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# 잎새버섯 균사체를 이용한 감초추출발효물의 플라보노이드 생성과 항염 활성 연구

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# Production of Flavonoid Compounds and Anti-inflammatory Property of Fermented Licorice Extract with the Basidiomycete *Grifola frondosa* HB0071

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**요 약**: 리퀴리티게닌과 이소리퀴리티게닌은 감초의 주요 플라보노이드 성분이다. 이들 플라보노이드는 수용성 감초 추출물과 β-glucosidase를 생성하는 잎새버섯 HB0071 균사체 발효배양을 통하여 생산하였다. 감초추출물 내 리퀴리티게닌과 이소리퀴리티게닌은 잎새버섯 발효배양 동안 현저히 증가하였다. 이 균주의 β-glucosidase의 활성은 배양 96시간을 기준으로 최고 91.5 mU/mL로 확인되었으며, 감초추출발효물로부터 생성된 리퀴리티게닌과 이소리퀴리티게닌의 함량은 HPLC 분석을 통하여 최대 568.5 μg/mL과 89.6 μg/mL로 확인되었다. 본 연구에서는 감초추출물의 잎새버섯 발효 전·후의 시료가 처리된 각질형성세포를 이용하여 자외선 UVB에 조사로 발현된 염증유발인자(COX-2)와 사이토카인(IL-1β, IL-6) 모두 감초추출발효물(FLEx)에서 농도의존적으로 발현이 억제되는 것을 확인하였다. 결론적으로 리퀴리티게닌과 이소리퀴리티게닌의 함량이 증가된 감초추출발효물은 자외선으로부터 손상된 피부 염증반응을 완화시켜줄 것으로 사료된다.

**Abstract**: Liquiritigenin and isoliquiritigenin are the major flavonoids present in licorice. These flavonoid compounds were prepared by submerged culture of *Grifola frondosa* (*G. frondosa*) HB0071 mycelia producing β-glucosidase in the aqueous extract of licorice. The contents of liquiritigenin and isoliquiritigenin were increased during the fermentation. This fungus produced a high β-glucosidase (activity of 91.5 mU/mL), thereby achieving high amounts of liquiritigenin and isoliquiritigenin (568.5 μg/mL and 89.6 μg/mL), respectively at 96 h. A reversed- phase high-performance liquid chromatography method was established for simultaneous determination of liquiritigenin and isoliquiritigenin in fermented licorice extract (FLEx). The anti-inflammatory activities were investigated by licorice extract (LEx) before and after fermentation with *G. frondosa* HB0071. The treatment of UVB-irradiated HaCaT keratinocytes with FLEx resulted in a dose-dependent decrease in the expression level of cyclooxygenase-2 (COX-2) mRNA. Furthermore, FLEx dose-dependently decreased mRNA of the pro-inflammatory cytokines of IL-1β and IL-6 in UVB-irradiated HaCaT cells. These results suggest that FLEx may mitigate the effects of skin inflammation by reducing UVB-induced adverse skin reactions.

***Keywords***: *Licorice, Grifola frondosa HB0071, β-glucosidase, flavonoids anti-inflammation*

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## 1. Introduction

*G. frondosa* is a Basidiomycete fungus belonging to the order Aphyllopherales, and the family Polyporaceae. *G. frondosa* has been frequently used to treat spleen and stomach ailments, to calm the nerves and the mind, and to treat hemorrhoids. Its dried fruit body has been used in the production of health foods, including teas, whole powders, powders of hot water extracts, granules, and drinks[1]. Previous work demonstrated that various biological and physicochemical properties of the extracellular polysaccharides of *G. frondosa*, which are optimization of submerged culture conditions[2], antioxidant activity, stimulation of collagen biosynthesis, cell proliferation activity, and inhibitory effect on matrix metalloproteinase (MMP-1) expression upon UVA-irradiation in cultured human dermal fibroblasts (HDFs)[3].

Licorice is a flavorful herb that has been used in food and medicinal remedies for thousands of years. It can be used for various purposes, such as bronchitis, ague, hepatitis, phthisis, and gastric ulcer[4]. Phytochemical investigation has demonstrated that the major bioactive components of licorice are flavonoids, isoflavones and triterpene saponins[5]. Among them, the remarkable flavonoids such as liquiritin, liquiritigenin (LTG), isoliquiritin, and isoliquiritigenin (ILG), are reported to have various biological activities, including antioxidative, anti-inflammatory, antimicrobial, antitumor, and others[6].

Phytochemicals are important secondary metabolites and are ubiquitous in all plants. They are usually synthesized in plants as a defense against pathogenic attack or environmental stress such as UV exposure or hyperhydracity[7]. Most phenolics that are found in plants, however exist conjugated to sugar (primarily glucose) as glycosides. Several studies have revealed that aglycones are superior to glycosides in various bioactivities, due to their effective absorption[8]. There is interest in increasing the amounts of aglycone components in licorice. Therefore, if free aglycones (liquiritigenin and isoliquiritigenin) in licorice root are released from their glycosides (liquiritin and isoliquiritin), then the anti-inflammatory functionality of these phytochemicals could be improved.

The skin is directly exposed to the environment and is therefore highly vulnerable to UV radiation from the sun. Especially, UVB is a major cause of cutaneous inflammatory disorders, such as erythema and edema[9]. Many investigators have reported that UVB promotes skin inflammation by inducing production of pro-inflammatory cytokines, such as tumor necrotic factor (TNF)-$\alpha$, interleukin (IL)-1$\alpha$, IL-1$\beta$, IL-6, IL-8, IL-10, and prostaglandins (PG), including PGE2 in cultured human keratinocytes[10]. These pro-inflammatory cytokines are associated with various inflammatory skin diseases. Kim et al.[11] reported that isoliquiritigenin-isolated licorice extract effectively inhibited lipopolysaccharide (LPS)-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions and iNOS, COX-2, TNF-$\alpha$, and IL-6 transcriptions in RAW 264.7 macrophages.

