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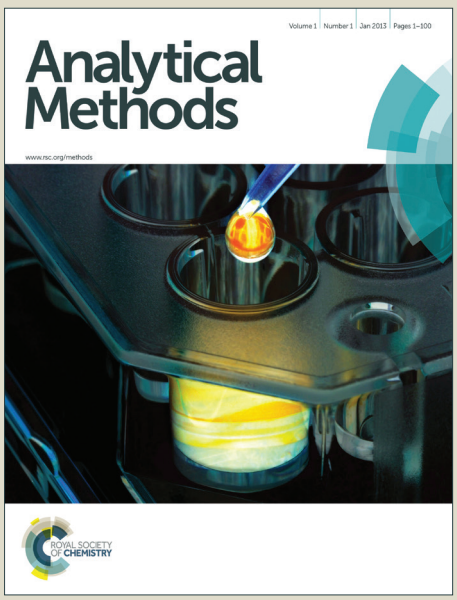

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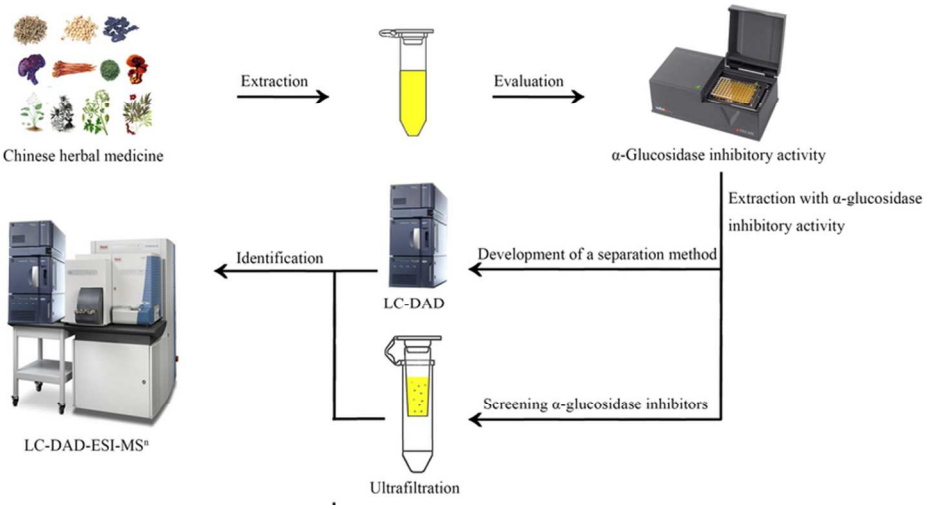

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Screening and Structural Characterization of Potential α-Glucosidase Inhibitors from *Radix Astragali* Flavonoids Extract by Ultrafiltration LC-DAD-ESI-MS^n

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Abstract

Inhibition of intestinal α-glucosidase activity is one important mechanism for the management of diabetes mellitus (DM). Identifying plants with α-glucosidase inhibitory activities, and screening active compounds (α-glucosidase inhibitors) in them, has become a popular field of research in the treatment of DM. In the present study, we used a vitro assay of ultraviolet spectrophotometry to evaluate the α-glucosidase inhibitory activity of *Radix Astragali* flavonoids extract (RAFE). Then the ultrafiltration liquid chromatography with photodiode array detection coupled to electrospray ionization tandem mass spectrometry (ultrafiltration LC-DAD-ESI-MS^n) was used to screen the active compounds in RAFE. As a result, the concentration (final) required for 50% enzyme inhibition (IC50) value of RAFE was calculated as 2.888 mg/mL. Through ultrafiltration LC-DAD-ESI-MS^n analysis, seven compounds were identified as potential active compounds. They were calycosin-7-O-β-D-glucoside, biochanin A, calycosin-7-O-β-D-glucoside-6″-O-malonate, ononin, calycosin, formononetin-7-O-β-D-glucoside-6″-O-malonate and formononetin. Then, two of the potential active compounds, the biochanin A and formononetin were evaluated α-glucosidase inhibitory activities. Their IC50 values were calculated as 0.020 mM and 0.027 mM respectively, while that of reference drug acarbose was evaluated as 0.382 mM.

