รายงานวิจัยฉบับสมบูรณ์

โครงการ

สารที่มีฤทธิ์ยับยั้ง แอลฟากลูโคซิเดสหรือแอลฟาอะไมเลสจากสมุนไพร 14 ชนิดที่เป็นส่วนประกอบในตำรับยาแผนไทยรักษาโรคเบาหวาน

α-glucosidase or **α**-amylase inhibitors from 14 medicinal plants constituted in Thai folk antidiabetes formularies

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ผู้ช่วยศาสตราจารย์ ดร. ภญ. ชิตชไม โอวาทพารพร ที่ปรึกษาโครงการวิจัย

งานวิจัยฉบับนี้ได้รับการสนับสนุนจาก

คณะการแพทย์แผนไทยและเงินรายจากมหาวิทยาลัยสงขลานครินทร์

ประจำปีงบประมาณ 2557 รหัสโครงการ TTM560523s-013130

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บทคัดย่อ

สารต้านเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลส สามารถนำมาใช้ในการรักษาโรคเบาหวานประเภทที่ 2 ได้ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อทดสอบฤทธิ์ยับยั้งการทำงานของเอนไซม์ดังกล่าวจากสมุนไพรเดี่ยวและตำรับ 9 ขนานในตำรับยาแผนไทยรักษาโรคเบาหวานของหมอพร (กรมหลวงชุมพรเขตอุดมศักดิ์) และตำรับยาโรงพยาบาลวังน้ำเย็น ผลการศึกษาพบว่าสมุนไพร 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 ไมโครกรัมต่อมิลลิตร ตามลำดับ และผลการยับยั้งเอนไซม์แอลฟาอะไมเลสสูงสุด คือ ใบหูกวาง (*Terminalia catappa*), เปลือกต้นไข่เน่า (*Vitex glabrata)*, ลูกใต้ใบ (*Phyllanthus amarus*), แก่นกำแพงเจ็ดชั้น (*Salacia chinensis*) และแก่นขี้เหล็ก (*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 ไมโครกรัมต่อมิลลิตร ตามลำดับ ส่วนผลการทดสอบฤทธิ์ยับยั้งเอนไซม์ในตำรับ พบว่าตำรับยาขนานที่ TFD-02 มีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลสสูงสุดที่ IC_{50} เท่ากับ 1.99±2.87 และ 12.58±2.63 ไมโครกรัมต่อมิลลิตร นอกจากนี้การศึกษาองค์ประกอบทางเคมีของเปลือกต้นไข่เน่า สามารถแยกสารบริสุทธิ์ได้ทั้งหมด 6 ชนิด คือ lupeol (**1**), α-amyrin (**2**), β-amyrin (**3**), butulin (**4**) and betulibic acid (**5**) และ scopoletin (**6**) ซึ่งสาร lupeol มีฤทธิ์ดีที่สุดในการยับยั้งเอนไซม์แอลฟากลูโคซิเดสที่ค่า IC_{50} เท่ากับ 7.4 ไมโครโมลาร์ และ สาร α-amyrin มีฤทธิ์ดีที่สุดในการยับยั้งเอนไซม์แอลฟาอะไมเลสที่ค่า IC_{50} เท่ากับ 32.33 ไมโครโมลาร์ จากการศึกษาในครั้งนี้พบว่าสารทั้งหมดที่แยกได้ยังไม่เคยมีรายงานของการศึกษาในเปลือกต้นไข่เน่า ดังนั้นผลจากการศึกษาครั้งนี้สามารถนำมาเป็นข้อมูลสนับสนุนถึงประสิทธิภาพของสมุนไพรชนิดนี้ในการรักษาโรคเบาหวานของหมอพื้นบ้าน และควรมีการศึกษาถึงประสิทธิภาพของสาร lupeol เพื่อพัฒนาเป็นผลิตภัณฑ์ยาจากธรรมชาติที่ใช้ในการรักษาโรคเบาหวานประเภทที่ 2 ต่อไป

สัญญาเลขที่ **TTM560523s-013130**

Title: α-glucosidase or α-amylase inhibitors from 14 medicinal plants constituted in Thai folk antidiabetes formularies

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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 twoThai folk anti-diabetes formularies including Mor Phon's recipe and the recipe of Wang Nam Yen hospital. Furthermore folk medicinal formulas from MorPhon'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} were 5.01±1.51, 11.22±1.70, 14.12±1.59, 15.84±1.34 and 25.11±1.44 µg/mL, respectively. For α-amylase inhibitory activity were *Terminalia catappa, Vitex glabrata, Phyllanthus amarus, Salacia chinensis* and *Senna siamea* with IC_{50} were 8.91±2.92, 14.54±1.37, 17.78±2.34, 19.56±1.38 and 20.89±1.87, respectively. For the formulas the best both activities were found using extract from formula TFD-02 with IC_{50} were 1.99 ±2.87 and 12.58±2.63 µg/mL, respectively. Furthermore, the chemical constituents of *V. glabrata* stem bark extract were isolated by chromatographic techniques to obtain six know compounds as lupeol (**1**), α-amyrin (**2**), β-amyrin (**3**), butulin (**4**) betulibic 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 α-amyrin with IC_{50} values of 32.33 µM, respectively. This study is the first report of isolation of compounds from this plant 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 activity are interesting to view to be developed as new drug for treatment of type 2 diabetes patients.

ACKNOWLEDGEMENTS

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Dr. Chonlatid Sontimuang

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LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
β	beta
δ	chemical shift in ppm
g	gram
mg	milligram
IC_{50}	half maximal inhibitory concentration
L	liter
mL	millitter
M	molar
mM	millimolar
μ	micro
μM	micromolar
μg	microgram
m/z	mass-over-charge ratio
MS	mass spectroscopy
NMR	nuclear magnetic resonance
IR	infrared
λ_{max}	maximum wavelength
UV-VIS	ultraviolet-visible
°C	degree of Celsius
%	percentage
DMSO-d_6	deuterated dimethylsulfoxide
$CDCl_3$	deuterat

INTRODUCTION

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, Guariguata 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 their 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 displayed chemical compounds isolated from the bark such as 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).

Objectives

The objectives of this study are:

1. Selected medicinal plants from Mor Phon's recipe.

2. Evaluation of α-glucosidase and α-amylase inhibitory activities of medicinal plants from Mor Phon's recipe.

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

REVIEW OF LITERATURES

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 (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 has 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).

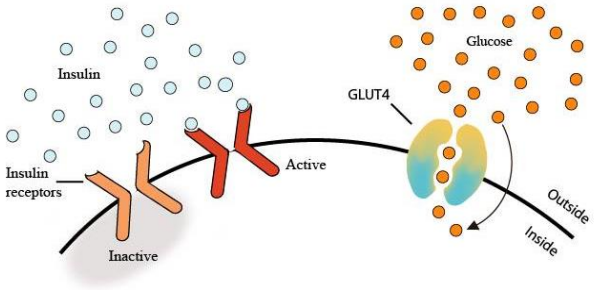


Figure 1 Action of insulin on muscle and adipose tissue

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

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.

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)

Oral antidiabetic drugs

Currently, there are six types of commercially available oral antidiabetic drugs for type 2 diabetes mellitus 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. (Silvio and Inzucchi, 2002; Williams and Pickup, 1999; Territory organization, 2012; Wass and Stewart, 2011)

In vitro studies on anti-diabetic activity

The study of anti-diabetic agents can affect several pathways of glucose metabolism evaluated to *in vitro* such as study of glucose uptake, α-glucosidase and α-amylase inhibitory activity and DPP-4 inhibitory activity

α-Glucosidase and α-amylase inhibitory activity

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 activity were measured by determining the color from the hydrolysis of substrate and using spectrophotometric method (Rao and Jamil, 2011)

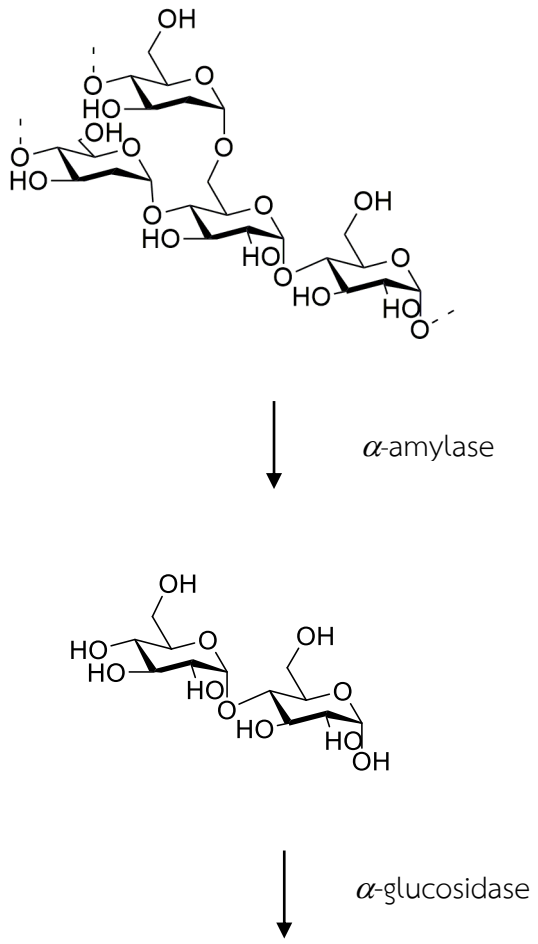
α-Glucosidase and α-amylase enzymes

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-Amylase enzyme (EC.3.2.1.1) is the enzyme secreted by salary glands and pancreas in the 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 salary 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.

