Study of α-Glucosidase and α-Amylase Inhibitory Activities of Thai Folk Anti-Diabetes Remedies and Phytochemical Study of *Vitex glabrata* Stem Bark and its Chemical Constituents

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Thesis Title Study of α-Glucosidase and α-Amylase Inhibitory Activities of Thai Folk Anti-Diabetes Remedies and Phytochemical Study of *Vitex glabrata* Stem Bark and its Chemical Constituents

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ชื่อวิทยานิพนธ์ การศึกษาฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลส ของตำรับยาไทย และการศึกษาพฤกษเคมีของเปลือกต้นไข่เน่าและสาระสำคัญ
ผู้เขียน นางสาวพิชชานันท์ เธียรทองอินทร์
สาขาวิชา การแพทย์แผนไทย
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บทคัดย่อ

ด้วยสารต้านเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลส สามารถนำมาใช้ในการรักษาโรคเบาหวานประเภทที่ 2 ได้ วัตถุประสงค์ของการศึกษาเพื่อทดสอบฤทธิ์ยับยั้งการทำงานของเอนไซม์ดังกล่าวจากสมุนไพรเดี่ยวและตำรับ 9 ขนานในตำรับยาแผนไทยรักษาโรคเบาหวานของหมอพร (กรมหลวงชุมพรเขตอุดมศักดิ์) และตำรับยาโรงพยาบาลวังน้ำเย็น ผลการศึกษาพบว่า สมุนไพร 5 ชนิด ที่มีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสสูงสุด คือ แก่นกำแพงเจ็ดชั้น (*Salacia chinensis*), เปลือกต้นไข่เน่า (*Vitex glabrata*), แก่นขี้เหล็ก (*Senna siamea*), ใบหูกวาง (*Terminalia catappa*) และลูกใต้ใบ (*Phyllanthus amarus*) โดยมีค่า IC_{50} เท่ากับ 5.01±1.51, 11.22±1.70, 14.12±1.59, 15.84±1.34 และ 25.11±1.44 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และผลการยับยั้งเอนไซม์แอลฟาอะไมเลสสูงสุด คือ ใบหูกวาง (*Terminalia catappa*), เปลือกต้นไข่เน่า (*Vitex glabrata)*, ลูกใต้ใบ (*Phyllanthus amarus*), แก่นกำแพงเจ็ดชั้น (*Salacia chinensis*) และ แก่นขี้เหล็ก (*Senna siamea*) โดยมีค่า IC_{50} เท่ากับ 8.91±2.92, 14.54±1.37, 17.78±2.34, 19.56±1.38 และ 20.89±1.87 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ส่วนผลการทดสอบฤทธิ์ยับยั้งเอนไซม์ในตำรับ พบว่าตำรับยาขนานที่ TFD-02 มีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลสสูงสุดที่ IC_{50} เท่ากับ 1.99±2.87 และ 12.58±2.63 ไมโครกรัมต่อมิลลิลิตร นอกจากนี้การศึกษาองค์ประกอบทางเคมีของเปลือกต้นไข่เน่า สามารถแยกสารบริสุทธิ์ได้ทั้งหมด 6 ชนิด คือ lupeol (**1**), β-amyrin (**2**), α-amyrin (**3**), betulin (**4**), betulinic acid (**5**) และ scopoletin (**6**) ซึ่งสาร lupeol มีฤทธิ์ดีที่สุดในการยับยั้งเอนไซม์แอลฟากลูโคซิเดสที่ค่า IC_{50} เท่ากับ 7.4 ไมโครโมลาร์ และ สาร β-amyrin มีฤทธิ์ดีที่สุดในการยับยั้งเอนไซม์แอลฟาอะไมเลสที่ค่า IC_{50} เท่ากับ 32.33 ไมโครโมลาร์ จากการศึกษาในครั้งนี้พบว่าสารทั้งหมดที่แยกได้ยังไม่เคยมีรายงานของการศึกษาในเปลือกต้นไข่เน่า ดังนั้นผลจากการศึกษาครั้งนี้สามารถนำมาเป็นข้อมูลสนับสนุนถึงประสิทธิภาพของสมุนไพรชนิดนี้ในการรักษาโรคเบาหวานของหมอพื้นบ้าน และควรมีการศึกษาถึงประสิทธิภาพของสาร lupeolเพื่อพัฒนาเป็นผลิตภัณฑ์ยาจากธรรมชาติที่ใช้ในการรักษาโรคเบาหวานประเภทที่ 2 ต่อไป

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ABSTRACT

α-Glucosidase and α-amylase inhibitors are used in the treatment of type 2 diabetes mellitus. This study aims to identify the α-glucosidase and α-amylase inhibitors from two Thai folk anti-diabetes formularies including Mor Phon's recipe and the recipe of Wang Nam Yen hospital. Furthermore folk medicinal formulas from Mor Phon's recipe were also assessed. The results indicated five plants whose ethanolic extracts exhibited highest α-glucosidase inhibitory activity were *Salacia chinensis, Vitex glabrata, Senna siamea, Terminalia catappa* and *Phyllanthus amarus* with IC_{50} of 5.01±1.51, 11.22±1.70, 14.12±1.59, 15.84±1.34 and 25.11±1.44 µg/mL, respectively. Five highest α-amylase inhibitory activity were *Terminalia catappa, Vitex glabrata, Phyllanthus amarus, Salacia chinensis* and *Senna siamea* with IC_{50} of 8.91±2.92, 14.54±1.37, 17.78±2.34, 19.56±1.38 and 20.89±1.87, respectively. The formulas that showed the best activities were found using extract from formula TFD-02 with IC_{50} were 1.99±2.87 and 12.58±2.63 µg/mL for α-glucosidase and α-amylase, respectively. Furthermore, the chemical constituents of *V. glabrata* stem bark extract were isolated by chromatographic techniques gave six known compounds as lupeol (**1**), β-amyrin (**2**), α-amyrin (**3**), betulin (**4**), betulinic acid (**5**), and scopoletin (**6**). The best of α-glucosidase and α-amylase inhibitory activity was found in lupeol with IC_{50} values of 7.4 µM and β-amyrin with IC_{50} values of 32.33 µM, respectively. The result from this study is the first report of isolation of the compounds from this plant with potential α-glucosidase and α-amylase inhibitory activity. This finding could be used to support the use of this plant by Thai traditional doctors for treatment of diabetes. Furthermore lupeol which showed the highest activity is an interesting compound to be developed as a new drug for treatment of type 2 diabetes patients.

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CHAPTER 1

INTRODUCTION

1.1 Rationale

Diabetes mellitus is a group of disorders characterized by hyperglycemia. Importantly, if chronically, it will affect much the quality of patients' lives. Worldwide, the prevalence of diabetic patients in 2011 was up to 366 million and will be rising to 552 million in 2030 (Whiting et al., 2011). In Thailand, diabetes mellitus have been also increasing from 2.3% to 7.7% during 1991 to 2009 (Deerochanawong and Ferrario, 2013). Type 2 diabetes mellitus (T2DM) is the most common diabetes as about 90-95% and is usually caused either by insulin resistance or insulin deficiency (American Diabetes Association, 2011). Hyperglycemia or high blood glucose level is usually presented in clinical, particularly after the meal (post-prandial hyperglycemia) due to carbohydrate diet. The recommendation of American Diabetes Association (American Diabetes Association, 2013) suggested that 2-hour after glucose intake, post-prandial plasma glucose greater than 200 mg/dl is considered as diabetes patients. Moreover post-prandial hyperglycemia in T2DM is a major risks for microvascular and macrovascular complication, such as, neuropathy, retinopathy, and nephropathy (Aryangat and Gerich, 2010; Campos, 2012).

The management of post-prandial hyperglycemia can be achieved by disturbing carbohydrate-digesting enzyme including α-glucosidase and α-amylase. These enzymes located in intestinal lumen that function to breakdown the starch and oligosaccharides to monosaccharide, glucose (Ortiz-Andrade et al., 2007; Shang et al., 2012). Inhibition of α-glucosidase, glucose absorption can be delayed and results to lowering blood glucose levels. Modern drugs such as acarbose, miglitol and voglibose are α-glucosidase inhibitors and therapeutically accepted to improve the post-prandial hyperglycemia in

diabetic treatment, however the side effects including diarrhea, flatulence, bloating and nausea can be adversely presented. (Hollander, 1992). For example, gastrointestinal problems including flatulence (12%) and diarrhea (8%) are existing with administration acarbose in T2DM (Holman et al., 1999).

Thai traditional medicines have been reported to have several recipes for diabetes treatment. World Health Organization promotes the uses of natural products based on traditional knowledge and encourages the development of herbal medicinal products for primary health care (Bailey and Day, 1989; Chokevivat and Chuthaputti, 2005). In 2007, it revealed that the costs in overall of diabetes treatment in the U.S. were high to $174 billion in which just the medication costs were up to $116 billion. This affected to economic losses, public health problems and decreasing the lives' quality of diabetes patients (American Diabetes Association, 2008). Additionally, in Thailand, there were a number of reports that the cost of diabetic patients with complications is more than without complications (Deerochanawong and Ferrario, 2013).

Currently, many groups of researchers are interested in the study of the inhibition of α-glucosidase and α-amylase from medicinal plants, for example the methanolic extracts of leaves of *Terminalia* species (*Terminalia arjuna*, *Terminalia ballerica*, *Terminalia chebula*, *Terminalia catapa*, *Terminalia kaerbachii* and *Terminalia microcarpa*) were investigated *in vitro* for α-glucosidase activity (Anam et al., 2009). Some of there extracts showed the potential that can be used to reduce blood glucose level.

In this study, we aimed to screen the α-glucosidase and α-amylase inhibitors from thirty-seven plants selected from two folk medicinal recipes namely Mor Phon's recipe and recipe of Wang Nam Yen hospital and eight anti-diabetes folk medicinal formulas from Mor Phon's recipe. Furthermore, since our screening results suggested the potential of *Vitex. glabrata* in inhibiting both digestive enzymes, we were interested in the phytochemical study of *V. glabrata*.

Vitex glabrata R.Br. is belonging in Verbenaceae family, and locally called “Khai-nao”. The stem bark and root have long been used as antidiarrheal agent, tonic, antipyretic, astringents, anthelmintic and treating gastrointestinal disorders and the leaves can promote lactation (Luecha et al., 2009; Sukamran et al., 1999). Previously, ethanol extracts of *V. glabrata* leaves demonstrated anti-inflammatory activity, anti-estrogenic, antioxidant and hepatoprotective activity (Luecha et al., 2009; Chouhan et al., 2012; Sridevi et al., 2012). The phytochemical studies of this plant have previously been reported of chemical compounds isolated from the bark including ecdysteroids, 11α, 20-dihydroxyecdysone, 7-dehydrocholesterol, pterosterone, and 20-hydroxyecdysone from the leaves, such as khainaoside A, khainaoside B and khainaoside C (Weerawattanametin et al., 1986; Suksamrarn et al., 1999; Luecha et al., 2009).

1.2 Objectives

The objectives of this study are:

1. Evaluation of α-glucosidase and α-amylase inhibitory activities of medicinal plants from Mor Phon’s recipe and the recipe of Wang Nam Yen hospital.

2. Evaluation of α-glucosidase and α-amylase inhibitory activity of Thai folk anti-diabetes formulas from Mor Phon’s recipe and the recipe of Wang Nam Yen hospital.

3. Isolation of the chemical constituents from the stem bark of *V.glabrata* and investigation for their α-glucosidase and α-amylase inhibitory activities.

CHAPTER 2

REVIEW OF LITERATURES

2.1 Diabetes Mellitus

Diabetes mellitus is the metabolic disorder characterized by hyperglycemia or high blood glucose level. It is usually caused by the deficiency of insulin secretion, insulin action or both. The clinical symptoms of diabetes mellitus are presented as weight loss, polyuria, thirst, blurring of vision and complication of renal failure, neuropathy, foot ulcers and prolong illness will lead to microvascular and macrovascular diseases (WHO, 1999).

A healthy individual should have fasting plasma glucose (FPG) less than 100 mg/dL (5.6 mmol/L) or 2-h plasma glucose (Oral Glucose Tolerance, OGTT) less than 140 mg/dL. After meal the blood glucose level is high so insulin is produced by β-cell in the pancreas to normalize the glucose level. It increases plasma membrane glucose transporters of glucose from bloodstream into the muscle, liver and adipose tissue. In addition it converts glucose to glycogen in the muscle and the liver for storage of the nutrients. Finally, the level of glucose in the blood will comedown, insulin secretion will slow down or stop, resulting in the body to come to homeostasis. In patients with diabetes, the absence or in sufficient production of insulin causes high blood glucose levels or hyperglycemia.

The insulin is secreted by the β-cell of the pancreas. The insulin levels in the portal vein and systemic circulation are changed by the meals and blood glucose levels. In fasting, the insulin secretion is low, so called as basal insulin, in contrast, after meal insulin levels will be high, so called prandial insulin. The insulin have function to decrease blood glucose levels in the bloodstream by binding to the insulin receptor locating at surface of cell with specificity of α-subunits, which sticks into the cell and the tail which has the tyrosine kinase. This is activated process of autophosphorylation. The first of proteins to be phosphorylated are insulin receptor-substrate1 (IRS-1) and receptor-substrate2 (IRS-2). The phosphorylate of IRS-1 stimulate GTPase and kinase protein and

then stimulate phosphor-inositide-3 kinase resulting in glucose transfer of glucose transporter type 4 (GLUT-4, is the transporter of glucose in muscle and adipose tissue) to the cell membrane surface (**Figure 1**). After that the glucose will be stored in the muscle cell, liver cell and adipose tissue in the form of glycogen by increasing glycogen synthesis and decreasing gluconeogenesis of the liver cell, increasing fatty acid and triacylglycerol synthesis and decreasing β-oxidation of fatty acid of the adipose tissue. In contrast, the glucagon hormone functions to increase gluconeogenesis resulting in high blood glucose levels. In patients with diabetes, with the high blood glucose level, the kidney will be reabsorped after that glucose will be defecated in the urine (glycosuria), thereby the kidney must increase urine production (polyuria) and increase fluid loss resulting in increasing thirst (polydipsia) (Lee and pilch, 1994; Wass and Stewart, 2011; Akkarachaiyasit, 2008).

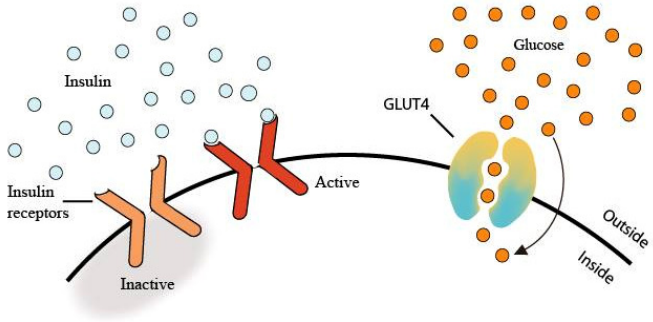


Figure 1 Action of insulin on muscle and adipose tissue

(Adapted from www.http://musom.marshall.edu/graphicdesign/ibooks.)

2.2 Diagnosis of diabetes mellitus

The American Diabetes Association classified diabetes mellitus into four types as followed (American Diabetes Association, 2013; Wass and Stewart, 2011):

1) Type 1 diabetes

This was previously known as insulin dependent diabetes mellitus (IDDM), or juvenile-onset diabetes mellitus, which was mostly onset in childhood and adolescence.The number of diabetic patients worldwide was found to be type 1 diabetes

approximately only 5-10% of all diabetics. It is caused by β-cells of the islets of Langerhans in the pancreas which are destroyed from immune (immune-mediated) usually leading to absolute insulin deficiency and resulting in high blood glucose levels or hyperglycemia, lipolysis, ketosis, acidosis and proteolysis. The clinical signs and symptoms of type 1 diabetes mellitus are polyuria, polydipsia, drowsiness, decrease conscious level, weight loss, skin infection, visual disturbances and respiratory infection. In addition, the patients have trends to be sick of other autoimmune diseases such as Grave's disease, Addison's disease, vitiligo, Hashimoto's thyroiditis, myasthenia gravis, celiac sprue, alopecia, serositis and pernicious anaemia. The control of blood glucose levels for type 1 diabtes patients can be managed with insulin, diet and physical activity.

2) Type 2 diabetes

This was previously known as non-insulin dependent diabetes mellitus (NDDM), or adult-onset diabetes mellitus which was mostly onset in adult. The numbers of type 2 diabetes patients are approximately 90-95% of all diabetes patients worldwide for which most patients have obesity. The cause was due to insulin resistance with relative to insulin deficiency or the defect in insulin secretion resulting to high blood glucose levels or hyperglycemia. The risk factors of type 2 diabetes patients are increased by age, obesity, family history, physical activity, hypertension and dyslipidaemia. The patients of this type can develop to macrovascular (coronary, cerebrovascular or peripheral arterial disease) and microvascular diseases (retinopathy, nephropathy and neuropathy). Therefore, this type of patients should be managed with physical activity, diet, oral anti-diabetic drugs or insulin in combination with oral anti-diabetic drugs.

3) Other specific types of diabetes

The other specific types of diabetes rise from many causes: genetic defects of β-cell function (caused by mutations of glucokinase gene, which is relative to insulin secretion); genetic defects of insulin action (caused by mutations of insulin receptor); disease of the exocrine pancreas (the pancreas which has been destroyed may result in β-cell disfunction such as pancreatitis, trauma, infection, pancreatic-tomy and pancreatic carcinoma); drug-or chemical–induced (defect of insulin action such as vacor, pentamidine, nicotinic acid, glucocorticoids, thyroid hormone diazoxide, β-adrenergic

agonists, thiazides, dilantin and α-interferon); Endocrinopathies (many hormones can defect insulin action such as growth hormone, cortisol, glucagon, epinephrine causing various diseases: acromegaly, Cushing's syndrome, glucagonoma, phaeochromocytoma, hyperthyroidism, somatostatinoma); Infections (occurring to β-cell which was damaged by virus such as congenital rubella, cytomegalovirus); uncommon forms of immune-mediated diabetes which is another genetic syndromes sometimes associated with diabetes.

4) Gestational diabetes mellitus (GDM)

The gestational diabetes mellitus is found in the early stage of pregnancy. If the weight is not controlled, a pregnant woman can develop a type 2 diabetes mellitus. The diagnosis of GDM using 100-g or 75-g oral glucose tolerance test. The 100-g glucose load are fasting blood glucose level, 1 hour, 2 hour and 3 hour with ≥95, ≥180, ≥155 and ≥140 mg/dL, respectively, whereas the 75-g glucose load are fasting blood glucose level, 1 hour and 2 hour with ≥95, ≥180 and ≥155 mg/dL, respectively. However the pregnant woman with the low risk factors of GMD can be excluded from the test. These include age of <25 years old, normal body weight, no family history, no history of abnormal glucose metabolism, no history of abnormal labor, with the exception for members of ethnic group with high prevalence of diabetes.

2.3 Criteria of diagnosis of diabetes mellitus

The diagnosis of diabetes mellitus is mainly relied on plasma glucose level including 1) fasting plasma glucose (FPG, the patients must not eat for 8 hours before the test) 2) 2-h plasma glucose in the 75-g OGTT (measure at 2 hour after loading of 75 g anhydrous glucose dissolved in water) and 3) symptoms + random plasma glucose (the patients have symptoms including polyuria, polydipsia and unexplained weight loss). A healthy individual should have fasting plasma glucose (FPG) less than 100 mg/dL (5.6 mmol/L) or 2-h plasma glucose (OGTT) less than 140 mg/dL (American Diabetes Association, 2013). In contrast, the patients of diabetes mellitus have fasting plasma glucose (FPG) or 2-h plasma glucose (OGTT) and symptoms+random plasma glucose greater than 126, 200 and 200 mg/dL, respectively as shown in **Table 1**.

Table 1 Criteria for diagnosis of diabetes mellitus

Test	Normal	Diabetes Mellitus
- Fasting Plasma Glucose (FPG)	<100 mg/dL (5.6 mmol/L)	≥126 mg/dL (≥7.0 mmol/L)
- 2-h plasma glucose in the 75-g OGTT	<140 mg/dL (7.8 mmol/L)	≥ 200 mg/dL (≥11.1 mmol/L)
- Symptoms + random plasma glucose	-	≥200 mg/dL (≥11.1 mmol/L)

2.4 Oral anti-diabetic drugs

Currently, there are six types of commercially available oral antidiabetic drugs for type 2 diabetes mellitus treatment including biguanide (e.g. metformin), alpha-glucosidase inhibitor (e.g. acarbose), sulfonylurea (e.g. acetohexamide), glitinide (e.g.repaglinide), thiazolidinedione (e.g. rosiglitazone), and dipeptidyl peptidase-4 (DPP-4) inhibitor (e.g. vildagliptin). Each type has a different mechanism of action in controlling blood glucose level of type 2 diabetes patients and side-effects as shown in **Table 2**. (Silvio and Inzucchi, 2002; Williams and Pickup, 1999; Territory organization, 2012; Wass and Stewart, 2011)

2.5 The theory of Thai traditional medicine in diabetes mellitus treatment

In Thai traditional medicine our body contains four elements (Thai language called “tard”) including earth, water, wind and fire. In healthy people, there four elements are in balanced. However, in illness people, they have deficiency of either of the four elements in the body. In the past, the traditional doctors (morboran or morphaenboran) examined and diagnosed patients of diabetes mellitus by examine the characteristic of urine. If the patients have "sweet urine" meaning that they have high blood glucose level. The patient’s history such as symptoms, usual behaviors of life will be performed along with the physical examinations. Nowadays the examination and diagnosis of diabetes patients can be used in combination both Thai traditional medicine and modern medicine (Chokevivat and Chuthaputti, 2005).

Table 2 The oral diabetes drugs for treatment type 2 diabetes patients

Drug Class	Mechanism of action	Side effect	Drug name
Biguanide	Reduce glucose production from the liver	Abdominal pain, nausea, and diarrhea	Metformin
Sulfonylurea	Stimulates the β-cells of pancreas to release more insulin	Hypoglycemia, weight gain	-Acetohexamide -Chlopropamide -Glibenclamide -Gliclazide -Glipizide
Thiazolidinedione	Increase glucose uptake by the skeleton muscle cells	Weight gain, swelling (edema), increased risk of congestive heart failure	- Rosiglitazone - Pioglitazone
Alpha-glucosidase inhibitor	Inhibit carbohydrate absorption by intestinal	Abdominal pain, diarrhea, flatulence	-Acarbose -Voglibose -Miglitol
Meglitinides	Stimulates the β-cells of pancreas to release more insulin	Hypoglycemia, weight gain	Repaglinide
Dipeptidyl peptidase-4 (DPP-4) inhibitor	Inhibit DPP-4 enzyme not to breakdown GLP-1 (increase GLP-1 release) resulting to increase insulin secretion and decrease glucagon secretion	Rash (Stevens-Jonhson syndrome), acute pancreatitis	-Vildagliptin -Sitagliptin

1) The causes of diabetes patients by the traditional doctors.

In the theory of Thai traditional medicine, there are many factors that can cause diabetes as described below. (Wiwatchankit, 1996; เพ็ญนภา ทรัพย์เจริญ, พ.ศ. 2546; มูลนิธิฟื้นฟูส่งเสริมการแพทย์แผนไทยเดิมฯ อายุรเวทวิทยาลัย (ชีวกโกมารภัจจ์), พ.ศ. 2541)

- **Tardsamuthan** (ธาตุสมุฏฐาน) (The four elements in the body). Substances that are solid can be said to have the qualities of the earth element, they affect the organs such as skin, muscle, bone, tendon, fat, small and large intestine, liver, and other solid organs in the body. Substances that are liquid are of the water element, they affect the blood, bile, saliva, lymph, urine, and other liquid organs in the body. Movement is the quality of the wind element which has function to the movement in the body, respiratory system, digestive system, excretion, motion of limbs and joints, sexuality and aging. Finally heat is the quality of the fire element which has function to heat in the body, the body temperature, metabolism and circulatory system. In diabetes patients, the causes of illness are from the onset of the fire element (Phathapita of part) because this element controls digestive system, liver or bile system and metabolism. It is related to the pancreas, which is an organ producing insulin and glucagon. For people with diabetes, the high blood glucose levels are resulted from deficiency of fire element. Moreover imbalance of other elements will help promoting the disease: imbalance of the wind element, which function to movement in the body relative circulatory system, respiration system and motion of the limbs and joints; imbalance of the water element from liquid in the body such as blood system and bladder or urinary tract system; imbalance of the earth element from solid in the body such as muscle and skin.

- **Aryusamuthan** (อายุสมุฏฐาน) is a different period of life, which is divided into 3 groups. The first group is during the age of 0-15 years old, the ailment arises from water element and causes the symptoms such as cold and diarrhea. The second group is during the age of 16-30 years old, the ailment arises from fire element and the cause symptoms in heart, circulatory system and digestive system. The third group is during the age of 30 years old and over, the ailment arises from wind element and causes the symptoms such as in respiratory system.

- **Utusamuthan** (อุตุสมุฏฐาน) is a season which is divided into 3 groups e.g. the hot season so called “khimhantarudu (คิมหันตฤดู)” is the fire element origin of disease, the rainy season so called “wasatarudu (วสันตฤดู)” is the wind element origin of disease and the cold season so called “hemantarudu (เหมันตฤดู) is the water element origin of disease”. Each group affects differently to human health.

- **Kalasamuthan** (กาลสมุฏฐาน) is a period of the day that has different effects to human health comprising 3 periods. The first period of the day is during 6-10 a.m. and the night during 6-10 p.m. with the ailment from water element origin of disease. The second period of the day is during 10 a.m.-2 p.m. and the night during 10 p.m.-2 a.m. with the ailment from fire element origin of disease. The third period of the day is during 2-6 p.m. and the night during 2-6 a.m. with the ailment from wind element origin of disease.

- **Prathetsamuthan** (ประเทศสมุฏฐาน) is a geographic location, that affect differently to people’ lives. It is divided into 4 groups: hot location (such as mountains, high area) with the fire element origin of disease; warm location (such as water, pebble and sand area) with the water element origin of disease; cool location (such as rain, sludge) with the wind element origin of disease and cold location (such as salt water, sludge) with the earth element of disease.

- **Inappropriate behaviors** (พฤติกรรมที่ก่อให้เกิดโรค) include 8 factors including inappropriate eating such as eat too much or too little; over sexual activity and overwork; imbalance of activity in life (sleep, stand, walk); living in different weathers (hot and cold); type of food, water and quality of sleep; urination and over expression.

From above, the theory of Thai traditional medicine suggested that many factors may causes diabetes: Tardsamuthan (beginning with the fire element imbalance and further advancing to wind, water and earth), Aryusamuthan (age 16-30 years old, the ailment from fire element), Utusamuthan (season), Kalasamuthan (period of the day 10 a.m.-2 p.m. and night 10 p.m.-2 a.m. the ailment from fire element), Prathetsamuthan (geographical location) and inappropriate behaviors**.** All factors are related and promoting the occurrence of diabetes mellitus in humans.

2) The symptoms of diabetes patients (Wiwatchankit, 1996)

Diabetes patients usually have several symtoms including:

- Urinary frequency and polyuria
- Feeling very thirsty
- Weight loss
- Fatigue
- Blurred vision
- Skin infection

3) The complications of diabetes mellitus (Wiwatchankit, 1996)

Diabetes patients usually suffer from a number of complications such as

- Retinopathy (eye damage)
- Neuropathy (nerve damage)
- Nephropathy (kidney damage)
- Heart and blood vessel diseases

4) Thai traditional anti-diabetes formularies (สำนักงานปลัดกระทรวงสาธารณสุข, ม.ป.ป.)

Normally, Thai traditional doctors recommend diabetic patients control blood glucose level by exercising, eat proper diet to control four elements balances in the body. One therapeutic approach to the decrease of high blood glucose level or post-prandial hyperglycemia is by using Thai traditional anti-diabetes recipes.

In Thai traditional medicine, there are three principal of tastes including hot, cool and mild. Hot-taste; used for treatment of disease due to the wind element, such as *Zingiber officinale* Roscoe. (rhizome). Cool-taste; used for treatment of disease due to the fire element, such as *Coccinia grandis* (L.) Voigt. (leaves)., and mild-taste; used for treatment of disease due to the water element and to adjust blood tonicity, such as *Aquilaria crassna* Pierre ex Lecomte. (heartwood). There are ten tastes of herbal medicines have been identified, including astringent, oily, salty, sweet, bitter, toxic (mao-bua), sour, hot/spicy, fragrant/cool and tasteless. These tastes of herbal medicines provide the relationship between diagnosis and herbal therapy as shown in **Table 3**.

Table 3 Ten tastes of herbal and the treatment in Thai traditional medicine

Taste	Treatment	Example of herbal
Astringent	Diarrhea, *wound healing*	**Garcinia mangostana Linn. (pericarp)**
Oily	Tonic, treat sore muscle, joint and tendon	*Sesamum indicum* L. (seed)
Salty	Skin disease, constipation	*Avicennia marina* (Forsk.) Vierh. (stem)
Sweet	Cardiac tonic, exhaustion, haematinic	*Carthamu stinctorius* L. (flower)
Bitter	Stimulate appetite, fever, liver disease	*Tinospora crispa* (L.) Miers ex Hook.f.& Thomson.
Toxic	Skin disease, anti-helminthics, detoxifier	*Cassia alata* (L.) Roxb. (leaves)
Sour	Laxative, cough, constipation	*Acacia concinna* (Willd.) D. C. (leaves)
Hot/spicy	Digestive system, sweating, flatulence	*Syzygium aromaticum* (L.) Merr. & L.M.Perry. (flower)
Fragrant/cool	Tonic (Cardiac and female)	*Aquilaria crassna* Pierre ex Lec.
Tasteless	Diuretic, kidney disease	*Pluchea indica* (L.) Less.

In most anti-diabetes formularies, the tastes of herbal are bitter, tasteless, astringent and fragrant/cool. These tastes will stimulate fire element and tonic of the water, wind and earth elements. A number of Thai folk medicinal recipes have been known to have anti-diabetes activity and some of them have been used successfully to treat diabetes patients. For example:

4.1) Thai traditional anti-diabetes recipes of KromLuang-Chomphon or Mor Phon (ธรรมนิตย์ ชำนาญ, พ.ศ. 2552)

Admiral KromLuang. ChumphonKhetUdomSak, Prince of Chumphon or Mor Phon was dedicated as “father of royal Thai navy”. He interested in studying Thai

traditional medicine in order to help poor people. He had done a lot of researches to determine the potential use of Thai traditional medicine to treat various diseases by using animals as a test model. He was generous and offered free medicine to many people.

Since, 1915 the prince had written a traditional medicine textbook known as "พระคัมภีร์ อติสาระวรรคโบราณะกรรมและปัจจุบันนะกรรม" which included contents with formularies (herbal components, method of preparations, administration and properties of recipes). At present this ancient textbook was kept at the Naval Museum. All formularies are useful for the treatment of patients with various diseases. For example recipes which have been known to have anti-diabetes activity and some of them have been used successfully to treat diabetes patients as described below.

Thai Folk Anti-Diabetes Formula 1 (TFD-01) from Mor Phon's recipe.

Herbal components : *Zea mays* L. (corn silk)
Method of preparations : Infusion: dry and then roast to light yellow, followed by addition of water.
Administration : Drink everyday.

Thai Folk Anti-Diabetes Formula 2 (TFD-02) from Mor Phon's recipe.

Herbal components : *Lagerstroemia speciosa* (L.) Pers. (leaves)
Method of preparations : Decoction: cut fresh leaves into small pieces and boil in water.
Administration : Drink everyday.

Thai Folk Anti-Diabetes Formula 3 (TFD-03) from Mor Phon's recipe.

Herbal components : *Senna siamea* Lam. (heartwood)
Table salt
Method of preparations : Decoction: cut dried heartwood into small pieces, clean and boil in water, then add some table salt and continue heating. Pour the mixture in the container with closed lid

and then keep in the bottom of the container of milled rice for 3 days. (Protecting from light)

Administration : Given three times a day.

Thai Folk Anti-Diabetes Formula 4 (TFD-04) from Mor Phon's recipe.

Herbal components : *Vitex glabrata* R. Br. (stem bark)
Table salt

Method of preparations : Decoction: cut the stem bark into small pieces followed by addition to hot water and some table salt, then continue heating until the amount of water has been reduced to a 1/3 of the beginning.

Administration : Given twice a day (for 7 days).

Thai Folk Anti-Diabetes Formula 5 (TFD-05) from Mor Phon's recipe.

Herbal components : *Abutilon hirtum* Lam. (whole plants)
Mimosa pudica L. (whole plants)
Each plant is weighed equally.

Method of preparations : Infusion: dry and then roast the plant to light yellow, followed by addition of water.

Administration : Drink everyday.

Thai Folk Anti-Diabetes Formula 6 (TFD-06) from Mor Phon's recipe.

Herbal components : *Terminalia catappa* L. (leaves)

Method of preparations : Decoction: cut fresh leaves into small pieces, clean and boil in water.

Administration : Drink everyday.

Thai Folk Anti-Diabetes Formula 7 (TFD-07) from Mor Phon's recipe.

Herbal components : *Pandanus amaryllofolius* Roxb. (leaves)
Tectona grandis L. f. (leaves)
Each plant is weighed equally.

Method of preparations : Decoction: cut dried plants into small pieces, clean and boil in water.

Administration : Drink everyday for 1 month.

Thai Folk Anti-Diabetes Formula 8 (TFD-08) from Mor Phon's recipe.

Herbal components : *Phyllanthu samaras* Schumach. (whole plants)
Smilax corbularia Kunth Subsp. (rhizome)
Smilax glabra Wall. ex Roxb. (rhizome)
Each plant is weighed equally.

Method of preparations : Decoction: cut each plant into small pieces followed by addition to hot water and continue heating until the amount of water has been reduced to a 1/3 of the beginning.

Administration : Drink everyday.

4.2) Thai traditional antidiabetes recipes of Wang Nam Yen hospital (Sa Kaeo province).