Medicinal herbs have long been processed via microbial fermentation. Studies have demonstrated that fermentation not only alters the original bioactivities of medicinal herbs, resulting in new treatment effects, but also enhances the original treatment efficacy[12]. It is known that $\beta$-glucosidase from *Bacillus subtilis* natto can convert isoflavone glucosides to their aglycones[13]. Numerous studies have revealed that the biological effects of isoflavone stem from their aglycones[14]. In this study, *G. frondosa* fermentation of licorice extract might convert flavonoid glycosides to aglycones by its $\beta$-glucosidase activity. However, few reports have paid attention to the changes in the components of licorice by fermentation with basidiomycete mushrooms as well as with *G. frondosa*.

The objective of the work was to develop liquiritigenin and isoliquiritigenin production from a submerged culture of *G. frondosa* HB0071 using licorice extract as substrate. The effects of fermented licorice extract with *G. frondosa* HB0071 on inflammatory responses were investigated using in vitro UVB-irradiated HaCaT keratinocytes.

## 2. Materials and Methods

### 2.1. Microorganism, Media and Extraction

*G. frondosa* HB0071 was isolated from the mountainous district in Chungbuk province, Korea. The stock culture was maintained on potato dextrose agar (PDA) slants. Unless otherwise mentioned, slants were incubated at 27 ℃ for 5 days and then stored at 4 ℃. The seed culture was grown in a 250 mL flask containing 50 mL of medium containing 30 g/L glucose, 6 g/L yeast extract, 2 g/L polypeptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $K_2HPO_4$, and 0.2 g/L $MnSO_4 \cdot 5H_2O$ at 27 ℃ on a rotary shaker incubator at 120 rpm for 5 days. Licorice was purchased from herb markets in Chungbuk province, Korea. Dried licorice (100 g) was treated with 1 L of distilled water under reflux (4 h, twice). Licorice extract was filtered and evaporated in vacuo to yield a raw residue (yield: 6.05 %).

### 2.2. Fermentations

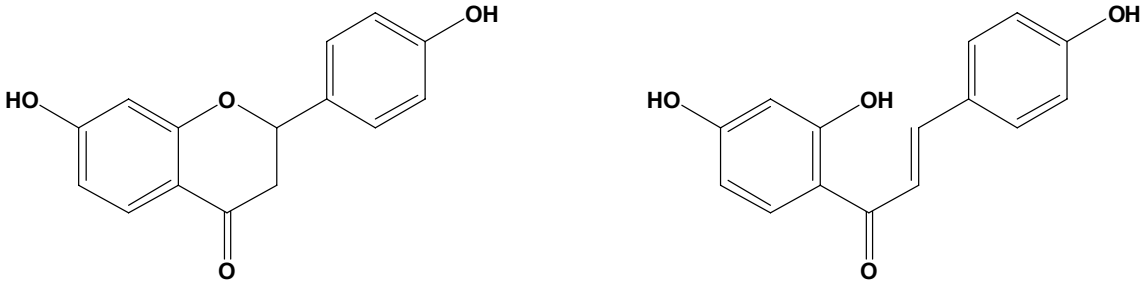
Fermentation conditions were prepared as previously described[15]. Aqueous licorice extract, as the carbon source instead of glucose, was used to produce β-glucosidase and flavonoid aglycones. Fermentation were conducted at 27 ℃, aeration rate 1.0 vvm, agitation speed 150 rpm, pH 5.5, and working volume 3-L for 5 days. All fermentation experiments were performed in triplicate at least.

### 2.3. β-glucosidase Assay

β-glucosidase from the fermented *G. frondosa* mycelia with licorice extract was extracted at every 24 h intervals and mycelium was removed by centrifugation (10,000 rpm, 20 min, 4 ℃). The supernatant was concentrated (12,000 rpm, 20 min, 4 ℃) using centrifugal filter (Vivaspin 500, 10,000 MWCO, Vivaproducts, USA). The concentrated supernatant was resuspended in 100 mM acetate buffer (pH 5.0) to give the 5-fold concentrated crude enzymes of the initial supernatant. β-glucosidase activity was routinely assayed by using a 1 mL reaction mixture containing 5 mM p-nitrophenyl-β-D-glcucoside (pNP βG) (Sigma Chemical, St. Louis, MO, USA), 100 mM acetate buffer (pH 5.0), and an appropriate dilution of enzyme preparation. After 30 min of incubation at 50 ℃, the reaction was stopped by adding 2 mL of 1 M $Na_2CO_3$, and the p-nitrophenol release was monitored at 400 nm. One unit of activity was the amount of enzyme that released 1 μmol of *p*-nitrophenol per min under the assay condition[16].

