รายงานวิจัยฉบับสมบูรณ์

การสำรวจสมุนไพรในตำรับยาสมุนไพรรักษาเบาหวานที่มีฤทธิ์ยับยั้งการทำงานของเอนไซม์ Dipeptidyl Peptidase IV

Screening for Dipeptidyl Peptidase IV (DPP-4) inhibitors from Thai herbal medicines use in traditional anti-diabetes recipe

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โครงการวิจัยนี้ได้รับทุนสนับสนุนจากเงินรายได้

มหาวิทยาลัยสงขลานครินทร์

ประจำปีงบประมาณ 2558 **รหัสโครงการ PHA580295S**

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ชื่อโครงการวิจัย

(ภาษาไทย) การสำรวจสมุนไพรในตำรับยาสมุนไพรรักษาเบาหวานที่มีฤทธิ์ยับยั้งการทำงานของเอนไซม์ Dipeptidyl Peptidase IV

(ภาษาอังกฤษ) Screening for Dipeptidyl Peptidase IV (DPP-4) inhibitors from Thai herbal medicines use in traditional anti-diabetes recipe

คณะนักวิจัยและหน่วยงานต้นสังกัด

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Finally, the most special thanks to scholarship supports from Prince of Songkla University for all financial support.

Dr. Panupong Puttarak

บทคัดย่อ

เบาหวานชนิดที่ 2 เป็นโรคที่มีอุบัติการณ์การเกิดที่เพิ่มสูงขึ้นมากกว่า 10 เท่าเมื่อเทียบกับ 10 ปีที่ผ่านมา โดยปัจจุบันได้มีการพัฒนายามากขึ้น โดยเอนไซม์ Dipeptidyl Peptidase IV (DPP-4) จัดเป็นอีกหนึ่งเป้าหมายที่น่าสนใจในการพัฒนายาหรือสารยับยั้ง เพื่อช่วยควบคุมระดับน้ำตาลในเลือดในผู้ป่วยโรคเบาหวานชนิดที่ 2 งานวิจัยนี้มีวัตถุประสงค์เพื่อประเมินฤทธิ์ยับยั้งการทำงานของเอนไซม์ดังกล่าวของพืชที่อยู่ในสูตรตำหรับรักษาเบาหวานของกรมหลวงชุมพรเขตอุดมศักดิ์หรือหมอพร ซึ่งยังไม่มีการรายงานผลการยับยั้งการทำงานของเอนไซม์ DPP-4 มาสกัดด้วยวิธีแช่สกัด และทดสอบฤทธิ์ยับยั้งการทำงานของเอนไซม์ DPP-4 โดยใช้ gly-pro-*p*-nitroanilide เป็นซับสเตรท ตามงานวิธีการทดลองของงานวิจัยก่อนหน้านี้

จากผลการทดลองพบว่า สมุนไพรทั้ง 14 ชนิดมีฤทธิ์การยับยั้งการทำงานของเอนไซม์ DPP-4 ที่ความเข้มข้น 50 μg/mL ที่แตกต่างกัน โดยสารสกัดอินทนินน้ำ สามารถยับยั้งเอนไซม์ได้สูงที่ร้อยละ 71.07±0.07 รองลงมาเป็น หูกวาง ยับยั้งเอนไซม์ได้ร้อยละ 69.89±0.43 ในขณะที่สารมาตรฐาน diprotin A สามารถยับยั้งได้ร้อยละ 90.07±0.39

จากการทดลองพบว่าสมุนไพรทุกชนิดที่ศึกษามีฤทธิ์ในการยับยั้งการทำงานของเอนไซม์ DPP-4 โดยเฉพาะสารสกัดอินทนินน้ำและหูกวางที่มีฤทธิ์ยับยั้งการทำงานของเอนไซม์ DPP-4 ที่สูง และการศึกษานี้ถือเป็นการศึกษาแรกที่รายงานผลยับยั้งการทำงานของเอนไซม์ DPP-4 ของสมุนไพรในสูตรตำรับนี้ ซึ่งเป็นข้อมูลสนับสนุนถึงประสิทธิภาพของสมุนไพรในสูตรตำรับนี้ในการรักษาโรคเบาหวานของหมอพื้นบ้านโดยออกฤทธิ์ผ่านกลไกการยับยั้งเอนไซม์ DPP-4

Abstract

Type 2 diabetes mellitus (T2DM) patients has dramatically increased almost 10 times within the past 10 years. Several groups of drug have been developed to treat T2DM including dipeptidyl peptidase-IV inhibitor. KromLuangChomphon folk recipewas used as alternative anti-diabetes recipe; however, no scientific data on DPP-IV inhibitory activities of this recipe has beenevaluated. In the present study, 14 selected medicinal herb extracts from this recipe were prepared by maceration with ethanol and investigated for their DPP-IV inhibitory activity using gly-pro-*p*-nitroanilide as substrate.

The result demonstrated that all extracts exhibited DPP-IV inhibitory activity but in different levels. The highest inhibitory activities at 50 μg/mL were detected in *Lagerstroemia speciose* (L.) Pers. (71.07±0.07%) and *Terminalia catappa*L. (69.89±0.43%), while diprotin A (standard) gave 90.07±0.39% inhibition.

All selected herbs especially leaves of *T. catappa* and *L. speciose* showed ability to be used as DPP-IV inhibitor. Furthermore, our results is the first report of DPP-IV inhibitory activity of fourteen herb extracts which are the main ingredients in KromLuangChomphon folk recipe. These finding also support the potential use of these recipe as alternative treatment of diabetes through a new mechanism.

Introduction and Objectives

Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia which may result from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes may cause long-term organs damage, dysfunction or failure especially eyes, kidneys, nerves, heart disease, stroke and serious wound (American Diabetes Association, 2008). There are four types of DM have been classified, Type 1 DM is associated with β-cell destruction which usually leading to absolute insulin deficiency. The patient of this type is insulin dependence. Type 2 DM (T2DM) is usually caused by insulin resistance with relative to insulin deficiency or the defect in insulin secretion. The patients of this type can control blood glucose in a normal level by using anti-diabetic drugs. Type 3 is gestational DM is a type of DM occurs during pregnancy and no need of any treatment. Type four is other specific type of DM which may cause by genetic defect (American Diabetes Association, 2008). Nowadays, Type 2 DM patients are the world's fifth leading cause of death and it was estimated to reach 366 million people in 2030 (Wild et al., 2004), which accounted for 90-95% of all diabetics (American Diabetes Association, 2008). There are two processes causing type 2 DM including insulin resistance, impaired insulin action, and beta-cell malfunction resulting in high blood glucose level. . Gastrointestinal enzymes such as α-glucosidase, α-amylase and dipeptidyl peptidase-IV (DPP-4) also play important role on blood glucose level. DPP-4 enzyme is a new drug target in DM treatment. This enzyme is a membrane bound enzyme involving in incretin system. DPP-IV inhibitors improved insulin secretion, suppress glucagon release, and lowering blood glucose. Long term DM is often associated with secondary complications including cerebral ischemia, heart disease, high blood pressure and etc. (Fuchs et al., 2014) due to over production of free radicals and lack of antioxidant process. Furthermore, in diabetes patients oxidative stress always produced resulting in free radicals with plays an important role in those complications. Moreover, DM patients have also malfunction of antioxidant defend system give rise to increasing in oxidative stress. High level of free radicals causes multi-organ damage and many complications in DM patients. Traditional antidiabetic remedies can be used as alternatives for the treatment of diabetes or reinforcements to the currently used treatments. They may even reduce the risk of the disease. Large amounts can be consumed in everyday diet, which is a positive aspect. Currently, there are a large number of plants and natural biomolecules have been reported in literatures for their antidiabetic effects (Arulselvan et al., 2014). From the recent reviewed