Key words: α-glucosidase inhibitor; ultrafiltration; LC-MS^n; *Radix Astragali*; diabetes

Introduction

Diabetes mellitus (DM), as one of the most serious and chronic metabolic diseases, has dramatically increased due to excessive intake of high carbohydrate diets.[1] It was proposed that prevention of digestion of carbohydrates after food uptake would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation.[2,3] Carbohydrate digestion primarily occurs in small intestine through the action of pancreatic α-amylase, yielding both maltose and isomaltose oligosaccharides. These oligosaccharides and other natural oligosaccharides like sucrose are finally hydrolyzed by intestinal α-glucosidases to release absorbable monosaccharide suitable for absorption.[4] The inhibitors of α-glucosidases, like acarbose, can delay digestion of oligosaccharides both yielded from carbohydrate and from natural source. These inhibitors can prolong overall carbohydrate digestion time and cause a reduction in the rate of glucose absorption, and ultimately blunting the postprandial plasma glucose rise.

So far, many studies have devoted to screen plants with α-glucosidase inhibitory activities and identify active compounds from them.[5-8] Among plants screening for α-glucosidase inhibitory activities, research on Chinese herbal medicine (CHM) has become a topic of interest due to a lot of these herbs possessing great inhibitory activities and low toxicities. More and more studies were carried out to find the naturally α-glucosidase inhibitory extracts or compounds from CHM.[9-11] Among the methods for evaluating α-glucosidase inhibitory activities, ultraviolet spectrophotometry with 4-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate is widely used for easy, low-cost and high-throughput.[12] For screening active compounds (α-glucosidase inhibitors) in extracts, a high-throughput and effective screening method has been developed recent years. The method was ultrafiltration combined with liquid chromatography-tandem mass spectrometry (ultrafiltration LC-MS^n), which based on the ligands (active compounds) contained in the extracts bound to the receptor of α-glucosidase.[11,13]

In this research, we aimed to study the α-glucosidase inhibitory activity and active compounds from a common CHM named *Radix Astragali*, which has long been favored as an antidiabetic plant for treatment of type 2 diabetes.[14,15] As known, there are many hypoglycemic medicines preparated from *Radix Astragali* in China, such as "Huangqi" hypoglycemic granules, "Shenqi" hypoglycemic granules and "Zhenqi" hypoglycemic capsule ("qi" is an abbreviation of the *Radix Astragali in China*). However, no report has previously screened α-glucosidase inhibitors from *Radix Astragali*. In this study, the α-glucosidase inhibitory activity of *Radix Astragali* was evaluated by ultraviolet spectrophotometry with pNPG as a substrate, and then ultrafiltration LC-MS^n was applied to identify potential active compounds in it.

Experimental

Reagents and chemicals

α-Glucosidase (EC 3.2.1.20) and pNPG (EC 223.189-3) were obtained from Sigma Chemical Company (St. Louis, MO, USA); Biochanin A and formononetin were taken from Shanghai Ronghe Medical Technology Development Company (Shanghai, China). Acarbose (tablet) was purchased from Bayer HealthCare Company (Leverkusen, Germany). HPLC grade methanol, acetonitrile, dimethylsulfoxide (DMSO) and formic acid were taken from TEDIA (Fairfield, OH, USA); D101 macroporous resin was supported from Tianjin Agricultural Chemical Company (Tianjin, China); Water was obtained from Milli-Q water purification system (Milford, MA, USA); Solvents and all other chemicals not mentioned were analytical grade.

Materials and sample preparation

Radix Astragali was purchased from Tongrentang drugstore (Changchun, China) and authenticated by Prof. shumin Wang (Changchun University of Chinese Medicine). Voucher specimens were deposited in our laboratory.

