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감초 신품종 및 약전 수재감초의 항알러지 효과 비교 연구

강윤미[1#], 김원남[2], 진종식[3], 이종현[4], 장재기[5], 이정훈[5*], 안효진[1*]

1: 상지대학교 한의과대학 약리학교실, 2: 세명대학교 한의과대학 본초방제학교실,
3: 전북대학교 환경생명자원대학 한약자원학과, 4: 동덕여자대학교 약학대학 약학과,
5: 농촌진흥청 국립원예특작과학원 약용작물과

The Comparative Study of Anti-allergic Effect by *Glycyrrhiza* New Varieties and Official Compendia

Yun-Mi Kang[1#], Wonnam Kim[2], Jong-Sik Jin[3], Jong-Hyun Lee[4]
Jae Ki Chang[5], Jeonghoon Lee[5*] and Hyo-Jin An[1*]

1 : Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 220-702, Republic of Korea
2 : Division of Pharmacology, College of Korean Medicine, Semyung University, 65 Semyung-ro, Jecheon, Chungbuk, 27136, Republic of Korea
3 : Department of Oriental Medicine Resources, Jeonbuk National University, 79 Gobong-ro, Iksan, Jeollabuk-do 54596, Republic of Korea
4 : Department of Pharmacy, College of Pharmacy, Dongduk Women's University, 60 Hwarang-ro 13-gil, Seongbuk-gu, Seoul 02748, Republic of Korea
5 : Herbal Crop Research Division, NIHHS, RDA, Eumseong, Chungbuk, 27709, Republic of Korea

ABSTRACT

Objective : The genus *Glycyrrhiza* has been used in food and traditional herbal medicine. Many experimental studies reported that *Glycyrrhiza* species possess several pharmacological properties. *Glycyrrhiza* new varieties WONGAM and SINWONGAM have been developed by Korea Rural Development Administration doing research for registration on Ministry of Food and Drug Safety. During the evaluations about pharmacological effect of *Glycyrrhiza* new varieties WONGAM and SINWONGAM, we focused the anti-allergic effect in this study.

Methods : We investigated the anti-allergic effect of WONGAM and SINWONGAM compared with *Glycyrrhiza uralensis* Fischer and *G. glabra* L. using anti-dinitrophenyl-immunoglobulin E (IgE)/human serum albumin-stimulated RBL-2H3 cells, phorbol 12-myristate 13-acetate plus calcium ionophore A23187-stimulated HMC-1 cells and compound 48/80-induced anaphylaxis mice model. We analyzed the effect on the expression of various cytokines, and IgE from mast cells and the underlying molecular mechanisms of WONGAM and SINWONGAM in presented models.

Results : WONGAM and SINWONGAM showed the inhibitory effect on the histamine release from rat peritoneal mast cells or human mast cells without cytotoxicity. WONGAM and SINWONGAM blocked anaphylactic shock and decreased the IgE production. Furthermore, WONGAM and SINWONGAM inhibited the productions of TNF-α and IL-6 in compound 48/80-induced anaphylaxis mice model.

*Corresponding author : Hyo-Jin An, Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do, 26339, Republic of Korea.
· Tel : +82-33-738-7503 · Fax : +82-33-730-0679 · E-mail : sangjipharm@gmail.com
Jeong Hoon Lee, Herbal Crop Research Division, NIHHS, RDA, Eumseong, Chungbuk, 27709, Republic of Korea
· Tel : +82-43-871-5670 · Fax : +82-43-871-5659 · E-mail : artemisia@korea.kr
#First author : Yun-Mi Kang, Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do, 26339, Republic of Korea.
· Tel : +82-33-735-3881 · Fax : +82-33-730-0679 · E-mail : yunmi6115@naver.com
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Conclusion : These results indicated that WONGAM and SINWONGAM would have protect effect on allergic responses through the inhibition of allergic mediators and pro-inflammatory cytokines. This study may facilitate the development on *Glycyrrhiza* new varieties for allergy.

