SUPPLEMENTARY MATERIAL

Effect of *Agave americana* L. on the Human, and *Aspergillus oryzae* S2 α-Amylase Inhibitions

Mouna Sahnoun[1], *, Samir Bejar[1], Lobna Daoud, Leila Ayadi [2], Faiçal Brini[3] and Walid Saibi[3]

[1] Laboratory of Microbial Biotechnology and Engineering Enzymes (LMBEE), Centre of Biotechnology of Sfax (CBS), University of Sfax, Sidi Mansour Road Km 6, P.O. Box 1177, Sfax 3018, Tunisia; [2] Preparatory Institute for Engineering Studies, Sfax (IPEIS), University of Sfax, Menzel Chaker Road Km 0.5 P.O. Box 3018, Tunisia; [3] Biotechnology and Plant Improvement Laboratory Centre of Biotechnology of Sfax University of Sfax, B.P 1177, 3018 Sfax, Tunisia

Abstract: Among phenolic compounds, *Agave americana* L. extract contained puerarin (38.4 %) and *p*-coumaric acid (12.29 %). From the Lineweaver–Burk plots, *p*-coumaric acid and puerarin demonstrated a competitive and a non competitive inhibitions towards human α-amylase activity respectively. *p*-Coumaric acid exhibited a higher human inhibitory activity with an IC_{50} of 98.8µM which was about 2.3 times than acarbose. Puerarin (IC_{50}=3.87 µM) and *p*-coumaric acid (IC_{50}=10.16 µM) also showed an excellent inhibition for *Aspergillus oryzae* S2 α-amylase activity. The inhibitions of the described biocatalysts compounds towards both amylases were significantly decreased when they were pre-incubated with starch. The binding modes of these compounds were evaluated *in silico*. The binding efficiency order of these molecules in terms of polar contact numbers for both enzymes was in agreement with the *in vitro* studies. These findings provided a rational reason to establish the isolated compounds capability as therapeutic target for hyperglycaemia modulation and antifungal therapy.

Table S1

Table S1. Effect of *Agave americana* L. extract concentrations on α-amylase activities. Data expressed as means ± SD. n = 3. Means followed by the same letter within line are non-significantly different ($P < 0.05$). (1) For Human α-amylase, for (2) *Aspergillus oryzae* S2 α-amylase.

Agave americana L. extract (µL)	0	10	50	100	200	300	400
Residual activity (1) (%)	100	115.3±0.25 [b]	123.94±0.24 [d]	131.75±0.12 [e]	124±0.21 [d]	119±0.26 [c]	113±0.29 [a]
Residual after heating (100°C, 10min) (1) (%)	100	101.3±0.64 [a]	107.94±0.34 [d]	115.35±0.53 [f]	110±0.62 [e]	105±0.51 [c]	102±0.38 [b]
Residual activity (2) (%)	100	112.4±0.43 [b]	118.5±0.27 [d]	125.8±0.42 [f]	119.6±0.47 [e]	113.5±0.91 [c]	108±0.73 [a]
Residual after heating (100°C, 10min) (2) (%)	100	100.5±0.21 [a]	105.1±0.37 [b]	109.7±0.28 [d]	106.8±0.72 [c]	101.3±0.81 [a]	100.4±0.68 [a]

Human α-amylase activity (1)

Aspergillus oryzae S2 activity (2)

Table S2

Table S2. *In silico* study results of the competitive interactions of *p*-coumaric acid and puerarin towards amylases.

Distance (Å)	Types of bond	Number of interactions				
		Puerarin	Acarbose		*p*-coumaric acid	
		A. oryzae α-amylase	*A. oryzae* α-amylase	Human α-amylase	*A. oryzae* α-amylase	Human α-amylase
3,88903	Salt Bridge	1	1	0	2	0
>3,1	Attractive Charge	2	1	0	5	0
>1,7	Conventional Hydrogen	10	12	27	9	17
>2.2	Carbon Hydrogen	5	11	4	2	3
>3,6	Pi-Anion	1	2	0	1	0
>3.6	Pi-Donor Hydrogen	1	0	6	0	1
>3.5	Pi-Sigma	2	2	0	0	0
>4,7	Pi-Pi T-shaped	2	0	2	3	1
5,06773	Pi-Pi Stacked	1	0	0	1	0
>4.2	Pi-Alkyl	4	1	4	1	0
4,14937	Pi-Sulfur	0	0	0	0	0
2,40917	Pi-Lone Pair	0	0	0	0	1
Total interactions		**29**	**30**	**43**	**24**	**23**

Figure S4

Figure S4. Reverse phase HPLC chromatogram of *Agave americana* L. extract.

