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In vitro anti-rotavirus activity of polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*

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ABSTRACT

We evaluated the ability of six polyphenols isolated from the roots of *Glycyrrhiza uralensis* to inactivate rotaviruses, specially G5P[7] and G8P[7]. Upon finding that all polyphenols possessed anti-rotavirus activity, we evaluated whether these properties were attributable to direct inhibition of the binding of rotavirus to cells and/or to inhibition of viral replication. Using the virucidal assay, we found that all six compounds directly inhibited rotavirus binding, with activity being dependent on the type of virus. The 50% effective inhibitory concentrations (EC_{50}) of the six compounds were 18.7–69.5 μM against G5P[7] and 14.7–88.1 μM against G8P[7], respectively. Five of the six compounds inhibited hemagglutination activity. Moreover, the CPE inhibition assay showed that five compounds inhibited viral replication with EC_{50} values of 12.1–24.0 μM against G5P[7] and 12.0–42.0 μM against G8P[7], respectively. RT-PCR showed that the compounds suppressed viral RNA synthesis in TF-104 cells. Interestingly, the anti-rotavirus activities of four compounds were attributable to inhibition of both viral absorption and viral replication. These results suggest that compounds isolated from the roots of *G. uralensis* may be potent anti-rotavirus agents in vivo, acting by inhibiting both viral absorption and viral replication.

1. Introduction

Group A rotaviruses (GARV), members of the Reoviridae family, are major pathogens that cause severe, acute dehydrating diarrhea in young children and in a wide variety of domestic animals.[1–3] Rotaviruses infect virtually all children within the first five years of life and are responsible for about 611,000 deaths per year worldwide, mostly in developing countries.[4] The clinical signs of rotavirus infection include acute gastroenteritis with fever, vomiting, abdominal pain, diarrhea, dehydration, and rhinitis.[1] Although the anti-rotavirus vaccines Rotarix (GlaxoSmithKline) and Rota Teq (Merck) may prevent rotavirus infections in infants and young children, vaccine effectiveness in preventing rotavirus infections in populations at highest risk in developing countries remains to be assessed. The only known treatment for rotavirus gastroenteritis is replacement of fluids and electrolytes lost by vomiting and diarrhea.[1] However, patients with prolonged diarrhea may require enhancement of the antiviral immune response and virus-specific treatments.

Synthetic compounds, such as 1-β-D-ribofuranosyl-1,2,4,-triazole-3-carboxamide (ribavirin), 3-deazaguanine,[5] and isoprinosine,[6] have been shown to be active against simian rotavirus. Among natural compounds, theaflavins extracted from black tea have been found to inhibit human and bovine rotaviruses,[7] and flavonoids from *Citrus aurantium* are active against human rotavirus.[8] However, these compounds are not currently available for human or animal use, and diarrhea caused by rotaviruses thus remains uncontrolled. Thus, new compounds are urgently needed to control rotavirus infection.

Glycyrrhiza uralensis is a traditional medicinal herb grown in various parts of the world, and has long been used to treat fever, liver ailments, dyspepsia, constipation, gastric ulcers, sore throat, asthma, and bronchitis. In addition, the roots of this plant are used as flavoring and sweetening agents in tobacco, chewing gum, candies, and beverages.[9] The major bioactive components of *G. uralensis* roots are flavonoids and pentacyclic triterpene saponins, including liquiritin, isoliquiritigenin, liquiritin apioside, glycyrrhizin, and glycyrrhizic acid.[10] These constituents of *G. uralensis* have been found to exhibit anticancer, anti-diabetic, anti-inflammatory, anti-malarial, anti-bacterial, antioxidant, and estrogenic properties.[11,12] Also, isolated glycyrrhizin and derivatives thereof have shown activity against a variety of viruses, including herpes simplex type 1 (HSV-1);[13] hepatitis A, B, and C viruses (HAV, HBV, and HCV);[14] HIV-1;[15] and influenza viruses.[16] In contrast, the

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anti-rotavirus activities of polyphenols isolated from the plant have not previously been tested. We therefore evaluated the ability of six compounds isolated from *G. uralensis* to inhibit porcine and bovine rotaviruses. We also tested the mechanisms of action of the compounds, by determining whether they directly inhibit rotavirus binding to cells and/or inhibit viral replication.

2. Results and discussion

We isolated six naturally occurring compounds from the ethanol extract of *G. uralensis* rhizomes. Chemical structures were determined based on comprehensive spectral analysis using 1D- and 2D-NMR. The six isolated compounds **1–6** were identified as licocoumarone (**1**), glyasperin C (**2**), 2′-methoxyisoliquiritigenin (**3**), glycyrin (**4**), licoflavonol (**5**), and glyasperin D (**6**) (Fig. 1).[17] We hypothesized that antiviral compounds isolated from *Glycyrrhiza* would act via two pathways: (1) blockage of virus absorption to cells, and (2) inhibition of viral replication after entry. Time-of-addition experiments were performed to determine the stage at which polyphenols from *G. uralensis* exerted inhibitory activities. Compounds were incubated with TF-104 cells at two distinct time points: for 1 h at 4 °C prior to virus infection (virucidal assay), and at 1 h after virus entry (CPE inhibition assay).