As reported, flavonoids possess the highest inhibitory activities related to a number of hydroxyl groups in the molecule of the compound.[16] So in the present study, the inhibitory

activity of *Radix Astragali* flavonoids extract (RAFE) was evaluated, and the potential α-glucosidase inhibitors were screened from it.

Radix Astragali were ground to 10-20 mesh and extracted by ultrasonic wave with 20 times of 70% ethanol for 20 min twice. After that, the sample was centrifuged at 8,000 rpm for 10 min and the supernatant was collected. Then the collected solution was dried by vacuum and the residue was dissolved by water to a concentration, 0.2 g/mL (in terms of starting material). Continuously, the solution was used to enrich RAFE through a D101 macroporous resin column. Firstly, it was eluted with 5 times of column volume (CV) distilled water to remove carbohydrates. Subsequently, 5 CV 70% ethanol was used to elute flavonoids from the column. The eluent was collected, condensed and lyophilized by using a freeze drier. As a result, the yield of the RAFE was 2.1% (w/w, the amount of RAFE/*Radix Astragali* power mass).

α-Glucosidase inhibitory activity assay

The assay was performed in the 96-well plates according to the modified method described by Watanabe.[17] Both α-glucosidase and pNPG were dissolved in the 100 mM phosphate buffer (pH 6.8) and passed through a 0.45 μm filter. The samples were dissolved to various concentrations by DMSO. Then, 10 μL of the sample solution was mixed with 50 μL α-glucosidase solution (0.2 U/mL). After incubation for 5 min at 37°C, 40 μL of pNPG (2 mM) was added as a substrate and incubated for an additional 5 min at 37°C. The amount of released product nitrophenyl was measured on a Tecan GENios multifunctional microplate reader (Mäanedorf, Switzerland) at 405 nm. The inhibition (%) of the test sample on α-glucosidase could be calculated as

$$\text{Inhibition (\%)} = [(A_{S1}-A_{S0})/(A_{C1}-A_{C0})]\times 100$$

where A_{S1}, A_{S0}, A_{C1}, and A_{C0} are the absorbance of the sample at terminal time, the absorbance of the sample at zero time, the absorbance of the blank control at terminal time and the absorbance of the blank control at zero time, respectively. The measurements were performed in triplicates.

Screening of α-glucosidase inhibitors from RAFE by ultrafiltration

The screening procedure was performed according to the modified method of Li.[11] The RAFE and the α-glucosidase were dissolved in the 10 mM ammonium acetate buffer (pH 6.8). The RAFE (10 mg/mL, 10 μL) was incubated for 30 min at 37°C with a-glucosidase (20 U/mL, 90 μL). After incubation, the mixture was filtered through a Microcon YM-10 centrifugal filter (Millipore, MA, USA) containing a regenerated cellulose ultrafiltration membrane with a 10,000 MW cut off at room temperature. The filter was washed with 100 μL ammonium acetate buffer by centrifugal force at 10,000 rpm for 15 min in order to remove the unbound compounds. This process was repeated two times. After that, the bound ligands were released by adding 100 μL of methanol-water (50:50; v/v) (pH 3.3) and centrifuged at 10,000 rpm for 15 min. This process was repeated two times. Then the ultrafiltrated solvent was removed under vacuum, further, the released ligands for analysis were re-dissolved in 50 μL methanol-water (10:90; v/v). The control experiment was carried out with denatured enzyme.

Structural characterization of α-glucosidase inhibitors from RAFE by LC-DAD-ESI-MS^n

After ultrafiltration, the released ligands were identified by LC-DAD-ESI-MS^n which consisted of liquid chromatography with photodiode array detection coupled to an ion trap mass spectrometer with an electrospray ionization source.

