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GLUT4 Translocation Active Flavonoids from *Caragana jubata*

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Abstract: Two new isoflavones, caraganin E (**1**) and caraganin F (**2**), were purified from 75% ethanolic extract of *Caragana jubata* roots, in company with ten known compounds. The chemical structures of two novel isoflavones were elucidated by NMR, HR-ESIMS and circular dichroism (CD). GLUT4 translocation activity was tested in L6 cells for all isolated compounds. Among them, compound **5** exhibited the best activity, increasing the fluorescence intensity by 3.25 folds. The results of the exploration may help us understand the chemotaxonomic variety of natural products in *Caragana jubata* and enhance the diversity of flavonoids.

Keywords: *Caragana*; *Caragana jubata*; flavonoids; GLUT4 translocation.

1. Introduction

Diabetes, a chronic metabolic disease mainly caused by insulin resistance, absolute or relative insulin deficiency, or a combination of both, is characterized by elevated blood glucose levels. The prevalence of type 2 diabetes (T2D) and its associated complications is expected to increase and presents a substantial threat to global health and the economy [1,2]. This issue is especially of concern in China due to the considerable rise in the number of people with diabetes owing to lifestyle changes driven by rapid economic growth, enhanced immunity to infectious diseases, and genetic predisposition [3].

As insulin resistance is the primary metabolic issue associated with T2D, there has been a profound interest in using insulin-sensitizing medications to treat this condition [4,5]. Insulin-responsive glucose transporter 4 (GLUT-4) is a key target for therapeutic development [6,7]. The growing body of evidence stating that GLUT-4 translocation could alleviate insulin resistance likely underlies the methods employed to discover new lead compounds with antidiabetic properties [8,9].

A cell-based GLUT4 translocation test method was developed to evaluate the potential of plant parts and extracts. Stable L6 myotubes expressing GLUT4-mOrange cDNAs were utilized in this technique. The intention was to examine the possibility of obtaining hypoglycemic medications from natural sources [10-15]. An ethyl acetate fraction derived from *Caragana jubata* exhibited substantial activity in promoting GLUT4 translocation while screening a library of plant extracts comprising 800 different biotas. *C. jubata* (Pall.) Poir. belongs to the family of legumes and is native to regions such as Tibet, Qinghai, and Gansu in China. According to the Highland Chinese Herbal Medicine Treatment

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Manual, *C. jubata* is known for its heat-clearing, detoxifying, and blood pressure-lowering properties, making it a popular remedy for conditions such as mammary carbuncles, sores, and hypertension. Two previously undescribed compounds, caraganin E (**1**) and caraganin F (**2**) were obtained from the 75% ethanol extract of *C. jubata*, along with ten known chemicals via a phytochemical study performed using bioassays. Furthermore, the separation and structure identification of two novel compounds and the determination of their absolute configuration using optical rotation and distinctive absorption in CD spectra were discussed. In addition, the GLUT-4 transmembrane activity was evaluated.

2. Materials and Methods

2.1. General Experimental

Semi-preparative HPLC separations were carried out on a Waters 2535 instrument with a 2998 photodiode array detector equipped with GALAK BF-CN columns (5 μm, 10 × 150 mm; 5 μm, 20 × 150 mm) and a Nacalai ODS column (5 μm, 10 × 250 mm). TLC: silica gel plate (Sil GF254, 0.20-0.25 mm; Qingdao Marine Chemical, Ltd., Qingdao, China). A Bruker AV-600 MHz spectrometer was used to monitor the NMR spectra. A Rudolph Autopol IV spectrometer was employed to obtain optical rotations. A UH5300 spectrometer and a Nicolet Magna FT-IR 750 spectrometer, a Circular dichroism were applied to analyze the UV, IR, and CD spectra, respectively.

2.2. Plant Material

In 2018, experimental materials were collected in Yushu County, Qinghai Province. They were identified as *Caragana jubata* (pall.) Poir. by Prof. Duojie of Qinghai Tibetan Medicine Institute. The *C. jubata* samples (No. SC0885) are stored in the herbarium of the South-Central Minzu University in Wuhan, Hubei Province, within the School of Pharmaceutical Sciences.

