ORIGINAL ARTICLE

A NEW ESCULETIN GLYCOSIDE FROM *CALENDULA OFFICINALIS* (*ASTERACEAE*) AND ITS BIOACTIVITY

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Abstract

A new coumarin glycoside, neoisobaisseoside, was isolated from the flowers of *Calendula officinalis* L. (*Asteraceae*), together with three known compounds identified as isobaisseoside, haploperoside A and haploperoside D. The structure of neoisobaisseoside was characterized as 6,7-dihydroxycoumarin-7-*O*-(2′-*O*-α-l-rhamnopyranosyl)-β-d-glycopyranoside or esculetin-7-*O*-neohesperiodoside, based on UV-, NMR-spectroscopy and mass-spectrometric data. Investigation of the biological properties of neoisobaisseoside demonstrated its amylase and α-glucosidase inhibiting activity as well as its ability to reduce the production of advanced glycation end-products formed in the Maillard reaction. These facts indicate that neoisobaisseoside could be a potential anti-diabetic agent.

Rezumat

O nouă glicozidă cumarinică, neoizobaisseozida, a fost izolată din florile de *Calendula officinalis* L. (*Asteraceae*) împreună cu trei compuși cunoscuți, identificați ca isobaisseozidă, haploperozida A și haploperozida D. Structura neoizobaisseozidei a fost caracterizată ca 6,7-dihidroxicumarin-7-O-(2'-O-α-L-ramnopiranozil)-β-D-glicopiranozidă sau esculetin-7-O-neohesperiodozidă, pe baza spectroscopiei UV și a spectroscopiei de masă. Investigarea proprietăților biologice ale neoizobaisseozidei a demonstrat activitatea inhibarea activității amilazei și α-glucozidazei, precum și capacitatea de a reduce apariția produșilor finali de glicare avansată prin reacția Maillard. Rezultatele obținute indică faptul că neoizobaisseozida prezintă un potențial anti-diabetic.

Keywords: *Calendula officinalis*, neoisobaisseoside, coumarins, antidiabetic activity

Introduction

Calendula officinalis L. (marigold) is a known medicinal plant of the *Asteraceae* family, which has been used for more than a century as a pharmaceutical remedy in most countries due to its wide spectrum of biological activities [1]. Previously recorded scientific data have demonstrated the antidiabetic potential of drugs (preparations) and individual compounds from *C. officinalis*. It has been shown that the methanolic extract of marigold flowers and two oleanolic acid 3-monodesmosides (glycosides D and F) showed potent hypoglycaemic activity after a single oral administration [2]. Administration of the hydroalcoholic extract of *C. officinalis* in rats with alloxan and streptozotocin-induced diabetes resulted in significant reductions in blood glucose and serum lipids and in the level of sugar in the urine [3, 4]. A total extract of *C. officinalis* flower heads may inhibit the Maillard reaction by reducing the accumulation of advanced glycation end products (AGEs) [5]. Our previous study using leaves of *C. officinalis* demonstrated an inhibitory effect on amylase caused by phenolic components such as coumarins and coumarin glycosides [6-11]. As part of our ongoing research into the chemical composition and bioactivities of *C. officinalis*, this study presents the isolation of a new compound, neoisobaisseoside (**1**) and three known coumarin glycosides isobaisseoside (**2**), haploperoside A (**3**) and haploperoside D (**4**) and an investigation of their antidiabetic potential.

Materials and Methods

Materials

Plant material. Plants of *C. officinalis* (variety "Greenheart Orange") were harvested in open-field conditions from the Institute of General and Experimental Biology (IGEB) plantations (2012 - 2014) [6]. The flowers were dried in a convective drying oven UT-4610 (Ulab, Sankt-Petersburg, Russia) at 40°C (20 - 24 h) to a humidity level of 9 - 12%. The flowers were ground in an A11 basic analytical mill (IKA®-WerkeGmbH & Co. KG, Staufen, Germany) and then sieved using an ERL-M1 sieving machine (Zernotekhnika, Moscow, Russia) to achieve an average particle diameter of 0.5 mm.

