ฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดสที่สูงของพืชสกุลซิซิฟุสบางชนิด

Potent α–glucosidase inhibitory activity of some *Ziziphus* plants

Natthakaln Lomchoey[1], Audchara Saenkham[1,2], Santhana Nakapong[3] and Sunit Suksamrarn[1,2,*]

[1] Department of Chemistry and [2] Center of Excellent for Innovation in Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand

[3] Department of Chemistry and Center of Excellent for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

[*]Corresponding Author, E–mail: sunit@g.swu.ac.th

บทคัดย่อ

จากการศึกษาฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดสของสารสกัดจากพืชสกุลซิซิฟุสสี่ชนิด โดยศึกษาสารสกัดชั้นเอทิลแอซิเตตและชั้นเมทานอลของส่วนเปลือกรากและเปลือกต้นตะครอง รากและเปลือกต้นพุทรา รากเล็บเหยี่ยว และเปลือกต้นเล็บแมว พบว่าสารสกัดเมทานอลของพืชทั้งสี่ชนิด แสดงฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดสที่สูงมาก ในช่วง IC_{50} 0.007±0.006 – 0.031±0.0018 มิลลิกรัมต่อมิลลิลิตร ซึ่งดีกว่าอะคาร์โบส 79 – 350 เท่า ส่วนสารสกัดชั้นชั้นเอทิลแอซิเตตแสดงฤทธิ์ในช่วง IC_{50} 0.014±0.001 – 8.772±0.234 มิลลิกรัมต่อมิลลิลิตร จากการแยกสารโดยเทคนิคคอลัมน์โครมาโทกราฟี พบ betulinic acid (**1**) ในปริมาณมากในสารสกัดชั้นเอทิลแอซิเตต และพบว่าสารสกัดชั้นเมทานอลมีแทนนินเป็นองค์ประกอบหลัก สาร **1** แสดงฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดส (IC_{50} 0.031±0.0027 มิลลิกรัมต่อมิลลิลิตร) ดีกว่าอะคาร์โบส 79.2 เท่า รายงานนี้เป็นครั้งแรกที่ได้ศึกษาฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดสของสารสกัดจากพืชสกุลซิซิฟุสในประเทศไทย

ABSTRACT

α–Glucosidase inhibitory activity of crude extracts of four Thai *Ziziphus* plants was investigated using the root bark and stem bark of *Z. cambodiana*, the root and stem bark of *Z. mauritiana*, the root of *Z. oenoplia* var. *oenoplia* and the stem bark of *Z. oenoplia* var. *brunoniana*. The results showed that the MeOH extracts displayed high potent α–glucosidase inhibitory activity with IC_{50} range of 0.007±0.006 – 0.031±0.0018 mg/mL, about 79 – 350 folds

more potent than standard drug acarbose. While the EtOAc extracts possessed IC_{50} ranges of 0.014±0.001 – 8.772±0.234 mg/mL in the same test. Column chromatographic isolation and TLC screening led to obtain betulinic acid (**1**) as major product from EtOAc extract and highest quantity of tannin was found in MeOH extracts. Compound **1** (IC_{50} of 0.031±0.0027 mg/mL) exhibited 79.2 times more potent α–glucosidase inhibition than that of acarbose. This work is the first report of α–glucosidase inhibitory property of Thai *Ziziphus* plants crude extracts.

คำสำคัญ: ตะครอง พุทรา เล็บเหยี่ยว เล็บแมว ฤทธิ์ยับยั้งเอนไซม์แอลฟา–กลูโคซิเดส
Keywords: *Z. cambodiana, Z. mauritiana, Z. oenoplia,* α–glucosidase inhibitory activity

INTRODUCTION

Diabetes mellitus is characterized by chronic high blood glucose concentration or known as hyperglycemia, a metabolic disorder causing by an insufficiency in insulin secretion, insulin action, or both. One of key approaches to control diabetes is reducing glucose level by inhibition of intestinal α–glucosidase and salivary α–amylase, enzymes that catalyze the digestion of carbohydrate to absorbable monosaccharide (Taj Eldin et al., 2012). α–Glucosidase inhibitors interfere with the digestion of carbohydrates, achieving better glycemic control. Acarbose has been considered for treatment of diabetes to decrease postprandial hyperglycemia through delayed carbohydrate digestion. Recently, α–glucosidase inhibitors have been classified as a new class of anti–diabetic drug and medicinal plants have been regarded as a good source of potent α–glucosidase inhibitors (Yin et al., 2014).

