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Probing the interactions of hemoglobin with antioxidant flavonoids via fluorescence spectroscopy and molecular modeling studies

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ABSTRACT

Steady state and time resolved fluorescence spectroscopy, combined with molecular modeling computations, have been used to explore the interactions of two therapeutically important flavonoids, fisetin (3,7,3′,4′-OH-flavone) and 3-hydroxyflavone (3-HF), with normal human hemoglobin (HbA). Distinctive 'two color' fluorescence signatures and fairly high fluorescence anisotropy ($r = 0.12–0.28$) of fisetin and 3-HF reveal their specific interactions with HbA. Binding constants estimated from the fluorescence studies were $\approx 4.00 \times 10^4\ M^{-1}$ and $9.83 \times 10^3\ M^{-1}$ for fisetin and 3-HF respectively. Specific interactions with HbA were further confirmed from flavonoid-induced static quenching of the protein tryptophan fluorescence as indicated by: (a) bimolecular quenching constant $K_q \gg$ diffusion controlled limit (b) closely matched values of Stern–Volmer quenching constant and binding constant (c) $\tau_o/\tau \approx 1$ (where τ_o and τ are the unquenched and quenched tryptophan fluorescence lifetimes respectively). Molecular docking and electrostatic surface potential calculations reveal contrasting binding modes of fisetin and 3-HF with HbA.

1. Introduction

Flavonoids, which are polyphenolic compounds ubiquitous in plants of higher genera, are gaining increasing attention as potentially important therapeutic drugs with high potency and low systemic toxicity [1–4]. Many recent studies, both in vivo and in vitro, have established that flavonoids are powerful antioxidants effective against a wide range of free radical mediated and other diseases including various types of cancers, tumors, diabetes mellitus, atherosclerosis, ischemia, neuronal degeneration, cardiovascular ailments, and AIDS [5,6]. Among dietary flavonoids, fisetin (3,7,3′,4′-OH flavone) (Scheme 1), which belongs to the most abundant chemical class among naturally occurring flavonoids, namely flavonols (3-hydroxy-flavones), has been the focus of especial interest for its multifunctional therapeutic potential. This dietary flavonoid (which is especially abundant in strawberries and also present in other fruits and vegetables) has been reported to possess remarkable anti-carcinogenic effects as well as anti-oxidant and anti-inflammatory activities. In particular, fisetin causes apoptosis in cancer cells, suppresses inflammatory processes in human mast cells [7], inhibits thrombin- and cathepsin G induced platelet aggregation [8], induces cell cycle arrest, acts as a free radical scavenger [9], and inhibits the proliferation of HIV by inactivating HIV1 proteinase [10]. The inhibitory potential of fisetin and other related flavonoids against non enzymatic glycosylation of hemoglobin has also been established [11–13]. Furthermore, fisetin enhances long-term memory, and thus may be useful for treating patients with memory disorders [14]. Similar to polyhydroxy substituted plant flavonoids like fisetin, the unsubstituted flavonol 3-hydroxyflavone (of synthetic origin), was recently shown to exhibit significant antioxidatant effects against membrane lipid peroxidation in red blood cell membrane as well as in model biomembranes [15,16].

On a different scenario, much recent attention has focused on the remarkable fluorescence properties of flavonoids. In particular, many commonly occurring flavonoids have been found to exhibit 'two color' (in 'blue-violet' and 'yellow-green' regions) fluorescence emission, which arise due to ultrafast photo-induced excited state intramolecular proton transfer (ESIPT) [17,18]. The relative contributions between the two colors are strongly modulated by the local environment of the fluorophore. Taking advantage of such remarkable intrinsic fluorescence behavior, interesting uses of 3-HF, fisetin, and related flavonoid derivatives as exquisitely sensitive fluorescence probes for exploring their local environments in different biorelevant systems (including proteins, biomembranes and nano-vehicles for drug delivery) have been extensively demonstrated in our previous studies [19–23].

Normal human hemoglobin (HbA), the major protein component of red blood cells, is a physiologically important globular protein which carries oxygen from the lungs to different tissues. Moreover, HbA reversibly binds many endogenous and exogenous molecules including various drugs [24]. Thus, HbA can play an important role in

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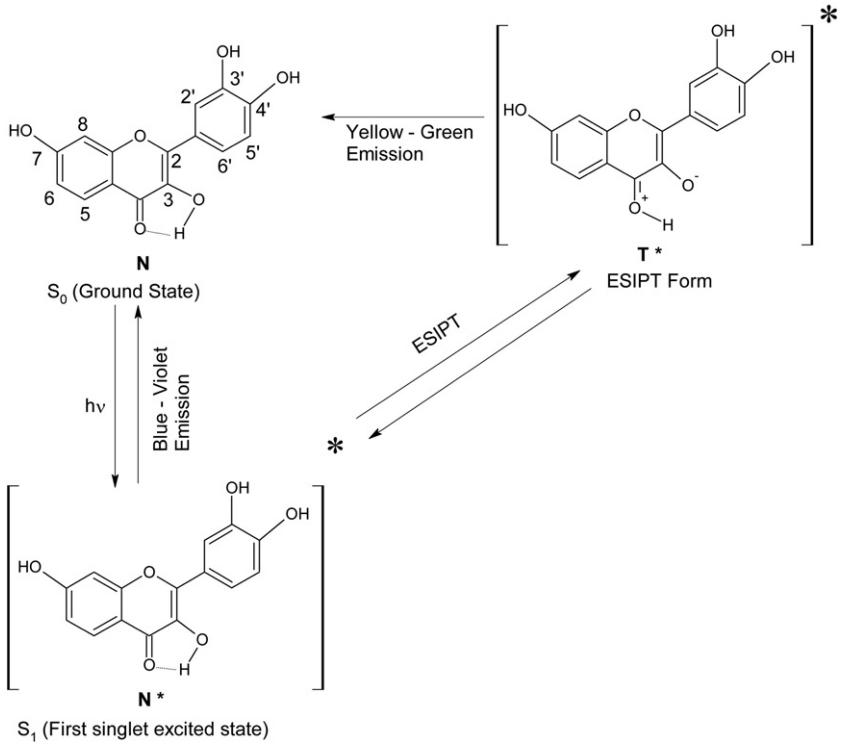

Scheme 1. Excited state intramolecular proton transfer (ESIPT) in fisetin (3,7,3′,4′-OH-flavone).

the distribution and bioavailability of flavonoids [25]. Another important aspect concerns the glycosylation of hemoglobin. Glycosylated hemoglobin (HbAIc, HbAIa + b, minor hemoglobins of human erythrocytes) is formed continuously from normal human hemoglobin (HbA) and the non-enzymatic HbA glycosylation level is known to increase in diabetes. Since the glycosylation process is an oxidative reaction, the flavonoid antioxidants are expected to prevent this reaction, and as already mentioned, some flavonoids (e.g. fisetin) have been actually reported to do so [11,13]. Thus the knowledge of the binding characteristics of therapeutically important flavonoids with HbA and other relevant functionally important cellular proteins is crucial for understanding the mechanism of their drug actions. However, to date very little is known about the specific interactions of flavonoids with HbA.

