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**Screening of flavonoids in Flower buds of *Sophora japonica* L. with
high activity against α -amylase and α -glucosidase and inhibitory
mechanism research**

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Abstract:

In this article, a method was established to screen compounds with enzyme activity inhibition in vitro using chromatographic analysis. The flower buds of *Sophora japonica* L. (FBSJ) were found to contain abundant flavonoids. And then these flavonoids were screened for the high inhibition activity against α -amylase and α -glucosidase using HPLC fingerprint and activity difference analysis. Consistent conclusions were drawn from multiple techniques, including the reported technique, IC50 data, and our method. The inhibitory mechanism was furtherly analyzed through enzyme inhibition kinetics, circular dichroism (CD), fluorescence spectrometry, molecular docking, and molecular dynamics. Among the six flavonoid components studied, quercetin acted as a competitive inhibitor against α -amylase, while kaempferol showed a mixed manner of inhibition against α -glucosidase. Molecular docking and molecular dynamics simulations demonstrated that quercetin and kaempferol had higher binding energy and bind more tightly. Generally, flavonols exhibited higher inhibitory activity than their corresponding flavonol glycosides against α -amylase and α -glucosidase. Quercetin and kaempferol in FBSJ showed potential as inhibitors of α -amylase and α -glucosidase. This study not only presented a novel method for screening compounds with high activity, but also provided a theoretical basis for studying the application and mechanism of flavonoids against α -amylase and α -glucosidase in FBSJ.

Key Words: *Sophora japonica*L., flavonoids, α -amylase, α -glucosidase

1. Introduction

Type 2 Diabetes Mellitus (T2DM) ranks first in the class of diabetes mellitus and causes an increase in blood sugar, while inhibition of digestive enzymes (α -amylase and α -glucosidase) that increase blood sugar is among important approaches used in the treatment of T2DM¹. Various plants were identified and used in the treatment of diabetes because of the interesting biological properties of their constituent, which had the potential for inhibiting activity of α -amylase and α -glucosidase²⁻⁴.

Flavonoids, which are polyphenolic substances commonly found in plants, play an important role in the human diet, and a great number of studies have extensively reported on the diverse biological activities of these compounds⁵. Flavonoids exhibited multifunctional anti-diabetic effects by modulating specific cellular signaling networks, thereby improving diverse aspects of the carbohydrate metabolic pathway, such as α -glucosidase, glucose transport, aldose reductase, and glucose uptake by cells⁶⁻⁸. An effective approach to managing postprandial hyperglycemia levels is the use of α -glucosidase and α -amylase inhibitors⁹. Dietary interventions involving flavonoids were used to develop practical dietary guidelines for individuals who are healthy, as well as to alleviate symptoms in patients with T2DM¹⁰.

Flower Buds of *Sophora japonica* L. (FBSJ), also known as *Flos Sophorae Immaturus*, is a traditional Chinese medicine (TCM) used to treat a variety of disorders such as hypertension and arteriosclerosis. Flavonoids such as quercetin, kaempferol, genistein, rutin and isorhamnetin had been extracted from *S. japonica*^{11, 12}. The components of TCM are complex, with multitudinous chemicals contributing to its effectiveness. This complexity makes it challenging to identify the functional

components of TCM. However, fingerprints spectra based on HPLC had been used for comprehensively analyze the varieties and quantities of compounds in plants and evaluate the quality of TCM^{13, 14}. Circular dichroism (CD), in combination with fluorescence quenching technology, is usually applied to study the binding of flavonoids to proteins and provides fundamental insights into protein-ligand interactions, conformation, and structural modifications of flavonoids to proteins¹⁵. Molecular simulation techniques, such as molecular docking and molecular dynamics, have gained importance in interaction research and have been used to investigate the mechanism of inhibitor-enzyme interactions, aiding in the understanding of the molecular biology perspective of the mechanism of action¹⁶.

A successful drug candidate needs to be efficacious without unacceptable adverse effects and during drug development, one needs to select drug candidates with the most appropriate drug-like properties¹⁷. Various screening methods have been developed and applied in a wide range of fields. The spectrum-effect relationship between HPLC fingerprint of natural products and bioactivities was widely reported¹⁸⁻²¹. However, these methods often require specialized software and complex data processing. Therefore, there is an urgent need to develop a simple and feasible screening and comparison method. This article aims to assess the inhibitory potentials of the extract against α -amylase and α -glucosidase, and to develop a new screening method using compounds found in FBSJ. The compounds that exhibit significant inhibitory effects were verified and the inhibitory mechanism was preliminarily studied.

2. Materials and methods

2.1 Materials

Flower buds of *Sophora japonica* L obtained from the campus of Henan University. I was permitted to pick flower buds of *Sophora japonica* L. as materials for experiments, which was planted in the campus of Henan University.

2.2 Reagents

Porcine pancreas α -amylase and yeast α -glucosidase were purchased from Bomei Biochemical Co.(Hefei, Anhui Province). 4-Nitrophenyl- α -D-glucopyranoside (*p*NPG) was purchased from Beijing Kuerhuaxue Co. Rutin, quercetin, kaempferol, isorhamnetin, kaempferol-3-O-rutinoside and narcissoside were purchased from Chengdu Alfa Biotechnology Co. Acarbose Tablets were purchased from Bayerhealthcare Co.

2.3 Determination of flavonoids in samples

The effective components in *S. japonica* were extracted by ethanol with different concentrations of 30%, 50%, 70%, 90% and ethyl acetate. Standard flavonoids were dissolved in methanol and flavonoids in samples were extracted by ethanol. The flavonoids in samples were separated using a Waters HPLC system (Milford, Massachusetts, USA) with a C18 Agilent column. Gradient elution conditions: (A) and 0.05% phosphoric acid (B) and according to the condition: 0-10 min, 24%-40% (A); 10-20 min, 40%-50% (A); 20-35 min, 50%-75% (A); 35-40 min, 75%-80% (A); 40-50 min, 80%-95% (A); 50-55 min, 95%-95% (A); 55-80 min, 95%-24% (A). The samples were analyzed qualitatively and quantitatively using standard solutions.

2.4 Inhibition activity assays of enzyme

Determination of α -amylase inhibition activity follows the report and changed properly²². Firstly, 20 μ L of samples at different concentrations were added to 150 μ L of 0.58 U/mL of α -amylase, which was then incubated at 37 °C for 5 min. Then, after adding 150 μ L of 1% starch and 15 min of incubation at 37°C to the mixture. Finally, 250 μ L DNS was added and then boiled for 5 min. After the solution was diluted to the appropriate concentration, the absorbance at 540 nm was measured using a microplate reader.

Determination of α -glucosidase inhibition activity follows the report and is changed properly²³. Firstly, 50 μ L α -glucosidase of 2 U/mL, 50 μ L inhibitor at different concentrations and 80 μ L buffer solution were added, which was then incubated at 37 °C for 10 min. Then, 50 μ L of *p*NPG (20 mM) was added to mix and shake evenly, and the reaction was continued at 37°C and the experiment was terminated by 100 μ L of Na₂CO₃ after 20min. After the solution was diluted to the appropriate concentration, the absorbance at 540 nm was measured using a microplate reader. The following equation was used to determine the inhibition rate:

$$\text{Inhibition rate\%} = 1 - \left[\frac{(A_3 - A_4)}{(A_1 - A_2)} \right] \times 100 \quad \text{equation 1}$$

A₁ is blank group: enzyme + buffer; A₂ is blank control group: buffer + buffer; A₃ is sample group: enzyme + inhibitor; A₄ is sample control group: buffer + inhibitor. The IC₅₀ value represented the inhibitor concentration required when the enzyme activity was semi-inhibited. Acarbose was the control.

