



Inhibitory effect of skullcap (*Scutellaria baicalensis*) extract on ovalbumin permeation *in vitro* and *in vivo*

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ABSTRACT

Scutellaria baicalensis Georgi (skullcap) has been widely used as a dietary ingredient. The purpose of this study was to reveal novel function of skullcap and its mechanism on allergen permeation in intestinal epithelial cells. Intestinal epithelial Caco-2 cell monolayers were used to evaluate the inhibitory effect of skullcap on ovalbumin (OVA) permeation by measuring transepithelial electrical resistance (TEER) and the quantity of permeated OVA. TEER increased and the OVA flux decreased in a dose-dependent manner through up-regulating tight junction-related proteins in cells incubated with increasing concentrations of skullcap extract. In the *in vivo* study, the amounts of OVA from orally ingested albumen reduced on administration of the skullcap extract. We also revealed for the first time that the active component of skullcap extract for inhibition of OVA permeation was baicalein. These findings demonstrated that skullcap extract might attenuate a food allergic response by inhibiting allergen permeation *in vitro* and *in vivo*.

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1. Introduction

Scutellaria baicalensis Georgi (skullcap) is a species of flowering plants of the Lamiaceae family. Skullcap is used extensively as a dietary ingredient and as a traditional herbal medicine in oriental countries such as China, Japan, and Korea. In particular, the root of skullcap is an important medicinal herb material and has been widely used to treat inflammation, cancer, and bacterial and viral infections of the respiratory tract and gastrointestinal tract (GIT) (Lu, Joerger, & Wu, 2011; Ye, Xui, Yi, Zhang, & Zhang, 2002; Yoon et al., 2009). Skullcap extract inhibited the expression of pro-inflammatory factors, including inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, prostaglandin E2 (PGE2), IL-1 β , IL-6, and tumour necrosis factor (TNF)- α by downregulating

NF- κ B and the mitogen-activated protein (MAP) kinase signaling pathway in RAW 264.7 cells (Kim et al., 2009). Recently, a study demonstrated the anti-allergic effect of skullcap by showing that skullcap treatment suppressed passive cutaneous anaphylaxis in rats and 48/80-induced histamine release in rat peritoneal mast cells (Jung et al., 2012). Although studies on the physiological functions of skullcap, including anti-inflammatory and anti-allergic effects, have progressed actively, the detailed functions and mechanisms underlying skullcap's effects have not been elucidated.

Allergy is a hypersensitivity disorder of the immune system; it develops when the immune system reacts to normally harmless substances in the environment. The incidence of allergic diseases including atopic dermatitis, asthma, and anaphylaxis are continuously increasing. In particular, food allergy is an increasing clinical problem and has been estimated to affect 5–6% of children and 3–4% of the adult population (Sampson, 1999; Venter et al., 2008). Eggs, milk, wheat, peanuts, soybeans, and rice are commonly known food allergens (Tanabe, 2007). Although the allergic responses triggered by orally ingested food allergens are generated through various complicated mechanisms, the first among several steps is allergen permeation into the GIT. The intestinal epithelium has not only an absorption function for dietary factors but also a barrier function to restrict the permeation of noxious substances. However, some dietary proteins like allergens can cross the intestinal epithelium via paracellular diffusion pathways, and they cause allergic immune responses through access to effector T cells,

Abbreviations: BBB, Blood–brain barrier; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GIT, gastrointestinal tract; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; JAM, junctional adhesion molecule; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEAA, non-essential amino acids; OM, ovomucoid; OVA, ovalbumin; SDS, sodium dodecylsulphate; TEER, transepithelial electrical resistance; TJ, tight junction; ZO, zonula occludens.

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dendritic cells, and mast cells (Kaminogawa, Hachimura, Nakajima-adachi, & Totsuka, 1999). The major determinant of paracellular diffusion is the intercellular tight junction (TJ). The TJ is generally expressed in intestinal epithelial cells, and it is located around the apical end of the lateral cell membrane. Modulation of TJ can control the permeability of food factors or allergens via paracellular diffusion pathways.

Cells of the human adenocarcinoma cell line, Caco-2, which are derived from a colon carcinoma, spontaneously undergo the process of differentiation that leads to the formation of monolayers. The cell monolayers have been used as an *in vitro* model that mimics the human intestinal epithelium. (Grasset, Pinto, Dussaulx, Zweibaum, & Desjeux, 1984; Hidalgo, Raub, & Borchardt, 1989) The cells have been mainly used to estimate the bioavailability or transport of dietary factors because the cells express nutrient transporters and TJ proteins (Konishi, Kobayashi, & Shimizu, 2003; Shimizu, Tsunogai, & Arai, 1997). In this study, we established an *in vitro* system to evaluate food allergen permeation by using Caco-2 cell monolayers, and we investigated the inhibitory effects of natural product extracts derived from food and plant on the permeation of a food allergen, ovalbumin (OVA), across Caco-2 cell monolayers to attenuate food allergy.

2. Material and methods

2.1. Materials

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, non-essential amino acids (NEAA), Dulbecco's phosphate-buffered saline (D-PBS), and Hank's balanced salt solution (HBSS) were purchased from WelGENE (Daegu, Korea). Ovalbumin (OVA), bile salts (mixture of sodium cholate and sodium deoxycholate), horseradish peroxidase (HRP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulphate (SDS) were purchased from Sigma (St. Louis, MO, USA). The primers for real-time PCR were obtained from Macrogen (Seoul, Korea), and 3-(4-hydroxyphenyl) propionic acid was purchased from Wako Pure Chemicals Inc., Ltd. (Osaka, Japan). RNeasy® Mini Kit, QuantiTect® Reverse Transcription Kit, and 2 × Rotor-Gene SYBR® Green master mix were purchased from QIAGEN (Valencia, CA, USA).

2.2. Sample preparation

The 14 samples that were used in this study were purchased from Kyeong-dong Oriental Pharmacy (Seoul, Korea) and identified by Professor Y. Bu, Department of Herbal Pharmacology, Kyung Hee University. The specimen (KFRI-SL-101) has been kept in functional materials research group, Korea Food Research Institute. Each extract was obtained by microwave extraction in 70% ethanol for 5 min. The ethanol extract was concentrated under vacuum in a rotary evaporator. The concentrated extract was finally lyophilised (–50 °C), and kept at 4 °C until needed. The dried ethanol extract was dissolved in saline prior to use. This fraction was used in all *in vitro* and *in vivo* studies.