The inhibition of α-glucosidase and α-amylase enzyme 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 miglito are found to be associated with various side effects of abdominal pain, gas, diarrhea and flatulence. Nowadays many groups of researchers are working to find new drugs from natural sources for treatment of type 2 diabetes mellitus.



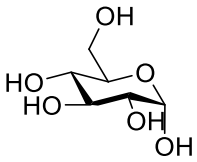
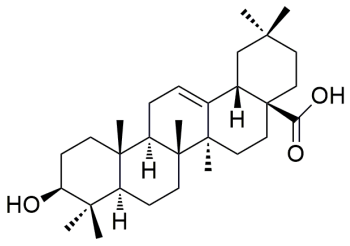


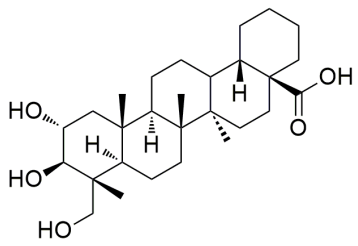
Figure 2 The digestion of carbohydrate by α-amylase and α-glucosidase enzymes

α-Glucosidase and α-amylase inhibitors from medicinal plants

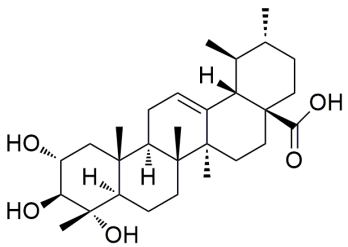
Thai traditional medicines or other alternative medicines have reported various medicinal plants for treatment of diabetes. In this study 37 medicinal plants from Mor Porn's recipes and the recipe of Wang Nam Yen hospital were selected. In the previous reports, thirteen medicinal plants were investigated for α-glucosidase and α-amylase activities. The methanolic and aqueous extract of *Lagerstroemia speciosa.* (leaves) had a strong inhibitory 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 isolated from the ethyl acetate of the leaves of *L. speciosa* are oleanolic acid, arjunolic acid, asiatic acid, maslinic acid, corosolic acid, and 2 3 - hydroxyursolic acid. The corosolic acid showed high α-glucosidase inhibition with the IC_{50} values of 7 μM. (Hou *et al*., 2009)

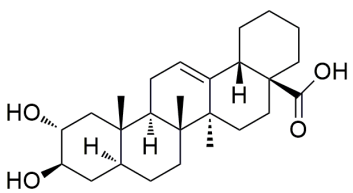


Oleanolic acid

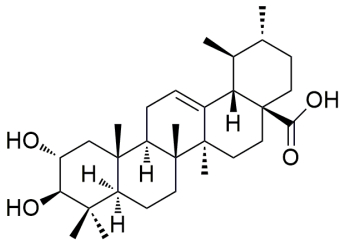


Arjunolic acid

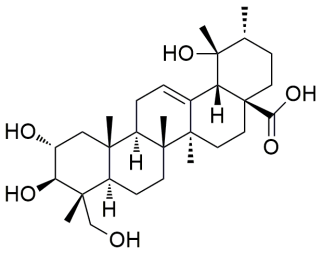




Asiatic acid



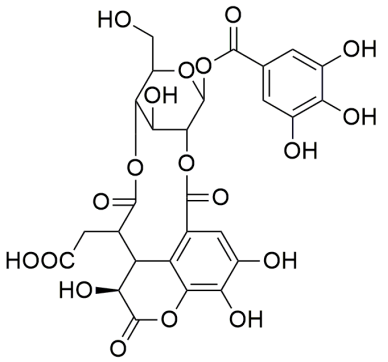
Maslinic acid



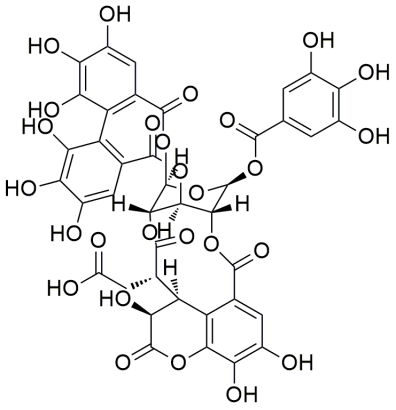
Corosolic acid

2,3-Hydroxyursolic acid

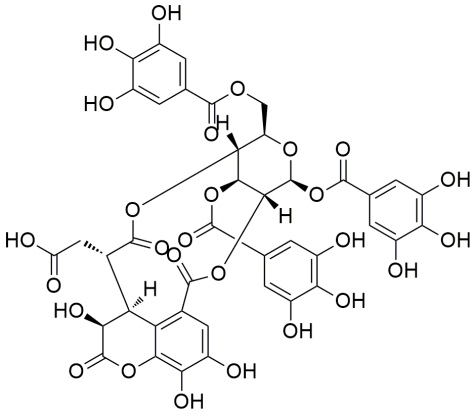
From aqueous methanolic extract of *Terminalia chebula* was found chebulanin, chebulagic acid, and chebulinic acid. These compounds showed potent inhibition of rat intestine maltose with the IC_{50} values of 690 μM, 97 μM and 36 μM, respectively (Gao *et al*., 2007). The methanolic extracts of *Cyperus rotundus* L. (tubers) exhibited a potent inhibition of α-glucosidase activity with the 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-glucosidaseat 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 the 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 activity with the IC_{50} values of 17.2 mg/mL and 50.9 mg/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 oxidase with the IC_{50} values of 56 μM and 50 μM, respectively (Varghese *et al*., 2012).



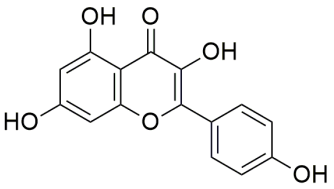
Chebulanin



Chebulagic acid



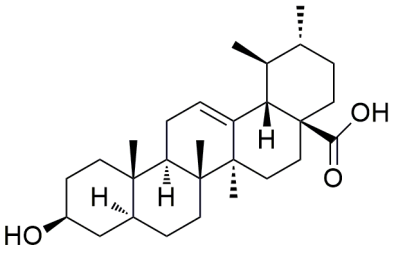
Chebulinic acid



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 the IC_{50} values of 36.05 μg/mL and 48.92 μg/mL, respectively (Tamil *et al*., 2010). Moreover, from isolation of the hexane extract of *P. amarus* was found dotriacontanyl docosanoate, triacontanol and mixtures of oleanolic acid and ursolic acid, from which 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 the IC_{50} values of 2.01 μg/mL. (Ali *et al*., 2006)



Ursolic acid

Figure 3 Structures of compounds from medicinal plants with α-glucosidase and α-amylase inhibitory activity

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 *V. cannabifolia* from the fruit to treat analgesia; *V. agnus-castus* to treat diuretic and stomachache; *V. trifolia* to treat fever and inflammation (Ganapaty *et al.*, 2005; Yanaski *et al.*, 2008; Meena *et al.*, 2010). The biological activities of this genus have been reported for various activities, ie aqueous extracts of *V. doniana* leaves exhibited anti-diabetic activity by decreasing blood glucose level (Ezekwesili *et al*., 2012). The new flavonoid glycoside isolated from the leaves of *V. negundo* exhibited antifungal activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformanas* (Sathiamoorthy *et al*., 2007). The groups of chemical compounds isolated from this genus from the previous reports are iridoids, flavonoids, diterpenoids, triterpenoids and sterols (Meena *et al.*, 2010).

MATERIALS AND METHODS

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., th. 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-H_2SO_4 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. For bioactivity activity testing α-glucosidase enzymes and *p*-nitrophenyl-α-D-glucopyranoside were bought from Sisco Research Laboratories Pvt. Ltd., India while acarbose, α-amylase enzyme and starch azure were purchased from Sigma, Sigma-Aldrich, Germany.

Plant materials

All plants to be prepared the extracts as either of single plant (Table 2) were selected from Mor Phon's recipe. The plants materials were harvested at Khuan Niang, Songkla province and were bought from Thai traditional drug stores, Hatyai, Songkla. All of them 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 smaller and were further dried in hot-air oven at 50-55 ^{o}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 2 Selected medicinal plant to be screened α-glucosidase and α-amylase inhibitory activities

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	heartwood	Songkla province
8.	*Terminalia catappa* L.	หูกวาง	leaves	Songkla province
9.	*Vitex glabrata* R. Br.	ไข่เน่า	stem bark	Songkla province
10.	*Zea mays* L.	ข้าวโพด	corn silk	Songkla province

11.	*Abutilon hirtum* Lam.	ครอบจักรวาล	whole plants	Songkla province
12.	*Acanthus ebracteatus* Vahl.	เหงือกปลาหมอ	whole plants	Songkla province
13.	*Lagerstroemia speciosa* (L.) Pers.	อินทนิลน้ำ	leaves	Songkla province
14.	*Salacia chinensis L.*	กำแพงเจ็ดชั้น	heartwood	Songkla province

The crude extracts preparation

Extracts of single plants

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 to that plant powder. The crude extracts were finally combined and were kept at -20 °C in the refrigerator until use.

Extraction and 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 H_2O 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 finally give six fractions (VH1 to VH6). VH1 (470 mg) was 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). Fractions 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).

Chemical Characteristics

Lupeol (**1**) **:** white needles; IR (KBr) ν_{max}: 3339, 2945, 2826, 1454, 1379 cm^{-1}; ^1H 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 (*brs*), 4.66 (*brs*); ^{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*: 426.9; $[M]^+$ ($C_{30}H_{50}O$).