2.5 correlation analysis

The concentration-effect curves of several different solvent extracts for inhibiting α -amylase and α -glucosidase activities were studied. Grey Relation analysis (GRA) was performed based on the peak areas of the HPLC fingerprint profiles of different extracts and the IC₅₀ of the inhibited enzyme activities^{24, 25}. Based on the concurrent area of the HPLC fingerprint of different extracts and the IC₅₀ of the inhibitory enzyme activity, the integration method was calculated by comparing the peak area of each component and the IC₅₀ value of the inhibitory enzyme activity. In addition, the method included the work of establishing the correlation between each component and the inhibitory enzyme activity and speculating the substances related to enzyme inhibition.

2.6 Kinetic assay for inhibition action

For the general determination of inhibition kinetics, the same steps as for the testing of enzyme activity were employed. The Lineweaver-Burk curve was used to examine the type of inhibition¹². It was obtained using the equations:

$$\text{Competitive inhibition: } \frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \quad \text{equation 2}$$

$$\text{Uncompetitive inhibition: } \frac{1}{v} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \quad \text{equation 3}$$

$$\text{Noncompetitive inhibition: } \frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \quad \text{equation 4}$$

$$\text{Mixed inhibition: } \frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{is}} \right) \quad \text{equation 5}$$

$$\text{Slope} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \quad \text{equation 6}$$

$$Y_{\text{intercept}} = \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{is}} \right) \quad \text{equation 7}$$

Where v is the reaction rate; K_m , K_i and K_{is} represent the Michaelis-Menten, free and bound enzyme inhibition constants; $[S]$ stands for substrate concentration; $[I]$ represents the concentration of the inhibitor.

2.7 Fluorescence spectrum analysis

Fluorescence quenching test for the interaction between flavonoids in FBSJ had been conducted of α -amylase and α -glucosidase, and the method was slightly modified²⁶: The α -amylase (0.58 U / mL) and α -glucosidase (2 U / mL) and different concentrations of quercetin / kaempferol was kept in 298 K、304 K、310 K water bath for 5 min. Under the excitation wavelength of 280 nm and the scanning wavelength of 300-500 nm, the width of the excitation and emission slit was 5.0 nm. The information about K_{SV} , K_q and K_a and quenching type derived from the Stern-Volmer equation as follows:

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad \text{equation 8}$$

$$\lg \frac{F_0 - F}{F} = \lg K_a + n \lg [Q] \quad \text{equation 9}$$

Where F_0 and F represent the fluorescence intensities with or without flavonoids and τ_0 is 10^{-8} s; K_{SV} , K_q represent the quenching constants and diffusion collision quenching rate constants; $[Q]$ represents the concentration of flavonoids.

2.8 Calculation of thermodynamic parameters

The temperature-dependent interactions were investigated by calculating the interactions between inhibitors and enzymes using the following equations:

$$\ln K_a = -\frac{1}{T} \left(\frac{\Delta H^\circ}{R} \right) + \frac{\Delta S^\circ}{R} \quad \text{equation 10}$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_a \quad \text{equation 11}$$

where K_a (Lmol^{-1}) was the binding constant. R ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) was the gas constant. ΔS° and ΔH° are the values to evaluate the binding force and ΔG° is the value to evaluate the spontaneity of the interaction.

2.9 Circular dichroism (CD) spectrum analysis

The α -amylase and α -glucosidase were mixed with flavonoid samples in different proportions and scanned by CD spectrum. Afterwards, the combined solutions were scanned from 190 to 260 nm in wavelength. The results of the measurements were then calculated using CDNN software.

2.10 Molecular docking

Utilizing AutoDock 4.0, molecular docking simulations were carried out. The structure of flavonoid compounds from PubChem to download. Protein data for α -amylase (PDB ID: 1OSE) were obtained from the protein database, and α -glucosidase was obtained from homology modeling. Before docking, the enzyme was applied to remove all water molecules, add hydrogen atoms and distribute the Gasteiger charge to generate the PDBQT file. Finally, the docked complexes were visualized and analyzed by PyMOL^{27, 28}.

2.11 Molecular dynamics

Molecular dynamics (MD) simulations at 100 ns were performed using GROMACS 2020. The Amber99 SB force field was used for the proteins in the study, and the topologies of α -amylase and α -glucosidase were prepared by the pdb2gmx module of GROMACS 2020. The topology and coordinate parameter files of quercetin and kaempferol were generated by simulation via ACPYPE Server (<https://www.bio2byte.be/acpype/status/>), and the physiological environment of the proteins was simulated by adding the TIP3P aqueous model to the protein complex system, which were all simulated using dodecahedral solvation boxes, and sodium ions were

added to neutralize the overall charge. The simulations were performed by conjugate descent combined with the most rapid descent method for 50,000 steps each to minimize energy. The V-rescale thermostat was used for temperature coupling to obtain a correct trajectory distribution of the canonical ensemble. The Berendsen pressure control method was applied to slowly heat the system temperature to 300 K within 10 ns. After the heating is completed, the NPT ensemble ($T = 300$ K and $P = 1$ atm) is used for 100 ns simulation balance under periodic boundary conditions²⁹.

2.12 Statistical analysis

All data were analyzed and presented as mean $\pm$ standard deviation of the result. Three experiments were performed for each data. All the statistical analysis was carried out using the SPSS 19.0 and GraphPad Prism 8 embracing the significant difference.

3. Results and discussion

3.1 Identification of flavonoids in FBSJ and establishment of HPLC fingerprint

Figure 1 showed the fingerprints of flavonoids in FBSJ by extracting with different solvents. The total of six common peaks were calibrated from six samples. Compared with the peak diagram of the reference substance, the six components were identified as rutin, kaempferol-3-O-rutinoside, narcissoside (isorhamnetin-3-O-rutinoside), quercetin, kaempferol and isorhamnetin according to the peak time, which was consistent with the reports^{30, 31}. The flavonoids in FBSJ include three flavonols and their corresponding flavonol glycosides, which contributed to comparing the activities of flavonols and their corresponding glycosides. Combined with the fingerprints, it was evident that the main chromatographic peak was rutin in various ethanol solutions

extract of FBSJ, but the main chromatographic peak was quercetin in ethyl acetate extract of FBSJ. It was shown that the extraction of FBSJ with different solvents enzyme inhibition rate (expressed by half inhibition rate concentration IC₅₀) and the order of α -amylase inhibition rate was ethyl acetate > 30% ethanol > 90% ethanol > 50% ethanol > 70% ethanol > water, and the order of α -glucosidase inhibition rate was ethyl acetate > 70% ethanol > 90% ethanol > 50% ethanol > 30% ethanol > water (Table 1). The inhibition effect of the extract of FBSJ with ethyl acetate was significant, compared with the other extracts, although the inhibition of acarbose was the most obvious. Based on the data in Figure 1, quercetin was the main constituent in ethyl acetate, which probably meant that quercetin was the favorite natural compound of inhibition activity.

