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Article

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1 **Erythritol attenuates postprandial blood glucose by inhibiting α -glucosidase**

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20

21

22 **Abstract**

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

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43 **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 2040¹. 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 regulation⁵. However, clinicians still depend on fasting blood
58 glucose and glycated hemoglobin to guide treatment⁶. It is reported that a linear
59 relationship between the risk of death from cardiovascular disease and the oral
60 glucose tolerance is observed⁷⁻⁸. Meanwhile, another study confirms postprandial
61 hyperglycemia to be a major risk factor for cardiovascular disease in individuals with
62 type 2 DM⁹⁻¹⁰. It is reported that postprandial hyperglycemia may promote
63 cardiovascular disease by increasing oxidative stress¹¹. Furthermore, it is suggested
64 that postprandial hyperglycemia is a common phenomenon for individuals without
65 DM¹². Adequate control of postprandial glucose levels is thus very important and

66 should be given more attention¹³.

67 In type 2 DM, α -glucosidase inhibitors are commonly used to control
68 postprandial hyperglycemia¹⁴. The enzyme α -Glucosidase is a carbohydrase located
69 on the surface intestinal epithelial cells, where it catalyses the hydrolysis of
70 oligosaccharides into monosaccharides (such as glucose) to facilitate absorption. By
71 significantly delaying intestinal glucose absorption, α -glucosidase inhibitors can
72 reduce postprandial hyperglycemia to lower the risk of late diabetic complications¹⁴.
73 Because of its tolerability and cardiovascular benefits, the International Diabetes
74 Federation (IDF) have recommended α -glucosidase inhibitors as a first line therapy
75 for DM¹⁵.

76 Many sugar derivatives such as sugar alcohols, due to their low-energy character,
77 have been developed into food additives as substitutes for glucose¹⁶. The sugar
78 alcohol, erythritol exists naturally in algae, wine, sake, beer, pears, grapes,
79 watermelons, and mushrooms and is a C4 polyol, which was approved in 1997 by the
80 FDA to be listed in GRAS (Generally Recognized As Safe List). More than 90% of
81 ingested erythritol is not metabolized by the human body and is excreted unchanged
82 in the urine. Consequently, erythritol has a potential use as a sweetener that may be
83 added to the diets of DM patients¹⁷.

84 In a previous study, the effects of erythritol on blood glucose in streptozotocin
85 (STZ)-induced DM rats was assessed. It was found that erythritol had a long-term
86 blood glucose controlling capacity, that led to reduced kidney damage caused by
87 DM¹⁸. Here we demonstrate that that erythritol is effective for controlling

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

90

91 **Materials and methods**

92 **Chemicals and reagents**

93 For the outlined studies, α -Glucosidase from *Saccharomyces cerevisiae* (EC
94 3.2.1.20), erythritol, p-nitrophenyl- α -D-glucopyranoside (pNPG) and acarbose were
95 obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other
96 chemicals used were of analytical grade.

97

98 **Animals**

99 Thirty SPF grade male Swiss albino male mice (balb/c strain), eight weeks old,
100 were obtained from the SLAC Company Limited (Shanghai, China). Diabetes was
101 induced by a single intravenous injection of aqueous alloxan monohydrate (200
102 mg/kg). After 48 h, animals with the serum glucose level within the range (17-24
103 mmol/L) were selected for the further study. All the animals were housed 5 per cage at
104 25 °C with a 12 h light/12 h dark cycle. All experiments were approved by the Animal
105 Ethics Committee of Chinese Academy of Sciences and were performed according to
106 Guidelines for the Care and Use of Laboratory Animals. The animal ethical approval
107 number is NWIPB-2016-33.

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

110 plus erythritol solution (erythritol 500 mg/Kg/d), DM plus water (DM Cn), DM plus
111 acarbose solution group (acarbose 4 mg/Kg/d) and DM plus erythritol solution
112 (erythritol 500 mg/Kg/d). Erythritol was dissolved in water (vehicle) and was
113 administered by intragastric administration with 0.5g starch after fasting for 12 hours.
114 After intragastric administration, the blood glucose levels were measured at 0, 30, 60,
115 100, 140 and 180 min.

