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An investigation of molecular dynamics simulation and molecular docking: Interaction of citrus flavonoids and bovine β -lactoglobulin in focus

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Abstract

Citrus flavonoids are natural compounds with important health benefits. The study of their interaction with a transport protein, such as bovine β -lactoglobulin (BLG), at the atomic level could be a valuable factor to control their transport to biological sites. In the present study, molecular docking and molecular dynamics simulation methods were used to investigate the interaction of hesperetin, naringenin, nobiletin and tangeretin as citrus flavonoids and BLG as transport protein. The molecular docking results revealed that these flavonoids bind in the internal cavity of BLG and the BLG affinity for binding the flavonoids follows naringenin>hesperetin>tangeretin>nobiletin. The docking results also indicated that the BLG–flavonoid complexes are stabilized through hydrophobic interactions, hydrogen bond interactions and π – π stacking interactions. The analysis of molecular dynamics (MD) simulation trajectories showed that the root mean square deviation (RMSD) of various systems reaches equilibrium and fluctuates around the mean value at various times. Time evolution of the radius of gyration, total solvent accessible surface of the protein and the second structure of protein showed as well that BLG and BLG–flavonoid complexes were stable around 2500 ps, and there was not any conformational change as for BLG–flavonoid complexes. Further, the profiles of atomic fluctuations indicated the rigidity of the ligand binding site during the simulation.

Keywords

Citrus flavonoids

Bovine β -lactoglobulin

Binding affinity

Molecular docking

Molecular dynamics simulation

1. Introduction

Citrus fruits have a determining role in human health. Due to the delicious flavors and healthy benefits of these fruits, they play a major role in human diet. Their flavonoids are actually among the main components of these fruits. Flavonoids have the same skeleton but have different classifications that are generated upon the modifications of the nucleus of these compounds. Flavonoids display antioxidant [1], [2] and anti-carcinogenic activity [3], [4], [5]. They are mainly detected in human serum and urine. It is thus clear that the study of bioactivity of flavonoids is essential to achieve a full biological significance. Below is a description of citrus flavonoid:

Naringenin (Fig. 1a) is the predominant flavonoid in grapefruit. Its antioxidant activity on angiotensin II-treated VSMCs and its role in cell growth have been investigated previously [6], [7].

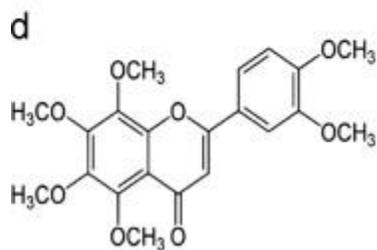
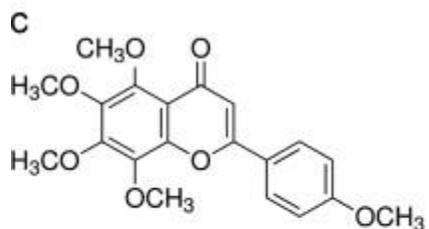
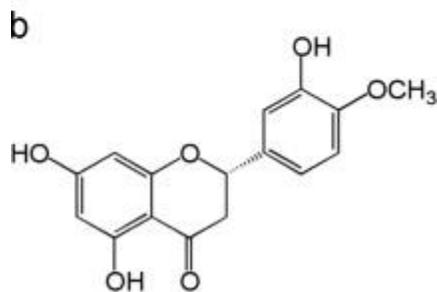
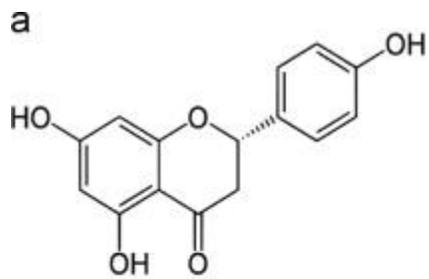


Fig. 1. Chemical structure of citrus flavonoids. (a) Naringenin, (b) hesperetin, (c) tangeretin and (d) nobiletin.

