

Inhibition Mechanism of 3-Hydroxyflavones Against α -Glucosidase

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ABSTRACT

3-Hydroxyflavones belong to a subgroup of flavonoids that exhibit a broad range of bioactivities. In this respect, diverse 3-hydroxyflavones have been isolated from natural sources and synthesized in laboratories through different chemical routes to search for analogs with promising bioactivities. As part of our ongoing research into the α -glucosidase inhibition of synthesized 3-hydroxyflavones, we herein screened for the α -glucosidase inhibitory activity of 3-hydroxyflavones. Among the tested compounds (**1-5**), for the first time, two fluorinated derivatives (compounds **4** and **5**), bearing fluoro substituents at the 4'- and 3',4'-positions of the B ring were active, presenting IC₅₀ values of 326.47±0.79 μ M and 373.74±1.83 μ M, respectively. The intrinsic fluorescence spectra of α -glucosidase were quenched gradually with increasing amounts of inhibitors **4** and **5**, indicating both inhibitors were bound to α -glucosidase. Furthermore, when 8-anilino-1-naphthalenesulfonic acid (ANS), an extrinsic fluorescence probe was added to the enzyme solution, the fluorescence intensity of [α -glucosidase-ANS] complex was reduced, suggesting that 3-hydroxyflavone **4** and **5** interacted with the enzyme via hydrophobic domain. Finally, inhibitory kinetic analysis using Lineweaver-Burk plots was also applied for both selected inhibitors. It was found that compounds **4** and **5** acted as competitive inhibitors of α -glucosidase.

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1. Introduction

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia and can be classified as type 1 (insulin-dependent DM) and type 2 (non-insulin-dependent DM) [1]. It has been reported that type 2 DM is the most prevalent, accounting for over 90% of all diabetes cases, which relates to a high glucose level in blood [2]. α -Glucosidase is a carbohydrate-hydrolyzing enzyme that catalyzes the conversion of oligo- and disaccharides into glucose. Therefore, an effective strategy for controlling type 2 DM is to retard glucose release in blood by using α -glucosidase inhibitors. Currently, several oral drugs such as voglibose, acarbose and miglitol have been used to treat type 2 DM via the inhibition of α -glucosidase [3]. However, the use has been associated with side effects, including abdominal distension, diarrhea, and liver damage [4]. Hence, it is necessary to develop new α -glucosidase inhibitors for safer and more effective treatment.

Flavonoids have attracted much attention due to their diverse and promising biological activities [5]. 3-Hydroxyflavones, known as (3-hydroxy-2-phenylchromen-4-one) (Figure 1), are a subgroup of flavonoids and have been widely found in vegetables, medicinal plants and herbs. Several studies have reported that 3-hydroxyflavones exhibited inhibitory effects against α -glucosidase [6], [7]. Structure-activity relationship analyses of a panel 3-hydroxyflavones indicated that the 3-OH group on the C ring, the 3',4'-dihydroxy groups on the B ring, and the C2=C3 double bond mainly contributed to the α -glucosidase inhibition [6].

In general, the previous results on the inhibition of 3-hydroxyflavones against α -glucosidase found in the literature have focused on the effects of methoxy (-OCH₃), hydroxy (-OH) groups in rings on the

inhibitory activity. Interestingly, several 3-hydroxyflavones containing fluorine (F) have demonstrated enhanced biological activities compared with their non-fluorinated counterparts [8], [9]. However, fluoro-substituted analogs have been rarely isolated from natural sources. Thus, much effort has been undertaken to synthesize fluorinated 3-hydroxyflavones for bioassay evaluation [9]. In this context, the present study aims to investigate the α -glucosidase inhibitory activity of 3-hydroxyflavones containing $-\text{OCH}_3/-\text{OH}/-\text{F}$ substituents on the B ring. Moreover, the interactions and inhibition types of active 3-hydroxyflavones were elucidated using spectrofluorimetric analysis.

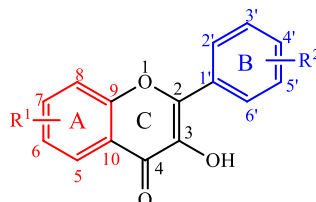


Figure 1. Backbone of 3-hydroxyflavones. R^1 and R^2 refer to substituents in A and B rings.