116

117 **Assessment of α -Glucosidase activity**

118 The α -glucosidase activity assay was performed according to a slightly modified
119 method of that previously reported¹⁹. The α -glucosidases (0.35 U/mL) and substrate
120 (1.5 mM p-nitrophenyl- α -D-glucopyranoside) were dissolved in 0.1 M sodium
121 phosphate buffer, pH 7.0. For this 50 μ L of acarbose or erythritol was pre-incubated
122 with 100 μ L of α -glucosidase at 37°C for 10 min. The substrate solution (100 μ L) was
123 then added to the reaction mixture and incubated at 37°C for 20 min. The reaction was
124 then terminated by adding 1 ml of 1 M Na₂CO₃. The absorbance at 405 nm was
125 determined using a microplate Reader (EnVision; PerkinElmer, USA). All samples
126 were analyzed in triplicate and the percentage of activity was calculated as:

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

127

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

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

134

135 **Molecular modeling**

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

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

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

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

170

171 **Results and discussion**

172 **Reduction of postprandial blood glucose by erythritol**

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

175 140 min after intragastric administration of starch. The blood glucose increased and
176 peaked at 30 minutes in all the groups. Compared with the sham operation plus water
177 group (Sham Cn), the sham plus acarbose solution (acarbose 4 mg/Kg/d) and sham
178 plus erythritol solution (erythritol 500 mg/Kg/d) groups both displayed a decrease in
179 average blood glucose but the differences between the three groups were not
180 considered to be significant (Figure 1A). Meanwhile, the area under the
181 concentration-time curve (AUC) of the above three groups did not differ significantly
182 (Figure 1B). The results indicated that erythritol and acarbose did not induce any
183 significant postprandial blood glucose changes in healthy animals. However, in the
184 DM-model animals, the postprandial hypoglycemic effect of erythritol and acarbose
185 became significant (Figure 1C). A significant decline in AUC was observed for both
186 erythritol and acarbose-treated groups, demonstrating both drugs to exert an *in vivo*
187 postprandial hypoglycemic effect (Figure 1D). This finding was contrary to a previous
188 report that found erythritol treatment to have no effect on blood glucose²⁰. However,
189 in that study fasting blood glucose levels and not postprandial blood glucose were
190 measured. In a very recent report a postprandial hypoglycemic effect of erythritol was
191 shown *in vivo*²¹. However, this study focused specifically on the effects of erythritol
192 on insulin resistance.

193

194 **Inhibition of α -glucosidase activity by erythritol**

195 The ability of erythritol to inhibit α -glucosidase was assessed. As shown in Figure
196 2, erythritol exhibited a strong inhibitory effect displaying an IC₅₀ 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 reactions performed 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 gradients, which intersected at the same point on Y-axis (indicative of an increased K_m
214 and unchanged V_{max}), indicating competitive inhibition. The K_i 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

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

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

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

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

265 In summary, we have shown using an alloxan-induced DM mouse model that
266 erythritol treatment significantly reduces postprandial blood glucose at the dose of
267 500 mg/Kg compared with acarbose (4 mg/Kg), but has no significant effects on
268 healthy animals. Biochemical analysis indicated that erythritol exerts its
269 hypoglycemic effect by inhibiting α -glucosidase in the competitive manner. Molecular
270 docking of erythritol to the α -glucosidase, isomaltase from *S. cerevisiae* revealed that
271 the C1 hydroxyl and C4 hydroxyl groups of erythritol may form hydrogen-bonds with
272 Asp69, Arg446, Asp215, Arg213 and Asp352 residues though H113 and H132 water
273 molecules to competitively occupy the active pocket. An energy decomposition study
274 indicated that electrical free energy provided the majority of the binding free energy
275 and the polar solvent free energy provided the majority dissociation energy. The Asp
276 215, Asp69 and Arg446 residues contributed the strongest total binding free energy. It
277 is hoped that these findings will stimulate further work into the role of erythritol in the
278 management of DM and its potential use as a therapeutic food additive.

279

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289

290 **Figure captions**

291 **Figure 1 (A)** After intragastric administration of erythritol (500 mg/Kg) and acarbose
292 (4 mg/Kg) to healthy mice, the blood glucose was monitored at different time points.

