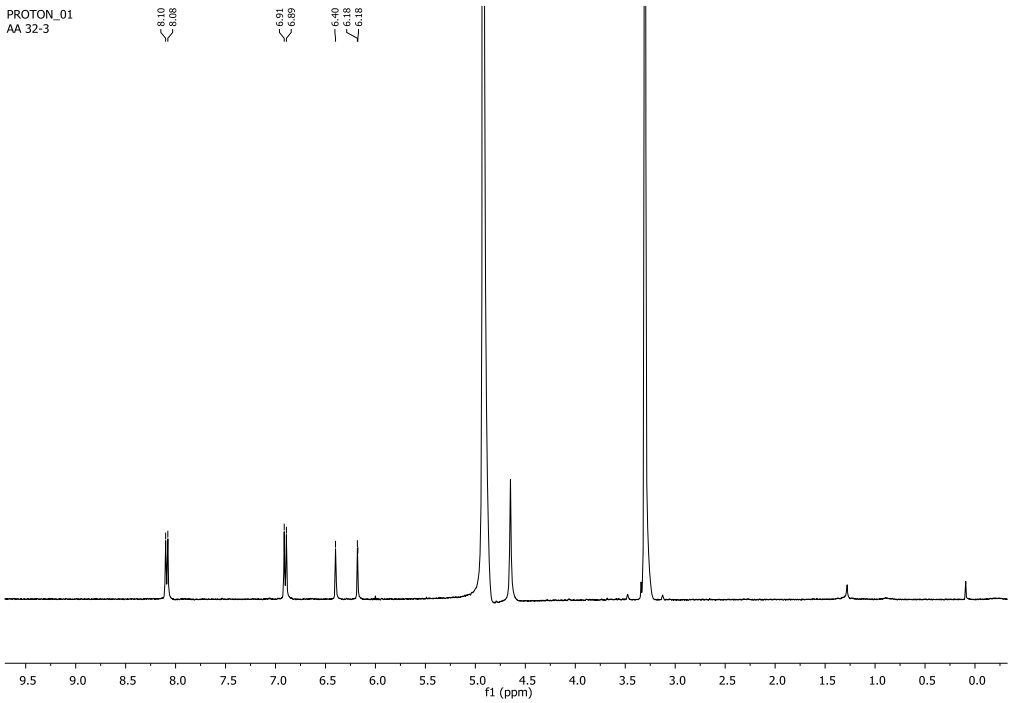


Figure S-2.3 The ^{1}H NMR (methanol-d_4) spectrum of Keampferol (**2**).

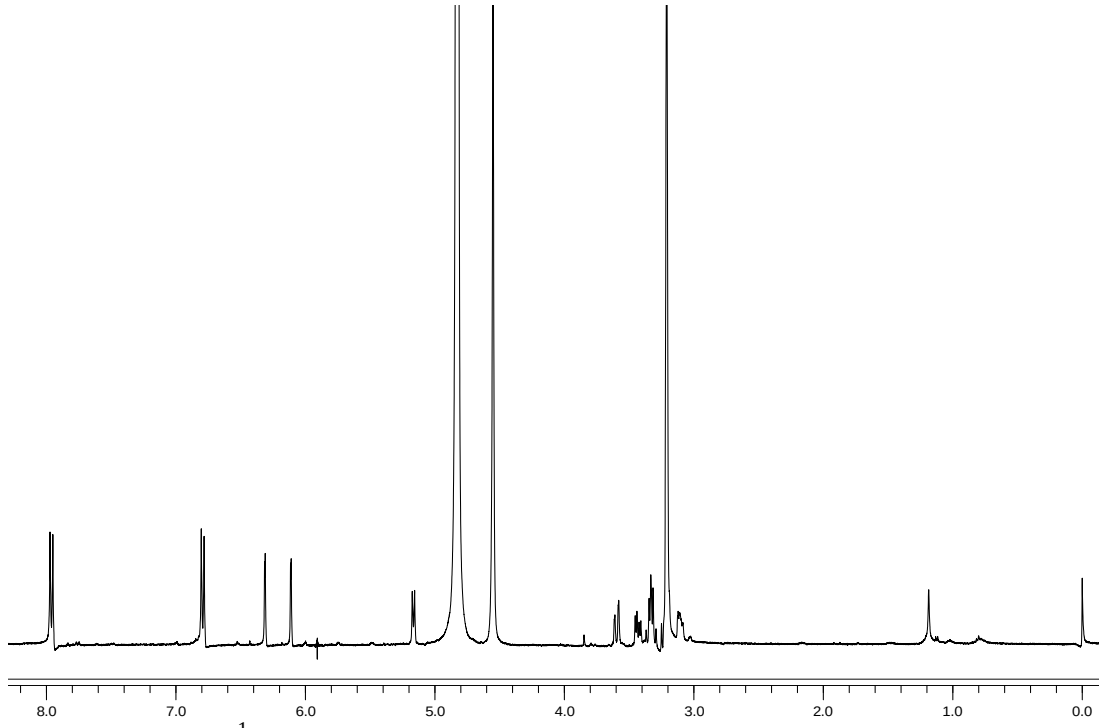


Figure S-2.4 The ^{1}H NMR (methanol-d_4) spectrum of Kaempferol-3-*O*-β-glucopyranoside (**3**).

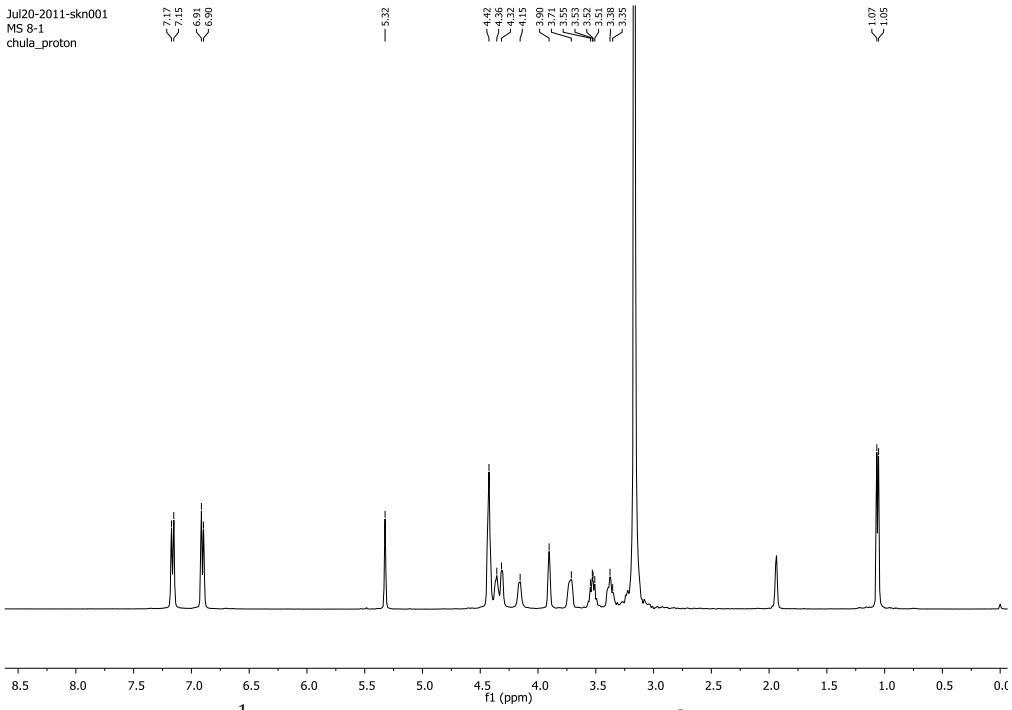


Figure S-2.5 The ^{1}H NMR (acetone-d_6) spectrum of 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**).