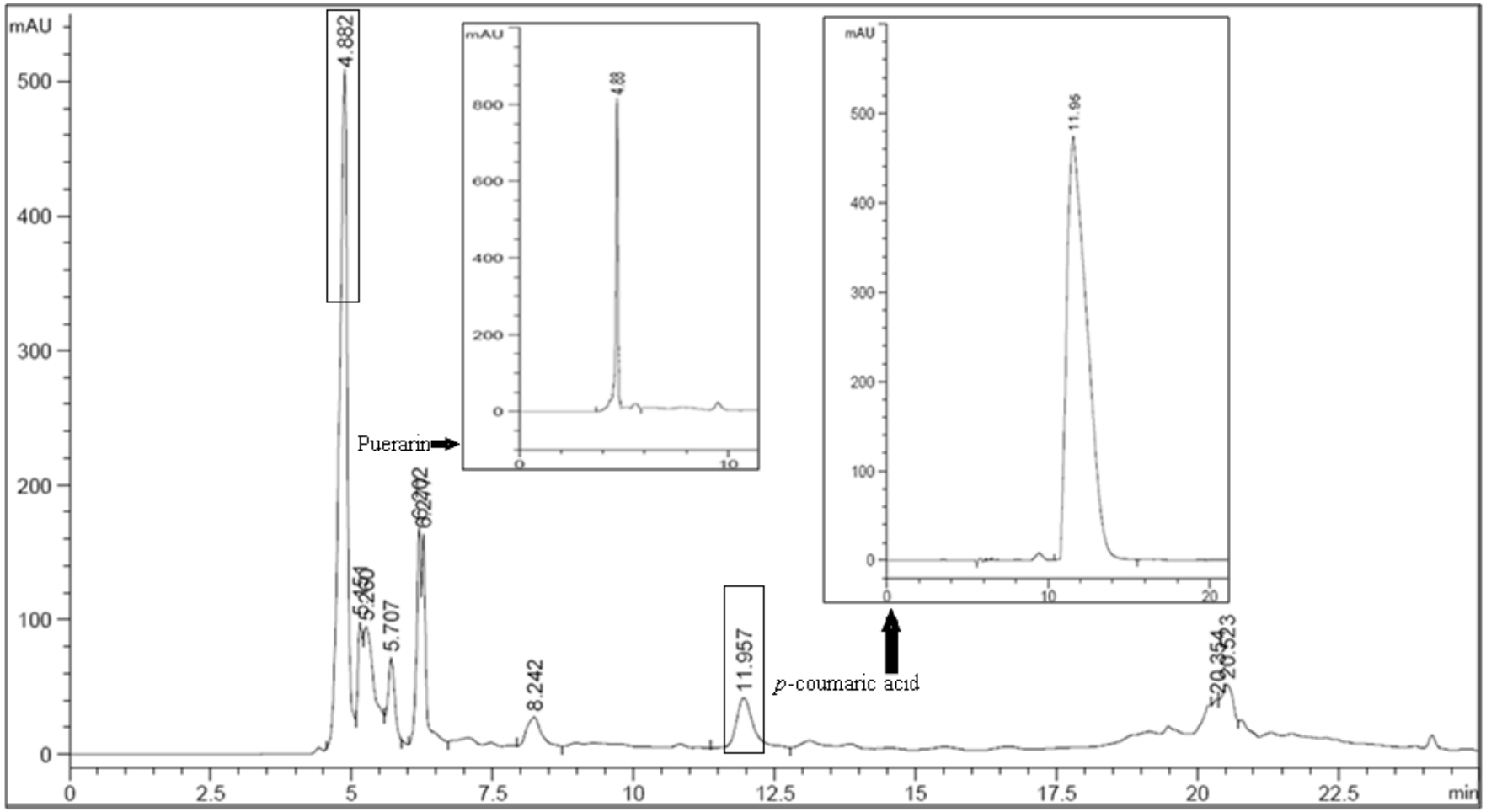


Figure S5

Figure S5. Determination of the type of inhibition observed with *p*-coumaric towards human α-amylase (A), and *Aspergillus oryzae* S2 α-amylase (B). Reverse values of activity (V) and substrate concentration ([S]) were plotted in absence of *p*-coumaric acid (filled diamond), and in presence of three different concentrations of the compound: 2µM (filled square), 30µM (filled triangle), 60µM (filled circle). The Dixon plot was used to calculate the K_i of *p*-coumaric acid towards human α-amylase (C), and *Aspergillus oryzae* S2 α-amylase (D). Reverse values of activities (V) were plotted in presence of *p*-coumaric acid ([I]) at different concentration values, with the use of four different concentrations of the substrate expressed as (mg/ml): 5 (filled diamond), 10 (filled square), 12.5 (filled triangle), and 20 (filled rectangle).