2.3. Cell culture

The Caco-2 cells were cultured at 37 °C in humidified air containing 5% CO₂. The cells were maintained in a 100-mm dish with DMEM containing 1000 mg/l of glucose and supplemented with 10% FBS, 1% NEAA, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were seeded at a density of 2×10^5 cells/

ml on a 12-transwell or 24-well plate (Costar, Corning, NY, USA) and allowed to grow for 3 weeks for the experiments; the medium was changed every 2–3 days.

2.4. Measurement of transepithelial electrical resistance

The monolayers of Caco-2 cells were used from day 21 after seeding. The integrity of the Caco-2 cell monolayers was checked by measuring the transepithelial electrical resistance (TEER) by using a Millicell-ERS device (Millipore, Bedford, MA, USA). The monolayers of Caco-2 cells were used when their TEER values were in the range 300–500 Ω cm^{–2}. The cell monolayers were washed twice with Hank's balanced salt solution (HBSS), and then they were pre-incubated for 30 min at 37 °C in a CO₂ incubator to stabilise the cell monolayers. The cell monolayers were treated with each sample and bile salts (mixture of sodium cholate and sodium deoxycholate) for 60 min, and OVA was added for 3 h at 37 °C. After 3 h of incubation with OVA the TEER of the cell monolayers was measured, and the medium on the basolateral side of the monolayer, which contained the permeated OVA, was collected for enzyme-linked immunosorbent assay (ELISA).

2.5. Elisa

The monolayers of Caco-2 cells in the 12-transwell plate were pre-incubated with bile salts and skullcap extract for 1 h and then treated with 400 µg/ml OVA for 3 h in HBSS. After incubation, the level of permeated OVA on the basolateral side of the monolayer was determined using a commercially available ELISA method. Primary and secondary antibodies for ELISA were obtained from OVA-immunised rabbits. The secondary antibodies were conjugated with horseradish peroxidase (HRP) according to the protocol (Nakane & Kawaoi, 1974). The flash count between HRP and 3-(4-hydroxyphenyl) propionic acid was detected fluorometrically using Fluoroskan Ascent Microplate Fluorescence Reader (Thermo Labsystems, Waltham, MA, USA) set to excitation 320 nm and emission 405 nm wavelengths.

2.6. Cytotoxicity assay

The cytotoxicity of Caco-2 cells was determined by performing MTT assay. Briefly, the cells were seeded in 96-well plates at a density of 2×10^5 cells/ml and incubated with various concentrations of the skullcap extract for 3 h. The cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS) (200 mg/l KCl, 200 mg/l KH₂PO₄, 8000 mg/l NaCl, 2160 mg/l Na₂HPO₄·7H₂O), and 100 µl of MTT solution (2 mg/ml) was added to each well. After incubation at 37 °C under 5% CO₂ for 4 h, the cells were dissolved in 100 µl of 10% SDS (in 10 mM HCl). The amount of formazan was determined by measuring absorbance at 595 nm using an Epoch microplate reader (BioTek, Winooski, VT, USA).

2.7. Real-time reverse transcription PCR

Total RNA was isolated from the cells using the RNeasy® Mini Kit according to the manufacturer's instructions. Reverse transcription of the RNA was performed using the QuantiTect® Reverse Transcription Kit. First-strand cDNA was prepared from 1 µg of total RNA. The real-time PCR reaction was performed in a volume of 25 µl containing 0.1 µg of cDNA, 1 µM of each primer, and 2 × Rotor-Gene SYBR® Green master mix. Each sample was denatured at 95 °C for 5 min and subsequently subjected to 40 cycles of 5 s each of denaturation at 95 °C and 10 s at the appropriate annealing temperature (60 °C). The primer sequences are shown in Table 1. The real-time PCR reactions were run on Rotor-Gene Q (QIAGEN, Valencia, CA, USA). The gene expression

Table 1
Sequences of primers used for real-time PCR.

Gene	Forward primer	Reverse primer	Genebank No.
Claudin-1	CCAGTCAATGCCAGGTACGAAT	TGGTGTGGGTAAGAGTTGTT	AF134160
Occludin	ACAGAGCAAGATCACTATGAGACA	TGTTGATCTGAAGTGATAGGTGGA	NM_002538
ZO-1	TGGTGTCTACCTAATTCACACTCA	CGCCAGCTACAAATATTCCAACA	ZM_175610
JAM-1	AACACTGGGACATACACTTGT	CGATGAGCTTGACCTTGACCT	AF111713
β -actin	TCATGAAGTGTGACGTGGACATC	TGCATCCTGTCGGCAATG	NM_001101

Table 2
Yield (% w/w) of ethanol extracts and solvent fractions from skullcap.

Skullcap (<i>Scutellaria baicalensis</i>)		Yield (% w/w)			
70% Ethanol extract	Hexane Fr.	Chloroform Fr.	Ethyl acetate Fr.	Butanol Fr.	Water Fr.
45.68	2.6	4.26	5.74	40	47.4

levels were normalised to the expression level of the housekeeping gene (β -actin). Relative gene expression changes, calculated using the 2-delta delta CT method, are reported as number-fold changes compared to those in the control samples.

2.8. Animals

Six-week-old male ICR mice, weighing approximately 29–31 g, were purchased from OrientBio Inc. (Kyeonggi, Korea). The mice were housed in an air-conditioned room ($23 \pm 2^\circ\text{C}$) with a 12-h light/dark cycle. They were allowed free access to food and tap water. All animals received humane care, and the study protocols were approved and performed in accordance with the guidelines for the animal use and care of Korea Food Research Institute.

2.9. Solvent fractionation of skullcap extract

The resultant ethanol extract of skullcap was further fractionated with different solvents. Dried skullcap extract was suspended in distilled H_2O , and then sequentially partitioned with hexane, chloroform, ethyl acetate, butanol, and water. All solvent fractions were evaporated until dry. The detailed method and yields of the solvent fractions are shown in Table 2.

2.10. Determination of total phenolic content

Measurement of total phenolic content (TPC) was determined using the Folin–Ciocalteu method (Singelton, Orthifer, & Lamuela-Raventos, 1999). 100 μl of samples (100 $\mu\text{g}/\text{ml}$) were oxidised with 50 μl of 50% Folin–Ciocalteu reagent (Sigma, St. Louis, MO, USA). After incubation at room temperature for 3 min, the reaction was neutralised with 100 μl of 10% sodium carbonate (Shinyo pure chemical, Osaka, Japan). After 1 h, the absorbance of test solutions was measured spectrophotometrically at 700 nm. TPC was calculated using tannic acid (Sigma, St. Louis, MO, USA) as a standard.

