JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Gymnemic Acids Inhibit Sodium-Dependent Glucose Transporter 1

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ABSTRACT: To evaluate the activity of botanicals used in Chinese Traditional Medicine as hypoglycemic agents for diabetes type II prevention and/or treatment, extracts prepared from 26 medicinal herbs were screened for their inhibitory activity on sodium-dependent glucose transporter 1 (SGLT1) by using two-electrode voltage-clamp recording of glucose uptake in *Xenopus laevis* oocytes microinjected with cRNA for SGLT1. Showing by far the strongest SGLT1 inhibitory effect, the phytochemicals extracted from *Gymnema sylvestre* (Retz.) Schult were located by means of activity-guided fractionation and identified as 3-*O*-*β*-D-glucuronopyranosyl-21-*O*-2-tigloyl-22-*O*-2-tigloyl gymnemagenin (**1**) and 3-*O*-*β*-D-glucuronopyranosyl-21-*O*-2-methylbutyryl-22-*O*-2-tigloyl gymnemagenin (**2**) by means of LC-MS/MS, UPLC-TOF/MS, and 1D/2D-NMR experiments. Both saponins exhibited low IC_{50} values of 5.97 (**1**) and 0.17 μM (**2**), the latter of which was in the same range as found for the high-affinity inhibitor phlorizin (0.21 μM). As SGLT1 is found in high levels in brush-border membranes of intestinal epithelial cells, these findings demonstrate for the first time the potential of these saponins for inhibiting electrogenic glucose uptake in the gastrointestinal tract.

KEYWORDS: *diabetes, SGLT1, inhibitor, Gymnema sylvestre, gymnemic acid V, gymnemic acid XV*

INTRODUCTION

Hyperglycemia is considered a major pathogenic factor in the development of acute and chronic diabetic complications such as diabetic ketoacidosis, retinopathy, nephropathy, and stroke.[1] Any practical and effective ways of maintaining long-time perfect glycemic control are lacking, thus leaving diabetics susceptible to developing acute and chronic complications such as cardiovascular diseases, blindness, amputation, and kidney failure.[2,3] Today, oral medication with euglycemic agents, such as sulfonylureas and thiazolidine derivatives,[4−6] combined with lifestyle intervention are applied to slow the progression of diabetic complications.[7−9] However, agents for glycemic control, targeting either insulin secretion or insulin action, can have significant adverse effects,[9] and thus additional treatment options are required. Lowering postprandial blood sugar excursions therefore becomes a focus in diabetes research and prevention programs.

Glucose is transported through biological membranes by the facilitative (GLUT) and sodium-dependent (SGLT) glucose transporters, which belong to the SLC2A and SLC5A gene families, respectively.[10] Facilitative glucose transport is mediated by 13 members of the GLUT protein family comprising GLUT1−12 and HMIT, which belong to a superfamily of transmembrane segment transporters identified in various tissue cells including liver, kidney, heart, brain, intestine, etc.[11] GLUT1, GLUT2, and GLUT4 have been reported to play a key role in glucose sensing in insulin secretion and for glucose uptake into insulin-sensitive organs.[12−14] The sodium/glucose cotransporters SGLT1 and SGLT2 are located primarily in epithelial cells of the intestine and kidney and transport glucose by the energy generated from the electrochemical gradient.[15] Exhibiting a high affinity to glucose and galactose, SGLT1 is predominantly located at the brush border membrane of the small intestine and carries these monosaccharides from the intestinal lumen into enterocytes.[15]

Both types of glucose transporters are currently targets in diabetes therapy. Among the GLUT protein family, for example, one strategy is to increase *m*RNA and protein expression of GLUT4 in the insulin-sensitive tissues or to increase the translocation of GLUT4.[16,17] For the sodium-dependent glucose transporters, the major strategy is to decrease glucose absorption by inhibiting or attenuating the transporter activity. SGLT1 in the kidney is responsible for only 2% of glucose reabsorption,[15] with the majority of glucose reabsorbed via SGLT2. Inhibitors of SGLT2 have been developed and are in clinical use and effectively increase glucose excretion via urine.[18] In contrast to kidney, the intestine only expresses SGLT1 that mediates glucose uptake through the apical membrane of intestinal epithelial cells. It could by its location be considered a promising target for diabetes prevention and/or therapy via specific inhibitors provided via the oral route.

To date, there are only a few studies published on natural SGLT1 inhibitors; for example, the dihydrochalcone phlorizin from apples was identified as a competitive inhibitor of glucose transporters including SGLT1 and was reported to normalize plasma glucose levels independent of insulin in diabetic animals.[19,20] To prevent its partial hydrolysis in the gastrointestinal (GI) tract, phlorizin was poly-*γ*-glutamylated and found to significantly decrease glucose-induced hyperglycemia.[21] Studies using two-electrode voltage clamp experiments

Received: April 14, 2014
Revised: May 22, 2014
Accepted: May 23, 2014
Published: May 23, 2014

