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Six flavonoids inhibit the antigenicity of β-lactoglobulin by noncovalent interactions: A spectroscopic and molecular docking study

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ABSTRACT

It is practical to inhibit the allergenicity of β-lactoglobulin (β-LG) using natural products acting via noncovalent interactions; however, the mechanism of the effect has not been investigated in detail. Herein, the comprehensive noncovalent mechanism of inhibition of the antigenicity of β-LG by six flavonoids (kaempferol, myricetin, phloretin, epigallocatechin-3-gallate (EGCG), naringenin, and quercetin) was investigated by spectroscopic and molecular docking methods. Our results indicate that six flavonoids reduced the antigenicity of β-LG in the following order: EGCG > phloretin > naringenin > myricetin > kaempferol > quercetin, with antigenic inhibition rates of 72.6%, 68.4%, 59.7%, 52.3%, 51.4% and 40.8%, respectively. Six flavonoids induced distinct conformational changes in β-LG, which were closely associated with a decline in antigenicity of β-LG. The flavonoids bound to specific antigen epitopes in the β-sheet and β-turn of β-LG to induce a decrease in the antigenicity of the protein.

1. Introduction

Food hypersensitivity has been recognized by the World Health Organization as the sixth main disease affecting human health (Kazemi, Taheri-Kafrani, Motahari, & Kordesedehi, 2018). Milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soybean are the eight major food allergens (Zhou et al., 2016). Allergy to cow milk protein is one of the most common childhood food allergies with an incidence of 2–3% in newborns under 2 years of age and 1% in adults (Kurpiewska et al., 2019). β-Lactoglobulin (β-LG) is considered as the major allergen in cow milk.

β-LG is composed of 162 amino acid residues and is a globular protein with a monomeric molecular weight of 18.4 kDa. β-LG consists of two α-helices and eight antiparallel β-sheets, which establish a calyx-shaped β-barrel structure (Liu et al., 2018). The structure of β-LG contains two main types of epitopes, including linear or sequential epitopes and conformational epitopes. The former are contiguous sequences of amino acid residues determined by the primary structure of the protein, and the latter are discontinuous sequences, which are contingent in the tertiary or quaternary structure of the protein (Rahaman, Vasiljevic, & Ramchandran, 2015).

Numerous strategies have recently been used to reduce the allergenicity of β-LG, involving heat treatment (Bloom et al., 2014), high-pressure treatment (Kurpiewska et al., 2019), hydrolysis (Peram, Loveday, Ye, & Singh, 2013), and binding of natural products via noncovalent/covalent interactions (Abd El-Maksoud et al., 2018; Wu et al., 2018). However, heating and hydrolysis may destroy useful β-LG functions and taste (Wu et al., 2013); high pressure may increase the antigenicity of β-LG (Meng, Bai, Gao, Li, & Chen, 2017). Binding with natural products may be a potentially safe and feasible protein-modifying method because of the direction and specificity of the modification.

Flavonoids are a series of compounds with C6-C3-C6 as the basic carbon framework (Gholami & Bordbar, 2014) with a wide range of potentially beneficial bioactivities (Pinela et al., 2016). So far, covalent interactions between flavonoids and β-LG have been investigated as a ways to lower protein antigenicity, such as glycation (Zhong et al., 2014), phosphorylation (Enomoto et al., 2007), PEGylation (Yu et al., 2007), and other methods (The references are shown in *Supplementary Material*). However, there are only a few studies on reducing the antigenicity of β-LG by noncovalent interactions (Plundrich, Cook, Maleki, Fourches, & Lila, 2019; Zhong et al., 2019), and the reversible binding method is simple and does not change the solvent environment of the protein. Thus, it is necessary to use the noncovalent binding of natural compounds to reduce the antigenicity of β-LG and to determine the mechanism of the effect.

Our aim was to use experimental and computational approaches to explore the mechanism of β-LG antigenicity reduction by six flavonoids through noncovalent interactions. We selected six flavonoids (kaempferol, myricetin, phloretin, epigallocatechin-3-gallate (EGCG), naringenin,

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and quercetin) to investigate the influence of the number of hydroxyl groups on the phenyl (B ring), hydrogenation of the C2 = C3 double bond and replacement of the gallate group on the interactions between each flavonoid and β-LG. Finally, the mechanism of reduction in β-LG antigenicity was assessed by multispectral and molecular docking methods. This work provides theoretical and practical support for a solution of the milk allergy problem.

2. Materials and methods

2.1. Materials and chemicals

β-LG (purity ≥ 90%, molecular weight 18.4 kDa), kaempferol (purity ≥ 95%), myricetin (purity ≥ 96%), phloretin (purity ≥ 98%), EGCG (purity ≥ 98%), naringenin (purity ≥ 98%), and quercetin (purity ≥ 95%) were acquired from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Sodium phosphate was obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China), and anhydrous ethanol was from Chengdu Jinshan Chemical Reagent Co., Ltd. (Chengdu, Sichuan, China). All reagents and solvents were of the analytical reagent grade, and ultrapure water was used throughout the experiment.

2.2. Preparation of various β-LG-flavonoid complexes

An β-LG stock solution (1.0×10^{-5} mol/L) was prepared with phosphate-buffered saline (PBS) (0.2 mol/L, pH 6.0, 6.5, 7.0, 7.5, and 8.0) and was refrigerated overnight. The flavonoid stock solutions (5.0×10^{-5} mol/L) were prepared by dissolving kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin in 10 mL of an 80% (v/v) ethanol–water solution; then, the mixtures were diluted to working concentrations with PBS for the experiments. All stock solutions were stored at 4 °C (Li et al., 2018). Various reaction parameters, including molar ratios of β-LG-flavonoid mixtures (1:1–1:5), reaction time (5–25 min), and reaction pH (pH 6.0–8.0), were considered to optimize the reaction conditions at room temperature.

