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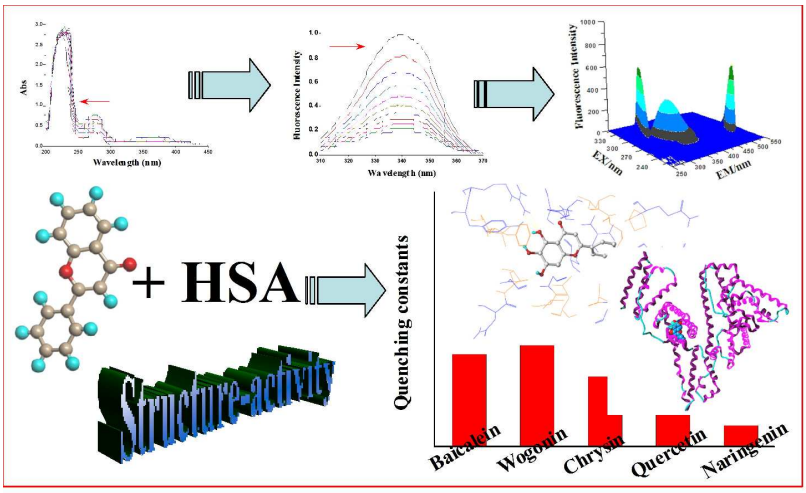
Graphical abstract



In this study, the influence about the introduction of functional groups on the interaction between flavonoids and human serum albumin has been studied detailed; Quenching degree for different flavonoids on HSA fluorescence intensity is differ from each other; The conformation change of HSA by flavonoids has been considered through multiple methods; Molecular docking method has been introduced to verify that the related experimental results.

Study of the Structure-Activity Relationship of Flavonoids Based on Their Interaction with Human Serum Albumin

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Abstract

Structure-activity relationship (SAR) study helps in understanding biological effects of a compound, thus contributing to the development of new drugs. On the other hand, the study of protein-drug interaction is very important for understanding mechanism behind versatile bioactivities of drugs. Flavonoids have been known for their numerous biological activities. To examine the potential of flavonoids as therapeutics, we systematically investigated SAR of flavonoids based on their interaction with human serum albumin (HSA). Our study demonstrates that all the studied flavonoids (baicalein, wogonin, chrysin, naringenin, and quercetin) bind to HSA at the subdomain IIIA. Molecular docking was employed to investigate binding sites and surrounding environment of flavonoids on HSA. We found that number and position of hydroxyl groups, conjugated structure, and functional groups are responsible for differences in the interactions between different flavonoids and HSA. Our results together provide further molecular level understanding of protein-polyphenol binding and a strategy for SAR studies.

Keywords: Flavonoids, structure-activity relationship, human serum albumin, fluorescence spectroscopy, molecular docking.

1. Introduction

Flavonoids, a large group of plant metabolites (polyphenolic molecules) containing a benzo-γ-pyrane structure with two phenyl rings and one heterocyclic ring, have been known for numerous biological activities and low toxicity.[1-3] More than 5000 natural flavonoids have been identified in plants as well as in various dietary sources that are presumed to have potential health benefits.[4] Flavonoid compounds often attracts phytochemists and experimental biologists because of their interesting chemical properties and biological activities.[5-7] Baicalein, wogonin, and chrysin are flavonoid belonging to the flavone subgroup, while chrysin, naringenin, and quercetin are flavonoid belonging to different subgroups — flavones, dihydroflavones, and flavonols, respectively. Moghaddam et al.[8] reported that baicalein exhibits significant antiviral effects against in vitro replication of dengue virus-2 in Vero cells, functioning at different stages of virus replication. Wogonin exhibits anti-angiogenesis and has been reported to inhibit cell growth and induce apoptosis in various cancer cell lines.[9] Chrysin has been reported to possess antioxidant, anti-inflammatory, vasodilatory, and anticancer properties.[10] Naringenin, a grapefruit flavonoid, dose-dependently inhibits HCV production without affecting intracellular levels of the viral RNA or protein and prevents metabolic deregulation induced by dietary cholesterol both in the presence and absence of dietary fat.[11,12] Quercetin has also drawn increased attention as an potential chemopreventive agent that significantly reduces cancer risk by intervening with the antiapoptotic proteins of the Bcl-2 family and recently has been shown to inhibit proliferation in orthotopically transplanted pancreatic xenografts.[13,14] All these are flavonoids showing different biological activity because of differences in their structures (Table 1). The differences in number and distribution of substituents across the three rings are responsible for their wide

range pharmacological properties. Therefore, investigating SAR is very important for understanding numerous biological activities and design of flavonoids based drugs.

To obtain information regarding absorption, distribution, metabolism, and excretion (ADME) of pharmaceutical agents within the body, it is very important to study interactions between drugs and serum proteins.[15,16] Human serum albumin (HSA), most abundant (around 60%) of all plasma proteins, is a carrier for therapeutic and diagnostic agents, contributes to apparent solubility of hydrophobic drugs in plasma and modulates their transportation to cells.[17-20] Therefore, drug binding sites and drug binding constants to HSA are important factors that determine pharmacokinetics and pharmacological effects of a drug. Hence, we selected HSA to study the SAR of flavonoids.

Baicalein, wogonin, chrysin, naringenin, and quercetin were purposely chosen to compare the interaction between different flavones as well as different flavonoids with HSA. Although, the study about selected flavonoids and HSA have been reported, but most of them are just stay at the investigation about interaction, with the help of interaction to study the SAR systematically is quite rare. Here, comparisons about the differences in quenching mechanism, comparing thermodynamic parameters, binding sites, and binding constants have been associated with their structure for the SAR discussion. This study may not only improve our understanding regarding the mechanism behind versatile bioactivities of polyphenols but also help in providing a theoretical basis for subsequent development of flavonoids as potential therapeutics and a strategy for SAR studies.