Key words : Allergic response, *Glycyrrhiza*, *Glycyrrhiza* new varieties, Mast cell, Anaphylaxis

Ⅰ. Introduction

Glycyrrhiza species (Licorice) are one of the most commonly used medicinal plants belonging to the Leguminosae family, and its members are now commonly used having a long history of traditional medicines and folk remedies. Licorice and its extracts are currently used in pharmaceutical and food industries such as functional foods and food supplements[1]. Licorice and several of its important constituents are widely used in traditional medicine and for industrial purposes. Licorice contains chemical compounds such as triterpene saponins, flavonoids, coumarins and other phenolics[2]. Previous studies have identified triterpenoids, such as glycyrrhizin, and flavonoids, such as liquiritigenin, as the major components of licorice[3]. Many studies have shown that *Glycyrrhiza* species and its components have a number of pharmacological effects including anti-allergic[4], anti-inflammation[5], anti-asthmatic[6], anti-ulcer[7], anti-viral[8], estrogenic[9] and anti-carcinogenic activities[10].

Licorices are listed in the Korean Pharmacopoeia as *Glycyrrhiza uralensis* Fisch. (감초), *Glycyrrhiza glabra* L. (광과감초), and *Glycyrrhiza inflata* Batal. (창과감초). *Glycyrrhiza* species are the most demanding herbal medicine and widely used in various herbal medicinal preparations, pharmaceuticals and cosmetics industries. However, but more than 5,000 tons are imported every year and the domestic self-sufficiency rate is less than 5 %. WONGAM and SINWONGAM are breeding variety from Rural Development Administration, which are hybrid of *G. glabra* × *G. uralensis*. *G. uralensis* and *G. glabra* are difficult to cultivate in Korea, and when it cultivated in Korea, the content of Glycyrrhizin (2.5 %) and liquiritigenin (0.7 %) were not meet the standard of the Korea pharmacopoeia. However, the breeding varieties, WONGAM and SINWONGAM, meet the standard. These have been developed to overcome the problems such as low productivity and early leaf fall and to supplement the functionality of the origin plants. It was reported that WONGAM has much higher yields (227 %) and glycyrrhizin content than *G. uralensis*[11]. The suitability of the WONGAM for cultivation in Korea was demonstrated, through the study on the growth, yield, and pharmacological properties of the WONGAM. Moreover, it was suggested that extension the current cultivation area to the south central region can the improve the productivity of this cultivar[12]. As mentioned above, many studies have been conducted on the ingredients and efficacy of single *Glycyrrhiza* species, but there is a lack of research on the *Glycyrrhiza* new varieties. Recently, Lee SE *et al*. conducted to compare the *Glycyrrhiza* varieties radix including WONGAM and SINWONGAM for their anti-oxidation, anti-inflammation and cognition improvement effects. It is reported that SINWONGAM exhibited potent anti-oxidant, anti-inflammatory and N-methyl-D-aspartate receptor inhibitory activities with higher total phenol and licochalcone A contents than the other cultivars[13].

Allergic response is described as a hypersensitivity disorder that is caused by environmental substances known as allergens. Mast cell have a critical role as a mediator in allergic response such as anaphylaxis, rhinoconjunctivitis and urticaria. Mast cells activated by IgE release a spectrum of chemical mediator such as pro-inflammatory cytokines at the affected site[14]. IgE, mast cells, basophils and eosinophils are essential components of allergic inflammation. Antigen-specific IgE production, with subsequent fixation of IgE to Fcε RI receptors on mast cells and basophils, is central to the initiation and propagation of immediate hypersensitivity reactions[15]. Anaphylaxis is a fatal systemic hypersensitivity reaction characterized by life-threatening problems involving airway, breathing and circulation. It is considered that anaphylaxis is can be triggered by imbalance of immune response including immune effector cells, antibodies and mediators[16]. The major mechanism underlying allergic anaphylaxis is mediated by IgE. After exposure to the allergen, the production of allergen-specific IgE by B cells was triggered by sensitization with a series of signals. Subsequently, the antigen-allergen-specific IgE complex binds to the Fcε RI receptor on mast cells and/or basophils and activates and induces degranulation of these cells. In consequence, activated mast cells release preformed mediators, enzymes and cytokines and *de novo* synthesized mediators such as histamine, leukotrienes, tryptase, pro-inflammatory cytokines prostaglandins, platelet-activating factor[17]. However, there are no therapies available to cure allergic

diseases completely. Therefore, anti-allergic ingredients derived from medicinal plant without side effects would be a suitable alternative anti-allergic strategy.

As part of the study for the Korean Pharmacopoeia registration of *Glycyrrhiza* new varieties, the present study was conducted to investigate the anti-allergic activity of *Glycyrrhiza* new varieties and the efficacy homogeneity with the *Glycyrrhiza* species listed in the Korean Pharmacopoeia were compared using RBL-2H3 cells, HMC-1 cells and compound 48/80-induced anaphylaxis mice model.