### 2.4. Analysis of Flavonoid Compounds by HPLC

The contents and compositions of flavonoid, including liquiritigenin and isoliquiritigenin from fermented and non-fermented licorice extract were determined quantitatively by HPLC. HPLC analysis was carried out by the modified method of Sakakibara et al.[17], usinga Alliance Waters 2695 (Waters Co., Milford, MA, USA) system consisting of degasser, quaternary pump, auto sampler, column oven and diode array detector (Waters 996). The compounds were eluted using a Xterra RP18 column (250 × 4.6 mm i.d., Waters Co., Milford, MA, USA), joined with a guard column (10 × 4.0 mm i.d.) and detected at 275 nm and 370 nm with a flow rate of 0.8 mL/min. the column temperature was set at 35 ℃. In order to isolate the region of liquiritigenin and isoliquiritigenin by flavonoid compounds with much more retention time, the elution was carried out with gradient mobile phase (A) aqueous acetic acid (6 mM) and (B) methanol, starting from 80 % A to 40 % A for 0 ~ 110 min, 40 ~ 0 % A for 10 min and 80 % A for 10 min. All extracts were completely diluted with the same amount of methanol and filtered through 0.45 μm PTFE syringe filters for injection to HPLC. Fractionation and purification of liquiritigenin and isoliquiritigenin (Figure 1) isolated from the analytical Xterra RP18 column were performed by the preparative HPLC using a Xterra RP18 preparative column (10 μm particle size, 250 × 10 mm). For the mobile phase, solvent A was methanol while solvent B was $H_2O$. the elution conditions were 0 ~ 10 min of 40 ~ 70 % A to B; 10 ~ 20 min of 70 ~ 75 % A to B; 20 ~ 30 min of 70 ~ 100 % A to B. The flow rate was 2.5 min/mL and injection volume was 100 μL. The flavonoid compounds peaks




Liquiritigenin Isoliquiritigenin

**Figure 1.** Molecular structures of liquiritigenin and isoliquiritigenin isolated from fermented licorice extract with *G. frondosa* HB0071.

were collected manually. The fractions, which represented two flavonoids collected from the eluent of preparative HPLC, were lyophilized and the corresponding purified liquiritigenin and isoliquiritigenin powders were obtained. Electrospray ionization mass spectrometry (ESI-MS) (negative-ion mode) data were collected using a ion-trap time-of-flight mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan) and NMR data such as $^{1}H$, $^{13}C$, HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond correlation) spectra of liquiritigenin and isoliquiritigenin dissolved in CD3OD were recorded using a 500 MHz spectrometer. (Bruker, Ettlingen, Germany).

### 2.5. Cell Culture

Human keratinocyte cell line, HaCaT cell, was maintained at 37 ℃ in a humidified atmosphere containing 5 % $CO_2$ in Eagle's minimum essential medium supplemented with 10 % heat inactivated FBS, 2 mM glutamine, penicillin (100 IU/mL), and streptomycin (100 $\mu$g/mL). For experiments, cell ($5 \times 10^4$ cells/mL) were seeded in culture dish, and maintained in a tissue culture incubator.

### 2.6. Assay of Cell Viability

The effects of licorice extract (LEx) and fermented licorice extract (FLEx) on cell viability were evaluated using the Cell Counting Kit-8 (CCK-8) produced by Dojindo Laboratories (Tokyo, Japan). HaCaT cells were plated at a density of $3 \times 10^4$ cells per well in a 96-well plate, and incubated at 37 ℃ for 24 h. The cells were treated with various concentrations of LEx or FLEx, and incubated at 37 ℃ for an additional 24 h. After incubation, 10 $\mu$L of CCK-8 solution was added to each well and incubated under the same conditions for another 2 h. The resulting color was assayed at 450 nm using a microplate reader (Model ELX 800, BIOTEK Inc., Winooski, VT, USA).

### 2.7. UVB Irradiation, RNA Isolation and RT-PCR

HaCaT cells were seeded into 96-well plates and cultured overnight. UVB irradiation doses were 40 mJ/cm$^2$ and the radiation intensity was measured using UV radiometer (EKO, JAPAN). RNA was extracted using a Intron easy-BLUETM Total RNA Extraction Kit (iNtRON Biotechnology, Korea) according to the supplier's instructions. A reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to synthesize cDNA. PCR was then performed with each cDNA of COX-2, IL-6, IL-1$\beta$ and GAPDH (the internal standard)fragments, primers, and Tag DNA polymerase. The primers used were as follows: 5′-TCC ACA GCC AGA CGC CCT CA-3′ (forward) and 5′-GGC CCA GCC CGT TGG TGA AA-3′ (reverse) for COX-2; 5′-AGC GCC TTC GGT CCA GTT GC-3′ (forward) and 5′-GCT TCG TCA GCA GGC TGG CA-3′ (reverse) for IL-6; 5′-ATC GCA CGC TCC GGG ACT CA-3′ (forward) and 5′-AAG GGC TGG GGA TTG GCC CT-3′ (reverse) for IL-1$\beta$ 5′-ATT GTT GCC ATC AAT GAC CC-3′ (forward) and 5'-AGT AGA GGC AGG GAT GAT

GT-3′ (reverse) for GAPDH. Reactions were carried out in an automatic heat-block DNA thermal cycler (ASTEC PC801, ASTEC Inc, Tokyo, Japan) for 22 cycles: denaturation for 60 s at 94 ℃ annealing for 60 s at 60 ℃ extension for 60 s at 72 ℃. PCR products were electrophoresed on a 1.5 % agarose gel in TAE (40 mM Tris acetate, 1 mM EDTA) and visualized by ethidium bromide staining. Gel images were scanned using a CS analyzer 2.0 (ATTO, Tokyo, Japan).

### 2.8. Statistical Analysis

All experiments were performed in triplicate. Data were presented as meanstandard error (SE). Statistical comparison was conducted using Student's *t*-test after ANOVA. The results are considered to be significant when $p < 0.05$.

