

Assessment of Dermal Safety of *Scutellaria baicalensis* Aqueous Extract Topical Application on Skin Hypersensitivity

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Abstract

Scutellaria baicalensis has been used as a traditional herbal medicine for bronchitis, hepatitis, and allergic diseases. The root of *Scutellaria baicalensis* contains active flavonoid components, including baicalin, baicalein, wogonoside, and wogonin, which have pharmaceutical properties. In the present study, the anti-allergic properties of a standardized aqueous extract of *S. baicalensis* were evaluated, and the skin toxicity of its dermal application was also determined. The *in vivo* and *in vitro* assays were performed by using the β -hexosaminidase assay in rat basophilic leukemia cells (RBL-2H3) and cutaneous skin reaction in BALB/c mice, respectively. In addition, the acute dermal irritation/corrosion test was carried out in New Zealand white rabbits, and the skin sensitization test was conducted by Buhler's method in Hartley guinea pigs to estimate the safety of the standardized aqueous extract of *S. baicalensis* for topical application. β -Hexosaminidase release in RBL-2H3 was markedly decreased following treatment with the standardized aqueous extract of *S. baicalensis*. It also ameliorated antigen-induced ear swelling compared with the control group in BALB/c mice. In the toxicological studies, it did not induce any dermal irritation/corrosion in rabbits or skin sensitization in guinea pigs. Although still limited, these results concerning the toxicological effects of *S. baicalensis* could be an initial step toward the topical application of *S. baicalensis* extracts on hypersensitive skin.

Key words

Scutellaria baicalensis · Lamiaceae · topical application · skin hypersensitivity · dermal toxicity · β -hexosaminidase

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

Scutellaria baicalensis Georgi (Lamiaceae) is a widely used traditional herbal medicine that contains various flavonoids. The plant root is used for the treatment of inflammatory diseases, fever, and allergies and as a smooth muscle relaxer [1–3]. The 4 major active flavonoid components are baicalin, baicalein, wogonoside, and wogonin [1], which have anti-inflammatory, antioxidant, and antitumor activities [4–6].

Recent research has demonstrated that the oral administration of *S. baicalensis* extracts prevented chemical-induced hypersensitivity [7–9]. However, systemic treatment usually requires a higher dose of this medicinal plant-derived extract to achieve the optimal therapeutic level, thus increasing expense and toxicity risk. Therefore, topical application is especially attractive for dermatologists because of the potential for maximum efficacy with minimal systemic adverse effects [10]. However, dermal toxicity, such as skin irritation and sensitization, should be considered prior to topical application of medicinal plant extracts. Although the beneficial effects of *S. baicalensis* in cases of skin hypersensitivity have been widely studied, little is known about its efficacy or toxicity when applied topically. Therefore, we investigated the effects of topical application of standardized *S. baicalensis* aqueous extract (WSBE) on skin hypersensitivity by using *in vivo* and *in vitro* assays. Furthermore, dermal toxicity tests were also performed to determine the dermal irritation/corrosion and sensitization effects of topical application of WSBE in animal models.

Topical drug application is simple and has several advantages, including a high local drug concentration and minimal risk of side effects [11]. Many drugs used in skin disease, including glucocorticosteroids and nonsteroidal anti-inflammatory drugs, have been formulated for topical application. In the development of a topical agent, assessment and evaluation of the dermal efficacy and safety of a substance are important initial steps. In this context, an *S. baicalensis* extract reported to reduce chemical-induced hypersensitivity when administered orally was tested for its antiallergic, dermal irritation/corrosion, and sensitization effects following dermal application.

