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감초 신품종 및 약전 수재감초의 면역조절 효과 비교 연구

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The Comparative Study of Immunomodulatory Effect by *Glycyrrhiza* New Varieties and Official Compendia

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ABSTRACT

Objective : The genus *Glycyrrhiza* has been used in food and traditional herbal medicine. *Glycyrrhiza* new varieties Wongam and Sinwongam have been developed by Korea Rural Development Administration and investigated to register on Korean Pharmacopoeia of the Ministry of Food and Drug Safety. The aim of this study is to investigate the immunomodulatory effect of Wongam and Sinwongam comparing with listed *Glycyrrhiza* species (*Glycyrrhiza uralensis* Fischer and *G. glabra* Linne) for evaluations about pharmacological effect of *Glycyrrhiza* new varieties.
Methods : We studied the immunomodulatory effect of Wongam and Sinwongam compared with *G. uralensis* and *G. glabra* using THP-1 cell *in vitro* model. The cells were treated with phorbol 12-myristate 13-acetate (PMA) for differentiation and stimulated with lipopolysaccharides (LPS) to induce immune activation. We analyzed and compared the effects *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species using nitric oxide (NO) assay, western blot, and reverse transcription-quantitative polymerase chain reaction analysis.
Results : Wongam and Sinwongam showed no cytotoxicity in THP-1 cells. Wongam and Sinwongam, and listed *Glycyrrhiza* species increased NO production, and cyclooxygenase (COX)-2 expression with or without LPS in differentiated THP-1 macrophages. Furthermore, Wongam and Sinwongam and listed *Glycyrrhiza* species upregulated the mRNA expressions of T helper type 1 (Th 1)-associated cytokines in LPS-stimulated THP-1 macrophages.

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Conclusion : These results indicated that Wongam and Sinwongam would have effect of enhancing immune response through the increase of NO and COX-2 expression, and activate Th1-associated cytokines. The findings of this study suggest the wide applicability of *Glycyrrhiza* new varieties.

Key words : Immunomodulatory effect, *Glycyrrhiza* new varieties, Wongam, Sinwongam, immune response

Ⅰ. Introduction

The importance of immunity has recently emerged and attracted more attention since the global pandemic disease break out including severe acute respiratory syndrome, influenza, and coronavirus. People who are weakened or impaired immune system have a higher the risk of infection. The immune system is divided in categories: non-specific natural or innate immunity and specific acquired or adaptive immunity[1]. Innate immune response, the first line of defense against non-self pathogens, consists of physical barriers, biochemical and cellular defenses against pathogens. The main purpose of the innate immune response is to immediately prevent infection, to eliminate invader pathogens, and to stimulate the acquired immune response. On the other hand, adaptive immune response, the second line of defense against non-self pathogens, is specific to the pathogen presented[2].

Innate immune cells comprise a broad range of cell types including natural killer (NK) cell, neutrophil, monocyte, and macrophage[3]. In particular macrophages are specialized cells in detection, phagocytosis, and removal of pathogens and bacteria. Moreover, macrophages play a role as an antigen presenting cell that mediate specific immune response to T cells[4]. In addition, macrophages secrete high levels of reactive oxygen species and produce cyclooxygenase (COX)-2, nitric oxide synthase (iNOS), and nitric oxide (NO), that can kill phagocytosed bacteria. Various cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, and IL-12 considered as important factors of innate immunity, are released from activated macrophages and promote immune responses of other immune cells[5,6]. The activated macrophages are known to have major roles in host defense against various microbial pathogens, hence, the researches on immune-regulation have been actively conducted using activated macrophages as an instrument[7].

Licorice is one of the most widely used medicinal herbs, and the root has been used therapeutically for many centuries[8]. A lot of experimental studies reported that *Glycyrrhiza* species possess several pharmacological properties such as anti-microbial[9], anti-viral[10], anti-inflammatory[11], anti-ulcer[12], anti-tumor[13], anti-oxidant[14], anti-depressant[15], and anti-diabetic activities[16,17]. It was reported that licorice and its components exhibit immunomodulatory effect in different models[18-21]. *Glycyrrhiza* new varieties Wongam and Sinwongam have been developed by Korea Rural Development Administration and investigated to register on Korean Pharmacopoeia of the Ministry of Food and Drug Safety. Wongam and Sinwongam are breeding variety, which are hybrid of *G. glabra* × *G. uralensis*. We are performing research to evaluate the homogeneity of the *Glycyrrhiza* new varieties Wongam and Sinwongam and *Glycyrrhiza* species listed in the Korean Pharmacopoeia as part of the study for the Korean Pharmacopoeia registration of *Glycyrrhiza* new varieties. We reported the comparative study of anti- allergic effect by *Glycyrrhiza* new varieties and official compendia[22]. No study to date about immunomodulatory effect on *Glycyrrhiza* new varieties has examined yet. In the present study, therefore, we evaluated the immunomodulatory effects of *Glycyrrhiza* new varieties, compared with that of *Glycyrrhiza* species listed in the Korean Pharmacopoeia using THP-1 macrophage *in vitro* model.

