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COMPARISON OF BIOLOGICAL ACTIVITIES OF *GLYCYRRHIZA GLABRA* AND *G. URALENSIS*

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Abstract - The biological activities of *Glycyrrhiza* (GLs) extracts (GL-1, *Glycyrrhiza glabra* from Eumseong, Korea; GL-2, *G. uralensis* from Eumseong, Korea; GL-3, *G. uralensis* from Yeongcheon, Korea; GL-4, *G. uralensis* from Neimenggu, China: GL-5, *G. uralensis* purchased from Korea Medicine Herbal Association, Korea) were investigated. *G. uralensis* (GLs-2, -3, -4, and -5) extracts exhibited higher free radical scavenging activity against DPPH and OH radicals than *G. glabra* (GL-1). In addition, all GLs had antibacterial activity against *E. coli*, *S. aureus*, and *H. pylori*. GL-3 inhibited the growth of *E. coli* and *S. aureus,* while GL-1 had antibacterial activity against *H. pylori*. All GL extracts tested inhibited the lipopolysaccharide- and interferon-γ-induced inflammatory activity of RAW 264.7 cells. *G. glabra* and *G. uralensis* reduced NO generation. GL-3 also inhibited the growth of AGS human gastric adenocarcinoma cells. GLs-3 and -4 showed the inhibition of rat lens aldose reductase. GL-4 had a higher total content of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**). *G. uralensis* (GLs-2, -3, -4, and -5) is thus more effective than *G. glabra* (GL-1).

Key words: disease; food industry; licorice; natural component

Introduction

Licorice (liquorice) is the name given to the roots and stolons of some *Glycyrrhiza* species that have been used worldwide as herbal medicine for over 4 000 years. The pharmaceutical effects of licorice are known to include anti-inflammatory, antivirus, antiulcer and anticarcinogenic activities (Wang and Nixon 2001). In addition, licorice extract can be used as a food addictive in tobaccos, chewing gums and candies (Fu et al., 2005).

There are different species of licorice, including *G. glabra* (European licorice) and *G. uralensis* (Chinese licorice), that contain species-specific flavonoids (Nomura et al., 2002). *G. uralensis*, *G. glabra* and *G. inflate* are recognized as medicinal plants in China, while *G. uralensis* and *G. glabra* are considered as beneficial herbs in Japan (Kondo et al., 2007) and *G. uralensis* is recognized as a medicinal plant in Korea. In previous studies, *G. glabra* has been shown to have antiulcer, expectorant, diuretic, antipyretic (Lata et al., 1999), antimicrobial, and anxiolytic ac-

tivities (Ambawade et al., 2001). Moreover, an ethanol extract from *G. uralensis* has been shown to be beneficial in diabetes, abdominal obesity and hypertension in animal models (Mae et al., 2003).

Licorice flavonoids are mainly flavones, isoflavones, chalcones, bihydroflavones, and bihydrochalcones. Glycyrrhizin (**1**) and glycyrrhetinic acid (**2**), major components of licorice, have well-known hepatoprotective properties (Lin et al., 1999). In addition, glabridin (**3**) has been reported to exhibit multiple pharmacological activities, including cytotoxic (Fukai et al., 2000), antimicrobial (Fukai et al., 2002), estrogenic and antiproliferative activities against human breast cancer cells (Tamir et al., 2001). Glabridin also influences melanogenesis, inflammation (Yokota et al., 1998), low-density lipoprotein (LDL) oxidation, and can protect mitochondrial function from oxidative stress (Fuhrman et al., 2000). Isoliquiritigenin (**4**), a simple chalcone-type flavonoid, has anti-oxidative (Haraguchi et al., 1998) and anticarcinogenic effects (Takahashi et al., 2004).

In this study, we compare the licorice species *G. glabra* and *G. uralensis* by measurement of their biological activities and analysis of active components.

MATERIALS AND METHODS

Plant materials

GLs-1 and -2 were *G. glabra* and *G. uralensis*, respectively, cultivated at the National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea. GLs-3 and -4 were *G. uralensis* collected at Yeongcheon, Korea and Neimenggu, China, respectively. GL-5 was *G. uralensis* purchased from Korea Medicine Herbal Association, Korea.

