Inhibitors of α-amylase and α-glucosidase from *Andromachia igniaria* Humb. & Bonpl.

Mariela Beatriz Vera Saltos[a], Blanca Fabiola Naranjo Puente[b], Immacolata Faraone[c], Luigi Milella[c], Nunziatina De Tommasi [d], Alessandra Braca[a,e,*]

[a]*Dipartimento di Farmacia, Università di Pisa, Via Bonanno 33, 56126, Pisa, Italy*

[b]*Departamento de Ciencias de la Vida, Universidad de las Fuerzas Armadas, ESPE, Av. General Rumiñahui s/n,Sangolqui, Ecuador*

[c]*Dipartimento di Scienze, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy*

[d]*Dipartimento di Farmacia, Università di Salerno, Via Giovanni Paolo II, 84084 Fisciano (SA), Italy*

[e]*Centro Interdipartimentale di Ricerca "Nutraceutica e Alimentazione per la Salute", Università di Pisa, via del Borghetto 80, 56124 Pisa, Italy*

* Corresponding author. Tel.: +39 050 2219688; fax: +39 050 2220680.

E-mail address: alessandra.braca@farm.unipi.it (A. Braca).

Abstract

Two new flavonoids, (2*S*)-3',4',7,8-tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**1**) and 3',4',7,8-tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**2**) along with fourteen known compounds were isolated from *Andromachia igniaria* aerial part extracts. Their structures were determined via spectroscopic analyses including 2D NMR. The hypoglycemic properties of all extracts and pure compounds were evaluated measuring α-amylase and α-glucosidase inhibitory effects. The *n*-butanol among the extracts was found to be the best inhibitor of both α-amylase and α-glucosidase enzymes, while among compounds the most active were: bidenoside F and luteolin 4'-*O*-β-D–glucopyranoside as α-amylase inhibitors; eriodictyol, butein and okanin as α-glucosidase inhibitors. Results demonstrated that *A. igniaria* can represent an important natural source with high biological values, helpful to control postprandial hypoglycemia.

Keywords: Andromachia igniaria, Asteraceae, flavonoids, α-amylase activity, α-glucosidase activity

1. Introduction

Andromachia is a genus of flowering plants in the Asteraceae family, which was recognized in 1819 as a synonym of *Liabum* and placed in the Vernonieae (Robinson and Brettell, 1973). Few information are reported about previous investigations carried out on some species from Central and South America of this genus, that resulted basically on the isolation of some sesquiterpene lactones, triterpenes, and flavonoids (Bohlmann et al., 1984; Jakupovic et al., 1988; Juarez et al., 1995). As a part of our continuing chemical and biological analyses of plants from Ecuadorian flora, *A. igniaria* Humb. & Bonpl., synonym *Liabum igniarum* Less., was selected for a phytochemical and biological study. This species is widely distributed in the Andes mainly from Colombia and Ecuador, between 2000 and 3500 m a.s.l.; it's a shrub, 1-3 m high, with bracts separated by petioles, opposite petiolated leaves, with abundant white pubescent in the underside, top inflorescence, ligulate yellow flower and the fruit is an achene. The name "*igniaria*", that means "of fire", comes from the intense yellow color of the inflorescences. The leaves are used to feed guinea pigs, as well as to prepare a special sweet bread called "pan de leche". In popular medicine people use the leaves to wash the body for purification, to treat headache or to heal wounds. Nevertheless, no phytochemical study on this species was reported in the literature.

Recently, low molecular weight plant-derived molecules such as luteolin and green tea polyphenols have been shown to be helpful for treating hyperglycemia (Lo Piparo et al., 2008). Diabetes mellitus is a major chronic disease induced by an unsuitable balance of blood glucose with a significant impact on health. Type 1 diabetes is an insulin-dependent disease, while type 2 diabetes is a non-insulin-dependent disease characterized by hyperglycemia which results from insufficient or inefficient insulin secretion. The first can effectively be controlled by the regulation of insulin while the management of type 2 diabetes is more difficult. One of the available therapy is directed to decrease postprandial hyperglycemia by delaying the absorption of glucose, accomplished by inhibition of starch breakdown enzymes such as α-amylase and α-glucosidase. In

fact the inhibition of these two enzymes by pharmaceutical agents (e.g. acarbose) is an accepted clinical strategy for managing postprandial glycemia in patients affected by type 2 diabetes. The increasing prevalence of type 2 diabetes mellitus and the negative clinical outcomes observed with the commercially available anti-diabetic drugs have led to the investigation of new therapeutic approaches focused on controlling postprandial glucose levels. The use of carbohydrate digestive enzyme inhibitors from natural resources could be a possible strategy to block dietary carbohydrate absorption with less adverse effects than synthetic drugs, and plant-derived materials have been demonstrated to be alternative to synthetic drugs due to their bioactive substances (Lee et al., 2015).

Based on this evidence, in this paper, we describe the isolation and structural characterization of two new natural flavonoids, (2*S*)-3',4',7,8-tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**1**) and 3',4',7,8-tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**2**), together with fourteen known compounds (**3-16**) (**Fig. 1**), and their hypoglycemic properties measuring α-amylase and α-glucosidase inhibitory effects.

2. Results and Discussion

The aerial parts of *A. igniaria* were sequentially extracted with solvents of increasing polarity giving *n*-hexane (ER), chloroform (CR), chloroform-methanol (9:1) (CMR), and methanol (MR) residues. The MR was partitioned between *n*-BuOH (BuR) and H_2O (WR). To evaluate the potential biological activity of *A. igniaria*, all extracts were tested for their ability to inhibit α-amylase and α-glucosidase enzymes at different concentrations. CMR, BuR and MR extracts demonstrated to be able to inhibit the two enzymes in a concentration-dependent manner as reported in **Figure 2**, while the ER, CR and WR were inactive (data not shown). Acarbose, used as reference drug, was more effective than CMR, BuR and MR extracts to inhibit α-amylase (**Fig. 2a**), whereas CMR and BuR extracts exerted higher α-glucosidase inhibitory activity than acarbose (**Fig. 2b**)

demonstrating the biological potential of this species. Among all extracts, BuR was found to be the most active inhibitor of both enzymes, showing the lowest IC_{50} values, 327.97 ± 23.03 μg/mL for α-glucosidase, sensibly lower than acarbose (1104.29 ± 82.22 μg/mL) and 140.31 ± 9.14 μg/mL for α-amylase, in this case higher than acarbose (IC_{50} = 7.42 ± 0.71 μg/mL). For this reason BuR and CMR were further analysed for their secondary metabolite content by different chromatographies to afford two new (**1**-**2**) and fourteen known compounds (**3**-**16**).