2.3. Determination of antigenicity

The antigenicity of all samples (control and treated) was evaluated by sandwich enzyme-linked immunosorbent assays (ELISAs) using a bovine β-LG ELISA quantification kit (Jianglai biotechnology CO., Ltd, Shanghai, China) (Rahaman et al., 2015). Each well of a 96-well microtiter plate was coated with a capture antibody diluted in 0.05 M phosphate-buffered solution. After overnight incubation at 4 °C, the plate was washed 5 times with ELISA washing solution. Then, the wells were filled with 350 μL of a blocking solution to block the residual free binding sites. After incubation for 30 min at room temperature, the blocking solution was discarded, and the plate was washed 5 times to remove unbound antibody. Then, 50 μL of the standards or samples diluted in the sample diluent under various conditions was added; 50 μL of the sample diluent was immediately added to the blank wells. Then, 100 μL of horseradish peroxidase-conjugated β-LG-detection antibody was added to each well. After incubation for 1 h and subsequent washing, the wells were filled with 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, and the color was allowed to develop for 15 min in the dark at 37 °C. The reaction was stopped by adding stop solution (2 M H_2SO_4, 50 μL), and the absorbance was measured using a microplate reader (iMark, Bio-Rad Laboratories). The antigenicity of the β-LG-flavonoid complexes was calculated based on the standard curve (the range of linear correlation was 6.25–200 μg/mL of β-LG) as equivalent micrograms of protein per milliliter of the sample.

2.4. Intrinsic fluorescence spectroscopy measurements

The fluorescence investigation of the complexes of the protein (1.0×10^{-5} mol/L) and various flavonoids (5.0×10^{-5} mol/L) under the corresponding optimal binding conditions was performed according to the method described by Wang et al. with some modifications (Wang et al., 2019). An RF-6000 spectrophotometer (Shimadzu, CO., Ltd, Japan) was used for fluorescence spectroscopy measurements at 25 °C in a quartz cell with a path length of 1.0 cm. Intrinsic protein fluorescence was determined at a 295 nm excitation wavelength, and the emission spectra were recorded from 300 to 500 nm. The width of slit for excitation and emission was set at 5.0 nm. To avoid the effects of intrinsic flavonoids fluorescence, the intensities of the flavonoids in the samples were automatically subtracted from the emission intensities of the corresponding β-LG-flavonoids complexes. All fluorescence experiments were performed in triplicate.

2.5. Fourier transform infrared (FTIR) spectroscopy measurements

FTIR spectra of six flavonoids and β-LG under the corresponding optimal conditions from 4000 to 400 cm^{-1} were recorded at 25 °C using a Nicolet iS50 FTIR spectrophotometer (Thermo Fisher Technology CO., Ltd, USA) (Abd El-Maksoud, Abd El-Ghany et al., 2018). The spectrometer was fitted with a KBr beam splitter. All spectra were obtained by the attenuated total reflection method with a resolution of 4 cm^{-1} using 32 scans. The spectral processing procedure involved collection of the spectra of KBr under the same conditions. The absorbance of KBr was subtracted from the spectra of the samples to obtain the FTIR spectra. The obtained spectral data were baseline corrected and normalized according to the protein peak from 1600 cm^{-1} to 1700 cm^{-1}.

2.6. Far-UV circular dichroism (far-UV CD) spectroscopy measurements

The spectra of the β-LG-flavonoid complexes in solution under the corresponding optimal conditions at 25 °C were recorded with a Chirascan V100 spectrophotometer (Applied Photophysics Ltd, UK) in 1.0 mm path length cells. The far-UV CD spectra of β-LG in the presence or absence of flavonoids were recorded from 190 to 250 nm (the scan rate was 30 nm/min and the response time was 1 s) under constant nitrogen flushing at room temperature and the spectrum of PBS was subtracted. Three scans were recorded for each spectrum. Various secondary structures of β-LG were analyzed based on the CD spectroscopic data by the online CONTIN program in DichroWeb (Whitmore & Wallace, 2004).

2.7. Molecular docking

Molecular docking was used to investigate the binding of the flavonoid ligands (kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin) with the receptor (β-LG) by Discovery Studio 2019 (Xiong, Liu, Zhou, Zou, & Chen, 2016). The docking mode was based on a semiflexible mechanism, i.e., the receptor was defined as rigid, and the ligand was defined as flexible. The X-ray crystal structures of β-LG (PDB ID: 3NPO & 5IO5) were downloaded from the Protein Data Bank (Berman et al., 2000); these structures were selected because they had no missing atoms or crystallized ligands and were available at a reasonable resolution (2.20 Å and 2.85 Å, respectively). The β-LG structures with two PDB codes above were pretreated by removing water molecules and adding Gasteiger charges and all hydrogen atoms before docking. The 3D structures of the ligand molecules were obtained from PubChem (Kim et al., 2019). The ligand molecules were optimized, and the protein was cleaned to obtain the conformation with the minimum energy. The ligand molecules were placed in the CHARMM force field of the receptor (β-LG). Other parameters were set to their default values. The conformation with the highest −CDOCKER energy ($-E_{CD}$) and highest −CDOCKER interaction energy ($-IE_{CD}$) was assumed to be the optimal conformation.

2.8. Statistical analysis

The mean values were determined based on 3 individual experiments. Statistical data were processed by one-way ANOVA using Microsoft Office Excel version 2019. Statistical significance level was defined at $p < 0.05$.

Table 1
Inhibitory effects of six flavonoids on the antigenicity of β-LG under the corresponding optimal conditions.

Flavonoids	Reaction time (min)	Reaction pH	Molar ratio (β-LG: flavonoid)	Antigen conc. (μg/ml)	Inhibition ratio [a] (%)
Kaempferol	25	6.0	1:4	29.16	51.4
Myricetin	15	6.0	1:4	28.62	52.3
Phloretin	15	6.0	1:3	18.96	68.4
EGCG	20	6.0	1:2	16.44	72.6
Naringenin	15	6.0	1:5	24.18	59.7
Quercetin	25	6.0	1:5	35.52	40.8

[a] Inhibition ratio was calculated as the difference of the original antigen concentration and the residual antigen concentration divided by the original antigen concentration.