Here we have explored the interactions of two representative flavonoids namely fisetin (3,7,3′,4′-OH flavone) and 3-hydroxyflavone (3-HF) with normal human hemoglobin (HbA) using the intrinsic flavonoid fluorescence as well as flavonoid-induced quenching of the protein tryptophan fluorescence. Furthermore, we present for the first time, molecular modeling studies which provide critical insights regarding the interaction processes.

2. Materials and methods

2.1. Materials

Lyophilized powder of human hemoglobin, 3-HF, fisetin, and phosphate buffer were purchased from Sigma Chemicals, USA. Solvents used were of spectroscopic grade and obtained from Sigma-Aldrich.

2.2. Interaction of flavonoids with HbA

Concentrated methanolic flavonoid solutions were added to 1 ml solutions of various concentrations of HbA. The final concentrations of methanol were kept <1% (by volume) in all samples. After adding the probe, the solutions were allowed to equilibrate for 1 h at 25 °C before carrying out spectroscopic measurements.

2.3. Spectroscopic measurements

Steady state absorption and fluorescence spectra were recorded with a Cecil model 7500-spectrophotometer and Varian Cary Eclipse spectrofluorimeter respectively. The fluorescence readings were taken by exciting the samples and measuring the emissions at relevant wavelengths and appropriate blanks were subtracted for respective measurements. A quartz cuvette of 1 cm path length was used in all experiments. The fluorescence anisotropy (r) values were obtained using the expression,

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G is the sensitivity factor of the detection system [26]. Each intensity value used in this expression represents the computer-averaged values of ten successive measurements.

Time resolved fluorescence decay measurements were carried out with an Edinburgh Instruments nano-second time correlated single photon counting (TCSPC) spectrometer, using 295 nm diode excitation source (IBH, UK, nano-LED, pulse FWHM ~ 1.2 ns). An emission monochromator was used to block scattered light and isolate the emissions. Fluorescence intensity decay curves were deconvoluted with the instrument response function and fitted to a multiexponential decay function, $F(t) = \sum_i A_i \exp(-t/\tau_i)$, where A_i and τ_i represent the amplitudes and times of the decay components such that $\sum_i A_i = 1$. The goodness of fit was estimated by using χ^2 values.

Average lifetimes $\langle\tau\rangle$ for biexponential decays were calculated from the decay times and preexponential factors using the expression [26]:

$$\langle\tau\rangle = \frac{A_1\tau_1^2 + A_2\tau_2^2}{A_1\tau_1 + A_2\tau_2}$$

All spectral measurements were carried out at room temperature (298 K).

2.4. Molecular modeling

Molecular modeling studies were carried out to elucidate the interaction of 3-HF and fisetin with normal human hemoglobin HbA. 3-D atomic coordinates of the protein were obtained from the Brookhaven Protein Data Bank (PDB id 2D60). An online server QsiteFinder [27] was used to identify the probable binding pockets of HbA while Adaptive Poisson-Boltzmann Solver (APBS) software [28] was used to calculate electrostatic surface potential on the hemoglobin. Default values for charge (full charge), salt concentration (0.0), interior dielectric (2.0), and exterior dielectric (80.0) were used.

AutoDock 4 [29] was employed to dock the flavonoids with the receptor (HbA) using the same 3-D atomic coordinates of the receptor protein as already mentioned (PDB id 2D60). All the hetero atoms were deleted and non-polar hydrogens were merged. Kollman united-atom charge model was applied and atomic solvation parameters and fragmental volumes were added to the protein. Grid maps used by the empirical free-energy scoring function in AutoDock were generated. A grid map of $100\times100\times100$ grid points in size with a grid-point spacing of 0.375 Å was created for the protein. The map was centered such that it covered the entire functional binding site predicted by QsiteFinder. The ligand (flavonoid) molecular structures were created using HYPERCHEM [30] molecular builder module and optimised using AM1 semi-empirical method to an rms convergence of 0.001 kcal/ mol with Polak-Ribiere conjugate gradient algorithm implemented in the HYPERCHEM 7.5 package. For both 3-HF and fisetin, rotatable bonds were assigned and partial atomic charges were calculated using Gasteiger–Marsili method after merging non-polar hydrogen. 100 docking runs were done and for each run, a maximum of 2,500,000 GA operations were performed on a single population of 150 individuals. The weights for crossover, mutation and elitism were default parameters (0.80, 0.02 and 1 respectively).

3. Results

3.1. Electronic absorption and steady state and time resolved fluorescence results

Fig. 1A and B presents the fluorescence excitation and emission spectra of fisetin and 3-HF respectively in presence of HbA; the corresponding absorption spectra are shown in the respective insets. The emission spectra of both fisetin and 3-HF consist of 'two color' fluorescence bands, namely a yellow-green emission band along with a high energy band in the blue-violet region. While the blue-violet fluorescence is assigned to the S_1 ($\pi\,\pi^*$)$\rightarrow S_o$ normal (non-proton-transferred) emission, the large Stokes shifted green fluorescence is attributable to emission from a tautomer species generated by an excited state intramolecular proton transfer (ESIPT) process occurring along the internal H-bond (i.e. C(4) = O–HO–C(3)) of the molecule (Scheme 1) [17]. The "blue-violet" and "yellow-green" fluorescence emissions occur from **N*** and **T*** species respectively (Scheme 1). The emission spectrum of fisetin consists of overlapping dual fluorescence bands (N.B.—the overlap arises due to strong charge transfer character of the normal emission and consequent solvatochromic red shift) consisting of the tautomer and normal (non proton transferred) emissions (with λ_{em}^{max} ca. 540 nm and 495 nm respectively) of comparable magnitudes. The absorption spectrum of fisetin after binding with HbA does not show any change in peak position indicating that no additional species of fisetin is generated.