Compound **1** was obtained as a yellow powder, analyzed for the molecular formula $C_{23}H_{24}O_{12}$ by HRESI-MS at *m/z* 491.482 $[M-H]^-$ (calcd for $C_{23}H_{23}O_{12}$, 491.1190), which was further confirmed by ^{13}C NMR and DEPT spectra. The ESI MS spectrum of compound **1** showed a quasimolecular ion peak at *m/z* 491 $[M-H]^-$ and one peak at *m/z* 287 $[M-H-204]^-$, due to the loss of one acetylated hexose moiety. The UV absorption bands at 328 and 284 nm were suggestive of a flavanone skeleton. In the ^1H NMR spectrum, three aromatic proton signals (δ 7.04, d, *J* = 2.0 Hz; δ 6.92, dd, *J* = 8.5, 2.0 Hz; δ 6.82, d, *J* = 8.5 Hz) indicated an ABX system on ring B, and these proton signals were assigned to H-2', H-6' and H-5' by HSQC analysis. Likewise, another two aromatic protons signals (δ 7.40, d, *J* = 8.0 Hz; δ 6.90, d, *J* = 8.0 Hz) were readily assigned to H-5 and H-6 of ring A. The ^1H and ^{13}C NMR spectra (**Table 1**) displayed also signals for an oxygenated methine doublet of doublets at δ 5.45 (1H, dd, *J* = 12.0, 3.0 Hz, H-2), two methylene doublet of doublets at δ 3.16 (1H, dd, *J* = 17.0, 12.0 Hz, H-3_{ax}) and 2.81 (1H, dd, *J* = 17.0, 3.0 Hz, H-3_{eq}), that were assigned with the help of 1D TOCSY and DQF-COSY, together with signals of a sugar residue and an acetyl group. These 1D NMR data, in combination with the observed 2D NMR correlations, suggested that compound **1** was a flavanone having isookanin as aglycone (Agrawal, 1989). The structure of the monosaccharide moiety of compound **1** was deduced using hydrolysis followed by trimethyl silylation and GC-analysis, 1D-TOCSY and DQF-COSY experiments. Thus, the chemical shifts of the sugar resonances were attributable to one β-glucopyranosyl unit esterified at C-6 position. The substitution sites of the glucose and acetyl residues were also confirmed by the HMBC correlations between δ 4.98 (H-1_{glc}) and 152.2 ppm (C-7) and δ 4.45, 4.28 (H_2-6_{glc}) and 172.0 ppm ($COCH_3$).

The stereochemistry of C-2 was determined as *S* on the basis of a negative Cotton effect at 280 nm in the CD spectrum of **1** (Slade et al., 2005). Thus, the structure of compound **1** was unambiguously elucidated as (2*S*)-3',4',7,8-tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside or 6''-*O*-acetyl-flavanomarein.

The molecular formula of compound **2** ($C_{23}H_{22}O_{12}$) was established by ^{13}C NMR and HR-ESIMS spectra (*m/z* 489.1041 for $[M-H]^-$). Its NMR spectral data suggested that the acetylated sugar moiety of compound **2** was superimposable to that of compound **1**, while the aglycone was the point of difference. In fact, ^{1}H and ^{13}C NMR spectra (**Table 1**) revealed the presence of an additional aromatic signal (δ_H 6.78 s, δ_C 115.5), which could be easily assigned to ring C, and was consistent with the presence of 3',4',7,8-tetrahydroxyflavone as aglycone (Yang et al., 2012). The configuration of the sugar moiety was determined as reported for compound **1**. Thus, the structure of **2** was elucidated as 3',4',7,8-tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside.

The following known compounds were identified by spectral analysis and comparison with published spectroscopic data: rutin (**3**) (Agrawal, 1989), kaempferol 3-*O*-β-D-glucopyranoside (**4**) (Agrawal, 1989), 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene 8,10,12-triyne (**5**) (Rücker et al., 1992), eugenyl *O*-β-D-glucopyranoside (**6**) (Fujita et al., 1994), 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**7**) (Chiang et al., 2007), eriodictyol (**8**) (Agrawal, 1989), okanin (**9**) (Chokchaisiri et al., 2009), butein (**10**) (Chokchaisiri et al., 2009), luteolin (**11**) (Agrawal, 1989), bidenoside F (**12**) (Li et al., 2005), caffeic acid methyl ester (**13**) (Saleem et al., 2004), okanin 4'-*O*-β-D-glucopyranoside (**14**) (Hoffmann and Holzl, 1988), luteolin 4'-*O*-β-D-glucopyranoside (**15**) (Nawwar et al., 1994), and caffeic acid (**16**) (Saleem et al., 2004).

