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ORIGINAL PAPER

The Interaction of Flavonoid-Lysozyme and the Relationship Between Molecular Structure of Flavonoids and Their Binding Activity to Lysozyme

Ran Yang · Lanlan Yu · Huajin Zeng · Ruiling Liang · Xiaolan Chen · Lingbo Qu

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Abstract In this work, the interactions of twelve structurally different flavonoids with Lysozyme (Lys) were studied by fluorescence quenching method. The interaction mechanism and binding properties were investigated. It was found that the binding capacities of flavonoids to Lys were highly depend on the number and position of hydrogen, the kind and position of glycosyl. To explore the selectivity of the bindings of flavonoids with Lys, the structure descriptors of the flavonoids were calculated under QSAR software package of Cerius2, the quantitative relationship between the structures of flavonoids and their binding activities to Lys (QSAR) was performed through genetic function approximation (GFA) regression analysis. The QSAR regression equation was K_A= 37850.460+1630.01Dipole +3038.330HD-171.795MR. (r= 0.858, r^2_{CV} = 0.444, $F_{(11,3)}$=7.48), where K_A is binding constants, Dipole, HD and MR was dipole moment, number of hydrogen-bond donor and molecular refractivity, respectively. The obtained results make us understand better how the molecular structures influencing their binding to protein which may open up new avenues for the design of the most suitable flavonoids derivatives with structure variants.

Keywords Flavonoids · Fluorescence quenching · Molecular modeling techniques · Quantitative structure-activity relationship (QSAR)

R. Yang · L. Yu · R. Liang · X. Chen · L. Qu (✉)
Department of Chemistry, Zhengzhou University, Zhengzhou, China
e-mail: qulingbo@zzu.edu.cn

R. Yang
e-mail: yangran@zzu.edu.cn

H. Zeng
School of Pharmaceutical Science, Zhengzhou University, Zhengzhou, China
email: zenghuajin@sina.com.cn

Introduction

Current research has shown a surge in the utilization of natural components for the development of pharmaceutical agents. The potent antioxidant and anticancer activities of polyphenols have attracted scientists in both medicinal and pharmaceutical fields in recent times. Flavonoids, a group of important polyphenols that occur ubiquitously in foods of plant origin, have been proved to exhibit broad pharmaceutical activities such as antiproliferative, anti-tumour, anti-flammation, allergies and so on [1, 2]. The effects of flavonoids have been exclusively explained by their bindings to or interferences with enzymes, receptors, transporters and signal transduction systems [3]. In this case, it is very helpful to study the interaction of flavoniods and proteins for understanding their effects.

Lys, also called muramidase, is an antimicrobial protein widely distributed in various biological fluids and tissues including avian egg and animal secretions, human milk, tears, saliva, airway secretions, and secreted by polymorphonuclear leukocytes [4]. It has been many physiological and pharmaceutical functions, such as anti-inflammatory, anti-viral, immune modulatory, antihistaminic and anti-tumor activities [5, 6]. So it is

extensively used in the pharmaceutical and food fields. Another important function of Lys is their ability to carry drugs. It can cure some illness via the binding with these drugs. In view of the multifarious functions of this protein and its important practical role from a medicinal point of view, studies on the interactions between drugs and Lys have important meaning on realizing the transport and metabolism process of drugs. The interactions between Lys and some flavonoids, including puerarin [7], baicalein [8], myricetin [9], naringein and naringin [10] have been reported. However, among these papers, more attentions are focused on the descriptions of chemical characters of the interactions between flavonoids and Lys. Since the basic structure of flavonoids allows a multitude of substitution patterns: phenolic hydroxyls, O-sugars, methoxy groups, sulphates and glucuronides, over 6,000 different flavonoids have been described [11]. The problem how these substitutes influence the bonding of flavonoids with Lys has not been investigated in the literature.

In this study, twelve structurally flavonoids (Table 1) were selected to study their binding behaviors to Lys. The interaction mechanism and binding properties were investigated by fluorescence quenching method. It was

Table 1 The chemical structures of investigated flavonoids

Substances	R_3	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
Flavones							
7-hydrogen flavone	H	H	H	OH	H	H	H
Apigenin	H	OH	H	OH	H	H	OH
Baicalein	H	OH	OH	OH	H	H	H
Luteolin	H	OH	H	OH	H	OH	OH
Flavonols							
Icariin	OG[a]	OH	H	OG[b]	C_5H_9	H	OCH_3
Quercetin	OH	OH	H	OH	H	OH	OH
Rutin	OR[c]	OH	H	OH	H	OH	OH
Flavanones							
Naringerin	H	OH	H	OH	H	H	OH
Naringin	H	OH	H	OG[d]	H	H	OH
Isoflavines							
Daidzein	H	H	H	OH	H	H	OH
Genistein	H	OH	H	OH	H	H	OH
Puerarin	H	H	H	OH	OG[e]	H	OH