The genus *Ziziphus* is a group of spiny shrubs or thorny scandent and small trees that belongs to the buckthorn family Rhamnaceae The *Ziziphus* plants are widespread in tropical and subtropical areas with a family of 58 genera and 900 species worldwide (Bhattacharyya and Johri, 1998), particularly in arid regions (Gardner et al., 2000) with 100 species distributed in the tropical America, Africa, the Mediteranean region, Indo–Malaysia, and Australia, and also the tropical parts of India, Nepal, Pakistan, Bangla Desh and Sri Lanka (Bhattacharyya and Johri, 1998). In Thailand there are only ten *Ziziphus* plants of nine species (Smitinand, 2014).

Various medicinal properties are attributed to many species of the Rhamnaceous *Ziziphus* in the traditional medicine. A tea from *Z. mucronata* leaves is traditionally used in a combination with other herbs for the treatment of diabetes mellitus in southern Africa, and the methanol extract of

the bark exhibited weak α–amylase inhibitory activity (<30%), while the aqueous extract stimulated the enzyme (Da Costa Mousinho, 2013). The aqueous extract of *Z. mauritiana* fruits and its nonpolysaccharide fraction were found to exhibit significant antihyperglycemic and hypoglycemic activities in glucose overloaded hyperglycemic rats (Jarald et al., 2009). The proanthocyanidin–containing MeOH extract of jujube (*Z. jujuba* fruits) possessed strong *In vitro* anti–diabetic activity against two enzymes, α–glucosidase and α–amylase, with percentage inhibition values of about 93.8% and 91.34%, respectively (Gao et al., 2015). The root bark of *Z. oenoplia* has been used for treatment of ulcers, anti–infectious, anti–diabetic and diuretic in Thai folk medicine (Bunyapraphatsara and Chokechaijaroenporn, 2000). Phytochemical studies revealed that the genus *Ziziphus* to be a rich source of lupane–typed triterpene, especially a major constituent **1** (Suksamrarn et al., 2006). Other types of compounds discovered include cyclopeptides, saponins (Renault et al., 1997), flavonoids (Maciuk et al., 2003), flavonoid glycosides (Li, et al., 2007), steroids (Chauhan and Srivastava, S.K., 1978), pectin, lipids (Goyal et al., 2012) and aliphatic compounds (Srivastava, and Srivastava, 1979). Some *Ziziphus* saponins were reported to be haemolytic, anxiolytic, sedative and sweetness inhibiting agent. Certain cyclopeptide alkaloids exhibited sedative, hypoglycemic, anti–infectious, anti–plasmo-dial, anti–microbial, anti–diabetic, diuretic, anti–inflammatory, analgesic and anti–convul-sant activities (Goyal et al., 2012). This work aims to evaluate α–glucosidase inhibition potential of four Thai *Ziziphus* species that include *Z. brunoniana* Clarke ex Brand. or *Z. oenoplia* Mill. var. *brunoniana* (Nam lep maeo), *Z. oenoplia* Mill. var. *oenoplia* (Nam lep yiao), *Z. cambodiana* Pierre (Takhrong), *Z. mauritiana* Lam. or *Z. jujube* Lam. (Phutsa).