The LC-DAD analysis was carried out on ACQUITY UPLC H-Class System (Waters, Milford, MA, USA) using a Kinetex C18 analytical column (2.1 mm×100 mm, 1.7 μm, 100A, Phenomenex). The injection volume was 5 μL. The column temperature was kept at 35°C. The mobile phases A and B were acetonitrile and 0.1% formic acid aqueous, respectively. The flow rate was set to 0.4 mL/min and the eluting procedure was as follows: t=0 min, 10% A; t=0~10 min, 10%~30% A; t=10~12 min, 30%~40% A; t=12~14 min, 40% A; t=14~15 min, 40%~90% A; t=15~20 min, 90% A.

Mass spectrometric detection was carried out on LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in positive and negative ion modes. In positive ion

mode, the electrospray voltage was 4.0 kV. The capillary voltage was optimized to 15 V and the tube lens offset to 150 V. The sheath and auxiliary gas flows (both nitrogen) were adjusted to 50 and 2 arbitrary units, respectively. The capillary temperature was 250°C. The collision energy was set to 25 to 30 V. In negative ion mode, the electrospray voltage was 3.0 kV. The capillary voltage was optimized to -15 V and the tube lens offset to -150 V. Other parameters were the same as the positive ion mode.

Results and discussion

Evaluation of α-glucosidase inhibitory activity of RAFE

Seen as Fig. 1, a dose-dependent relationship was found between the concentration of RAFE and the α-glucosidase inhibitory activity. The concentration (final) required for 50% enzyme inhibition (IC50) value of RAFE was calculated as 2.888 mg/mL. This assay indicated that one mechanism underlying the hypoglycemic effects of *Radix Astragali* was the inhibition of α-glucosidase activity.

Development of a LC-DAD method for the separation of RAFE

Before ultrafiltration, a LC-DAD separation method is needed to develop to identify the compounds that contained in the RAFE. In order to achieve a satisfactory separation, the gradient elution procedure was optimized. As shown in Fig. 2A, under optimum separation conditions a total of nine constituents were well separated within 13 min. Because most constituents in the analyte had the highest absorbances between 240 to 280 nm, a wavelength of 250 nm was selected for detecting. Later, this LC-DAD separation method coupled with ESI-MS^n was used to analysis the ligands of α-glucosidase from RAFE after ultrafiltration.

Screening active compounds (α-glucosidase inhibitors) from RAFE by ultrafiltration LC-DAD-ESI-MS^n

As mentioned previously, the ligands specifically bound to a-glucosidase were extracted. While the ligands nonspecifically bound to the ultrafiltration membrane were determined by carrying out a control experiment with denatured enzyme. The screening results are shown in Fig. 2B. The LC-chromatograms of experimental group and control group are shown as the solid curve and the dashed curve respectively. As shown in Fig. 2B, the peak area of

experimental group was larger than that of control group. This difference of peak areas between the experimental group and the control group indicated the specific binding to α-glucosidase of compounds in RAFE. As shown, there were seven peaks appearing such differences for peak areas. These peaks were 1, 2, 4, 5, 7, 8 and 9. As each peak represents a compound, then seven components were screened as potential active compounds by ultrafiltration. The data of LC-retention time (t_R), DAD-λ_{max} and ESI-MS^n *m/z* of these seven compounds are summarized in Table 1, and their chemical structures are shown in Fig. 3.

The DAD spectrum of peak 1 shows that the peaks of the λ_{max} were 220, 248 and 285 nm, respectively. In the negative- and positive-ion ESI-MS, quasimolecular ion peaks were observed at *m/z* 477 $[M + H]^+$, *m/z* 491 $[M + HCOO]^-$ respectively. In the MS^2 data, the *m/z* 477 gave the product ion at *m/z* 285 by neutral loss of 162 Da corresponding to losing a hexose residue. In the MS^3 data, the *m/z* 285 directly gave the product ions at *m/z* 270, 253, 241, 229, 225, 214 and 137, respectively, and was identified as calycosin. By comparing the reference compounds, the compound of peak 1 was identified as calycosin-7-O-β-D-glucoside.