Table 1 Inhibition effects of different extracts of FBSJ on enzymes

Samples	α -amylase inhibition IC ₅₀ (mg/mL)	α -glucosidase inhibition IC ₅₀ (mg/mL)
Acarbose	0.95 ± 0.01	0.00033 ± 0.0001
30% ethanol extract	13.59 ± 0.03	0.63 ± 0.02
50% ethanol extract	14.68 ± 0.05	0.61 ± 0.01
70% ethanol extract	20.09 ± 0.12	0.29 ± 0.02
90% ethanol extract	14.36 ± 0.03	0.43 ± 0.03
ethyl acetate extract	10.16 ± 0.01	0.09 ± 0.01
Water extract	44.26 ± 0.16	0.94 ± 0.03

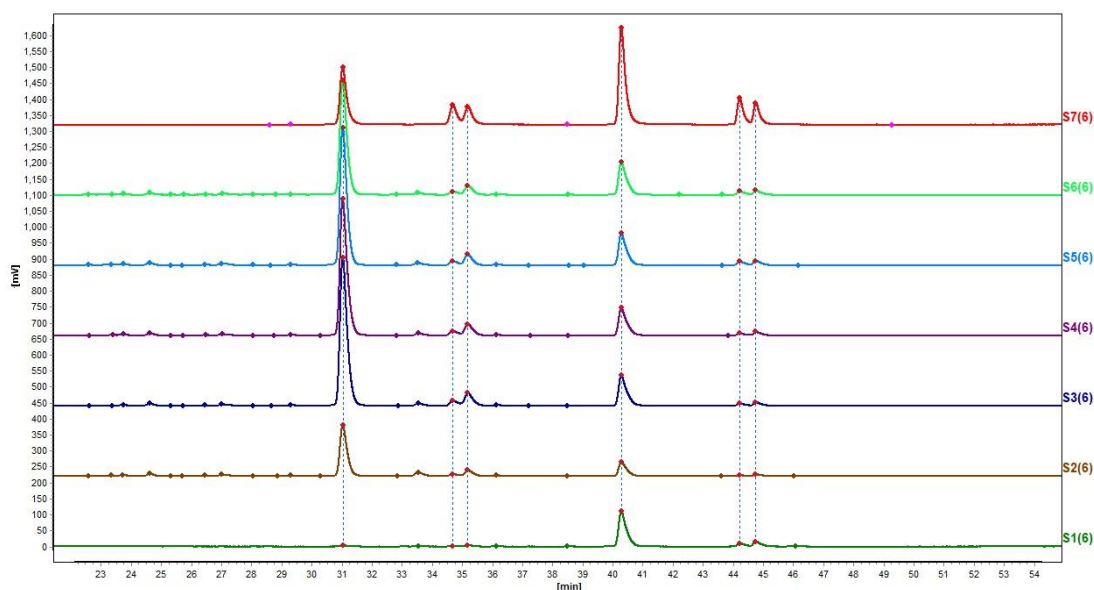


Figure 1. HPLC fingerprint of extract with different solvents, S1. ethyl acetate extract, S2 Water extract, S3 90% ethanol extract, S4 70% ethanol extract, S5 50% ethanol extract, S6 30% ethanol extract, S7 Standard mixed solution

3.2 correlation analysis

The peak area of six common peaks, which were represented by P1-P6, were established as the comparison sequence and the IC₅₀ value of enzyme inhibition as the reference sequence³². According to the Reference³³⁻³⁵, using the enzyme inhibition rate as a pharmacodynamic index, the results were shown in Table 2. The correlation order with α -amylase is P5, P4, P3, P6, P2, P1, the correlation order with α -glucosidase is P5, P4, P6, P3, P2, P1. Among the six characteristic peaks of the HPLC pattern, the correlations of peaks were all greater than 0.6, indicating that the inhibition of two enzyme's activities of FBSJ were jointly exerted³⁶. Peak P4 (quercetin), peak P5 (kaempferol), and peak P6 (isorhamnetin) are more closely related, indicating that quercetin, kaempferol and isorhamnetin may have a better inhibitory effect. The use of our method (integral method) was an overall method between the changes in the content of each substance in different solvent extracts and their enzyme

inhibition activity. The change of each substance in different solvent extracts and the change of the total inhibition rate - up- or down-regulation were compared, according to the significant difference (p) of the peak areas of the fingerprint profiles of different solvent extracts. If the change of the substance and the change of the total inhibition rate were the same and $p < 0.01$, score was 2 points, and if $p < 0.05$, score was 1 point, on the contrary, the opposite change scores -1 point. The results are shown in Table 3-5, it can be seen that the highest scores were obtained for P4, P5 and P6, indicating that quercetin, kaempferol and isorhamnetin may have significant effects on the inhibition of two enzyme's activities. From the above results, the results of the GRA and our analytical method (integral method) were in general agreement with the IC₅₀ of each substance, indicating the reference value of our method (integral method) and its relative simplicity and ease of operation^{37, 38}. Except for isorhamnetin, the result of new method is a high fit with that of GPA and IC₅₀. Perhaps, the lower concentration of isorhamnetin led to the system error. The analysis led to the conclusion that quercetin and kaempferol may be better inhibitors of two enzymes. From the IC₅₀ of each flavonoid, it can be inferred that flavonols have inhibitory activity than their corresponding flavonol glycosides against α -amylase and α -glucosidase under the same conditions, this was consistent with the structural requirements of previous studies on enzyme activity³⁹.

Table 2 IC50 values and GRA analysis of flavonoids from FBSJ extracts

Peak	α -amylase		α -glucosidase	
	Correlation Coefficient	IC50(mg/ml)	Correlation Coefficient	IC50(mg/ml)
P5 (Kaempferol)	0.787	0.33±0.05	0.678	0.19±0.05
P4 (Quercetin)	0.783	0.29±0.03	0.669	0.06±0.01
P6 (Isorhamnetin)	0.777	1.1±0.10	0.664	0.21 ±0.06
P3 (Narcissoside)	0.781	>5	0.644	5.04±0.02
P2 (Kaempferol-3-rutinoside)	0.777	>5	0.64	3.00±0.03
P1 (Rutin)	0.772	ND	0.626	0.99±0.01

Table 3 Comparative index of different solvent extracts of various substances and inhibition of α - amylase activity.

	30% ethanol	50% ethanol	70% ethanol	90% ethanol	ethyl acetate	water
30% ethanol	--	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↑ 6 ↑ I ↓ **	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↓ **	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↓ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↓ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
50% ethanol	--	--	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↓ **	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↓ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
70% ethanol	--	--	--	1 ↑ 2 ↑ 3 ↑ 4 ↑ 5 ↓ 6 ↓ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↑ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
90% ethanol	--	--	--	--	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↑ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
ethyl acetate	--	--	--	--	--	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
water	--	--	--	--	--	--

Table 4 Comparative index of different solvent extracts of various substances and inhibition of α -glucosidase activity.

	30% ethanol	50% ethanol	70% ethanol	90% ethanol	ethyl acetate	water
30% ethanol	--	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↑ 6 ↑ I ↑ *	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↑ **	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↓ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
50% ethanol	--	--	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↓ **	1 ↑ 2 ↑ 3 ↑ 4 ↓ 5 ↓ 6 ↓ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↓ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
70% ethanol	--	--	--	1 ↑ 2 ↑ 3 ↑ 4 ↑ 5 ↓ 6 ↓ I ↓ **	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↑ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
90% ethanol	--	--	--	--	1 ↓ 2 ↓ 3 ↓ 4 ↑ 5 ↑ 6 ↑ I ↑ **	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
ethyl acetate	--	--	--	--	--	1 ↓ 2 ↓ 3 ↓ 4 ↓ 5 ↓ 6 ↓ I ↓ **
water	--	--	--	--	--	--

1 represents Rutin, 2 represents Kaempferol-3-O-rutinoside, 3 represents Narcissoside, 4 represents Quercetin, 5 represents Kaempferol, 6 represents Isorhamnetin, ↑ / ↓ represents contrast peak area or inhibition activity rise or fall. *represents $p < 0.05$, **represents $p < 0.01$

Table 5 Integral method score table

Compound	α -amylase(score)	α -glucosidase(score)
P4 (Quercetin)	27	15
P5 (Kaempferol)	15	14
P6 (Isorhamnetin)	21	20
P4 (Rutin)	6	11
P2 (Kaempferol-3-O-rutinoside)	6	11
P3 (Narcissoside)	6	11

3.3 Inhibition types of flavonoids

Kaempferol and quercetin's kinetics to inhibit the enzymes α -amylase and α -

glucosidase was investigated using Lineweaver-Burk plots and to derive the type of inhibition^{40, 41}. As seen in Figure 2a-d, several lines were intersected on the y-axis, showing that quercetin acted as a competitive inhibitor against α -amylase, while several lines were intersected in the second quadrant or third quadrant, indicating that quercetin inhibits α -glucosidase in a mixed manner and kaempferol was a mixed manner of α -amylase/ α -glucosidase^{42, 43}. The slope and inhibitor concentration were linear (inset of Figure 2a-d), proving that they were only able to bind to one inhibition site on α -amylase and α -glucosidase⁴⁴.