Interestingly, in case of 3-HF, HbA addition induces the appearance of a long-wavelength band (at ~410 nm) observed in the absorption and excitation spectra (Fig. 1B: Inset). The presence of the long-wavelength absorption and an isosbestic point (at ca. 367 nm) suggest

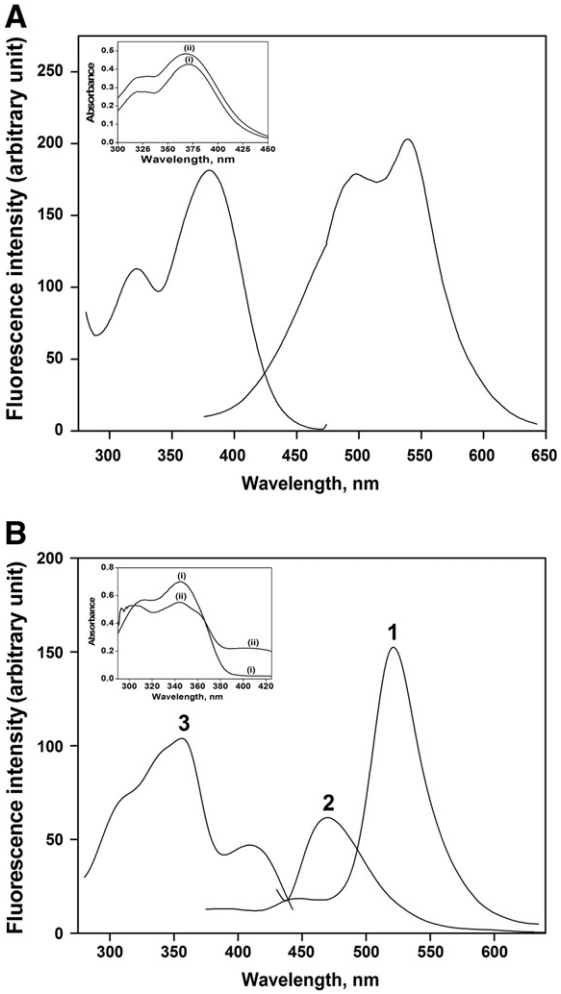

Fig. 1. (A) Fluorescence excitation (at $\lambda_{em} = 520$ nm) and emission spectra of fisetin (with $\lambda_{ex} = 360$ nm) in 3.0 μM HbA at 25^0 C, Inset: Absorption spectra of fisetin in absence (i) and presence (ii) of HbA. [Fisetin] $= 1.5\times10^{-5}$ M. (B) Fluorescence excitation ($\lambda_{em} = 540$ nm) (3) and emission spectra of the neutral (at $\lambda_{ex} = 345$ nm) (1) and anionic species (at $\lambda_{ex} = 410$ nm) (2) of 3-Hydroxyflavone in 3.0 μM HbA at 25^0 C, Inset: Absorption spectra of 1.5×10^{-5} M 3-HF in absence (i) and presence (ii) of HbA. [3-HF] $= 1.5\times10^{-5}$ M. (N.B.—Absorption spectra were obtained as the absorption of flavone–HbA mixture minus the absorption of HbA alone.)

the existence of two distinct species. The selective excitation of the 410 nm band results in emission at ca. 470 nm (Fig. 1B). On the other hand, upon excitation at 345 nm, the emission of 3-HF in HbA is characterized by dual fluorescence bands ($\lambda_{em}^{max} = 410$ nm and 520 nm) with predominantly tautomer (green) emission, indicating that 3-HF molecules are present in environments with limited accessibility of water, permitting efficient ESIPT process (N.B.—The weak band at ~440 nm in curve 1 of Fig. 1 is due to Raman scattering from the aqueous solvent medium). Such emission behavior is reminescent of previous studies of 3-HF in model membranes and in aprotic solvents containing traces of methanol [15,31,32] and is suggestive of the existence of the neutral (3-HFN) and anionic forms (3-HFA) of 3-HF in HbA environment. Because the long-wavelength absorption (assigned to the anionic species) can be observed in HbA environment but not in water, we infer that the anionic form is generated by HbA mediated deprotonation of the 3-OH group of the molecule in the ground state.

Fluorescence anisotropy (r) serves as a sensitive indicator for monitoring fluorophore binding to motionally constrained regions of proteins [26]. The pronounced increase in the 'r' values of fisetin as well as for 3-HF in presence of HbA suggests their binding to motionally constrained site(s) in the protein matrix (Table 1).

Table 1
Fluorescence emission properties of flavonoids in presence of HbA.

Flavonoid	λ_{em}^{max} (nm)		Fluorescence anisotropy (r)[#]
	I_1^*	I_2^*	
Fisetin	540	495	0.14
3-Hydroxyflavone (neutral)	520	410[+]	0.12
3-Hydroxyflavone (anion)	470		0.28

[*] I_1 and I_2 stand for ESIPT tautomer and normal fluorescence respectively. $\lambda_{ex} = 360$ nm for fisetin, 345 nm and 410 nm for the neutral and the anionic species of 3-HF respectively.
[+] Very low intensity.
[#] Fluorescence anisotropy was monitored at the emission maxima of the respective fluorophore.

The presence of multiple emissive species of fisetin and 3-HF in HbA has been directly tested by fluorescence anisotropy. With only one emissive species the fluorescence anisotropy should not change over the fluorescence excitation and emission bands belonging to a particular electronic transition [18,26]. In case of fisetin no significant change of fluorescence anisotropy across the excitation or emission band (Fig. 2) clearly indicates that the emission bands arise from essentially the same ground state species of fisetin (i.e. the neutral form of fisetin). However, the excitation anisotropy spectra (a plot of steady state anisotropy versus excitation wavelength) (Fig. 2A) show that the anisotropies of 3-HF in HbA undergo considerable change upon altering the excitation wavelength, with a sharp increase toward the long-wavelengths of the absorption bands. Emission anisotropy (Fig. 2B) shows steady and significant decrease with increasing emission wavelength in the entire emission band region. The dependence of fluorescence anisotropy with excitation and emission wavelength is due to the selective excitation and emission of two separate species, namely 3-HFN and 3-HFA which show distinct absorption and emission spectra (emission of 3-HFA is blue shifted relative to 3-HFN whereas the absorption of 3-HFA is red shifted relative to 3-HFN).

Quenching studies of protein tryptophan fluorescence by ligands is a convenient means for exploring ligand–protein interactions [33–36]. Fig. 3 shows quenching of HbA fluorescence in the presence of increasing concentrations of the flavonoids. Fig. 4A presents corresponding Stern–Volmer plots based on the equation,

$$\frac{F_0}{F} = 1 + K_{SV}[Flavonoid]$$

where K_{SV} is the Stern–Volmer quenching constant for the quenching of HbA tryptophan fluorescence by flavonoid [26]. The Stern–Volmer plots are essentially linear for flavonoid concentrations upto 25 μM (Fig. 4A) which indicates that only one type of quenching occurs [26]. However, at higher flavonoid concentrations (>25 μM), the Stern Volmer plots become non-linear (Fig. 4A: Inset), which may be attributed to two complicating factors: (i) existence of more than one type of quenching mechanisms at higher flavonoids concentrations, (ii) attenuation of tryptophan fluorescence due to inner filter effect arising from significant absorption of flavonoids in the tryptophan emission region with these (>25 μM) quencher (flavonoid) concentrations. Hence, further analysis has been restricted to this flavonoid concentration range.

