Coumaroyl and feruloyl flavonoid glycosides from the male flowers of *Ginkgo biloba* L. and their inhibitory activity against α-glucosidase

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ABSTRACT

Four flavonoid glycosides containing coumaroyl or feruloyl groups were isolated from the male flowers of *Ginkgo biloba* L., and compounds 3 and 4 were identified as novel compounds. The inhibitory activities against α-glucosidase were investigated by docking studies, *in vitro* assays and kinetic studies. The docking results showed that all compounds mainly formed hydrogen-bond and π-π-stacking interactions with α-glucosidase. Compound 4 had the lowest binding energy and maximum number of hydrogen bonds. Subsequently, the *in vitro* assays showed that compound 4 exhibited the strongest inhibitory potency. Finally, the kinetic studies indicated the inhibitory mode of compounds 1–4 against α-glucosidase were mixed types of competitive and non-competitive. Together, these findings suggested that the isolated flavonoid glycosides in this study, especially compound 4, have potential as α-glucosidase inhibitors.

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1. Introduction

The leaves of *Ginkgo biloba* L. are important resours of natural compounds and biological activities. New compounds or functions from *Ginkgo* leaves are still being discovered (Eleftheriou and Zacharia 2021; Wang et al. 2021). The sarcotestas of *G. biloba* L. possess about 75% of the total fruit weight, and have been reported to provide significant inhibition of proliferation (Zhou et al. 2020). The pollen is also important organ of *G. biloba* L., and usually used as nutritional supplements based on the high contents of nutritional components, such as vitamins, amino acids and unsaturated fatty acids (Li et al. 2019a). Meanwhile, *G. biloba* pollen contains abundant flavonoid substances, the contents of which are about 1.42 times higher than the value in *G. biloba* leaves (Li et al. 2010). The male flowers with catkin blossom of *G. biloba* are carriers of pollen grains, so they should share similar bioactive substances. Its reported that *Ginkgo* flowers have a good performance in anti-inflammatory and anti-cancer activities due to flavonoid glycosides, such as bilobetin and isoginkgetin (Li et al. 2019a, 2019b). Although such above information has achieved, most resource of *Ginkgo* flowers are used only for the purpose of pollination at present. However, the falling price and high labor costs widely cause the rot of *Ginkgo* nuts on the trees, which eventually limit the pollination purpose of *Ginkgo* flowers in the end. Most of *Ginkgo* flowers are wasted every year in Rugao City, Nantong City, Jiangsu Province (China), which are the main producing areas. In this case, other purpose of *Ginkgo* flowers should be studied and developed in order to achieve sustainable development for *Ginkgo* flowers. To realise this objective, we researched the chemical compositions of *Ginkgo* flowers. As the results, four flavonoid glycosides containing coumaroyl or feruloyl groups were isolated. Many coumaroyl flavonoid glycosides have been proven to possess antidiabetic effects (Zhang et al. 2014; Ikechukwu and Ifeanyi 2016), and coumaroyl group and its approximate, caffeoyl group, have been considered important in the inhibiting of α-glucosidase activity (Yoshida et al. 2008; Sun et al. 2016). Besides, the other organs or parts of *G. biloba* L., such as seed coats and leaves, have showed strong α-glucosidase inhibition values (Chen et al. 2020). Hence in current study, the inhibitory activity of these four compounds against α-glucosidase was evaluated in vitro and their binding interactions with α-glucosidase were explored using docking studies.