sixty natural products were reported to have antidiabetic activity in many mechanisms (Arulselvan et al., 2014) including DPP-IV inhibition mechanism. Therefore, many researchers are searching for DPP-IV inhibitors lead compounds from natural sources for new drug discovery. Examples of were previous reported methanolic extracts of *Magifera indica* leaves (Yogisha et al., 2010) and *Berberisaristata* barks(Chakrabarti et al., 2011), water and ethanol extracts of *Dodonaeaviscosa* (L.) Lacq. aerial parts(Veerapur et al., 2010), *Castanospermum australe* seeds (Bharti et al., 2012) and *Pilea microphylla* (L.) whole plants (Bansal et al., 2012) were demonstrated to have DPP-IV inhibitory and antioxidant activities. They also have been tested in diabetic rat models and the results showed that they could be used effectively in blood glucose lowering and comparable to the animal models that have been tested with DPP-IV antidiabetic drugs.

In this study, thirteen plants are selected from Thai folk anti-diabetes remedies namely Krom-Luang-Chomphon or MorPhon's. This folk medicinal recipe has been previously used in Thai traditional medicine to treat diabetes patients. However, no information of DPP-IV inhibitory activity of these thirteen plant extracts have been previously reported.

Objectives

1. To understand the therapeutic activity through DPP-4 pathway of selected medicinal plants that been used as traditional herbal recipe for the treatment of DM.

2. To determine the potency of selected crude medicinal plant extracts for inhibition of DPP-4 activity.

3. To support the effectiveness of Thai herbal medicine recipe for using as alternative glycemic control agents.

Review literatures

1. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia which may result from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes may cause long-term organ damage especially eyes, kidneys, nerves, heart disease, stroke and serious wound (American Diabetes Association, 2008). There are four types of DM have been classified, Type 1 DM is associated with β-cell destruction which usually leading to absolute insulin deficiency. The patient of this type is insulin dependent. Type 2 DM which accounts for about 90-95% of diabetes patients are usually caused by insulin resistance with relative to insulin deficiency or the defect in insulin secretion. The patients of this type can have blood glucose in a normal level by using anti-diabetic drugs or insulin. Type 3 is gestational DM is a type of DM occurs during pregnancy and no need of any treatment. Type four is other specific type of DM which may cause by genetic defect (American Diabetes Association, 2008).

2. Type 2 diabetes mellitus

T2DM is possibly the world's fastest growing metabolic syndrome of multiple etiologies causing hyperglycemia (Al-Masri et al., 2009; Nyenwe et al., 2011). According to the World Health Organization(World Health Organization, 1999), T2DM is the world's fifth leading cause of death and it was estimated in 2000 that there were 171 million diabetic patients. Incidences of T2DM for 2030 are estimated to reach 366 million people, which accounted for 90-95% of all diabetics (American Diabetes Association, 2008). The progression of T2DM is a human hyperglycemic state characterized by insulin resistance with relative to insulin deficiency or insulin secretion (Wild et al., 2004; World Health Organization, 1999).

Exogenous insulin, drug and lifestyle modification can control blood sugar in T2DM. There are many different drug mechanisms for DM treatment including stimulate insulin secretion, reduce glucose absorption, and metabolism (Wass et al., 2011).

3. Oral antidiabetic drugs

Nowadays, there are many types of commercially available oral anti-diabetic drugs for the T2DM treatment such as sulfonylureas (e.g. chlorpropamide and glibenclamide), biguanide (e.g. metformin), thiazolidinediones, α-glucosidase inhibitors (e.g. acarbose), glucagon like

peptide-1 (GLP-1) analogues (e.g. lariglutide, pramlintide and exenatide), sodium-glucose cotransporter-2 (SGLT2) inhibitors (e.g. dapagliflozin), bile acid sequestrants (e.g. colesevelam), dopamine-2 agonists (e.g. bromocriptine quick release) and dipeptidyl peptidase-IV inhibitors (e.g. sitagliptin and sexagliptin) groups. However, some group of anti-diabetic drugs had side effects such as hypoglycemia, weight gain, hyperinsulinemia, lactic acidosis, heart failure, stomach pain and nausea. DPP-4 inhibitors are antidiabetic agents which are currently developed and are of interests since they did not have evidence to show hypoglycemia and weight gain in diabetic patients (Wass et al., 2011).

4. Role of DPP-4, GLP-1 and GIP in diabetes mellitus patients

Normally, blood glucose level is increased after food ingestion. Glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are then released from intestine to stimulate insulin secretion and reduce glucagon secretion from pancreas. They could also increase insulin sensitivity and inhibit glucagon production from liver, therefore, reduce blood glucose level (Figure 1).

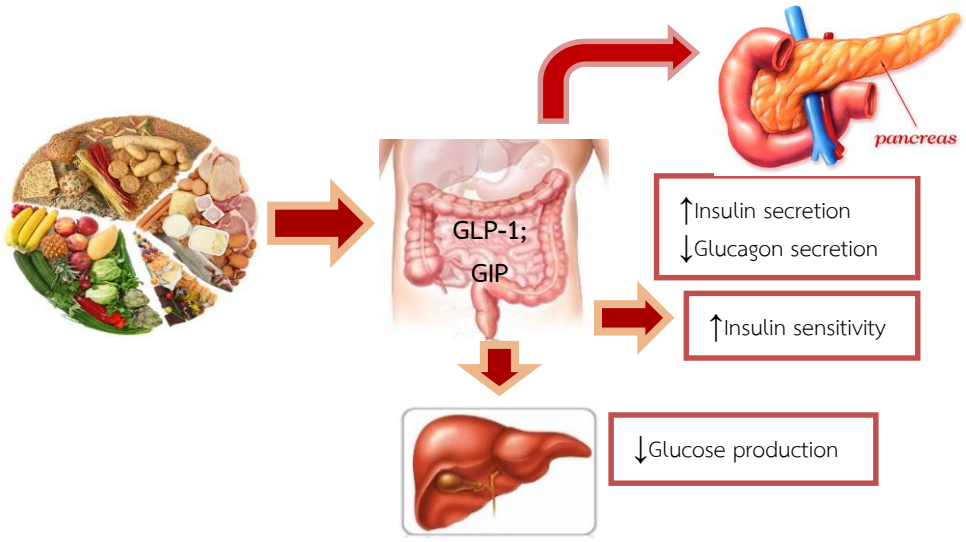


Figure 1 Function of GLP–1 and GIP (This picture is modified fromNTU-Taida team (2012)).

