Screening and Structural Characterization of α-Glucosidase Inhibitors from Hawthorn Leaf Flavonoids Extract by Ultrafiltration LC-DAD-MS^n and SORI-CID FTICR MS

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In vitro α-glucosidase inhibition assays and ultrafiltration liquid chromatography with photodiode array detection coupled to electrospray ionization tandem mass spectrometry (ultrafiltration LC-DAD-ESI-MS^n) were combined to screen α-glucosidase inhibitors from hawthorn leaf flavonoids extract (HLFE). As a result, four compounds were identified as α-glucosidase inhibitors in the HLFE, and their structures were confirmed to be quercetin-3-O-rha-(1-4)-glc-rha and C-glycosylflavones (vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside and vitexin) by high-resolution sustained off resonance irradiation collision-induced dissociation (SORI-CID) data obtained by Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS). Several other C-glycosylflavones (vitexin, isovitexin, orientin, isoorientin) and their aglycones apigenin and luteolin were evaluated by in vitro assays, and were found to possess strong α-glucosidase inhibitory activities as well. Moreover, the substituent groups on the flavones had a great impact on the enzyme inhibition activity. C-3′-OH of the B-ring of flavones in particular increased the α-glucosidase inhibition activity, whereas C-glycosylations at C-6 or C-8 of the A ring weakened the inhibition activity. (J Am Soc Mass Spectrom 2009, 20, 1496–1503) © 2009 American Society for Mass Spectrometry

Type 2 diabetes mellitus has been recognized as a serious global health problem, often resulting in substantial morbidity and mortality. α-Glucosidase inhibitors are usually used in the treatment of type 2 diabetes mellitus. They act by a reversible inhibition of α-glucosidase, an enzyme present in the brush border of the small intestine. The inhibition of intestinal α-glucosidases could delay the digestion and absorption of carbohydrates and, consequently, suppress postprandial hyperglycemia [1]. Although strong synthetic α-glucosidase inhibitors (i.e., acarbose) are available, they usually cause significantly adverse gastrointestinal events [2]. For this reason, naturally occurring α-glucosidase inhibitors, particularly those from the Chinese herbal medicines, have been explored as viable, low toxicity alternatives for treating type 2 diabetes mellitus. More than 100 herbal medicines have been reported to show great potency in inhibiting the α-glucosidase activity and equivalent efficacies to synthetic drugs in managing diabetes [3–5].

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Flavonoids are a group of natural polyphenolic derivatives and are ubiquitous in fruits, vegetables, and traditional Chinese herbal medicines. For centuries, preparations containing flavonoids as the principal physiologically active constituents have been used to treat human diseases [6]. Some of such flavonoid-containing preparations have been shown to have significant effects on diabetes and diabetic syndromes by the following mechanisms: antioxidant activity [7], α-glucosidase inhibitory effect [8], and insulin-like effect [9]. In addition, flavonoids have also been found to have antiplatelet aggregation and aldose reductase inhibitory activities, which may have helped to reduce the severity of the diabetic syndrome [10, 11].

Hawthorn (*Crataegus oxyacantha L.*) is a traditional medicinal plant, and its leaf has been used as a traditional medicine to treat irregular heartbeat, high blood pressure, chest pain, hardening of the arteries, and congestive heart failure. Hawthorn leaf extracts contain different subgroups of flavonoids, including flavones and flavonols. So far, more than 60 flavonoids have been isolated and identified from hawthorn, which are mainly derivatives of (1) apigenin, (2) luteolin, (3) kaempferol, (4) quercetin, and (5) naringin [12]. These compounds have

1044-0305/09/$32.00
doi:10.1016/j.jasms.2009.04.003

Published online April 14, 2009
Received November 25, 2008
Revised April 3, 2009
Accepted April 4, 2009

lipid-lowering, antioxidant, anti-inflammatory and LDL oxidation inhibitory properties, and protective effect against diabetes [13]. Recent studies have also shown that the intake of certain types of flavonoids, including quercetin and myricetin is inversely associated with the risk of incident type 2 diabetes [14]. Isoquercitrin, isorhamnetine-3-O-rutinoside, and vitexin have shown inhibitory activities on α-glucosidase from the rat intestine [15].

Hawthorn leaf flavonoids extracts (HLFE) have been widely studied for their roles in preventing lipid disorder, treatment of heart failure, and lowering blood pressure, using either bioassays or instrumental analysis such as LC/MS [16–20]. However, the inhibitory effect of hawthorn leaf flavonoids on α-glucosidase activity has not been evaluated by either of these two methods. Conventional methods use bioassay-guided separation techniques to identify bioactives. Natural α-glucosidase inhibitors have been screened using in vitro enzymatic assays, followed by time-consuming isolation of the active compounds [21, 22]. In recent years, mass spectrometry-based assays have attracted great attention for high-throughput screening. These methods, which include quantitative MALDI-FT-MS [23], enzyme affinity selection procedure followed by ESI-FT-MS [24], frontal affinity chromatography-MS [25], size-exclusion chromatography-MS [26], capillary electrophoresis-MS [27], and pulsed-ultrafiltration MS [28–30], all have been successfully used to determine potentially active compounds. Ultrafiltration LC-MS/MS is a powerful tool to screen biologically active compounds from botanical extracts because of its high throughput on-line screening ability, and its high sensitivity and selectivity necessary for the characterization of compounds present at low concentrations in highly complex matrices without time-consuming purification procedures [28–30].