All the isolated compounds were tested to evaluate their starch breakdown enzyme inhibition ability against α-amylase and α-glucosidase. The inhibition of these two enzymes has been measured since it's currently used as a pharmacological approach directed to decrease postprandial hyperglycemia by delaying the absorption of glucose. Results were expressed as IC_{50} (mM) and compared with reference compound acarbose (**Fig. 3**). Several isolated compounds showed an

interesting α-amylase (**Fig. 3a)** and α-glucosidase (**Fig. 3b)** inhibitory activity; in particular among flavonoids, monoglycosylated derivatives (compounds **1**, **2**, **4**, **12**, **14**, and **15**) reported the highest inhibitory activity against α-amylase. Interestingly, acetylene derivatives **5** and **7** were also strong *in vitro* inhibitors of α-amylase (IC_{50} = 0.05 ± 0.003 and 0.05 ± 0.006 mM, respectively), to the best of our knowledge there aren't reports about the α-amylase inhibitory activity of such structures. In the α-amylase inhibition assay all tested compounds reported IC_{50} values higher than acarbose but lower than the extract they come from, except compound **3** which presents the highest IC_{50}value (0.90 ± 0.08 mM) accordingly to previous study (Jo et al., 2009). Instead, in the α-glucosidase inhibition test, all compounds reported IC_{50} values lower than acarbose and extracts. The chalcones **9** and **10** showed the highest α-glucosidase inhibition activity with IC_{50} values around 0.02 mM. Also in this case compound **3** has the highest IC_{50} (0.40 ± 0.03 mM). On the other hand, eugenyl *O*-β-D-glucopyranoside (**6**) showed no inhibitory capacity against both tested enzymes. In conclusion, both CMR and BuR extracts and their pure compounds showed a higher inhibitory capacity against α-glucosidase than α-amylase. Okanin (**9**) and butein (**10**), belonging to the class of chalcones, reported a good *in vitro* α-glucosidase inhibition, while flavonoids and the two acetylenes glycosylated derivatives (**5** and **7**), were more effective against α-amylase. The two new compounds **1** and **2** demonstrated to be able to inhibit α-glucosidase better than acarbose, but they needed to be more concentrated than the reference standard to reach the α-amylase IC_{50} value.

The activity demonstrated by *A. ignaria* extracts appears to be of interest compared to other previously investigated extracts, belonging to the same family. For example, *Matricaria chamomilla* hot water extract demonstrated an α-amylase IC_{50} value that is one order of magnitude higher than *A. ignaria* BuR (5200 µg/mL *vs* 140.31 µg/mL respectively) (Kato et al., 2008). Finally, the *A. ignaria* polar extracts might be used for the design of novel functional foods with blood-glucose-lowering potential, which could be useful as a complement of other antidiabetic drugs.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, bandwidth 2.0 nm. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. ESI-MS were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. HR-ESIMS were acquired in positive and negative ion mode on a Q-TOF premier spectrometer (Waters-Milford). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ and NTS (Naturstoffe reagent)-PEG (Poliethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID10A refractive index detector and a Shimadzu injector, using a C_{18}μ-Bondapak column (30 cm x 7.8 mm, 10 μm, Waters-Milford) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses were performed using a Dani GC 1000 instrument. All spectrophotometric measurements were done in 96-well microplates on a UV/VIS spectrophotometer SPECTROstar^{Nano} (BMG Labtech) and each reaction was performed in triplicate.

3.2.Chemicals

Sodium phosphate, sodium chloride, potassium sodium tartrate tetrahydrate, 3,5-dinitrosalicylic acid, sodium hydroxide, α-amylase from hog pancreas starch, α-glucosidase from *Saccharomices cerevisiae*, potassium phosphate monobasic, 4-nitrophenyl α-D-glucopyranoside, acarbose, NTS (Naturstoffe reagent), PEG (Poliethylene glycol 4000), cerium disulfate and sulfuric acid were acquired from Sigma-Aldrich (Milano, Italy). Solvent as *n*-hexane, *n*-butanol, methanol, hydrochloric acid, chloroform and glacial acetic acid were purchased from VWR (Milano, Italy).

3.3 Plant material

Aerial parts of *A. igniaria* were collected in Tumbaco, Ecuador in September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 9371 *Andromachia igniaria* /1) was deposited at Herbarium Horti Botanici Pisani, Pisa, Italy.

3.4. Extraction and isolation

The dried and powdered aerial parts (580 g) of *A. igniaria* were in sequence extracted for 48 h using *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH by exhaustive maceration (3 x 2 L), to give 8.2 (ER), 14.9 (CR), 6.3 (CMR) and 23.1 g (MR) of the respective residues. The MR extract was partitioned between *n*-BuOH (BuR) and H_2O (WR) to afford a *n*-BuOH residue. The BuR fraction (4 g) was submitted to Sephadex LH-20 column (3 x 100 cm, flow rate 1.0 mL/min) using MeOH as eluent to obtain sixteen major fractions (A-P) grouped by TLC. Fraction G (109.2 mg) was subjected to RP-HPLC with MeOH-H_2O (45:55) as eluent yielding a new flavonoid (2*S*)-3',4',7,8-tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**1**, 4.3 mg, t_R = 10 min), and caffeic acid methyl ester (**13**, 1.9 mg, t_R = 14 min), rutin (**3**, 2.7 mg, t_R = 15 min) and kaempferol 3-*O*-β-D-glucopyranoside (**4**, 0.9 mg, t_R = 21 min) . Fractions A (215.2 mg), B (136.8 mg), N (20.3 mg) and P (25 mg) were separately purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to give

3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene 8,10,12-triyne (**5**, 2.2 mg, t_R = 35 min), eugenyl *O*-β-D-glucopyranoside (**6**, 1.5 mg, t_R = 10 min) from fraction A, 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**7**, 0.8 mg, t_R = 38 min) from B, eriodictyol (**8**, 1.9 mg, t_R = 12 min) from N, and okanin (**9**, 1.4 mg, t_R = 13 min), butein (**10**, 17.3 mg, t_R = 22 min) and luteolin (**11**, 0.3 mg, t_R = 25 min) from fraction P. Fractions E (70.9 mg), I (143.3 mg), J (424.9 mg) and L (67.9 mg) were also subjected to to RP-HPLC with MeOH-H_2O (45:55) as eluent to obtain bidenoside F (**12**, 1.3 mg, t_R = 13 min) from E, kaempferol 3-*O*-β-D-glucopyranoside (**4**,1.8 mg, t_R = 20 min), from J, okanin 4'-*O*-β-D-glucopyranoside (**14**, 14.2 mg, t_R = 12 min) and luteolin 4'-*O*-β-D-glucopyranoside (**15**, 1.1 mg, t_R = 23 min) from fraction L.