2. Materials and Methods

2.1. Materials

Human serum albumin (defatted HSA, approx. 99%) and Tris (hydroxymethyl

aminomethane) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Baicalein and woginion were obtained from National Institutes for Food and Drug Control (Beijing, China), while chrysin, naringenin and quercetin were obtained from Chengdu MUST Bio-Technology Co., Ltd. (Chengdu, China). Ibuprofen was obtained from Hubei Biocause Pharmaceutical Co., Ltd. (Hubei, China, the purity no less than 99.7%). Warfarin was obtained from Trust Chemical Industry Co., Ltd. (Nanjing, China, the purity no less than 98%). Tris buffer had purity no less than 99.5%, and NaCl, HCl, etc. were all of analytical purity. All the chemicals were of high-purity grade and used as purchased without further purification except for HSA solution; HSA was dissolved in buffer solution one day before experiments. Stock solution with high concentration of flavonoids was first dissolved in N,N,-dimethyl amide, then dissolved in tris-HCl buffer solution (0.05 mol·L^{-1} Tris, 0.15 mol·L^{-1} NaCl, pH 7.4). Sample masses were accurately weighted on a microbalance (Sartorius, ME 215S) with a resolution of 0.1 mg. Appropriate blanks, run under the same conditions, were subtracted from each sample spectrum.

2.2. Measurement of Fluorescence Spectra

All fluorescence spectra of HSA-flavonoids system were recorded on a LS-55 spectrofluorimeter (Perkin Elmer, USA) equipped with 1.0 cm quartz cell and a thermostat bath. The width of the excitation slit was set at 11 nm, and the emission slit was set at 7.5 nm. An excitation wavelength of 295 nm was chosen because of the exclusive excitation of the intrinsic tryptophan fluorophores. Synchronous fluorescence spectra were also recorded at 298 K in the wavelength range of 250–320 nm at the fixed intervals of $\Delta\lambda$ = 15 nm and 60 nm, respectively. The three-dimensional (3D) fluorescence spectra to study HSA-flavonoids interactions were recorded at 298 K at the excitation wavelength range of 250–320 nm and the

emission wavelength range of 200–600 nm, for solutions prepared as described above. Appropriate blanks corresponding to the buffer were subtracted to correct the fluorescence background; reproducibility of the experiments is good.

2.3. Absorption Spectra Measurements

Absorption spectra of HSA in the absence and presence of flavonoids were measured at 298 K in the wavelength range of 200–500 nm (0.1 nm intervals and three scans) on a UV9000S spectrophotometer (Shanghai, China) equipped with 1.0 cm quartz cell. The concentration of HSA was 1×10^{-5} mol·L^{-1}, and the concentration of flavonoids was increased from 0 to 2×10^{-5} mol·L^{-1} at an increment of 2×10^{-6} mol·L^{-1}. Appropriate blanks corresponding to the tris buffer were subtracted to correct for background.

2.4. Molecular Docking Study

2.4.1. Protein and Ligand Preparation

Molecular docking was conducted by a Surflex Dock program in the Sybyl–X 2.1.1 package. The three-dimensional structure of HSA was obtained from the Protein Data Bank (http://www.pdb.org/, PDB ID: 1H9Z).[21] All H were added randomly, and charge was applied using the AMBER method. The structures of flavonoids (baicalein, wogonin, chrysin, naringenin, and quercetin) were first drawn using Sybyl–X 2.1.1 package, and then energy and charge were optimized using a tripos force field and Gasteiger-Huckel method, respectively.

2.4.2. Docking Simulations

The small molecule located in the crystal structure of HSA (PDB ID: 1H9Z) at site I and site II have been extracted before docking simulations, then two different kind of ProtoMol in ligand mode keeping the threshold and bloat at 0.50 and 0.0 generated,

following with the docking simulations between two ProtoMol and flavonoids, respectively. Ring flexibility was considered, while other parameters were determined through a number of attempts during the docking program. In the end, two docking result have been compared for respectively.

3. Results and Discussion

3.1. Comparisons of Fluorescence Characteristics during Binding of Different Flavonoids to HSA

The measurement of intrinsic fluorescence quenching of protein has been widely used to elucidate the mechanism of its interaction with a ligand or drug molecule.[22,23] Figure 1 illustrates fluorescence spectra of HSA in presence of different flavonoids; all the spectra were recorded under same condition (buffer: tris-HCl buffer, pH 7.4; temperature: 298 K). We observed a remarkable decrease in the fluorescence intensity upon addition of all flavonoids to HSA solution. Interestingly, the quenching degree for flavones (baicalein, wogonin, and chrysin) are almost equal; however, there exists a big difference when compared with other two subgroups (dihydroflavones and flavonols) containing substituent group at ring-C. The quenching degrees of these two subgroups are equal but significantly less than that of flavones (baicalein, wogonin, and chrysin). Therefore, the quenching degree of flavonoids to HSA is related to the presence of substituent group at ring-C. Curve ***l*** in Figure 1 shows that all flavonoids individually do not affect the fluorescence intensity of the HSA-flavonoids complex. The insets in Figure 1 show that the fluorescence quenching of HSA by flavonoids is proportional to the concentration of flavonoids within the investigated concentration range. The plot of quenching constant vs. concentration for all the studied flavonoids (Figure 1F) clearly shows that different flavonoids exhibit different quenching constants associated with number as well as position of substituent. For flavones,

quenching constants are mainly related to the total number of substituent, e.g., the number of substituent is three for both baicalein and wogonin, while it is two for chrysin, and the quenching constants for the former two are larger than chrysin. Moreover, quenching constants for different flavonoids are mainly associated with the number of substituent at ring A and C; more substituent at ring A leads to higher quenching constants but opposite to ring C.

The decrease in fluorescence intensity of a fluorophore induced by a variety of molecular interactions, including energy transfer, molecular rearrangements, excited-state reactions, ground-state complex formation (static quenching), and dynamic quenching.[24,25] Static and dynamic quenching are principal mechanisms of fluorescence quenching.[26] In order to understand whether quenching is dynamic or static, we performed quenching experiments at different temperatures (292, 298, 302, and 310 K) to explore the mechanism and thermodynamics of flavonoids binding and observed a decrease in relative fluorescence intensities (RFI). The data were analyzed according to the Stern-Volmer equation:[27]

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

where F_0 and F denote the steady-state fluorescence intensity in the absence and presence of quencher, respectively; K_{SV} is the Stern-Volmer quenching constant; [Q] is the concentration of the quencher. Hence, equation (1) can be applied to determine K_{SV} by linear regression of a plot of F_0/F against [Q].

Static quenching is decreased at higher temperatures, leading to dissociation of weakly bound complexes. Thus, the decrease in K_{SV} with increasing temperature (Table 3) suggests that the quenching of HSA fluorescence by all flavonoids is initiated by complex formation (static quenching) rather than by dynamic quenching.

For static quenching, we analyzed the data using the modified Stern-Volmer

equation:[27]

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \quad (2)$$

where F_0 and F denote the steady-state fluorescence intensity in the absence and presence of quencher, respectively; K_a is the associative binding constant; [Q] is the concentration of quencher; f_a is the fraction of accessible fluorescence.