2. Materials and Methods

2.1. Materials

3-Hydroxyflavonols (**1-5**) were synthesized by our group. The chemical structures of compounds **1-5** (Figure 2) were elucidated using nuclear magnetic resonance (NMR) spectroscopy (500 or 600 MHz for ^1H NMR, 125 or 150 MHz for ^{13}C NMR). The multiplicities were denoted as follows: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), td (triplet of doublets). Mass spectrometry (MS) measurements were performed on an Agilent 1200 series LC-MSD. Synthetic procedures for 3-hydroxyflavones, along with the NMR, MS data of compounds **4** and **5**, were previously reported [10]. α -Glucosidase from *Saccharomyces cerevisiae* Type I, lyophilized powder, ≥ 10 units mg^{-1} protein, *p*-nitrophenyl- α -D-glucopyranoside (*p*NPG, $\geq 99\%$) and 8-anilino-1-naphthalenesulfonic acid (ANS, $\geq 97\%$) were purchased from Sigma-Aldrich (St., Louis, USA).

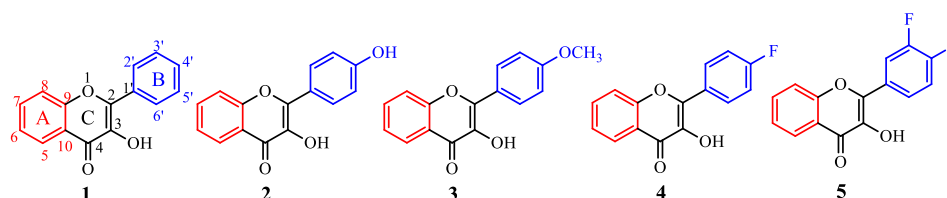


Figure 2. Chemical structures of 3-hydroxyflavones (**1-5**).

3-Hydroxy-2-phenyl-4H-chromen-4-one (1): Yield 16%, yellow solid, $\text{C}_{15}\text{H}_{10}\text{O}_3$ [238.1 g/mol]; $R_f = 0.51$ (Hex/EtOAc = 9/1); m.p. 170.6 $^\circ\text{C}$. ^1H -NMR (600 MHz, CDCl_3): $\delta = 7.02$ (s, 1 H, OH), 7.42 (td, $^3J(\text{H,H}) = 8.4$ Hz, $^4J(\text{H,H}) = 1.2$ Hz, 1 H, H4'), 7.48 (m, 1 H, H6), 7.54 (t, $^3J(\text{H,H}) = 7.2$ Hz, 2 H, H3', H5'), 7.60 (d, $^3J(\text{H,H}) = 8.4$ Hz, 1 H, H8), 7.71 (m, 1 H, H7), 8.26 (d, $^3J(\text{H,H}) = 8.4$ Hz, 3 H, H5, H2', H6') ppm. ^{13}C -NMR (150 MHz, CDCl_3): $\delta = 118.32$ (C8), 120.69 (C10), 124.55 (C4'), 125.51 (C5'), 127.79 (C2', C6'), 128.63 (C3', C5'), 130.22 (C6), 131.12 (C1'), 133.66 (C7), 138.48 (C3), 144.94 (C2), 155.50 (C9), 173.52 ($>\text{C}=\text{O}$) ppm. ESI-MS calcd for $[\text{M}+\text{H}]^+$: 239.1; found: m/z 238.9 $[\text{M}+\text{H}]^+$.

3-Hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (2): Yield 19%, yellow solid, $\text{C}_{15}\text{H}_{10}\text{O}_4$ [254.1 g/mol]; $R_f = 0.50$ (Hex/EtOAc = 7/3); m.p. 243.6 $^\circ\text{C}$. ^1H -NMR (500 MHz, $\text{DMSO}-d_6$): $\delta = 6.95$ (d, $^3J(\text{H,H}) = 9.0$ Hz, 2 H, H3', H5'), 7.46 (td, $^3J(\text{H,H}) = 8.0$ Hz, $^4J(\text{H,H}) = 1.0$ Hz, 1 H, H6), 7.73 (d, $^3J(\text{H,H}) = 8.0$ Hz, 1 H, H8), 7.78 (m, 1 H, H7), 8.10 (dd, $^3J(\text{H,H}) = 7.0$ Hz, $^4J(\text{H,H}) = 1.5$ Hz, 3 H, H5, H2', H6'), 9.31 (s, 1 H, 4'-OH), 10.10 (s, 1 H, 3-OH) ppm. ^{13}C -NMR (125 MHz, DMSO): $\delta = 115.43$ (C3', C5'), 118.24 (C8), 121.34 (C10), 121.97 (C1'), 124.42 (C6), 124.69 (C5), 129.56 (C2', C6'), 133.38 (C7), 137.78 (C3), 146.11 (C2), 154.38 (C9), 159.11 (C4'), 172.51 ($>\text{C}=\text{O}$) ppm. ESI-MS calcd for $[\text{M}+\text{H}]^+$: 255.1; found: m/z 254.9 $[\text{M}+\text{H}]^+$.