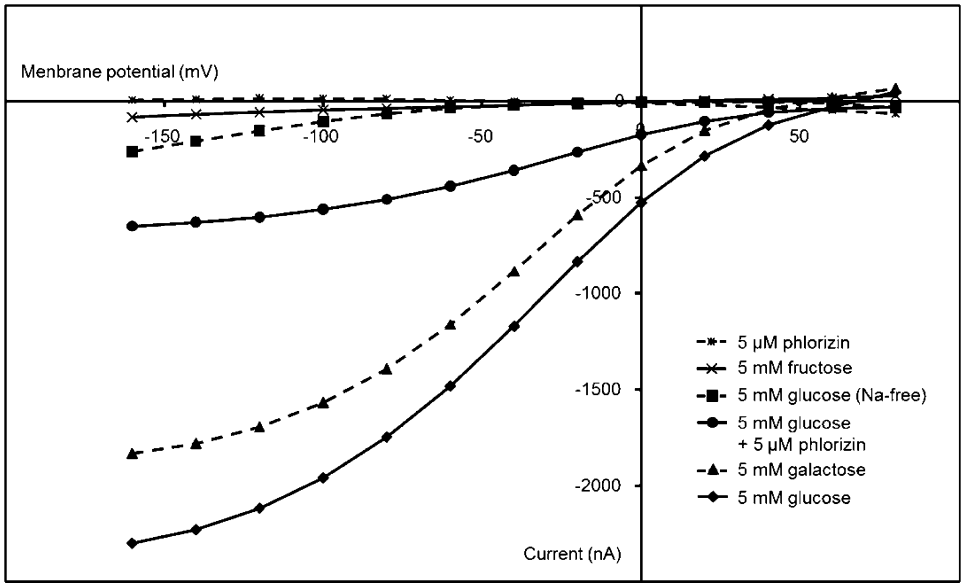

Figure 1. Comparative analysis of transporter currents induced by glucose, fructose, and galactose (5 mM each), and phlorizin (5 μM) and in a glucose/phlorezin mixture in *Xenopus* oocytes expressing hSGLT1. Data are mean values of 3−10 oocytes.

on *Xenopus* oocytes that express the human sodium-coupled glucose transporter hSGLT1 revealed that the inward current evoked by transport of α-methyl-D-glucopyranoside by SGLT1 is potently inhibited by the simultaneous application of flavonoid glycosides as well as by some aglycones.[22] Moreover, the green tea polyphenols epicatechin gallate and epigallocatechin gallate were found to have a modest SGLT1 inhibitory activity.[23]

Some medicinal herbs have been used in Asia for many centuries as hypoglycemic agents, for example, Huang Lian (*Rhizoma coptidis*), Mai Dong (*Radix ophiopogonis*), Fo-ti, Xuan Shen (*Radix scrophulariae*), Tian Men Dong (*Asparagi radix*), *Gymnema sylvestre* R. Schult, and Luo Han Guo (*Momordica grosvenori*).[24] However, their bioactive phytochemicals as well as their mechanisms of action remain elusive. The objective of the present study was to evaluate the SGLT1 inhibiting activity of a series of botanicals used in traditional Chinese medicine as hypoglycemic agents. Functional analysis was performed by two-electrode voltage-clamp recordings of glucose-associated transport currents in *Xenopus laevis* oocytes microinjected with the cRNA for hSGLT1 to identify the phytochemicals with SGLT1 inhibitory activity.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: α-methyl-D-glucopyranoside (α-MDG), glucose, galactose, fructose, and phorizin as well as all chemicals used for preparation of the bath solution were obtained from Sigma-Aldrich (Taufkirchen, Germany). The bath solution (pH 7.5) contained 88 mM NaCl, 1 mM KCl, 0.8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.3 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, and 10 mM HEPES. Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). All solvents were of HPLC grade (Merck, Darmstadt, Germany). Water for HPLC separation was purified by means of a Milli-Q Water Advantage A 10 water system (Millipore, Molsheim, France).

Medicinal Herbs. *Polygonatum sibiricum* Redouté (H1), *Polygonatum odoratum* (Mill.) Druce (H2), *Lycium chinense* Mill. (H3), *Arctium lappa* L. (H4), *Epimedium sagittatum* Maxim (H5), *Paeonia lactiflora* Pall. (H6), *Lycium barbarum* L. (H7), *Atractylodes macrocephala* Koidz. (H8), *Ligustrum lucidum* W. T. Aiton (H9), *Atractylodes macrocephala* Koidz. (H10), *Ophiopogon japonicus* (L.f.) Ker Gawl. (H11), *Rehmannia glutinosa* Steud. (H12), *Siraitia grosvenori* (H13), *Polygonatum odoratum* (Mill.) Druce (H14), *Angelica sinensis* (Oliv.) Diels (H15), *Eriobotrya japonica* (Thunb.) Lindl. (H16), *Cornus officinalis* Siebold & Zucc. (H17), *Panax ginseng* C. A. Mey. (H18), *Epimedium sagittatum* Maxim. (H19), *Coptis chinensis* Franch. (H20), *Cornus officinalis* Siebold & Zucc. (H21), *Paeonia lactiflora* Pall. (H22), *Panax ginseng* C. A. Mey. (H23), *Scrophularia ningpoensis* Hemsl. (H25), and *Paeonia lactiflora* Pall. (H26) were purchased from the Chinese Medicine Co. Tong Ren Tang (Beijing, China), *Gymnema sylvestre* (Retz.) R. Br. ex Schult (H24) was purchased from Magic Herbs (Ellerau, Germany).

Preparation of Oocytes. Maintenance of *Xenopus laevis* and the procedures of oocyte harvest were approved by the local authority for animal care in research (Regierung von Oberbayern, approval 211-2531.3-9/99). The surgical procedure and oocyte incubation were performed following the protocol reported earlier.[22] Oocytes were injected with 23 ng of hSGLT1-cRNA and incubated for 3−4 days at 17 °C. A solution of α-methyl-D-glucopyranoside (1 mM) was diluted with bath solution used as transport substrate for hSGLT1. In addition, oocytes that were not pretreated by cRNA injection and were lacking hSGLT1 were used as control.