2.11. Determination of total flavonoid content

Measurement of total flavonoid content (TFC) was based on the method described by Moreno, Isla, Sampietro, and Vattuone (2000). Briefly, 0.1 ml of ethanol solution (containing 0.5 mg of sample) was added to eppendorf tubes containing 20 μl of 10% aluminium nitrate (Junsei, Tokyo, Japan), 20 μl of 1 M potassium acetate (Junsei, Tokyo, Japan), and 860 μl of ethanol. After incubation at room temperature for 40 min, the absorbance of test solutions was measured spectrophotometrically at 415 nm. TFC was calculated using quercetin (Sigma, St. Louis, MO, USA) as a standard.

2.12. Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). A statistical analysis was performed using the SAS statistical software package (SAS Institute, Cary, NC, USA). Differences between the experimental data were assessed by 1-way analysis of variance (ANOVA), followed by Duncan's multiple-range test; a value of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of natural product extracts on intestinal barrier function in monolayers of Caco-2 cells

We examined the effects of extracts from 14 natural products derived food and plant (sickle senna, Chinese twinleaf, black pepper, cocklebur, magnolia, wrinkled giant hyssop, dandelion, green tea, nepeta herb, turmeric, skullcap, beefsteak plant, cheonggukjang, and fenugreek) on the intestinal barrier function by measuring TEER in Caco-2 cell monolayers. The control included 0.4% DMSO instead of extract in HBSS. Of the 14 natural product extracts, skullcap significantly increased the TEER value in Caco-2 cell monolayers (Fig. 1). This result indicates that the skullcap enhances the intestinal barrier function. In contrast, the other 13 natural product extracts did not change the TEER value in the Caco-2 cell monolayers.

3.2. Effects of skullcap extract on TEER and OVA flux in Caco-2 cell monolayers

We investigated the effects of skullcap extract on the intestinal barrier function by measuring TEER in the Caco-2 cell monolayers. The TEER increased in a dose-dependent manner in the cells that were incubated with increasing concentrations (100, 200, and 400 $\mu\text{g}/\text{ml}$) of skullcap extract for 3 h (Fig. 2A). We also investigated the skullcap extract ability to inhibit allergen permeation in Caco-2 cells. We used OVA, the major allergen in egg; OVA permeated the basolateral side of the monolayer via paracellular diffusion after 3 h, which was detected using ELISA. The OVA flux decreased in the cells incubated with 100, 200, and 400 $\mu\text{g}/\text{ml}$ of skullcap extract in a dose-dependent manner (Fig. 2B). This result indicates that skullcap extract may inhibit the allergic response by suppressing OVA permeation.

3.3. Effects of skullcap extract on TEER and cytotoxicity in Caco-2 cell monolayers

We investigated the effects of skullcap extract on TEER over time in the Caco-2 cell monolayers. The skullcap extract increased

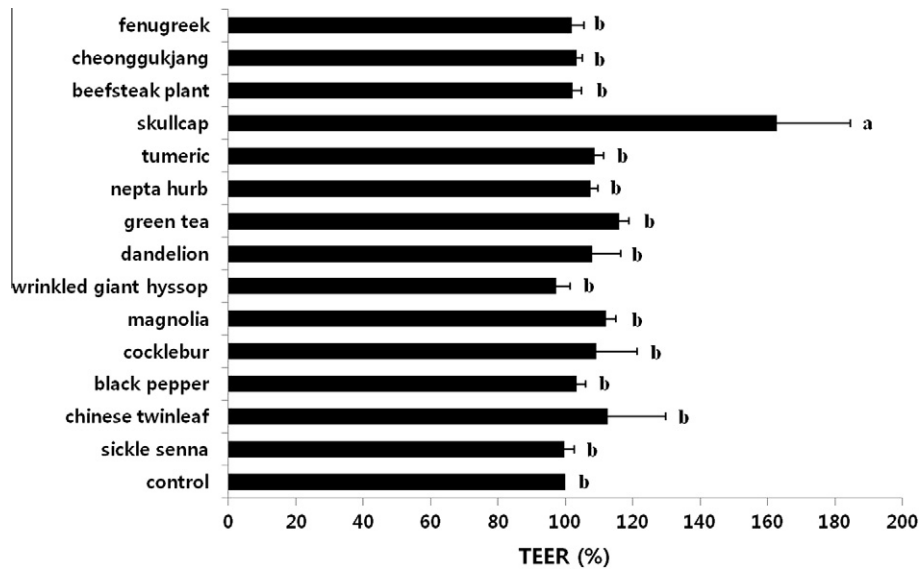


Fig. 1. Effects of natural product extracts on intestinal barrier function in Caco-2 cell monolayers. The intestinal barrier function was measured in terms of TEER in Caco-2 cell monolayers for 3 h. Fourteen natural product extracts (sickle senna, Chinese twinleaf, black pepper, cocklebur, magnolia, wrinkled giant hyssop, dandelion, green tea, nepeta herb, turmeric, skullcap, beefsteak plant, cheonggukjang, and fenugreek) were screened using TEER values in Caco-2 cell monolayers. Control included 0.4% DMSO instead of extract in HBSS. Each value is presented as mean $\pm$ SD ($n = 3$). ^{a,b}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TEER, transepithelial electrical resistance.

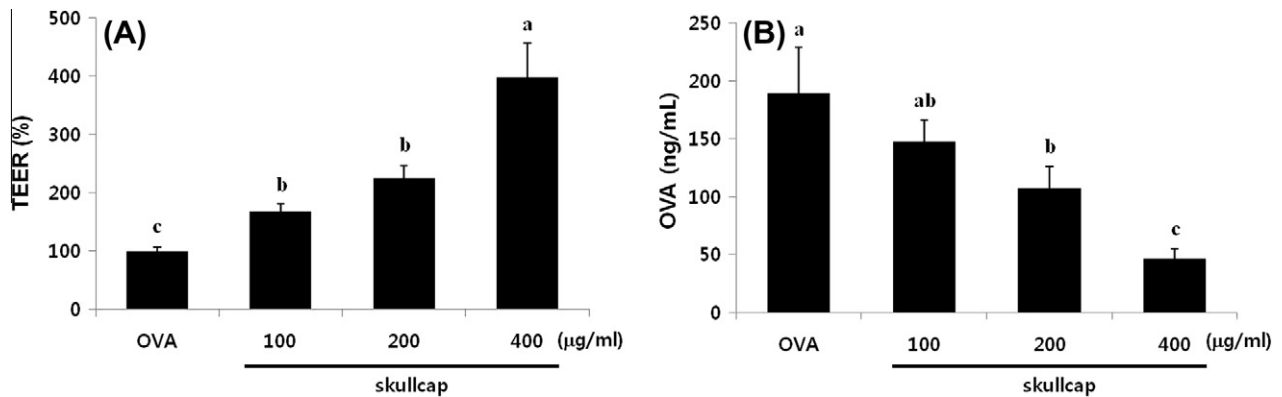


Fig. 2. Effects of skullcap extract on TEER and ovalbumin (OVA) flux in Caco-2 cell monolayers. The Caco-2 cell monolayers were incubated with 100, 200, and 400 μ g/ml skullcap extract for 3 h. TEER was measured at 3 h (A), and OVA flux on the basolateral side of the monolayer via paracellular diffusion for 3 h was detected using ELISA (B). Each value is presented as mean $\pm$ SD ($n = 3$). ^{a-c}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TEER, transepithelial electrical resistance; OVA, ovalbumin.

the TEER value in a time-dependent manner (Fig. 3A). The TEER value rapidly increased to 183% of the initial value during the first 5 h and then gradually decreased to 135% at 10 h.