Figure 2 presents modified Stern-Volmer plots for the HSA-flavonoid systems at different temperatures. Related results in Table 3 show that K_a is inversely correlated with temperature, similar to K_{sv} discussed above; this is in accordance with static quenching. High binding constants for protein ligand interactions remain within the range of 10^6–10^8 L·mol^{-1}.[28] The range of binding constants of the HSA-flavonoid systems is 5×10^4–1.65×10^5 L·mol^{-1} at 298 K, as obtained in the present study, indicating a relatively weak interaction between flavonoids and HSA. Lower binding constants observed at high temperature suggests that HSA might weakly bind fewer flavonoids molecules at body temperature than at room temperature, thus making release of flavonoids easier from plasma protein to target organs. We noted an interesting observation regarding the effect of temperature on different flavonoids. As evident from the results presented in Figure 2F and Table 3, the effect of temperature on flavonoids is related to the number of hydroxyl groups and space steric hindrance, and the order is as follows: quercetin > naringenin > chrysin > baicalein > wogonin. Due to intramolecular and intermolecular hydrogen bonds, the number of hydroxyl groups on ring C is the most important factor for different flavonoids to get affected by temperature, e.g., there are two hydroxyl groups on ring C in quercetin, and thus, it is mostly affected by temperature. In case of flavone, space steric hindrance is the most important factor, e.g., the methoxyl group on ring B in wogonin exerts biggest space hindrance among all the studied flavones, resulting in the lowest possibility for

forming complex, and thus, wogonin has the weakest influence of temperature.

In protein-ligand binding, four types of non-covalent interactions, such as hydrogen bonding, van der Waals forces, electrostatic and hydrophobic binding interactions, play important roles and these interactions can be studied by thermodynamic parameters.[29] When temperature variation is negligible, enthalpy change (ΔH) of a system is constant, ΔH and ΔS (entropy change) can be evaluated from the van't Hoff equation:[27]

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where K_a is analogous to associative binding constant at corresponding temperature, and R is the gas constant.

The free energy change (ΔG) can then be evaluated from the following equation:[27]

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Figure 3A shows the van't Hoff polts and Table 3 lists the thermodynamic parameters for the HSA-flavonoid interaction. The negative ΔG values indicate that the binding of all the studied flavonoids to HSA is spontaneous. According to Ross,[30] if positive enthalpy change and entropy change imply hydrophobic interaction, then negative enthalpy change and positive entropy change suggest electrostatic force, and negative enthalpy change and entropy change reflect the van der Waals force or H-bonding. Table 3 shows that ΔH values for all flavonoids are negative, suggesting the binding reaction as exothermic. We found that a negative ΔH but a positive ΔS (except quercetin) for all the studied flavonoids; therefore, we can infer that the binding between flavonoids (baicelin, wogonin, chrysin and naringenin) and HSA is mainly driven by electrostatic interactions. Hydroxyl groups present on flavonoids could form intermolecular hydrogen bonds, thereby strengthening the formation of

HSA-flavonoid complex and contributing to strong quenching efficacy. This again indicates that hydrogen bond is existed in the interactions between flavonoids and HSA. Negative values about both enthalpy change and entropy change for quercetin, reflecting the binding forces are van der Waals force and hydrogen bond. The binding of flavonoids to HSA is enthalpy-entropy driven except for quercetin, where the binding is predominantly enthalpy driven. Different binding forces work for different flavonoids owing to their different structures. In case of quercetin, there are four phenolic hydroxyl groups and one hydroxyl group connected at double bond next to the ketonic oxygen, easily forming intermolecular hydrogen bonds. Owing to the hydroxyl group, connected at double bond next to the ketonic oxygen, the electronic configuration of quercetin is different from other flavonoids studied, and thus, van der Waals force plays predominant role in the binding of quercetin to HSA.

We obtained a linear regression between ΔH and ΔS by plotting enthalpy change (ΔH) and entropy change (ΔS), and this is called the "enthalpy-entropy compensation" (Figure 3), suggesting the following relationship exists when flavonoids bind to HSA:

$$\Delta H\ (\mathrm{kJ\cdot mol^{-1}}) = -27.83 + 0.27\ \Delta S\ (\mathrm{J\cdot mol^{-1}\cdot K^{-1}})$$

If we divide the axis into four quadrants, the enthalpy-entropy compensation line does not pass the second quadrant, where the value of enthalpy change (ΔH) is positive but entropy change (ΔS) is negative, according to equation (4). Therefore, we can infer that the binding process between flavonoids and HSA is always spontaneous in the investigated range.

3.2. Identification of Flavonoid's binding sites on HSA and Comparison among the Five Studied Flavonoids

3.2.1. Site Marker Competitive Experiments

Majority of ligands and drugs bind to HSA with considerable affinity. All of these

compounds bind either at the Sudlow's site I, located at the subdomain IIA, or at the Sudlow's site II, positioned at the subdomain IIIA.[31] In the present study, we used well-characterized HSA binders, such as warfarin and ibuprofen, as site marker fluorescence probes for monitoring site I and site II of serum albumin, respectively. In order to locate the binding sites of flavonoids on HSA, we measured fluorescence quenching at fixed HSA-marker molar ratios (1:1) as a function of varying flavonoids concentration. Upon addition of flavonoids, the intensity of fluorescence decreased for both HSA: warfarin and HSA: ibuprofen system (Figure S1). In addition, a remarkable red shift was observed for warfarin, which can be explained by an increasing polarity of the region surrounding the tryptophan site; however, decrease in fluorescence intensity was slighter compared to the other HSA-flavonoid systems (Table 4). These results together suggest that the binding between flavonoids and HSA is affected by both warfarin and ibuprofen. Interestingly, there is a remarkable change in the quenching constants after adding warfarin (Table 4), which could partly be due to interaction between flavonoids and warfarin. Figure S2 illustrates the modified Stern-Volmer plots for the HSA-flavonoids (site makers) systems, while Table 4 lists the variable binding constants calculated in the presence of warfarin and ibuprofen. As evident from the results presented in Figure S2 and Table 4, the binding constants become larger after adding warfarin (except naringenin), which might be due to the interaction between flavonoids and warfarin, increasing the value of binding constants post warfarin addition. In order to confirm this assumption, we performed an experiment replacing HSA by tris. The results shown in Table 4 indicate that even under the excitation wavelength of HSA, flavonoids could decrease the fluorescence intensity of warfarin. The binding constant is the least for naringenin, which may well explained why the binding constant is smaller after adding warfarin only for

