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Biological Activities of Licorice F1 Lines and Content Analysis of Phytochemical Constituents

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Abstract – The biological activities of licorice F1 (*Glycyrrhiza glabra* × *G. uralensis*) lines (G) were investigated, revealing strong radical scavenging activity targeting 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (·OH) radicals. At a concentration of 100 μg/mL, most of the licorice F1 lines scavenged DPPH and ·OH by more than 80%. Gs-1, -2, and -6 can be considered good scavengers of DPPH radical and G-7 have higher antioxidant activity against ·OH radical. In addition, licorice F1 lines exerted effective anti-microbial activities against *Escherichia coli* (Gs-12, -17, and -18) and *Staphylococcus aureus* (Gs-3, -4, -5, -21, and -26). Moreover, Gs-2, -20, -31, and -32 effectively inhibited the growth of *Helicobacter pylori*. Among licorice F1 lines, Gs-25 exhibited high anti-inflammatory effects on nitric oxide produced by lipopolysaccharide- and interferon-γ-activated RAW 264.7 cells. Furthermore, Gs-1, -12, and -20 inhibited the growth of AGS human gastric adenocarcinoma cells by more than 60% at a concentration of 100 μg/mL and Gs-5, -11, -19, and -32 showed inhibitory effects against rat lens aldose reductase (IC_{50} values, 1.69, 6.07, 6.12, and 4.54 μg/mL, respectively). The total content of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**) in licorice F1 lines was high in Gs-11, -15, and -30. The present study therefore indicated that Gs-2, -26, -31, and -32 of licorice F1 possessing strong anti-oxidative, anti-microbial, anti-inflammatory, anti-cancer, and aldose reductase inhibitory effects may be used as a possible source material for natural health supplements in the future.
Keywords – Licorice, Analysis, Bioassay, Industrial application, Medicinal plant

Introduction

Licorice is a perennial herb belonging to Leguminosae. Approximately 18 licorice species are recognized around the world; these species generally thrive in Asia, Europe, Australia, and the United States.[1] Licorice has been used for medicinal purposes for at least 4000 years.[2] Among naturally growing species, *Glycyrrhiza glabra* (European licorice), *G. echinata* (Russian licorice), and *G. uralensis* (Chinese licorice) are primarily cultivated as medicinal herbs in Europe, Russia, and the Far East, respectively. Recent papers have described the beneficial effects of licorice, including the inhibition of histamine-induced ulceration, detoxification, immunomodulatory activity, and anti-oxidant, anti-microbial, anti-platelet, anti-viral, anti-biotic, anti-inflammatory, anti-allergic, anti-cancer, and blood pressure-reducing effects,[3-13] as well as its side effects such as hypertension, edema, and hypokalemia-induced myasthenia gravis.[14] *G. uralensis*, *G. glabra*, and *G. inflata* are recognized as medicinal plants in China, whereas in Japan, *G. uralensis* and *G. glabra* are considered beneficial herbs.[15] *G. uralensis* is recognized as a medicinal plant in Korea.

The main active ingredient of licorice is glycyrrhizin.[16] One problem associated with cultivated licorice is that its glycyrrhizin content is generally lower (< 2.5%) than that in its naturally growing counterparts, which could be > 8.0% in mature plants.[17,18] Glycyrrhizin (**1**) and glycyrrhetinic acid (**2**) have anti-inflammatory, anti-ulcer, anti-virus, and hepatoprotective activities.[19-21] Glabridin (**3**) is one of licorice flavonoids known for its beneficial effects on the skin because of its anti-inflammatory and

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skin whitening properties.[22] Other licorice flavonoids, such as liquiritin, liquiritigenin, isoliquiritin, and isoliquiritigenin (**4**) have reported anti-inflammatory, anti-allergic, and anti-tumor effects.[20,23]

This study analyzes the biological activities of licorice F1 (*G. glabra* × *G. uralensis*) lines.