3. Results and discussion

3.1. Different flavonoids have different inhibitory effects on the antigenicity of β-LG

The effects of noncovalent interactions of six flavonoids with β-LG on the antigenicity of β-LG was investigated under various reaction conditions, including various reaction times (5–25 min), molar ratios (β-LG: flavonoid = 1:1–1:5), and pH values (6.0–8.0); the antigenicity was assayed by ELISAs. Table 1 shows the optimal binding conditions and the results obtained by the ELISAs. EGCG had the more profound inhibitory effect on the antigenicity of β-LG compared to that of other five flavonoids at the 20 min reaction time, pH 6.0, and a molar ratio of 1:2. The residual antigen concentration was 16.44 μg/ml, corresponding to 72.6% inhibition, versus the original antigen concentration of 60.0 μg/ml. Quercetin had the lowest inhibitory effect on the antigenicity of β-LG with only 40.8% inhibition. Four flavonoids, namely, kaempferol, myricetin, phloretin, and naringenin, inhibited antigenicity by 51.4%, 52.3%, 68.4%, and 59.7%, respectively. These results indicate that antigenicity of β-LG can be reduced to a variable extent via noncovalent binding of six flavonoids.

3.2. Changes in the microenvironment of the tryptophan residue

The binding of small molecular ligands may cause changes in the microenvironment around the protein. Intrinsic fluorescence spectroscopy was used to analyze the changes in the microenvironment of the key residue to probe the conformational changes in β-LG induced by various flavonoids via noncovalent interactions. β-LG contains two tryptophan residues, Trp19 and Trp61; Trp19 is located in the epitope region and Trp61 is located outside of the epitope region (Kurpiewska et al., 2019). The data shown in Fig. 1A indicate that six flavonoids induced the fluorescence quenching of the protein at the excitation wavelength of 295 nm, indicating that the microenvironment of the tryptophan residue is altered to provide a higher chance of interaction of the tryptophan residue with the ligand. Kaempferol, myricetin, phloretin, and quercetin induced higher fluorescence quenching than that induced by EGCG and naringenin, suggesting that these four compounds have a higher impact on the microenvironment of tryptophan in β-LG. However, these differences in the fluorescence quenching do not provide a reasonable explanation for the differences in the inhibition of antigenicity of β-LG for each flavonoid.

3.3. No new covalent bonds were formed between β-LG and flavonoids

The six flavonoids studied were successfully bound to β-LG by noncovalent interactions. As shown in Fig. 1B, no new FTIR spectral signals appeared after the formation of the β-LG-flavonoid complexes according to the comparison with the spectra of native β-LG, indicating that no new covalent bonds were formed between β-LG and flavonoids. Native β-LG and the flavonoid-modified β-LG had an infrared signal near 1480 cm^{-1} and a wide band at 1600–1700 cm^{-1}, which are mainly attributed to C—N stretching, N—H bending and C═O stretching vibrations in the peptide chain (Ferraro, Madureira, Sarmento, Gomes, & Pintado, 2015).

The data of Fig. 1C indicate that in the region of the amide I band (1600–1700 cm^{-1}), the peak position in the native β-LG was 1637.54 cm^{-1}. After various flavonoids were added to the β-LG solution, the peak positions shifted to 1638.87, 1645.32, 1648.11, 1647.85, 1638.90, and 1646.89 cm^{-1} for kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin, respectively, implying that the binding of the flavonoids altered the C—N stretching and N—H bending of β-LG.

These results indicate that the formation of the β-LG-flavonoid complexes resulted in conformational changes in β-LG, which influenced the antigenicity of the protein. In general, the spectral changes in the amide I band range are considered to be related to random coils in the secondary structure (Bhattacharjee et al., 2005). EGCG binding to β-LG with the formation of the complex enhanced the spectral intensity within the region from 1637 to 1648 cm^{-1}, indicating that the random coil of β-LG was expanded. The maximum wavenumber of the peak representing EGCG binding to β-LG was shifted by approximately 10 cm^{-1} from 1637.54 cm^{-1} to 1647.85 cm^{-1}. According to the antigenicity assay, EGCG was the strongest antigenicity inhibitor of β-LG; thus, we hypothesized that the epitopes of β-LG are mainly located in other secondary structures and do not include the random coil.

3.4. Changes in the secondary structure of β-LG induced by the binding of flavonoids

Far-UV CD spectroscopy was used to investigate the mechanism of flavonoid influence on the secondary structure of β-LG. The data of Fig. 2 indicate that the CD spectrum of native β-LG has a broad negative region at 216 nm, which is a typical feature of a β-sheet. The two negative bands at 208 nm and 222 nm and a positive band at 192 nm are characteristic of a α-helical structure. The contents of the secondary structures detected in the native β-LG were as follows: 49.5% α-helix, 27.9% β-sheet, 9.3% β-turn, and 13.3% random coil. After binding with kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin, the α-helix content was decreased from 49.5% to 13.5%, 15.1%, 8.2%, 21.4%, 16.0%, and 17.3%, respectively, and there was a significant increase in the β-sheets, from 27.9% to 38.5%, 42.1%, 42.5%, 32.5%, 41.1%, and 38.0%, respectively; the content of random coil was increased from 13.3% at the baseline to 24.3%, 18.5%, 25.9%, 22.6%, 20.7%, and 21.1%, respectively. These data indicate that binding of the six flavonoids induced changes in the secondary structure of β-LG; the most distinct feature of the changes induced by the formation of the β-LG-flavonoid complexes was the trending of transition from the α-helix to the β-sheet and random coil.

The inhibitory effects of these six flavonoids on the antigenicity of β-LG were closely related to the decline in the α-helix content. Phloretin induced the largest decline in the content of α-helix, from 49.5% to 8.2%, compared with that detected for the other five flavonoids. The largest α-helix shift induced by phloretin resulted in a remarkable inhibition of antigenicity of β-LG, which indicates that the conformational epitopes are more likely to be in the α-helical structure similar to the observation of Clement, Boquet, Frobert, Bernard, & Grassi (2002). Additionally, the α-helical structure had a larger decline (from 49.5% to 13.5%) in the case of kaempferol than that of quercetin (from 49.5% to 17.3%). The inhibitory effect of kaempferol on the antigenicity of β-LG was stronger than that of quercetin confirming that the α-helix content in β-LG is closely correlated with antigenicity of the protein.