The DAD spectrum of peak 2 shows that the peaks of the λ_{max} were 226 and 318 nm, respectively. In the negative- and positive-ion ESI-MS, quasimolecular ion peaks were observed at *m/z* 285 $[M + H]^+$, *m/z* 283 $[M - H]^-$, respectively. So the molar mass of this compound was 284. In the MS^2 data, the *m/z* 285 directly gave the product ions at *m/z* 270, 253, 241, 229, 225, 214 and 152, respectively. By comparing the reference compounds, the compound of peak 2 was identified as biochanin-A.

The DAD spectrum of peak 4 shows that the peaks of the λ_{max} were the same as peak 1, i.e., 220, 248 and 285 nm, respectively. In the positive-ion ESI-MS, quasimolecular ion peak was observed at *m/z* 533 $[M + H]^+$. In the MS^2 data, the *m/z* 533 directly gave the product ion at *m/z* 285 by expelling 248 Da neutrual fragment corresponding to malonylglucosyl. In the MS^3 data, the *m/z* 285 gave rise to the same product ions as that of calycosin. This result indicats that the only difference between peaks 4 and 1 was the substituted group. Thus, peak 4 was tentatively identified as calycosin-7-O-β-D-glucoside-6″-O-malonate.

The DAD spectrum of peak 5 shows that the peaks of the λ_{max} were 240 and 300 nm, respectively. In the negative- and positive-ion ESI-MS, quasimolecular ion peaks were observed at *m/z* 431 $[M + H]^+$, *m/z* 475 $[M + HCOO]^-$, respectively. In the MS^2 data, the *m/z* 431 gave the product ion at *m/z* 269. In the MS^3 data, the *m/z* 269 gave the product ions at *m/z* 254, 237, 225, 213, 209, 198 and 136, respectively. By comparing the reference compounds, a conclusion can be reached that the compound corresponding to peak 5 was ononin.

The DAD spectrum of peak 7 shows that the peaks of the λ_{max} were 216 and 277 nm, respectively. In the negative- and positive-ion ESI-MS, quasimolecular ion peaks were observed at *m/z* 285 $[M + H]^+$, *m/z* 283 $[M - H]^-$ respectively. So the molar mass of this compound was 284. In the MS^2 data, the *m/z* 285 directly gave the product ions at *m/z* 270, 253, 241, 229, 225, 214 and 137, respectively. By comparing the reference compounds, the compound of peak 7 was unambiguously identified as calycosin.

The DAD spectrum of peak 8 shows that the peaks of the λ_{max} were 220, 248 and 290 nm, respectively. In the positive-ion ESI-MS, ion peak was observed at *m/z* 517. In the MS^2 data, the *m/z* 517 generating *m/z* 269 to be needed the losses of 248 Da neutral fragment. In the MS^3 data, the production of *m/z* 269 was the same as the fragmentation pathway with the *m/z* 269 of ononin (peak 5). So the compound of peak 8 was identified as formononetin-7-O-β-D-glucoside-6″-O-malonate.

The DAD spectrum of peak 9 shows that the peaks of the λ_{max} were 220 and 248 nm, respectively. In the negative- and positive-ion ESI-MS, quasimolecular ion peaks were observed at *m/z* 269 $[M + H]^+$, *m/z* 267 $[M - H]^-$, respectively. So the molar mass of this compound was 268. In the MS^2 data, the *m/z* 269 gave the product ions at *m/z* 254, 237, 225, 213, 209, 198 and 136, respectively. By comparing the reference compounds, the compound corresponding to peak 9 was formononetin.