The plants are harvested and dried in a cool place, well-ventilated area. After drying, approximately 200 grams of dried plant material were prepared as specimens to be stored in the herbarium at a constant temperature and humidity of 10 degrees Celsius. The remaining samples were crushed and extracted using 75% ethanol, and the dried ethanol extract was preserved in a refrigerator at -20°C.

2.3. Extraction and Isolation

The dry roots of 9 kg of *C. jubata* were cut, crushed, and extracted with 75% ethanol at rt (6 × 15 L, 7 d each). The extract was subsequently concentrated under vaccum to yield 2.08 kg of extract. They were separated by macroporous resin column chromatography, eluted with a water-ethanol gradient, concentrated under reduced pressure, and placed in a test tube. Six components (Fr.1-Fr.6) were obtained by thin-layer chromatography (TLC) detection. A part of Fr.3 (1.17 kg) was purified by column chromatography (CC) with silica gel (300-400 mesh), concentrated by dichloromethane-methanol gradient elution under reduced pressure, and placed in a test tube. Seven components (Fr.3.1-Fr.3.7) were obtained by TLC detection.

A silica gel column (100-200 mesh) was selected to separate Fr.3.4 (303 g), eluted by a dichloromethane-methanol gradient, concentrated on a rotary evaporator and transferred to a test tube. Six components (Fr.3.4.1-Fr.3.4.6) were obtained by TLC detection. 225 g of Fr.3.6 was isolated by a medium pressure silica gel CC (GF254), with a dichloromethane-methanol gradient, reduced pressure concentrated and transferred to a test tube. Five components (Fr.3.6.1-Fr.3.6.5) were obtained by TLC detection.

Fr.3.4.2 was separated using semi-preparative HPLC to obtain **5**-**7** as well as Sephadex LH-20; Fr.3.4.4 was isolated with Sephadex LH-20 and HPLC to isolate the new compound **2**. Fr.3.6.1 yielded new compounds **1** and compounds **11**-**12**, which were isolated through HPLC and HPLC. Fr.3.4.2, Fr.3.6.3, and Fr.3.6.5 were separated by HPLC to obtain compound **4**, compounds **3** and **9**-**10**, and compound **8**, respectively.