However, the α-helix content is probably not the only factor contributing to the antigenicity of β-LG. EGCG had the most significant inhibitory effect on the antigenicity of β-LG among the six flavonoids; however, a decrease in the α-helix content of β-LG after binding with EGCG was the smallest (45.9% to 21.4%) indicating that the

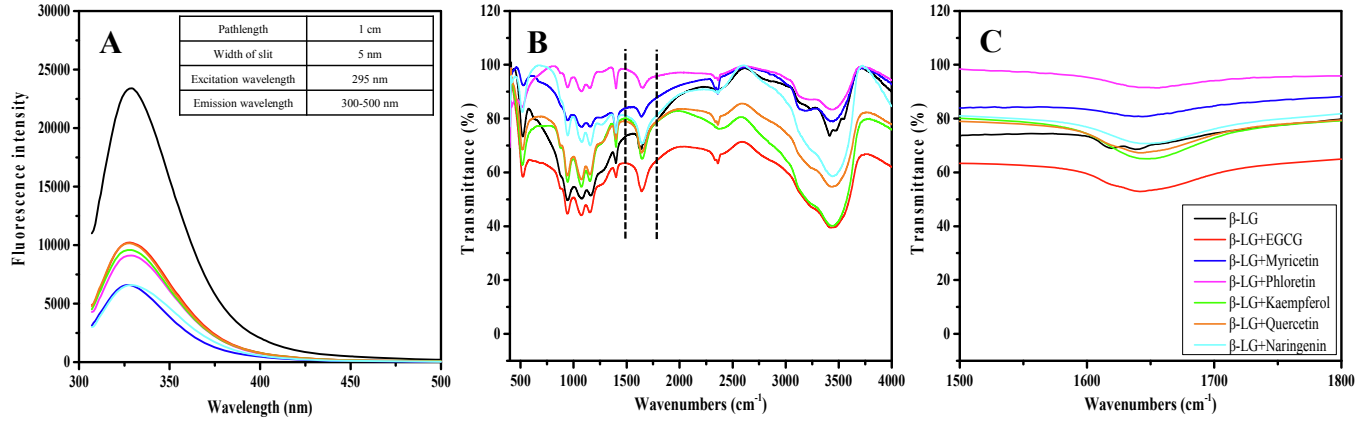

Fig. 1. Fluorescence emission spectra of β-LG (5.0 μM) at 298 K (A), FTIR spectra of β-LG in the 4000–400 cm^{-1} region (B), and the region from 1800 to 1500 cm^{-1} (including the amide I band ranging from 1700 to 1600 cm^{-1}) at 298 K (C).

antigenicity of β-LG is related to other factors in addition to the α-helix content, such as the molecular structure and the binding position relative to the epitopes. Additionally, there was no significant difference in the contents of the α-helices and β-sheets after the formation of the β-LG-flavonoid complexes of naringenin and myricetin with β-LG; however, a significant difference in the inhibitory effect of these two compounds on the antigenicity of β-LG was observed. The inhibitory effect of naringenin on the antigenicity of β-LG was stronger than that of myricetin. This result required consideration of the structures of the tested molecules and their interaction modes with β-LG.

3.5. Six flavonoids bind to the outer surface of β-LG

Molecular docking is an effective and accurate calculation simulation method for predicting binding mode and affinity between the acceptor protein and ligand molecules at the molecular level (Zeng, Ding, Hu, Zhang, & Gong, 2019). We used molecular docking to study the interactions and binding modes of β-LG and flavonoids. Two major hydrophobic ligand-binding sites are known to exist on the monomer form of β-LG (Cheng, Liu, Prasanna, & Jing, 2017): site I is located in the central hydrophobic cavity of the β-barrel structure called the calyx. Site II is located in the slit on the outer surface of β-LG, and the main amino acid residues in the site II include Trp19, Try20, Try42, Gln44, Gln59, Gln68, Leu156, Glu157, Glu158, and His161. The flavonoids (kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin) were docked to site I and site II to probe the potential interaction modes. The values of $-E_{CD}$ and $-IE_{CD}$ for site I were 9.28, 12.32, 19.29, 33.01, 17.67, and 9.64 and 16.25, 17.89, 27.26, 35.30, 24.62, and 10.59, respectively; the values of $-E_{CD}$ and $-IE_{CD}$ for site II were 5.63, 12.95, 27.72, 45.71, 22.53, and 10.03 and 14.15, 18.71, 35.04, 49.55, 30.34, and 12.86, respectively. Therefore, the $-E_{CD}$ and $-IE_{CD}$ values indicate that the flavonoids bind to the outer surface rather than to the hydrophobic cavities of β-LG; moreover, site II is the most suitable binding site, which is consistent with the conclusion of Li et al. (2018). All six flavonoids were able to successfully bind to the β-sheet and β-turn regions of the outer surface of β-LG close to the α-helical region (Fig. 3A-F).

3.6. Multiple driving forces ensure the stability of the β-LG-flavonoid complexes

Noncovalent interactions mainly include hydrogen bond, hydrophobic interaction, van der Waals interaction and electrostatic interaction (Bijari, Ghobadi, & Derakhshandeh, 2017). Moreover, pi-pi interaction also participates in the formation of noncovalent interactions (Rastegari, Karbalaei-Heidari, Yousefi, Zeinali, & Nabavizadeh, 2016). The results of Table 2 indicate that kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin interact with 8, 8, 13, 16, 15, and 6 amino acid residues of β-LG forming 2, 5, 6, 12, 4, and 4 hydrogen bonds with β-LG, respectively. Notably, Thr18 forms hydrogen bonds with five flavonoids (kaempferol, myricetin, phloretin, EGCG, and quercetin), and in all these hydrogen bonds (Fig. 3A-F), Thr18 acts as a hydrogen donor and the flavonoid acts as hydrogen acceptor. It should be noted that Gln and Glu make important contributions to the hydrogen bonding between the flavonoids and β-LG. For example, Glu44, Glu45, Glu157, and Gln59 act as hydrogen acceptors to form hydrogen bonds with EGCG. Additionally, phloretin forms hydrogen bonds with Glu44, Glu45, and Gln59 as hydrogen acceptors and forms hydrogen bonds with Gln159 as a hydrogen donor.