However, to prevent hypoglycemia due to high level of GLP-1 and GIP, dipeptidyl peptidase-IV (DPP-4) enzyme is released to inactivate GLP-1 and GIP to make blood glucose concentration in normal level.

GLP-1 is an incretin hormone secreted by intestinal L-cells of the distal small intestine in response to food intake. The active from of GLP-1 is a 30 amino acids peptide (Figure 2), which binding to the GLP-1 receptor on pancreatic β-cells will stimulate insulin gene

expression, insulin bio-synthesis and glucose-dependent insulin release. It also inhibits glucagon release, promote satiety, slow gastric emptying and promotes growth the pancreatic *β*-cells (Havale et al., 2009).

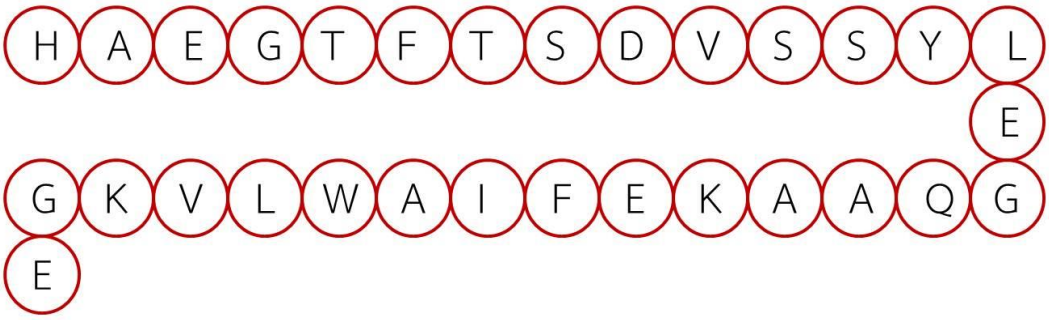


Figure 2 Structure of GLP-1(in circle are amino acid constituents of GLP-1) (This picture is modified from Havale et al. (2009)).

The function of GIP is to neutralize stomach acid to protect the small intestine from acid damage and reduce the rate at which food is transferred through the stomach, and inhibits the gastrointestinal motility and secretion of acid. The active from of GIP is a 42 amino acids peptide (Figure 3) is secreted by K cells from the upper small intestine within minutes of nutrient ingestion, facilitates the rapid disposal of ingested nutrients. Both, GLP-1 and GIP play a major role in glucose homeostasis (Havale et al., 2009).

DPP-4 is a member of a family of protease that includes dipeptidyl peptidase-8, dipeptidyl peptidase-9 and fibroblast activation protein (FAP). DPP-4 is dipeptidyl peptidase-IV, CD-26 and adenosine deaminase complexing protein. It is a serine protease enzyme, plays a major role in glucose metabolism by cleaving incretin including GIP and GLP-1 by cutting two amino acids from *N*-terminal of peptide making the GLP-1 and GIP inactive (Figure 4).

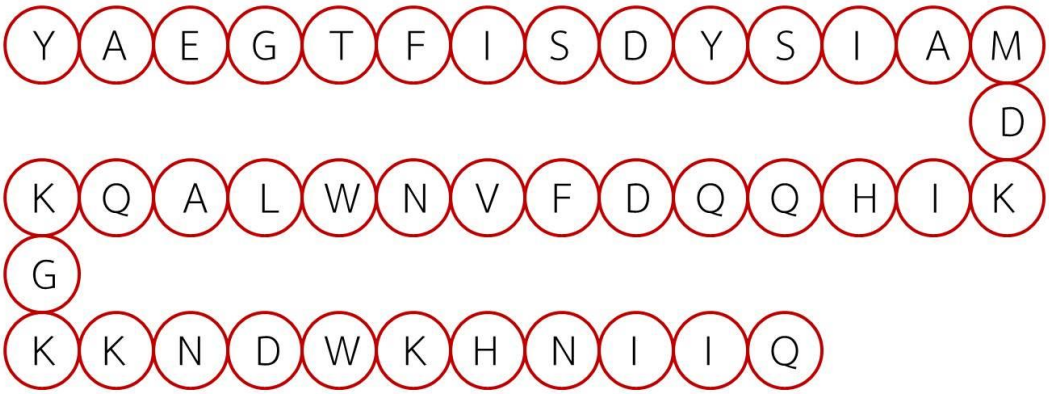
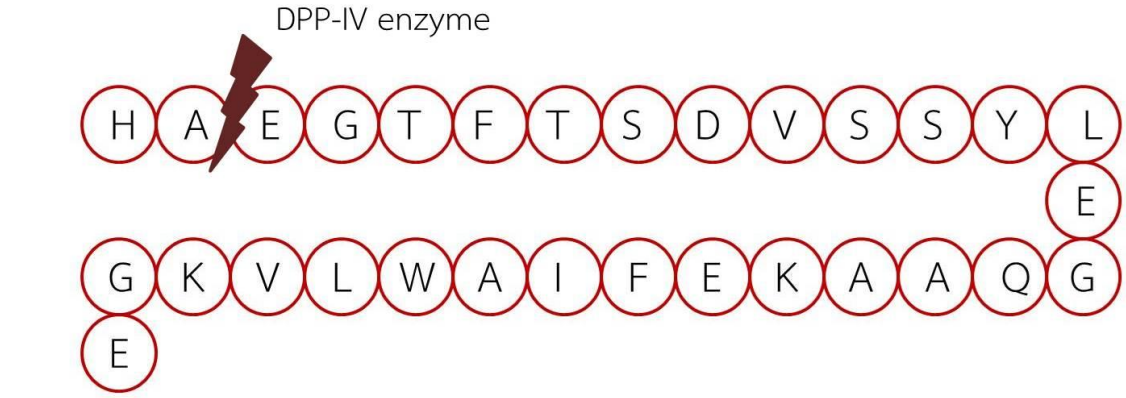
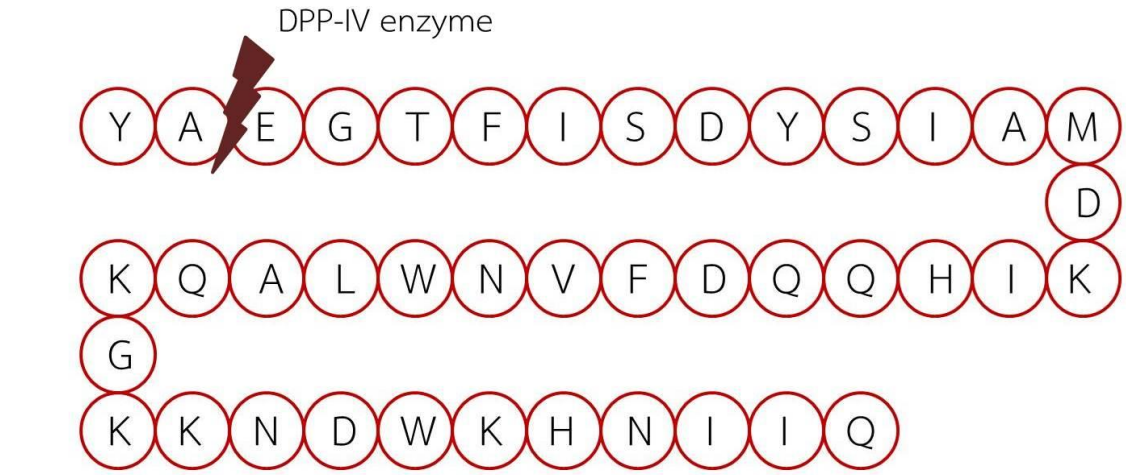


Figure 3 Structure of GIP (in circle are amino acid constituents of GIP.) (This picture is modified from Havale et al. (2009)).



