Songklanakarin J. Sci. Technol.
42 (2), 359-364, Mar. - Apr. 2020

http://www.sjst.psu.ac.th

Original Article

Highly potent α-glucosidase inhibitors from *Pterocarpus indicus* and molecular docking studies

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Received: 12 August 2018; Revised: 19 November 2018; Accepted: 1 January 2019

Abstract

The phytochemical investigation of *Pterocarpus indicus* barks led to isolation of twelve compounds (**1**-**12**), including two major triterpenoids (**1** and **2**), one quinone derivative (**3**), three phenolic compounds (**4**-**6**) and six flavonoids (**7**-**12**). A major active compound (**1**) was derivatized to one new analogue (**1d**) and five known derivatives (**1a**-**1c** and **1e**-**1f**). All isolated compounds (**1**-**12**) and modified analogues (**1a**-**1f**) were evaluated for their α-glucosidase activity. In this regard, compounds **1** and **11** exhibited potent inhibitory activity towards yeast α-glucosidase when compared to the positive control (acarbose). In addition, the α-glucosidase (maltase and sucrase) inhibitory activity of all compounds (**1**-**12** and **1a**-**1f**) was also evaluated. Only compound **11** showed moderate inhibitory activity towards rat intestinal α-glucosidase. Further study on mechanism underlying yeast α-glucosidase inhibition indicated that **1** and **11** could retard the enzyme function by noncompetitive and mixed manners, respectively. The experimental results were also confirmed by docking analysis. Therefore, these compounds have emerged as promising molecules for diabetic therapy.

Keywords: *Pterocarpus indicus*, lupeol, clycosin, α-glucosidase inhibitory activity, molecular docking calculation

1. Introduction

Pterocarpus indicus Wild. is a member of the genus *Pterocarpus* (Leguminosae) and widely distributed over tropical and subtropical south Asia as Malaysia, Philippines, Brunei, Indonesia and Thailand (Hartati, Angelina, Meilawati, & Dewijanti, 2016). Previous phytochemical investigations have indicated that *P. indicus* mainly comprises procycnidin-type tannins, flavonoids and polyphenols (Hartati *et al.*, 2016), with some of these having promising antibacterial (Khan & Omoloso, 2013), antiallergic (Cha, 2016), antimicrobial (Ragasa, De Luna, & Hofilena, 2005), antiplasmin (Takeuchi *et al.*, 1986) and antitumor (Endo & Miyazaki Y, 1972) activities.

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In the Philippines, herbal pills and teas made from *P. indicus* extracts have been popularized for treating a wide range of diseases and ailments including menstrual pain, leprosy, rheumatoid arthritis, flu and diabetes (Thomson, 2006). Interestingly, there has been no report on the isolation and characterization of active compounds in this plant as potential antidiabetic inhibitors. Therefore, this study was carried out to identify active α-glucosidase inhibitory constituents from *P. indicus*. We report herein the isolation and structural characterization of twelve compounds (**1**-**12**), including two major triterpenoids (**1** and **2**), one quinone derivative (**3**), three phenolic compounds (**4**-**6**) and six flavonoids (**7**-**12**) from *P. indicus* bark. Moreover, a modification of the most potentially active compound (**1**) was prepared, and the binding conformations and important interactions between α-glucosidse and potent inhibitors were studied.

2. Material and Methods

2.1 General experimental procedures

The ^{1}H and ^{13}C NMR spectra were acquired by Bruker AVANCE 400 spectrometers. TLC was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer), and spots were performed using UV or dipped in 3% (v/v) anisaldehyde followed by heating. 4-nitrophenyl-α-D-glucopyranoside (*p*-NPG) and α-Glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae* and rat intestinal acetone powder were purchased from Sigma-Aldrich. Acarbose was supplied by Bayer Vitol Leverkusen. Enzymatic activity was taken on a Sunrise microplate reader spectrophotometer.

2.2 Plant material

The bark of *P. indicus* was collected in April 2016, from Mahasarakham Province, Thailand, and identified by Dr. Suttira Khumkratok. A voucher specimen (Khumkratok no.1-15) was deposited at the Walai Rukhavej Botanical Research Institute, Mahasarakham University.