General equipment. Elemental (C/H/O) composition was determined using a MAT 8200 spectrometer (Thermo Finnigan, Waltham, MA, USA). UV spectra were recorded using a SF-2000 spectrophotometer (OKB Specter, St. Petersburg, Russia). MS spectra were recorded on an LCQ mass spectrometer (Thermo Finnigan). NMR spectra were recorded on a VXR 500S spectrometer (Varian, Palo Alto, CA, USA). Column chromatography was performed over Sephadex LH-20 (25 - 100 μm, Pharmacia, Uppsala, Sweden) and polyamide Woelm (Waters Associates, Inc., Framingham, MA, USA). Microcolumn HPLC-UV analysis (MC-HPLC-UV) was performed on a Milichrom A-02 high-performance liquid chromatograph (Econova, Novosibirsk, Russia) and preparative HPLC (prep. HPLC) was performed on a Summit high-performance liquid chromatograph (Dionex, Sunnyvale, CA, USA). GC/MS data was obtained using a 5973N gas-chromatograph with a 5973N MSD mass-selective detector (Agilent Technologies, Santa-Clara, CA, USA).

Isolation of compounds **1 - 4**. Isolation was preformed using a *n*-butanol fraction, which was isolated and pre-chromatographed as described previously [5, 8]. Milled flowers of *C. officinalis* (1.4 kg) were extracted successively using 96, 70, and 50% ethanol (×3, 1:20, 40°C). Combined extracts were concentrated to an aqueous residue and extracted completely with hexane, chloroform, ethyl acetate, and *n*-butanol (*n*-BuOH) to obtain the *n*-BuOH fraction with a yield of 4.51% (63.14 g). The fraction (60 g) was separated by column chromatography on polyamide (4 × 100 cm, H_2O - ethanol eluent, 100:0 → 0:100). A sub-fraction obtained by elution with H_2O - ethanol (60:40) was separated on Sephadex LH-20 (3 × 70 cm, ethanol - H_2O eluent, 96:4 → 0:100). Sub-fractions of similar composition were combined and then chromatographed on prep. HPLC: column LiChrosorb PR-18 (250 × 4.6 mm, ∅ 5 μm; Merck); eluents: H_2O (A), MeCN (B); elution program (% B): 5 - 80 min 5 - 35%; ν 1 mL/min; column temp. 35°C; UV-detector, λ 350 nm. Fractions with retention times 50 - 52 (A), 57 - 60 (B), 65 - 67 (C) and 69 - 72 minutes (D) were collected. Repeated separation and recrystallization from ethanol (70%) of fraction A resulted in compound **1** (22 mg). Analogous separations allowed the isolation of 14 mg of **2** from fraction B, 23 mg of **3** from fraction C, and 20 mg of **4** from fraction D.

Acidic hydrolysis. A mixture of **1** (2 mg) and 5 mL of CF_3COOH - Me_2CO (5:95) was shaken at 100°C for 2 h and then concentrated in vacuum to dryness. The residue was dissolved in 1 mL of 70% ethanol and passed through polyamide patron (5 g) pre-conditioned with methanol (50 mL) and water (60 mL). Elution with water (100 mL) and methanol (150 mL) gave carbohydrate and coumarin fractions, respectively. Both fractions were concentrated and analysed by HPLC: a) carbohydrates as derivatives with 3-methyl-1-phenyl-2-pirazoline-5-one [12]: MC-HPLC-UV; column ProntoSIL-120-5-C18 AQ (2 × 75 mm, ∅ 5 μm; Metrohm AG); eluent: 100 mM CH_3COONH_4 (pH 4.5) (A), MeCN (B); elution program (% B): 0 - 20 min 20 - 26%; ν 150 μL/min; column temp. 35°C; UV-detector λ 250 nm; t_R(glucose) 12.53 min, t_R(rhamnose) 8.51 min; b) coumarins without pre-treatment: MC-HPLC-UV; column ProntoSIL-120-5-C18 AQ (2 × 75 mm, ∅ 5 μm; Metrohm AG); eluent: 0.2 M $LiClO_4$ 0.006 M $HClO_4$ (A), MeCN (B); elution program (% B): 0 - 9 min 0 - 80%, 9 - 15 min 80 - 100%; ν 150 μL/min; column temp. 35°C; UV-detector λ 340 nm; t_R(esculetin) 10.24 min, t_R(scopoletin) 12.64 min.