(A)



(B)

Figure 4 Displays how DPP-IV makes GLP-1(A) and GIP(B) to inactive forms.

5. Role of DPP-4 inhibitors

In T2DM patient, after food is taken high glucose level in blood is a result to stimulate releasing of GLP-1 and GIP. The latter will stimulate insulin secretion and inhibit glucagon release. However, patients with T2DM have insulin resistance or impairment of insulin secretion, therefore, hyperglycemia will be observed. If DPP-IV is also released that will make GLP-1 and GIP inactive giving rise to very high blood glucose level. So, when DPP-IV inhibitor is given, DPP-4 could not then remove a couple the *N*-terminal amino acids of GLP-1 and GIP. They are still in active from to stimulate insulin secretion, improve insulin

sensitivity and inhibit glucagon release, blood glucose is then reduced (Figure 5)(McAuley, 2014).

The commercially available DPP-4 inhibitors can be used as monotherapy and in conjunction with insulin, metformin, sulfonylureas, and thiazolidinediones in patients with T2DM(Wass et al., 2011).Nowadays, there are 4 groups of DPP-IV Inhibitor has been developed and most of the DPP-IV inhibitors are structurally distinct (Figure 6). First group is substrate-like inhibitors such as Vildagliptin (Galvus®), Saxagliptin (Onglyza®), and Denagliptin. The second group is triazolepyrazine such as Sitagliptin (Januvia®). The third group is xanthine-based compounds such as Linagliptin (Tradjenta®). The last group is quinazolinone-base compounds such as Alogliptin (Nesina®) (Levien et al., 2009). The first marketed DPP-IV inhibitor was sitagliptin, which was approved by the FDA in 2006.

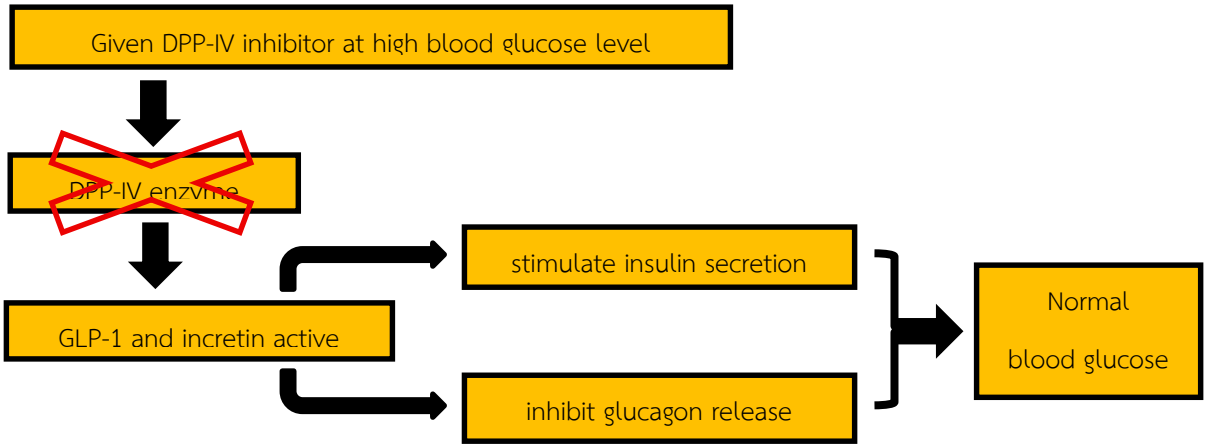
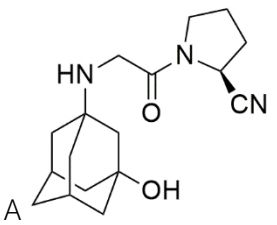
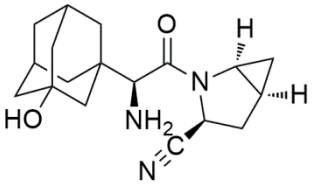


Figure 5 Diagram displays the effect of DPP-4 inhibition process.



A



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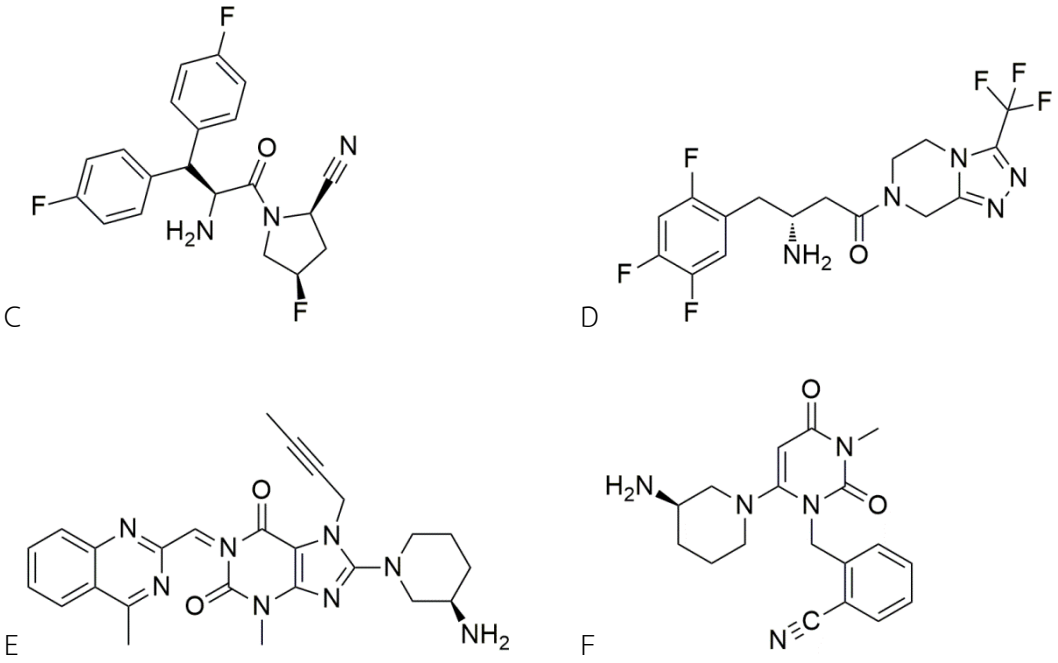


Figure 6 Structures of current DPP-4 inhibitor drugs. (A) Vildagliptin, (B) Saxagliptin, (C) Denagliptin, (D) Sitagliptin, (E) Linagliptin and (F) Alogliptin.