2.3 Extraction and isolation

Air-dried and finely powdered barks of *P. indicus* (4.0 kg) was sequentially extracted with EtOH at ambient temperature for 6 days. The solvent extract was evaporated *in vacuo* to obtain crude EtOH extract (35.8 g). This concentrated EtOH extract was subjected to open column chromatography (CC) over silica gel (Merck Art 7730) and eluted with eight mobile phases of hexane:CH_2Cl_2:MeOH (8:2:0, 5:5:0, 0:1:0, 0:9:1, 0:7:3, 0:5:5, 0:3:7 and 0:0:1) to give six major fractions (A-F). Fraction A was subjected to Si CC eluting with a CH_2Cl_2 and hexane (0:10-10:0) gradient to obtain **1** (12.3 g). Fraction B was subjected to Sephadex LH-20 (20% MeOH:CH_2Cl_2) to give **2** (15.1 mg). Fraction C was chromatographed on Si CC eluting with a EtOAc and hexane gradient (2:8-10:0) to afford **3** (4.3 mg) and **4** (1.8 mg). Fraction D was separated on Si CC eluting with using MeOH:CH_2Cl_2 (3:1) and Sephadex LH-20 (MeOH:CH_2 Cl_2, 5:5) to afford three subfractions (D1-D3). Subfraction D1 was chromatographed on Si CC eluting with a EtOAc and hexane gradient (4:6-10:0) to give **5** (5.5 mg) and **6** (2.3 mg). After preparative TLC purification using MeOH:CH_2Cl_2 (1:9) of subfraction D2 to give **7** (2.7 mg), **8** (2.4 mg) and **9** (1.5 mg). Finally, fraction E was purified by preparative TLC (3% MeOH:CH_2Cl_2) to obtain **10** (3.8 mg), **11** (5.7 mg) and **12** (4.7 mg), respectively. The chemical structure of all isolated compounds (**1**-**12**) was determined using various spectroscopic techniques.

2.4 Nucleophilic acyl substitution of 1

Briefly, 4-(dimethylamino) pyridine (DMAP), triethylamine (TEA) and several anhydrides were added to the major triterpenoid (**1**) in CH_2Cl_2. The reaction mixture was stirred for 1 h at room temperature, then diluted with CH_2Cl_2, washed with brine and dried over solid anhydrous Na_2SO_4. Na_2SO_4 was removed by filtration and then CH_2Cl_2 was removed by evaporation. Finally, the residue was purified by chromatotron to yield the derivatives **1a**-**1f**.

2.5 α-Glucosidase inhibitory assay

The yeast α-glucosidases inhibition assay was modified from a previous colorimetric method (Schäfer & Högger, 2007). The sample (10 µL) was mixed with α-glucosidase (0.1 U/mL) in 1 mM phosphate buffer (pH 6.9) and incubation added 10 min at 37 ºC. The mixture was added with 50 mL substrate solution (1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG)) followed by additional incubation for 20 min, and terminated by adding 100 µL of 1 M Na_2CO_3. Enzymatic activity was monitored by measuring the absorbance at 405 nm. The percent inhibition was calculated using the following equation.

$$\frac{(A_0\text{-}A_1)}{A_0} \times 100$$

where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample.

In addition, the previous colorimetric method (Barham & Trinder, 1972) was also modified to determine inhibitory activity of all compounds against intestinal (maltase and sucrase) α-glucosidases. A 10 µL of sample (1 mg/mL in DMSO) and substrate solution (maltose: 10 mM, 20µL; sucrase: 100 mM, 20 µL, respectively) in 0.1 M phosphate buffer (pH 6.9) were pre-incubated at 37 °C (20 min for maltase and 60 min for sucrase). The mixture was heated in an oven at 80 °C for 10 min to quench the reaction. The concentration of glucose released from the reaction was converted to quinoneimine using a commercial glucose assay kit (SU-GLLQ2, Human, Germany). The absorbance of final product was determined at 503 nm, and the percentage inhibition was determined using the expression above.

Determination of the inhibition modes of the samples against α-glucosidase was performed according to our previous method (Sichaem *et al.*, 2017).

2.6 Molecular docking calculation

The molecular structure of compounds **1**, **1a** and **11** have been modeled and further were geometrically optimized at PM6 by using MOPAC2016 program (Stewart, 2016). The docking calculation of these inhibitors is similar to our previous report (Sichaem *et al*., 2017). The docking results

showing the lowest binding free energy were further subjected for molecular interaction analyses by focusing on the intermolecular hydrogen bonding.