Ⅱ. Materials and Methods

1. Chemicals and reagents

Extractions of *Glycyrrhiza* species were provided from Korea Rural Development Administration. For the present study, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS; *Escherichia coli*, serotype 055:B5), [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), Griess reagent and all other chemicals were purchased from Millipore Sigma (Billerica, MA, USA). Roswell Park Memorial Institute (RPMI) 1640 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 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). COX-2, and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TNF-α, IL-6, IL-2, IL-10, IL-12, and GAPDH

oligonucleotide primers were purchased from Bioneer Corporation (Daejeon, South Korea).

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 distilled water at 100℃ for 4.5 h. The extracts were concentrated under reduced pressure in a rotary evaporator at 70℃ for 3 h. 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. Cell viability assay

Cells were seeded in a 96-well culture plate at 1×10^5 cells/㎖ in culture medium. Cells were treated with medium containing various concentrations of *Glycyrrhiza* species samples. After incubating for 24 h, THP-1 cells were treated with 20 ㎕ of MTS for 4 h and absorbance was measured at 490 ㎚ using a microplate reader.

4. THP-1 cell differentiation and stimulation

THP-1 cell was purchased from Korea Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were grown at 37℃ in RPMI 1640 supplemented with 10 % FBS, penicillin (100 U/㎖) and streptomycin (100 ㎍/㎖) in a humidified atmosphere of 5 % CO_2. THP-1 cells were seeded at a density of 5×10^5 cell per well and incubated for 4 h and then, treated with 100 ng/㎖ of PMA for cell differentiation. After incubation for 48 h, the cells were exposed to *Glycyrrhiza* species samples with or without LPS (10 ㎍/㎖) for 24 or 48 h.

5. NO assays

NO content was determined indirectly by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilamide in 5% phosphoric acid, 1% α-naphthylamide in H_2O). NO production from THP-1 cells was a form of NO_2 that exists in culture media. A 50 ㎕ amount of cell culture media was mixed with 50 ㎕ of Griess reagent in a 96-well plate, incubated at room temperature for 15 min, and then measured at 540 ㎚ using an automatic microplate reader (Titertek Multiskan).

6. Western Blot Analysis

The cells were resuspended in a commercial lysis buffer (PRO-PREP, Intron Biotechnology, Seoul, Republic of Korea) and incubated for 20 min at 4℃. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Aliquots of each protein sample (30 ㎍) were separated on a sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated for 1 h with 5% skim milk at room temperature, followed by incubation overnight with a 1:1000 dilution of primary antibody at 4℃. Blots were washed three times with Tween 20/Tris-buffered saline (T/TBS) and incubated with a 1:2500 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with T/TBS and then developed by enhanced chemiluminescence (GE Healthcare, Waukesha, WI, USA).

7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated from the cells or liver tissues using an Easy Blue kit (Intron Biotechnology, Inc., Seoul, Korea) according to the manufacturer's protocol. Total RNA was quantified using an Epoch micro-volume spectrophotometer system (BioTek Instruments, Inc., Winooski, VT, USA). cDNA was obtained using isolated total RNA (2 ㎍), d(T)16 primer, and Avian Myeloblastosis Virus reverse transcriptase with genomic DNA remover. The relative gene expression was quantified using RT-qPCR analysis (Real Time PCR System 7500; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with SYBR Premix Ex Taq. Fold changes of gene expression were calculated using the comparative quantification cycle method. The Cq values of target genes TNF-α, IL-6, IL-2, IL-10, and IL-12 were normalized to that of GAPDH using the ABI Gene Express 2.0 programme (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

8. Statistical analysis

The data are expressed as the mean ± standard deviation of triplicate experiments. Statistically significant 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 THP-1 cells

To investigate whether *Glycyrrhiza* species samples were cytotoxic to the THP-1 cells, the MTT assay was performed. The extracts of Wongam, Sinwongam, *G. uralensis*, *G. glabra* at concentrations up to 1000 ㎍/㎖ exhibited no significant cytotoxicity to the THP-1 cells after 24 h of incubation when compared with non-treated THP-1 cells. Rather, the result showed the increasing trend in cell viability (Figure 1). Accordingly, we used 500 ㎍/㎖ of Wongam, Sinwongam, *G. uralensis*, and *G. glabra* for subsequent experiment.