Van der Waals force is another factor involved in the maintenance of the stability of the β-LG-flavonoid complexes. Leu, Glu, and Gln are the key amino acid residues in the van der Waals interactions with the studied flavonoids. Additionally, Leu is the main contributor to the hydrophobic interactions between the flavonoids and β-LG. EGCG formed the largest number of hydrogen bonds with β-LG compared to other five flavonoids; a total of 9 amino acid residues of β-LG are involved in the interaction with EGCG (Table 2). Moreover, van der Waals interactions of EGCG included 9 amino acid residues (Tyr20, Ser21, Tyr42, Leu57, Gln68, Leu156, Glu157, Glu158, and Gln159). This result partly explains why EGCG has the strongest ability to inhibit the antigenicity of β-LG. Additionally, Tyr20 and Glu157 were involved in a significant interaction with EGCG via hydrogen bonding and van der Waals interactions.

To confirm the reliability of the docking results, we used another crystal structure of β-LG (PDB ID: 5IO5) for parallel docking comparison (Fig. S1 and Table S1). Consistent with the docking results obtained using the 3NPO structure, Gln and Glu played an important role in the hydrogen bond interactions, including Glu45, Glu157, and Glu158 in the case of EGCG and Glu44, Glu45, Gln59, Glu158, and Gln159 in the case of phloretin. Van der Waals forces make a considerable contribution to the maintenance of the stability of the β-LG-flavonoid complexes, and Thr, Leu, Glu, and Gln of β-LG are the main contributors. Additionally, Leu plays an important role in the hydrophobic interactions. EGCG formed more hydrogen bonds than five other flavonoids; these hydrogen bonds involved 7 amino acid residues, and the van der Waals interactions involved 10 amino acid residues. The docking results based on two crystal structures of β-LG indicate that the interactions between six flavonoids and β-LG include hydrogen bonding, van der Waals interactions and hydrophobic interaction with Leu, Glu. Gln, etc.

3.7. Flavonoids interact with the antigenic epitopes of β-LG

The binding positions of flavonoids on β-LG were crucial to reduce its antigenicity, so the relationships between the binding positions of six flavonoids and the epitope region of β-LG were analyzed. It has been reported that the epitopes of β-LG involve 27 amino acid residues:

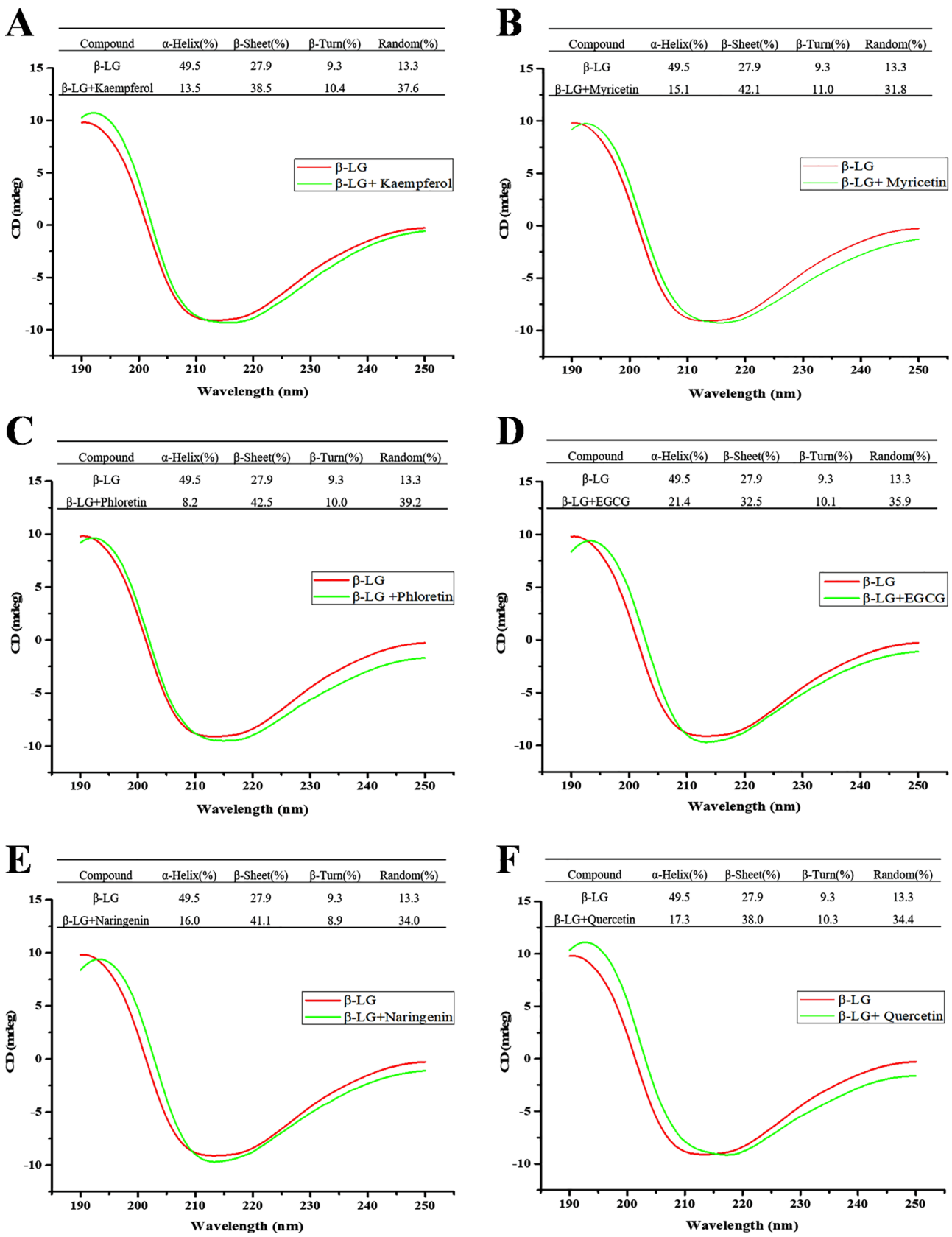

Fig. 2. Circular dichroism (CD) spectra of β-LG in the presence of six flavonoids. (The insets show the fractions of α-helices, β-sheets, β-turns and random coils in the absence and in the presence of flavonoids.)

