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Screening α-glucosidase inhibitors from mulberry extracts via DOSY and relaxation-edited NNR

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ABSTRACT

Inhibition of the α-glucosidase activity is a therapeutic approach for diabetes. In this study, an effective strategy for screening α-glucosidase inhibitors based on Nuclear magnetic resonance (NMR) techniques was developed to screen and identify α-glucosidase inhibitors from Mulberry leaf extract. As a result, deoxynojirimycin, as a potential α-glucosidase inhibitor, was found. The study suggested that our strategy was a powerful tool for screening and identification of α-glucosidase inhibitors in complex samples. Furthermore the interaction between α-glucosidase and its inhibitor was studied by NMR.

1. Introduction

Diabetes mellitus, which is one of the main diseases associated with glucose and fat metabolism, becomes an important issue due to its threat to human health and life. There are two main types of diabetes: insulin-dependent diabetes mellitus (IDDM) (type 1 diabetes) [1] and non-insulin-dependent diabetes mellitus (NIDDM) (type 2 diabetes) [2]. IDDM results from autoimmune destruction of insulin-producing beta cells of the pancreas. The subsequent lack of insulin leads to increased blood and urine glucose. The classical symptoms are polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss. NIDDM is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency [3]. But the same character of two kinds of diabetes mellitus is the postprandial hyperglycemia. Thus the control of postprandial hyperglycemia is needy in diabetes mellitus [4,5], because excessively high blood glucose may cause the induction and/or progression of diabetic complications and the promotion of hyperinsulinemia [6].

After a high carbohydrate meal, massive quantities of glucoses enter into blood. For healthy populations, blood glucose level could be adjusted to a normal level by metabolism. For the diabetics, they usually are in postprandial hyperglycemia state. If the hydrolysis process of oligosaccharides into glucose could be inhibited, postprandial hyperglycemia will be controlled. The α-glucosidase is a critical enzyme located in the brush-border surface membrane of intestinal cells that digests disaccharides such as maltose, sucrose, and lactose into glucose. Thus if the activity of the α-glucosidase and the hydrolysis of carbohydrates were inhibited, it will be beneficial for diabetics to control the blood glucose. Small molecules that can interact with the α-glucosidase have the potential to decrease postprandial hyperglycemia by inhibiting the activity of the α-glucosidase and could become a kind of medications to delay the absorption of carbohydrate. It is reported that α-glucosidase inhibitors have been used against diabetes mellitus [7] in clinical practice.

A good case in point is acarbose [8], which is the inhibitor of α-glucosidase used first in clinic. It is isolated from *Streptomyces sp.* (see Fig. 1). Acarbose can competitively bind to the active site of α-glucosidase and inhibit the activity of α-glucosidase reversibly. Based on this principle, many researchers try to find out some drugs with high efficacy and low toxicity. How to screen α-glucosidase ligands efficiently has become attractive and challenging for scientists. Bio-target recognition is one of the most important methods. At present, several techniques have been applied in identifying α-glucosidase ligands [9]. But there are

Abbreviations: IDDM, Insulin-dependent diabetes mellitus; NIDDM, Non-insulin-dependent diabetes mellitus; NMR, Nuclear magnetic resonance; DOSY, Diffusion ordered spectroscopy; MLE, Mulberry leaf extract; TSP-d_4, 3-(trimethylsilyl) propionic acid-d_4; DNJ, Deoxynojirimycin; CPMG, Carr-Purcell-Meiboom-Gill; MW, molecular weigh

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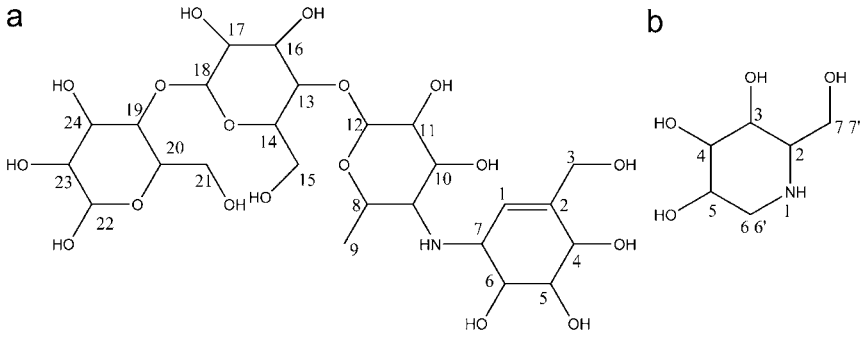

Fig. 1. The structure of (a) acarbose and (b) DNJ.

some shortcomings in these methods, for example, some active components that are difficult to be separated could not be identified and discovered as a new ligand.

