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Posted Date: 11 March 2025

doi: 10.20944/preprints202503.0700.v1

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Article

Inhibition Mechanism of Sanggenone D/Kuwanon G Against α -Glucosidase and the Regulation of Glucose via GLUT4 Pathway

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Abstract: Inhibition of α -glucosidase activity is considered to be an effective treatment for type 2 diabetes. The inhibitory mechanisms of sanggenone D and kuwanon G on α -glucosidase were investigated and the pathways for hypoglycemic effects were explored in the current study. The outcomes indicated that sanggenone D (IC_{50} : 4.51×10^{-5} mol/L) and kuwanon G (IC_{50} : 3.83×10^{-5} mol/L) inhibited α -glucosidase activity by non-competition/anti-competition mixed inhibition and competitive inhibition, respectively. Besides, the secondary structure of α -glucosidase were altered by static quenching, and exhibited the decrease in α -helix, β -antiparallel content, while increase in β -sheet content. Furthermore, the interaction forces between sanggenone D/kuwanon G and α -glucosidase were hydrophobic interactions and hydrogen bonds as evidenced by molecular docking. The binding affinity, stability and binding energy aligned with the results of IC_{50} . Cyclization in sanggenone D structure resulted in a decrease in the number of phenolic hydroxyl groups and thus a reduction in the formation of hydrogen bonds, which ultimately diminished the binding affinity of sanggenone D to α -glucosidase. In addition, western blot results showed that sanggenone D and kuwanon G regulated glucose metabolism by activating of GLUT4 pathway.

Keywords: α -Glucosidase; Inhibition mechanisms; Molecular docking; Western blotting; Structure-activity relationship

1. Introduction

Diabetes mellitus is a metabolic condition resulting from impaired insulin secretion, characterized by elevated fasting blood glucose, excessive thirst, hunger, urination, and weight loss. Management of the condition generally involves ongoing medication to regulate blood sugar levels [1]. According to statistics, approximately 9 % of adults worldwide are affected by diabetes, with more than 90 % of diabetes patients are type 2 diabetics (T2DM) [2]. The clinical manifestations of T2DM are hyperglycemia and insulin resistance. When the feedback pathway of insulin action and insulin secretion is destroyed, all insulin-sensitive organs in the human body will be greatly affected, leading to the failure of synthesize glycogen with glucose normally, and resulting in increased glucose concentration in the blood and the formation of hyperglycemia [3].

Alpha-glucosidase is a key digestive enzyme that degrades starch into monosaccharides, and many compounds show their biological activities of decreasing blood glucose by inhibiting α -glucosidase [4,5]. At present, the common drugs used as α -glucosidase inhibitors include: acarbose, sitagliptin, miglitol [6]. However, many α -glucosidase inhibitors are prone to cause hypoglycemia, gastrointestinal dysfunction, increased insulin resistance, liver toxicity and other adverse side effects. As a result, the search for safe, effective and economical dietary anti-diabetic compounds is of great importance [7].

Mulberry as a valuable plant, its different parts (leaves, branches, roots) have been widely utilized for centuries in the management of diabetes, rheumatics, arthritis and other diseases [8,9]. Mulberry leaves contain polyphenolic compounds, such as flavonoids, which consist of two benzene rings connected by a heterocyclic six-membered pyrone ring. Their structure is generally believed to be closely associated with their biological activities, including antioxidant, cardiovascular protective, and anticancer effects [10,11]. Studies have shown that rats taking mulberry leaf extract for 5 weeks reduced blood sugar levels in the body by 5 %, and oral administration of 0.8 g and 1.2 g mulberry leaf powder significantly inhibited post-meal blood sugar and insulin secretion [12,13].

Mulberry resources are rich in prenylated flavonoids, such as D and G, each containing one prenyl group. Wen's research indicated that compared to non-prenylated flavonoids, prenylated flavonoids exhibit significantly enhanced activities in areas such as immunomodulation, cardiovascular protection, and improvement of metabolic diseases. Moreover, the structure-activity relationship of a single prenyl group in flavonoids shows activity ranked as follows: C-8 prenyl > C-3 prenyl > C-6 prenyl [14]. Prenylation significantly enhanced the inhibitory effects of parent compounds like quercetin, genistein, and chalcone on α -glucosidase activity [15].

Currently, there was limited research focused on the target and mechanism of inhibition of α -glucosidase by sanggenone D and kuwanon G. In this study, enzymatic kinetics, Fourier transform infrared spectra, fluorescence spectroscopy and molecular docking techniques were applied to explore the inhibitory effect and mechanism of α -glucosidase inhibition by sanggenone D and kuwanon G. Besides, the high glucose model of human hepatocellular carcinoma HepG2 cells was established to investigate the effect of sanggenone D and kuwanon G on reducing the glucose level, and the pathway of their action was explored by western blotting. The results would provide useful reference for the application of sanggenone D and kuwanon G in hypoglycemic functional food.

2. Materials and Methods

2.1. Materials

Alpha-glucosidase, acarbose and p-nitrophenyl- α -D-glucopyranoside were sourced from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China. Sanggenone D and kuwanon G were sourced from Baoji Chenguang Biotechnology Co., Ltd., Baoji, China with purity of > 98%. HepG2 cells were sourced from Yuji Biotechnology Co., Ltd., China. Trypsin, fetal bovine serum, double antibody, non-essential amino acid and High-glucose Dulbecco's modified Eagle's medium (DMEM) were sourced from Casbad, California, USA. CCK-8 kit, glucose assay kit, total cholesterol (TC) kit, triglyceride (TG) kit were sourced from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Analytical grade reagents with purity of > 99.8% were used for the experiment, along with ultra-pure water.

2.2. Inhibitory Activity

Samples of 50 μ L serial dilution concentration (sanggenone D: 50, 100, 200, 400, 600, 800 μ g/mL; kuwanon G: 50, 100, 200, 250, 300, 400 μ g/mL; acarbose: 25, 50, 75, 100, 125, 250 ng/mL) were put into a 96-well plate, then 50 μ L of 1 U/mL α -glucosidase solution (prepared in pH 6.8, 0.1 mol/L PBS) was added, and incubated at 37 °C for 10 min. Subsequently, 50 μ L of 2 mmol/L p-NPG was added, and the mixture was incubated at 37 °C for 20 minutes. Finally, 50 μ L of 1 mol/L Na₂CO₃ was added into

an ice bath for 5 min to terminate the reaction. The absorbance was measured at 405 nm and the α -glucosidase inhibitory activity was calculated according to formula (1):

$$\text{Inhibition rate of enzyme activity/\%} = \left(1 - \frac{A_a - A_b}{A_c - A_d}\right) \times 100\% \quad (1)$$

Aa is the absorbance of the sample group, Ab is the absorbance of the sample background group (equal volume of buffer instead of enzyme solution), Ac is the absorbance of the control group (equal volume of buffer instead of the sample solution), and Ad is the absorbance of the control background group (equal volume of buffer instead of the sample and enzyme solution).

