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Article

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Erythritol attenuates postprandial blood glucose by inhibiting α-glucosidase

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Abstract

Diabetes mellitus (DM) is a serious metabolic disorder where impaired postprandial blood glucose regulation often leads to severe health complications. The natural chemical, erythritol is a C4 polyol approved by FDA for use as a sweetener. Here we examined a potential role for erythritol in the control of postprandial blood glucose levels in DM. An anti-postprandial hyperglycemia effect upon erythritol administration (500 mg kg^{-1}) was demonstrated in alloxan-induced DM model mice by monitoring changes in blood glucose after intragastric administration of drugs and starch. We also found that erythritol most likely exerts its anti-postprandial hyperglycemic activities by inhibiting α-glucosidase in a competitive manner. This was supported by enzyme activity assays and molecular modelling experiments. In the latter experiments it was possible to successful dock erythritol into the catalytic pocket of α-glucosidase, with the resultant interaction likely to be driven by electrostatic interactions involving Asp 215, Asp69 and Arg446 residues. This study suggests that erythritol may not only serve as a glucose substitute but may also be a useful agent in the treatment of diabetes mellitus to help manage postprandial blood glucose levels.

Key words: Diabetes mellitus; postprandial blood glucose; erythritol; α-glucosidase;

44 competitive inhibition.

45

46

47 **Introduction**

48 Diabetes mellitus (DM), is a disorder characterized by high blood glucose levels
49 (>126 mg/dL) that affects 415 million people worldwide. DM is recognized as a
50 serious public health concern with the total number of those affected estimated to
51 increase to 642 million by 2040[1]. DM is associated with serious health complications
52 including kidney failure, cardiovascular disease, visual impairment, cognitive decline
53 and premature death[2,3-4]. Therefore, new effective therapies for DM are urgently
54 required.

55 Type 2 DM is identified as a staged reduction of insulin secretion in response to
56 food glucose intake; hence, its primary pathological phenomenon is impaired
57 postprandial glucose regulation[5]. However, clinicians still depend on fasting blood
58 glucose and glycated hemoglobin to guide treatment[6]. It is reported that a linear
59 relationship between the risk of death from cardiovascular disease and the oral
60 glucose tolerance is observed[7-8]. Meanwhile, another study confirms postprandial
61 hyperglycemia to be a major risk factor for cardiovascular disease in individuals with
62 type 2 DM[9-10]. It is reported that postprandial hyperglycemia may promote
63 cardiovascular disease by increasing oxidative stress[11]. Furthermore, it is suggested
64 that postprandial hyperglycemia is a common phenomenon for individuals without
65 DM[12]. Adequate control of postprandial glucose levels is thus very important and

should be given more attention[13].

In type 2 DM, α-glucosidase inhibitors are commonly used to control postprandial hyperglycemia[14]. The enzyme α-Glucosidase is a carbohydrase located on the surface intestinal epithelial cells, where it catalyses the hydrolysis of oligosaccharides into monosaccharides (such as glucose) to facilitate absorption. By significantly delaying intestinal glucose absorption, α-glucosidase inhibitors can reduce postprandial hyperglycemia to lower the risk of late diabetic complications[14]. Because of its tolerability and cardiovascular benefits, the International Diabetes Federation (IDF) have recommended α-glucosidase inhibitors as a first line therapy for DM[15].

Many sugar derivatives such as sugar alcohols, due to their low-energy character, have been developed into food additives as substitutes for glucose[16]. The sugar alcohol, erythritol exists naturally in algae, wine, sake, beer, pears, grapes, watermelons, and mushrooms and is a C4 polyol, which was approved in 1997 by the FDA to be listed in GRAS (Generally Recognized As Safe List). More than 90% of ingested erythritol is not metabolized by the human body and is excreted unchanged in the urine. Consequently, erythritol has a potential use as a sweetener that may be added to the diets of DM patients[17].

In a previous study, the effects of erythritol on blood glucose in streptozotocin (STZ)-induced DM rats was assessed. It was found that erythritol had a long-term blood glucose controlling capacity, that led to reduced kidney damage caused by DM[18]. Here we demonstrate that that erythritol is effective for controlling

postprandial hyperglycemia in alloxan-induced DM model mice and show that it likely mediates this effect by acting as a competitive inhibitor of α-glucosidase.