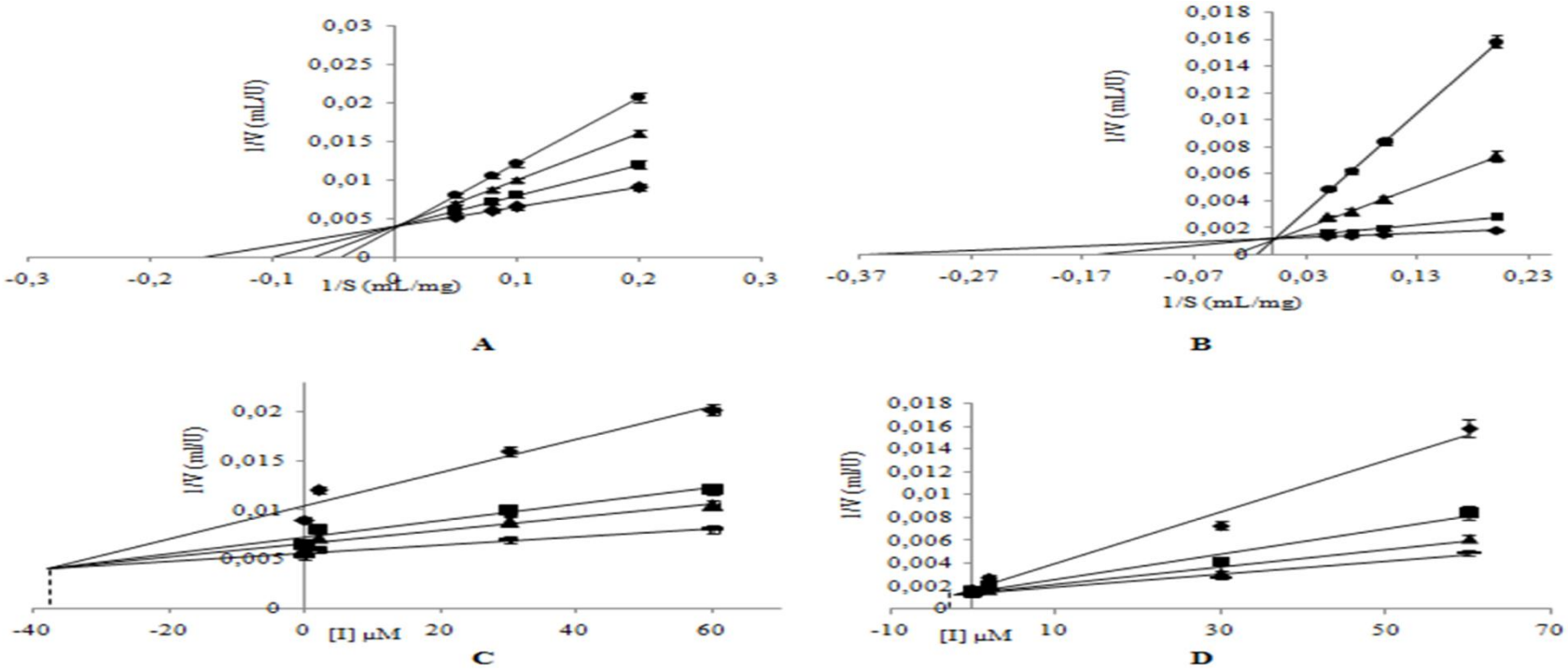


Figure S6

Figure S6. The determination of the inhibition type observed with puerarin towards human α-amylase (A), and *Aspergillus oryzae* S2 α-amylase (B). Reverse values of α-amylase activity (V) and starch concentration ([S]) are plotted in the absence of puerarin (filled square), and in the presence of four different concentrations of this compound: 6µM (filled triangle), 9µM (filled diamond), 19µM (filled circle), and 48µM (filled filled rectangle).The Dixon plot was involved to calculate the K_i of puerarin towards human α-amylase (C), and *Aspergillus oryzae* S2 α-amylase (D). Reverse values of activities (V) were plotted in presence of puerarin ([I]) at different concentration values, through the use of four concentrations of the substrate expressed as (mg/ml): 5 (filled diamond), 10 (filled square), 12.5 (filled triangle), and 20 (filled rectangle).