2.3. Inhibition Kinetics Assay

The inhibition assay of α -glucosidase was adapted from MORTEZA with some modification [16]. 50 μ L α -glucosidase solution (1 U/mL) was added to 96-well plates, and 50 μ L sample solution with different concentrations (sangenone D: 0, 20, 100, 200 μ g/mL; kuwanon G: 0, 10, 20, 40 μ g/mL) was incubated at 37 $^{\circ}$ C for 10 min. Subsequently, different concentrations of pNPG solution were added and incubated at 37 $^{\circ}$ C for 20 min. Finally, sodium carbonate solution (50 μ L, 2 mmol/L) was added to terminate the reaction. Colorimetry was performed at 405 nm using a spectrophotometer to determine the change in absorbance of the enzymatic reaction system. The reversible inhibitory effect types of sangenone D and kuwanon G on the enzyme were analyzed by the Lineweaver-Burk equation. The relevant parameters were calculated using Formulas (2)–(4).

$$\frac{1}{V} = \frac{K_m}{V_m} \left(1 + \frac{[I]}{K_I}\right) \times \frac{1}{S} + \frac{1}{V_m} \left(1 + \frac{[I]}{K_{IS}}\right) \quad (2)$$

$$\text{Slope} = \frac{K_m}{V_m} + \frac{K_m[I]}{V_m K_I} \quad (3)$$

$$Y - \text{intercept} = \frac{1}{V_m} + \frac{[I]}{K_{IS} V_m} \quad (4)$$

In the formula, V represents the reaction rate, and Vm represents the maximum reaction rate. [I] is the concentration of the compound sample, and [S] is the substrate concentration. Km represents the Michaelis constant, and Ki and Kis represent the inhibitory constants of the compound sample on α -glucosidase and the α -glucosidase-substrate complex, respectively.

2.4. Multi-Fluorescence Spectroscopy

100 μ L of 1 U/mL α -glucosidase extract was mixed with PNPG solutions of different concentrations (sangenone D: 0, 1, 5, 10, 20, 50, 60 μ g/mL; kuwanon G: 0, 1, 5, 10, 20, 40, 60 μ g/mL) and incubated at three temperatures of 298 K, 304 K, and 310 K for 5 min, the mixture were scanned with fluorescence spectroscopy (SpectraMax i3x, Molecular Devices, LLC., CA, USA). The excitation wavelength was set at 280 nm, the emission wavelength was 300 - 500 nm, and the widths of both excitation and emission slits were 5 nm. The fluorescence quenching parameters were calculated according to the following equations.

Stern-Volmer dynamic collision quenching equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_q \tau_0 [Q] \quad (5)$$

$$\lg \frac{F_0 - F}{F} = \lg K_a + n \lg [Q] \quad (6)$$

F₀ and F represent the fluorescence intensity peaks of α -glucosidase without and with the compound, respectively; [Q] is the sample concentration; τ_0 is the protein's average lifetime (10⁻⁸ s).

2.5. FT-IR Measurements

Fourier transform infrared spectroscopy (VERTEX 7.0, BRUKER Co., Ltd., Germany) was used to detect the secondary conformation of α -glucosidase before and after sample treatment by infrared

amide I (1600 cm⁻¹-1700 cm⁻¹) type curve fitting. In the experiment, the α -glucosidase (1 U/mL) was mixed with G (100 μ g/mL) and D (150 μ g/mL) in equal volume respectively [17].

2.6. Molecular Docking

Molecular docking techniques were employed to investigate the binding conformation of small molecules and proteins, and the binding energy, binding site and binding mode of the interaction between sangenone D, kuwanon G and α -glucosidase were analyzed. The crystal structure of the α -glucosidase protein used for docking was acquired from the PDB database (PDB ID: 5NN4), while the 3D structures of the small molecules were retrieved from PubChem and subjected to energy minimization using the MMFF94 force field.

AutoDock Vina 1.1.2 software was used to perform molecular docking. Before docking, the α -glucosidase protein (EC 3.2.1.20) was processed using PyMol 2.5.2, which involved eliminating water molecules, salt ions, and small molecules. Subsequently, position the docking box with its center aligned to the centroid of the ligand in the original crystal structure. Additionally, all processed small molecules and the receptor protein were converted into PDBQT format. During docking, the global search precision was set to 32, with all other parameters set to their default values. The docking conformation with the highest score was considered the binding conformation, and the docking results were subsequently visualized and analyzed using PyMol 2.5.2. [18,19].

The small molecule-protein complex obtained through docking was used as the initial structure for all-atom molecular dynamics simulations, which were carried out using AMBER 18 software [20]. Prior to the simulation, energy optimization of the system was performed using 2500 steps of steepest descent and 2500 steps of conjugate gradient methods. During the simulation, the nonbonded cutoff distance was set to 10 Å, and the Particle Mesh Ewald (PME) method was applied to calculate long-range electrostatic interactions. The SHAKE algorithm was used to constrain hydrogen bond lengths, and the Langevin algorithm was employed for temperature control. The collision frequency (γ), system pressure, and integration timestep were set to 2 ps⁻¹, 1 atm, and 2 fs, respectively. Trajectories were saved every 10 ps for subsequent analysis.

The protein-ligand binding free energies for all systems were computed via the MM/GBSA approach [21]. MD trajectories of 90 to 100 ns were employed for the calculations in this research, with the detailed formula as follows.

$$\begin{aligned}\Delta G_{bind} &= \Delta G_{complex} - (\Delta G_{receptor} + \Delta G_{ligand}) \\ &= \Delta E_{internal} + \Delta E_{VDW} + \Delta E_{elec} + \Delta G_{GB} + \Delta G_{SA}\end{aligned}\quad (7)$$

In formula (7), $\Delta E_{internal}$, ΔE_{VDW} and ΔE_{elec} represent internal energy, van der Waals action and electrostatic interaction, respectively. Internal energy includes E_{bond} , E_{angle} and $E_{torsion}$. ΔG_{GB} and ΔG_{SA} are collectively referred to as the free energies of solvation. G_{GB} is the free energy of polar solvation and G_{SA} is the free energy of non-polar solvation.

2.7. Cell Experiment

2.7.1. HepG2 Cell Cultivation

DMEM complete medium was configured according to 88 % DMEM high glucose medium, 10 % fetal bovine serum, 1 % double antibiotics and 1 % non-essential amino acids. HepG2 cells were seeded in the complete medium and incubated at 37 °C with 5 % CO₂. When the cell confluence grew to 80 % - 90 %, the passage was carried out at a ratio of 1:3. According to the method of Cao et al., hyperglycemic model was established through insulin induction [22].

2.7.2. Toxicity Test of HepG2 Cells

HepG2 cells in logarithmic growth stage were inoculated with 4×10^4 cells per well in a 96-well plate and cultured at 37 °C and 5 % carbon dioxide for 24 h. After the cell coverage reached 80 %, 100 μ L of sangenone D and kuwanon G with different concentrations were added for 24 h. Then, 100 μ L

CCK-8/ complete culture solution (1:9) was added to each well, and the absorbance at 450 nm was measured by enzymometer after 1 h culture.

$$\text{Cell survival rate (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \% \quad (8)$$

2.7.3. Determination of Cellular Glucose, TC and TG Content

The sample concentration used in this experiment was determined by CCK8 experiment. HepG2 cells were inoculated with 2×10^5 in a 12-well plate and cultured for 24 h. After being attached to the wall, the samples containing different concentrations were replaced and the cells were treated for 24 h. The relevant indexes were determined by kit method according to the instructions of the manufacturer.

2.7.4. Western Blotting

The tissue proteins were extracted with cold total protein extraction buffer (TPEB) and protein concentration was determined by bicinchoninic acid assay (BCA) method. 20 μg of protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was washed with PBST for 5 min, then incubated with 5% skim milk at room temperature for 1 h. Subsequently, the membrane was cleaned with PBST five times for 5 min and incubated overnight at 4°C with the primary antibody. Finally, the membrane was incubated with the secondary antibody at room temperature for 1 h.

2.8. Statistical Analysis

The experiments were conducted in triplicate, and data analysis was performed with Graphpad software (GraphPad Software Co., Ltd., Santiago, CA, USA). One-way ANOVA was used to assess differences, with statistical significance set at $p < 0.05$.