EXPERIMENT

Plant Materials: root bark of *Z. cambodiana* [or ZC(RB)] collected from Chamni District, Burirum Province in March, 2001; the stem bark of *Z. cambodiana* [or ZC(SB)] collected from Nangrong District, Burirum Province in March, 2007; the root and stem bark of *Z. mauritiana* [or ZM(R) and ZM(SB)] (collected from Samchuk District, Suphanburi Province in June 2005; the root of *Z. oenoplia* var. *oenoplia* [or ZOO(R)] collected from Takhli District, Nakornsawan Province in April 2011; and the stem bark of *Z. oenoplia* var. *brunoniana* [or ZOB(SB)] collected from Nangrong District, Buriram Province in May 2013; and their respective voucher specimens Wicharn Wisetsri 001, Napaporn Charoenrum 001, Jessada Netsawangwicha 002, Kimyong Chokepaiboon 001, and Napaporn Charoenram 002, have

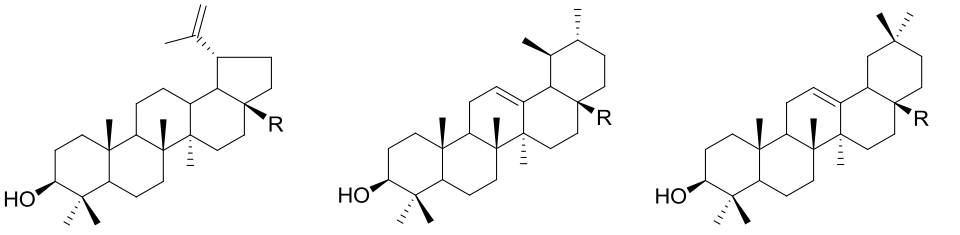
been identified and deposited at the Natural Product Unit, Faculty of Science, Srinakharinwirot University, Thailand.

Extraction and isolation: Individual air–dried plant material was ground and successively extracted with EtOAc (3 times) and then MeOH (3 times) by maceration for each 7 days. The filtrates were concentrated to dryness under reduced pressure to yield their corresponding extracts. Thus, from the root bark of *Z. cambodiana* or ZC(RB) (10 kg), EtOAc (brown solid 144 g, 1.4% based on dry plant material) and MeOH (red–brown solid 1500 g, 15.0%) extracts were yielded as described previously (Suksamrarn et al., 2006). From the stem bark of *Z. cambodiana* or ZC(SB) (4.7 kg), EtOAc (brown solid 57.0 g, 1.2%) and MeOH (red–brown solid 485.3 g, 10.3%) extracts were obtained (Saenkham, 2015). *Z. mauritiana* root or ZM(R) (4.5 kg) yielded the EtOAc (brown solid 29.5 g, 0.6%) and MeOH (red–brown solid, 45.6 g, 1.0%) extracts (Panseeta et al., 2011). *Z. mauritiana* stem bark or ZM(SB) (3.5 kg) yielded the EtOAc (brown solid 35.0 g, 1.4%) and MeOH (red–brown solid, 140.0 g, 5.6%) extracts (Lomchoey, 2011). *Z. oenoplia* var. *oenoplia* root or ZOO(R) (3.5 kg) yielded the EtOAc (brown solid 28.3 g, 0.8%) and MeOH (red–brown solid, 407.0 g, 11.6%) extracts. *Z. oenoplia* var. *brunoniana* stem bark or ZOB(SB) (3.9 kg) yielded the MeOH extract (red–brown solid, 328.0 g, 8.4%). A portion of MeOH extract (29 g) of ZOB(SB) was subjected to QCC using gradient system of *n*–hexane, DCM, EtOAc and MeOH as eluting solvent to yield 8 fractions (A–H), in which triterpene and tannin were found in fractions B–F (19.0 g, 64.2% based on MeOH extract or 5.3% based on plant material) and G–H (2.73 g, 9.2% based on MeOH extract or 0.8% based on plant material), respectively.

Phytochemicals identification in the extract was performed by TLC screening technique and by comparison with authentic compounds as described previously. Common *Ziziphus* triterpenes display violet spots (Suksamrarn et al., 2006) and *Ziziphus* cyclopeptide alkaloids give blue color (Panseeta et al., 2011) with anisaldehyde–H_2SO_4 reagent. Tannins show orange–brown color with vanillin–HCl reagent (Pegg et al., 2008).