Hesperetin (Fig. 1b) is another citrus flavonoid specifically found in peel. It has a protective role against lead-induced damage in rats and cardiac remodeling induced by pressure overload [8], [9]. Nobiletin (Fig. 1c), isolated from citrus peels, like in tangerine. Nobiletin has anti-inflammatory effects and inhibits cartilage degradation [10], [11].

Tangeretin (Fig. 1d) is found in tangerine and other citrus peels, inhibits agonist-induced integrin, α IIb β 3 inside-out and outside-in signaling, intracellular calcium mobilization, and granule

secretion [12]. The results of Chong et al. suggest that tangeretin can be appropriate for chemosensitization of cancerous cells which lack the ability of DSB-repair [13].

The delivery of flavonoids and their metabolites are poorly understood. The interaction of these flavonoids and a transport protein such as bovine β -lactoglobulin (BLG) could be a valuable factor to control their transport to biological sites.

Lipocalins are small proteins with the ability to bind and transfer small hydrophobic ligands [14], [15]. The core structure of these proteins consists of an eight stranded antiparallel β -barrel that defines an internal cavity for binding the ligands. Bovine β -lactoglobulin (BLG) is the major whey protein, with several genetic variants [16], [17]. It consists of 162 amino acids with about 18.4 kDa of molecular weight [18]. The high affinity of BLG to hydrophobic ligands such as steroids, fatty acids, retinoids, vitamin D, and cholesterol, has been verified [19], [20], [21].

Unfortunately, the clinical application of flavonoids is limited due to their poor water solubility and low bioavailability caused by their hydrophobic ring structure. Besides, an important physicochemical property of BLG is its ability to bind in-vitro several physiologically relevant ligands. Therefore, BLG can serve as a potential transport and depot protein, especially for poor water-soluble molecules. Bohin et al. [22] have confirmed the efficacy of milk proteins as carriers for flavonoids. Also, finding novel bioactive ligand molecules of BLG is of biotechnological and pharmacological importance. This contributes to deeper understanding of molecular recognition properties of this lipocalin. However, BLG exhibits significant resistance against both gastric and simulated duodenal digestions. Therefore, it seems a proper nominee for the safe delivery and protection of pH sensitive drugs in stomach. Moreover, the high stability of BLG at acidic condition guarantee the low delivery of hydrophobic ligands in acidic condition of stomach.

We have previously studied the interaction of three polyphenol flavonoids (quercetin, quercitrin and rutin) and BLG [23]. In the present study, however, we examined the interaction and protein conformation of BLG with hesperetin, naringenin, tangeretin and nobiletin, using molecular docking and molecular dynamics simulation studies. Finding new biologically active ligands to bind BLG is very important, and not only can contribute to the better understanding of the molecular properties of this protein, but also might pave the way for future studies about the transport of drugs to biological sites.

2. Experiment

2.1. Molecular docking

All of the docked conformations of flavonoid–BLG complexes were generated through using ArgusLab 4.0.1 molecular docking program [24]. Comparison of the results of ArgusLab and Genetic Optimization for Ligand Docking (GOLD) showed a little difference in their docking accuracies [24], [25], [26]. Also, the accuracy of ArgusLab was checked by redocking the dodecyl sulfate to BLG. After docking the dodecyl sulfate back into BLG, the RMSD value between docked dodecyl sulfate and reference dodecyl sulfate was 2.4 Å and the interactions between docked dodecyl sulfate and BLG were similar to those of original modes, suggesting that ArgusLab is suitable for docking of ligands to BLG.