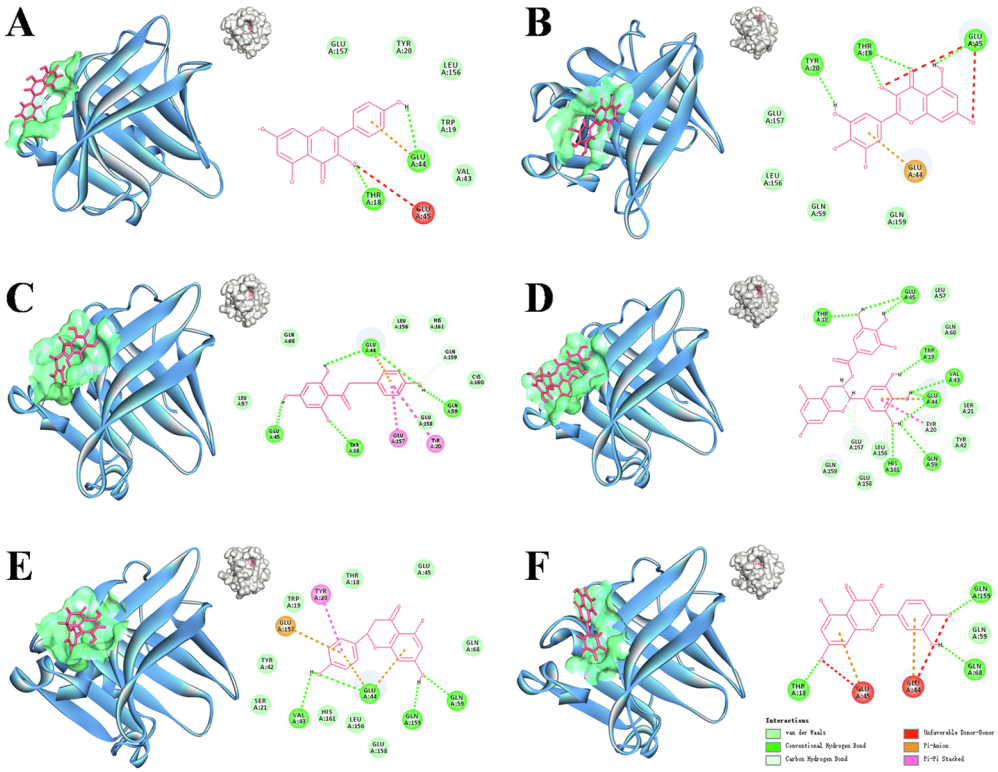

Fig. 3. The 3D interaction modes of the six flavonoids with β-LG (PDB code 3NPO). A, B, C, D, E, and F are the complexes of β-LG with kaempferol, myricetin, phloretin, EGCG, naringenin and quercetin, respectively. In the 2D schematic diagrams, the light green circles correspond to the amino acid residues interacting with the flavonoids via van der Waals forces, the green dotted lines indicate hydrogen bonds, and the purple and orange dotted lines indicate pi-pi stacking and pi-cation interactions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Trp19-Tyr20 (β-sheet A), Val43-Lys47 (β-sheet B), Leu57-Gln59 (β-sheet C), Cys66-Gln68 (β-sheet D), and Leu149-Ile162 (β-turn) (Bogahawaththa, Chandrapala, & Vasiljevic, 2019; Kurpiewska et al., 2019). Table 2 summarizes the binding patterns of the flavonoids to β-LG. The 16 amino acids that interact with EGCG are located in the β-sheet (A, B, C, and D) and β-turn of β-LG with the highest crossing rate of 59.26% demonstrating that EGCG has the strongest inhibitory effect on the antigenicity of β-LG. Phloretin and naringenin interacted with the β-sheet (A, B, C, and D) and β-turn of β-LG involving 13 amino acid residues and 15 amino acid residues, respectively. Additionally, the binding sites of kaempferol, myricetin, and quercetin had a low crossing rate (29.63%, 29.63%, and 22.22%, respectively) with the antigen epitopes of β-LG; however, these compounds were bound to the β-sheets (A and B), β-sheets (A, B, and C), β-sheets (B, C, and D), and β-turn, respectively. Therefore, six flavonoids mainly interact with the antigenic epitopes in the β-sheets and β-turns of β-LG to mask the original epitopes, thereby reducing β-LG antigenicity. The studied flavonoids did not interact with the amino acid residues in the α-helix structure in β-LG; if they had interacted, then the conformational changes and the resulting changes in the content of the α-helical structures would have been caused by binding of the flavonoids to induce dissociation and destruction of the conformational epitopes, thus reducing the antigenicity of β-LG.

The results of the docking of six flavonoids with the other crystal structure of β-LG (5IO5) (Table S1) indicated that the flavonoids bind to the epitope regions in the β-sheet and β-turn and interact directly with 13, 12, 15, 18, 14, and 11 amino acid residues of β-LG, respectively. EGCG and phloretin interacted with β-sheets A, B, C, D, and H, β-turn and β-sheets A, B, and C and β-turn, respectively, with the top crossing rates of 66.67% and 55.56%, respectively, indicating significant inhibition of the antigenicity of β-LG. The binding modes of four other compounds, kaempferol, myricetin, naringenin, and quercetin, were mainly concentrated in the β-sheets (A, B, and C) and β-turn with the crossover rates of 48.15%, 44.44%, 51.85%, and 41.74%, respectively. These results indicate that six flavonoids bind to the β-sheet and β-turn structures of β-LG to partly shield or destroy the epitopes, reducing their antigenicity, and they do not directly interact with the α-helical structure of β-LG, consistent with the docking results of the 3NPO protein structure.