3. Results and Discussion

3.1. α -Glucosidase Inhibition Rate

The properties of essential groups in enzyme molecules are changed by the influence of certain substances, resulting in the reduction or loss of enzyme activity called inhibition. As shown in Figure 1, the inhibition rate of α -glucosidase by acarbose (positive control), sangenone D and kuwanon G increased with higher compound concentration in a dose-dependent manner. The semi-inhibitory concentration values of acarbose, sangenone D and kuwanon G were 3.10×10^{-7} mol/L, 4.51×10^{-5} mol/L and 3.83×10^{-5} mol/L, respectively, which indicated that kuwanon G had higher inhibitory activity on α -glucosidase than sangenone D, while lower than acarbose. Although there is a certain gap compared to positive drugs, the activity is relatively strong when compared with other reported natural product compounds. For example, the IC_{50} of ferulic acid for α -glucosidase was 0.866 mg/mL [23], and the IC_{50} of the dodecyl-acylated derivatives of phlorizin and polydatin for α -glucosidase were 55.10 and 70.95 μM , respectively [24]. Compared with kuwanon G, sangenone D was cyclized, which weakened the activity of isopentenyl group and reduced one phenolic hydroxyl group, resulting in less hydrogen bonding with amino acid residues at the active site of α -glucosidase, herein, the decreased inhibitory activity, which was also verified in subsequent molecular docking simulations [25].

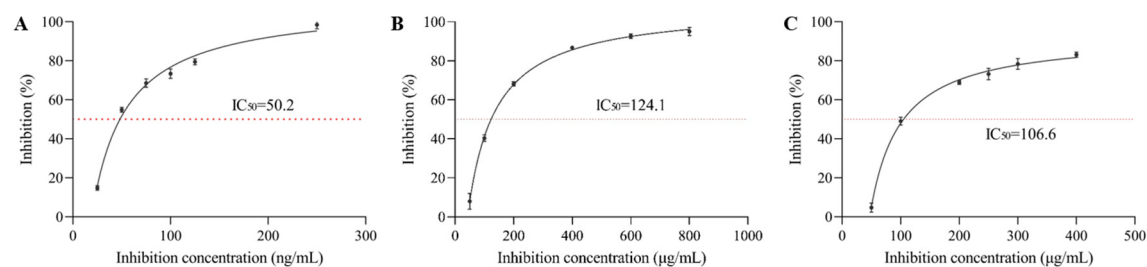


Figure 1. The inhibitory effect of different concentrations of (A) acarbose, (B) sangenone D and (C) kuwanon G on α -glucosidase activity.

3.2. Inhibition Type on α -Glucosidase Activity

The inhibition type of α -glucosidase was determined according to the intersections in the Lineweaver Burk diagram [26]. The Lineweaver-Burk curves of sangenone D and kuwanon G with α -glucosidase were exhibited in Figure 2. The double reciprocal curves of different concentrations of sangenone D intersected in the third phase quadrant. The slope of the line increased with the increase of sangenone D concentration, while both K_m and the V_m decreased, which indicated the mixed inhibition of non-competitive and anti-competitive [27]. The double reciprocal curves of kuwanon G intersected in the first quadrant of the phase diagram. With the increase of kuwanon G concentration, the K_m value increased while the V_m value decreased, which represented the competitive inhibition type [17]. The curves in the Figure 2 showed a strong linear correlation, suggesting that the two phenolic compounds interact with α -glucosidase through one or a group of binding sites [28]. The reason for the different inhibition types might be that cyclization lead to a change from a single competitive inhibition to a mixed inhibition, which weakened its inhibitory effect. However, due to little change in the overall structure, cyclization does not lead to a change in the number of inhibitory sites, which was still one or a group of binding sites. According to Table 1, the K_{is} values of sangenone D and kuwanon G were lower than K_i values, suggesting that their interaction with α -glucosidase-substrate complex was weaker than α -glucoglycinase-substrate complex [29].

Table 1. K_m , V_m , K_i and K_{is} values of the compounds inhibiting α -glucosidase activity.

	concentration ($\mu\text{g/mL}$)	K_m	V_m	K_i	K_{is}
Sangenon D	0	78.49	5.02	252.95	9.86
	20	17.18	1.00		
	100	1.57	0.071		
	200	0.89	0.027		
Kuwanon G	0	1.73	0.27	83.46	50.89
	10	2.17	0.29		
	20	5.68	0.63		
	40	15.98	1.49		

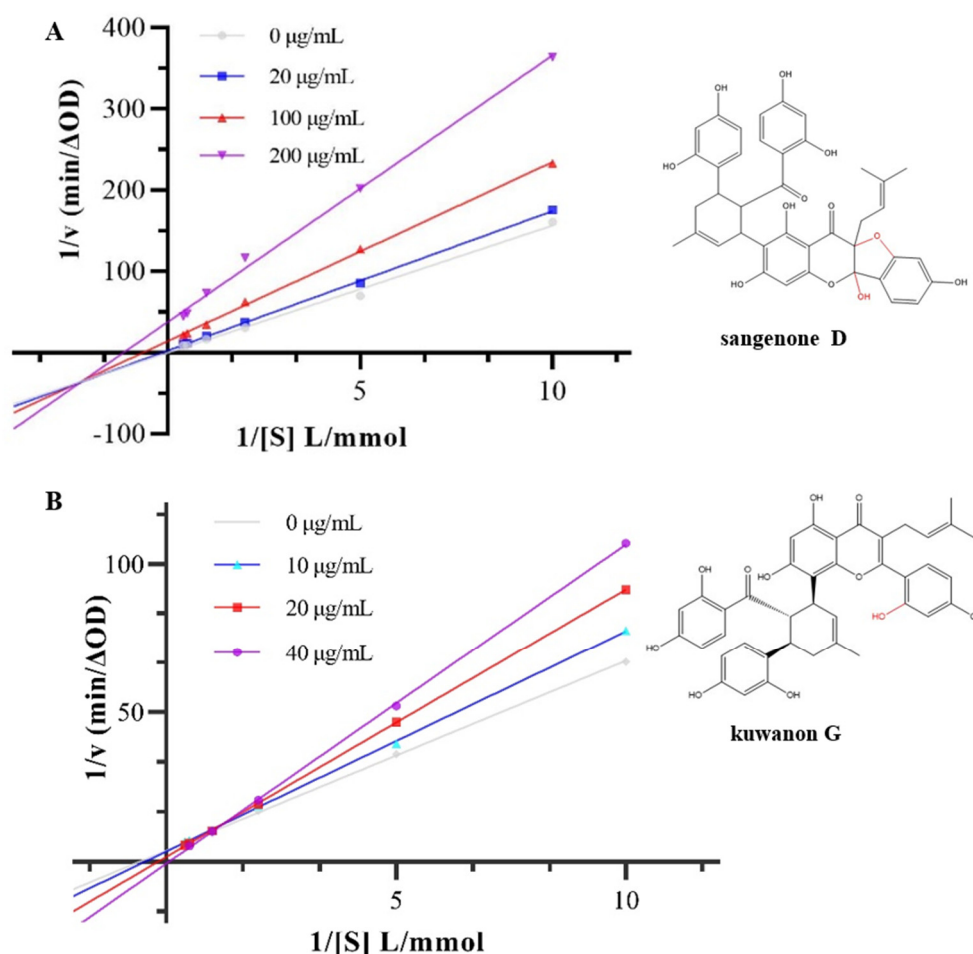


Figure 2. Inhibition kinetic of hyperoside on α -glucosidase by Lineweaver-Burk plots (A) sangenone D, (B) kuwanon G.

3.3. Fluorescence Spectroscopy Analysis of Binding Mechanism and Properties

The aromatic amino acids in α -glucosidase produced endogenous fluorescence when exposed to a specific excitation wavelength. The fluorescence intensity together with the position of the maximum emission wavelength were affected by the enzyme folding state and the environment surrounding the aromatic amino acid residues [30]. As shown in Figure 3A,B, under 280 nm excitation, the α -glucosidase solution displayed notable fluorescence between 300 and 500 nm, with the peak occurring at 340 nm. The fluorescence intensity of α -glucosidase declined progressively as the concentration of sangenone D and kuwanon G increased, and the maximum emission wavelength of the mixed solution shifted blue with regularity. These results indicated that sangenone D and kuwanon G could quench the endogenous fluorescence of the enzyme by interacting with α -glucosidase.