The Caco-2 cells were incubated with 10–800 μ g/ml of skullcap extract. We evaluated the cell damage and cytotoxicity by microscopic observation and by performing MTT assay. No significant change in the cell morphology was evident under microscopy after the cells had been treated with skullcap extract. Further, none of the treatments led the cells to produce a significant amount of formazan (Fig. 3B). These results show that the cells used in our studies maintained their normal state.

3.4. Effects of skullcap extract on TJ-related protein mRNA expression in Caco-2 cell monolayers

The expression of TJ-related protein mRNA was examined by real-time PCR analysis using whole-cell mRNA extracts after incubation with skullcap extract for 0.5 h. Claudin-1, occludin, zonula occludens (ZO)-1, and junctional adhesion molecule

(JAM)-1 were selected and evaluated as targets for modulation of TJ. The skullcap extract significantly increased the mRNA levels of occludin, ZO-1, and JAM-1 in a dose-dependent manner (Fig. 4B–D). Although claudin-1 mRNA expression level did not significantly change compared to that in the control, an increased tendency of claudin-1 mRNA expression was observed in Caco-2 cell monolayers (Fig. 4A).

3.5. Effects of skullcap extract on albumen absorption in ICR mice

Next, we examined the effect of skullcap extract on albumen absorption in ICR mice. Mice were fed 200 mg/kg body weight of skullcap extract by oral administration and then 500 mg/kg body weight of albumen was given to the mice after fasting overnight. The sera samples from each group were obtained by collecting blood from the orbital venous plexus (Fig. 5A). We observed water intake and changes in body weight for 1 week. Neither the water intake nor the body weight of the skullcap extract-treated group (group 3) differed from the control group (group 1) or albumen

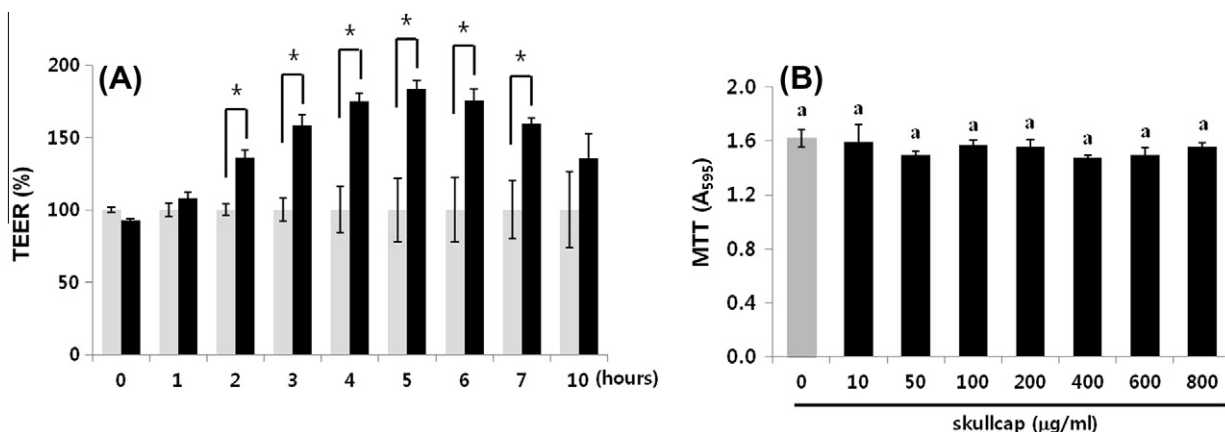


Fig. 3. Effects of skullcap extract on TEER and cytotoxicity in Caco-2 cell monolayers. The effect of skullcap extract on TEER in Caco-2 cell monolayers was examined in a time-dependent manner (A). The cells were also incubated with 10–800 µg/ml of skullcap extract to evaluate cell damage and cytotoxicity using MTT assay (B). Each value is presented as mean $\pm$ SD ($n = 3$). Bars are significantly different from the control at $*P < 0.05$. Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TEER, transepithelial electrical resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