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

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

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

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

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

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

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

312

313

314 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

315 VDWAALS is total van der waals free energy. EEL is total electrical free energy.

316 EGB is total polar solvation energy. ESURF is total non-polar solvation energy.

317 DELTA G gas=VDWAALS+EEL. DELTA G solv= EGB+ ESURF. DELTA TOTAL=

318 VDWAALS+ EEL+ EGB+ ESURF.

319

320

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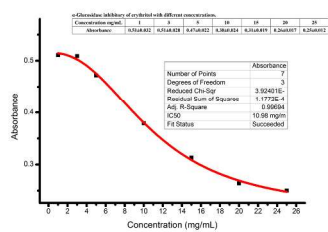
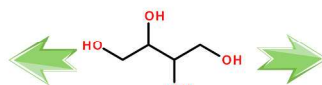
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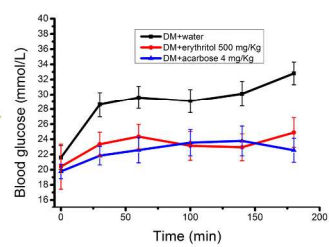
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Inhibiting α -glucosidase

Erythritol



Reducing postprandial blood glucose

Graphic abstract

244x78mm (300 x 300 DPI)

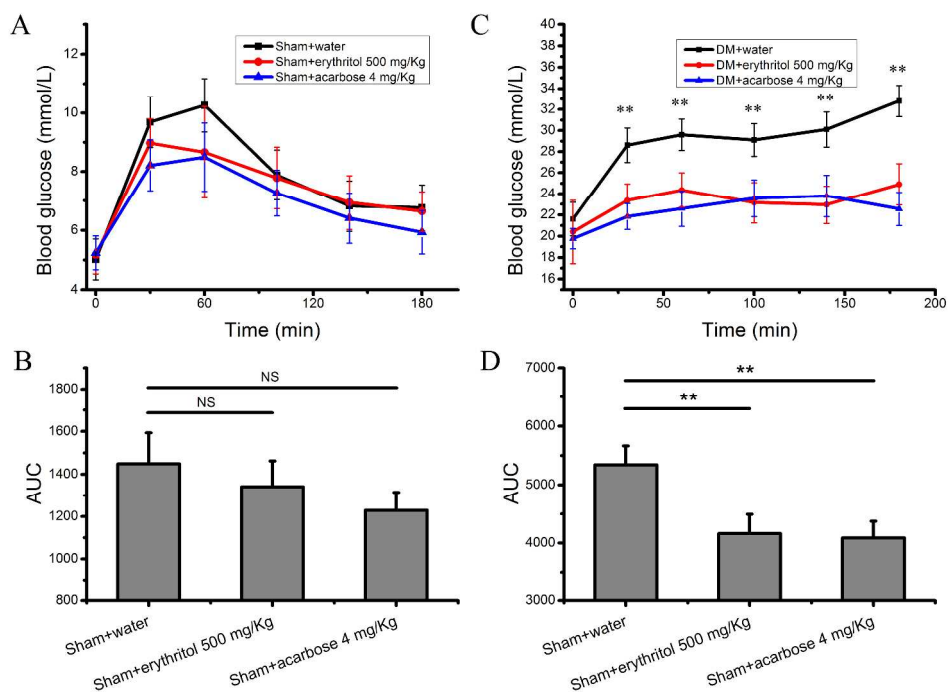


Figure 1 (A) After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to healthy mice, the blood glucose was monitored at different time points. (C) After intragastric administration of erythritol (500 mg/Kg) and acarbose (4 mg/Kg) to diabetes mellitus mice, the blood glucose 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.

584x406mm (300 x 300 DPI)