[a] O-Rhamnoglucoside

[b] O-Glucopyranoside

[c] O-Rutinose

[d] O-Glucopyranoside-(1,2)-Rhamnoglucoside

[e] O-Glucoside

found that the number and position of hydrogen, the kind and position of glycosyl influenced the binding potency of flavonoids to Lys significantly. To explore the selectivity of the bindings of flavonoids to Lys, the

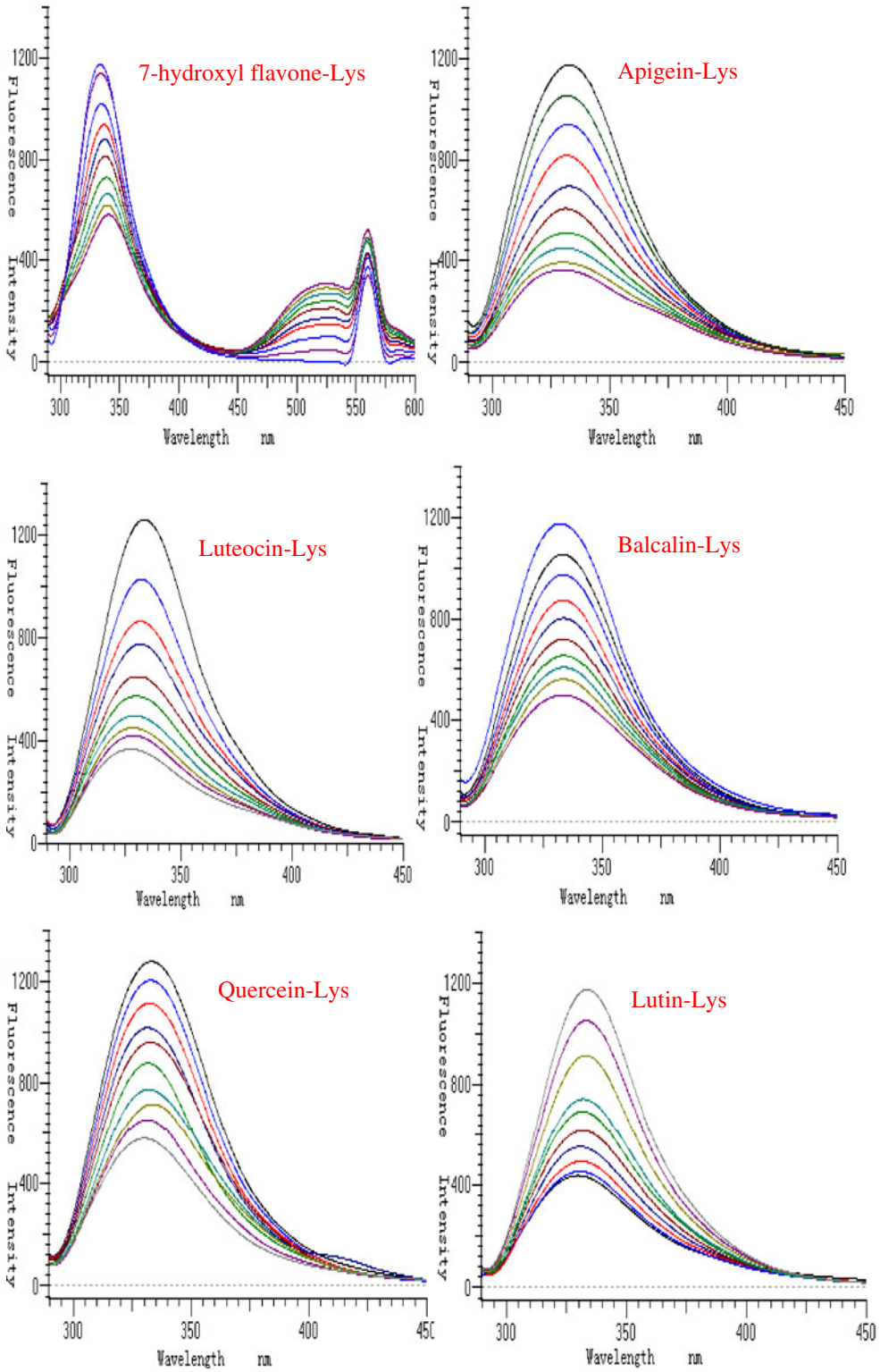


Fig. 1 The effects of flavonoids on the fluorescence spectra of Lys. Peak at 334 nm from up to down $C_{7\text{-OH Flavone}}=C_{Apigein}=C_{Luteocin}=C_{Qurcetin}=C_{Rutin}=C_{Icariin}=C_{Naringein}=C_{Naringin}=C_{Daidzein}=C_{Genistein}=C_{Puerarin}=$ (0, 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0, 27.0)$\times10^{-6}$ mol L^{-1}; $C_{Baicalin}=C_{Rutin}=$(0, 3.5, 7.0, 10.5, 14.0, 17.5, 21.0, 24.5, 28.0, 31.5)$\times10^{-6}$ mol L^{-1}; $C_{Lys}=1.0\times10^{-6}$ mol L^{-1} in all the case