Wang Nam Yen hospital was selected as one of eight hospitals which have good performance in Thai traditional medicine therapy from department for development of Thai traditional and alternative medicine. The hospital offers treatment with diagnosis, physical examination. The treatments including Thai massage (nuad Thai), hot herbal compresses and herbal steam baths, aromatherapy, traditional Thai midwifery (treatment for mother and child), herbal medicine and meditation. The hospital has opened special clinics for specific diseases such as Psoriasis disease clinic, midwifery clinic, chronic diseases clinic (diabetes mellitus, high blood pressure and hypercholesterolemia). The hospital uses a combination of Thai traditional medicine and modern medicine to treat their patients.

Currently, the hospital has developed a herbal garden named as “Herb garden Pennapha” with collection of plants of more than 500 species. This hospital has produced and sold herbal medicines for patients, particularly the anti-diabetes formularies, whose recipes were prescribed to treat patients with diabetes which resulted in blood glucose level reduction and increasing insulin sensitivity of liver cell. The recipes are described below:

Thai Folk Anti-Diabetes Formula 9 (TFD-09) from Mor Phon’s recipe.

Herbal components : *Abutilon hirtum.* Lam. (whole plants)
Acanthus ebracteatus Vahl. (whole plants)
Albizia myriophylla Benth. (heartwood)
Andrographis paniculata (Burm.f.) Wall. ex Nees. (leaves)
Cappari smicracantha DC. (heartwood)
Caryota mitis Lour. (stem)
Cyperus rotundus L. (tubers)
Harrisonia perforata (Blanco). Merr. (stem)
Homalomena aromatic Schott. (stem)
Hydnophytum formicarum Jack. (tubers)
Imperata cylindrica (L.) P Beauv. (root)
Lagerstroemia speciosa (L.) Per. (leaves)
Orthosiphon aristatus (Blume) Miq. (whole plants)
Pandanus odoratissimus L. f. (stem)
Rhina canthusnasutus (L.) Kurz. (whole plants)
Salacia chinensis L. (heartwood)
Smilax corbularia Kunth. (rhizome)
Smilax glabra Roxb. (rhizome)
Solanum indicum L. (stem)
Terminalia arjuna (Roxb.) (fruits)
Terminalia bellirica (Gaertn.) Roxb. (fruits)
Terminalia chebula Retz. (fruits)
Tinospora cripa (L.) Miers ex. Hook. f & Thomso. (stem)
Tribulus terrestris L. (whole plants)
Urceola rosea (Hook. & Arn) (stem)
Ureceola minutiflora (Pierre). (stem)

Method of preparations : The dried plant was cut into small pieces and ground with an electric grinder to get fine powder and then filled into capsules.

Administration : Take 3-4 capsules, three times a day (before meals).

2.6 *In vitro* and *in vivo* studies on anti-diabetic activity

The study of anti-diabetic agents can affect several pathways of glucose metabolism which were evaluated *in vivo* using animal models such as streptozotocin-induced diabetes and alloxan-induced diabetes and *in vitro* study including glucose uptake, α-glucosidase and α-amylase inhibitory activities and DPP-4 inhibitory activity

***In vivo* studies**

1. Streptozotocin-induced diabetes mellitus

Streptozotocin (STZ) is a chemical used for induced diabetes in animals model. STZ can damaged pancreatic β-cell and free radical degeneration, resulting in reduction in insulin secretion and high amount of free radicals that couse diabetes mellitus (Sharma et al., 2013).

2. Alloxan-induced diabetes mellitus

Alloxan is a chemical used in animals model, which induced degeneration of pancreatic β-cell to stimulate diabetes mellitus.

The alloxan gave maximum blood glucose levels during acute hyperglycaemia plase occur in 45 min, while the streptozotocin need 120 min (Sharma et al., 2013).

In vitro studies

1. Glucose uptake

Glucose uptake activity was evaluated by measuring the rate of uptake of radioactively tagged 2-deoxy glucose in differentiated 3T3 L1 cells in cell lines of adipocytes and rat L6 muscle engineered to over-express GLUT4. After fasting, the cells were treated with insulin and plant extracts. Then these ligands will bind to the receptors on the surface of the cells. These triggered the translocation of glucose transporters to the cell surface. By measuring this uptake rate by using liquid scintillation counter help to

analyze the glucose uptake activity and the effect of plant extract on the glucose uptake activity (Thorat et al., 2012).

2. α-Glucosidase and α-amylase inhibitory activities

The inhibiting of key enzymes including α-glucosidase and α-amylase, which play roles to break specifically carbohydrate down into absorbable monosaccharide or glucose resulting to improve blood glucose level after meal (postprandial hyperglycemia). α-Glucosidase and α-amylase activities were measured by determining the color from the hydrolysis of substrate and using spectrophotometric method (Rao and Jamil, 2011).

3. Dipeptidyl peptidase-4 (DPP-4) inhibitory activity

Dipeptidyl peptidase-4 (DPP-4) is an enzyme found in the capillary bed of the intestinal mucosa. DPP-IV cleaves the alanine and proline from the *N*-terminal ends of GLP-I and GIP making them biologically inactive. Administration of DPP-IV inhibitors, block the enzyme and thereby prolongs the half life and biological activity of GLP-I. This is one of the recent therapies used in the treatment of Type 2 diabetes (Chakrabarti et al., 2011).

Several herbs from Mor Porn's recipe and recipe of Wang Nam Yeng hospital have been reported to have anti-diabetic activity. However, different mechanisms of action could be involved (**Table 4**).

2.7 α-Glucosidase and α-amylase enzymes

α-Glucosidase enzyme (EC.3.2.1.20, maltase) is the enzyme that hydrolyzes α-1,4 glycosidic bond in carbohydrate digestion (disaccharides such as maltose, sucrose and lactose into monosaccharides or glucose), whose location is in the brush-border surface membrane of intestinal cells in human (Gao et al., 2008; Melo et al., 2006). α-mmylase enzyme (EC.3.2.1.1) is the enzyme secreted by salivary glands and pancreas in humans, that can hydrolyze starch at α-1,4 glycosidic bond into oligosaccharides and maltose (Feng, 2011; Nater, 2009). The human digestive system begins with digestion of carbohydrate by using α-amylase enzymes secreted from the salivary glands and the pancreas in the small intestine to oligosaccharides or disaccharides. α-Glucosidase enzyme (maltase) is a final step for the breakdown of oligosaccharides and maltose into monosaccharides or glucose as shown in **Figure 2**.

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
1.	*Albizia myriophylla* Benth.	Heartwood	Against cough, tonic	Hypoglycemic activity in streptozotocin-nicotinamide induced diabetes rats, α-glucosidase inhibitory activity	Saat et al., 2012; Tunsaringkarn et al., 2009
2.	*Abutilon hirtum* Lam.	Whole plants	Against cough, treat diabetes, antipyretic and to adjust blood tonicity	PPARγ agonist activity, increase glucose utilization via GLUT1	Krisanapun et al., 2011
3.	*Acanthus ebracteatus* Vahl.	Whole plants	Treat dermatitis and diuretic	Decreased blood glucose level in rats with alloxan induced diabetes	Venkataiah et al., 2013
4.	*Andrographis paniculata (Burm. f.) Wall. Ex Nees.*	Whole plants	Treat dysentery, ulcerative colitis, diabetes	α-Glucosidase and α-amylase inhibitory activities, hyperglycemic, glucose uptake	Subramanianl et al., 2008; Augustine et al., 2014
5.	*Capparis micracantha* D. C.	Heartwood	Fever, tonic diuretic	No information	วุฒิ วุฒิธรรมเวช, พ.ศ.

2540

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
6.	*Caryota mitis* Lour.	Tubers	Itch, cardiac tonic	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
7.	*Cyperus rotundus* L.	Tubers	Diuretic, fever, thirst, diabetes	α-Glucosidase inhibitory activity, decreased blood glucose level in rats with alloxan induced diabetes	Bachhawat et al., 2011; Raut and Gaikwad, 2006
8.	*Diospyros rhodocalyx* Kurz.	Stem bark	Tonic, diabetes	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
9.	*Harrisonia perforata* (Blanco) Merr.	Stem	Fever, diarrhea	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
10.	*Homalomena aromatica* Schott.	Tubers	Diuretic, liver disease	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
11.	*Hydnophytum formicarum* Jack.	Tubers	Assuage liver	α-Glucosidase inhibitory activity	Ahmad et al., 2011

			damage, tonic		
12.	*Imperata cylindrica* (L.) P. Beauv.	Rhizome	Diuretic, fever	Decreased blood glucose level in mice with streptozotocin induced diabetes	**Jue et al., 2012**
13.	*Lagerstroemia speciosa* (L.) Pers	Leaves	Diuretic, diabetes	α-Glucosidase inhibitory activity,	

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
				hypoglycemic activity, glucose uptake	Hou et al., 2009; Saha et al.,2009; Liu et al., 2001
14.	*Mimosa pudica L.*	Whole plants	Diabetes, diuretic	α-Glucosidase inhibitory activity, decreased blood glucose level in	Suryadevara et al., 2009; Azmi et al.,

				mice with alloxan induced diabetes	2011
15.	*Orthosiphon aristatus* Miq.	Whole plants	Diuretic, kidney disease,	Decreased blood glucose level in rat with streptozotocin induced diabetes	Mohamed et al., 2011
16.	*Pandanus amaryllofolius* Roxb.	Leaves	Diuretic, diabetes, rheumatic	decreased blood glucose level in mice with streptozotocin induced diabetes	Sasidharan et al., 2011
17.	*Pandanus odoratissimus* L.f.	Stem	Diuretic, detoxing, purifying the blood	Decreased blood glucose level in rat with alloxan induced diabetes	Savitha et al., 2012

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
18.	*Phyllanthus amarus*	Whole plants	Diuretic, diabetes, fever, liver disease	α-Amylase inhibitory activity, hypoglycemic activity, decreased blood glucose level in rat with alloxan induced diabetes	Tamil et al., 2010; Mbagwu et al., 2011; Shetti et al., 2012

19.	*Rhinacanthus nasutus* (L.) Kurz.	Leaves	Treat skin disease	Decreased blood glucose level in rat with streptozotocin induced diabetes	Rao and Naidu, 2010
20.	*Senna alata* L.	Leaves	Treatment of skin disease, urinary stone, ringworm, laxative	α-Glucosidase inhibitory activity	Varghese et al., 2012
21.	*Salacia chinensis* L.	Stem	Tonic of joint and muscle, diabetes	α-Glucosidase inhibitory activity, decreased blood glucose level in rat with streptozotocin induced diabetes	Yoshikawa et al., 2003; Sellamuthu et al., 2009

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
22.	*Senna siamea* Lam.	Leaves Heartwood	Laxative, diabetes, diuretic	Decreased blood glucose level in rat with alloxan induced diabetes	Mohammed et al., 2012

23.	*Smilax corbularia* Kunth	Rhizome	Tonic of joint and muscle, diuretic	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
24.	*Smilax glabra* Roxb.	Rhizome	Fever, diabetes diuretic, cancer	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
25.	*Solanum indicum* L.	Fruits	Against cough, diuretic, diabetes, tonic of gall bladder	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
26.	*Terminalia* arjuna.	Fruits	Laxative, carminative	α-Glucosidase inhibitory activity	Anam et al., 2009
27.	*Terminalia bellirica* (Gaertn.) Roxb.	Fruits	Laxative, fever, carminative	α-Glucosidase and α-amylase inhibitory activities, decreased blood glucose level in rat with alloxan induced diabetes, glucose uptake	Malina et al., 2012; Sabu and Kuttan, 2009

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference
28.	*Terminalia catappa* L.	Leaves	Treat tonsillitis, rheumatism liver	α-Glucosidase and α-amylase inhibitory activities, decreased blood glucose level in rat	Anam et al., 2009;

			disease	with alloxan induced diabetes	Ahmed et al., 2009
29.	*Terminalia chebula* Retz var.chebula.	Fruits	Laxative, carminative	α-Glucosidase inhibitory activity, decreased blood glucose level in rat with streptozotocin induced diabetes	Anam et al., 2009; Rao and Nammi, 2006
30.	*Tinospora cripa* (L.) Miers ex Hook. f &Thomson.	Stem	Diuretic ,fever, cures jaundice and liver	Increased insulin levels in diabetic rats	Noor and Ashscoff, 1989
31.	*Tribulus terrestris* L.	Whole plants	Diuretic, kidney disease	α-Glucosidase inhibitory activity, inhibiting oxidative stress and decreased blood glucose level in rat with streptozotocin induced diabetes	Lamba et al., 2011; Amin et al., 2006
32.	*Urceola minutiflora* (Pierre).	Stem	Would healing, cancer, diabetes	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540

Table 4 Medicinal plants of Mor Porn's recipe and recipe of Wang Nam Yeng hospital used in anti-diabetic treatment (cont.)

No.	Scientific name	Part of use	Thai traditional use	Anti-diabetic activity	Reference

33.	*Urceola rosea* (Hook & Arn.).	Stem	Would healing, cancer, diabetes	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
34.	*Vitex glabrata* R. Br.	Stem bark	Diarrhea, fever, anthelmintic, diabetes	No information	วุฒิ วุฒิธรรมเวช, พ.ศ. 2540
35.	*Zea mays* L.	Corn silk	Diuretic, kidney disease	Reduced hyperglycemia in alloxan-induced diabetes mice	Guo et al., 2009

The inhibition of enzymes α-glucosidase and α-amylase can delay the absorption of carbohydrate digestion resulting in reducing of blood glucose level. This has been used in the treatment of type 2 diabetes mellitus (Andrade, 2007; Karthic et al., 2008). The inhibitors of these enzymes such as acarbose, voglibose and miglitol are found to be associated with various side effects of abdominal pain, diarrhea and flatulence. Nowadays many groups of researchers are working to find new drugs from natural sources for treatment of type 2 diabetes mellitus by using the mechanism of α-glucosidase and α-amylase inhibitory activities.

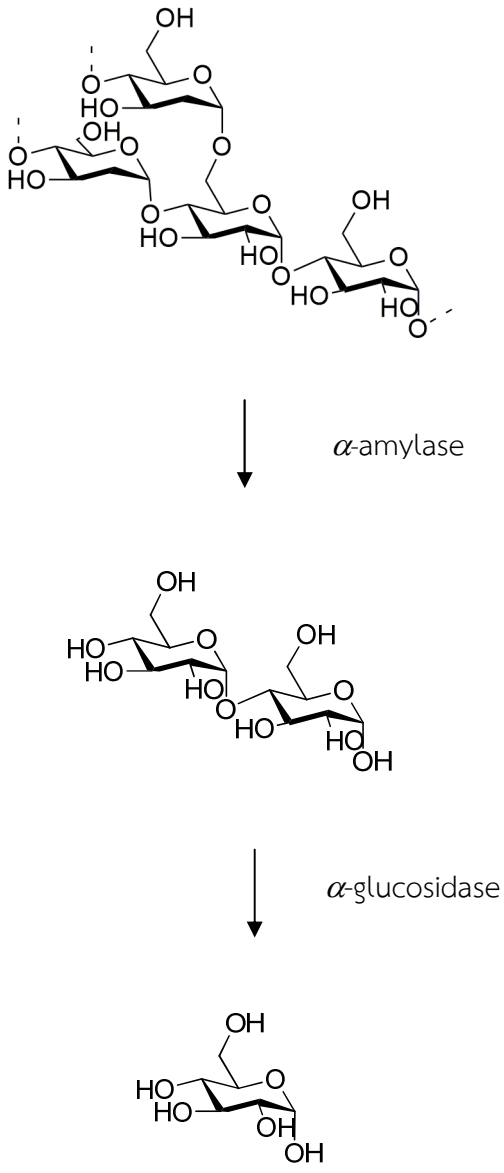
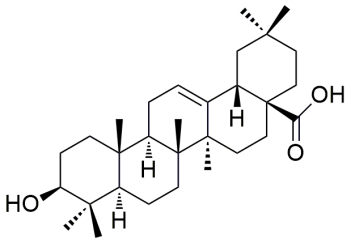


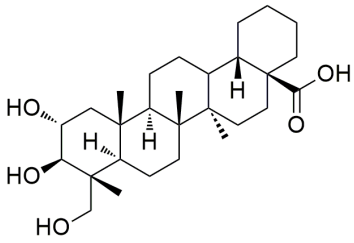
Figure 2 The digestion of carbohydrate by α-amylase and α-glucosidase enzymes

2.8 α-Glucosidase and α-amylase inhibitors from medicinal plants

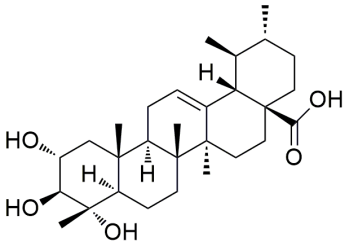
A number of Thai traditional medicines or other alternative medicines have been reported to have ability for treatment of diabetes. In this study thirty-seven medicinal plants from Mor Porn's recipes and recipe of Wang Nam Yen hospital were selected. Thirteen medicinal plants were previously investigated for α-glucosidase and α-amylase activities and are reviewed below. The methanolic and aqueous extracts of *Lagerstroemia speciosa.* (leaves) had a strong inhibition effect on α-glucosidase and the effect was found to be higher than acarbose (a standard α-glucosidase inhibitor) at a concentration of 1 mg/mL (Rungprom et al., 2009). Six triterpenes were isolated from the ethyl acetate extract of the leaves of *L. speciosa* which are oleanolic acid, arjunolic acid, asiatic acid, maslinic acid, corosolic acid, and 23-hydroxyursolic acid. Corosolic acid showed high α-glucosidase inhibition with IC_{50} values of 7 μM (Hou et al., 2009).



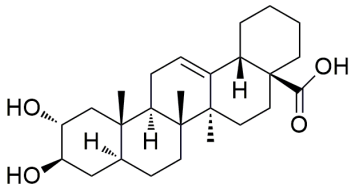
Oleanolic acid



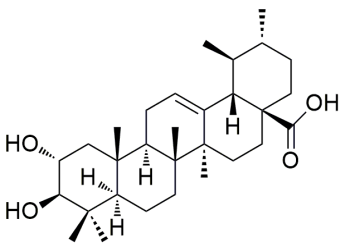
Arjunolic acid



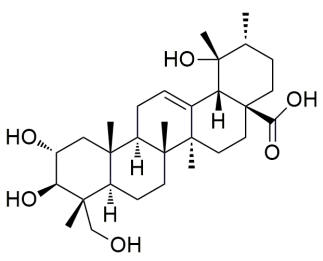
Asiatic acid



Maslinic acid

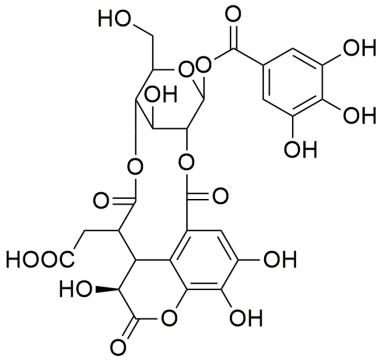


Corosolic acid

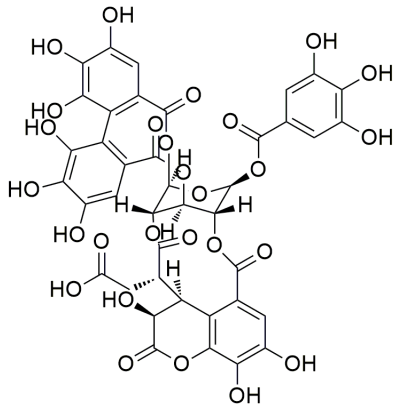


23-Hydroxyursolic acid

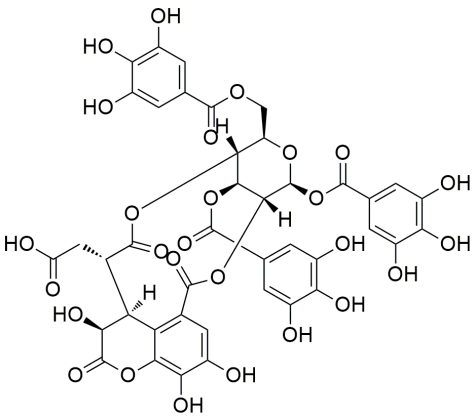
From aqueous methanolic extract of *Terminalia chebula* found chebulanin, chebulagic acid, and chebulinic acid. These compounds showed potent inhibition of rat intestine maltose with IC_{50} values of 690 μM, 97 μM and 36 μM, respectively (Gao et al., 2007). The methanolic extracts of *Cyperus rotundus* L. (tubers) exhibited a potent inhibition of α-glucosidase activity with IC_{50} value of 3.98 μg/mL which was more active than voglibose (Bachhawat et al., 2011). In addition the aqueous extract of the branch of *Albizia myriophylla* showed 9% inhibitory effect on the α-glucosidase at a concentration of 1 mg/mL (Tunsaringkarn et al., 2009). The ethanol extract of the fruits of *Tribulus terrestris* showed moderate α-glucosidase inhibition of 59% at a concentration of 30 μg/mL (Lamba et al., 2011). The methanol extract of the stem of *Salacia chinensis* showed strong inhibitory effect on the α-glucosidase with IC_{50} value of 133 μg/mL, (better than acarbose and volgibose with IC_{50} value of >400 μg/mL) (Yoshikawa et al., 2003). Moreover the ethanol extracts of the leaves of *Andrographis paniculata* displayed α-glucosidase and α-amylase inhibitory activities with the IC_{50} values of 17.2 μg/mL and 50.9 μg/mL, respectively (Subramanianl et al., 2008). The previous study on isolation of the leaves of *Senna alata* afforded kaempferol which showed the strong inhibitory effect on the α-glucosidase and α-amylase with IC_{50} values of 56 μM and 50 μM, respectively (Varghese et al., 2012).



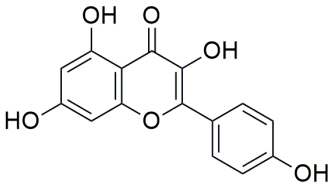
Chebulanin



Chebulagic acid

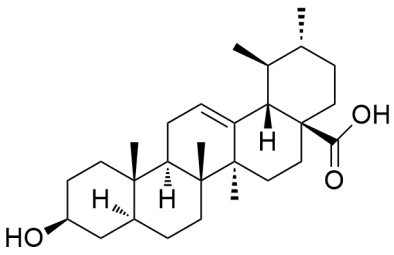


Chebulinic acid

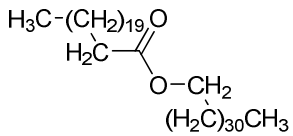


Kaempferol

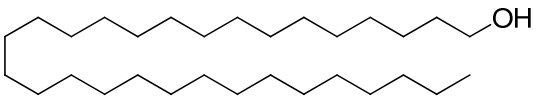
The ethanol extract of the leaves of *Terminalia catappa* demonstrated 10% inhibitory activity of α-amylase (Koffi et al., 2010). The ethanol and hexane extract of the leaves of *Phyllanthus amarus* showed high α-amylase inhibition with IC_{50} values of 36.05 μg/mL and 48.92 μg/mL, respectively (Tamil et al., 2010). Moreover dotriacontanyl docosanoate, triacontanol and mixtures of oleanolic acid and ursolic acid were isolated from hexane extract of *P. amarus.* Dotriacontanyl docosanoate and a mixture of oleanolic acid and ursolic acid have been reported from this plant for the first time. The mixture of oleanolic acid and ursolic acid (2:1) showed the highest α-amylase inhibition with IC_{50} values of 2.01 μg/mL. (Ali et al., 2006)



Ursolic acid



Dotriacontanyl docosanoate



Triacontanol

2.9 Pharmacology of genus *Vitex*

The genus *Vitex* is in Verbenaceae family. It consists about 250 species around the world, which are commonly found in tropical areas. Several species have long been used traditionally for the treatment of various illnesses such as fruit of *Vitex cannabifolia* was used for treatment analgesia; *Vitex agnus-castus* was used for diuretic and stomachache; *Vitex trifolia* was used to treat fever and inflammation (Ganapaty et al., 2005; Yanaski et al., 2008; Meena et al., 2010). The various biological activities of this genus have been reported, for example aqueous extracts of *Vitex doniana* leaves exhibited anti-diabetic activity by decreasing blood glucose level (Ezekwesili et al., 2012). Vitegnoside isolated from the leaves of *Vitex negundo* also exhibited anti-fungal activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformanas* (Sathiamoorthy et al., 2007). A groups of chemical compounds were isolated from this genus have been previously reported such as iridoids, flavonoids, diterpenoids, triterpenoids and steroids (Meena et al., 2010).

2.10 Chemical constituents of genus *Vitex*

In our study, we selected *Vitex glabrata* for isolation of pure compounds because it showed high potential of α-glucosidase and α-amylase inhibitory activities. This plant is in the genus *Vitex* and Verbenaceae family. A number of reports have previously been shown that terpenoids, flavonoids, iridoid glycosides and steroids were isolated from this plant and are summarized in **Table 5**.

Table 5 Chemical constituents of *Vitex* species

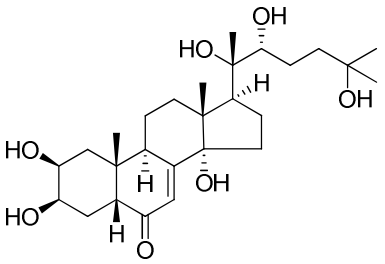
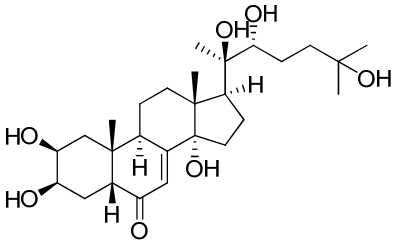
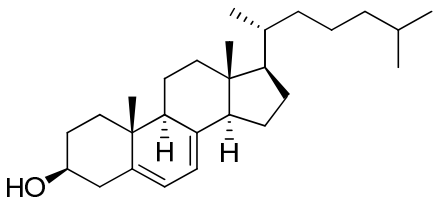
Plant (parts)	Compound	Structures	Chemical group	References
V. glabrata (Bark)	Ecdysterone		Steriod	Werawattanametin et al., 1986
V. glabrata (Bark)	20-Hydroxyecdysone		Steriod	Werawattanametin et al., 1986
V. glabrata (Bark)	7-Dehydrocholesterol		Steriod	Werawattanametin et al., 1986

Table 5 Chemical constituents of *Vitex* species (cont.)

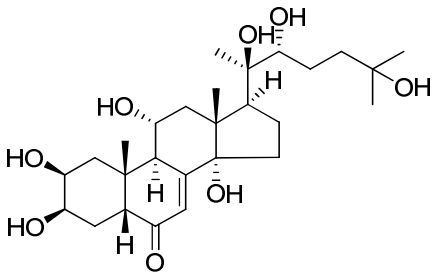
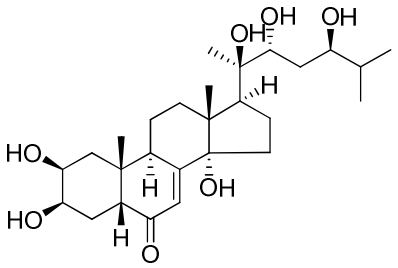
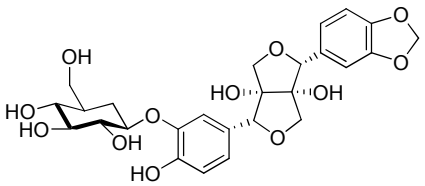
Plant (parts)	Compound	Structures	Chemical group	References
V. glabrata (Bark)	11α,20-Dihydroxyecdysone (turkesterone)		Steriod	Werawattanametin et al., 1986
V. glabrata (Bark)	Pterosterone		Steroid	Suksamrarn et al., 1999
V. glabrata (Bark)	Khainaoside A		Lignan glycoside	Luecha et al., 2009

Table 5 Chemical constituents of *Vitex* species (cont.)

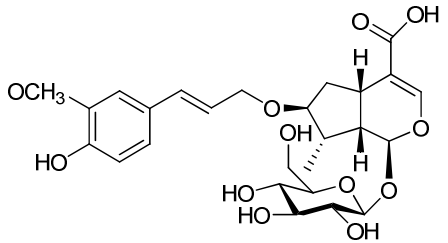
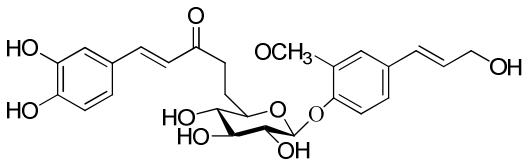
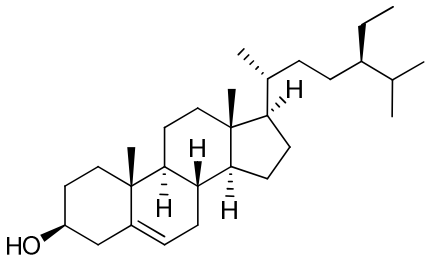
Plant (parts)	Compound	Structures	Chemical group	References
---	---		Lignan glycoside	Luecha et al., 2009
V. glabrata (Leaves)	Khainaoside C		Lignan glycoside	Luecha et al., 2009
V. quinata (Leaves)	*β*-Sitosterol		Steroid	Cheng et al., 2007

Table 5 Chemical constituents of *Vitex* species (cont.)

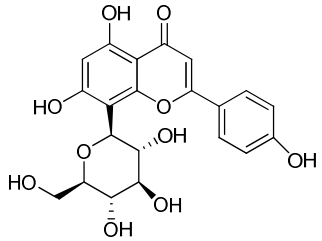
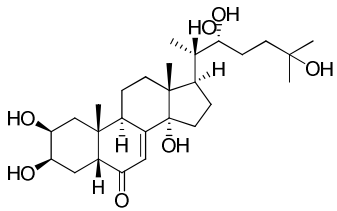
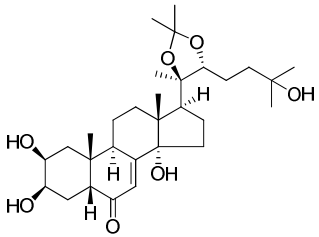
Plant (parts)	Compound	Structures	Chemical group	References
V. quinata (Leaves)	Vitexin		Flavone C-glycoside	Cheng et al., 2007
V. quinata (Leaves)	20-Hydroxyecdysone		Steroid	Cheng et al., 2007
V. quinata (Leaves)	20,22-Monoacetonide		Steroid	Cheng et al., 2007

Table 5 Chemical constituents of *Vitex* species (cont.)

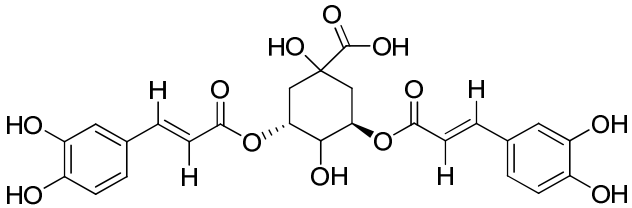
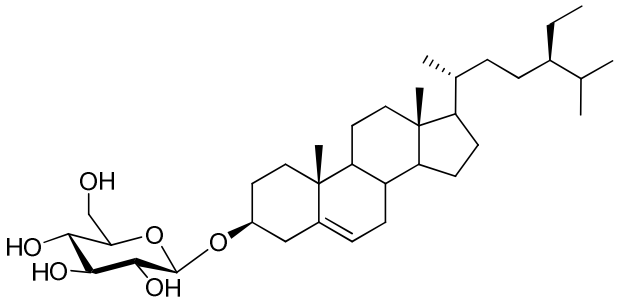
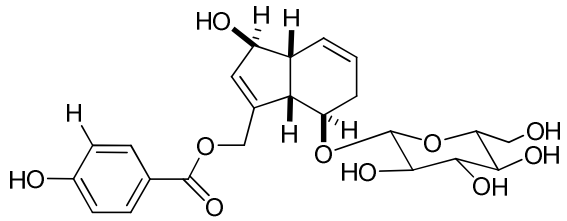
Plant (parts)	Compound	Structures	Chemical group	References
V. quinata (Leaves)	3,5-*O*-Dicaffeoylquinic acid		Polyphenolic	Cheng et al., 2007
V. quinata (Leaves)	Daucosterol		Steroid saponin	Cheng et al., 2007
V. peduncularis (Stem bark)	Agnuside		Iridoid glycoside	Suksamrarn et al., 2002

Table 5 Chemical constituents of *Vitex* species (cont.)

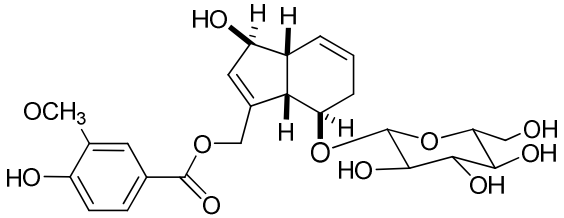
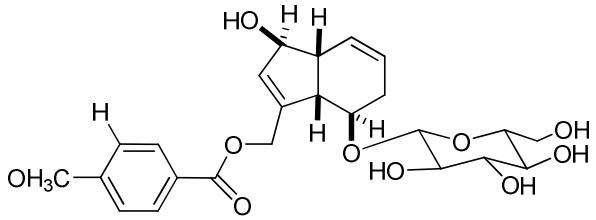
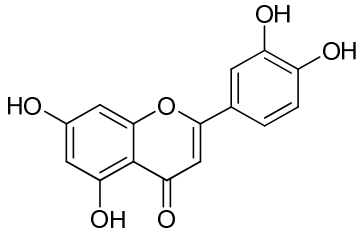
Plant (parts)	Compound	Structures	Chemical group	References
V. peduncularis (Stem bark)	Pedunculariside		Iridoid glycoside	Suksamrarn et al., 2002
V. peduncularis (Stem bark)	Limoniside		Iridoid glycoside	Suksamrarn et al., 2002
V. pinnata (Bark)	Luteolin		Flavonoid	Ata et al., 2009

Table 5 Chemical constituents of *Vitex* species (cont.)