Eventhough, DPP-IV inhibitors are commercial available, but there are still expensive and show some side effect such as inflammation of the pancreas, upper respiratory tract infection (URI) and urticarial or angioedema (Wass et al., 2011). Therefore, many researchers are looking for DPP-4 inhibitors lead compounds from natural sources for new drug discovery.

6. DPP-4 inhibitors from natural sources

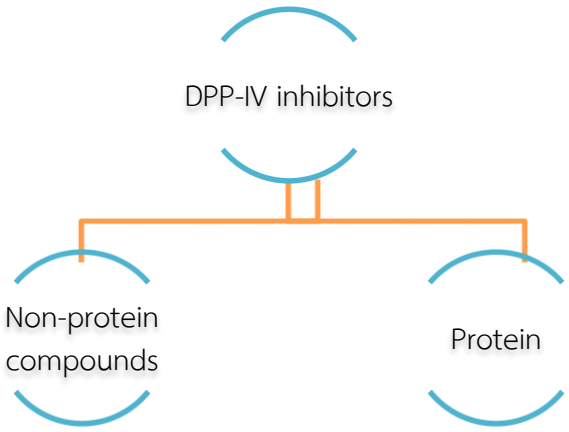


Figure 7 Groups of DPP-4 inhibitors from natural sources.

There are two groups of compounds found in natural sources that have DPP-IV inhibitory activity including non-protein and protein compounds (Figure 7). In this review, only the non-protein compounds DPP-IV inhibitors from plants are focused.

6.1 Berberine

Berberine ($[C_{20}H_{18}NO_4]^+$) (Figure 8) is a major active constituent commonly found in dried rhizomes of many plants. This compounds is an alkaloid has been found in many plant such as *Hydrastis canadensis*, *Coptis chinensis*, *Berberis aquifolium*, *Berberis vulgaris* and *Berberis aristata* (Ronald Steriti, 2010).

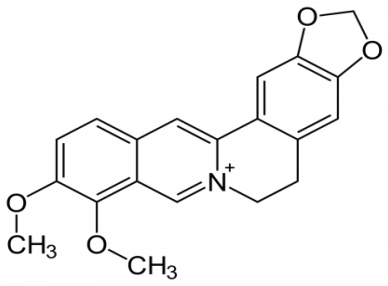


Figure 8 Structure of Berberine.

The *in vitro* activity of berberine in DPP-4 inhibition was tested and the results were expressed as the concentration of berberine that inhibited enzyme activity by 50% (IC_{50}). The inhibitory action of berberine on DPP-4 was estimated by IC_{50} of 13.3 mM. On the other hand, the IC_{50} of the positive control (P32/98(Ile-thiazolidide)) was estimated to be 131 nM (Al-Masri et al., 2009).

6.2 *Berberis aristata*

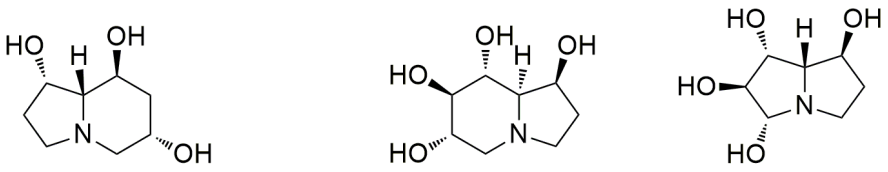
The methanolic extract of *B. aristata* was used to determine the DPP-4 inhibitory activity *in vitro* and compared with the positive control diprotin A. The result showed that IC_{50} value of the extract was 14.46 μg/mL, whereas,diprotin A has IC_{50} value of 1.54 μg/mL (Chakrabarti et al., 2011).

6.3 *Pilea microphylla* (L.)

Bansal and co-worker (2012) studied the anti-diabetic, anti-hyperlipidemic and antioxidant effects of the flavonoid rich fraction of *Pilea microphylla* (L.) in high fat diet/streptozotocin-induced diabetes in mice. Anti-diabetic tests showed that *P. microphylla* (L.) extract could inhibit dipeptidyl peptidase IV (DPP-4) *in vitro* with an $IC_{50}$of 520.4 ± 15.4 μg/mL. The result from animal models found that *P. microphylla* (L.) extract could reduce blood glucose level and enhance insulin secretion similar to that of sitagliptin anti-diabetic drug (Bansal et al., 2012).

6.4 *Castanospermum australe*

The ethanolic extract of *C. australe* seeds were used to determine the DPP-4 inhibitory activity *in vitro* and compared with the positive control diprotin A. The result showed that IC_{50} value of the extract was 13.96 μg/mL, whereas diprotinAshowed IC_{50} value of 1.54 μg/mL. The ethanolic extract was then determined for the chemical compositions by using HPLC (column Phenomenex IB-SIL 5 NH_2 250 mm × 22.5 mm i.d.; mobile phase consisting of MeCN-H_2O (90:10) as mobile phase A and MeCN-H_2O (50:50) as mobile phase B). The result revealed that the main compositions are alkaloids compounds namely, 7-deoxy-6-*epi*-castanospermine, castanospermine and australine (Figure 9) (Bharti et al., 2012).



7-deoxy-6-*epi*-castanospermine castanospermine australine

Figure 9 Chemical structures of 7-deoxy-6-*epi*-castanospermine, castanospermine and australine.

6.5 *Magifera indica*

The methanolic extract of *M. indica* leaves were used to determine the DPP-4 inhibitory activity *in vitro* and compared with the positive control diprotin A. The result showed that IC_{50} value of the extract and diprotin A were 182.7 *μ*g/mL and 19.71 μg/mL, respectively(Yogisha et al., 2010).

6.6 *Dodonae aviscosa*

The water and ethanol extracts of *D. viscosa* (L.) Jacq. aerial parts were used to determine the DPP-IV inhibitory activity *in vitro* and compared with the positive control diprotin A. The result showed %inhibition value of the water and ethanol extracts were 6.6% and 11.7%, respectively at 200 *μ*g/mL, whereas diprotin A had IC_{50} value of 39 μg/mL (Veerapur et al., 2010).

7. Plant extracts of antidiabetic recipes of Krom-Luang-Chomphon or MorPorn's.

In this study, Krom-Luang-Chomphon or MorPorn'srecipe was of interested, since, these recipes were known to be used successfully in diabetic treatment.