The known crystal structure of BLG (PDB ID: 3NPO) was taken from the RSCB protein data bank (www.rcsb.org) and was prepared in pH=7.5. The *R*-value of this file was 0.216, a sufficient capacity for further studies. The water molecules in the used pdb file are not structural; thus, the ArgusLab suite was used to remove them. Actually, Becke three-parameter Lee–Yang–Parr (B3LYP) hybrid density functional theory with the basis set of 6–31 g** was employed to optimize the flavonoids structures, using the GAMESS quantum chemistry software (USA) [27]. The blind docking approach was used and the whole protein was taken as a potential binding site. Also, Ascore scoring method in ArgusLab using high precision, 0.4 Å grid resolution, and maximum 200 candidate poses were employed for docking calculations. During all docking process, the structure of BLG kept rigid and all the torsional bonds of ligands were set free (flexible docking). Ascore scoring function ranked the docked conformations on the basis of their free binding energy. The lowest free binding energy conformation of each flavonoid–BLG complex was left for further studies.

2.2. Molecular dynamics simulation

The lowest free binding energy conformation of each complex was considered as the initial conformation for the MD studies. All MD studies were carried out using GROMACS 4.5.5 (University of Groningen, Netherlands) package [28], [29] and GROMOS96 43a1 force field [30], [31]. The Dundee PRODRG2.5 server was used to generate the topology parameters of flavonoid [32]. The partial atomic charges of flavonoids were calculated using GAMESS quantum

chemistry software (USA) [27] at the level of HF/6-31 g**. The complex was located in the cubic box with the periodic boundary conditions. The box volume was 294.14 nm³ (6.6504×6.6504×6.6504 nm³) and the minimum distance between protein surface and the box was 1.0 nm. The box filled with extended simple point charge (SPC), water molecules [33], and the solvated systems were neutralized by adding 8 sodium ions (Na⁺). Energy minimization was done through using the steepest descent method for 8 ps. Then, the system was equilibrated for 40 ps at the temperature of 300 K. Finally, a 10 ns MD simulation was carried out at 1 bar and 300 K. Berendsen thermostat [34] at 300 K, the particle mesh Ewald (PME) method [35], [36] for long range electrostatics, and a 7 Å cut off for van der Waals interactions and Coulomb interactions were used. The equation of motions was integrated by the leap-frog algorithm with the 2 fs time step. The atomic coordinates were recorded to the trajectory file every 0.5 ps for later analysis. Finally, an all-bond constrain was used to keep the ligand from drifting in MD.

3. Results and discussion

3.1. Molecular docking studies

BLG has three potential binding sites, with the internal cavity as the main binding site for the hydrophobic ligands [37]. Previous studies on the interaction of polyphenol flavonoids and BLG show that quercetin and quercitrin bind in this cavity [23]. In the present study, the binding mode of citrus flavonoids, i.e. hesperetin, naringenin, tangeretin and nobiletin, at the active site of BLG was examined. The docking results revealed that hesperetin, naringenin, tangeretin and nobiletin bind in the internal cavity of BLG (Fig. 2). Fig. 3 shows the interactions of flavonoids within the internal cavity of BLG. This binding site is considerably hydrophobic, and naringenin is in hydrophobic interaction with Val (41), Val (43), Pro (38), Leu (39), Leu (58), Met (107), Ile (56), and Ile (84). However, despite the main role of hydrophobic interactions, this site focused on some hydrogen-bond interactions where the –OH groups of naringenin interact with oxygen of Pro (38) and nitrogen of Asn (90). In addition, the π - π stacking interaction with Phe (105) and dipolar interactions with Asn (90) and Gln (120) could stabilize the naringenin in its binding site. Fig. 3b shows that hesperetin is in hydrophobic interaction with Ile (12), Ile (56), Leu (46), Leu (54), Val (15), Val (41), Val (43), Val (92), and Met (107). However, the –OH of hesperetin is in hydrogen-

bond interaction with oxygen of Val (41). The π - π stacking interaction with Phe (105) and dipolar interactions with Gln (120) can also stabilize the hesperetin in its binding site. Fig. 3c shows that tangeretin is in hydrophobic interaction with Ile (71), Ile (56), Leu (39), Leu (58), Pro (38), Val (41), and Met (107). At the same time, the $-OCH_3$ of tangeretin are in hydrogen-bond interactions with nitrogen of Lys (69). The π - π stacking interaction with Phe (105) and dipolar interactions with Lys (69) can stabilize the tangeretin in its binding site. Finally, Fig. 3d indicates that nobiletin is in hydrophobic interaction with Asn (90), Ile (71), Ile (56), Ile (84), Leu (39), Leu (46), Val (41), Val (43), and Met (107). However, the $-OCH_3$ of nobiletin is in hydrogen-bond interaction with nitrogen of Asn (90). Furthermore, the π - π stacking interaction with Phe (105) and dipolar interactions with Gln (120), Lys (60), and Lys (69) can stabilize hesperetin. The near residues for each flavonoids are listed in Table 1.