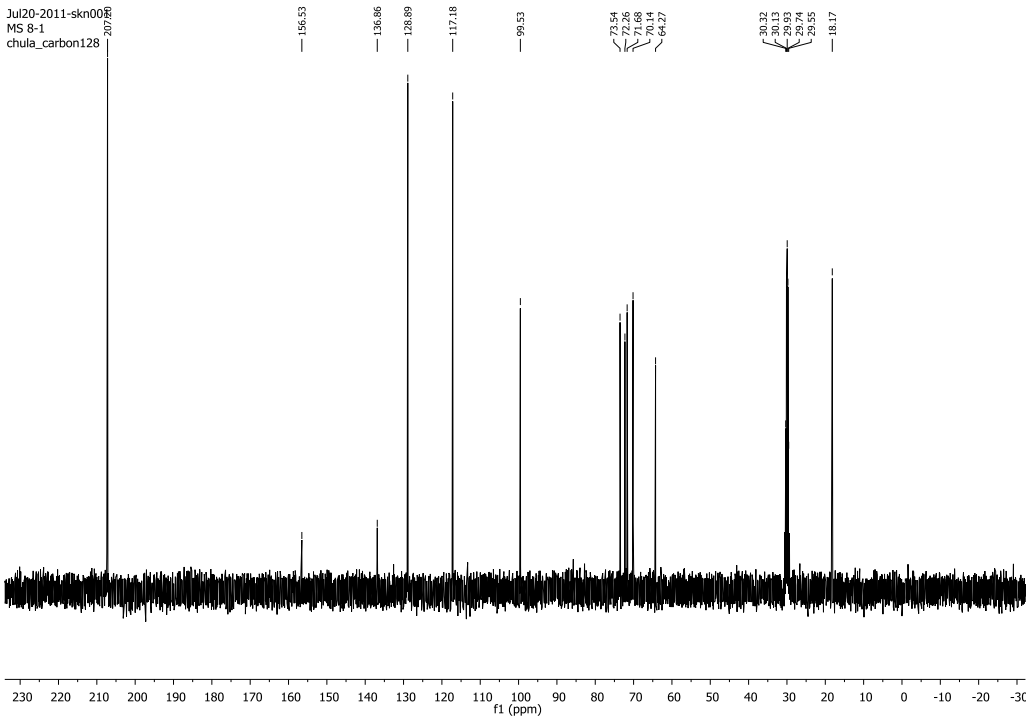


Figure S-2.6 The ^{13}C NMR (acetone-d_6) spectrum of 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**).

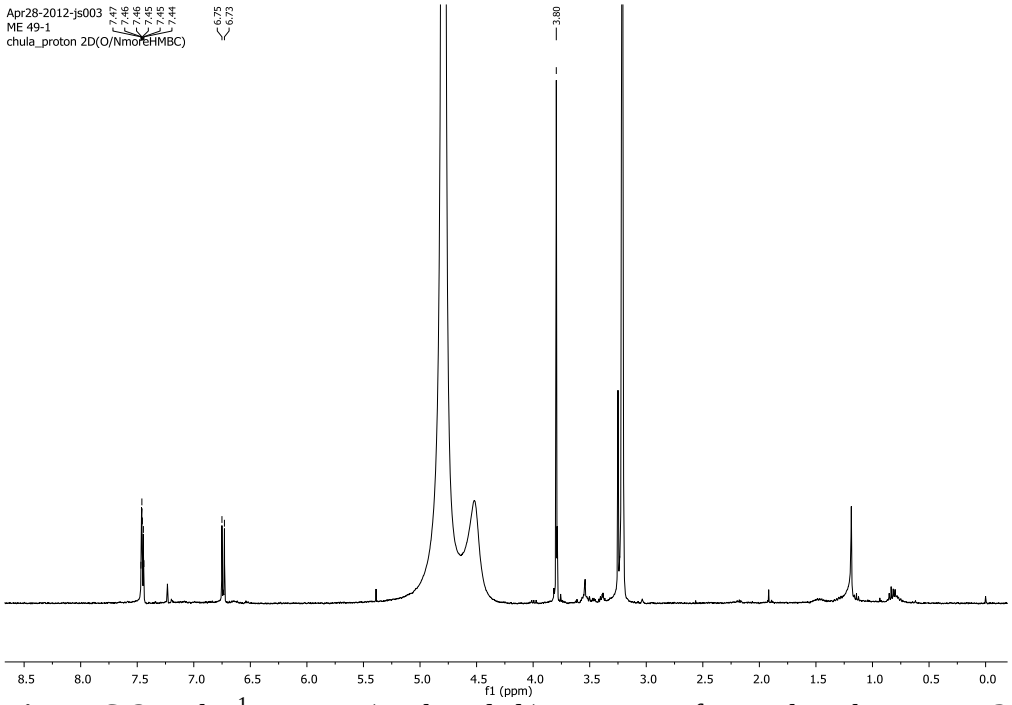


Figure S-2.5 The ^{1}H NMR (methanol-d_4) spectrum of 4-Methoxybenzene-1,3-diol (**5**).