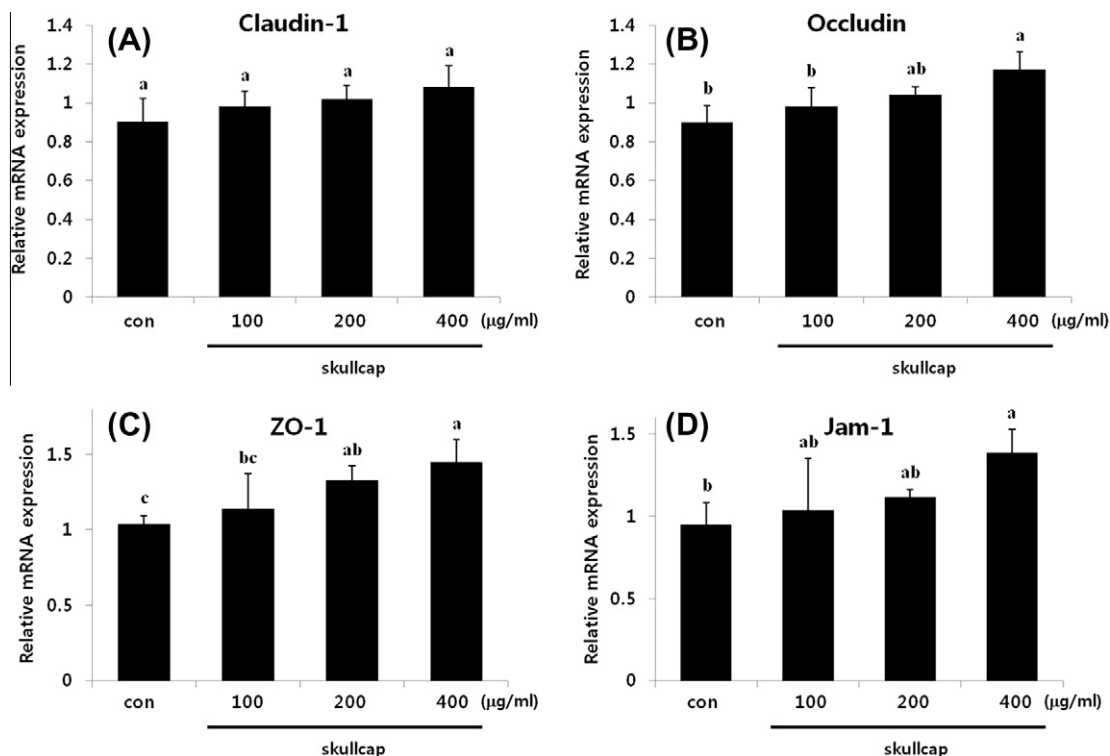


Fig. 4. Effects of skullcap extract on tight junction (TJ)-related protein mRNA expression in Caco-2 cell monolayers. Expression of TJ-related protein mRNA was examined by real-time PCR analysis using whole-cell mRNA extracts after incubation with skullcap extract for 0.5 h. Claudin-1 (A), occludin (B), ZO-1 (C), and JAM (D) were evaluated as targets for modulation of TJ. Each value is presented as mean $\pm$ SD ($n = 3$). ^{a-c}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TJ, tight junction; ZO-1, zonula occludens-1; JAM, junctional adhesion molecule.

group (group 2) for 7 days (data not shown). We investigated the amount of OVA and ovomucoid (OM) metabolised from the albumen in sera. Skullcap extract significantly inhibited the OVA and OM production induced by the administration of albumen in mice (Fig. 5B and C). Thus, the skullcap extract may attenuate various allergen-induced gastrointestinal diseases by suppressing allergen permeation.

3.6. Effects of skullcap extract fractions on TEER and OVA flux in Caco-2 cell monolayers

We examined the effects of skullcap extract fractions on the intestinal barrier function by measuring TEER in Caco-2 cell

monolayers. Among five fractions (hexane, chloroform, ethyl acetate, butanol and water), hexane and ethyl acetate fractions significantly increased TEER value in Caco-2 monolayers (Fig. 6A). We also investigated the ability of skullcap extract fractions to inhibit OVA permeation using ELISA. The OVA flux was decreased by only ethyl acetate fraction (Fig. 6B). This result demonstrated that the inhibitory effect of skullcap extract on allergen permeation was caused by compounds in ethyl acetate fraction.

It has been known that ethyl acetate fraction includes many phenolic compounds and flavonoids. We investigated TPC and TFC of each fraction. As a result, we confirmed that ethyl acetate fraction included the most phenolic compounds and flavonoids by TPC and TFC assay (Table 3). We suggest that the effects of

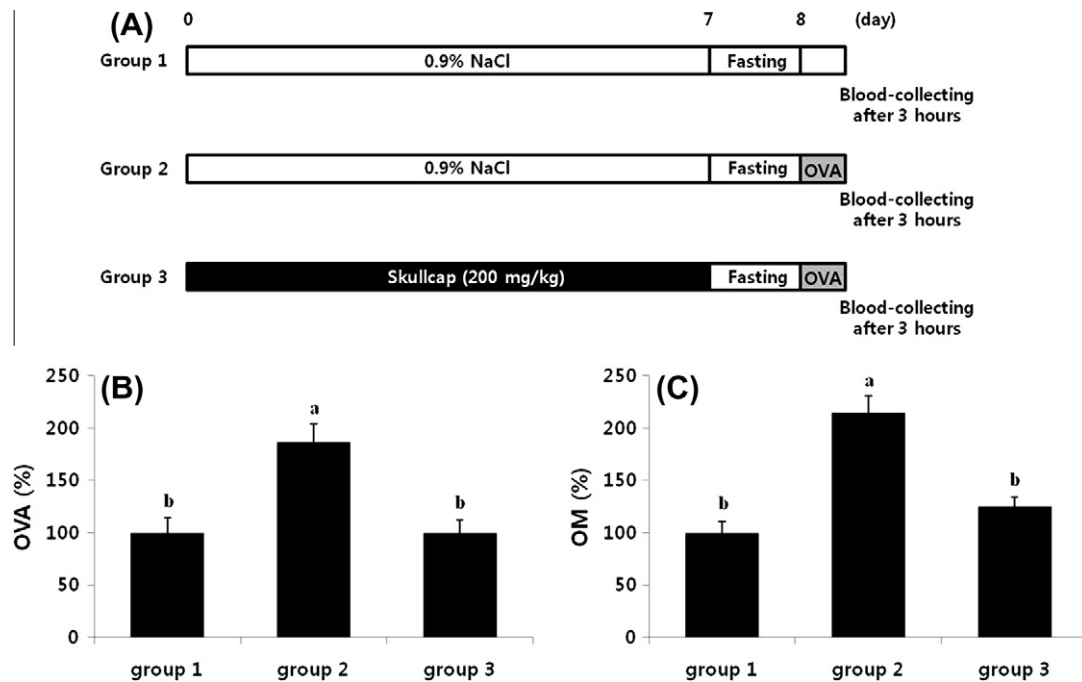


Fig. 5. Effects of skullcap extract on albumen absorption in ICR mice. ICR mice were fed 200 mg of skullcap extract/kg body weight by oral administration for a week and then 500 mg albumen/kg body weight was given to the mice after fasting overnight. The mice were divided into 3 groups: control (group 1), albumen (group 2), and skullcap extract (group 3) (A). The sera from each group were obtained from blood collected from the orbital venous plexus of the mice. The amounts of OVA (B) and OM (B) metabolised from albumen were detected using ELISA. Each value is presented as mean $\pm$ SD ($n = 3$). ^{a,b}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: OVA, ovalbumin; OM, ovomucoid.