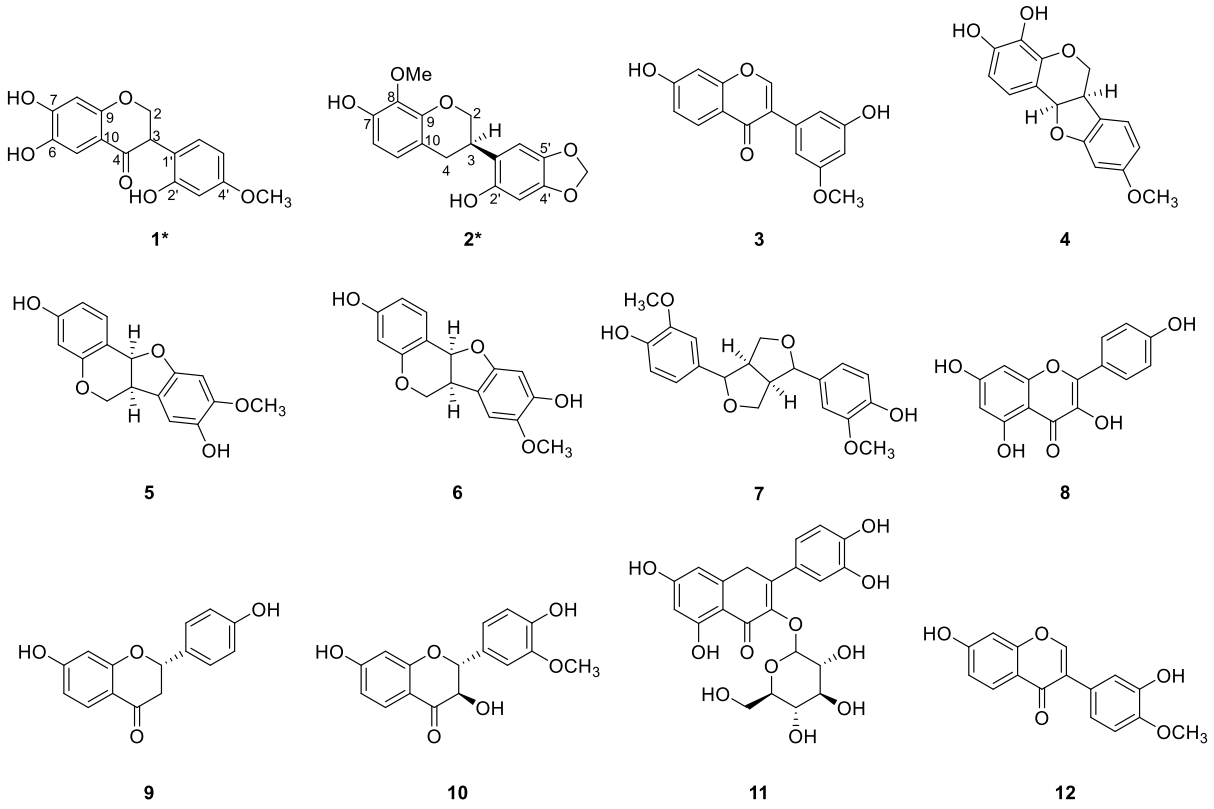

Figure 1. The chemical structures of compounds **1-12**

3. Results and Discussion

3.1. Structural Characterization

Compound **1** was isolated as brown oils. The molecular formula of **1** was assigned as $C_{16}H_{14}O_6$ on the basis of the HR-ESIMS pseudomolecular ion at *m/z* 301.0731 $[M-H]^-$ (calculated for 301.0718), corresponding to 10 index of hydrogen deficiency (IHD) in **1**. IR (KBr) spectroscopy of **1** clearly exhibited the specific absorptions of the hydroxyl (3416 cm^{-1}), carboxyl (1654 cm^{-1}), and aromatic double bond (1616, 1519 cm^{-1}) functional groups. Its UV spectrum displayed the characteristic absorptions at 225, 280, and 345 nm. The ^1H nuclear magnetic resonance (NMR) spectrum of **1** displayed three double doublets at δ_H 4.11 (1H, dd, J = 10.8, 5.3 Hz, H-3), 4.38 (1H, dd, J = 10.8, 5.3 Hz, H-2α), and 4.53 (1H, t, J = 10.8, Hz, H-2β), two isolated singlet protons at δ_H 7.23 (1H, s) and 6.36 (1H, s), and a ABX coupling system [δ_H 6.90 (1H, d, J = 8.4 Hz), 6.40 (1H, d, J = 2.5 Hz), and 6.37 (1H, dd, J = 8.4, 2.5 Hz)], which suggested that it was a dihydroisoflavonoid. Compound **1** exhibited 16 carbon signals, which included 1 methyl group, 1 methylene group, 8 quaternary carbons (comprising 6 oxygenated aromatic carbons, 1 carbonyl carbon, and 2 aromatic carbons), and 6 methine carbons, as indicated by the ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectroscopic data (Table 1). Careful analysis of NMR data (Table 1) suggested that **1** shared an identical framework with the known natural product (3*R*)-vestitone [16] The only difference was that two isolated singlet protons at δ_H 7.23 (1H, s) and 6.36 (1H, s) in the A ring of **1** replaced an ABX coupling system [δ_H 7.74 (1H, d, J = 8.8 Hz), 6.48 (1H, dd, J = 8.8, 2.2 Hz), and 6.31 (1H, d, J = 2.2 Hz)] in the A ring of (3*R*)-vestitone. This observation indicates that two isolated aromatic protons at δ_H 7.23 (1H, s) and 6.36 (1H, s) could be ascribed to H-5 and H-8. This inference was supported by the primary heteronuclear multiple bond correlation (HMBC) of H-5 with C-4 at δ_C 194.9, C-6 at δ_C 142.1, C-7 at δ_C 155.7, and C-9 at δ_C 159.5 as well as that of H-8 with C-10/C-6/C-7/C-9. Furthermore, the aromatic proton at δ_H 6.90 (1H, d, J = 8.4 Hz) exhibited considerable ROESY correlations with H_2-2 (δ_H 4.53, 1H, dd; 4.38, 1H, dd) and H-3 (δ_H 4.11, 1H, dd), signifying that the proton at δ_H 6.90 was attached to C-6'. The combined analysis