Materials and methods

Chemicals and reagents

For the outlined studies, α-Glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), erythritol, p-nitrophenyl-α-D-glucopyranoside (pNPG) and acarbose were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Animals

Thirty SPF grade male Swiss albino male mice (balb/c strain), eight weeks old, were obtained from the SLAC Company Limited (Shanghai, China). Diabetes was induced by a single intravenous injection of aqueous alloxan monohydrate (200 mg/kg). After 48 h, animals with the serum glucose level within the range (17-24 mmol/L) were selected for the further study. All the animals were housed 5 per cage at 25 ℃ with a 12 h light/12 h dark cycle. All experiments were approved by the Animal Ethics Committee of Chinese Academy of Sciences and were performed according to Guidelines for the Care and Use of Laboratory Animals. The animal ethical approval number is NWIPB-2016-33.

The thirty mice were randomly allocated into six groups: sham operation plus water group (Sham Cn), sham plus acarbose solution (acarbose 4 mg/Kg/d), sham

plus erythritol solution (erythritol 500 mg/Kg/d), DM plus water (DM Cn), DM plus acarbose solution group (acarbose 4 mg/Kg/d) and DM plus erythritol solution (erythritol 500 mg/Kg/d). Erythritol was dissolved in water (vehicle) and was administered by intragastric administration with 0.5g starch after fasting for 12 hours. After intragastric administration, the blood glucose levels were measured at 0, 30, 60, 100, 140 and 180 min.

Assessment of α-Glucosidase activity

The α-glucosidase activity assay was performed according to a slightly modified method of that previously reported[19]. The α-glucosidases (0.35 U/mL) and substrate (1.5 mM p-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M sodium phosphate buffer, pH 7.0. For this 50 μL of acarbose or erythritol was pre-incubated with 100 μL of α-glucosidase at 37℃ for 10 min. The substrate solution (100 μL) was then added to the reaction mixture and incubated at 37℃ for 20 min. The reaction was then terminated by adding 1 ml of 1 M Na_2CO_3. The absorbance at 405 nm was determined using a microplate Reader (EnVision; PerkinElmer, USA). All samples were analyzed in triplicate and the percentage of activity was calculated as:

$$\text{Percentage activity (\%)} = \frac{A_{405}\ (\text{sample})}{A_{405}\ (\text{control})} \times 100\%$$

Kinetic studies were also performed based on the α-glucosidase assay described above. The concentration of α-glucosidase was kept constant at 0.1 unit/mL and the pNPG concentrations varied from 0.11 to 0.51 mM in the absence and presence of

erythritol (5, 10 and 15 mg/mL). The type of inhibition was determined using V_{max} and K_m values obtained using a Lineweaver-Burke plot obtained by plotting velocities of reaction (vertical axis) and substrate concentrations (horizontal axis) reciprocally.

Molecular modeling

Molecular docking studies were performed using Discovery studio v2.5 software (Accelrys Inc, San Diego, USA). The 3D structure of the α-glucosidase, isomaltase from *Saccharomyces cerevisiae* (PDB: 3AJ7) was obtained from Protein Data Bank (www.rcsb.org). This structure was chosen based on its very high resolution (1.3 Å). Crystallographic disorders and unfilled valence atoms were corrected by alternate conformations and valence monitor tools. The molecular docking of erythritol was performed using the Libdock method. To begin the docking, hydrogen bonds were added firstly, and then the energy was minimized by CHARMM force field. After the above steps of preparations, the active site of the enzyme was identified as the binding site to start the docking protocol.

The AMBER 11.0 software was employed to run all the molecular dynamics studies. Erythritol was minimized by the Discovery studio v2.5 software. General Amber Force Field (GAFF) parameters were firstly assigned to the ligands, while partial charges were calculated using the AM1-BCC method of AMBER 11.0. The erythritol-enzyme complex was charge neutralized by adding 10 sodium counter ions, and then were surrounded by a periodic box of TIP3P water molecules extending up to 10 Å from the solute. First, energy minimizations using a steepest descent method,

followed by the conjugate gradient method, were performed for each system. Then, each system was gradually heated from 0 K to 300 K within 30 ps. This was followed by a further 500 ps of equilibration at 300 K carried out to obtain a stable density. Afterward, an unconstrained production phase was initiated and continued for 40 ns in an NPT ensemble at 1 atm and 300 K. During the simulations, the long-range electrostatic interactions were evaluated by the Particle Mesh Ewald (PME) algorithm. The cutoff distance for the long-range van der Waals interaction was set to 8 Å. The SHAKE method was applied to con-strain the bond lengths of hydrogen atoms attached to heteroatoms. The time step used for the MD simulations was set to 2.0 fs and the trajectory files were collected every 1 ps for the subsequent analysis.