Instruments and reagents

An evaporator was obtained from EYELA (Tokyo, Japan) and methanol from Sam Chun Pure Chemical Co. (Pyeongtaek, Korea). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2-deoxyribose used to investigate radical-scavenging activity were obtained from Sigma Chemical Co. (MO, USA) and hydrogen peroxide (H_2O_2) was purchased from Junsei Chemical Co. (Tokyo, Japan). Adenocarcinoma gastric stomach (AGS) and RAW 264.7 cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Welgene (Daegu, Korea). The lipopolysaccharide (LPS) used in this study was from Sigma Chemical Co. (MO, USA) and interferon-gamma (IFN-γ) was from Pepro Tech (NJ, USA). Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT), 3,3-tetramethylene glutaric acid (TMG) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (MO, USA). The standard compounds of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**) and isoliquiritigenin (**4**) were purchased from Sigma-Aldrich Co. (MO, USA).

Preparation of methanol (MeOH) extracts of GLs

Ten grams of dried GLs were extracted with MeOH (200 mL × 3) under reflux conditions and the solvent was evaporated *in vacuo*. Each individual MeOH extract (1.0 mg) was dissolved in DMSO (1 mL).

DPPH and hydroxyl (OH) radical-scavenging activity

In a 96-well microplate, 100 μL of each sample were added to an ethanol solution of DPPH (60 μM) according to the method described by Hatano et al., (1989). After vortexing, the mixture was incubated for 30 min at room temperature and absorbance was measured at 540 nm. The DPPH radical-scavenging activity was recorded as a percentage (%) compared to the control. Scavenging of OH radicals was measured according to the method of Chung et al., (1997). The reaction mixture contained 10 mM $FeSO_4 \cdot 7H_2O_2$-EDTA, 10 mM 2-deoxyribose and the sample solutions. After incubation at 37°C for 4 h, the reaction was stopped by adding 2.8% trichloroacetic acid and 1.0% thiobarbituric acid solution. The solution was boiled for 20 min and then cooled in a

water bath. OH scavenging activity was measured at 490 nm.

Antibacterial activity

Escherichia coli and *Staphylococcus aureus* were provided by the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). Trypticase Soy Agar (TSA) was purchased from BD Difco (NJ, USA), and disc paper was obtained from Adabantec (Tokyo, Japan). The TSA culture medium contained 15 g pancreatic digest of casein, 5 g papaic soybean digest, 5 g NaCl, 15 g sodium chloride and 15 g agar in 1 L of distilled water. Microaerophilic conditions were maintained at 37°C. *Helicobacter pylori*, provided by the Korean Type Culture Collection (KTCC, Daejeon, Korea), were cultured in Brucella broth (Difco, NJ, USA) containing 10% horse serum (Welgene, Daegu, Korea) and, for testing, were grown on a medium prepared with (per liter) BD Bactodextrose (1 g), BD Bactoyeast extract (2 g) (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA), sodium chloride (5 g), and sodium bisulfate (0.1 g). Antibacterial activity against *S. aureus*, *E. coli* and *H. pylori* was measured by the disc agar method (Davidson and Parish, 1989). Plates of medium were spread with 0.1 mL of culture broth, and 15 and 30 μg/30 μL of the fractions and compounds were pipetted onto sterile filter paper discs (8 mm). Inhibition zones were determined after 24 h at 37°C.

Cell culture

AGS cells were maintained in RPMI-1940 medium and RAW 264.7 cells were cultured in DMEM containing 100 $U \cdot mL^{-1}$ of penicillin/streptomycin and 10% FBS at 37°C in a 5% CO_2 incubator. Cells were sub-cultured weekly with 0.05% trypsin-EDTA in phosphate buffered saline.

Cell viability assay

After confluence had been reached, the cells were plated at a density of 5×10^4 cells/well into 96-well plates, incubated for 2 h and then treated with LPS (1 μg/mL) and IFN-γ (10 ng/mL). Samples were treated for 24 h. After incubation, cell viability was determined using the MTT assay. MTT solution was added to each 96-well plate, the plates were incubated for 4 h at 37°C, and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 mL of DMSO and the absorbance of each well was read at 540 nm (Mosmann 1983).

Measurement of nitrite

Nitric oxide (NO) production was assayed by measuring the accumulation of nitrite using a microplate assay method based on the Griess reaction (Sreejayan and Rao 1997). RAW 264.7 cells were seeded in 96-well plates (5×10^4 cells/well) and LPS (1 μg/mL) and IFN-γ (10 ng/mL) were added. After incubating the samples for 24 h, 100 μL of culture supernatant was allowed to react with 100 μL of Griess reagent and the mixture was incubated at room temperature for 15 min. The optical density of the samples was measured at 540 nm using a microplate reader (Chiou et al., 1997).