## 3. Results and Discussion

### 3.1. Fermentation for Flavonoid Aglycones Production

Licorice (*Glycyrrhizae radix*) is one of the oldest and most frequently used botanicals in oriental medicine. A large number of components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids and chalcones[6]. We investigated the transformation of flavonoid glycosides of licorice extract to liquiritigenin (aglycone of liquiritin) and isoliquiritigenin (aglycone of isoliquiritin) production using β-glucosidase produced from a submerged culture of *G. frondosa* HB0071. In plants, flavonoids are usually found in conjugated forms through hydroxyl groups with sugar as glycosides.

To obtain bioactive flavonoids by enzymatic hydrolysis from different beverages and cereals, many investigators have studied submerged fermentation of microorganism and some basidiomycete mushrooms for β-glucosidase production[18]. Figure 2 shows the typical time profiles of mycelia growth and β-glucosidase activity in the fermented licorice extract with *G. frondosa* HB0071. The maximum mycelial biomass and β-glucosidase activity were 25.3 g/L and 91.5 mU/mL, respectively. To examine whether liquiritigenin and isoliquiritigenin in licorice extract were increase by the fermentation by *G. frondosa*, the detected major peaks in licorice extract and fermented licorice extract were analyzed by HPLC. Figure 3 shows the HPLC chromatogram at 327 nm of licorice extract (Figure 3A) fermented with *G. frondosa* HB0071 mycelium (Figure 3B) for 96 h. As shown in Figure 3B, the new peaks of compound 1 (C1) and compound 2 (C2) were detected and identified by their retention times and UV spectra and by comparison with liquiritigenin and isoliquiritigenin as standards (Figure 3C). The isolated C1 and C2 were identified by MS, $^{1}$H NMR, and $^{13}$C NMR analyses and were characterized as liquiritigenin and isoliquiritigenin, respectively. Their characteristics were analyzed and showed the follows: (1) liquiritigenin: colorless amorphous powder; negative ESI-MS ($C_{15}H_{12}O_4$) m/z : 257 [M-H]; $^{1}$H-NMR (500 MHz, METHANOL-D4) : δ 5.38 (1H, dd, J=3.0, 13.0 Hz, H-2), 3.05 (1H, dd, J=13.0, 16.5 Hz, Ha-3), 2.47 (1H, dd, J=3.0, 16.5 Hz, Hb-3), 7.73 (1H, d, J=8.5 Hz, H-5), 6.49 (1H, dd, J=2.5, 8.5 Hz, H-6), 6.35 (1H, d, J=2.5 Hz, H-8), 7.32 (2H, d, J=8.5 Hz, H-2′, 6′), 6.81 (2H, d, J=8.5 Hz, H-3′, 5′); $^{13}$C-NMR (125 MHz, METHANOL-D4) : δ 81.2 (C-2), 45.1 (C-3), 193.6 (C-4), 130.0 (C-5), 111.9 (C-6), 167.1 (C-7), 103.9 (C-8), 165.7 (C-9), 115.0 (C-10), 131.5 (C-1′), 129.1 (C-2′, 116.4 (C-3′, 159.1 (C-4′, 116.4

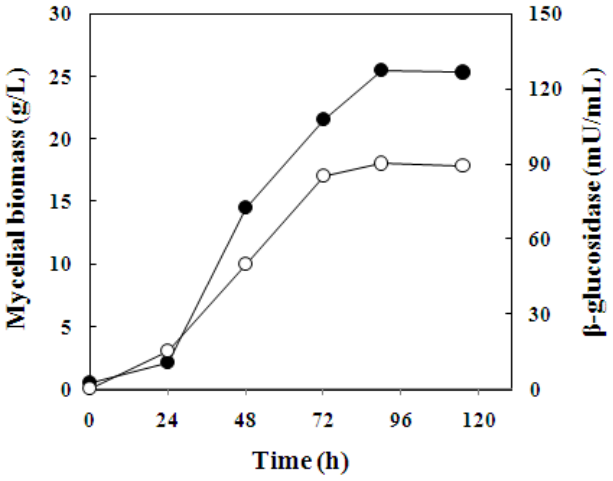



**Figure 2.** Typical time profiles of mycelial growth and β-glucosidase activity in the licorice extract fermentation with *G. frondosa* HB0071 in a 5-L stirred-tank fermenter. (●) mycelial biomass, (○) β-glucosidase activity.