The interaction between inhibitor and each residue was computed using the MM/GBSA decomposition process by the mm_pbsa program in AMBER 11.0. The binding interaction of each inhibitor–residue pair includes three energy terms: van der Waals contribution (vdw), electrostatic contribution (ele) and polar solvation contribution (Polar E) and non-polar solvation contribution (non-Polar E). All energy components were calculated using the 300 snapshots extracted from the MD trajectory from 1.0 to 4.0 ns.

Results and discussion

Reduction of postprandial blood glucose by erythritol

The postprandial hypoglycemic effect of erythritol in an alloxan-induced DM mouse model was analyzed. Blood glucose was measured before and 30, 60, 100 and

140 min after intragastric administration of starch. The blood glucose increased and peaked at 30 minutes in all the groups. Compared with the sham operation plus water group (Sham Cn), the sham plus acarbose solution (acarbose 4 mg/Kg/d) and sham plus erythritol solution (erythritol 500 mg/Kg/d) groups both displayed a decrease in average blood glucose but the differences between the three groups were not considered to be significant (Figure 1A). Meanwhile, the area under the concentration-time curve (AUC) of the above three groups did not differ significantly (Figure 1B). The results indicated that erythritol and acarbose did not induce any significant postprandial blood glucose changes in healthy animals. However, in the DM-model animals, the postprandial hypoglycemic effect of erythritol and acarbose became significant (Figure 1C). A significant decline in AUC was observed for both erythritol and acarbose-treated groups, demonstrating both drugs to exert an *in vivo* postprandial hypoglycemic effect (Figure 1D). This finding was contrary to a previous report that found erythritol treatment to have no effect on blood glucose[20]. However, in that study fasting blood glucose levels and not postprandial blood glucose were measured. In a very recent report a postprandial hypoglycemic effect of erythritol was shown *in vivo* [21]. However, this study focused specifically on the effects of erythritol on insulin resistance.

Inhibition of α-glucosidase activity by erythritol

The ability of erythritol to inhibit α-glucosidase was assessed. As shown in Figure 2, erythritol exhibited a strong inhibitory effect displaying an IC_{50} value of 6.43

197 mg/mL (52.7 mM). This suggests that the mechanism by which erythritol exerts its
198 postprandial hypoglycemic effect is potentially through direct inhibition of
199 α-glucosidase. In healthy animals, under the hypoglycemic effect of insulin, it is
200 likely that blood glucose concentrations are controlled so well that erythritol is unable
201 to influence postprandial blood glucose (Figure 1A and 1B). However in the
202 DM-model mice, in the absence of sufficient glycemic control by insulin, a reduction
203 in the production of intestinal glucose by erythritol-mediated α-glucosidase inhibition
204 could explain the significant difference observed.

205 To determine the mechanism of inhibition kinetic studies were performed. For
206 these, erythritol was added at three different concentrations (i.e. 5, 10 and 15 mg/mL)
207 and the reacations performed a at five different pNPG (substrate) concentrations (i.e.
208 0.11–0.51 mM). Product formation was first plotted against time to obtain initial
209 velocities, which were calculated by taking the linear part of the increasing
210 absorbance. The reciprocal velocities were subsequently plotted against the reciprocal
211 of substrate concentration to construct Lineweaver-Burk plots. The Lineweaver–Burk
212 plots for α-glucosidase inhibition by erythritol generated linear data with different
213 granients, which intersected at the same point on Y-axis (indicative of an increased K_m
214 and unchanged V_{max}), indicating competitive inhibition. The Ki value was calculated
215 to be 38.98 mM at 5 mg/ml (Figure 3).

216

217 **Computational modeling of the α-glucosidase-erythritol complex**

218 A Ramachandran Plot, often used as a first check to verify predicted torsion

219 angles in proteins, showed low energy conformations for φ (phi) and ψ (psi), which
220 were used to represent the torsion angles on either side of alpha carbons in peptides.
221 The Ramachandran Plot provided the local backbone conformation of each residue by
222 graphical expression form. The φ and ψ torsion angles of a residue were represented
223 as the points on the Ramachandran Plot, which also included a representation of the
224 favorable and unfavorable regions for residues to aid determination of whether
225 individual residues were built correctly. As shown in Figure 4, most of the residues
226 resided in either the core or allowed region with the exception of 9 residues (marked
227 as red triangles), which overall indicated that the quality of the docking model was
228 very good.