In the present paper, we used an in vitro model to examine the inhibitory activity of HLFE on α-glucosidase, and then ultrafiltration LC-MS to screen the ligands of α-glucosidase. As a result, four compounds with α-glucosidase inhibitory effects were observed and identified by on line LC-DAD-MS^n analysis, and their identities were further confirmed to be quercertin-3-O-rhamnosyl-(1-4)-glcosylrhamnoside, vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, and vitexin by accurate mass data taken at high mass resolving power with an FT-MS. Because three of the four α-glucosidase inhibitors were C-glycosylflavones, additional C-glycosylflavones (vitexin, isovitexin, orientin, isoorientin) and their prototypal compounds, apigenin, and luteolin were tested, and found to be strong α-glucosidase inhibitors as well, particularly those with a hydroxy substituent at C-3′.

Experimental

Materials

The HLFE was purchased from Xi'an Acetar Biology and Technology Co., Ltd. (Xi'an, China) with total flavones >40%. Standard apigenin, vitexin, quercetin, and luteolin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and isovitexin and orientin were purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Isoorientin was from Beijing Seajet Scientific Co., Ltd. (Beijing, China), and α-glucosidase (E.C. 3.2.1.20) from yeast was purchased from Fluka (Bueke, Switzerland). Acetonitrile was of HPLC grade from Fisher Scientific (Loughborough, UK); acetic acid was HPLC grade from TEDIA (Fairfield, OH); water was purified in-house using a Milli-Q water purification system (Milford, MA). Solvents and all other chemicals, otherwise not mentioned, were purchased from Beijing Shiji (Beijing, China).

Methods

α-Glucosidase Inhibition Assay

The enzyme inhibition studies were carried out spectrophotometrically in a 96-well microplate reader using a procedure reported by Li et al. [31]. A total 60 μL reaction mixture containing 20 μL of 100 mM phosphate buffer (pH 6.8), 20 μL of 2.5 mM *p*-nitrophenyl α-D-glucopyranoside (PNP-G) (Biochem) in the buffer, and 20 μL of standard flavonoid in 50% methanol were added to each well, followed by 20 μL of 10 mM phosphate buffer (pH 6.8) containing 0.2 U/mL α-glucosidase to the mixture of treatment terminated wells. The plate was incubated at 37 °C for 15 min, and then adding 80 μL of 0.2 mol/L sodium carbonate solution to stop the reaction. Right after that, absorbance was recorded at 405 nm with a Tecan GENios multifunctional microplate reader (Männedorf, Switzerland). Controls contained the same reaction mixture except the same volume of phosphate buffer was added instead of a flavonoid solution. Acarbose (Bayer) was dissolved in water and used as a positive control. The inhibition (%) was calculated as: $(A_1 - A_2)/A_1 \times 100\%$, where A_1 is the absorbance of the control, and A_2, the absorbance of the sample.

Screening by Ultrafiltration LC-MS

One μL of 20 mg/mL HLFE sample solution was incubated for 0.5 h at 37 °C with 4 μL of 250 μM α-glucosidase in 10 mM ammonium acetate buffer (pH 6.86), in a total volume of 100 μL. After incubation, each mixture was filtered through a Microcon (Millipore, Bedford, MA) YM-10 centrifugal filter containing a regenerated cellulose ultrafiltration membrane with a 10,000 MW cutoff by centrifugation at 10,000 *g* for 10 min at room temperature. The filter was washed three times to remove the unbound compounds by centrifugation with 100 μL aliquots of ammonium acetate buffer (pH 6.86) at room temperature. The bound ligands were released by adding 100 μL of methanol/water (50:50; vol/vol) (pH 3.30) followed by centrifugation at 10,000 *g* for 15 min; this process was repeated

three times. The solvent in the ultrafiltrates were removed under vacuum, and the released ligands were used for LC-MS analysis. A control experiment in which enzyme omitted was also carried out before each screening experiment.

LC-MS^n

The released ligands were redissolved in 40 μL of methanol/water (50:50; vol/vol). Aliquots (20 μL) of this reconstituted ligand solution were analyzed using LC-MS, which consisted of a Waters (Milford, MA) 2690 HPLC system coupled to an ion trap mass spectrometer with an electrospray ionization interface (Finnigan MAT, San Jose, CA). HPLC separations were carried out using a C18 column (150 mm × 4.6 mm, 5 μm, Dikma, Beijing, China) and a C18 guard column (Dikma). The column temperature was kept at 25 °C. The flow rate was set to 0.5 mL/min and the eluting gradient was as follows: [acetonitrile (A) and 0.5% acetic acid in water (B)]: 0–10 min, 20% A; 10–15 min, 20%–30% A; 15–20 min, 30% A. The mass spectrometer was operated in the negative ion mode with source voltage of 5.0 kV. The metal capillary voltage was set to 4.5 V and temperature at 250 °C, the sheath gas (N_2) flow-rate was 50 arb, the scan range was *m/z* 100–1500 Da; the maximum injection time was 200 ms.