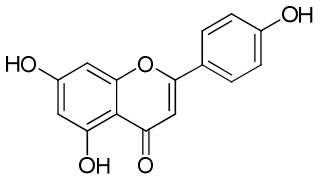
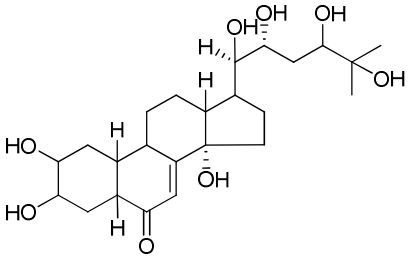
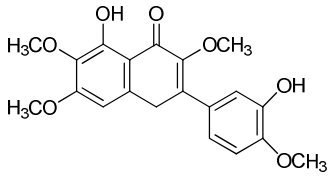
Plant (parts)	Compound	Structures	Chemical group	References
V. pinnata (Bark)	Apigenin		Flavonoid	Ata et al., 2009
V. canescens (Root bark)	24-epi-Abutasterone		Steroid	Suksamrarn et al.,1997
V. agnus-castus (Aerial part)	Casticin		Flavonoid	Mesaik et al., 2009

Table 5 Chemical constituents of *Vitex* species (cont.)

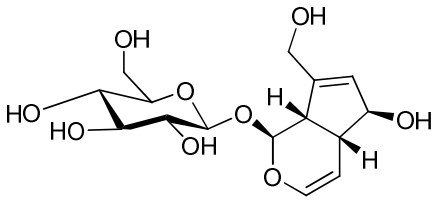
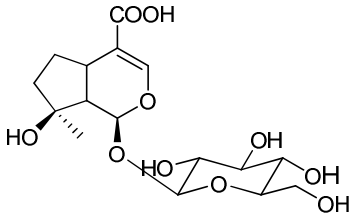
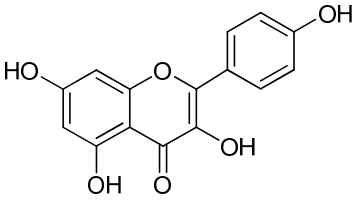
Plant (parts)	Compound	Structures	Chemical group	References
V. agnus-castus (Flowering stem)	Aucubin		Iridoid glycoside	Kuruüzüm-Uz et al., 2003
V. agnus-castus (Flowering stem)	Mussaenosidic acid		Iridoid glycoside	Kuruüzüm-Uz et al., 2003
V. cymosa (Leaves)	Kampferol		Flavonoid	Leitao et al., 2011

Table 5 Chemical constituents of *Vitex* species (cont.)

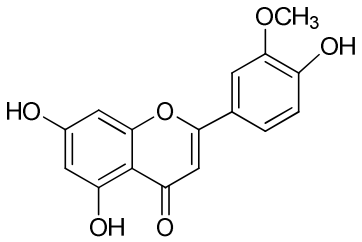
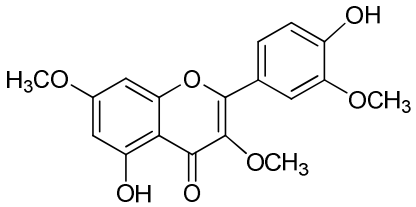
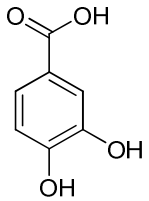
Plant (parts)	Compound	Structures	Chemical group	References
V. cymosa (Leaves)	3'-*O*-Methyl-luteolin		Flavonoid	Leitao et al., 2011
V. cymosa (Leaves)	Pachypodol		Flavonoid	Leitao et al., 2011
V. cymosa (Leaves)	Protocatechuic acid		Phenolic	Leitao et al., 2011

Table 5 Chemical constituents of *Vitex* species (cont.)

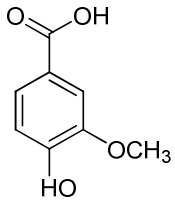
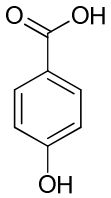
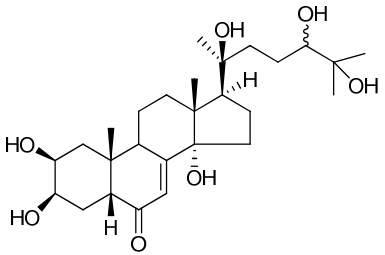
Plant (parts)	Compound	Structures	Chemical group	References
V. cymosa (Leaves)	Vanillic acid		Phenolic	Leitao et al., 2011
V. cymosa (Leaves)	*p*-Hydroxybenzoic acid		Phenolic	Leitao et al., 2011
V. scabra (Stem bark)	24-epi-Pinnatasterone		Steroid	Suksamrarn et al., 2002

Table 5 Chemical constituents of *Vitex* species (cont.)

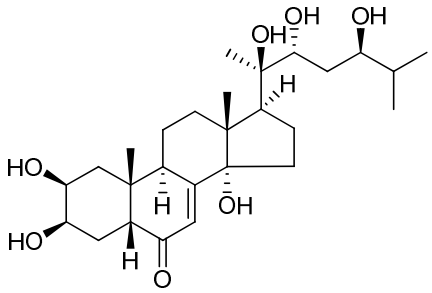
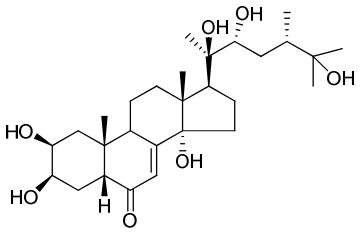
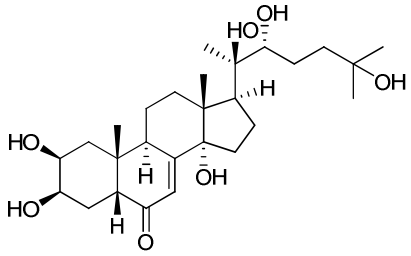
Plant (parts)	Compound	Structures	Chemical group	References
V. scabra (Stem bark)	Pterosterone		Steroid	Suksamrarn et al., 2002
V. scabra (Stem bark)	24-epi-makisterone A		Steroid	Suksamrarn et al., 2002
V. scabra (Stem bark)	20-Hydroxyedsterone		Steroid	Suksamrarn et al, 2002

Table 5 Chemical constituents of *Vitex* species (cont.)

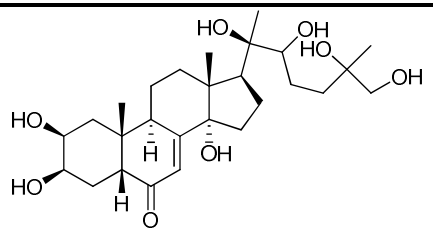
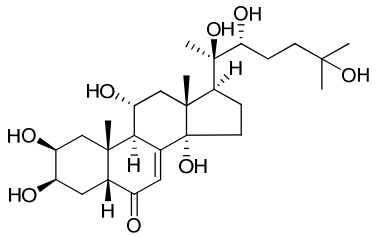
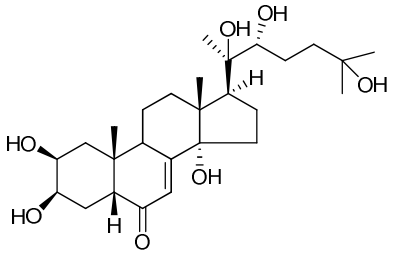
Plant (parts)	Compound	Structures	Chemical group	References
V. scabra (Stem bark)	Polypodine B		Steroid	Suksamrarn et al., 2002
V. scabra (Stem bark)	20,26- Dihydroxyedsterone		Steroid	Suksamrarn et al., 2002
V. scabra (Stem bark)	Turkesterone		Steroid	Suksamrarn et al.,2002

Table 5 Chemical constituents of *Vitex* species (cont.)

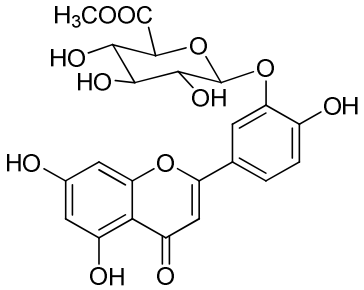
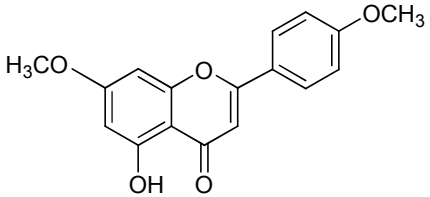
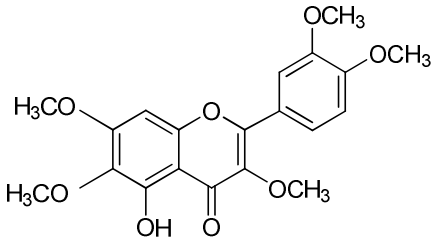
Plant (parts)	Compound	Structures	Chemical group	References
V. negundo (Leaves)	Vitegnoside		Flavonoid glycoside	Gautam et al., 2008
V. negundo (Leaves)	5-Hydroxy-7,4'dimethoxyflavones		Flavonoid	Gautam et al., 2008
V. negundo (Leaves)	5-Hydroxy-3,6,7,3',4'-pentamethoxyflavones		Flavonoid	Gautam et al., 2008

Table 5 Chemical constituents of *Vitex* species (cont.)

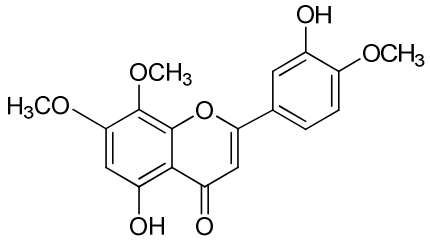
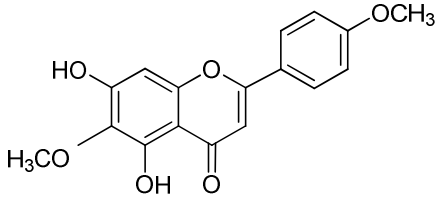
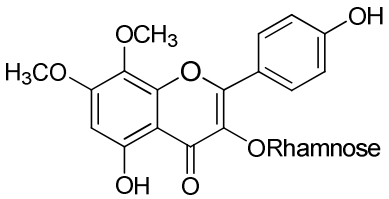
Plant (parts)	Compound	Structures	Chemical group	References
V. negundo (Leaves)	5, 3'-Dihydroxy-7, 8, 4'-trimethoxyflavanone		Flavonoid	Gautam et al., 2008
V. negundo (Leaves)	5,7-Dihydroxy-6,4'dimethoxyflavanone		Flavonoid	Gautam et al., 2008
V. negundo (Leaves)	7, 8-Dimethylherbacetin-3-rhamnoside		Flavonoid	Gautam et al., 2008

Table 5 Chemical constituents of *Vitex* species (cont.)

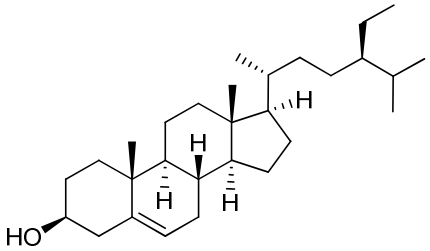
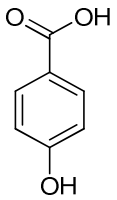
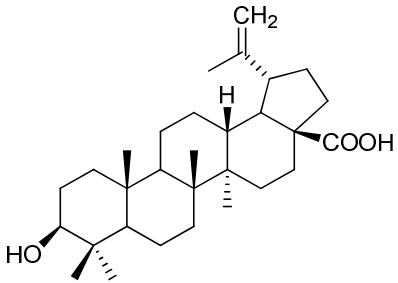
Plant (parts)	Compound	Structures	Chemical group	References
V. negundo (Bark)	β-Sitosterol		Steroid	Dhakal et al., 2009
V. negundo (Bark)	*p*-Hydroxybenzoic acid		Phenolic	Dhakal et al., 2009
V. negundo (Leaves)	Betulinic acid		Triterpenoid	Chandramu et al., 2003

Table 5 Chemical constituents of *Vitex* species (cont.)

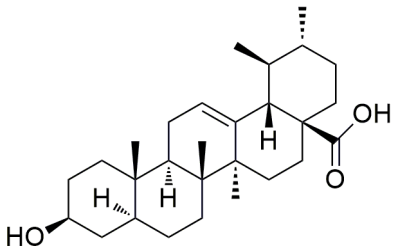
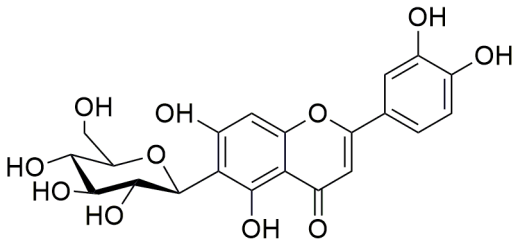
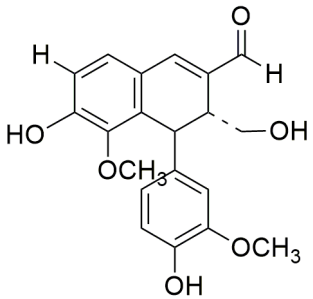
Plant (parts)	Compound	Structures	Chemical group	References
V. negundo (Leaves)	Ursolic acid		Triterpenoid	Chandramu et al., 2003
V. negundo (Leaves)	Iso-orientin		Flavonoid glycoside	Sathiamoorthy et al., 2007
V. negundo (Seed)	Vitedoin A		Phenyldihydronapthalene-type lignin	Ono et al., 2004

Table 5 Chemical constituents of *Vitex* species (cont.)

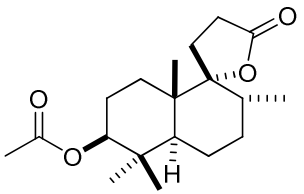
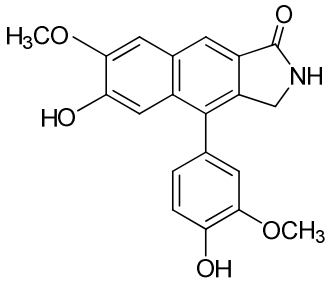
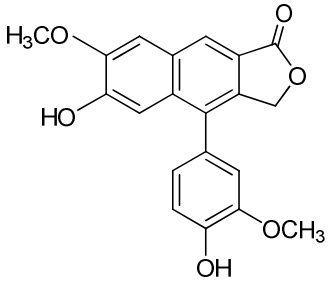
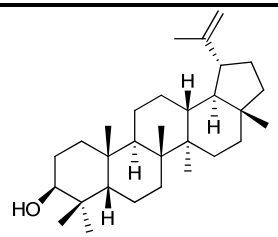
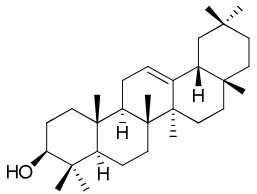
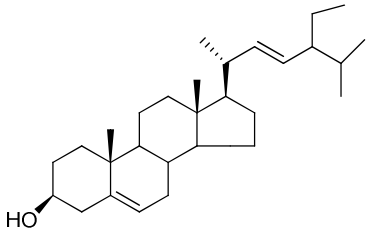
Plant (parts)	Compound	Structures	Chemical group	References
V. negundo (Seed)	Vitedoin B		Diterpenoid	Ono et al., 2004
V. negundo (Seed)	Vitedoamine A		Lignan alkaloid	Ono et al., 2004
V. negundo (Seed)	Detetrahydroconidendrin		Lignan phenol	Ono et al., 2004

Table 5 Chemical constituents of *Vitex* species (cont.)

Plant (parts)	Compound	Structures	Chemical group	References
V. parviflora (Leaves)	β-Amyrin		Triterpenoid	Ragasa et al., 2003
V. parviflora (Leaves)	Lupeol		Triterpenoid	Ragasa et al., 2003
V. parviflora (Leaves)	Stigmasterol		Steroid	Ragasa et al., 2003

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and instruments

The solvents for extractions and isolation including *n*-C_6H_{14}, CH_2Cl_2, EtOAc, *n-BuOH*, EtOH, MeOH and *MeCN were purchased from Labscan Asia co., Thailand. All solvents were as commercial grade which distilled prior uses except n-BuOH and MeCN as analytical grade that no distillation prior uses. Chemical purification was performed mainly by column chromatography using SiO_2 (Silicycle*® Inc., Canada*),* Sephadex LH-20 (*Silicycle*® Inc., Canada), and Diaion HP-20 (Sigma-Aldrich, Germany). Thin layer chromatography was performed on *SiO_2* GF_{254} pre-coated on aluminium sheet (0.20 mm thickness) and visualized by UV lamp (UVGL-58 Handheld, Cambridge, UK) at 254 and 365 nm, by iodine vapor, and by anisaldehyde-sulfuric acid spraying reagent. HPLC experiment was operated on Waters® 1525 with Binary HPLC pump (model), autosample (waters 2707), and photodiode array detector (waters 2998) using semi-preparative reversed-phase column (Luna 10 µM, C_{18} 100 A, 250x10 mm, Phenomenex®, USA). IR spectra were measured with KBr disc on FT-IR spectrophotometer (Spectrum One, Perkin Elmer Ltd., UK). EIMS were recorded as low resolution on MAT 95 XL mass spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, with Varian Unity Inova 500 FT-NMR spectrometer. The chemical shift (δ) of resonance signal as part per million scale (ppm) was compared with residual solvents as internal standard where residual $CHCl_3$, DMSO and MeOH signals were set at 7.25, 2.50, 3.35 ppm for ^{1}H and 77.0, 39.5, and 49.0 ppm for ^{13}C, respectively. Biological activity testing were performed using α-glucosidase and *p*-nitrophenyl-α-*D*-glucopyranoside from Sisco Research Laboratories Pvt. Ltd., India while acarbose, α-amylase and starch azure were purchased from Sigma, Sigma-Aldrich, Germany.

3.2 Plant materials

All plants which were used in preparation of the extracts either as single plant (**Table 6**) or folk preparations (**Table 7**) were selected from Mor Phon's recipe and the recipe of Wang Nam Yen hospital. The plants materials were harvested at Khuan Niang, Songkla province in July 2012 and were bought from Thai traditional drug stores, Hatyai, Songkla. Their specimens were identified and deposited at Faculty of Traditional Thai Medicine, Prince of Songkla University. Plant samples were cleaned by tap water to remove soils and other contaminants. After being air-dried, they were chopped into small piecies and were further dried in hot-air oven at 50-55 $^{\circ}$C for 48 hr. The dried samples were powdered by electrical grinder and these plant powders were kept at dry place and avoid of light.

Table 6 Selected medicinal plants used for α-glucosidase and α-amylase inhibitory activities screening

No.*	Medicinal plants		Parts of use	Source
	Scientific name	Thai name		
1.	*Diospyros rhodocalyx* Kurz.	ตะโกนา	Stem bark	Songkla province
2.	*Mimosa pudica* L.	ไมยราบ	Whole plants	Songkla province
3.	*Pandanus amaryllofolius* Roxb.	เตยหอม	Leaves	Songkla province
4.	*Phyllanthus amarus* Schumach. & Thonn.	ลูกใต้ใบ	Whole plants	Songkla province
5.	*Rhinacanthus nasutus* (L) Kurz.	ทองพันชั่ง	Leaves	Songkla province
6.	*Senna alata (*L.) Roxb.	ชุมเห็ดเทศ	Leaves	Songkla province
7.	*Senna siamea* Lam.	ขี้เหล็ก	Leaves	Songkla province
8.	*Senna siamea* Lam.	ขี้เหล็ก	Heartwood	Songkla province
9.	*Terminalia catappa* L.	หูกวาง	Leaves	Songkla province
10.	*Vitex glabrata* R. Br.	ไข่เน่า	Stem bark	Songkla province
11.	*Zea mays* L.	ข้าวโพด	Corn silk	Songkla province

Table 6 Selected medicinal plants used for α-glucosidase and α-amylase inhibitory activities screening (cont.)

No.*	Medicinal plants		Parts of use	Source
	Scientific name	Thai name		
12.	*Abutilon hirtum* Lam.	ครอบจักรวาล	Whole plants	Songkla province
13.	*Acanthus ebracteatus* Vahl.	เหงือกปลาหมอ	Whole plants	Songkla province
14.	*Andrographis paniculata* (Burm. f.) Wall. ex Nees.	ฟ้าทะลายโจร	Leaves	Songkla province
15.	*Albizia myriophylla* Benth.	ชะเอมไทย	Heartwood	Songkla province
16.	*Capparis micracantha* DC.	แส้ม้าทะลาย	Heartwood	Songkla province
17.	*Caryota mitis Lour.*	เต่าร้าง	Tubers	Songkla province
18.	*Cyperus rotundus* L.	แห้วหมู	Tubers	Songkla province
19.	*Harrisonia perforata* (*Blanco*).	คนทา	Stem	Songkla province
20.	*Homalomena aromatica* Schott.	เต่าเกียด	Tubers	Songkla province
21.	*Hydnophytum formicarum* Jack.	หัวร้อยรู	Tubers	Songkla province
22.	*Imperata cylindrica* (L.) P Beauv.	หญ้าคา	Root	Songkla province
23.	*Lagerstroemia speciosa* (L.) Pers.	อินทนิลน้ำ	Leaves	Songkla province
24.	*Orthosiphon aristatus* Miq.	หญ้าหนวดแมว	Whole plants	Songkla province
25.	*Pandanus odoratissimus* L. f.	ลำเจียก	Stem	Songkla province
26.	*Rhinacanthus nasutus* (L.) Kurz.	ทองพันชั่ง	Whole plants	Songkla province
27.	*Salacia chinensis* L.	กำแพงเจ็ดชั้น	Heartwood	Songkla province
28.	*Smilax corbularia Kunth.*	ข้าวเย็นเหนือ	Rhizome	Songkla province
29.	*Smilax glabra* Roxb.	ข้าวเย็นใต้	Rhizome	Songkla province
30.	*Solanum indicum* L.	มะแว้งต้น	Fruits	Songkla province
31.	*Terminalia arjuna* (*Roxb.*) Wight. & Arn.	สมอเทศ	Fruits	Songkla province
32.	*Terminalia bellirica* (Gaertn) Roxb.	สมอพิเภก	Fruits	Songkla province
33.	*Terminalia chebula* Retz.	สมอไทย	Fruits	Songkla province
34.	*Tinospora cripa* (L.) Miers ex Hook. f & Thomson.	บอระเพ็ด	Stem	Songkla province

Table 6 Selected medicinal plants used for α-glucosidase and α-amylase inhibitory activities screening (cont.)

No.*	Medicinal plants		Parts of use	Source
	Scientific name	Thai name		
35.	*Tribulus terrestris* L.	โคกกระสุน	Whole plants	Songkla province
36.	*Ureceola minutiflora* (Pierre)	มวกขาว	Stem	Songkla province
37.	*Urceola rosea* (Hook. & Arn)	มวกแดง	Stem	Songkla province

*Plants No. 1-11 and 12-37 are selected from Mor Phon's recipe and recipe of Wang Nam Yen hospital, respectively.

Table 7 Major components of the selected Thai Folk Anti-Diabetes Formulas (TFD) and their parts of use

No.	Herbal components		Parts of use
	Scientific name	Thai name	
TFD-01	*Zea mays* L.	ข้าวโพด	Corn silk
TFD-02	*Lagerstroemia speciosa* (L.) Pers.	อินทนิลน้ำ	Leaves
TFD-03	*Senna siamea* Lam.	ขี้เหล็ก	Heartwood
	Table salt	เกลือแกง	
TFD-04	*Vitex glabrata* R. Br.	ไข่เน่า	Stem bark
	Table salt	เกลือแกง	
TFD-05	*Abutilon hirtum* Lam.	ครอบจักรวาล	Whole plants
	Mimosa pudica L.	ไมยราบ	Whole plants

Table 7 Major components of the selected Thai Folk Anti-Diabetes Formulas (TFD) and their parts of use (cont.)

No.	Herbal components		Parts of use
	Scientific name	Thai name	
TFD-06	*Terminalia catappa* L.	หูกวาง	Leaves
TFD-07	*Tectona grandis* L. F.	สัก	Leaves
	Pandanus amaryllofolius Roxb.	เตยหอม	Leaves
TFD-08	*Phyllanthus amarus* Schumach.	ลูกใต้ใบ	Whole plants
	Smilax corbularia Kunth.	ข้าวเย็นเหนือ	Rhizome
	Smilax glabra Roxb.	ข้าวเย็นใต้	Rhizome
TFD-09	*Abutilon hirtum.* Lam.	ครอบจักรวาล	Whole plants
	Acanthus ebracteatus Vahl.	เหงือกปลาหมอ	Whole plants
	Albizia myriophylla Benth.	ชะเอมไทย	Heartwood
	Andrographis paniculata (Burm.f.)	ฟ้าทะลายโจร	Leaves
	Caryota mitis Lour.	แส้ม้าทะลาย	Heartwood
	Cyperus rotundus L.	แห้วหมู	Tubers
	Harrisonia perforata (Blanco). Merr.	คนทา	Stem
	Homalomena aromatic Schott.	เต่าเกียด	Tubers
	Hydnophytum formicarum Jack.	หัวร้อยรู	Tubers
	Imperata cylindrical (L.) P Beauv.	หญ้าคา	Root
	Lagerstroemia speciosa (L.) Per.	อินทนิลน้ำ	Leaves
	Orthosiphon aristatus (Blume) Miq.	หญ้าหนวดแมว	Whole plants
	Pandanus odoratissimus L.f.	ลำเจียก	Stem
	Rhina canthusnasutus (L.) Kurz.	ทองพันชั่ง	Whole plants

Table 7 Major components of the selected Thai Folk Anti-Diabetes Formulas (TFD) and their parts of use (cont.)

No.	Herbal components		Parts of use
	Scientific name	Thai name	
	Salacia chinensis L.	กำแพงเจ็ดชั้น	Heartwood
	Smilax corbularia Kunth.	ข้าวเย็นเหนือ	Rhizome
	Smilax glabra Roxb.	ข้าวเย็นใต้	Rhizome
	Solanum indicum L.	มะแว้งต้น	Fruits
	Terminalia arjuna (Roxb.)	สมอเทศ	Fruits
	Terminalia bellirica (Gaertn.) Roxb.	สมอพิเภก	Fruits
	Terminalia chebula Retz.	สมอไทย	Fruits
	Tinosporacripa (L.) Miers ex. Hook. f &Thomso.	บอระเพ็ด	Stem
	Tribulus terrestris L.	โคกกระสุน	Whole plants
	Urceola rosea (Hook. & Arn).	เถามวกแดง	Stem
	Ureceola minutiflora (Pierre).	เถามวกขาว	Stem

3.3 The crude extracts preparation

3.3.1 Single plant extraction process.

About 30 g of each plant powder was macerated with approximately 150 mL EtOH at room temperature for 2-3 days. The filtrate was collected by filtering though Whatman® No.1 filtering paper and then removed the solvent by rotary evaporator. This maceration was repeated 2-3 times. The crude extracts were finally combined and kept in closed bottles at -20 $^{\circ}$C in the refrigerator until use.

3.3.2 Preparation of the extracts from the selected Thai traditional recipes.

About 150 g of each plant powder was mixed and boiled with approximately 1500 mL of water for about 90 min. After cooling down, it was filtered though a filtering paper (Whatman® No. 1) and the filtrate were freeze-dried to obtain the extract. The extracts were kept in well-closed bottles and stored at -20 °C in the refrigerator until use.

3.4 Extraction and chemical isolation of the stem bark of *V.glabrata*

The 40 g of methanolic extract of *V.glabrata* was dissolved with 1,000 ml of 10% MeOH in water and then was exhaustively and sequentially partitioned with 1,000 ml of each *n*-C_6H_{14}, EtOAc, *n*-BuOH, and H_2O, respectively and finally obtained four fractions including VH (3.01 g), VE (21.15 g), VB (5.80 g), and VW (7.90 g). VH (2.5 g) was chromatograped using SiO_2 and stepwise gradient by starting with 100% *n*-C_6H_{14} to 100% EtOAc and continued by 2.5-40% of MeOH in EtOAc to give six fractions (VH1 to VH6). VH1 (470 mg) was further separated by Sephadex LH-20 using 20% MeOH in CH_2Cl_2 to obtained VH1-1 to VH1-4. VH1-3 (75 mg) was further chromatographed using SiO_2 and 30% EtOAc in *n*-C_6H_{14} to give six fractions (VH1-3A to VH1-3F). VH1-3C (61 mg) was subjected to purify by semi-preparative HPLC using RP-18 column to yield **1** (6.4 mg), **2** (3.7 mg) and **3** (3.6 mg). Fractions VH2 (822 mg) was isolated over Sephadex LH-20 with 20% MeOH in CH_2Cl_2 to afford VH2-1 (92 mg) and VH2-2 (730 mg). VH2-2 was purified over SiO_2 with 30%, 50% and 100% of EtOAc in *n*-C_6H_{14}, respectively to yield ten fractions (VH2-2.1 to VH2-2.10). Fraction VH2-2.4 (163 mg) was subjected to semi-preparative HPLC using RP-18 column and 10% H_2O in MeOH to yield **4** (5 mg), **5** (3 mg).

EtOAc extract (VE) (20 g) was chromatograped over Diaion HP-20 and eluted with stepwise gradient of 50% H_2O in MeOH, 100% MeOH, 80% EtOAc in MeOH and 100% EtOAc, respectively to afford VE1 to VE6. VE3 (1 g) was further subjected to Sephadex LH-20 with 100% MeOH to yield three fractions (VE3A to VE3C). VE3B (400 g) was chromatograped over SiO_2 with 10% MeOH in CH_2Cl_2 to yield five fractions (VE3B.1 to VE3B.5). VE3B.1 (89 mg) was fractionated by Sephadex LH-20 to afford **6** (3 mg).

3.5 Isolated compounds characterization

Pure compounds (1-6) from the isolation process were well characterized by using spectroscopic methods and their data are summarized below.

Lupeol (**1**) **:** white needles; IR (KBr) ν_{max}: 3339, 2945, 2826, 1454, 1379 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ0.72 (*s*), 0.75 (*s*), 0.79 (*s*), 0.90 (*s*), 0.93 (*s*) and 0.99 (*s*), 1.65 (*s*), 3.17 (*dd*), 4.54 (*m*), 4.66 (*d*); ^{13}C NMR (125 MHz, $CDCl_3$): δ14.5, 15.3, 15.9, 16.1, 17.9, 18.2, 19.2, 20.8, 25.0, 27.4, 27.5, 27.9, 29.7, 34.2, 35.5, 37.1, 37.9, 38.6, 38.8, 39.9, 40.7, 42.7, 42.9, 47.9, 48.2, 50.3, 55.2, 78.9, 109.3, 150.9; EIMS *m/z*: 426 $[M]^+$ ($C_{30}H_{50}O$).

β-amyrin (**2**)**:** white solid; IR (KBr) ν_{max}: 3249, 2946, 1464, 1385, 1036, 995 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ0.83 (*s*), 0.93 (*s*), 0.96 (s), 1.01 (*s*), 1.05 (*s*), 1.08 (*s*), 3.19 (*dd*), 5.15 (*t*); EIMS *m/z*: 426 $[M]^+$ ($C_{30}H_{50}O$).

α-amyrin (**3**): white solid; IR (KBr) ν_{max}: 3450, 2946, 1637, 1477, 1036, 993 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ0.77 (*s*), 0.90 (*s*), 0.93 (*s*), 0.95 (*s*), 0.96 (*s*) and 1.03 (*s*), 3.19 (*dd*), 5.09 (*t*); EIMS *m/z*: 426 $[M]^+$ ($C_{30}H_{50}O$).

Betulin (**4**): white needles; IR (KBr) ν_{max} : 3450, 2943, 1452, 1008 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ0.74 (*s*), 0.79 (s), 0.96 (*s*), 0.97 (*s*), 0.99 (*s*), 1.65 (*s*), 3.16 (*dd*), 4.55 (*m*), 4.66 (*d*); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.7, 15.3, 15.9, 16.0, 18.4, 19.0, 20.7, 25.1, 27.0, 27.3, 27.9, 29.1, 29.6, 33.9, 34.1, 37.1, 37.2, 38.6, 38.8, 40.8, 42.6, 47.7, 48.0, 48.7, 50.3, 55.2, 60.5, 78.9, 109.6, 150.6; EIMS *m/z*: 442 $[M]^+$ ($C_{30}H_{50}O_2$).

Betulinic acid (5): white solid; IR (KBr) ν_{max} : 3446, 2941, 1687, 1455, 1377, 1043 cm^{-1}: ^{1}H NMR (500 MHz, CD_3OD): δ 0.74 (s), 0.84 (*s*), 0.94 (*s*), 0.95 (*s*), 0.99 (*s*), 1.02 (*s*), 1.68 (*s*), 4.51 (*br s*), 4.76 (*br s*); ^{13}C NMR (125 MHz, CD_3OD): δ 14.4, 15.5, 15.9, 16.7, 19.4, 19.5, 26.9, 28.0, 28.6, 30.4, 30.7, 31.7, 33.0, 35.6, 38.2, 38.3, 39.6, 39.9, 40.0, 41.9, 48.4, 49.4, 49.5, 52.0, 56.9, 79.6, 110.0, 152.1; EIMS *m/z*: 456 $[M]^+$ ($C_{30}H_{48}O_3$).

Scopoletin (**6**): yellow solid; IR (KBr) ν_{max} : 3341, 1704, 1608, 1566, 1510 cm^{-1} ; ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.77 (*s*), 6.09 (*d*), 6.65 (*s*), 7.10 (*s*), 7.83 (*d*); ^{13}C NMR (125 MHz, DMSO-d_6): δ56.8, 102.0, 109.9, 112.2, 112.3, 146.1, 147.3, 151.6, 153.6, 164.1.