NMR is powerful in the screening based on bio-target recognition, because of its unique ability to provide information on the structural, thermodynamic, and kinetic aspects of the binding reactions between ligands and targets [10,11]. Besides diffusion ordered spectroscopy (DOSY) [12], the change of transverse relaxation rate (R_2) of small molecule is an attractive probe of ligand binding [13].

It is known that the natural resources are perfect treasure-house for discovering active compounds with low toxicity and high efficacy [14]. The multiple biological activities of mulberry [15] (*Morus alba* L.) leaf have been reported. Water extract of mulberry leaf decreased blood glucose by 61% in alloxan-induced diabetic mice [15]. Ethanolic Mulberry leaf extract (MLE) also suppressed postprandial blood glucose in normal Wistar rats. Although it is known that mulberry is rich in anthocyanins, polyphenol, crude protein, fat and carbohydrate [16], these components which could regulate blood glucose need to be proved. In this work, we used the NMR techniques and took α-glucosidase as target to screen active components in MLE in order to elucidate the mechanism of MLE regulating blood glucose. Meanwhile the activity of the ligand was tested by bioassay. This was the first time to screen the ligand of α-glucosidase from extracts by NMR successfully, which supplied hints for researchers to screen the ligand of α-glucosidase.

2. Material and methods

2.1. Samples

MLE was obtained from Shanxi Pengzhan Technology Co. Ltd., in which the concentration (w/w) of deoxynojirimycin (DNJ) was 10%. α-Glucosidase (EC 3.2.1.20), D_2O, maltose and 3-(trimethylsilyl) propionic acid-d_4 (TSP-d_4) sodium salt were purchased from Sigma Co. (USA). DNJ was purchased from national standard substance net. Glucose oxidase reagent was purchased from Biosino Bio-Technology & Science Inc. Other chemicals were of analytical reagent grade and ultra-pure water was used throughout the experiments. The buffer solution used was phosphate buffer (100 μM K_2HPO_4/KH_2PO_4, 90% H_2O/10% D_2O, pH 6.8).

2.2. Methods

2.2.1. NMR

NMR experiments were performed on a Bruker AVANCE 600 spectrometer equipped with a 5 mm BBI probe capable of delivering 50 G/cm z-field gradients. All experiments were carried out at 310 K. The pulse program stebpgp1s19 was applied for DOSY measurement. For all DOSY experiments, 32k data points in the F2 dimension and 32 data points (diffusion dimension) in the F1 dimension were collected with 2 s relaxation delays, while the diffusion time (Δ) and the gradient length (δ) were set to 50 and 2 ms, respectively. The data analyses were applied to the raw experimental data using the standard 2D DOSY processing protocol in TOPSPIN (Bruker, Version 2.0) software with logarithmic scaling in the F1 dimension (diffusion coefficient). The HSQC and HMBC spectra were obtained using hsqcphpr and hmbcgplpndprqf pulse programs, respectively. For each FID, 64 transients were collected with 2 s relaxation delays. C–H coupling constant was set to 145 Hz, while the long range C–H coupling constant for HMBC was 6.25 Hz. The acquisition data size were 2048×128, and 2048×1024 points were used in Fourier transformation.

The relaxation-edited NMR experiments utilized a [D/pre-saturation-90x- (Δ-180y-Δ) n-acquire] pulse sequence, in which the Carr-Purcell-Meiboom-Gill (CPMG) sequence was used for the spin-lock [17,18]. For all relaxation-edited experiments the following variables were used: pre-saturation water suppression was applied in a 5 s pre-acquisition delay, P_{90} was measured and set up for each sample, $\Delta = 1.5$ ms, and $2 \times n \times \Delta =$ total spin-lock time. The spectra were collected with 32k data points and 16 scans.