The K_i and K_{is} values of quercetin, kaempferol and the two enzymes were calculated according to equation (2) - (7)⁴⁵. The pertinent outcomes were displayed in Table 6, the K_i value of kaempferol was less than the K_{is} value, proving that kaempferol binds more tightly to α -amylase/ α -glucosidase than the enzyme-substrate interaction. However, quercetin inhibited α -amylase competitively, indicating that quercetin binds directly to α -amylase and competes with the substrate, and the K_i values (0.085 ± 0.002 mg/ml) of quercetin were higher than the K_{is} values (0.058 ± 0.007 mg/ml), indicating that α -glucosidase binds more to the substrate complex than to the free enzyme⁴⁶.

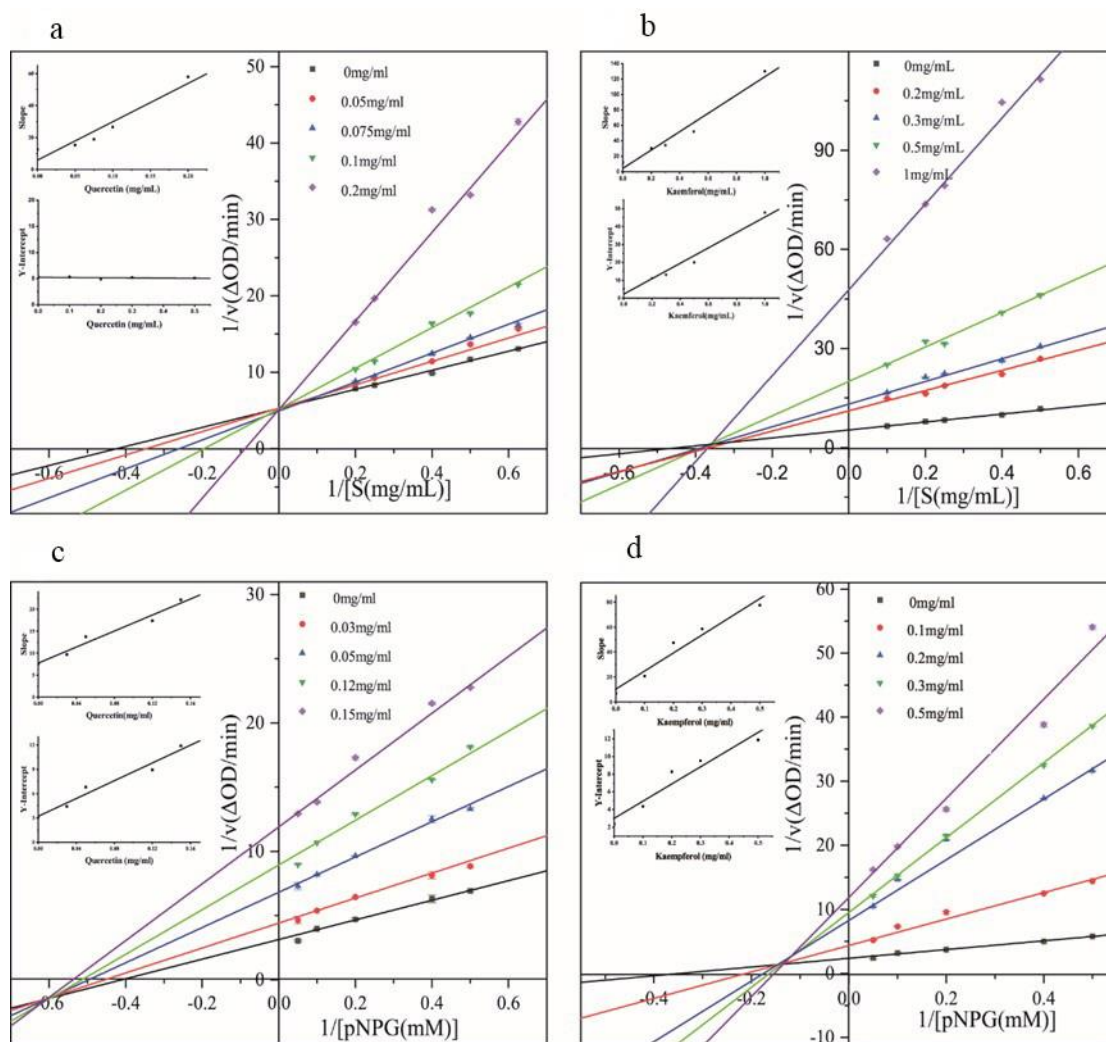


Figure 2. The inhibition kinetics of α -amylase of quercetin (A) and kaempferol (B) and α -glucosidase of quercetin (C) and kaempferol (D) were plotted, Lineweaver–Burk plots were drawn. The flavonoid concentrations are listed in the corresponding legend entries. The secondary plots (in the insert) show the slope and y-intercept in relation to the flavonoid compound, respectively.

Table 6 Detailed kinetic parameters of quercetin, kaempferol

Concentrations		K_i (mg/ml)	K_{is} (mg/ml)	Inhibition Type
α -amylase	Quercetin	0.024±0.003	-	Competitive
	Kaempferol	0.036±0.0003	0.051±0.006	Mixed
α -glucosidase	Quercetin	0.085±0.002	0.058±0.007	Mixed
	Kaempferol	0.071±0.003	0.155±0.012	Mixed

3.4 Fluorescence quenching

Figure 3 showed the fluorescence emission spectra of quercetin and kaempferol at different temperatures with α -amylase and α -glucosidase. The fluorescence intensity gradually reduced as quercetin and kaempferol concentrations rose, suggesting that the inhibitors had a noticeable impact on the α -amylase and α -glucosidase⁴⁷. The fluorescence intensity of the inhibitor's binding to the enzyme decreases with increasing inhibitor concentration and the more pronounced the quenching effect on the enzymes.

Stern-Volmer analysis was performed to reveal the type of quenching and binding constants. Usually, mainly includes two different kinds of mechanisms between inhibitors and enzymes: static and dynamic quenching⁴⁸. The F_0/F pair [Q] was plotted according to equations (8)-(9) to calculate the values^{16, 49}. The K_q values were all greater than $2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ and these values gradually decreased with increasing temperature (Table 7), these results suggested that the quenching mechanism of quercetin and kaempferol was static. The K_a values of quercetin and kaempferol considerably altered with the rise in temperature and were consistent with the change of K_{sv} values, indicating that quercetin and kaempferol form complexes with enzymes that decrease in stability with the increase in temperature. This was mostly caused by the ground state complexes between the enzyme, quercetin, and kaempferol inhibiting endogenous fluorescence. Both quercetin and kaempferol had binding sites that are close to 1, suggesting that there was just one type of binding site⁵⁰. This was consistent with the kinetic results.