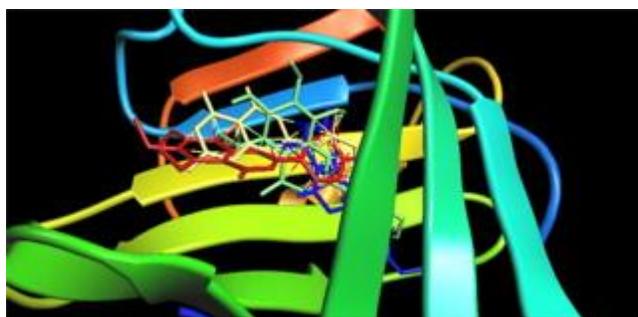
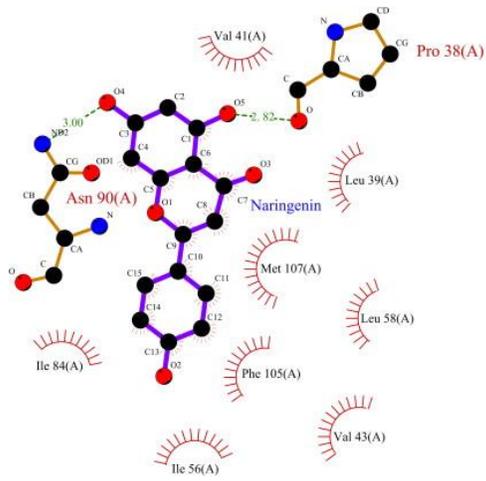


Fig. 2. Superposition of the docking poses of naringenin (blue), hesperetin (green), tangeretin (yellow) and nobiletin (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

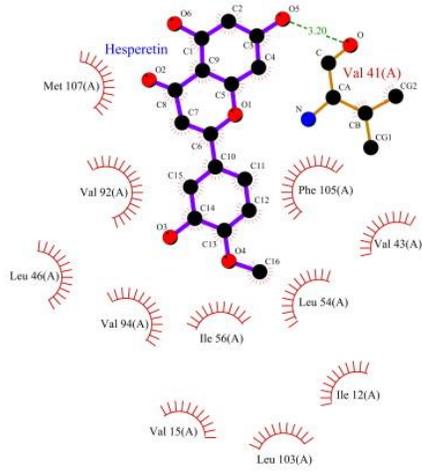
a



Key

- ● Ligand bond
- ● Non-ligand bond
- ● Hydrogen bond and its length
- Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

b



Key

- ● Ligand bond
- ● Non-ligand bond
- ● Hydrogen bond and its length
- Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