Electrophysiology. Two-electrode voltage clamp experiments were performed as described previously in the literature[22] with oocytes placed in an open chamber and superfused (3 mL/min) with bath solution or with solutions containing the substrate. Oocytes were voltage-clamped, and transport current was measured at −60 mV using a TEC-05 amplifier and CellWorks software (npi electronic, Tamm, Germany). Current−voltage relationships were measured in a potential range from −160 to +80 mV, and the current generated by the transport at a given membrane potential was calculated as the difference of the currents measured in the presence and absence of substrate. The kinetic parameters of transport K_m and I_{max} (nA) were calculated from least-squares fits of at least three data points based on the Michaelis−Menten equation. The inhibition rate (IR) was calculated using the formula IR = [current(I) − current(0)]/(current(s) −current(0)), where current(0) is the transport current applied with bath solution and current(s) and current(I) are transport currents applied with α-MDG in the absence and presence of inhibitor, respectively. Dixon-type kinetic studies were performed at a membrane potential of −60 mV. To avoid errors due to the replenishment of the oocytes with α-MDG, the application time of the substrates was limited to 15 s at an increased (7 mL/min) perfusion speed. Activity of hSGLT1 in oocytes was measured for the monosaccharides glucose, fructose, and galactose (5 mM each) in bath solutions in the absence

or presence of phlorizin (5 μM) or as described above (Figure 1). In addition, 5 mM glucose in a sodium-free bath solution as well as a binary mixture of 5 mM glucose and 5 μM phlorizin in bath solution was analyzed.

Botanical extracts and subfractions as well as purified individual compounds were solubilized in DMSO at concentrations of 50−200 mM and were diluted with the α-MDG solution. The final concentration of DMSO in the test solutions was not higher than 1% (v/v).

Screening of Medicinal Herbs for SGLT1 Inhibitors. An aliquot of each ground botanical (10 g dry weight) was extracted with *n*-pentane (3 × 50 mL), followed by methanol/water (7:3, v/v; 3 × 50 mL) and methanol (3 × 50 mL). After removal of the solvents in a vacuum, aliquots (12 mg) of the pentane extractables (fraction I) and the methanol extractables (fraction II), respectively, were dissolved in small amounts of DMSO and made up with α-MDG solution to 50 mL to perform the hSGLT1 experiments (Figure 2). The final concentration of DMSO was not higher than 1% (v/v).

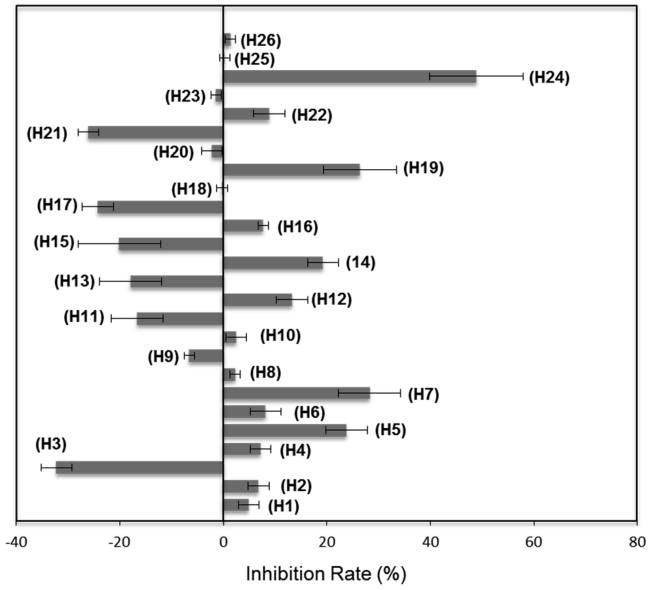

Figure 2. SGLT1 inhibitory activity of the methanol soluble fractions II of *Polygonatum sibiricum* Redouté (H1), *Polygonatum odoratum* (Mill.) Druce (H2), *Lycium chinense* Mill. (H3), *Arctium lappa* L. (H4), *Epimedium sagittatum* Maxim (H5), *Paeonia lactiflora* Pall. (H6), *Lycium barbarum* L. (H7), *Atractylodes macrocephala* Koidz. (H8), *Ligustrum lucidum* W. T. Aiton (H9), *Atractylodes macrocephala* Koidz. (H10), *Ophiopogon japonicus* (L.f.) Ker Gawl. (H11), *Rehmannia glutinosa* Steud. (H12), *Siraitia grosvenori* (H13), *Polygonatum odoratum* (Mill.) Druce (H14), *Angelica sinensis* (Oliv.) Diels (H15), *Eriobotrya japonica* (Thunb.) Lindl. (H16), *Cornus officinalis* Siebold & Zucc. (H17), *Panax ginseng* C. A. Mey. (H18), *Epimedium sagittatum* Maxim. (H19), *Coptis chinensis* Franch. (H20), *Cornus officinalis* Siebold & Zucc. (H21), *Paeonia lactiflora* Pall. (H22), *Panax ginseng* C. A. Mey. (H23), *Gymnema sylvestre* (Retz.) Schult (H24), *Scrophularia ningpoensis* Hemsl. (H25), and *Paeonia lactiflora* Pall. (H26).

Isolation and Identification of SGLT1 inhibitors from *Gymnemna sylvestre* (H24). Dried leaves of *Gymnemna sylvestre* (300 g) were ground, extracted with pentane (2 × 2L), and, finally, extracted with methanol/water (7:3, v/v; 2 × 2L), followed by methanol (2 × 2L). The latter two polar extracts were combined, solvents were separated by means of a Genevac Rocket evaporation system (Genevac Limited, Ipswich, UK), and the remaining residue was lyophilized to provide polar fraction II (104 g, 34.7% yield). Fraction II was dissolved in water (in sum 1 L), and 20 mL was applied each time onto a PP cartridge (i.d. = 40 mm, l = 150 mm) filled with RP18 material (LiChroprep RP18, 25−40 μm mesh material (Merck) and separated by MPLC by flushing the column with water (400 mL, fraction II-1), followed by methanol (400 mL, fraction II-2). After lyophilization, fractions II-1 and II-2 were both used for the SGLT1 bioassay. Fraction II-2 (51.7 g, 17.2%) showing SGLT1 inhibition was further separated by normal phase column chromatography using silica gel (400 × 30 mm, 5% water) as stationary phase and the following chloroform/methanol/water mixtures (v/v/v) as the mobile phase: fraction II-2/A (7:3:0.5), fraction II-2/B (6.9:3.1:0.57), fraction II-2/C (6.7:3.3:0.64), fraction II-2/D (6.6:3.4:0.71), fraction II-2/E (6.4:3.6:0.78), fraction II-2/F (6.3:3.7:0.86), fraction II-2/A (6.1:3.9:0.93), and fraction II-2/H (6:4:1) (Figure 3). An aliquot (1 g)