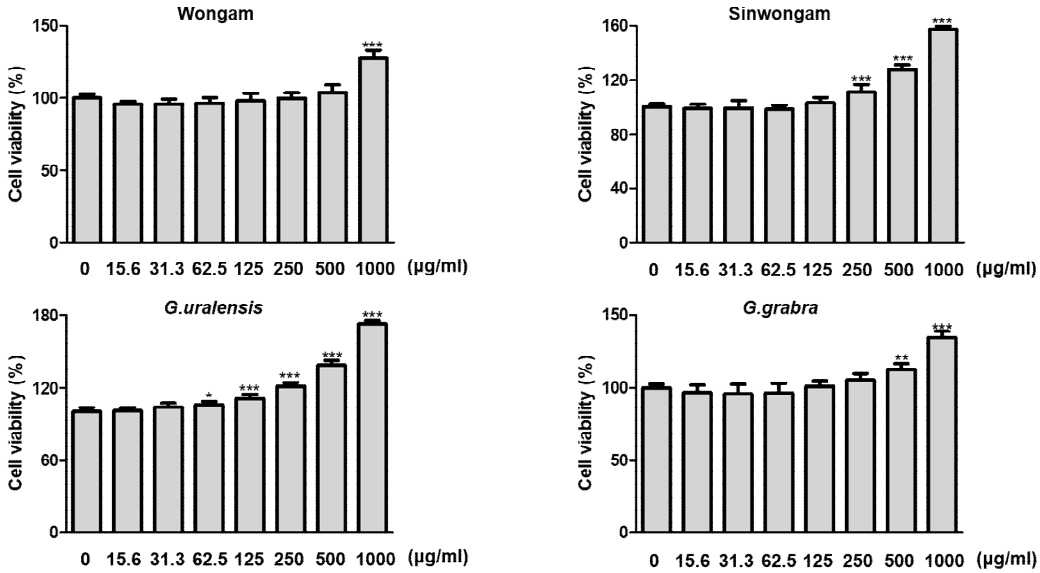

Figure 1. Effects of four *Glycyrrhiza* species samples on cell viability on THP-1 cells. Cells were treated with different concentrations of *Glycyrrhiza* species samples for 24 h and their viability was determined using MTS assay. The data shown represent mean ± S.D. of three independent experiments.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

2. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species increase the level of NO production from differentiated THP-1 macrophages

THP-1 monocytes were treated with PMA for 48 h for differentiation into macrophages to study immune responses. In order to examine the immunomodulatory effect of *Glycyrrhiza* species, the level of NO production from differentiated THP-1 cells was measured. Treatment of Wongam, Sinwongam, *G. uralensis*, and *G. glabra* induced a significant increase in NO levels compared with PMA- differentiated THP-1 cells. The NO level induced by Wongam treatment for 48 h was higher than other *Glycyrrhiza* species (Figure 2A). Moreover, LPS stimulation increased the NO level compared to PMA-differentiated cells, whereas the higher levels of NO were observed in groups treated with Wongam, Sinwongam, or *G. glabra*. Among *Glycyrrhiza* species, Sinwongam resulted in higher NO level in LPS-stimulated THP-1 macrophages (Figure 2B).

3. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species increased the COX-2 expression differentiated THP-1 macrophages

LPS activates the differentiated THP-1 cells and induces the expressions of immune-associated genes and proteins. To investigate the effects of *Glycyrrhiza* species samples on COX-2 protein expression, western blot analysis was conducted. In the absence of LPS stimulation, Wongam, Sinwongam, *G. uralensis*, and *G. glabra* increased the protein level of COX-2 with

significance compared with the non-treated THP-1 cells under the both of time conditions (24 and 48 h). COX-2 expression was more induced markedly by LPS stimulation, however, all *Glycyrrhiza* species samples enhanced the expression in activated THP-1 macrophages. Notably, the Sinwongam revealed stronger protein expression of COX-2 in LPS-stimulated THP-1 macrophages for 24 h (Figure 3A). The expressions of COX-2 were more prominent under the LPS stimulation for 24 h (Figure 3A) than 48 h (Figure 3B).