SORI-CID FTICR MS

The HLFE was diluted into proper concentration, and then introduced to the IonSpec (Irvine, CA) 7.0 T FTICR mass spectrometer with a Waters Z-spray source. The capillary voltage was set at −3.5 kV. The ions were accumulated for 1.5 s in a hexapole ion guide. After accumulation, the ions were transferred to the ICR cell. The operating software was IonSpec99 version 7.5.10.64. All acquisitions were performed on a 1024 K dataset and 1 scan. During detection, a broad band analysis mode was used in the mass range of *m/z* 108–1500 Da, source temperature of 100 °C, and probe temperature of 80 °C. Tandem mass spectra were obtained by sustained off resonance irradiation (SORI) CID with nitrogen as collision gas after isolation of the appropriate precursor ions (±1 Da); 100 ms N_2 was added as collision gas.

Results and Discussion

α-Glucosidase Inhibitors in HLFE by an In Vitro Enzyme Assay and Ultrafiltration LC-DAD-MS

The in vitro α-glucosidase assay was conducted as a prescreening of the activity for the high-throughput ultrafiltration LC-MS based assay. The enzyme inhibition assay showed that the HLFE inhibited 86% of the enzyme activity at 25 μg/mL, significantly higher than that of the well known synthetic α-glucosidase inhibitor, acarbose (63.90% inhibition at 250 μg/mL) (Table 1). The α-glucosidase inhibitory activity of HLFE was further confirmed by ultrafiltration LC-DAD-MS, which also provided enzyme inhibition activities of individual flavonoids present in the extract.

Table 1. Inhibition activity of flavones against a-glucosidase

Samples	Inhibition[a,b] (%)	IC_{50} (μM)[a,e]
HLFE	89.86[c]	7.1 μg/mL
Acarbose	63.90[d]	228.16
Apigenin	46.22	21.85
Vitexin	19.91	25.11
Isovitexin	30.20	23.26
Luteolin	60.42	13.07
Orientin	38.22	23.30
Isoorientin	47.72	19.68

[a]The result was an average of three determinations.
[b]Inhibition by 25 μM flavone.
[c]Inhibition by 25 μg/mL HLFE.
[d]Inhibition by 250 μg/mL acarbose, namely 387.5 μM.
[e]Concentration required for 50% inhibition of the enzyme activity under the assay conditions.

The ultrafiltration LC-MS screening is an affinity screening method based on MS. When a mixture of compounds is injected into the ultrafiltration chamber containing a macromolecular receptor, in our study, α-glucosidase, the ultrafiltration membrane retains the macromolecules and the ligand–receptor complexes but allows unbound, low molecular weight compounds to elute from the chamber. In this manner, the ultrafiltration chamber functions as a solution-phase extraction device, retaining ligands that can tightly bind to a receptor, e.g., α-glucosidase, while unbound compounds will be washed away. After that, the ligand–receptor complex is disrupted by addition of organic solvent or changing pH value, and then the released ligands can be identified by LC-MS analysis [29].

The receptor and ligand concentrations also determine the number of "hits" that might be obtained, so incubation of equimolar concentrations of the hawthorn leaf flavonoids were carried out at three different concentrations of α-glucosidase (5, 10, and 20 μM). After incubation with α-glucosidase and ultrafiltration affinity purification, the trapped ligands originally from the HLFE were analyzed by LC-DAD-MS. The LC-MS spectra for this experiment are shown in Figure 1. At 5 μM α-glucosidase, only two components, eluting at 8.43 and 10.03 min, showed binding affinity to the receptor, α-glucosidase (see Figure 1a). However, when the α-glucosidase concentrations were increased to 10 and 20 μM, up to four ligands were observed (Figure 1b and 1c). Moreover, the amount of ligands bound to the enzyme (receptor) was also found to be greater at increased α-glucosidase concentrations (Figure 1b and 1c). Higher receptor concentrations typically result in larger numbers of hits because weaker ligands can also be identified together with the high-affinity compounds [28]. Botanical extracts usually contain multiple ligands; therefore excess receptor is required to minimize the competition between ligands of different affinities, and to detect more potential ligands.