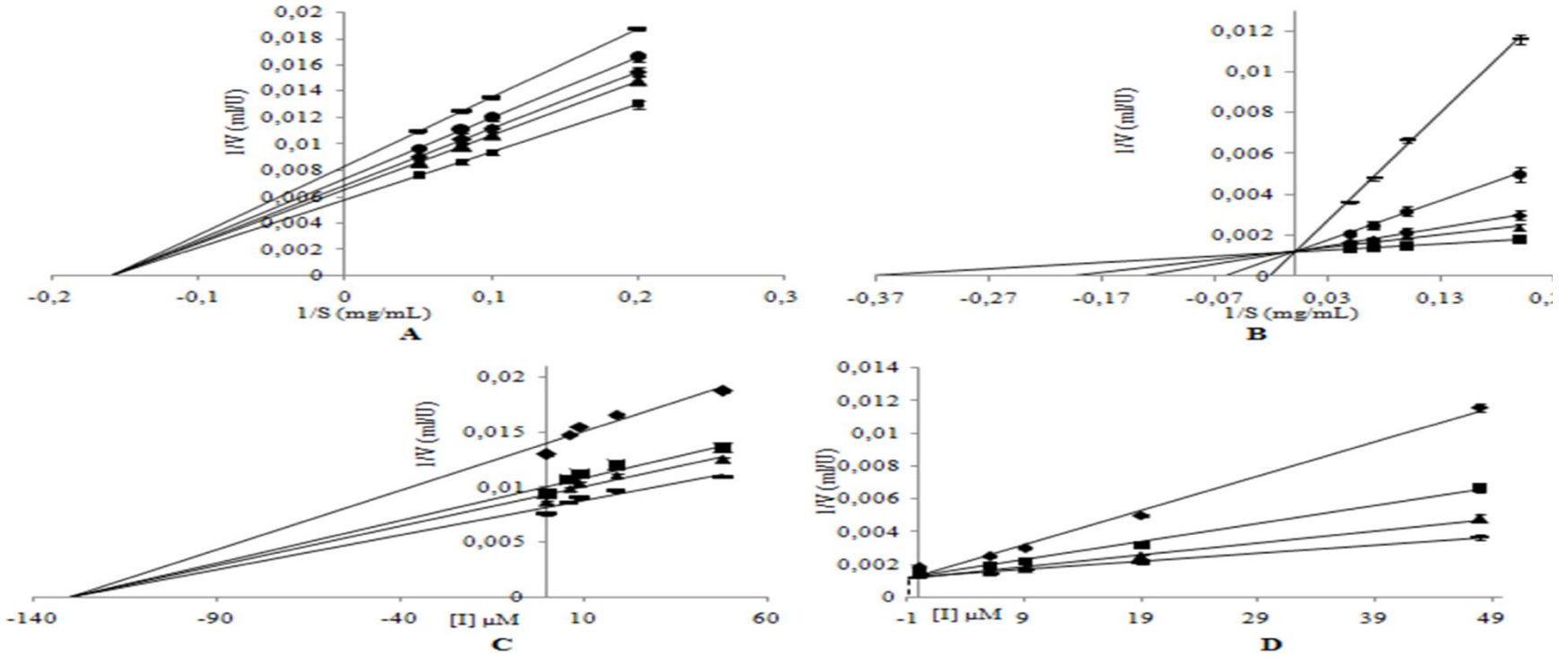
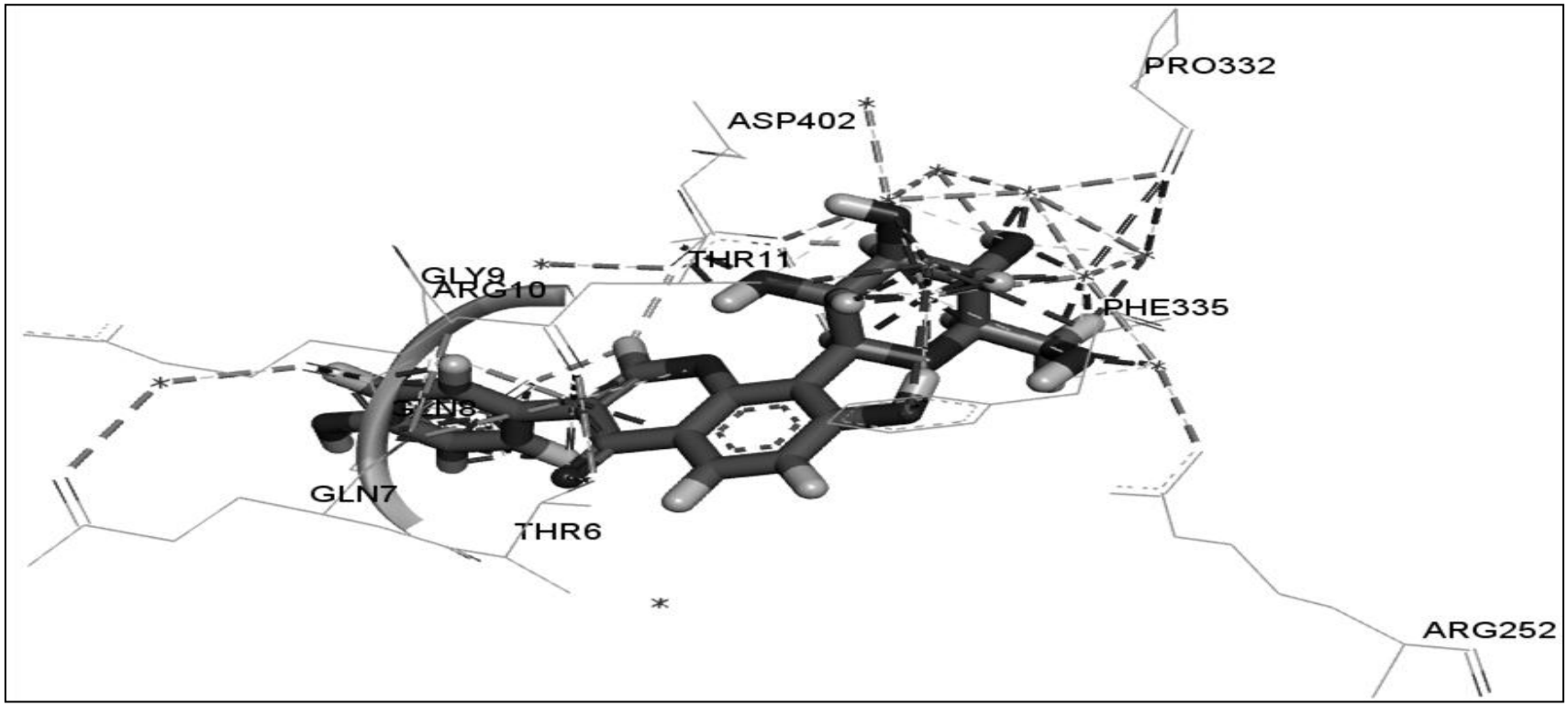
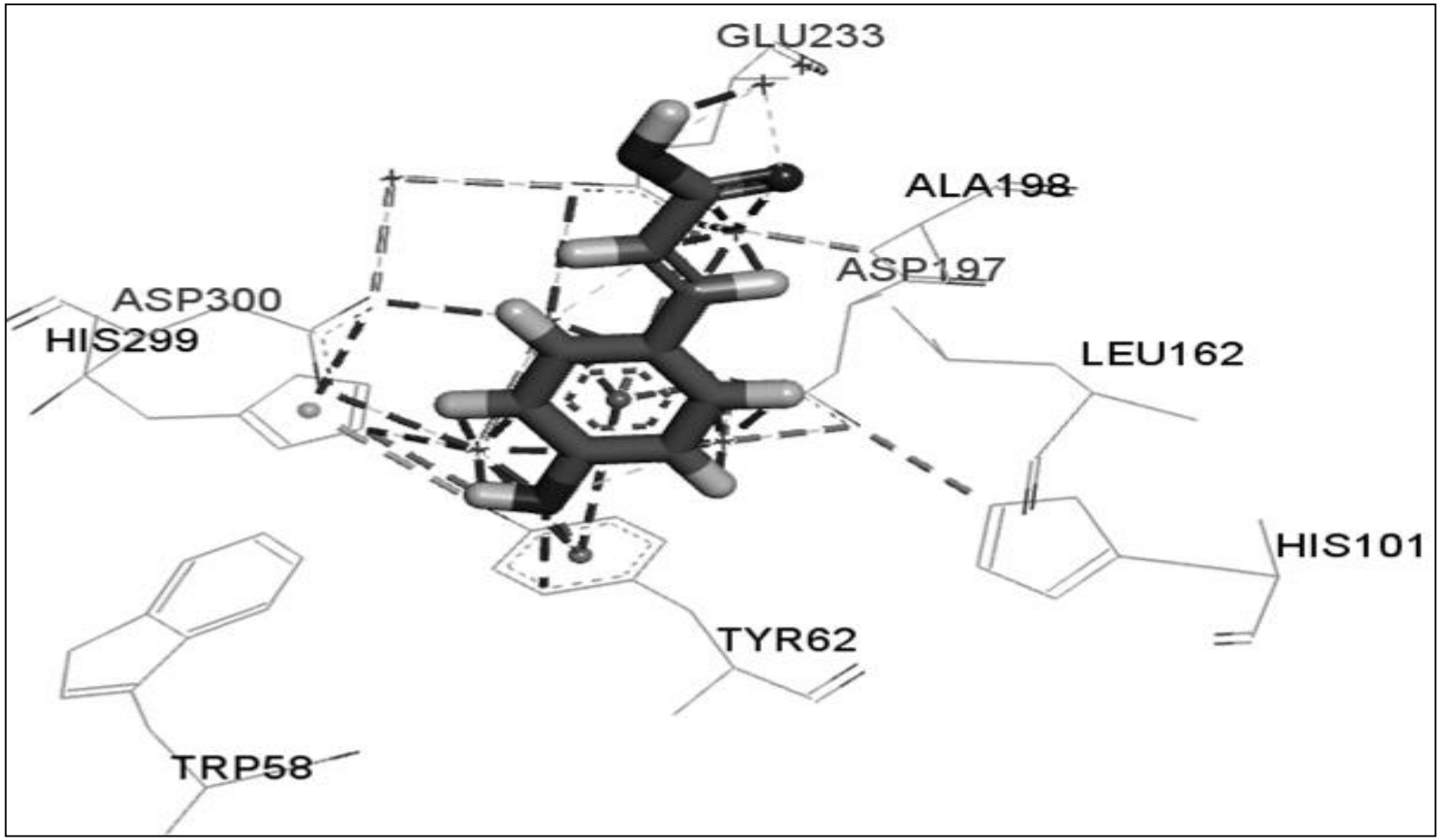