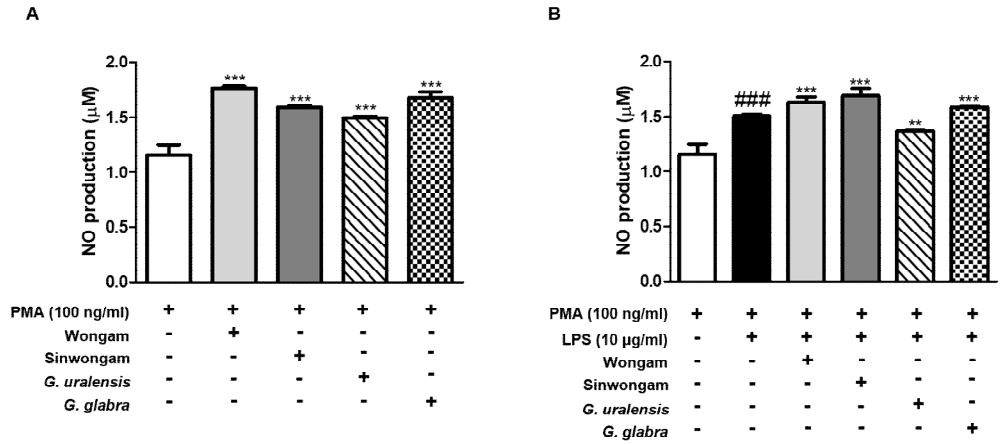

Figure 2. Effects of four *Glycyrrhiza* species samples on NO production in THP-1 macrophages. (A) THP-1 cells differentiated by PMA were incubated with *Glycyrrhiza* species samples for 48 h. (B) THP-1 cells differentiated by PMA were incubated with *Glycyrrhiza* species samples with LPS (10 ㎍/㎖) for 48 h. NO levels were determined with Griess reagent. 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 (A) control group or (B) LPS-stimulated cells.

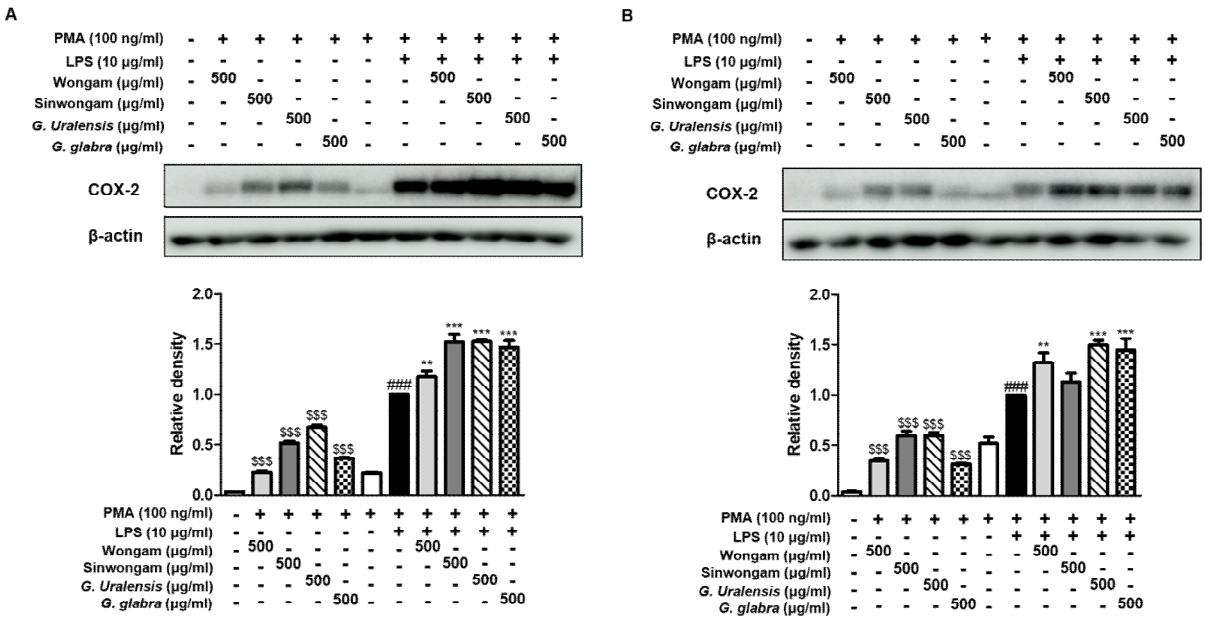

Figure 3. Effects of four *Glycyrrhiza* species samples on COX-2 protein expression in THP-1 macrophages. (A) THP-1 cells differentiated by PMA were incubated with *Glycyrrhiza* species samples or samples plus LPS (10 ㎍/㎖) for 24 h or (B) 48 h. The protein level of COX-2 was determined by western blot analysis using specific antibodies. Densitometric analysis was performed using Bio-Rad Quantity One software. The data shown represent mean ± S.D. of three independent experiments. $^{\$\$\$}p < 0.001$ vs the non-treated group, $^{###}p < 0.001$ vs the differentiation group, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs LPS-stimulated cells.