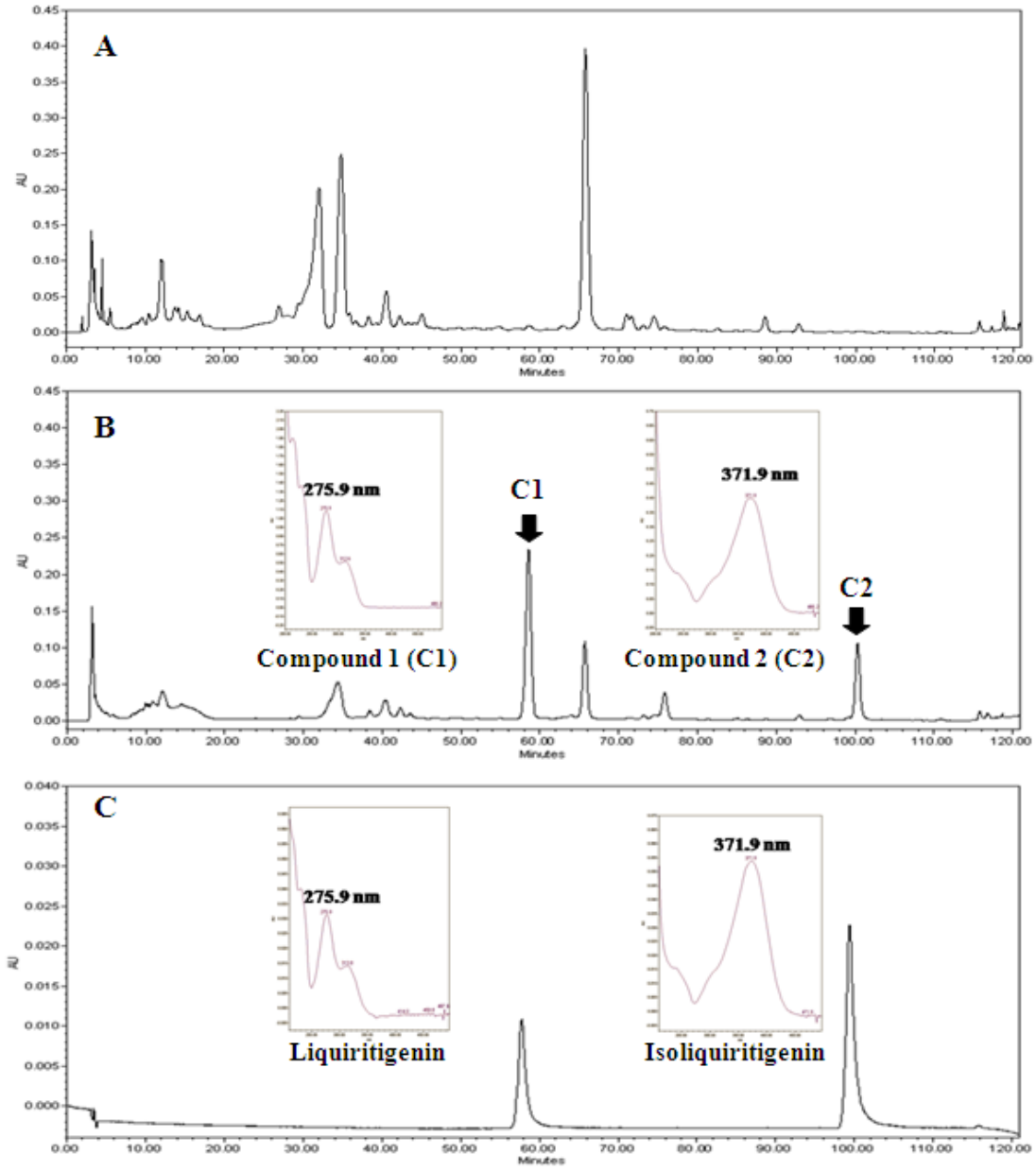



**Figure 3.** HPLC chromatograms of aqueous extract of licorice (A), fermented licorice extract (B) and mixture of standards (C) at 327 nm. The UV spectra of compound 1, 2, liquiritigenin and isoliquiritigenin were obtained using a PDA over the range 210 to 400 nm, respectively, in (B) and (C). Standards (liquiritigenin and isoliquiritigenin) were purchased from Chromadex Inc. (St. Santa Ana, CA, USA).

(C-5′, 129.1 (C-6′. (2) isoliquiritigenin: yellow amorphous powder; negative ESI-MS ($C_{15}H_{12}O_4$) m/z : 257 [M-H]: $^1$H-NMR (500 MHz, METHANOL-D4) : $\delta$ 7.62 (2H, d, J=8.5 Hz, H-2,6), 6.84 (2H, d, J=8.5 Hz, H-3,5), 7.62 (1H, d, J=15.0 Hz, H-$\alpha$), 7.79 (1H, d, J=15.0 Hz, H-$\beta$), 6.28 (1H, d, J=2.5 Hz, H-3′), 6.41 (1H, dd, J=2.5, 8.5 Hz, H-5′), 7.97 (1H, d, J=8.5 Hz, H-6′) $^{13}$C-NMR (125 MHz, METHANOL-D4) : $\delta$128.0 (C-1), 131.9 (C-2), 117.0 (C-3), 161.7 (C-4), 117.0 (C-5), 131.9 (C-6), 118.5 (C-$\alpha$), 145.7 (C-$\beta$), 193.6 (C=O), 114.7 (C-1′, 166.8 (C-2′, 104.0 (C-3′, 167.7 (C-4′, 109.4 (C-5′, 133.5 (C-6′. These results were con-