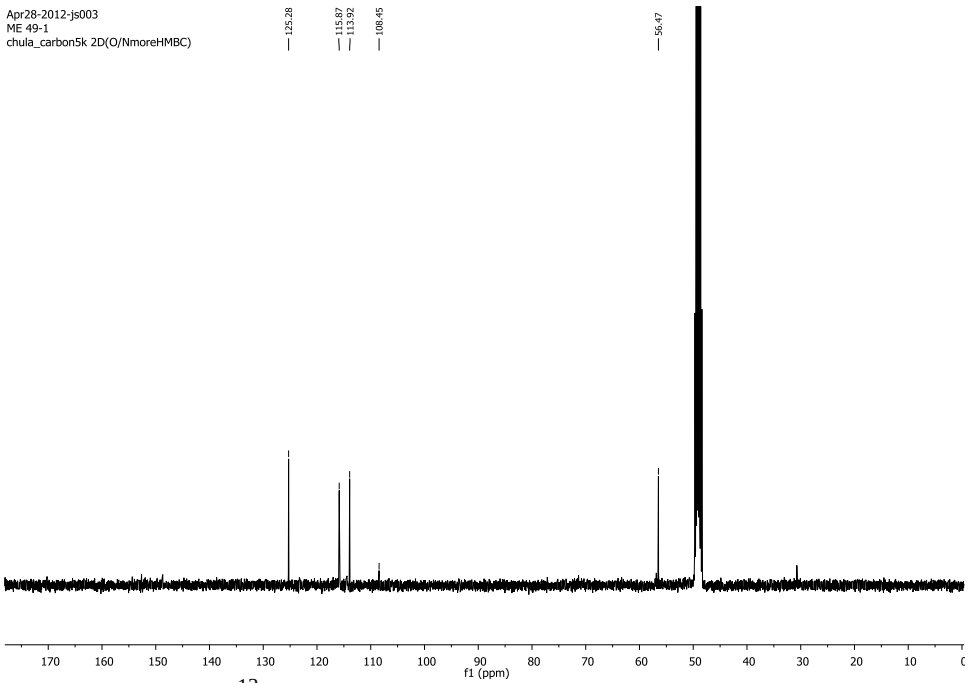


Figure S-2.6 The ^{13}C NMR methanol-d_4) spectrum of 4-Methoxybenzene-1,3-diol (**5**).

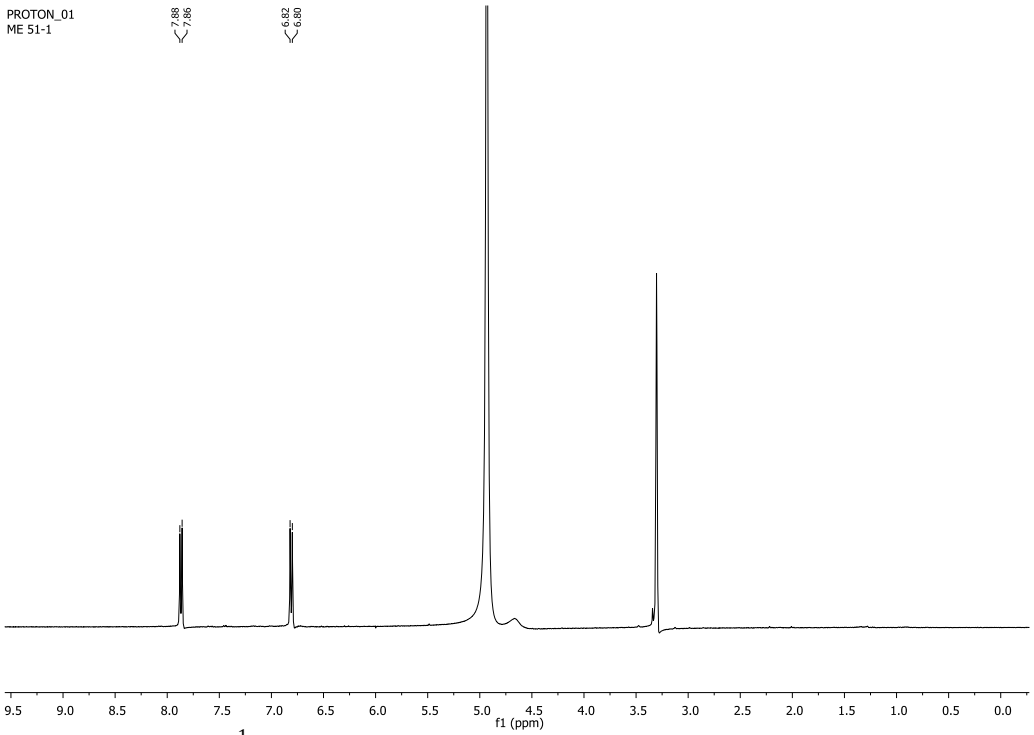


Figure S-2.7 The ^{1}H NMR (methanol-d_4) spectrum of *p*-hydroxybenzoic acid (**6**).

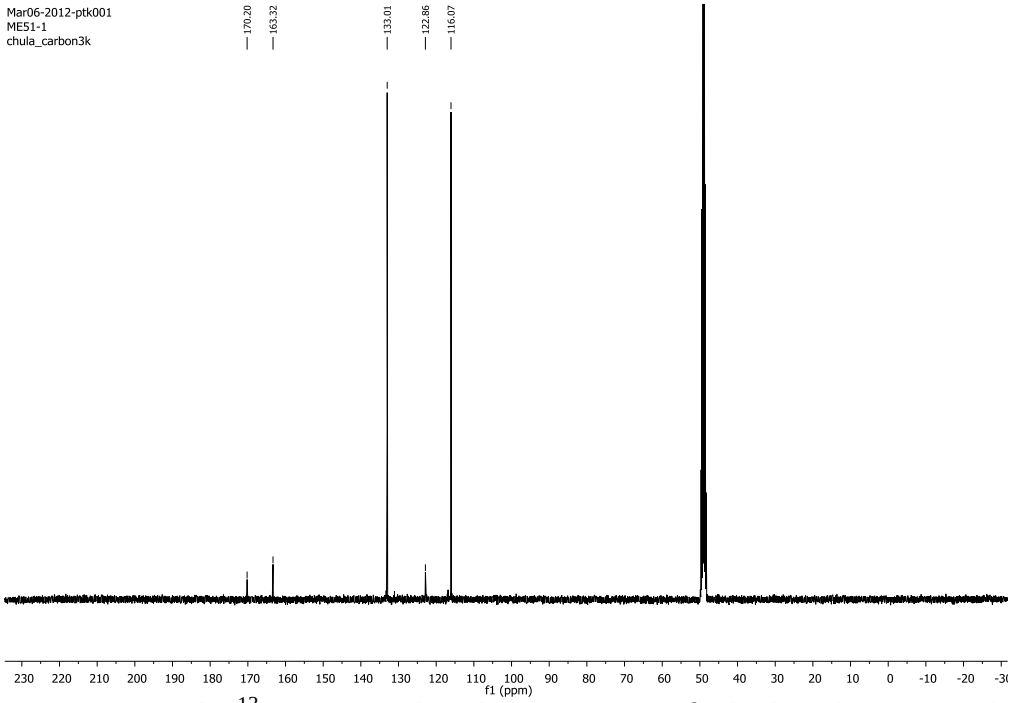


Figure S-2.8 The ^{13}C NMR methanol-d_4) spectrum of *p*-hydroxybenzoic acid (**6**).

