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## Antidiabetic Agents with $\alpha$ -glucosidase Inhibition and Antioxidant Capacity from the Shoots of *Clausena cambodiana* Guill

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Separation of CH<sub>2</sub>Cl<sub>2</sub> crude extract of the shoots of *Clausena cambodiana* Guill led to the isolation of four known compounds including quercetin (**1**), sinensetin (**2**), clausenidin (**3**) and  $\beta$ -sitosterol (**4**). Quercetin exhibited highest antioxidant capacity with IC<sub>50</sub> value 8.45 $\pm$ 1.42 mM, which are comparable to ascorbic acid. Clausenidin exhibited strong inhibitory activity specifically against sucrose with an IC<sub>50</sub> value 12.32 $\pm$ 3.60 mM, which are more potent inhibition than acarbose (IC<sub>50</sub> = 32.58 $\pm$ 3.92 mM) using as the positive control. In addition, the kinetic mechanism of **3** indicated that it retarded sucrose in a noncompetitive manner.

**Keywords:** *Clausena cambodiana* Gill, Clausenidin,  $\alpha$ -Glucosidase inhibitor, Antioxidant

### Introduction

Type II (T2DM) diabetes is a common metabolic disorder characterized by chronic hyperglycemia and impaired insulin secretion. Symptoms of T2DM include autooxidation of glucose and increases in oxidative stress, which precede diabetic complications such as blindness, nephropathy, nerve damage, amputation and cardiovascular disease (Johnston *et al.*, 1994). At present,  $\alpha$ -glucosidase inhibition is a primary mechanism of controlling postprandial T2DM, which is described by current antidiabetic drugs such as miglitol, voglibose and acarbose (Borges de Melo *et al.*, 2006). Recent evidence indicated that free radicals are implicated in T2DM complications, causing dysfunction of  $\beta$ -cells and insulin resistance.

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Elevated plasma glucose causes overproduction of free radicals and other reactive oxygen species that destroy cells through oxidative stress, which supports the goal of developing antidiabetic drugs with radical scavenging (Maritim *et al.*, 2003). Dual function agents which have both, antidiabetic ( $\alpha$ -glucosidase inhibitor) and radical scavenging capacities are particularly relevant for treatment of T2DM and its complications. In this study, we searched for antidiabetic agents having a dual mechanism from herbal medicine. Inspired by ethnopharmacological records, we screened *C. cambodiana* Guill for  $\alpha$ -glucosidase inhibition and radical scavenging capacity.

*Clausena cambodiana* Guill (Family: Rutaceae, Traditional name: Samui-Hom) is an herb commonly distributed throughout the South of Thailand. *C. cambodiana* Guill has been recognized as a rich source of various phytochemicals such as clausarin, dentatin, nordentatin, xanthoxyletin, dihydropyranocoumarin and clausenidin (Tangyuenyongwatthana *et al.*, 1992). *Clausena* species have been used for the treatment of human diseases such as cardiovascular disease, anti-inflammatory, antioxidants, anti-snake venom, anticancer, anti-HIV, and antiplatelet (Albaayit *et al.*, 2014). In addition, *Clausena* species has been found to possess anti-diabetic activity (Ojewole *et al.*, 2002). Nevertheless, to date there have been no report on the constituents and active components putatively responsible for retarding  $\alpha$ -glucosidase and radical scavenging capacity, which is the aim of this investigation.

## Materials and methods

### *Plant material*

The shoots of *C. cambodiana* Guill were purchased in May 2013 from local market at Nakhon Si Thammarat province, in the southern of Thailand.