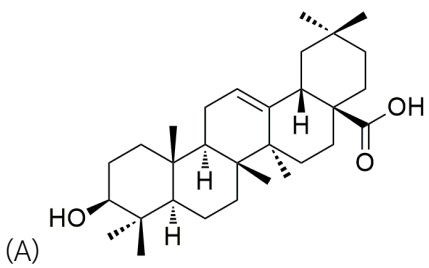
Krom-Luang-Chomphon or MorPorn's recipe contains 13 herbal plants, 4 of them have been previously reported to have α-glucosidaseand α-amylase inhibitory activities and the results are summarized in Table 1 (α-glucosidase inhibitory activity) and Table 2 (α-amylase inhibitory activity) (Bachhawat et al., 2011; Elya et al., 2011; Gao et al., 2007; Hou et al., 2009; Lamba et al., 2011; Subramanian et al., 2008; Suryadevara et al., 2009; Tamil et al., 2010; Tunsaringkarn et al., 2009; Varghese et al., 2013; Yakugakkai, 2003).

Table 1 α-glucosidase inhibitory activity of the reported herbal medicines.

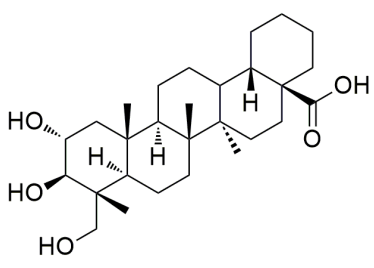
Medicinal plants	Compound/Crude extracts	Activities	Ref.
Lagerstroemia speciosa (Leaves)	Oleanolic acid (A)	IC_{50} = 14 μM	Hou et al. (2009)
	Arjunolic acid (B)	IC_{50} =38 μM	
	Asiatic acid (C)	IC_{50}=61 μM	
	Maslinic acid (D)	IC_{50} = 12 μM	
	Corosolic acid (E)	IC_{50} = 8 μM	
	23-hydroxyursolic acid (F)	IC_{50} = 16 μM	
Senna alata(L.) Roxb. (Leaves)	Kaempferol(G)	IC_{50} = 56 μM	Varghese et al. (2013)
	Kaempferol 3-*O*-gentiobioside (H)	IC_{50} = 50 μM	
Mimosa pudica L. (Whole plants)	EtOAc extracts	IC_{50} =229.7 μg/mL	Suryadevara et al. (2009)

Table 2 α-amylase inhibitory activity of the reported herbal medicines.

Medicinal plants	Compound/Crude extracts	Activity	Ref.
Phyllanthus amarus Schumach. &Thonn. (Whole plants)	EtOH extracts	IC_{50}=36.05 μg/mL	Tamil et al. (2010)
	Hexane extracts	IC_{50}=48.92 μg/mL	
	Oleanolic acid:Ursolic acid (2:1)	IC_{50}=2.01 μg/mL	
	Oleanolic acid (I)		
	Ursolicacid (J)		



(A)



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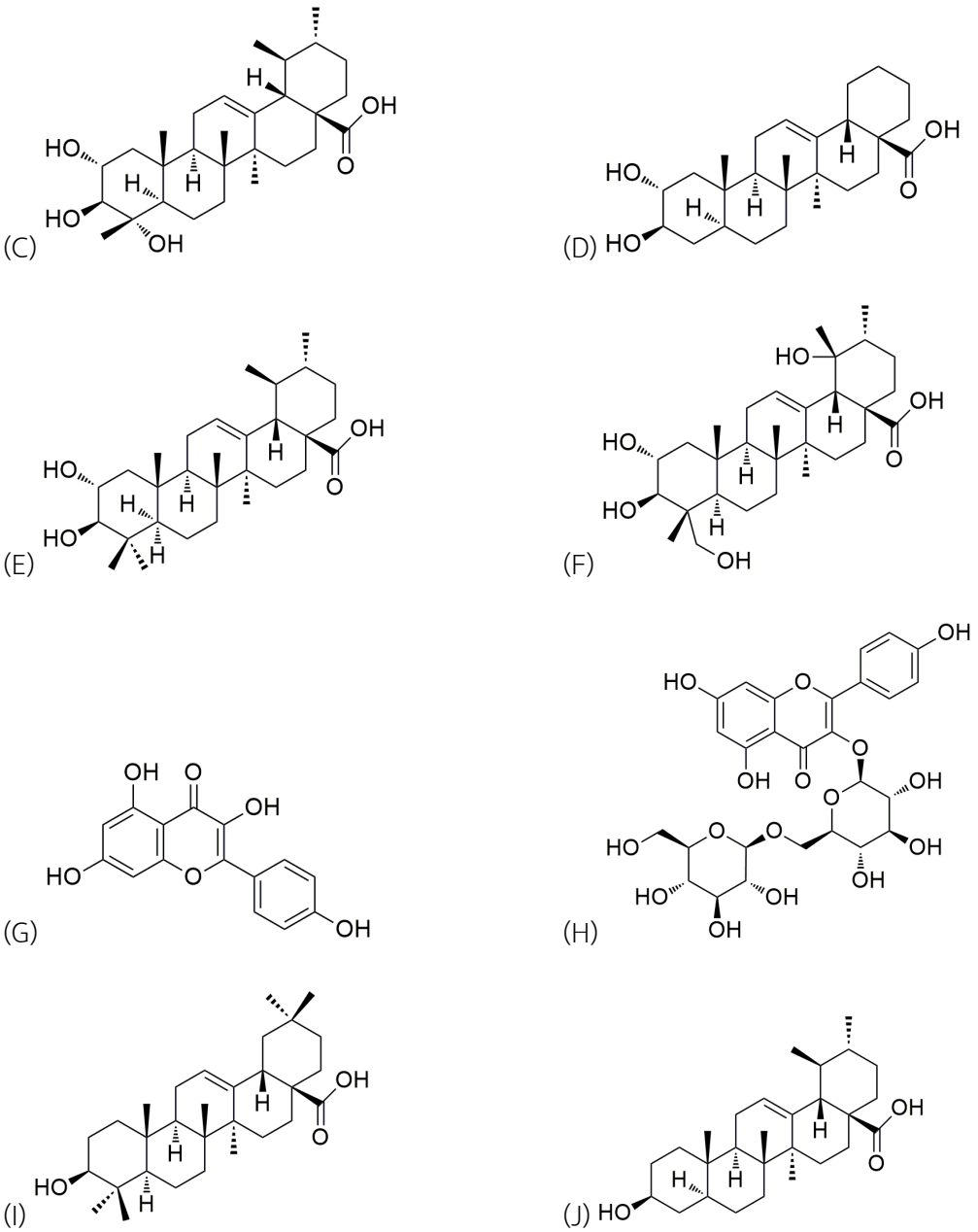


Figure 10 Structures of compounds from medicinal plants against α-glucosidase and α-amylase inhibitory activities according to the results displayed in Table 1 and Table 2

Materials and methods

1. Chemicals and instruments

Dipeptidyl peptidase–IV (DPP-4) enzyme was purchased from Merck, Germany. Analytical grade gly-pro-*p*-nitroanilide-*p*-toluene sulfonate salt and diprotin A were purchased from Sigma-Aldrich, Switzerland. Tris (hydroxymethyl) was purchased from Sigma-Aldrich, Germany. The solvents for extractions and analysical including ethanol, methanol and acetic acid were purchased from Labscan, Thailand. All solvents were as analytical grade no distillation prior uses.