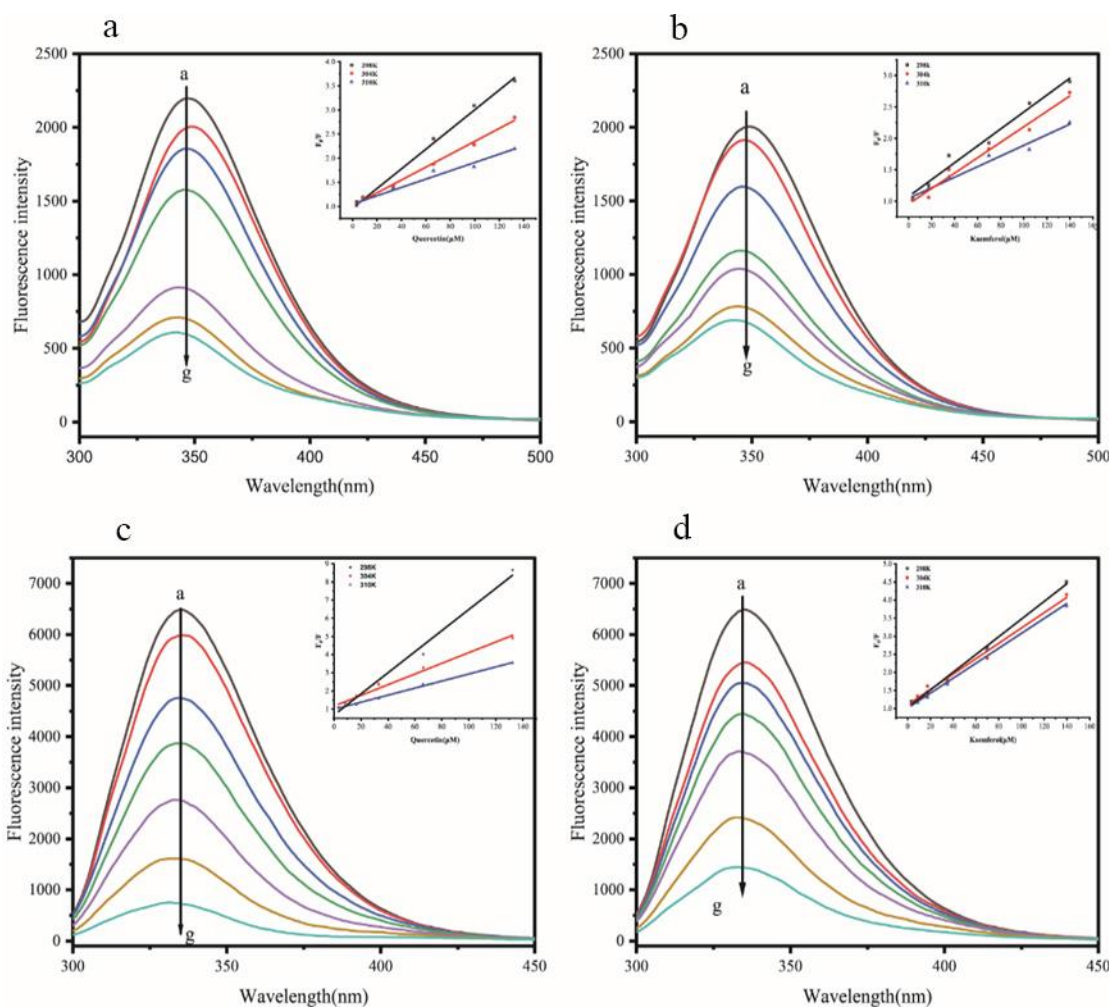


Figure 3. Fluorescence quenching of quercetin and kaempferol on α -amylase and α -glucosidase. (A) Fluorescence quenching spectra of α -amylase caused by quercetin (0, 3.31, 8.28, 16.56, 33.11, 66.22, 132.45×10^{-6} mol/L from curve a to g) at 298K. (B) Fluorescence quenching spectra of α -amylase caused by kaempferol (0, 3.49, 8.73, 17.47, 34.94, 69.87, 139.75×10^{-6} mol/L from curve a to g) at 298 K. (C) Fluorescence quenching spectra of α -glucosidase caused by quercetin (0, 3.31, 8.28, 33.11, 66.22, 99.34, 132.45×10^{-6} mol/L from curve a to g) at 298 K. (D) Fluorescence quenching spectra of α -glucosidase caused by kaempferol (0, 3.49, 17.47, 34.94, 69.87, 104.81, 139.75×10^{-6} mol/L from curve a to g) at 298 K. The secondary plot is the Stern-Volmer plot.

Table 7 Quercetin, kaempferol with α -amylase and α -glucosidase on the binding constants, binding sites, and thermodynamic parameters at 298, 304, and 310 K.

System	T(K)	K_q ($\times 10^{12} \text{Lmol}^{-1}$)	K_a ($\times 10^4 \text{Lmol}^{-1}$)	n	ΔH° (KJmol $^{-1}$)	ΔG° (KJmol $^{-1}$)	ΔS° (J mol $^{-1}$ k $^{-1}$)
Quercet	298K	2.04 $\pm$ 0.02	0.83 $\pm$ 0.03	0.91		-22.41 $\pm$ 1.02	
in- α - amylase	304K	1.34 $\pm$ 0.03	0.54 $\pm$ 0.02	0.90	-60.39 $\pm$ 2.52	-21.65 $\pm$ 2.54	-127.45 $\pm$ 3.52
	310K	0.86 $\pm$ 0.02	0.32 $\pm$ 0.01	0.88		-20.88 $\pm$ 1.32	
Kaemfe	298K	1.35 $\pm$ 0.05	1.51 $\pm$ 0.02	1.00		-24.48 $\pm$ 0.58	
rol- α - amylase	304K	1.23 $\pm$ 0.03	1.20 $\pm$ 0.04	1.25	-126.08 $\pm$ 4.57	-22.43 $\pm$ 1.24	-340.96 $\pm$ 5.76
	310K	0.85 $\pm$ 0.03	0.21 $\pm$ 0.01	1.20		-20.38 $\pm$ 2.65	
Quercet	298K	5.81 $\pm$ 0.02	25.12 $\pm$ 0.22	1.17		-30.71 $\pm$ 3.02	
in- α - glucosi dase	304K	2.94 $\pm$ 0.01	4.37 $\pm$ 0.04	1.02	-204.95 $\pm$ 3.68	-27.21 $\pm$ 1.61	-584.70 $\pm$ 6.52
	310K	1.94 $\pm$ 0.03	1.02 $\pm$ 0.02	0.94		-23.69 $\pm$ 4.32	
Kaemfe	298K	2.45 $\pm$ 0.04	0.33 $\pm$ 0.03	0.80		-19.75 $\pm$ 2.32	
rol- α - glucosi dase	304K	2.12 $\pm$ 0.04	0.05 $\pm$ 0.04	0.85	-168.35 $\pm$ 5.09	-16.75 $\pm$ 2.65	-498.67 $\pm$ 6.35
	310K	2.07 $\pm$ 0.03	0.02 $\pm$ 0.02	0.90		-13.76 $\pm$ 1.53	

3.5 Thermodynamic parameters

Typically, small molecules interact with enzymes noncovalently, which can be calculated by thermodynamic parameters including electrostatic interactions, hydrogen bonding, van der Waals forces and hydrophobic interactions. Negative values of ΔG° imply that the binding process of quercetin and kaempferol was spontaneous with α -amylase and α -glucosidase. ΔH° was calculated as a negative value indicating that the interaction of quercetin and kaempferol was an exothermic process with α -amylase and α -glucosidase (Table 7). $\Delta H^\circ < 0$ and $\Delta S^\circ < 0$ demonstrate that the principal forces that bind quercetin and kaempferol to two enzymes may be hydrogen bonds and van der Waals forces, in accordance with the guidelines put forth by Ross and Subramanian⁵¹.