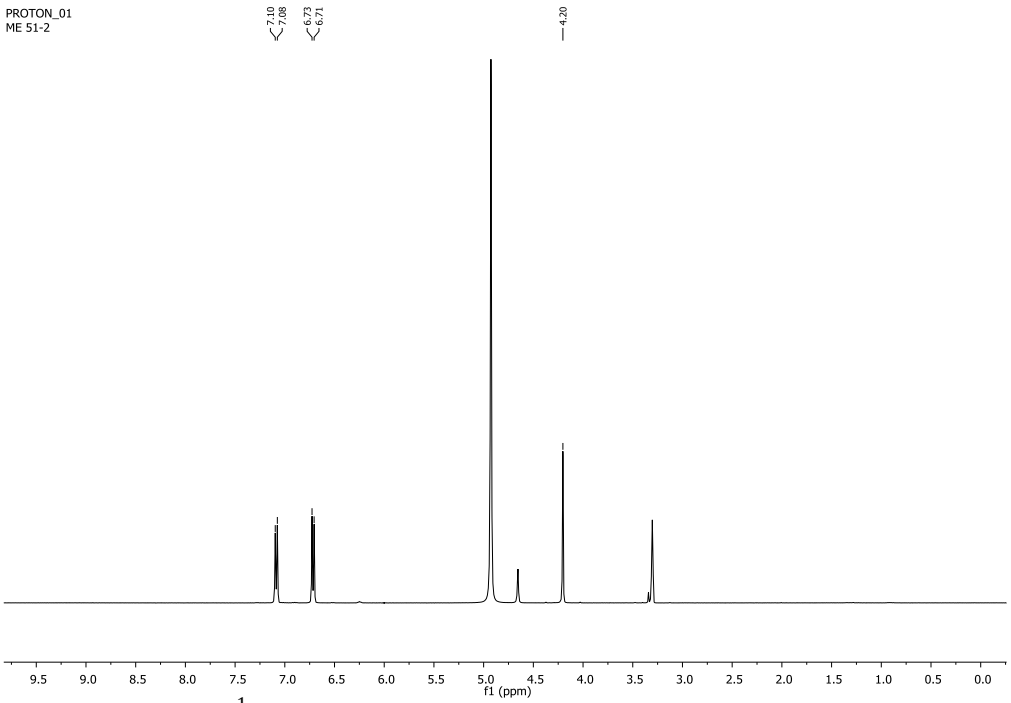


Figure S-2.7 The ^{1}H NMR (methanol-d_4) spectrum of *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**).

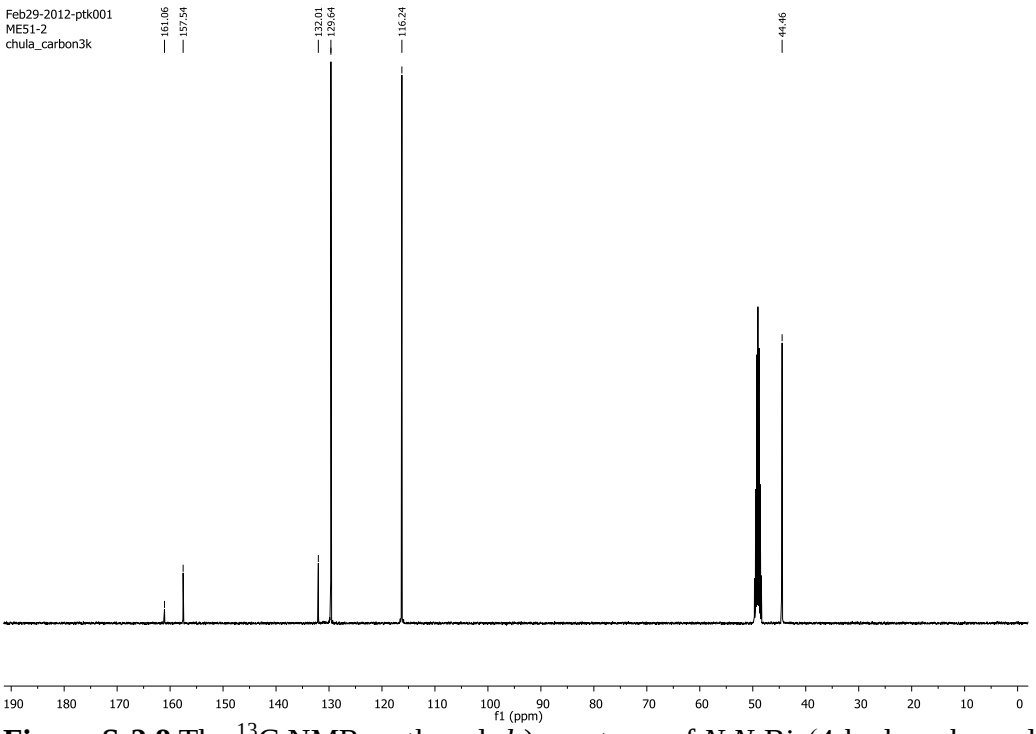


Figure S-2.8 The ^{13}C NMR methanol-d_4) spectrum of *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**).

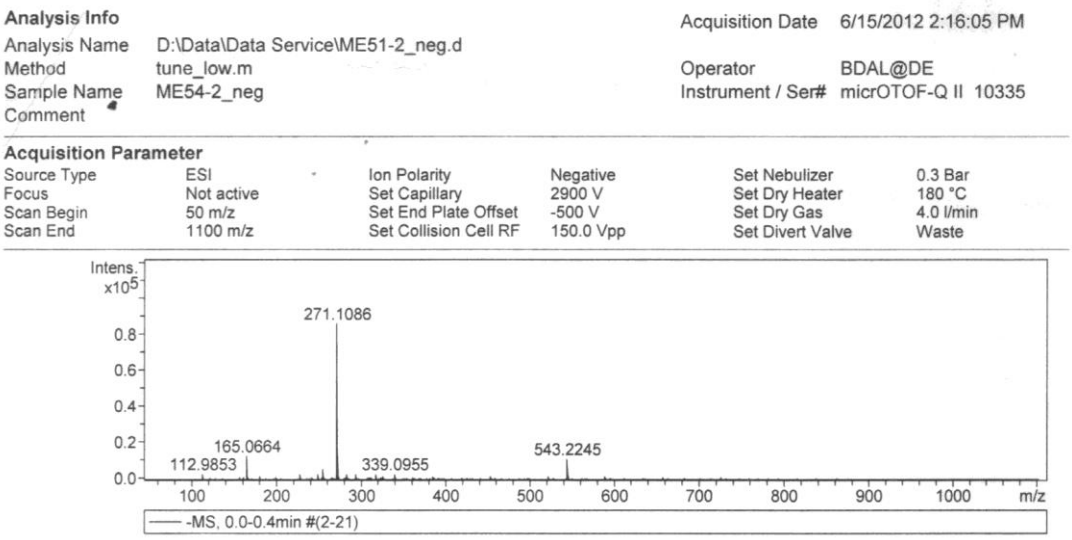


Figure S-2.9 Mass spectrum of *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**).