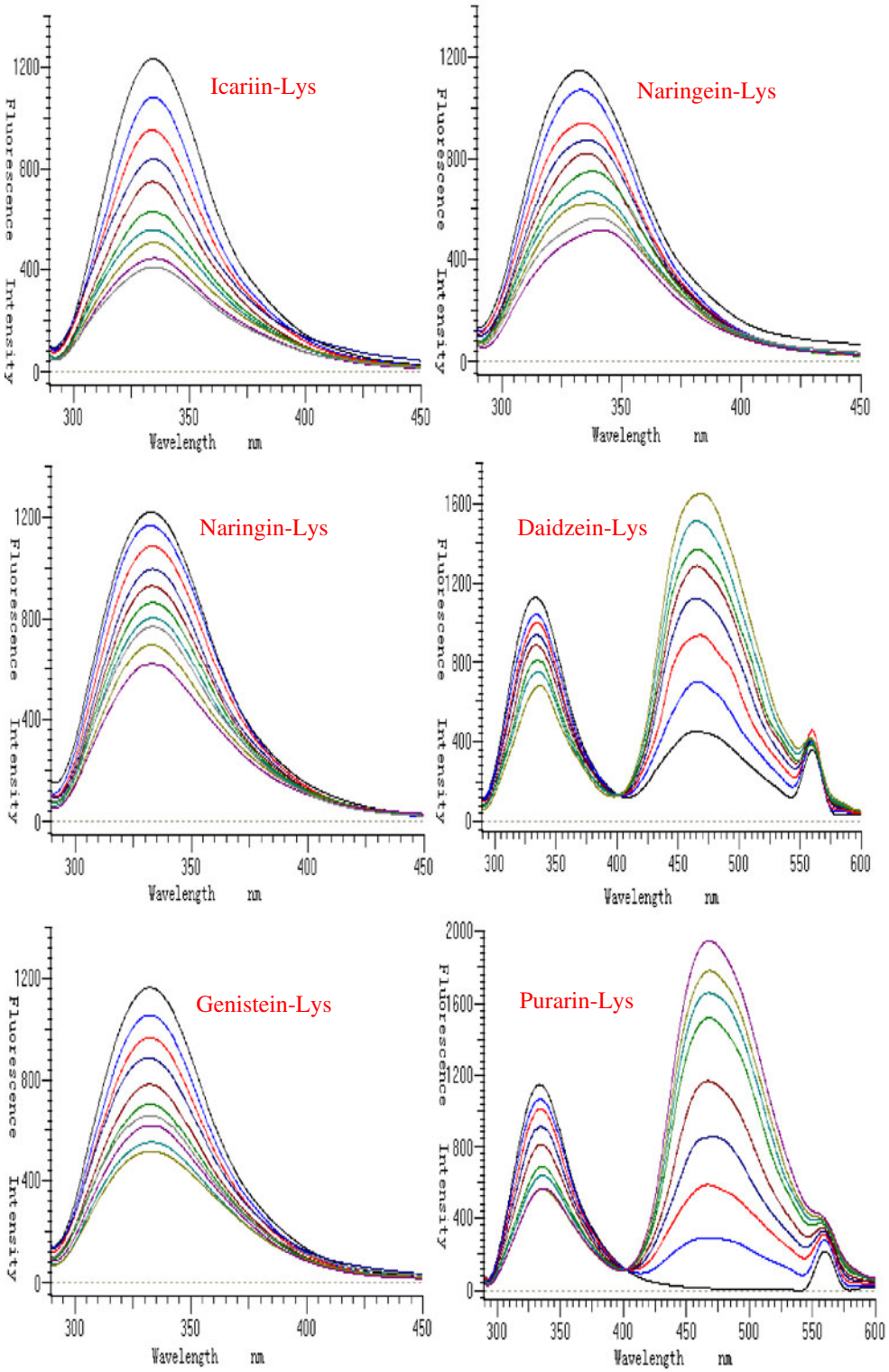


Fig. 1 (continued)

quantitative relationship between the structures of flavonoids and their binding activities to Lys (QSAR) was performed through GA regression method. The work described in this paper is an attempt to explore the effects of structure characters on the drug-protein interaction by molecular modeling techniques.

Experimental

Apparatus and Reagents

All fluorescence measurements were carried out on a F-4500 Spectrofluorimeter (Hitatch). A HP-8453 visible ultraviolet spectrophotometer (HP, USA) was used for scanning the UV spectra of flavonoids. The temperature was controlled with a TB-85 thermostat water-bath at 288 K. All pH measurements were made with a pHS-3C digital pH-meter with a combined glass electrode.

Crystal of hen egg white Lys was purchased from Sigma Chemical Co (America), and was used without further purification. The twelve natural flavonoids were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Tris-HCl (0.025 mol L^{-1}) buffer solution containing 0.1 mol L^{-1} NaCl was used to keep the pH of the solution at 7.20. Lys stock solution of 1.0×10^{-4} mol L^{-1} was prepared with Tris-HCl buffer solution and kept in the dark at 4 °C. The stock solution (2.0×10^{-3} mol L^{-1}) of twelve flavonoids were prepared in absolute methanol and diluted with Tris-HCl buffer to obtain the appropriate assay solutions. All other materials were analytical reagent grade and double distilled water was used throughout.

