Xanthine Oxidase Inhibitory Mechanism of Fisetin and Hesperitin

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Summary: Fisetin and hesperitin are two common flavonoids in plant medicines. In this paper, the mechanism of xanthine oxidase (XO) inhibition was systematically studied by combining experimental and theoretical methods. The HPLC results suggested that the XO inhibitory activity of fisetin (IC_{50}, 0.140 mM) was superior to that of hesperitin (IC_{50}, 0.635 mM). The spectrofluorimetry results showed flavonoids could induce the static fluorescence quenching of XO, indicating that they played the inhibitory activity by forming the complexes with XO. We showed the paramount force of fisetin and XO was hydrophobic; in the complex of hesperidin and XO, hydrogen bonding and van der Waals force were crucial forces. We used Autodock software for molecular docking. The results suggested that both fisetin and hesperitin entered the active pocket of XO, and the complexes were maintained by hydrogen bonding and hydrophobic interaction, which coincided with the experimental results.

Keywords: Fisetin; Hesperitin; Xanthine oxidase; Interaction; Spectrofluorimetry; Molecular docking.

Introduction

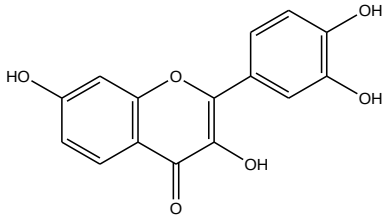
Gout is an inflammatory disease which caused by an overreaction of the body's immune system to the formation of needle-like crystals in soft tissues such as joint membranes or tendons in excess of uric acid [1,2]. Xanthine oxidase (XO) is a crucial enzyme which participated in the metabolism of nucleic acids in humans [3]. The main mechanism of XO is to catalyze the oxidation of hypoxanthine and xanthine to produce uric acid and free radicals which is closely related to the formation of gout [4]. At present, the main treatment for gout is to control serum uric acid content with the XO inhibitors [5]. Allopurinol is an XO inhibitor used in clinic. However, it has numerous and serious side effects, for example inducing allergies, damaging myocardial function and liver, etc. [6,7]. Thus, to search safe and efficient XO inhibitors is imminent matter.

Flavonoids widely exist in plant medicines and plant-derived foods [8]. It's worth noting that they have numerously outstanding pharmacological activities as an example antioxidant [9], protect cardiovascular [10], anti-aging [11] and anticancer, etc. [12]. Fisetin and hesperitin (Fig 1) are two common flavonoids in plant medicines. As we know, the two molecules have not been comparably explored as XO inhibitors. In view of this, the XO inhibition of fisetin and hesperitin were systematically compared by using the experimental and theoretical methods.

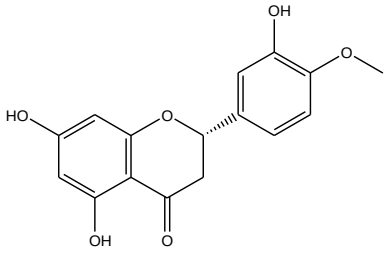
Experimental

Chemicals

Xanthine oxidase (purity≥99%) from bovine milk and xanthine (purity≥99%) were the product of Sigma-Aldrich (St. Louis, Missouri). Allopurinol (purity=98%), uric acid (purity=99%), fisetin (purity>96%), hesperitin (purity≥98%) were the product of Aladdin (Shanghai, China). The experiment pure water was purchased from the Wahaha (Zhengzhou, China). Methanol (HPLC grade) was the product of Avantor J.T. Baker.



Fisetin



Hesperitin

Fig. 1: Chemical structure of fisetin and hesperitin.