***In vitro* α–glucosidase inhibitory activity assay.** The α–glucosidase inhibition assay was modified from Elya et al. (Elya et al., 2012) For primary screening, 10 μL of crude extracts, which dissolved in DMSO to the concentration of 1 mg/mL, were pre-incubated with 0.1 unit of *Saccharomyces cerevisiae* α-glucosidase in 50 mM phosphate buffer pH 6.8 at 37°C for 10 min. Then 20 μL of 10 mM p-nitrophenyl-α-d-glucopyranoside solution (p-NPG) in potassium phosphate

buffer was added and further incubated for 15 min. After incubation, 100 μL of 100 mM Na_2CO_3 was added into the reaction mixture to stop the reaction and the changes in absorbance were measured at a wavelength of 405 nm. For IC_{50} determination, the reactions were prepared in the same manner as the primary screening using different sample concentration ranging from 0.0001-1 mg/mL. Acarbose was used as a standard inhibitor. The α–glucosidase activity in the presence of inhibitors was expressed as a percentage of the uninhibited enzyme activity, and plotted versus inhibitor concentration. Non-linear regression was performed using a GraphPad Prism 5 software. The IC_{50} value was defined as the concentration of compound that give 50% inhibition of maximal activity. All data were expressed as mean ± standard deviation of triplicate determinations.



Betulinic acid (**1**): R = CO_2H
Lupeol (**2**): R = CH_3
Betulin (**3**): R = CH_2OH

α-Amyrin (**4**): R = CH_3
Ursolic acid (**6**): R = CO_2H

β-Amyrin (**5**): R = CH_3
Oleanolic acid (**7**): R = CO_2H

Figure 1 Structures of pentacyclic triterpenes

RESULTS AND DISCUSSION

Four Thai *Ziziphus* plants collected from different locations included the root bark and stem bark of *Z. cambodiana*, the root and stem bark of *Z. mauritiana*, the stem bark of *Z. oenoplia* var. *brunoniana* and the root of *Z. oenoplia* var. *oenoplia* (Table 1), from the TLC–screening chromatograms of all extracts and by comparison with authentic common triterpenes, showed that betulinic acid (**1**) and tannin were the two major components in both EtOAc– and MeOH–extracts, respectively. Chromatographic isolation and purification of each extract yielded high quantity of **1** in overall averages of 0.41% (based on dried plants) and 0.37%, from the EtOAc– and MeOH– soluble fractions, respectively. The highest amount of tannin was found in 7% from the polar fractions (G–H) of the ZOB(SB) methanolic extract.

The structure of **1** was characterized by comparison of its spectroscopic data with the reported values (Suksamrarn et al., 2006).

In vitro anti–diabetic activity of the crude *Ziziphus* extracts was investigated against α–glucosidase enzyme. The percentage inhibition, IC_{50} values and the selectivity ratios are collected in the Table 1. The EtOAc– and MeOH–extracts possess α–glucosidase inhibitory activity with IC_{50} value ranges of 0.014±0.001 – 8.772±0.234 mg/mL and 0.007±0.006 – 0.031±0.0018 mg/mL, respectively. All extracts displayed inhibitory effect more potent than positive control, except for the EtOAc extract of *Z. mauritiana* root [ZM(R)] which exhibited 3.6 times weaker. Interestingly, the MeOH extract of *Z. oenoplia* var. *brunoniana* stem bark [ZOB(SB)] showed the highest inhibition with 350.7–fold stronger than that of standard. A triterpene enriched fraction obtained from ZOB(SB) methanolic soluble part displayed enzymatic inhibitory potency 61–time weaker than that of MeOH extract, however, it possessed 5.7 folds more potent than acarbose.