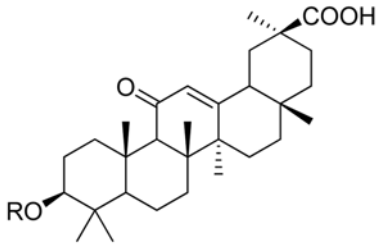
Experimental

Plant materials – European licorice (*G. glabra* L.; a female parent) and Chinese licorice (*G. uralensis* Fisch; a male parent) were planted in the greenhouse and artificially crossed in May 2007. In September 2007, crossed and germinated seeds were retrieved and sown in the greenhouse. In June 2008, stolons were separated from F1 licorice seedlings and cultivated, resulting in 32 clonal lines of interspecific hybrids. Stolons were separated from the 32 F1 lines and transplanted to a field at National Institute of Horticultural and Herbal Science, RDA, Eumseong 369-873, Korea in May 2011. In April 2012, the roots of the F1 lines were collected.

Instruments and reagents – The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2-deoxyribose used to investigate radical scavenging activity were obtained from Sigma Chemical Co. (MO, USA). In addition, 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). The Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT), and 3,3-tetramethylene glutaric acid (TMG) were obtained from Sigma Chemical Co. (MO, USA). Glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**) (Fig. 1) were purchased from Sigma-Aldrich Co. (MO, USA).

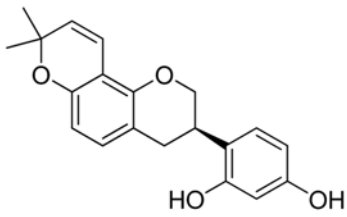
Preparation of methanol (MeOH) extracts – Ten grams of dried licorice F1 lines were extracted with MeOH (200 mL × 3) under reflux conditions and the solvent was evaporated *in vacuo* with EYELA (Tokyo, Japan). Each individual MeOH extract (1.0 mg) was dissolved in DMSO (1 mL).

DPPH and hydroxyl (·OH) radical scavenging activity – In a 96 micro-well plate, 100 μL of samples were added to an methanol solution of DPPH (60 μM) according to the method detailed by Hatano *et al.*[24] After



1 R= GlcA^{2}GlcA

2 R= H



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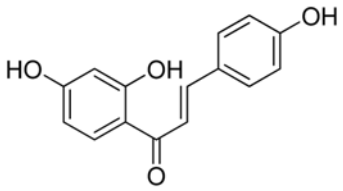

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Fig. 1. Structures of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**).

vortexing, the mixture was incubated for 30 min at room temperature and absorbance was measured at 540 nm. The DPPH radical scavenging ability was recorded as a percentage (%) compared to the control. Scavenging of ·OH radicals was measured according to the method given by Chung *et al.*[25] The reaction mixture contained 10 mM $FeSO_4$ $7H_2O_2$-EDTA, 10 mM 2-deoxyribose solution, and the sample solutions. After incubation at 37 ºC for 4 hr, the reaction was stopped by adding 2.8% trichloroacetic acid (TCA) and 1.0% thiobarbituric acid (TBA) solution. The solution was boiled for 20 min and then cooled in a water bath. ·OH scavenging activity was measured at 490 nm.

Anti-microbial activity – *Escherichia coli* and *Staphy-*

lococcus aureus were provided by 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 Advantec (Tokyo, Japan). The TSA culture medium contained 15 g pancreatic digest of casein, 5 g papaic digest of soybean, 5 g NaCl, 15 g sodium chloride, and 15 g agar in 1 L of distilled water. Microaerophilic conditions were maintained at 37 ºC. *Helicobacter pylori*, provided by 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 Bacto dextrose (1 g), BD Bacto yeast extract (2 g) (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA), sodium chloride (5 g), and sodium bisulfate (0.1 g). The antibacterial activity against *S. aureus*, *E. coli* and *H. pylori* was tested by the disc agar method.[26] 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 hr at 37 ºC.

Cell culture – AGS cells were maintained in RPMI-1940 medium and RAW 264.7 cells were cultured in DMEM containing 100 U/mL of penicillin-streptomycin and 10% FBS at 37 ºC 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 for 2 hr and treated with LPS (1 μg/mL) and IFN-γ (10 ng/mL). The samples were treated in the wells for 24 hr. After incubation, cell viability was determined using MTT assay. MTT solution was added to each 96-well plate, the plates were incubated for 4 hr at 37 ºC, and then 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.[27]

Measurement of nitrite – The amount of nitric oxide (NO) production was assayed by measuring the accumulation of nitrite using a microplated assay method based on the Griess reaction.[28] 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 hr, 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.[29]

Aldose reductase (AR) inhibitory activity – 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 ºC, 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.[30] 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. IC_{50} is the concentration of an inhibitor that results in a 50% inhibition of enzyme activity. IC_{50} values were calculated from the least-squares regression line of the log of the concentration plotted against residual activity.