Carbohydrate analysis. The configuration of monosaccharides (D/L) was determined after derivatisation with 1-(trimethylsilyl)imidazole using a previously published method [13] with some modifications, followed by GC/MS analysis [9]. Retention times of reference compounds: D-rhamnose 11.07 min; L-rhamnose, 11.16; D-glucose, 15.25; and L-glucose, 15.39 min were used for comparison. The retention times of compound hydrolysates co-injected with standard D-glucose and L-rhamnose did not differ. Methylation was realised in K_2CO_3–dimethylformamide–MeI medium using a previously published method [14], followed by GC/MS analysis of the methylation products [15].

Bioactivity methods

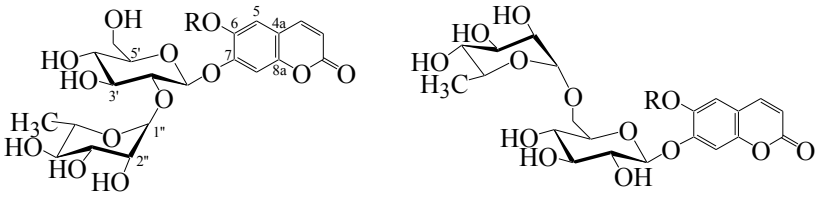
Amylase inhibitory activity was assayed according to a previously published spectrophotometric protocol using amylase from *Aspergillus niger* with starch as a substrate [10]. The α-glucosidase inhibition assay was performed using a spectrophotometric method using α-glucosidase from *Saccharomyces cerevisiae* and *p*-nitrophenyl-α-d-glucopyranoside as substrate [16]. Measurement of the inhibitory effect on advanced glycation end products (AGE) formation was carried out by the fluorimetric method of Matsuura *et al*. [17] with slight modifications [18]. Acarbose (Sigma Aldrich, Germany) was used as a positive control.

Results and Discussion

The *n*-butanol fraction of *C. officinalis* flowers was separated by column chromatography and preparative HPLC to yield four compounds **1** - **4** which were identified by comparison of their UV, ^1H and ^{13}C NMR spectra and MS data with those reported in the literature as the known coumarin glycosides isobaisseoside (scopoletin-7-*O*-rutinoside; **2**) [19], haploperoside A (esculetin-7-*O*-rutinoside; **3**) and haploperoside D (scopoletin-7-*O*-neohesperidoside; **4**) [20] (Figure 1). In the following, we present the elemental analysis for compound 1.

Neoisobaisseoside (**1**). $C_{21}H_{26}O_{13}$ {*m*/*z* 487.312 ($[M+H]^+$; calcd. 487.442)}. UV (MeOH, λ_{max}, nm):