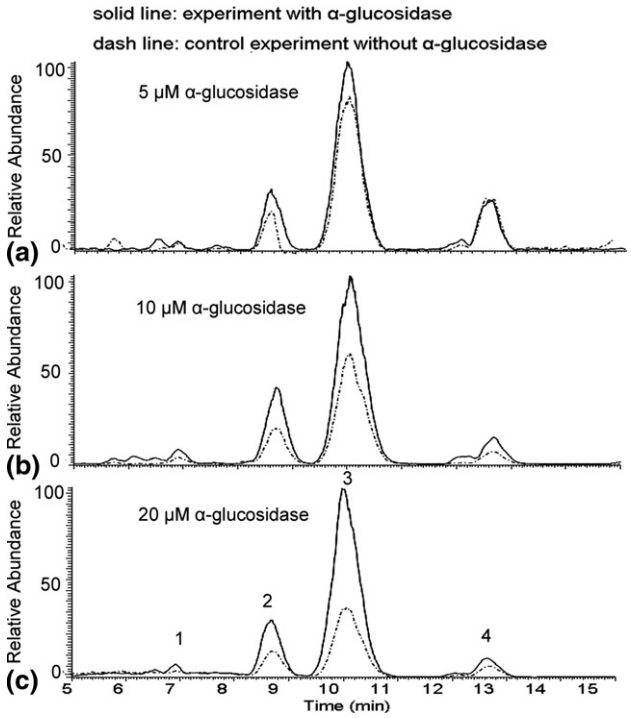


Figure 1. Ultrafiltration LC-MS screening of HLFE for binding to α-glucosidase.

LC-DAD data showed that Compound 1 had two λ_{max} at around 254 and 353 nm, while Compounds 2, 3, and 4 had nearly the same UV spectra with their λ_{max} at around 267 and 335 nm, indicating Compounds 2–4 were structurally similar but different from Compound 1. The negative ion ESI-MS produced deprotonated ions of Compounds 1–4 at *m/z* 755 (t_R = 6.27 min), 593 (t_R = 8.43 min), 577 (t_R = 10.03 min), and 431 (t_R = 12.28 min), respectively. These compounds (ligands) were identified by LC-MS^n and SORI-CID FTICR MS.

Structural Characterization of α-Glucosidase Inhibitors from HLFE by LC-MS^n and SORI-CID FTICR MS

LC-MS^n and SORI-CID FTICR MS experiments were both carried out to identify compounds that bound to α-glucosidase in the enzyme and ultrafiltration LC-MS assays. The main fragment ions were *m/z* 609, 591, 489, 300, and 301 in the MS^2 spectrum of Compound 1, as shown in Figure 2a. The two ions at *m/z* 300 and 301 suggested that Compound 1 might contain a quercetin (MW 302) skeleton, and this observation was confirmed by matching the tandem MS spectra of the ion at *m/z* 301 with those of the standard quercetin (data not shown). The $[M - H - 146]^-$ ion at *m/z* 609 and the $[M - H - 164]^-$ ion at *m/z* 591 were suggestive of a deoxyhexose residue presents at the terminal site of a sugar chain. Detailed fragmentation ions and their relationships with the molecular structure of each compound were analyzed by high-resolution SORI CID, and listed in Table 2. Ablajan et al. [32] reported an elaborate MS study on a series of flavonoid compounds with a quercetin skeleton. Based on their results, different glycosylation positions can significantly affect the fragmentation patterns of quercetin glycosides in the nega-

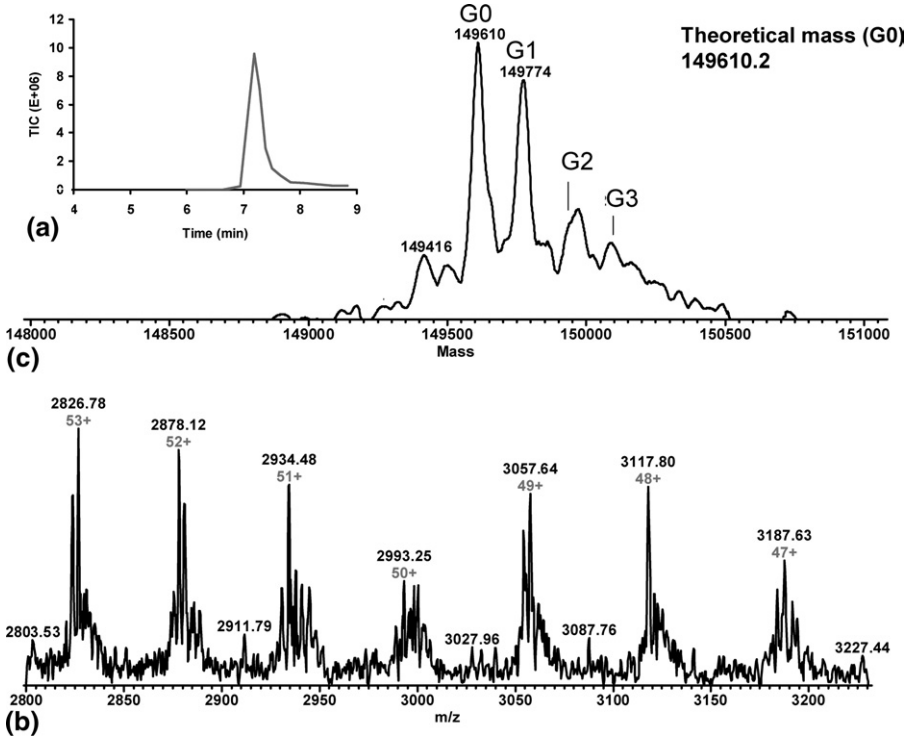


Figure 2. MS^n spectra of Compound 1 in the negative ion mode.