HPLC analysis – 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 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 gradient elution program decreased solvent B from 55% to 35% for 35 min, the isocratic elution system for 10 min, and then to 0% for 25 min. The injection volume was 10 μL and the flow rate was 1 mL/min. The UV chromatograms were recorded at 254 - 350 nm for analysis. All the injections were performed in triplicate.

Results and Discussion

Anti-oxidant activities – The DPPH radical and hydroxyl radical scavenging assay is widely used to evaluate the antioxidant activity of different samples. DPPH is a stable free radical that donates an electron or hydrogen radical to become a stable diamagnetic molecule.[31] According to Kim *et al.*,[9] ethanol extract of licorice root has greater anti-oxidative activity than α-tocopherol and a similar level to that of the butylated hydroxytoluene and butylated hydroxylanisole. Therefore, we investigated whether the licorice F1 lines have a hydrogen-donating ability to DPPH radical scavenging. The DPPH radical contains an odd electron that is responsible for the

Table 1. Anti-oxidative activities of licorice F1 lines.

Sample	Radical scavenging activity (%)	
	DPPH	·OH
G-1	81.40 ± 0.66^{a}	87.96 ± 0.10^{abc}
G-2	81.91 ± 0.43^{a}	86.81 ± 0.08^{bcd}
G-3	77.65 ± 0.42^{cdef}	87.31 ± 0.13^{bc}
G-4	74.29 ± 0.76^{l}	87.69 ± 0.09^{bc}
G-5	76.49 ± 0.16^{ghi}	86.92 ± 0.13^{bcd}
G-6	80.36 ± 0.33^{b}	83.49 ± 1.80^{f}
G-7	72.09 ± 0.72^{no}	89.51 ± 0.14^{a}
G-8	69.12 ± 0.47^{p}	81.06 ± 1.43^{hijk}
G-9	75.45 ± 0.65^{ij}	83.60 ± 1.37^{f}
G-10	67.44 ± 0.53^{q}	80.40 ± 1.82^{ijk}
G-11	76.74 ± 0.28^{fghi}	81.10 ± 1.06^{hijk}
G-12	75.97 ± 0.40^{hij}	87.65 ± 0.19^{bc}
G-13	77.91 ± 0.33^{cde}	79.36 ± 0.91^{k}
G-14	78.17 ± 0.55^{cd}	81.60 ± 0.79^{ghij}
G-15	67.31 ± 0.65^{q}	79.51 ± 0.78^{k}
G-16	71.83 ± 0.71^{o}	86.38 ± 0.32^{cd}
G-17	73.26 ± 0.44^{m}	88.46 ± 0.28^{ab}
G-18	68.60 ± 0.79^{p}	86.73 ± 0.92^{bcd}
G-19	54.91 ± 1.33^{u}	86.84 ± 0.98^{bcd}
G-20	78.55 ± 0.26^{c}	83.49 ± 1.82^{f}
G-21	76.87 ± 0.42^{efgh}	80.83 ± 1.96^{hijk}
G-22	58.40 ± 0.52^{t}	87.35 ± 0.11^{bc}
G-23	60.59 ± 0.47^{s}	82.37 ± 1.33^{fgh}
G-24	65.76 ± 0.55^{r}	83.91 ± 0.84^{ef}
G-25	77.13 ± 0.52^{defg}	82.52 ± 0.64^{fgh}
G-26	75.32 ± 0.47^{jk}	82.14 ± 0.49^{fghi}
G-27	74.68 ± 0.68^{kl}	87.89 ± 0.08^{abc}
G-28	60.98 ± 0.89^{s}	81.56 ± 0.36^{ghij}
G-29	71.83 ± 0.52^{o}	83.22 ± 0.56^{fg}
G-30	72.87 ± 0.53^{mn}	80.02 ± 0.69^{jk}
G-31	77.78 ± 0.55^{cdef}	85.26 ± 0.41^{de}
G-32	76.74 ± 0.53^{fghi}	86.92 ± 0.24^{bcd}
Ascorbic acid* (IC_{50})	2.49 ± 0.01	0.55 ± 0.01