2. Results and discussion

Three coumaroyl (compounds 1, 2, 3) and one feruloyl (compound 4) flavonoid were isolated from the male flowers of *Ginkgo biloba* L. (Figure 1). Compounds 3 and 4 were identified as new compounds in further assays. Compounds 1 and 2 were identified as quercetin 3-O-(6-O-trans-p-coumaroyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (Kang et al. 1990) and isorhamnetin 3-O-(6-O-trans-p-coumaroyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (Tang et al. 2001), respectively, based on published reports. MS analysis of compound 3 consistently showed a molecular formula of C_{36}H_{36}O_{17}, and nineteen degrees of unsaturation were calculated from the molecular formula. ¹H NMR spectra (Supplemental data, Table S2) demonstrated a typical B-ring of flavonoids
at δ_H 6.12 and 6.28. Moreover, methyl at δ_H 0.95 and methylene at δ_H 4.20, 4.22 indicated the possible existence of rhamnose and glucose, respectively. Meanwhile, two sets of AABB-type signals were assigned at δ_H 6.83, 7.60 and 6.62, 7.42, wherein one of them was the signal from the aromatic protons from the C-ring in flavonoids. ^1H NMR spectra also exhibited a pair of cis-olefinic protons at δ_H 5.26 and 6.31 (J = 12.9 Hz). ^13C NMR spectra (Supplemental data, Table S2) showed two carbonyls at δ_C 167.8 and 179.6. The carbons at δ_C 17.6 and 64.3 maybe the substitute positions of rhamnose and glucose, respectively. Finally, a kaempferol aglycone, rhamnose, glucose, and cis-coumaroyl were confirmed by comparing the NMR spectroscopic data acquired here to acylated flavonol glycosides from Gouania longipetala (Gossan et al. 2015). HMQC correlation (Supplemental data, Figure S2) shows that δ_H 5.55, 4.34, and 4.36 were assigned to δ_C 103.3 (C-1'), 83.8 (C-2'), and 107.0 (C-1'), respectively, and δ_H 4.20 and 4.22 were assigned to δ_C 64.3 (C-6'). Furthermore, two resonant protons at δ_H 4.34 and 4.36 were correlated with carbons of signals at δ_C 83.8 (C-2') and 107.0 (C-1'), respectively, indicating that the anomeric proton of glucose was coupled to the C-2' of rhamnose from HMBC correlations (Supplemental data, Figure S2). Additionally, an anomeric proton signal at δ_H 5.55 and a –CH3 signal at δ_H 4.20 and 4.22 were correlated with signals at δ_C 137.4 (C-3) and 167.8 (C-9'), respectively. Compound 3 was finally identified as kaempferol 3-O-(6-O-cis-p-coumaroyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside.

MS analysis of compound 4 consistently showed a molecular formula of C_{37}H_{38}O_{19}, which displayed nineteen degrees of unsaturation. NMR spectroscopic analysis of compound 4 (Supplemental data, Table S2) exhibited a molecular structure similar to that
of compound 3. However, when compared with compound 3, two groups of ABX-type signals were observed at δH 6.82, 7.12, 7.22, and 6.57, 6.69, 6.73. A pair of trans-olefinic protons at δH 5.94 and 7.26 (J = 15.9 Hz) differed from the trans-olefinic protons of compound 1 and 2. A methoxyl group was detected at δH 3.64 and δC 56.11. δC 83.8 (C-2) also indicated the same relationship of substituents between rhamnose and glucose with compound 3. HMBC correlation indicated that the aromatic proton H-30/C0/C18/C18/C18 in the distal aromatic ring was replaced with a methoxyl group, and a trans-feruloyl was generated (Supplemental data, Figure S2). Compound 4 was finally identified as quercetin 3-O-(6-O-trans-p-feruloyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (Tang et al. 2011).

α-Glucosidase is a hydrolyzing enzyme that catalyses the hydrolysis of starch and certain disaccharides to generate postprandial hyperglycemia (Yousefi et al. 2015). Inhibition of the enzymatic activity of α-glucosidase can reduce the release of free glucose (Hara et al. 1996). Hence, α-glucosidase has been considered a crucial target to discover and develop alternative hypoglycemic drugs (Zhang et al. 2021). In the current study, intermolecular interactions between isolated compounds and α-glucosidase were investigated using molecular docking. The hydrogen atoms from acarbose mainly formed hydrogen bonds with the oxygen atoms within GLN 1561, ASP 1279, HIS 1584, ASP 1526, ARG 1510, A 1157, and MET 1421 amino acid residues (Supplemental data, Figure S17).