3.8. Structural differences of the flavonoids result in differences in β-LG antigenicity reduction

Structural characteristics of six flavonoids were assessed to determine the reasons for differential effects of the compounds on the antigenicity of β-LG. The molecular basis of declined antigenicity of β-LG was reported to be associated with dissociation and destruction of the epitope structures or with lowered accessibility of the epitopes (Besler, Steinhart, & Paschke, 2001). According to these results, all studied flavonoids bind to the antigenic epitope region of β-LG, which explains why the flavonoids can affect the antigenicity of β-LG. On the one hand, some epitopes are shielded by the binding of the flavonoids resulting in a reduction in antigenicity. On the other hand, this effect can be predominantly attributed to the conformational changes in β-LG caused by the flavonoids subsequently inducing the corresponding changes in the epitopes. However, the effects of six flavonoids on the antigenicity of β-LG were significantly different, and the essential reason for this difference was attributed to the structural differences between the compounds.

EGCG was the strongest inhibitor of antigenicity among six flavonoids, which was the result of the efficient binding of EGCG to β-LG. During the binding process, the B ring of the compound is embedded into the slit of β-LG to interact with it, while the exposed A and C rings and the gallic acid moiety may have sheltered the epitopes adjacent to the binding sites (Glu45, Glu157, etc.). Phloretin is a linear molecule, which contains several rotating single bonds to ensure that the structure can to rotate to attain a suitable pose in its complex with β-LG, suggesting that their proximity promotes β-LG binding with phloretin to inhibit β-LG antigenicity effectively. Myricetin, kaempferol, and quercetin are flavonols with variable number of hydroxyl groups on the B ring. Hydroxylation of the B ring of flavonols was shown to have little effect on their binding affinity to β-LG (Xiao et al., 2011), indicating that the inhibitory effects of myricetin and kaempferol on the

Table 2
The binding patterns of six flavonoids to β-LG.

Flavonoids	The number of interacting amino acids	Amino acids forming H-bond	Amino acids involved in van der Waals interactions	Amino acids involved in hydrophobic interactions	Secondary structures involved in interaction [a]	The crossing rate [b]
Kaempferol	8	Thr18, Glu44	Trp19, Tyr20, Val43, Leu156, Glu157	Val43, Leu156	β-sheet (A, B) and β-turn	29.63
Myricetin	8	Thr18, Tyr20, Glu45	Gln59, Leu156, Glu157, Gln159	Leu156	β-sheet (A, B, C) and β-turn	29.63
Phloretin	13	Thr18, Glu44, Glu45, Gln59, Gln159	Leu57, Gln68, Leu156, Glu158, Cys160, His161	Leu57, Leu156	β-sheet (A, B, C, D) and β-turn	48.15
EGCG	16	Thr18, Trp19, Tyr20, Val43, Glu44, Glu45, Gln59, Glu157, His161	Tyr20, Ser21, Tyr42, Leu57, Gln68, Leu156, Glu157, Glu158, Gln159	Trp19, Ser21, Val43, Leu57, Leu156,	β-sheet(A, B, C, D) and β-turn	59.26
Naringenin	15	Val43, Glu44, Gln59, Gln159	Thr18, Trp19, Ser21, Tyr42, Glu45, Gln68, Leu156, Glu158, His161	Trp19, Ser21, Val43, Leu156,	β-sheet (A, B, C, D) and β-turn	55.56
Quercetin	6	Thr18, Gln68, Gln159	Gln59	none	β-sheet (B, C, D) and β-turn	22.22

[a] The distribution of antigenic epitopes in β-sheets and β-turns was reported as follows: Trp19-Tyr20 (β-strand A), Val43-Lys47 (β-strand B), Leu57-Gln59 (β-strand C), Cys66-Gln68 (β-strand D), and Leu149-Ile162 (β-turn) (Bogahawaththa et al., 2019; Kurpiewska et al., 2019).
[b] The crossing rate is the number of amino acids associated with an epitope (in β-sheets A, B, C, and D and in β-turn) divided by the number of amino acids involved in interaction of six flavonoids with β-LG.

antigenicity of β-LG are not significantly different ($P > 0.05$). Quercetin can easily react with water and form hydrates with variable molecular weight, thus making it difficult to effectively bind to β-LG; hence, only a minimal reduction in the antigenicity of β-LG was observed. Naringenin is a flavanone compound. Hydrogenation of the C2 = C3 double bond destroys the near-planar structure of the B and C rings and twists the B ring closer to the C ring (Edenharder, von Petersdorff, & Rauscher, 1993), improving the efficiency of its binding to β-LG. This feature can explain stronger inhibition of the antigenicity of β-LG by naringenin compared with that of the other flavonols (myricetin, kaempferol, and quercetin).

4. Conclusions

A promising approach was used to reduce the antigenicity of β-LG via the noncovalent binding of six flavonoids. The results indicate that the studied flavonoids dock to the outer surface rather than to the central hydrophobic cavity of β-LG via various driving forces. Interestingly, six flavonoids are mostly bound to the epitope regions of the β-sheets and β-turn in β-LG. Within the same modification range, the order of the flavonoid potency of β-LG antigenicity inhibition was as follows: EGCG > phloretin > naringenin > myricetin > kaempferol > quercetin. Differences in the inhibition of the antigenicity of β-LG are due to differences in the interaction modes between the flavonoids and β-LG, masking or altering the epitope region or structure of β-LG, which is ultimately attributed to the structural differences of the studied molecules. This study clarifies the binding patterns of the studied flavonoids with β-LG and the mechanism of inhibition of its antigenicity, which can expand the applications of β-LG, provide a theoretical basis for the development of hypoallergenic foods.

CRediT authorship contribution statement

Pei Pu: Data curation, Methodology, Formal analysis. **Xin Zheng:** Software, Investigation, Methodology. **Linna Jiao:** Validation. **Lang Chen:** Visualization, Validation. **Han Yang:** Software, Validation, Resources. **Yonghong Zhang:** Software, Resources, Validation. **Guizhao Liang:** Conceptualization, Methodology, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128106.

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Six flavonoids inhibit the antigenicity of β-lactoglobulin by noncovalent interactions: A spectroscopic and molecular docking study

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Table S1

The binding patterns of six flavonoids to β-LG.