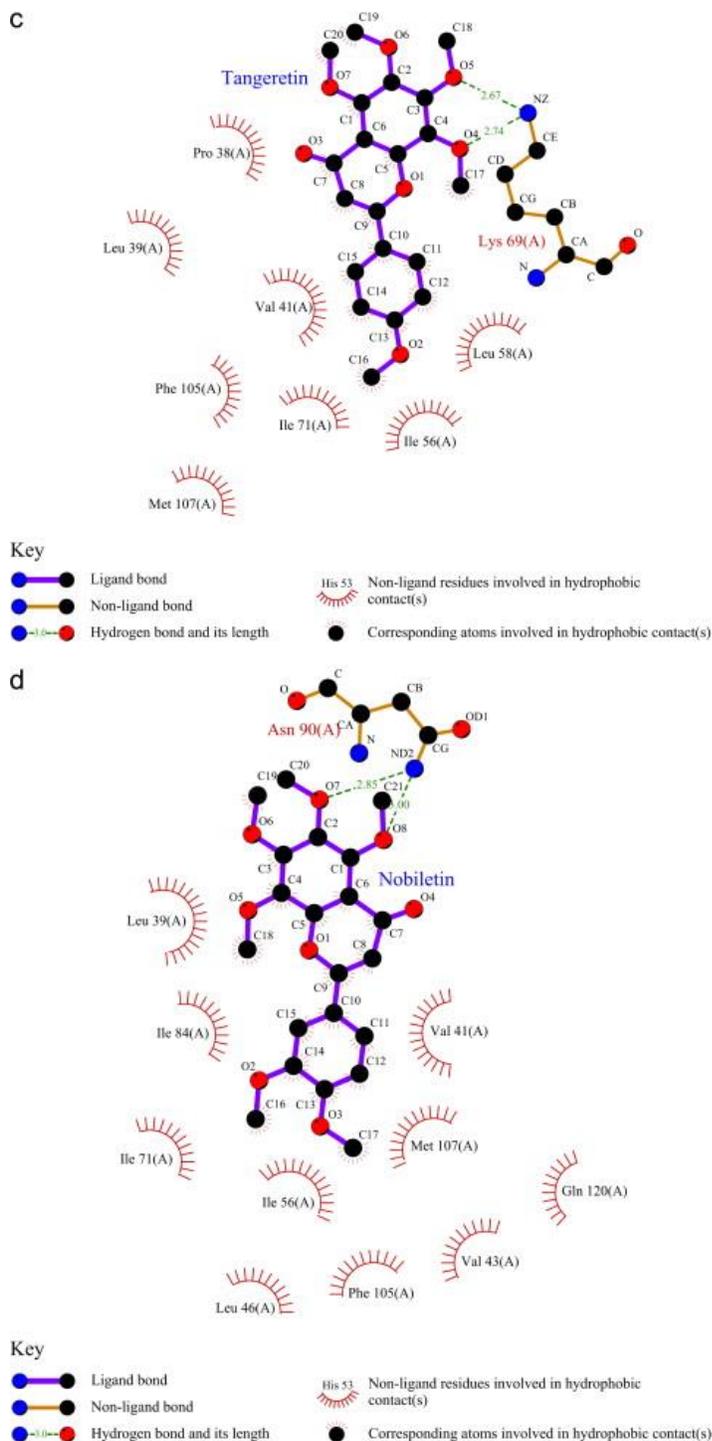


Fig. 3. The docking poses of the BLG–flavonoid complexes. Naringenin (a), hesperetin (b), tangeretin (c) and nobiletin (d). H-bonds (as highlighted by the line in green colors) formed between ligands and BLG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1. Residues within 3 Å distance from each flavonoid.

Flavonoid	Residues
Naringenin	Ala (73), Asn (90), Gln (120), Ile (12), Ile (56), Ile (71), Ile (84), Leu (39), Leu (46), Leu (54), Leu (58), Leu (103), Leu (122), Met (107), Phe (82), Phe (105), Pro (38), Val (15), Val (41), Val (43), Val (92), Val (94)
Hesperetin	Ala (73), Gln (120), Ile (12), Ile (56), Ile (71), Ile (84), Leu (46), Leu (54), Leu (103), Leu (122), Met (107), Phe (82), Phe (105), Val (15), Val (41), Val (43), Val (92), Val (94)
Tangeretin	Ile (56), Ile (71), Ile (84), Leu (39), Leu (46), Leu (54), Leu (58), Leu (103), Leu (122), Lys (69), Met (107), Phe (105), Pro (38), Val (41), Val (43), Val (92)
Nobiletin	Asn (90), Gln (120), Ile (56), Ile (71), Ile (84), Leu (39), Leu (46), Leu (54), Leu (58), Leu (122), Lys (60), Lys (69), Met (107), Phe (105), Pro (38), Tyr (42), Val (41), Val (43), Val (92)