3.6 CD spectra analysis

Circular dichroism is an effective tool for examining protein conformational changes brought on by ligands. As shown in Figure 4 shows that according to the properties of the protein, α -amylase and α -glucosidase have two negative bands at approximately 207 and 228 nm and 208 and 220 nm, respectively⁵³. For α -amylase, the content of α -helix increased from 14.25% to 14.72% (quercetin), 14.57% (kaempferol), 15.50% (isorhamnetin), 15.35% (rutin), 15.93% (kaempferol-3-O-rutinoside) and 15.90% (narcissoside), however, the β -sheet content decreased from the initial content of 47.46% to 46.16% (quercetin), 46.16% (kaempferol), 45.92% (isorhamnetin), 42.81% (rutin), 42.49% (kaempferol-3-O-rutinoside) and 45.92% (narcissoside) (Table 8). For α -glucosidase, the content of α -helix decreased from 24.54% to 23.47% (quercetin), 22.66% (kaempferol), 23.13% (isorhamnetin), 22.51% (rutin), 23.16% (kaempferol-3-O-rutinoside) and 22.77% (narcissoside), however, the β -sheet content increased from the initial content of 24.30% to 27.00% (quercetin), 28.90% (kaempferol), 28.10% (isorhamnetin), 28.30% (rutin), 27.00% (kaempferol-3-O-rutinoside) and 27.20% (narcissoside), which may be due to the alteration of the enzyme activity and structure by the inhibitor⁵⁴. These results suggest that flavonoid compounds induce changes in the secondary structure of enzymes, blocking the formation of active sites or hindering substrate binding, thereby affecting the activity of enzymes^{55, 56}. This is in line with the fluorescence data, which show that these substances have an impact on the enzyme's

structure and imply that they affect the enzyme activity.

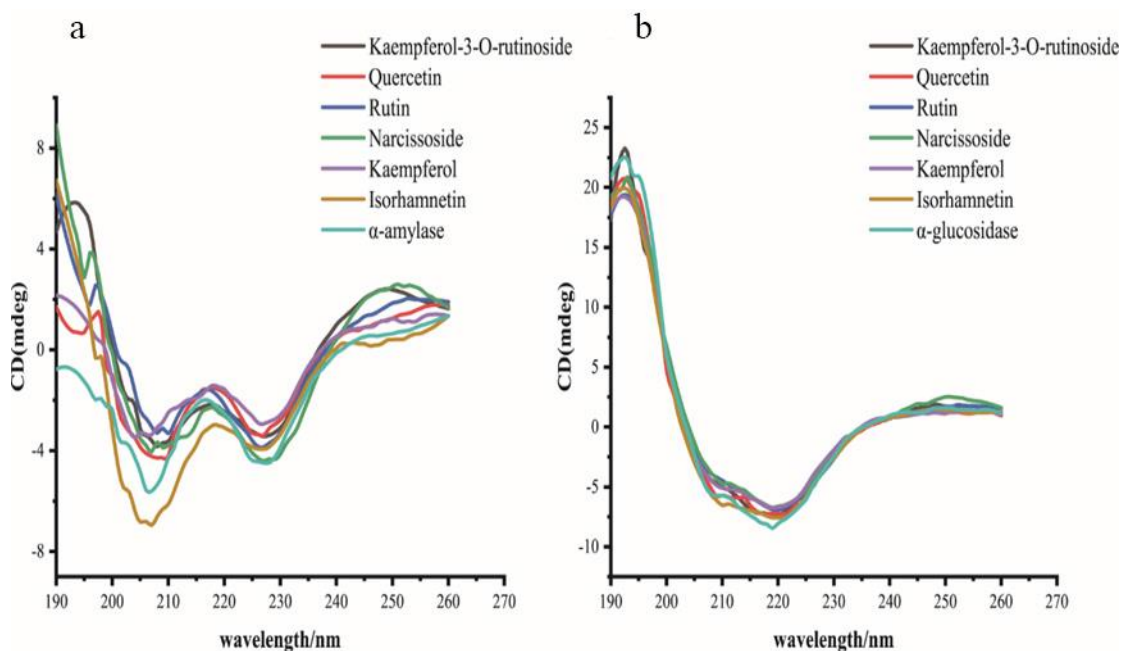


Figure 4 CD spectra of flavonoids- α -amylase/ α -glucosidase systems. (a) Flavonoids- α -amylase; (b) Flavonoids- α -glucosidase

Table 8 The contents of secondary structures of α -amylase and α -glucosidase in *S. japonica*

	α -helix(%)	β -sheet(%)	β -turn(%)	Random Coil(%)
α -amylase	14.25	47.46	11.92	26.45
α -amylase: quercetin=4:1	14.72	46.16	12.13	26.92
α -amylase: kaempferol=4:1	14.57	46.16	11.84	27.42
α -amylase: isorhamnetin=4:1	15.50	45.92	12.88	25.76
α -amylase: rutin=4:1	15.35	42.81	11.81	29.25
α -amylase: kaempferol-3-O-rutinoside=4:1	15.93	42.49	12.44	29.21
α -amylase: narcissoside=4:1	15.9	41.97	12.23	30.05
α -glucosidase	24.54	24.30	15.11	37.05
α -glucosidase: quercetin=3:1	23.47	27.00	14.89	36.19
α -glucosidase: kaempferol=3:1	22.66	28.90	14.67	35.84
α -glucosidase: isorhamnetin=3:1	23.13	28.10	14.79	35.77
α -glucosidase: rutin=3:1	22.51	28.30	14.23	36.93
α -glucosidase: kaempferol-3-O-rutinoside=3:1	23.16	27.00	14.41	37.10
α -glucosidase: narcissoside=3:1	22.77	27.20	14.25	37.49

3.6. Molecular docking analysis

The 3D structure of α -glucosidase derived from homology modeling, Verify-3D

assay revealed that 95.35% of the amino acid residues had an average 3D/one-dimensional fraction ≥ 0.2 , indicating that 95.35% of the target amino acid sequence was compatible with other amino acid residues (Figure 5). The detection results of Ramachandran plot program showed that 99.6% of the residues in the model were in the optimal and permissive regions, of which 89.7% were in the optimal region and 9.9% were in the permissive region, indicating the reasonableness of amino acid dihedral angle in the model. In conclusion, the structure of the model is reasonable and reliable.