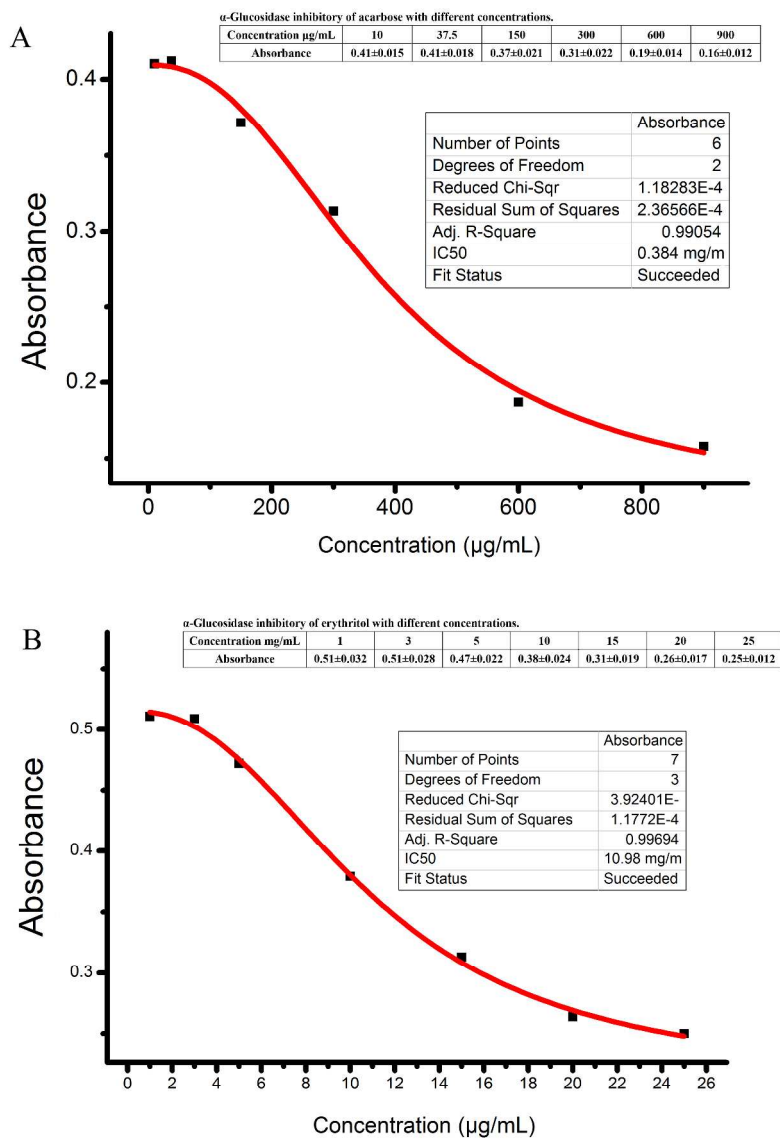


Figure 2 the inhibition data of acarbose (A) and erythritol (B) with different concentrations was fitted with logistic function to calculate the IC₅₀ value. The detailed inhibition data was at the head of each figures.

296x419mm (300 x 300 DPI)

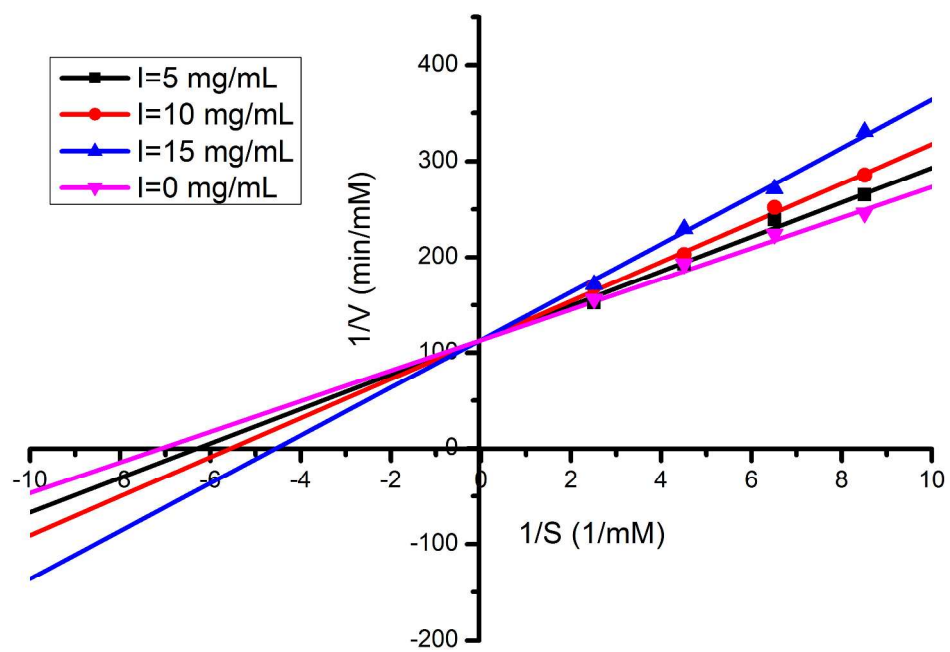


Figure 3 Lineweaver-Burk plot of erythritol against α -glucosidase at different concentrations of pNPG.