### *Extraction and isolation*

The shoots of *C. cambodiana* (720 g) were extracted with MeOH successively at room temperature to give MeOH extract, which was further partitioned with CH<sub>2</sub>Cl<sub>2</sub> to yield crude extract of CH<sub>2</sub>Cl<sub>2</sub>. The crude extract of CH<sub>2</sub>Cl<sub>2</sub> was further separated by Sephadex LH-20 with 10% CH<sub>2</sub>Cl<sub>2</sub>-MeOH to yield eight fractions. Fraction **2** was further purified by column chromatography on silica gel eluting with 20% EtOAc-*n*-hexane to afford clausenidin (**3**, 150 mg) and  $\beta$ -sitosterol (**4**, 250 mg). Fractions 5-8 were further separated by Sephadex LH-20 (MeOH) to afford quercetin (**1**, 130 mg), and silica gel eluting with 60% EtOAc-*n*-hexane to yield sinensetin (**2**, 80 mg).

### ***α-Glucosidase inhibition assay***

The α-glucosidase inhibition was investigated using a protocol previously described by Damsud (Damsud *et al.*, 2013). Briefly, a sample (10 μL) was mixed with α-glucosidase (0.1 U/ml) in 1 mM phosphate buffer (pH 6.9), followed by pre-incubation at 37°C for 10 min. The reaction mixture was added with 50 μL of *p*-nitrophenyl-α-D-glucopyranoside (PNPG) and then incubated at 37°C for 20 min. The reaction was quenched by adding Na<sub>2</sub>CO<sub>3</sub> (100 μL). The release of *p*-nitrophenoxide from PNPG was detected by a microplate reader at 405 nm (Sunrise microplate reader). The percent inhibition of reaction was calculated using the following equation. The % inhibition =  $[(A_0 - A_1)/A_0] \times 100$ , where: A<sub>1</sub> and A<sub>0</sub> are absorbance with and without the sample, respectively.

In addition, the inhibition against rat intestinal maltase and sucrase by these isolated compounds was determined using our previous protocol (Damsud *et al.*, 2013). Briefly, a sample (10 μL) of test and substrate maltose (10 mM, 20 μL) and sucrose (10 mM, 20 μL) in 1 mM phosphate buffer (pH 6.9) was incubated at 37°C (20 min, maltase and 60 min, sucrose) The release of glucose, after derivatization by commercial Glu-kit (Human, Germany), was determined on a microplate reader at 503 nm, and the percent inhibition was calculated using the expression in the aforementioned equation.

### ***Kinetic study***

The kinetic mechanism of enzyme (sucrase) was carried out. Briefly, 0.3 U/mL of rat intestinal (sucrase) was mixed with of 2 (1.83 and 3.14 mM). Lineweaver-Burk plots were used to determined the type of inhibition whereas the  $K_i$  and  $K'_i$  values were investigated from secondary plots (slope= $v_s[I]$  and interception  $v_s[I]$ ) of Lineweaver-Burk plots, respectively.

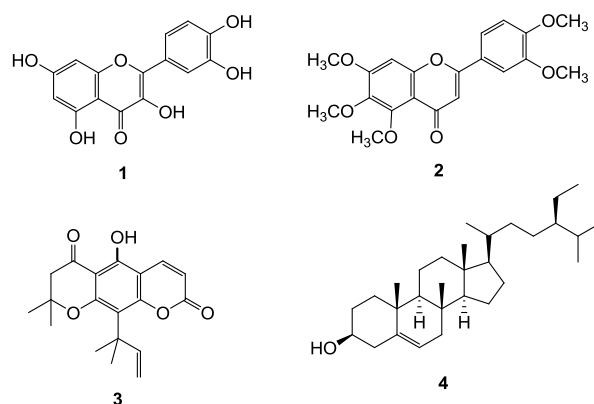
### ***DPPH radical scavenging assay***

DPPH radical scavenging activity of each of the extracts was determined by the method of Ramadhan (Ramadhan *et al.*, 2015). Briefly, 10 μL of each of the samples at different concentrations (50, 25, 10, 1.25 mg/mL) was mixed with 500 μL DPPH (0.01mM in the methanol). The reaction mixture was then incubated at room temperature in the dark for 30 min. All measurements were performed in triplicate. DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm and expressed as the inhibition percentage

of free radicals by the samples after calculation using the expression in  $\alpha$ -glucosidase activity.