Ⅱ. Materials and Methods

1. Chemicals and reagents.

Extractions of *Glycyrrhiza* species were provided from Korea Rural Development Administration. For the present study, anti-dinitrophenyl immunoglobulin E antibody (anti-DNP IgE), dinitrophenyl human serum albumin (DNP-HAS), phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 (Calcimycin), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), dimethyl sulfoxide (DMSO) and all other chemicals were purchased from Millipore Sigma (Billerica, MA, USA). Iscove's modified Dulbecco's medium (IMDM), Dulbecco's Modified Eagles medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). The histamine enzyme-linked immunosorbent assay (ELISA) kit was obtained from Enzo life Sciences, Inc. (Farmingdale, NY, USA). The ELISA kits for tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IgE were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

2. Sample preparation

The extracts of WONGAM, SINWONGAM, *G. uralensis*, *G. glabra* were obtained from Korea Rural Development Administration. The four samples were extracted with water. The extract was concentrated under reduced pressure. The decoction was filtered, lyophilized and stored at 4℃. The yields of the dried extract from the starting crude of WONGAM, SINWONGAM, *G. uralensis* and *G. glabra* were 8.8 %, 9.13 %, 6.53 % and 7.71%, respectively. To prepare the sample for the *in vitro* experiment, the extract powder that resulted from the drying process was dissolved in distilled water.

3. RBL-2H3 cell sensitization and stimulation

RBL-2H3 was purchased from Korea Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were grown at 37℃ in DMEM supplemented with 10 % FBS, penicillin (100 U/㎖) and streptomycin (100 ㎍/㎖) in a humidified atmosphere of 5 % CO_2. RBL-2H3 cells were seeded at a density of 1×10^5 cell per well and incubated with 50 ng/㎖ of anti-DNP-IgE overnight for cell sensitization. After washing with PBS three times, the cells were exposed to *Glycyrrhiza* species samples for 1 h and then stimulated with 100 ng/ml of DNP-HAS for 4 h.

4. HMC-1 cell culture and sample treatment.

HMC-1 cells were provided by Professor Jong-Sik Jin (Chonbuk University, Republic of Korea) and were grown at 37℃ in IMDM supplemented with 10 % FBS, penicillin (100 U/㎖) and streptomycin (100 ㎍/㎖) in a humidified atmosphere with 5 % CO_2. HMC-1 cells were seeded at a density of 1×10^6 cells per well, treated with *Glycyrrhiza* species samples for 30 min at 37° C in humidified air with 5 % CO_2 and then stimulated with 40 nM of PMA and 1 ㎛ of A23187 (PMACI) at 37℃. The OA was dissolved in DMSO. The cells were treated with DMSO as a control.

5. Cell viability assay

Cells were seeded in a 96-well culture plate at 5×10^4 cells/㎖ in culture medium. Cells were treated with medium containing various concentrations of *Glycyrrhiza* species samples. After incubating for 24 h, HMC-1 cells were treated with 20 ㎕ of MTS for 4 h and absorbance was measured at 490 ㎚ using a microplate reader. After incubating for 24 h, RBL-2H3 cells were treated with 50 ㎕ of MTT (5 ㎍/㎖) for 4 h. The formazan precipitated was dissolved in DMSO and absorbance was measured at 540 ㎚ using a microplate reader.

6. Histamine assay

Culture media were collected after treatment with *Glycyrrhiza* species samples and stored at −80℃. Histamine release was measured using ELISA kits according to the manufacturer' s protocol.

7. Compound 48/80-induced anaphylactic shock model.

A total of 35 male ICR mice (6 weeks old; 20-25 g body weight) were obtained from Charles River Laboratories