of ^{1}H-^{1}H COSY correlations and three coupling constants of the ABX system at ring B showed that two aromatic protons at δ_H 6.40 (1H, d, J = 2.5 Hz) and 6.37 (1H, dd, J = 8.4, 2.5 Hz) were located at C-5' and C-3', severally. Moreover, crucial HMBC correlations were noted, including signals of the MeO moiety at δ_H 3.72 to C-4' (δ_C 161.7), H-3' to C-1' (δ_C 116.0), C-2' (δ_C 157.6), C-4' and C-5' (δ_C 105.9), H-5' to C-1', C-3' and C-4', H-6' to C-3 (δ_C 49.8), C-2' and C-4', H_2-2 to C-1', and H-3 to C-6' and C-2', which allowed the assigning of all carbons at ring B. The optical rotation test results of compound **1** demonstrated that $[\alpha]_D^{20}$ = +1.4, and there was no apparent trend in the CD spectrum, which indicated that it did not exhibit optical activity and was a racemate. **1** is therefore an undescribed novel compound, and elucidated as 6,7,2'-trihydroxy-4-methoxy-dihydroisoflavanone (Figure 1), namely caraganin E.

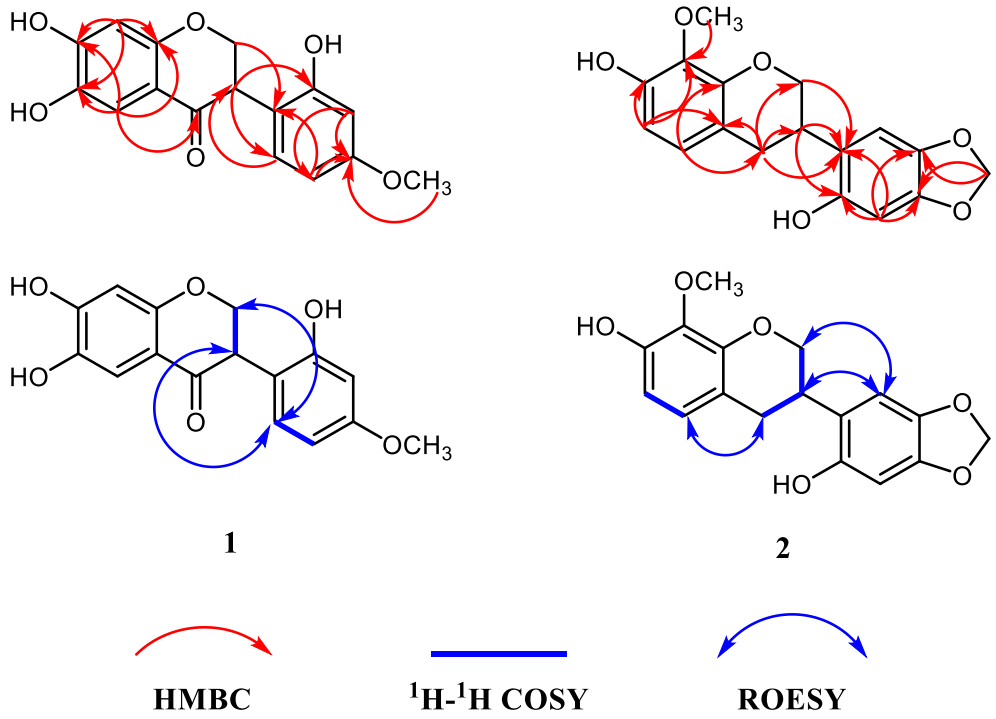

Figure 2. The key HMBC, ^{1}H-^{1}H COSY and ROESY correlations of compounds **1** and **2**