3.6 Biological activity testing

3.6.1 α-Glucosidase inhibitory activity testing

α-Glucosidase inhibitory assay was modified from the method of Bachhawat et al (2011). The reaction mixtures were performed in 96 well plates containing 50 μL of sample mixed with 50 μL of the enzyme (0.57 unit/mL) and incubated at 37 $^{\circ}$C for 10 min. Then, 50 μL of the *p*-nitrophenyl-α-*D*-glucopyranoside (5 mM) as substrate was placed to the mixture and incubated at 37 $^{\circ}$C for 20 min. The reaction was stopped by addition of 50 μL of 1 M Na_2CO_3 solution. The absorbance was measured at 405 nm using UV/Vis absorbance spectrophotometer microplate reader (Biotek Power, Biotex Instrument, Vermont, USA). %Inhibition is calculated by following equation (1);

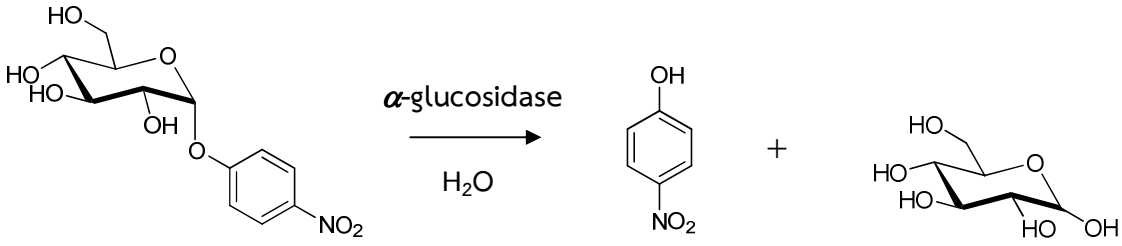
$$\%\text{inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (1)$$

Where $A_{control}$ = absorbance of the negative control at 405 nm

A_{sample} = absorbance of the sample at 405 nm

IC_{50} = a concentrations providing 50 % inhibition were determined from the graph ploted between % inhibitions against sample concentrations.

The principle of α-glucosidase inhibitory assay using spectrophotometic method. The crude extracts were pre-incubated with the enzyme and then adding the *p*-nitrophenyl-α-*D*-glucopyranoside (PNPG) as substrate. The activity of this method was measured by determining the colour of the release of *p*-nitrophenol arising from the hydrolysis of substrate PNPG by α-glucosidase reaction is shown in **Figure** 3 (Rao and Jamil, 2011; Guo et al., 2010).



p-nitrophenyl-*α*-*D*-glucopyranoside (PNPG) *p*-nitrophenol *D*-glucopyranoside

Figure 3 The α-glucosidase-catalyzed reaction using PNPG as a substrate

3.6.2 α-Amylase inhibitory activity testing

α-Amylase inhibitory assay was adapted from the method of Hansawasdi et al (2000). Starch azure (2 mg) was suspended in 200 μL of a 50 mM Tris-HCl buffer (pH 6.9) containing 10 mM $CaCl_2$ and the solution was boiled for 10 min at 100 $^{\circ}$C and cool down. The starch solution was pre-incubated at 37 $^{\circ}$C for 5 min. Sample was dissolved in 100% of dimethyl sulfoxide (1 mL), and was added 100 μL of α-amylase (1.6 unit/mL) solution was used as enzyme in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM NaCl. The reaction was incubated at 37 $^{\circ}$C for 10 min and stopped by adding 500 μL of 50% acetic acid. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4 $^{\circ}$C. The absorbance was measured at 595 nm using by UV/Vis absorbance spectrophotometer microplate reader (Biotek Power, Biotex Instrument, Vermont, USA). %Inhibition is calculated by equation (2);

$$\%\text{inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (2)$$

Where $A_{control}$ = absorbance of the negative control at 595 nm

A_{sample} = absorbance of the sample at 595 nm

IC_{50} = a concentrations providing 50 % inhibition were determined from the graph ploted between % inhibitions against sample concentrations.

The principle of α-amylase inhibitory assay using colorimetric method. The starch azure as the substrate, which is cleaved by α-amylase into soluble colour products

that can be detected by spectrophotometically at 595 nm to give a direct measurement of α-amylase activity in the sample (Rinderknecht et al., 1967).

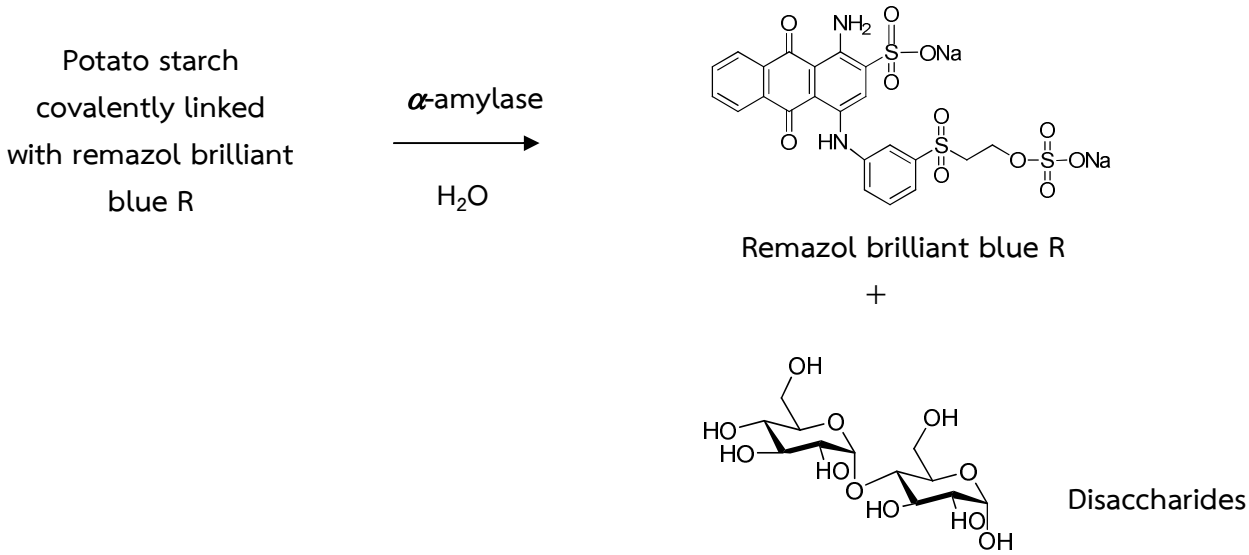


Figure 4 The α-amylase-catalyzed reaction using starch azure as a substrate

3.7 Statistical analysis

The experimental data were reported as mean±SD. To compare them to each other, one-way analysis of variance (one-way ANOVA) was performed with 95% confident level using SPSS software.

CHAPTER 4

RESULTS AND DISCUSSIONS

Traditional medicines have long been utilized from the ancient time. A number of traditional recipes were reported to use successfully to treat both acute and chronic disease. In Thailand, Thai traditional doctors have gained their knowledge thoughout their practice to get effective recipes to cure many diseases, and recorded those recipes in several ways. In this study, we are interested in anti-diabetic recipes of Mor Phon or KromLuang-ChumphonKhetUdomSak ' s recipes and recipes that have been used in Wang Nam Yen hospital. These recipes have been known to be effective in the treatment of diabetic patients, however, non of the reports was found in the mechanism of action of the recipes. Since one of the machisms that could be involved in anti-diabetic treatment is α-glucosidase and α-amylase inhibitory activities which could lead to reduction of blood sugar level, we were then would like to investigate for their potential in anti-diabetic therapy. Hence, the result in this chapter comprise of two parts. The first part is the screening results of α-glucosidase and α-amylase inhibitory activities of each plant which is the composition in the selected anti-diabetic recipes to find out the plants that have high α-glucosidase and α-amylase inhibitory activities. The second part was investigation fot the potential compounds that play major roles in α-glucosidase and α-amylase inhibitory activities of *V. glabrata* which was obtained from the first part.

4.1 Assessment of α-glucosidase and α-amylase inhibitory activities of Thai folk anti-diabetes formularies of Mor Phon and Wang Nam Yen hospital.

4.1.1 α-Glucosidase and α-amylase inhibitory activities of selected plants.

α-Glucosidase and α-amylase inhibitory activities were performed using plant samples which were chosen from two recipes as following.

1) Mor Phon's recipes. This recipe is usually used to treat diabetes mellitus by blood glucose lowering, diuretic, tonic, and stimulate liver or kidney and laxative.

Therefore, plants that have been reported to have one or more of these activities were selected. The eleven items of plant were selected from this recipe.

2) Recipe of Wang Nam Yen hospital. Twenty-six plants were colleted from this recipe.

The results indicated first top ten plants whose ethanolic extracts exhibited high α-glucosidase inhibitory activity were *V. glabrata*, *S. siamea, P. amarus, T. catappa*, *S. chinensis, M. pudica, L. speciosa, D. rhodocalyx, U. minutiflora, and Z. mays.* (**Figure 6**), while IC_{50} of *S. chinensis*, *V. glabrata*, *S. siamea, T. catappa*, and *P. amarus* against α-glucosidase were 5.01±1.51, 11.22±1.70, 14.12±1.59, 15.84±1.34, and 25.11±1.44 μg/mL (**Figure 8**), respectively. The top ten plants that showed high α-amylase inhibitory activity (**Figure 7**) were *P. amarus, M. pudica, V. glabrata*, *D. rhodocalyx, R. nasutus, S. siamea, O. ristatus, T.* arjuna, *T. catappa,* and *C. mitis*. The IC_{50} values of *T. catappa, V. glabrata, P. amarus, S. chinensis, and S. siamea* against α-amylase were 8.91±2.92, 14.54±1.37, 17.78±2.43, 19.56±1.38, and 20.89±1.87 μg/mL (**Figure 9**), respectively.

The result from this investigation demonstrated that the selected plants from recipes of Mor Phon and Wang Nam Yen hospital have potential to be used in diabetic treatment. As previously known that both recipes were used successfully in blood glucose lowering ability in diabetic patients. One of the possible mechanisms to give that affect could be α-glucosidase and α-amylase inhibitory activities. All of the plants that belong to the recipes have shown to have both α-glucosidase and α-amylase inhibitory activities but in different levels. Anti-diabetic recipes of Mor Phon and Wang Nam Yen hospital are either used as single or as combination of a number of plants, therefore, blood glucose lowering ability could be due to the effect of inhibitory activity of single plant or synergistic effect of plants compositions in the recipes. Out finding was similar to the pervious report. For example, ethanolic extract of stem of *S. chinensis* was reported to have α-glucosidase inhibitory activity with IC_{50} values of 5.01±1.51 μg/mL (Yoshikawa et al., 2003). Further isolation of this plant gave mangiferin (**Figure 5)** which was found to have blood glucose lowering ability in streptozotocin-induced diabetes rats (Sellamuthu et al., 2012). The result could support the utilization of stem and roots of *S. chinensis* which has indication to be used for anti-diabetic (Farnsworth et al., 1992). Morover, in Thai traditional medicine as *S.*

chinensis has cool taste, it is therefore can be used to balance the fire element in diabetic patients (สำนักงานปลัดกระทรวงสาธารณสุข, ม.ป.ป.).

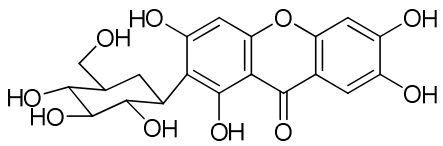


Figure 5 Chemical structure of mangiferin

Leaves of *T. catappa* have tasteless and it has indication for the treatment of tonsillitis, perspiration, rheumatism, digestive disorder and liver disease. Morover, antioxidant, anti-cancer, anti-diabetic, anti-inflammatory, anti-bacterial, anti-tumor of this plant were reported (Mandloi et al., 2013; Akharaiyi et al., 2011; Saroja et al., 2011). Result from our study found that ethanolic extract form leaves of this plant gave α-glucosidase inhibitory activity (IC_{50} values of 15.84±1.34 μg/mL) which was consistent with the report of Anam et al (2009), with IC_{50} values of 3.43±0.98 μg/mL. In addition the aqueous and cold extract of the leaves exhibited anti-hyperglycemic activity in alloxan-induced diabetes rats (Ahmed et al., 2005).

The ethanolic extract of *P.amarus* exhibited strong α-amylase inhibitory activity with IC_{50} value of 17.78±2.43 μg/mL. Similar results were obtained in previous reports of Tamil et al. (2010), which the ethanolic extract showed α-amylase inhibitory activity with IC_{50}value of 36.05±4.01 μg/mL. Nowover, the extract of aerial parts showed excellent α-amylase and α-glucosidase inhibitory activities with IC_{50} values of 2.15±0.1 and 0.2±0.02 mg/mL, respectively (Okoli et al., 2011). Therefore, the hypoglycemic effect was observed when using ehtanolic extract administered to alloxan induced diabetic mice (Shetti et al., 2012). This plant has long been known to have several pharmacological activities including anti-inflammatory, anti-oxidant, anti-arthritic, anti-diabetic and hepatoprotective activities (Kiemer et al., 2003). In china and India, a decoction of whole plant have been used to promote urination and treat jaundice (Wiart et al., 2006). In Thai traditional medicine, this plant has bitter taste and is used for the treatment of fever, digestive disorder, jaundice and liver disease and promotes diuretic (สำนักงานปลัดกระทรวงสาธารณสุข, ม.ป.ป.).

The ethanolic extract of heartwood of *S. siamea* showed potent α-glucosidase and α-amylase inhibitory activities with the IC_{50} values 14.12±1.59 and 20.89 ±1.87 µg/mL, respectively. Our finding is the first report of α-glucosidase and α-amylase inhibitory activities of heartwood of this plant. An α-glucosidase inhibitory activity of this plant was previously reported by Mun'im et al (2013), but their test sample was the ethanolic extract from leaves with IC_{50} of 28.4 ppm lower than acarbose (503.91 ppm). Hypoglycemic effect was found in the administration of methanolic extract of flower of this plant in alloxan induced diabetic rats (Pushpavathi et al., 2013). In Thai traditional medicine, this plant has bitter taste and indications for the treatment of fever, insomnia, diabetes and diuresis, haemagogue, promote fire element and as tonic (สำนักงานปลัดกระทรวงสาธารณสุข, ม. ป. ป; Tripathi et al., 1991).

The ethanolic extract from bark of *V. glabrata* showed excellent activities against both α-glucosidase and α-amylase enzymes. It IC_{50} values were 11.22±1.70 and 14.54±1.37 µg/mL, respectively. This is the first report on the activity of this plant. In Thai traditional medicine, this plant has astringent taste and has been used to treat digestive disorder, anti-diabetes and anthelmintic. It is therefore the result from our study could be used to support the indication. The theory of Thai traditional medicine classified the herbal medicinal by taste. Normally, herbal can be divided into ten tastes: astringent, oily, salty, sweet, bitter, toxic, sour, hot/spicy, fragrant/cool and tasteless. As four elements are associated with several organs and several tastes. The medicinal plants from Mor Phon's recipes and recipe of Wang Nam Yen hospital known to have anti-diabetes activity. The results of this study demonstrated that the selected plants which have strong activities against both enzymes are bitter, tasteless and fragrant/cool. The bitter taste is known to be good for circulatory system, blood system, promoting digestive system, and stimulating the liver, gall bladder, stimulating the pancreas to release the enzyme, promoting appetite, and to counteract heart disease. According to the phytochemical study the bitter taste was found in alkaloid and glycoside. Alkaloids have bitter taste and have been reported to show effect on the *nervous system, digestive system, blood circulatory system, and act as anti-cancer, and anti-inflammatory* (Jacobsen and salguero, 2003; วุฒิ วุฒิธรรมเวช, พ.ศ. 2540). From the literature review found that diabetes is originated from deficiency of fire element. Therefore, to treat diabetes patients, bitter taste medicinal plants must be employed for

stimulating and help to adjust tonicity of the fire element and tasteless medicinal plants is used for increasing diuretic. It may decrease high blood glucose levels.

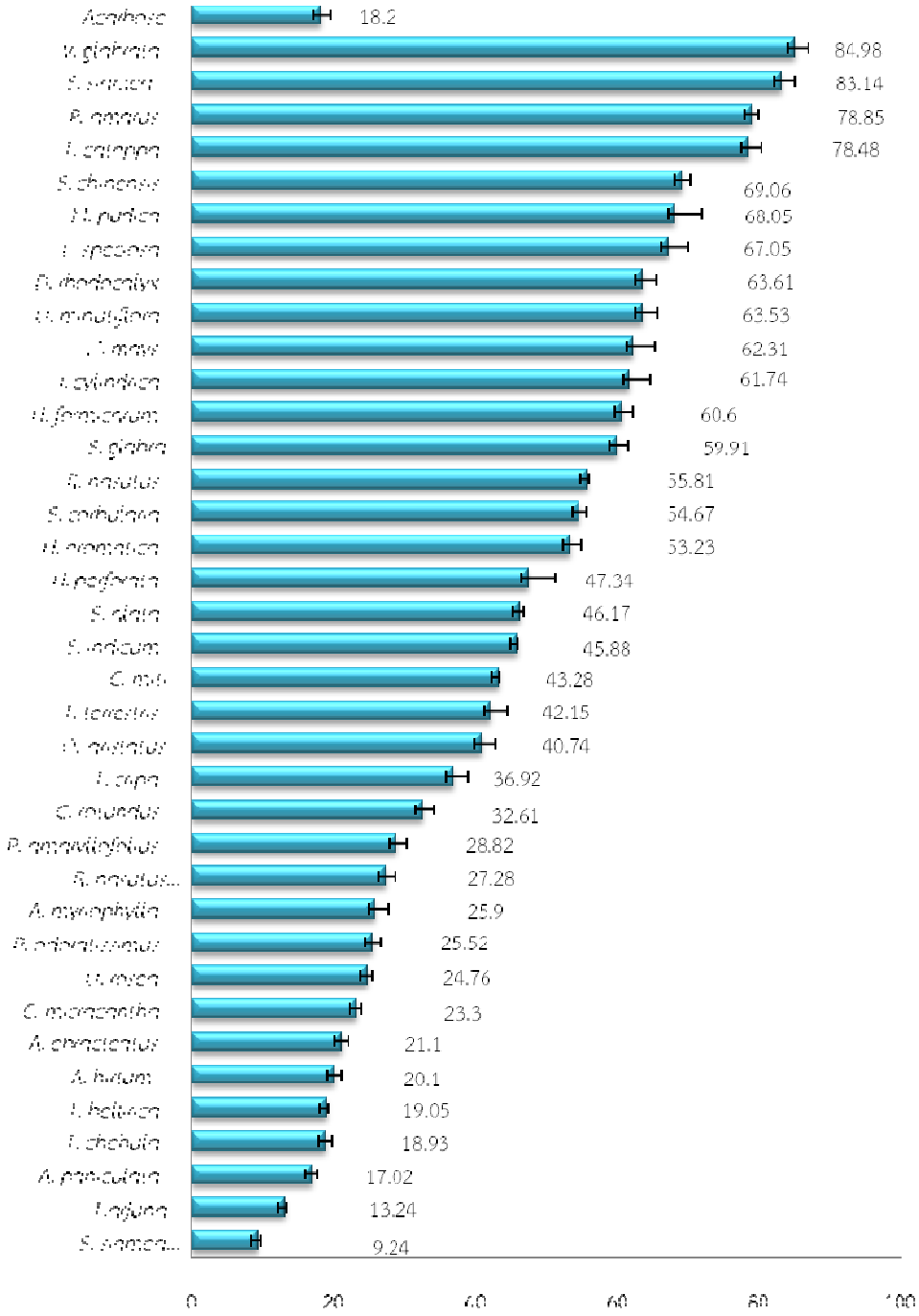


Figure 6 Percentage of α-glucosidase inhibitory activity of the selected thirty-seven herbs

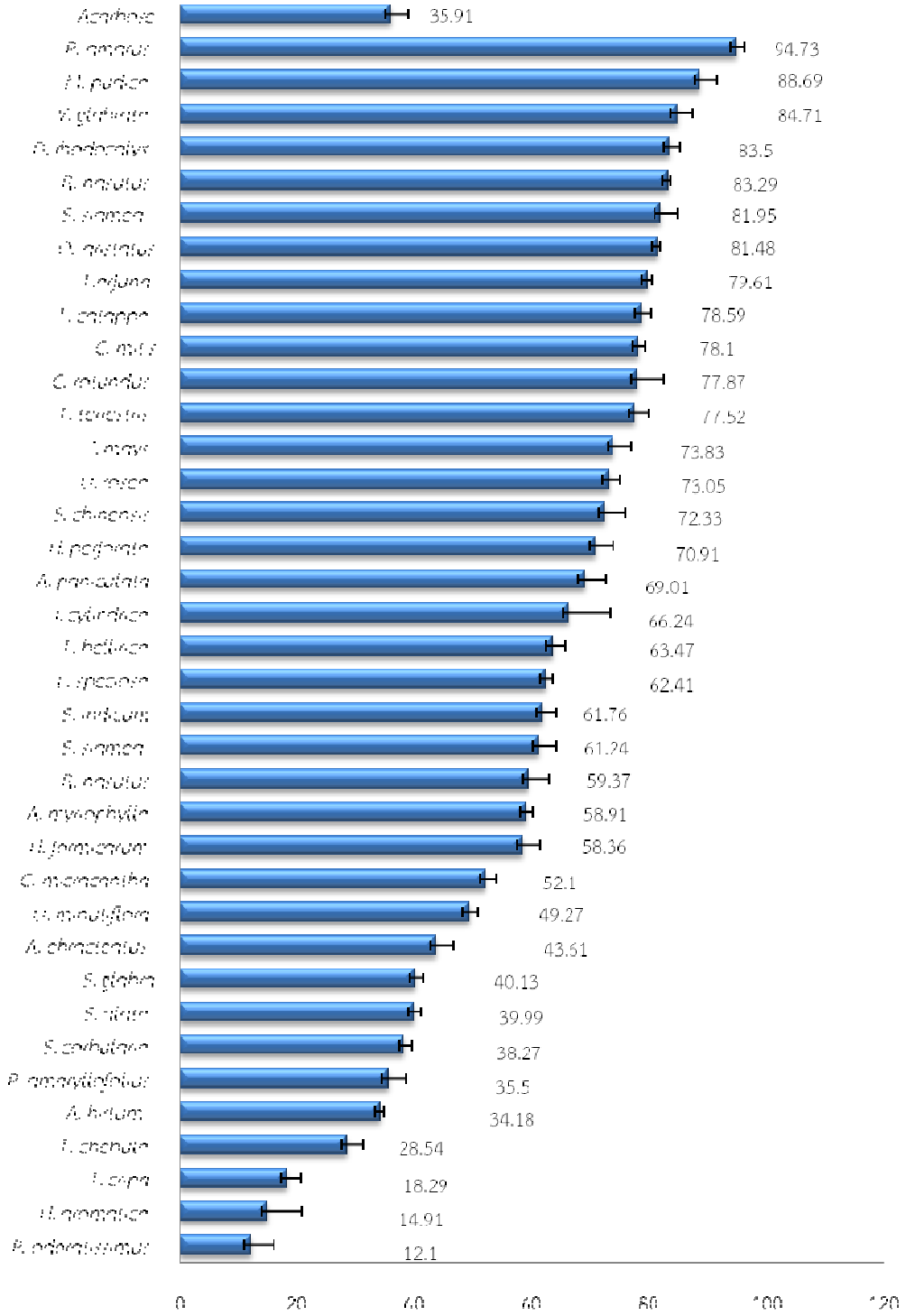


Figure 7 Percentage of α-amylase inhibitory activity of the selected thirty-seven herbs

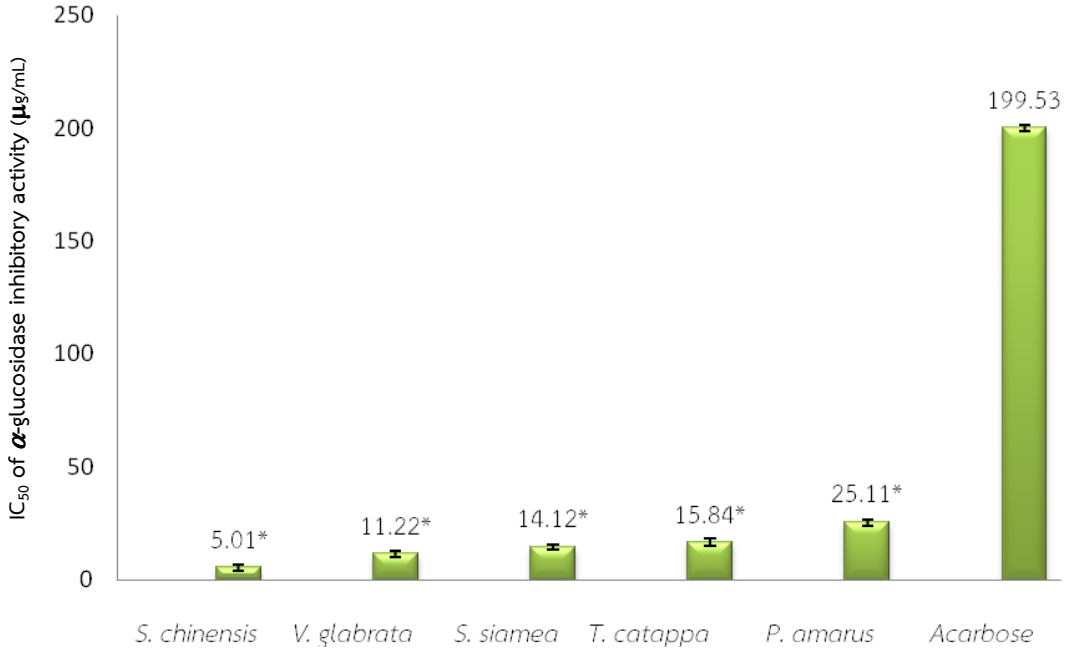


Figure 8 The IC_{50} of α-glucosidase inhibitory activity of five samples selected from Thai folk antidiabetes formularies. * at $p<0.05$.

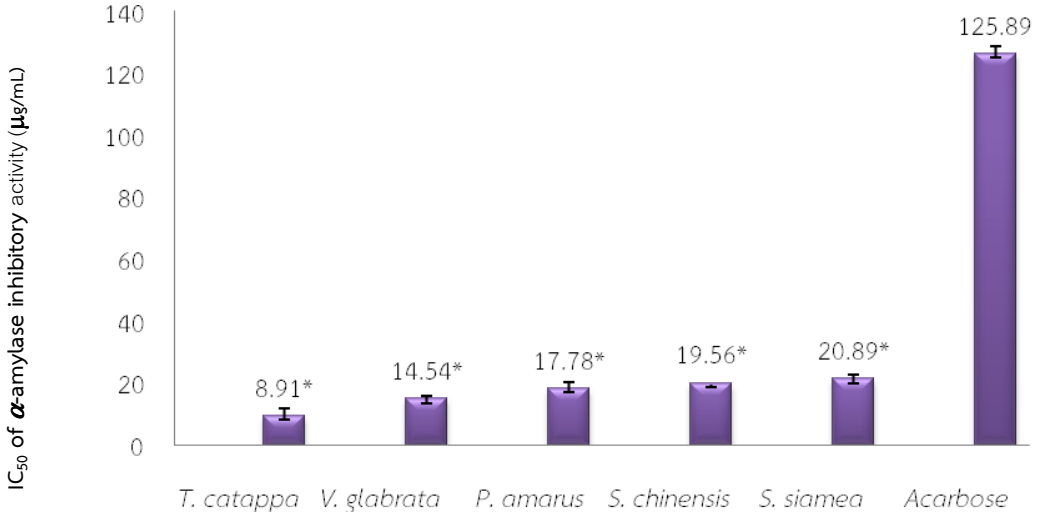


Figure 9 The IC_{50} of α-amylase inhibitory activity of five samples selected from Thai folk antidiabetes formularies. * at $p<0.05$.

4.1.2 α-Glucosidase and α-amylase inhibitory activities of selected formulas from Mor Phon's recipe and the recipe of Wang Nam Yen hospital.

Many recipes of Thai traditional medicines which have been used for the treatment of various diseases usually come from traditional doctors or ancient textbooks. Most of recipes contain many medicinal plants, however some of them may consist of only one medicinal plant. Thai folk medicinal formulas have long been used successfully to treat patients, especially Mor Phon's recipe and the recipe of Wang Nam Yen hospital which were prescribed to treat patients with diabetes. Nine anti-diabetes folk medicinal formulas from these recipe were investigated for α-glucosidase and α-amylase inhibitory activity. The selection process is considered from the results of the preliminary screening tests in section 4.1.1. The plants composition which showed strong α-glucosidase and α-amylase inhibitory activities such as *V. glabrata*, *S. siamea, T. catappa*, *P. amarus*, *L. speciosa, Z. mays, M. pudica* except *P. amaryllofoliu* showed weak activity. The procedure for preparation of the decoction was performed according to the prescribed procedure of the recipes.

The result (**Table 8**) indicated that all nine Thai folk anti-diabetes formulas decoctions exhibited both α-glucosidase and α-amylase inhibitory. The strong activities of α-glucosidase inhibitory were found using extracts from formulas TFD-02 and -08 with IC_{50} of 1.99 ±2.87 and 2.81 ±3.04 µg/mL, respectively, whereas strong activities of α-amylase inhibitory were achieved from the formula TFD-02 and -04, with the IC_{50} values of 12.58±2.63 and 14.12±4.15 µg/mL, respectively. All formulas exhibited inhibitory activity significantly better than acarbose at $p<0.05$ (positive control) which showed the IC_{50} values against α-glucosidase and α-amylase of 199.53±1.72 and 125.89±2.72 µg/mL, respectively as shown in **Table 8**.

Interestingly, the best formula from this result was TFD-02 which contained leaves of *L. speciosa*. This finding was supported by the results from previous study indicating that aqueous and methanolic leave extracts of *L. speciosa* were found to have high activity against α-glucosidase (% inhibition of 22% at a concentration of 1 mg/mL) (Rungprom et al., 2010). The formula TFD-08 which consisted equal amount of *P.amarus, S. corbularia, and S.glabra* showed high activity against α-glucosidase which their % inhibition

valves were 78.85±0.95%, 54.67±1.81% and 59.91±2.97%, respectively at a concentration of 25 µg/mL. Eventhough, *S. corbularia* and *S.glabra* showed moderate activity against α-glucosidase but formula TFD-08 still showed high activity against α-glucosidase. That could be due to *P.amarus* constituent of this formula which has high activity played a major role in the activity.

The formula of TFD-05 containing *A. hirtum* and *M. pudica* showed moderate activity against α-glucosidase and weak activity against α-amylase (**Table 8**). From the results of screening test (**Figure 6-7**) when tested individually, *A. hirtum* showed weak activity (% inhibition of 20.1±1.45% at a concentration of 25µg/mL) while *M. pudica* showed high activity (% inhibition of 68.05±1.67% at a concentration of 25µg/mL) (**Figure 6**). The reason of the moderate activity of formula TFD-05 may be due to antagonistic effect upon mixing of the two plants. The formulas TFD-03 (*S. siamea* (heartwood), and table salt) and -04 (*V. glabrata* (stem bark) and table salt) displayed strong activity against both enzymes. Strong inhibitory activity of both plants was also observed when each plant was tested individually. *V. glabrata* gave α-glucosidase and α-amylase inhitory activities with IC_{50} of 11.22±1.70 and 14.54±1.37 µg/mL, respectively. The IC_{50} values of *S. siamea* against α-glucosidase and α-amylase inhitory activities were 14.12±1.59 and 20.89±1.87 µg/mL, respectively. Additional of table salt to both formula of TFD-03 and TDF-04 may provide positive effect in diabetic treatment, since, it is known in Thai traditional medicine that table salt would help in tonicity adjustment of four elements. Morover, table salt could counteract with the bowel disease, therefore, blood glucose lowering effect could be a result (สำนักงานปลัดกระทรวงสาธารณสุข, ม.ป.ป).

The formula TFD-07 consists of *P. amaryllofolius* and *T. grandis* displayed weak activity against α-glucosidase and moderate activity with α-amylase. The same result was obtained from individually testing of each plant (**Figure 6-7**). In the previous report of Sasidharan et al. (2011) demonstrated that *P. amaryllofolius* (leaves) extract could induce the regenerative β-cells of pancreas, therefore, blood glucose reduction was achieved. Moreover, leaves of *T. grandis* displayed significant anti-hyperglycemic activity in streptozotocin-induced diabetic rat which gave comparable result to metformin antidiabetic

drug (Shukla et al., 2010). However, no report was found in *T. grandis* α-glucosidase and α-amylase inhibitory activities, our finding are therefore the first to report this activity.

Formula TFD-09 was selected from recipe of Wang Nam Yen hospital composes of twenty-six medicibal plants (**Table 7**). The result displayed strong both activities against α-glucosidase and α-amylase inhibitory activities. When each plant was tested individually, each plant showed varying in activities as shown in **Figure 6-7**. However, when the formula TFD-09 was tested, the result indicated high inhibitory activities. This may be due to synergistic effect of the chemical constituents of these plants.

In 2011, Rojjanarattanangkoo and Srijaroen investigated for total phenolic and flavonoid contents of the decoction of TFD-09 pill found that it contained total phenolic content 316.3±6 mg/g gallic acid equivalent and flavonoid content 88.18±0.46 mg/g rutin equivalent. Moreover, they found that the decoction of this formula have moderate antioxidant activity with IC_{50} of 78.34 µg/mL when tested using DPPH radical scavenging assay. It was hence summarized that flavonoids the major content of the formula could play a major role in antioxidant activity.

Our present study indicated that all nine formulas except TFD-07 and -05 exhibited high potential inhibitory activity against α-glucosidase and α-amylase. These provide scientific information to support the use of these formulas by Thai traditional doctor (Mor Phon' recipe and the recipe of Wang Nam Yen hospital) for treatment of diabetes. Furthermore, the results from the screening activity, the stem bark of *V. grablata* exhibited strong α-glucosidase and α-amylase inhibitory activities and this was the first report of anti-diabetic activity of this plant. It is therefore utilization of this formula in diabetic treatment could be due to the antioxidant property of the formula. Further, utilization of stem bark of *V. glabrata* would interesting to find the active compound which play a major role in these activities. Therefore, the phytochemical investigation was performed by several chromatographic techniques.