Values are mean ± SD.
*Ascorbic acid was used as a positive control.
$^{a\sim u}$Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

absorbance at 540 nm and also for the visible deep purple color. A higher % indicates better scavenging activity or antioxidant potential. Among 32 kinds of licorice F1, 24 kinds of licorice demonstrated more than 70% DPPH radical scavenging activities at concentrations of 100 μg/mL. Gs-1, -2, and -6 exerted the strongest scavenging activity, showing 81.40%, 81.91%, and 80.36%, respectively (Table 1). ·OH, the most reactive and toxic radical, can damage adjacent biomolecules. It is produced by the fenton reaction between O_2^-, H_2O_2, and one of the most reactive nitrogen species, $ONOO^-$. The ·OH reduction by the extracts is shown in Table 1. The ·OH scavenging effect of licorice F1 at a concentration of 100 μg/mL was greater than 70%, indicating good potential as free radical scavengers. Moreover, over 85% scavenging was shown in Gs-1, -2, -3, -4, -5, -6, -7, -12, -16, -17, -18, -19, -22, -27, -31, and -32. In particular, G-7 had the highest ·OH radical scavenging ability, indicating 89.51%. These results demonstrate that Gs-1, -2, and -6 can be considered good scavengers of DPPH radical and G-7 have higher antioxidant activity against ·OH radical. These screening of the extracts using the DPPH and ·OH radical method proved to be effective for the selection of those which could have an antioxidant activity. Further studies on the chemical composition of those extracts are essential to characterize them as biological antioxidants.

Anti-microbial activities – *S. aureus* and *E. coli* are recognized as major food-borne pathogens that produce a wide array of toxins, causing various types of diseases.[32] Also, infection from Gram-negative bacteria *H. pylori* is strongly associated with gastric cancer and gastric adenocarcinoma.[33] It has been reported that ethanol extract from licorice roots exerts a very strong growth inhibition on gram positive and negative bacteria.[10] The anti-microbial activities of licorice F1 lines against *E. coli, S. aureus*, and *H. pylori* are shown in Table 2. The results indicate that antibacterial effects of 32 kinds of licorice F1 at a concentration of 30 μg/30 μL treated for 24 hr. The highest zones of growth inhibition more than 18 mm against *E. coli* were observed in Gs-12, -17, and -18. In particular, the highest inhibition zone was observed about 22 mm in G-18 against *E. coli*. On the other hand, the results for *S. aureus*, a Gram-positive bacterium, showed that Gs-3, -4, -5, -21 and -26 produced zones of growth inhibition greater than 16 mm. The antibacterial effect against *H. pylori*, a known cause of gastritis and gastric cancer, showed that licorice F1 including Gs-20, -31, and -32 produced zones of inhibition greater than 12 mm. Most extracts possessed remarkable antibacterial activity against gram positive and gram negative bacteria compared with penicillin as a positive control. In almost extracts, the inhibition zone for *E. coli* and *S. aureus* was greater than the zone for *H. pylori*. However, the extracts differ significantly in their activity between *E. coli*, *S. aureus* and *H. pylori*. The reason of these differences may be the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the gram-negative consists of a multilayer. The results presented in the study showed that

Table 2. Anti-microbial activities of licorice F1 lines.

Sample	Inhibition zone (mm)		
	E. coli	*S. aureus*	*H. pylori*
G-1	13	12	8
G-2	13	13	10
G-3	8	17	8
G-4	11	16	8
G-5	8	18	8
G-6	12	12	8
G-7	13	14	8
G-8	12	12	8
G-9	8	8	8
G-10	11	11	10
G-11	11	13	8
G-12	18	13	8
G-13	12	13	8
G-14	14	8	8
G-15	11	13	8
G-16	15	11	8
G-17	18	8	8
G-18	22	8	8
G-19	8	12	9
G-20	11	12	14
G-21	8	18	8
G-22	11	13	9
G-23	13	13	9
G-24	13	8	8
G-25	11	8	9
G-26	13	17	9
G-27	13	15	8
G-28	12	12	8
G-29	11	14	8
G-30	12	13	8
G-31	13	12	12
G-32	15	13	13
Penicillin*	25	23	17

*Penicillin (15 μg/μL) was used as a positive control.