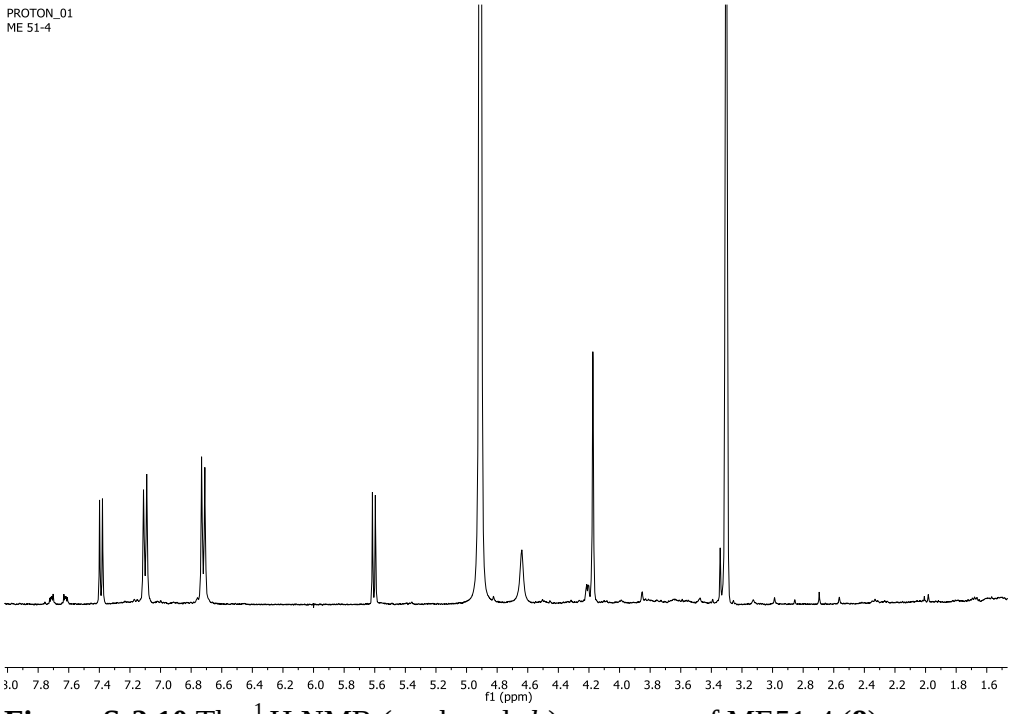


Figure S-2.10 The ^{1}H NMR (methanol-d_4) spectrum of ME51-4 (**8**).

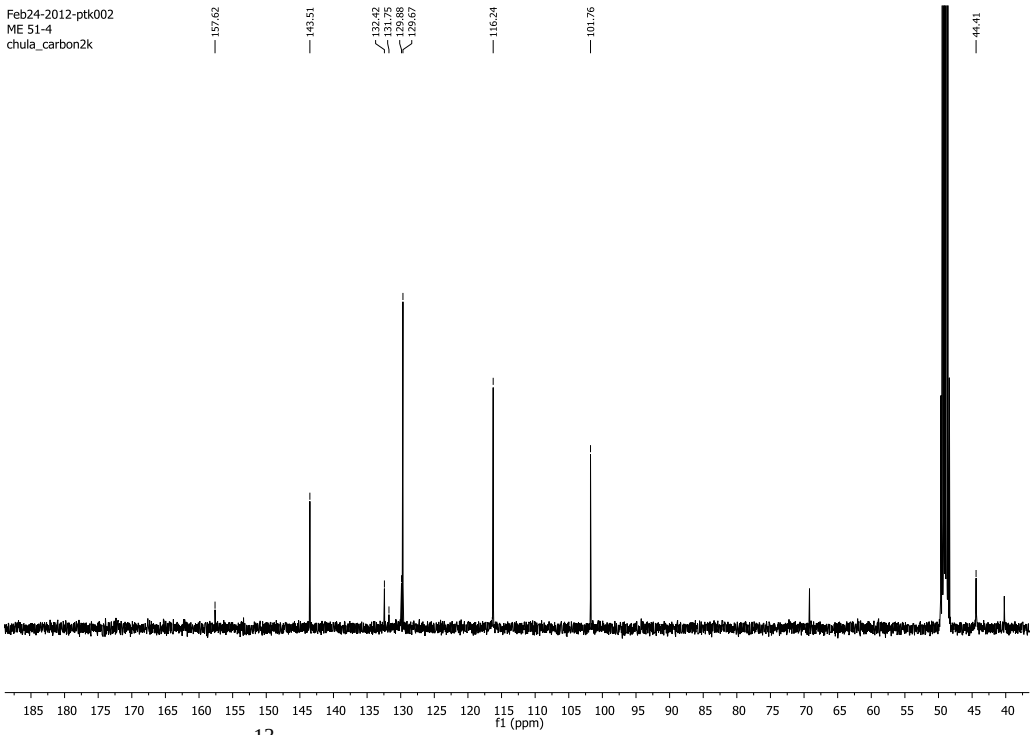


Figure S-2.11 The ^{13}C NMR methanol-d_4) spectrum of ME51-4 (**8**).

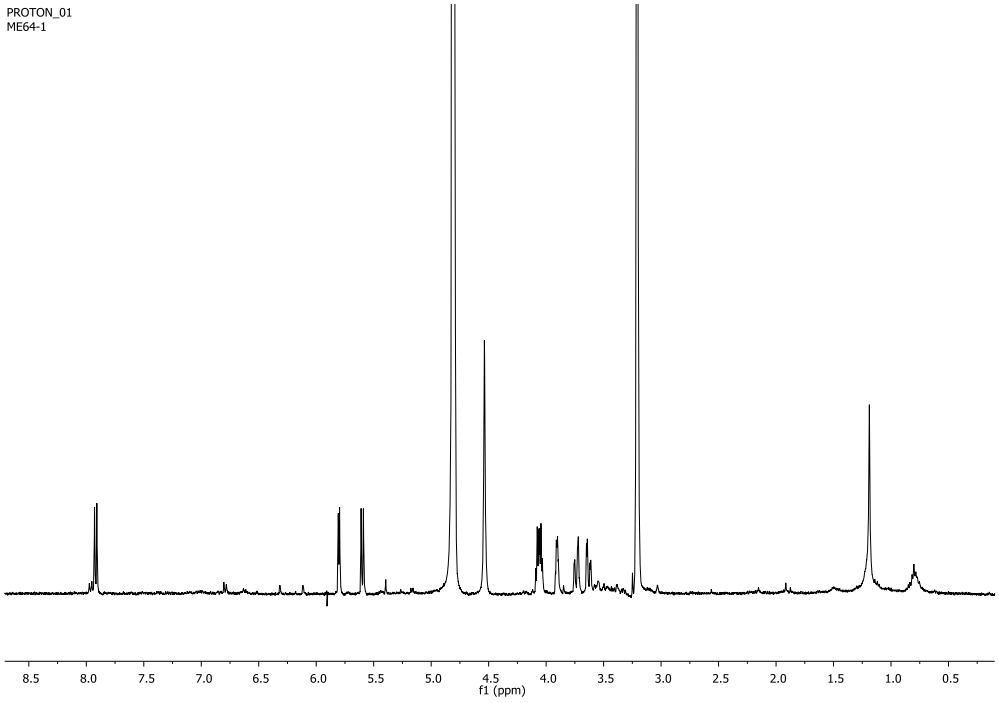


Figure S-2.12 The ^{1}H NMR (methanol-d_4) spectrum of uridine (**9**).

