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Bioactivities of the Herb Extracts Used for Gamhongroju, a Korean Liqueur

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Abstract

In this study, antioxidative and anti-inflammatory activities of the herb (cinnamon, clove, glehnia root, ginger, violet-root cromwell, licorice, citrus peel and longan) extracts used for gamhongroju, one of the popular liqueurs in Korea, were investigated. Twenty grams of individual herbs were extracted in 60% purified ethanol and freeze-dried. A mixture of the individual herb extracts (HEM) was separately prepared. Cytotoxicity of the individual extracts and HEM on murine RAW264.7 macrophage cells were examined along with their recovering activity on H_2O_2-treated RAW264.7 cells. Antioxidant and anti-inflammatory activities of the extract-treated cells were determined by measuring superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and Trolox equivalent antioxidant capacity (TEAC), nitric oxide (NO) and prostaglandin E2 (PGE2) levels. Violet-root cromwell extract showed the least cytotoxicity in terms of treated concentration. Most of the extracts, below levels of cytotoxicity, recovered the H_2O_2-treated cells. Treatment with some of the extracts increased SOD and GPx activities and TEAC levels while a majority inhibited the production of NO and PGE2 in lipopolysaccharide (LPS)-treated cells.

Key words: gamhongroju, herb, antioxidation, anti-inflammatory activity, antioxidant enzyme activity

INTRODUCTION

Medicinal herbs and spices have been used worldwide to make liqueur for dessert and health. Recently, preserving traditional medicinal liqueurs, such as the popular Korean liqueur gamhongroju, has become of interest. In Korea, medicinal liqueurs have been manufactured by extracting or distilling medicinal herbs. Gamhongroju is manufactured, in a similar fashion to other Korean medicinal liqueurs, by distilling rice-fermented must three times and adding honey and a combination of extracted herbs. Gamhongroju is usually created from the herbal mixture of cinnamon, glehnia root, clove, ginger, violet-root cromwell, licorice, citrus peel and longan. According to a book of traditional Korean dietary life, gamhongroju has a very sweet and impressive flavor and a purplish red color (1).

Antioxidant, anti-inflammatory, anti-fungal, and anti-proliferative enzyme activities of the herbs have been extensively studied. Cinnamon, ginger and clove had high antioxidant activities among 1,113 herbs tested (2,3). The anti-inflammatory and anti-cancer properties of cinnamon resulted from the inhibition of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) production, and tumor cell proliferation (4). Cancer cell growth and cell cycle distribution patterns responded in a dose-dependent manner to cinnamon extract (5). Clove had chemopreventative potential to lung cancer in view of its apoptogenic and anti-proliferative properties (6). 6-Gingerol, a major pungent agent of ginger, exhibited dose-dependent inhibition of NO production and significantly reduced inducible NO synthase (iNOS) in LPS-stimulated J774.1 cells (7).

Antioxidant activities of the other herbs and their constituents were also investigated (8-10). Quercetin, isoquercetin, rutin, chlorogenic acid and caffeic acid are the major constituents for the antioxidant activity found in glehnia root (11,12). Longan seed extract had a higher antioxidant activity than longan pericarp extract (9), although both demonstrated anti-cancer activities in HepG2, A549, SGC 7901, Colo 320DM, SW480 and HT-29 cancer cell lines (13,14). Licorice had anti-proliferative effects on breast and prostate cancer cells and inhibited *Helicobacter pylori* activity (15,16). Aqueous licorice extract had significant anti-inflammatory activity, and combination therapy of licorice with famotidine showed higher anti-ulcer activity than either of them alone (17). Isoliquiritigenin, a compound derived from licorice, exhibited prevention of 1,2-dimethylhydrazine-induced co-

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lon and lung tumors in mice when administered at a dose of 300 mg/kg (18).

Citrus peel extract had a NO inhibitory effect in RAW 264.7 cells, later attributed to the compound nobiletin found concentrated within the peel (19). Nobiletin also markedly inhibited hepatitis C virus activity in MOLT-4 cells, NF-κB DNA-binding activity and reactive oxygen species (ROS) generation in LPS-induced RAW264.7 cells, and TNF-α generation in human monocytes (20-22). Herbs and spices containing polyphenols and phenolic acids, coumarins, terpenoids and alkaloids possess antimicrobial activity (23-25). Recently, the methylene chloride fraction from *Glehnia littoralis* extract exhibited anti-inflammatory activity via suppression of NF-κB and mitogen-activated protein kinase (MAPK) activity (26). Violet-root cromwell displayed anti-fungal and anti-HIV activities (27-29).