229 Next, we carried out a Libdock protocol to study the interactions between the
230 α-glucosidase enzyme and its inhibitor erythritol. During catalysis the Glu277 residue
231 of the enzyme forms a hydrogen bond with O1 of the glucose saccharide, with a bond
232 distance of 2.8 Å. The lengths of hydrogen bonds between Asp352 OD1 and O3 of the
233 glucose residue and between OD2 and O2 are 2.7 and 2.5 Å, respectively. Asp69 OD2
234 and Arg442 NH1 form hydrogen bonds with O4 of the glucose residue. Glu277,
235 Asp352, Asp69 and Arg442 together form the active site of α-glucosidase. For
236 erythritol (shown in Figure 5), a hydrogen-bond network was found at the two ends of
237 the molecule. The hydroxyl group on the C1 carbon atom connected to Asp69 and
238 Arg446 by hydrogen-bonds through the H113 water molecule. Another hydroxyl
239 group on the C4 carbon atom made similar connections with Asp215, Arg213, Asp352
240 through H132 water molecules. It was clearly observed in our model that the active

site of the enzyme was occupied by erythritol. We speculated that these H-bonds provide the main electrostatic means to aid erythritol binding at the active pocket and facilitate its demonstrated inhibitory activity.

In order to gain more information as to the chemical properties of the erythritol-enzyme interactions, the binding free energy was decomposed into inhibitor-residue pairs. The resultant quantitative data (shown in Table 1) was very useful for understanding how erythritol could bind in the active pocket of α-glucosidase. It was demonstrated that the major binding energy was electrical free energy and the majority dissociation energy was polar solvation free energy. The van der Waals free energy, electrical free energy and non-polar solvation free energy were sufficient to overcome the polar solvation free energy to promote erythritol binding.

To deepen our understanding on forces stabilizing the binding of erythritol to the enzyme, we decomposited the free energy of the residues in the active pocket proposed to interact with erythritol (Figure 6). It was demonstrated that Asp 215, Asp69 and Arg446 residues were likely to contribute the largest total binding free energy. By decomposition, we found that these three residues provided a high proportion of electrical free energy to promote erythritol binding in the active pocket, which we propose is provided by hydrogen-bonds. Moreover, these three residues undertake the largest polar solvent decomposition free energy. For Asp 352, Val 109 and Arg213 residues, the most important binding energy was solar solvent free energy, while the main decomposition free energy was electrical free energy. In addition, we found that all the four kinds of energy could promote inhibitor-binding by His351 and

Val216 residues, in which van der Waals and polar solvent free energy provided the most energy.

In summary, we have shown using an alloxan-induced DM mouse model that erythritol treatment significantly reduces postprandial blood glucose at the dose of 500 mg/Kg compared with acarbose (4 mg/Kg), but has no significant effects on healthy animals. Biochemical analysis indicated that erythritol exerts its hypoglycemic effect by inhibiting α-glucosidase in the competitive manner. Molecular docking of erythritol to the α-glucosidase, isomaltase from *S. cerevisiae* revealed that the C1 hydroxyl and C4 hydroxyl groups of erythritol may form hydrogen-bonds with Asp69, Arg446, Asp215, Arg213 and Asp352 residues though H113 and H132 water molecules to competitively occupy the active pocket. An energy decomposition study indicated that electrical free energy provided the majority of the binding free energy and the polar solvent free energy provided the majority dissociation energy. The Asp 215, Asp69 and Arg446 residues contributed the strongest total binding free energy. It is hoped that these findings will stimulate further work into the role of erythritol in the management of DM and its potential use as a therapeutic food additive.

Acknowledgments

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Discovery, Evaluation and Transformation of Active Natural Compounds, Strategic Biological Resources Service Network Program of Chinese Academy of Sciences (No. ZSTH-027), Major Special Science and Technology Projects in Qinghai Province (2014-GX-A3A-01).

Figure captions

Figure 1 (A) After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to healthy mice, the blood glucose was monitored at different time points. **(B and D)** the area under concentration-time curve (AUC) were calculated to evaluate the postprandial blood glucose changes. **(C)** After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to diabetes mellitus mice, the blood glucose was monitored at different time points.

Figure 2. Inhibition of α-glucosidase by acarbose **(A)** and erythritol **(B).** Data was fitted using a logistic function to calculate the IC_{50} values. The detailed inhibition data is presented.

Figure 3. Lineweaver-Burk plot of erythritol-mediated α-glucosidase inhibition. Data is plotted as a double reciprocal of initial reactions velocities versus concentration of the substrate, pNPG.

Figure 4. Ramachandran contours were used to test the quality of the presented molecular model. Green dots indicate the position of residues in the core region, while red triangles highlight the outlying residues.