## Results and Discussion

The active  $\text{CH}_2\text{Cl}_2$  extract of *C. cambodiana* Guill was repeatedly chromatographed using a mixture of  $\text{MeOH-CH}_2\text{Cl}_2$ , yielding four major fractions. Fraction 2-4 were purified using the combination of Sephadex LH-20 and silica gel column chromatography, affording the two flavonoids quercetin(1) (Thanakosai *et al.*, 2012), sinensetin(2) (Damsud *et al.*, 2013), together with the pyranocoumarin and triterpenoid named clausenidin(3) (Tangyuenyongwatthana *et al.*, 1992) and  $\beta$ -sitosterol(4) (Habib *et al.*, 2007) as shown in (Fig 1), respectively. Their structures were elucidated by NMR and MS analysis, and by comparison of their spectroscopic data with these reported in the literature.



**Fig 1.** Structures of compounds 1-4

All compounds were further evaluated for their anti-oxidant activity and inhibitory effects against  $\alpha$ -glucosidase from two different sources baker's yeast (type I) and rat intestinal (type II), maltase and sucrase (Table 1). Compound (1) exhibited highest antioxidant capacity with  $\text{IC}_{50}$  value of  $8.45 \pm 1.42$  mM. Compounds 1 and 4 inhibited  $\alpha$ -glucosidase from baker's yeast with  $\text{IC}_{50}$  values of  $270.25 \pm 27.25$  and  $220.24 \pm 31.25$  mM, respectively, while compounds 2 and 3 showed no inhibition. From these results, it might be proposed that the presence of hydroxyl (-OH) groups on the structure (compound 1), it may be enhanced  $\alpha$ -glucosidase inhibition compare to compound 2 (Tadera *et al.*, 2006).

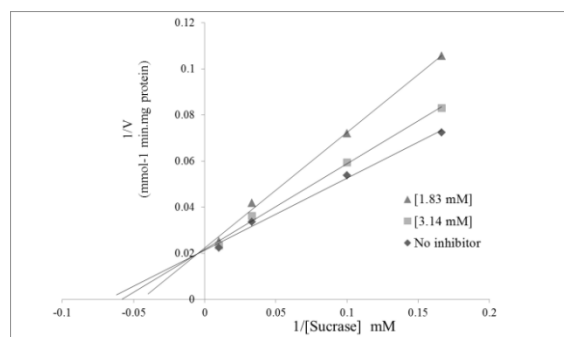
**Table 1.**  $\alpha$ -Glucosidase inhibition and anti-oxidant activities of compounds 1-4

Compound	IC <sub>50</sub> (mM)			
	Baker's yeast	Maltase	Sucrase	DPPH <sup>a</sup>
1	270.25±27.25	32.81±2.04	125.47±5.68	8.45±1.42
2	NI <sup>b</sup>	NI <sup>b</sup>	NI <sup>b</sup>	23.76±2.78
3	NI <sup>b</sup>	152.87±12.21	12.32±3.60	45.75±5.52
4	220.24±31.25	NI <sup>b</sup>	NI <sup>b</sup>	67.43±4.21
Acarbose	1.20±0.18	3.10±0.54	32.58±3.92	NI <sup>b</sup>
Ascorbic acid	NI <sup>b</sup>	NI <sup>b</sup>	NI <sup>b</sup>	2.45±0.45

<sup>a</sup>The values are expressed as SC<sub>50</sub> (mM)