Table 1 α–Glucosidase inhibitory activity of *Ziziphus* extracts and of betulinic acid (**1**)

Plant species	Sources	% Inhibition[a]		IC_{50} (mg/mL) (selectivity[c])	
		EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.
Z. cambodiana (ZC)	Root bark (RB)	79.97±0.95	91.25±0.50	0.026±0.003 (94.4)	0.016±0.001 (153.4)
	Stem bark (SB)	81.66±0.15	65.64[b]±3.04	0.044±0.001 (55.8)	–
Z. mauritiana (ZM)	Root (R)	75.74±3.85	76.80±4.29	8.772±0.234 (0.28)	0.031±0.0018 (79.2)
	Stem bark (SB)	79.69±0.50	91.07±0.76	0.026±0.014 (94.4)	0.010±0.002 (245.5)
Z. oenoplia var. *oenoplia* (ZOO)	Root (R)	86.50±2.36	44.27±0.83	0.014±0.001 (175.3)	–
Z. oenoplia var. *brunoniana* (ZOB)	Stem bark (SB)	–	92.84±0.30	–	0.007±0.006 (350.7)
	Triterpene rich fraction	84.56±0.38		0.429±0.114 (5.7)	
Betulinic acid (**1**)		98.62±2.64		0.031±0.0027 (79.2)	
Acarbose[d]		85.46±0.66		2.455±0.0126	

[a] Sample concentration: 10 mg/mL of crude sample or 5 mM Acarbose

[b] Partial dissolve in DMSO [c] Selectivity = IC_{50} of acarbose/IC_{50} of extract [d] Positive control

Polyphenol and tannin have been known to inhibit α–glucosidase enzyme (Kim et al., 2010, Kavitha et al., 2015, Omar, 2012). Tannin enriched fraction contributed to the α–amylase inhibitory activity of its corresponded water extracts of *Rubi fructus* which supported by IC_{50} values of 8.9 μg/mL and 59.4 μg/mL, respectively (Kim et al., 2010). The tannin containing ethanolic extract of *Nilgirianthus ciliatus* showed higher α–glucosidase inhibitory effect (IC_{50} 21.90 mg/mL) than inhibition against α–amylase (IC_{50} 462.49 mg/mL) (Kavitha et al., 2015). Hydrolyzable tannins isolated from the seeds of *Eugenia jambolana* were found to inhibit α–glucosidase (Omar et al., 2012). α–Glucosidase inhibition of natural isolated pentacyclic triterpenes seemed variable. Betulinic acid (**1**), α– and β–amyrins (**2**–**3**), ursolic acid (**4**) and oleanolic acid (**5**) (Figure 1) from *Pelliciera rhizophorae* leaves, were very potent inhibitors with the respective IC_{50} values of 2.37, 1.45, 0.02, 1.08 and 0.98 μM, when compared with the reference drug, acarbose (IC_{50} 217.7 μM), i.e., **1** was almost 92–fold more active than control (Lopez et al., 2015). However, betulinic acid (**1**) of *Euonymus alatus* twigs was only 3.7–fold more active than the standard drug, where as its analogues, lupeol (**6**) and betulin (**7**), were inactive (>150 μM) (Choi et al., 2015). In contrast, the triterpene acids (compounds **1**, **4** and **5** and their related derivatives) purified from the apple peels was much less potency, the most active compound, betulinic acid (**1**), showed 28–fold less α–glucosidase inhibition than control (He et al., 2014). Nevertheless, in our test, the isolated **1** exhibited very strong anti-α–glucosidase effect with IC_{50} 0.031±0.0027 mg/mL, the activity of which was 79 times more potent than that of acarbose (Table 1). The most abundant of **1** and tannin in the respective *Ziziphus* EtOAc– and MeOH–extracts, therefore, play important role for high α–glucosidase inhibitory effect. These results consistent with the ethanopharmacological use of *Z. oenoplia* root bark as antidiabetic agent in Thai folk medicine (Bunyapraphatsara and Chokechaija-roenporn, 2000), and also corresponded with previous reports based on α–glucosidase inhibitory property of *Z. mauritiana* fruit extracts (Jarald et al., 2009).

CONCLUSION

This work provides the antidiabetic potency against α–glucosidase of Thai *Ziziphus* plant extracts. The high content of **1** and/or tannin in the extracts is in part responsible for α–glucosidase inhibitory property. This finding also supported and suggested the ethnopharmacological information of these medicinal plants that can be used as anti–diabetic agents in folk medicine

and further make value addition toward Thai *Ziziphus* plants.

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