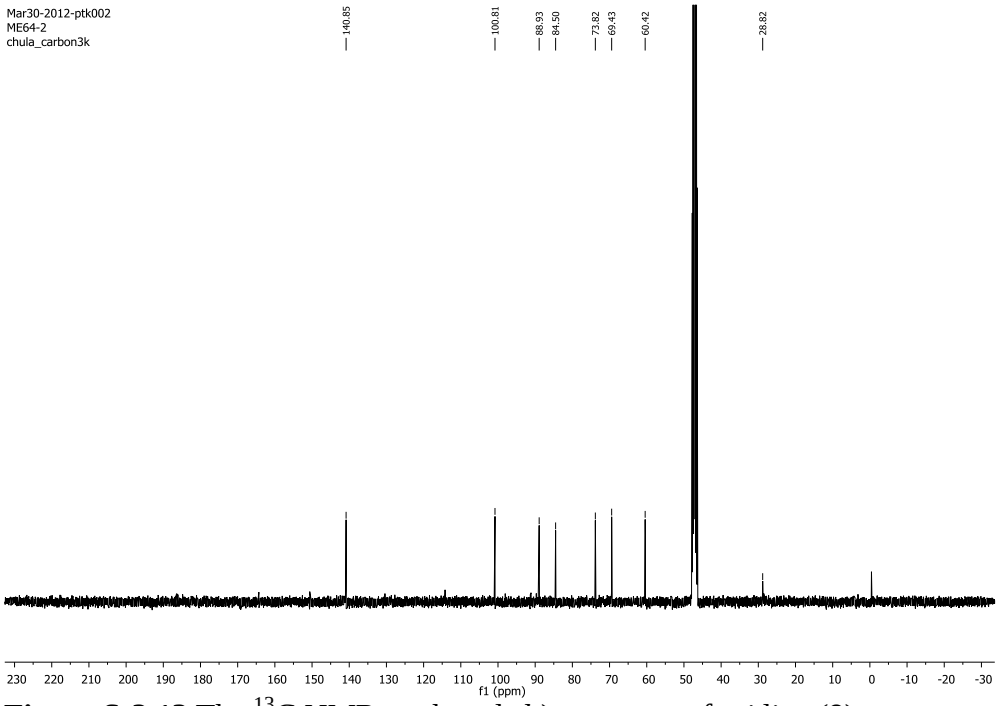


Figure S-2.13 The ^{13}C NMR methanol-d_4) spectrum of uridine (**9**).

CHAPTER III

α-GLUCOSIDASE INHIBITORY ACTIVITY OF THE ISOLATED COMPOUNDS

3.1 α-Glucosidase inhibitory activity of the isolated compounds

The α-glucosidase inhibitory activity of compounds **1-9** isolated from *Moringa oleifera* Lam. was evaluated by colorimetric method and the results are shown in Table 3.1-3.3.

Table 3.1 α-Glucosidase (baker' yeast) inhibitory activity of compounds **1-9**

	% inhibition at various concentration (mg/mL)				IC_{50}[a]
compounds	0.01	0.1	1	10	
1	9.85±3.85	10.04±1.31	1.14±2.60	-47.73±3.16	-
2	29.52±1.52	40.53±0.98	85.9±1.09	ND	0.047±0.04
3	ND	6.56±1.09	28.24±0.46	62.70±0.72	0.523±0.09
4	4.79±3.11	-3.59±2.99	-2.79±3.46	-71.66±1.83	-
5	-10.34±2.92	-17.24±0.99	3.02±1.71	-7.11±2.45	-
6	5.56±3.82	8.42±2.83	14.94±1.52	-8.62±2.45	-
7	8.71±1.18	7.01±1.42	12.88±1.99	51.52±0.32	2.180±0.06
8	-14.73±1.39	-17.85±1.77	-20.54±1.77	5.80±0.38	-
9	-15.17±1.15	-12.05±3.30	-21.87±2.41	-44.64±1.34	-
acarbose[b]	5.17±3.19	3.25±2.71	9.57±3.46	47.59±0.66	0.480±0.01

[a] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of enzyme activity (mM).
[b] Standard control
ND is 'not determined'

Table 3.1 showed α-glucosidase (baker' yeast) inhibitory activities of isolated compounds from leaves and seeds of *Moringa oleifera* Lam; in which the PNP-glucoside was used as a substrate. Apparently, kaempferol (**2**) and kaempferol-3-*O*-β-glucopyranoside (**3**), which was isolated only from leaves aqueous extract, showed

high inhibition. At the concentration of 1 mg/ml, kaempferol (**2**) and kaempferol-3-*O*-β-glucopyranoside (**3**) revealed percent inhibition of 85.9±1.09 and 28.24±0.46%, respectively. Further examination of inhibition of **2** and **3** at various concentrations led to the IC_{50} value of 0.047±0.04 and 0.523±0.09 mM, respectively. Noticeably, **2** demonstrated about 11 time more potent inhibition than its glucoside **3**, thus indicate indicating that sugar moiety of kaempferol-3-*O*-β-glucopyranoside (**3**) is not critical in exerting inhibition of against baker's yeast glucosidase.

In addition, *N*,*N*-Bis(4-hydroxybenzyl)urea (**7**), the first natural urea analogues, surprisingly demonstrated inhibitory effect against baker's yeast glucosidase (IC_{50} 2.180±0.06 mM) whereas other related urea congeners **8** and **9** revealed no inhibition. To the best of our knowledge, nitrogen containing compounds reported from this plant have not been documented for α-glucosidase inhibitory effect. *N*,*N*-Bis(4-hydroxybenzyl)urea (**7**) was reported by Tomita and coworkers as a by-product resulted from condensation of methylolphenols and urea under acidic condition (Tomita, 1992).

On the other hand, *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**), 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**), 4-Methoxybenzene-1,3-diol (**5**), *p*-hydroxybenzoic acid (**6**), ME51-4 (**8**) and uridine (**9**) showed no inhibitory effect against α-glucosidase from baker' yeast, even at high concentration of 10 mg/ml.