Table 3. Anti-inflammatory activities of licorice F1 lines.

Sample	NO generation (%)	Cell viability (%)
G-1	36.61 ± 1.85^{i}	47.59 ± 0.65^{tu}
G-2	51.07 ± 1.98^{ef}	38.21 ± 0.50^{v}
G-3	30.29 ± 1.01^{j}	38.95 ± 0.42^{s}
G-4	56.79 ± 4.04^{d}	47.41 ± 0.26^{tu}
G-5	51.51 ± 2.82^{ef}	65.71 ± 0.48^{o}
G-6	62.89 ± 2.29^{c}	47.89 ± 0.73^{stu}
G-7	36.45 ± 1.24^{i}	81.28 ± 0.76^{i}
G-8	51.07 ± 2.49^{ef}	72.84 ± 0.12^{m}
G-9	30.07 ± 1.05^{j}	79.24 ± 0.57^{j}
G-10	36.61 ± 1.85^{i}	91.42 ± 0.64^{e}
G-11	51.07 ± 1.98^{ef}	47.29 ± 1.19^{u}
G-12	30.29 ± 1.01^{j}	48.59 ± 0.26^{st}
G-13	53.44 ± 4.55^{de}	68.42 ± 0.31^{n}
G-14	44.86 ± 3.46^{gh}	87.42 ± 0.37^{g}
G-15	28.51 ± 1.33^{j}	68.43 ± 0.38^{n}
G-16	42.95 ± 6.82^{h}	75.76 ± 1.73^{l}
G-17	39.88 ± 4.34^{i}	77.98 ± 0.30^{k}
G-18	50.65 ± 5.27^{ef}	79.60 ± 0.73^{j}
G-19	48.87 ± 1.44^{f}	88.65 ± 2.23^{f}
G-20	62.29 ± 1.02^{c}	64.57 ± 2.74^{op}
G-21	36.34 ± 1.45^{i}	102.97 ± 1.32^{b}
G-22	69.82 ± 2.08^{b}	104.58 ± 0.44^{a}
G-23	70.08 ± 1.87^{b}	103.96 ± 1.18^{ab}
G-24	68.06 ± 4.66^{b}	63.96 ± 0.96^{p}
G-25	20.62 ± 0.11^{k}	96.57 ± 0.49^{d}
G-26	21.50 ± 0.11^{k}	57.46 ± 0.78^{q}
G-27	22.10 ± 0.28^{k}	64.86 ± 0.49^{op}
G-28	21.11 ± 0.25^{k}	54.16 ± 0.33^{r}
G-29	20.40 ± 0.11^{k}	75.00 ± 0.72^{l}
G-30	22.16 ± 0.38^{k}	73.46 ± 0.18^{m}
G-31	20.67 ± 0.00^{k}	81.60 ± 0.47^{i}
G-32	20.84 ± 0.11^{k}	83.21 ± 0.43^{h}
Control	100.00 ± 2.81^{a}	99.46 ± 0.57^{c}
Normal	47.77 ± 1.89^{fg}	100.00 ± 1.42^{c}
AMT*	64.61 ± 1.60	

Values are mean ± SD.
*AMT (10 μg/μL) was used as a positive control.
[a~v]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

the licorice F1 extracts having properties for the treatment of infections.

Anti-inflammatory activities: The treatment of RAW 264.7 cells with LPS/IFN-γ causes synthesis and secretion of NO. NO, an important inflammatory and neuro-transmission mediator, has high reactivity as a free radical and has the physiology for immune reactions in low concentrations, but high concentrations of NO result in many pathological responses including inflammation.[34,35] Therefore, effective inhibition of NO accumulation by inflammatory stimuli represents a beneficial therapeutic effect.[36] A recent study reported that methanol, water, and ethanol extracts of *G. uralensis* exhibited the greatest efficacy in inhibiting NO production.[37,38] Prior to the inhibition assay of NO formation, the cell viability of the extracts on RAW 264.7 macrophages was determined by MTT test. As shown in Table 3, there was no significant

Table 4. Anti-cancer activities of licorice F1 lines.