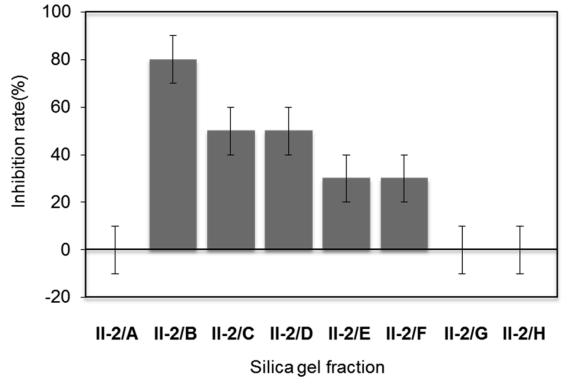

Figure 3. Inhibition of the hSGLT1 transport currents induced by α-methylglucose (1 mM) in the presence of fractions II-2/A−II-2/H isolated from *Gymnema sylvestre*. Data are mean values of 3−10 oocytes.

of fraction II-2/B was dissolved in acetonitrile/0.1% formic acid (30:70, v/v) and fractionated by means of preparative RP-HPLC. Twenty-eight fractions were collected individually in several runs, and the corresponding fractions were combined (Figure 4). HPLC fractions 22 and 24, showing the highest SGLT1 inhibitory activity, were separated from solvent using a Genevac Rocket evaporation system (Genevac Limited) and freeze-dried to yield gymnemic acids V (**1**) and XV (**2**), the structures of which were identified by means of LC-MS/MS, UPLC-TOF-MS, and 1D/2D-NMR experiments.

*Gymnemic acid V, **1**, Figure 5:* LC-MS (ESI$^-$), *m/z* 845 (100, [M − H]$^-$), 881 (12, [M + Cl]$^-$); MS/MS (DP = −64 V), *m/z* (%) 637 (89), 175 (74), 157 (100); LC-TOF-MS, *m/z* 845.4672 ([M − H]$^-$, measured), *m/z* 845.4687 ([M − H]$^-$, calcd for $C_{46}H_{69}O_{14}$); ^{1}H NMR (500 MHz, pyridine-d_5; COSY, *J*-RESOLVED, ROESY), δ 0.84−1.00 [m, 1H, H−C(1a)], 0.89 [s, 3H, H−C(25)], 0.92 [s, 3H, H−C(26)], 0.95 [s, 3H, H−C(24)], 1.02 [s, 3H, H−C(29)], 1.18−1.40 [m, 3H, H−C(6a,7a,19a)], 1.26 [s, 3H, H−C(30)], 1.30 [s, 3H, H−C(27)], 1.40−1.50 [m, 1H, H−C(1b)], 1.46 [d, 3H, *J* = 7.0 Hz, H−C(4‴)], 1.51−1.57 [m, 1H, H−C(15a)], 1.60 [d, 3H, *J* = 7.0 Hz, H−C(4″)], 1.62−1.73 [m, 4H, H−C(5,6b,7b,9)], 1.74−1.86 [m, 8H, H−C(11,5″,5‴)], 1.92−2.07 [m, 2H, H−C(2a,15b)], 2.14−2.27 [m, 1H, H−C(19b)], 2.27−2.35 [m, 1H, H−C(2b)], 3.11 [dd, 1H, *J* = 4.0 Hz, 14.0 Hz, H−C(18)], 3.74 [d, 1H, *J* = 10.8 Hz, H−C(23a)], 4.02 [d, 1H, *J* = 12 Hz, H−C(28a)], 4.10−4.19 [m, 1H, H−C(2′)], 4.19−4.28 [m, 1H, H−C(3′)], 4.23 [d, 1H, *J* = 12 Hz, H−C(28b)], 4.30−4.40 [m, 2H, H−C(3,23b)], 4.52−4.62 [m, 2H, H−C(4′,5′)], 4.99−5.08 [m, 1H, H−C(16)], 5.25 [d, 1H, *J* = 7.9 Hz, H−C(1′)], 5.33−5.39 [m, 1H, H−C(12)], 5.82 [d, 1H, *J* = 12.0 Hz, H−C(21)], 6.34 [d, 1H, *J* = 12.0 Hz, H−C(22)], 6.95−7.03 [m, 2H, H−C(3″,3‴)]; ^{13}C NMR (125 MHz, pyridine-d_5; HSQC, HMBC), δ 12.2 [C(5″)], 12.2 [C(5‴)], 13.6 [C(24)], 14.1 [C(4‴)], 14.2 [C(4″)], 16.2 [C(25)], 16.9 [C(26)], 18.1 [C(6)], 19.8 [C(30)], 23.9 [C(11)], 26.0 [C(2)], 27.4 [C(27)], 29.2 [C(29)], 32.5 [C(7)], 36.6* [C(10)], 36.8* [C(15)], 36.8 [C(20)], 38.8 [C(1)], 40.2 [C(8)], 42.6 [C(4)], 42.6 [C(18)], 43.5 [C(14)], 45.8 [C(19)], 47.1 [C(9)], 47.4 [C(5)], 48.0 [C(17)], 59.8 [C(28)], 64.4 [C(23)], 67.0 [C(16)], 73.5