Table 3.2 Sucrase inhibitory activity of compounds **1-9**

	% inhibition at various concentration (mg/mL)					
compounds	0.01	0.1	1	5	10	IC_{50} [a]
1	11.92±1.96	11.57±1.49	11.74±0.17	ND	18.08±0.72	-
2	22.20±1.22	44.61±2.14	79.74±0.35	ND	ND	0.030±0.05
3	ND	26.31±1.67	45.8±1.07	75.35±1.73	ND	0.359±0.14
4	12.19±1.62	13.32±0.73	16.25±1.35	ND	11.28±1.34	-
5	16.28±2.99	12.43±1.37	-1.15±1.74	ND	0.66±2.06	-
6	12.59±0.61	12.77±1.04	5.53±1.16	ND	-15.67±0.19	-
7	11.86±0.26	9.84±0.62	2.70±0.26	ND	-11.23±3.07	-
8	16.34±1.19	13.84±0.09	12.71±0.51	ND	7.09±4.12	-
9	14.46±0.68	14.40±1.03	15.14±0.68	ND	24.67±0.59	-
acarbose [b]	15.73±0.73	42.52±2.19	54.32±2.19	ND	59.83±0.92	0.0023±0.01

[a] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of enzyme activity (mM).
[b] Standard control
ND is 'not determined'

Table 3.3 Maltase inhibitory activity of compounds **1-9**

	% inhibition at various concentration (mg/mL)					
compounds	0.01	0.1	1	5	10	IC_{50} [a]
1	10.21±1.66	14.87±0.49	13.54±2.7	ND	26.94±1.24	-
2	10.57±0.67	29.59±1.76	79.74±0.35	ND	ND	0.094±0.08
3	ND	40.2±2.09	94.06±1.73	96.04±0.56	ND	0.077±0.04
4	13.52±2.01	5.37±0.36	4.64±1.58	ND	13.34±3.79	-
5	15.48±2.21	12.02±3.81	2.40±2.03	ND	19.57±1.41	-
6	6.49±1.10	6.11±2.74	0.76±2.04	ND	-14.84±0.76	-
7	20.97±1.42	17.48±2.56	6.28±0.67	ND	-5.47±2.05	-
8	14.62±4.33	12.56±2.50	13.17±0.31	ND	10.69±2.28	-
9	12.26±1.26	12.14±1.37	14.56±2.67	ND	26.88±.93	-
acarbose [b]	25.12±1.03	48.25±3.28	60.38±2.12	ND	82.18±0.45	0.0015±0.01

[a] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of enzyme activity (mM).
[b] Standard control
ND is 'not determined'

From Tables 3.2 and 3.3, only kaempferol (**2**) and kaempferol-3-*O*-β-glucopyranoside (**3**) are still the most potent α-glucosidase inhibitors against rat intestinal sucrase and maltase at submillimolar level, the same as determined against

baker's yeast glucose. Kaempferol (**2**) inhibited sucrase with IC_{50} value of 0.030±0.05 mM, which was 12 time more potent than that of **3** (IC_{50} 0.359±0.14 mM). However, inhibitory activity of **2** and **3** against maltase turned out to be comparable, with IC_{50} value of 0.094±0.08 and 0.077±0.04 mM, respectively. On the other hand, *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**), 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**), 4-Methoxybenzene-1,3-diol (**5**), *p*-hydroxybenzoic acid (**6**), *N*,*N*-Bis(4-hydroxybenzyl)urea (**7**), ME51-4 (**8**) and uridine (**9**) showed no inhibitory effect, even at a high concentration of 10 mg/ml.

Generally, the presence of glucose moiety in **3** also reduced its potency to inhibit α-glucosidase. The observed results were significant in baker's yeast α-glucosidase and sucrase. To the best of our knowledge, the effect of sugar moiety in flavonoid glycosides on glucosidase inhibition has not been documented.

In 2010, Heng Xu reported the inhibitory effect of flavonoids on α-glucosidase (baker' yeast). The result showed that the kaempferol are mixed-type inhibitors against α-glucosidase (baker' yeast). In addition, the hydroxyl groups in B-ring of flavonoids were showned to play an important role in the catalytic activity of enzyme. Moreover, the results show that the B ring of flavonoids plays a more important role than A and C ring in the inhibition of α-glucosidase (baker' yeast). The flexibly and relatively small volume of B ring may be the main factors (Xu, 2010).

From the result, kaempferol (**2**) and kaempferol-3-*O*-β-glucopyranoside (**3**) were considered to be active components isolated from leaves aqueous extract of *M. oleifera* Lam. Thus, our study also suggested that the use of aqueous extract *M. oleifera* leaves, possibly as herbal fusion, is more effective than seeds in suppressing blood glucose level.

3.2 α-Glucosidase inhibitory assay

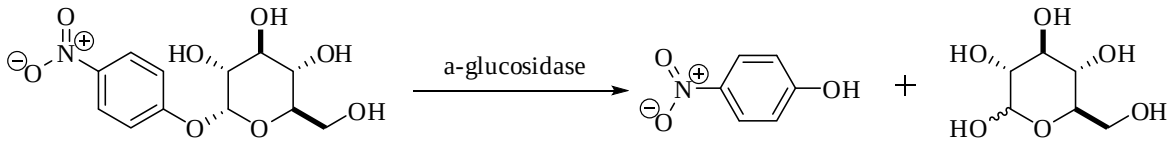
3.2.1 Chemical and equipment

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 substrate solution *p*-nitrophenyl α-D-glucopyranoside was prepared in 0.1 M phosphate buffer, adjusted to pH 6.9, to simulate a model of intestinal fluid. Briefly, yeast glucosidase was dissolved in 0.1 M phosphate buffer, pH 6.9, to yield 57 U/mL stock-solution, and further diluted with 0.1 M phosphate buffer to get 1 U/mL. Acarbose (Glucobay® 50 N 1; Bayer Vital, Leverkusen, Germany) as a synthetic inhibitor of α-glucosidase was obtained from a local pharmacy. Bio-Rad microplate reader model 3550 UV was used to measure the absorbance at 405 nm of enzyme reaction.

3.2.2 Baker's yeast α-glucosidase inhibitory activity

The α-glucosidase inhibition assay was performed according to the slightly modified method of Wacharasindhu and coworker. The α-glucosidase (0.1 U/mL) and substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M phosphate buffer, pH 6.9. 10 µL of synthesized compounds (1 mg/mL in DMSO) was pre-incubated with 40 µL of α-glucosidase at 37 °C for 10 min. A 50 µL substrate solution was then added to the reaction mixture and incubated at 37 °C for 20 min, and terminated by adding 100 µL of 1 M Na_2CO_3. Enzymatic activity was quantified by measuring the absorbance at 405 nm (Bio-Red microplate reader model 3550 UV).



p-nitrophenyl-α-D-glucopyranoside *p*-nitrophenol glucose

Figure 3.1 The hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside by α-glucosidase from baker's yeast

The percentage inhibition was calculated according to the equation shown below.

$$\% \text{ inhibition} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100$$

Where, A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The IC_{50} value was determined from a plot of percentage inhibition versus sample concentration. Acarbose® was used as standard control.