Table 2. Accurate product ions mass data of Compounds 1 to 4

Measured *m/z*	Fragment ions	Theory *m/z*	Formula element $[M-H]^-$	Error (ppm)
Compound 1				
755.20581>	$[M-H]^-$	755.20402	$C_{33}H_{40}O_{20}^{-1}$	2.37
489.10254	$^{0,2}X_1^-$	489.10385	$C_{23}H_{22}O_{12}^{-1}$	−2.68
343.04625	$^{0,2}X_0^-$	343.04594	$C_{17}H_{12}O_8^{-1}$	0.90
301.03566	Y_0^-	301.03538	$C_{15}H_9O_7^{-1}$	0.93
300.02786	Y_0^-–H	300.02755	$C_{15}H_8O_7^{-1}$	1.03
271.02505	Y_0^-–CH_2O	271.02481	$C_{14}H_7O_6^{-1}$	0.86
255.03031	Y_0^-–CO–H_2O	255.02990	$C_{14}H_7O_5^{-1}$	1.61
Compound 2				
593.15032>	$[M-H]^-$	593.15119	$C_{27}H_{29}O_{15}^{-1}$	−1.48
413.08676	Z_1^-	413.08781	$C_{21}H_{17}O_9^{-1}$	−2.54
293.04458	$^{0,2}X_0Z_1^-$	293.04555	$C_{17}H_9O_5^{-1}$	−3.31
Compound 3				
577.15455>	$[M-H]^-$	577.15628	$C_{27}H_{29}O_{14}^{-1}$	−3.00
457.11304	$^{0,2}X_0^-$	457.11402	$C_{23}H_{22}O_{10}^{-1}$	−2.14
413.08589	Z_1^-	413.08781	$C_{21}H_{17}O_9^{-1}$	−4.65
311.05496		311.05611	$C_{17}H_{11}O_6^{-1}$	−3.70
293.04440	$^{0,2}X_0Z_1^-$	293.04555	$C_{17}H_9O_5^{-1}$	−3.92
Compound 4				
431.09864>	$[M-H]^-$	431.09837	$C_{21}H_{19}O_{10}^{-1}$	0.63
311.05673	$^{0,2}X_0^-$	311.05611	$C_{17}H_{11}O_6^{-1}$	2.64
283.06189	$^{0,2}X_0^-$–H_2O	283.06120	$C_{16}H_{11}O_5^{-1}$	2.44

tive ion mode. Quercetin C-3′-O-glycoside only gives a product ion at *m/z* 301; quercetin C-3-O-glycoside gives product ions of *m/z* 300 and 301, both of which had high relative abundance. However, for quercetin C-7-O-glycoside, while these two ions (*m/z* 300 and 301) were still detected, the relative abundance of the ion *m/z* 300 was markedly lower than that of the ion *m/z* 301. We therefore concluded that the oligosaccharide chain of Compound 1 is likely linked at the C-3 position because of the high relatively abundance of the two aforementioned ions (Figure 2a). As shown in Figure 2a, the $[M - H - 146 - 120]^-$ ion of *m/z* 489 indicates that the deoxyhexose unit is linked to the hexose residue, and the neutral loss of 120 Da corresponds to the cross-ring cleavage of the hexose at 0,2 position. In the MS^3 spectrum of the ion *m/z* 489 (Figure 2b), fragment ions of *m/z* 343, 325, and 281 are detected with neutral losses of 146, 164, and 208 Da, respectively. The neutral losses of 146 and 164 Da indicate the existence of an additional deoxyhexose. The trisaccharide moiety in Compound 1 containing one hexose and two deoxyhexoses is considered to be linked as follows: deoxyhexose–hexose–deoxyhexose. The MS^3 experiment of ion *m/z* 591 was also performed, and the data are shown in Figure 2c. The base peak of *m/z* 355 corresponds to the cross-ring cleavage of the deoxyhexose moiety at 0,3 site along with loss of a molecular H_2O. This result further proves the linear linkage of the trisaccharide moiety. The cross-ring cleavage of the deoxyhexose moiety at 0,3 site also suggests that the linkage of between the deoxyhexose to hexose is a 1–4 linkage. The linkage between the hexose residue and the terminal deoxyhexose moiety could not be determined because of lack of a similar cleavage at the 0,2 site. As glucose and rhamnose are the main types of hexose and deoxyhexose in hawthorn leaves [12], respectively, we have tentatively given the identity of Compound 1 as quercetin-3-O-rha- (1–4)-glc-rha [18], and the structure is shown in Figure 2.