Fluorescence Measurement

The fluorescence measurements were carried out by successive addition of the solution of the flavonoid to a fixed amount of Lys (to give a final concentration 1.0×10^{-6} mol L^{-1}) in each tube. The final volume was made up to 5.0 mL with pH 7.20 Tris-HCl buffer. Thus, a series of solutions containing different amount flavonoids and a definite amount of Lys were obtained. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths of 290–450 nm) at 288 K. All solutions were mixed thoroughly and kept 20 min before measurements.

QSAR Building

The binding capacities of the twelve flavonoids were taken from the results obtained in our work. The 2D structures of the molecules were drawn using the Chem 3D software. The pre-optimization was conducted using molecular mechanics (MM2) procedure included in Chem 3D, and then the molecular structures were transferred into QSAR software package of Cerius2 (Accelrys Inc 2002) to calculated the structural descriptors including highest occupied molecular orbital energy (E_{HOMO}), lowest unoccupied molecular orbital energy (E_{LUMO}), dipole moment (Dipole), hydrogen-bond acceptor (HA), hydrogen-bond donor (HB), molar refractivity (MR), molecular surface area (Area), density (Density), molecular volume (V_m), molecular weight (MW), principal moment of inertia (PMI), radius of gyration (RG), number of rotatable bonds (RB) and the octanol/water partition coefficient (LogP). The QSAR model was built through genetic function approximation regression analysis.

Table 2 Parameters of interaction between flavonoids and lysozyme

Flavonoids	Parameters					
	*K*sv/(L mol^{-1})	K_A/(L mol^{-1})	*n*	*J* ($cm^3\cdot$ $moL^{-1}\cdot$L)	R_0 (nm)	*r* (nm)
Flavones						
7-hydrogen flavone	4.37×10^4	3.91×10^4	1.18	1.02×10^{-14}	2.45	4.03
Apigenin	8.84×10^4	6.63×10^4	1.35	1.88×10^{-14}	2.72	3.36
Baicalein	10.9×10^4	6.72×10^4	1.37	0.81×10^{-14}	2.37	3.27
Luteolin	9.74×10^4	8.56×10^4	1.13	1.90×10^{-14}	2.73	3.14
Flavonols						
Icariin	8.12×10^4	6.46×10^4	1.26	1.99×10^{-14}	2.75	3.71
Quercetin	4.31×10^4	4.03×10^4	1.17	1.52×10^{-14}	2.63	3.79
Rutin	6.18×10^4	5.35×10^4	1.30	1.81×10^{-14}	2.71	3.98
Flavanones						
Naringerin	5.23×10^4	4.83×10^4	1.10	0.35×10^{-14}	2.06	3.21
Naringin	4.15×10^4	3.24×10^4	1.11	0.31×10^{-14}	2.02	3.30
Isoflavines						
Daidzein	3.57×10^4	3.28×10^4	0.99	0.26×10^{-14}	1.95	3.31
Genistein	4.83×10^4	4.95×10^4	1.13	0.38×10^{-14}	2.08	3.29
Puerarin	4.18×10^4	4.24×10^4	1.17	0.23×10^{-14}	2.08	3.14

Results and Discussion

Fluorescence Quenching

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching [12]. Such decrease in fluorescence intensity is called fluorescence quenching. In order to investigate the binding of the twelve flavonoids to Lys, the fluorescence emission spectra were recorded in the range of 300–500 nm upon excitation at 280 nm. Figure 1 shows the fluorescence emission spectra of Lys in the presence of various concentrations of flavonoids at 288 K. Under the same condition, no fluorescence spectra of flavonoids itself was observed. It was found that the addition of flavonoids not only led to a significant reduction in the fluorescence signal but also a conspicuous changes of peak shape, such as wavelength shifts and occurrence of isoactinic point in the emission spectra of Lys, which referred to a change in the conformation of tryptophan micro-region [13].

To confirm quenching process of flavonoids to the fluorescence of Lys, the Stern–Volmer equation shown in Eq. (1) was applied [14].