At first, we utilized the virucidal assay to identify compounds blocking viral adsorption to cells (Fig. 2A). Ethanol extracts of *G. uralensis* rhizomes showed anti-rotavirus activity with EC_{50} value of 24.5 μg/mL against bovine rotavirus G8P[7] (KJ56-1; SI = 15.0) and 22.4 μg/mL against porcine rotavirus G5P[7] (KJ205-1; SI = 16.4). Therefore, cells were incubated with various concentrations of each compound and subsequently incubated with two kinds of bovine rotavirus G8P[7] and porcine rotavirus G5P[7] for 1 h. We found that isolated polyphenols **1**, **2**, and **4–6**, except for compound **3**, inhibited bovine rotavirus (G8P[7]) with EC_{50} values ranging from 14.7 to 88.1 μM, as shown in Table 1. Glyasperin C (**2**), licoflavonol (**5**), and glyasperin D (**6**) inhibited G8P[7] absorption with EC_{50} values of 14.7, 15.5, and 17.6 μM, respectively (SI = 2.9, 5.5, and 5.7, respectively). When evaluated biological activity against G5P[7], the chalcone derivative **3** inhibited virus absorption with an EC_{50} value of 69.5 μM (SI = 1.9), and the other isolated polyphenols (**1**, **2**, and **5**) had EC_{50} values of 26.1, 18.7, and 36.8 μM, respectively. As a result, the flavans (**2** and **6**) and flavone (**5**) were more potent at inhibiting virus absorption than those of phenylbenzofuran (**1**) and coumarin (**4**) against bovine rotavirus G8P[7]. In contrast, glycyrin (**4**) and glyasperin D (**6**), with methoxy group substitutions at the C-7 position, had no effect against porcine rotavirus G5P[7].

We found that compounds **1**, **2**, and **4**–**6** completely abrogated virus infectivity, with compounds **2** and **5** inhibiting both G5P[7] and G8P[7] in the virucidal assay. Hence, these compounds may act by interfering with the binding of virus particles to the sialic acid receptor at the cell surface. To extend these experiments, we used HI assays to determine whether the compounds inhibited

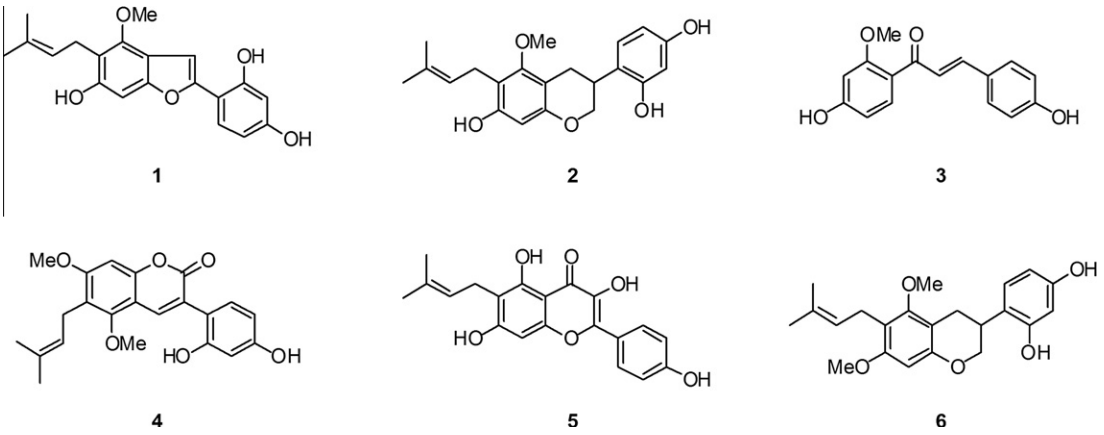

Figure 1. Chemical structures of compounds **1**–**6** isolated from the roots of *Glycyrrhiza uralensis*.