Inhibition of aldose reductase (AR)

Rat lenses (one lens per 0.5 mL of sodium buffer) were removed from Sprague-Dawley rats (weighing 250-280 g) and preserved until use by freezing. The rat lenses were homogenized and centrifuged at 10 000 rpm (4°C, 20 min) and the supernatant was used as an enzyme source. AR activity was spectrophotometrically determined by measuring the decrease in the absorption of β-NADPH at 340 nm for a 4 min period at room temperature in a quartz cell with DL-glyceraldehydes as the substrate (Sato and Kador 1990). The assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 0.1 M sodium phosphate buffer (pH 6.2), 1.6 mM β-NADPH, and the test samples (in DMSO), with 0.025 M DL-glyceraldehyde as the substrate.

HPLC analysis

The residue was dissolved in 1 mL of MeOH and then filtered with a Whatman 0.2-μm nylon syringe

filter. The resulting solution was used for HPLC analysis. A μBondapak C18 (3.9 × 300 mm, 10 μm) column was used for simultaneous analysis. The mobile phase was 0.05% trifluoroacetic acid + acetonitrile (solvent A) and 0.05% trifluoroacetic acid (solvent B). The elution program used a flow rate of 1 mL/min and decreased solvent B from 55% to 35% for 35 min, eluted with 35% B for 10 min, and then ran at 0% B for 25 min. The injection volume was 10 μL. The UV chromatograms were recorded at 254-350 nm for analysis. All the injections were performed in triplicate.

RESULTS AND DISCUSSION

Antioxidant activities

The DPPH radical and hydroxyl radical scavenging assay has been used extensively in recent years for evaluating antioxidant activity. DPPH is a stable free radical at room temperature, and produces a violet solution in ethanol. The presence of the antioxidants leads to a less strongly colored solution (Mensor 2001). The DPPH radical contains an odd electron that is responsible for absorbance at 540 nm and for the visible deep purple color. A higher percent in our assay indicates better scavenging activity or an antioxidant potential. As shown in Table 1, *G. uralensis* (GLs-2, -3, -4, and -5) had a DPPH radical-scavenging activity of more than 70% at concentrations of 100 μg/mL. *G. glabra* (GL-1) had less scavenging activity (33.33%) at these concentrations. OH is the most reactive species of activated oxygen produced from superoxide (O_2^-) and H_2O_2 under a variety of stress conditions and is involved in numerous cellular processes including inflammation, cell death and killing of micro-organisms in pathogen-defense reactions (Winterbourn 1981; Halliwell and Gutteridge 1984; Babbs et al., 1989; McCormick et al., 1994). The ·OH scavenging activity of GL extracts at a concentration of 100 μg/mL was greater than 75%. *G. uralensis* (GLs-2, -3, -4, and -5) in particular had a scavenging activity greater than 80% under the same conditions. These results suggest that *G. uralensis* may be effective scavengers of DPPH and OH radicals.

Anti-bacterial activities

S. aureus is one of the most common Gram-positive bacteria causing food poisoning. It does not originate in the food itself, but from the humans who contaminate foods after they have been processed. *E. coli*, a Gram-negative bacterium, can also cause serious cases of food poisoning (Rauha et al., 2000). Infection with *H. pylori*, another Gram-negative bacterium, can lead to a variety of gastrointestinal disorders, including chronic gastritis, peptic ulcer disease and gastric cancer (Kusters et al., 2006); therefore, preservatives to eliminate its growth are needed. The antibacterial activities of the GL extracts against *E. coli, S. aureus* and *H. pylori* are shown in Table 2. Results represent the antibacterial effects of GL extracts at concentrations of 15 and 30 μg/30 μL given for 24 h. The largest inhibition zone was observed after treatment with the GL-3 extract (20 mm), which was similar to penicillin (25 mm), used as a positive control against *E. coli*. GLs-3 and -5 produced *S. aureus* growth inhibition zones greater than 15 mm. Overall, the largest inhibition zones were observed with the application of 30 μg/30 μL GL-3 against *E. coli* and *S. aureus*, which produced 20 and 19 mm clearing, respectively. GLs-1, -3 and -5 produced zones of *H. pylori* inhibition greater than 13 mm. GL-1 in particular showed significant antibacterial activity, producing an inhibition zone of 15 mm against *H. pylori* at a concentration of 15 μg/30 μL. These results indicate that GL-3 at the concentration of 30 μg/30 μL is potent against *E. coli* and *S. aureus*. GL-1 at a concentration of 15 μg/30 μL had the strongest antibacterial activity against *H. pylori*.