3.2.3 Rat intestinal α-glucosidase inhibitory activity

Rat intestinal α-glucosidase inhibitory activity was determined according to the method of Adisakwattana and coworker. with slight modification. The crude enzyme solution prepared from rat intestinal acetone powder was used as a source of maltase and sucrase. Rat intestinal acetone powder (1 g) was homogenized in 30 mL of 0.9%NaCl solution. After centrifugation (12,000*g* × 30 min), the aliquot was subjected to assay. A 10 µL of synthesized compounds (1 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 assay 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 percentage inhibition was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The IC_{50} value was determined from a plot of percentage inhibition versus sample concentration. Acarbose® was used as standard control.

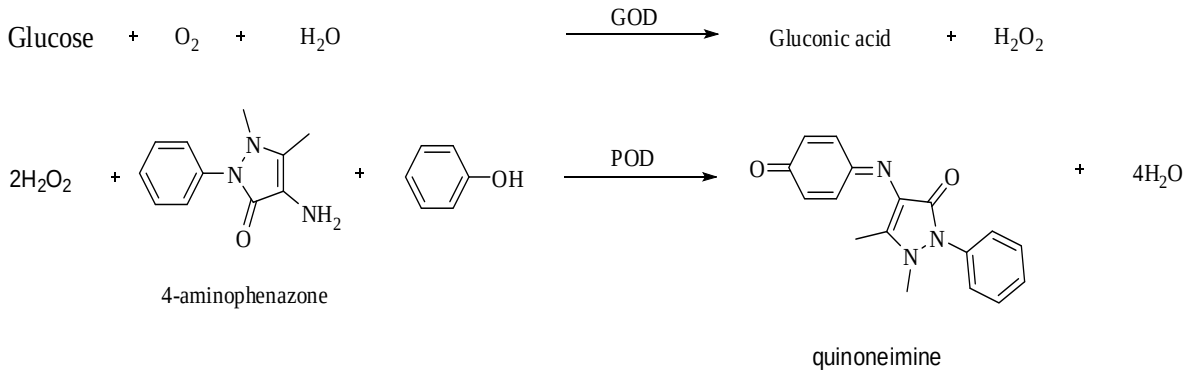


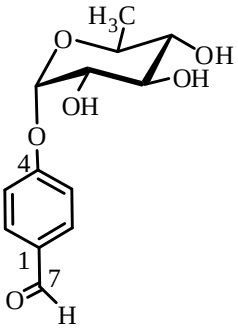
Figure 3.2 The reaction principle of α-glucosidase from rat small intestine

CHAPTER IV

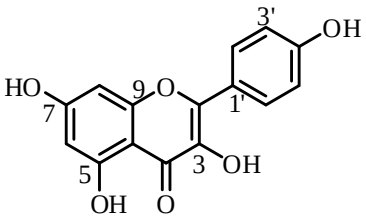
CONCLUSION

In search of α-glucosidase inhibitors from medicinal plants, bioactive components from leaves and seeds of *Moringa oleifera* were identified. The isolation of the leaves aqueous extract afforded four compounds named *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**), keampferol (**2**), kaempferol-3-*O*-β-glucopyranoside (**3**) and 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**). The isolation of MeOH extract from leaves afforded six compounds named 4-methoxybenzene-1,3-diol (**5**), *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**2**) *p*-hydroxybenzoic acid (**6**), *N*,*N*-Bis(4-hydroxybenzyl) urea (**7**), uridine (**9**) as well as a new amide derivative named ME51-4 (**8**). In addition, the isolation of MeOH extract from seeds of *Moringa oleifera* as *p*-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**), 1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**) and uridine (**9**). The structures of all isolated substances from leaves and seeds of *M. oleifera* were summarized in Figure 4.1.

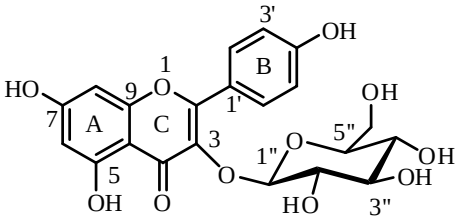
The inhibitory activities of isolated compounds against α-glucosidases were evaluated using colorimetric method. Kaempferol (**2**) and kaempferol-3-*O*-β-glucopyranoside (**3**) are among the most potent α-glucosidase inhibitor, which broadly inhibited sucrase and maltase as well as α-glucosidase from baker' yeast. However, kaempferol (**2**) exhibited more slightly improved inhibition than kaempferol-3-*O*-β-glucopyranoside (**3**). More interestingly, the finding of the active compounds **2** and **3** in leaves aqueous extract implies that consumption of *M. oleifera* leaves in form of herbal infusion would result in more effective control of blood glucose level. Our results were also concise well with previous reports of hypoglycemic effect of *M. oleifera* in animal models. Although kinetic study of **2** in yeast α-glucosidase was documented, further investigation in mammalian glucosidase is required since these enzymes have different features.



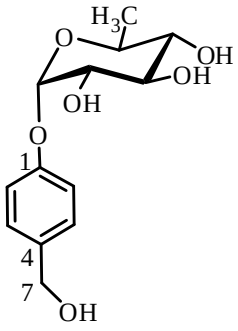
p-hydroxybenzaldehyde-*O*-α-L-rhamnopyranoside (**1**)



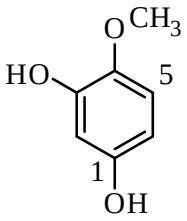
Kaempferol (**2**)



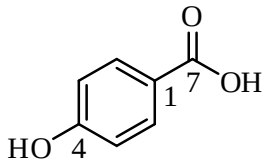
kaempferol-3-*O*-β-glucopyranoside (**3**)



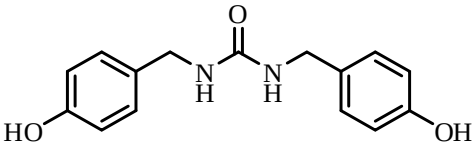
1-*O*-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (**4**)



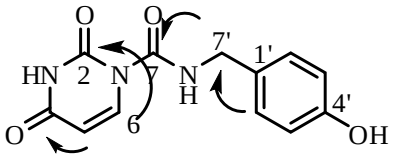
4-Methoxybenzene-1,3-diol (**5**)



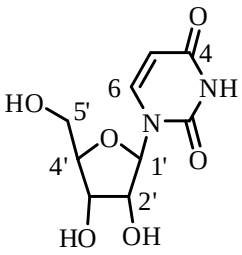
p-hydroxybenzoic acid (**6**)



N,N-Bis(4-hydroxybenzyl)urea (**7**)



ME51-4 (**8**)



Uridine (**9**)

Figure 4.1 The chemical structures of active compounds from leaves and seeds of *Moringa oleifera.*

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