3. Results and Discussion

The dried *P. indicus* bark was extracted with EtOH. This crude extract was fractionated and purified using chromatographic techniques to furnish two major triterpenoids, lupeol (**1**) (Sichaem *et al.*, 2017) and canophyllol (**2**) (Duarte *et al.*, 2009), one quinone derivative, 2,6-dimethoxy-*p*-benzoquinone (**3**) (Inoshiri *et al.*, 1986), three phenolic derivatives, vanillic acid (**4**) (Xu, Liu, & Xu, 1995), *trans*-4-hydroxymellein (**5**) and *cis*-4-hydroxymellein (**6**) (Hussain *et al.*, 20 15), six flavonoids that can be classified into two pterocarpin derivatives, (6α*R*,11α*R*)-medicarpin (**7**) (Sichaem, Ruksilp, Sawasdee, Khumkratok, & Tip-pyang, 2018) and (6α*R*,11α*R*) -3,8-dihydroxy-9-methoxypterocarpan (**8**) (Bezuidenhoudt, Brandt, & Ferreira, 1987), together with four isoflavones, afromosin (**9**) (Fukai, Wang, Inami, & Nomura, 1990), formononetin (**10**) (Sichaem *et al.*, 2018), clycosin (**11**) (Morikawa, Xu, Matsuda, & Yoshikawa, 2006) and 8-*O*-methylretusin (**12**) (Sichaem *et al.*, 2018), by comparing their NMR spectral data to those formerly published and assured through co-TLC with authentic samples (Figure 1).

α-Glucosidases are mainly divided into two groups, types I (baker's yeast) and II (mammals), on the basis of the difference in primary structure (Oki *et al*., 1999). In this study, all isolated compounds (**1-12**) were determined for their yeast α-glucosidase inhibitory activity, compounds **1** and **11** showed highly potent inhibitory activity towards yeast α-glucosidase with the IC_{50} values of 37.2 and 39.8 µM, respectively. On the other hand, compounds **6-9** and **12** exhibited significant inhibitory activity with the IC_{50} values in the range of 119-311µM when compared to the standard agent (acarbose, IC_{50} 526 µM). Moreover, when the rat intestinal α-glucosidase (maltase and sucrase) inhibitory activity of all isolated compounds (**1-12**) was evaluated, only compound **11** showed moderate inhibitory activity towards rat intestinal α-glucosidase with the IC_{50} values of 67.7 µM (maltase) and 103 µM (sucrase). Compounds **8** and **12** exhibited weak inhibitory activity against maltase and sucrase with the IC_{50} values in the range of 152-209 µM.

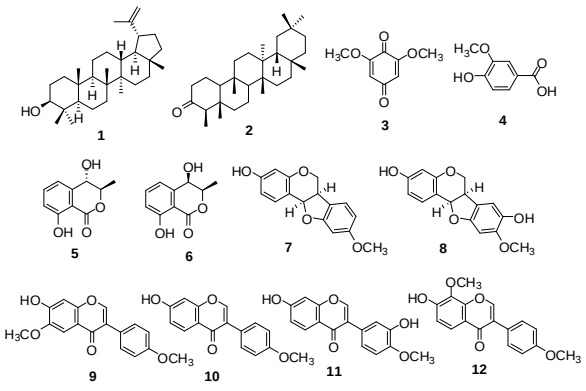


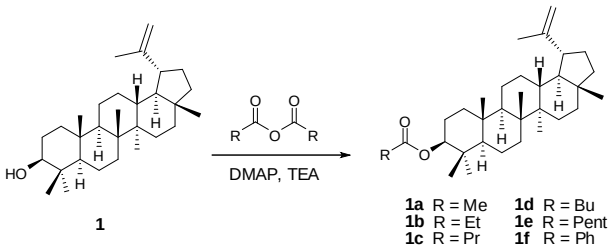
Figure 1. Structures of **1-12** isolated from the bark of *P. indicus.*

Compound **1** exhibited more powerful yeast α-glucosidase inhibitory activity than other compounds (Table 1). Interest in the results above led us to study the structural modification and anti α-glucosidase activity of its derivatives (**1a-1f**) from this lupine-type skeleton (**1**). In the course of chemical modifications, a new compound (**1d**) and five known analogues (**1a-1c** and **1e-1f**) were obtained from the derivatization of **1** by the nucleophilic acyl substitution with various anhydrides of the hydroxyl group at C-3 of **1** (Scheme 1).