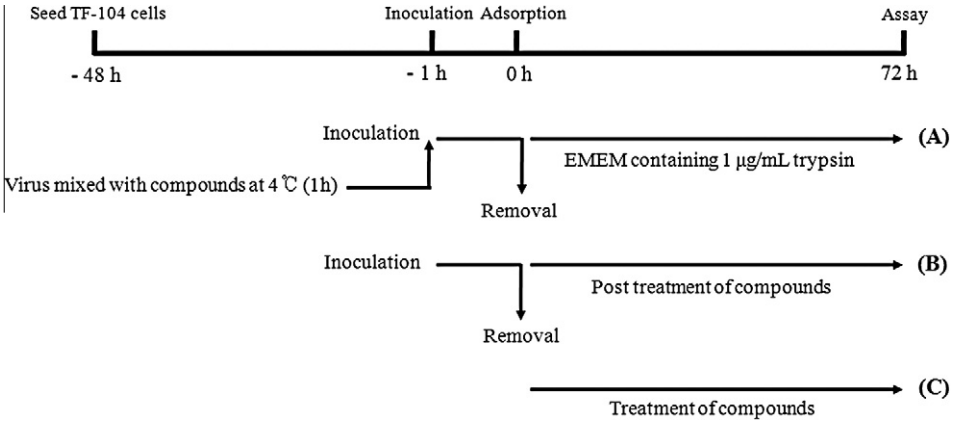

Figure 2. Anti-rotaviral assay strategies with compounds of *Glycyrrhiza uralensis*. Porcine (G5P[7]) and bovine (G8P[7]) rotavirus inoculation after virus incubation with compounds at 4 °C for 1 h in virucidal assay (A), post treatment of compounds after viral infection in CPE inhibition assay (B), and serial dose treatment of compounds in cytotoxicity assay (C).

Table 1
In vitro anti-rotavirus activities of EtOH extract and compounds **1**–**6** against KJ56-1 (bovine rotavirus, G8P[7]) and KJ205-1 (porcine rotavirus, G5P[7]) on TF-104 cells using the virucidal assay and CPE inhibition assay

Extract or compound	CC_{50}[a] (μM)	Virucidal assay				CPE inhibition assay			
		KJ56-1 (G8P[7])		KJ205-1 (G5P[7])		KJ56-1 (G8P[7])		KJ205-1 (G5P[7])	
		EC_{50}[b] (μM)	SI[c]	EC_{50}[b] (μM)	SI[c]	EC_{50}[b] (μM)	SI[c]	EC_{50}[b] (μM)	SI[c]
EtOH extract[d]	368.6	24.5	15.0	22.4	16.4	14.3	25.8	27.4	13.5
Licocoumarone (**1**)	54.5	37.8	1.4	26.1	2.1	22.3	2.5	43.6	1.3
Glyasperin C (**2**)	42.8	14.7	2.9	18.7	2.3	12.0	3.6	12.1	3.6
2′-Methoxyisoliquiritigenin (**3**)	>133.3	–	–	69.5	1.9	41.8	>3.2	24.0	5.6
Glycyrin (**4**)	>133.3	88.1	1.5	–	–	>100	–	–	–
Licoflavonol (**5**)	85.3	15.5	5.5	36.8	2.3	25.9	3.3	–	–
Glyasperin D (**6**)	68.1	17.6	5.7	–	–	42.0	1.6	–	–
Genistein	174.5	–	–	–	–	99.6	1.8	–	–

[a] CC_{50}: mean (50%) value of cytotoxic concentration.
[b] EC_{50}: mean (50%) value of effective concentration.
[c] SI: selective index, CC_{50}/EC_{50}.
[d] EtOH extract: μg/mL.

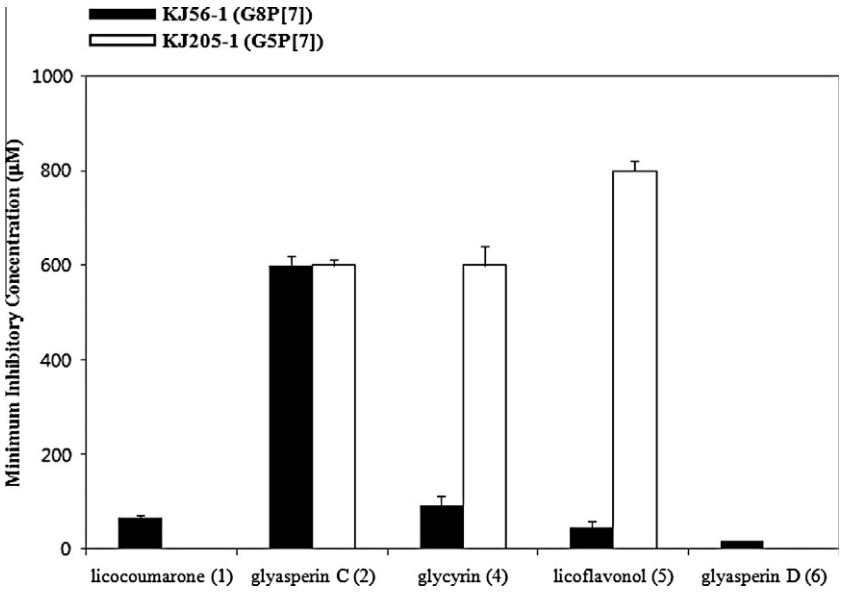

Figure 3. Hemagglutination inhibitory activity of compounds **1**, **2**, and **4**–**6**. Four HAU of porcine (G5P[7]) and bovine (G8P[7]) rotavirus were incubated with two-fold dilutions of compounds **1**, **2**, and **4**–**6** or PBS (negative control), and human RBC for 1 h at room temperature. The minimum concentration of each compound that completely inhibited viral adsorption was determined.