XO Inhibitory Assay

The XO suppression measurement was based on previous reports [13]. The solution of xanthine oxidase, xanthine and flavonoids were prepared with the phosphate buffered saline (PBS,0.2 M, pH=7.5). 0.5

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mL sample (flavonoid or allopurinol) of different concentrations, 0.5 mL of 40 μg/mL xanthine oxidase solution and 2 mL PBS were mixed at 37 °C incubated 30 minutes, and then adding xanthine solution (1 mL, 0.067mg/ml), making the reaction continuing at 37°C for 15 min. There is one more point, the reaction was terminated by adding 2 mL of methanol. The production of uric acid in the mixture was determined by using an Agilent 1260 HPLC with a C_{18} column at 30 °C. The flow rate of the mobile phase was controlled as 0.8 mL/min. The mobile phase consisted of water (98%) and methanol (2%). 10 μL of uric acid entered the column and detected at 290 nm [14]. The content of uric acid for the flavonoid (C_{sample}) was determined by using the external standard method. The PBS content was calculated as $C_{control}$. The calculation formula of XO inhibitory activity was as follow:

$$\text{XO inhibitory activity} = \frac{C_{control} - C_{sample}}{C_{control}} \times 100\%$$

Fluorescence spectroscopy

The interaction behavior between the sample and xanthine oxidase was investigated with fluorophotometer [15]. 4 mL of xanthine oxidase solution (40 μg/mL) was reacted with 1 mL sample liquor of different concentration. The reaction conditions were as follows: the reaction time was 10 minutes; the temperature was 30 ℃ and 37 ℃. The parameters of the fluorophotometer as both slits of 5nm, excitation wavelength of 280nm and scanning range of 300-450nm were set. After the completion of the reaction, the fluorescence spectrum of the solution was scanned and recorded.

Molecular Docking

We adopted Autodock 4.2 [16] to analyze the pattern of flavonoid and xanthine oxidase. Both the 3D structures of fisetin and hesperitin were established and optimized by adopting the PM6 method of MOPAC 2016 software (http://openmopac.net/home.html). The crystal structure of xanthine oxidase (ID:1FIQ) was obtained from the RCSB PDB database [2,17]. For docking, the xanthine oxidase structure was first dehydrated, then the polar hydrogen was added. The grid points in three directions were 60, 60, 60 Å, and the entire active center was covered by the 0.375 Å lattice. The possible docking modes were obtained by Lamarck genetic algorithm (LGA) and scored by semi-empirical energy function.

Results and Discussion

XO inhibitory activity

To avoid the interference of the ultraviolet absorbance of flavonoids, the XO inhibitory activities of fisetin and hesperitin were determined based on HPLC. The decrease of substrate xanthine and the increase of product uric acid could be well monitored by HPLC (Fig 2). By comparing the production of uric acid, their XO inhibitory activities could be determined. As shown in Fig 3, fisetin, hesperitin and allopurinol showed the strong XO inhibitory activities. The level of XO inhibitory activities relied on the concentration of sample. The supreme inhibitory activity was found by allopurinol (IC_{50}, 0.040 mM). The inhibitory performance of fisetin (IC_{50} 0.140 mM) was higher than that of hesperitin (IC_{50} 0.635 mM).

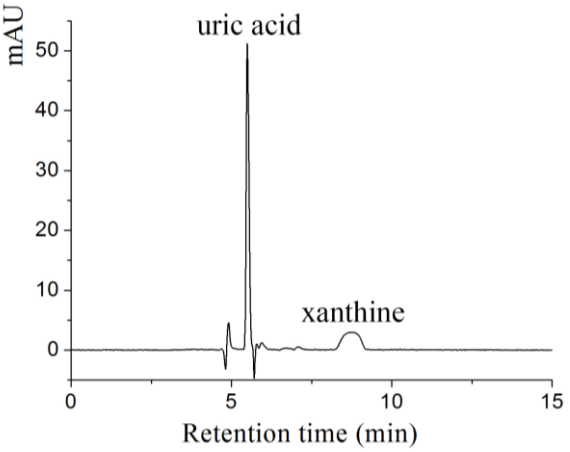

Fig. 2: The HPLC profile for the XO inhibitory activity.

Table-1: Quenching constants between flavonoids and XO.