231, 290, 347. ESI-MC, *m/z*: 487 $[M+H]^+$, 341 $[(M+H)–rhamnose]^+$, 309 $[(rhamnosyl\text{-}glycose+H)–H_2O]^+$, 179 $[esculetin+H]^+$, 147 $[(rhamnose+H)–H_2O]^+$. NMR ^1H (500 MHz, $CDCl_3$, δ_H, ppm, *J*/Hz): 1.21 (3H, d, 6.0, H-6″), 3.61 - 4.40 (10H, m, H-2′–H-6′, H-2″–H-5″), 4.52 (1H, d, 2.0, H-1″), 5.57 (1H, d, 8.0, H-1′), 6.10 (1H, d, 9.5, H-3), 6.75 (1H, c, H-8), 7.05 (1H, c, H-5), 7.83 (1H, d, 9.5, H-4). NMR ^{13}C (125 Hz, $CDCl_3$, δ_C, ppm, DEPT): 17.5 (CH_3, C-6″), 60.2 (CH_2, C-6′), 68.1 (CH, C-5″), 69.5 (CH, C-4′), 70.0 (CH, C-2″), 70.3 (CH, C-3″), 71.6 (CH, C-4″), 75.2 (CH, C-3′), 76.8 (CH, C-5′), 77.5 (CH, C-2′), 97.5 (CH, C-1′), 99.7 (CH, C-1″), 102.3 (CH, C-8), 111.0 (C, C-8a), 112.2 (CH, C-3), 112.6 (CH, C-5), 143.2 (C, C-6), 144.2 (CH, C-4), 148.4 (C, C-4a), 150.7 (C, C-7), 161.2 (C, C-2).



1: R = H
4: R = CH_3

2: R = CH_3
3: R = H

Figure 1.
Structures of compounds **1** - **4** isolated from *C. officinalis* flowers

Compound **1** was obtained as a white amorphous powder. The molecular structure of **1** was established as $C_{21}H_{26}O_{13}$ by positive fast atom bombardment (FAB) mass spectrometry. The UV spectrum exhibited absorption maxima at 231, 290 and 347 nm, typical for coumarins [21]. Esculetin, D-glucose and rhamnose were identified by acidic hydrolysis of **1** indicating that the compound was an esculetin glycoside. Positive ESI-MS of **1** displayed a protonated molecular ion at *m/z* 487 ($[M+H]^+$); intrinsic fragments of a de-rhamnosylated moiety at *m/z* 341 ($[(M+H)–rhamnosyl]^+$); a protonated esculetin ion at *m/z* 179; as well as a rhamnose ion at *m/z* 147; and a rhamnosyl-glucose ion at *m/z* 308 suggesting a rhamnosyl-glucose structure for the carbohydrate moiety of glycoside **1** [22]. Methylation of **1** followed by hydrolysis and GC/MS analysis of the methylated products gave 3,4,6-tri-*O*-methyl-glycopyranose and 2,3,4-tri-*O*-methyl-rhamnopyranose in a 1:1 ratio confirming the nature of the carbohydrate moiety as a 2-*O*-rhamnopyranosyl-glucopyranose or neo-hesperidose [6]. The identification of the aglycone moiety showed to be esculetin-7-*O*-substituted by comparison with known ^1H- and ^{13}C-NMR data of esculetin-7-*O*-β-d-glycopyranoside (cichoriin) [23] and esculetin-7-*O*-rutinoside (haploperoside A) [20]. The presence of a neohesperidose moiety in the molecule of **1** could be clearly deduced from the two anomeric proton duplets at δ_H 5.57 and 4.52 with *J*-values of 8.0 and 2.0, which were characteristic for β-glucopyranose and α-rhamnopyranose.

Downfield location of H-1″ of rhamnose was indicative of 1″ → 2′ interglycosidic linkage [6]. The ^{13}C-NMR spectrum was characterized by the presence of 21 carbon resonances, nine of which were typical for an esculetin moiety and 12 carbon resonances were assigned to the rhamnoglucosyl moiety [23]. The location of the terminal rhamnosyl fragment on C-2′ of the glucosyl fragment was evidenced from the downfield shift of its resonance to δ_C 77.5 and the β-upfield shift of both C-1′ and C-3′ to 97.5 and 75.2 [6, 9].