The binding constant (K_a), free binding energy (ΔG°), and the structural volume (V) of flavonoids are listed in Table 2. The comparison of binding energies shows that $-\text{OCH}_3$ groups interfere negatively with binding of flavonoids to BLG. The results reveal how the substitution of $-\text{OH}$ groups with $-\text{OCH}_3$ groups causes an increase in the volume of flavonoids, while their binding constant decreases and the structural volume of flavonoids increases. It is to be added that as the structural volume of the ligand increases, the orientation of the ligand in its binding site gets inappropriate, and this can affect the fitting of ligand in the binding site. The inappropriate orientation of ligand leads to the reduction of both hydrophobic interactions and the stability of BLG–flavonoid complexes. Similar results have been observed in the interaction of rutin and BLG [23].

Table 2. Docking summary of BLG with flavonoids.

Flavonoid	K_a (M^{-1})	ΔG° (kcal mol $^{-1}$)	V (\AA^3)
Naringenin	1.004×10^8	-10.91	727.25
Hesperetin	4.542×10^7	-10.44	804.38
Tangeretin	5.141×10^6	-9.15	1011.95
Nobiletin	6.550×10^5	-7.93	1080.08

3.2. MD simulation studies

The root mean square deviation (RMSD) of the backbone of BLG was obtained to examine the stability of trajectories for unliganded BLG and BLG–flavonoids complexes (Fig. 4). A glance at the analysis of Fig. 4 shows that the RMSD of various systems reached equilibrium and fluctuated around the mean value at various times. These times were about 1100, 3500, 3600, 4000 and 5500 ps, respectively for BLG–tangeretin, unliganded BLG, BLG–hesperetin, BLG–nobiletin and BLG–naringenin complex.

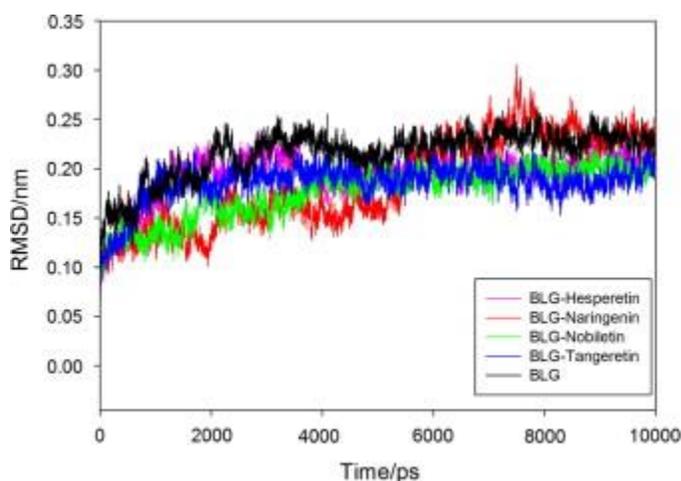


Fig. 4. Time dependence of RMSD. RMSD values for unliganded BLG and BLG–flavonoid complexes.

The radius of gyration (R_g) for the BLG and BLG–flavonoid complexes were also specified and plotted versus simulation time to examine the protein compactness (Fig. 5). The results showed

that the R_g of all systems stabilizes after about 2500 ps, indicating that the MD simulation equilibrated after 2500 ps. The initial value of R_g for all systems was about 1.44 nm, which approves earlier experimental results [38]. Also, Fig. 5 shows that the R_g value of BLG does not depend upon the complexation with flavonoids. This indicates that the environment of BLG did not change during its interaction with flavonoids.