Fluorescence quenching occurs in two forms: static quenching and dynamic quenching. In static quenching, non-fluorescent products are formed through the interaction of quenchers with fluorophores, with the quenching constant decreasing as temperature rises. Dynamic quenching occurs when excited fluorescent molecules collide with quenchers, leading to a decrease in fluorescence intensity, and its quenching constant increases with rising temperature [31]. The type of Stern-volmer quenching curve, the change of static quenching constant at different temperatures and the apparent quenching rate constant are the important basis for judging the quenching type [32]. The Stern-Volmer quenching curve exhibited a nice linear correlation when F_0/F is plotted against $[Q]$, and the quenching constant (K_{sv}) value decreases as the temperature increases. Moreover, the

K_{sv} value aligned with the α-glucosidase inhibitory activity, following the order: kuwanon G > sangenone D (Figure 3C,D). In addition, the quenching rate constant (K_q) values for both compounds exceeded the maximum diffusion-controlled quenching constant of 2.0×10^{10} L/(mol·s), illustrating that the quenching of α-glucosidase by sangenone D and kuwanon G were of the static quenching type [33]. As shown in Table 2, K_a value was negatively correlated with temperature, which was consistent with the change of K_{sv}, indicating that sangenone D, kuwanon G and α-glucosidase form an unstable complex, and the binding force of the interaction between the two was destroyed by the increase of temperature, and the stability of the complex decreased with the increase of temperature, which accorded with the general characteristics of static quenching type. In addition, at the experimental temperature, the value of n was close to 1, which could be inferred that there was only one binding site on the α-glucosidase for sangenone D and kuwanon G.

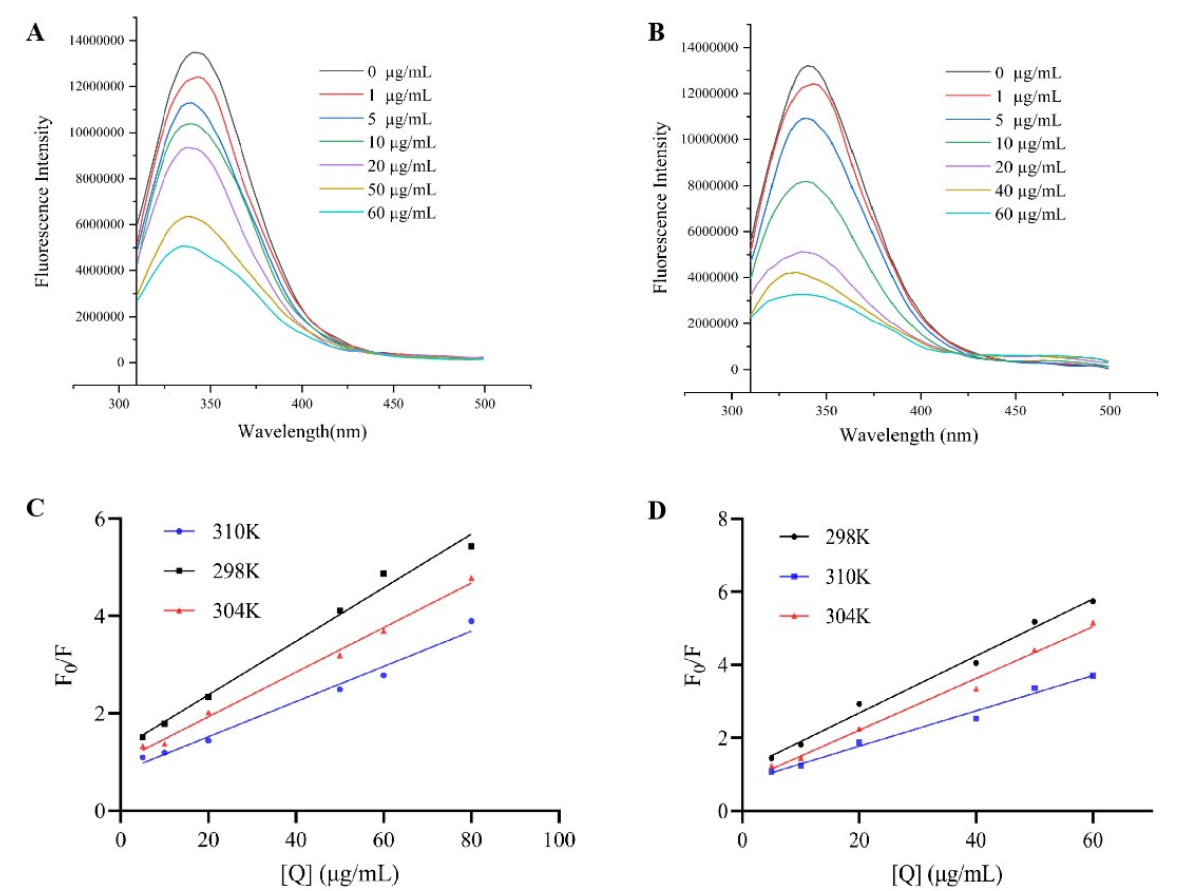


Figure 3. The effect of different concentration compounds on fluorescence spectra (A) sangenone D, (B) kuwanon G; Stern-Volmer plots indicating α-glucosidase fluorescence quenching at different temperature (C) sangenone D, (D) kuwanon G.

Table 2. K_{sv}, K_q, K_a and n of compounds on α-glucosidase at three temperatures.

	T(K)	K _{sv} (×10 ⁴ L/mol)	R _a	K _q (×10 ¹² L/mol)	K _a (×10 ³ L/mol)	n	R _b
Sangenon D	298	3.90 ± 0.04	0.98	3.90 ± 0.04	7.28 ± 0.21	0.81	0.98
	304	3.24 ± 0.02	0.99	3.24 ± 0.02	16.73 ± 0.14	0.93	0.99
	310	2.56 ± 0.02	0.98	2.56 ± 0.02	180.18 ± 0.30	1.22	0.98
Kuwanon G	298	5.41 ± 0.01	0.99	5.41 ± 0.01	36.85 ± 0.17	0.96	0.99
	304	4.91 ± 0.03	0.99	4.91 ± 0.03	194.98 ± 0.42	1.15	0.99
	310	3.35 ± 0.01	0.98	3.35 ± 0.01	1984.27 ± 0.33	1.43	0.98

Note: R_a is the correlation coefficient for the K_{sv} value. R_b is the correlation coefficient for the K_a value.

3.4. FT-IR Analysis

The secondary structure of the protein was altered upon interaction with phenolic compounds [34]. The effects of sangenone D and kuwanon G on the secondary conformation of α -glucosidase were analyzed using FT-IR spectroscopy. Because the amide I band produced by the C=O double bond stretching vibration is more responsive to changes in protein secondary structure than the amide II band, this study mainly used the amide I band to quantitatively analyze the change of α -glucosidase secondary structure [35]. As shown in Figure 4A, after adding sangenone D and kuwanon G to α -glucosidase, the peak position of the amide I band moved left from 1656 cm^{-1} to 1639 cm^{-1} and 1637 cm^{-1} , respectively. Besides, The free α -glucosidase contained 26.32% α -helix, 20.96 % β -sheet, 19.02 % β -turn, 9.53 % β -antiparallel, and 24.18 % random coil, respectively (Figure 4B). With the addition of sangenone D and kuwanon G, α -helix of the α -glucoglycase complex was reduced to 15.20 %, 19.46 %, β -antiparallel was reduced to 2.67 %, 2.17 %, while β -sheet was increased to 39.16 %, 36.82 %, respectively. These findings indicated that the secondary conformation of α -glucosidase was altered by sangenone D and kuwanon G (Figure 4C,D). The secondary structure around the catalytic site of α -glucosidase was crucial for maintaining enzyme activity stability [36]. With the addition of inhibitors, α -helix content dropped significantly and β -sheet content rose obviously, which were speculated to relate to the decrease of α -glucosylase activity. The effect of sangenone D and kuwanon G on secondary structure was different obviously, which might be the reason for their different inhibitory activities on α -glucosidase.