Compound **2** was supplied as an amorphous powder. Its formular was determined as $C_{17}H_{16}O_6$ basing on the HR-ESI-MS data *m/z* 317.1020 $[M+H]^+$ (calculated for 317.1020), indicating 10 IHD in the molecule. The infrared spectrum exhibited absorption bands at 3410, 2916, 1732, and 1620 cm^{-1}, signifying the presence of hydroxyl, chelated carbonyl, and aromatic ring functionalities. The ^{1}H NMR data of **2** displayed two methylenes [δ_H 4.30 (1H, ddd, J = 10.2, 3.4, 1.8 Hz), 3.98 (1H, t, J = 10.0 Hz); δ_H (2.91 1H, dd, J = 15.6, 10.6 Hz), 2.80 (1H, dd, J = 15.6, 5.2 Hz)], one methine δ_H 3.48 (1H, m), two isolated singlet aromatic protons at δ_H 6.60 (1H, s) and 6.40 (1H, s), and two vicinal coupling aromatic protons at δ_H 6.65 (1H, d, J = 8.4 Hz, H-5) and 6.37 (1H, d, J = 8.4 Hz, H-6). The ^{13}C NMR and DEPT spectroscopic data of compound **2** presented 17 carbon signals, including 1 methyl, 3 methylenes, 5 methines, 8 quaternary carbons (6 oxygenated aromatic carbons and 2 aromatic carbons), and 6 methines (Table 1). The NMR data were compared to those in the literature, which revealed that it was almost identical to the known compound hildegardiol, both of which were isoflavan analogs.[17] The major difference was that two vicinal coupling aromatic protons at δ_H 6.65 (1H, d, J = 8.4 Hz, H-5) and 6.37 (1H, d, J = 8.4 Hz, H-6) in the A ring of **2** replaced two isolated singlet protons at δ_H 6.74 (1H, s, H-7) and 6.43 (1H, s, H-8) in the A ring of hildegardiol, which was determined using the pertinent ROESY correlations of H-5 at δ_H 6.65 to H_2-4 at δ_H 2.91 (1H, dd) and 2.80 (1H, dd). Similarly, the important correlations of the aromatic proton at δ_H 6.60 to H_1-2 and H_2-2 (δ_H 4.30, 1H, ddd, J = 10.1, 3.4, 1.8 Hz; 3.98, 1H, t, J =10.1 Hz) and H-3 (δ_H 3.48, 1H, m) were noted in the ROESY spectrum, which suggested that two aromatic protons at δ_H 6.60 (1H, s) and 6.40 (1H, s) could be attributed to H-6' and H-3',

respectively. At approximately 240 and 280 nm, the CD spectra of compound **2** demonstrated a negative and a positive Cotton effect, respectively (Figure 3). This finding implies that compound **2** possessed a 3*R* configuration [17]. Therefore, the new compound was identified as (3*R*)-7,2'-dihydroxy-8-methoxy-4',5'-methylenedioxyisoflavan (Figure 1), namely caraganin F.

In addition, the structures of the ten known compounds as 7,3'-dihydroxyl-5'-methoxyisoflavone (**3**),[18] (6a*R*,11a*R*)-3,4-dihydroxy-9-methoxy-pterocarpan (**4**),[19] (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (**5**),[19] lespedezol D_1 (**6**),[20] pinoresinol (**7**),[21] kaempferol (**8**),[22] liquiritigenin **(9)**,[23] erycibenin D **(10)**,[24] quercetin 3-*O*-β-D-glucopyranoside **(11)**[25] and calycosin **(12)**,[26] by comparing the spectroscopy data and physical and chemical properties with those reported in the literature (Figure 1).

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data in CD_3OD for Compounds **1**-**2**.

Position	**1**		**2**	
	δ_H, *J* (Hz)	δ_C	δ_H, *J* (Hz)	δ_C
2	4.53, t, (10.8) 4.38, dd, (10.8, 5.3)	72.1	4.30, ddd, (10.2, 3.4, 1.8) 3.98, t, (10.0)	71.0
3	4.11, dd, (10.8, 5.3)	49.8	3.48, m	33.1
4	-	194.9	2.91, dd, (15.6, 10.6) 2.80, dd, (15.6, 5.2)	31.4
5	7.23, s	112.0	6.65, d, (8.4)	125.2
6	-	142.1	6.37, d, (8.4)	109.2
7	-	155.7	-	149.7
8	6.36, s	104.0	-	136.9
9	-	159.5	-	149.1
10	-	114.8	-	116.1
1'	-	116.0	-	120.6
2'	-	157.6	-	150.9
3'	6.40, d, (2.5)	102.5	6.40, s	98.6
4'	-	161.7	-	147.8
5'	6.37, dd, (8.4, 2.5)	105.9	-	142.1
6'	6.90, d, (8.4)	131.7	6.60, s	107.8
4'-OCH_3	3.72, s	55.6	-	-
8-OCH_3	-	-	3.78	61.0
OCH_2O	-	-	5.80, d, (2.8)	102.0