(Harlan Laboratories, Inc., Wilmington, MA, USA) and maintained under constant conditions at a temperature of 20-25℃, humidity of 40-60 % and a 12 h light/dark cycle. The mice were randomly assigned to one of seven groups (n = 5 per group). The ICR mice were injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS) or compound 48/80 (24 ㎎/㎏ dissolved in PBS). Four *Glycyrrhiza* species samples, disodium cromoglycate (DSCG; Sigma-Aldrich; EMD Millipore, Billerica, MA, USA), or PBS was dissolved in saline and injected i.p. at doses of 25 ㎎/㎏ DSCG and 100 ㎎/㎏ *Glycyrrhiza* species samples for 1 h prior to the compound 48/80 injection. Survival was monitored for 1 h following the induction of anaphylactic shock. Survival data were analyzed using the Kaplan-Meier method and log rank test. Following the assessment of animal survival, blood was collected from the heart of each mouse to measure serum cytokine production. The blood was allowed to clot for 1 h at room temperature and then centrifuged for 20 min at 3,000×*g* at 4℃ to obtain serum. Following collection of blood samples from the mice, the mice were sacrificed by cervical dislocation. All procedures were performed in accordance with university guidelines and approved by the Ethical Committee for Animal Care and the Use of Laboratory Animals, Korean Medicine, Sangji University (Wonju, Korea; approval no. 2019-10).

8. IgE and Cytokine assays.

Blood was collected from each mouse at the end of the experiment. Serum was obtained by centrifugation at 1700×*g* for 30 min and stored at −80℃ until analysis. The levels of TNF-α, IL-6 and IgE were measured using ELISA kits according to the manufacturer's protocol.

9. Statistical analysis.

The data are expressed as the mean ± standard deviation of triplicate experiments. Statistically signifi cant differences were compared using one-way analysis of variance and Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS statistical analysis software (version 19.0, IBM SPSS, Armonk, NY, USA).

Ⅲ. Result

1. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species exhibit no direct cytotoxicity on RBL-2H3 cells

To investigate whether *Glycyrrhiza* species samples were cytotoxic to the RBL-2H3 cells, the MTT assay was employed. *Glycyrrhiza* species samples at concentrations up to 1000 ㎍/㎖ exhibited no significant cytotoxicity to the RBL-2H3 cells after 24 h of incubation when compared with control (Figure 1).

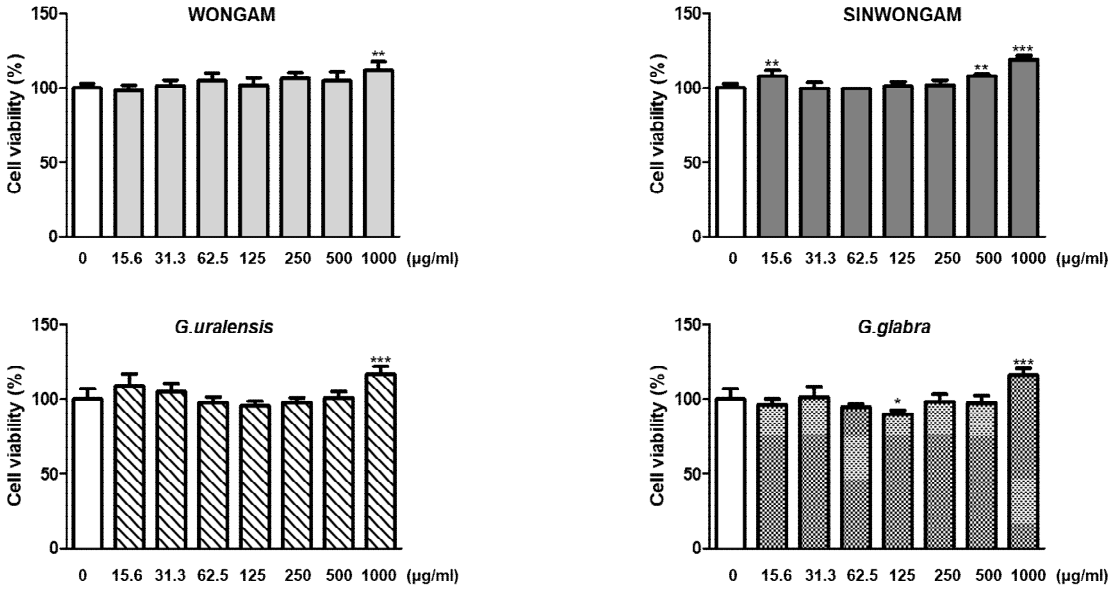

Figure 1. Effect of four *Glycyrrhiza* species samples on cell viability on RBL-2H3 cells. RBL-2H3 cells were treated with different concentrations of *Glycyrrhiza* species samples for 24 h and their viability were determined using MTT assay. The data shown represent mean ± S.D. of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs non-treated cells.

2. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species suppress the release of histamine from RBL-2H3 cells

To determine the allergy suppressive effect of *Glycyrrhiza* species samples in rat-derived mast cells, the release of histamine, indicator of degranulation, was examined. The release of histamine were high in the DNP-IgE/HAS group. However, all *Glycyrrhiza* species samples except high concentration of *G. glabra*, inhibited the histamine release in DNP-IgE/HAS-stimulated RBL-2H3 cells (Figure 2).

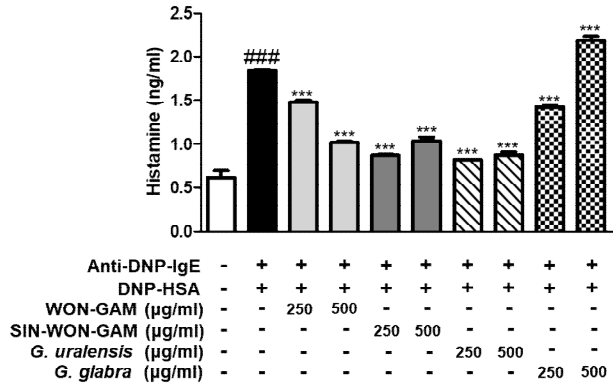

Figure 2. Effect of four *Glycyrrhiza* species samples on the histamine release in IgE-antigen complex-stimulated RBL-2H3 cells. RBL-2H3 cells were sensitized with anti-DNP IgE (50 ng/㎖). After overnight incubation, the cells were pretreated with samples for 1 h and then challenged with DNP-HSA (100 ng/㎖). Histamine release was measured using ELISA kit. The data shown represent mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs the control group, $^{***}p < 0.001$ vs sensitized with anti-DNP-IgE and stimulated with DNP-HAS group.

3. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species exhibit no direct cytotoxicity on HMC-1 cells

The effects of *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species samples on the cell viability in human mast cells were measured by MTS to ensure that the decreased levels of mast cell granule were not due to the cell death. Various concentrations of *Glycyrrhiza* species samples decreased cell viability as concentration increased, but cell viability remained higher than 80 % in HMC-1 cells. WONGAM, SINWONGAM, *G. uralensis* and *G. glabra* displayed the cell viability at 92.62 %, 86.41 %, 89.44 % and 87.02 % in the higher concentration, respectively. Thus, *Glycyrrhiza* species samples did not show cytotoxicity in tested concentration in HMC-1 cells (Figure 3).

4. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species suppress the release of histamine from HMC-1 cells

To investigate regulatory effects of *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species on histamine release from human mast cells, we measured histamine levels in PMACI-stimulated HMC-1 cells. The results showed that all samples significantly inhibited the PMACI-induced histamine release. The inhibition effect of SINWONGAM was similar to *G. uralensis* and *G. glabra* showed better effect on PMACI-stimulated HMC-1 cells (Figure 4).