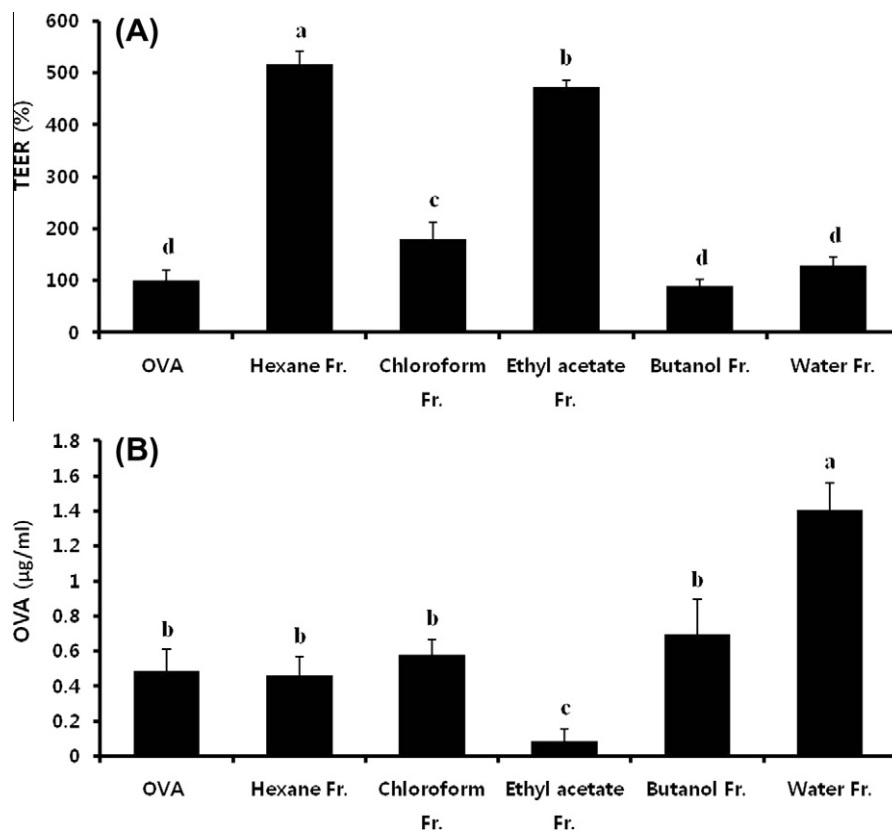


Fig. 6. Effects of skullcap extract fractions on TEER and OVA flux in Caco-2 cell monolayers. Five fractions (hexane, chloroform, ethyl acetate, butanol and water) from skullcap extract were prepared. The Caco-2 cell monolayers were incubated with 400 µg/ml of each skullcap fraction for 3 h. TEER was measured at 3 h (A), and OVA flux on the basolateral side of the monolayer via paracellular diffusion for 3 h was detected using ELISA (B). Each value is presented as mean $\pm$ SD ($n = 3$). ^{a-d}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TEER, transepithelial electrical resistance; OVA, ovalbumin.

Table 3

Total phenols content and total flavonoids content (% w/w) of solvent fractions from skullcap.

Skullcap (<i>Scutellaria baicalensis</i>)	Total phenols content (% w/w)	Total flavonoids content (% w/w)
Hexane fraction	17.17 ± 0.19	4.61 ± 0.02
Chloroform fraction	46.87 ± 1.01	15.71 ± 0.22
Ethyl acetate fraction	71.32 ± 1.65	28.83 ± 0.79
Butanol fraction	13.38 ± 0.07	5.24 ± 0.06
Water fraction	9.40 ± 0.13	2.66 ± 0.03

skullcap on the intestinal barrier function and allergen permeation are caused by multiflora or single flavonoid(s) such as baicalin, baicalin, and wogonin.

3.7. Effects of single component (baicalin and baicalein) on TEER and OVA flux in Caco-2 cell monolayers

We analysed the components in ethyl acetate fraction by HPLC. The HPLC was carried out with a Jasco PU-980 liquid

chromatography system (JASCO, Tokyo, Japan) equipped with multi wavelength detector Jasco MD-2010 plus (JASCO, Tokyo, Japan). As a result, the ethyl acetate fraction included 9 components such as baicalin and baicalein (Fig. 7A). We investigated the effects of baicalin and baicalein on OVA permeation across Caco-2 cell monolayers. Baicalein enhanced the TEER value and inhibited OVA permeation, but not baicalin (Fig. 7B–E). We suggest that baicalein in skullcap acts as an active component to suppress OVA permeation across Caco-2 cell monolayers.

4. Discussion

One of the most important functions of the gastrointestinal epithelium is to maintain a physiological barrier by forming protein complex networks that block the diffusion of pathogens, toxins, and allergens from the external environment into the tissues. TJ is a key determinant of intestinal barrier function in the protein complex networks that are connected to epithelial cells. TJs are organised by specific interactions between various intracellular proteins and transmembrane proteins: interactions have been