Figure 5. Molecular model of erythritol-binding at the catalytic active pocket of

α-glucosidase. The interacting protein residues, Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216 are highlighted.

Figure 6. Decomposition of the calculated free energies of the Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216 residues, which are proposed to contribute to the binding of erythritol to α-glucosidase.

Table 1 Total binding free energy decomposition (kJ/mol)

Energy Component	Average	Std. Dev.
VDWAALS	-9.8652	3.3202
EEL	-69.2587	6.6086
EGB	58.9938	4.1258
ESURF	-3.2684	0.1329
DELTA G gas	-79.1239	5.4402
DELTA G solv	55.7253	4.1494
DELTA TOTAL	-23.3986	4.9532

VDWAALS is total van der waals free energy. EEL is total electrical free energy. EGB is total polar solvation energy. ESURF is total non-polar solvation energy. DELTA G gas=VDWAALS+EEL. DELTA G solv= EGB+ ESURF. DELTA TOTAL= VDWAALS+ EEL+ EGB+ ESURF.

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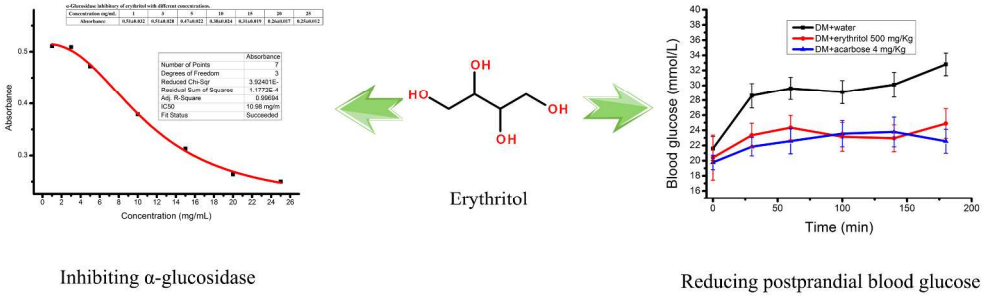

Inhibiting α-glucosidase

Reducing postprandial blood glucose

Graphic abstract

244x78mm (300 x 300 DPI)

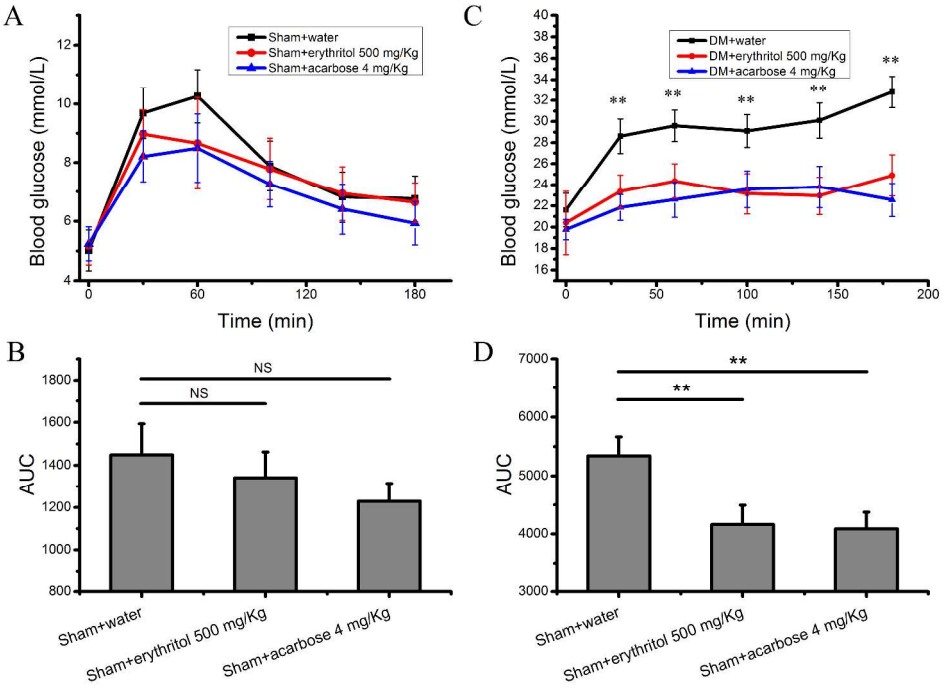

Figure 1 (A) After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to healthy mice, the blood glucose was monitored at different time points. (C) After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to diabetes mellitus mice, the blood glucose was monitored at different time points. (B and D) the area under concentration-time curve (AUC) were calculated to evaluate the postprandial blood glucose changes.