4. *Glycyrrhiza* new varieties and listed *Glycyrrhiza* species upregulate the expressions of T helper type 1 (Th 1)-associated cytokines

IL-2 is classified as Th1 cytokine along with TNF-α, IL-6 and IFN-γ, and it plays a role as an immune stimulant to other cells such as NK cells[23]. IL-12 is known as a potent inducer of the differentiation of Th1 cells[24], and IL-10 up-regulates the production of NO

in LPS-activated macrophages[25]. Next, we measured the mRNA levels of Th1-associated cytokines involved in potentiation of the immune responses, including TNF-α, IL-6, IL-2, IL-12, and IL-10. The mRNA expression of TNF-α increased by LPS stimulation, whereas its level was significantly higher upon Wongam, Sinwongam, *G. uralensis*, *G. glabra* treatment. The Sinwongam treated group displayed the most statistically significant upregulation of TNF-α (Figure 4A). In the case of IL-6 expression, it was significantly increased by LPS stimulation compared with control, however, the levels were decreased by treatment with Wongam, Sinwongam, and *G. uralensis* rather than LPS stimulation group, and increased slightly by *G. glabra* treatment (Figure 4B). The mRNA level of IL-2 was also increased by LPS stimulation compared to control, but it was upregulated by Wongam, Sinwongam, and *G. uralensis*, not *G. glabra* (Figure 5A). All *Glycyrrhiza* species samples significantly induced upregulation of IL-12 and IL-10 levels, which were increased by LPS (Figure 5B and C).

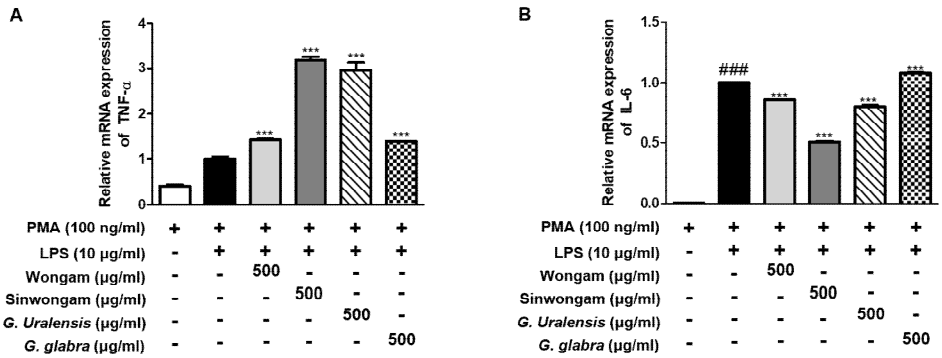

Figure 4. Effects of four *Glycyrrhiza* species samples on mRNA expressions of TNF-α, and IL-6 in differentiated THP-1 macrophages. THP-1 cells differentiated by PMA were incubated with *Glycyrrhiza* species samples with LPS (10 ㎍/㎖) for 6 h. Total RNA prepared form the dorsal tissue, and the levels of (A) TNF-α, and (B) IL-6 were determined by qRT-PCR. 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 LPS-stimulated cells.

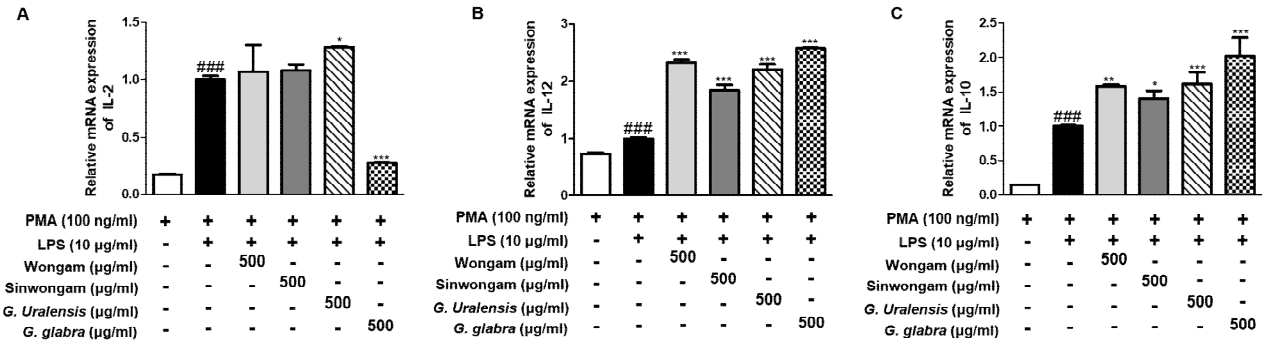

Figure 5. Effects of four *Glycyrrhiza* species samples on mRNA expressions of IL-2, IL-12, and IL-10 in differentiated THP-1 macrophages. THP-1 cells differentiated by PMA were incubated with *Glycyrrhiza* species samples with LPS (10 ㎍/㎖) for 6 h. Total RNA prepared form the dorsal tissue, and the levels of (A) IL-2, (B) IL-12, and (C) IL-10 were determined by qRT-PCR. The data shown represent mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs LPS-stimulated cells.