Table 8 IC_{50} of α-glucosidase and α-amylase inhibitory activities of nine Thai Folk Anti-Diabetes Formulas (TFD)

No	IC_{50} (µg/mL)	
	α-glucosidase	**α-amylase**
TFD-01	17.78 ±3.58	28.18±3.04
TFD-02	1.99 ±2.87	12.58±2.63
TFD-03	3.54 ±3.77	19.95±2.62
TFD-04	6.30 ±2.31	14.12±4.15
TFD-05	24.54 ±6.99	121.29±2.47
TFD-06	3.98±7.78	79.43±4.39
TFD-07	75.68±3.12	39.81±2.08
TFD-08	2.81 ±3.04	20.79±2.59
TFD-09	4.46±2.18	35.67±1.89
Acarbose	199.53±1.72	125.89±2.7

4.2 Phytochemical study of the stem bark of *V.glabrata*

The dried powder of stem barks of *V.glabrata* (400 g) was extracted using maceration with MeOH. The methanolic crude extract was exhuaustively partitioned by the sequence in polarity using *n*-C_6H_{14}, EtOAc, *n*-BuOH and water (**scheme 1**). Each fraction was obtained after evaporation in 3.01, 21.15, 5.8, and, 7.9 g, respectively. These fractions were further determined for their α-glucosidase and α-amylase inhibitory activities. The result **(Table 9)** demonstrated that hexane and ethyl acetate fractions provided high inhibitory activity against both α-glucosidase and α-amylase. Hexane fraction at 25 µg/mL gave 83.86 ± 1.03 and 67.86 ± 2.45 %inhibition of α-glucosidase and α-amylase, respectively. Ethyl acetate fraction at 25 µg/mL gave 86.44 ± 2.15 and 63.30 ± 1.34 %inhibition of α-glucosidase and α-amylase, respectively. Moderate activity of *n*-buthanoland water fractions against both enzymes were achieved. It is therefore, hexane and ethyl acetate fractions were selected for further isolation by chromatographic techniques.

Isolation process of hexane fraction was displayed in **scheme 2**. The fraction was first isolated using silica gel column chromatography eluted sequentially from 100% hexane to ethyl acetate followed by 2.5-40% methanol in ethyl acetate. Two fractions were obtained from the first step, VH1 (400 mg) and VH2 (822 mg). Both fractions were further purified by sephadex-LH20 using 20% methanol in dichloromethane as the diluent to provide VH1-3 (75 mg) and VH2-2 (730 mg), respectively.

VH1-3 was then subjected to silica gel column chromatography using 30% ethanol in hexane as the mobile phase to yield VH1-3C (61 mg) which was further purified by reverse phase column chromatography using 100% methanol as the mobile phase. Three pure compounds (compound 1-3) were obtained from HPLC purification process and they were characterized by H^{1}-NMR, C^{13}-NMR, IR and EIMS techniques. From the spectroscopic data and compared to that previously reported data (Reynolds et al., 1986; Lima et al., 2004; Rao et al., 2012; Kushiro et al., 1998) found that compound 1-3 were lupeol, β-amyrin and α-amyrin, respectively.

VH2-2 was further purified by using silica gel column chromatography using as sequence of mobile phase start from 30% ethanol in hexane, 50%ethyl acetate in hexane and 100% ethyl acetate to provide V2-2.4 (163 mg). The latter was further purified by HPLC using 10% water in methanol as a mobile phase to give two pure compounds, compounds 4 and 5. The spectroscopic data revealed that compound 4 and 5 were betulin and betulinic acid. These compounds were previously reported to isolate from *Betula pendula* and the spectroscopic data were similar that we obtained (Tinto et al., 1992; Kwon et al., 2003; Macias et al., 1994)

Another fraction that is used for isolation was ethylacetate fraction (VE, 20g) (**Scheme 3**). It was first isolated by using Diaion HP-20 column chromatography using sequential of 50% water in methanol, 80% ethylacetate in methanol and 100% ethyl acetate as the eluents. VE3 (1g) was achieved and subjected to further purify by sephadex LH-20 column chromatography using 100% methanol as the mobile phase to provide VE3B in 400 mg. The latter was further separated by silica gel column chromatography using 10% methanol in dichloromethane as the mobile phase gave VE3B.1 in 89 mg. Pure compound 6

(3 mg) was finally obtained by purification of the VE3B.1 by sephadex LH-20 using methanol as the eluent.

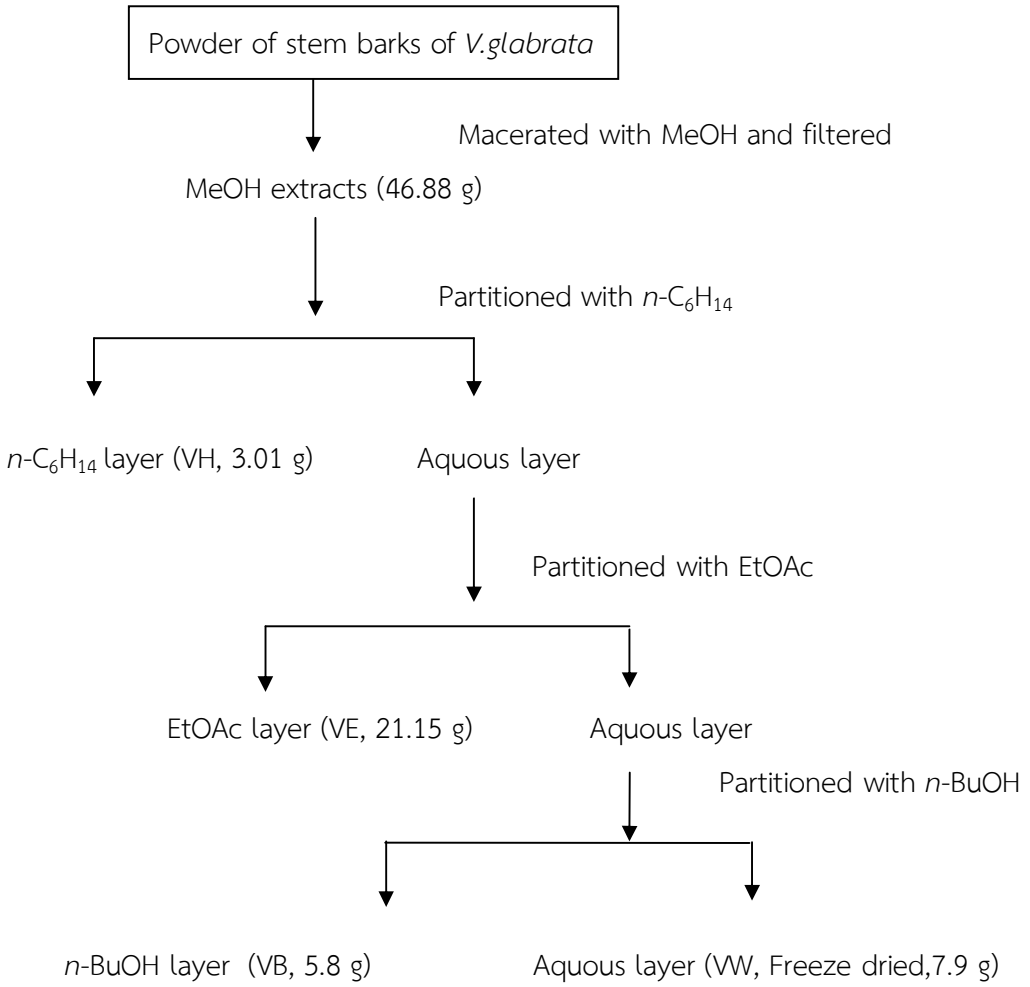
Compound 6 was characterized by H^{1}-NMR, C^{13}-NMR, FT-IR and EIMS and compared the spectroscopic data **(Table 13)** with the previously reported data (Silva et al., 2001; Lin et al., 2002), found the compound 6 is scopoletin.

Pure compounds from our isolation process of hexane and ethylacetate fractions were differed from the compounds which were previously obtained from previous reports. Weraeattanametin and co-workers (1986) could isolate ecdysteroids, 20-hydroxyeecdysone, 7-dehydrocholesterol and 11α,20-dihydroxyecdysone (turkesterone) from bark of *V. glabrata*. Moreover, pterosterone was isolated also from bark of *V. glabrata* reported by Suksamrarn et al., 1999. The different in the types of isolated compounds from our study to that previously reports could be due to the different in isolation process including types of solvent, steps of isolation process and isolation techniques. Interestingly, betulinic acid was found to be detected in the extracts of *Vitex negunda* leaves (Chandramu et al., 2003), β-amyrin and lupeol were isolated from leaves of *Vitex parriflora* (Ragasa et al., 2003). Our study is the first to report the isolation of these pure compounds (compound 1-6) from the stem bark of *Vitex glabrata*. These compounds are non-polar compounds and were well fractionated to the non-polar solvents such as hexane and ethylacetate.

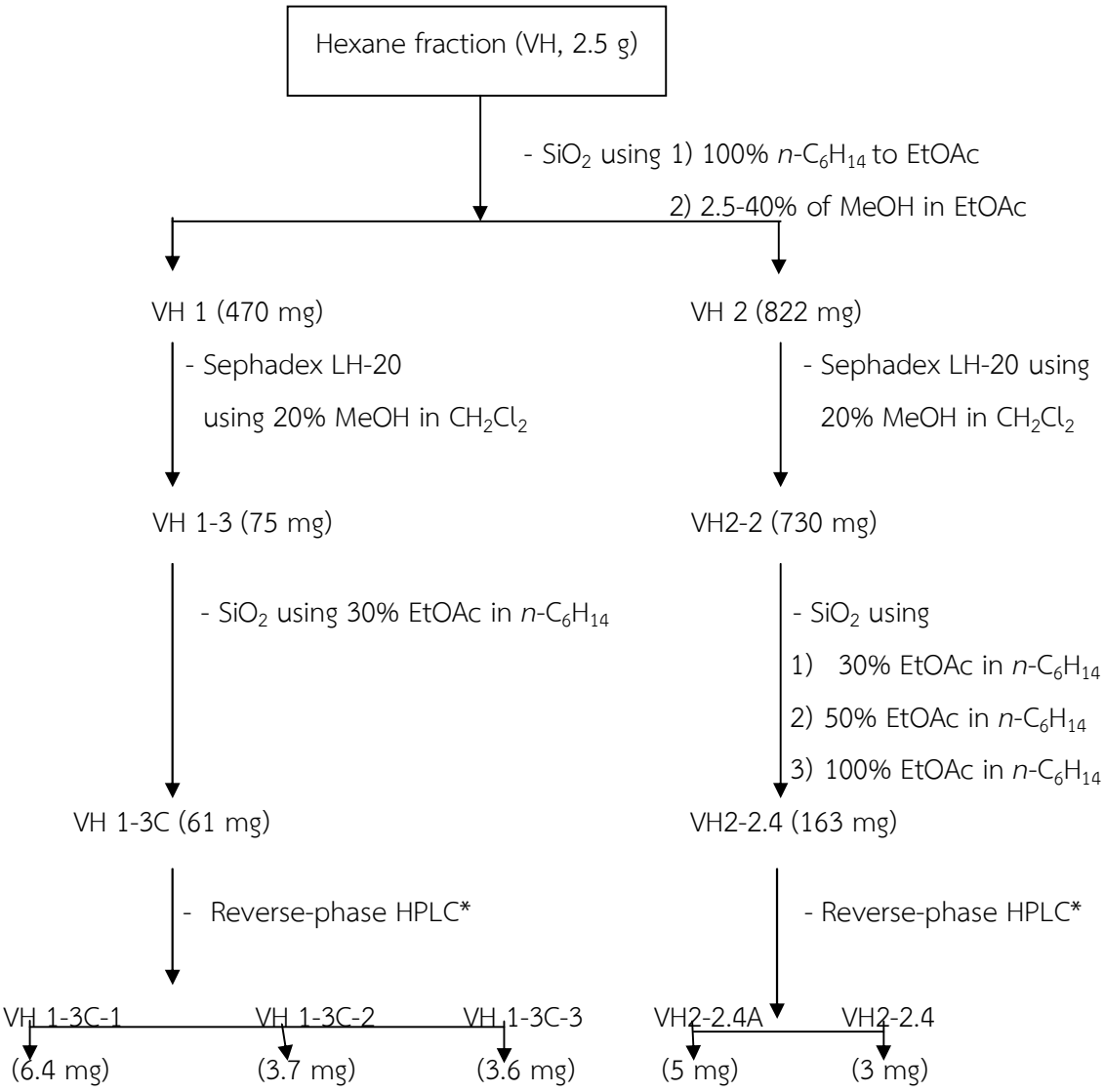
Table 9 Yield and percentage of α-glucosidase and α-amylase inhibitory activities of each fraction obtained from partition process.

Fraction	% yield	% inhibition*	
		α-glucosidase activity	α-amylase activity
Hexane fraction (VH)	7.52	83.86±1.03	67.86±2.45
EtOAc fraction (VE)	52.87	86.44±2.15	63.3±1.34
n-BuOH fraction (VB)	14.50	49.46+3.18	61.5±2.01
Water fraction (VW)	19.75	46.52±1.89	51.91±3.05

*at 25 μg/mL.



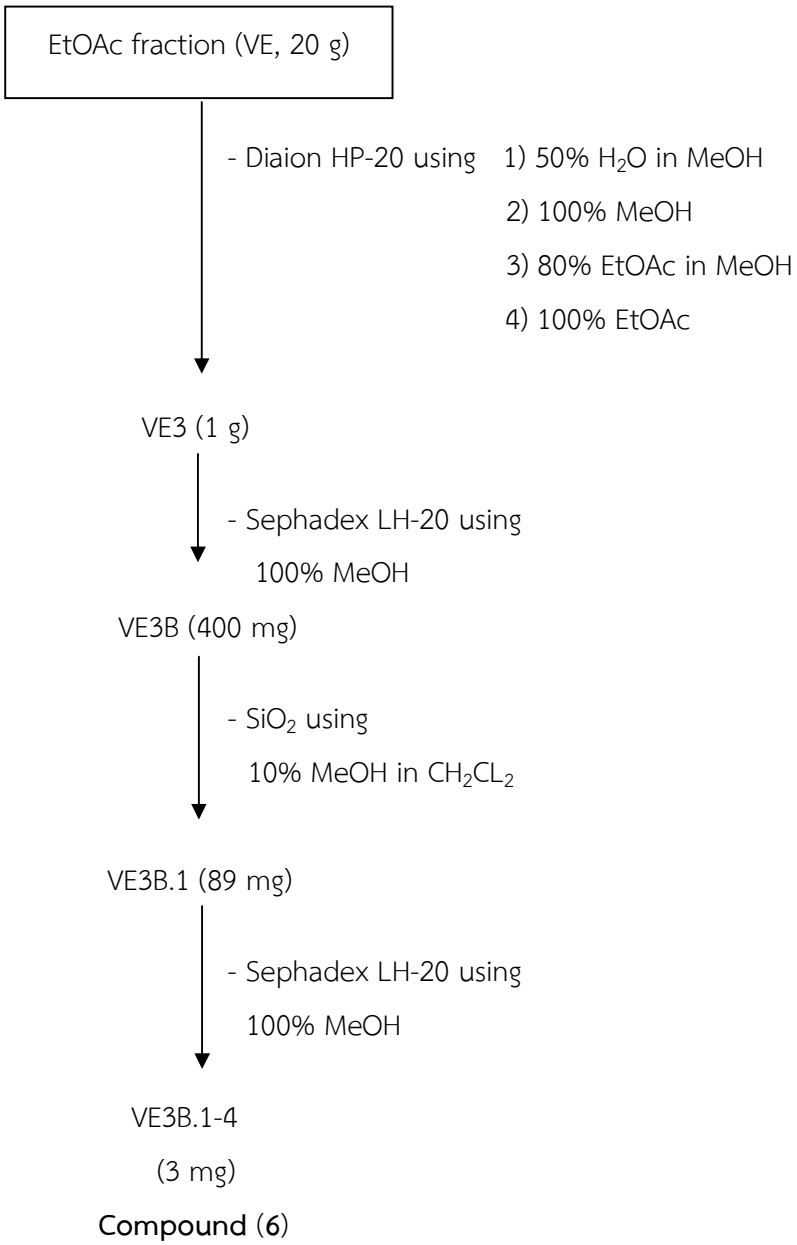
Scheme 1 Diagram displays the extraction process of *V. glabrata* stem bark



Compound (1) Compound (2) Compound (3) Compound (4) Compound (5)

*Note HPLC chromatographic system Column: semi-preparative reversed-phase colum
Pump : Binary HPLC pump
Detector: Photodiode array detector
Frow rate: 4 mL/min
Mobile phase: 100% MeOH
Injection volume: 100 μL

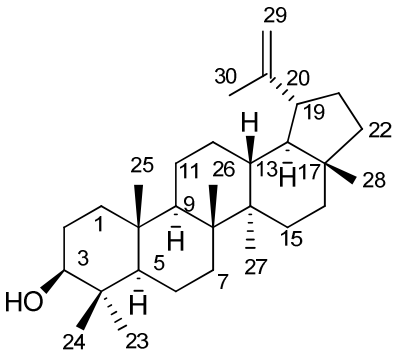
Scheme 2 Diagram displays the isolation process of hexane fraction from *V. glabrata* stem bark



Scheme 3 Diagram displays the isolation process of ethylacetate fraction from *V. glabrata* stem bark

4.2.1 Structure elucidation of pure compounds

4.2.1.1) Lupeol (**1**)



Compound (**1**) was obtained as white needles. It gave a purple spot with anisaldehyle-sulfulic acid test, which suggested that compound **1** could be a triterpene skeleton. It showed a molecular ion peak at *m/z* 426 $[M]^+$ in EIMS, corresponding to a molecular formula $C_{30}H_{50}O$. The IR spectrum showed absorption bands of hydroxyl group (O-H) at 3339 cm^{-1} and double bond (C=C) at 1637 cm^{-1}.

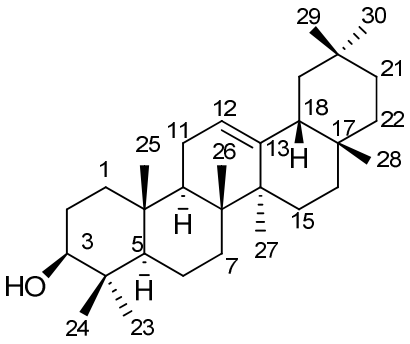
The ^{13}C NMR (125 MHz, $CDCl_3$) spectral data (**Table 10**) of compound **1** exhibited a total of 30 carbon signals including seven methyl (δ 14.5, 15.3, 15.9, 16.1, 17.9, 19.2 and 27.9), eleven methylene (δ 18.2, 20.8, 25.0, 27.4, 27.5, 29.7, 34.2, 35.5, 38.6, 39.9 and 109.3), six methine (δ 37.9, 47.9, 48.2, 50.3, 55.2 and 78.9), and six quaternary carbons (δ 37.1, 38.8, 40.7, 42.7, 42.9, and 150.9), respectively.

The ^1H NMR (500 MHz, $CDCl_3$) spectral data (**Table 10**) showed characteristic of lupane-type triterpenoids as seven methyl singlet signals at δ 0.72, 0.75, 0.79, 0.90, 0.93 and 0.99 including one vinylic methyl at δ 1.65, two protons of an isopropenyl moiety at δ 4.54 (*m*) and 4.66 (*d, J* = 2.5 Hz), and an oxymethine proton at δ 3.17 3.17 (*dd*, *J* = 11.5, 5.0 Hz). From the comparison of its ^1H NMR spectral with the previous report (Reynolds et al., 1986), the compound **1** was identified as lupeol.

Table 10 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compound (**1**) ($CDCl_3$) and lupeol ($CDCl_3$)

Position	Compound (1)		Lupeol	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	0.91 (*m*)	38.6	0.91 (*t*), 1.68 (*d*)	38.7
2	1.55 (*m*)	27.4	1.54 (*q*), 1.61 (*d*)	27.4
3	3.17 (*dd*, 11.5, 5.0)	78.9	3.18 (*dd*)	79.0
4	-	38.8	-	38.8
5	0.66 (*d*)	55.2	0.69 (*d*)	55.3
6	1.38 (*m*), 1.53 (*m*)	18.2	1.39 (*q*), 1.54 (*d*)	18.3
7	1.42 (*m*)	34.2	1.41 (*m*)	34.2
8	-	40.7	-	40.8
9	1.28 (*m*)	50.3	1.28 (*d*)	50.4
10	-	37.1	-	37.1
11	1.25 (*m*), 1.42 (*m*)	20.8	1.25 (*q*), 1.42 (*d*)	20.9
12	1.04 (*m*)	25.0	1.07 (*q*), 1.68 (*d*)	25.1
13	1.67 (*m*)	37.9	1.67 (*t*)	38.0
14	-	42.7	-	42.8
15	1.56 (*m*)	27.5	1.01 (*q*), 1.71 (*t*)	27.4
16	1.53 (*m*)	35.5	1.38 (*t*), 1.49 (*d*)	35.5
17	-	42.9	-	43.0
18	1.36 (*m*)	48.2	1.37 (*t*)	48.2
19	2.34 (*m*)	47.9	2.39 (*m*)	47.9
20	-	150.9	-	150.9
21	1.93 (*m*)	29.7	1.33 (*m*), 1.93 (*m*)	29.8
22	1.19 (*m*), 1.42 (*m*)	39.9	1.20 (*m*), 1.42 (*m*)	40.0
23	0.93 (*s*)	27.9	0.98 (*s*)	28.0
24	0.72 (*s*)	15.3	0.77 (*s*)	15.4
25	0.79 (*s*)	16.1	0.84 (*s*)	16.1
26	0.99 (*s*)	15.9	1.04 (*s*)	16.0
27	0.90 (*s*)	14.5	0.97 (*s*)	14.5
28	0.75 (*s*)	17.9	0.79 (*s*)	18.0
29	4.54 (*m*),4.66 (*d,* 2.5 Hz)	109.3	4.56 (*m*), 4.69 (*m*)	109.3
30	1.65 (*s*)	19.2	1.69 (*s*)	19.3

4.2.1.2) β-amyrin (**2**)

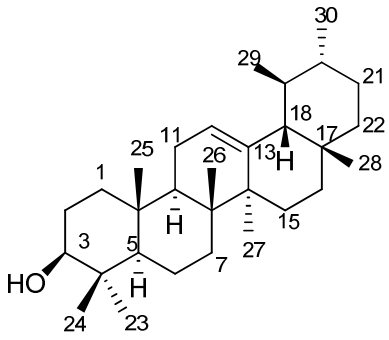


Compound (**2**) was obtained as a white solid. It gave a purple spot with anisaldehyle-sulfuric acid test, which suggested that compound **2** could be a triterpene skeleton. IR spectrum exhibited absorption band of hydroxyl group (O-H) at 3249 cm^{-1}. It showed a molecular ion peak at *m/z* 426 $[M]^+$ in EIMS, corresponding to a molecular formula $C_{30}H_{50}O$. Moreover, compound **2** also displayed a EIMS base peak at *m/z* 218 (100) $[M\text{-}208]^+$. From previous report (Fingolo et al., 2013), it showed that most of the oleanene and ursene triterpene skeletons normally found characteristic base peak at *m/z* 218 in the EIMS spectrum. Look closely for more detail on the mass fragmentation pattern in the EIMS spectrum of compound **2**, it showed that there is a big different ratio of relative intensity between the peaks at *m/z* 189 and 203 (Fingolo et al., 2013). It was a 1:1 ratio of *m/z* 189 and 203 for the α-amyrin, whereas it was 1:2 ratio of *m/z* 189 and 203 for the β-amyrin. EIMS spectrum of compound **2** displayed 1:2 ratio of the peaks between at *m/z* 189 and 203, which strongly suggested that compound **2** could be a β-amyrin.

The ^{1}H NMR (500 MHz, $CDCl_3$) spectral data of compound **2** showed characteristic of olenane-type triterpenoids as eight methyl groups at δ 0.96 (*s*), 1.08 (*s*), 1.05 (*s*), 1.01 (*s*), 0.83 (*s*), 0.93 (*s*), and two unidentified peaks (ranging from 0.70 to 1.00 ppm), an oxymethine proton at δ 3.19 (*dd*, *J* = 11.5, 4.5 Hz) and an cyclic-olefinic proton at δ 5.15 (*t*, *J*

= 4.5 Hz), respectively. From the comparison of its ^{1}H NMR spectral with the previous report (Lima et al., 2004; Rao et al., 2012), the compound was identified as β-amyrin.

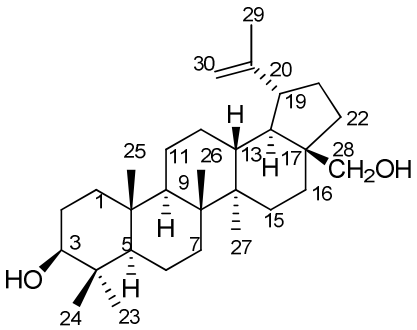
4.2.1.3) α-amyrin (**3**)



Compound (**3**) was obtained as a white solid. It gave a purple spot with anisaldehyle-sulfuric acid test, which suggested that compound **3** could be a triterpene skeleton. IR spectrum exhibited absorption band of hydroxyl group (O-H) at 3450 cm^{-1}. It showed a molecular ion peak at *m/z* 426 $[M]^{+}$ in EIMS, corresponding to a molecular formula $C_{30}H_{50}O$. Moreover, compound **2** also displayed a EIMS base peak at *m/z* 218 (100) $[M\text{-}208]^{+}$. EIMS spectrum of compound **3** displayed 1:1 ratio of the peaks between at *m/z* 189 and 203, which strongly suggested that compound **3** could be a α-amyrin ((Fingolo et al., 2013).

^{1}H NMR (500 MHz, $CDCl_3$) spectral data of compound **3** showed characteristic oleanane- type triterpenoids as six methyl singlet signals at δ 0.77 (*s*), 0.90 (*s*), 0.93 (*s*), 0.95 (*s*), 0.96 (*s*) and 1.03 (*s*), an oxymethine proton at δ 3.19 (*dd*, *J* = 11.0, 5.0 Hz) and an cyclic-olefinic proton at δ 5.09 (*t*, *J* = 4.0 Hz), respectively. From the comparison of its ^{1}H NMR spectral with the previous report (Kushiro et al., 1998), the compound was identified as α-amyrin.

4.2.1.4) Betulin **(4)**



Compound (4) was obtained as white needles. It gave a purple spot with anisaldehyle-sulfuric acid test indicating that compound **4** could be a triterpene skeleton. The IR spectrum showed absorption bands of hydroxyl group (O-H) at 3450 cm^{-1} and double bond (C=C) at 1643 cm^{-1}. It showed a molecular ion peak at *m/z* 442 $[M]^+$ in EIMS, corresponding to a molecular formula $C_{30}H_{50}O_2$.

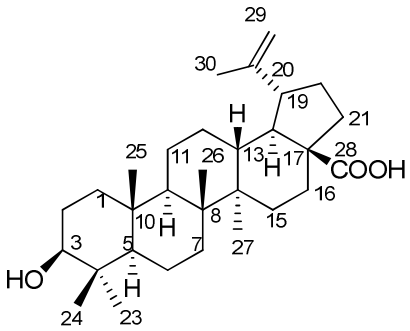
The ^{13}C NMR (125 MHz, $CDCl_3$) spectral data (**Table 11**) of compound **4** exhibited a total of 30 carbon signals at δ 14.7, 15.3, 15.9, 16.0, 18.4, 19.0, 20.7, 25.1, 27.0, 27.3, 27.9, 29.1, 29.6, 33.9, 34.1, 37.1, 37.2, 38.6, 38.8, 40.8, 42.6, 47.7, 48.0, 48.7, 50.3, 55.2, 60.5, 78.9, 109.6 and 150.6. Thus it appeared in the ^{13}C NMR spectrum signals at δ 150.9 and 109.3 ppm corresponding to olifinic carbons at C-20 and C-29 of the lupane skeleton.

The ^{1}H NMR (500 MHz, $CDCl_3$) spectral data (**Table 11**) of compound **4** showed characteristic oleanane- type triterpenoids as six methyl singlet signals at δ 0.74, 0.79, 0.96, 0.97, 0.99 and 1.65, two protons of an isopropenyl moiety at δ 4.55 (*m*) and 4.66 (*d*, *J* = 2.0 Hz), the non-equivalent oxymethylene protons at δ 3.78 (*dd*, *J* = 11.0, 1.5 Hz) and 3.30 (*d*, *J* = 11.0 Hz), and an oxymethine proton at δ 3.16 (*dd*, *J* = 11.5, 5.0 Hz), respectively. From the comparison of its ^{1}H NMR spectral with the previous report (Tinto et al., 1992), the compound was identified as betulin. The pharmacological activity of betulin has been reported as anti-inflammatory, anti-malaria, and anti-tumor (Sami et al., 2006).

Table 11 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compound (**4**) ($CDCl_3$) and betulin ($CDCl_3$)

Position	Compound (4)		Betulin	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	0.90 (*m*), 1.71 (*m*)	38.8	0.90 (*m*), 1.70 (*m*)	38.8
2	1.57 (*m*)	27.3	1.58 (*m*)	27.2
3	3.16 (*dd*, 11.5, 5.0)	78.9	3.19 (*dd*, 10.8, 5.1)	78.9
4	-	38.8	-	38.9
5	0.66 (*m*)	55.2	0.68 (*m*)	55.3
6	1.40 (*m*)	18.4	1.41 (*m*)	18.3
7	1.05 (*m*), 1.39 (*m*)	34.1	1.04 (*m*), 1.40 (*m*)	34.3
8	-	40.8	-	40.9
9	1.26 (*m*)	50.3	1.27 (*m*)	50.4
10	-	37.1	-	37.2
11	1.33 (*m*), 1.49 (*m*)	20.7	1.28 (*m*), 1.46 (*m*)	20.9
12	1.68 (*m*)	25.1	1.68 (*m*)	25.3
13	1.64 (*m*)	37.2	1.67 (*m*)	37.3
14	-	42.6	-	42.7
15	1.13 (*m*), 1.61 (*m*)	27.0	1.11 (*m*), 1.66 (*m*)	27.0
16	1.23 (*m*), 1.95 (*m*)	29.1	1.20 (*m*), 1.98 (*m*)	29.2
17	-	48.0	-	47.8
18	1.59 (*m*)	48.7	1.60 (*m*)	48.8
19	2.35 (*dt*)	47.7	2.38 (*dt*, 10.5, 5.7)	47.8
20	-	150.9	-	150.6
21	1.91 (*m*)	29.6	1.91 (*m*)	29.8
22	1.80 (*m*), 1.88 (*m*)	33.9	1.80 (*m*), 1.88 (*m*)	34.0
23	0.96 (s)	27.9	0.97 (s)	28.0
24	0.74 (s)	15.3	0.76 (s)	15.4
25	0.79 (s)	15.9	0.82 (*s*)	16.1
26	0.99 (s)	16.0	1.02 (*s*)	16.0
27	0.97 (s)	14.7	0.98 (*s*)	14.8
28	3.78 (*dd*, 11.0, 1.5), 3.30 (*d*, 11.0)	60.5	3.33 (*d*, 10.8), 3.80 (*dd*, 10.8, 1.5)	60.2
29	4.55 (*m*), 4.66 (*d*, 2.0)	109.3	4.58 (*m*), 4.68 (*d*, 2.1)	109.6
30	1.65 (s)	19.2	1.68 (*s*)	19.1

4.2.1.5) Betulinic acid (**5**)



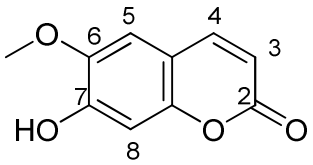
Compound (5) was obtained as white needles. It gave a purple spot with anisaldehyle-sulfuric acid test indicating that compound **5** could be a triterpene skeleton. The IR spectrum showed absorption bands of hydroxyl group (O-H) at 3446 cm^{-1} and carbonyl group at 1687 cm^{-1}. It showed a molecular ion peak at *m/z* 456 $[M]^+$ in EIMS, corresponding to a molecular formula $C_{30}H_{48}O_3$.

The ^{13}C NMR (125 MHz, CD_3OD) spectrum of compound **5** exhibited a total of 30 carbon signals including six methyl (δ 14.4, 15.5, 15.9, 19.4, 19.5 and 28.0), eleven methylene (δ 16.7, 26.9, 28.0, 28.6, 30.4, 30.7, 31.7, 33.0, 35.6, 38.3, and 110.0), six methine (δ 38.2, 48.4, 49.4, 49.5, 52.0 and 79.6), and six quaternary carbons (δ 39.6, 39.9, 40.0, 41.9, 56.9 and 152.1). Thus it appeared in the ^{13}C NMR spectrum signals at δ 152.1 and 110.0 ppm corresponding to olifinic carbons at C-20 and C-29 of the lupane skeleton. The ^{1}H NMR (500 MHz, CD_3OD) spectral data of compound **5** showed characteristic of lupane-type triterpenoids as summarized in **Table 12**. From the comparison of its ^{1}H NMR spectral with the previous report (Kwon et al., 2003; Macias et al., 1994), the compound was identified as betulinic acid.