naringenin. In case of ibuprofen, the binding constant decreases like quenching constant, suggesting flavonoids could bind to HSA at the subdomain IIIA. Another experiment has been done to investigate the binding site of flavonoids on HSA, by performing the site marker competitive experiments in reverse order, we found that at fixed HSA-flavonoid molar ratios (1:1), the fluorescence intensity decreased after adding ibuprofen, and the order of quenching constants is as follows: blank (3.05×10^4 $L \cdot mol^{-1}$) > naringenin (1.86×10^4 $L \cdot mol^{-1}$) > baicalein (1.35×10^4 $L \cdot mol^{-1}$) > chrysin (1.29×10^4 $L \cdot mol^{-1}$) > wogonin (1.13×10^4 $L \cdot mol^{-1}$) > quercetin (0.91×10^4 $L \cdot mol^{-1}$), indicated that ibuprofen compete the binding site of flavonoids on HSA, as ibuprofen is a typical molecule bind at Sudlow's site II, we assume the binding site of flavonoids is same with ibuprofen.

3.2.2. Definition of the Binding Site of Flavonoids to HSA by Molecular Docking

To understand the efficacy of a biologically active drug molecule to function as a therapeutic agent, the knowledge of its binding location in the model transport protein environment is very crucial and important.[32] Herein, the binding site and the residues involved in interactions of flavonoids with HSA has been explored with molecular docking studies using Sybyl–X 2.1.1 package. The lowest energy docked structure clearly showed that flavonoids bind to HSA both at the subdomain IIA (Sudlow's sites I) and subdomain IIIA (Sudlow's sites II) with the corresponding docking score pK_d listed in Table 5. Since higher score reflects greater binding constant between a compound and docking site,[33,34] flavonoids principally bind on site II (subdomain IIIA) of HSA, which is consistent with the assumption before. Ibuprofrn, known as subdomain IIIA molecule, has been docked with HSA at the same time with pK_d = 6.92, no obvious difference when compared with the results of flavonoids, means that the result aforementioned is credible. At the subdomain IIIA, different flavonoids

interact differently with different amino acid residues. The results, presented in Figure 4 and Table 5, explain the differences between those interactions. As shown in Figure 4, flavonoids are represented by ball-stick model, and the amino acid residues within the range of 5 Å around flavonoids are expressed in line mode and distinguished by hydrophobicity, where the blue and brown stand for weaker and stronger hydrophobicity, respectively. The hydrogen bonding between flavonoids and residues are shown by yellow ines, and two planes are shown vividly by purple rectangle (Figure 4A). Baicalein is not planar and its C-ring is slightly tilted over the AB-ring plane allowing efficient entry through the bind site of a plane angle (68.11^{o}). As evident from Figure 4A, five hydrogen bonds are formed between baicalein and amino acid residues; among those five hydrogen bonds, two are built by the hydrogen atom (H) linked with C-5, C-7 of baicalein with oxygen atom (O) of TRP 411 and LEU430, two are built by O atom linked with C-5 with two H atoms of ARG411, and the other one is formed between O atom linked with C-4 and H atom of ASN391. The bond lengths of the above mentioned hydrogen bonds are 2.168 Å, 1.957 Å, 2.464 Å, 2.680 Å, and 2.449 Å, respectively. Meanwhile, it is worthy to note that the number of hydrogen bond and position for chrysin are same as baicalein with only difference in the lengths of hydrogen bonds (2.072 Å, 1.878 Å, 2.414 Å, 2.608 Å, and 2.466 Å, respectively). Moreover, Figure 4C reveals that chrysin is not planar and it has a plane angle of 66.08°. Wogonin tilts its C-ring from AB-ring plane to enter into the cavity better with a plane angle at 89.96°. There are three hydrogen bonds formed between wogonin and amino acid residues of HSA; two hydrogen bonds are formed by O atom linked with C-4 and C-5 with H atom of ASN391 and ARG410, while the third one formed between H atom linked with C-7 and O atom of LEU410, and the corresponding bond lengths are 2.287 Å, 1.974 Å, and 2.107 Å, respectively. The

results for baicalein, wogonin and chrysin suggest that the presence of methoxyl group at C-8 of wogonin is responsible for the difference; introduction of methoxyl group decreases electronegativity of the adjacent hydroxyl oxygen atom, which is detrimental for the formation of hydrogen bonds. Figure 4D shows that quercetin has a plane angle (83.60°), and eight hydrogen bonds are formed between quercetin and amino acid residues; one hydrogen bond is formed by H atom linked with C-3 of quercetin with O atom of PRO384, two hydrogen bonds are built by the O atom linked with C-5 of quercetin with two H atoms of ASN391, one is formed between O atom linked with C-7 and H atom of TYR411, three hydrogen bonds are existed between O atom linked with C-11 and two H atoms of ARG384 and another H atom of ARG485, the eighth one is formed between O atom linked with C-12 and H atom of SER342, and the corresponding bond lengths are 2.074 Å, 1.808 Å, 2.581 Å, 2.622 Å, 1.920 Å, 2.727 Å, 2.098 Å, and 1.813 Å, respectively. For naringenin, a plane angle at 53.70° was also found. We identified five hydrogen bonds for naringenin; two are formed between O atom linked with C-4 and H atom of ASN391, H atom linked with C-5 and O ASN391of ASN391,one was formed between O atom linked with C-5 and H atom of ARG410, one was built between O atom linked with C-7 and H atom of LEU430, the fifth one was formed between O atom linked with C-12 and H atom of ARG485, and the corresponding bond lengths are 2.453 Å, 2.009 Å, 2.182 Å, 1.900 Å, and 2.728 Å, respectively. The differences found in different flavonoids might be due to the number of hydroxyl, e.g., quercetin generates maximum hydrogen bonds due to presence of maximum number of hydroxyl groups among the studied flavonoids. In addition to electrostatic interactions (the main force), docking results also reveal that hydrogen bonds and hydrophobic interactions (Table 5) are also involved in baicalein, wogonin, chrysin and naringenin system, respectively.

Moreover, Table 5 also shows that hydrophobic interaction is existed in HSA-quercetin system. Furthermore, docking results indicated that the differences in the main binding forces associated with the number of hydroxyl group present in flavonoids.