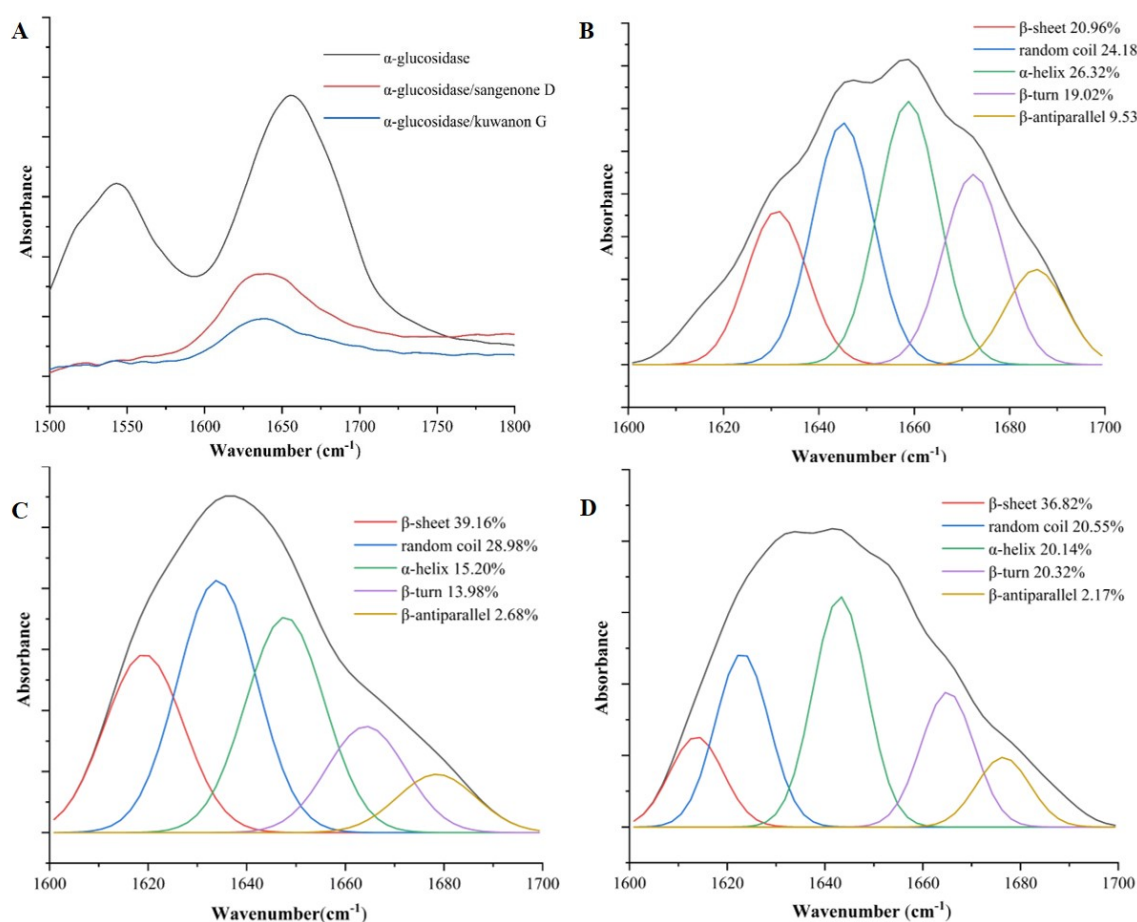


Figure 4. The FT-IR spectra of α -glucosidase-inhibitor(A).The effect of sangenone D and kuwanon G on the secondary conformation of α -glucosidase. (B) α -glucosidase black control, (C) sangenone D, (D) kuwanon G.

3.5. Molecular Docking

3.5.1. Docking Mode Analysis

To further investigate the inhibition mechanism and exacted binding site on α -glucosidase, sangenone D and kuwanon G were molecular-docked with α -glucosidase, respectively. The negative binding affinity indicated the spontaneous binding between the compound and α -glucosidase, with a larger absolute value indicating a stronger binding force and improved docking effect [37]. In this study, the values of binding affinity of sangenone D/ α -glucosidase and kuwanon G/ α -glucosidase were -7.73 and -7.94 kcal/mol, respectively, indicating that kuwanon G had a better inhibitory effect on α -glucosidase, which was consistent with the previous IC_{50} results.

As demonstrated in Figure 5, sangenone D and kuwanon G attach to the active pocket inside the α -glucosidase protein. As could be observed from the mutual detail map, the binding sites of sangenone D complex were PRO-253, VAL-230, ALA-327, PRO-326, PRO-131, VAL-236, PH-90, PRO-85, VAL-84, PH-129, ASN-233, SER-251, SER-88, and SER-325 amino acid construction. Among them, there was hydrophobic interaction with PRO-253, VAL-230, ALA-327, PRO-326, PRO-131, VAL-236, PH-90, PRO-85, VAL-84 and PH-129, and hydrogen bonding with PH-129 and ASN-233 (Figure 5A). In the same way, the binding sites of kuwanon G complex are VAL-230, PRO-253, LEU-252, PRO-326, PRO-85, VAL-84, VAL-236, PH-90, ALA-237, PRO-238, PRO-130, PRO-131, SER-325, and SER-88 amino acid construction. Among them, hydrophobic effects were observed between kuwanon G and VAL-230, PRO-253, LEU-252, PRO-326, PRO-85, VAL-84, VAL-236, PH-90, ALA-237, PRO-238, PRO-130, and PRO-131, and hydrogen bonding occurred with SER-325 and SER-88 at the site (Figure 5B). These interactions were the primary factor that ensures the stable combination between small molecules and α -glucosidase. In a word, these results indicated that the compounds interacted with the amino acid residues of α -glucosidase, thereby occupying the active site and inhibiting the activity of the enzyme. Besides, the hydrophobic forces and hydrogen bonds were the main driving forces of the interaction [38].