The docking results of compounds 1–4 with α-glucosidase obtained the best conformation with binding energies of −10.1 kcal mol⁻¹, −9.9 kcal mol⁻¹, −9.8 kcal mol⁻¹ and −10.2 kcal mol⁻¹, respectively. All of these values were much lower than the binding free energy of acarbose, −8.3 kcal mol⁻¹. Thus, the low binding energies indicated that these four compounds are potential α-glucosidase inhibitors. The visualised in 2D and 3D diagrams of docking studies for compounds 1–4 were showed in Figure S18. Compound 1 constructed hydrogen bond interactions with ASP 1420, LYS 1460, ASP 1157 and THR 1528 residues of α-glucosidase. In addition, the benzene ring in coumaroyl presented π–π stacking interactions with TYR 1251, PHE 1559 and PHE 1560 amino acid residues. Compound 2 formed hydrogen bonds with LYS 1460, THR 1586, ASP 1157 and GLN1561 amino acid residues of α-glucosidase. Besides, TYR 1251, PHE 1559 and PHE 1560 residues within α-glucosidase showed π–π stacking interactions with the benzyl rings. Compound 3 constructed hydrogen bond interactions with ASP 1279 and LYS 1164 residues. Moreover, π–π stacking interactions were identified between the benzyl ring and TRY 1251, PHE 1560 residues. Compound 4 generated hydrogen bonds with ASP 1279, GLN 1158, LYS 1164, LYS 1460, PRO 1160 and THR 1528 amino acid residues. Additionally, π–π stacking interactions were found between benzyl rings and the TYR 1251, PHE 1560 residue.

An in vitro α-glucosidase inhibition assay was performed to verify the docking results. The results showed that isolated compounds 1–4 displayed IC₅₀ values of 3.58 ± 0.79, 2.74 ± 0.11, 3.24 ± 0.77 and 1.82 ± 0.09 mM, respectively. Compound 4 had the highest inhibitory activity, which was similar to that of acarbose (1.21 ± 0.03, p > 0.5) and generally consistent with the docking results.

The kinetic studies showed that Lineweaver–Burk plots of acarbose intersected at the y-axis (Figure S19A), thereby indicating that acarbose competitively inhibited α-glucosidase. This result was consistent with previous reports (Lee et al. 2017). However, Lineweaver–Burk plots of compounds 1–4 did not intersect the x- or y-axis, thereby indicating the mixed inhibition of compounds 1–4 (Figure S19).
3. Experimental

3.1. General experimental procedures

All NMR measurements were performed on a Bruker DPX-400 spectrometer using standard Bruker pulse programs (Bruker BioSpin GmbH, Rheinstetten, Germany) with a solvent peak as reference. Chemical shifts were presented in ppm, and the coupling constant was expressed in Hz. A Q Exactive Orbitrap MS (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate MS data. Semipreparative HPLC was performed on an HPLC system connected to a LabAllance pump and detector (LabAllance, Tianjin, China) using a reversed-phase C18 column (50 × 250 mm, 10 μm, Kromasil, Stockholm, Sweden).

HPLC grade acetonitrile and p-nitrophenyl-α-glucopyranoside (pNPG) were obtained from Sigma–Aldrich, MO, USA. Acarbose was obtained from Bayer & Co., Inc., Beijing, China. All other chemicals and reagents were analytical reagent grade.

3.2. Plant material

The male flowers of G. biloba L. were collected from Pizhou (Jiangsu, China; 34°07′–34°40′N, 117°35′–118°10′E, A: 20–32 m) in April 2014 and identified by the corresponding author. The specimens (Gbf-201401) were stored at the Traditional Chinese Medicine Specimen Museum, School of Pharmaceutical Science, Wuhan University, China.

3.3. Extraction and isolation

For the details of the isolation of compounds 1–4 see the Supplementary material.

**Compound 1**: Amorphous yellow powder. [α]20 D =−59.1 (c 0.23, MeOH). The NMR data, see supplementary material, Table S1. HRESIMS m/z 757.1969 [M + H]+ (calcd C36H37O18, 757.1980), 779.1775 [M + Na]+ (calcd C36H36O18Na, 779.1799).