2.3. α-Glucosidase inhibition assay

α-Glucosidase assays were performed according to the method of Rossi et al. [19] with a slight modification. Sample solution was pre-incubated with 4 μM α-glucosidase solution at 37 °C for 30 min. After preincubation, 2 μL of 400 mM maltose solution was added in 0.1 M phosphate buffer(pH 6.8). At last glucose oxidase reagent was added to determine the amount of glucose produced by the α-glucosidase in the reaction. Absorbance readings were recorded at 505 nm by multiskan Mircoplate reader (Thermo MK3). The α-glucosidase inhibitory activity was expressed as inhibition percentage and was calculated as follows:

$$\text{Inhibitory rate} = (A_0 - A_1)/(A_0 - A_B)$$

where A_0, A_1 and A_B were the absorbance of the control, the sample and blank, respectively.

3. Results and discussion

In this work, DOSY and relaxation-edited NMR experiments, which have been proven to be robust tools to study complexes involving target/ligand recognitions and interactions [13], are used to study the interaction of α-glucosidase with its inhibitors. Compared with previous techniques, our strategy is not only helpful to detect the interaction of targets with ligands, but also used to obtain structural information on ligands.

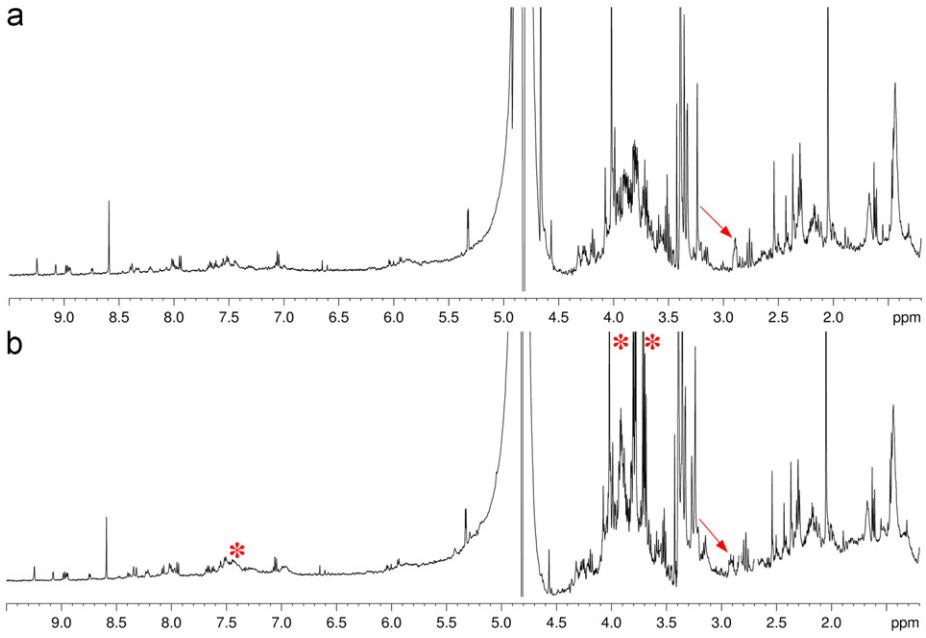

Fig. 2. The relaxation-edited NMR spectra of (a) 9.97 mg ml^{-1} mulberry extract and (b) 9.97 mg ml^{-1} mulberry extract plus 0.02 mM α-glucosidase. The spin-lock time of each experiment was 30 ms. The resonances attenuated when applying CPMG spin-lock in the presence of α-glucosidase were marked with red arrow. The resonances marked with "*" were from α-glucosidase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1. Identification of α-glucosidase inhibitors in MLE

In this study, the effect of MLE on α-glucosidase was investigated. The results showed that the extracts of the MLE had α-glucosidase inhibitory activity.