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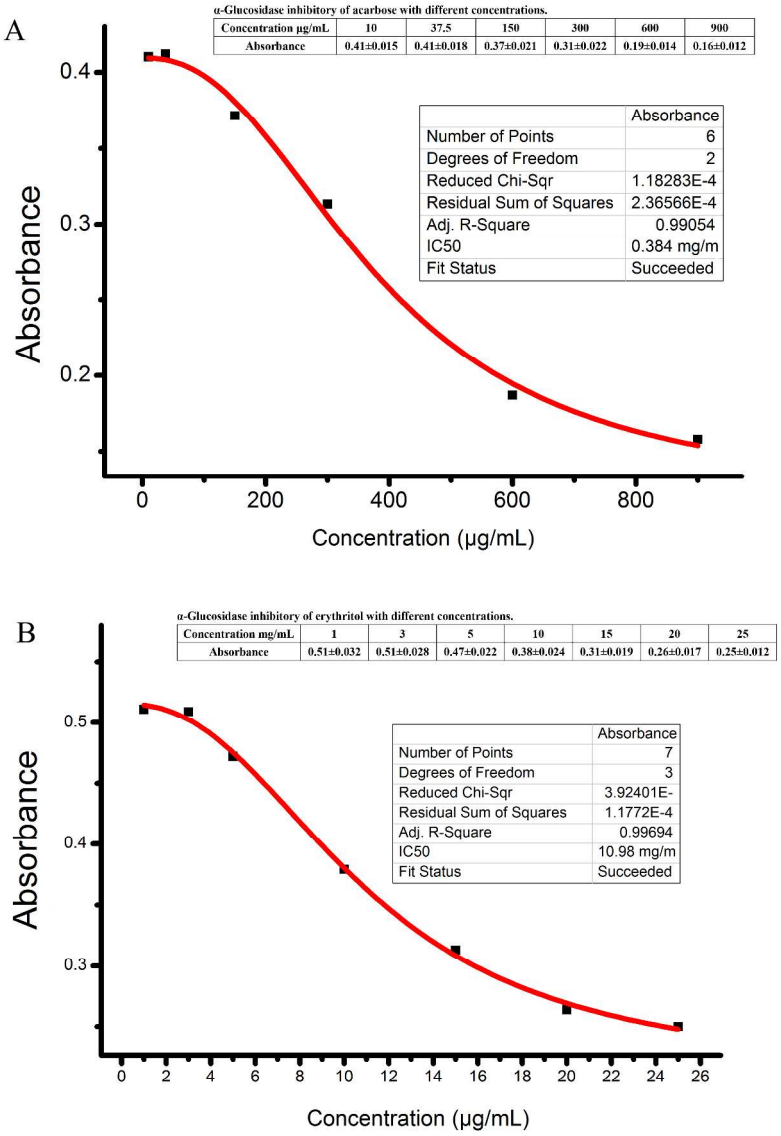

Figure 2 the inhibition data of acarbose (A) and erythritol (B) with different concentrations was fitted with logistic function to calculate the IC50 value. The detailed inhibition data was at the head of each figures.

296x419mm (300 x 300 DPI)