rotavirus-induced hemagglutination. Rotaviruses can bind to O-type hRBCs, resulting in hemagglutination. Incubation of 4 HAU of rotavirus with various concentrations of the six compounds 1–6 for 1 h at room temperature showed that compounds **1**, **2**, and **4**–**6** completely inhibited viral adsorption onto hRBCs at concentrations of 17.2–800 μM, depending on virus type (Fig. 3). Compound **6** has proved to be the most effective with minimum inhibitory concentration of 17.2 μM against KJ56-1 (G8P[7]), but ineffective against KJ205-1 (G5P[7]). Also, licocoumarone (**1**) inhibited viral absorption of G8P[7] but not G5P[7]. Glyasperin C (**2**), glycyrin (**4**), and licoflavonol (**5**) were effective against both G8P[7] and G5P[7] but showed low inhibitory effect against G5P[7]. Especially, glycyrin (**4**) and licoflavonol (**5**) showed 6–17-fold greater activity against G8P[7] than against G5P[7]. Interestingly, the HI activity of compound **2** against both G5P[7] and G8P[7] was 40-fold lower than was the activity shown by the virucidal assay. In contrast, compound **1**, which exhibited a weak anti-rotavirus effect on the virucidal assay, showed high activity against G8P[7] on the HI assay. These results suggest that compounds **1**, **2** and **4**–**6** may interfere with a very early stage of viral infection, possibly by interacting directly with the virus particle.

Using the CPE inhibition assay, we tested the ability of the six compounds **1**–**6** to inhibit replication of rotaviruses G8P[7] and G5P[7] in TF-104 cells (Fig. 2B). In preliminary experiments, ethanol extracts of *G. uralensis* were found to inhibit viral replication, with an EC_{50} value of 14.3 μg/mL against G8P[7] (SI = 25.8) and an EC_{50} value of 27.4 μg/mL against G5P[7] (SI = 13.5). Especially, flavan derivative **2** exhibited both rotaviruses G8P[7] and G5P[7] with EC_{50} values of 12.0 and 12.1 μM, respectively. Also, compounds **1** and **3** having benzopyren and chalcon moieties showed moderate activities with EC_{50} values of 22.3 and 41.8 μM against G8P[7] and EC_{50} values of 43.6 and 24.0 μM against G5P[7], respectively. Also, compounds **5** and **6** exhibited low activities against G8P[7], however, had no detectable effect on G5P[7]. When we utilized immunofluorescence assay to investigate the inhibition of virus replication by glyasperin C (**2**), we observed green fluorescence in early virus-infected (Fig. 4B and E), but not in mock-infected, TF-104 cells (Fig. 4A and D). However, treatment of cells with 30 μM glyasperin C (**2**) reduced the number of fluorescence-positive cells infected with G8P[7] and G5P[7] (Fig. 4C and F). As results, compound (**2**) exerts antiviral activity by two mechanisms, blockage of viral attachment and virus replication. But compound