The K_{SV} values obtained are ca. $9.32 \pm 0.72 \times 10^3\ M^{-1}$ and $3.76 \pm 0.86 \times 10^4\ M^{-1}$ (Table 2) for 3-HF and fisetin respectively which indicate that HbA tryptophan fluorescence is efficiently quenched by the flavonoids. We further performed lifetime measurements of HbA tryptophan fluorescence both in presence and absence of the flavonoids. Table 3 shows that the protein tryptophan fluorescence decay is bi-exponential with decay times of 0.725 ns and 3.087 ns. It is noteworthy that in presence of 3-HF and fisetin, no significant changes in the tryptophan fluorescence decay parameters are observed (Table 3). Thus

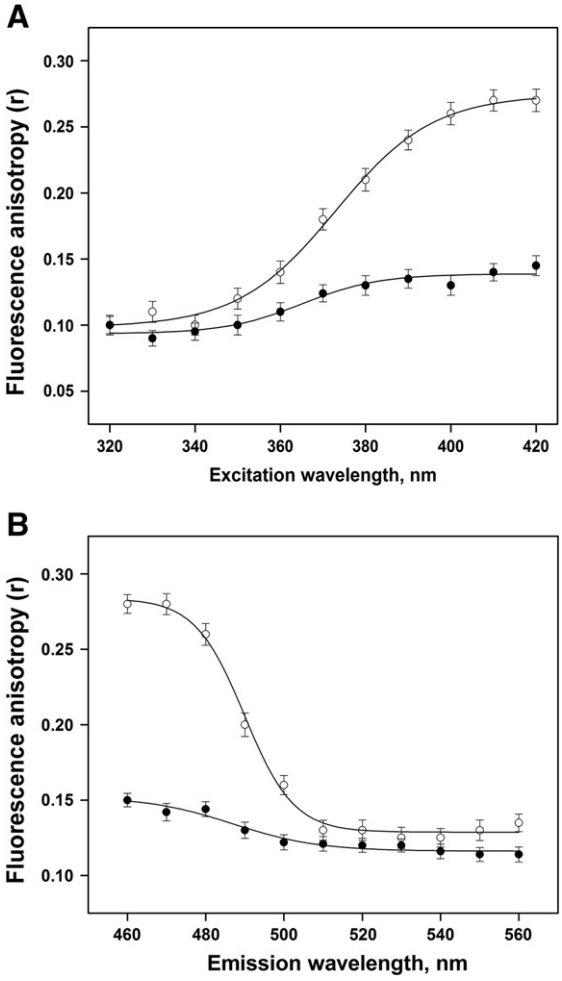

Fig. 2. (A) Fluorescence anisotropy of the flavonoids as a function of excitation wavelength; $\lambda_{em} = 520$ nm, (B) Fluorescence anisotropy of the flavonoids as a function of emission wavelength; $\lambda_{ex} = 370$ nm; (●) for fisetin and (○) for 3-HF. The data points are the average of three independent measurements, ± indicates standard deviation.

the average lifetimes (τ) computed from the decay parameters remain essentially unchanged. This indicates that a static mechanism is principally responsible for the observed steady-state fluorescence quenching, implying that fisetin and 3-HF do indeed bind to hemoglobin.

The apparent binding constant '*K*' and the number of binding site (s) '*n*' were estimated from fluorescence titration studies, using the plot of $\mathrm{Log}(F_o - F)/F$ vs. $\mathrm{Log}(1/([D_t] - (F_o - F))[P_t]/F_0)$ [37] (Fig. 4B) which is based on the equation:

$$Log\frac{F_0 - F}{F} = n\ LogK - n\ Log\left(\frac{1}{[D_t] - (F_0 - F)[P_t]\,/\,F_0}\right)$$

where F_0 and F are the fluorescence intensity of HbA in absence and presence of flavonoid (D) respectively, $[D_t]$ is the total flavonoid concentration and $[P_t]$ is the total protein concentration, n is the number of binding sites and K is the binding constant. Table 2 shows the binding constants '*K*' and number of binding sites '*n*' for the binding of fisetin and 3-HF with HbA within the studied concentration range of the flavonoids.

3.2. Far ultraviolet Circular Dichroism (CD) spectroscopic studies

To investigate the possible effect of the flavonoids on the secondary structure of HbA, we used far-UV CD spectroscopy. The CD spectrum of HbA in aqueous buffer (in the absence of flavonoids) has two characteristic peaks of negative ellipticity at 208 nm and

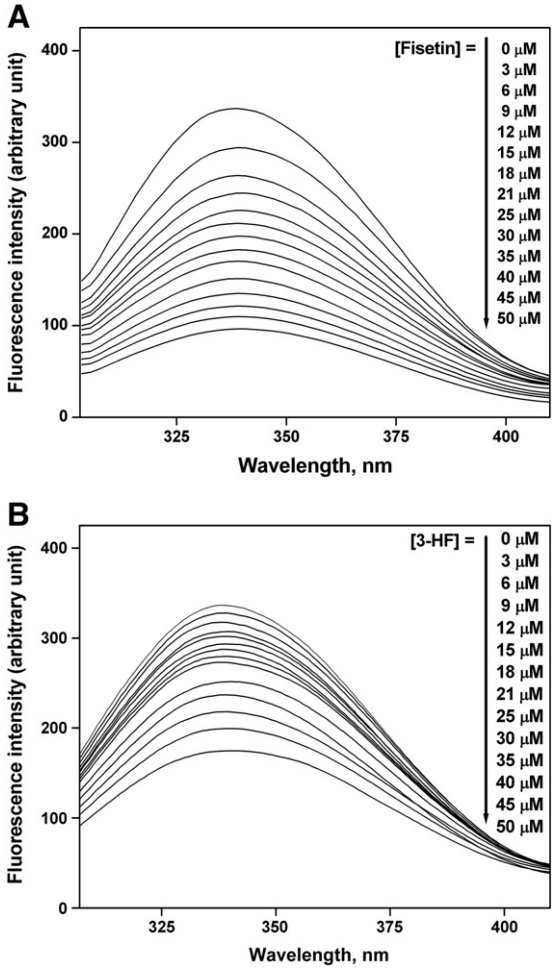

Fig. 3. Quenching effect of the flavonoids on HbA (3.0 μM) fluorescence ($\lambda_{ex} = 280$ nm), (A) for fisetin and (B) for 3-Hydroxyflavone.