Anti-inflammatory activity

LPS, a principle component of the outer membrane of Gram-negative bacteria, is an endotoxin (Hewett and Roth 1993). Macrophages activated by inflammatory agents such as LPS are known to produce NO (Moncada and Higgs 1993). LPS and IFN-γ can synergistically stimulate cells to produce a large amount of NO (Nathan 1992). NO is involved in various biological processes including inflammation and immunoregulation (Ialenti et al., 1992; Stichtenoth and

Table 1. Antioxidative activities of GLs.

Sample	Radical scavenging activity (%)	
	DPPH	·OH
GL-1	33.33 ± 0.45	79.48 ± 2.48
GL-2	71.32 ± 0.35	87.85 ± 0.50
GL-3	77.26 ± 0.43	82.99 ± 2.18
GL-4	77.52 ± 0.80	87.77 ± 0.38
GL-5	73.26 ± 0.71	85.65 ± 1.41

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)

Table 2. Antibacterial activities of GLs.

Sample	Concentration (μg/μL)	Inhibition zone (mm)		
		E. coli	*S. aureus*	*H. pylori*
GL-1	15	14	12	15
	30	16	13	8
GL-2	15	12	13	8
	30	13	8	8
GL-3	15	19	13	14
	30	20	19	13
GL-4	15	12	13	11
	30	16	12	8
GL-5	15	13	15	8
	30	12	12	13
Penicillin	15	25	23	17
	30	25	28	17

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)

Frolich 1998). In order to screen the GL extracts for anti-inflammatory activity, we tested their ability to inhibit the expression of proinflammatory cytokines stimulated by LPS/IFN-γ. As shown in Table 3, there was no significant difference between the normal and control groups in terms of cell viability, which was measured at about 100% and 99.46%, respectively. RAW 264.7 cells were incubated with the inflammatory mediators, LPS and IFN-γ, which induced the generation of NO. Secretion of NO from RAW264.7 macrophage cells in the normal group decreased 47.77% as compared to the control (100%). All extracts markedly inhibited LPS/IFN-γ-induced NO generation to less than 22%. Thus, our results suggest that *G. glabra* and *G. uralensis* may be useful in the treatment of inflammatory disease.

Anticancer activity

In previous experiments, we confirmed that the extracts significantly inhibited the growth of *H. pylori*. We used the MTT method to test whether these extracts moderated the growth of AGS cells. Cell viability was measured by detecting purple formazan that was metabolized from MTT by mitochondrial dehydrogenases, which are active only in live cells. Cells were incubated for one day and then treated with GL extracts (100 μg/mL), which all caused some inhibition of gastric cancer cell growth. In particular, GL-3 had the greatest cancer cell growth inhibiting effect (45.73%) of the various extracts. GL-1 had the least effect, inhibiting AGS cell growth rate by 7.47%. These experiments show that GL-3 (*G. uralensis*) has

Table 3. Anti-inflammatory activities of GLs.

Sample	NO generation (%)	Cell viability (%)
GL-1	21.72 ± 0.11	78.47 ± 0.78
GL-2	20.89 ± 0.00	60.60 ± 0.49
GL-3	21.28 ± 0.11	71.90 ± 0.41
GL-4	21.28 ± 0.11	89.00 ± 0.43
GL-5	20.84 ± 0.11	35.39 ± 1.04
Control	100.00 ± 2.81	99.46 ± 0.57
Normal	47.77 ± 1.89	100.00 ± 1.42

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)

Table 4. Anticancer activities of GLs.

Sample	AGS cell growth inhibition rate (%)
GL-1	7.47 ± 0.15
GL-2	28.55 ± 0.40
GL-3	45.73 ± 0.32
GL-4	19.65 ± 0.59
GL-5	22.04 ± 0.38

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)

Table 5. AR inhibition by GLs.

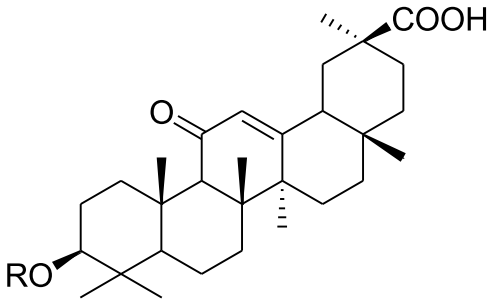
Sample	Concentration (μg/mL)	AR inhibition (%)
GL-1	10	1.92
GL-2	10	22.78
GL-3	10	61.44
GL-4	10	66.59
GL-5	10	55.72
TMG	10	73.55

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)

Table 6. Contents of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**) in GLs.