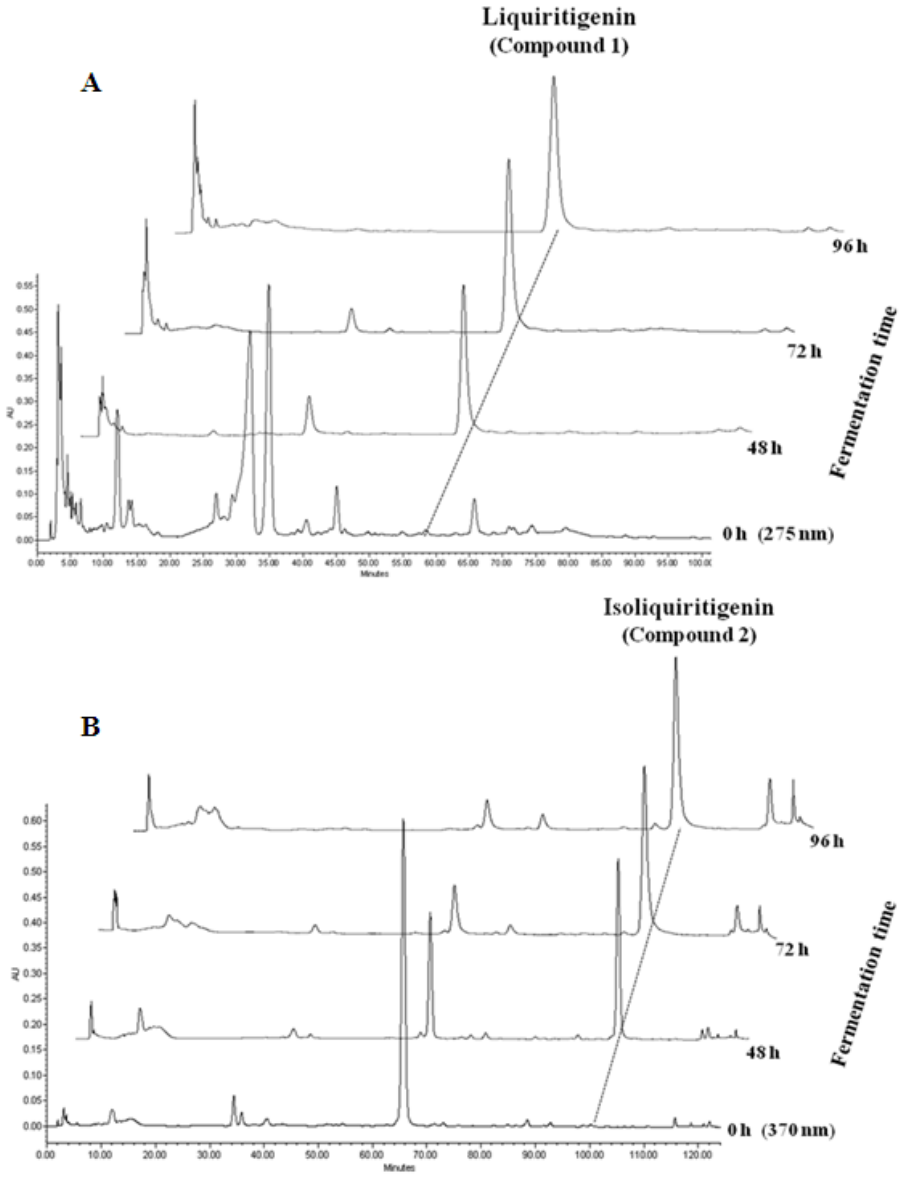



**Figure 4.** Time profile of the changes in (A) liquiritigenin (compound 1) and (B) isoliquiritigenin (compound 2) during licorice extract of fermentation by *G. frondosa* HB0071. HPLC profile with PDA detector at (A) 275 nm and (B) 370 nm, respectively.

sistent with the literature with respect to liquiritigenin and isoliquiritigenin[19]. The molecular structures of the above two flavonoids from purified fermented licorice extract are shown in Figure 1.

To begin to study the anti-inflammatory activities of aqueous licorice extracts before and after fermentation with *G. frondosa* HB0071, the changed contents and yield of aglycones on the bioconversion were investigated. As shown in Table 1, the contents of liquiritigenin and isoliquiritigenin increased from 1.92 and 0.72 μg/mL at the start of the process to 568.54 and 89.62 μg/mL after 96 h of fermentation, respectively. Figure 4 shows that the contents of liquiritigenin (compound 1) and isoliquiritigenin (compound 2) increased significantly during fermentation by *G. frondosa*. This could be attributed to being hydrolyzed

**Table 1.** Changes of Liquiritigenin and Isoliquiritigenin Contents During the Fermentation of the Aqueous Extract of Licorice (10 %, w/v) with *G. frondosa* HB0071

| Time (h) | Liquiritigenin (μg/mL) | Isoliquiritigenin (μg/mL) |
|---|---|---|
| 0 | 1.92 ± 0.01 | 0.72 ± 0.01 |
| 24 | 41.10 ± 0.63 | 2.91 ± 1.02 |
| 48 | 346.47 ± 0.50 | 52.87 ± 0.30 |
| 72 | 508.31 ± 0.39 | 82.18 ± 0.51 |
| 96 | 568.54 ± 1.01 | 89.62 ± 0.84 |
| 120 | 457.42 ± 0.66 | 79.20 ± 0.50 |

Results are presented as the average of triple determination with means ± SE.