Figure S7

Figure S7. Details about the interaction between puerarin (A) *p*-coumaric acid (B) and acarbose (C) with *Aspergillus oryzae* S2 α-amylase. (For understanding of this figure, the reader is referred to Table S3).



A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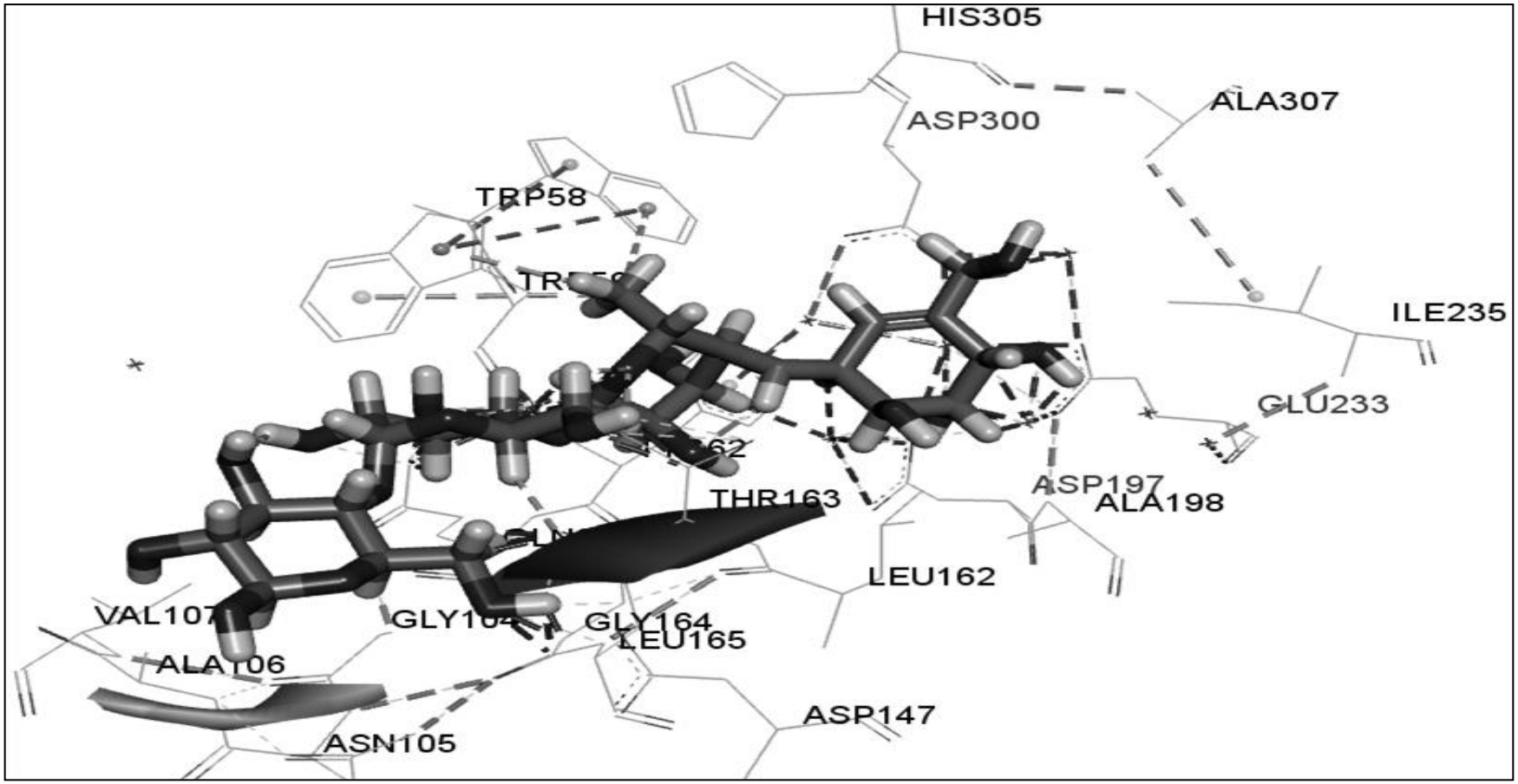
ALA198
ASP197
ASP300
HIS299
LEU162
HIS101
TYR62
TRP58