3.3. Conformational Changes of HSA in The Presence of Flavonoids

3.3.1. Absorption Spectroscopy (AS)

Studies of the conformation changes of HSA and the interaction drug-protein mechanism can be made by UV-visible spectrophotometry.[35] In this work, absorption spectroscopy was employed to gain insight into conformational changes occurring in HSA while adding different flavonoids. Table 6 presents the observations obtained from absorption studies. We found decrease in maximum absorption wavelength (except wogonin), suggesting increased polarity (or decreased hydrophobicity) in the region surrounding tryptophan and tyrosine residues; as for wogonin, the increased hydrophobicity results from the introduction of methoxyl group.

3.3.2. Synchronous Fluorescence Spectroscopy (SFS)

Characteristic information regarding micro-environmental changes in the vicinity of fluorophores can be investigated using synchronous fluorescence spectroscopy.[35] In the current study, we studied micro-environmental changes in the vicinity of tyrosine and tryptophan residues using SFS at the fixed intervals ($\Delta\lambda$) of 15 nm and 60 nm, respectively. The red shift in the spectra (Figure S3B) indicates that hydrophobicity around tryptophan is slightly decreased in the presence of flavonoids (except wogonin) when $\Delta\lambda$ = 60 nm;[36] for wogonin, a minor blue shift appears, which might be due to the presence of methoxyl group, responsible for strong hydrophobicity. When $\Delta\lambda$ = 15 nm, the red shift indicates that the hydrophobicity around tyrosin is slightly decreased in presence of flavonoids except for quercetin and naringenin (Parts A_4 and A_5); there

might be other types of interaction for quercetin and naringenin causing a minor blue shift (no shift appears because of the number of hydroxyl connected to ring-C). Table 6 presents the detailed results of SFS study. These results together suggest that in the presence of flavonoids, the polarity surrounding tryptophan and tyrosine residues slightly changes and this is in accordance with the results obtained from absorption spectra.

3.3.3. The 3D Fluorescence Spectroscopy (TDFS)

More information regarding micro-environmental and conformational changes occurring in HSA during flavonoids binding were obtained by recording 3D fluorescence spectra of the studied samples.[37] Figure 5 illustrates the 3D fluorescence spectra for the HSA-flavonoids systems with peak information summarized in Table 6. Peak-a results from the Rayleigh scattering of water ($\lambda_{ex} = \lambda_{em}$), while peak-b is the second-order scattering peak ($\lambda_{em} = 2\lambda_{ex}$) (Figure 5). In addition, the strong peak-1 mainly reflects the characteristics of chromophores (tryptophan and tyrosine residues) of HSA, while peak-2 corresponds to the polypeptide backbone structure of has (Figure 5).[38,39] The results show that the fluorescence intensity of both peak-1 and peak-2 are quenched when flavonoids are added; the fluorescence quenching is accompanied with stokes shift. For peak-1, the order of the decrease in fluorescence intensity among the studied flavonoids is as follows: wogonin > chrysin > quercetin > baicalein > naringenin. Moreover, Table 6 shows the extent of fluorescence quenching is nearly same for all the studied flavonoids, except naringenin. The extent of quenching is the smallest for naringenin. Absence of double bond at C_2 and C_3 in naringenin structure might be the reason for less quenching; however, this particular structural characteristic is responsible for the biggest change in peak position and stokes shift. Baicalein system exhibits highest quenching among the studied

flavonoids; however, stokes shift increases for all upon adding flavonoids, except baicalein. Such difference might result from the position of hydroxyl group on the rings; for baicalein, three hydroxyl groups are located adjacently on ring-B, and this not only greatly influences polypeptide backbone structure but also increases polarity. Turn to peak-2, the quenching ratio of the fluorescence intensity was prodigious indicated a disturbance of the polypeptide backbone structure of HSA caused by flavonoids. The 3D fluorescence spectroscopy study suggests that conformational changes do occur in HSA upon adding flavonoids. The main influence factor for peak-1 is the conjugated structure of the flavonoids, while the position of hydroxyl group is responsible for the change in peak-2.

4. Conclusions

The present work describes the structure-activity relationships of flavonoids based on the investigations on the interaction between flavonoids and HSA. We noted many similarities, such as static quenching procedure, spontaneous binding reaction, same binging site, in the interaction of different flavonoids with HSA; the same skeleton structure in flavonoids could be the reason for such similarities. However, many marked differences were also seen. Our experimental results indicate that the number of substituent group at ring A and ring C is responsible for the different degree of fluorescence quenching, while the number of hydroxyl group is the primary reason for differences in the binding forces. In addition, degree of conjugation and the position of hydroxyl group are the main factor affecting the conformation of HSA. The study of SAR provides a promising foundation for the design of novel flavonoids-based medicines and a further molecular level understanding of the interaction between flavonoids and HSA.

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Captions:

Figure 1. Comparison of fluorescence characteristics when flavonoids bind to HSA. Concentration of HSA = 5.0 $\times 10^{-6}$ mol·L^{-1}; Concentration of flavonoids/(10^{-6}), *a*–*k*: from 0.0 to 10.0 mol·L^{-1} at an increment of 1.0 mol·L^{-1}. Figures A-E display the emission spectra of HSA in the present of different flavonoids, the insets correspond to the fluorescence intensity quenching of HSA at various concentrations of flavonoids. Figure F illustrates the comparison of quenching constants (K_{SV}) for different systems.

Figure 2. Comparison of Modified Stern-Volmer plots when flavonoids bind to HSA. Figures A–E display modified Stern-Volmer plots of five syetems. Figure F is the comparison of the effect of temperature on different systems.

Figure 3. Figure A is van't Hoff polts of HSA-flavonoids system, Figure B is enthalpy-entropy compensation plots for different HSA-flavonoid system.

Figure 4. Energy-minimized structure details of the interaction between flavonoids and HSA at subdomain IIIA. Figures A–E stand for baicalein, wogonin, chrysin, quercetin and naringenin syetem, respectivity.

Figure 5. The 3D fluorescence spectra of free HSA (A) and the HSA-flavonoids (B–F). *c* (HSA) = 5×10^{-6} mol·L^{-1}; *c* (flavonoids) = 5×10^{-6} mol·L^{-1}.