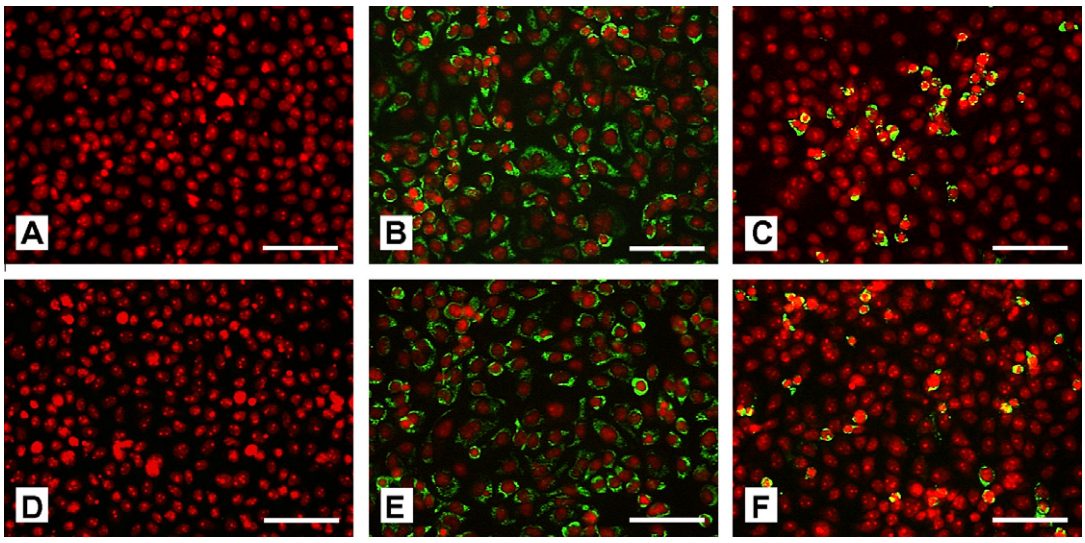

Figure 4. Immunofluorescence assay of the anti-rotavirus effects of representative compound **2**. TF-104 cells were grown on 8-well chamber slides and were infected with rotaviruses (G8P[7] and G5P[7]) at 0.01 MOI for 1 h. The solution was removed and replaced by EMEM containing 1 μg/mL trypsin and 30 μM of a compound **2**. (A and D) Mock-inoculated TF-104 cells, showing no immunofluorescence reaction. (B) Bovine rotavirus (G8P[7])-infected TF-104 cells without compound **2**, showing many positive cells (green). (C) Bovine rotavirus (G8P[7])-infected TF-104 cells with compound **2**, showing fewer positive cells than appear in (B). (E) Porcine rotavirus (G5P[7])-infected TF-104 cells without compound **2**, showing many positive cells (green). (F) Porcine rotavirus (G5P[7])-infected TF-104 cells with compound **2**, showing fewer positive cells than appear in (E). Bars: A–F, 100 μm.

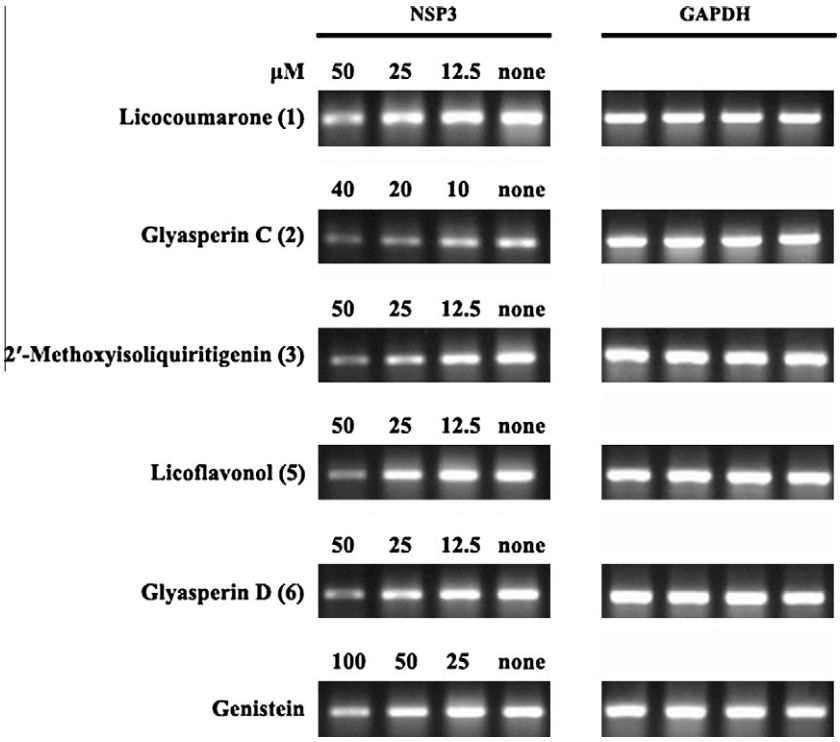

Figure 5. Effect of compounds **1**–**3**, **5** and **6** on rotavirus RNA synthesis in infected cells, as shown by RT-PCR analysis. Confluent cells were infected with rotavirus (G8P[7]) at 0.01 MOI. After 1 h, the solution was removed and replaced by EMEM containing 1 μg/mL trypsin and compounds **1**–**3**, **5** and **6** at various concentrations. Cells were harvested 18 h after infection, and total RNA (cellular and viral) was isolated from cell pellets. RT-PCR was performed using primers specific for viral RNA (NSP3) and cellular RNA (GAPDH).

(**2**) showed stronger inhibitory activity, virus replication step than virus binding step.

As viral mRNA synthesized in mid-stage, their syntheses were compared between drug-treated and untreated infected cells. RNA extraction was performed at 18 h after rotavirus infection and RT-PCR analysis was performed using primers specific for viral NSP3. Cells infected with rotaviruses G8P[7] and cultured in the absence of any isolated compound showed expression of viral mRNAs (Fig. 5). In contrast, the compounds **1**–**3** and **5**–**6** dramatically decreased viral mRNA synthesis at high concentration (40–50 μM). GAPDH mRNA signals from the same sample revealed no significant difference between the drug-untreated and -treated cells (Fig. 5). These findings indicate that compounds **1**–**3** and **5**–**6** decrease the synthesis of viral mRNA.