Compound 4 (t_R = 12.18 min) was identified as vitexin by comparing mass spectra and the fragmentation pattern with those of the standard. In the MS^2 spectrum of deprotonated vitexin, only two product ions were observed, $^{0,2}X_0^-$ ion of *m/z* 311 and $^{0,3}X_0^-$ ion of *m/z* 341, both are considered to originate from the cross-ring cleavage of the glucose residue by losing C4H8O4 (120 Da) and C3H6O3 (90 Da), respectively (Figure 3a and Table 2). All of these data matched those of vitexin. The spectra were simpler because vitexin contains a C-glycoside bond that is less readily cleaved. The only product ions detected were those from the cross-ring cleavage (Figure 3).

Compounds 2 and 3 have similar UV absorbance as Compound 4, which indicates they both possess the vitexin skeleton. This conclusion was supported by the following MS^n data. As shown in Figure 4a and Table 2, the $[M - H]^-$ ion *m/z* 593 of Compound 2 produced a base peak ion of *m/z* 413 (Z_1^-) by losing 180 Da, suggesting the presence of a hexose moiety, and the ion *m/z* 413 matched the dehydrated vitexin ion. In addition, a minor product ion of *m/z* 473 ($^{0,2}X^-$) by losing 120 Da from the $[M - H]^-$ ion, demonstrates the cleavage of the hexose ring at 0,2 position (Figure 4a), more importantly, the linkage of hexose residue to hexose residue is at the 1–2 position. MS^3 experiments of ions of *m/z* 413 and 473 were further performed (Figure 4b and 4c). In the MS^3 spectrum of ion of *m/z* 413, the product ion *m/z* 293 ($^{0,2}XZ_1^-$) was from losing 120Da due to the cleavage at the 0,2 site of the hexose residue. MS^3 of *m/z* 473 produced *m/z* 311 as a result of a neutral loss of 162 Da, further suggesting the existence of a hexose unit in

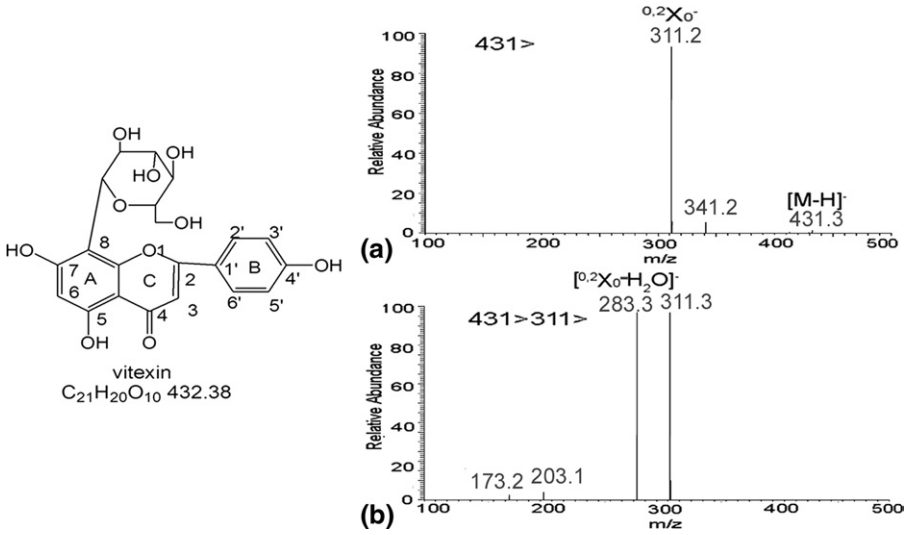


Figure 3. MS^n spectra of Compound 4 in the negative ion mode.

Compound 2. Compound 2 was therefore identified as vitexin-2″-O-glucoside [19].

MS^2 of the $[M - H]^-$ ion (*m/z* 577) of Compound 3 produced *m/z* 413 (Z_1^-), which matched the loss of a deoxyhexose unit (Figure 5a and Table 2). The ion *m/z* 457 ($^{0,2}X_0^-$) was a result of the cross-ring cleavage of the hexose moiety at 0,2, indicating the linkage between this hexose and the deoxyhexose to be at the 1–2 site. The ion of *m/z* 413 gave rise to the fragment ion *m/z* 293 in the MS^3 experiment (Figure 5b), and the latter [*m/z* 293 ($^{0,2}X_0Z_1^-$)] was similarly suggestive of the cross-ring cleavage at 0,2 site as discussed above. In addition, the MS^3 experiment of the $^{0,2}X_0^-$ ion (*m/z* 457) produced the base peak ion *m/z* 311 (Figure 5c), further confirming the existence of a deoxyhexose moiety. Compound 3 can be identified as vitexin-2″-rhamnoside [19].