3-Hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (3): Yield 20%, yellow solid, $\text{C}_{16}\text{H}_{12}\text{O}_4$ [268.1 g/mol]; $R_f = 0.43$ (Hex/EtOAc = 8/2); m.p. 231.8 $^\circ\text{C}$. ^1H -NMR (600 MHz, CDCl_3): $\delta = 3.90$ (s, 3

H, -OCH₃), 6.94 (s, 1 H, 3-OH), 7.06 (d, ³J(H,H) = 9.0 Hz, 2 H, H3', H5'), 7.41 (t, ³J(H,H) = 7.8 Hz, 1 H, H6), 7.58 (d, ³J(H,H) = 8.4 Hz, 1 H, H8), 7.69 (td, ³J(H,H) = 7.2 Hz, ⁴J(H,H) = 1.2 Hz, 1 H, H7), 8.24 (d, ³J(H,H) = 9.0 Hz, 3 H, H2', H6', H5'). ¹³C-NMR (150 MHz, CDCl₃): δ = 55.41 (-OCH₃), 114.11 (C3', C5'), 118.17 (C8), 120.75 (C10), 123.58 (C1'), 124.42 (C6), 125.42 (C5), 129.53 (C2', C6'), 133.34 (C7), 137.64 (C3), 145.32 (C2), 155.32 (C9), 161.13 (C4'), 173.15 (>C=O) ppm. ESI-MS calcd for [M+H]⁺: 269.1; found: *m/z* 268.9 [M+H]⁺.

2.2. *In vitro* α-glucosidase assay and kinetics inhibition

The α-glucosidase inhibition assay of 3-hydroxyflavones (**1-5**) was evaluated following previously described protocols [11]. Acarbose was used as the reference standard. To determine the IC₅₀ values (the half maximal inhibitory concentration), the absorbance (A) of reaction mixtures containing inhibitors at various concentrations (**acarbose**: 60-150 μM, **1**: 100-200 μM, **2**: 100-350 μM, **3**: 48-228 μM, **4**: 165-385 μM, **5**: 220-385 μM) was measured at 405 nm using an Elisa microplate reader (JP Selecta, 2100C, Spain). Each inhibitor concentration was tested in three separate wells per 96-well plate and in duplicate (*n* = 2×3). The inhibition percentage, I (%) was calculated by the following equation: I (%) = [(A_{control} - A_{3-hydroxyflavones treated})/A_{control}] × 100%. The IC₅₀ (μM) values were determined by regression analysis using OriginPro 9.0 software.

The inhibition types of α-glucosidase by compounds **4** and **5** were determined by Lineweaver-Burk plots derived from equation (1). The optical density (OD) was measured in 96-well microplates containing a fixed enzyme concentration (0.3 U/mL) and increasing concentrations of *p*NPG (0.05; 0.1; 0.15; 0.2) in the absence and presence of **4** and **5**. The maximum reaction velocity, *v*_{max} and Michaelis-Menten constants, *K*_m were determined from Lineweaver-Burk plots.

$$\frac{1}{v} = \frac{K_m}{v_{max}} \frac{1}{[S]} + \frac{1}{v_{max}} \quad (1)$$

Here, *v* is the enzyme reaction rate. *v*_{max} and [S] denote the maximum reaction velocity and the substrate concentration, respectively. *K*_m is the Michaelis-Menten constant.