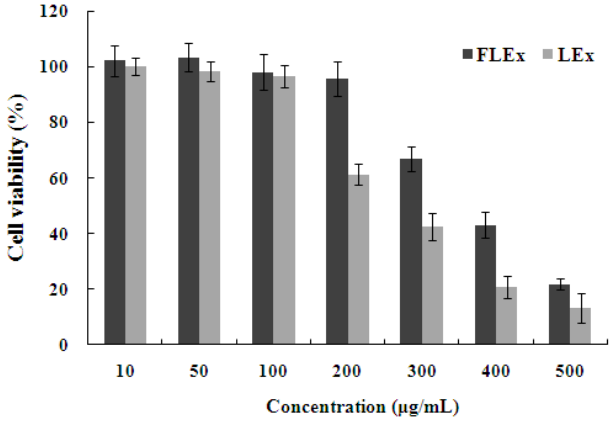


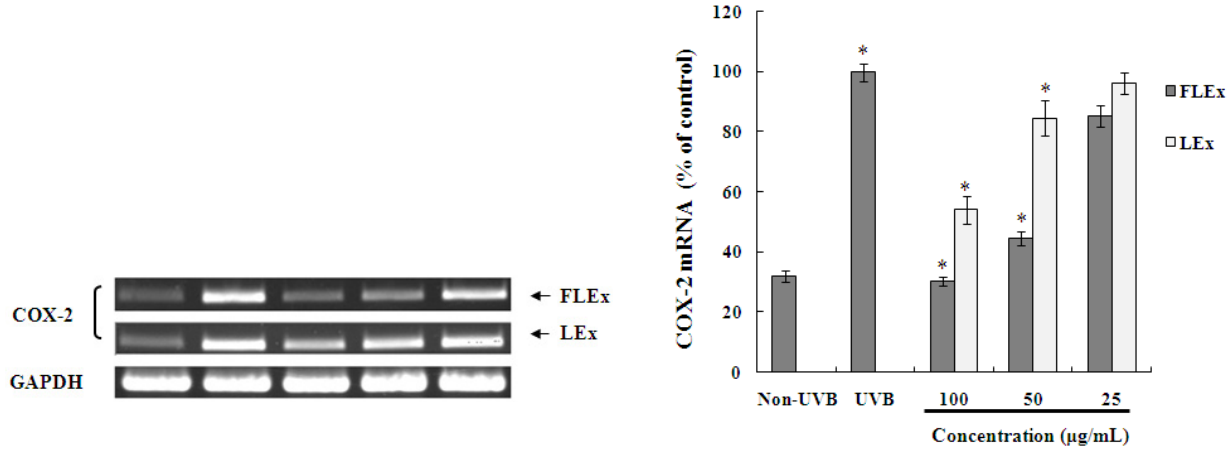

**Figure 5.** Effects of LEx and FLEx on cell viability in HaCaT cell using a Cell Counting Kit. The cell viability is significant ($p < 0.05$) and the values are mean ± S.E.

and used as a carbon source for mycelial growth and metabolite production. Many stuides have been carried out on β-glucosidase, which catalyzes the hydrolysis of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety[20]. Miura et al.[21] reported that the basidiomycete, *Ganoderma lucidum* could convert isoflavone glycosides to aglycones by its β-glucosidase activity. In plants, natural phenolics are usually found as glycosides, and the sugar species and binding forms show great variety[22]. Several investigators reported that licorice contains most glycoside compounds, such as glycyrrhizin, liquiritin, and isoliquiritin, but aglycones are relatively low from results of HPLC analysis [23]. Recently, Li et al.[24] reported that the liquiritin, isoliquiritin, liquiritigenin, and isoliquiritigenin contents of 17 licorice samples from different locations in China were 7.3 ~ 23.2, 2.1 ~ 7.7, 0.1 ~ 1.9 and 0.1 ~ 0.7 mg/g, respectively.

In this study, there were a greater amount of liquiritin and isoliquiritin than liquiritigenin and isoliquiritigenin in aqueous licorice extracts due to the poor water-solubility characteristics of flavonoid aglycones (data not shown). Interestingly, however, liquiritigenin and isoliquiritigenin contents increased after fermentation (Table 1). This may be due to the fact that the hydrolytic enzyme β-glucosidase produced by *G. frondosa* HB0071 catalyzes the release of flavonoid aglycones from licorice extract substrate, thereby resulting in an increase in aglycone content.

### 3.2. Effect of Fermented Licorice Extract on UVB-Irradiated HaCaT Keratinocytes

Reactive oxygen species (ROS) such as hydroxyl and superoxide radicals produced by sunlight, ultraviolet, chemical reactions, and metabolic processes have a wide variety of pathological effects on cellular processes[25]. Exposure of skin to UVB radiation results in a variety of biological effects, including inflammation, sunburn cell formation, immunologic alterations, and photo-aging[26]. Keratinocytes respond to the major changes in the inflammation and immune-modulation observed after UVB exposure, at least via the UVB-induced release of inflammatory mediators, such as cytokines and prostaglandins[10]. COX-2 is a pivotal player in inflammatory processes, and UV radiation is a known stimulus for COX-2 expression in skin cells. Fernau et al.[27] reported that COX-2 expression in HaCaT human keratinocytes was observed only upon exposure of cells to UVB (280 ~ 320 nm) but not to UVA radiation (320 ~ 400 nm). In the present study we found that treatment of fermented licorice extract (FLEx) with HaCaT keratinocytes suppressed UVB-induced reduction of cell viability and production of inflammatory mediators.




(A) (B)

**Figure 6.** Inhibitory effects of LEx and FLEx on UVB-induced mRNA expression of COX-2. HaCaT cells were cultured in the presence of various concentrations of LEx and FLEx (25 ~ 100 μg/mL) for 24 h, respectively. * $p < 0.05$ compared with UVB (UVB: 40 mJ/cm$^2$). Total RNA extracted from HaCaT cells was analyzed by RT-PCR and each lane in (A) corresponds to each bar in (B). The COX-2 data were normalized to the GAPDH transcript control.

HaCaT cells were treated with various concentrations of licorice extract and FLEx for 24 h, and the cell viability was tested by a CCK-8 assay. As shown in Figure 5, neither LEx nor FLEx (under 100 μg/mL) had a significantly cytotoxic effect on the HaCaT cells, whereas FLEx had less cytotoxicity compared with LEx at higher concentrations (above 200 μg/mL).

To investigate whether LEx and FLEx inhibit UVB-induced COX-2 expression, HaCaT cells were exposed to UVB (40 mJ/cm$^2$). Thus, in the present study the effects of LEx and FLEx upon UVB irradiation were examined by measuring the steady-state COX-2 mRNA level in relation to GAPDH mRNA levels. As shown in Figure 6, the expression of COX-2 mRNA in UVB-irradiated HaCaT cells was significantly reduced by FLEx in a dose-dependent manner, while LEx-treated COX-2 mRNA had a relatively lower level compared with that of FLEx. These results are associated with the higher concentration of liquiritigenin and isoliquiritigenin produced from licorice extract with *G. frondosa* HB0071 than that of licorice extract, which has a higher ratio of flavonoid glycosides. In addition, mRNA expression of IL-1β and IL-6 in UVB-irradiated HaCaT keratinocytes was determined by RT-PCR analysis. Treatment of UVB-irradiated HaCaT cells with FLEx concentrations of 25, 50, and 100 μg/mL dose dependently reduced the elevated IL-1β and IL-6 mRNA expression significantly, as shown in Figure 7. Remarkably, the expression of IL-6 mRNA in UVB-irradiated HaCaT cells was almost completely inhibited (by about 90 %) at a FLEx concentration of 100 μg/mL.