To furtherly understand the binding pattern of the flavonoid components of FBSJ, we used molecular docking to mimic the interaction of the compounds with α -amylase and α -glucosidase and determined the optimal binding active component based on the binding energy generated according to the docking process. The higher the binding energy, the higher the stability of the polymer and the tighter the binding. According to the outcome of the molecular docking, quercetin and kaempferol had the highest binding energy for both enzymes. The energies of quercetin and kaempferol were -5.7 kcal/mol and -4.63 kcal/mol for α -amylase, and -5.15 kcal/mol and -5.06 kcal/mol for quercetin and kaempferol for α -glucosidase (Table 9)^{57, 58}. Thus, from an energetic

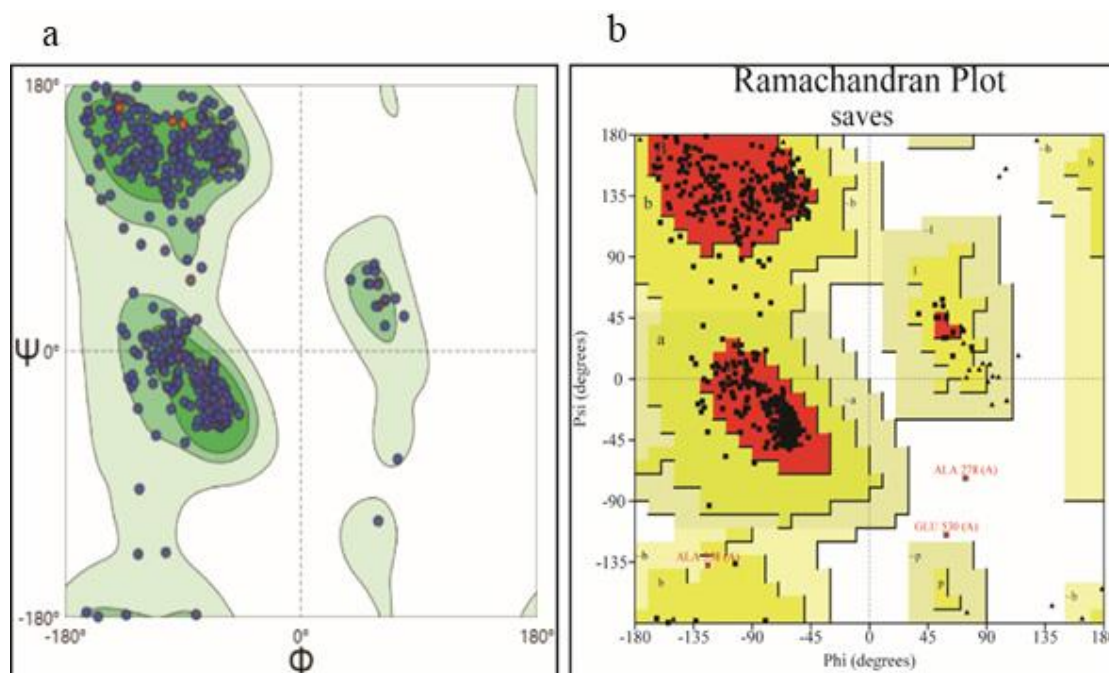


Figure 5. Ramachandran plot model quality evaluation diagram

Table 9 Flavonoids in *S. japonica* L. molecular docking evaluation table

number	compound	α -amylasebinding energy (kcal/mol)	α -glucosidasebinding energy (kcal/mol)
1	Quercetin	-5.70	-5.15
2	Rutin	-3.71	-3.27
3	Isorhamnetin	-4.54	-4.74
4	Kaempferol-3-O-rutinoside	-3.20	-2.51
5	Kaempferol	-4.63	-5.06
6	Narcissoside	-3.14	-2.19

point of view, quercetin and kaempferol had better binding affinity, which agreed with the outcome of the previous correlation analysis and with the IC₅₀ results for each substance.

As shown in Figure6, for α -amylase, quercetin can form hydrogen bonds with Asp197. Additionally, it interacts hydrophobically with Asp300, Gln63, His305, Trp59, Leu165, Tyr62, Trp58 and His299. Kaempferol can establish hydrogen bonding

interactions with Glu233 and His299. It also forms hydrophobic interactions with residues Asp300, Asp197, Gln63, Trp59, Leu165, Tyr62, Leu16 and Aln198. When binding α -amylase, quercetin and kaempferol impede the entry of the substrate or occupy the starch binding site, thus further inhibiting α -amylase activity. Asp197, Glu233, and Asp300 were identified as the three primary active site residues of α -amylase in earlier research, and they are essential to the catalytic process. Quercetin and kaempferol form hydrogen bonding interactions and interact with key active site residues, suggesting that quercetin and kaempferol are located within the active cavity of α -amylase. As for α -glucosidase, quercetin formed hydrogen bonds with Thr307, Glu304 and Gln322, while quercetin formed hydrophobic forces with His279, Pro309, Ser308, Thr301 and Val305. In addition, kaempferol formed hydrogen bonds with Lys422, Glu402 and Asp406, while kaempferol formed hydrophobic forces with Tyr413, Ile401, Val409, Tyr404, Lys410 and Glu405. Glu277, Asp352 and Glu411 residues in α -glucosidase were the main active site residues and they are essential to the catalytic process⁵⁹. However, neither quercetin nor kaempferol did not interact with the key residues of α -glucosidase mentioned, most likely as a result of their being combined around the active site. In conclusion, Flavonoids in FBSJ interact hydrophobically and through hydrogen bonds and α -amylase and α -glucosidase have a significantly influenced on the inhibitory activity⁶⁰.

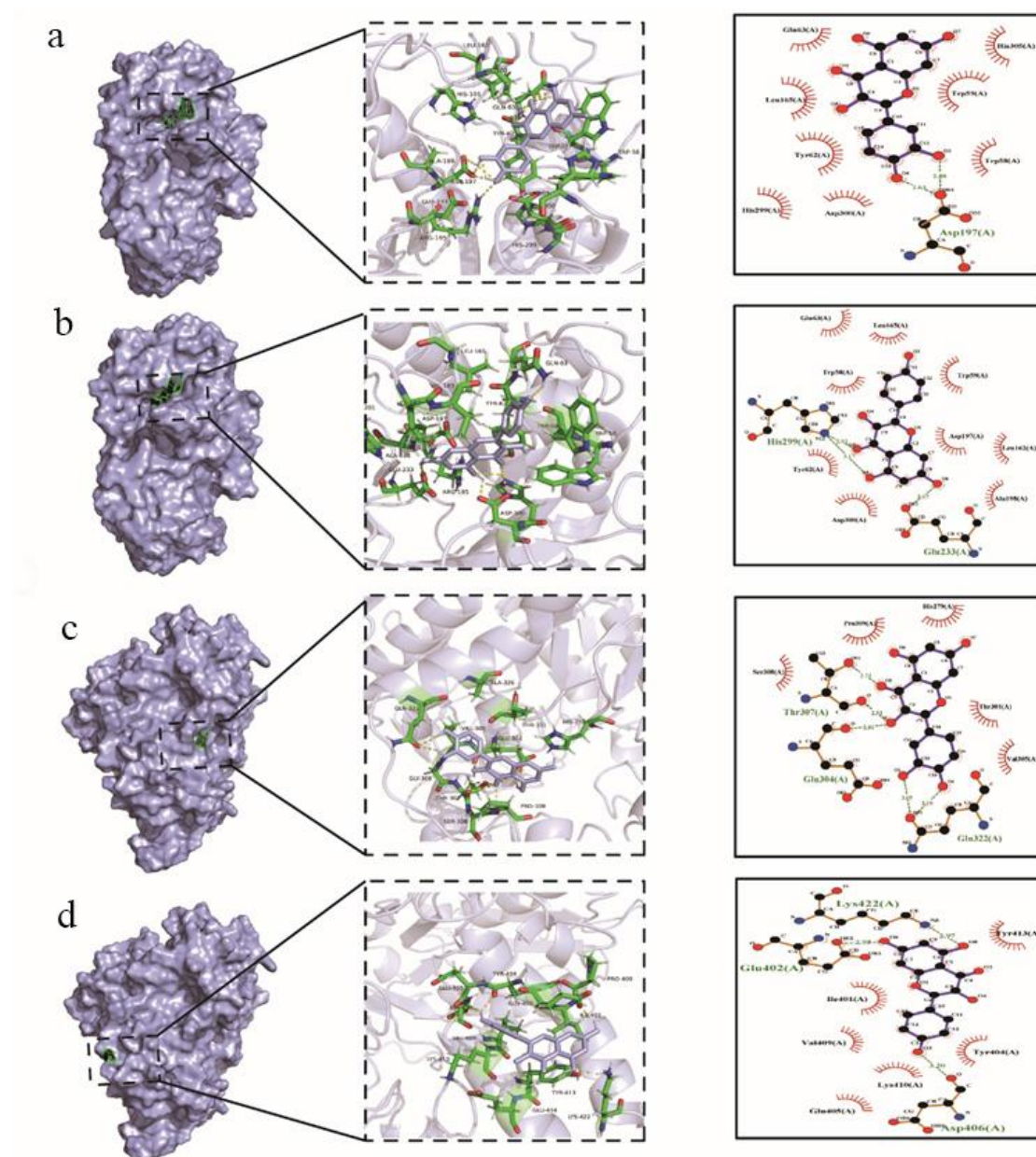


Figure6The molecular docking results between quercetin and kaempferol and α -amylase and α -glucosidase. The 3D binding diagram of quercetin- α -amylase (a)、kaempferol- α -amylase (b)、 quercetin- α -glucosidase (c) and kaempferol- α -glucosidase (d) (left) and the corresponding 2D interaction diagram (right).