Flavonoid	T(°C)	Stern-Volmer equation	R^2	K_q ($L \cdot Mol^{-1} \cdot s^{-1}$)
Fisetin	30	$F_0/F=1.9328\times10^4x+1$	0.9843	1.9328×10^{12}
	37	$F_0/F=1.3474\times10^4x+1$	0.9800	1.3474×10^{12}
Hesperitin	30	$F_0/F=1.9844\times10^4x+1$	0.9929	1.9844×10^{12}
	37	$F_0/F=1.2436\times10^4x+1$	0.9907	1.2436×10^{12}

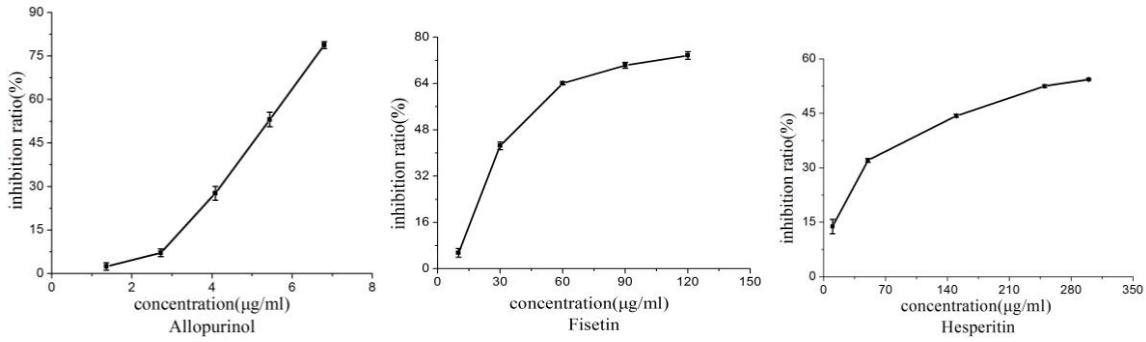

Fig. 3: XO inhibitory activities of allopurinol, fisetin and hesperitin.

Table-2: Binding constants between flavonoids and XO.

Flavonoid	T(°C)	Lineweaver-Burk equation	R^2	K_a ($L \cdot Mol^{-1}$)	n
Fisetin	**30**	**lg[(F_0-F)/F]=1.0253lg[Q]+ 4.4071**	**0.9878**	**2.5527×10^4**	**1.0253**
	37	**lg[(F_0-F)/F]=1.1483lg[Q]+ 4.8231**	**0.9931**	**6.6527×10^4**	**1.1483**
Hesperitin	**30**	**lg[(F_0-F)/F]=1.0987lg [Q]+ 4.5151**	**0.9979**	**2.2734×10^4**	**1.0987**
	37	**lg[(F_0-F)/F]=1.0604lg[Q]+ 4.3603**	**0.9969**	**2.2909×10^4**	**1.0604**

Fluorescence spectroscopy

Although fisetin and hesperitin have the similar structures, their inhibitory performance is different from each other. To clarify the underlying mechanism, their effect on the fluorescence spectra were investigated by fluorescent spectrometer. By detecting the change of internal fluorescence intensity in xanthine oxidase solution before and after adding the sample, the interaction can be inferred. It was found that both fisetin and hesperitin could induce the fluorescence quenching of XO in concentration-dependent manner at 30 °C and 37 °C (Fig 4 and 5). The fluorescence quenching process can be divided into static quenching and dynamic quenching [18]. The static quenching is associated with the formation of enzyme and inhibitor complexes. The dynamic quenching is the collision and energy exchange between the enzyme and the inhibitor. The quenching rate constant (K_q) of the sample with XO could be calculated (Table 1) according to the Stern-Volmer equation [19].

$$\frac{F_0}{F} = K_q \tau_0 [Q] + 1$$

where, F_0 was the fluorescence intensity of XO without the flavonoid. F was the fluorescence intensity of XO presence of the flavonoid. [Q] was flavonoid concentration, K_q was the quenching rate constant of the enzyme. τ_0 was the average life span of tryptophan in the enzyme system ($\tau_0 = 10^{-8}$ s).