α-amyrin (**2**)**:** white solid; IR (KBr) ν_{max}: 3249, 2946, 1464, 1385, 1036, 995 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ0.74 (*d*), 0.79 (*d*), 0.83 (*s*), 0.93 (*s*), 0.96 (s), 1.01 (*s*), 1.05 (*s*), 1.08 (*s*), 3.17 (*dd*), 5.15 (*t*); EIMS *m/z*: 426.9; $[M]^+$ ($C_{30}H_{50}O$).

β-amyrin (**3**): white solid; IR (KBr) ν_{max}: 3450, 2946, 1637, 1477, 1036, 993 cm^{-1}; ^1H 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*: 426.9; $[M]^+$ ($C_{30}H_{50}O$).

Betulin (**4**): white needles; IR (KBr) ν_{max} : 3450, 2943, 1452, 1008 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ0.74 (*s*), 0.79 (s), 0.96 (*s*), 0.97 (*s*), 0.99 (*s*), 1.65 (*s*), 3.17 (*dd*), 4.55 (*d*), 4.65 (*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, 48.7, 50.3, 55.2, 58.4, 60.5, 78.9, 109.6, 150.4; EIMS *m/z*: 442.9; $[M]^+$ ($C_{30}H_{50}O_2$).

Betulinic acid (5): white solid; IR (KBr) ν_{max} : 3446, 2941, 1687, 1455, 1377, 1043 cm^{-1}: ^1H 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*), 4.5 (*brs*), 4.76 (*brs*); ^{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, 30.8, 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, 78.6, 110.0, 152.1; EIMS *m/z*: 456.8; $[M]^+$ ($C_{30}H_{48}O_3$).

Scopoletin (**6**): yellow solid; IR (KBr) ν_{max} : 3341, 1704, 1608, 1566, 1510 cm^{-1} ; ^1H NMR (500 MHz, DMSO-d_6): δ 3.77 (*s*), 6.09 (*d*), 6.59 (*s*), 7.10 (*s*), 7.83 (*d*); ^{13}C NMR (125 MHz, CD_3OD): δ 56.8, 102.0, 109.9, 112.2, 112.3, 146.1, 147.3, 151.6, 153.6, 164.1.

Biological activity testing

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory assay was modified from the method of Bachhawat *et al* (2011). The reaction mixtures in 96 well plates containing 50 µL of sample were mixed with 50 µL of enzyme (0.57 unit/mL) and incubated at 37 °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 °C for 20 min. The reaction was stopped by addition of 50 µl of 1 M Na_2CO_3 solution. The absorbance measured at 405 nm using UV/Vis absorbance spectrophotometer microplate reader. % inhibition is calculated by following equation (1);

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

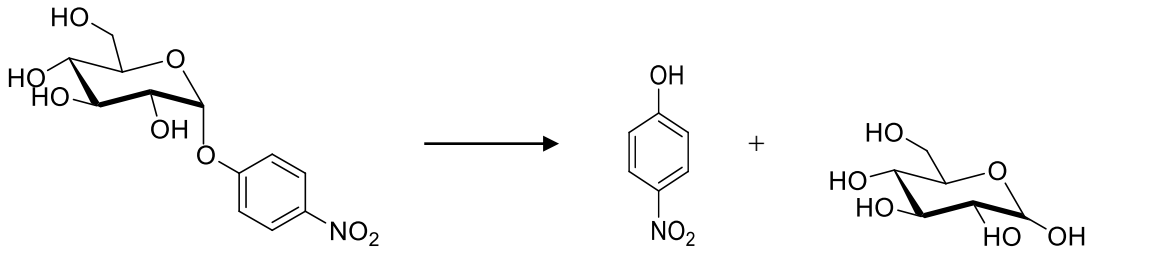
Where $A_{control}$ = absorbance of the negative control

A_{sample} = absorbance of the sample

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

The principle of α-glucosidase inhibitory assay using spectrophotometric 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 color of the release of *p*-nitrophenyl arising from the hydrolysis of substrate PNPG by α-glucosidase reaction is shown in Figure 4. (Rao and Jamil, 2011; Guo *et al*., 2010.)



PNPG ***p*-nitrophenyl** **D-glucopyranoside**

Figure 4 20

α-Amylase inhibitory activity

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 °C . The starch solution was pre-incubated at 37 °C for 5 min(Hansawasdi, Kawabata et al. 2000). 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 °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 °C. The absorbance measured at 595 nm using by UV/Vis absorbance spectrophotometer microplate reader. % inhibition is calculated by equation (1).

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

products. And can detect spectrophotometrically at 595 nm to give a direct measurement of α-amylase activity in the sample. (Rinderknecht et al., 1967)

Statistical analysis

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

RESULTS AND DISCUSSIONS

Thai traditional medicine can provide interesting knowledge of pharmacological- active medicinal plants. Although, these medicinal plants or recipes prescribed by folk doctor. There have been widely used for long time ago, it is still lacking how they work pharmacologically. It is the challenge task to identify the mechanism of action. As of diabetes treatment, α-glucosidase and α-amylase are key targets to lower the blood glucose after meal. Therefore, in this study we aimed to investigate the anti-α-glucosidase and α-amylase plants which selected from reliable recipes including Mor Phon's recipe and the recipe of Wang Nam Yen hospital. Hence, this chapter is comprised of two parts as 1) screening of α-glucosidase and α-amylase inhibitory plants and 2) phytochemical studies of the stem bark of *V. glabrata.*

Table 3 % yield of plant extract

No	Plant	Part used	Before Extract (g)	After Extract (g)	% yield
1	*Vitex glabrata* R.Br (ไข่เน่า)	Bark	30	3.12	10.4
2	*Acanthus ebracteatus* Wall. (เหงือกปลาหมอ)	Whole plant	30	1.63	5.43
3	*Zea mays* L. (ข้าวโพด)	Silk	30	1.28	4.27
4	*Mimosa pudica* Linn. (ไมยราบ)	Aerial parts	30	2.33	7.77
5	*Pandanus odorus* Ridl (เตยหอม)	Leaves	30	4.24	14.13
6	*Diospyros rhodocalyx* Kurz. (ตะโกนา)	Bark	30	1.35	4.5
7	*Cassia siamea* Britt. (ขี้เหล็ก)	Stem	30	1.21	4.03
8	*Phyllanthus amarus*. (ลูกใต้ใบ)	Aerial parts	30	2.07	6.9

9	*Abutilon polyandrum* W & A (ครอบจักรวาล)	Aerial parts	30	1.06	3.54
10	*Terminalia catappa* Linn. (หูกวาง)	Leaves	30	6.99	23.3
11	*Cassia alata* L. (ชุมเห็ดเทศ)	Leaves	30	8.22	27.4
12	*Legerstroemia speciosa* Pers. (อินทนิลน้ำ)	Leaves	30	5.09	16.96
13	*Rhinacanthus nasutus* Kurz. (ทองพันชั่ง)	Leaves	30	2.60	8.67
14	*Salacia chinensis* L. (กำแพงเจ็ดชั้น)	Stem	30	4.36	14.53

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

The plants to be tested the α-glucosidase and α-amylase inhibitory activities were selected the results indicated first fourteen plants whose ethanolic extracts exhibited highest α-glucosidase inhibitory activity (Table 4) including, *V. glabrata*, *S. siamea, P. amarus, T. catappa*, *S. chinensis, M. pudica, L. speciosa, D. rhodocalyx., , Z. Mays*, . While IC_{50} of *S. chinensis*, *V. glabrata*, *S. siamea, T. catappa*, *P. Amarus*, P. *amaryllofolius*, R. *nasutus*, S. *alata* and A. *ebracteatus*. Fourteen plants whose ethanolic extracts exhibited highest α-amylase inhibitory activity (table 4) were *V. glabrata*, *S. siamea, P. amarus, T. catappa*, *S. chinensis, M. pudica, L. speciosa, D. rhodocalyx., , Z. Mays* .

Table 4. % inhibition of α-glucosidase and α-amylase, 25 µg/mL

No	Plants	% inhibition of α-glucosidase activity (µg/mL)	% inhibition of α-amylase activity (µg/mL)
01	*Vitex glabrata* R.Br (ไข่เน่า)	84.98±0.59	84.71±1.51
02	*Acanthus ebracteatus* Wall. (เหงือกปลาหมอ)	21.10±3.01	43.61±3.85
03	*Zea mays* L. (ข้าวโพด)	65.31±1.02	73.83±1.16
04	*Mimosa pudica* Linn. (ไมยราบ)	28.24±1.67	88.69±0.56
05	*Pandanus odorus* Ridl (เตยหอม)	28.82±2.88	35.50±6.1
06	*Diospyros rhodocalyx* Kurz. (ตะโกนา)	63.61±1.09	83.50±2.32
07	*Abutilon polyandrum* W & A (ครอบจักรวาล)	53.33±1.45	74.18±5.97
08	*Phyllanthus amarus*. (ลูกใต้ใบ)	74.85±0.79	94.73±1.37
09	*Senna siamea* Lam. (แก่นขี้เหล็ก)	83.14±0.15	81.95±7.17
10	*Terminalia catappa* L. (หูกวาง)	78.48±0.14	78.59±1.84
11	*Senna alata* L. (ชุมเห็ดเทศ)	46.17±2.11	39.99±3.14
12	*Legerstroemia speciosa* (L.) Pers. (อินทนิลน้ำ)	69.05±0.25	62.41±10.84
13	*Rhinacanthus nasutus* (L.) Kurz. (ใบทองพันชั่ง)	55.81±1.76	83.29±1.28
14	*Salacia chinensis* L (กำแพงเจ็ดชั้น)	69.06±0.78	72.33±2.92
15	Acarbose (25 µg/mL)	34.00±0.45	33.00±0.79

In the present study, we can observe medicinal plants which can be used to support the mechanism in blood glucose lowering by the α-glucosidase and α-amylase inhibitory activity, namely *S. chinensis, T. catappa, P. amarus, V. glabrata,* and *S. siamea.* It is likely to describe as following:

Generally, the stem and roots of *S. chinensis* have toxic, astringent and fragrant/ cool tastes. In Thai traditional medicine, a decoction of stem has been prepared for blood tonic, anti- diabetic, anti- inflammatory, carminative, haemagogue, and relief of rheumatism, (Farnsworth *et al*., 1992; กองการประกอบโรคศิลปะ กระทรวงสาธารณสุข, มปป.) As the ethanolic extract of stem showed potent activity against α-glucosidase with IC_{50} values of 5.01±1.51 μg/mL. It was supported by previous study of Yoshikawa *et al* (2003). Moreover, the mangiferin compound isolated from this plant was found to decrease blood glucose level after injection to streptozotocin- induced diabetes rats, indicating its anti-diabetic property (Sellamuthu *et al*., 2012).