The CMR extract (6 g) was subjected to Sephadex LH-20 column (3 x 100 cm, flow rate 1 mL/min) eluting with MeOH to give eleven major fractions (A-K) grouped by TLC, together with pure luteolin (**11**, 11.6 mg). Fraction E (232 mg) was purified by RP-HPLC with MeOH-H_2O (55:45) as eluent to afford 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene 8,10,12-triyne (**5**, 1 mg, t_R = 23 min). Fraction G (130.7 mg) was purified by RP-HPLC with MeOH-H_2O (2:3) as eluent yielding compound **1** (1 mg, t_R = 16 min). Fraction H (108 mg) was subjected to RP-HPLC with MeOH-H_2O (45:55) as eluent to give compound **1** (1.1 mg, t_R = 10 min) and caffeic acid (**16**, 5.3 mg, t_R = 6 min). Finally fraction I (34.1 mg) was purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to afford a new flavonoid 3',4',7,8-tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**2**, 2 mg, t_R = 18 min). All the compounds met the criteria of ≥ 95% purity, as inferred by HPLC and NMR analyses.

3.4.1.(2S)-3',4',7,8-tetrahydroxyflavanone 7-O-(6''-O-acetyl)-β-D-glucopyranoside (**1**): yellow amorphous powder; $[\alpha]_D^{25}$ -40 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε): 328 (4.19), 284 (3.90); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) – 5200 (280 nm); ^{1}H and ^{13}C NMR data, see **Table 1**; ESI MS *m/z* 491 $[M-H]^-$, 473 $[M-H-18]^-$, 287 $[M-H-204]^-$, HR ESIMS $[M-H]^-$ 491.1182 (calcd for $C_{23}H_{23}O_{12}$ 491.1190), 355.1042 $[M-H-134]^-$, 287.0993 $[M-H-204]^-$.

*3.4.2. 3',4',7,8-tetrahydroxyflavone 7-O-(6''-O-acetyl)-β-D-glucopyranoside (**2**)*: yellow amorphous powder; $[\alpha]_D^{25}$ -35 (*c* 0.078, MeOH); UV (MeOH) λ_{max} (log ε): 258 (4.07), 321 (3.85); ^{1}H and ^{13}C NMR data, see **Table 1**; ESI MS *m/z* 489 $[M-H]^-$; HR ESIMS *m/z* 489.1041 $[M-H]^-$ (calcd for $C_{23}H_{21}O_{12}$ 489.1033), 355.0972 $[M-H-134]^-$, 285.0944 $[M-H-204]^-$.

*3.5. Acid hydrolysis of compounds **1** and **2***

A solution of each compound (2.0 mg) in HCl 1 N (1 mL) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-Chirasil-Val column (0.32 mm x 25 m). Temperatures of the injector and detector were both 200°C. A temperature gradient system was used for the oven, starting at 100°C for 1 min and increasing up to 180°C at a rate of 5°C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

3.6. Hypoglycemic activity assays

3.6.1. α-Amylase inhibitory activity

The α-amylase inhibitory activity was assayed using 10 µL of 20 mM sodium phosphate buffer (pH 6.9 with 6 mM NaCl) containing 0.5 mg/mL of α-amylase (50 Units/mg), the solution was incubated at 25°C for 10 min with 10 µL of sample, resuspended in 15% methanol/buffer solution, at different concentrations. After this preincubation, 10 µL of 1% starch solution in 20 mM of sodium phosphate buffer, used as substrate, was added to each sample and the reaction mixtures

were again incubated at 25 °C for 10 min. The reaction was stopped with 20 µL of dinitrosalicylic acid color reagent. Subsequently the test tubes were incubated in a boiling water bath for 10 min and cooled at room temperature. After the last incubation step the reaction mixture was diluted adding 300 µL of distilled water and the absorbance was measured at 540 nm. The absorbance of blank (enzyme solution was added during the boiling) and negative control (sodium phosphate buffer in place of sample) were recorded. Acarbose was dissolved in 15% methanol/buffer solution at different concentrations, and it was used as positive control. Analyses were performed in triplicate and the final sample absorbance (A540 nm) was obtained by subtracting its corresponding blank reading (Ranilla et al., 2010). The inhibitory activity was calculated by using the formula and compared to the positive control:

$$\%\in hibition = \frac{(A\,540\,NegativeControl - A\,540\,Sample)}{A\,540\,NegativeControl} * 100$$

The concentration of the sample required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by nonlinear regression analysis.

3.6.2. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was assessed using the procedure previously reported with slight modification (Vinholes et al., 2011). In each well 130 µL of 10 mM phosphate buffer pH 7.0 and 60 µL of substrate (2.5 mM 4-nitrophenyl α-D-glucopyranoside in 10 mM phosphate buffer) were added to 40 µL of sample dissolved in 15% methanol/buffer solution at different concentrations. The reaction was initiated by the addition of 20 µL of enzyme (0.28 U/mL in 10 mM phosphate buffer) and the plates were incubated at 37 ºC for 10 min. The absorbance was measured at 405 nm before the addition of the enzyme ($T_{0'}$) and after 10 minutes of incubation ($T_{10'}$). Acarbose was dissolved in 15% methanol/buffer solution (different concentrations were used), and it was used as positive control. A negative control absorbance (phosphate buffer in place

of sample) was also recorded. The inhibitory activity was calculated by using the following formula:

$$\%\in hibition=\frac{\left(A405\,NegativeControl_{T10',-T0'}-A405\,Sample_{T10',-T0'}\right)}{A540\,NegativeControl_{T10',-T0'}}*100$$

The concentration of the sample required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by nonlinear regression analysis.

3.6.3. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were considered statistically significant when p values ≤ 0.05. Statistical analyses were performed using GraphPad Prism 5 Software (San Diego, CA, USA) and the IC_{50} values were estimated by nonlinear curve-fitting and presented as their respective 95% confidence limits.

References

Agrawal, P., 1989. Carbon-13 NMR of flavonoids. Amesterdam: Elsevier 39.