Collectively, our results show that the anti-rotavirus effects of the compounds were dependent both on the chemical skeletons and substituents, and the type of rotavirus. For example, licocoumarone (**1**), licoflavonol (**5**), and glyasperin D (**6**) were more active against G8P[7] than G5P[7]. In contrast, the chalcone derivative 2′-methoxyisoliquiritigenin (**3**) had a twofold higher activity against G5P[7] compared to G8P[7]. In the CPE inhibition assay, compound **6** showed no activity, a somewhat surprising result. This is similar to the demonstrated virucidal assay activity, in that a methoxy motif at C-7 in the B-ring had a very negative effect on anti-rotavirus activity.

Ribavirin and interferon both showed anti-rotavirus activity, and dipyridamole has been reported to possess antiviral activity against representatives of different virus families (e.g., Picornaviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, Herpesviridae, and Poxviridae).[5,18–20] Cimentidine and famodine not only have anti-HIV activity, but also exhibit therapeutic effects against herpes simplex virus infection.[21,22] Although these drugs act by inhibiting rotavirus replication and adsorption, any side-effects have not been examined in clinical studies. Although immunoglobulins have also been used to treat rotavirus diarrhea,[23–25] these drugs are very costly and side-effects are uncertain. Antiviral drugs from natural sources, including *G. uralensis*, marine sponges, soy, and *Stevia rebaudiana* may be ideal candidates because such drugs are less toxic, have fewer side-effects, and are more effective and less costly than are commercially available anti-rotavirus agents.[26–28] We have shown here that compounds isolated from *G. uralensis* may be superior anti-rotavirus agents compared with other known chemical synthetic compounds. We are now attempting to determine the mechanisms by which these compounds act against rotaviruses.

3. Materials and methods

3.1. Isolation of polyphenols from *G. uralensis*

Dried rhizomes of *G. uralensis* (8.8 kg) were purchased from a local market in Jeongeup, Korea, pulverized to a fine powder, and extracted with MeOH for one week at room temperature. The MeOH extract was concentrated on a rotary evaporator, and the dried extract (590 g) was dissolved in distilled water and sequentially extracted with organic solvents in order of increasing polarity: hexane (5.5 g), chloroform (180 g), and ethyl acetate (140 g). The chloroform layer was chromatographed on silica gel using mixtures of hexane/EtOAc of increasing polarity (100:0→0:100), yielding nine fractions (C1–C9). Fraction C8 (20.7 g) was purified on silica gel with hexane/EtOAc (100:0→0:100) to yield five subfractions. Subfraction C8.3 (6.8 g) was chromatographed on RP-18 with MeOH to yield four subfractions. Sub-subfraction C8.3.2 (1.2 g) was subjected to passage over RP-18 with MeOH/H_2O (100:0→0:100) to yield compound **2** (13 mg). The ethyl acetate fraction (140 g) was chromatographed over silica gel and eluted in a step gradient with $CHCl_3$/MeOH (100:0→0:100) to yield eight fractions (E1–E8). Fraction E1 (15.9 g) was again chromatographed over silica gel ($CHCl_3$/MeOH, 100:0→0:100) to yield six subfractions. Subfraction E1.3 (10.0 g) was rechromatographed using the same solvent gradient to yield five sub-subfractions. Sub-subfraction E1.3.4 (1.0 g) was subjected to passage over RP-18 with ACN/H_2O (1:1) to yield compound **4** (7 mg). Compounds **1** (20 mg) and **5** (5 mg) were purified from sub-subfraction E1.3.5 (1.2 g) on Sephadex LH-20, using MeOH. Subfraction E3.4 (13.0 g) was chromatographed on Sephadex LH-20 with MeOH to yield four sub-subfractions. Subfraction E1.4. (10.0 g) was chromatographed on RP-18 with ACN/H_2O (1:1) to yield compound **3** (11 mg). Sub-subfraction E3.4.1 (500 mg) was purified on Sephadex LH-20 with MeOH to yield compound **6** (5 mg).

Licocoumarone (***1***). Obtained as yellow needles; mp 179–181 °C; LC–MS *m/z* 339 $[M-H]^+$; ^1H NMR (500 MHz, acetone-d_6) δ 1.51, 1.65 (each 3H, s, *gem*-dimethyl at C10, H-11, H-12), 3.27 (2H, d, J = 7.09 Hz, H-8), 5.13 (1H, t, H-9), 6.36 (1H, dd, J = 2.32, 8.55 Hz, H-5′), 6.42 (1H, d, J = 2.30 Hz, H-3′), 6.63 (1H, s, H-7), 7.16 (1H, d, J = 0.66 Hz, H-3), 7.55 (1H, d, J = 8.55 Hz, H-6′).