Fig. 2 showed when α-glucosidase was added into mulberry extract, the intensities of some resonances in the spectrum were attenuated. This result suggested the existence of α-glucosidase ligands in the extracts. It is well-known when small molecular ligand binds to macromolecular target [10], the R_2 of target and bound ligand is much larger than that of free ligand ($R_{2,\text{bound-ligand}} \gg R_{2,\text{free-ligand}}$). As a result, the observed R_2($R_{2,\text{obs}}$) of bound molecule, which is the weighted average of $R_{2,\text{bound-ligand}}$ and $R_{2,\text{free-ligand}}$, will become much larger relative to free small molecule. Herein, ligand resonances in relaxation-edited NMR were attenuated upon the addition of α-glucosidase, because the applying of CPMG spin-lock reduced or even eliminated fast relaxing resonances. Therefore the attenuated resonances were resulted from the ligand of α-glucosidase.

To validate the results from relaxation-edited NMR studies, DOSY experiments about α-glucosidase and mulberry extract were conducted. The diffusion coefficient of the peak (δ 3.555) in Mulberry extract is 7.11×10^{-10} m^2 s^{-1}. When α-glucosidase was added into the Mulberry extract, the diffusion coefficient of the peak (δ 3.555) was changed into 5.50×10^{-10} m^2 s^{-1} which was close to the diffusion of α-glucosidase (3.57×10^{-10} m^2 s^{-1}) under the same solution conditions. When small molecular ligand binds to macromolecular target, the diffusion coefficient of bound ligand is much smaller than that of free ligand. It is well-known that the diffusion coefficients of molecules depend on the sizes of molecules which could be judged by the molecular weigh (MW) under the identical condition. The larger the MW, the smaller the diffusion coefficient. Because the MW of bound ligand is much larger than that of free small molecule, the change of diffusion coefficient could detect the bound ligand. As demonstrated in Table 1, there were some components in MLE binding to α-glucosidase, which was fully consistent with the results obtained from relaxation-edited NMR studies.

Table 1
Diffusion coefficients for Mulberry extract in the absence and presence of α-glucosidase.

Sample	δ (ppm)	D (m^2 s^{-1})
Mulberry extract	3.555	$(7.11 \pm 0.05) \times 10^{-10}$
α-glucosidase	1.006	$(3.57 \pm 0.05) \times 10^{-10}$
Mulberry extract + α-glucosidase	3.579	$(5.50 \pm 0.16) \times 10^{-10}$

Since it is evident that there were α-glucosidase ligands in mulberry extracts, the next step was to find out which component was bound to α-glucosidase. According to the attenuation of resonances in relaxation-edited NMR spectra and the change of diffusion coefficients in DOSY experiment, HMQC and HMBC spectra were utilized to elucidate the structure of this component (Fig. 3 and Table 2). Through the elucidation of the structure, the framework structure of DNJ was identified. The result was almost the same with the previous report [20].

3.2. Interaction of DNJ and α-glucosidase

In order to testify the above result, the interaction of DNJ between α-glucosidase was studied by relaxation-edited NMR experiments.

The result of relaxation-edited NMR was shown in Fig. 4 and Table 3. The result proved that DNJ was the ligand of α-glucosidase. Also it showed that DNJ could be detected by relaxation-edited NMR experiments from MLE. The change of R_2 of all protons in DNJ suggested strong binding affinity of DNJ towards α-glucosidase, which was proved by the measure of dissociation constant between DNJ and α-glucosidase subsequently.

NMR method proved to be very efficient in study of ligand-target interaction [21]. According to the research of Luo et al. [22], it was assumed that the binding sites in a protein were independent and had the same dissociation constant (K_d). At the meantime, each