Table 12 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compound (**5**) (CD_3OD) and betulinic acid (CD_3OD)

Position	Compound (5)		Betulinic acid	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	0.87 (*m*), 1.66 (*m*)	38.3	0.88 (*m*), 1.65 (*m*)	38.7
2	1.56 (*m*), 1.61 (*m*)	28.0	1.57 (*m*), 1.61 (*m*)	27.4
3	3.11 (*dd*)	79.6	3.19 (*dd*)	79.0
4	-	39.6	-	38.9
5	0.70 (*br d*)	52.0	0.69 (*m*)	55.3
6	1.33 (*m*), 1.51 (*m*)	16.7	1.36 (*m*), 1.51 (*m*)	18.3
7	1.39 (*m*)	33.0	1.38 (*m*)	34.3
8	-	40.0	-	40.7
9	1.27 (*m*)	49.5	1.26 (*m*)	50.5
10	-	38.2	-	37.2
11	1.25 (*m*), 1.42 (m)	26.9	1.23 (*m*), 1.43 (*m*)	20.9
12	1.70 (*m*)	28.6	1.69 (*m*)	25.5
13	2.22 (*m*)	39.9	2.22 (*m*)	38.4
14	-	41.9	-	42.4
15	1.15 (*m*), 1.52 (*m*)	30.4	1.15 (*m*), 1.51 (*m*)	30.6
16	1.36 (*m*), 2.26 (*m*)	31.7	1.40 (*m*), 2.25 (*m*)	32.2
17	-	56.9	-	56.3
18	1.61 (*m*)	48.4	1.58 (*m*)	46.9
19	3.00 (*m*)	49.4	3.01 (*m*)	49.3
20	-	152.1	-	150.4
21	1.45 (*m*), 1.89 (*m*)	30.7	1.42 (*m*), 1.91 (*m*)	29.7
22	1.43 (*m*), 1.92 (*m*)	35.6	1.41 (*m*), 1.93 (*m*)	37.0
23	0.95 (*s*)	28.0	0.97 (*s*)	28.0
24	0.74 (*s*)	15.9	0.75 (*s*)	15.4
25	0.84 (*s*)	19.4	0.82 (*s*)	16.0
26	0.94 (*s*)	15.5	0.94 (*s*)	16.1
27	0.99 (*s*)	14.4	0.98 (*s*)	14.7
28	-		-	180.4
29	4.51 (*br s*), 4.76 (*br s*)	110.0	4.61 (*br s*),4.74 (*br s*)	109.7
30	1.68 (*s*)	19.5	1.69 (*s*)	19.4

4.2.1.6) Scopoletin **(6)**



Compound (**6**) was obtained as yellow solid. The UV-Vis spectrum showed absorption bands at 208, 228 and 344 nm. The IR spectrum exhibited characteristic absorption bands of hydroxyl group (O-H) at 3341 and carbonyl group (C=O) at 1704 cm^{-1}.

The ^{1}H NMR (500 MHz, DMSO-d_6) spectrum (**Table 13**) of compound **6** showed two characteristic olefinic protons of coumarin moiety at 7.83 (*d*, *J* = 9.5 Hz) and 6.09 (*d*, *J* = 9.5 Hz), two aromatic protons at δ 7.10 (*s*) and 6.65 (*s*), and a methoxy groups at δ 3.77 (*s*), respectively. The ^{13}C NMR (125 MHz, DMSO-d_6,) spectrum of compound **6** exhibited 10 carbon signals at δ 56.8, 102.0, 109.9, 112.2, 112.3, 146.1, 147.3, 151.6, 153.6 and 164.1. From the comparison of its ^{1}H NMR spectral with the previous report (Silva et al., 2001; Lin et al., 2002), compound was identified as scopoletin.

Table 13 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compound (**6**) (DMSO-d_6) and scopoletin

Position	Compound (6)		scopoletin	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	-	-	-	-
2	-	164.1	-	161.0
3	6.09 (*d*, 9.5)	112.3	6.19 (*d*)	113.3
4	7.83 (*d*, 9.5)	147.3	7.60 (*d*)	146.0
5	7.10 (*s*)	112.2	6.92 (*s*)	112.1
6	-	151.6	-	151.9
7	-	146.1	-	144.6
8	6.59 (*s*)	102.0	6.85 (*s*)	103.7
9	-	109.9	-	110.0
10	-	153.6	-	151.0
6-OMe	3.77 (*s*)	56.8	3.95 (*s*)	56.7

4.3 α-Glucosidase and α-amylase inhibitory activities of pure compounds 1-6.

The pure compounds (compound 1-6) were utilized to investigate for their inhibitory activities against α-glucosidase and α-amylase using similar procedure to that used with the extracts. Acarbose was used as positive control in each experiment.

All compounds found to have both α-glucosidase and α-amylase inhibitory activities but in different extents. Their IC_{50} values are summarized in **Table 14**. The results demonstrated that compounds 1-6 have high α-glucosidase inhibitory activity and much higher than acarbose positive control. Lupeol (**1**) and β-amyrin (**2**) were found to be the best among the tested compounds. From α-amylase inhibitory activity testing all compounds except betulin (**4**) showed high activity and again much better than acarbose.

Table 14 IC_{50} of pure compounds for α-glucosidase and α-amylase inhibitory activities

Compound	IC_{50} of α-glucosidase inhibitory activity		IC_{50} of α-amylase inhibitory activity	
	(µg/mL)	(µM)	(µg/mL)	(µM)
Methanol extract	11.22±1.70		14.54±1.37	
Lupeol (**1**)	3.16±1.34	7.4±1.34	19.95±1.06	46.81±1.06
β-myrin (**2**)	3.71±2.10	8.69±2.10	13.80±1.56	32.33±1.56
α-amyrin (**3**)	6.02±0.91	14.1±0.91	28.18±1.82	66.07±1.82
Betulin (**4**)	10.02±1.24	22.58±1.24	126.80±1.59	284.35±1.59
Betulinic acid (**5**)	12.86±0.97	28.15±0.97	56.23±1.54	123.12±1.54
Scopoletin (**6**)	10.14±1.96	52.76±1.96	15.86±2.53	82.53±2.53
Acarbose	199.53±1.72	308.63±1.72	125.89±2.72	194.72±2.7

The results of this study indicated that compounds of pentacyclic triterpene group were strong inhibitors of α-glucosidase and α-amylase, especially lupeol which showed the best activity. Lupeol has been reported to be isolated from several plant species and has been previously reported to have α-glucosidase inhibitory activity. Lupeol was isolated from acetone extract from acetone extract of *Terminalia sericea* and exhibited strong α-glucosidase and α-amylase inhibitory activities with IC_{50} values of 54.5 and 140.72 μM, respectively (acarbose IC_{50} values were 93.2 and 60.25 μM, respectively) ((Nkobole et al., 2011). It was also isolated from the acetone extract of root bark of *Euclea undulate* and found to have α-glucosidase inhibitory activity with IC_{50}value of 14.69 μM (acarbose IC_{50} value was 7.35 μM) (Deutschlander et al., 2011). Moreover lupeol has been reported to exhibit various pharmacological activities such as anti-artgritic, anti-microbial, anti-protozoal, anti-cancer, anti-diabetic, anti-inflammatory, cardioprotechtivie, and hepatoprotective activity (Siddique and Saleem, 2011). It is therefore our finding would be used to support the activity of this compound to treat diabetes.

Both α-amyrin and β-amyrin have been reported to exhibit various pharmacological activities including anti-microbial, anti-inflammatory, anti-ulcer, anti-oxidation and anti-diabetic (Santos et al., 2012; Vazquez et al., 2012). As for anti-diabetic activity, these compounds exhibited moderate to good α-glucosidase and α-amylase inhibitory activities (Wei et al., 2012) in agreement with the present study.

This is the first report of isolation of betulin from *V. glabrata,* This compound has been reported to exhibit several activities such as anti-proliferative, anti-inflammatory, anti-cancer and anti-diabetic activity (Dehelean et al., 2012; Sharma et al., 2011). The study of betulin isolated from *Betula pendula* showed to have inhibition of α-amylase activity and was found to be a competitive inhibitor (Llyina et al., 2014). However the study of betulin isolated from *Ruellia tuberasa* indicated that it was an effective inhibitor on α-amylase activity from pancrease of rat and human and the molecular docking result showed that this compound was a non-competitive inhibitor (Wulan et al., 2014). The pharmacological activities of betulinic acid as anti-inflammatory, anti-malarial, anti- HIV, anti-cancer, anti-bacterial, anti-obese and anti-diabetic activities were reported (Moghaddam et al., 2012). The study of anti-diabetic activity on the α-glucosidaseand α-amylase inhibitory activity of

betulinic acid isolated from *Dillenia indica* showed significant activities for both enzymes (Kumar et al., 2013).

Scopoletin was the coumarin type compound. It's activities including anti-oxidant, anti-inflammatory, anti-cholinesterase, anti-diabetic activities were previously reported (Mogana et al., 2013). Scopoletin was isolated from *Hortia longifolia* and found to have strong inhibitory activity against α-glucosidase enzyme with IC_{50} value of 4.63 μM (Queiroz et al., 2013).

The results from inhibitory activities demonstrated that all pentacyclic triterpenes showed the high inhibitory activity against α-glucosidase and much better than acarbose and scopoletin. When their chemical structures were taken into the consideration, pentacyclic triterpene moiety could be an important part in the interaction with the active site of α-glucosidase enzyme. Morover, the hydroxyl group in their structures may play an important role in enzyme binding capacity which may be due to through the hydrogen bonding. It is interesting that pentacyclic triterpenes having substitions at C-17 either ethanolic (betulin) or carboxylic (betulinic acid) groups, reduction in inhibitory activities upto 2-3 times were observed. It is therefore, the substitution at C-17 position could prevent inhibitor enzyme interaction which may be due to steric effect. Scopoletin may have less interaction with the active site of enzyme which may be due to the small size of molecule that could be eassly complete by the substrate. Similar results were observed with α-amylase inhibitory activity, where pentacyclic triterpenes could inhibit α-amylase better than acarbose except betulin. It is therefore compounds with pentacyclic triterpene moiety with hydroxyl group at C-13 still be an important structure in inhibition activity. It is however the compounds having substitution at C-17 position (betulin and betulinic acid) may reduce the interaction with the active site of α-amylase enzyme. Ethanolic substitution group at C-17 position of betulin may prevent the interaction of the compound with the active site of enzyme better than carboxylic group in betulinic acid, resulting in higher IC_{50} valve (Uddin, 2012)

Scopoletin have moderate inhibitory activity against α-amylase. It could inhibit α-amylase less than lupeol, β-amyrin and α-amyrin but better than betulin and betulinic

acid. It is therefore, type of scopoletin-α-amylase interaction would be difeerent from scopoletin-α-glucosidase interaction.

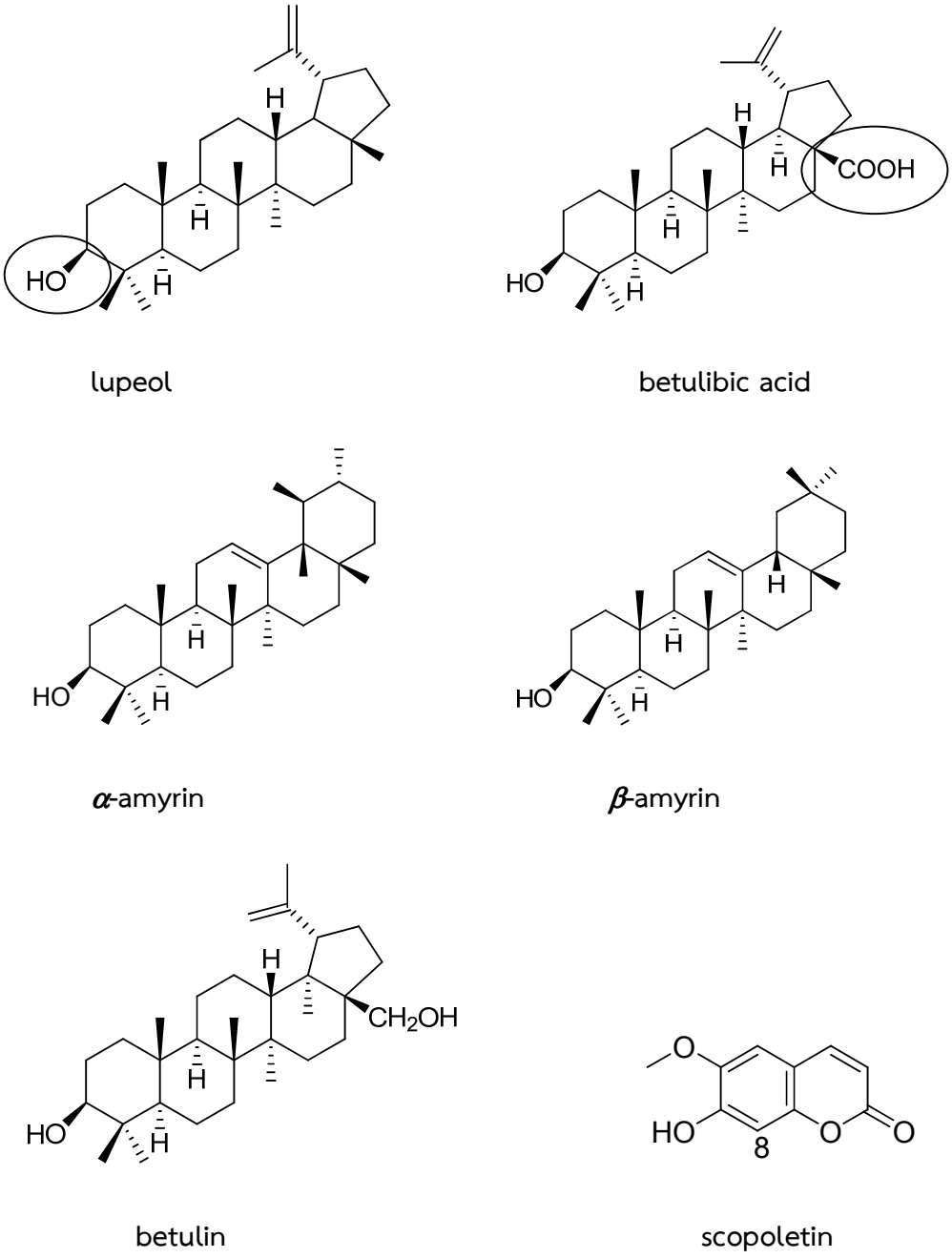


Figure 10 The chemical structures of compound (1-6)

CHAPTER 5

CONCLUSION

The present have demonstrated the information of anti-diabetic mechanism of the selected Thai folk medicinal recipes. MorPhon's recipes consisted of 11 plants whereas the recipe from Wang Nam Yen hospital contained 26 plants. Eight recipes MorPhon's anti-diabetic recipes and one recipe from Wang Nam Yen hospital were selected to test for their ability in α-glucosidase and α-amylase inhibitory activites. The result demonstrated that all recipes have α-glucosidase and α-amylase inhibitory activities and their IC_{50} values of all recipes were much lower than acarbose, a positive control. These result could support the potential utilization of their recipes indiabetic treatment. Blood glucose lowering ability after administration of these recipes could be α-glucosidase and α-amylase inhibitor which are composed in the recipes.

Testing α-glucosidase and α-amylase inhibitory activity of each plant showed that all plants have inhibitory capacity but in different extent. *S. chinensis*, *V. glabrata*, *S. siamea, T. catappa*, and *P. amarus* exhibited most five potent of α-glucosidase inhibitory activity, by IC_{50} 5.01±1.51, 11.22±1.70, 14.12±1.59, 15.84±1.34, and 25.11±1.44 μg/mL, respectively and *T. catappa, V. glabrata, P. amarus, S. chinensis, and S. siamea* exhibited most five potent of α-amylase inhibitory activity, by IC_{50} 8.91±2.92, 14.54±1.37, 17.78±2.43, 19.56±1.38, and 20.89±1.87 μg/mL, respectively. These plants have astringent, biter, tasteless or fragrant/cool tastes which are known in Thai traditional medicine that these tastes could help to regulate the fire element in diabetic patients and would result in blood glucose lowering capacity. The result from this study could be used to support the successfulness of utilization of both anti-diabetic recipes in scientific point of view. However, the utilization of these recipes in diabetic treatment should be prescribed by traditional doctors consideration or used in combinational therapy.

The most active plant, *V. glabrata* gave the highest α-glucosidase and α-amylase inhibitory activity, it was further phytochemically isolated and obtained five triterpenoids compounds including, lupeol (**1**), β-amyrin (**2**), α-amyrin (**3**), betulin (4), betulinic acid (**5**), and one coumarin compound, scopoletin (**6**). Lupeol showed the highest α-glucosidase and α-amylase inhibitory activity with IC_{50} 7.4, and 46.81 μM, respectively. Triterpenoids compounds exhibited better inhibitory activity than scopoletin which their structures could be important in enzyme interaction.

This is the first report of isolation of compounds from *V. glabrata* with potential α-glucosidase and α-amylase inhibitory activity. This finding supports the use of this plant by Thai traditional doctors for treatment of diabetes. Furthermore lupeol which showed highest activities are interesting to be further studied in vivo for toxicity and anti-diabetic activity. New drug development can be performed using the finding compounds as the leads compounds for future anti-diabetic treatment.

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APPENDIX

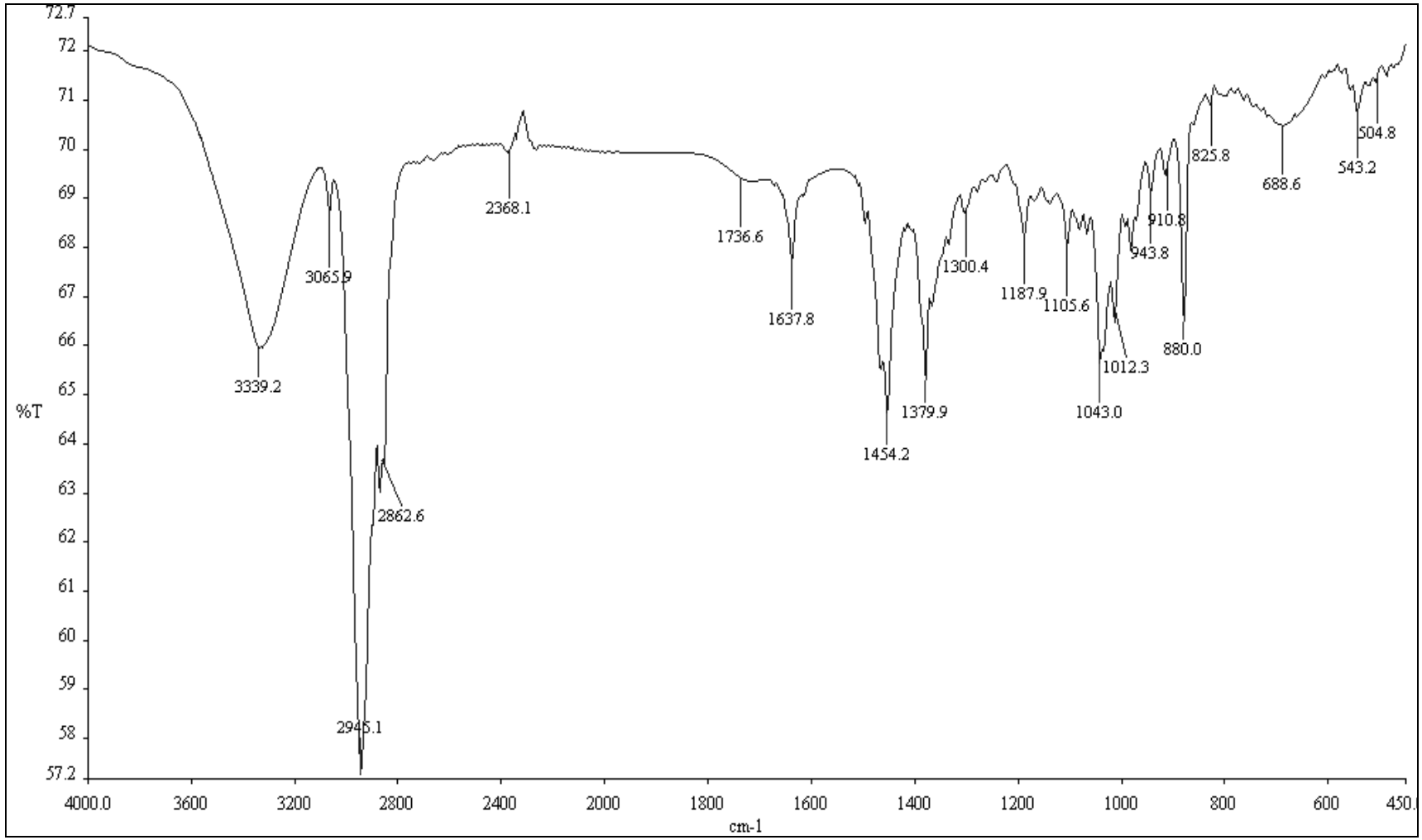
α-Glucosidase and α-amylase inhibitory activities of thirty-seven samples

No.	Medicinal plants			% inhibition*	
	Scientific name	Thai name	Part of use	α-glucosidase	α-amylase
1.	Acarbose			18.59±0.45	33.14±0.79
2.	*Abutilon hirtum* Lam.	ครอบจักรวาล	Whole plants	20.1±1.45	34.18±5.79
3.	*Acanthus ebracteatus* Vahl.	เหงือกปลาหมอ	Whole plants	21.10±3.01	43.61±3.85
4.	*Albizia myriophylla* Benth.	ชะเอมไทย	Heartwood	25.90±4.84	58.91±3.60
5.	*Andrographis paniculata* (Burm. f.) Wall. ex Nees.	ฟ้าทะลายโจร	Whole plants	17.02±0.92	69.01±1.68
6.	*Capparis micracantha* DC.	แส้ม้าทะลาย	Stem	23.30±2.04	52.10±2.0
7.	*Caryota mitis* Lour.	เต่าร้าง	Tubers	43.28±2.31	78.10±3.52
8.	*Cyperus rotundus* L.	แห้วหมู	Tubers	32.61±3.94	77.87±4.71
9.	*Diospyros rhodocalyx* Kurz.	ตะโกนา	Stem bark	63.61±1.09	83.50±2.32
10.	*Harrisonia perforata*	คนทา	Stem	47.34±0.75	70.91±1.19
11.	*Homalomena aromatic*	เต่าเกียด	Tubers	53.23±1.51	14.91±7.37
12.	*Hydnophytum formicarum* Jack.	หัวร้อยรู	Tuers	60.60±1.49	58.36±0.34
13.	*Imperata cylindrica* (L.) P Beauv.	หญ้าคา	Root	61.74±1.16	66.24±0.75
14.	*Lagerstroemia speciosa* (L.) Pers.	อินทนิลน้ำ	Leaves	67.05±0.25	62.41±10.8
15.	*Mimosa pudica* L.	ไมยราบ	Whole plants	68.05±1.67	88.69±0.56
16.	*Orthosiphon aristatus* Miq.	หญ้าหนวดแมว	Whole plants	40.74±0.01	81.48±12.8
17.	*Pandanus amaryllofolius* Roxb.	เตยหอม	Leaves	28.82±2.88	35.50±3.1
18.	*Pandanus odoratissimus* L.f.	ลำเจียก	Stem	25.52±1.63	12.10±7.16
19.	*Phyllanthus amarus* Schum. & Thonn.	ลูกใต้ใบ	Whole plants	78.85±0.79	94.73±1.37

20.	*Rhinacanthus nasutus* (L).	ทองพันชั่ง	Leaves	55.81±1.76	83.29±1.28
21.	*Rhinacanthus nasutus* (L).	ทองพันชั่ง	Whole plants	27.28±1.06	59.37±7.61
22.	*Salacia chinensis* L.	กำแพงเจ็ดชั้น	Stem	69.06±0.78	72.33±2.92

α-Glucosidase and α-amylase inhibitory activities of thirty-seven samples (cont.)

No.	**Medicinal plants**			**% inhibition***	
	Scientific name	**Thai name**	**Part of use**	**α-glucosidase**	**α-amylase**
24.	*Senna siamea* Lam.	ขี้เหล็ก	Leaves	9.24±3.85	61.24±1.25
25.	*Senna siamea* Lam.	ขี้เหล็ก	Heartwood	84.14±0.15	81.95±7.17
26.	*Smilax corbularia Kunth.*	ข้าวเย็นเหนือ	Rhizome	54.67±1.81	38.37±2.24
27.	*Smilax glabra* Wall. ex Roxb.	ข้าวเย็นใต้	Rhizome	59.91±2.99	40.13±7.05
28.	*Solanum indicum* L.	มะแว้งต้น	Fuits	45.88±9.18	61.76±2.9
29.	*Terminalia arjuna* (*Roxb.*) Wight & Arn.	สมอเทศ	Fruits	13.24±1.86	79.61±0.37
30.	*Terminalia bellirica* (Gaertn) Roxb.	สมอพิเภก	Fruits	19.05±3.81	63.47±7.79
31.	*Terminalia catappa* L.	หูกวาง	Fruits	78.48±0.14	78.59±1.84
32.	*Terminalia chebula* Retz.	สมอไทย	Fruits	18.93±1.18	28.54±2.31
33.	*Tinospora cripa* (L.) Miers ex Hook. f & Thomson.	บอระเพ็ด	Stem	36.92±0.49	18.29±1.16
34.	*Tribulus terrestris* L.	โคกกระสุน	Whole plants	42.15±0.11	77.52±1.57
35.	*Ureceola minutiflora* (Pierre)	มวกขาว	Stem	63.53±0.72	49.27±1.6
36.	*Urceola rosea* (Hook.& Arn)	มวกแดง	Stem	24.76±2.77	73.05±6.67
37.	*Vitex glabrata* R. Br.	ไข่เน่า	Stem bark	84.98±0.59	84.71±1.51
38.	*Zea mays* L.	ข้าวโพด	Corn silk	62.31±1.02	73.83±1.16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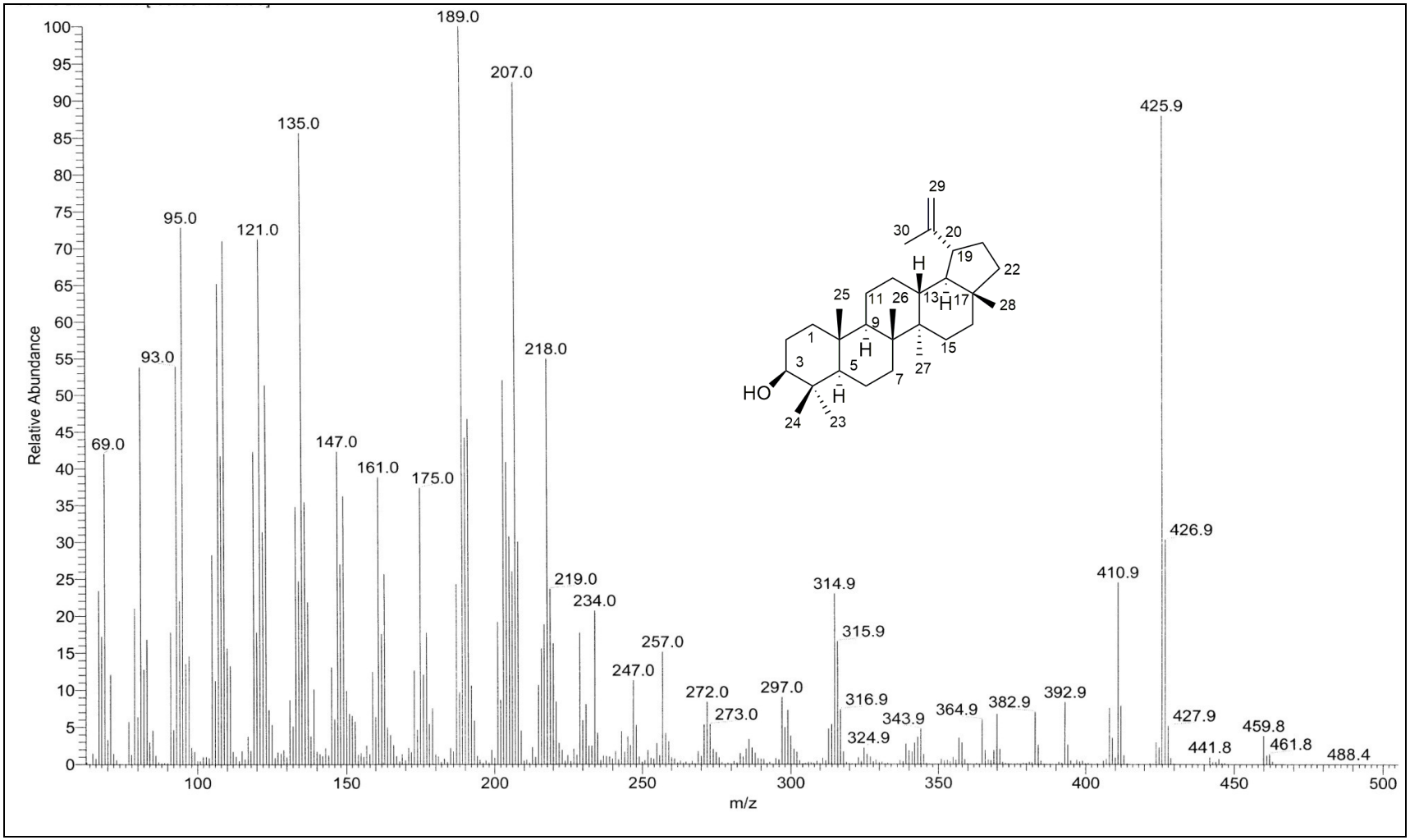
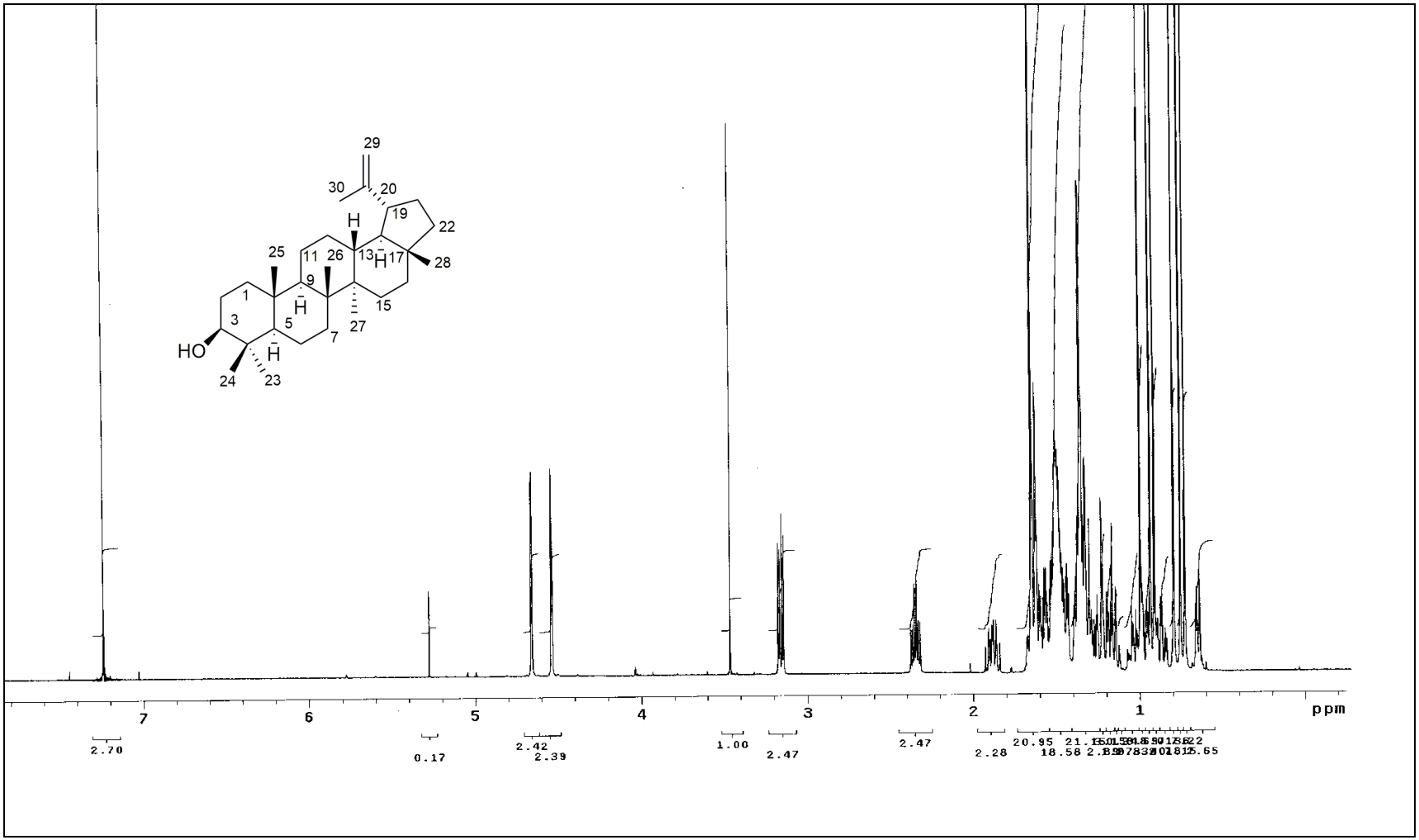
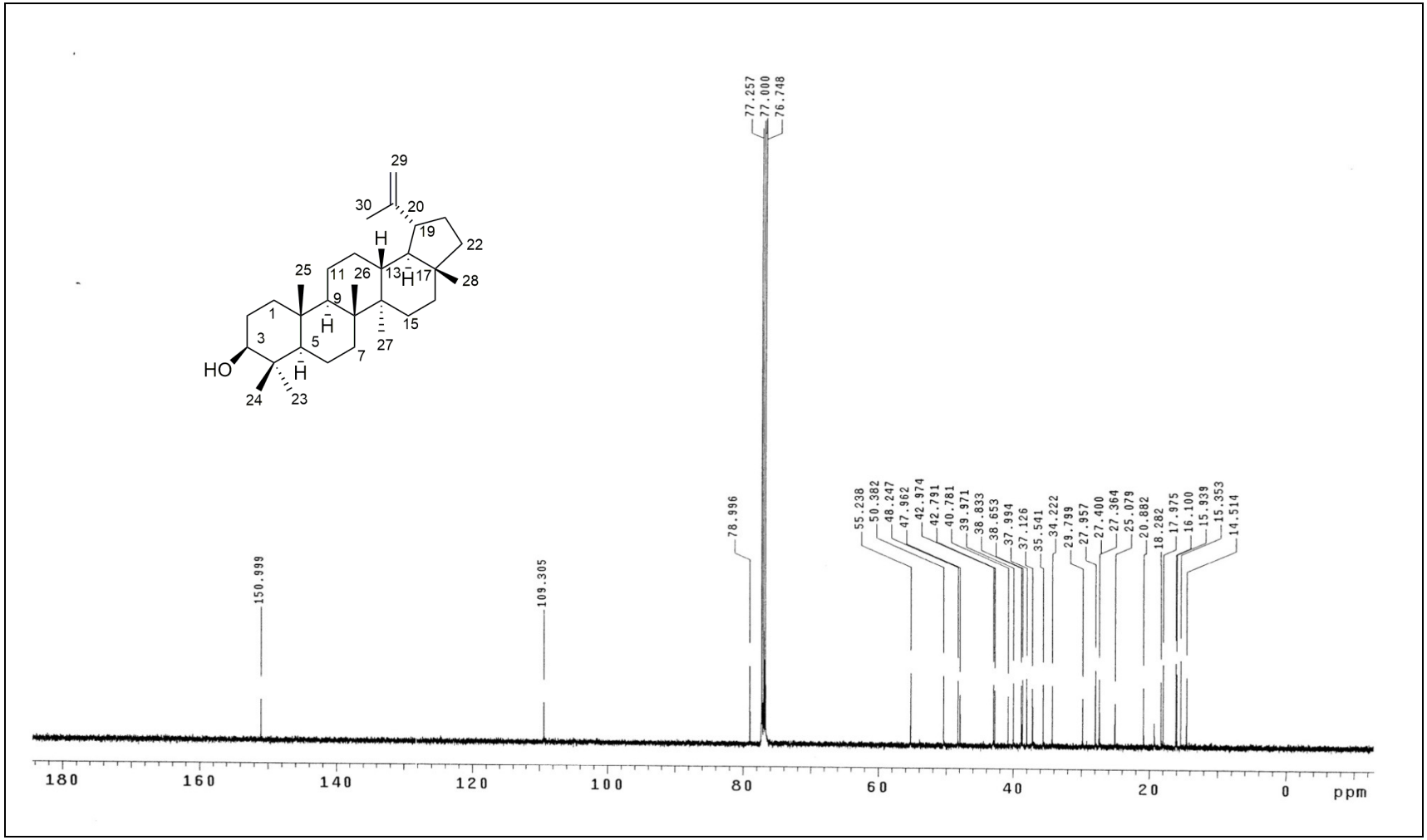