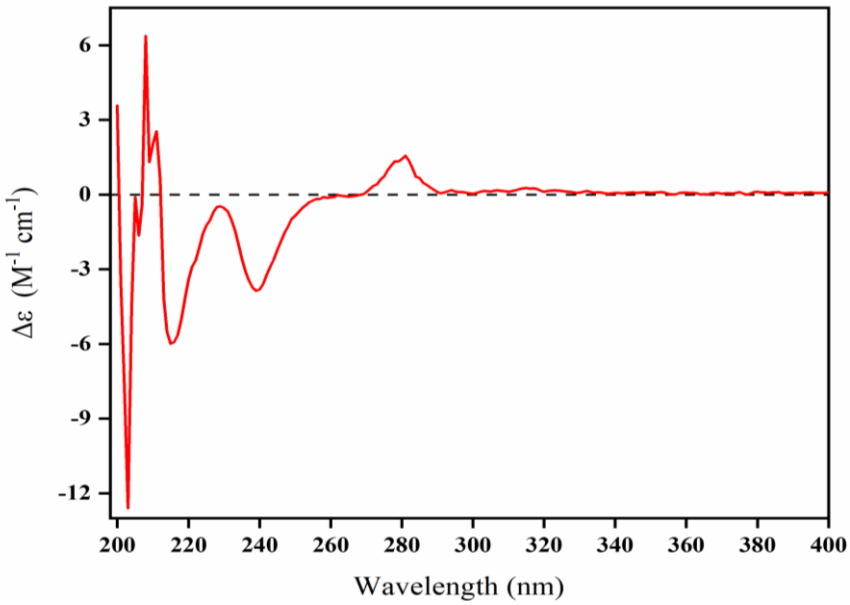

Figure 3. The CD spectrum of compound **2**

3.2. GLUT4 Translocation Assay

Immunofluorescence studies were performed using the isolated compounds in GV348-myc-GLUT4-mOrange L6 cells. Laser confocal imaging was used to quantify the levels and distribution of fluorescein isothiocyanate-myc (green fluorescence) and GLUT4-mOrange (red fluorescence) in the cytoplasm. The statistics of GLUT4-mOrange fluorescence intensity in the cells showed a significant increase in GLUT4 expression in L6 cells. According to the experimental findings, the consumption of novel chemicals **1** and **2** increased the fluorescence intensity by 1.72 and 1.80 times, respectively. Compounds **3-12** augmented the fluorescence intensity by 2.1, 2.08, 3.25, 2.73, 1.61, 2.24, 1.59, 2.44, 2.61, and 1.25 times, respectively, compared with the control group. Compound **5** exhibited the highest activity. This finding indicates that at 50 μg/mL, these compounds could considerably improve GLUT4 expression and translocation in L6 cells.

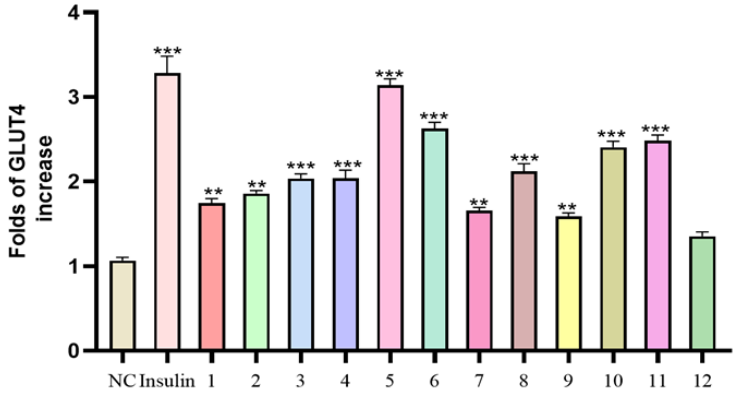

Figure 4. GLUT4 translocation activities of compounds **1-12** at 50 μg/mL

4. Conclusion

From the entire dried roots of *C. jubata*, two previously undescribed isoflavone compounds (**1** and **2**) were isolated, along with ten known compounds (**3-12**). The structures of these compounds were determined on the basis of spectral analysis and comparisons with relevant literature date. In addition, compound **5** had the potential to significantly enhance GLUT4 expression and translocation in L6 cells. This could potentially serve as a theoretical model for developing innovative antidiabetic medications.

The observations from these investigations broaden our understanding of Caragana's chemical composition. Moreover, the results from this study provide a solid basis for examining the pharmacological effects of this plant.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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