Glyasperin C (***2***). Obtained as a colorless amorphous; LC–MS *m/z* 355 $[M-H]^+$; ^1H NMR (500 MHz, methanol-d_4) δ 1.50 (3H, s, H-13), 1.59 (3H, s, H-12), 2.60 (1H, dd, J = 15.9, 10.79 Hz, H-4a), 2.71 (1H, m, H-4b), 3.08 (2H, m, H-9), 3.19 (1H, m, H-3), 3.51 (OCH_3), 3.76 (1H, t, J = 10.11 Hz, H-2a), 4.01 (1H, m, H-2b), 5.04 (1H, t, J = 1.24, 2.48 Hz, H-10), 5.93 (1H, s, H-5), 6.10 (1H, dd, J = 2.42, 8.30 Hz, H-5′), 6.16 (1H, d, J = 2.40 Hz, H-3′), 6.72 (1H, d, J = 8.3 Hz, H-6′).

2′-Methoxyisoliquiritigenin (***3***). Obtained as a yellowish solid; mp 89–92 °C; LC–MS *m/z* 269 $[M-H]^+$; ^1H NMR (500 MHz, methanol-d_4) δ 3.78 (3H, s, OMe), 6.37 (2H, m, H-3, H-5), 6.78 (2H, H-3′, H-5′), 7.49 (2H, H-6, H-β), 7.86 (2H, H-2′, H-6′), 7.87 (1H, d, J = 15.0 Hz, H-α).

Glycyrin (***4***). Obtained as colorless needles; mp 197–202 °C; LC–MS *m/z* 381 $[M+H]^+$; ^1H NMR (500 MHz, DMSO-d_6) δ 1.63 (3H, s, H-12), 1.73 (3H, s, H-13), 3.28 (2H, s, J = 7.15 Hz, H-9), 3.77 (3H, s, OMe), 3.88 (3H, s, OMe), 5.10 (1H, br t, H-10), 6.28 (1H, dd, J = 2.6, 8.3 Hz, H-5′), 6.37 (1H, d, J = 2.6 Hz, H-3′), 6.88 (1H, s, H-8), 7.13 (1H, d, J = 8.6 Hz, H-6′), 7.85 (1H, s, H-4).

Licoflavonol (***5***). Obtained as a pale yellow amorphous; mp 120–130 °C; LC–MS *m/z* 353 $[M-H]^+$; ^1H NMR (500 MHz, acetone-d_6) δ 1.66, 1.78 (each 3H, s, *gem*-dimethyl at C11, H-12, H-13), 3.31 (2H, m, H-9, overlap with methanol peak), 5.24 (1H, m, H-10), 6.42 (1H, s, H-8), 6.89 (2H, s, H-2′, H-6′), 8.07 (2H, s, H-3′, H-5′).

Glyasperin D (***6***). Obtained as a colorless amorphous; LC–MS *m/z* 369 $[M-H]^+$; ^1H NMR (500 MHz, acetone-d_6) δ 1.63, 1.74 (each 3H, s, *gem*-dimethyl at C11, H-12, H-13), 2.80–2.97 (2H, m, H-4a, H-4b), 3.24 (2H, m, H-9), 3.40 (1H, m, H-3), 3.69 (C5–OCH_3), 3.76 (C7–OCH_3), 3.99–4.22 (2H, m, H-2a, H-2b), 5.16 (1H, t, H-10), 6.21 (1H, s, H-8), 6.33 (1H, dd, J = 2.3, 8.3 Hz, H-5′), 6.46 (1H, d, J = 2.3 Hz, H-3′), 6.97 (1H, d, J = 8.3 Hz, H-6′).

3.2. Cells and viruses

Fetal rhesus monkey kidney (TF-104) cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 U/mL amphotericin B.[29] The rotaviruses KJ56-1 (bovine rotavirus, G8P[7]) and KJ205-1 (porcine rotavirus, G5P[7]) were preactivated with 10 μg/mL trypsin (1:250; GIBCO Invitrogen Corporation, California) for 30 min at 37 °C before being inoculated onto confluent TF-104 cells and infected cells were maintained in the presence of 1 μg/mL trypsin (1:250; GIBCO Invitrogen). Prof. Kyoung Oh Cho (Chonnam National University, Korea) provided us viruses that were isolated from the fecal samples of Korean calf and pig with diarrhea.

3.3. Virucidal assay

Each compound was mixed with 0.01 multiplicity of infection (MOI) virus at various concentrations and incubated at 4 °C for 1 h. The mixture were inoculated onto near confluent TF-104 cell monolayers (1×10^5 cells/well) for 1 h with occasional rocking; each concentration of compounds was assayed in triplicate. The solution was removed. The cells washed two times using PBS and replaced with EMEM containing 1 μg/mL trypsin. The cultures were incubated for 72 h at 37 °C under 5% CO_2 atmosphere until the cells in the infected, untreated control well showed complete viral CPE as observed by light microscopy (Fig. 2A). The 50% effective concentration (EC_{50}) was calculated by regression analysis.