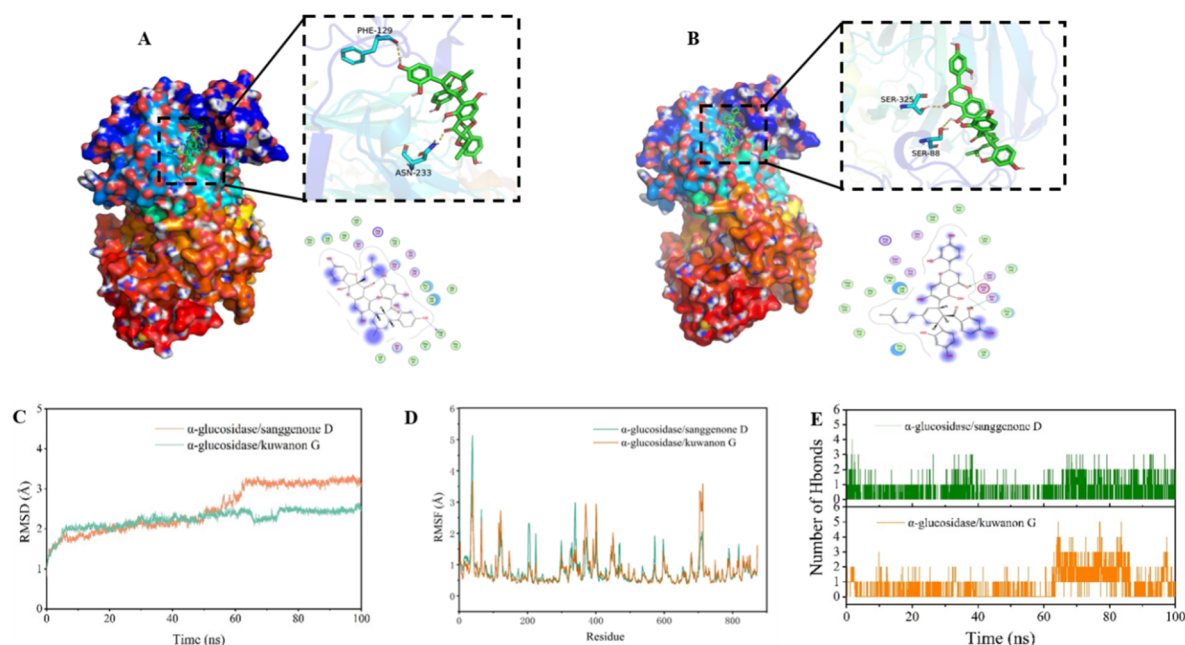


Figure 5. 3D binding diagram and molecular dynamics simulation of α -glucosidase with sangenone D and kuwanon G. (A) sangenone D, (B) kuwanon G, (C) Root mean square deviation (RMSD), (D) Root mean square fluctuation value (RMSF) based on molecular dynamics simulation, (E) number of hydrogen bond.

3.5.2. MD Simulation

The root mean square deviation (RMSD) in molecular dynamics simulations indicates the movement of the complex. A higher RMSD with larger fluctuations suggest more intense motion, while a lower RMSD indicate less movement [39]. As shown in Figure 5C, RMSD fluctuated less during the simulation of α -glucosidase/sanggenon D and α -glucosidase/kuwanon G complexes, which meant that the complex structure did not disintegrate, indicating a high stability in the binding of the small molecules to the protein. Additionally, the fluctuation of α -glucosidase/kuwanon G was relatively small, suggesting that kuwanon G bound more stably to the protein with better inhibition effect, which was consistent with the results of IC₅₀.

In molecular dynamics simulations, the root mean square fluctuation (RMSF) indicated the changes in protein flexibility [40]. After binding to the protein, the drug typically reduces the protein’s flexibility, so as to stabilize the protein and play the role of inhibiting enzyme activity. As illustrated in Figure 5D, apart from the local region of the protein, the RMSF of the protein was less than 2 angstroms, indicating that the main structural rigidity of α -glucosidase was very high, which might be due to the binding effect of the enzyme and the small molecules sanggenon D and kuwanon G with inhibitory effects.

Hydrogen bonding was among the strongest types of non-covalent interactions. The hydrogen bond count between ligand molecules and proteins was monitored over 100 ns during the molecular dynamics simulations. As depicted in Figure 5E, the number of hydrogen bonds formed by α -glucosidase/sanggenon D and α -glucosidase/kuwanon G in the simulation process ranges from 0 to 6. Most of the α -glucosidase/sanggenon D data were concentrated within 1-2, while most of the α -glucosidase/kuwanon G complex data were concentrated within 2-3. In general, the inhibitory activity of flavonoids on α -glucosidase was correlated with the quantity and location of hydroxyl functionalities in the compounds, which in turn affects the number of hydrogen bonds [33]. According to the present result, kuwanon G formed more hydrogen bonds with the α -glucosidase than sanggenon D, which contributed to understanding why kuwanon G has a better inhibitory effect than sanggenon D.

3.5.3. MM-GBSA Result

The binding energy was computed using the MM-GBSA method based on molecular dynamics simulation trajectories, offering a more precise assessment of the interaction between small molecules and target proteins. As shown in Table 3, the combined energies of α -glucosidase/sanggenon D and α -glucosidase/ kuwanon G were -9.83 ± 2.44 and -13.83 ± 2.94 kcal/mol. A negative number indicated that both molecules had an affinity for the target protein, and a lower value for α -glucosidase/kuwanon G complex represented a stronger binding than α -glucosidase/sanggenon D. In addition, the main contribution of α -glucosidase/sanggenon D and α -glucosidase/ kuwanon G combination were van der Waals energy.

Table 3. Binding free energies and energy components predicted by MM/GBSA (kcal/mol).

System name	α -glucosidase/sanggenon D	α -glucosidase/ kuwanon G
ΔE_{vdw}	-22.55 ± 4.19	-26.16 ± 2.43
ΔE_{elec}	109.95 ± 4.95	59.73 ± 5.51
ΔG_{GB}	-94.59 ± 3.78	-44.70 ± 6.19
ΔG_{SA}	-2.63 ± 0.51	-2.69 ± 0.26
ΔG_{bind}	-9.83 ± 2.44	-13.83 ± 2.94

ΔE_{vdw} : van der Waals energy. ΔE_{elec} : electrostatic energy. ΔG_{GB} : electrostatic contribution to solvation. ΔG_{SA} : non-polar contribution to solvation. ΔG_{bind} : binding free energy.

3.6. CCK-8 Assay

Cell survival assay is essential for assessing sample concentration and cytotoxicity. The cytotoxicity test results of HepG2 treated with sanggenon D and kuwanon G are shown in Figure 6. When the concentrations of sanggenon D and kuwanon G were less than 100 and 80 $\mu\text{g/mL}$, respectively, the survival percentage of HepG2 cells remained above 90 %, reflecting that sanggenon D and kuwanon G were safe for HepG2 cells within this concentration range. This provided a reference for selecting the appropriate concentration range for subsequent experiments.

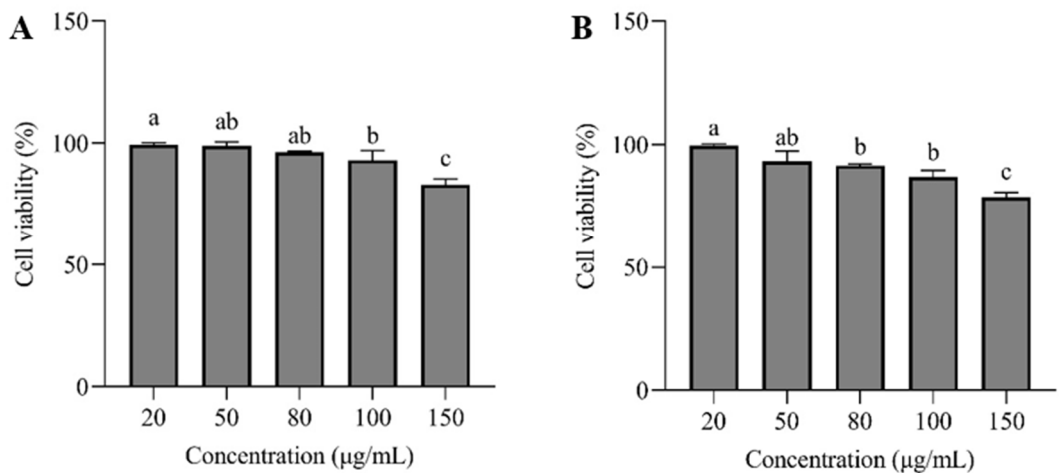


Figure 6. Survival rate of HepG2 cells treated with (A) sanggenone D and (B) kuwanon G.

3.7. Glucose, TC, TG Contents

In Figure 7A–F, the model group showed significantly higher levels of intracellular glucose, TC, and TG compared to the blank group. Compared with the model group, the contents of glucose, TC and TG in HepG2 cells were significantly reduced following the treatment with different concentrations of sanggenon D and kuwanon G, and no significant difference was shown between samples with different concentrations, which exhibited better inhibitory effect than the positive control basically.