Flavonoids	The number of interacting amino acids	Amino acids forming H-bond	Amino acids involved in van der Waals interactions	Amino acids involved in hydrophobic interactions	Secondary structures involved in interaction [a]	The crossing rate [b]
Kaempferol	13	Val43, Gln59	Trp19, Ser21, Tyr42, Leu156, Glu158, Gln159, Cys160, His161	Trp19, Ser21, Val43, Leu156	β-sheet(A, B, C) and β-turn	48.15
Myricetin	12	Trp19, Leu156, Glu158, Gln159	Tyr20, Tyr42, Val43, Gln59, Cys160, His161	Trp19, Val43, Leu156	β-sheet(A, B, C) and β-turn	44.44
Phloretin	15	Tyr20, Val43, Glu44, Glu45, Gln59, Leu156, Glu158, Gln159	Thr18, Trp19, Ser21, Tyr42, Glu157, Gln159, Cys160, His161	Trp19, Ser21, Val43, Leu156	β-sheet(A, B, C) and β-turn	55.56
EGCG	18	Thr18, Trp19, Glu45, Leu156, Glu157, Glu158, His161	Thr18, Ser21, Tyr42, Val43, Gln59, Gln68, Pro126, Gln155, Gln159, Cys160	Trp19, Ser21, Val43, Pro126, Leu156,	β-sheet(A, B, C, D, H) and β-turn	66.67
Naringenin	14	Val43, Gln59, Glu157, Gln159	Thr18, Trp19, Ser21, Tyr42, Leu156, Glu158, Cys160, His161	Trp19, Ser21, Val43, Leu156,	β-sheet(A, B, C) and β-turn	51.85
Quercetin	11	Trp19, Tyr20, Gln59, Gln159	Thr18, Val43, Leu156, Glu158, His161	Trp19, Val43, Leu156	β-sheet(A, B, C) and β-turn	40.74

[a] The distribution of antigenic epitopes in β-sheets and β-turns was reported as follows: Trp19-Tyr20 (β-strand A), Val43-Lys47 (β-strand B), Leu57-Gln59 (β-strand C), Cys66-Gln68 (β-strand D), and Leu149-Ile162 (β-turn) (Bogahawaththa, Chandrapala & Vasiljevic, 2019; Kurpiewska et al., 2019).

[b] The crossing rate is the number of amino acids associated with an epitope (in β-sheets A, B, C, and D and in β-turn) divided by the number of amino acids involved in interaction of six flavonoids with β-LG.

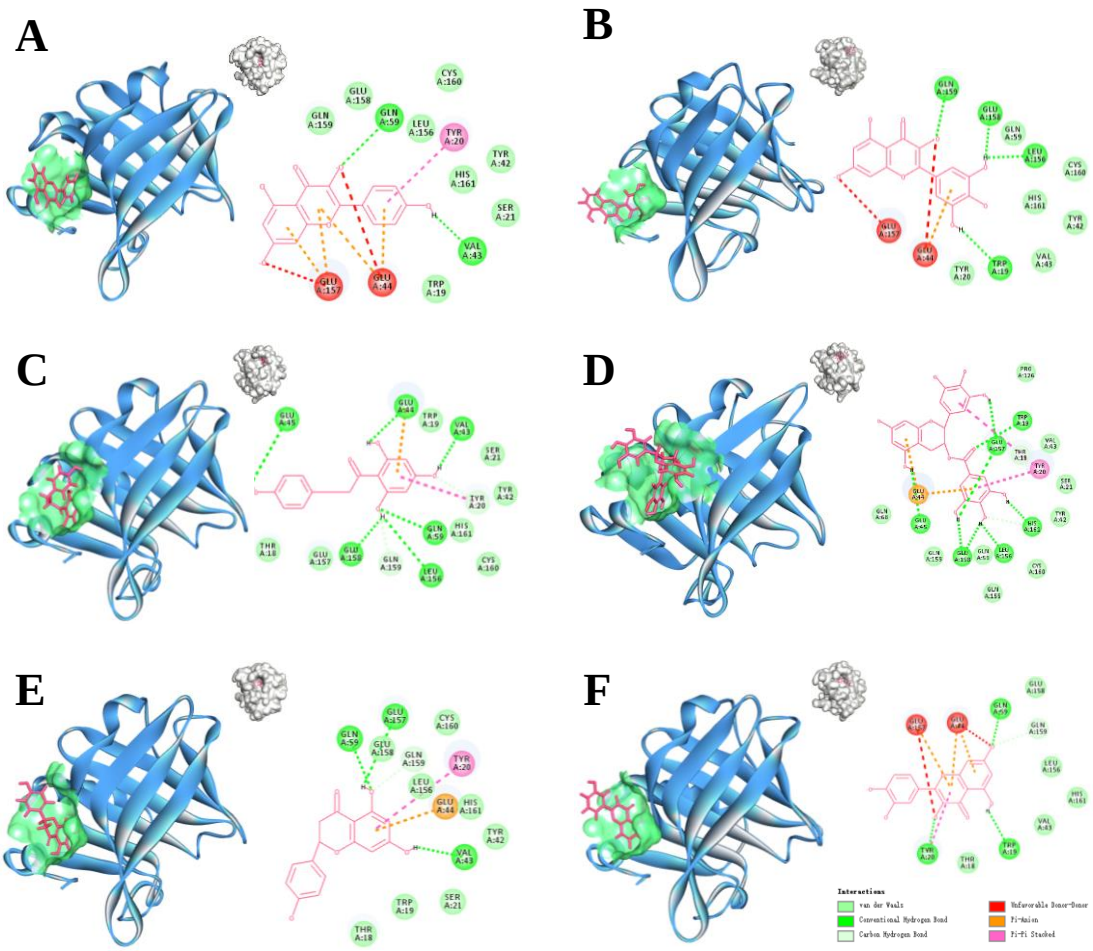

Fig. S1. The 3D interaction modes of the six flavonoids with β-LG (PDB code 5IO5). A, B, C, D, E, and F are the complexes of β-LG with kaempferol, myricetin, phloretin, EGCG, naringenin, and quercetin, respectively. In the 2D schematic diagrams, the light green circles correspond to the amino acid residues interacting with the flavonoids via van der Waals forces, the green dotted lines indicate hydrogen bonds, and the purple and orange dotted lines indicate pi-pi stacking and pi-cation interactions, respectively.

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