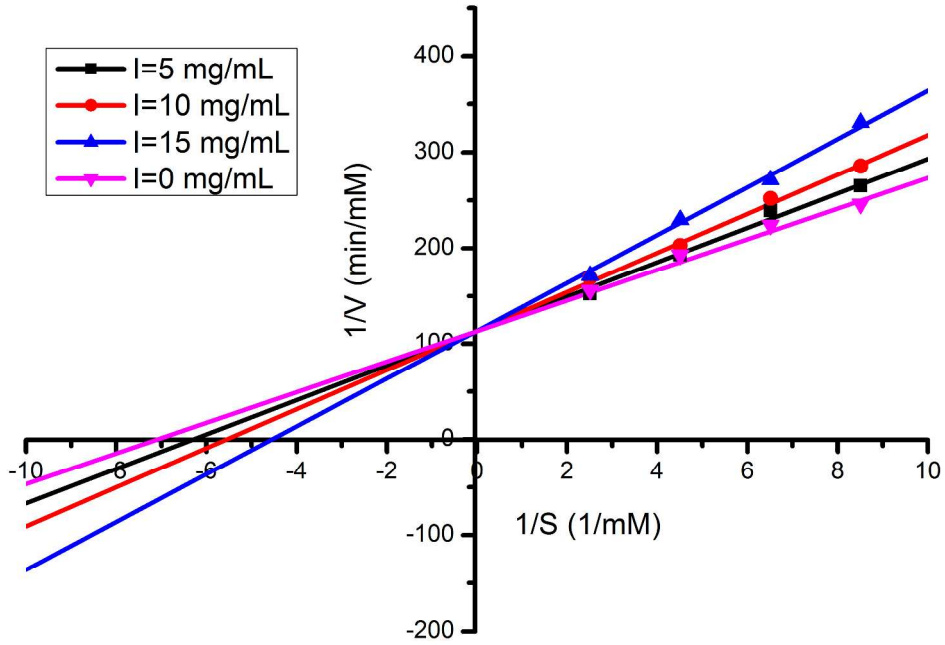

Figure 3 Lineweaver-Burk plot of erythritol against α-glucosidase at different concentrations of pNPG.

288x200mm (300 x 300 DPI)

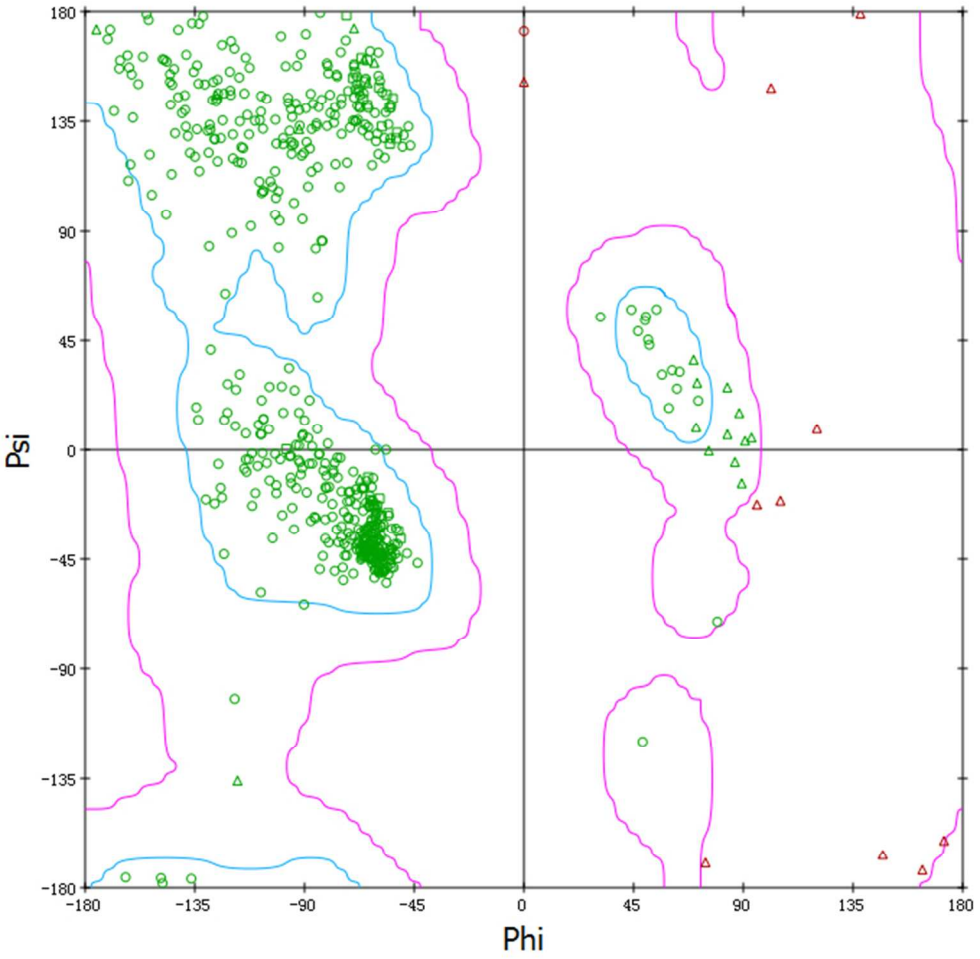

Figure 4 Ramachandran contours is used to test the quality of newly developed model. Green dots donate the position of residues in core region. While, red spots sign highlights the outliers.