Thus, compound **1** was identified as a new 6,7-di-hydroxycoumarin-7-*O*-(2′-*O*-α-l-rhamnopyranosyl)-β-d-glucopyranoside or esculetin-7-*O*-neohesperidoside, which was empirically named, as neoisobaisseoside. Previously, the presence of some coumarins has been shown in *C. officinalis*, such as umbelliferone, esculetin, scopoletin [24], scopolin (scopoletin-7-*O*-glucoside) [25], cichoriin (esculetin-7-*O*-glucoside) and esculin (esculetin-6-*O*-glucoside) [10]. This is the first case of detection of coumarin biosides in *C. officinalis*.

The investigation of the biological activity of compounds **1**–**4** showed that IC_{50} values of esculetin biosides **1** and **3** against amylase/α-glucosidase were 92.51/86.11 and 93.18/85.06 μM respectively, much lower than the IC_{50} of the reference compound acarbose (318.20/455.81 μM) indicating that both compounds strongly suppressed the digestive enzymes (Table I). Aglycone esculetin was the most active compound with IC_{50} values 32.31 and 44.18 μM against amylase and α-glucosidase. Compounds **2** and **4** showed the lowest activity against both enzymes although their aglycone scopoletin inhibited amylase and α-glucosidase with IC_{50} values of 127.62 and 162.40 μM, respectively.

Table I

Inhibitory effect on amylase (Amy), α-glucosidase (Glu) and AGEs formation (AGE) of compounds **1 - 4**, esculetin, scopoletin and acarbose[a]

Compound	Assay		
	Amy, IC_{50}, μM	Glu, IC_{50}, μM	AGE, %[b]
1	92.51 ± 2.59*	86.11 ± 2.23*	12.69 ± 0.35*
2	> 500	> 500	< 5
3	93.18 ± 2.70*	85.06 ± 2.38*	14.87 ± 0.43*
4	> 500	> 500	< 5
Esculetin	32.31 ± 0.93*	44.18 ± 1.28*	45.47 ± 1.54*
Scopoletin	127.62 ± 3.31*	162.40 ± 4.87*	< 5
Acarbose	318.20 ± 9.54	455.81 ± 13.67	< 5

[a]Average of three analyses (± SD); [b]inhibitory activity at a dose of 0.5 mg/mL.
Values with asterisk (*) indicate statistically significant differences among acarbose group at $p < 0.05$ by one-way ANOVA.

When the enzyme inhibitory activities of compounds **1 - 4** were compared, it was found that the scopoletin derivatives were less potent than esculetin derivatives. Esculetin derivatives may be ineffective due to the negative impact of a methoxy-group located at the C-6 of the coumarin nucleus. A negative effect on α-amylase inhibition by compounds with a methoxy-group located at the *ortho*-position to a carbohydrate substituent has been shown previously for flavonoid structures [26]. This phenomenon is shown for the first time for coumarin glycosides.

AGE inhibitory activities of esculetin biosides **1** and **3** and esculetin were higher compared with **2**, **4** and scopoletin. That evidence demonstrated the important role of the hydroxyl group at the C-6 of the coumarin nucleus on AGE inhibition. The introduction of a carbohydrate moiety at the C-7 position of esculetin decreased AGE inhibitory activity but the nature of the sugar (rutinose or neohesperidose) had no effect on the inhibition.

Conclusions

The application of phenolic compounds as inhibitors of amylase and α-glucosidase in the treatment of diabetic disorders is related to their ability to reduce the postprandial blood glucose level [27]. High levels of glucose lead to the accumulation of advanced glycation end products (AGEs) resulted in the development of pathological conditions associated to diabetes such as nephropathy, neuropathy, cardiovascular disease and atherosclerosis [28]. Thus, the search for compounds that can inhibit these negative processes is important. The present study revealed that the glycosylated esculetin derivatives isolated from *C. officinalis* flowers may be considered as promising antidiabetic agents.

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