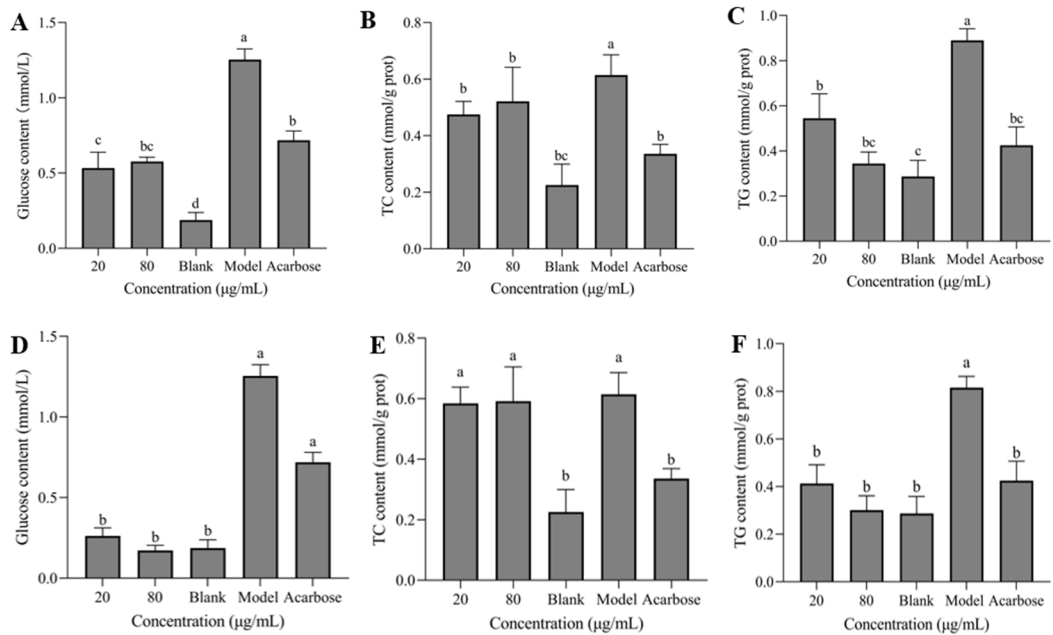
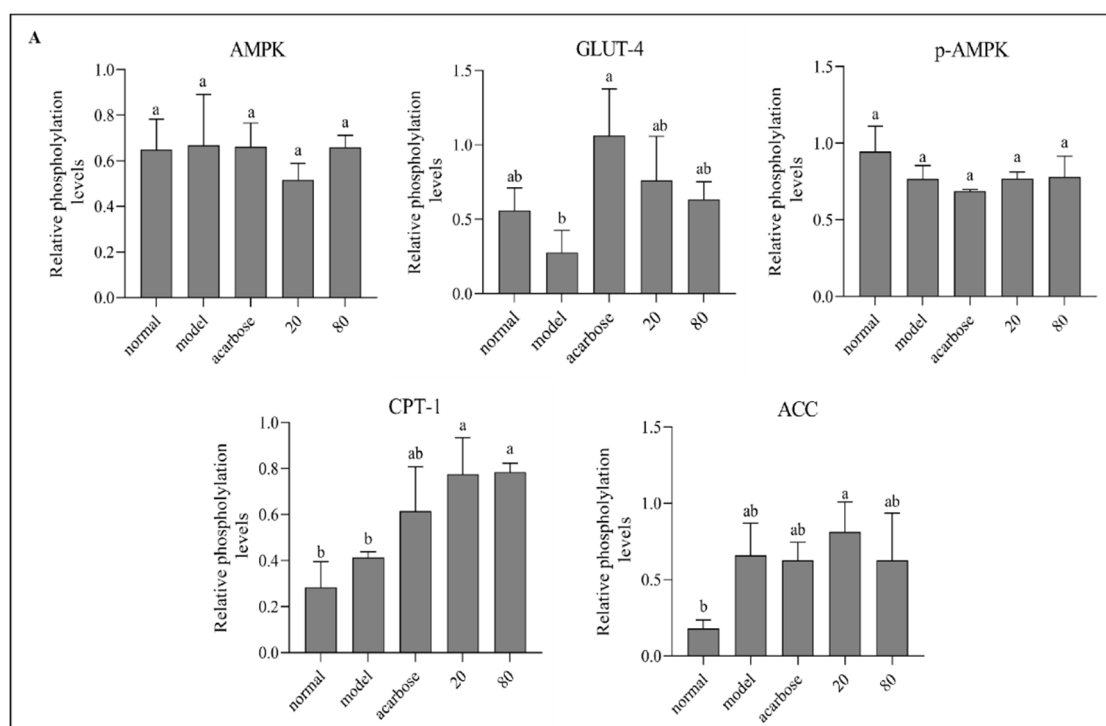


Figure 7. Effect of sangenone D on glucose content in supernatant (A) and intracellular TC (B) and TG (C) content; effect of kuwanon G on glucose content in supernatant (D) and intracellular TC (E) and TG content (F).

3.8. Western Blot Analysis

The primary pathological basis of T2DM is insulin resistance, characterized by decreased insulin sensitivity and impaired glucose metabolism in insulin-targeted tissues [41]. Multiple pathways were involved in IR biological signal transduction. Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is a metabolic regulator, widely involved in the glycolipid metabolic process, has become a new clinical treatment target for metabolic diseases based on IR [42]. Glucose transporter type 4 (GLUT4), an insulin-modulated glucose transporter in the AMPK pathway, enhances insulin sensitivity and glucose tolerance in adipose tissue when highly expressed [43]. Therefore, the corresponding dietary intervention targeting AMPK-GLUT4 signaling pathway is beneficial for improving the diabetic metabolic and reduce insulin resistance. Studies have shown that polyphenols such as quercetin mainly enhance glucose absorption in muscle and adipose cells by activating the AMPK pathway, which translocates GLUT4 to the plasma membrane [44,45]. Korean red pepper polyphenol extract stimulated muscle cell uptake of glucose by activating AMPK, which regulating! peroxisome proliferator-activated receptor gamma (PPAR- γ) and acetyl-CoA carboxylase (ACC) expression negatively [46]. p-Coumaric acid promoted the phosphorylation of AMPK, increased the phosphorylation of ACC and the mRNA expression of carnitine palmitoyltransferase1 (CPT-1) in L6 skeletal muscle cells, thereby facilitating the β -oxidation of fatty acids, reducing the deposition of triglycerides, and promoting glucose absorption of cells [47]. Figure 8 describes the protein expression levels of AMPK, GLUT4, p-AMPK, ACC and CPT-1 in HepG2 cells after sample treatment with different concentrations. Compared with the model group, GLUT4 expression was notably up-regulated in both groups, and p-AMPK was significantly promoted in kuwanon G group, indicating that AMPK phosphorylation was significantly activated in kuwanon G group, which might be one of the reasons why kuwanon G obtained better inhibitory effect than sanggenon D. In addition, both Sanggenon D and kuwanon G significantly upregulated the expression of CPT-1, and CPT-1 could promote the decomposition and oxidation of fatty acids, which corresponded to the decrease of TC and TG results. The mechanism of action in regulating glucose metabolism was summarized in Figure 9.