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, respectively, [Q] is the concentration of the quencher, τ_0 is the fluorescence lifetime without the quencher and its value is 10^{-8} s [15], K_q is the quenching rate constant of biomolecule and K_{sv} is the

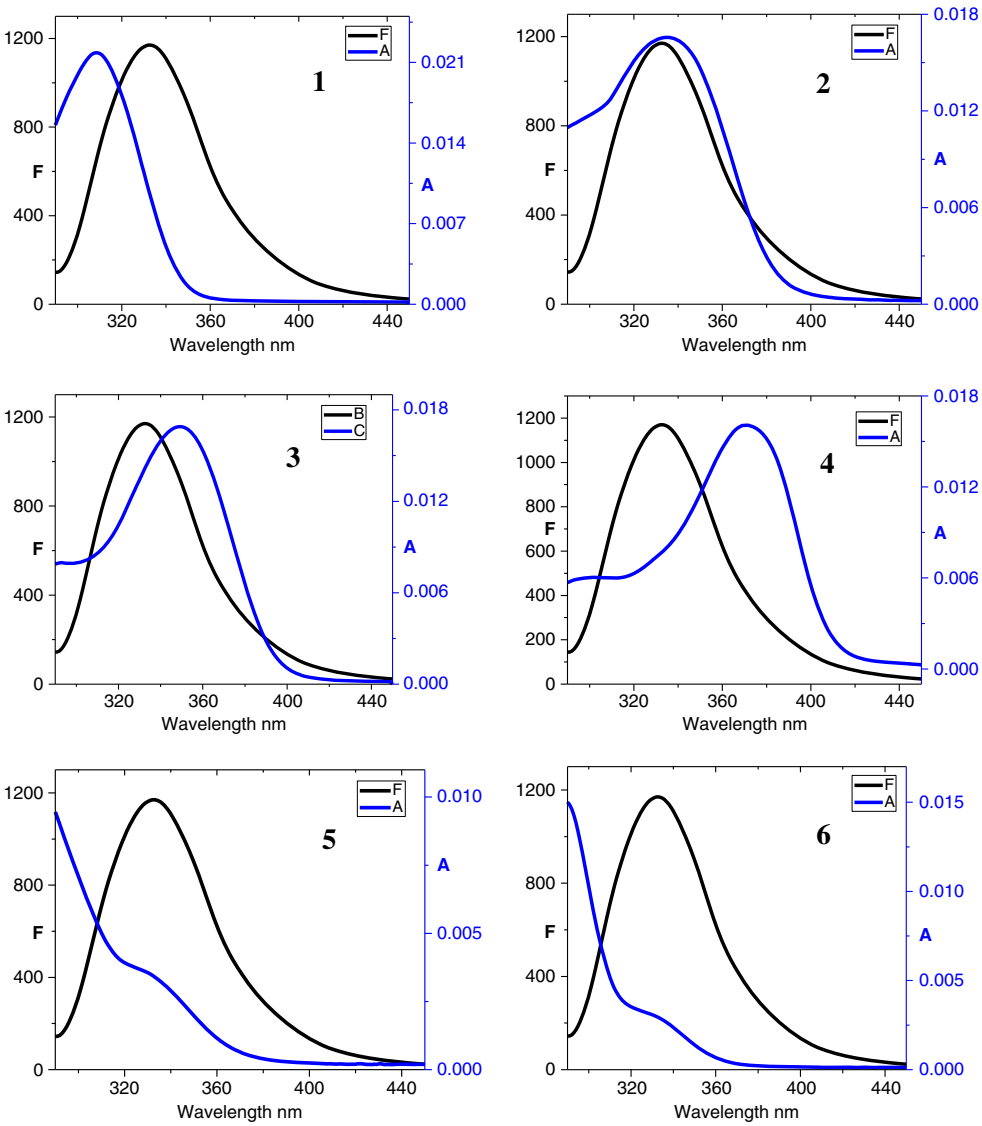
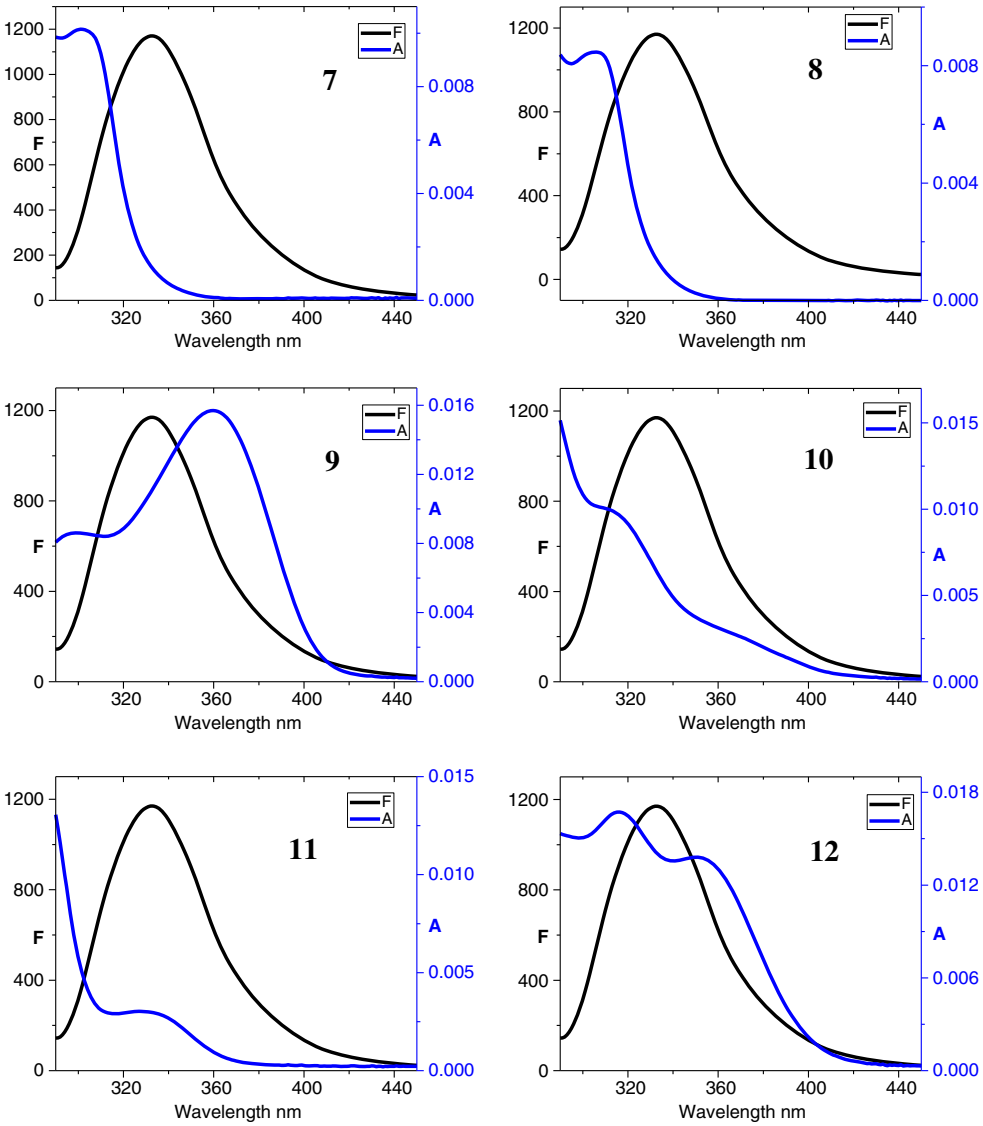