For *T. catappa,* its leaves have tasteless. Thai traditional medicines have used this plant to treat tonsillitis, perspiration, rheumatism, digestive disorder and liver disease. The biological activities of this plant were reported as antioxidant, anti-cancer, anti- diabetic, anti- inflammatory, anti- bacterial, anti- tumor, and it also showed hepatoprotective activity (Mandloi *et al*., 2013; Akharaiyi *et al*., 2011; Saroja *et al*., 2011). As for α-glucosidase inhibitory activity, our results displayed that ethanolic extract from leaves gave strong activity (IC_{50} values of 15.84±1.34 μg/mL) which was consistent with pervious report of Anam *et al* (2009). For α-amylase inhibitory activity the ethanolic extract of the leaves displayed strong activity (IC_{50} values of 8.91 ±2.92 μg/mL) and also consistent with that of Koffi *et al* (2010). In addition, the aqueous and cold extract of the leaves exhibited anti-hyperglycemic activity in alloxan-induced diabetes rats (Ahmed *et al*., 2005). These results can be used to support anti-diabetic activity.

P. amurus. The whole plant has bitter taste. In China and India, a decoction of the whole plant has been used to promote urination and treat jaundice (Wiart, 2006). Thai traditional medicine has used this plant to treat fever, digestive disorder, jaundice and liver disease and promote diuretic for long time ago. (กองการประกอบโรคศิลปะ กระทรวงสาธารณสุข, มปป.) Previous reports on pharmacological activities of this plant revealed anti- inflammatory, antioxidant, anti- arthritic, anti-diabetic activity, and also hepatoprotective activity (Kiemer *et al*., 2003; Harish *et al*., 2006; Mail *et al*., 2011). The ethanolic extract of the leaves of this plant displayed

potential hypoglycemic activity on alloxan induced diabetic mice (Shetti *et al*., 2012). Our study of ethanolic extract of this plant exhibited strong inhibitory activity against α-amylase (IC_{50} values of 17.78 ±2.43 μg/mL) consistent with the previous report of Tamil *et al* (2010) (The ethanolic extract with IC_{50} values of 36.05±4.01 μg/mL). Moreover, the extract of aerial parts showed moderate to good α-amylase and α-glucosidase inhibitory activity with IC_{50} of 2.15±0.1 and 0.2±0.02 mg/mL, respectively (Okoli *et al*., 2011).

V. glabrata. The bark has astringent taste. Thai traditional medicines have used the plant to treat digestive disorder, anti-diabetes and anthelminthic. The result of this study indicated strong inhibitory activities against both α-glucosidase and α-amylase. This is the first report of the activity of this plant against these two enzymes.

S. siamea. The heartwood has bitter taste and has long been used in Thai traditional medicine to treat fever, insomnia, diabetes and diuresis, haemagogue, promote fire element and as tonic (กองการประกอบโรคศิลปะ กระทรวงสาธารณสุข, มปป; Tripathi *et al*., 1991). Pushpavathi *et al* (2013) reported potent anti-diabetic activity of the methanolic extract of flower in alloxan induced diabetic rats. The result of this study indicated the ethanolic extract of heartwood to exhibit potent α-glucosidase and α-amylase inhibitory activity with the IC_{50} values 14.12$\pm$1.59 and 20.89 $\pm$1.87 μg/mL, respectively. This is the first report for α-amylase and α-glucosidase inhibitory activities from the heartwood. There was previous report on ethanolic extract from the leaves against α-glucosidase inhibitory activity with IC_{50} of 28.4 ppm more potent than acarbose (Mun'im *et al*., 2013).

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 is associated with several organs and several tastes. The medicinal plants from these recipes have been known to have anti-diabetes activity and from results of this study, that strong both activities, so the tastes of most herbals 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 about causes agents of diabetes patients, it can be concluded that diabetes is originated from deficiency of fire element. Therefore, to treat diabetes patients, the use of bitter taste medicinal plants must be employed for stimulating and tonic of the fire element and use tasteless medicinal plants for increasing diuretic. It may decrease high blood glucose levels.

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 is associated with several organs and several tastes. The medicinal plants from these recipes have been known to have anti-diabetes activity and from results of this study, that strong both activities, so the tastes of most herbals 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 about causes agents of diabetes patients, it can be concluded that diabetes is originated from deficiency of fire element. Therefore, to treat diabetes patients, the use of bitter taste medicinal plants must be employed for stimulating and tonic of the fire element and use tasteless medicinal plants for increasing diuretic. It may decrease high blood glucose levels.

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

The dried stem barks of *V.glabrata* (400 g) were extracted using maceration with MeOH. After that the methanolic crude extract was exhuaustively partitioned by the sequence in polarity using *n*-C_6H_{14}, EtOAc, *n*-BuOH and water (**scheme 1**) to afford crude extract and after evaporation obtain the weight of 3.01, 21.15, 5.8, and, 7.9 g, respectively.These fractions were further determined against α-glucosidase and α-amylase inhibitory activity whose results showed high potential for α-glucosidase activity for hexane and ethyl acetate extract with 83.86±1.03 and 86.44$\pm$2.15 µg/mL, respectively as shown in Table 5. The hexane and ethyl acetate extract were selected separate using chromatography technique to obtained six know compound, lupeol (1), α-amyrin (2), β-amyrin (3), butulin (4) and betulinic acid (5) from *n*-C_6H_{14} fraction and scopoletin (6) from EtOAc fraction. These pure compounds were elucidated by spectroscopic data including, infrared spectra (IR), nuclear magnetic resonance spectra (NMR), and electron impact mass spectra (EIMS) and along with compare with those reported in the literature for reliable results.

Table 5 Yield and percentage of α-glucosidaseand α-amylase inhibitory activity of crude extracts

Fraction	% yield	% inhibition*	
		α-glucosidase activity	α-amylase activity
n-C_6H_{14} (VH)	7.52	83.86±1.03	67.86±2.45
EtOAc (VE)	52.87	86.44±2.15	63.3±1.34
n-BuOH (VB)	14.50	49.46+3.18	61.5±2.01
Aqueous (VW)	19.75	46.52±1.89	51.91±3.05

*at 25 µg/mL.

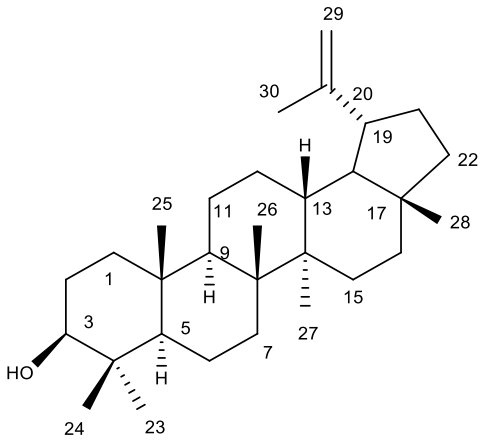
Structure elucidation of pure compound

Compound (**1**) was obtained as white needles. It gave a purple anisaldehyle-sulfulic acid test. The molecular formula was $C_{30}H_{50}O$ by EIMS (*m/z* 426.9; $[M]^+$). The IR spectrum (KBr) ν_{max} showed absorption band of hydroxyl group at 3339 cm^{-1} and double bond at 1637 cm^{-1}.

The ^1H NMR spectral data ($CDCl_3$, 500 MHz) 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 (*brs*) and 4.66 (*brs*). And the oxymethine proton at δ 3.17 (*dd*).

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

Compound (**1**) was identified as lupeol by comparison of the data with the previous study (Reynolds *et al*., 1986).