Both the K_q values of fisetin and hesperitin were superior to the maximum dynamic quenching rate constant (Table 1). The constant was 2.0×10^{10} L mol^{-1} s^{-1}. Hence, the intrinsic fluorescence quenching of XO with fisetin or hesperitin was caused by the static fluorescence quenching, which suggested that they could played the inhibitory role by forming the complexes with XO [20].

To further understand their interaction behavior, the binding constants (K_a), the number of binding sites (n) between the flavonoid and XO were computed by the double-logarithm formula [19]:

$$\lg\frac{F_0 - F}{F} = n\lg[Q] + \lg K_a$$

As shown in Table 2, all the n were about 1, indicating that fisetin or hesperitin could interact with XO at the molar ratio of 1:1. Both binding constant (K_a) of fisetin and hesperitin against XO increased with the increasing temperature. The K_a of fisetin/XO was greater than hesperitin/XO, which meant that the binding of fisetin with XO had advantage over hesperitin with XO.

To investigate the interaction force between the flavonoid and XO, the thermodynamic parameters of the complex were obtained according to the following formulas [21]:

$$\ln\frac{Ka_{T_2}}{Ka_{T_1}} = \frac{\Delta H\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}{R}$$

$$\Delta G = -RT\ln K_a$$

where, ΔG, ΔH and ΔS respectively represented free energy change, enthalpy change and was entropy change.

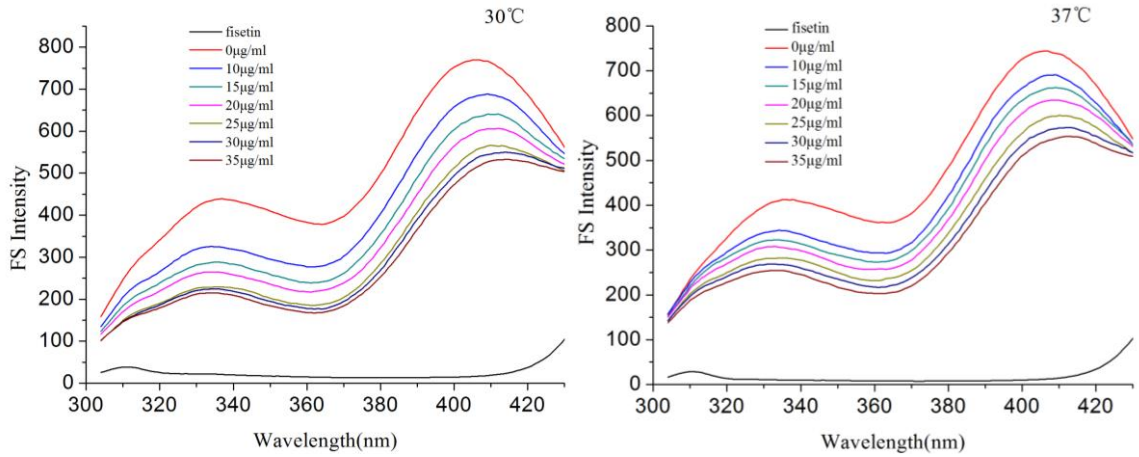

Fig. 4: Effects of fisetin on the fluorescence spectra of XO at 30°C and 37°C.