Sample	AGS cell growth inhibition rate (%)
G-1	61.30 ± 0.29[e]
G-2	58.83 ± 1.23[d]
G-3	34.82 ± 0.95[j]
G-4	28.75 ± 0.76[l]
G-5	22.60 ± 0.44[o]
G-6	57.71 ± 0.17[e]
G-7	20.33 ± 0.85[p]
G-8	37.90 ± 0.45[i]
G-9	31.03 ± 0.22[k]
G-10	15.16 ± 0.59[r]
G-11	46.15 ± 0.51[f]
G-12	65.36 ± 0.26[a]
G-13	41.13 ± 0.76[h]
G-14	25.98 ± 0.73[m]
G-15	21.61 ± 0.48[o]
G-16	28.71 ± 0.74[l]
G-17	20.43 ± 0.36[p]
G-18	18.55 ± 0.98[q]
G-19	4.89 ± 0.33[t]
G-20	63.34 ± 0.40[b]
G-21	37.62 ± 0.36[i]
G-22	11.30 ± 0.79[s]
G-23	14.48 ± 0.67[r]
G-24	11.54 ± 1.09[s]
G-25	26.02 ± 0.61[m]
G-26	43.11 ± 0.13[g]
G-27	24.86 ± 0.34[n]
G-28	17.55 ± 0.31[q]
G-29	20.45 ± 1.25[p]
G-30	37.68 ± 0.29[i]
G-31	29.55 ± 0.49[l]
G-32	24.58 ± 0.47[n]
5- Fluorourasil*	58.77 ± 0.75

Values are mean ± SD.
*5-Fluorourasil (5 μg/μL) was used as a positive control.
[a–t]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

difference in cell viability between the normal group and the control group, measured at about 100% and 99.46%, respectively. These results demonstrated that LPS/IFN-γ has no effect on cell viability caused by an endotoxin. However, the extracts less than 80% of cell viability were supposed to have toxicity. To investigate the anti-inflammatory effect of licorice F1 lines (100 μg/mL), we examined whether extracts could modulate NO synthesis in LPS/IFN-γ-stimulated cultures of the RAW 264.7 cells.

Table 5. AR inhibition of licorice F_1 lines.

Sample	Concentration (μg/mL)	AR inhibition (%)	IC_{50} (μg/mL)
G-1	10	61.92	–
G-2	10	56.62	–
G-3	10	54.30	–
G-4	10	51.51	–
G-5	10	77.31	1.69
	5	62.83	
	1	43.34	
G-6	10	44.81	–
G-7	10	49.13	–
G-8	10	49.51	–
G-9	10	64.74	–
G-10	10	45.70	–
G-11	10	82.37	6.07
	5	38.88	
	1	11.26	
G-12	10	18.23	–
G-13	10	63.77	–
G-14	10	28.04	–
G-15	10	43.30	–
G-16	10	43.90	–
G-17	10	25.67	–
G-18	10	27.94	–
G-19	10	73.67	6.12
	5	41.76	
	1	20.64	
G-20	10	49.29	–
G-21	10	65.59	–
G-22	10	69.55	–
G-23	10	35.84	–
G-24	10	65.59	–
G-25	10	8.46	–
G-26	10	27.12	–
G-27	10	55.43	–
G-28	10	50.00	–
G-29	10	25.55	–
G-30	10	29.75	–
G-31	10	41.21	–
G-32	10	77.96	4.54
	5	47.45	
	1	5.51	
TMG*	10	73.55	2.01
	5	66.54	
	1	38.43	

* TMG was used as a positive control.