Bohlmann, F., Umemoto, K., Jakupovic, J., King, M.R., Robinson, H., 1984. Sesquiterpenes from *Liabum floribundum.* Phytochemistry 23, 1800-1802.

Chiang, Y.M., Chang, C.L.T., Chang, S.L., Yang, W.C., Shyur, L.F., 2007. Cytopiloyne, a novel polyacetylenic glucoside from *Bidens pilosa*, functions as a T helper cell modulator. J. Ethnopharmacol. 110, 532-538.

Chokchaisiri, R., Suaisom, C., Sriphota, S., Chindaduang, A., Chuprajob, T., Suksamrarn, A., 2009. Bioactive flavonoids of the flowers of *Butea monosperma*. Chem. Pharm. Bull. 57, 428-432.

Fujita, T., Funayoshi, A., Nakayama, M., 1994. A phenylpropanoid glucoside from *Perilla frutescens*. Phytochemistry 37, 543-546.

Hoffmann, B., Holzl, J., 1988. New Chalcones from *Bidens pilosa*. Planta Med. 54, 52-54.

Jakupovic, J., Schuster, A., Bohlmann, F., Dillona, M.O., 1988. Guaianolides and other constituents from *Liabum floribundum*. Phytochemistry 27, 1771-1775.

Jo, S., Ka, E., Lee, H., Apostolidis, E., Jang, H., Kwon, Y., 2009. Comparison of antioxidant potential and rat intestinal a-glucosidases inhibitory activities of quercetin, rutin, and isoquercetin. Int. J. Appl. Res. Nat. Prod. 2, 52-60.

Juarez, B.E., Mendiondo, M.E., Seeligmann, P., 1995. Flavonoids from leaves and flowers of *Liabum polymnioides* and L-Candidum (Asteraceae) - Chemotaxonomical significance. Biochem. Syst. Ecol. 23, 335-336.

Kato, A., Minoshima, Y., Yamamoto, J., Adachi, I., Watson, A.A., Nash, R.J., 2008. Protective effects of dietary chamomile tea on diabetic complications. J. Agric. Food Chem. 56, 8206-8211.

Lee, H.-W., Lee, H.-S., Park, J.-H., Cheong, J.-J., Kwon, H.-B., Kim, K.O., Ku, C.-S., Kim, M.-J., Park, Y.J., Ryu, H.W., 2015. 2-Hydroxyquinoline and its structural analogs show antidiabetic effects against α-amylase and α-glucosidase. J. Appl. Biol. Chem. 58, 1-3.

Li, S., Kuang, H.-X., Okada, Y., Okuyama, T., 2005. New flavanone and chalcone glucosides from *Bidens bipinnata* Linn. J. Asian Nat. Prod. Res. 7, 67-70.

Lo Piparo, E., Scheib, H., Frei, N., Williamson, G., Grigorov, M., Chou, C.J., 2008. Flavonoids for controlling starch digestion: structural requirements for inhibiting human α-amylase. J. Med. Chem. 51, 3555-3561.

Nawwar, M.A., Hussein, S.A., Merfort, I., 1994. Leaf phenolics of *Punica granatum*. Phytochemistry 37, 1175-1177.

Ranilla, L.G., Kwon, Y.I., Apostolidis, E., Shetty, K., 2010. Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. Biores. Technol. 101, 4676-4689.

Robinson, H., Brettell, R.D., 1973. Tribal revisions in the Asteraceae. III. A new tribe, Liabeae. Phytologia 25, 404-407.

Rücker, G., Kehrbaum, S., Sakulas, H., Lawong, B., Goeltenboth, F., 1992. Acetylenic glucosides from *Microglossa pyrifolia*. Planta Med. 58, 266-269.

Saleem, M., Kim, H.J., Jin, C., Lee, Y.S., 2004. Antioxidant caffeic acid derivatives from leaves of *Parthenocissus tricuspidata*. Arch. Pharm. Res. 27, 300-304.

Slade, D., Ferreira, D., Marais, J.P., 2005. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. Phytochemistry 66, 2177-2215.

Vinholes, J., Grosso, C., Andrade, P.B., Gil-Izquierdo, A., Valentao, P., de Pinho, P.G., Ferreres, F., 2011. In vitro studies to assess the antidiabetic, anti-cholinesterase and antioxidant potential of *Spergularia rubra*. Food Chem. 129, 454-462.

Yang, X.W., Huang, M.Z., Jin, Y.S., Sun, L.N., Song, Y., Chen, H.S., 2012. Phenolics from *Bidens bipinnata* and their amylase inhibitory properties. Fitoterapia 83, 1169-1175.

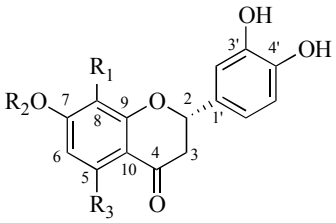
Legend for Figures

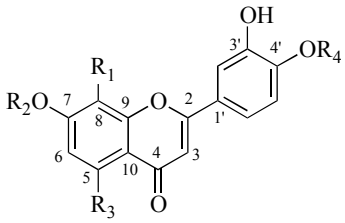
Fig. 1 Chemical structure of isolated compounds **(1-16)**: (2*S*)-3',4',7,8-Tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**1**); 3',4',7,8-Tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**2**); Rutin (**3**); Kaempferol 3-*O*-β-D-glucopyranoside (**4**); 3-β-D-Glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene 8,10,12-triyne (**5**); eugenyl *O*-β-D-glucopyranoside (**6)**; 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**7**); Eriodictyol (**8**); Okanin (**9**); Butein (**10**); Luteolin (**11**); Bidenoside F (**12**); Caffeic acid methyl ester (**13**); Okanin 4'-*O*-β-D-glucopyranoside (**14**); 4'-*O*-β-D-glucopyranoside (**15**); Caffeic acid (**16**).