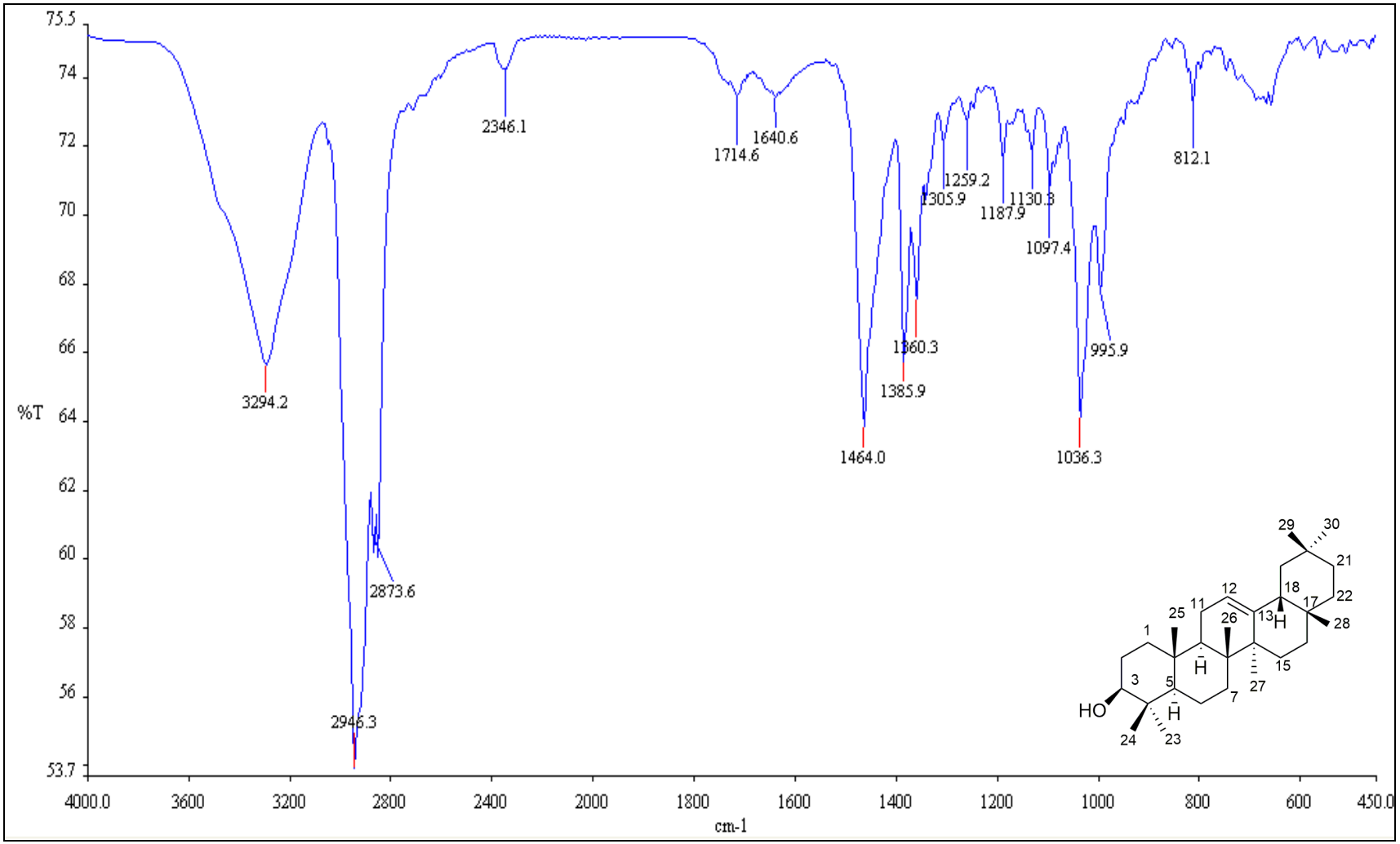
EIMS spectrum of lupeol (**1**)



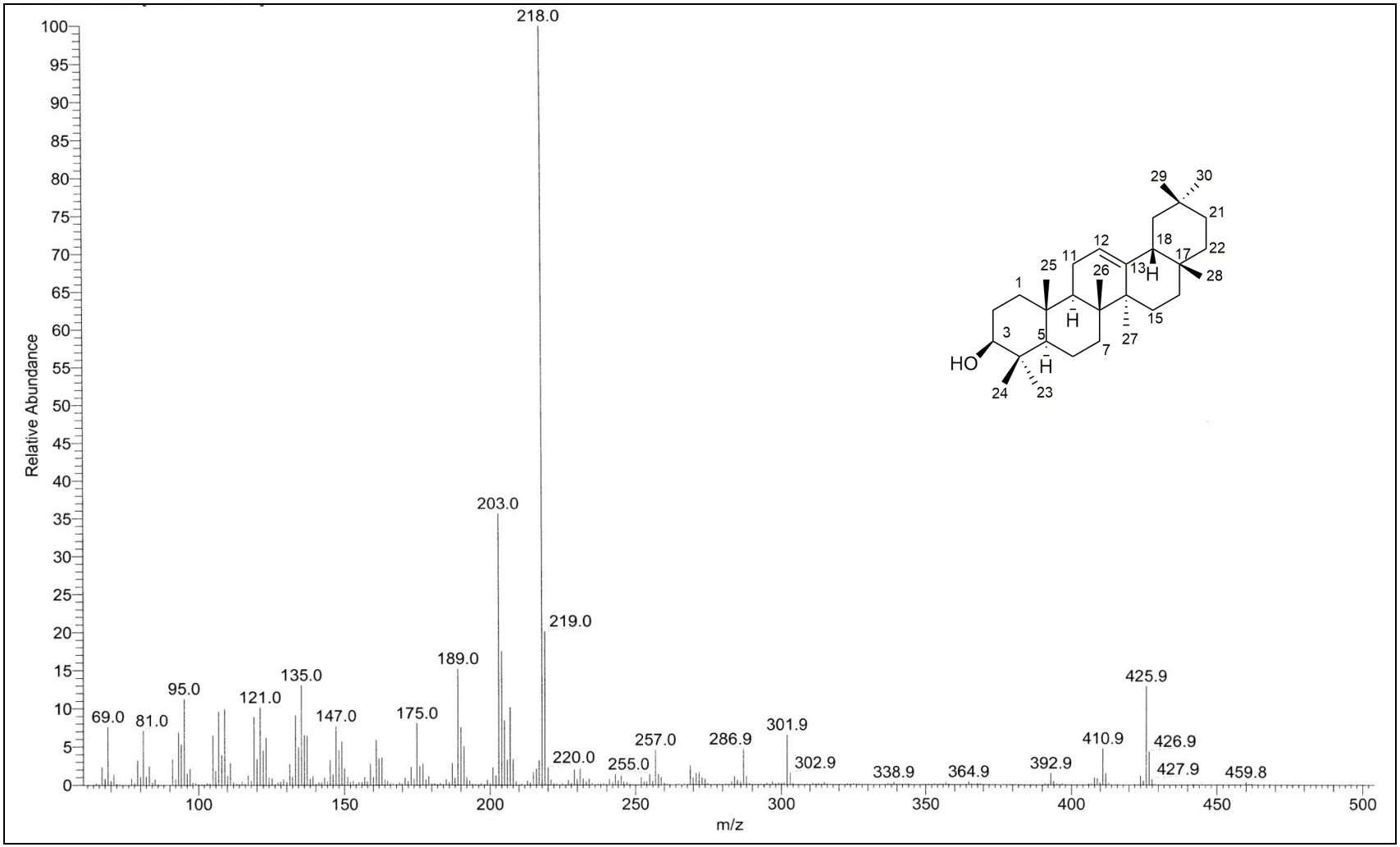
^{1}H NMR (500 MHz, $CDCl_3$) spectrum of lupeol (**1**)



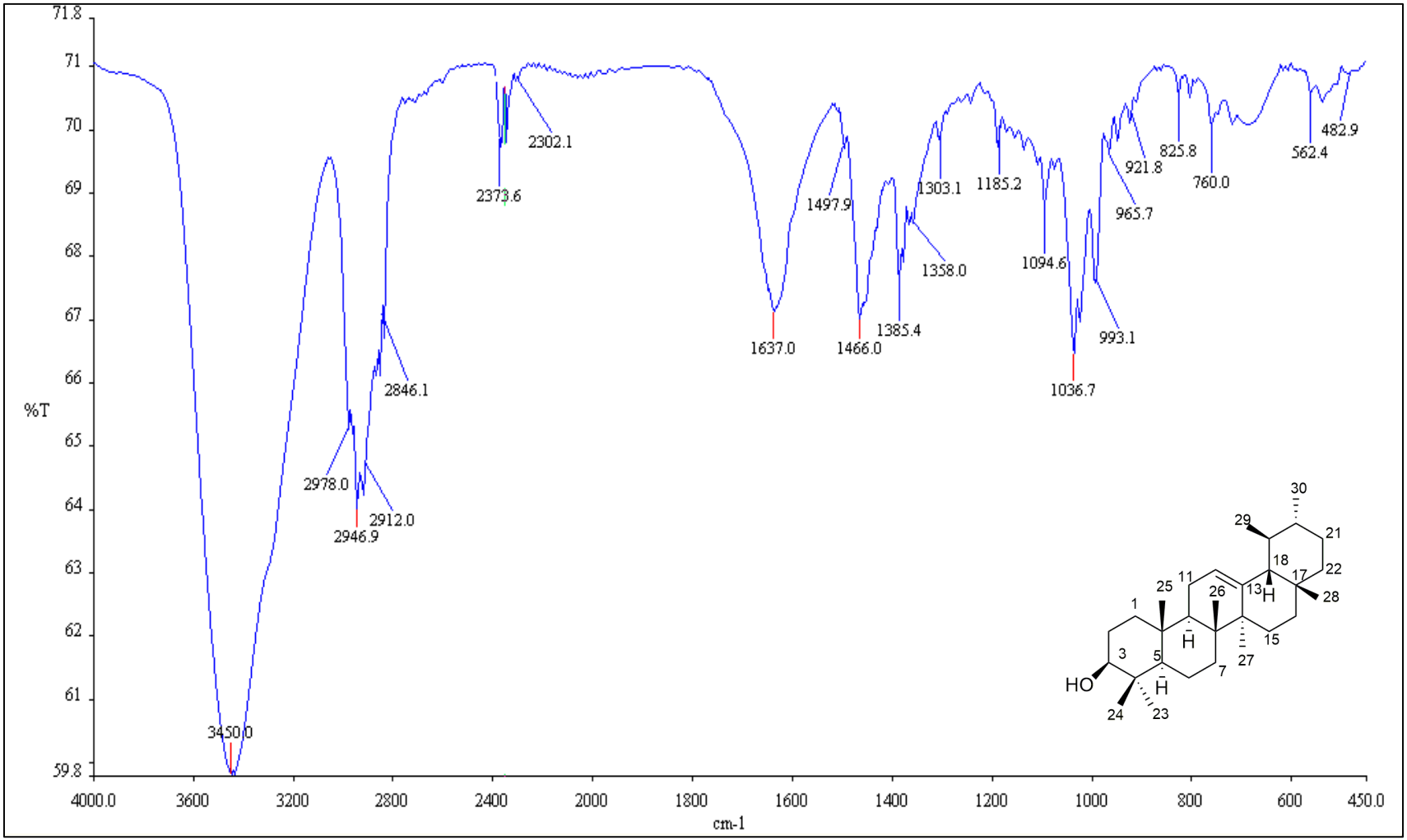
^{13}C NMR (125 MHz, $CDCl_3$) spectrum of lupeol (**1**)



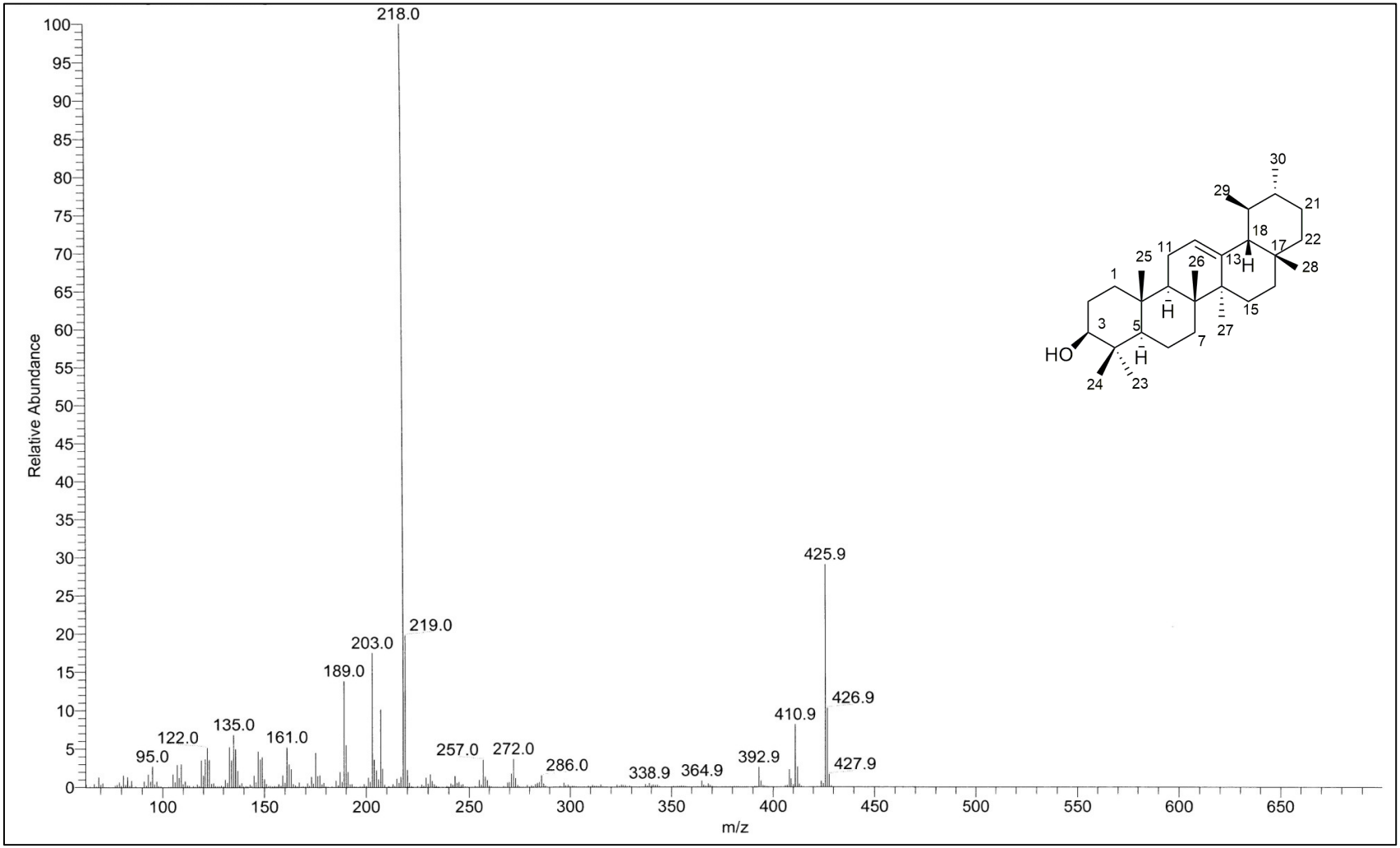
IR (KBr) spectrum of β-amyrin (**2**)



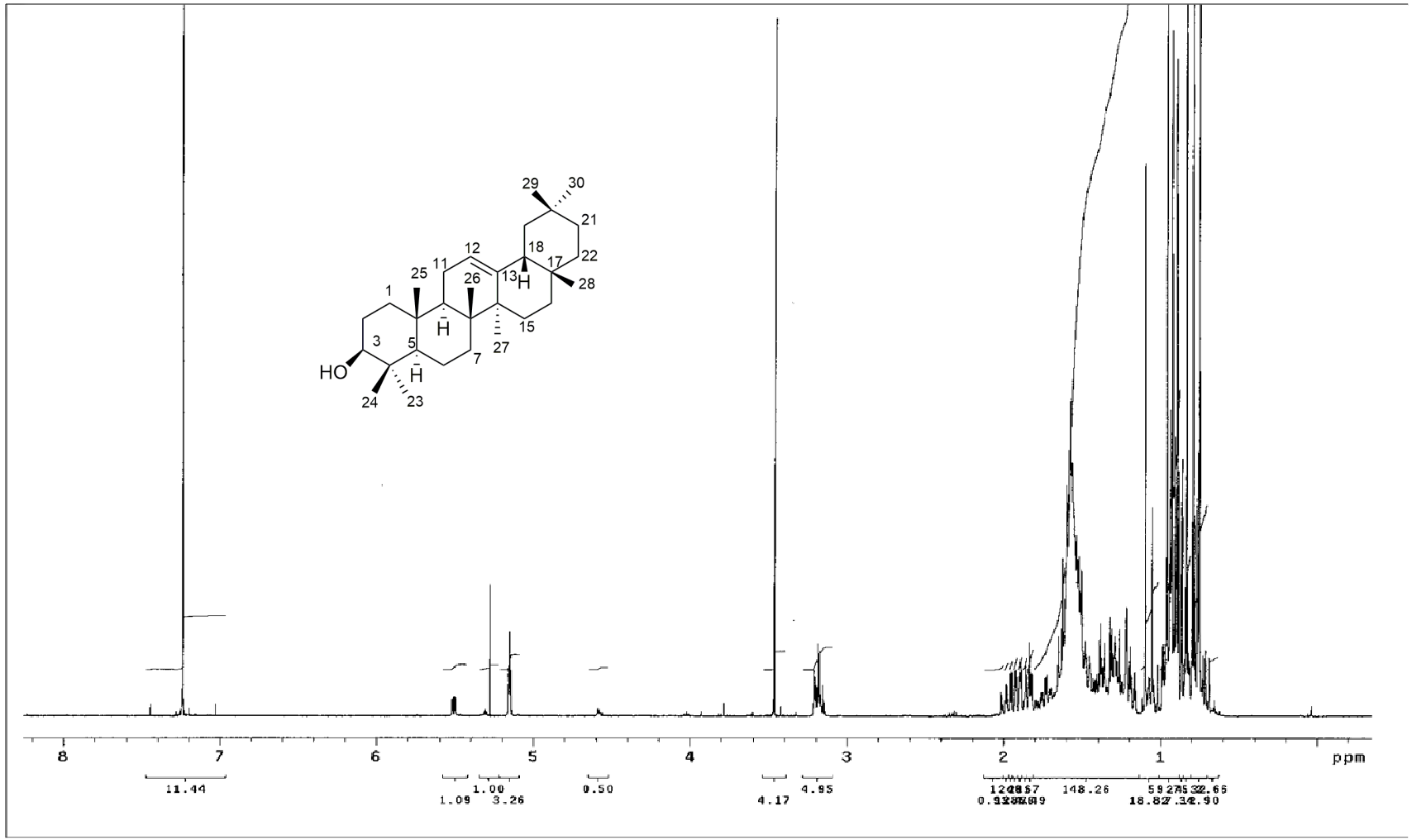
EIMS spectrum of β-amyrin (**2**)



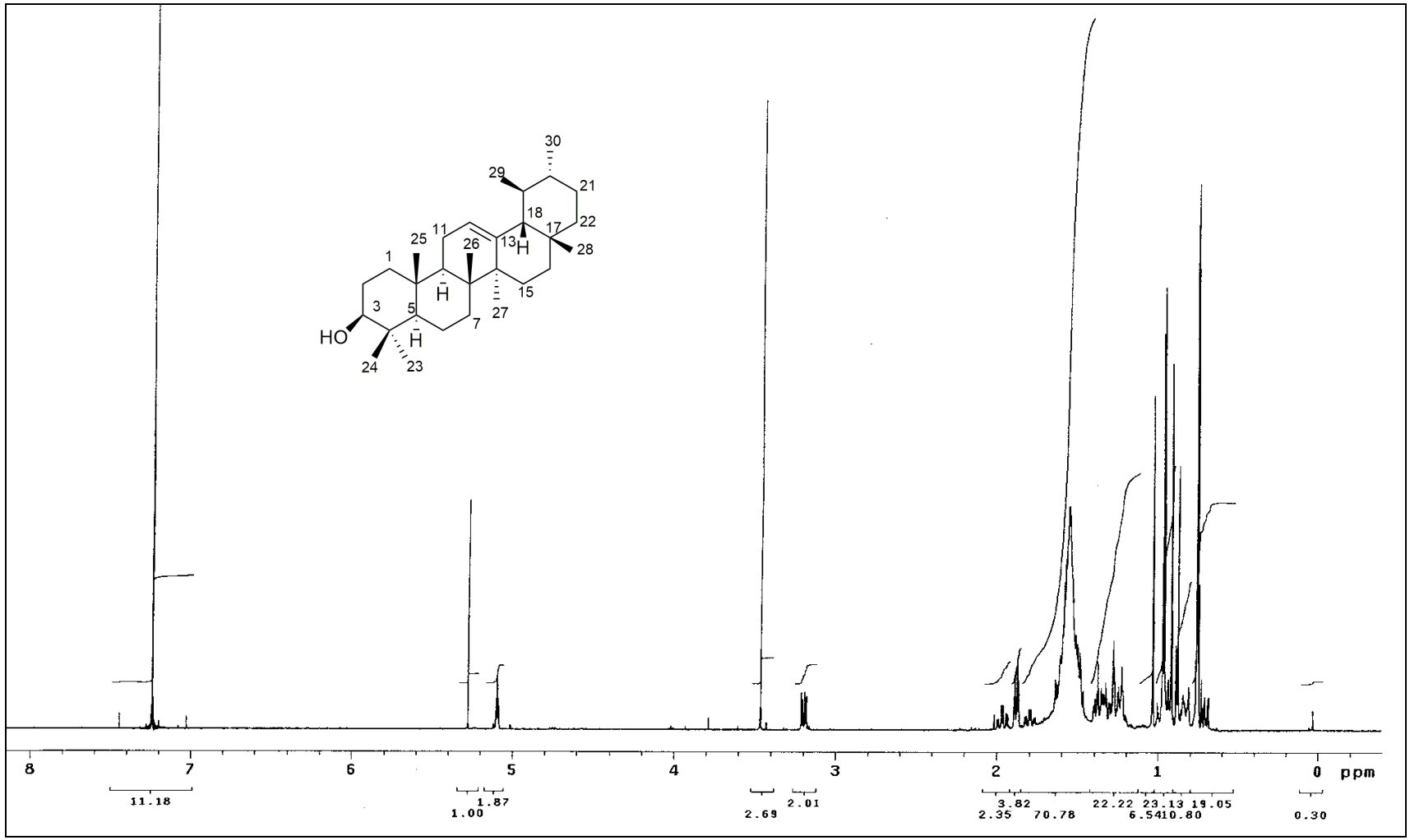
IR (KBr) spectrum of α-amyrin (**3**)



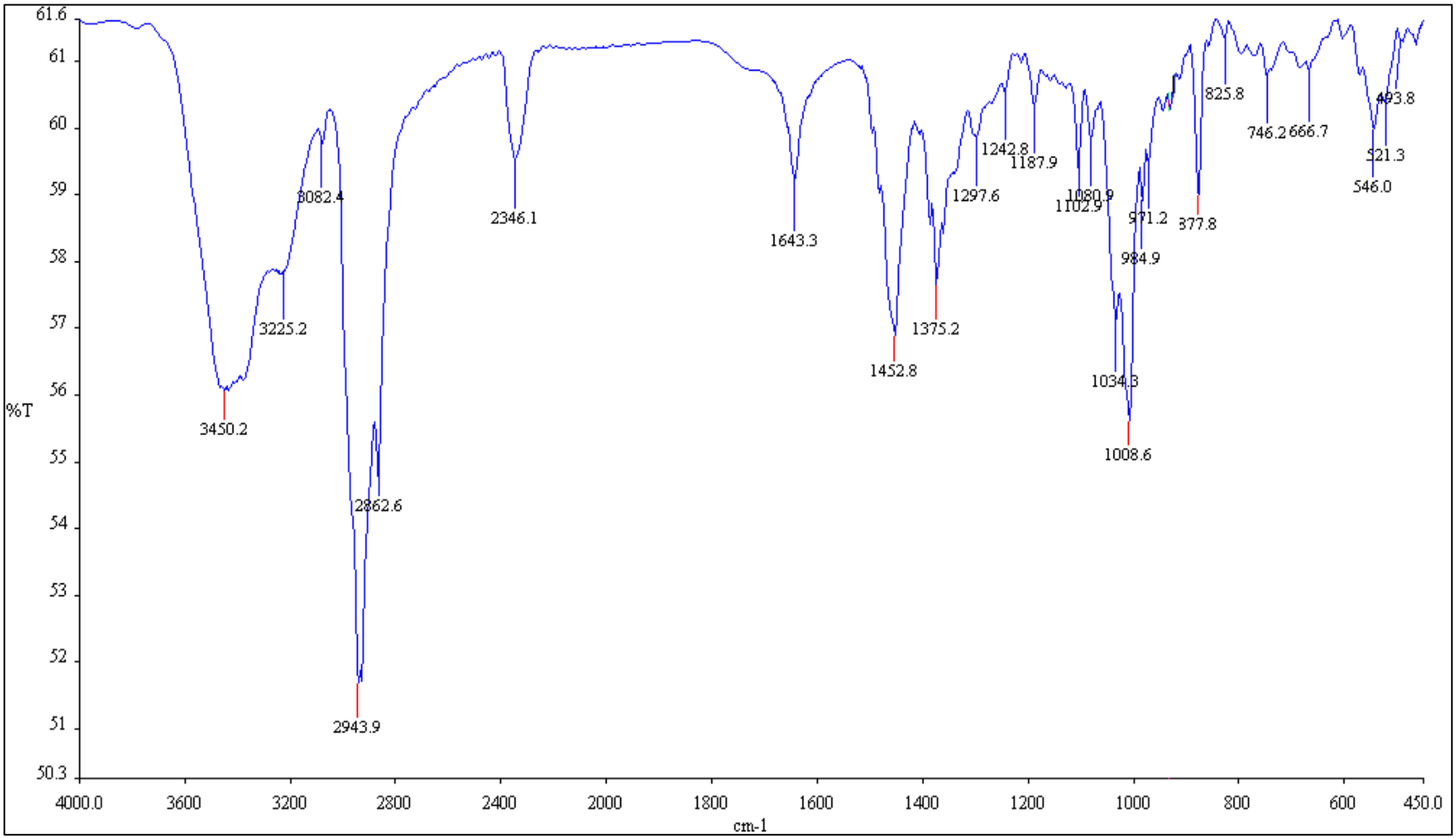
EIMS spectrum of α-amyrin (**3**)

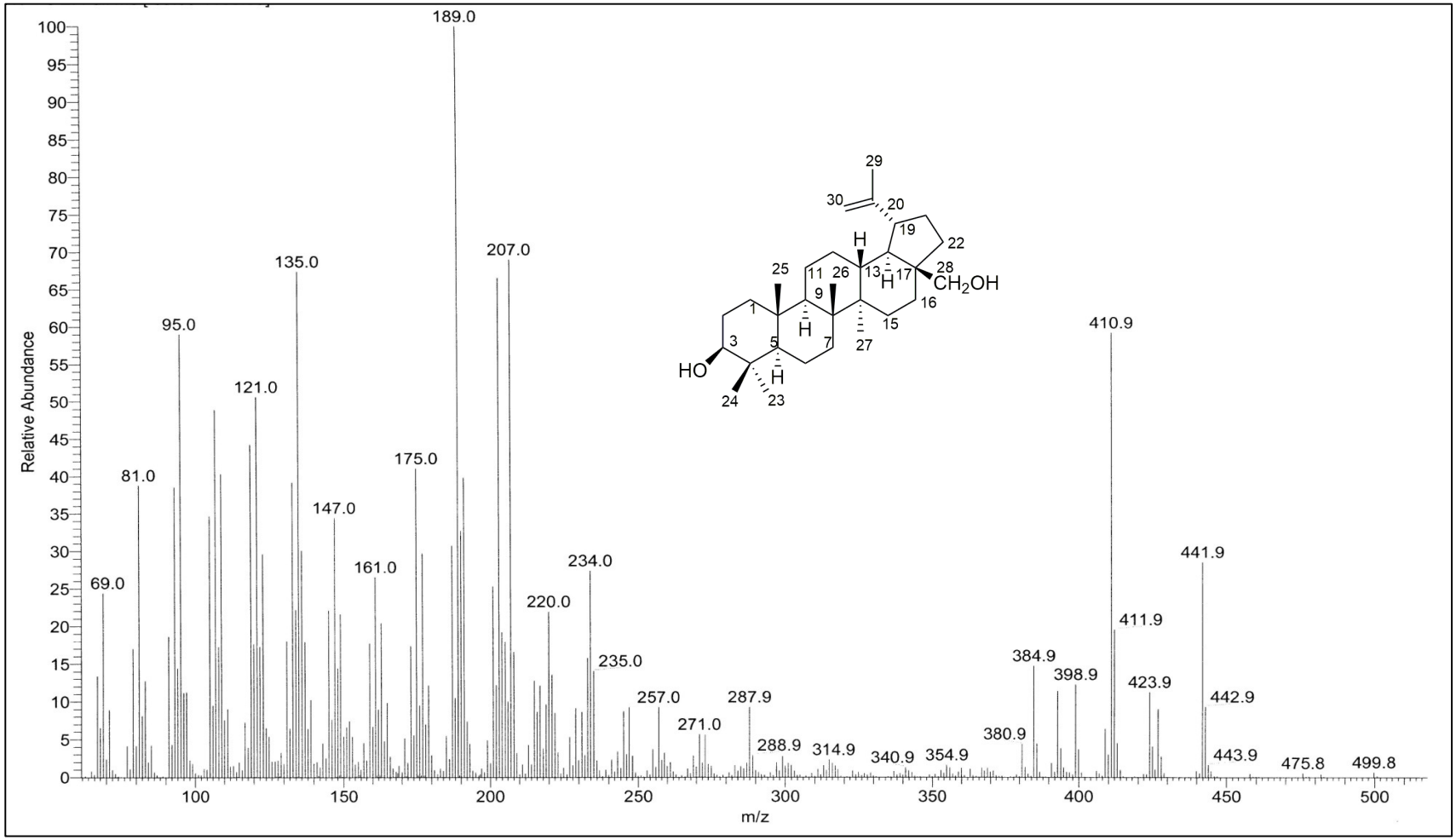


^{1}H NMR (500 MHz, $CDCl_3$) spectrum of β-amyrin (**2**)