Table 1. The α-glucosidase inhibitory activity of all naturally isolated compounds (**1-12**) and modified analogues (**1a-1f**).

Compound	IC_{50} (µM)		
	Baker's yeast	Maltase	Sucrase
1	37.2	NI	NI
2	NI[a]	NI	NI
3	NI	NI	NI
4	NI	NI	NI
5	NI	NI	NI
6	311	NI	414
7	288	NI	NI
8	119	152	177
9	148	NI	NI
10	NI	NI	NI
11	39.8	67.7	103
12	162	209	172
1a	490	NI	NI
1b	441	NI	NI
1c	NI	NI	NI
1d	NI	NI	NI
1e	NI	NI	NI
1f	NI	NI	NI
Acarbose	526	7.9	10.9

[a] No inhibition (inhibitory effect less than 30% at concentration of 10 mg/mL)



Scheme 1. Modified reaction of lupeol (**1**).

All derivatives (**1a-1f**) were determined for their α-glucosidase activity (Table 1). Modified compounds **1a-1f**, which lacked the hydroxyl group at C-3, had a lower α-glucosidase inhibitory activity than the original (**1**). This investigation confirmed that the hydroxyl group at C-3 might be involved in anti α-glucosidase activity of **1**. In addition, the enhancement of the hydrophobic side chains at C-3 also led to a decrease in antidiabetic activity.

From the above results, lupeol (**1**) showed the most potential inhibitory activity toward in α-glucosidase. To gain insight into the mechanism underlying the inhibitory effect of

1, a kinetic study was also conducted. In the kinetic study, the K_m and V_{max} values were calculated from the Lineweaver-Burk plot (Figure 2A). The analysis revealed that V_{max} value decreased with increasing concentration of **1** while K_m remained constant. These results suggested that **1** inhibited yeast α-glucosidase in a noncompetitive manner.

Envisage the mechanism underlying this inhibition, a kinetic study of **11** was performed. The Lineweaver-Burk plots indicated that **11** showed linearity at each concentration examined. The kinetic analysis revealed that V_{max} decreased with increasing concentrations of **11** while K_m increased (Figure 2B).

The *in vitro* activity elucidation of compound **1** occurred in a noncompetitive manner, while **11**, which shared a similar core structure with daidzein (Wu *et al.*, 2012) and genistein (Lee *et al.*, 2001), showed mixed mode of inhibition. In this work, therefore, molecular docking calculations were studied on the active site and region beyond that which defined almost the whole protein. The molecular binding of compounds of interest is shown in Figure 3A; compounds **1** and **1a** have a similar binding conformation at the same binding site, while compound **11** bound at a different binding site. This is due to the difference in their molecular structures. Molecular binding analyses revealed that compound **1** (IC_{50} 37.2 µM) has a binding free energy of -6.78 kcal/mol, while compound **1a** showed -6.46 kcal/mol. This result was in qualitative accordance with the biological activity testing (IC_{50} 37.2 and 490.66 µM, respectively). The binding conformation of **1** is shown in Figure 3B; it formed a H-bond between hydroxyl group (OH) and backbone C=O of Phe157 with the distance of 2.95 Å. This interaction disappeared in 1a in which the -OH was replaced by acetyl group (Figure 3C). Compound **11** showed binding free energy of -6.76 kcal/mol and the molecular binding is displayed in Figure 3D. We found that **11** formed H-bonds between hydroxyl and methxyl of **11** and the sidechain of Asn314 with the distance of 2.89 and 2.93 Å, respectively.