Few studies are available regarding comparative research on bioactivities of various herb extracts. In this study, antioxidant and anti-inflammatory enzyme activities of individual herb extracts (cinnamon, clove, glehnia root, ginger, violet-root cromwell, licorice, citrus peel and longan), and the combined herbal mixture (HEM), used for making gamhongroju were determined.

MATERIALS AND METHODS

Materials

Cinnamon (*Cinnamomum cassia*), glehnia root (*Glehnia littoralis* Fr. Schmidt et Miquel), licorice (*Glycyrrhiza uralensis* Fischer), citrus peel (*Citrus unshiu*) and longan (*Dimocarpus longan* Lour) were purchased from Kyungdong Market (Seoul, Korea). Clove (*Eugenia aromaticum*) and violet-root cromwell (*Lithospermum erythrorhizon*) were obtained from Samhong Market (Seoul, Korea) and ginger (*Zingiber officinale* Roscoe) from a local market (Seoul, Korea). These herbs were stored at 4°C before use. A 60% purified ethanol (PE) solution was made by diluting 95% ethanol (Pretanol A; Duksan Pure Chemical Co. Ltd., Ansan, Korea). Murine RAW264.7 macrophages were purchased from Korean Cell Bank (Seoul, Korea).

Sample preparation

Twenty grams of each herb was extracted in PE for 90 days at room temperature. Separately, HEM was made by mixing 150 mL each of the glehnia root, ginger, licorice, citrus peel and longan extracts, 30 mL of the cinnamon and violet-root cromwell extracts, and 15 mL of the clove extract. HEM and the individual herb extracts were concentrated using a vacuum rotary evaporator (Eyela Co., Tokyo, Japan) and freeze-dried with a freeze dryer (Ilshin Lab Co., Seoul, Korea). The equal weights of the freeze-dried extracts were dissolved in water (10 mg/mL). Each of these solutions was dissolved in a medium for studying antioxidant activities in cell cultures. The murine RAW264.7 macrophage cells were cultured in Dulbecco's Modified Eagle Medium containing 4.5 g/L D-glucose, 4 mM L-glutamine and 110 mg/L sodium pyruvate (Gibco, Grand Island, NY, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco).

Cytotoxicity in RAW264.7 cells

The RAW264.7 cells were cultured at a density of 5×10^3 cells/well in 96-well plates incubating at 37°C in 5% CO_2 for 24 hr. The medium was aspirated and replaced with fresh media containing different concentrations of the freeze-dried extracts for 24 hr. The medium was discarded, and 10 μL tetrazolium salt 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide reagent (MTT; Sigma Chemical Co., St. Louis, MO, USA) in 100 μL serum free medium was added to the wells and the plates were stored for 4 hr at 37°C in 5% CO_2. 100 μL dimethyl sulfoxide (DMSO; Samchun Pure Chemical Co., Ltd., Pyeongtaek, Korea) was added to each well and the plates were stored for 20 minutes at room temperature. Absorbance was measured at 540 nm using an ELISA microplate reader (Model 680, Bio-Rad Laboratories, Richmond, CA, USA).

$$\text{Cell viability (\%)} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Antioxidant activity

Antioxidant activity was measured by the H_2O_2 assay. The RAW264.7 cells were cultured at a density of 7×10^4 cells/well in flat-bottom 96-well plates incubated at 37°C in 5% CO_2 for 24 hr. Media with different concentrations of freeze-dried extracts were added to the wells. After 4 hr, 14 μL 1 mM H_2O_2 was added to each of the sample wells and 14 μL phosphate buffered saline (PBS; Bio-Rad Laboratories) was added to each of the control wells. After 20 hr, the medium was replaced with 10 μL MTT in 100 μL serum free medium followed by 3 hr incubation. DMSO (100 μL) was added to each well and the plates were stored for 20 min at room temperature. Absorbances were measured at 540 nm using the ELISA microplate reader (Model 680).

Antioxidant enzyme activities

The RAW264.7 cells were cultured in 6×15 dishes at a density of 4×10^6 cells/mL. After 24 hr, 2 mL media was replaced with different concentrations of freeze-dried extracts. After 24 hr, the dishes were washed with PBS and the cells were harvested in e-tubes with 1 mL PBS.