Sample	Content (%)				
	1	**2**	**3**	**4**	Total
GL-1	2.29 ± 0.01	0.12 ± 0.01	0.37 ± 0.01	-	2.78 ± 0.03
GL-2	0.86 ± 0.01	0.07 ± 0.01	1.05 ± 0.02	0.07 ± 0.01	2.05 ± 0.05
GL-3	1.90 ± 0.01	0.09 ± 0.01	1.29 ± 0.01	0.10 ± 0.01	3.38 ± 0.04
GL-4	2.96 ± 0.01	0.13 ± 0.01	0.22 ± 0.01	0.14 ± 0.01	3.45 ± 0.04
GL-5	2.49 ± 0.01	0.13 ± 0.01	0.39 ± 0.01	0.08 ± 0.01	3.09 ± 0.04

GL-1: *G. glabra* (Eumseong, Korea), GL-2: *G. uralensis* (Eumseong, Korea), GL-3: *G. uralensis* (Yeongcheon, Korea), GL-4: *G. uralensis* (Neimenggu, China), GL-5: *G. uralensis* (Korea Medicine Herbal Association, Korea)



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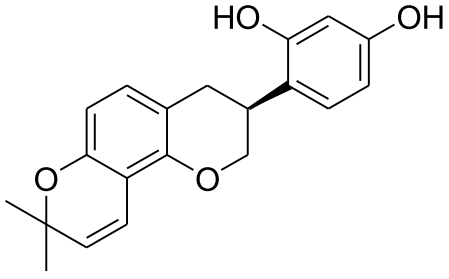
2 R= OH



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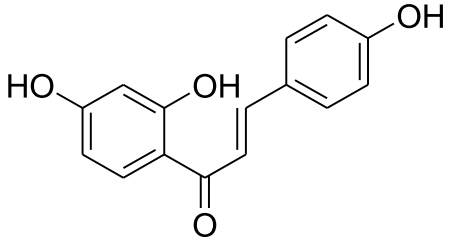

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Fig. 1. Structures of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**).

a significant antiproliferative effect on AGS cells (Table 4).

AR inhibition

The MeOH extracts of the GLs were tested for their ability to inhibit rat lens AR activity, and the results are shown in Table 5. GLs-1, -2, -3, -4 and -5 inhibited AR activity by 1.92, 22.78, 61.44, 66.59 and 55.72%, respectively. *G. uralensis* was a better inhibitor of AR than *G. glabra*. GLs-3 and -4 were more effective inhibitors than the other GL species, but were still less effective than the positive control, TMG. In a previous study, Daehwanggamchoeumja (Rhei Radix et Rhizoma, Glycyrrhizae Radix, or *Glycine max*) reduced diabetic metabolic dysfunction (glucose, triglyceride, total cholesterol, HDL cholesterol, total protein, albumin, creatine and blood urea nitrate) (Go et al., 2002). GLs are thus expected to have a certain therapeutic effect on diabetic metabolic dysfunction.

Content of compounds 1-4

The HPLC separation of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**) and isoliquiritigenin (**4**) for qualitative and quantitative analysis was performed using a reverse phase system. The GL extracts tested contained 0.86-2.96, 0.07-0.13, 0.22-1.29 and 0.07-0.14% of compounds **1-4**, respectively. In previous studies, glycyrrhizin (**1**) in licorice was detected at 3.68% from *G. inflata*, 4.67-5.8% from *G. uralensis* and 5.37% from *G. glabra* (Sabbioni et al., 2006; Yu et al., 2007; Xie et al., 2010; Kojoma et al., 2011). In our study, the total content of compounds **1-4** was the highest in GL-4 (3.45%) and the total content of compounds **1-4** in *G. uralensis* was higher than that of *G. glabra* (Table 6). In *G. uralensis*, the content of glycyrrhizin (**1**) in Korean samples was less than that in Chinese samples. However, the content of glabridin (**3**) in Korean samples was higher than that in Chinese samples.

The extracts from *G. glabra* and *G. uralensis* display various biological benefits including antioxidant, antibacterial, anti-inflammatory, anticancer

and AR inhibitory activities. Taken together, our data suggest that the biological activities of *G. uralensis* may be stronger than those of *G. glabra*, except for its antibacterial activity against *E. coli*. Considering these results, *G. uralensis* could be useful in the food industry and helpful as a natural supplement in the treatment of a number of diseases.

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