183x176mm (96 x 96 DPI)

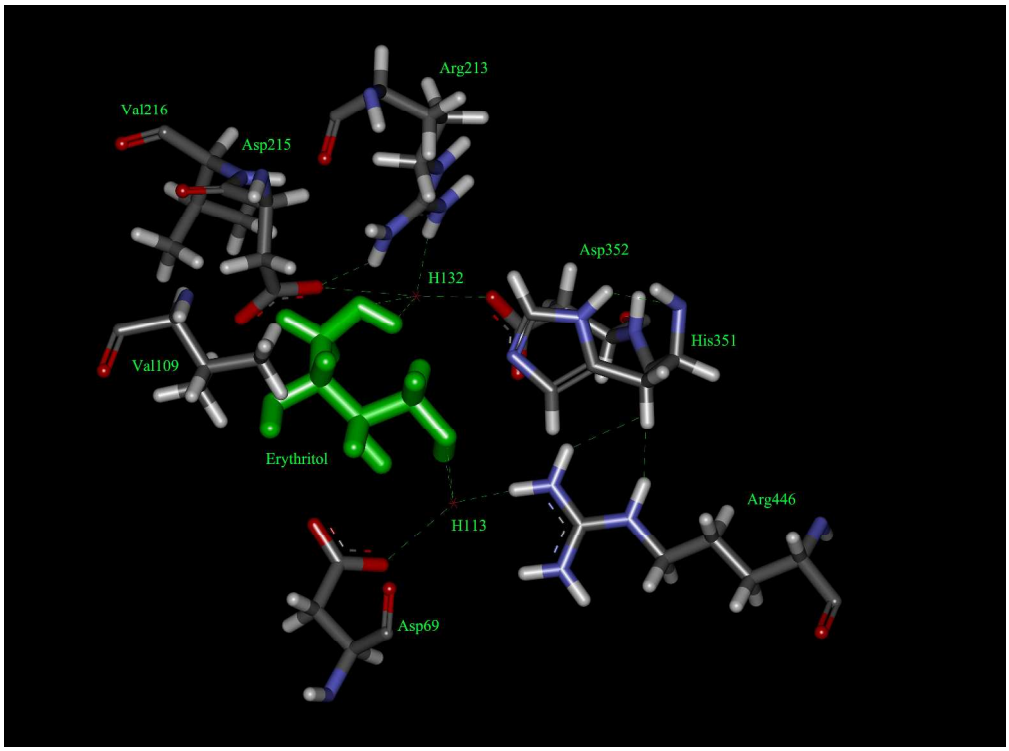

Figure 5 the interaction mode of erythritol in the catalytic active pocket of α-glucosidase with Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216.

1587x1174mm (96 x 96 DPI)

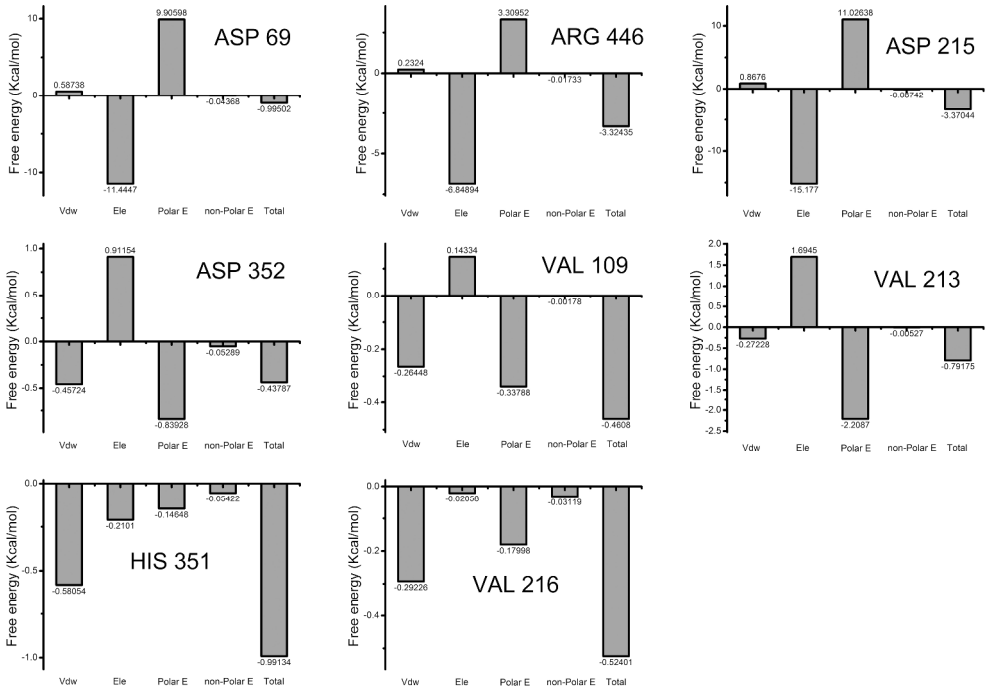

Figure 6 Decomposition of the free energy of Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216.

423x298mm (300 x 300 DPI)