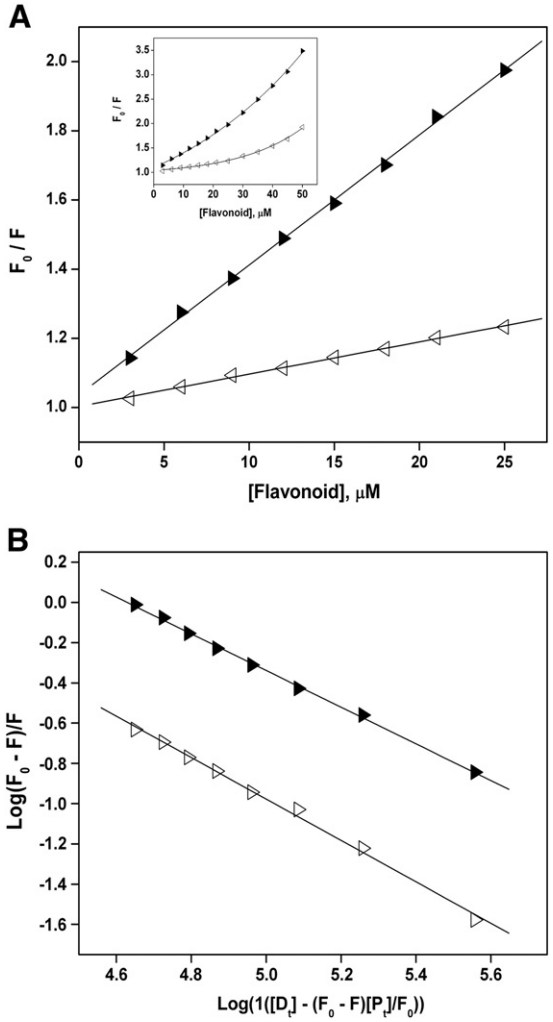

Fig. 4. (A) Stern–Volmer plots of the HbA tryptophan quenching data, upto 25 μM quencher (flavonoid) concentration; Inset: Stern–Volmer plots of the HbA tryptophan quenching data, for the flavonoid concentration range of 0–50 μM, ( ) for fisetin and (Δ) for 3-HF, (B) The plot of $\log(1/([D_t]-(F_0-F))[P_t]/F)$ vs. $\log(F_0-F)/F$ for Fisetin ( ) and 3-HF (Δ) (the concentration of HbA, $[P_t] = 3.0\times10^{-6}$ M).

222 nm indicating its predominantly α-helical secondary structure (Fig. 5). As shown in the figure, the CD spectrum of the HbA remains essentially unchanged upon addition of different concentrations of flavonoids (hemoglobin concentration remaining fixed).

3.3. Molecular modeling studies

We carried out computational studies for ligand binding site prediction using Q-SiteFinder, and found that hemoglobin possesses a total of ten probable binding pockets. Out of these, four are comparatively large binding sites which are located in each HbA chain. In general, the 'A' chain (α1, α2 chains) with its comparatively larger molecular volume of ~653 Å^3 is the usual ligand binding site. Interestingly, analysis of our docking results reveals that the flavonoid binding site is located in the 'D' chain of HbA (β2 chain) which has a relatively smaller molecular volume of 261 Å^3 (Fig. 6). The flavonoid binding cavity is enclosed by helix 4 and helix 5, while the C-terminal segment of helix 6 and the N-terminal segment of helix 7 form the base of the cavity. The list of residues lining the flavonoid binding site is given in Table 4. An especially interesting feature, shown in Fig. 6, is the position of nearby Trp 37 (β chain) residue (which is the emitting fluorophore in HbA [38]) occurring just 12.6 Å^3 away from the binding cavity.

The binding pockets of HbA have been further characterised by calculating the electrostatic surface potential using Poisson–Boltzmann equation (Fig. 6B). It is observed that the surface electrostatics of the flavonoid binding pocket of HbA is dominated by positively charged regions which contain the positively charged residue, Lys 66 (Table 4). In addition, there are two histidine residues (His 63 and His 92) in the flavonoid binding pocket (Table 4). The deprotonated form of the histidine side chain is capable of abstracting the 3-OH proton of 3-HF molecules. In this connection it is noteworthy that, since the anionic form of 3-HF molecules is stabilized in the positively charged region of the binding pocket of HbA, the consequence of the dramatically higher dielectric constant of the HbA binding pocket is that the energy of singly charged 3-HF anion (3-HFA) in HbA is significantly lower than that in aqueous solution. This difference in energy (ΔE) can be treated as a perturbation to the dissociation constant by [39]

$$\Delta pK_a = \frac{\Delta E}{2.303 k_B T}$$

Table 2
Binding parameters for flavonoid-HbA interactions.

Flavonoid	$^{\dagger}K_{SV}$, M^{-1}	$^{\dagger}K_q$, $M^{-1}\,s^{-1}$	$^{\dagger}K$, M^{-1}	$^{\dagger}n$
3-HF	$9.32\pm0.72\times10^3$	$3.45\pm0.72\times10^{12}$	$9.83\pm0.94\times10^3$	1.01
Fisetin	$3.76\pm0.86\times10^4$	$13.92\pm0.86\times10^{12}$	$4.00\pm0.88\times10^4$	0.97

$^{\dagger}K_{SV}$, the Stern–volmer quenching constant; K_q, the bimolecular quenching constant; K, the binding constant and n, the number of binding sites.

Table 3
Fluorescence lifetime of HbA tryptophan at different flavonoid concentrations.

[Flavonoid], μM	A_1	τ_1 (ns)	A_2	τ_2(ns)	χ^2
0 (HbA in Buffer)	0.775	0.725 ± 0.016	0.225	3.087 ± 0.024	1.01
3-HF, 12 μM	0.804	0.721 ± 0.012	0.196	2.960 ± 0.02	1.166
3-HF, 24 μM	0.784	0.740 ± 0.017	0.216	3.075 ± 0.14	1.053
Fisetin, 12 μM	0.793	0.690 ± 0.008	0.207	3.032 ± 0.017	1.121
Fisetin, 24 μM	0.792	0.693 ± 0.011	0.208	3.202 ± 0.031	1.09

λ_{ex} = 295 nm, λ_{em} = 335 nm.
HbA concentration was 3 μM.

Consequently, the pK_a for a 3-HF buried in the positive electrostatic potential of the HbA pocket should be lower than that in aqueous solution (where $pKa \approx 9$).

Molecular docking has been further employed to understand binding of both the flavonoids (fisetin and 3-HF) in HbA in atomistic detail. It is evident from the docking results (Fig. 7) that both the flavonoids are docked with the HbA in such a way that the B-ring is tilted by ~45° relative to the chromone moiety (formed by the A and C rings) of the polyphenols. The chromone part interacts with helix 4 and helix 5 while the phenyl ring is dipped into the cavity and interacts with helix 6 and helix 7. The docking studies suggest that the van der Waals interactions play an important role in the binding process. It is further evident from the docking results that while no hydrogen bonding possibilities exist in case of 3-HF, for fisetin there are two hydrogen bonding possibilities namely, between His 92 of HbA and the 7-OH group of fisetin and between HbA Val 134 and the 4′-OH group of fisetin. For both the flavonoids the internal hydrogen bond (namely the C(4) = O.....C(3)-OH intramolecular hydrogen bond) is preserved in the respective complexes with HbA. Table 5 lists all the interacting residues of HbA with 3-HF and fisetin. Assuming that van der Waals interactions are possible when a particular residue falls within 6 Å radii of a particular flavonoid, it is evident that Leu 31, Gly 136 and Tyr 130 specifically interact with 3-HF while Phe 42, Leu 105, Phe 71 specifically interact with fisetin through van der Waals interaction.