The IgE-mediated cutaneous reaction in BALB/c mice was used to assess the antiallergic effect of topical WSBE *in vivo*. Previously, Song et al. reported that oral administration of *S. baicalensis* ameliorated atopic dermatitis via the inhibition of mast cell infiltration [12]. In the present cutaneous reaction test, WSBE ameliorated ear lobe thickening caused by DNFB (1-fluoro-2,4-dinitrobenzene) sensitization in a dose-dependent manner after 24 h of stimulation ($p < 0.05$; ● Fig. 3). In addition, the WSBE treatment reduced epidermal thickness up to 31% compared with the control group in H&E staining (● Fig. 4). These results were confirmed by the *in vitro* β -hexosaminidase release test. RBL-2H3 (rat basophilic leukemia) cells have been widely used for testing novel mast cell stabilizers [13]. In addition, β -hexosaminidase, an enzyme in the secretory granules of mast cells, is considered a degranulation marker and has been widely used in the screening of antiallergic agents [14]. The inhibitory effect of WSBE on β -hexosaminidase release in RBL-2H3 is shown in ● Fig. 2. Azelastine hydrochloride was selected as a positive control. WSBE decreased β -hexosaminidase release in stimulated RBL-2H3 cells, as compared with the challenged control cells (19%, 34%, and 60% in 125, 250, and 500 ppm, respectively; $p < 0.05$). These results were consistent with previous studies using other mast cells [7,8]. In addition, according to Bak et al. (2011), the root bark extract of *S. baicalensis* showed a concentration-dependent inhibitory effect on antigen-induced degranulation in mast cells [15]. Previously, Jung et al. (2012) reported that oral administration of *S. baicalensis* slightly inhibited the IgE-dependent cutaneous reaction [7]. In this study, topical application of *S. baicalensis* extracts showed a remarkable inhibitory effect on the cutaneous reaction, by approximately 40% compared with the control group (● Fig. 3).

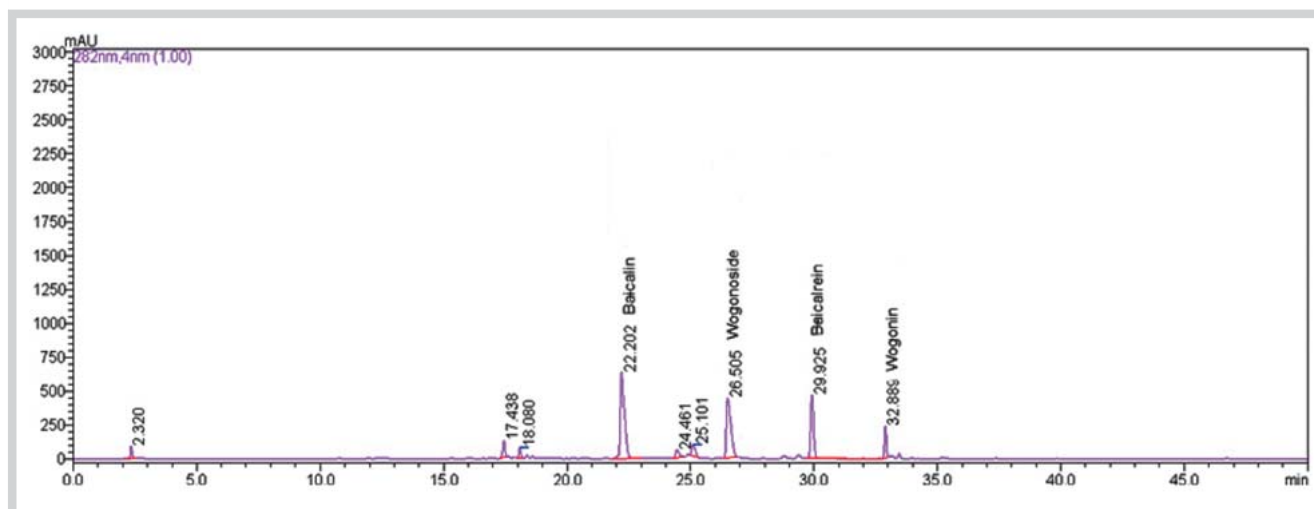


Fig. 1 Fingerprint analysis of WSBE using high-performance liquid chromatography (Waters ACQUITY UPLC™ system). Separation was achieved on YMC C₁₈ reversed-phase column (150 mm × 4.6 mm, 5.0 μm, YMC) using a mobile phase composed of A (0.1% formic acid in distilled water) and B (acetonitrile). The gradient program consisted of solvent A and B (0–4 min,

0% B; 4–12 min, from 0 to 20% B; 12–24 min, from 20 to 30% B; 24–32 min, from 30 to 65% B; 32–36 min, from 65 to 100% B; 36–42 min, hold on 100% B; 42–50 min, reconstitute 0% B). Column temperature was maintained at 40 °C with a flow rate of 1 mL/min. The detection wavelength was set at 276 nm. (Color figure available online only.)