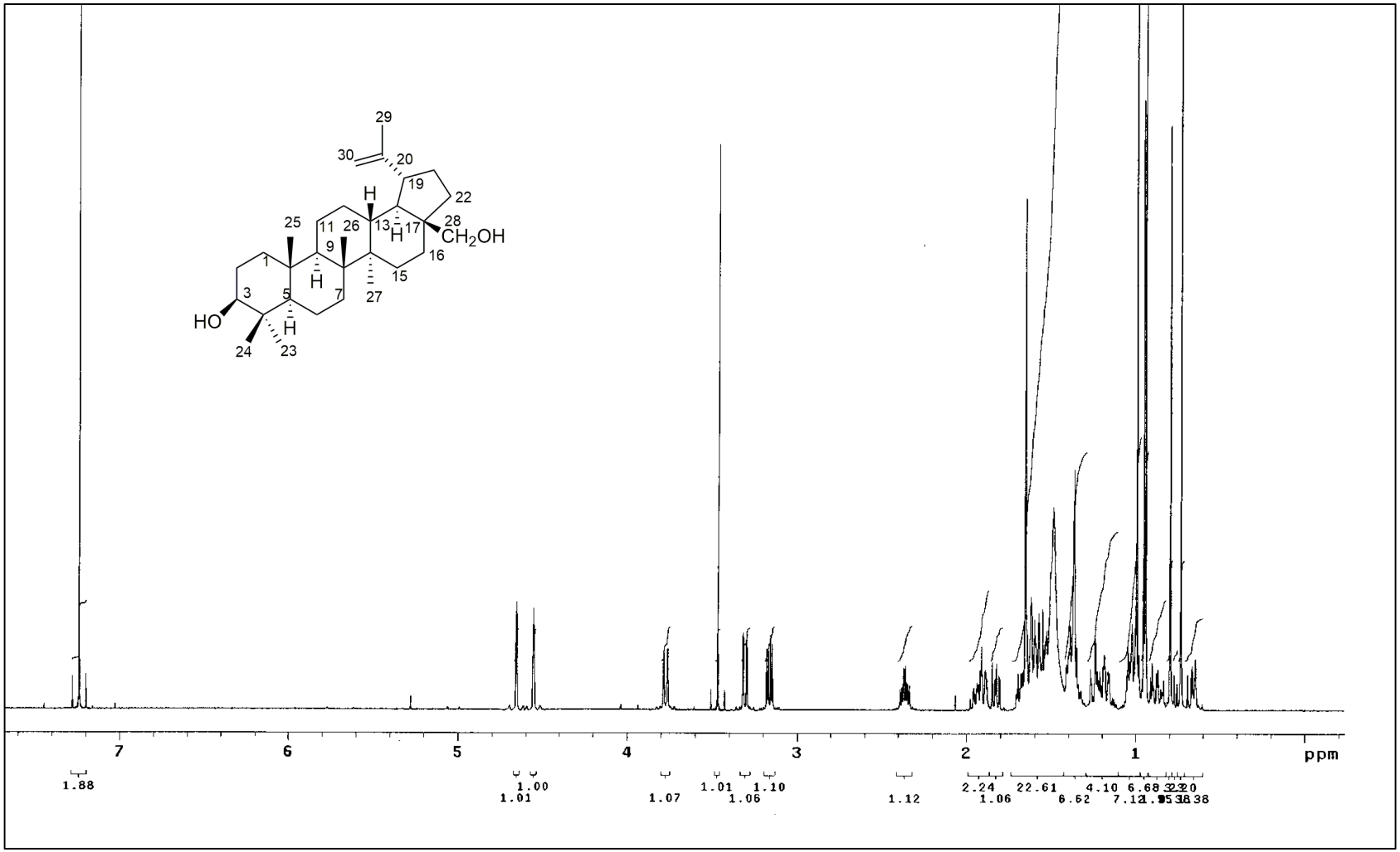


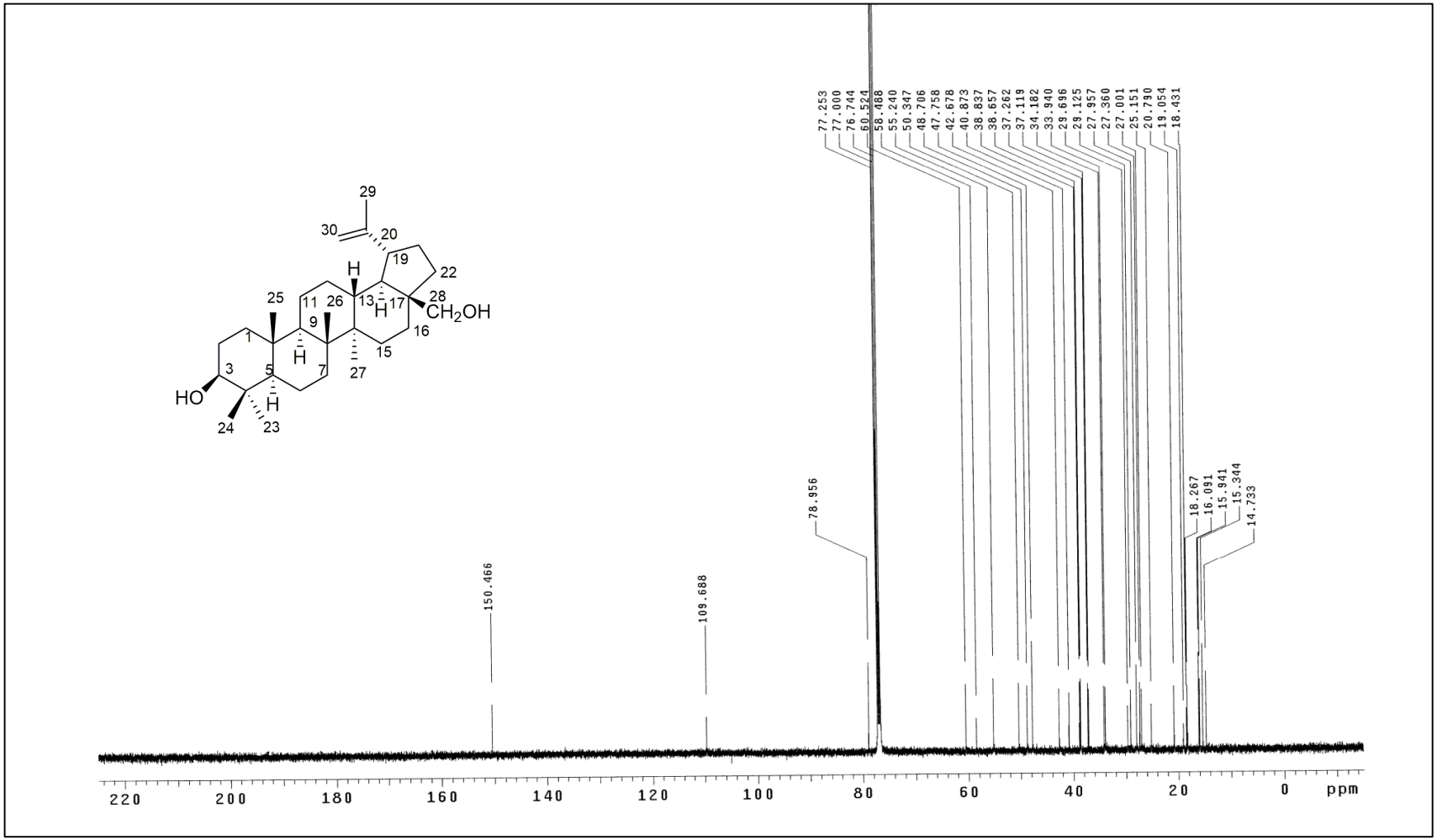
^{1}H NMR (500 MHz, $CDCl_3$) spectrum of α-amyrin (**3**)


61.6
61
60
59
58
57
56
55
54
53
52
51
50.3
%T
3450.2
3225.2
3082.4
2943.9
2862.6
2346.1
1643.3
1452.8
1375.2
1297.6
1242.8
1187.9
1102.9
1080.9
1034.3
1008.6
984.9
971.2
825.8
377.8
746.2
666.7
546.0
521.3
493.8
4000.0
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
450.0
cm-1

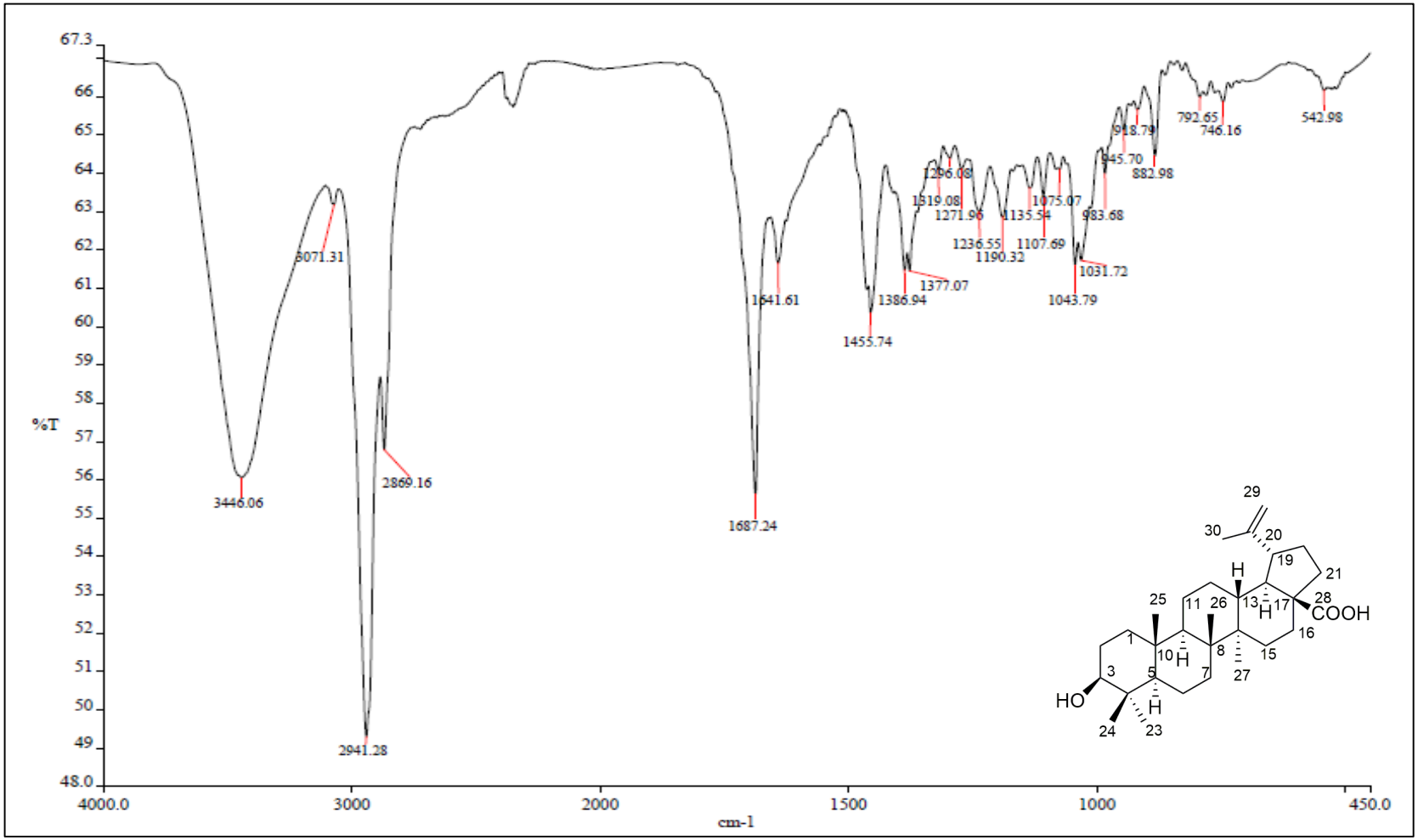


EIMS spectrum of betulin **(4)**

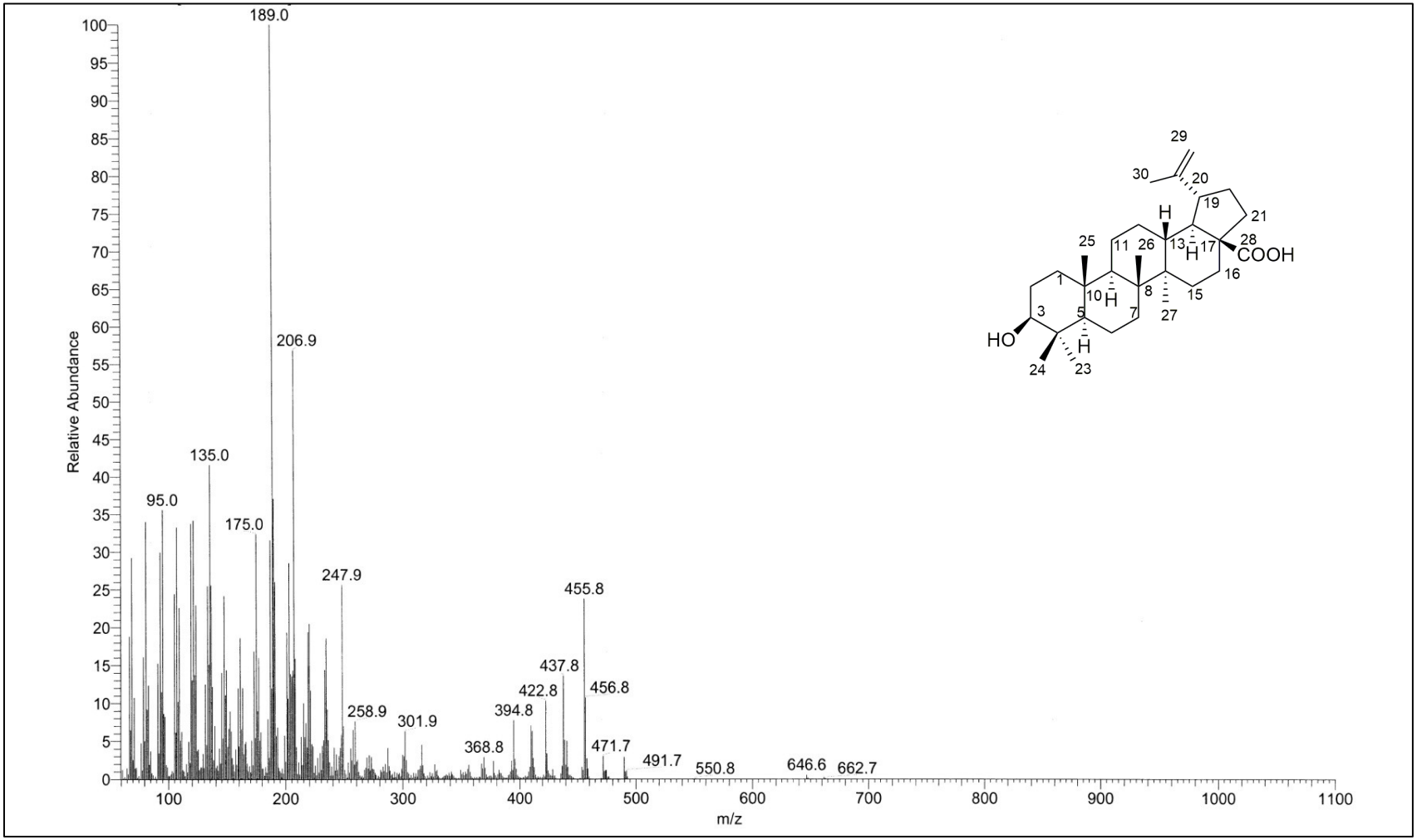

29
30
20
19
22
H
25
11
26
13
H
17
28
CH2OH
1
9
16
H
15
3
5
7
27
HO
H
24
23
7
6
5
4
3
2
1
ppm
1.88
1.00
1.01
1.07
1.01
1.10
1.06
1.12
2.24
1.06
22.61
6.62
4.10
7.12



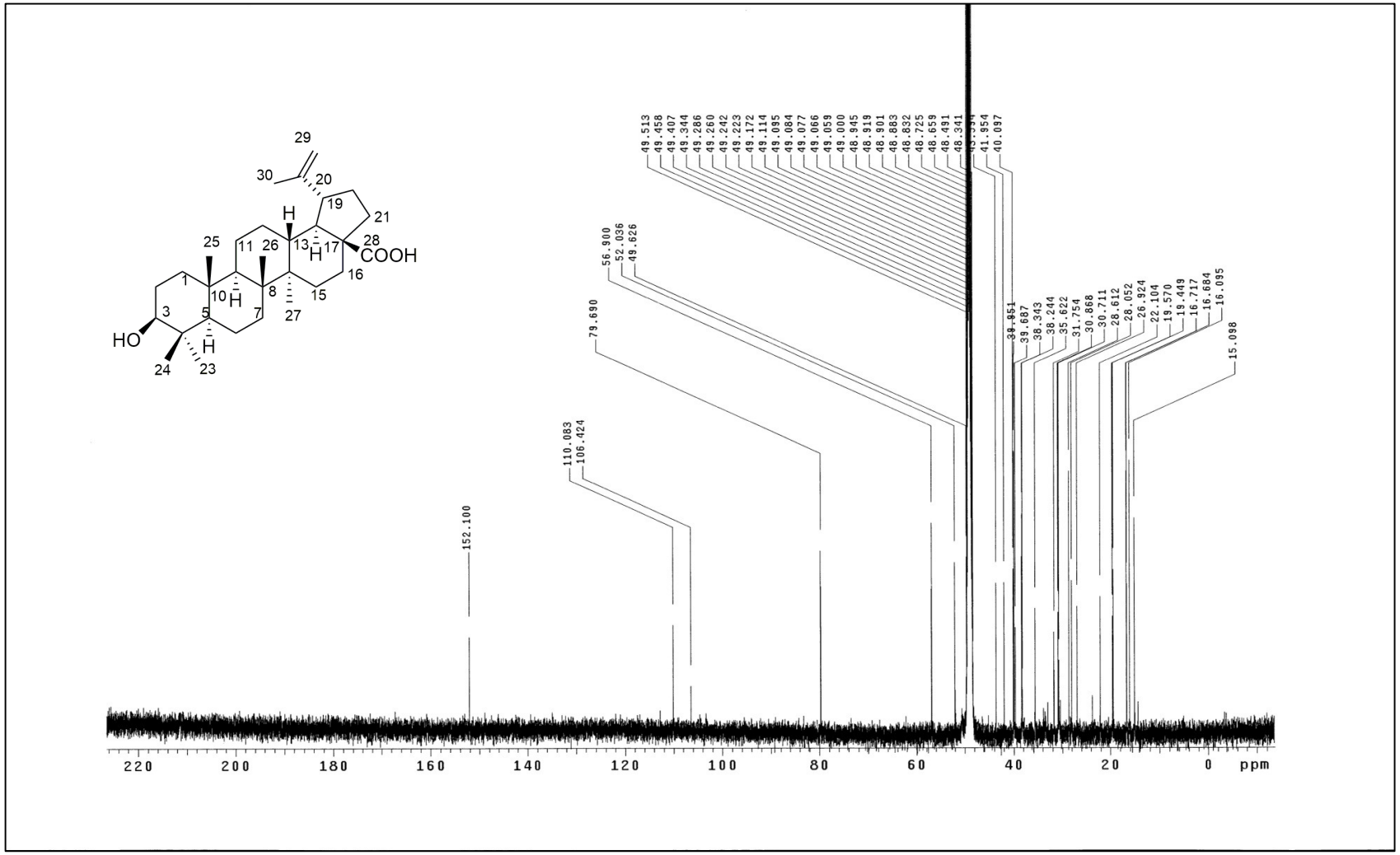
^{13}C NMR (125 MHz, $CDCl_3$) spectrum of betulin **(4)**



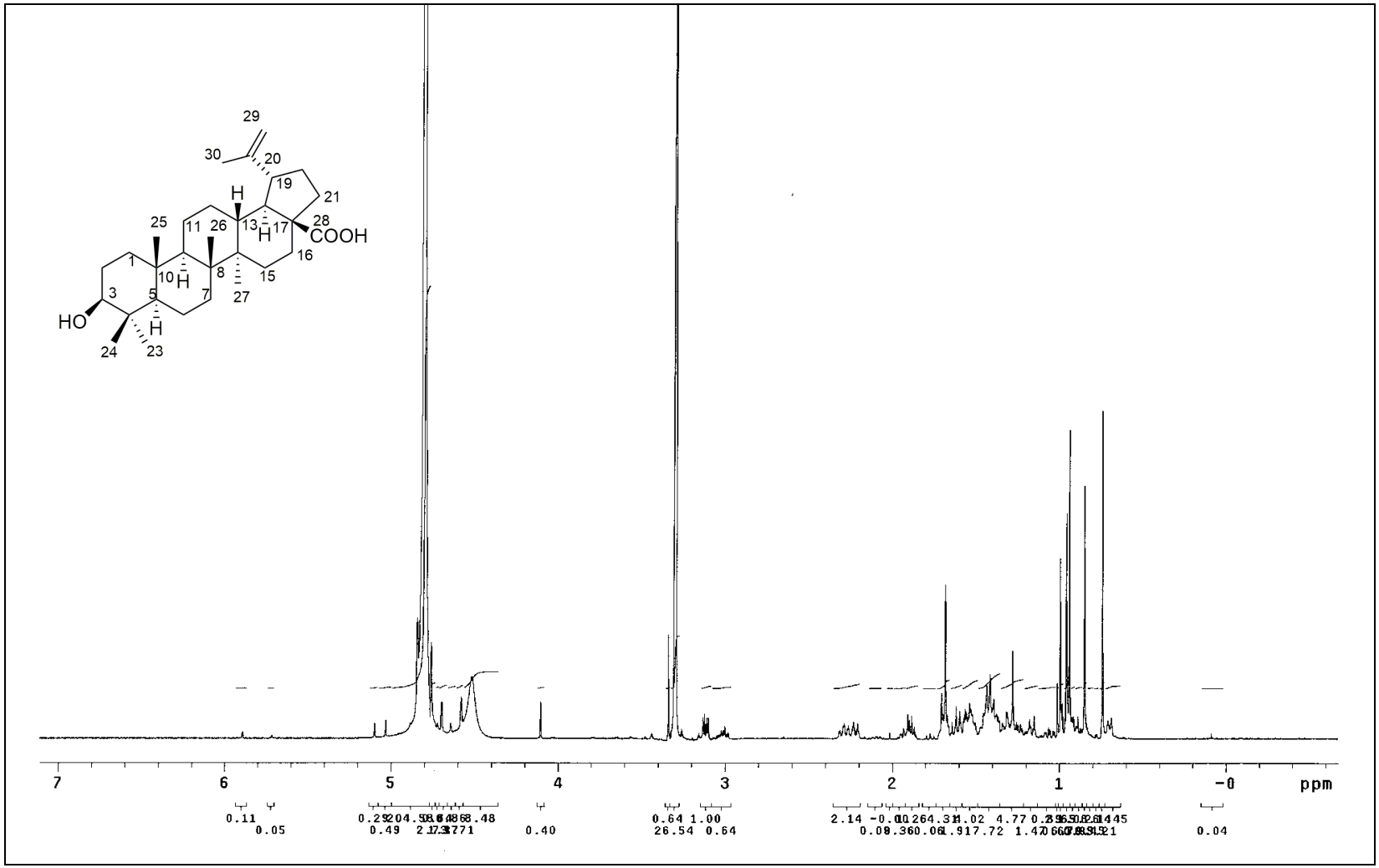
IR (KBr) spectrum of betulinic acid (**5**)



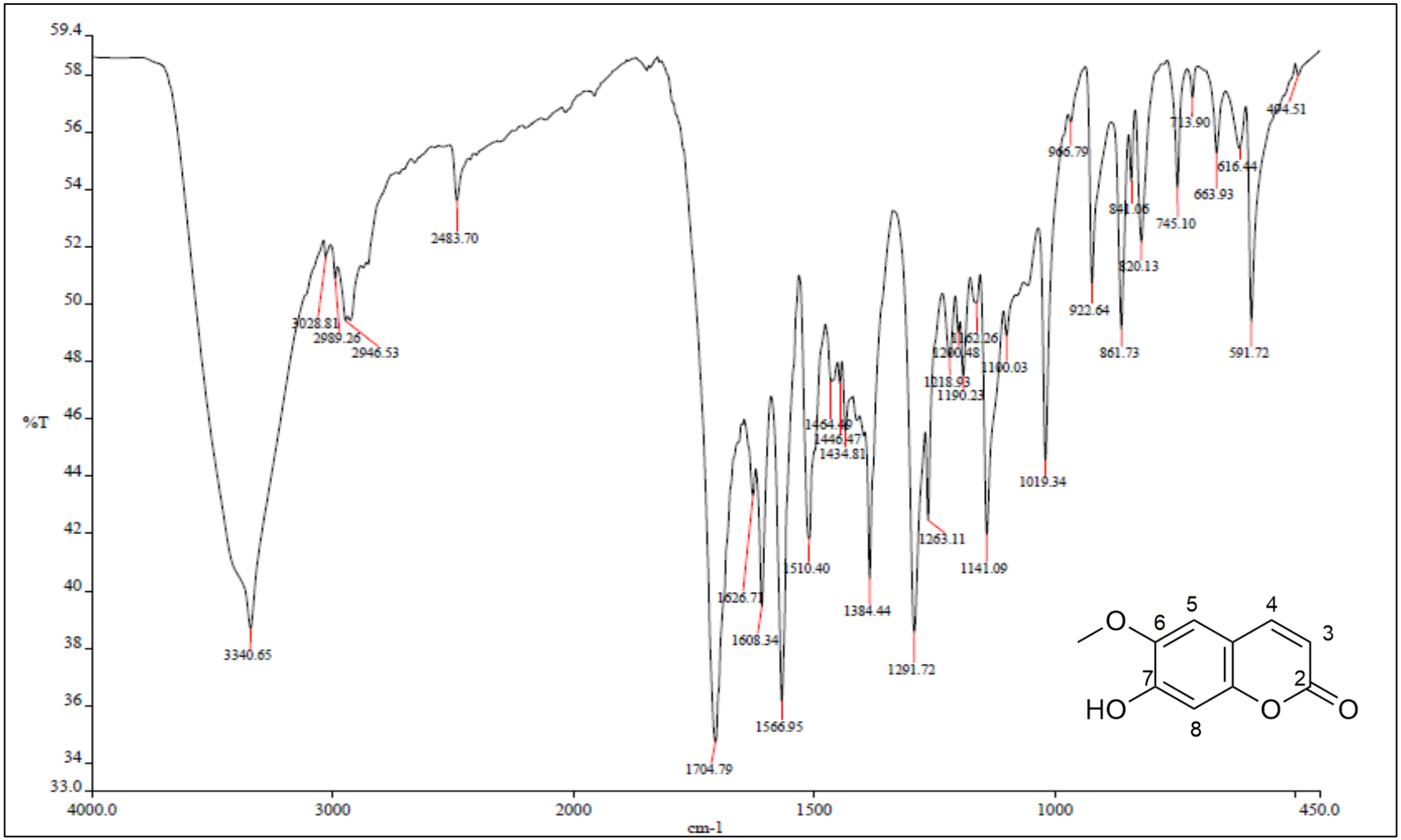
EIMS spectrum of betulinic acid (**5**)



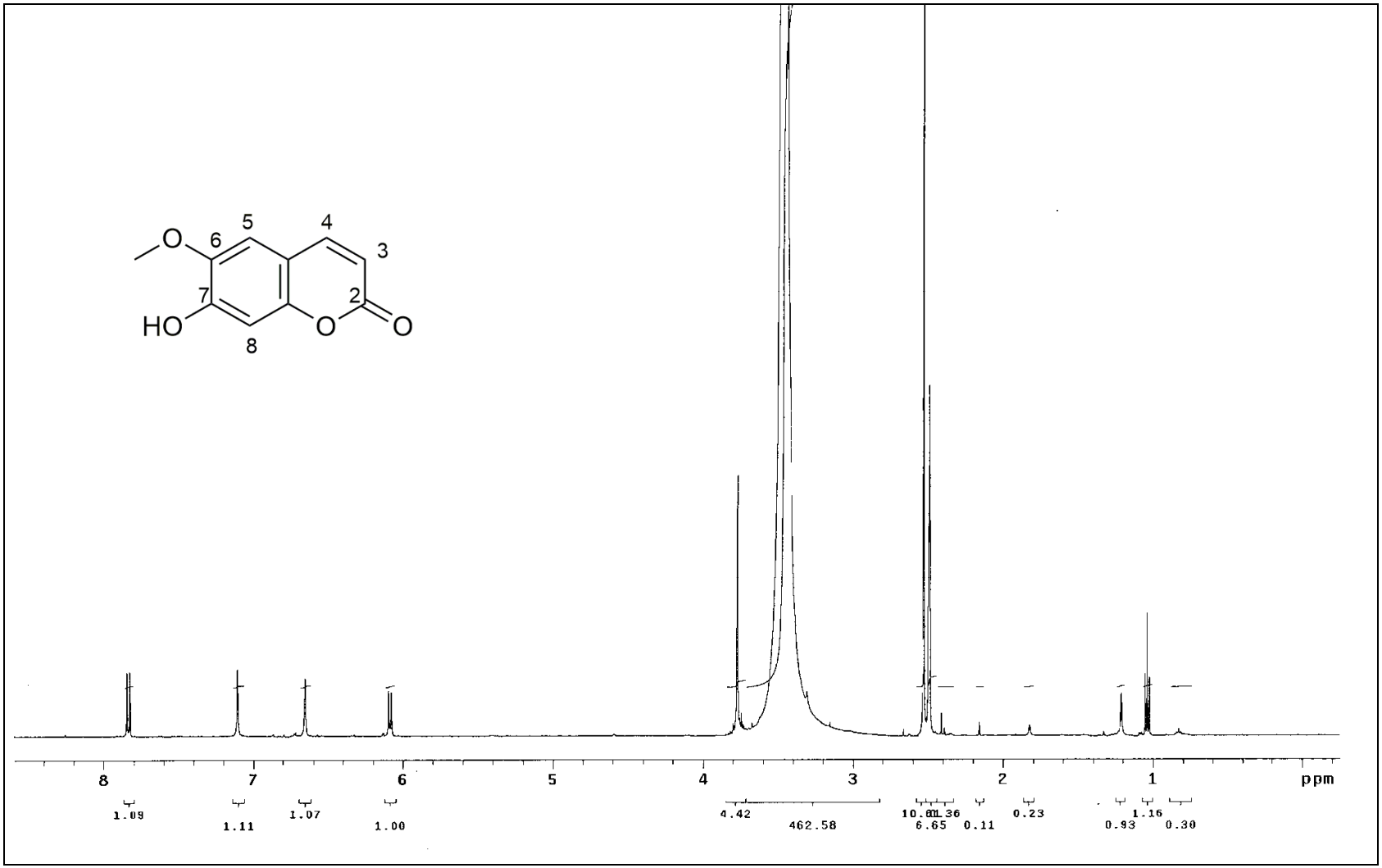
^{13}C NMR (125 MHz, CD_3OD) spectrum of betulinic acid (**5**)



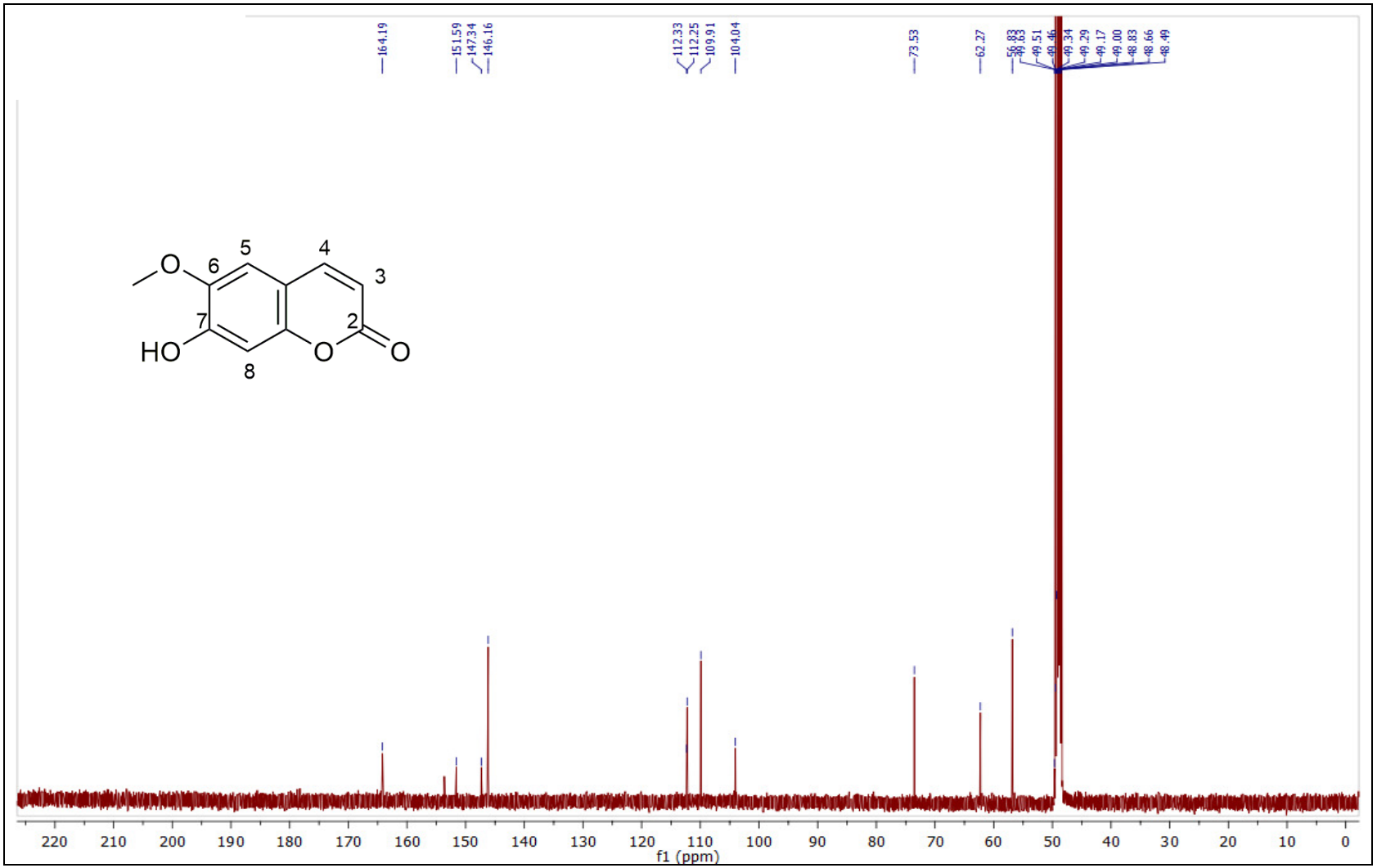
^{1}H NMR (500 MHz, CD_3OD) spectrum of betulinic acid (**5**)



IR (KBr) spectrum of scopoletin (**6**)



^{1}H NMR (500 MHz, DMSO-d_6) spectrum of scopoletin **(6)**



^{13}C NMR (125 MHz, DMSO-d_6) spectrum of scopoletin **(6)**

VITAE

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Educational Attainment

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Work-position and Address

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List of Publication and Proceedings

Thiantongin, P; Sontimuang, C. and Ovatlarnporn, C. 2014. α-Glucosidase and α-amylase inhibitory activities of Thai folk antidiabetes formularies. Proceeding of the 3rd CDD International conference. Pavilion Queen's Bay Krabi Ao Nang Beach, Thailand, May 1-3, 2014.