The e-tubes were centrifuged at 12,000 rpm for 10 sec and PBS was removed. The solution made from radioimmune precipitation assay buffer (Biosesang Inc., Seongnam, Korea) and protease inhibitor cocktail #6 (Biosesang Inc.) at the ratio of 100:1 was added to the tubes and mixed using a vortex mixer (Wisemix® VM-10, Daihan Scientific Co., Ltd., Seoul, Korea) for 10 sec. The samples were incubated on ice for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected for the enzyme assays. Superoxide dismutase (SOD) activity was determined using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) by the manufacturer's protocol. Ten μL SOD standard and the samples were added to 96-well plates followed by diluted radical detector (200 μL). Twenty μL diluted xanthine oxidase was added and the samples were gently mixed. Absorbance was measured at 450 nm using an ELISA microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA) every two min for 20 min. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Glutathione peroxidase (GPx) activity in the samples was determined using a Glutathione Peroxidase Assay Kit (Cayman Chemical) by the manufacturer's protocol. Fifty μL co-substrate mixture and 120 μL assay buffer were added in background or non-enzymatic wells. Fifty μL co-substrate mixture and 100 μL assay buffer were added in positive control or sample wells. Twenty μL diluted GPx was added in the positive control wells and 20 μL of the samples were added in the samples wells. The reaction started when 20 μL cumene hydroperoxide was treated to all the wells. Absorbance was measured every min at 340 nm using the ELISA microplate reader (Spectramax 190). One unit is defined as the amount of enzyme oxidizing 1.0 nmol NADPH to NADP+ per min at 25°C.

Ferric reducing antioxidant power (FRAP) of the antioxidant enzyme activity was measured by the method of Benzie and Strain (30). The FRAP measures ability to reduce the ferric ion to ferrous products, indicated by the colored ferrous tripyridyl triazine complex. All reagents were diluted with Chelex-treated (2 g/L) Milli-Q water (Chelex 100 sodium form; Sigma Chemical Co.). TPTZ reagent was made by diluting 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ; Sigma Chemical Co.) in 40 mM HCl (Samchun Pure Chemical Co.). FRAP reagent was made by adding 20 mL 300 mM sodium acetate buffer (pH 3.6; Sigma Chemical Co.), 2 mL TPTZ reagent and 2 mL 20 mM $FeCl_3$ (anhydrous; Duksan Pure Chemicals Co. Ltd.) and incubated at 37°C for 10 min. Twenty μL of the samples and standards were added to a 96-well plate. Pre-warmed FRAP reagent (180 μL) was added to each well and stored at 37°C for 10 min. The absorbance was measured at 550 nm using the ELISA microplate reader (Spectramax 190). FRAP values were determined by trolox(+/-)-6-hydroxy-2,5,6,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma Chemical Co.) equivalent antioxidant capacity (TEAC) using a Trolox standard curve.

Anti-inflammatory activities

NO was measured by a method from Izumi et al. (31). The RAW264.7 cells were seeded in 96-well plates (2×10^6 cells/well) and allowed to adhere for 24 hr. Media was discarded, and new media with different concentrations of freeze-dried extracts were added to the wells treated or not treated with 100 ng/mL lipopolysaccharides (LPS 0111:B4; Sigma Chemical Co.) from *Escherichia coli*. The plates were incubated for 24 hr and 100 μL macrophage culture supernatant was taken from each well and mixed with 100 μL Griess reagent (mixing 1% (w/v) sulfanilamide and 0.1% (w/v) naphthlethylenediamine dihydrochloride in 2.5% (w/v) phosphoric acid, 1:1). After 20 min, absorbance was measured at 540 nm using the ELISA microplate reader (Spectramax 190). NO level was determined by a sodium nitrite (Sigma Chemical Co.) standard curve.

For measuring prostaglandin E2 (PGE2), the RAW264.7 cells were cultured at a density of 2×10^6 cells/well in 96-well plates for 24 hr. Media with different concentrations of freeze-dried extracts were added to the wells with or without 100 ng/mL LPS. A PGE2 ELISA kit (Cayman Chemical) was used to measure the amount of PGE2 metabolites produced.

Statistical analysis

The experiments were repeated 3 times and the results were expressed as mean±standard error. Statistical analyses were processed with SPSS program (version 12.0 SPSS, Chicago, IL, USA) using unpaired *t*-tests or one-way ANOVA. Significances were determined by Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Cytotoxicity of herb extracts in RAW264.7 cells

Cytotoxicities of the freeze-dried HEM and the eight individual herb extracts were determined on murine RAW264.7 macrophages (Table 1). The RAW264.7 cell viability IC_{50} value of the violet-root cromwell extract was the highest (6,124 μg/mL (freeze-dried extract/ medium)), showing the lowest cell cytotoxicity ($p<0.05$), followed by the HEM, glehnia root and clove extracts. The ginger extract had the lowest IC_{50} value (67 μg/mL), showing the highest cell cytotoxicity.