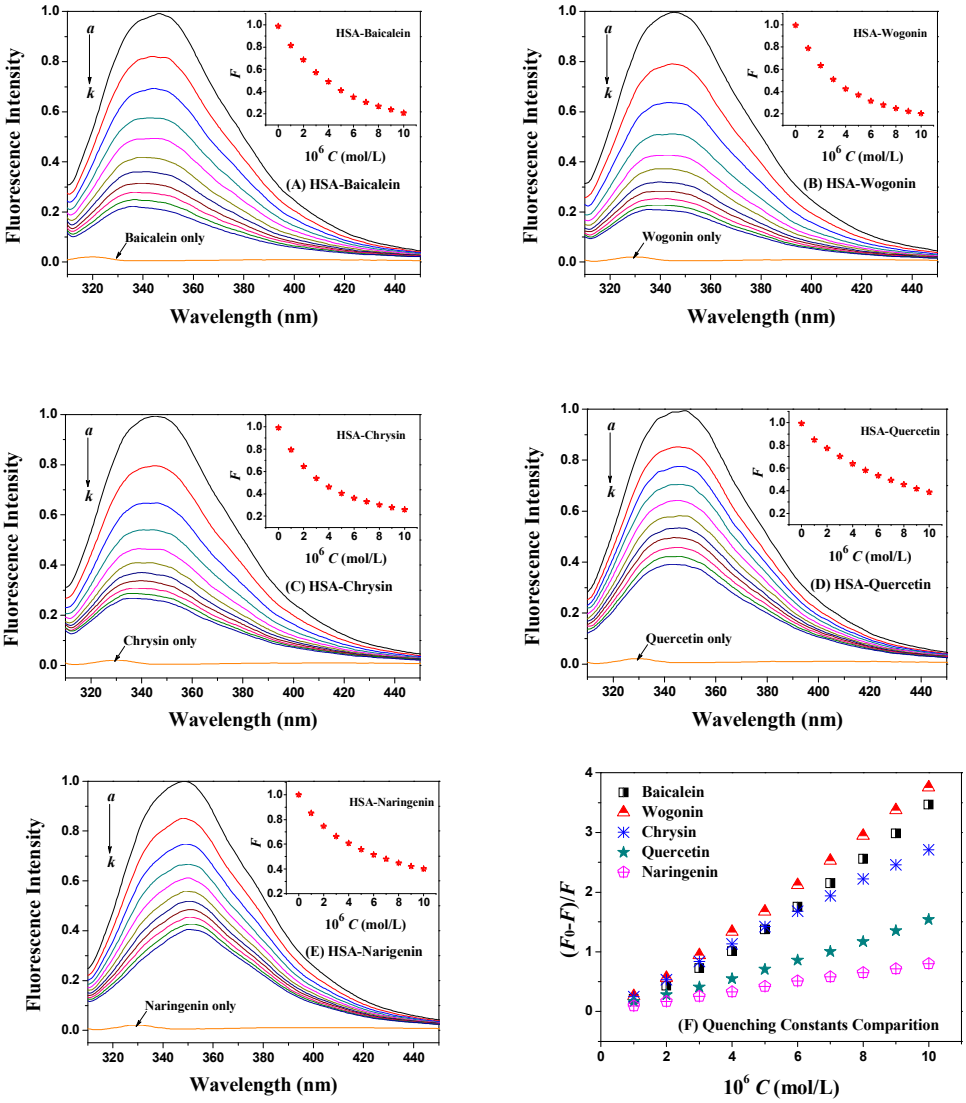


Figure 1

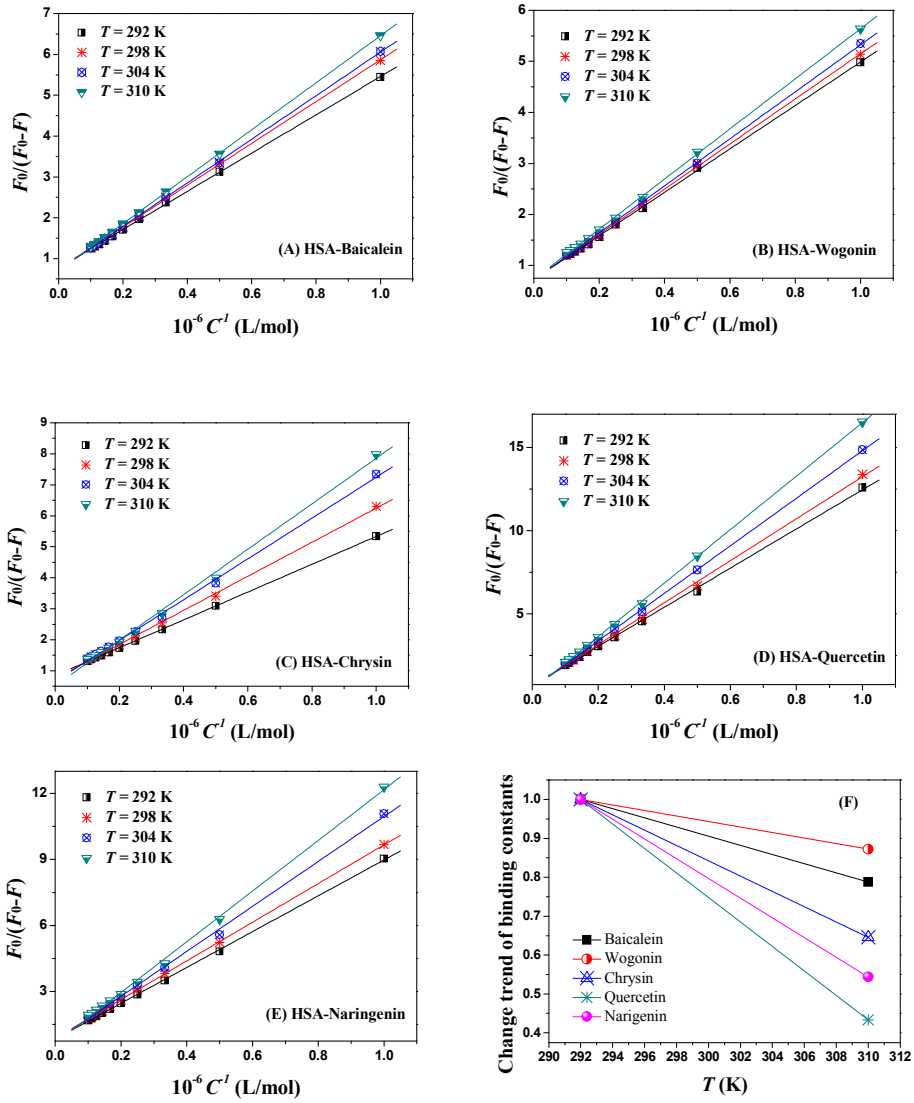


Figure 2

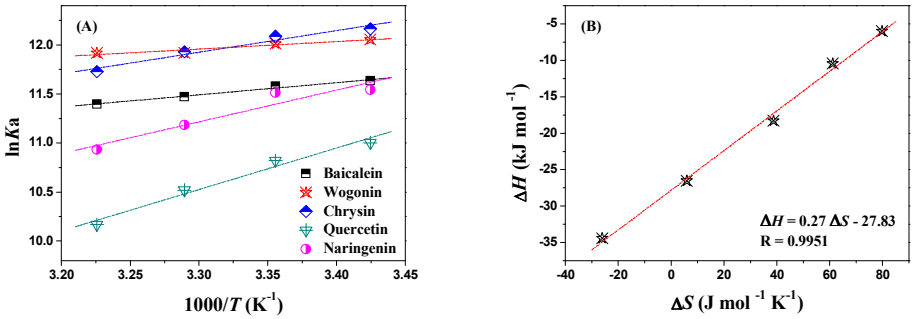


Figure 3

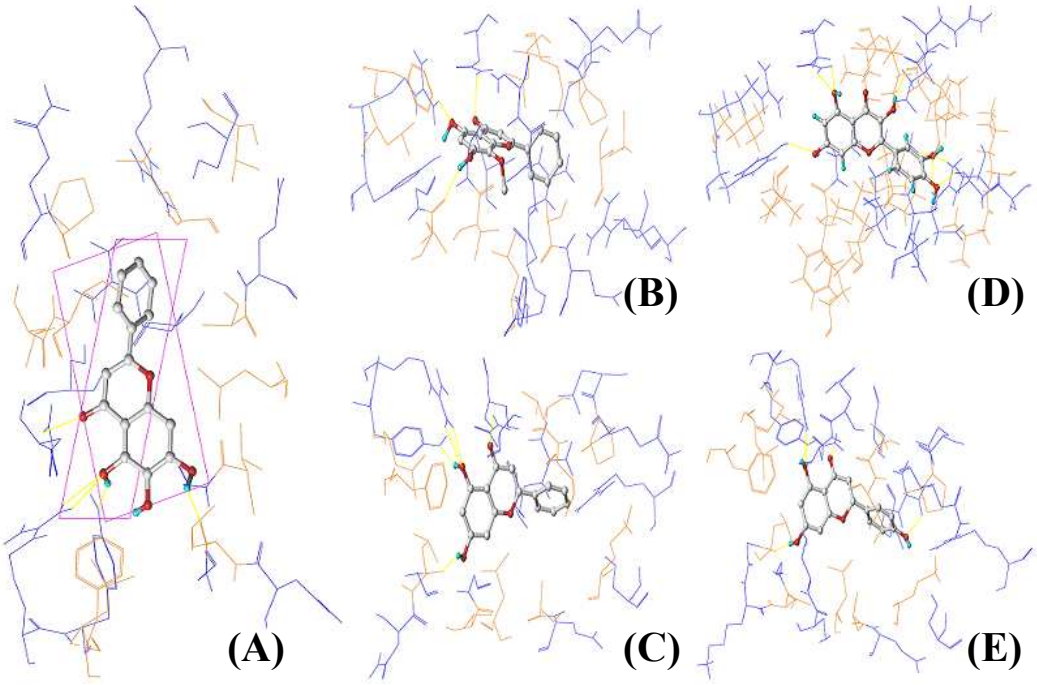


Figure 4

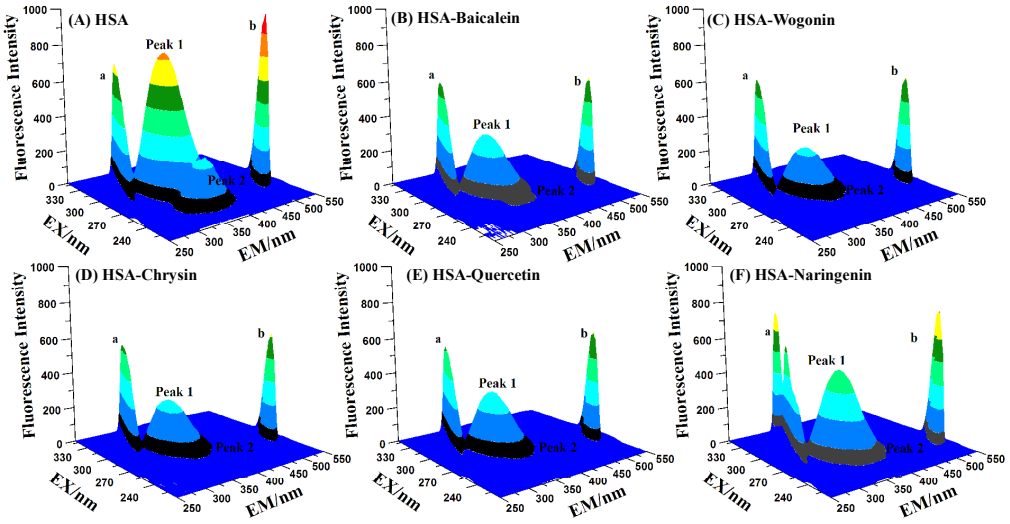


Figure 5

Table 1 Structure of flavonoids studied

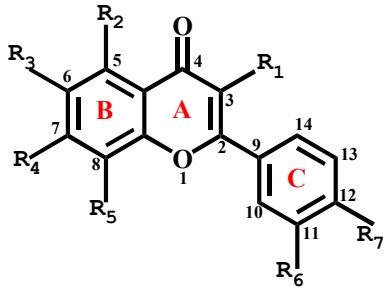
Structure	Flavonoids	C_2-C_3	R_1	R_2	R_3	R_4	R_5	R_6	R_7
	**Baicalein**	C=C	H	OH	OH	OH	H	H	H
	Wogonin	C=C	H	OH	OH	H	OCH_3	H	H
	Chrysin	C=C	H	OH	H	OH	H	H	H
	Quercetin	C=C	OH	OH	H	OH	H	OH	OH
	Naringenin	C-C	H	OH	H	OH	H	H	OH

Table 2. The comparison of wavelength shift and decrease degree in different systems