Ⅳ. Discussion

Licorice contains a large number of biologically active compounds, and flavonoids and triterpenoid are the main components which show broad biological activities[26]. Among those components, glycyrrhizin, a triterpenoid saponin, has been reported to increase the activity of dendritic cells and proliferation of T cells with increased interferon (IFN)-γ and IL-10 cytokines production[27]. Moreover, it has reported that glycyrrhetinic acid induces the expression of toll-like receptor (TLR)-4 along with its downstream signaling molecules[28]. These molecules have been known as the important role in modulating innate immune responses against pathogens. In addition, liquiritigenin, aglycone from licorice, has an immunomodulating activity that mediates macrophage activation and induces dominant Th1 type cytokine production from the activated $CD4^+$ Tcells[21]. The present study showed that Wongam and Sinwongam induced NO production level and upregulated the innate immune-associated cytokines in LPS-stimulated THP-1 macrophages. These results were comparable with that of

the listed *Glycyrrhiza* species in Korean Pharmacopoeia. These findings imply that Wongam and Sinwongam have an efficacy homogeneity in immunomodulatory effect with listed *Glycyrrhiza* species. Notably, *Glycyrrhiza* new varieties have a higher content of glycyrrhizin (3.96 %) than *G. uralensis* (1.90 %). Therefore, in line with the results of previous studies, it is expected that Wongam and Sinwongam have a better effect on the various pharmacological activities mentioned above as well as immunomodulating activity.

LPS is a component of cell wall of gram negative bacteria, and play a role as a potent inducer of COX-2 expression in activated macrophages[29]. This response is mediated by TLR4 that leads to stimulation intracellular signaling cascades such as mitogen activated protein kinase and nuclear factor-κB pathways[30]. In this study, Wongam and Sinwongam, and listed *Glycyrrhiza* species significantly induced NO production level in both conditions with/without LPS treatment (Figure 2A and B). However, the protein level of iNOS was not detected in western blot analysis (data not shown). This seems to be related to the low level of NO production, which was less than 2 μM in all groups. By comparison with iNOS expression, the strong protein expressions of COX-2 were observed in *Glycyrrhiza* species-treated group in THP-1 macrophages. Overexpression of COX-2 metabolizes the accumulation of prostaglandin E_2 and is mainly associated with inflammation, but if the expression increases within the appropriate level, it promotes the immune response[31,32]. However, COX-2 protein levels were significantly enhanced by treatment with *Glycyrrhiza* species compared to LPS treated THP-1 macrophages. This data revealed that *Glycyrrhiza* species activate THP-1 macrophages in condition of LPS stimulation. Since these factors including are involved in inflammatory networks and are inducible by inflammatory stimuli, future research should consider the potential effects of Wongam and Sinwongam in connection with immunopotentiation and inflammation more carefully.

Th1 cells are critical in the innate immune response and play an important role in host defense against intracellular viral and bacterial pathogens. These cells are a lineage of CD4+ effector T cell that secrete TNF-α/β, IFN-γ, IL-2, IL-12 and IL-10. These cytokines promote NO production, cytotoxic T lymphocyte proliferation, and macrophage activation, leading to the phagocytosis and destruction of microbial pathogens[33,34]. As a result of mRNA expression of these cytokines in this study, IL-12 and IL-10 levels were shown similar increased trend in LPS-stimulated THP-1 macrophages, that levels are significantly higher than LPS-treated group. In addition, Wongam, Sinwongam, and *G. uralensis* upregulated the mRNA expressions of TNF-α and IL-2, while IL-6 expression was downregulated by Wongam, Sinwongam, and *G. uralensis*. IL-10 is also known as an anti-inflammatory cytokine which regulates cytokine-balance and limits the overt inflammation preventing tissue damage[35]. As licorice has a well-documented anti-inflammatory property, further studies should investigate the effect of Wongam, Sinwongam on anti-inflammatory action and more specific Th1 cell-mediated immune responses. These further researches on *Glycyrrhiza* species might extend the explanations of the immunomodulatory effect of Wongam and Sinwongam.

Ⅴ. Conclusion

In conclusion, this study showed that *Glycyrrhiza* new varieties Wongam and Sinwongam have an immunomodulatory effect and enhance immune function by regulating the NO, COX-2, and immune-associated cytokines derived from activated macrophages. Compared to listed *Glycyrrhiza* species, the higher NO level was induced by Wongam treatment in differentiated THP-1 cells, and the Sinwongam treatment activated immune responses increasing COX-2, and TNF-α expression in LPS-stimulated THP-1 macrophages. The findings of this study imply that Wongam and Sinwongam have an immunomodulation efficacy homogeneity with listed *Glycyrrhiza* species in the Korean Pharmacopoeia, thus suggest the wide applicability of *Glycyrrhiza* new varieties Wongam and Sinwongam.