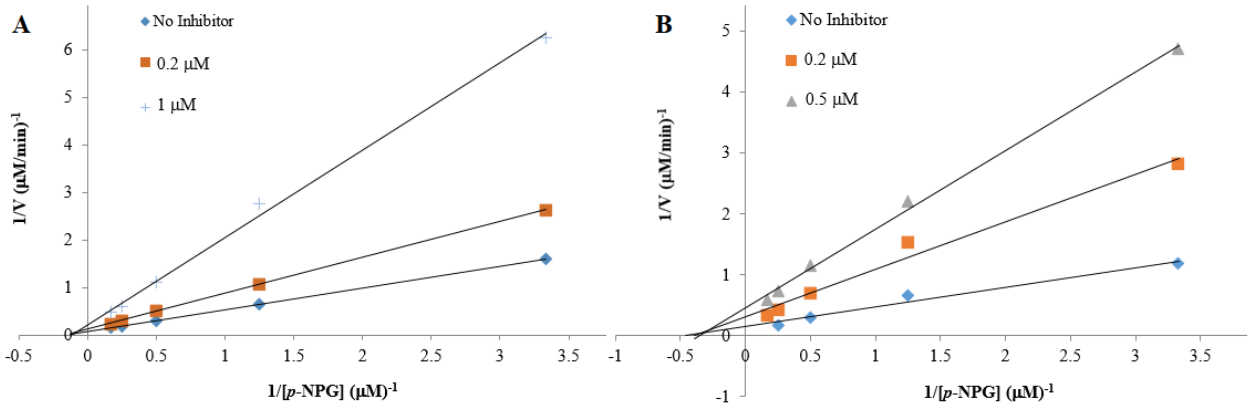


Figure 2. Lineweaver-Burk plots for inhibitory activity of **1** (A) and **11** (B) against yeast α-glucosidase.

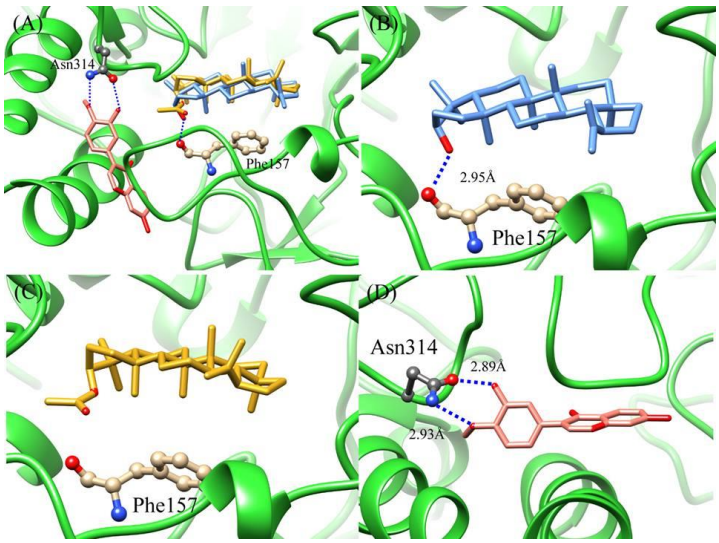


Figure 3. Comparison the binding conformation of compounds **1** (blue), **1a** (yellow) and **11** (pink) (A) bound yeast α-glucosidase. The magnification of binding pose of **1** (B), **1a** (C) and **11** (D) was studied. All compounds are shown in stick model, while the amino acids are depicted as ball and stick model. The hydrogen bonding interactions are shown as dashed blue line. The molecular conformations were prepared using the Chimera software (Pettersen *et al*., 2004) (For colour in this Figure, the reader is referred to the web version).

4. Conclusions

This is the first report on mainly *α*-glucosidase inhibitors of *P. indicus* bark. Twelve natural compounds (**1**-**12**) were isolated, as well as one new modified analogue (1d) and five known derivatives (**1a**-**1c** and **1e**-**1f**) were also prepared. Compound **1** exhibited the highest activity against yeast *α*-glucosidase and was superior to the positive agent. The docking analyses showed that compound **1** formed H-bond with Phe157. In the other hand, this interaction disappeared with a less potent inhibitor. Compound **11**, which is a different class of inhibitor compared to **1** and **1a**, showed H-bond with Asn134. This highlights the role of the H-bond for these inhibitors. Further investigation should determine the additive effects of the active compounds in order to develop new antidiabetic drugs.

Acknowledgements

This study was supported by Thammasat University Research Fund, Contract No. 4/2561. We also thank Dr. Suttira Khumkratok, Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham, Thailand, for collection, identification and deposition of the plant material. K.L. would like to thank RGJ Advanced Programme (RAP60 K003) and Bansomdejchaopraya Rajabhat University.

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