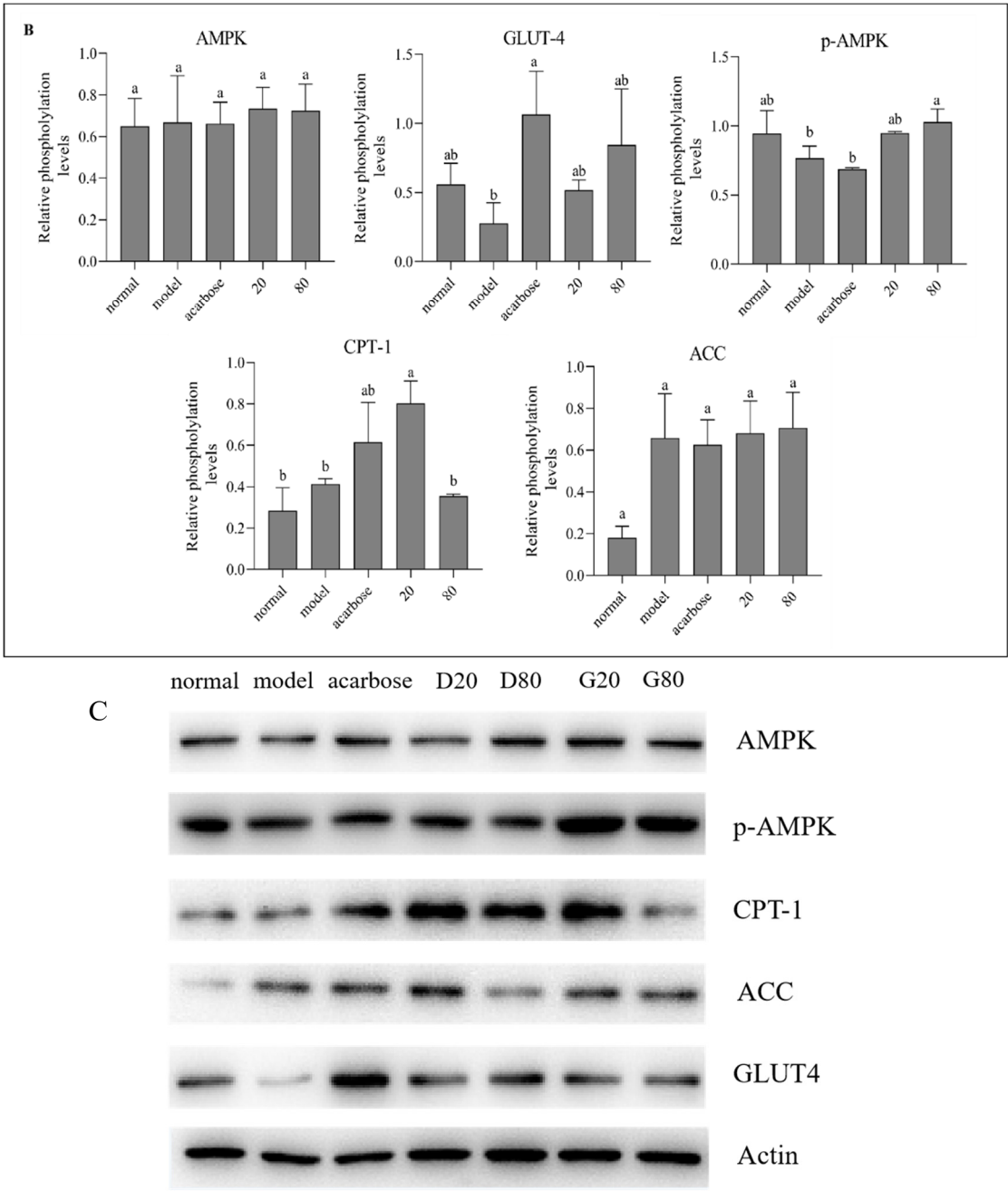


Figure 8. Effect of sanggenone D (A) and kuwanon G (B) on glucose transport related mRNA expression in HepG2 cells, Western blot images of AMPK, p-AMPK, CPT-1, ACC and GLUT4 (C).

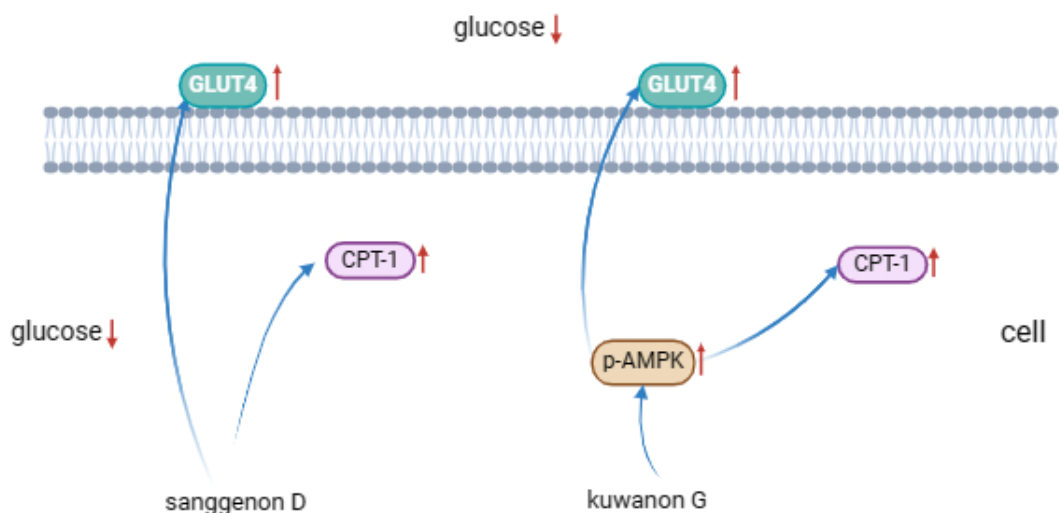


Figure 9. Mechanism of sanggenone D and kuwanon G effects on regulating glucose.

4. Conclusion

The inhibition mechanisms and the structure–activity relationships of sanggenon D and kuwanon G on α -glucosidase were analyzed in this study. The results suggested that two flavonoids could effectively inhibit α -glucosidase activity, and the inhibitory effect of kuwanon G was better than that of sanggenon D. The reason might be that cyclization weakened the activity of the isopentene group and removed a phenol hydroxyl group, resulting in the formation of fewer hydrogen bonds. Furthermore, fluorescence and FT-IR analyses indicated that both compounds changed the microenvironment and secondary structure of α -glucosidase through static quenching. Moreover, molecular docking results confirmed that the stability, binding energy, and affinity matched the inhibitory activity of the two compounds. Their main binding to α -glucosidase occurred spontaneously through hydrophobic interactions and hydrogen bonds with amino acid residues. Through the establishment of HepG2 cell high glucose model, it was verified that both sanggenon D and kuwanon G had a good hypoglycemic effect. Western blot results showed that sanggenon D and kuwanon G regulated blood glucose, and the mechanism might be related to the activation of GLUT4 pathway.

Abbreviations Used

T2DM, type 2 diabetics; DMEM, Dulbecco’s modified Eagle’s medium; TC, total cholesterol; TG, triglyceride; TPEB, total protein extraction buffer; BCA, bicinchoninic acid assay; PVDF, polyvinylidene difluoride; PBST, Phosphate Buffered Saline with Tween-20; RMSD, root mean square deviation; RMSF, root mean square fluctuation; AMPK, 5’-monophosphate AMP activated protein kinase; GLUT4, glucose transporter type 4; PPAR- γ , peroxisome proliferator-activated receptor gamma; ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase1.

Acknowledgments: This research received funding from the Aquaculture Collaborative Innovation Center of GDAAS [No.XT202502], and the Science & Technology Projects of Guangdong Province [No.2024A1515011122; No. 2023B0202010004]. The authors would like to acknowledge the contribution of Agricultural Products Processing Project of Guangdong Key Laboratory [No.202406], and the China Agriculture Research System of MOF and MARA [No.CARS-18-ZJ0503].

Declaration of Competing Interest: The authors declare that they have no financial interest or personal relationships that influenced the work reported in this paper.

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