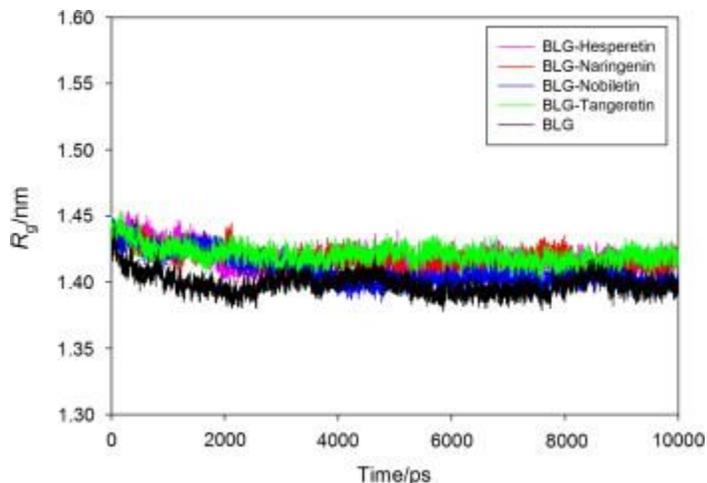


Fig. 5. Time evolution of the radius of gyration (R_g) during 10000 ps of MD simulation of BLG and BLG–flavonoids complexes.

Fig. 6 shows the changes in the total solvent accessible surface (SAS) of protein during the 10 ns simulation time. The variation of SAS represents that the trends for unliganded BLG and BLG–flavonoid complexes are similar to the variation of their R_g . This similarity validates the accuracy of MD simulation results.

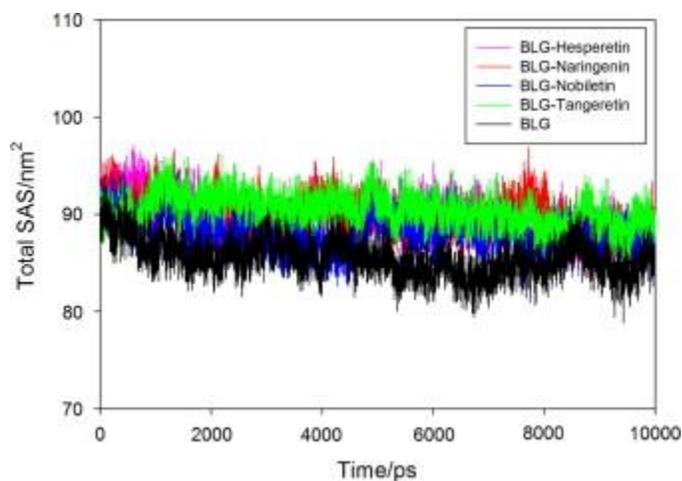


Fig. 6. Time evolution of the total solvent accessible surface (SAS) during 10000 ps of MD simulation of BLG and BLG–flavonoids complexes.

In addition, the amount of secondary structure components of unliganded BLG and BLG–flavonoid complexes revealed that the conformation of BLG does not change during flavonoids complexation (Fig. 7).

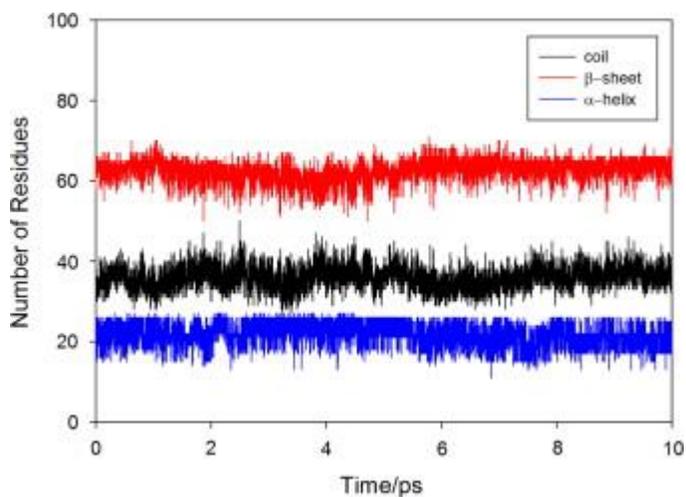


Fig. 7. Amount of the secondary structure components of BLG–hesperetin complex. (The results for unliganded BLG and other BLG–flavonoid complexes are not shown.)