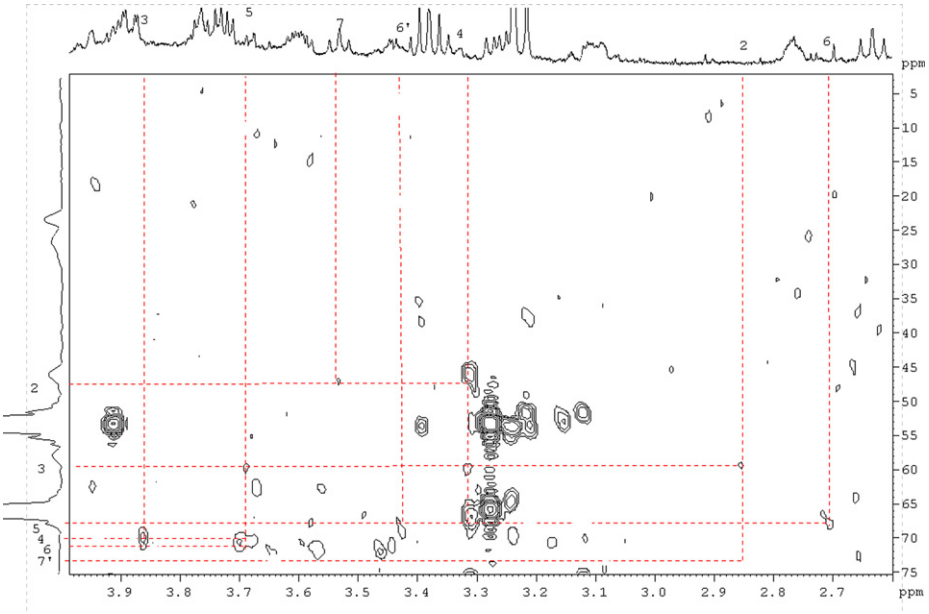

Fig. 3. The HMBC of MLE (C_{MLE}=3.2 mg/mL).

Table 2
Assignment of the framework of DNJ in MLE.

Carbon	δ_H^1 (ppm)	δ_C^{13} (ppm)	HMBC (H→C)
2	2.870	47.6	(2–7′,3)
3	3.860	59.7	(3–4,2)
4	3.319	70.5	(4–2,5)
5	3.690	55.1	(5–3,4,6)
6	2.710	63.0	(6–5)
6′	3.425	63.0	(6′–5)
7′	3.540	74.0	–
7	3.380	74.0	(7–2)

Table 3
T_2 of DNJ resonances in the absence and presence of α-glucosidase and the change of T_2 (-- T_2 could not been obtained due to the overlap of spectrum).

δ_H^1 (ppm)	T_{2DNJ} (ms)	$T_{2DNJ\ +\ \alpha\text{-glucosidase}}$ (ms)	Δ (ms)
4.001	1164	528	636
3.836	1209	–	–
3.698	1398	–	–
3.554	1503	954	549
3.348	1134	–	–
2.835	1524	747	777
2.714	1467	909	558

protein molecule had maximum n (the numbers of binding sites) available for accepting ligand molecules. In the case, these two equations were valid for relaxation to obtain K_d

$$T_{obs} = (1-X_B) \times T_F + X_B \times T_B \quad (1)$$

$$\frac{2(T_{obs}-T_F)}{T_B-T_F} = 1 + \frac{K_d}{C_p} \times \frac{C_p}{C_l} + n \times \frac{C_p}{C_l} - \left[\left(1 + \frac{K_d}{C_p} \times \frac{C_p}{C_l} + n \times \frac{C_p}{C_l}\right)^2 - 4 \times n \times \frac{C_p}{C_l}\right]^{1/2} \quad (2)$$

where X_B is the molar fraction of the binding ligand molecules, T_{obs} is the observed $1/R_{2,}$ in binding state, T_B and in free state, T_F, C_P and C_l denote the total concentrations of the protein and the ligand, respectively.

For testing the binding capacity of DNJ and α-glucosidase, a positive drug with definite α-glucosidase inhibition, acarbose, was used as a reference. By stimulating the data in Fig. 5 using Eq. (2), K_d was simultaneously obtained about the complex of acarbose and α-glucosidase and DNJ and α-glucosidase respectively, as listed in Table 4.

The K_d of the complex of DNJ and α-glucosidase was similar with that of the complex of acarbose and α-glucosidase, which showed that affinity of DNJ towards α-glucosidase was comparable with that of acarbose towards α-glucosidase. Furthermore, promising biological activity of DNJ in vivo was presented and its efficacy and dose profiles were better than those of acarbose [23], which was a powerful proof that DOSY and relaxation-edited NMR were effective to detect the inhibitor of α-glucosidase.

The content of ligand in the extracts and the K_d between ligand and α-glucosidase were key factors that influence the results [12]. In our work, a 10% (w/w) of DNJ in MLE could be detected by our strategy and the K_d between ligand and α-glucosidase was $(3.8 \pm 0.96) \times 10^{-5}\ M^{-1}$.