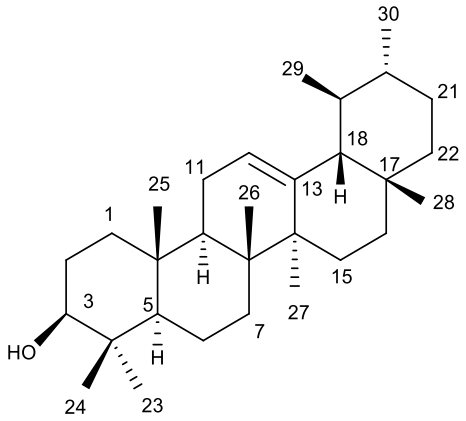
Lupeol

Table 6 NMR spectral data of compound (**1**) as compared with lupeol (in ($CDCl_3$)

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*)	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 (*brs*), 4.66 (*brs*)	109.3	4.56 (*m*), 4.69 (*m*)	109.3
30	1.65 (*s*)	19.2	1.69 (*s*)	19.3

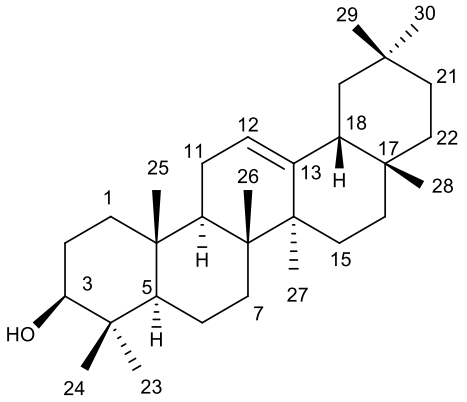
Compound (**2**) was obtained as a white solid. It gave a purple anisaldehyle-sulfuric acid test. The molecular formula was $C_{30}H_{50}O$ by EIMS (*m/z* 426.9; $[M]^+$). The IR spectrum (KBr) ν_{max} showed absorption band of at 3249 cm^{-1} for a hydroxyl group. The ^1H NMR spectral data ($CDCl_3$, 500 MHz) of this compound showed characteristic ursane-type triterpenoids as six methyl groups at δ 0.96 (*s*), 1.08 (*s*), 1.05 (*s*), 1.01 (*s*), 0.83 (*s*) and 0.93 (*s*), the carbinylic proton at δ 3.17 (*dd*) and the olefinic proton at δ 5.15 (*t*). This compound was identified as α-amyrin by comparison of the data with the previous study (Lima *et al*., 2004; Rao *et al*., 2012).



α-amyrin

Compound (**3**) was obtained as awhite solid. It gave a purple anisaldehyle-sulfuric acid test. The molecular formula was $C_{30}H_{50}O$ by EIMS (*m/z* 426.9; $[M]^+$). The

IR spectrum (KBr) ν_{max} showed absorption band of at 3450 cm^{-1} for a hydroxyl group. The ^{1}H NMR spectral data ($CDCl_3$, 500 MHz) of this compound showed characteristic oleanane- type triterpenoids as signals of six methyl groups at δ 0.77 (*s*), 0.90 (*s*), 0.93 (*s*), 0.95 (*s*), 0.96 (*s*) and 1.03 (*s*), and the carbinylic proton at δ 3.19 (*dd*) and the olefinic proton at δ 5.09 (*t*). This compound was identified as β-amyrin by comparison of the data with the previous literature (Kushiro *et al*., 1998).



β-amyrin

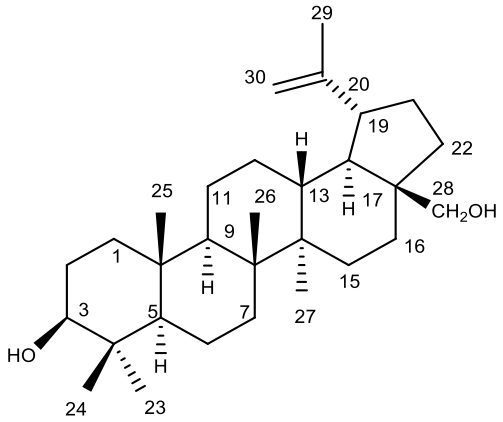
Both α-amyrin and β-amyrin have been isolated from various species and exhibited various pharmacological activities in vitro and in vivo, such as anti-microbial and anti-fungal, anti-cancer and anti-inflammatory (Rao *et al*., 2012).

Compound (4) was obtained as white needles. It gave a purple anisaldehyle-sulfuric acid test. The molecular formula was $C_{30}H_{50}O_2$ by EIMS (*m/z* 442.9; $[M]^+$). The IR spectrum (KBr) ν_{max} showed absorption band of hydroxyl group at 3450 cm^{-1} and double bond at 1643 cm^{-1}.

The ^{1}H NMR spectral data ($CDCl_3$, 500 MHz) of this compound showed six singlet signals of methyl groups 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 4.65 (*d*). The oxymethylene protons at δ 3.78 (*dd*) and 3.30 (*d*).

The ^{13}C NMR spectral data ($CDCl_3$, 125 MHz) of this compound showed signals of 30 carbons: 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. The methyl carbons appeared at δ14.7, 15.3, 15.9, 16.0, 19.2, 27.9, the olefinic carbons at δ150.9 and 109.3, the oxymethylene carbons at δ79.9 and 60.5. The ^{13}C NMR spectrum indicating a lupane-type triterpenoids and was identified as betulin by comparison of the data with previous literature. The pharmacological activity of this compound has been reported as anti-inflammatory, anti-malaria, and anti-tumor (Sami *et al*., 2006).



Betulin

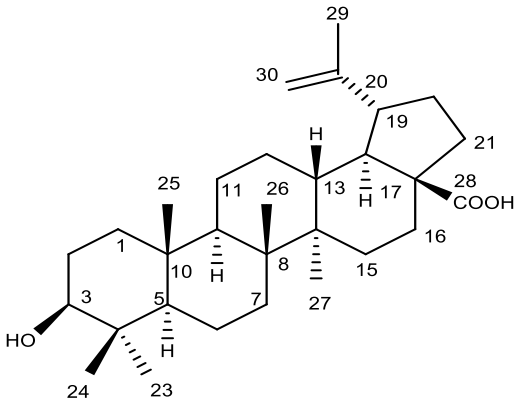
Table 7 NMR spectral data of compound (**4**) as compared with betulin (in ($CDCl_3$)

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*)	78.9	3.19 (*dd*)	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*)	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	3.30 (*dd*), 3.78 (*dd*)	60.5	3.31 (*d*) , 3.77 (*dd*)	60.2
29	4.55 (*m*), 4.65 (*d*)	109.3	4.58 (*m*), 4.68 (*d*)	109.6
30	1.65 (s)	19.2	1.68 (*s*)	19.1

Compound (5) was obtained as white needles. It gave a purple anisaldehyle-sulfuric acid test. The molecular formula was $C_{30}H_{48}O_3$ by EIMS (*m/z* 456.8; $[M]^+$). The IR spectrum (KBr) ν_{max} showed absorption band of hydroxyl group at 3446 cm^{-1} and a carbonyl group at 1687 cm^{-1}.

The ^1H NMR spectral data (CD_3OD, 500 MHz) showed characteristic of lupane- type triterpenoids as one vinylic methyl at δ 1.68, two protons of an isopropenyl moiety at δ4.51 (*br s*) and 4.76 (*br s*). The ^{13}C NMR spectrum (CD_3OD, 125 MHz) of this compound showed signals of the six methyl carbons appeared at δ14.4, 15.5, 15.9, 19.4, 19.5 and 28.0, eleven methylene carbons appeared at δ16.7, 26.9, 28.0, 28.6, 30.4, 30.7, 31.7, 33.0, 35.6, 38.3, and 110.0, six methine carbons appeared at δ38.2, 48.4, 49.4, 49.5, 52.0 and 79.6 and six quaternary carbons appeared at δ39.6, 39.9, 40.0, 41.9, 56.9, 152.1. This compound was identified as betulinic acid by comparison of the data with previous study (Kwon *et al*., 2003)



Betulinic acid

Table 8 NMR spectral data of compound (**5**) as compared with betulinic acid (in (CD_3OD)

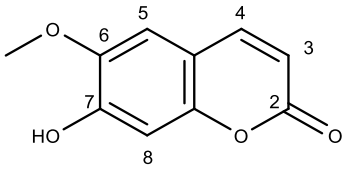
Position	Compound (5)		Betulinic acid	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	0.87 (*m*), 1.66 (*m*)	38.3	0.88 (*m*), 1.65 (*m*)	38.7
2	1.56 (*m*), 1.61 (*m*)	28.0	1.57 (*m*), 1.61 (*m*)	27.4
3	3.11 (*dd*)	79.6	3.19 (*dd*)	79.0
4	-	39.6	-	38.9
5	0.70 (*br d*)	52.0	0.69 (*m*)	55.3
6	1.33 (*m*), 1.51 (*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

Compound (6) was obtained as yellow solid. The UV spectrum showed maximum wavelength (λ_{max}) at 208, 228 and 344 nm. The IR spectrum (KBr) ν_{max} showed absorption bands characteristic of hydroxyl group at 3341 and carbonyl group at 1704 cm^{-1}.

The ^{1}H NMR spectrum (DMSO-d_6) of this compound showed signals of one methoxy groups at δ3.77 (*s*), and four olefinic protons at δ6.09 (*d*), 6.59 (*s*), 7.10 (*s*) and 7.83 (*d*). The ^{13}C NMR spectrum (CD_3OD) showed signals of ten carbons at δ56.8, 102.0, 109.9, 112.2, 112.3, 146.1, 147.3, 151.6, 153.6 and 164.1.

Compound (**6**) was identified as scopoletin by comparison of the data with the previous study. (Silva *et al*., 2001; Lin *et al*., 2002)



Scopoletin

Table 9 NMR spectral data of compound (6) as compared with 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*)	112.3	6.19 (*d*)	113.3
4	7.83 (*d*)	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
11	3.77 (*s*)	56.8	3.95 (*s*)	56.7

α-Glucosidase and α-amylase inhibitory activity of pure compounds

All the pure compounds showed both α-glucosidase and α-amylase inhibitory activities but in different extent as shown in Table 10. The results displayed strong α-glucosidase inhibitory activity for all pure compounds. The best activity was found in lupeol with IC_{50} values of 7.4 μM. The α-amylase inhibitory activities of all pure compounds were moderate to good except the butulin. The best activity was found in α-amyrin with IC_{50} values of 32.33 μM. The IC_{50} values of all compounds were

significantly better than acarbose at $p<0.05$ (a positive control) which against α-glucosidase and α-amylase were 308.63 and 194.72 μM, respectively.