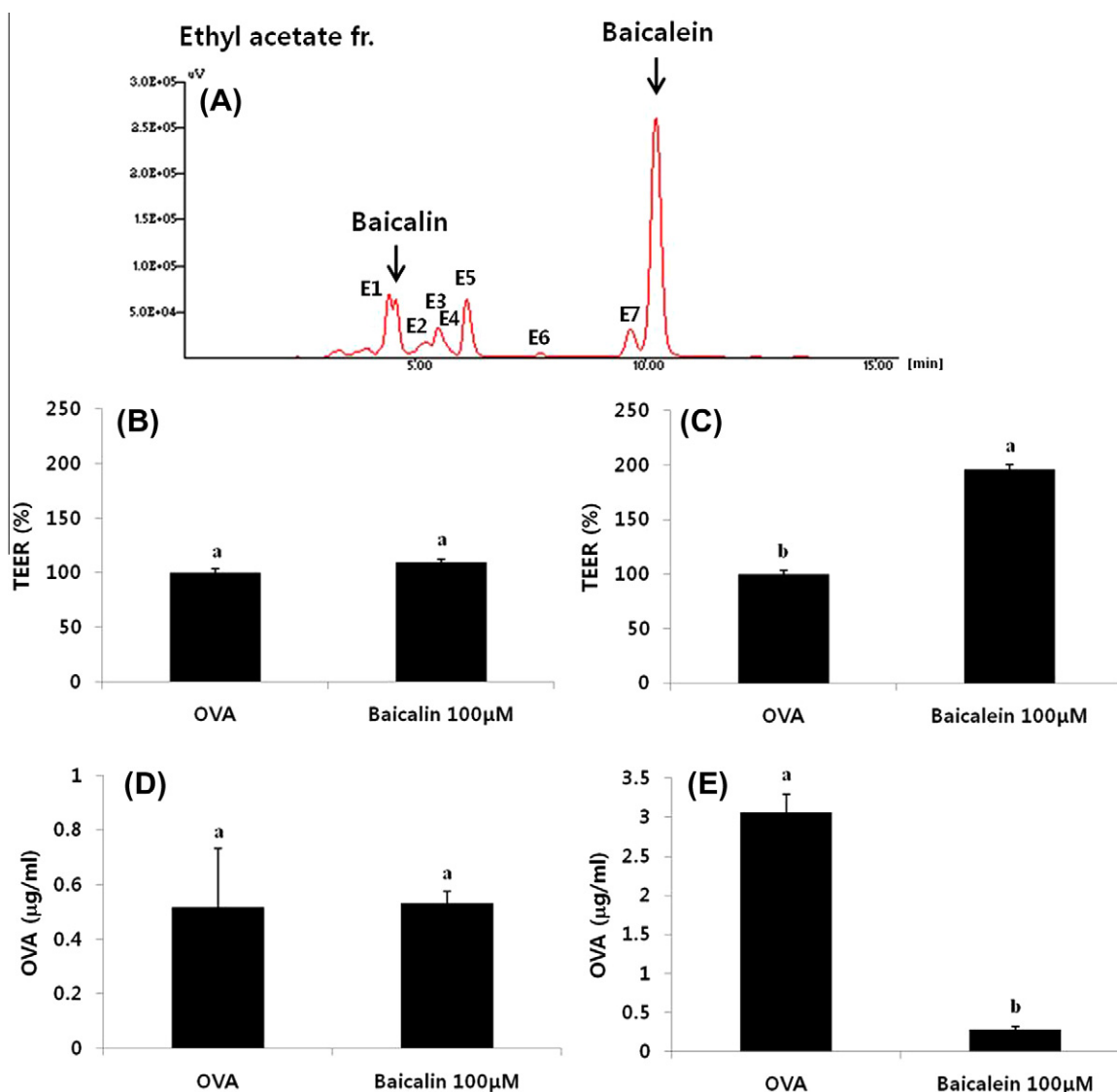


Fig. 7. Effects of single component (baicalin and baicalein) on TEER and OVA flux in Caco-2 cell monolayers HPLC chromatogram of ethyl acetate fraction recorded at 280 nm (A). The Caco-2 cell monolayers were incubated with 100 µM of baicalin or baicalein for 3 h. TEER was measured at 3 h (B and D), and OVA flux on the basolateral side of the monolayer via paracellular diffusion for 3 h was detected using ELISA (C and E). Each value is presented as mean ± SD ($n = 3$). ^{a,b}Values with different letters differ significantly ($P < 0.05$). Data were analysed using ANOVA followed by Duncan's multiple-range test. Abbreviations: TEER, transepithelial electrical resistance; OVA, ovalbumin.

identified with 3 transmembrane protein families: occludin (Furuse et al., 1993), claudins (Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998), and JAM (Martín-Padura et al., 1998). Intracellular proteins, such as ZO-1, ZO-2, ZO-3, cingulin, and 7H6 act to maintain the structure and function of TJs through interactions with transmembrane proteins (Gonzalez-Mariscal, Betanzos, Nava, & Jaramillo, 2003). The expression levels of these TJ-related proteins could be enhanced by food- and herbal plant-derived factors (Isobe, Suzuki, Oda, & Tanabe, 2008). As a result, the intestinal barrier function is enforced, and the body is protected from risks of inflammatory bowel disease (IBD) and food allergic disorder. For example, a cheese casein-derived peptide, Asn-Pro-Trp-Asp-Gln, inhibited OVA permeation by increasing occludin expression in Caco-2 cells and effectively suppressed OVA permeation in rats (Yasumatsu & Tanabe, 2010). Recently, quercetin and myricetin were reported to enhance the assembly of ZO-2, occludin, and claudin-1 and the expression of claudin-4 via PKC δ inhibition in studies about TJ regulation by flavonoids (Suzuki & Hara, 2009). Furthermore, kaempferol was reported to enhance the assembly of ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4 and the expression of ZO-2 and claudin-4 (Suzuki, Tanabe, & Hara, 2011). Our results showed that skullcap extract inhibited OVA permeation across Caco-2 cell monolayers by upregulating the expression of occludin, ZO-1, and JAM. The administration of skullcap extract suppressed OVA permeation in the serum of ICR mice (Figs. 4 and 5). These results showed for the first time that skullcap extract enhanced the intestinal barrier function by modulating the TJ-related proteins (occludin, ZO-1, and JAM). Consequently, skullcap extract may attenuate IBD and allergic disorders.

In this study, we added bile salts to the cells to mimic the gastrointestinal environment. It was reported that bile salt treatment of intestinal epithelial cells increased gut permeability by modulating TJ via epithelial growth factor receptor activation in Caco-2 cells (Raimondi et al., 2008). Despite the increased permeability observed in our study, the enhancing effect of TJ by skullcap extract overwhelmed the bile salt-induced depressed effect of TJ on TEER in Caco-2 cells. The enhancing effect of skullcap extract on the intestinal barrier function may not only prevent food allergic disorder by affording protection from allergens but also cure intestinal inflammatory disease by reducing the permeability of the inflammatory inducer. These effects of skullcap could be applied not only in intestinal epithelial cells but also in other epithelial or endothelial cells. Recently, it was reported that baicalin derived from skullcap reduced the permeability of the blood–brain barrier (BBB) by increasing the expression of claudin-5 and ZO-1 TJ proteins in brain microvascular endothelial cells (Zhu et al., 2012). This study suggested that the baicalin could suppress the inflammatory reaction and tissue injury of the brain by reinforcing the BBB. Our study showed that skullcap extract could inhibit allergen permeation by enhancing the intestinal barrier function via expression of TJ proteins. We expect the preventive anti-allergic effect to be observed in both intestinal epithelial cells and bronchial epithelial and epidermal cells through the inhibition of permeation of allergens such as pollen, dust, and ticks.

To reveal active components in skullcap extract, we investigated five fractions using different solvent from skullcap ethanol extract on OVA permeation in Caco-2 cells. As a result, the ethyl acetate fraction significantly enhanced the TEER value and inhibited the OVA permeation (Fig. 6). Fig. 6 and Table 3 showed that the active components in skullcap extract to inhibit OVA permeation were flavonoid classes. However chloroform fraction did not enhance the TEER value and inhibit the OVA permeation though the fraction included many phenols and flavonoids. We hypothesized that the reason was that flavonoids of the ethyl acetate fraction have more polar characteristics than that of chloroform fraction.