Table 1. Viability of murine RAW264.7 macrophage cells treated with herb extract mixture and individual herb extracts

Extract	Cell viability IC_{50} value (μg/mL)
HEM	5303±742[a]
Cinnamon	631±165[c]
Citrus peel	380±153[c]
Clove	1380±351[b]
Licorice	346±85[c]
Glehnia root	1668±637[b]
Violet-root cromwell	6124±578[a]
Longan	288±61[c]
Ginger	67±10[c]

μg/mL: freeze-dried extract/medium.
Expressed as concentration determining 50% cell viability to the control.
Values are means±standard error (n=3).
[a-c]Values with different superscripts are significantly different ($p<0.05$; one-way ANOVA and Duncan's multiple range test).

Antioxidant activity of herb extracts

H_2O_2 scavenging activities of the extracts at the concentrations showing no cytotoxicity were measured by cell viabilities (Table 2). The cell viability of the control added with H_2O_2 was only 84.7% due to radical damages. Most of the tested extracts showed H_2O_2 scavenging activity. H_2O_2 scavenging activity of the longan extract (5 μg/mL) was the lowest. The cell viability of the ginger extract at the lowest tested concentration, 3 μg/mL, was significantly higher than control ($p<0.05$), suggesting ginger extract exhibits a higher antioxidant activity, as previously discussed by Halvorsen et al. (2). The cinnamon, citrus peel and licorice extracts also had high H_2O_2 scavenging activities at relatively low concentrations (less than 10 μg/mL).

Antioxidant enzyme activities of herb extracts

SOD and GPx activities and TEAC were determined by adding the HEM and individual herb extracts to the RAW264.7 cells (Table 3). The SOD activities in extract-treated cells ranged from 2.86 to 9.34 U/mg protein. SOD activities of the HEM, licorice, glehnia root and violet-root cromwell extracts at the treated concentrations were significantly higher than that of control ($p<0.05$), while the longan extract showed the lowest SOD activity compared to the other extracts and the control.

The extracts-induced GPx activities in the treated cells,

Table 2. Effects of herb extract mixture and individual herb extracts on the viability of H_2O_2-treated RAW264.7 cells

Extract	Extract conc. (μg/mL)	Cell viability (%)	Extract conc. (μg/mL)	Cell viability (%)	Extract conc. (μg/mL)	Cell viability (%)
HEM	250	96.4±7.0*	500	96.9±5.1**	700	102.1±14.3*
Cinnamon	2	90.6±5.3	5	96.0±13.1*	10	100.8±18.1*
Citrus peel	5	95.5±7.5*	7.5	94.4±7.6*	10	99.7±15.7
Clove	5	90.1±5.4	7.5	98.9±7.3*	10	101.2±13.1**
Licorice	5	94.1±7.7*	10	96.5±3.3**	20	100.7±9.7**
Glehnia root	50	92.4±6.4*	100	95.4±7.3*	200	97.6±8.3**
Violet-root cromwell	500	90.5±5.3*	1000	94.3±6.8	2000	97.1±7.2**
Longan	5	89.2±10.2	7.5	93.5±8.0	10	89.4±2.2*
Ginger	2	90.9±5.2	3	100.4±8.2**	4	97.6±9.4*
Control[1)]		84.7±3.8				

μg/mL: freeze-dried extract/medium; Values are means±standard error (n=3).
*,**Significant as compared with the control (*$p<0.05$, **$p<0.01$).
[1)]H_2O_2-treated without sample. HEM: herb extract mixture.