**Compound 2**: Amorphous yellow powder. [α]20 D =−61.5 (c 0.20, MeOH). The NMR data, see supplementary material, Table S1. HRESIMS m/z 771.2123 [M + H]+ (calcd C37H39O18, 771.2136), 793.1933 [M + Na]+ (calcd C37H38O18Na, 793.1956).

**Compound 3**: Amorphous yellow powder. [α]20 D =−89.7 (c 0.23, MeOH). The NMR data, see supplementary material, Table S2. HRESIMS m/z 741.2017 [M + H]+ (calcd C36H37O17, 741.2031), 763.1824 [M + Na]+ (calcd C36H36O17Na, 763.1850).

**Compound 4**: Amorphous yellow powder. [α]20 D =−60.7 (c 0.22, MeOH). The NMR data, see supplementary material, Table S2. HRESIMS m/z 787.2053 [M + H]+ (calcd C37H39O19, 787.2086), 809.1867 [M + Na]+ (calcd C37H38O19Na, 809.1905).

3.4. Molecular docking

Intermolecular interactions were observed between isolated compounds and α-glucosidase. The PDB format of human intestinal α-glucosidase (ID: 3TOP) from the Protein Data Bank (https://www.rcsb.org/structure/3top) was used as the receptor protein. The A chain was used as the molecular target and saved in PDBQT format. AutoGridFR 1.0 was used to generate the active package. 3D structures of the isolated compounds
and acarbose were prepared using ChemDraw Ultra 7.0 and Chem3D Ultra 15.1 software. The docking studies were performed using Autodock Vina 1.1.2. The grid box was set as $22.5 \times 22.5 \times 22.5 \, \text{Å}$ with a grid spacing of 0.375 Å. The center of the grid corresponded to the active site pocket center. The docking results were analysed to obtain the best core, and the chosen outputs were viewed using BIOVIA Discovery Studio Client (v17.1.0.16143).

### 3.5. Isolation of $\alpha$-glucosidase

$\alpha$-Glucosidase was isolated from Sprague Dawley (SD) rats using previously described methods (Lee 2005). Enzymatic activity was determined by the release of $p$-nitrophenol per unit time (Lee and Kim 2001).

### 3.6. $\alpha$-Glucosidase inhibition assay

The inhibiting effect of $\alpha$-glucosidase was investigated using previously described methods (Dong et al. 2012). Acarbose was used as the standard. The inhibitory activity was calculated as inhibition (%):

$$\text{Inhibition (\%)} = \left[ 1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \right] \times 100$$

The results were presented as IC$_{50}$, and tests were performed in triplicate.

### 3.7. Kinetic analyses

The inhibition mode was determined using the Lineweaver–Burk plot (Lineweaver and Burk 1934).

### 3.8. Statistical analysis

The inhibitory activities and kinetic analyses of compounds 1–4 against $\alpha$-glucosidase were analyzed using IBM SPSS Statistics 20. Statistically significant data were further analyzed using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) post-hoc testing. Significant differences were considered based on $p < 0.05$ (Figure S19).

### 4. Conclusions

In this study, four flavonoid glycosides with similar structures containing coumaroyl or feruloyl groups were isolated from the male flowers of *G. biloba* L., and compounds 3 and 4 were identified as new compounds. Docking results showed that all compounds had low binding energy, mainly formed hydrogen bond interactions with ASP 1279 and LYS 1460, and established $\pi-\pi$ stacking interactions with TYR 1251. Compound 4 had the lowest binding energy and maximum number of hydrogen bonds. The *in vitro* $\alpha$-glucosidase inhibition assay revealed that compound 4 exhibited the strongest inhibitory potency, which was consistent with docking study predictions. The kinetic studies indicated the inhibitory modes of compounds 1–4 against $\alpha$-glucosidase were
mixed. To conclude, these findings suggested that the isolated flavonoid glycosides (especially compound 4) containing coumaroyl and feruloyl groups in this study have potential as α-glucosidase inhibitors.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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