In general, the major flavonoids of skullcap have been identified, such as baicalein, baicalin, and wogonin. Besides these components, it contains many constituents, and more than 60 structures (for example; wogonoside, oroxylin A, oroxylin A glucoside, paeoniflorin, glycyrrhizic acid, glycyrrhetic acid, liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin and ononin) have been identified (Li, Jiang, & Chen, 2004). We analysed the active components (flavonoids) in ethyl acetate fraction by HPLC; 9 components were detected (Fig. 7A). Among the 9 components, we examined the effects of baicalin and baicalein. Baicalein significantly inhibited OVA permeation, but not baicalin (Fig. 7B–E). In the future, 7 components (E1–E7) will be isolated from the ethyl acetate fraction and study the inhibitory effect and mechanism. In addition, studies in *in vivo* allergic disorder models are necessary to verify the anti-allergic effect of baicalein or skullcap extract via the inhibition of allergen permeation.

5. Conclusion

In this study, we demonstrated that skullcap extract that has been used as a dietary ingredient suppresses OVA permeation by up-regulating occludin, ZO-1, and JAM expression in intestinal epithelial cells. The inhibitory effect of skullcap extract on OVA permeation was also demonstrated in an *in vivo* study. We also revealed for the first time that the active component of skullcap extract for inhibition of OVA permeation was baicalein. Therefore, our results confirm the efficacy of skullcap as an anti-allergic agent and elucidate a novel mechanism for the anti-allergic effects of skullcap. These results can contribute to the prevention or/and cure of food allergic disorders and IBDs.

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References

- Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., & Tsukita, S. (1998). Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *The Journal of Cell Biology*, 141, 1539–1550.
- Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., et al. (1993). Occludin: A novel integral membrane protein localizing at tight junctions. *The Journal of Cell Biology*, 123, 1777–1788.
- Gonzalez-Mariscal, L., Betanzos, A., Nava, P., & Jaramillo, B. E. (2003). Tight junction proteins. *Progress in Biophysics and Molecular Biology*, 81, 1–44.
- Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A., & Desjeux, J. E. (1984). Epithelial properties of human colonic carcinoma cell line Caco-2: Electrical parameters. *American Journal of Physiology Cell Physiology*, 247, C260–C267.
- Hidalgo, I. J., Raub, T. J., & Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, 96, 736–749.
- Isobe, N., Suzuki, M., Oda, M., & Tanabe, S. (2008). Enzyme-modified cheese exerts inhibitory effects on allergen permeation in rats suffering from indomethacin-induced intestinal inflammation. *Bioscience, Biotechnology, and Biochemistry*, 72, 1740–1745.
- Jung, H. S., Kim, M. H., Gwak, N. G., Im, Y. S., Lee, K. Y., Sohn, Y., et al. (2012). Antiallergic effects of *Scutellaria baicalensis* on inflammation *in vivo* and *in vitro*. *Journal of Ethnopharmacology*, 141, 345–349.
- Kaminogawa, S., Hachimura, S., Nakajima-adachi, H., & Totsuka, M. (1999). Food allergens and mucosal immune systems with special reference to recognition of food allergens by gut-associated lymphoid tissue. *Allergy International*, 48, 15–23.
- Kim, E. H., Shim, B., Kang, S., Jeong, G., Lee, J. S., Yu, Y. B., et al. (2009). Anti-inflammatory effects of *Scutellaria baicalensis* extract via suppression of immune modulators and MAP kinase signaling molecules. *Journal of Ethnopharmacology*, 126, 320–331.
- Konishi, Y., Kobayashi, S., & Shimizu, M. (2003). Tea polyphenols inhibit the transport of dietary phenolic acids mediated by the monocarboxylic acid transporter (MCT) in intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry*, 51, 7296–7302.
- Li, H. B., Jiang, Y., & Chen, F. (2004). Separation methods used for *Scutellaria baicalensis* active components. *Journal of Chromatography B*, 812, 277–290.

- Lu, Y., Joerger, R., & Wu, C. (2011). Study of the chemical composition and antimicrobial activities of ethanolic extracts from roots of *Scutellaria baicalensis* Georgi. *Journal of Agricultural and Food Chemistry*, 59, 10934–10942.
- Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., et al. (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *The Journal of Cell Biology*, 142, 117–127.
- Moreno, M. I., Isla, M. I., Sampietro, A. R., & Vattuone, M. A. (2000). Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacology*, 71, 109–114.
- Nakane, P. K., & Kawaoi, A. (1974). Peroxidase-labelled antibody, a new method of conjugation. *Journal of Histochemistry and Cytochemistry*, 22, 1084–1091.
- Raimondi, F., Santoro, P., Barone, M. V., Pappacoda, S., Barretta, M. L., Nanayakkara, M., et al. (2008). Bile acids modulate tight junction structure and barrier function of Caco-2 monolayers via EGFR activation. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 294, 906–913.
- Sampson, H. A. (1999). Allergy clinical immunology disorders. *The Journal of Allergy and Clinical Immunology*, 103, 717–728.
- Shimizu, M., Tsunogai, M., & Arai, S. (1997). Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides*, 18, 681–687.
- Singelton, V. R., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology*, 299, 152–178.
- Suzuki, T., & Hara, H. (2009). Quercetin enhances intestinal barrier function through the assembly of zonula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. *The Journal of Nutrition*, 139, 965–974.
- Suzuki, T., Tanabe, S., & Hara, H. (2011). Kaempferol enhances intestinal barrier function through the cytoskeletal association and expression of tight junction proteins in Caco-2 cells. *The Journal of Nutrition*, 141, 87–94.
- Tanabe, S. (2007). Epitope peptides and immunotherapy. *Current Protein and Peptide Science*, 8, 109–118.
- Venter, C., Pereira, B., Voigt, K., Grundy, J., Clayton, C. B., Higgins, B., et al. (2008). Prevalence and cumulative incidence of food hypersensitivity in the first 3 years of life. *Allergy*, 63, 354–359.
- Yasumatsu, H., & Tanabe, S. (2010). The casein peptide Asn-Pro-Trp-Asp-Gln enforces the intestinal tight junction partly by increasing occludin expression in Caco-2 cells. *The British Journal of Nutrition*, 104, 951–956.
- Ye, F., Xui, L., Yi, J., Zhang, W., & Zhang, D. Y. (2002). Anticancer activity of *Scutellaria baicalensis* and its potential mechanism. *Journal of Alternative and Complementary Medicine*, 8, 567–572.
- Yoon, S. B., Lee, Y. J., Park, S. K., Kim, H. C., Bae, H., Kim, H. M., et al. (2009). Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. *Journal of Ethnopharmacology*, 125, 286–290.
- Zhu, H., Wang, Z., Xing, Y., Gao, Y., Ma, T., Lou, L., et al. (2012). Baicalin reduces the permeability of the blood–brain barrier during hypoxia *in vitro* by increasing the expression of tight junction proteins in brain microvascular endothelial cells. *Journal of Ethnopharmacology*, 141, 714–720.