The RAW 264.7 cells were treated the media containing LPS and IFN-γ, which generated NO. The results showed

that the NO generation rate of the non-treatment group was reduced to 47.77% compared to that of the treatment with LPS/IFN-γ group, at 100%. All extracts were found to have high inhibitory effects on NO release from RAW264.7 cells, averaging less than a 70% NO generation rate. We observed that LPS/IFN-γ-stimulated NO formation was inhibited as compared to the control. The extracts more than 80% of cell viability and less than 50% of NO generation rate were Gs-7, -10, -14, -19, -21, -25, -31, and -32. In particular, G-25 had the highest NO inhibitory activity, showing 20.62%. These results demonstrated that G-25 has anti-inflammatory properties through the inhibition of NO production.

Anti-cancer activities – The flavonoids isolated from *G uralensis* were reported to have antitumor and cytotoxic effects against human cancer cell lines HCT-116, HepG2, HeLa, SK-OV-3, SK-BR-3, MCF-7, and SK-MEL-5.[39,40] We investigated the anti-cancer effect of licorice F1 to determine whether these extracts moderated growth of AGS cells using MTT assay. Cell viability was measured with purple formazan that was metabolized from MTT by mitochondrial dehydrogenases, which are active only in live cells. Cells were incubated for one day and treated with licorice F1 extracts. Of the 32 kinds of licorice F1

Table 6. Contents of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**) in licorice F1 lines.

Sample	Content (%)				
	1	**2**	**3**	**4**	Total
G-1	3.26 ± 0.02	0.08 ± 0.00	0.36 ± 0.00	0.10 ± 0.00	3.80 ± 0.01
G-2	2.22 ± 0.01	0.06 ± 0.00	0.25 ± 0.00	0.09 ± 0.00	2.62 ± 0.00
G-3	3.36 ± 0.01	0.07 ± 0.00	0.32 ± 0.03	0.15 ± 0.00	3.90 ± 0.03
G-4	3.33 ± 0.01	0.09 ± 0.00	0.43 ± 0.00	0.11 ± 0.00	3.96 ± 0.01
G-5	2.22 ± 0.00	0.06 ± 0.01	0.24 ± 0.00	0.07 ± 0.00	2.59 ± 0.01
G-6	3.76 ± 0.01	0.10 ± 0.01	0.66 ± 0.02	–	4.52 ± 0.03
G-7	3.92 ± 0.01	–	0.52 ± 0.01	0.09 ± 0.00	4.53 ± 0.03
G-8	4.40 ± 0.02	0.11 ± 0.01	0.30 ± 0.01	0.08 ± 0.00	4.89 ± 0.04
G-9	3.33 ± 0.02	0.07 ± 0.00	0.34 ± 0.02	0.09 ± 0.00	3.83 ± 0.01
G-10	2.91 ± 0.01	0.05 ± 0.00	0.33 ± 0.01	0.18 ± 0.00	3.47 ± 0.02
G-11	3.84 ± 0.00	–	1.10 ± 0.01	0.13 ± 0.00	5.07 ± 0.02
G-12	2.85 ± 0.01	0.11 ± 0.00	0.81 ± 0.01	0.22 ± 0.00	3.99 ± 0.02
G-13	1.50 ± 0.01	–	0.51 ± 0.00	0.10 ± 0.00	2.11 ± 0.00
G-14	1.66 ± 0.01	–	0.77 ± 0.01	–	2.43 ± 0.00
G-15	4.37 ± 0.01	0.08 ± 0.00	0.51 ± 0.01	0.11 ± 0.00	5.07 ± 0.02
G-16	3.00 ± 0.00	0.08 ± 0.00	0.32 ± 0.00	0.09 ± 0.00	3.49 ± 0.00
G-17	2.83 ± 0.00	–	0.21 ± 0.00	0.17 ± 0.00	3.21 ± 0.00
G-18	2.53 ± 0.00	–	0.19 ± 0.00	0.11 ± 0.00	2.83 ± 0.00
G-19	2.12 ± 0.00	–	0.28 ± 0.01	0.11 ± 0.00	2.51 ± 0.01
G-20	3.38 ± 0.01	0.07 ± 0.00	0.72 ± 0.01	0.10 ± 0.00	4.27 ± 0.00
G-21	3.55 ± 0.00	–	0.54 ± 0.01	0.09 ± 0.00	4.18 ± 0.01
G-22	3.38 ± 0.01	–	0.42 ± 0.02	0.09 ± 0.00	3.89 ± 0.01
G-23	3.57 ± 0.03	–	0.24 ± 0.00	0.09 ± 0.00	3.90 ± 0.03
G-24	2.78 ± 0.01	–	0.35 ± 0.01	0.06 ± 0.00	3.19 ± 0.02
G-25	2.06 ± 0.00	–	0.42 ± 0.01	0.10 ± 0.00	2.58 ± 0.01
G-26	2.40 ± 0.01	0.05 ± 0.00	1.20 ± 0.00	0.09 ± 0.00	3.74 ± 0.01
G-27	3.58 ± 0.01	0.07 ± 0.00	0.38 ± 0.00	0.08 ± 0.00	4.11 ± 0.01
G-28	3.59 ± 0.01	–	0.38 ± 0.01	0.07 ± 0.00	4.04 ± 0.01
G-29	3.33 ± 0.01	0.06 ± 0.00	0.40 ± 0.01	0.08 ± 0.00	3.87 ± 0.00
G-30	5.63 ± 0.01	0.10 ± 0.00	0.45 ± 0.01	0.27 ± 0.00	6.45 ± 0.01
G-31	4.04 ± 0.00	–	0.53 ± 0.01	0.17 ± 0.00	4.74 ± 0.02
G-32	3.87 ± 0.01	–	0.37 ± 0.00	0.10 ± 0.00	4.40 ± 0.01