Table 10 IC_{50} of pure compounds for α-glucosidase and α-amylase inhibitory activity

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

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 species. This study was the first report on the isolation of lupeol from the bark of *V. glabrata.* From the previous reports lupeol has the effect on α-glucosidase and α-amylase inhibitory activities. Lupeol isolated from acetone extract of *T. sericea* showed strong activity against α-glucosidase and α-amylase with IC_{50} values of 54.5 and 140.72 μM, respectively (acarbose IC_{50} values 93.22 and 60.25 μM, respectively) whereas lupeol isolated from acetone extract of the root bark of *Euclea undulate* was found to inhibited α-glucosidase activity with IC_{50} value 14.69 μM. (acarbose IC_{50} values 7.35 μM) (Nkobole *et al.*, 2011; Deutschlander *et al.*, 2011). Moreover, lupeol has been reported to exhibit various pharmacological activities such as anti-artgritic, anti-

microbial, anti- protozoal, anti- cancerous, anti- diabetic, anti- inflammatory, cardioprotective, and hepatoprotective activity (Siddique and Saleem, 2011).

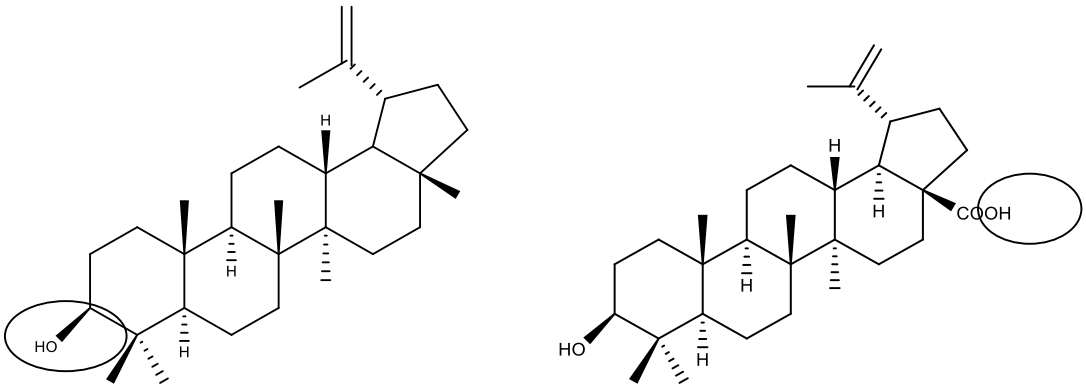
Both α- amyrin and β- amyrin have been reported to exhibit various pharmacological activities including anti- microbial, anti- inflammatory, anti- ulcer, anti-oxidation and anti- diabetic (Santos *et al.*, 2012; Vazquez *et al.*, 2012). As for anti-diabetic activity, these compounds exhibited moderate 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 inhibition of α- amylase activity in vitro and was the 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 in pancreatic of rat and human and the molecular docking result showed that this compound was the non- competitive inhibitor (Wulan *et al*., 2014). The previous study reported pharmacological activities of betulinic acid as anti-inflammatory, anti-malarial, anti- HIV, anti-cancer, anti-bacterial, anti- obese and anti- diabetic activity (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).

The compound scopoletin was the coumarin type whose reported activities were anti-oxidant, anti-inflammatory, anti-cholinesterase, anti-diabetic activity (Mogana *et al*., 2013). Scopoletin isolated from *HortiaLongifolia*was found to be strong inhibitor of α-glucosidase enzyme with IC_{50} value 4.63 μM (Queiroz *et al*., 2013).

In Addition, the chemical structure of pentaclyclic triterpene has position of hydroxyl group, may be this fuctional groups can contract or interactions between the ligand of α- glucosidase and α- amylase enzyme. Especially lupeol showed high activites, which the effects of hydroxyl group, while betulinic acid showed activities

less than lupeol. This may be due to betulinic acid have position of carboxylic acid group. The carboxylic acid group (COOH) replacing the hydrogen with active site of the enzyme, resulting decrease the effects of α-glucosidase and α-amylase inhibitory activities (Uddin *et al*., 2012).



Lupeol Betulibic acid

Figure 5 The chemical structure of compound, showing the chemically active centers

Isolation of the stem bark of *V. glabrata* afforded pentacyclic triterpenes namely lupeol, α-amyrin, β-amyrin, betulin, betulinic acid and coumarin namely scopoletin. This is the first report on isolation of these compounds. All compounds exhibited potent α-glucosidase and α-amylase inhibitory activity. Further research should be performed to develop these compounds to be used as potential anti-diabetic drug for treatment of type 2 diabetes patients.

CONCLUSIONS

The present study reported the screening of α-glucosidase and α-amylase inhibitory activities of fourteen plants items from Thai folk anti-diabetes formularies including MorPhon's recipe V. *glabrate*, S. *siamea*, T. *catappa*, P. *amarus*, S. *chinensis* exhibited most five potent of α-glucosidase inhibitory activity, by % inhibition 84.98$\pm$0.59, 83.14$\pm$0.15, 78.48$\pm$0.14, 74.85$\pm$0.79, and 69.06$\pm$0.78 μg/mL, respectively and P. amarus, M. *pudica*, V. *glabrate*, *D. rhodocalyx*, R. *nasutus* exhibited most five potent of α-amylase inhibitory activity, by % inhibition 94.73$\pm$1.37, 88.69$\pm$0.56, 84.71$\pm$1.51, 83.50$\pm$2.32, and 83.29$\pm$1.28 μg/mL, respectively. Furthermore, biological activity testing of recipe supported to those single plant activities. It is likely to explain that the tastes of these plants astringent, bitter, tasteless or fragrant/cool, which can regulate fire element. Hence, diabetes can be treated following the knowledge of Thai traditional medicine.

The most active plant, *V. glabrata* was further phytochemically isolated and obtained five triterpenoids compounds including, lupeol (**1**), α-amyrin (**2**), β-amyrin (**3**), betulin (4), betulinic acid (**5**), and one coumarin, scopoletin (**6**). Lupeol showed the highest α-glucosidase and α-amylase inhibitory activity with IC_{50} 7.4, and 46.81 μM, respectively.

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 studied *in vivo* for toxicity and anti-diabetic activity with a view to be developed as new drug for treatment of type 2 diabetes patients.

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APPENDEX

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Evaluation of *in vitro* α-amylase and α-glucosidase inhibitory potentials of 14 medicinal plants constituted in Thai folk antidiabetic formularies

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Abstract

The sporadic increase in the occurrence and prevalence of diabetes mellitus have compelled and vigorous search for alternative anti-diabetic therapeutic approach from medicinal plants and its bioactive. One of the major approach employed is the reduction of gastrointestinal glucose levels through the inhibition of carbohydrate digesting enzymes notably α-amylase and α- glucosidase. In this study, the ethanol extracts of 14 selected plants from Mor Porn's recipe were screened for their α-amylase and α- glucosidase inhibitory activity. The ethanolic extract from the stem of *Vitex glabrata* displayed the highest percentage inhibitory activity of 84.98 ± 0.59 and 84.71± 1.51 against α- glucosidase and α-amylase enzymes, respectively. Chemical investigation of the active extract of *V. glabrata* indicated that pentacyclic triterpenes were the major compounds responsible for the activity. The result obtained from this study suggests the potential use of *V. glabrata* as an alternative natural source for the treatment of diabetes mellitus.

Keywords

Anti-diabetic, *Vitex glabrata* R. Br; α-Amylase; α-Glucosidase; Mor Porn's recipe; Diabetes

Introduction

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia or high blood glucose level which affects the quality of patients' lives. Diabetes mellitus is triggered as a result of insufficiency insulin secretion, insulin action or both [1-2]. Globally, it is estimated that approximately 422 million people are living with diabetes and this number is envisaged to be doubled by 2030 [3-4]. 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 [5-6]. In Thailand, diabetes mellitus have been on the increase from 2.3% to 7.7% during 1991 to 2009. In fact, diabetes mellitus is the 10th leading cause of death among males (3.3 %) and it ranks 3rd among females (8.2 %) in Thailand [7-8]. Type 2 diabetes mellitus (T2DM) accounts for 90-95% of the global diabetic population. The increase in the blood glucose level associated with T2DM leads to damages in vital organs of the body and is also a major risk factor for microvascular and macrovascular complications such as, neuropathy, retinopathy, and nephropathy [9-10].

The management of diabetes mellitus is focused on regulating the blood glucose level so as to delay or impede the associated diabetic complications. Several pharmacological agents such as sulfonylureas, thiazolidinedione and α-glycosidase inhibitors (miglitol, voglibose and acarbose) have been approved and are widely used in the control of hyperglycemia. However, the side effects (including diarrhea, flatulence, bloating, nausea and soft feces in colon) associated with many of these antidiabetic agents as well as the high

cost have limited the success rate of these drugs [11-12]. Natural products especially traditional herbal plants have been considered as a suitable beneficial option for the treatment of diabetes. The World Health Organization (WHO) recommended the use of natural products based on traditional knowledge and encourages the development of herbal medicinal products for primary health care [13]. In addition, WHO has also encouraged the use of traditional medicinal plants for the treatment of diabetes due to their effectiveness and safety [14]. Thus, the focus on natural remedies which are considerably safer and more accessible as promising alternatives for the treatment of diabetes is imperative [15].