288x200mm (300 x 300 DPI)

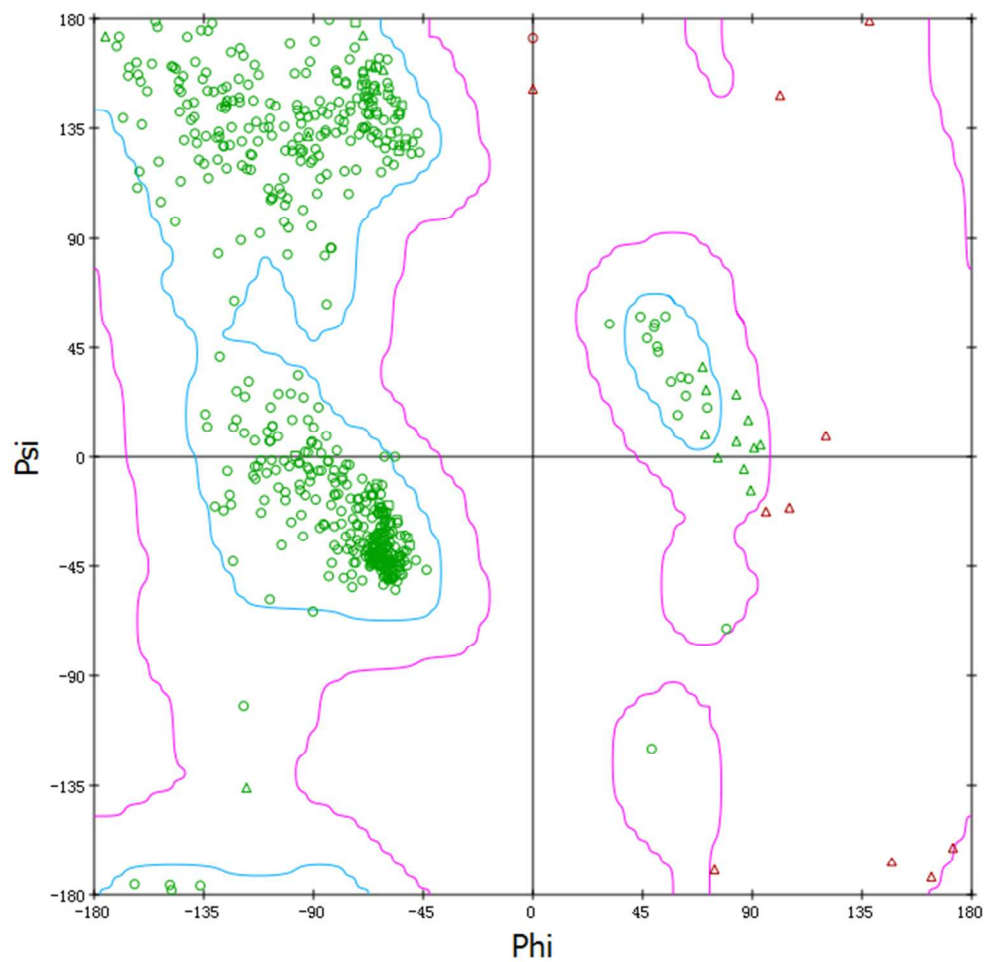


Figure 4 Ramachandran contours is used to test the quality of newly developed model. Green dots donate the position of residues in core region. While, red spots sign highlights the outliers.

183x176mm (96 x 96 DPI)