(100 μg/mL) cells were exposed to for 24 hr, Gs-1, -12, and -20 extracts reduced gastric cancer cell growth by more than 60%. In particular, G-12 was more effective in inhibiting AGS cell growth as chemosensitizer in gastric cancer, showing 63.34%. Although further investigations about apoptosis related this data, this study provides the clinical application potential for licorice F1, especially Gs-1, -12, and -20, in gastric cancer therapy (Table 4).

AR inhibitory activities – The MeOH extracts of licorice F1 lines were tested for their inhibitory effects on rat lens AR activity, and the results are shown in Table 5. The IC_{50} value of the licorice F1 Gs-5, -11, -19 and -32 was 1.69, 6.07, 6.12, and 4.54 μg/mL, respectively. The result of G-5 in particular was lower than the positive control, TMG (2.01 μg/mL). Consequently, the licorice F1 G-5 line has potential AR inhibitory effects. In a previous study, Daehwanggamchoeumja (Rhei Radix et Rhizoma, Glycyrrizae Radix, and *Glycine max*) had an effect on diabetic metabolic dysfunction (glucose, triglyceride, total cholesterol, HDL cholesterol, total protein, albumin, creatine, blood urea nitrate).[41] As a result, licorice is expected to have a certain therapeutic effect on diabetic metabolic dysfunction.

Content of phytochemical constituents – In the licorice F1 lines, the contents of glycyrrhizin (**1**), glycyrrhetinic acid (**2**), glabridin (**3**), and isoliquiritigenin (**4**) were 1.50 - 5.63, 0.05 - 0.10, 0.19 - 1.20, and 0.06 - 0.27%, respectively. In our study, the total content of compounds **1 - 4** was the highest in licorice F1 line (G-30, 6.45%) (Table 6). In previous studies, glycyrrhizin in licorice was detected at 3.68% from *G. inflata*, 4.67-5.8% from *G. uralensis*, and 5.37% from *G. glabra*.[42-45] It had also been reported that the glycyrrhizin level in licorice extract fermented with honey (5.19%) and nuruk (5.31%) was more than that in licorice alone (5.05%).[46] In our study, glycyrrhizin levels in 4 licorice F1 lines were more than 4%. Among them, the G-30 licorice F1 line had 5.63% glycyrrhizin and therefore is expected to have promising potential as a medicinal plant. In our results, it was not related to the content of compounds **1 - 4** and various biological activities. The MeOH extract of licorice was good biological activities. Additional compounds are related to the various biological activities.

The results of this study demonstrate that licorice F1 lines have anti-oxidant, anti-bacterial, anti-inflammatory, anti-cancer, and AR inhibitory activities. Moreover, licorice F1 lines can be useful as sources of natural antioxidants and as possible food supplements. Further experiments are needed for active compounds in the future.

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