A sizeable number of medicinal plants are used in Thai traditional medicinal system and have been reported to have scientific significance as sources of antidiabetic agents. The use of polyherbal preparations as treatment for diabetes has been widely employed in Thailand especially by traditional folk healers with several of the preparation showing excellent activities. In this present study, 14 medicinal plants were accessed for their antidiabetic activity, out of the 14 plants *Vitex glabrata* showed the highest inhibitory activity, thus affording us an opportunity to focus our interest in the plant. The genus *Vitex* belongs to the family Verbenaceae and it consists approximately 250 identified species which are commonly found in tropical areas. Several species in this genus have long been used traditionally for the treatment of various illnesses. For instance, *V .cannabifolia* is used as an analgesic, *V .agnus-castus* is used as a diuretic and for treating stomach ache, *V .trifolia* is used for treating fever and inflammation .[16-18] The biological activities of some species in this genus have been reported for various activities such as anti-diabetic and antifungal activity [19-20] .In this study *Vitex glabrata* R. Br., a plant belonging to the Verbenaceae family

was selected. *V. glabrata* is a medium size deciduous tree native to tropical and subtropical regions of Asia. In Thailand *V. glabrata* R.Br .is locally called "Khai-nao" and is used in Thai folk medicine as an antidiarrheal, antipyretic, astringents, anthelmintic, for treating gastrointestinal disorders and for promoting lactation [21-22] .The ethanol extracts from the leaves have been reported to have anti-inflammatory, anti-estrogenic, antioxidant and hepatoprotective activities [21, 23-24]. Previous phytochemical studies of this plant have reported the presence of ecdysteroids, 11α,20-dihydroxyecdysone, 7-dehydrocholesterol, pterosterone and 20-hydroxyecdysone isolated from the bark and khainaoside A, khainaoside B and khainaoside C isolated from the leaves of the plant [21-22, 25] .Thai traditional medicine which boast of an array of recipes for diabetes treatment .In this study 14 medicinal plants from Thai folk medicinal recipe Mor Porn's were selected and screened for their α-glucosidase and α-amylase inhibitory activity. The results indicated that the extracts from *V. glabrata* showed the highest inhibitory activity. Since there is no report available on anti-diabetic activity of the plant, we therefore explored the phytochemical constituents of *V. glabrata* using various chromatographic techniques and also evaluated the *in vitro* anti-diabetic activity of the isolated compounds.

Results

In vitro anti-diabetic assay of the selected of selected plants.

Several plants used as remedies in Thai traditional medicine have been reported to possess preventive effects against diabetes and its complications through various

mechanism including α-glucosidase and α-amylase inhibition. In other to ascertain the relevant ethno-medicinal claims of plants used as anti-diabetic remedies in the Mor Porn's recipe, the ethanol extract of 14 selected plants in this recipe were evaluated for their antidiabetic activity by accessing their α-glucosidase and α-amylase inhibitory activity (Table 1). The results from the α-glucosidase and α-amylase inhibitory assay of the 14 selected plants from the Mor Porn's recipe is shown in Table 1. The ethanol extracts of *Vitex glabrata, Senna siamea* Lam, *Terminalia catappa* L., *Phyllanthus amarus, Salacia chinensis* L, *Zea mays* L and *Diospyros rhodocalyx* Kurz displayed significant inhibitory activities against α-amylase (84.71 ± 1.51, 81.95 ± 7.17, 78.59 ± 1.84, 94.73 ± 1.37, 72.33 ± 2.92, 73.83 ± 1.16 and 83.50 ± 2.32, respectively) and α-glucosidase (84.71 ± 1.51, 83.14 ± 0.15, 78.48 ± 0.14, 74.85 ± 0.79, 69.06 ± 0.78, 65.31 ± 1.02 and 63.61 ± 1.09, respectively) at a concentration of 25 µg/mL. The inhibition percentage was approximately 2.5 fold higher than the standard drug acarbose (33.00 ± 0.79 and 34.00 ± 0.45 for α-amylase and α-glucosidase, respectively at 25 µg/mL).

In generally, all these extracts tested showed moderate to high inhibitory activity in the α-glucosidase and α-amylase and inhibitory assays (Table 1). Since *V. glabrata* displayed the highest inhibitory activity in both assay, the plant was chosen for further phytochemical studies in other to identify its bioactive constituents

Table 1 % .inhibition of α-glucosidase and α-amylase of the ethanol plant extracts at 25 µg/mL

Plant	% inhibition of α-glucosidase (µg/mL)	% inhibition *of* α-amylase (µg/mL)
Vitex glabrata R.Br	84.98 ± 0.59	84.71 ± 1.51
Acanthus ebracteatus Wall.	21.10 ± 3.01	43.61 ± 3.85
Zea mays L.	65.31 ± 1.02	73.83 ± 1.16
Mimosa pudica Linn.	28.24 ± 1.67	88.69 ± 0.56
Pandanus odorus Ridl	28.82 ± 2.88	35.50 ± 6.1
Diospyros rhodocalyx Kurz.	63.61 ± 1.09	83.50 ± 2.32
Abutilon polyandrum W & A	53.33 ± 1.45	74.18 ± 5.97
Phyllanthus amarus	74.85 ± 0.79	94.73 ± 1.37
Senna siamea Lam.	83.14 ± 0.15	81.95 ± 7.17
Terminalia catappa L.	78.48 ± 0.14	78.59 ± 1.84
Senna alata L.	46.17 ± 2.11	39.99 ± 3.14
Legerstroemia speciosa (L.) Pers.	69.05 ± 0.25	62.41 ± 10.84
Rhinacanthus nasutus (L.) Kurz.	55.81 ± 1.76	83.29 ± 1.28
Salacia chinensis L	69.06 ± 0.78	72.33 ± 2.92
Acarbose (25 µg/mL)	34.00 ± 0.45	33.00 ± 0.79

Characterization of isolated compounds from V. glabrata

Among the fractions obtained from the solvent partitioning of the crude ethanolic extract of the bark *V. glabrata*, it was demonstrated that the hexane and ethyl acetate fractions displayed the best α-glucosidase and α-amylase inhibitory activity (Table 2). Thus, the phytochemical study of hexane and ethyl acetate fractions obtained from the ethanol extracts from bark of *V. glabrata* led to the isolation and structural characterization of six pure bioactive molecules. The structures of the isolated compounds were elucidated based on spectroscopic data analysis (^{1}H, ^{13}C NMR and MS) and by comparison with previous literature. Five pentacyclic triterpenes and one coumarin were identified as lupeol (**1**) [25], α-amyrin (**2**) [26-27], β-amyrin (**3**) [28], betulin (**4**) [29], betulinic acid (**5**) [30] and scopoletin (**6**) [31-32]. The structures of the isolated compounds are shown in Fig. 1. This is the first report on the isolation of these compounds from *V. glabrata*.

Table 2. % inhibition of α-glucosidase and α-amylase of fractions obtained from solvent partitioning

Fractions	% inhibition at 25 µg/mL	
	α-glucosidase activity	*α*-amylase activity
n-C_6H_{14} (VH)	83.86 ± 1.03	67.86 ± 2.45
EtOAc (VE)	86.44 ± 2.15	63.3 ± 1.34
n-BuOH (VB)	49.46 ± 3.18	61.5 ± 2.01
Aqueous (VW)	46.52 ± 1.89	51.91 ± 3.05

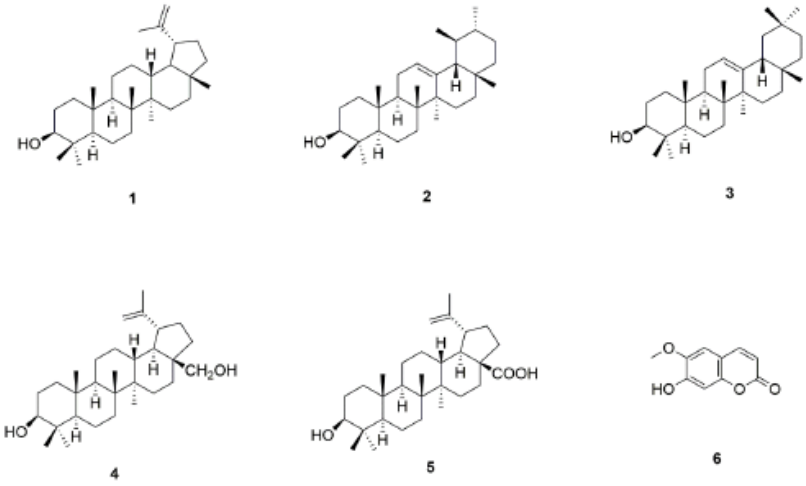

Figure 1. Structure of isolated compounds from the stem barks of *V. glabrata*.

Alpha glucosidase and α-amylase inhibitory activity of isolated compounds

The results from the *in vitro* anti-diabetic effect (α-glucosidase and α-amylase inhibitory activity) of the isolated compounds from *V. glabrata* are presented in Table 3. Out of the compounds tested, the IC_{50} values exhibited by lupeol (**1**) and α-amyrin (**2**) (α-glucosidase: IC_{50} values of 7.40 and 8.60 µM; and α-amylase inhibitory activity with IC_{50} values of 46.81 and 32.33 µM, respectively). Whereas scopoletin (**6**) had the weakest α-glucosidase activity (52.76 µM) and betulin (**4**) displayed the weakest α-amylase inhibitory activity (284.35 µM) (Table 3). All the isolated compounds displayed more potent inhibitory activity than the standard anti-diabetic drug acarbose. The standard acarbose had IC_{50} values of 308.63 µM for α-glucosidase and 194.72 µM for α-amylase inhibitory activity.