2.3. Fluorescence spectra measurements

The fluorescence spectra of α-glucosidase and the [α-glucosidase/ANS] complex were recorded on a Horiba spectrofluorometer (FluoroMax-4, Horiba, Japan). The experimental conditions were described in detail in our previous work [11], [12]. Solutions of α-glucosidase (2.5 U/mL) and compounds **4** (0-300 μM) or **5** (0-100 μM) were pre-incubated at 37 °C for 30 min. The α-glucosidase fluorescence was scanned in a wavelength range of 300-450 nm after excitation at 295 nm. The solutions consisting of α-glucosidase (2.5 U/mL) and ANS (12.5 μM) with or without inhibitor **4** (0-200 μM) or inhibitor **5** (0-100 μM), were excited at 375 nm to monitor their emissions in the range of 440-600 nm.

The Stern-Volmer constant (*K*_{SV}) and bimolecular quenching constant (*k*_q) were extracted from Stern-Volmer plot according to equation (2) [13].

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (2)$$

Where, *F*₀ and *F* are the fluorescence intensities of α-glucosidase in the absence and the presence of compounds **4** and **5**, respectively. [Q] are the concentrations of quenchers, **4** and **5**. The lifetime, *τ*₀ (*τ*₀ = 10⁻⁸ s) of α-glucosidase without inhibitors.

3. Results and Discussion

3.1. Inhibitory activity of 3-hydroxyflavones against α-glucosidase

The inhibitory activity of five 3-hydroxyflavones against α-glucosidase was evaluated, with acarbose, an antidiabetic drug, acting as a positive control to validate the assay. In our inhibition assay, IC₅₀ values (μM) for acarbose and compounds **1-5** were determined by plotting percent inhibition (I, %) against inhibitor concentration (**acarbose**: 60-150 μM; compounds **1-5**: 160-400 μM). As shown in

Table 1, the effects of 3-hydroxyflavones on α -glucosidase were strongly dependent on the substituents on the B ring. For compounds **1-3**, it was not possible to determine the IC_{50} values up to the inhibitor concentration of 400 μ M, indicating that 4'-OH and $-OCH_3$ groups are not relevant for inhibitory activity. The importance of the $-OH$ group position was highlighted in the literature. Based on *in vitro* and *in silico* structure-activity relationship studies on 3-hydroxyflavones, Proença et al. demonstrated that the presence of a catechol moiety, i.e., 3',4'-(OH)₂-positions on the B ring enhanced the α -glucosidase inhibitory activity with an IC_{50} of 54 μ M much lower than those of other tested compounds [6]. Moreover, the results also showed that substitution of 3'-OH or 4'-OH or 3',4'-(OCH₃)₂ groups at the B ring was insufficient to achieve the IC_{50} values.

In contrast, compound **4** ($IC_{50} = 326.47 \pm 0.79 \mu$ M) bearing a 4'-F substituent, demonstrated that the replacement of the $-OH/-OCH_3$ groups of 3-hydroxyflavones in the B ring with a fluorine atom enhanced the α -glucosidase inhibition. However, the introduction of a second $-F$ group into the 3'-position (**5**, $IC_{50} = 373.74 \pm 1.83$) in a B ring reduced activity compared with compound **4**. Due to the special properties of fluorine, the fluorination typically alters acidity, hydrogen bonding and lipophilicity that impact the physicochemical properties of a molecule such as absorption and distribution [14]. In other words, the effects of fluorine substituent in organic compounds on the bioactivities are of interesting and unpredictable. Herein, in continuation of our work on fluorinated flavonoids, we report for the first time that the fluorination of the B ring in 3-hydroxyflavones was found to be active against α -glucosidase.

Table 1. The IC_{50} values, inhibition mechanism, kinetic and quenching parameters of 3-hydroxyflavones against α -glucosidase.

Compound	IC_{50} (μ M)	Inhibition type	V_{max} ($mM \text{ min}^{-1}$)	K_{SV} ($\times 10^4 \text{ M}^{-1}$)	k_q ($\times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$)
1	ND				
2	ND				
3	ND				
4	326.47±0.79	Competitive	2.33	0.05	0.5
5	373.74 ±1.83	Competitive	2.82	0.3	3.0
Acarbose	127.74±3.96	Competitive			