3.3. Binding mechanism of the DNJ to the α-glucosidase

It was generally believed that the carbohydrate mimics containing nitrogen such as acarbose were protonated in the active site and acted as glycosidase inhibitors because of their ability to mimic the shape and/or charge of the presumed transition state for enzymatic glycoside hydrolysis [2]. Based on the structure of DNJ and α-glucosidase, three aspects as follow may be beneficial for the interaction between DNJ and α-glucosidase: firstly, a special feature of DNJ was the permanent positive charge carried by the nitrogen which was responsive for the ionic interaction between DNJ and α-glucosidase; secondly, there were four hydroxyls in the structure of DNJ, which made it a strong hydrogen-bond donor in the molecule mediating hydrogen-bond interaction with α-glucosidase. Furthermore, the weak steric hindrance for electrostatic attachment of DNJ on α-glucosidase surface was beneficial.

From the result of relaxation-edited NMR experiments, it was obtained that the protons of 2 had the greatest change of T_2, which suggested that the sites were close to the activity sites. Judging from the structure of DNJ, 2-position was near to the nitrogen, which

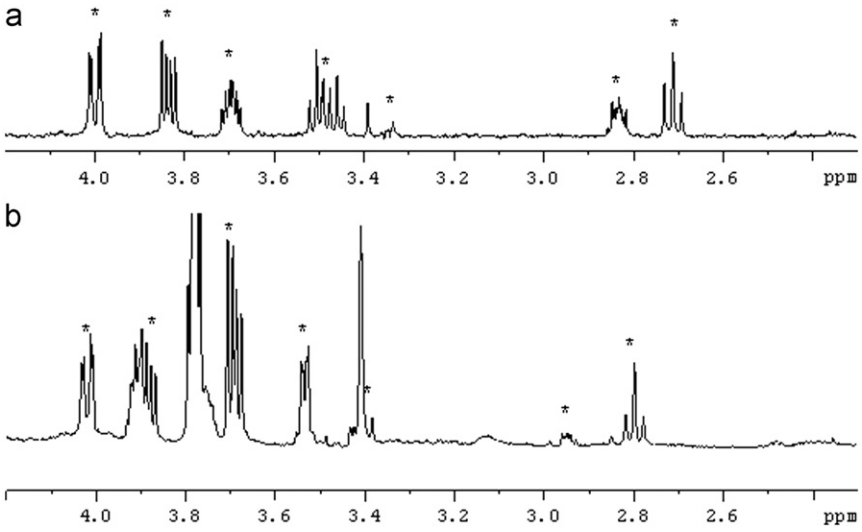

Fig. 4. The relaxation-edited NMR spectra of (a) 1.0 mM DNJ and (b) 1.0 mM DNJ + 19 μM α-glucosidase. The spin-lock time of each experiment was 30 ms. The resonances marked with "*" were from DNJ.

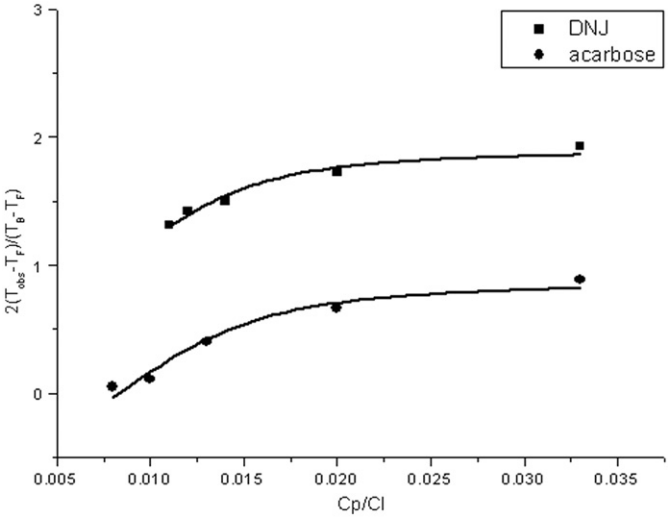

Fig. 5. Plots of the measured relaxation time as a function of the concentration ratio C_p/C_l for the DNJ/α-glucosidase system and acarbose/α-glucosidase system. Solid curves are the calculated results using Eq. (2).