Evaluation of α-glucosidase inhibitory activities of potential active compounds

Two of the potential active compounds from RAFE, the biochanin A and formononetin were evaluated α-glucosidase inhibitory activities in this study. Seen as Fig. 4, both biochanin-A

and formononetin were found to exhibit significant α-glucosidase inhibitory activities in a dose-dependent manner, and their inhibitory activities were much better than that of acarbose. By calculating, the IC50 values of biochanin A and formononetin were 0.020 mM and 0.027 mM, respectively, while that of acarbose was 0.382 mM.

Through this assay, the α-glucosidase inhibitory activities of the biochanin A and the formononetin were confirmed. The high α-glucosidase inhiibtory activities of them indicated they are the main active compounds in RAFE. **Discussion**

The ultraviolet spectrophotometry with pNPG as a substrate is an easy, low-cost, high-throughput and widely used method. However, this method has some flaws. As the procedure in this research, the determination was carried out at 405 nm. Under this absorption wavelength, many plant extracts have strong absorbance, especially the flavonoids extracts. The strong absorbance of extract itself might lead the inhibition assay to be failed, as the absorbance of the extract itself exceeding the maximum absorbance limit of the detector (2.5). So this ultraviolet spectrophotometry with pNPG as a substrate is needed to be further improved.

During screening of α-glucosidase inhibitors in plant extract, the oligosaccharides should be removed from the extract before ultrafiltration, especially removing the disaccharides such as maltose, isomaltose and sucrose. Because the disaccharides in the extract will act as ligands and bound to the acceptor of α-glucosidase, and eventually lead to the false positive result of α-glucosidase inhibition assay or the false high inhibitory activity of the sample. In addition, the bound between disaccharides and acceptor would competitively inhibit the bound between the target inhibitors and the acceptor, and eventually lead to the failure of screening α-glucosidase inhibitors from plants or CHM extracts. In addition, in the chromatogram, there might be a little difference for the peak areas between the sample (with α-glucosidase) and the control (with denatured α-glucosidase).This might be the main reason for the failure of ultrafiltration experiments.

In this study, seven potential active compounds were screened from RAFE by ultrafiltration LC-DAD-ESI-MS^n. It is better that all of them were evaluated the α-glucosidase inhibitory

activities. However, it's a pity that we couldn't obtain all standards. For example, the calycosin-7-O-β-D-glucoside-6″-O-malonate and the formononetin-7-O-β-D-glucoside-6″-O-malonate can't be obtained from the commodity business. So in the present study, we only chose two of the potential active compounds, biochanin A and formononetin which available in our laboratory, to evaluate the α-glucosidase inhibitory activitiies. In addition, by structure analysis [16], the biochanin A and formononetin might have the highest activities among the seven potential active compounds for possessing of the hydroxyl groups in C-5 and -7.

Conclusions

RAFE showed a dose-dependent relationship between the concentrations and the α-glucosidase inhibitory activity, and the IC50 value was calculated as 2.888 mg/mL (final concentration). This result indicates that one of the mechanisms for the hypoglycemic effects of *Radix Astragali* is the inhibition of α-glucosidase activity.

Through ultrafiltration LC-DAD-ESI-MS^n analysis, seven compounds were identified as potential α-glucosidase inhibitors. They were calycosin-7-O-β-D-glucoside, biochanin A, calycosin-7-O-β-D-glucoside-6″-O-malonate, ononin, calycosin, formononetin-7-O-β-D-glucoside-6″-O-malonate and formononetin. Two of the potential active compounds, biochanin A and formononetin have been comfirmed the α-glucosidase inhibitory activities, and their IC50 values were 0.020 mM and 0.027 mM (final concentration) respectively.

Acknowledgment

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Table 1 Data of a-glucosidase inhibitors from RAFE obtained by ultrafiltration LC-DAD-ESI-MS^n.