ND: Not Determined

3.2. Contacts of 3-hydroxyflavones and α -glucosidase

The *in vitro* assay of 3-hydroxyflavones **4** and **5** was found to be active against α -glucosidase. To further validate these results, the fluorescence intensity of α -glucosidase was monitored in the absence and presence of the inhibitors **4** and **5**. Fluorescence quenching has been widely used to study the enzyme-inhibitor interactions. The intrinsic fluorescence of the enzyme arises mainly from the aromatic residues, including tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) [13]. The π - π contacts of the indole ring of Trp and the aromatic rings of inhibitors can result in the fluorescence quenching of the enzyme. In other words, such quenching can be detected by excitation of Trp at 295 nm. As shown in Figure 3, when excited at 295 nm, the emissions of **4** and **5** were negligible, while the fluorescence spectrum of α -glucosidase was observed in a range from 300 to 450 nm with a maximum peak near 340 nm corresponding to the emission of the Trp residue of α -glucosidase [7], [13], [15], [16]. Upon treatment with inhibitors **4** and **5**, the fluorescence intensity of α -glucosidase was gradually quenched with increasing the concentrations of **4** (100-300 μ M) and **5** (50-100 μ M), suggesting direct evidence for the contacts between compounds **4**, **5** and α -glucosidase.

The Stern-Volmer constants, K_{SV} in eq. (1) were achieved by plotting F_0/F vs [inhibitor]. As shown in Table 1, the K_{SV} values are $0.05 \times 10^{-4} \text{ M}^{-1}$ and $0.3 \times 10^{-4} \text{ M}^{-1}$ for **4** and **5**, respectively. In addition, the bimolecular quenching constants (k_q), which reflect the efficiency of quenching, were interpreted from K_{SV} ($K_{SV} = k_q \times \tau_0$). The calculation of $k_q = K_{SV}/\tau_0$ ($\tau_0 = 10^{-8} \text{ s}$) for **4** and **5** yielded the values of 0.5×10^{11}

$M^{-1}s^{-1}$ and $3.0 \times 10^{11} M^{-1}s^{-1}$, respectively, suggesting that the encounters of both quenchers **4**, **5** and α -glucosidase are effective in quenching.

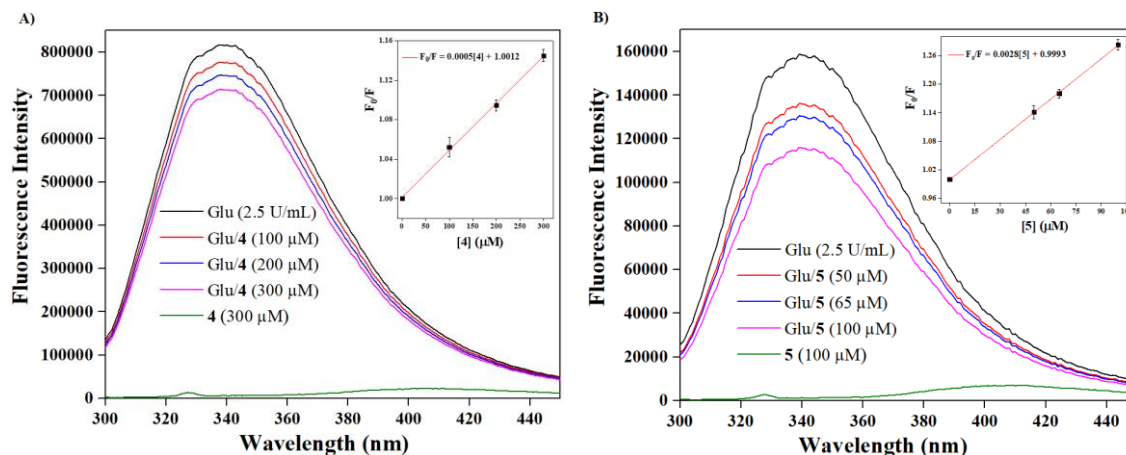


Figure 3. Fluorescence spectra of α -glucosidase (Glu, 2.5 U/mL) in the presence of the different concentrations of compounds **4** (panel A) and **5** (panel B). The insets refer to the Stern-Volmer plots (F_0/F vs inhibitor concentration). The regressive equations were also added to the plots.

3.3. Hydrophobic binding between 3-hydroxyflavones and α -glucosidase

Hydrophobic binding refers to a non-covalent interaction between a target enzyme and reversible inhibitors, which can be detected by fluorescence experiments. To evaluate the surface hydrophobicity of the enzyme, 8-anilino-1-naphthalenesulfonic acid (ANS) as an extrinsic fluorescence probe was added to the enzyme solution. The mixing hydrophobe and enzyme leads to the formation of an [enzyme/ANS] complex via hydrophobic contacts. When excited at 375 nm, this complex can emit fluorescence within the range from 400 to 600 nm [17]-[19].