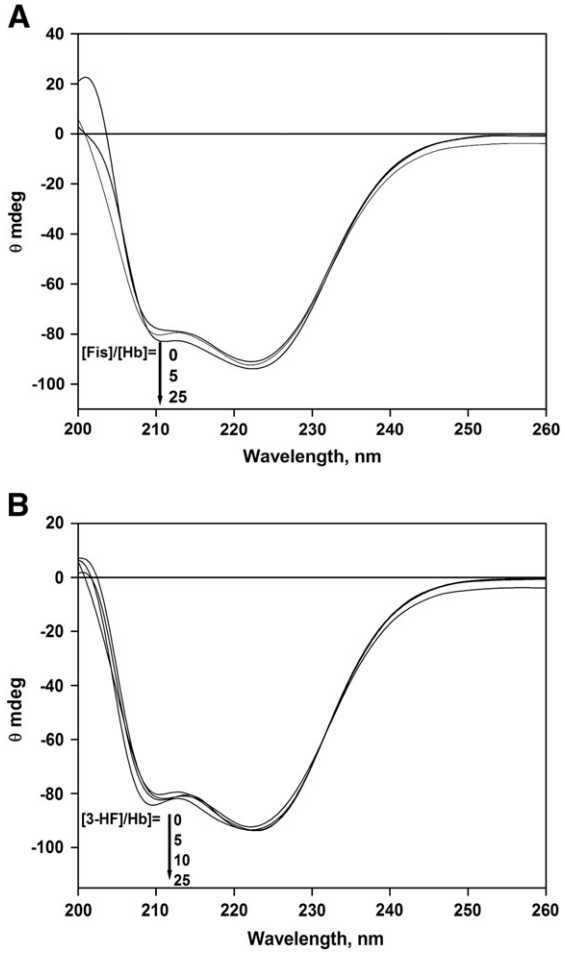

Fig. 5. Circular dichroism spectra of flavonoid-treated treated hemoglobin (HbA); (A) for fisetin and (B) for 3-HF and at different [Flavonoid]/[HbA] ratios. The concentration of HbA = 1×10^{-6} M in 0.01 M phosphate buffer, pH 7.4, was kept fixed, while the molar concentrations of flavonoids were varied accordingly.

Since the flavonoid binding site in HbA carries an intense positive potential, it is important to note the interaction between bound flavonoids with the positively charged residues in that pocket. The data presented in Table 4 show that all the positively charged residues (Lys 66, His 63, His 92 with protonated side chains) that contribute to the high positive potential of the flavonoid binding pocket of HbA interact with 3-HF. Table 5 shows that all the positively charged residues are within 6 Å distance from 3-HF. We have explicitly determined the atom to atom distance between hydrogen atom of C(3)-OH of 3-HF and the nitrogen atom of the basic residues. As shown in Fig. 7D, there are two nearby histidine residues with the calculated donor-acceptor distances between the 3-OH group of 3-HF and ε nitrogen of His 63 and His 92 being 6.1 Å and 7.6 Å respectively. The pKa of histidine side chain is in the range of 6.4–7.0, which suggests that it can readily accept a proton from the nearby 3-HF giving rise to the possibility of anion formation. We also note that there is a positively charged residue Lys 66 within 6 Å sphere and the calculated distance between 3-OH and the positively charged nitrogen of lysine is 13.3 Å which suggests the possibility of electrostatic interaction. Thus Lys 66 can increase the polarizability of the 3-OH bond. In contrast to the binding of 3-HF, in case of fisetin (Fig. 7B) it can be seen that the basic residues of HbA (Lys 66, His 63, His 92) in the flavonoid binding pocket interact with the C(7)-OH of the A ring of fisetin while the C(3)-OH group of fisetin is far away from the positively charged residues and faces towards the opening of the cavity. The pKa of C(3)-OH is lower than the other phenolic OH groups of the flavonoid. Also, the bond dissociation energy (BDE) calculations for the OH bonds of fisetin shown that the BDE of O(7)-H bond is ~1.5 times greater than that for the O(3)-H bond. Thus unlike the 3-OH group of 3-HF, the 7-OH group of fisetin possesses less probability of anion formation [12].

4. Discussion

The tautomer emission of flavonols is highly sensitive to external hydrogen-bonding perturbation, which can compete with the intramolecular H-bond, leading to decrease in tautomer fluorescence yield. Thus the remarkably enhanced tautomer fluorescence observed for 3-HF compared to fisetin suggests that, in the binding pocket of HbA the chromone moiety (which is the part of the molecule mainly relevant to the ESIPT process) of 3-HF is comparatively less accessible to water molecules. This conclusion is reinforced from the molecular docking studies which show that the desolvation energy is significantly higher for fisetin (+0.214 Kcal mol^{-1}) compared to that of 3-HF (+0.080 Kcal mol^{-1}).

The occurrence of significant amount of ground state anionic species of 3-HF in presence of HbA is evident from the absorption as well as the fluorescence excitation and emission spectra (Fig. 1). Presumably, 3-HF binds at a site predominantly rich in amino acids that can act as proton acceptors at the specific pH used (pH 7.4). Because 3-HF contains an electron withdrawing C O group adjacent to the –OH group, it can be expected to behave as a weak acid making proton abstraction and the consequent generation of the anion possible in HbA environment. This conclusion is reinforced by the picture emerging from the electrostatic surface potential calculations (Fig. 6B) which indicate that the flavonoid binding pocket of HbA contains positively charged regions.