3.7 Molecular dynamics simulation

The binding stability of quercetin / kaempferol- α -amylase and α -glucosidase complex was evaluated by root mean square deviation (RMSD), root mean square fluctuation (RMSF) and radius of gyration (Rg). The results of RMSD were used to

evaluate the dynamic changes of system stability⁶¹. In Figure 7a, the free α -amylase tended to be balanced at 10 ns, and the RMSD of quercetin- α -amylase complex fluctuated in the same region as that of free α -amylase, indicating that the binding of quercetin to α -amylase only slightly changed the degree of freedom of protein movement, and the stability was comparable to that of free α -amylase. The RMSD value of kaempferol- α -amylase complex was significantly higher than that of quercetin- α -amylase, and the stability was relatively poor. The flexibility of amino acid residues in Figure 7b showed RMSF. During the whole simulation process, the peptide chain of 105-111 residues in quercetin- α -amylase complex fluctuated greatly. The combination of kaempferol and α -amylase made the residues of 200-213 and 238 fluctuate greatly. Different protein ligand complexes have significant differences, reflecting the interaction between small molecules and proteins, which changes the protein conformation and may be related to the role of proteins. Rg is an important parameter to examine the denseness of the protein structure; the larger the Rg value of the system, the looser the protein structure^{62, 63}. As shown in Figure 7c, the binding of quercetin reduced the Rg value of α -glucosidase, indicating that quercetin binds to α -glucosidase and forms a more stable system, thus showing significant strong inhibitory activity.

The RMSD value of free α -glucosidase in Figure 7d fluctuated around 0.22 nm, and the RMSD value of α -glucosidase-kaempferol complex was significantly higher than that of α -glucosidase-quercetin complex. All ligand- α -glucosidase complexes in Figure 7e showed highly similar fluctuations. Among Lys233, His 279 and Ser281 residues, kaempferol- α -glucosidase showed higher fluctuations than quercetin- α -

glucosidase, indicating that the residue structure was flexible and unstable. The Rg value of the complex tended to be stable after 50 ns in the Rg diagram obtained in Figure 7f. The combination of quercetin and kaempferol ligand increased the Rg value of α -glucosidase, and the Rg of α -glucosidase-kaempferol complex was significantly higher than that of α -glucosidase-quercetin complex, which made the whole conformation loose and its structure unstable, resulting in a decrease in the catalytic activity of the active site of α -glucosidase.

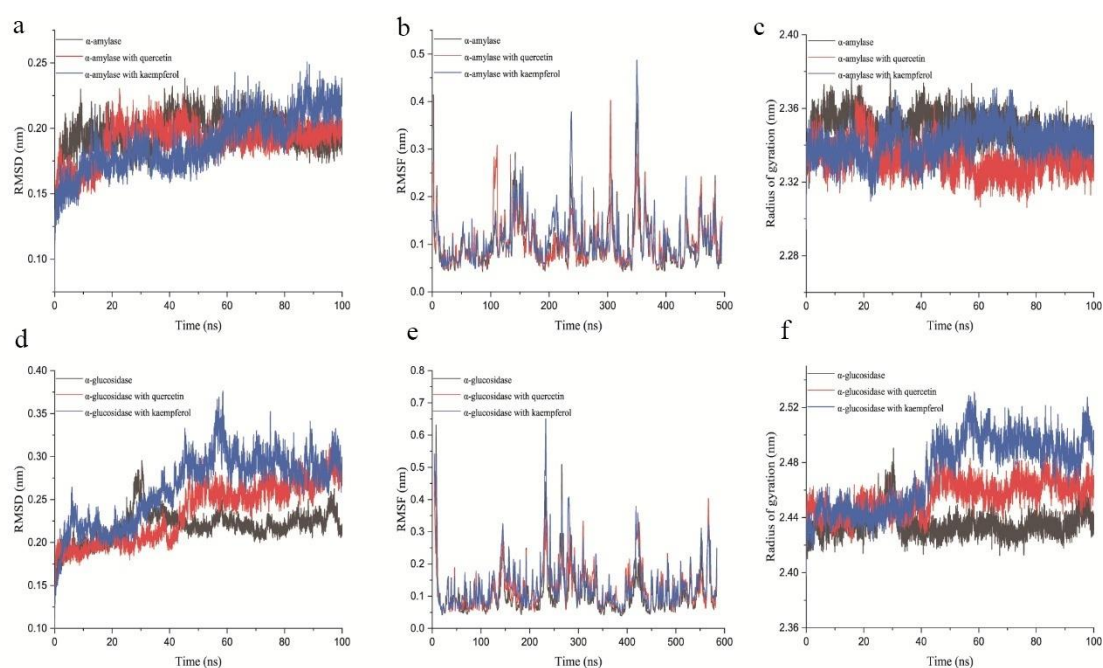


Figure 7. Molecular dynamics simulation of quercetin / kaempferol and α -amylase / α -glucosidase. Molecular dynamics simulation of quercetin, kaempferol with α -amylase (a-c) and α -glucosidase (d-f) for 100 ns.

4. Conclusions

In this study, we isolated and identified six flavonoids from FBSJ and then developed a method to screen compounds for their inhibition of α -amylase and α -glucosidase activity in vitro. The significance of the inhibition of compounds was analyzed through various methods. Additionally, we investigated the inhibitory mechanism using enzyme

inhibition kinetics, fluorescence spectrometry, circular dichroism, and molecular docking. The screening method we employed was simple and reliable, based on scoring for significance. Our analysis indicated that quercetin and kaempferol might be more effective inhibitors of α -amylase and α -glucosidase. These findings were consistent with the IC₅₀ and molecular docking results. Quercetin and kaempferol were found to act as competitive inhibitors against α -amylase and α -glucosidase enzymes. The study revealed that quercetin exhibited a mixed inhibition pattern against α -amylase, while kaempferol demonstrated a mixed inhibition pattern against both α -amylase and α -glucosidase. The effects of flavonoids in FBSJ were observed through fluorescence and CD spectra, which indicated a reduction in fluorescence intensity and structural changes in the enzyme. Molecular docking analysis revealed that quercetin and kaempferol exhibited higher binding energies and tighter interactions with the enzymes. Molecular dynamics simulations further provided insights into the structure and characteristics of the α -amylase-quercetin/kaempferol complexes and α -glucosidase-quercetin/kaempferol complexes. Notably, the α -amylase/ α -glucosidase-quercetin complex was found to be more stable compared to the α -amylase/ α -glucosidase-kaempferol complex. Overall, the findings suggest that quercetin and kaempferol present potential as inhibitors of α -amylase and α -glucosidase enzymes in FBSJ.

Statement

All data generated or analysed during this study are included in this published article.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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