3.4. Cytopathic effect (CPE) inhibition assay

The CPE inhibition assay used in this study has been previously described.[30] Briefly, virus at a 0.01 MOI was inoculated onto near confluent TF-104 cell monolayers (1×10^5 cells/well) for 1 h with occasional rocking. The solution was removed and replaced by EMEM containing 1 μg/mL trypsin and various concentrations of test compounds; all chemicals were tested in triplicate. The cells were cultured for 72 h at 37 °C under 5% CO_2 atmosphere until cells in the infected, untreated control well showed complete viral CPE as observed by light microscopy. Neutral red solution was added to each well to 0.034% (w/v), and plates were incubated for 2 h at 37 °C in the dark. The neutral red solution was removed, cells were washed with PBS (pH 7.4), and destaining solution (1% glacial acetic acid, 49% H_2O and 50% ethanol) was added to each well (Fig. 2B). The plates were incubated in the dark for 15 min at room temperature, and the absorbance of each well at 540 nm was read using a microplate reader. The EC_{50} of each compound was calculated by regression analysis.

3.5. Hemagglutination inhibition (HI) assay

A hemagglutination inhibition assay was employed to assess the effects of compounds on viral adsorption to target cells. Standardized solutions of human red blood cells (hRBC, type O) were prepared according to the WHO manual 2002 (WHO, 2002). A 25 μL aliquot of rotavirus solution containing 4 HAU was mixed with 25 μL of serial dilutions (in PBS, pH 7.4) of each compound and incubated for 1 h at room temperature. Fifty microliters of a 1% (v/v) hRBC suspension was added and samples were incubated for 1 h at room temperature.

3.6. Cytotoxicity assay

TF-104 cells (1×10^5 cells/well) were grown in 96-well plates for 48 h. The medium was removed and replaced by medium containing serial dilutions of compounds under test. After incubation for 72 h, the medium was removed, 5 μL MTT (3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) solution was added to each well, and plates were incubated at 37 °C for 4 h. The supernatant was removed, and 100 μL 0.04 M HCl-isopropanol was added to each well to dissolve formazan crystals (Fig. 2C). Using a microplate reader, the absorbance of each well was measured at 540 nm. After subtracting background absorbance at 655 nm, the 50% cytotoxic concentration (CC_{50}) of each compound was calculated by regression analysis.

3.7. Immunofluorescence assay (IFA)

TF-104 cells were grown on 8-well chamber slides (LAB-TEK, NUNC, USA), and the monolayers were infected with rotavirus at 0.01 MOI for 1 h (post treatment). The infecting solution was removed and replaced by EMEM containing 1 μg/mL trypsin and 30 μM of a compound **2** under test. All cells were cultured for 24 h at 37 °C in a 5% CO_2 atmosphere, washed three times with PBS (pH 7.4), and fixed in 80% acetone solution for 15 min at room temperature. After three times washed with PBS (pH 7.4), cells were incubated at 37 °C for 1 h with monoclonal antibody against rotavirus VP6 protein (Santa Cruz, California) diluted 1:50 in PBS (pH 7.4). After washing with PBS (pH 7.4), cells were incubated at 37 °C for 1 h with FITC-conjugated goat anti-mouse IgG antibody (Santa Cruz) diluted 1:100 in PBS (pH 7.4). Cells were washed with PBS (pH 7.4), stained with 500 nM propidium iodide solution for 10 min at room temperature, and washed three times with PBS (pH 8.0). Slides were mounted using antifade reagent (Molecular Probes, Eugene, OR) and visualized under a fluorescence microscope (Leica, New York).

3.8. RT-PCR analysis

TF-104 cells were grown to about 90% confluence, infected with rotavirus at 0.01 MOI, and cultured in the presence of various concentrations of test compounds. Medium was removed after 18 h. Cells were scraped off, washed twice with PBS, and collected by centrifugation (500*g* for 3 min). Total RNA was extracted from each cell preparation using an RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. One-step RT-PCR was performed in a 50 μL reaction volume containing 5 μL RNA template, 5 μL 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% [w/v] gelatin), 5 μL $MgCl_2$ (25 mM), 1 μL 10 mM deoxynucleoside triphosphates, 1 μL (50 pmol) of upstream and downstream primers, 0.5 μL avian myeloblastosis virus reverse transcriptase (5.0 U; Promega Corp., Madison, WIS), 0.5 μL RNasin RNase inhibitor (10 U; Promega Corp.), 0.5 μL *Taq* polymerase (2.5 U; Promega Corp.), and 30.5 μL of water. The specific rotavirus primers (NSP3 gene) were 5′-CAGTGGTTGATGCTCAAGATGGA-3′ (upstream) and 5′-TCATTGTAATCATATTGAATACCCA-3′ (downstream).[31] The GAPDH was used as internal control of cellular RNAs, with primer sequences of 5′-TCAACAGCGACACCCACTC-3′ (upstream) and 5′-CTTCCTCTTGTGCTCTTGCTG-3′ (downstream).[32] Amplification products were analyzed by 1% (w/v) agarose gel electrophoresis and visualized by UV irradiation of ethidium bromide-stained gels.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.073.

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