Table 4
Dissociation constant K_d for acarbose/α-glucosidase system and DNJ/α-glucosidase system determined by relaxation measurements.

δ_H^1 (ppm)	K_d (M^{-1})
Acarbose (H-9)	$(5.4 \pm 1.45) \times 10^{-5}$
DNJ (H-3)	$(3.8 \pm 0.96) \times 10^{-5}$

indicated nitrogen played a key role in the interaction between DNJ and α-glucosidase. The protonated amine with positive charge was beneficial for DNJ to enter the active sites of α-glucosidase which was covered with negative charge. The inference was in accordance with the previous report [2].

3.4. Identification of the effective component in MLE

The active component DNJ from mulberry extract was elucidated and its biological activity was examined. However, there were a lot of components in MLE, it is necessary to prove whether DNJ was the main component to contribute to the biological activity. Herein the inhibitory rate of MLE, DNJ and acarbose towards α-glucosidase were determined by α-glucosidase inhibition assay in order to find out the main effective component.

Table 5
The inhibitory rate of acarbose, MLE and DNJ on α-glucosidase.

	Concentration	The inhibitory rate on α-glucosidase (%)
Acarbose	0.48 g/L	65
MLE	10 g/L(DNJ 10% (w/w))	54
DNJ	0.97 g/L	65

As shown in Table 5, DNJ was a better inhibitor of α-glucosidase than MLE. In terms of content and activity, DNJ may be the main effective component in MLE. Meanwhile, the result indicated that DNJ was a comparable α-glucosidase inhibitor as acarbose. With the same inhibitory rate on α-glucosidase, the concentration of acarbose and DNJ was parallel.

The mass fraction of DNJ in MLE was 10% and the dissociation constant of DNJ/α-glucosidase complex was 3.8×10^{-5} M in current research. From the inhibitory rate experiment of α-glucosidase, DNJ may be the main effective component in MLE. Our study shows that the method could effectively been used to find inhibitors from extraction of plants.

3.5. Comparison of our strategy with other methods

The α-glucosidase is an important target in the research of type 2 diabetes mellitus. Some methods have been reported in exploring the inhibitor of α-glucosidase including UV and fluorescence methods [9] and mass spectrometry [24]. Additionally, there are some ligands lack chromophore in their molecules and therefore for them it is difficult to be detected by previous techniques. At that time, the superiority of NMR is obvious. Li et al., developed a HPLC-DAD-MS/MS and biochemical detection method for screening the α-glucosidase inhibitors [25]. The system generated both biological and chemical information of compounds, but it was not easy to obtain the structural information. Our strategy not only supplied the chemical information, but also was advantageous in the elucidation of structure based on

NMR. Dalvit et al., presented a NMR method for biochemical screening [26]. Although this method was efficient, it required the labeling of the substrate with a CF_3 moiety which limited the application of this method. Our strategy was not necessary for labeling to complete the screening, which is more convenient and rapid. The inhibitor of α-glucosidase was detected from MLE successfully and the antidiabetic effects of DNJ in type 2 diabetes mellitus had also been proved in animal models [23], which proved our strategy was effective.

4. Conclusion

In all, the strategy we proposed was effective and superior to previous methods in two aspects: firstly, the strategy could screen the inhibitor of α-glucosidase in mixtures, as long as the resonances of ligand can be detected by NMR, the ligand could be identified. Secondly, the process is time saving and labor saving, more importantly, our strategy can discover minor active components which may be lost in separation process. This was the first report of exploring the inhibitors of α-glucosidase by NMR from extraction of plants.

In this work, we successfully detected DNJ from MLE by the strategy. DNJ was the ligand of α-glucosidase [15] and in the work the result was proved by the inhibitory activity experiment of α-glucosidase. In addition, in vitro inhibitory activity of DNJ on α-glucosidase was comparable with acarbose. Compared with the previous detection method of the inhibitor of α-glucosidase, the strategy provides more information, which is more beneficial in screening of the ligand of α-glucosidase. It not only detected the ligand of α-glucosidase, but also gave the structural information of ligand and active sites at the same time. In summary, the strategy could screen and identify the ligand(s) of α-glucosidase simultaneously, which supplies hints for researches to screen the ligand(s) of α-glucosidase.

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