α-Glucosidase Inhibition Assay

Recent studies have shown that the intake of quercetin was inversely associated with the risk of incident type 2 diabetes. Isoquercitrin, isorhamnetine-3-O-rutinoside, and a C-glycosylate apigenin derivative vitexin have also been reported to have strong inhibitory activities against α-glucosidase [14, 15]. Our screening results confirmed that quercetin and vitexin and their glycosides, representing flavonols and flavones, are α-glucosidase inhibitors (Figure 1). C-glycosylflavones such as vitexin have been

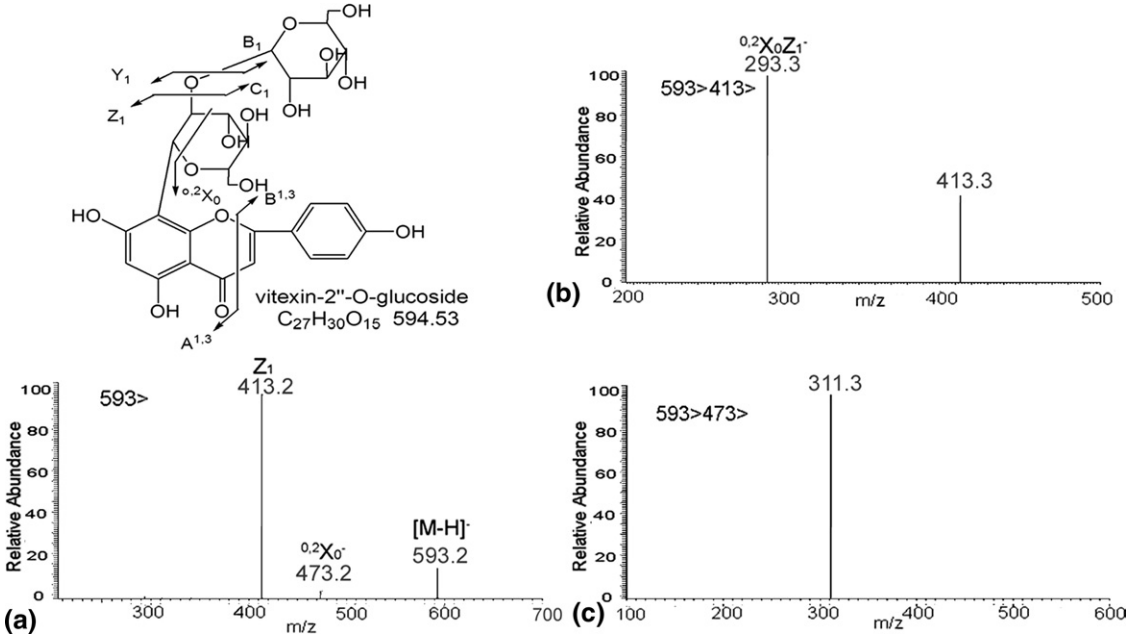


Figure 4. MS^n spectra of Compound 2 in the negative ion mode.

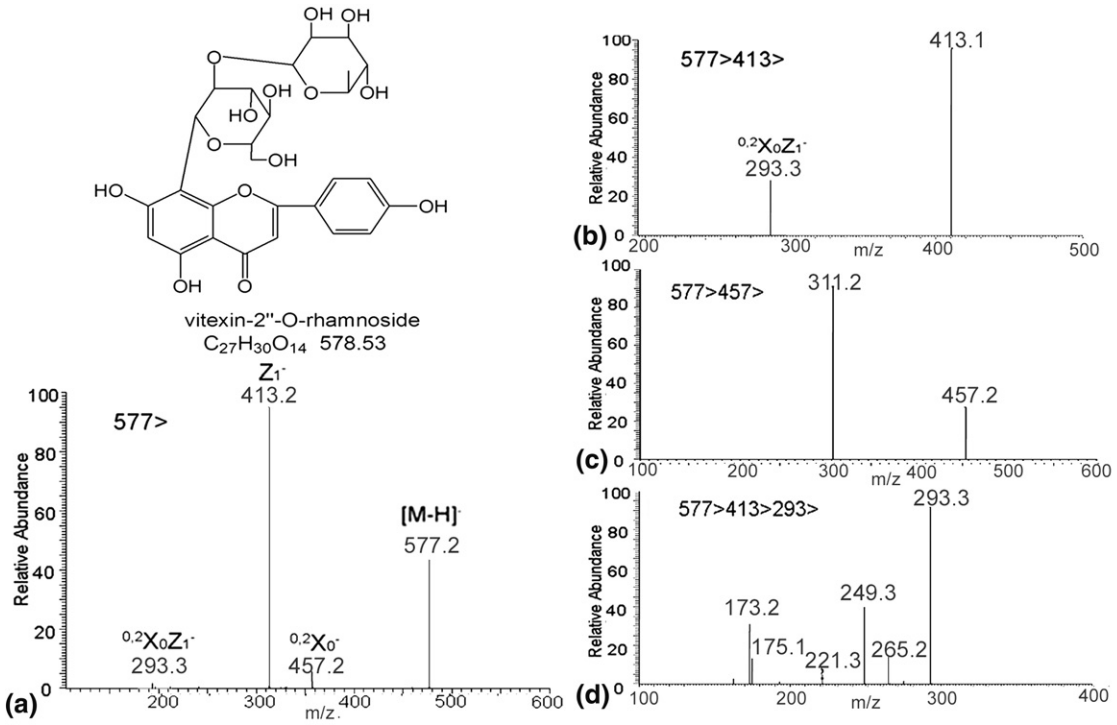
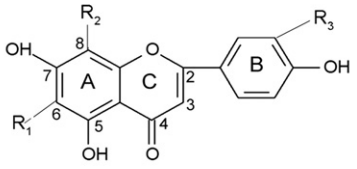