dx.doi.org/10.1021/jf501766u | J. Agric. Food Chem. 2014, 62, 5925−5931

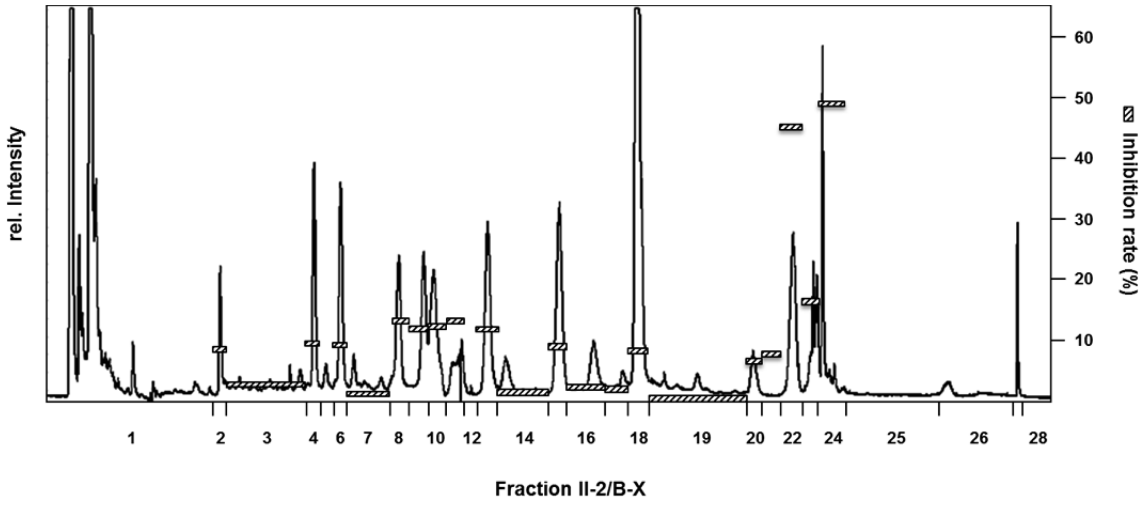

Figure 4. Inhibition of hSGLT1 transport currents induced by α-methylglucose (1 mM) in the presence of individual HPLC fractions in natural concentration ratios prepared from *Gymnema sylvestre* fraction II-2.

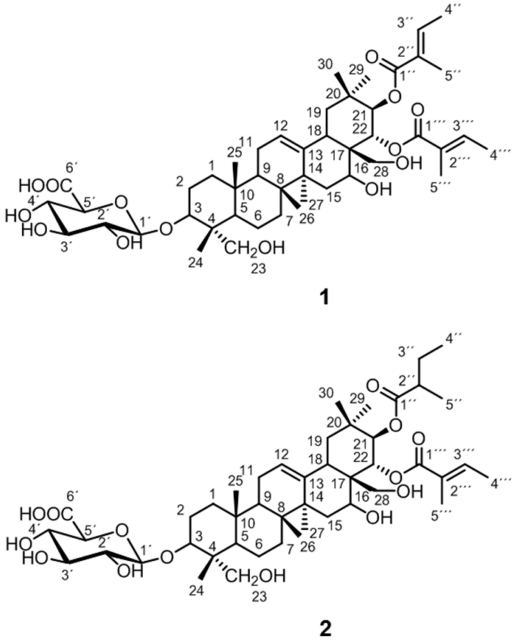

Figure 5. Chemical structures of hSGLT inhibitors gymnemic acid V (**1**) and gymnemic acid XV (**2**).

[C(4′)], 74.5 [C(22)], 75.4 [C(2′)], 76.6 [C(21)], 77.8 [C(5′)], 78.1 [C(3′)], 81.7 [C(3)], 106.2 [C(1′)], 123.8 [C(12)], 128.9 [C(2″)], 128.9 [C(2‴)], 137.7 [C(3″)], 137.9 [C(3‴)], 141.5 [C(13)], 167.5 [C(1‴)], 167.8 [C(1″)], 173.0 [C(6′)] (*, may be interchangeable).