Fig. 2 The overlap of the fluorescence emission spectra of Lys with the absorption spectra of (1) 7-OH Flavone, (2) Apigein, (3) Luteocin, (4) Quercetin, (5) Naringein, (6) Genistein, (7) Daidzein, (8)Puerarin, (9) Rutin, (10) Baicalin, (11) Naringin, (12) Icariin. The concentration of Lys and twelve flavonoids all were 1.0×10^{-6} mol L^{-1}

Fig. 2 (continued)



Stern–Volmer dynamic quenching constant. According to Eq. (1), the quenching constant of flavonoids to Lys were calculated and listed in Table 2. It is well known that the maximum scatter collision quenching constant of various quencher with the biopolymer is 2.0×10^{10} L mol^{-1} S^{-1} [16]. Obviously, the rate constant of Lys quenching procedure initiated by flavonoids all were greater than the K_q of the scatter procedure, which demonstrated that the above quenchings were the static quenching resulted from the formation of non-covalent compounds between Lys and flavonoids [17].

Energy Transfer

A non-radiation energy transfer will occur when two molecules satisfy the following preconditions [18]: (1) the energy donor can produce fluorescence light, (2) the fluorescence emission spectrum of donor overlaps enough with the absorption spectrum of acceptor, and (3) the maximum distance between donor and acceptor in general, should be less than 7 nm. According to Forster's non-radioactive resonance energy transfer theory, the energy transfer efficiency E is related not only to the distance (r) between acceptor and donor, but also to the critical energy transfer distance, that is

$$E = R_0^6/\left(R_0^6 + r^6\right) \tag{2}$$

where R_0 is the Forster critical distance, at which 50 % of the excitation energy is transferred to the acceptor and can be obtained from donor emission and acceptor absorption spectra using the Eq. (3)

$$\mathrm{R}_0^6 = 8.8 \times 10^{-25} K^2 \Phi n^{-4} J \tag{3}$$

where K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles; n is the average refractive index of medium; Φ is the fluorescence quantum yield of the

donor; J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which could be calculated by the following equation:

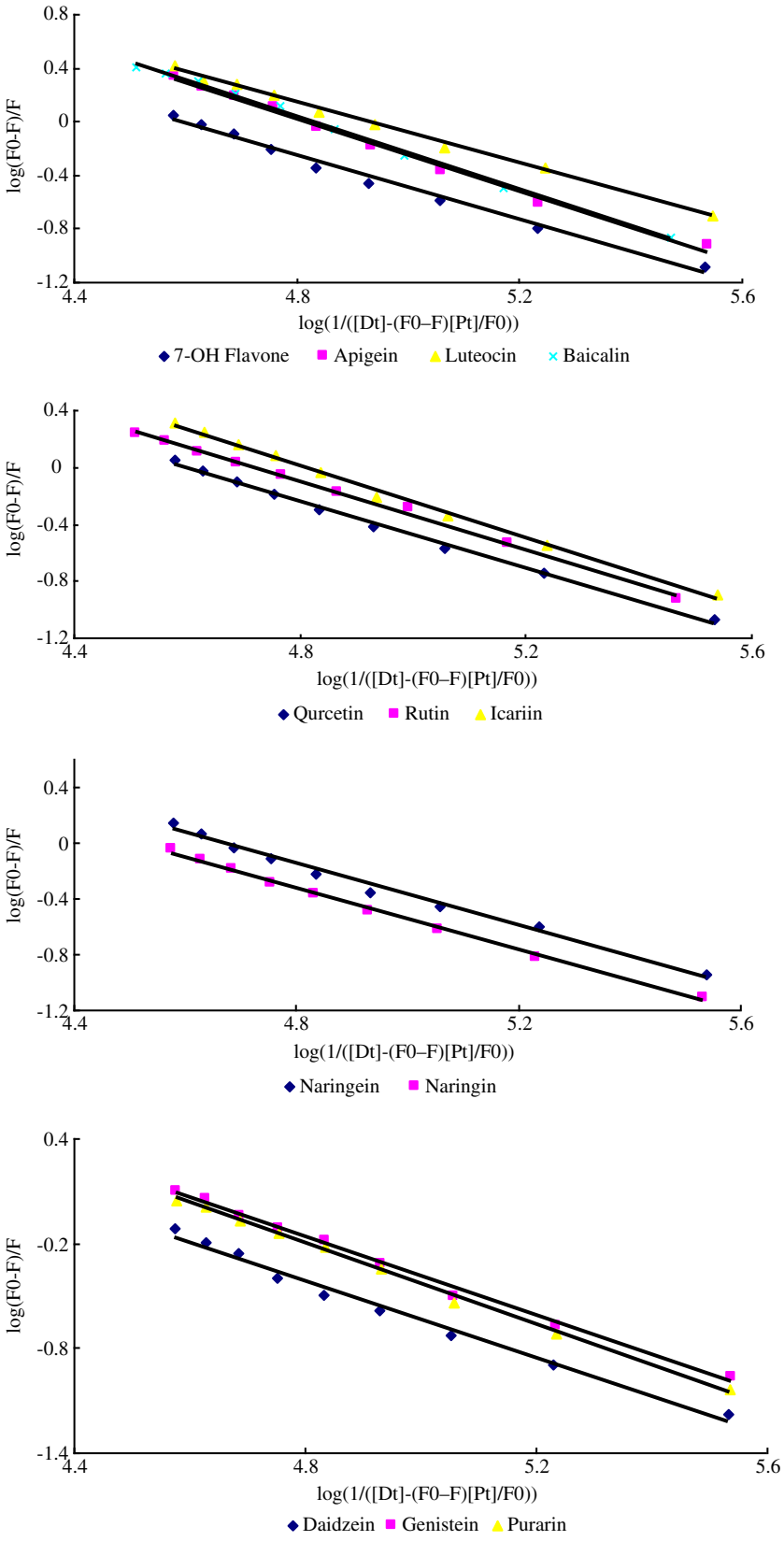


Fig. 3 The plots of $\log(F_0-F)/F$ Vs. $\log[1/([D_t]-(F_0-F)[Pt]/F_0)]$ of the flavonoid-Lys interaction

Table 3 Descriptors of the investigated flavonoids (I)

	E_H	E_L	ΔE_{H-L}	Dipole	HA	HB	RB
Flavones							
7-hydrogen flavone	−0.232	−0.063	0.169	5.392	3	1	2
Apigenin	−0.218	−0.064	0.150	6.457	5	3	4
Baicalein	−0.229	−0.085	0.144	5.726	11	6	10
Luteolin	−0.216	−0.064	0.153	7.536	6	4	5
Flavonols							
Icariin	−0.214	−0.074	0.140	7.234	15	8	17
Quercetin	−0.203	−0.067	0.136	5.183	7	5	6
Rutin	−0.209	−0.062	0.147	4.388	16	10	16
Flavanones							
Naringerin	−0.222	−0.053	0.168	5.116	5	3	4
Naringin	−0.223	−0.058	0.166	2.991	14	8	14
Isoflavines							
Daidzein	−0.207	−0.050	0.158	3.181	4	2	3
Genistein	−0.211	−0.055	0.156	3.627	5	3	4
Puerarin	−0.203	−0.046	0.157	5.668	9	6	9

$$J = \Sigma F_{(\lambda)}\varepsilon_{(\lambda)}\lambda^4\Delta\lambda / \Sigma F_{(\lambda)}\Delta\lambda \quad (4)$$

where F_λ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$ and ε_λ is the molar absorption coefficient of the acceptor at λ and its unit is 1 mol^{-1} cm^{-1}. The energy transfer efficiency E is

$$E = 1 - F/F_0 \quad (5)$$

The overlap of the absorption spectra of the flavonoids with fluorescence emission spectra of Lys was shown in Fig. 2. In the present case, K^2=2/3, n=136, Φ=0.14 [19]. According to Eqs. (2)–(5), r, R_0 and J of the flavonoid-Lys interaction could be calculated (Table 2). The values of R_0 and r for the twelve flavonoids were all less than 7 nm, which indicated that the energy transfer from Lys to all the flavonoids occurred with high probability [20].