Peak No.	LC-t_R (min)	DAD-λ_{max}	MW	M (+)	M (-)	MS^n (+)	Identification
1	4.7	220 248 285	446	447, 285	491, 283	MS^2 [447]: 285 MS^3 [447→285]: 270, 253, 241, 229, 225, 214, 137	Calycosin-7-O-β-D-glucoside
2	6.2	226 318	284	285	283, 268	MS^2 [285]: 270, 253, 241, 229, 225, 214, 152	Biochanin A
4	6.7	220 248 285	532	533, 285	283	MS^2 [533]: 285 MS^3 [533→285]: 270, 253, 241, 229, 225, 214, 137	Calycosin-7-O-β-D-glucoside-6″-O-malonate
5	7.5	240 300	430	431, 269	475, 267	MS^2 [431]: 269 MS^3 [431→269]: 254, 237, 225, 213, 209, 198, 136	Ononin
7	8.9	216 277	284	285	283, 268	MS^2 [285]: 270, 253, 241, 229, 225, 214, 137	Calycosin
8	9.4	220 248 290	516	517, 269	267	MS^2 [517]: 269 MS^3 [517→269]: 254, 237, 225, 213, 209, 198, 136	Formononetin-7-O-β-D-glucoside-6″-O-malonate
9	12.4	220 248	268	269	267	MS^2 [269]: 254, 198, 237, 209, 213, 225, 136	Formononetin

Figures

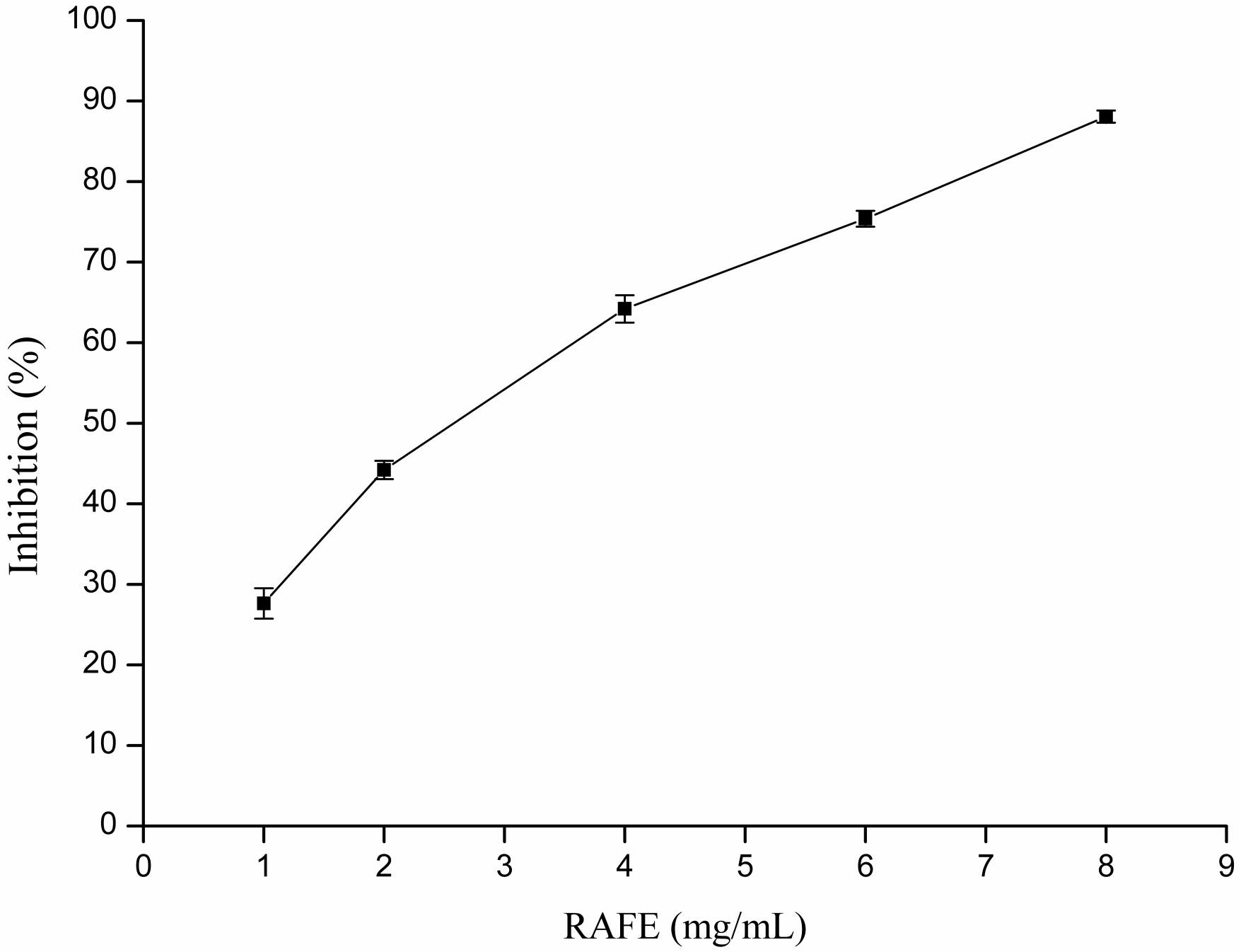
Figure 1. a-Glucosidase inhibitory activity of RAFE.