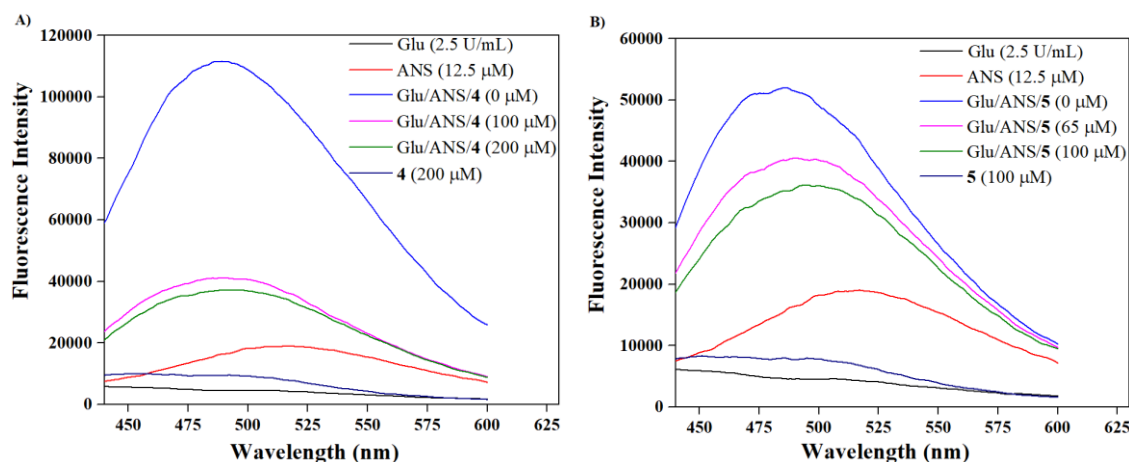


Figure 4. The emission spectra of [α -glucosidase/ANS] complexes in the absence and presence of compounds **4** (panel A) and **5** (panel B). The solutions were excited at 375 nm and their emission spectra were scanned from 440 to 600 nm.

The emission of the [α -glucosidase/ANS] complexes in the presence of varying concentrations of compounds **4** and **5** are shown in Figure 4. Upon excitation at 375 nm, there are barely any fluorescence signals of α -glucosidase and compounds **4**, **5**. However, a pronounced fluorescence signal in the range of 440–600 nm was observed after the addition of ANS to the α -glucosidase solution, confirming the effective formation of the [enzyme/ANS] complex. The maximum fluorescence intensity of the complexes was observed around 485 nm, which was distinct from that of free ANS emission. When treated with **4** and **5**, the fluorescence intensities were reduced remarkably with increasing inhibitor concentrations. These observations suggest that the inhibitors compete with ANS, resulting in a decrease

in [α -glucosidase/ANS] complex concentration. In other words, inhibitors bind to α -glucosidase through a hydrophobic domain.

3.4. Kinetic type of α -glucosidase inhibition by 3-hydroxyflavones

The enzyme inhibition was defined as a reduction of enzyme activity through the binding of an inhibitor to an active or/and second site on the enzyme [20]. In reversible inhibition, inhibitors can inhibit enzymes via three basic mechanisms of inhibition, including competitive, non-competitive and uncompetitive types. The type of inhibition can be determined from the Lineweaver–Burk plot, which is a graphical representation of reciprocals of both sides of the Michaelis–Menten equation (eq. 1) of enzyme kinetics [21]. As shown in Figure 5, an increase in slope, a decrease in the x-intercept ($1/[pNPG]$), and no change in the y-intercept ($1/v$) indicate that 3-hydroxyflavones **4** and **5** act as competitive inhibitors. A competitive inhibitor binds at the same position with a substrate ($pNPG$), i.e., a catalytic site. Consequently, the presence of an inhibitor results in a decrease in the enzyme's ability to bind with $pNPG$. As the inhibitor concentration increases, the rate of enzyme reaction decreases, i.e., $1/v$ increases. However, at infinitely high substrate concentrations ($1/[pNPG] \rightarrow 0$) the enzyme reaches the same v_{max} (v_{max} is unchanged) as the absence of the inhibitor. In other words, a series of straight lines crossing the y ($1/v$) axis at the same point.