2. Plant materials

Thirteen plants (Tables 3) were selected from KromLuangChomphon or Doctor Porn Thai folk anti-diabetes remedie which have indication to treat diabetes. All plants were purchased from KhuanNiang district, Songkhla and Thai traditional drug stores in Hatyai, Songkhla,Thailand.

Table 3 List of selected plants used in this study from KromLuangChomphon recipe.

Plants	Parts
Abutilon hirtum Lam. (AHW)	Whole plant
Acanthus ebracteatus Vahl. (AEW)	Whole plant
Diospyros rhodocalyx Kurz. (DRB)	Bark
Lagerstroemia speciosa (L.) Pers. (LSL)	Leaves
Mimosa pudica L. (MPW)	Whole plant
Pandanus amaryllofolius Roxb. (PAL)	Leaves
Phyllanthus amarus Schumach. &Thonn. (PAW)	Whole plant
Rhinacanthus nasutus (L) Kurz. (RNL)	Leaves
Senna alata (L.) Roxb. (SAL)	Leaves
Senna siamea Lam. Irwin &Barneby (SSB)	Buds
Senna siamea Lam.Irwin &Barneby (SSH)	Heartwood
Terminalia catappa L. (TCL)	Leaves
Vitex glabrata R.Br. (VGB)	Bark
Zea mays L. (ZMS)	Silk

3. Crude extracts preparation

The plant materials were rinsed thoroughly to remove any foreign matter with tap water and dried by hot air oven at 50 °C for 2 days. The dried plants were then ground with an electric grinder, weighed, and stored in desiccator at room temperature (25-30 °C) protected from light. Each plants powder (30 g) were macerated with ethanol (150 mL) for 2 days at room temperature and filtered through a filtering paper (Whatman® No. 1).The maceration processes were performed in triplicate. Each filtrate was pooled and evaporated to dryness under reduced pressure at 45 °C by rotary evaporator and the resulting crude extracts were kept in a refrigerator at -20 °C, protect from light until use.

4. Dipeptidyl peptidase-IV (DPP-4) inhibitory activity (modified from Al-Masri et al. (2009)

Diprotin A was used as positive standard and diluted to various concentrations by Tris-HCl buffer (50 mM, pH 7.5). Sample solution was prepared by dilution the extract with Tris-HCl buffer to have a final concentration of 50 μg/mL. 40 µL of Diprotin A solutions or sample solutions was transferred to each well of microplate, followed by additional of 20 μL of DPP-4 enzyme (0.05 U/mL). After adding the enzyme, the mixture was pre-incubated for 10 mins at 37 °C to enhance binding capacity of the inhibitor. This was followed by addition of 100 μL of Gly-pro-*p*-nitroanilide (GPPN 0.2 mM in Tris-HCl) as a substrate. The incubation was continued at 37 °C for 30 mins. The reaction was terminated by addition of 30 μL of 25% glacial acetic acid. The absorbance was then measured at 405 nm. The percentage of inhibition was calculated according to the following equation:

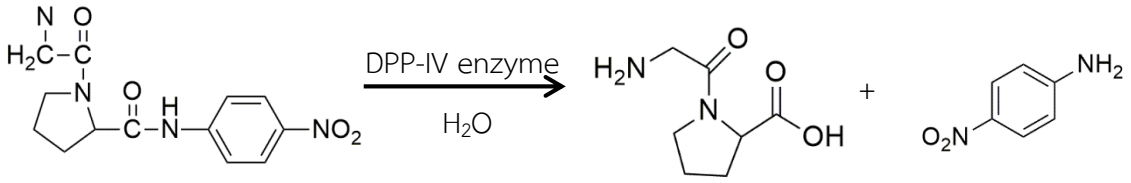
$$\%\text{Inhibition} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where $A_{control}$ = absorbance of DPP-IV solution

A_{sample} = absorbance of DPP-IV react with sample.

The tests are carried out in triplicate for each sample.

The principle of dipeptidyl peptidase-IV inhibitory assay using spectrophotometic method. The crude extracts were pre-incubated with the enzyme and then adding the gly-pro-*p*-nitroanilide (GPPN) as substrate. The activity of this method was measured by determining the color of the release of *p*-nitroaniline arising from the hydrolysis of substrate GPPN by dipeptidyl peptidase-IV reaction was shown in Figure 11 (Al-Masri et al., 2009).



gly-pro-*p*-nitroanilide (GPPN) gly-pro *p*-nitroaniline

Figure 11 The DPP-4 catalyzed reaction using GPPN as a substrate

5. Statistical analysis

The experimental data were reported as mean±SD. To compare the results among each other, one-way analysis of variance (one-way ANOVA) was performed with 99.50% confident level using SPSS software.

Results and discussions

1. DPP-IV inhibition

The dipeptidyl peptidase – IV activities of the ethanolic extracts from the selected herbs of anti-diabetes folk medicinal recipe was determined by ability to reduce the DPP-IV enzyme using diprotin A as the standard references and were expressed as IC_{50} (μg/mL). Diprotin A was found to have an IC_{50} of 2.07 ± 0.01 μg/mL (Figures 12). The DPP-IV inhibitory activities of the 14 extracts from 13 selected plantsare shown in Table 2. Inhibitory activity (%) of all samples at 50 μg/mL was in a range of 16.52 ± 0.11 to 71.07 ± 0.07 %, while diprotin A at 50 μg/mL gave 90.07 ± 0.39 % inhibition.

Table 4 Comparative DPP-IV screening activity of the ethanolic extracts from selected herbs of anti-diabetes folk medicinal recipeat 50 μg/mL.

Sample	%Inhibition	SD
Diprotin A	90.071	0.391
Lagerstroemia speciosa (L.) Pers. (leaves) (LSL)	71.066	0.067
Terminalia catappa L. (leaves) (TCL)	69.889	0.426
Senna siamea Lam. (buds) (SSB)	51.720	0.037
Senna siamea Lam. (heartwood) (SSH)	49.444	0.426
Phyllanthus amarus (whole plant) (PAW)	49.111	0.257
*Acanthus ebracteatus*Vahl. (whole plants) (AEW)	43.778	0.574
*Vitexgla brata*R.Br. (bark) (VGB)	38.778	0.426
Senna alata L. (leaves) (SAL)	30.201	0.360
Zea mays L. (silk) (ZMS)	30.020	0.049
Rhinacanthus nasutus (L.) Kurz. (leaves) (RNL)	28.966	0.080
Diospyros rhodocalyx Kurz. (bark) (DRB)	25.000	0.667
Mimosa pudica L. (whole plant) (MPW)	23.458	0.032
Pandanus amaryllofolius Roxb. (leaves) (PAL)	17.535	0.202
Abutilon hirtum Lam. (whole plant) (AHW)	16.523	0.111