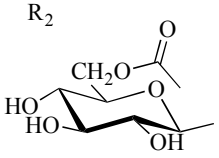
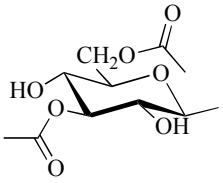
Fig. 2 Dose-dependent extracts α-amylase (**a**) and α-glucosidase (**b**) inhibition activities: **Acarbose**; Chloroform-methanol extract (9:1) (**CMR**), Methanol extract (**MR**) and *n*-BuOH extract (**BuR**).

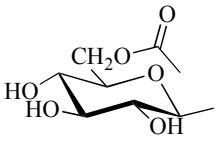
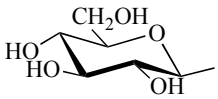
Fig. 3 α-amylase (**a**) and α-glucosidase (**b**) inhibition by isolated compounds (IC_{50} in mM, data are means ± SD from three experiments): **Acarbose**; (2*S*)-3',4',7,8-Tetrahydroxyflavanone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**1**); 3',4',7,8-Tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside (**2**); Rutin (**3**); Kaempferol 3-*O*-β-D-glucopyranoside (**4**); 3-β-D-Glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene 8,10,12-triyne (**5**); 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**7**); Eriodictyol (**8**); Okanin (**9**); Butein (**10**); Luteolin (**11**); Bidenoside F (**12**); Caffeic acid methyl ester (**13**); Okanin 4'-*O*-β-D-glucopyranoside (**14**); 4'-*O*-β-D-glucopyranoside (**15**); Caffeic acid (**16**).

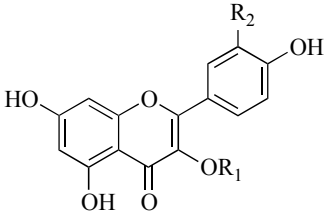
Fig. 1

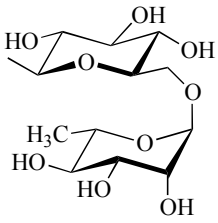
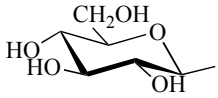


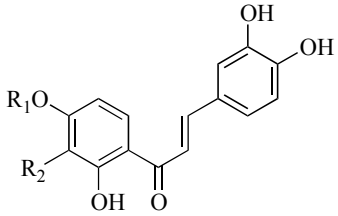


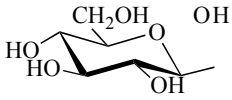
	R_1	R_2	R_3
1	OH		H
8	H	H	OH
12	OH		H

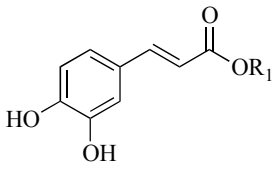
	R_1	R_2	R_3	R_4
2	OH		H	H
11	H	H	OH	H
15	H	H	OH	