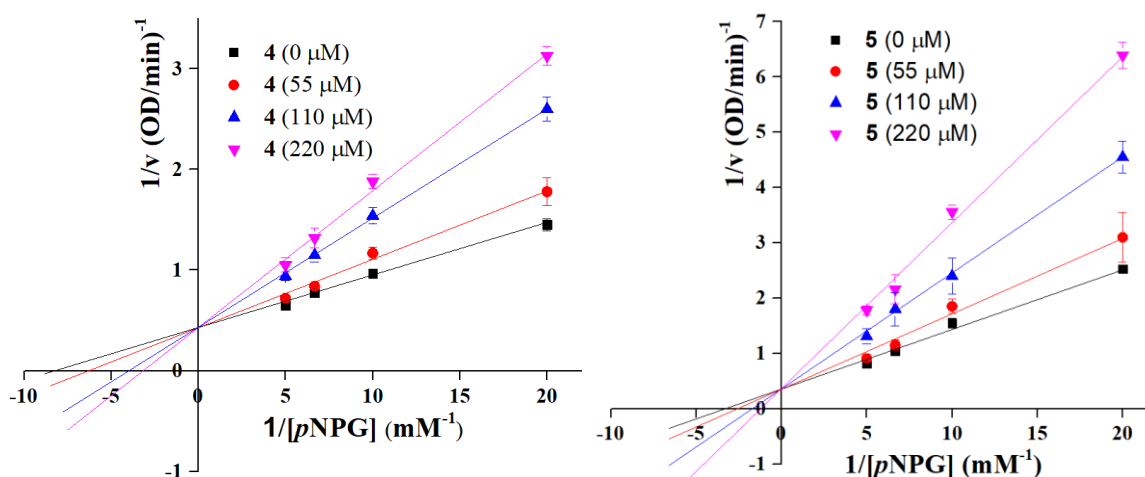


Figure 5. Lineweaver-Burk plots of α -glucosidase inhibition at different concentrations of $pNPG$ in the presence of various concentrations of 3-hydroxyflavones **4** (left) and **5** (right).

The parameter of K_m in the Michaelis–Menten equation (eq. 1) refers to the Michaelis-Menten constant, defined as the substrate concentration at half-maximum velocity ($v = v_{max}/2$). The K_m is related to the affinity of the enzyme for its substrate, with higher K_m values indicating lower affinity, i.e., more substrate is required to reach half of v_{max} , whereas low K_m describes that the enzyme has a high affinity toward its substrate [21]. The intercept on the x-axis of a Lineweaver–Burk plot corresponds to $-1/K_m$ (at $1/v = 0$), allowing for the interpretation of the K_m value. As depicted in Figure 5, the increase in concentrations of compounds **4** and **5** did not affect the v_{max} values (**4**: $v_{max} = 2.33 \text{ mM min}^{-1}$, **5**: $v_{max} = 2.82 \text{ mM min}^{-1}$), whereas it increased K_m values (**4**: from 0.12 to 0.31 mM, **5**: from 0.30 to 0.83 mM), supporting the finding that inhibitors **4** and **5** inhibit α -glucosidase activity competitively. In other words, when increasing the inhibitor concentrations, the affinity of the α -glucosidase for $pNPG$ is decreased due to the competition at the active site of the enzyme.

4. Conclusions

A panel of five 3-hydroxyflavones (**1-5**) was screened for the α -glucosidase inhibitory activity. The 3-hydroxyflavone structures **4** and **5** containing fluoro groups at 4'-, 3',4'-positions in the B ring are determinant factors for the α -glucosidase inhibition. Fluorescence analysis revealed that both compounds interact with α -glucosidase through hydrophobic binding. To elucidate the inhibition mechanisms, the Lineweaver–Burk plots indicated that 3-hydroxyflavones **4** and **5** inhibited α -glucosidase via competitive inhibition types, since the K_m values increased and the v_{max} values remained

constant with increasing concentrations of inhibitors **4** and **5**. Overall, Overall, the results indicate that fluorinated 3-hydroxyflavones represent promising candidates for the development of α -glucosidase inhibitors.

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Conflict of Interest

The authors declare no conflict of interest.

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