Kim et al.[11] reported that isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* reduced the LPS-induced expressions of inducible nitric oxide synthase (iNOS), COX-2, and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) at the protein and mRNA levels in a dose-dependent manner in macrophages. They also suggested that flavonoid glycosides are possibly due to their different affinities for cellular membranes because affinity for cellular membranes appears to play an important role in the cellular uptake of lipophilic compounds by passive diffusion and because isoliquiritin possesses a sugar moiety, which would reduce its lipophilicity and compatibility with cellular membranes.

Other investigators have reported that liquiritigenin significantly inhibited LPS-induced TNF-α, IL-1β, and IL-6 secretions. They found that the suppressive effects of liquiritigenin on IL-1β or IL-6 production were greater than its effect on TNF-α, which supports the possibility that liquiritigenin has a greater in

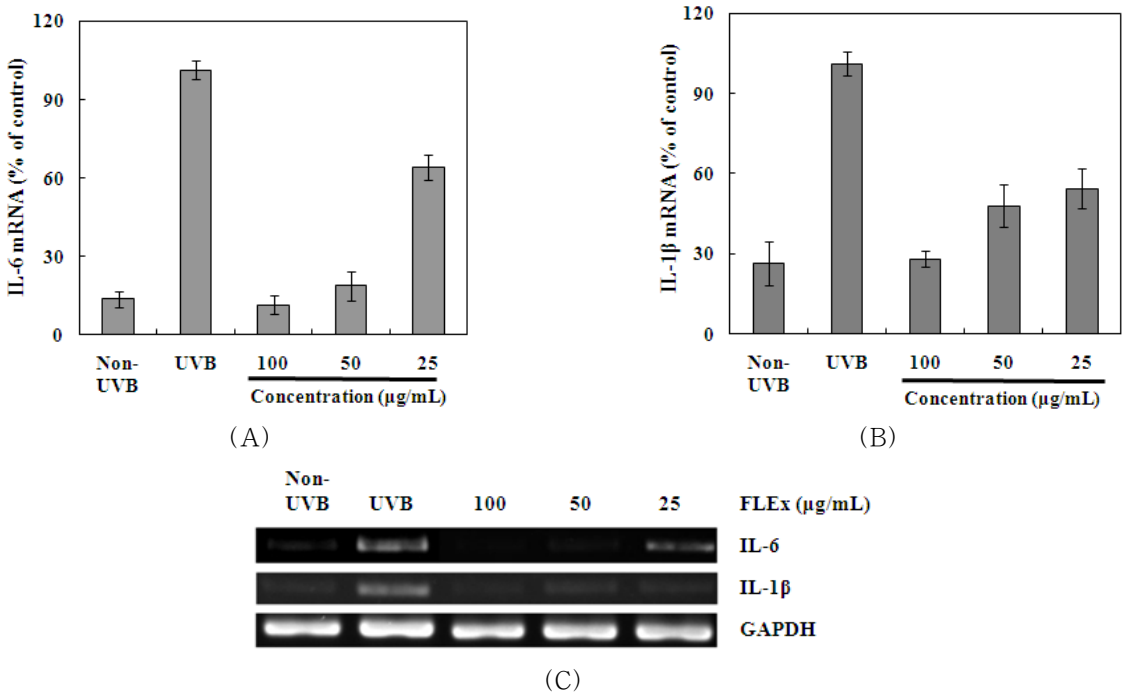



**Figure 7.** Inhibitory effect of FLEx on UVB-induced mRNA expression of IL-6 and IL-1$\beta$. HaCaT cells were cultured in the presence of various concentrations of LEx and FLEx (25 ~ 100 $\mu$g/mL) for 24 h, respectively. * $p < 0.05$ compared with UVB (UVB: 40 mJ/cm$^2$). Total RNA extracted from HaCaT cells was analyzed by RT-PCR and each lane in (A) and (B) corresponds to each bar in (C). The COX-2 data were normalized to the GAPDH transcript control.

hibitory effect on the production of secondary cytokines[28].

## 4. Conclusion

In summary, our results demonstrated that fermented licorice extract with *G. frondosa* HB0071 was very effective for the improvement of flavonoid aglycones (liquiritigenin and isoliquiritigenin) contents and inhibitory effects on UVB-induced inflammation in HaCaT keratinocytes. We also investigated that exopolysaccharide (EPS) was produced from submerged culture of aqueous licorice extract with *G. fronodsa* mycelia (data not shown). As we previously found, EPS has an inhibitory effect on MMP-1 expression in UVA-irradiated human dermal fibroblasts (HDFs) [3]. Therefore, EPS and other metabolites that produced FLEx could possibly affect the inhibitory action in photo-aging skin by reducing the MMP 1-related matrix degradation system. Our results suggest that FLEx, a mixture containing flavonoid aglycones and exo-polysaccharides produced by *G. frondosa* HB0071 mycelia, is a potential candidate to reduce UV-induced skin inflammation. In the future, combinations of other natural compounds may be considered for more efficient photoprotection, and further studies for elucidating overall biological functions of FLEx *in vivo* should be performed.

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