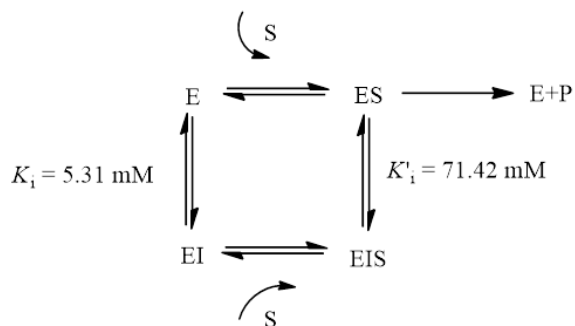
<sup>b</sup>No inhibition

Compound 3 exhibited strong inhibitory activity specifically against sucrase with an IC<sub>50</sub> value 12.32 ± 3.60 mM, which are more potent inhibition than acarbose (IC<sub>50</sub> = 32.58±3.92 mM) using as the positive control. In order to investigate the mechanism underlying components in 3, a kinetic study was carried out. The Lineweaver-Burk plots (Fig 2) of 3 against maltase showed linearity at each concentration examined (1.83 and 3.14 mM); all of which intersected at a single value in the second quadrant.

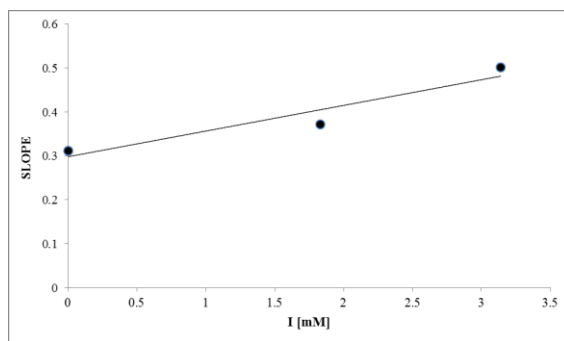
**Fig 2.** Lineweaver-Burk plots for inhibitory activity of 3 against sucrase.

The kinetic analysis revealed that  $V_{\max}$  decreased with increasing concentrations of 3 while  $K_m$  (14.63 mM) increased. This behavior indicated that 3 inhibited sucrase by two different pathways; competitive by forming enzyme-inhibitor (EI) and noncompetitive by forming enzyme-substrate-inhibitor (ESI) complexes (Fig 3). To determine the affinity of inhibitor in EI and ESI complexes, the secondary plots were constructed. The secondary plot

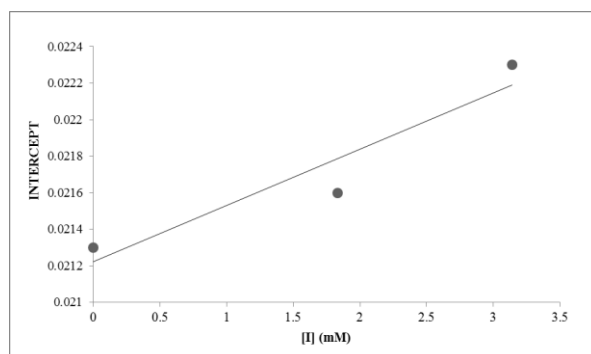
of slope vs inhibitor concentration revealed  $K_i$  value of 5.31 mM, while of Y-intercept vs inhibitor concentration showed  $K'_i$  value of 71.42 mM (Fig 4-5). The result suggested that 3 predominantly formed an ES complex rather than an EIS complex bound to the enzyme.



**Fig 3.** Proposed inhibitory mechanism of 3 against sucrase.



**Fig 4.** Secondary plot of slope vs [I] for deduction of  $K_i$  of 3 against sucrase



**Fig 5.** Secondary plot of intercept vs [I] for deduction of  $K'_1$  of 3 against sucrase.

## Conclusion

In conclusion, this study can be considered as the first detailed report. We have identified previously undocumented antidiabetic agents from *C. cambodiana*. Interestingly, certain isolated compounds namely, quercetin (1), sinensetin(2), clausenidin(3) and  $\beta$ -sitosterol(4), demonstrated both  $\alpha$ -glucosidase inhibition and antioxidant capacity, especially clausenidin(3) which exhibited strong  $\alpha$ -glucosidase inhibitory activity. It would be benefit in suppressing blood glucose and preventing the onset of diabetic complications. Moreover, it could be used solely as antidiabetic agent or combination with drugs such as acarbose and voglibose.

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