Binding Constant and Binding Sites

For the static quenching process, under the assumption that there are the same and independent binding sites n in the protein, the static quenching equation could be deduced as the following Eq. (6) [21]:

$$\log\frac{F_0 - F}{F} = \mathrm{n}\log K_A - \mathrm{n}\log\frac{1}{[\mathrm{Dt}] - (F_0 - F)[\mathrm{P_t}]/F_0} \quad (6)$$

where $[D_t]$, $[P_t]$ is the concentration of drug molecule and protein respectively. K_A is the binding constant. By plot of $\log(F_0{-}F)/F$ versus $\log(1/([Q_t] - (F_0 - F)[P_t]/F_0))$ (Fig. 3), the number binding sites n and binding constants K_A of the interactions between twelve flavonoids and Lys can be calculated (Table 2). By comparison of the binding constants of different flavonoids with Lys, it could be concluded that the amount and position of hydrogen-bond, double bond between C_2 and C_3, glycosylation and methylation were the main structure characters that influenced their binding potencies which were consistent with the structure features that determine the antioxidative activities [22–24]. This gives a clue that the determination of protein binding to structurally related compounds is a valuable tool for identifying the groups of a drug molecule.

Table 4 Descriptors of the investigated flavonoids (II)

	MW	Area	V_m	D	PMI	RG	log$P$	MR
Flavones								
7-hydrogn flavone	238.2	255.2	207.3	1.15	584.1	3.779	2.68	69.88
Apigenin	270.2	266.5	223.9	1.21	809.4	3.972	1.9	73.51
Baicalein	446.4	432.4	359.9	1.24	1837.6	4.825	0.02	107.21
Luteolin	286.2	283.1	232.2	1.23	901.4	4.013	1.51	75.32
Flavonols								
Icariin	676.7	736.7	584.4	1.16	3442.8	5.390	−0.13	171.28
Quercetin	302.2	297.4	241.4	1.25	919.6	3.991	0.35	76.51
Rutin	610.5	574.4	497.2	1.23	2942.9	5.017	−2.28	141.69
Flavanones								
Naringerin	272.3	287.2	230.0	1.18	814.3	3.888	1.63	71.67
Naringin	580.5	601.0	489.1	1.19	3663.6	5.752	−1.1	137.52
Isoflavines								
Daidzein	254.2	261.6	215.7	1.18	761.4	3.973	2.13	69.93
Genistein	270.2	274.9	223.8	1.21	804.1	3.978	1.74	71.74
Puerarin	416.4	421.4	348.1	1.20	1877.2	4.913	−0.49	104.19

QSAR Study

The relationship between the binding potencies (K_A) of twelve flavonoids to Lys and their structure descriptors (Tables 3 and 4) were built by GFA regression method.

The regression equation is K=37850.46 +1630.01 Dipole +3038.33HD-171.795MR (r=0.858, r^2_{CV} = 0.444, $F_{(11,3)}$= 7.48). Where, r, r^2_{CV} and F were correlation coefficient, cross-validated values and F-test value, respectively. The number of hydrogen-bond donor (HD) is a structural descriptor. For flavonoids, it was connection with the number and position of hydrogen bond. The heavy contribution of HD to binding suggested the significance of hydrogen bond, this were validated by our result. Dipole, a 3D electronic descriptor, displays the distribution of charge and the separation degree of negative charge and positive charge. The contribution of Diople to binding implied the probability of participations of electrostatic force in the binding. Molar refractivity (MR) is a combined measure of the size and polarizability of a molecular. The negative correlation suggested the size or polarizability of flavonoids is not good for their interaction with Lys, which agreed with the results that the large size of flavonoids would decrease their binding to bovine serum albumin [25].

The influence factors of small molecule on its binding to proteins had once been discussed by few researchers [26–28]. However, only single factor analysis was performed in these papers. Since the interactions between small molecule and proteins were very complicated, the multiparameter quantitative analysis on structure-binding relationship is very essential to explore the rules of drug-protein interaction. Except our research on the interaction between flavonoid and bovine serum albumin [29], no relevant QSAR of drugs structures and their binding activities had been reported.

Conclusion

In the work described on this paper, the interaction between Lys and twelve structurally different flavonoids were investigated on the base of the quenching effects of flavonoids on the intrinsic fluorescence of Lys. The binding capacities of different flavonoids to the Lys were calculated. It was found that the substitutes of flavonoids had an important influence on the magnitude of interaction. To explore the selectivity of binding of Lys with different flavonoids, the quantitative relationship between the structures of flavonoids and their binding activities to Lys was performed through GFA regression method. It was found that the dipole moment, the number of hydrogen-bond donors and molecular molar refractivity make more contributions to the flavonoid-lysozyme interaction. The obtained results make us understand better how the molecular structures influencing their binding to protein which may open up new avenues for the design of the most suitable flavonoids derivatives with structure variants.

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