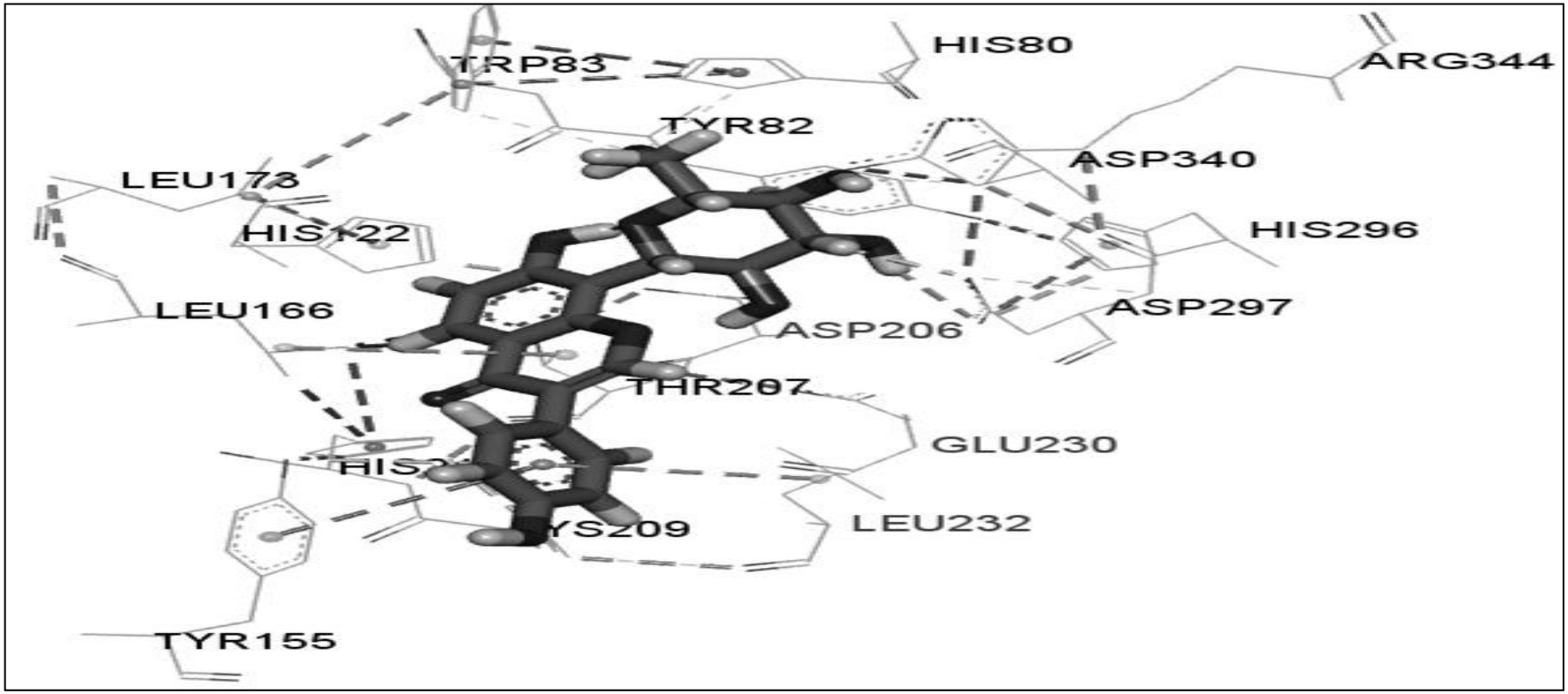
B


HIS305
ASP300
ALA307
TRP58
ILE235
GLU233
THR163
ASP197
ALA198
LEU162
VAL107
GLY164
LEU165
ALA106
ASP147
ASN105

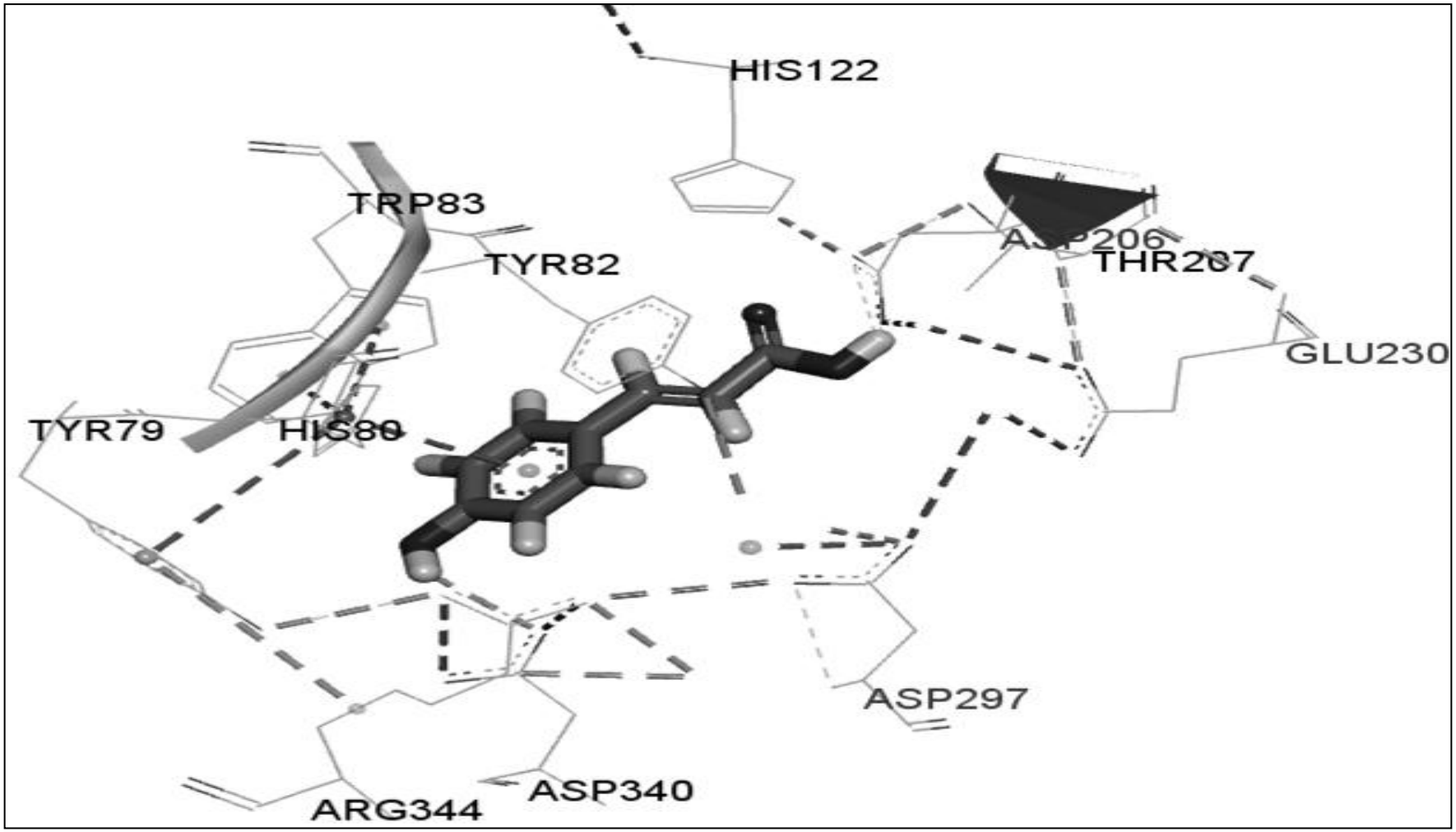
C

Figure S8

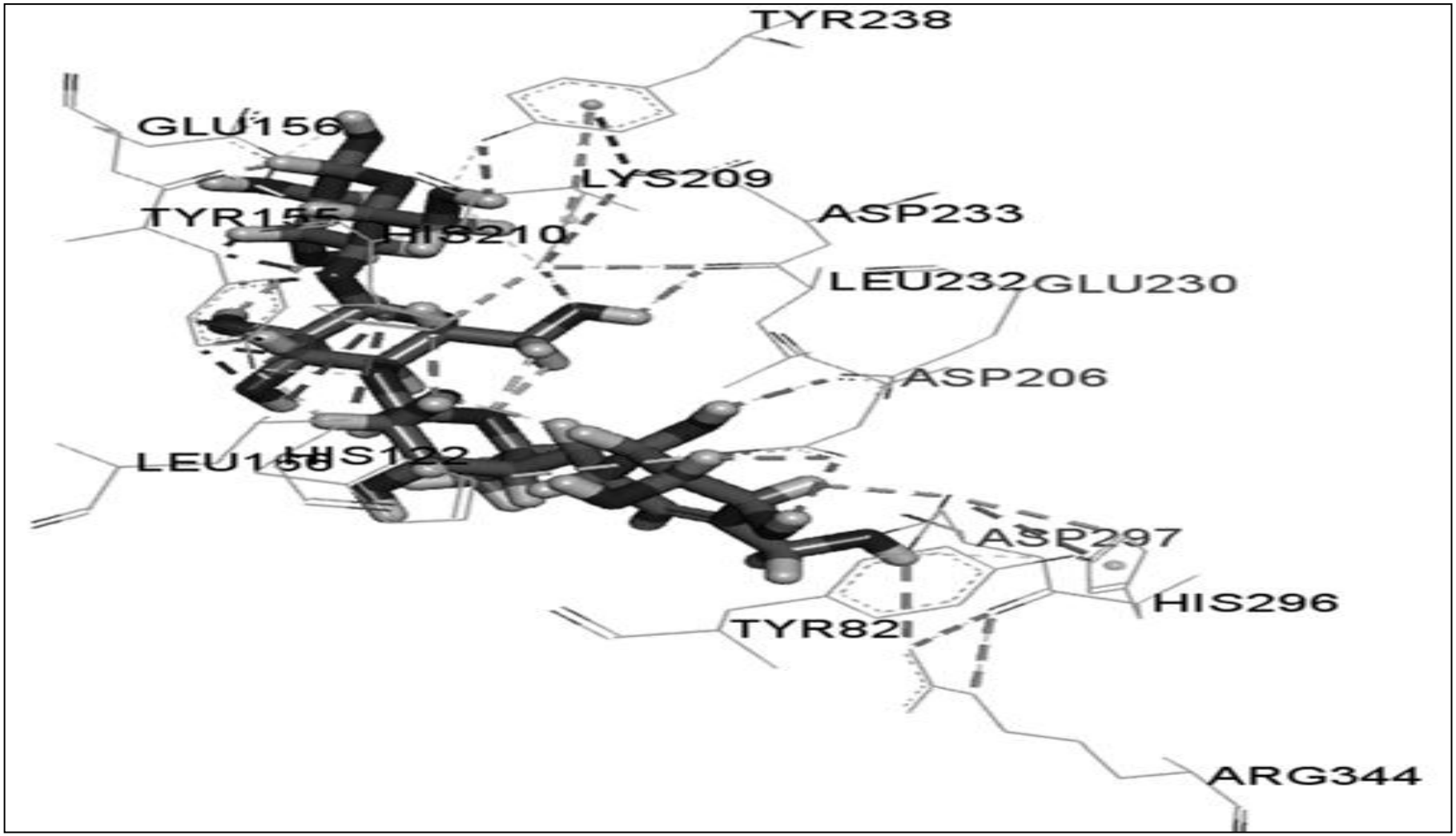
Figure S8. Details about the interaction between puerarin (A) *p*-coumaric acid (B) and acarbose (C) with human α-amylase. The binding mode was in the active site. (For understanding of this figure, the reader is referred to Table S3).



A


HIS122
TRP83
TYR82
THR207
GLU230
TYR79
HIS80
ASP297
ASP340
ARG344

B


TYR238
GLU156
LYS209
TYR155
HIS210
ASP233
LEU232
GLU230
ASP206
LEU158
HIS122
ASP297
HIS296
TYR82
ARG344

C

Experimental S9

Biological Materials and Chemical Materials

The leaves of *Agave americana* L. were obtained from Jelma State (the town of Sidi Bouzid-Tunisia). This studied plant was carefully identified under an accession number of (2016-1895*A). *Aspergillus oryzae* S2 used in this study was characterized in previous work (Sahnoun et al. 2011) as α-amylase (AmyA) producer. Human pancreatic α-amylase, puerarin, *p*-coumaric acid, and 3, 5-dinitrosalicylic acid were purchased from Sigma (St Louis, MO, USA). Puerarin and *p*-coumaric acid were dissolved in DMSO before use. The human pancreatic α-amylase was dissolved in phosphate buffer (100 mL, 0.2 M, pH 5.2).