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Notes

The authors declare no competing financial interest.

References

1. Calder, P.C. and S. Kew, The immune system: a target for functional foods? Br J Nutr. 2002;88 Suppl 2:S165-77.
2. Juan-Manuel Anaya, Yehuda Shoenfeld, Adriana

Rojas-Villarraga, Roger A. Levy, Ricard Cervera , editors. Autoimmunity: From Bench to Bedside, First edition. Bogota (Colombia): El Rosario University Press; 2013. Available from : URL : https://pubmed.ncbi.nlm.nih.gov/29087650.
3. Locati, M., A. Mantovani, and A. Sica, Macrophage activation and polarization as an adaptive component of innate immunity. Adv Immunol. 2013;120:163-84.
4. Elhelu, M.A., The role of macrophages in immunology. J Natl Med Assoc. 1983;75:314-7.
5. Arango Duque, G. and A. Descoteaux, Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014;5:491.
6. Pereira, A.C.A., R.J. Silva, P.S. Franco, A. de Oliveira Gomes, G. Souza, I.C.B. Milian, M. Ribeiro, A.M. Rosini, P.M. Guirelli, E.L.P. Ramos, T.W.P. Mineo, J.R. Mineo, N.M. Silva, E.A.V. Ferro, and B.F. Barbosa, Cyclooxygenase (COX)-2 Inhibitors Reduce Toxoplasma gondii Infection and Upregulate the Pro-inflammatory Immune Response in Calomys callosus Rodents and Human Monocyte Cell Line. Front Microbiol. 2019;10:225.
7. Leopold Wager, C.M. and F.L. Wormley, Jr., Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. Mucosal Immunol. 2014;7:1023-35.
8. Fiore, C., M. Eisenhut, E. Ragazzi, G. Zanchin, and D. Armanini, A history of the therapeutic use of liquorice in Europe. J Ethnopharmacol. 2005;99:317-24.
9. Sedighinia, F., A. Safipour Afshar, S. Soleimanpour, R. Zarif, J. Asili, and K. Ghazvini, Antibacterial activity of Glycyrrhiza glabra against oral pathogens: an in vitro study. Avicenna J Phytomed. 2012;2:118-24.
10. Fiore, C., M. Eisenhut, R. Krausse, E. Ragazzi, D. Pellati, D. Armanini, and J. Bielenberg, Antiviral effects of Glycyrrhiza species. Phytother Res. 2008;22:141-8.
11. Furuhashi, I., S. Iwata, S. Shibata, T. Sato, and H. Inoue, Inhibition by licochalcone A, a novel flavonoid isolated from liquorice root, of IL-1beta-induced PGE2 production in human skin fibroblasts. J Pharm Pharmacol. 2005;57:1661-6.
12. Aly, A.M., L. Al-Alousi, and H.A. Salem, Licorice: a possible anti-inflammatory and anti-ulcer drug. AAPS PharmSciTech. 2005;6:E74-82.
13. Dong, S., A. Inoue, Y. Zhu, M. Tanji, and R. Kiyama, Activation of rapid signaling pathways and the subsequent transcriptional regulation for the proliferation of breast cancer MCF-7 cells by the treatment with an extract of Glycyrrhiza glabra root. Food Chem Toxicol. 2007;45:2470-8.
14. Lateef, M., L. Iqbal, N. Fatima, K. Siddiqui, N. Afza, M. Zia-ul-Haq, and M. Ahmad, Evaluation of antioxidant and urease inhibition activities of roots of Glycyrrhiza glabra. Pak J Pharm Sci. 2012;25:99-102.
15. Wang, W., X. Hu, Z. Zhao, P. Liu, Y. Hu, J. Zhou, D. Zhou, Z. Wang, D. Guo, and H. Guo, Antidepressant-like effects of liquiritin and isoliquiritin from Glycyrrhiza uralensis in the forced swimming test and tail suspension test in mice. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32:1179-84.
16. Pastorino, G., L. Cornara, S. Soares, F. Rodrigues, and M. Oliveira, Liquorice (Glycyrrhiza glabra): A phytochemical and pharmacological review. Phytother Res. 2018;32:2323-2339.
17. Kwon, Y.J., D.H. Son, T.H. Chung, and Y.J. Lee, A Review of the Pharmacological Efficacy and Safety of Licorice Root from Corroborative Clinical Trial Findings. J Med Food. 2020;23:12-20.
18. Dziewulska, D., T. Stenzel, M. Smialek, B. Tykalowski, and A. Koncicki, The impact of Aloe vera and licorice extracts on selected mechanisms of humoral and cell-mediated immunity in pigeons experimentally infected with PPMV-1. BMC Vet Res. 2018;14:148.
19. Guo, A., D. He, H.B. Xu, C.A. Geng, and J. Zhao, Promotion of regulatory T cell induction by immunomodulatory herbal medicine licorice and its two constituents. Sci Rep. 2015;5:14046.
20. Ayeka, P.A., Y. Bian, P.G. Mwitari, X. Chu, Y. Zhang, R. Uzayisenga, and E.O. Otachi, Immunomodulatory and anticancer potential of Gan cao (Glycyrrhiza uralensis Fisch.) polysaccharides by CT-26 colon carcinoma cell growth inhibition and cytokine IL-7 upregulation in vitro. BMC Complement Altern Med. 2016;16:206.
21. Lee, J.Y., J.H. Lee, J.H. Park, S.Y. Kim, J.Y. Choi, S.H. Lee, Y.S. Kim, S.S. Kang, E.C. Jang, and Y. Han, Liquiritigenin, a licorice flavonoid, helps mice resist disseminated candidiasis due to Candida albicans by Th1 immune response, whereas liquiritin, its glycoside form, does not. Int Immunopharmacol. 2009;9:632-8.
22. Y.M. Kang, W.K., J.S. Jin, J.H. Lee, J.K. Chang, J. Lee, H.J. An, The Comparative Study of Anti-allergic Effect by Glycyrrhiza New Varieties and Official Compendia. Kor. J. Herbol. 2020;35:13-21.
23. Brubaker, J.O., C.M. Thompson, L.A. Morrison, D.M. Knipe, G.R. Siber, and R.W. Finberg, Th1-associated immune responses to beta-galactosidase expressed