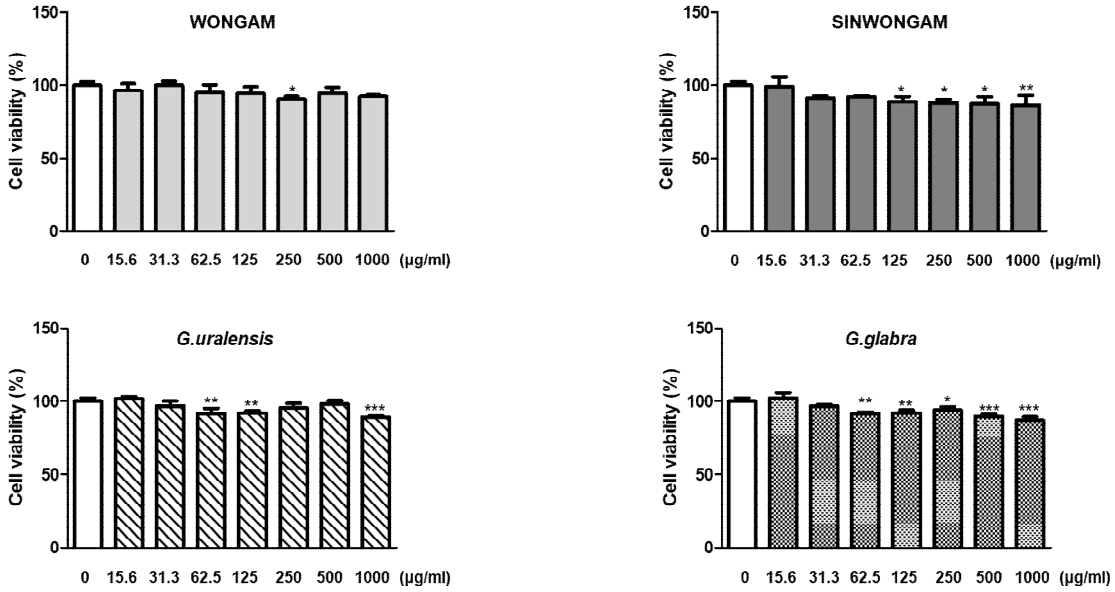

Figure 3. Effect of four *Glycyrrhiza* species samples on cell viability on HMC-1 cells. HMC-1 cells were treated with different concentrations of *Glycyrrhiza* species samples for 24 h and their viability were determined using MTS assay. The data shown represent mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs non-treated cells.

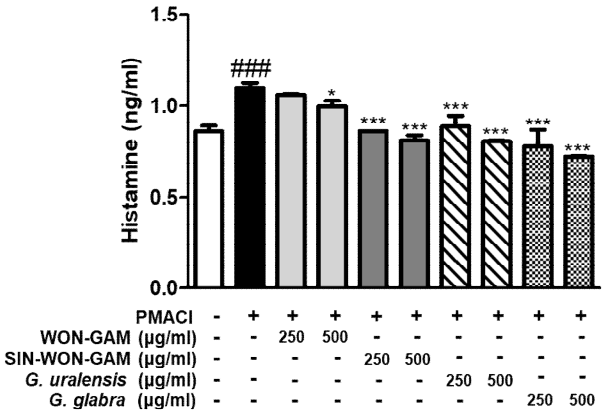

Figure 4. Effect of four *Glycyrrhiza* species samples on the histamine release in PMACI-stimulated HMC-1 cells. Cells were treated with *Glycyrrhiza* species samples for 30 min prior to the addition of PMACI and the cells were further incubated for 12 h. Histamine release was measured using ELISA kit. The data shown represent mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs the control group, *p < 0.05 and ***p<0.001 vs PMACI-treated group.

5. Effects of *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species on systemic anaphylaxis and IgE level

To examine the effect of *Glycyrrhiza* new varieties on allergic reaction *in vivo*, compound 48/80-induced systemic anaphylaxis in mice was tested. The intraperitoneal injection of compound 48/80 (24 ㎎/㎏), a mast cell degranulator, induces lethal systemic anaphylaxis, whereas the mortality rate was reduced in the mice administered *Glycyrrhiza* species samples (Figure 5A). In addition, treatment with compound 48/80 resulted in the increased serum IgE level in compound 48/80-induced systemic anaphylaxis mice. Consistent with these data, all samples suppressed the IgE level induced by compound 48/80 (Figure 5B). These results support the hypothesis that *Glycyrrhiza* new varieties inhibit compound 48/80-induced-anaphylaxis by blocking degranulation of mast cells.