Flavonoids	n(-OH) ring A	n(-OH) ring B	n(-OH) ring C	n ($-OCH_3$)	C_2-C_3	$\lambda-\lambda_0$ (nm)	Decrease degree (%)	$10^{-5}K_{SV}$ (L/mol)
Baicalein	0	3	0	0	C=C	–10.5	76.40	3.64
Wogonin	0	2	0	1	C=C	–5.5	77.14	3.95
Chrysin	0	2	0	0	C=C	–8.5	73.05	2.74
Quercetin	1	2	2	0	C=C	–4	60.95	1.53
Naringenin	0	2	1	0	C-C	4	60.31	0.79

Table 3 Constants for the interaction of flavonoids to HSA at different temperatures and the comparison about the effect of the temperature on the five systems

System	T (K)	$10^{-5}K_{SV}$ (L/mol)	$10^{-5}Ka$[a] (L/mol)	Ka[b] (H/L)	ΔH (kJ·mol^{-1})	ΔG (kJ·mol^{-1})	ΔS (J·mol^{-1}·K^{-1})
Baicalein	292	3.69	1.13	0.79	–10.42	–10.63	61.15
	298	3.43	1.07			–10.63	
	304	3.27	0.96			–10.63	
	310	3.11	0.89			–10.62	
Wogonin	292	5.63	1.72	0.87	–5.98	–29.28	79.79
	298	5.27	1.65			–29.75	
	304	4.71	1.56			–30.23	
	310	4.11	1.50			–30.71	
Chrysin	292	4.65	1.92	0.65	–18.29	–29.61	38.78
	298	4.26	1.78			–29.84	
	304	3.84	1.52			–30.08	
	310	3.50	1.24			–30.31	
Quercetin	292	1.09	0.60	0.43	–34.43	–26.82	-26.07
	298	1.04	0.50			–26.62	
	304	1.00	0.37			–26.50	
	310	0.93	0.26			–26.35	
Naringenin	292	1.50	1.03	0.54	–26.54	–28.28	5.96
	298	1.31	1.00			–28.32	
	304	1.24	0.72			–28.35	
	310	1.11	0.56			–28.39	

a is the binding constants calculated from modified Stern-Volmer equation;

b is refer to the ratio of binding constants at highest and lowest temperature studied.

Table 4 Stern-Volmer quenching constants and binding constants for the interaction of flavonoids with different systems

System	HSA		HSA+Warfarin		HSA+Ibuprofen		Warfarin	
	$10^{-5}K_{SV}$ (L/mol)	$10^{-5}Ka$ (L/mol)	$10^{-5}K_{SV}$ (L/mol)	$10^{-5}Ka$ (L/mol)	$10^{-5}K_{SV}$ (L/mol)	$10^{-5}Ka$ (L/mol)	$10^{-4}K_{SV}$ (L/mol)	$10^{-4}Ka$ (L/mol)
Baicalein	3.64	1.27	3.04	1.91	3.56	1.24	2.84	6.42
Wogonin	5.34	1.81	4.64	2.62	3.57	1.64	5.37	12.04
Chrysin	3.30	1.82	2.81	2.36	3.23	1.76	2.91	14.28
Quercetin	3.42	1.67	3.25	2.02	3.36	1.63	4.63	12.29
Naringenin	1.39	1.78	0.82	1.06	1.33	1.32	1.65	5.01

Table 5 The detailed information about the docking results

Baicalein	**site I**	**site II**	1[a]	PHE403, VAL344, LEU430, MET446, VAL433, PRO384, LEU453, ILE388, ALA449, LEU387
	4.21	6.08	2[b]	ASN391, ARG410, TRP411, LEU430
			3[c]	C_4, –0.4253; C_5, –0.3371; C_6, –0.3470; C_7, –0.3385
Wogonin	**site I**	**site II**	1[a]	PHE403, LEU407, LEU430, VAL433, LEU453, ALA449, MET446, LEU387, ILE388, PRO384
	5.16	6.16	2[b]	ASN391, ARG410, LEU410
			3[c]	C_4, –0.4250; C_5, –0.3395; C_7, –0.3391; C_8, –0.3345
Chrysin	**site I**	**site II**	1[a]	LEU407, PHE403, LEU430, VAL433, LEU453, ALA449, MET446, ILE388, PRO384, LEU387
	4.91	5.99	2[b]	ASN391, ARG410, TYR411, LEU430
			3[c]	C_4, –0.4247; C_5, –0.3325; C_7, –0.3350
Quercetin	**site I**	**site II**	1[a]	LEU407, VAL344, ILE388, LEU430, LEU345, ALA449, LEU457, LEU453, MET446, PHE488, PRO384, PRO486, LEU387
	5.13	6.45	2[b]	PRO384, ASN391, TYR411, ARG348, ARG485, SER342
			3[c]	C_3, –0.3400; C_4, –0.4153; C_5, –0.3327; C_7, –0.3353; C_{11}, –0.3371; C_{12}, –0.3363
Naringenin	**site I**	**site II**	1[a]	LEU349, ALA449, LEU407, MET446, PHE403, ILE388, LEU430, PRO384, VAL433, LEU397, LEU453
	4.83	5.45	2[b]	ARG485, ASN391, ARG410, LEU430
			3[c]	C_4, –0.3966; C_5, –0.3326; C_7, –0.3394; C_{12}, –0.3376

1[a] stands for the amino acid residues associated with hydrophobic interactions;

2[b] stands for the amino acid residues associated with the formation of hydrogen bond;

3[c] stands for the formal charge of oxygen atom O linked of different carbon atom C.

Table 6 Characteristic parameters associated with the conformational changes in HSA

Systems and parameters	AS	SFS		TDFS					
	Δλ (end-start)			Peak 1			Peak 2		
	–	15	60	Peak position (λ_{ex}, λ_{em})	Stokes shift (cm^{-1})	IF^a	Peak position (λ_{ex}, λ_{em})	Stokes shift (cm^{-1})	IF^a
HSA	–	–	–	278/344	0.69	1	233/344	1.38	1
HSA-Baicalein	–3	2	2	278/344	0.69	0.43	239/344	1.28	0.36
HSA-Wogonin	3	2	–1	278/346	0.71	0.35	233/345	1.39	0.05
HSA-Chrysin	–2	1	2	278/344	0.69	0.37	233/345	1.39	0.06
HSA-Quercetin	–1	–1	1	278/336	0.62	0.42	233/346	1.40	0.05
HSA-Naringenin	–1	0	2	263/348	0.93	0.56	212/325	1.64	0.03

IF^a stands for the relative fluorescence intensity values.