by a replication-defective herpes simplex virus. J Immunol. 1996;157:1598-604.
24. Huang, F.P., W. Niedbala, X.Q. Wei, D. Xu, G.J. Feng, J.H. Robinson, C. Lam, and F.Y. Liew, Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. Eur J Immunol. 1998;28:4062-70.
25. Jacobs, F., D. Chaussabel, C. Truyens, V. Leclerq, Y. Carlier, M. Goldman, and B. Vray, IL-10 up-regulates nitric oxide (NO) synthesis by lipopolysaccharide (LPS)-activated macrophages: improved control of Trypanosoma cruzi infection. Clin Exp Immunol. 1998;113:59-64.
26. Hosseinzadeh, H. and M. Nassiri-Asl, Pharmacological Effects of Glycyrrhiza spp. and Its Bioactive Constituents: Update and Review. Phytother Res. 2015;29:1868-86.
27. Bordbar, N., M.H. Karimi, and Z. Amirghofran, The effect of glycyrrhizin on maturation and T cell stimulating activity of dendritic cells. Cell Immunol. 2012;280:44-9.
28. Peng, L.N., L. Li, Y.F. Qiu, J.H. Miao, X.Q. Gao, Y. Zhou, Z.X. Shi, Y.L. Xu, D.H. Shao, J.C. Wei, and Z.Y. Ma, Glycyrrhetinic acid extracted from Glycyrrhiza uralensis Fisch. induces the expression of Toll-like receptor 4 in Ana-1 murine macrophages. J Asian Nat Prod Res. 2011;13:942-50.
29. Kang, Y.J., B.A. Wingerd, T. Arakawa, and W.L. Smith, Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J Immunol. 2006;177:8111-22.
30. Britt, R.D., Jr., M.L. Locy, T.E. Tipple, L.D. Nelin, and L.K. Rogers, Lipopolysaccharide-induced cyclooxygenase-2 expression in mouse transformed Clara cells. Cell Physiol Biochem. 2012;29:213-22.
31. Gandhi, J., L. Khera, N. Gaur, C. Paul, and R. Kaul, Role of Modulator of Inflammation Cyclooxygenase-2 in Gammaherpesvirus Mediated Tumorigenesis. Front Microbiol. 2017;8:538.
32. Kim, A.J., Y.O. Kim, J.S. Shim, and J.K. Hwang, Immunostimulating activity of crude polysaccharide extract isolated from Curcuma xanthorrhiza Roxb. Biosci Biotechnol Biochem. 2007;71:1428-38.
33. Leung, S., X. Liu, L. Fang, X. Chen, T. Guo, and J. Zhang, The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cell Mol Immunol. 2010;7:182-9.
34. Holley, M.M. and T. Kielian, Th1 and Th17 cells regulate innate immune responses and bacterial clearance during central nervous system infection. J Immunol. 2012;188:1360-70.
35. Steen, E.H., X. Wang, S. Balaji, M.J. Butte, P.L. Bollyky, and S.G. Keswani, The Role of the Anti-Inflammatory Cytokine Interleukin-10 in Tissue Fibrosis. Adv Wound Care (New Rochelle). 2020;9:184-198.