*Gymnemic acid XV, **2**, Figure 5:* LC-MS (ESI$^-$), *m/z* 847 (100, [M − H]$^-$); MS/MS (DP = −54 V), *m/z* (%) 847 (100), 175 (4), 157 (4), 639 (2); LC-TOF-MS, *m/z* 847.4842 ([M − H]$^-$, measured), *m/z* 847.4844 ([M − H]$^-$, calcd for $C_{46}H_{71}O_{14}$); ^{1}H NMR (500 MHz, pyridine-d_5; COSY, *J*-RESOLVED, ROESY), δ 0.83−0.98 [m, 4H, H−C(1a,4″)], 0.89 [s, 3H, H−C(25)], 0.92 [s, 3H, H−C(26)], 0.95 [s, 3H, H−C(24)], 0.99 [s, 3H, H−C(29)], 1.11 [d, 1H, *J* = 7.1 Hz, H−C(5″)], 1.16−1.40 [m, 3H, H−C(6a,7a,19a)], 1.23 [s, 3H, H−C(30)], 1.30 [s, 3H, H−C(27)], 1.39−1.50 [m, 2H, H−C(1b,3″a)], 1.52 [d, 3H, *J* = 7.0 Hz, H−C(4‴)], 1.55−1.60 [m, 1H, H−C(15a)], 1.61−1.78 [m, 4H, H−C(5,6b,7b,9)], 1.73−1.90 [m, 6H, H−C(11,3″b,5‴)], 1.95−2.10 [m, 2H, H−C(2a,15b)], 2.13−2.28 [m, 1H, H−C(19b)], 2.27−2.35 [m, 1H, H−C(2b)], 2.41 [sex., 1H, *J* = 6.9 Hz, H−C(2″)], 3.06 [dd, 1H, *J* = 4.0 Hz, 14.2 Hz, H−C(18)], 3.70 [d, 1H, *J* = 10.8 Hz, H−C(23a)], 4.00 [d, 1H, *J* = 11.3 Hz, H−C(28a)], 4.10−4.18 [m, 1H, H−C(2′)], 4.18−4.27 [m, 1H, H−C(3′)], 4.20 [d, 1H, *J* = 11.3 Hz, H−C(28b)], 4.28−4.40 [m, 2H, H−C(3, 23b)], 4.51−4.62 [m, 2H, H−C(4′, 5′)], 4.96−5.06 [m, 1H, H−C(16)], 5.25 [d, 1H, *J* = 7.4 Hz, H−C(1′)], 5.32−5.43 [m, 1H, H−C(12)], 5.73 [d, 1H, *J* = 12.0 Hz, H−C(21)], 6.27 [d, 1H, *J* = 12.0 Hz, H−C(22)], 7.07−7.14 [m, 1H, H−C(3‴)]; ^{13}C NMR (125 MHz, pyridine-d_5; HSQC, HMBC), δ 11.8 [C(4″)], 12.3 [C(5‴)], 13.6 [C(24)], 14.2 [C(4‴)], 16.2 [C(25)], 16.7 [C(5″)], 16.9 [C(26)], 18.0 [C(6)], 19.8 [C(30)], 23.9 [C(11)], 26.0 [C(2)], 27.0 [C(3″)], 27.4 [C(27)], 29.2 [C(29)], 32.5 [C(7)], 36.7* [C(15)], 36.8 [C(20)], 36.9* [C(10)], 38.7 [C(1)], 40.2 [C(8)], 41.6 [C(2″)], 42.6 [C(18)], 43.5 [C(4)], 43.5 [C(14)], 45.8 [C(19)], 47.1 [C(9)], 47.4 [C(5)], 48.1 [C(17)], 60.0 [C(28)], 64.4 [C(23)], 66.9 [C(16)], 73.5 [C(4′)], 74.3 [C(22)], 75.4 [C(2′)], 76.1 [C(21)], 77.8 [C(5′)], 78.1 [C(3′)], 81.7 [C(3)], 106.1 [C(1′)], 123.8 [C(12)], 128.9 [C(2‴)], 138.4 [C(3‴)], 141.5 [C(13)], 167.4 [C(1‴)], 173.0 [C(6′)], 176.2 [C(1″)] (*, may be interchangeable).

Medium-Pressure Liquid Chromatography (MPLC). MPLC separation of the polar *Gymnemna sylvestre* extract was performed on a Sepacore chromatography system (Buchi, Flawil, Switzerland) consisting of two C-605 pumps with a C-615 pump manager, a manual Rheodyne injection port (20 mL loop), and a C-660 fraction collector. Data acquisition was performed by means of Sepacore Control 1.0 software (Buchi). Chromatographic separation was performed using PP cartridges (i.d. = 40 mm, l = 150 mm) and LiChroprep RP18, 25−40 μm, mesh material (Merck). Operated at a flow rate of 40 mL/min, the following gradient was used for chromatography: Starting with pure water (for 10 min) (water washing fraction), the methanol content was increased to 100% within 2 min and kept at 100% for 12 min (methanol/water fraction). Both fractions were individually collected; the solvent was removed using a Genevac Rocket evaporation system (Genevac Limited) and freeze-dried twice.

High-Performance Liquid Chromatography (HPLC). For analytical as well as for preparative analyses of fraction B, the HPLC apparatus (Jasco, Groß-Umstadt, Germany) was equipped with two PU-2087 Plus pumps, an AS-2055 Plus autosampler, a Sedex LT-ELSD detector model 85 (Sedere, Alfortville, France; split ratio was set to 1 mL/min for the ELSD; gain = 7), and a Rh 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany). Data acquisition was performed by means of Chrompass 1.8.6.1 software (Jasco). Chromatographic separation was performed on a 250 × 21 mm i.d., 5 μm, NUCLEODUR C18 Pyramid column (Macherey-Nagel, Düren, Germany) operated with a flow rate of 20 mL/min. Using 0.1% formic acid in water (v/v) as solvent A and 0.1% formic acid in acetonitrile (v/v) as solvent B, chromatography was performed with the following gradient: 0 min, 30% B; 5 min, 30% B; 15 min, 40% B; 40 min, 40% B;

41 min, 45% B; 60 min, 45% B; 61 min, 65% B; 70 min, 65% B; 71 min, 100% B; 76 min, 100% B; 77 min, 30% B; 82 min, 30% B.

Liquid Mass Spectrometry (LC-MS). An API 4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany) with direct flow infusion was used to acquire electrospray ionization (ESI) mass spectra and product ion spectra. The MS system was operated in the full-scan mode detecting negative ions at an ion spray voltage at −4500 V. The MS/MS parameters were tuned for each compound to record fragmentation of the $[M - H]^-$ molecular ions into specific product ions. Data acquisition and instrumental control were performed with Analyst 1.4.2 software (AB Sciex).

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra of the target substances were measured on a SYNAPT G2 HDMS (Waters UK Ltd., Manchester, UK) in the negative ESI and resolution modus with the following parameters: capillary voltage, 2.8 kV; sampling cone, 30; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas, 5.0 L/h; and desolvation gas, 850 L/h. All saponins were dissolved in 1 mL water/methanol (90:10, v/v), and aliquots (1−5 μL) were injected into the UPLC/TOF-MS system. The samples were introduced into the instrument via an Acquity UPLC core system (Waters, Milford, MA, USA) consisting of a binary solvent manager, a sample manager, and a column oven. For chromatography, a 2 × 150 mm i.d., 1.7 μm, BEH C18 column (Waters) was operated with a flow rate of 0.4 mL/min at a temperature of 40 °C. The solvent system consisted of acetonitrile (A) and aqueous formic acid (0.1% in water, pH 2.5; B). The following gradient was used: 0 min, 50% B; 2 min, 100% B. The instrument was calibrated over a m/z range of 100−1200 using a solution of sodium formate (0.5 mM) in a 2-propanol/water mixture (90:10, v/v). All data were lock mass corrected using leucine enkephaline as the reference (m/z 556.2771, $[M + H]^+$). Data acquisition and interpretation were performed by using MassLynx software (version 4.1; Waters) and the tool "elemental composition".