The time averaged root mean square fluctuations (RMSF) of protein residues was obtained to analyze the local mobility of protein. The RMSF values versus residue numbers are illustrated in Fig. 8. These values are based on the 8900, 6500, 6400, 6000 and 4500 ps of trajectory, respectively for BLG–tangeretin, unliganded BLG, BLG–hesperetin, BLG–nobiletin and BLG–naringenin complex. The profiles of RMSF for unliganded BLG and BLG–ligand complex were similar. Also, RMSF profiles for BLG–ligand complexes showed that the fluctuations of residues in the internal cavity are lower than the others. This clearly indicates that the binding site remains approximately rigid during the MD simulation. Further, the comparison of the superposition of initial docking results (Fig. 2) and the average structure of the MD simulation (Fig. 9) indicated that flavonoids remain in the internal cavity of BLG during the simulation time.

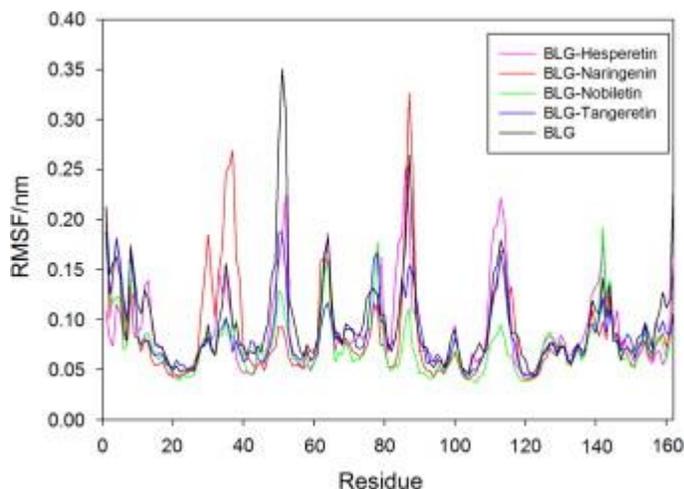


Fig. 8. The RMSF values of non-liganded BLG and BLG–flavonoids complexes were plotted against residue numbers.

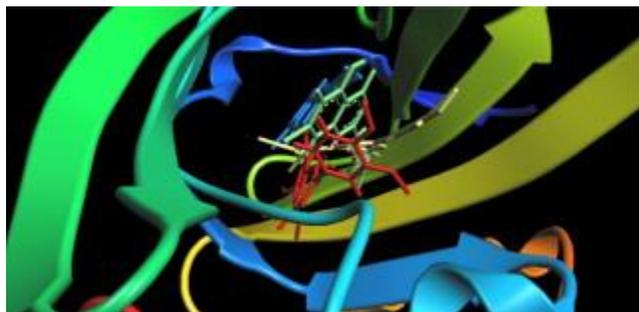


Fig. 9. Superposition of the average structure from the MD of naringenin (blue), hesperetin (green), tangeretin (yellow) and nobiletin (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Summary

Based on our molecular docking results, tangeretin, naringenin, hesperetin and nobiletin bind in the internal cavity of BLG. Among these flavonoids, naringenin has the lowest structural volume and its free binding energy is more negative than others. Therefore, the docking results represent that the hydrophobic interactions have a dominant role in the BLG–flavonoid complex stability. MD simulation showed the stabilization of BLG and BLG–ligand complexes to be around 2500 ps. Besides, BLG did not exhibit conformational changes during flavonoids binding. Also, the profiles of atomic fluctuations showed the rigidity of ligand binding site during the simulation. Despite to this fact that we have investigated the binding interaction of these flavonoids with BLG at physiological pH, it should be mentioned that pH does not have substantial effect on the binding affinity of BLG [39], therefore, it can be concluded that our obtained results certified the safe transferring of these flavonoids from stomach.

Conflict of interest

None.

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Fig. 9. Superposition of the average structure from the MD of naringenin (blue), hesperetin (green), tangeretin (yellow) and nobiletin (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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