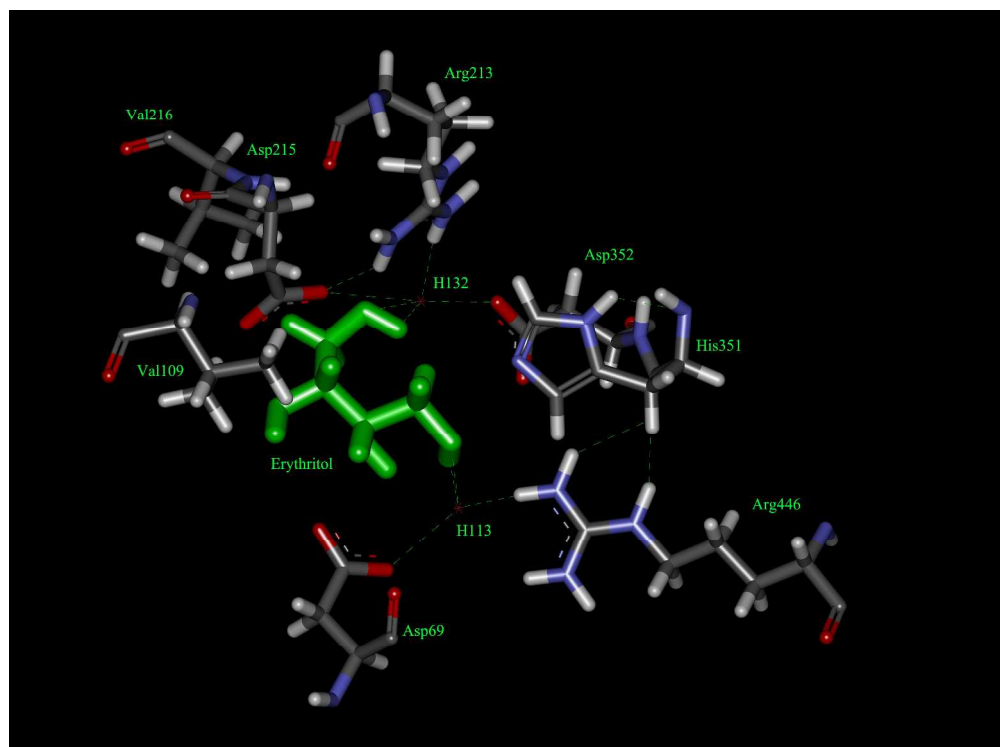


Figure 5 the interaction mode of erythritol in the catalytic active pocket of α -glucosidase with Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216.

1587x1174mm (96 x 96 DPI)

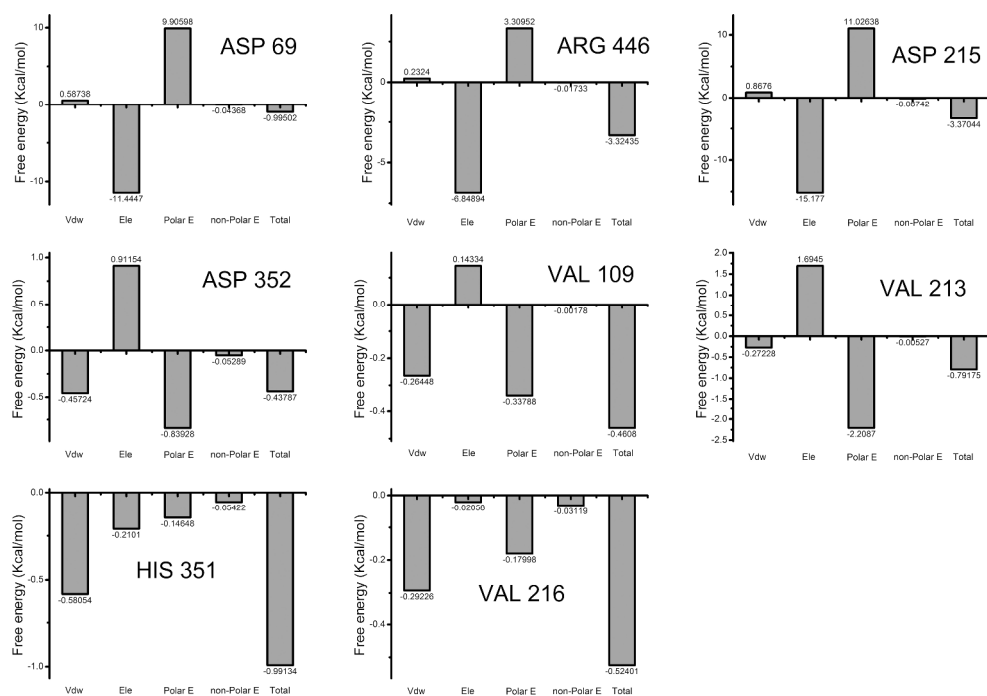


Figure 6 Decomposition of the free energy of Asp 215, Asp69, Arg446, Asp 352, Val 109, Arg213, His351 and Val216.

423x298mm (300 x 300 DPI)