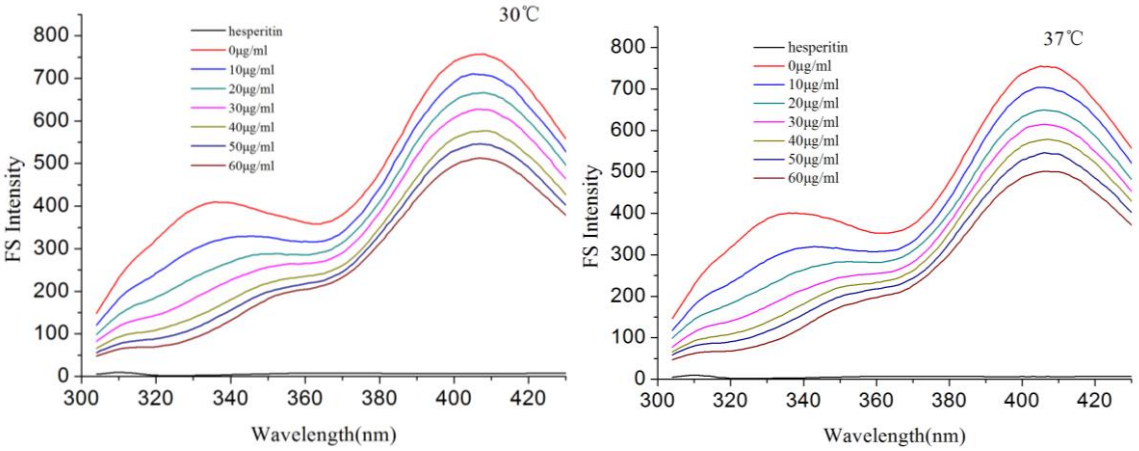

Fig. 5: Effects of hesperitin on the fluorescence spectra of XO at 30 °C and 37 °C.

Table-3 summarized the thermodynamic parameters. The negative ΔG suggested that the combination of the flavonoid with XO was spontaneous. For fisetin, the positive ΔH and ΔS values implied that its combination with XO was entropy-driven endothermic reaction, and the hydrophobic force was the foremost drive. But for hesperitin, the ΔH and ΔS values were negative, indicating that the becoming of the hesperitin/XO composite was enthalpy-driven exothermic reaction. During this process, the hydrogen bonding and van der Waals force were prominent forces.

Table-3: Thermodynamic parameters of flavonoids with XO.

Flavonoid	T(°C)	ΔH^0(KJ·mol^{-1})	ΔG^0(KJ·mol^{-1})	ΔS^0(J·mol^{-1}·K^{-1})
Fisetin	**30**	**106.97**	**-28.64**	**437.22**
	37		**-25.58**	
Hesperitin	**30**	**-39.85**	**-25.89**	**-45.03**
	37		**-26.20**	

Molecular docking

The combination mode of the flavonoid and XO was manifested by molecular docking. The structures with the lowest binding energy were exhibited in Fig 6a and 6b. The negative binding energy represented that the reaction could proceed spontaneously. The binding energy between fisetin and XO was -8.45 kcal/mol while the binding energy between hesperitin and XO was -8.03 kcal/mol, which coincided with the experimental results that fisetin possessed the higher XO binding capacity and inhibitory activity. As the Fig 6(a, b) showed, fisetin formed 8 hydrogen bonds with the residues of XO (Ser1080, Met1038, Gly1260, Thr1083, Val1259) while hesperitin formed six hydrogen bonds with the residues of XO (Phe798, Ser1080, Gln1040, Lys1045, Thr1083). It was also found that fisetin had the stronger hydrophobic interaction with XO (Fig 6c and 6d), which coincided with the thermodynamic results.