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D and 2D NMR spectra were acquired on a Bruker 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany). 2D NMR experiments COSY, TOCSY, *J*-RESOLVE, HMQC/HSQC, DEPT, ROESY, and HMBC were carried out using the pulse sequences taken from the Bruker software library. Pyridine-d_5 was used as solvent, and chemical shifts are reported in parts per million relative to the pyridine-d_5 solvent signals (^{1}H NMR δ 7.19, 7.55, and 8.71; ^{13}C NMR δ 123.5, 135.5, and 149.5). Data processing was performed by using Topspin NMR software (version 3.1 and 2.1; Bruker) and Mestre-C (Mestrelab Research, A Coruña, Spain).

Statistical Analysis. Processing of raw data was done using Excel 2010 (Microsoft Corp.). Quantitative data are expressed as mean ± standard deviation unless otherwise noted. IC_{50} values were determined using Prism 6 software (GraphPad, La Jolla, CA, USA). A nonlinear regression was calculated using the log dose−response variable slope model.

RESULTS AND DISCUSSION

For identification of potent SGLT1 inhibitors from hypoglycemic botanicals used in traditional Chinese medication, a two-electrode voltage-clamp screening was used that records electronic glucose transport in *Xenopus laevis* oocytes microinjected with the cRNA for hSGLT1.

Functionality Testing. After the membrane voltage had been set to −60 mV, transport currents generated by SGLT1 were determined for glucose, fructose, and galactose (5 mM each) as well as the SGLT1 inhibitor phlorizin (Figure 1). As expected, neither application of fructose nor application of glucose in sodium-free solution caused any current, whereas a current was recorded when glucose or galactose was applied in the presence of sodium ions. These data confirmed the sodium dependency as well as the sugar specificity of SGLT1 as a selective glucose and galactose transporter. In the presence of only 5 μM phlorizin, glucose-mediated currents were significantly reduced, thus confirming the inhibitory activity of this polyphenol.[25]

Screening of Botanicals for SGLT1 Inhibition. A total of 26 Chinese medical herbs (H1−H26) were extracted with *n*-pentane, followed by methanol/water and, after removal of the solvents in a vacuum, the pentane extractables (fraction I) as well as the methanol extractables (fraction II) were used in the SGLT1-based screening. Whereas none of the pentane extracts showed any effect in the assay (data not shown), distinct SGLT1 inhibitory activities were found for some of the methanol-soluble fractions II (Figure 2). A total of nine botanical extracts increased SGLT1 activity and glucose transport, among which wolfberry (*Lycium chinense* Mill.; H3) and *Cornus officinalis* Siebold & Zucc. (H21) showed the highest activity by increasing the current by >30%. In comparison, 14 extracts induced a significant decrease in cellular sugar transport; the highest activity was found for *Gymnema sylvestre* (Retz.) Schult (H24), showing a 48% SGLT1 inhibition, followed by Digupi (*Lycium barbarum* L.; H7), Yin yang huo (*Epimedium sagittatum* Maxim; H5), and *Epimedium sagittatum* Maxim (H19) with somewhat lower SGLT1 inhibitory activities between 25 and 30%. As the leaves of *Gymnema sylvestre* R. Schult were found to show the highest SGLT1 inhibitory activity (Figure 2) and to exhibit hypoglycemic activities in diabetic subjects,[26−32] this botanical was selected for isolation of phytochemicals.

Activity-Guided Identification of SGLT1 Inhibitors in *Gymnemna sylvestre*. To identify the SGLT1 inhibitors, fraction II prepared from *Gymnema sylvestre* was separated by means of MPLC using water (fraction II-1) followed by methanol (fraction II-2) as the solvent. After lyophilization, fractions II-1 and II-2 were both used for the SGLT1 bioassay. Fraction II-1 did not exhibit any significant activity, but fraction II-2 showed SGLT1 inhibition similar to the activity found for the total fraction II (data not shown), thus suggesting the active target molecules to be present in fraction II-2. The latter fraction was, therefore, further separated by means of silica gel chromatography to afford eight fractions, namely II-2/A−II-2/H, which were again tested for their SGLT1 inhibitory activity (Figure 3).

Subfraction II-2/B showed the highest SGLT1 inhibition rate of 80% and was, therefore, further fractionated by means of preparative RP-HPLC using an evaporative light scattering detector. A total of 28 HPLC fractions were collected, separated from solvent in a vacuum and, finally, used for analysis of their SGLT1 inhibitory activity in their natural concentration ratios (Figure 4). By far the highest SGLT1 inhibition rates of 45 and 48%, respectively, were found for HPLC fractions 22 and 24, followed by fractions 2, 4, 6, 8−11, 13, 15, 18, 20, 21, and 23, all of which exhibited inhibition rates >5%. With the aim of determining the molecular structure of the molecules imparting the most intense SGLT1 inhibitory activity, LC-MS/MS, UPLC-TOF-MS, and 1D/2D-NMR experiments focused on the phytochemicals isolated from HPLC fractions 22 and 24 in semipreparative scale.