Table 3. IC_{50} values of isolated pure compounds for α-glucosidase and α-amylase

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

Discussion

Diabetes mellitus is gradually become one of the serious metabolic disorder in the world due to the severity of its associated complications and the global occurrence of the disease [33]. Glucosidase inhibitors are agents which have the ability to reduce postprandial blood glucose level by hindering the activities of α-glucosidase and α-amylase which is are chiefly responsible for the degradation of carbohydrates. Natural products continue to play a pivotal role in drug discovery and the evaluation of medicinal plants in the treatment and prevention of diabetes is increasingly becoming important due to their beneficial effects as well as possible alternative sources for safer anti-diabetic therapy [34-35]. This present study investigated the anti-diabetic activity of 14 medicinal plants used as recipe in Thai anti-diabetic formulation. Out of these plants, *V. glabrata* displayed the highest *in vitro* anti-diabetic activity using α-glucosidase and α-amylase assay. Thus a bioassay guided fractionation of the extract from *V. glabrata* was pursued to obtain the bioactive constituents responsible for this activity.

The inhibition of key enzymes such as α-glucosidase and α-amylase which play vital roles in the breakdown of carbohydrate and polysaccharides into monosaccharides which is of paramount importance in postprandial hyperglycemia in diabetes. The damages to vital organs in the body caused by high blood glucose levels cannot be over emphasized as it accounts for several diabetic complications including atherosclerosis, diabetic wounds, retinopathy, neuropathy and nephropathy [36-37]. Impeding the activities of these enzymes limits the rate of conversion of starch and oligosaccharides leading to the reduction of

glucose absorption which is critical in the treatment of diabetes [38]. Our result indicated that the ethanol of *V. glabrata* exhibited the highest percentage of inhibition i.e. 84.98 ± 0.59 (α-glucosidase) and 84.71 ±1.51 (α-amylase) out of the 14 tested plant extracts. *P. amarus* showed a higher percentage of inhibition α-amylase enzyme (94.73±1.37) than *V. glabrata*, however its percentage of inhibition of α-glucosidase (74.85 ± 0.79) was much lower.

Numerous body of evidences have emphasized medicinal plants as a reservoir of bioactive compounds which are valuable asset with medicinal value for drug development. In a bid to identify and elucidate the major components responsible for the bioactivity observed in *V. glabrata*, a detailed study focusing on the chemical constituent was performed using various chromatographic methods, leading to the isolation of six compounds. Previous studies have indicated that some of the isolated compounds have been previously reported as anti-diabetic constituent's *in vitro* and *in vivo* studies [39-41]. The use of natural products as potential inhibitors of carbohydrate-degrading enzymes has been a continuous focus as an alternative approach for the prevention, management and treatment of diabetes mellitus. A number of previous studies have validated the α-glucosidase and α-amylase inhibitory activity of natural bioactive compounds from medicinal plants [42-44]. Pentacyclic triterpenes, a group of plant secondary metabolites are abundant in several medicinal plants and they display multiple bioactivities such as anti-inflammatory and anti-diabetic activities [45-46]. Our study demonstrated the inhibitory activities of the isolated compounds against the glucosidase enzymes with α-amyrin displaying the highest inhibitory activity among the six isolated compound.

Conclusion

In conclusion, this present study revealed that the ethanol extract from *V. glabrata* exhibited significant in vitro anti-diabetic activity. The result also suggests that *V. glabrata* can be exploited in the discovery of bioactive natural products for the treatment of diabetes mellitus.

Experimental section

Plant collection

The plants materials were either collected at Khuan Niang, Songkla province and or purchased from Thai traditional drug stores in Hat Yai, Songkla province, Thailand. The plants (*Vitex glabrata* R. Br, *Acanthus ebracteatus* Wall, *Zea mays* L., *Mimosa pudica* Linn., *Pandanus odorus* Ridl, *Diospyros rhodocalyx* Kurz, *Cassia siamea* Britt., *Phyllanthus amarus*, *Abutilon polyandrum* W & A, *Terminalia catappa* Linn, *Cassia alata* L, *Legerstroemia speciosa* Pers, *Rhinacanthus nasutus* Kurz and *Salacia chinensis* L.) were authenticated and deposited at Faculty of Traditional Thai Medicine, Prince of Songkla University. Fresh disease free plant samples were washed under running tap water, air-dried, chopped into smaller and oven dried 50 °C. The dried samples were pulverized to fine powder and stored in airtight containers at room temperature until further use.

Crude extract preparation

Thirty grams of each plant powder was macerated with ethanol (150 ml × 3) at room temperature for 3 days. The filtrate was collected by filtering though Whatman® No.1 filter paper and the solvent were evaporated under reduced pressure using a rotary evaporator. The resulting crude extracts were kept at -20 °C and used for the *in vitro* antidiabetic activity.

Extraction and isolation from the stem bark of V. glabrata

The ethanolic extract of *V. glabrata* (40 g) was dissolved in 10% MeOH (1 L) and then was exhaustively and sequentially partitioned with *n*-hexane, EtOAc and *n*-BuOH to obtain *n*-hexane soluble fraction (VH; 3.01 g), EtOAc soluble fraction (VE; 21.15 g) and *n*-BuOH soluble fraction (VB; 5.80 g). VH (2.5 g) was chromatographed using SiO_2 using stepwise gradient of *n*-hexane and EtOAc (100:0 to 100:0) and 2.5-40% MeOH in EtOAc to afford six fractions (VH1 to VH6). VH1 (470 mg) was further purified using Sephadex LH-20 (20% MeOH in CH_2Cl_2) to obtained four sub fractions (VH1-1 to VH1-4). VH1-3 (75 mg) was further chromatographed using SiO_2 eluting with n-hexane/EtOAc (70:30) to give six fractions (VH1-3A to VH1-3F). VH1-3C (61 mg) was subjected to semi-preparative HPLC using RP-18 column to yield compounds **1** (6.4 mg), **2** (3.7 mg) and **3** (3.6 mg).

Fractions VH2 (822 mg) was subjected to Sephadex LH-20 (20% MeOH in CH_2Cl_2) to afford VH2-1 (92 mg) and VH2-2 (730 mg). VH2-2 was purified over SiO_2 eluting with EtOAc in *n*-hexane (30, 50 and 100%) to yield ten fractions (VH2-2.1 to VH2-2.10). Fractions VH2-2.4 (163 mg) was subjected to semi-preparative HPLC using RP-18 column (90% MeOH)

to yield compounds **4** (5.0 mg) and **5** (3.0 mg). EtOAc extract VE (20 g) was subjected to Diaion HP-20 and eluted with stepwise gradient of 50-100% MeOH in water 80% EtOAc in MeOH and 100% EtOAc to afford VE1 to VE6. VE3 (1 g) was further subjected to Sephadex LH-20 (100% MeOH) to yield three fractions (VE3A to VE3C). VE3B (400 g) was further subjected to SiO2 column chromatography using 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 compound **6** (3 mg).

In vitro anti-diabetic assays

Alpha glucosidase inhibitory activity

The α-glucosidase inhibitory assay of the extracts and isolated compounds was performed based on the modified method of Bachhawat et al [47]. The reaction mixtures in 96 well plates containing 50 mL of sample was mixed with 50 mL of enzyme (0.57 unit/mL) and incubated at 37 °C for 10 min. Then, 50 mL of the *p*-nitrophenyl-α-D-glucopyranoside (5 mM) as substrate was placed to the mixture and incubated at 37 °C for an additional 20 min. The reaction mixture was stopped by adding 50 mL of 1 M Na_2CO_3 solution. The absorbance was measured at 405 nm using UV/Vis absorbance spectrophotometer microplate reader. The absorbance of blank and control samples was also determined. Acarbose was used as standard drug. The inhibition of α-glucosidase inhibitory activity was calculated using the following equation

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

Where $A_{control}$ corresponds to the absorbance of the solution without extract/compounds and A_{sample} corresponds to absorbance of the sample. IC_{50} is the concentration providing 50 %inhibition and was determined from the graph plotted between %inhibitions against sample concentrations.

Alpha amylase inhibitory assay

The α-amylase inhibitory assay was adapted from the method of Hansawasdi et al [48]. 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 °C. The starch solution was pre-incubated at 37 °C for 5 min. The sample was dissolved in 1 mL of dimethyl sulfoxide, and added to 100 µL of α-amylase (1.6 unit/mL) solution in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM NaCl. The reaction mixture was incubated at 37 °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 °C. The absorbance measured at 595 nm using by UV/Vis absorbance spectrophotometer micro-plate reader. % inhibition is calculated by equation

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

Where $A_{control}$ corresponds to the absorbance of the solution without extract/compounds and A_{sample} corresponds to absorbance of the sample. IC_{50} is the concentration providing

50 %inhibition and was determined from the graph plotted between %inhibitions against sample concentrations.

Statistical analysis

All data were expressed as the mean ± SD. Statistical analysis was performed using SPSS (version 17.0, SPSS Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for data comparison followed by post hoc analysis using Tukey test. Differences were considered significant at $P < 0.05$.

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Author Contribution Statement

All the authors contributed extensively to the work presented in this manuscript (design of the project, data analysis and manuscript preparation). C.S and C.O conceived and designed the experiment. C.S and O.J.O analyzed the data and prepared the manuscript. All authors read and approved the final version of the manuscript.

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