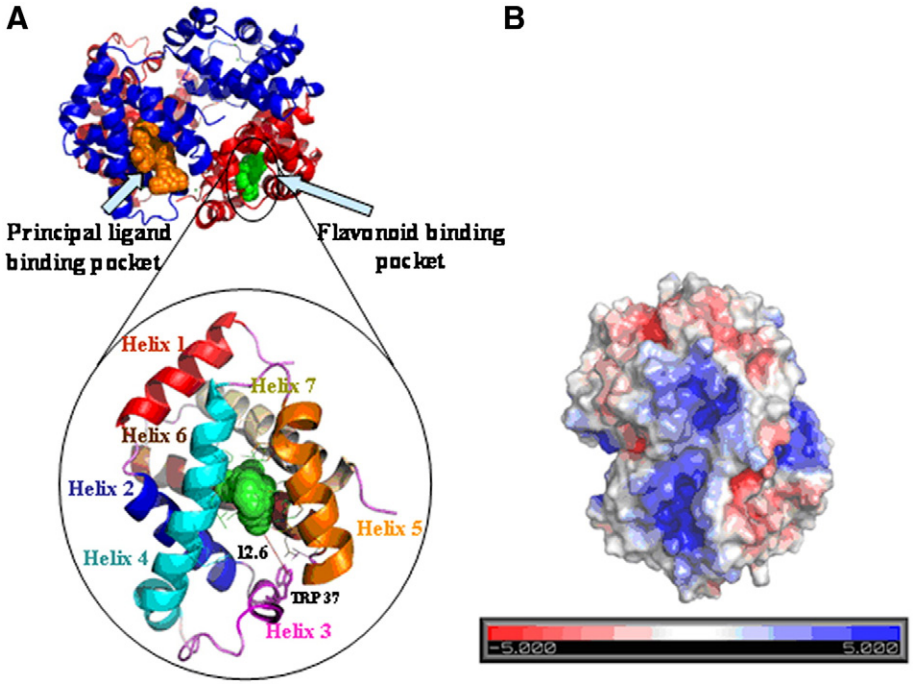

Fig. 6. (A) Predicted binding pockets of HbA. The orange colored pocket represents the principal ligand binding site (PDB id 2D60) while the green sphere represents flavonoid binding site. Details of the flavonoid binding site are shown in the magnified circle where different helices are shown in different colors. (B) Electrostatic surface potential of HbA. Blue and red colors are used to indicate the most positive and negative electrostatic potentials, respectively.

When bound to HbA, both fisetin and 3-HF show significantly high fluorescence anisotropy, indicating that the flavonoids are in environments where their mobilities are considerably reduced. The relatively higher fluorescence anisotropy value associated with 3-HFA compared to that of the neutral species is presumably due to its more restricted motion as a result of the electrostatic interaction with the positively charged amino acid residues of HbA (Table 4). Such high anisotropy value of 3-HFA is consistent with the results of the molecular modeling studies which suggest that 3-HF binds in a relatively positive potential site of HbA. This is reminiscent of our previous study of the interactions of the mono-hydroxyflavone (7-HF) with model membranes where significantly higher anisotropy value was obtained for the anionic species compared to the neutral 7-HF molecules [40]. Detailed analysis of the docking results of the binding of 3-HF reveals strong interactions with three positively charged residues, namely His 63, Lys 66, His 92 (with protonated side chains).

A linear Stern–Volmer plot (Fig. 4A) can be expected to arise from either collisional or static quenching [26]. However, the K_{SV} values obtained here are too large to be due to collisional quenching (Table 2), especially for an unquenched lifetime (τ_0) of tryptophan ca. 2.7 ns [26]. For this value of τ_o, the bimolecular quenching constant, K_q values are found to be $13.92 \pm 0.86 \times 10^{12}\ M^{-1}\ s^{-1}$ and $3.45 \pm 0.72 \times 10^{12}\ M^{-1}\ s^{-1}$ for fisetin and 3-HF respectively (Table 2) which are nearly 100-fold larger than the maximum value possible for diffusion-limited quenching in water ($2.0 \times 10^{10}\ M^{-1}\ s^{-1}$) [26]. Therefore, the quenching observed for HbA in presence of 3-HF and fisetin must be due to some specific interaction that increases the local concentrations of the flavonoids around the tryptophan residue(s) in hemoglobin. Thus, the large K_q value indicates that the observed quenching is static (and not dynamic) in nature, in the concentration ranges of the flavonoids we used. Fluorescence lifetime measurements were used to obtain further confirmation regarding the static nature of the quenching. (N.B.—For static quenching the complexed fluorophores are nonfluorescent, and the only observed fluorescence arises from the uncomplexed fraction. Therefore, the lifetime of the uncomplexed fluorophores (τ_0) remains unchanged and consequently $\tau_o/\tau = 1$ (τ is the lifetime in the presence of quencher). By contrast, for dynamic quenching $\tau_o/\tau = F_o/F$ where F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively [26,36]). The fact that no significant changes occur in the HbA tryptophan fluorescence decay parameters in presence of the flavonoids corroborate the conclusion drawn from the steady state fluorescence data that a static mechanism is principally responsible for the observed fluorescence quenching, implying that flavonoids do indeed bind to hemoglobin. Since the intrinsic fluorescence of human hemoglobin originates primarily from β-37 tryptophan [38], it seems reasonable to infer that the β-37 tryptophan residue is presumably at or near the binding site of the flavonoids. This conclusion is corroborated by the theoretical (docking) studies which show that the β-37 Trp residue occurs in close proximity to the flavonoid binding site (see Fig. 7) suggesting that the fluorescence of Trp 37 should be capable of 'sensing' ligand binding to that pocket.

For static quenching, the dependence of F_o/F on [Quencher] is identical to that observed for dynamic quenching except for the fact that the quenching constant is now the association constant [26]. The conclusion regarding the static quenching mechanism in the previous section derives additional credence from the observation that the values of K obtained here closely match with the values of K_{SV} calculated from the Stern–Volmer plots (Table 2).

The observation of only one binding site ($n \approx 1$, Table 2) for both the flavonoids in the β chain of tetrameric HbA ($\alpha_2\beta_2$) can be explained if we consider that only a certain fraction of the protein in solution is capable of binding the ligand [35].

Table 4
List of residues forming the flavonoid binding pocket of HbA.

Helix no.	Helix 4	Helix 5	Helix 6	Helix 7
Residues present in the binding pocket of Hemoglobin	His 63, Lys 66, Val 67, Leu 68, Ala 70, Phe 71	Phe 85, Leu 88, His 92, Val 98	Asn 102, Phe 103, Arg 104, Leu 106, Gly 107, Asn 108, Leu 110	Val 133, Ala 135, Val 137, Ala 138, Leu 141