Figure 2. LC-DAD chromatogram of RAFE and its ultrafiltration result.

A: LC-DAD chromatogram of RAFE; B: LC-DAD chromatograms of RAFE after ultrafiltration.

Figure 3. Chemical structures of the seven potential α-glucosidase inhibitors obtained from RAFE.

Figure 4. a-Glucosidase inhibitory activities of biochanin-A, formononetin and acarbose.



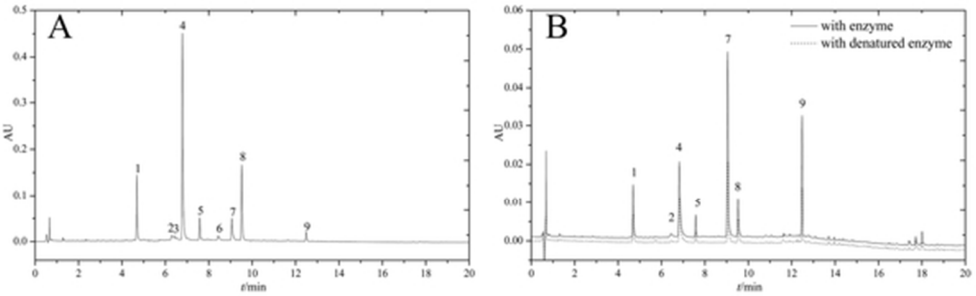


Figure 2. LC-DAD chromatogram of RAFE and its ultrafiltration result.
A: LC-DAD chromatogram of RAFE; B: LC-DAD chromatograms of RAFE after ultrafiltration.

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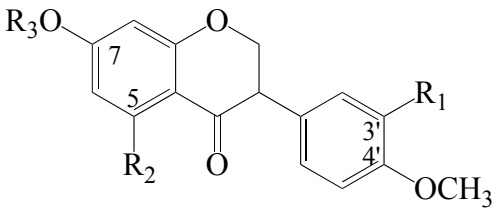

calycosin-7-O-¦Â-D-glucoside **(1)**: R_1=OH, R_2=H, R_3=glucosyl
biochanin A **(2)**: R_1=H, R_2=OH, R_3=H
calycosin-7-O-¦Â-D-glucoside-6¡å-O-malonate **(4)**: R_1=OH, R_2=H, R_3=6¡å-O-malonylglucosyl
ononin **(5)**: R_1=H, R_2=H, R_3=glucosyl
calycosin **(7)**: R_1=OH, R_2=H, R_3=H
formononetin-7-O-¦Â-D-glucoside-6¡å-O-malonate **(8)**: R_1=H, R_2=H, R_3=6¡å-O-malonylglucosyl
formononetin **(9)**: R_1=H, R_2=H, R_3=H