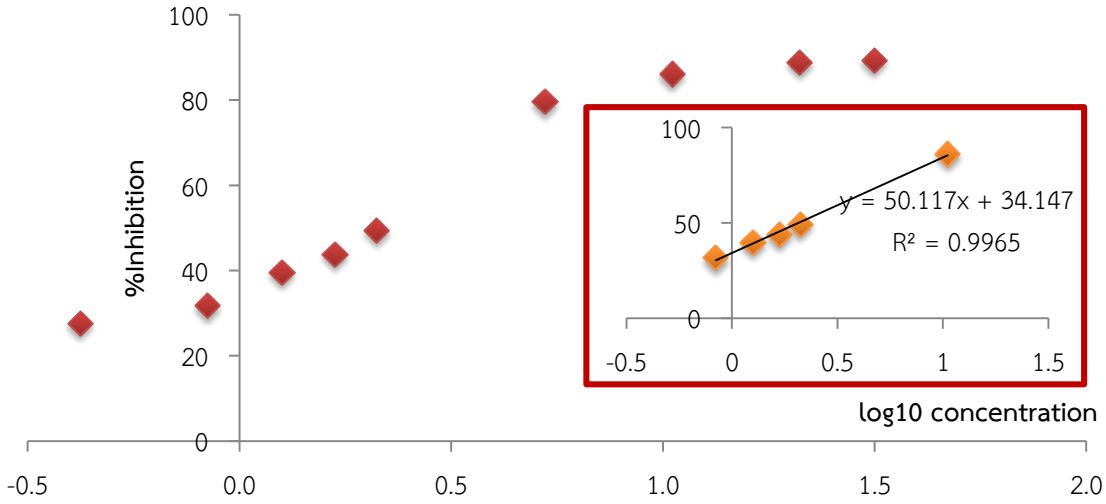


Figure 12 Calibration curve of Diprotin A from DPP-4 screening activities of the ethanolic plant extracts.

Most of the medicinal plants in this list were known to have antidiabetic effect in animal models such as LSL(Saumya et al., 2011), TCL (Ahmed et al., 2005), AHW (Krisanapun et al., 2011), PAW (Adeneye et al., 2006; Ali et al., 2006), SAL (Kolawole, 2006), ZMS (Guo et al., 2009), MPW (Sutar et al., 2009) and PAL (Sasidharan et al., 2011). A number of mechanisms have been reported in the hypoglycemic effect of these selected herbs for example glucosidase inhibitor (Anam et al., 2009), amylase inhibitor(Ali et al., 2006), activate glucose transporter 1 (GLUT1) (Krisanapun et al., 2011), increase in circulation of insulin level (Guo et al., 2009) and stimulating or regenerating effect of pancreatic β-cells (Ahmed et al., 2005). However, none of selected plants have been reported for DPP-IV inhibitory activities. Our result is the first report of DPP-IV inhibitory activity of these 14 herbs which are the ingredients in KromLuangChomphon folk recipe. Two plant extracts that gave high activity in our list were ethanolic extracts of LSL (%Inhibition = 71.07 ± 0.07 % at 50 μg/mL) and TCL (%Inhibition = 69.89 ± 0.43 % at 50μg/mL). Previously, LSL and TCL were found to have antidiabetic activity both in vivo and in vitro testing. LSL extracted has been extensively reviewed (Tanquilut et al., 2009) that water soluble LSL leaf extracts displayed hypoglycemic effect both in animal models and human subjects (Judy et al., 2003). The antidiabetic activity of the water LSL extract was mentioned to be due to its phytochemical compositions including corosolic acid, ellagitannins, lagersteroemin, flosin B and reginin A (Klein et al., 2007) or combination thereof. Moreover, hypoglycemic effect of standardized leaves extract of LSL (Glucesol®) was observed to have significant dose-dependent relationship in clinical study (Judy et al., 2003).Liu and co-workers (F. Liu et al., 2001; X. Liu et al., 2005) reported that blood-glucose lowering capability of LSL extracts could be due to glucose transport stimulation and adipocyte differentiation inhibition. Moreover, Hou and co-workers (Hou et al., 2009) proposed that antidiabetic activity of ethyl acetate extract of LSL could be due to α-glucosidase (Hou et al., 2009) and α-

amylase (Hou et al., 2009) inhibition. Corosolic acid was found to be the major active compound which played an important role. TCL was also demonstrated to have hypoglycemic effect in animal models both in rabbit (N'Guessan et al., 2011) and in rat models (Ahmed et al., 2005). The result from rabbit model showed that the aqueous decoction of TCL displayed a dose-dependent hypoglycemic effect, however the most effective dose was at 40 mg/mL. Not only the TCL extract could reduce hyperglycemia but also blood glucose level was brought up to the normal level after the treatment, however, no mechanism was proposed. Ahmed *et. al.* in 2005 (Ahmed et al., 2005) showed that TCL aqueous extract exhibited significant hypoglycemic effect in diabetic rat models without the change in body weight. In addition, Ahmed and co-worker suggested that blood glucose lowering capacity of capacity of TCL extracts could be according to the promotion of β-cells regeneration. Beside, TCL ethanolic extract was also shown to have α-glucosidase inhibitory activity which may be another role in hypoglycemic property (Anam et al., 2009). It is however, none of any report was found that LSL and TCL extracts have DPP-IV inhibitory activity. Our result is the first report to confirm that the LSL and TCL hypoglycemic activity could be due to their ability to inhibit DPP-IV enzyme. Two types of incretin hormones are responsible for glucose regulation process including glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Vilsbøll et al., 2004). DPP-IV enzyme is a soluble plasma enzyme presented in the capillary of gut mucosa (Lambeir et al., 2003) and many organs e.g. kidney, liver and intestine (Kieffer et al., 1999). This enzyme degrades GLP-1 and GIP making them biologically inactive (Deacon et al., 1995). DPP-IV inhibitors block the enzyme resulting in prolonging the half-life and biological activity of both incretin hormones. The latters could promote β-cells regeneration, insulin secretion, glycogenolysis thereby hypoglycemic effect would be the result (Fehmann et al., 1992).

Conclusions

Thirteen herbs of KromLuangChomphonThai folk anti-diabetes remedies were investigated for their dipeptidyl peptidase-IV inhibitory activities. The result from DPP-IV inhibitory activities testing found that leaves of *Lagerstroemia speciosa* (L.) Pers. gave the best activity.

This is the first report of dipeptidyl peptidase-IV (DPP–4) inhibitory activity of thirteen herbs from KromLuangChomphon Thai folk anti-diabetes remedies. Some selected herbs showed ability to be used effectively as DPP-IV inhibitory. These finding support the potential use of these recipe as alternative treatment of diabetes through a new mechanism.

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Comments and Suggestions

According to the results of this study, we found active herbal medicine that showed good DPP-4 inhibitor activities. Next study should continue the phytochemical study of active plant ingredient to isolate the active compounds that possibly have DPP-4 inhibitor activities or to gain the information for drug development as lead compounds forDPP-4 inhibitor drug and select as the biomarker of active herbal medicine. Furthermore we would suggest to identify the structure-activity relationships of pure isolated compounds and this may support in the discovery of lead compounds.