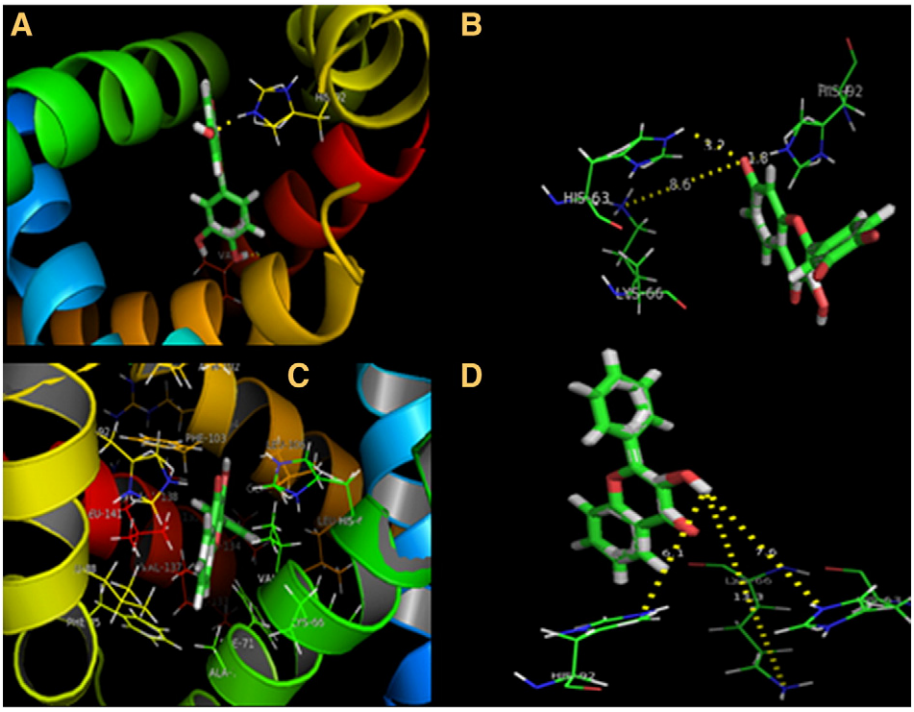

Fig. 7. Lowest energy docked conformation of fisetin and 3-HF with HbA. A, B: represent interaction profile of fisetin and C, D: represent interaction profile of 3-HF in the flavonoid binding pocket of HbA.

Far-UV circular dichroism (CD) spectra indicate that there is no significant change in the secondary structure of HbA (which is predominantly α-helical, with characteristic negative ellipticities at ca. 208 nm and 222 nm) upon binding of the flavonoids (fisetin, 3-HF).

Binding energies calculated by AutoDock reveals stronger binding of fisetin with HbA compared to that for 3-HF. AutoDock predicted free energy of binding is −8.80 Kcal/mol for fisetin whereas it is −8.04 Kcal/mol in case of 3-HF. Besides electrostatic and van der Waals interactions which is common in case of both the flavonoids, the main contributing factor for the difference in binding energies is the fact that two hydrogen bonding possibilities exist in case of fisetin (one between His 92 of HbA and 7-OH group and another between HbA Val 134 and 4′-OH group) but none for 3-HF (Fig. 7 and Table 5). This is in agreement with the significantly higher binding constant of fisetin relative to that of 3-HF as estimated through fluorescence studies.

Molecular docking studies predict differential binding of 3-HF and fisetin with HbA. Positively charged residues namely, His 63, His 92 (with protonated side chains) and LYS 66 can interact through electrostatic interaction with the 3-OH group of 3-HF. This probably explains the detection of the anionic species of 3-HF in HbA through fluorescence spectroscopy. The 3-OH residue of fisetin cannot find any interacting basic residues and hence anionic species of fisetin could not be detected for fisetin-HbA interaction.

5. Concluding remarks

Since the pharmacological actions of flavonoids in vivo are closely related to their binding with cellular targets including proteins, the investigation on binding of flavonoids with proteins is very significant. HbA has been found to play an important role in the distribution and bioavailability of flavonoids [25]. Besides, flavonoids are emerging as potentially useful drugs for the management of diabetes mellitus in view of their role in preventing non enzymatic glycosylation of HbA. With this scenario in mind we have explored the interactions of the poly hydroxy substituted naturally occurring flavonoid fisetin along with the simple mono hydroxylated flavonoid, 3-HF with HbA. For this we have fruitfully exploited the intrinsic flavonoid fluorescence as well as flavonoid-induced quenching of the protein tryptophan fluorescence. Molecular modeling studies have been further employed to provide detailed insights at atomistic level regarding the flavonoid recognition by HbA. The rationale of this study is that the small flavonoid molecules can easily penetrate the erythrocyte membrane and reaches inside the cells where hemoglobin is present in high concentrations.

In summary, our present study, using fluorescence spectroscopy and molecular modeling approaches, shows that fisetin and 3-HF interact differently with HbA. The spectroscopic data suggest that both the flavonoids bind with hemoglobin and quench its fluorescence. Based on

Table 5
Comparison of HbA–flavonoid specific interactions predicted by molecular docking.*

Name of the ligand	HbA amino acid residues falling within 6 Å from bound flavonoids	Estimated free energy of binding (Kcal/mol)	HbA-flavonoid hydrogen bonding interactions (Number and details)
3-HF	**Leu 31**, Leu 28, Val 98, Asn 102, Leu 96, Pro 100, His 92, Leu 91, Asn 139, Leu 141, Leu 88, Phe 85, **Gly 136**, Val 137, Ala 135, Ala 138, Phe 103, Arg 104, Leu 106, Asn 108, Gly 107, Val 134, Val 133, Val 111, Leu 110, **Tyr 130**, Gly 74, Ala 70, Ser 72, Gly 69, Leu 68, Lys 66, Val 67, His 63,	−8.04	None
Fisetin	Leu 28, Val 98, Asn 102, Leu 96, Pro 100, His 92, Asn 139, Leu 141, Leu 88, Phe 85, **Phe 42**, Val 98, Leu 96, Leu 91, **Leu 105**, Leu 106, Asn 108, Gly 107, Ala 135, Val 134, Val 137, Val 133, Gly 74, Ala 70, **Phe 71**, Ser 72, Gly 69, Ala 138, Phe 103, Arg 104, Val 67, Leu 28, His 63, Leu 110, Val 111, Lys 66, Leu 68,	−8.80	Two 1. His 92 of HbA and 7-OH of fisetin. 2. Val 134 of HbA and 4 -OH of fisetin.

*Residues that are specific for a particular flavonoid are shown in bold.

the Stern–Volmer equation, the quenching rate constants have been evaluated. Specific interactions with HbA are confirmed from the following lines of evidence : (a) bimolecular quenching constant $K_q \gg$ diffusion controlled limit (b) closely matched values of Stern–Volmer quenching constant and binding constant (c) $\tau_o/\tau = 1$ (where τ_o and τ are the unquenched and quenched tryptophan fluorescence lifetimes respectively). Far ultraviolet circular dichroism spectra of HbA (200 nm–260 nm) in presence of the flavonoids indicate essentially no change in the α-helix content (as revealed by the negative ellipticities at 208 nm) of flavonoid-treated hemoglobin. Molecular docking studies reveal that the position of Trp 37 (β' chain) residue, the emitting fluorophore in HbA is present just 12.6 Åaway from the binding cavity.

The present study demonstrates the usefulness of intrinsic fluorescence of the flavonoids together with that of hemoglobin (tryptophan), in combination with molecular modeling techniques, for obtaining a detailed picture of the differential binding of fisetin and 3-HF in critical detail. We can envision promising extension of this approach to other flavonoid derivatives, which would open the door to new avenues for the 'screening and design' of the most suitable flavonoid derivatives from among numerous structural variants of this new generation of rapidly emerging therapeutic drugs of immense importance in modern medicine.

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