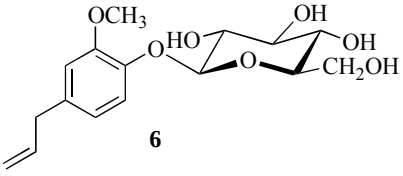
	R_1	R_2
3		OH
4		H



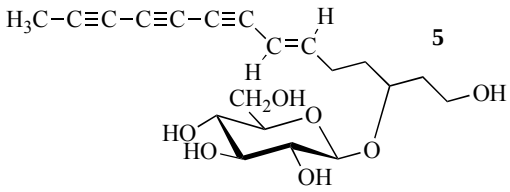
	R_1	R_2
9	H	OH
10	H	H
14		OH



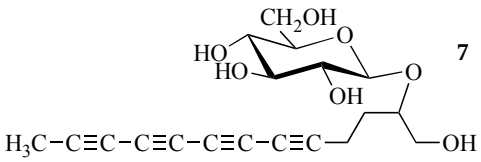
	R_1
13	CH_3
16	H



6



5



7

Fig. 2

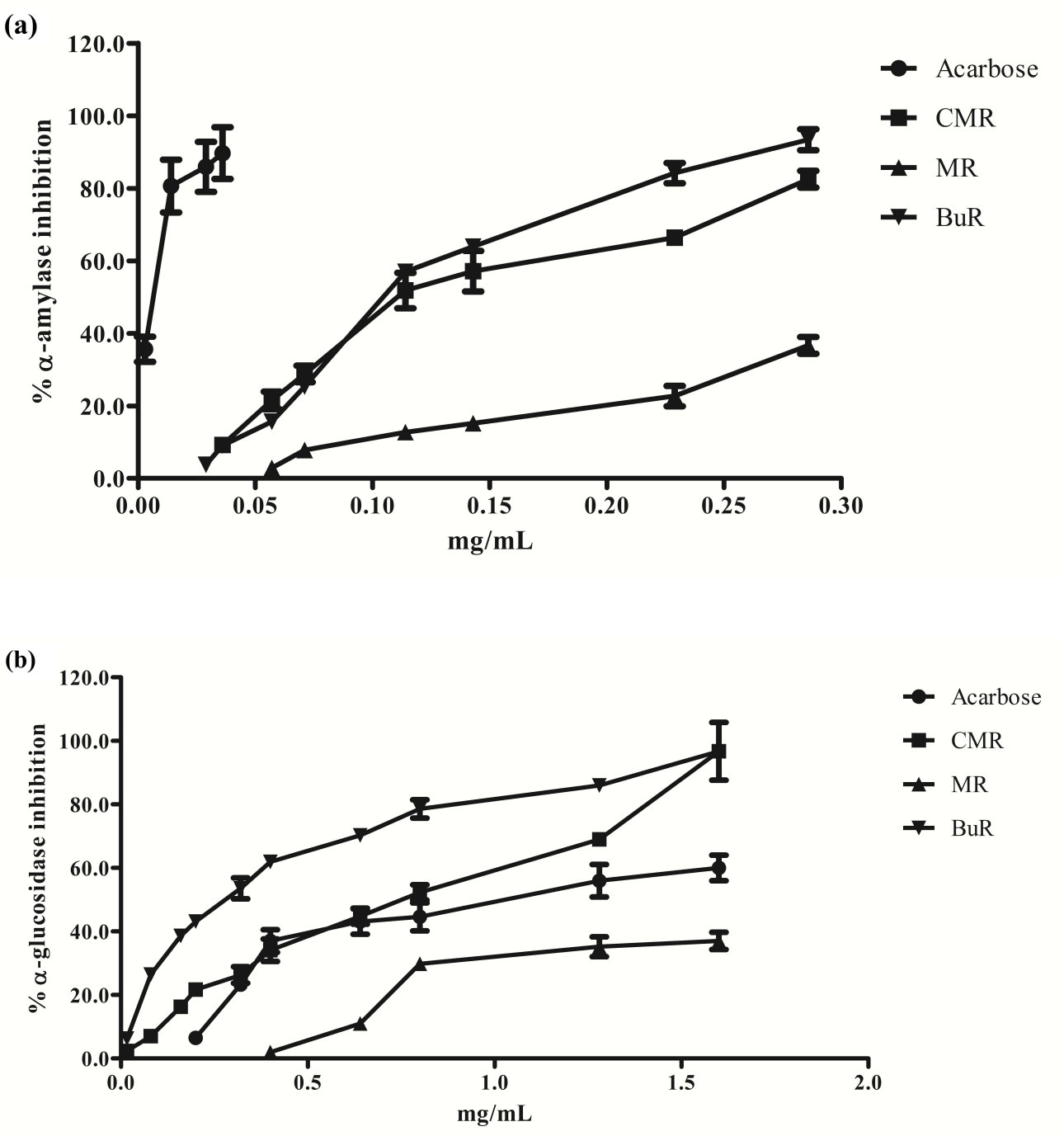
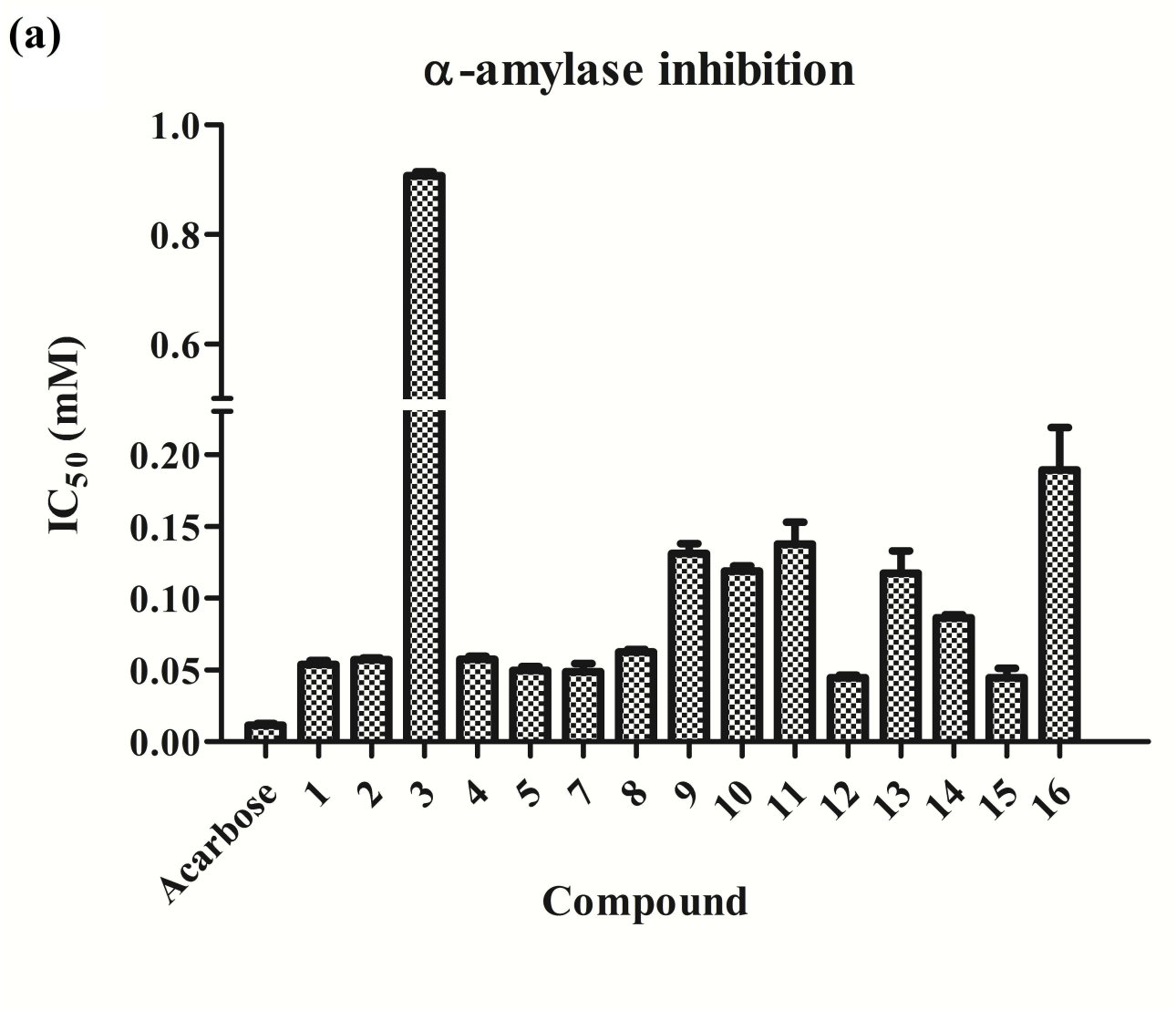

(a)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
% α-amylase inhibition
0.00
0.05
0.10
0.15
0.20
0.25
0.30
mg/mL
Acarbose
CMR
MR
BuR
(b)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
% α-glucosidase inhibition
0.0
0.5
1.0
1.5
2.0
mg/mL
Acarbose
CMR
MR
BuR

Fig. 3


(a)
α-amylase inhibition
IC50 (mM)
1.0
0.8
0.6
0.20
0.15
0.10
0.05
0.00
Acarbose
1
2
3
4
5
7
8
9
10
11
12
13
14
15
16
Compound