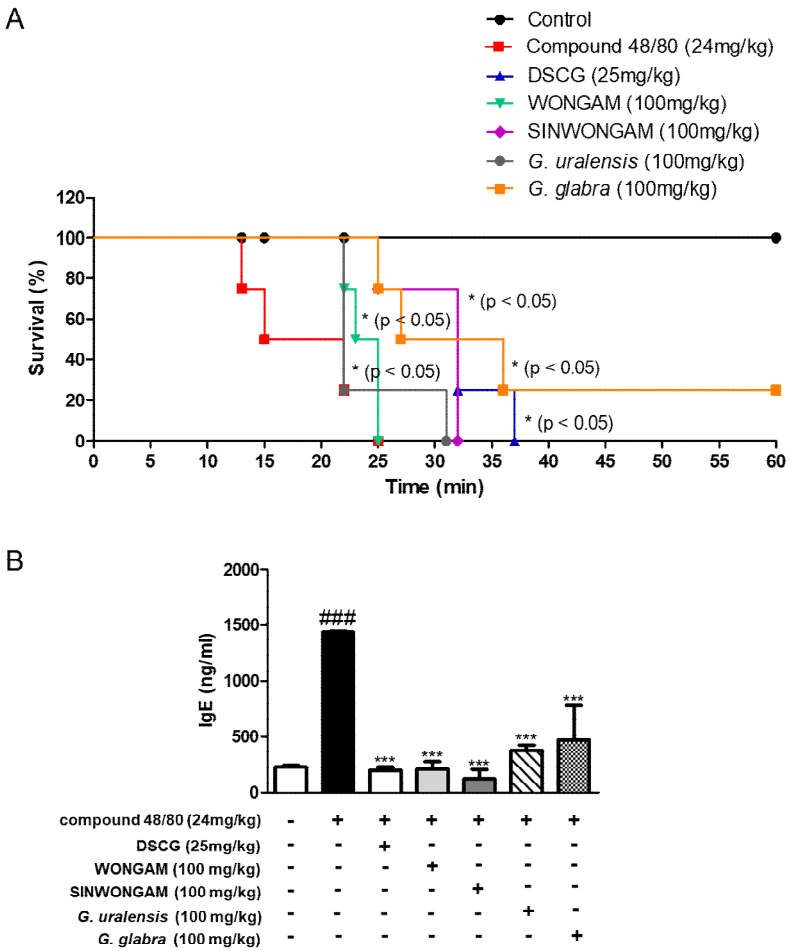

Figure 5. Effects of four *Glycyrrhiza* species samples on compound 48/80-induced mortality and IgE serum level in a mouse model of anaphylactic shock. Mice were injected with *Glycyrrhiza* species samples, DSCG and PBS as a vehicle (n = 5 per group) for 1 h before compound 48/80 injection (24 ㎎/㎏ i.p.). (A) Survival rates of these mice were monitored for 1 h. (B) IgE serum levels were measured using ELISA kit. The data shown represent mean ± S.D. of three independent experiments. $^{###}P < 0.001$ vs. the control group; *$P < 0.05$ and ***P < 0.001 vs. compound 48/80-treated group.

6. Effects of *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species on the production of pro-inflammatory cytokines

Mast cell activation could stimulate cytokines release; TNF-α and IL-6 are major key pro-inflammatory cytokines released during mast cell activation. Therefore, we examined the effect of samples on the production of TNF-α and IL-6 in serum of anaphylaxis mice. The result showed that pretreatment with samples and DSCG markedly suppressed the overexpression of TNF-α and IL-6 in compound 48/80-induced anaphylaxis shock mice. SINWONGAM had the greatest effect on the reduction of both cytokine production examined (Figure 6).

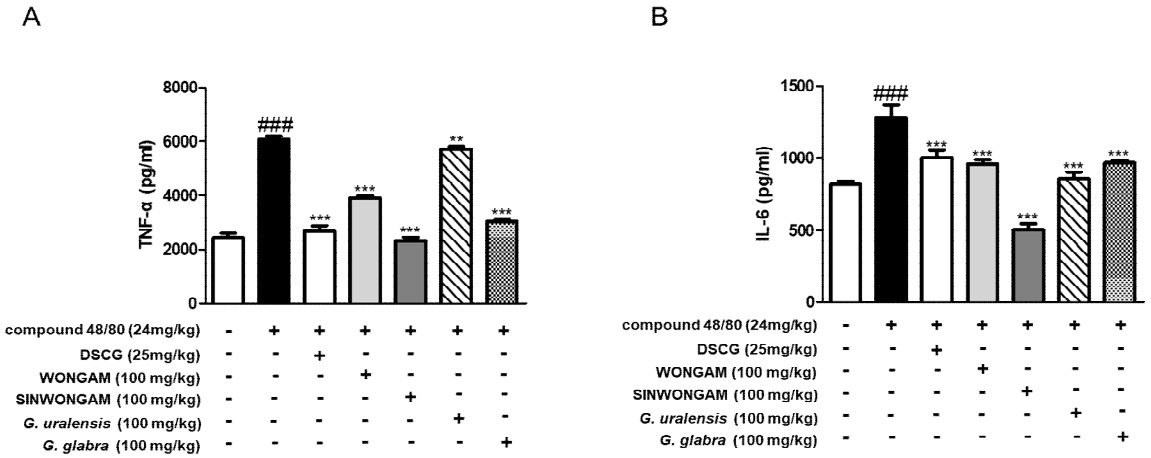

Figure 6. Effects of four *Glycyrrhiza* species samples on compound 48/80-induced pro-inflammatory cytokines in a mouse model of anaphylactic shock. (A) TNF-α and (B) IL-6 production from serum were measured using ELISA kit. The data shown represent mean ± S.D. of three independent experiments. $^{###}P < 0.001$ vs. the control group; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. compound 48/80-treated group.