LC-MS(ESI$^-$) analysis of compound **1** isolated from HPLC fraction 22 revealed an intense pseudomolecular ion ($[M - H]^-$) with m/z 845, thus suggesting a molecular mass of 846 Da. This was confirmed by LC-TOF-MS indicating an empirical formula of $C_{46}H_{70}O_{14}$. Additional LC-MS/MS experiments led to the identification of the daughter ion m/z 669 $[M - 176 - H]^-$ and indicated the cleavage of one galacturonic acid moiety as expected for gymnema saponins.[33]

For further structure elucidation and NMR signal assignment, 1D/2D-NMR experiments including COSY, DEPT, TOCSY, *J*-RESOLVED, ROESY, HSQC, and HMBC were performed. The ^{13}C NMR spectrum showed a total of 46 signals for compound **1** resonating between 12.3 and 173.0 ppm, among which 30 signals were well in alignment with the aglycone structure of gymnemagenin.[33−35] After identification of the aglycone of **1**, the following spectroscopic experiments were focused on the structure determination of the intact saponin. As expected for tiglic acid ester moieties, the HMBC experiment revealed two long-range couplings between proton H−C(21) and H−C(22) resonating at 5.80 and 6.34 ppm and the carbon atoms C(1″) and C(1‴) observed at 167.8 and 167.5 ppm, which showed heteronuclear connectivity with the methyl protons H−C(5″) and H−C(5‴) at 1.76−1.86 ppm. In addition, 1D/2D-NMR spectroscopic experiments led to the identification of an anomeric proton resonating at 5.25 ppm. This proton signal showed a large coupling constant of 7.9 Hz, thus indicating a *β*-configuration of the galacturonic acid moiety. The full assignment of the carbohydrate moieties as well as their linkage alignment was achieved by means of COSY, TOCSY, and HMBC experiments; for example, the HMBC spectrum showed long-range correlations between H−C(1′) and C(3). With all of these findings taken into account, the phytochemical in fraction 22 was identified as 3-*O*-*β*-D-glucuronopyranosyl-21-*O*-2-tigloyl-22-*O*-2-tigloyl gymnemagenin, known as gymnemic acid V (**1**, Figure 5). Although the structure of **1** is known in the literature,[34−36] the SGLT1 inhibitory activity of this saponin has not yet been reported.

Separation of fraction 24 by means of semipreparative HPLC revealed the SGLT1 inhibiting key phytochemical **2** in a purity of >98%, which showed a pseudomolecular ion ($[M - H]^-$) with *m*/*z* 847 in the LC-MS spectrum recorded by means of electrospray ionization. This was confirmed by LC-TOF-MS experiments indicating an empirical formula of $C_{46}H_{72}O_{14}$. Comparison of the ^1H, ^{13}C, homonuclear (COSY, TOCSY), and heteronuclear (HMSC, HMBC) as well as ROESY data of **2** with those of **1** confirmed a methylbuturyl residue instead of a tiglinoyl moiety attached to aglycone carbon C(22). Comparison of all spectroscopic data with those reported in the literature[33−36] led to the unequivocal identification of compound **2** as 3-*O*-*β*-D-glucuronopyranosyl-21-*O*-2-methylbutyryl-22-*O*-2-tigloyl gymnemagenin, known as gymnemic acid XV (Figure 5). The SGLT1 inhibitory activity of **2** has not been previously reported.

SGLT1 Inhibitory Activity of Gymnemic Acids V and XV. Prior to cell experiments, the purity of the test compounds was confirmed by HPLC-MS as well as ^1H NMR spectroscopy to be >98%. Recording of dose/response functions, followed by the determination of IC_{50} data through Graphpad (Graphpad Prism 6), revealed $LogIC_{50}$ values of −5.224 for gymnemic acid V (**1**) and −4.781 for gymnemic acid XV (**2**), thus corresponding to IC_{50} values of 5.97 *μ*M for gymnemic acid V and 0.17 *μ*M for gymnemic acid XV, the structures of which are differing by just by the tigloyl and methylbutyryl moieties at position 21 of **1** and **2**, respectively. Interestingly, the IC_{50} value of gymnemic acid XV (**2**) matched the low value of 0.21 *μ*M measured for the high-affinity SGLT1 inhibitor phlorizin in our oocyte assay (Table 1). The latter value was in the same range as found for phlorizin in other studies by means of a radioactive (0.11 *μ*M)[37] as well as a nonradioactive assay (0.17 *μ*M)[38] using COS-7 cells transiently expressing human hSGLT1, but was lower when compared to an IC_{50} value of 42 *μ*M found for phlorizin when using a stably transfected Chinese hamster vvary (CHO) cell assay.[39]

Table 1. IC_{50} Values for SGLT1 Inhibitory Activity of Phytochemicals

compound	IC_{50} value[a] (*μ*M)
gymnemic acid V (**1**)[b]	5.97 ± 0.52
gymnemic acid XV (**2**)[b]	0.17 ± 0.05
phlorizin	0.21 ± 0.03

[a]IC_{50} values were calculated from dose−response recordings. [b]Structures of compounds are given in Figure 5.

Although gymnemic acids have been reported to decrease blood glucose level in rats after an oral glucose load,[40] the mechanism of action was not yet clear. Using the hSGLT1 bioassay, this is the first report on gymnemic acids as high-affinity SGLT1 inhibitors, among which gymnemic acids V and XV were found as the key molecules with the strongest SGLT1 inhibitory activity in *Gymnema sylvestre*. As SGLT1 is found in high levels in brush-border membranes of intestinal epithelial cells, our findings demonstrate for the first time the potential of these saponins for the inhibition of electrogenic glucose uptake in the GI tract.

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Funding

We thank the Alexander von Humboldt Foundation for a postdoctoral fellowship.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Daniela Günzkofer for excellent technical assistance and Helene Prunkl for oocyte preparation. We acknowledge Kirsten Günther-Jordanland, Christian Schmid, and Dr. Oliver Frank for skillful acquisition of the NMR spectra.

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