(b)

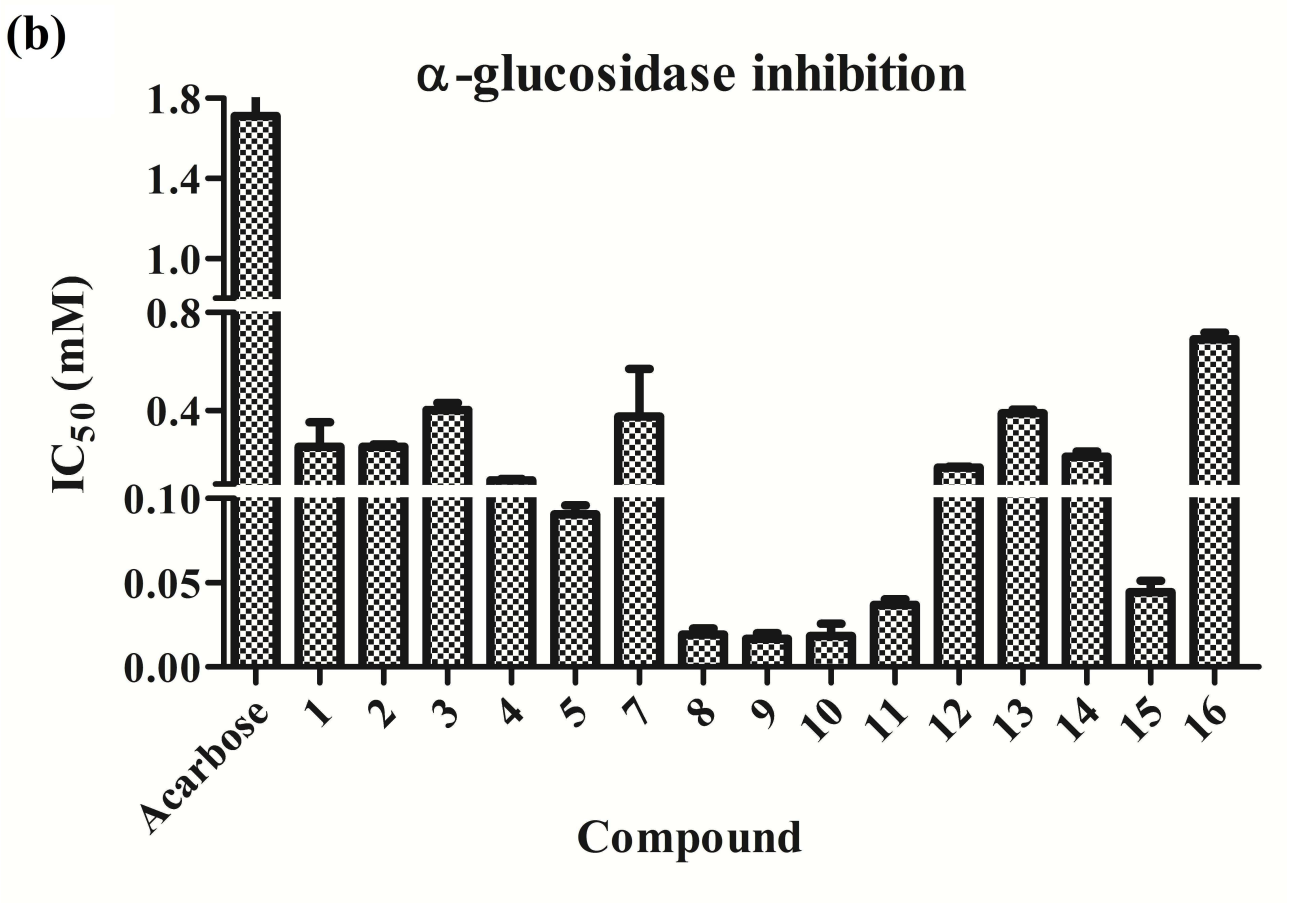

α-glucosidase inhibition
IC50 (mM)
1.8
1.4
1.0
0.8
0.4
0.10
0.05
0.00
Acarbose
1
2
3
4
5
7
8
9
10
11
12
13
14
15
16
Compound

Table1

^{1}H and ^{13}C-NMR data of compounds **1** and **2** (CD_3OD, 600 MHz, *J* in Hz)[a].

position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2	5.45 dd (12.0, 3.0)	81.5		154.0
3ax	3.16 dd (17.0, 12.0)	44.8	6.78 s	115.5
3eq	2.81 dd (17.0, 3.0)			
4		194.0		193.3
5	7.40 d (8.0)	118.3	7.22 d (8.0)	114.8
6	6.90 d (8.0)	111.0	7.09 d (8.0)	113.0
7		152.2		153.6
8		136.5		135.6
9		152.0		148.0
10		113.8		119.4
1’		131.5		125.6
2’	7.04 d (2.0)	115.3	7.60 d (1.8)	119.2
3’		145.0		147.7
4’		146.5		149.0
5’	6.82 d (8.5)	116.5	6.86 d (8.0)	116.7
6’	6.92 dd (8.5, 2.0)	118.6	7.39 dd (8.0, 1.8)	126.7
Glc 1	4.98 d (7.8)	102.6	5.00 d (7.8)	102.9
2	3.54 dd (9.0, 7.8)	74.7	3.60 dd (9.5, 7.8)	74.5
3	3.51 t (9.0)	77.6	3.50 t (9.5)	77.7
4	3.42 t (9.0)	71.6	3.43 t (9.5)	71.8
5	3.72 m	75.6	3.73 m	76.2
6a	4.45 dd (12.0, 3.0)	65.0	4.49 dd (12.0, 3.0)	65.0
6b	4.28 dd (12.0, 5.0)		4.30 dd (12.0, 4.5)	
$\underline{CO}CH_3$		172.0		172.7
$CO\underline{CH_3}$	2.14 s	20.8	2.10 s	21.1

[a]Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.