Figure 5. MS^n spectra of Compound 3 in the negative ion mode.

reported to have antioxidant, anti-virus, liver protection, and other activities [20], however, the inhibitory activities of C-glycosylflavones on α-glucosidase have rarely been reported. On the other hand, apigenin and luteolin are the two main flavone aglycones in hawthorn, and the only difference between them was the extra hydroxyl group at the C-3′ position (R_3) for luteolin (Figure 6). Vitexin and isovitexin are the corresponding C-6 and C-8 C-glycosylflavones of apigenin, respectively, and orientin and isoorientin are the C-6 and C-8 C-glycosylflavones of luteolin, respectively (Figure 6). We therefore tested these C-glycosylflavones (vitexin, isovitexin, orientin, and isoorientin) and their aglycones apigenin and luteolin for their α-glucosidase inhibitory activities.



Name	MW	R_1	R_2	R_3
apigenin	270	H	H	H
vitexin	432	H	glc	H
isovitexin	432	glc	H	H
luteolin	286	H	H	OH
orientin	448	H	glc	OH
isoorientin	448	glc	H	OH

Figure 6. Structures of flavones studied by an in vitro assay.

Table 1 shows the α-glucosidase inhibition rates (%) of the above mentioned flavone aglycones and their C-glycosylated derivatives (at 25 μM) and their IC_{50} values. All of the six tested compounds showed higher α-glucosidase inhibitory activity than acarbose, one of the most potent α-glucosidase inhibitor drugs. The inhibitory activity decreased in the following order: luteolin > isoorientin ≥ apigenin > orientin > isovitexin > vitexin (Table 1). Flavones with a hydroxyl group at C-3′ possessed stronger inhibition than the corresponding flavones without a C-3′ hydroxyl group, namely, luteolin > apigenin, orientin > vitexin, and isoorientin > isovitexin. The C-3′ hydroxylation of the B-ring apparently plays an important part in the α-glucosidase inhibitory activity of flavones. Moreover, the aglycones showed stronger activity than their respective C-glycosylated derivatives: the inhibition rate followed the order of luteolin > isoorientin > orientin for luteolin derivatives; and similarly, apigenin > isovitexin > vitexin for apigenin derivatives. Glycosylation at C-6 or C-8 weakens the inhibitory activity of flavones against α-glucosidase, although the C-6 glycosylation had relatively less impact than the C-8 glycosylation, as the inhibition rates were isovitexin > vitexin and isoorientin > orientin (Table 1). Similar results have been reported elsewhere by Tadera et al.: hydroxylations at the C-3 and C-5 positions on the A and C rings of flavones enhance the inhibitory activity, but for the B ring of flavones, C-4′-OH is crucial for the inhibitory activity, C-3′-OH and C-5′-OH are favorable to the inhibitory activity [33]; 5,6,7-trihydroxyflavone structure is crucial for the potent inhibitory activity, while

C-8 substituted flavone derivatives tend to decrease the inhibitory activity [34].

Conclusions

The ultrafiltration LC-DAD-MS^n method reported above is a valuable tool for effective screening of and identifying anti-α-glucosidase compounds in complex samples such as HLFE compared with the commonly used bioassay-guided column separation techniques. It is a high throughput assay technique that can significantly reduce the time, effort, and other resources than conventional approaches. Using accurate mass spectrometry and multistage tandem mass spectrometry data, ligands bound to the enzyme (α-glucosidase) were identified as quercertin-3-O-rha-(1–4)-glc-rha, vitexin-2″-O-glucoside, vitexin-2″-O-rhamnoside, and vitexin. Based on the activities of these ligands, similar C-glycosylflavones and corresponding aglycones were evaluated using in vitro assays, and their inhibitory activity toward α-glucosidase was validated. The 5,7,4′-trihydroxyflavone structure, such as in apigenin and luteolin derivatives, was crucial for the inhibitory activity; but an additional C-3′-OH substitute on the B-ring enhanced the activity. On the other hand, C-glycosylations at C-6 or C-8 weakened the inhibition. Our results and findings by others, such as the aldose reductase inhibitory activity of luteolin, apigenin, quercetin, and a series of corresponding glycosides [35], suggest that hawthorn leaf, a herbal medicine containing different types of flavonoids, may contain an important group of candidates as natural anti-type 2 diabetes drugs that warrant further investigation.

Acknowledgments

The authors acknowledge financial support for this work by the National Natural Science Foundation of China (30672600, 30472134), Science and Technology Planning Project of Jilin Province (20080736), and the Science and Technology Planning Project of Changchun City (2007GH27).

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