Effect of *Agave americana* L. Extract, Puerarin, and *p*-Coumaric Acid on the α-Amylase Activity

The α-amylase inhibition assay was determined with potato starch as substrate. Potato starch solution (1% (w/v)) was dissolved in phosphate buffer (100 mL, 0.2 M, pH 5.2) and gelatinized for 20 min at 80 °C. 100 µL of the above *Agave americana* L. extract or the isolated compounds was incubated with 350 µL phosphate buffer (100 mL, 0.2 M, pH 5.2) and 50 µL of the corresponded α-amylase (100 U/mL). The tubes were incubated in a water bath at 37 °C for 20 min, and added with 500 µL 1% (w/v) potato starch solution. The reaction mixture was incubated at 37 °C for 30 min and then the reaction was stopped with 3 mL of dinitrosalicylic acid reagent. Thereafter, the mixture was boiled for 10 min and cooled to room temperature. Next, the reaction mixture was diluted by adding 10 mL of deionized water and the absorbance was measured at 550 nm (Miller 1959). The α-amylase activity was determined as U/mL, where the unit was the amount of enzyme used to release one µmoL of glucose equivalent per minute under the assay conditions.

Denatured enzyme solution was obtained by boiling native enzyme in a water bath for 15 min. Percentage of inhibition towards α-amylase was calculated according to the equation below:

% Inhibition = (A control – A control blank) – (A sample – A sample blank) / (A control –A control blank)* 100.

Where the denatured enzyme was used as blank test. Control test was done in absence of inhibitor by replacing it by DMSO at the same amount. Sample test contained native enzyme and inhibitor. IC_{50} value (half maximal inhibitory concentration) was obtained graphically by an inhibition curve.

Influence of Pre-incubation with Potato Starch Solution

The effect of incubation order on α-amylase activity was evaluated using potato starch as substrate with the same method as described above, except that 100 µL of inhibitor and 500 µL of potato starch solution were kept first at 37 °C for 20 min, and added with 50 µL of α-amylase solution and 350 µL of phosphate buffer.

Influence of *Agave americana* L. Extract, Puerarin, and *p*-Coumaric Acid Concentrations

Residual α-amylase activity in the presence of *Agave americana* L. extract at different concentrations (10, 50, 100, 200, 300, and 400µL) was determined. The detection of the inhibition mode of the compound was carried out with increasing concentrations of potato starch as a substrate (0.5, 1, 1.25, 1.5, and 2% (w/v)). The catalysis kinetics of α-amylase at different concentrations in the presence of *p*-coumaric acid (2, 3, 6, 15, 30, and 60µM) and puerarin (6, 9, 19, and 48µM) were achieved. The Michaelis–Menten kinetic model: $V = V_{max}[S]/ K_m+[S]$ was employed to evaluate the effect of the inhibitor on starch digestion, where V is the reaction rate, [S] is the concentration of the substrate, V_{max} is the maximum enzyme reaction rate, and K_m is Michaelis–Menten constant. The inhibitory constant (K_i) were determined (Dixon 1953). All the experiments were repeated three to four times.

Biochemical Analyzes by High-Performance Liquid Chromatography (HPLC) of *Agave americana* L. Extract

Leaves of *Agave americana* L. were washed, freeze dried, harvested into a juice form, mixed with ethyl acetate (3V/V), sonicated (20 min) (Sonicator FS30HFisher Scientific, USA), centrifuged (28,620 × g/30 min/20°C) and the supernatant was recovered. The solvent was discarded under vacuum (40°C) with a rotary evaporator (BÜCHI Labortechnick AG, Switzerland) and the dried *Agave americana* extract was stored at 4°C until its use. *Agave americana* L. extract was dissolved in DMSO at 400µg/mL. *Agave americana* (L.) extract was submitted to a reverse phase HPLC-DAD (Agilent, Series 1260, Waldbronn. Germany). The instrument comprises an online degasser, a quaternary pump, an auto sampler and a thermostatically controlled column compartment. The separation was achieved on a ZORBAX Eclispe XDB-C18 column serial number USNH027266 (4.6mm I.D. x 300mm x 3.5 µm particle size) at a flow rate of 0.5 mL/min. The operating temperature was fixed at 40°C. The volume Fractions were eluted from the column with two mobile phases, namely A

(100% water) and B (100% acetonitrile), as follows: from 0 to 10 min (80% A, 20% B), from 10 to 30 min (50% A, 50% B), from 30 to 40 min (100% B), from 40 to 50 min (50% A, 50% B) and from 50 to 60 min (80% A, 20% B). Fractions were monitored at 280 nm. *p*-Coumaric acid and puerarin (Sigma) used as standards were dissolved in DMSO. The reproducibility of the retention time of the selected HPLC conditions was achieved by performing repeated injections (n = 10) of the mixture of the 10 standards at the concentration of 10µg/mL. The relative standard deviation (RSD) value for every compound for all retention times showed the excellent stability and performance of the method explored.

Docking Studies

The 3D structural model of the AmyA was generated as it was previously described (Sahnoun et al. 2016). The docking was performed with Autodock vina (Trott and Olson, 2010). The three-dimensional structure of human pancreatic α-amylase (PDB: 1HNY) was imported from the Protein Data Bank. The grid box of 70 x 20 x70 was used with a spacing of 1.0 Å. The Gasteiger charges were assigned to protein and ligand molecules. Exhaustiveness was set on 20. The best pose for the ligand was obtained.

Statistical Analyses

At least three replicates were performed for each analysis. The results are presented as x ± SD, where x refers to the mean of at least three replications and SD to the standard deviation. Student's t-test was used to determine the significance of differences between means. Significant difference was considered at $p < 0.05$. The data were analyzed by SPSS for windows (Version 11.0.1, 2001, LEAD Technologies, Inc., USA).

References

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Trott O, Olson AJ. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J Comput Chem 31(2): 455-461. doi: 10.1002/jcc.21334.