Table 3. Antioxidant enzyme activities in the RAW264.7 cells treated with herb extract mixture and individual herb extracts

Sample	Sample conc. (μg/mL)	SOD (U/mg protein)	GPx (U/mg protein)	TEAC (μM/mg protein)
HEM	250	6.10±0.05**	6.37±0.43	31.2±6.6*
Cinnamon	2	4.10±0.98	4.47±1.26	21.9±2.2*
Citrus peel	5	6.65±0.79	5.80±0.83	24.2±6.5
Clove	2	4.47±0.29	3.68±1.62	17.9±3.2
Licorice	5	6.63±0.66*	4.93±2.64	20.5±4.1
Glehnia root	50	7.55±0.28**	5.68±0.32	19.7±2.2
Violet-root cromwell	500	9.34±0.76**	3.96±0.15	20.5±2.4
Longan	5	2.86±1.05	6.21±1.10	17.2±2.0
Ginger	2	4.02±0.75	2.13±0.98	20.4±2.5
Control		4.32±0.12	3.68±0.87	17.7±2.0

μg/mL: freeze-dried extract/medium; Values are expressed as enzyme units per mg of protein±standard error (n=3).
*,**Significant as compared with the control (*$p<0.05$, **$p<0.01$).
HEM, herb extract mixture; SOD, superoxide dismutase; GPx, glutathione peroxidase; TEAC, Trolox equivalent antioxidant capacity.

Table 4. Effects of herb extract mixture and individual herb extracts on NO and PGE2 production in LPS-treated RAW264.7 cells

Extract	Extract conc. (μg/mL)	NO (μM)	PGE2 production (pg/mL)
HEM	250	17.3±1.9***	887±336**
Cinnamon	2.5	20.4±3.5*	1562±228*
Citrus peel	5	19.8±4.8	1018±8**
Clove	2	27.8±1.6	396±0**
Licorice	5	23.3±3.5	825±170**
Glehnia root	50	14.0±4.2**	1321±297*
Violet-root cromwell	500	17.5±3.1**	1261±174**
Longan	2	27.8±4.3	2208±0
Ginger	1	23.6±3.9	1747±66*
Control[1)]		25.7±3.7	2418±247
No LPS[2)]		2.0±0.9	40±5

μg/mL: freeze-dried extract/medium; Values are means±standard error (n=3).
*,**,***Significant as compared with the control (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
[1)]LPS-treated without sample. [2)]Not treated with LPS nor sample.
HEM, herb extract mixture; LPS, lipopolysaccharides from *Escherichia coli*; NO, nitric oxide; PGE2, prostaglandin E2.

ranging from 2.13 to 6.37 U/mg protein, were mostly higher than the control, although not significantly ($p>0.05$). Interestingly, the GPx activities in the cells treated with the cinnamon extract were not concentration-dependent ($p>0.05$). However, Sharma et al. (32) reported that GPx activities in the liver of mice treated orally with cinnamon for seven days increased in a dose-dependent manner.

Although the TEAC values for most of the extracts were higher compared to the control, only HEM and cinnamon extract were significantly greater ($p<0.05$). The TEAC value in the cells treated with the longan extract was the lowest (17.2 μM/mg protein) at the concentration of 5 μg/mL.

Anti-inflammatory activities of herb extracts

Anti-inflammatory effect was determined by LPS-induced NO and PGE2 production in RAW264.7 cells treated with the HEM and the eight individual herb extracts (Table 4). NO and PGE2 are involved in the development of inflammation (33,34). The concentrations of the HEM and individual herb extracts tested in this study had no noticeable cell cytotoxicities as discussed earlier. LPS-induced NO production was inhibited by all the extracts except clove and longan, where NO production was higher than the control but not significantly ($p>0.05$). HEM, glehnia root and violet-root cromwell extracts significantly inhibited NO production ($p<0.05$). In a study by Chung et al. (35), violet-root cromwell inhibited NO and TNF-α release and NF-κB at 1 mg/mL without cytotoxicity. Shikonins, naphtoquinone pigments from the violet-root cromwell, attenuate microglial inflammatory responses by inhibiting NF-κB (36). Interestingly, in a previous study, NO productions in RAW264.7 cells treated with the licorice and ginger extracts (at the concentration of 50 μg/mL (freeze-dried extract/medium)) were 9.2% and 10.1%, respectively, compared to that in the cells treated with LPS (100%) (37).

All the tested extracts significantly inhibited the LPS-induced PGE2 production in the RAW264.7 cells ($p<0.05$) except for the longan extract (Table 4). The clove extract showed the highest anti-inflammatory effect on LPS-induced PGE2 production (396 pg/mL) at the concentration of 2 μg/mL, followed by the licorice extract and HEM. These data agree with previous results conducted by Yoon et al. (26), who reported that *Glehnia littoralis* extract is a novel suppressor of the production of PGE2 (IC_{50} value 24.4 μg/mL). The effects appear to be mediated by inhibition of NF-κB and the MAPK pathway activation.

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