Ⅳ. Discussion

Licorice is a typical herbaceous perennial belonging to the Leguminosae family and is distributed in northeastern China, Siberia and Mongolia. Due to the development of the bio industry, licorice is becoming popular as a raw material for functional products such as medicines, foods and cosmetics as well as herbal medicines[1]. However, since 98 % of domestic licorice consumption depends on imports, it is urgent to revitalize the domestic licorice industry. Most of licorice distributed in Korea is imported from China or Mongolia. Also noteworthy, due to the desertification of Chinese licorice native land, which is a major importer, licorice production is gradually decreasing and domestic licorice demand is expected to increase in the future. WONGAM and SINWONGAM have been developed as new cultivars for localization of *Glycyrrhiza* species. It is expected to increase the domestic self-sufficiency rate of medicinal crops with the highest domestic demand and to spread them steadily.

To register the *Glycyrrhiza* new varieties in the pharmacopoeia, the efficacy homogeneity with existing *Glycyrrhiza* species should be evaluated. Since the anti-allergic effect of the listed *Glycyrrhiza* species has already been known, the allergy biomarkers was evaluated to determine the effect of *Glycyrrhiza* new varieties on allergic reaction using *in vitro* and *in vivo* model in this study. The results obtained showed all *Glycyrrhiza* species samples have no cytotoxicity and reduced histamine release in RBL-2H3 cells and HMC-1 cells, but the level of histamine in group of the high concentration of *G. glabra* was higher than other *Glycyrrhiza* species samples in DNP-IgE/HAS-stimulated RBL-2H3 cells. In anaphylaxis shock test, all *Glycyrrhiza* samples exhibited the protective effect against compound 48/80-induced anaphylactic shock, delaying the mortality and significantly inhibited the serum level of IgE. In particular, SINWONGAM was superior in terms of attenuating the expressions of mediators, the levels of which were similar to those in *Glycyrrhiza* species listed in the Korean Pharmacopoeia. These results suggest that *Glycyrrhiza* new varieties prevent IgE-mediated mast cell activation and suppress the production of histamine and pro-inflammatory cytokines, thus inhibit allergic reactions such as anaphylaxis. As a result, the safety and anti-allergic effects of *Glycyrrhiza* new

varieties were demonstrated to be homogeneous with the listed *Glycyrrhiza* species. To support this, further specific molecular mechanisms related to the components of *Glycyrrhiza* new varieties will be needed in future studies.

It is reported that anti-allergic and anti-inflammatory effect and several pharmacological activities of licorices. Among the licorice-containing ingredients, glycyrrhizin, 18β-glycyrrhetinic acid and liquiritigenin are known to inhibit anti-inflammatory and anaphylaxis [4,18-20]. The main constituent of roots is glycyrrhizin, a triterpenoid saponin that is almost 50 times sweeter than sucrose, being the primary active ingredient. The new *Glycyrrhiza* varieties have a 3.96 % glycyrrhizin content, which is much higher than *G. uralensis* (1.90 %). While *G. uralensis* has problems that the glycyrrhizin content is low at 2.0 % and the quantity is also low[21]. Therefore, it is expected that *Glycyrrhiza* new varieties has more effective various pharmacological activities than other origin of *Glycyrrhiza* species. The present comparative study of *Glycyrrhiza* new varieties and *Glycyrrhiza* species listed in the Korean Pharmacopoeia on *in vitro* and *in vivo* model concluded that *Glycyrrhiza* new varieties had a protective effect on acute allergic reaction as well as listed *Glycyrrhiza* species.

Ⅴ. Conclusion

This study showed that *Glycyrrhiza* new varieties WONGAM and SINWONGAM decreased the histamine release without cytotoxicity in both of rat peritoneal mast cell and human mast cell. WONGAM and SINWONGAM exhibited the protective effect against anaphylactic shock showing increasing the survival and suppressing IgE and cytokine production. These findings suggest that WONGAM and SINWONGAM might have anti-allergic effects which is expect to various allergic diseases and inflammation. We believe that our findings provide a valuable guide with respect to the usage of *Glycyrrhiza* new varieties.

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Notes

The authors declare no competing financial interest.

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