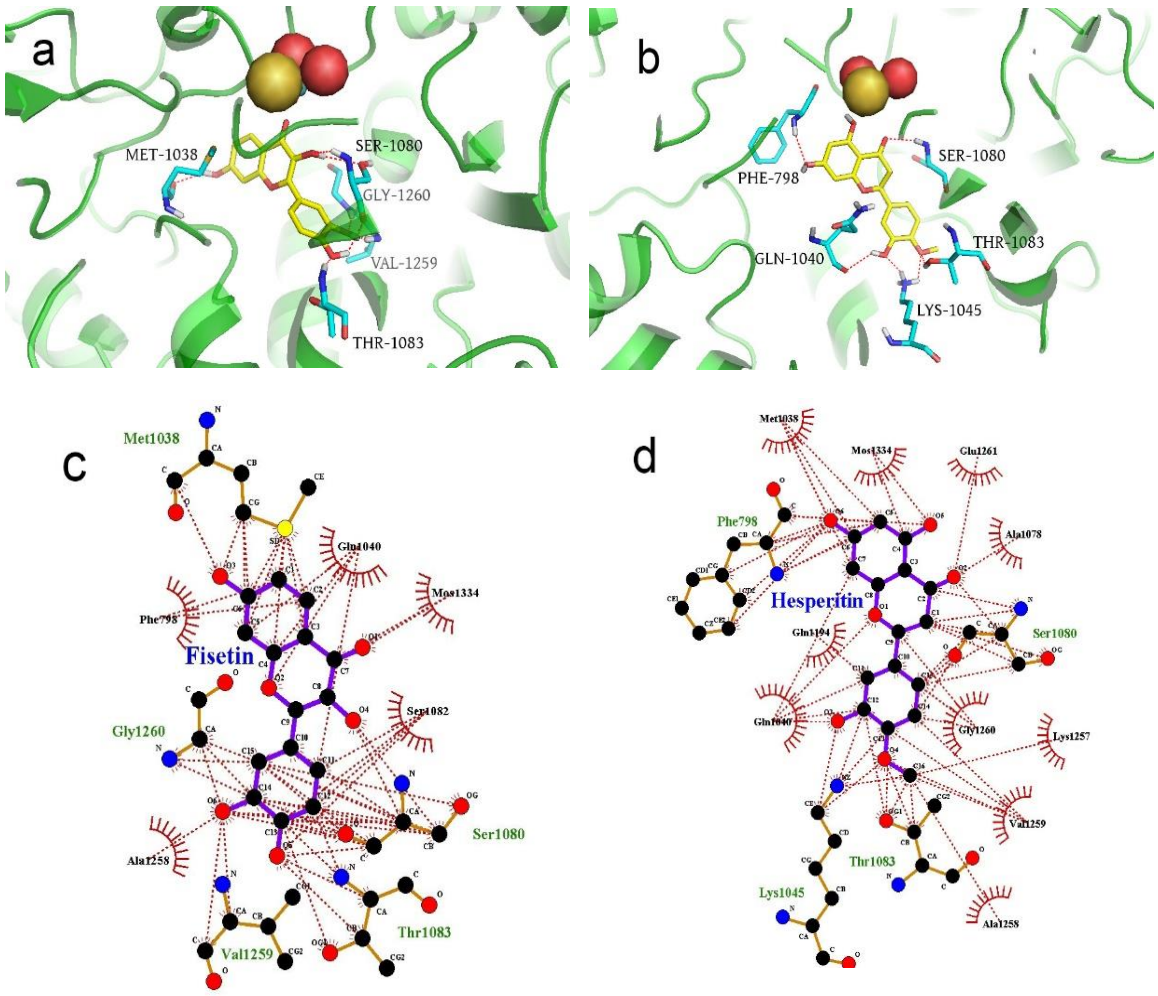

Fig. 6: The 3D schematic plot of hydrogen bond and hydrophobic interactions between fisetin (a, c), hesperitin (b, e) and XO. In the diagram of hydrogen bond interaction (a, b), the yellow globule represents molybdenum ion. The yellow molecular structure represents flavonoid ligand molecule (fisetin and hesperitin). The cyan structure represent the amino acids that form hydrogen bonds with flavonoid in the active site of xanthine oxidase. The red dotted lines donote intermolecular hydrogen bonds.In the hydrophobicity diagram (c,d), the red dotted lines represent hydrophobicity.

Conclusions

With this research, using a combination of experiment and theory, the inhibitory effects of fisetin and hesperitin on xanthine oxidase were studied. Fisetin exhibited the higher XO inhibitory activity, which attributed to its stronger binding capacity with XO. The molecular docking analysis also provided the binding modes and energies of fisetin and hesperitin with XO, which suggested both fisetin and hesperitin could enter the active center of XO with different orientations. The lower binding energy was found by fisetin, which could explain its high inhibitory capacity.

Acknowledgements

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