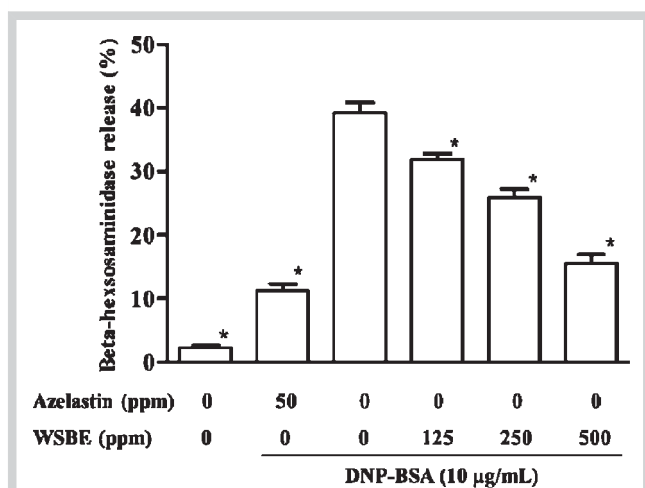


Fig. 2 Effects of WSBE on the antigen-induced degranulation of RBL-2H3. Azelastin was selected for the positive control. Values are expressed as means ± SEM. * $P < 0.05$, a significant difference compared with the antigen stimulated control.

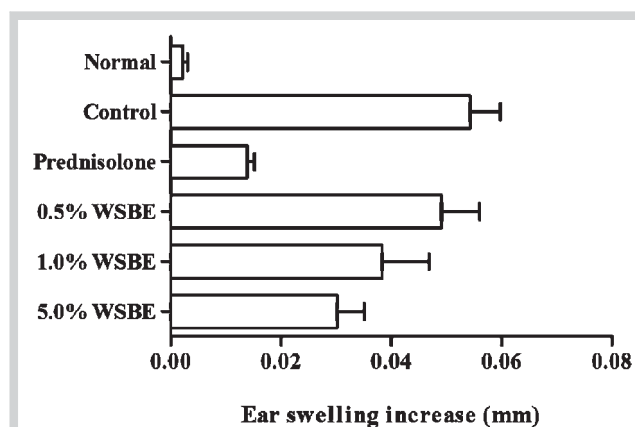


Fig. 3 Effects of WSBE on cutaneous reaction in the BALB/c mice ear ($n = 7$, each group). Mice were topically given 20 μL of D.W. (control), 1% prednisolone (positive control), and WSBE in D.W. (0.5%, 1.0%, and 5.0% WSBE) on each mouse ear lobe for three consecutive days. Values are expressed as means ± SEM. ^a Denotes statistically significant difference in comparison with the normal group and ^b denotes statistically significant difference in comparison with the control group ($p < 0.05$).

Skin irritation and sensitization information is indispensable for dermal toxicity screening when developing agents for topical application. During the dermal irritation/corrosion and skin sensitization test, there were no significant body weight changes, clinical signs, or mortality following topical application of WSBE to rabbits and guinea pigs during the experimental period. The scores for skin reactions to WSBE are presented in **Table 1**. In the acute dermal irritation/corrosion study, D.W.-treated skin did not show any erythema or edema. Also, in WSBE-treated group, only 1 out of 6 rabbits showed slight erythema with edema (scored as “1”), 1 h after removal of the patch. However, these resolved by 24 h post exposure. The PII value of WSBE-treated group was graded as “0” according to the Draize scoring scale,

which indicated that treatment with WSBE caused no irritation to rabbits at the dose employed [16]. Thus, under the conditions of this study, WSBE could be categorized as non-irritant.

In skin sensitization test, the skin reaction was observed 24 and 48 h after patch removal (**Table 2**). The guinea pigs in the positive control group [1% 1-chloro-2,4-dinitrobenzene (DNCB)] showed skin responses, which indicated that the skin sensitization tests were performed correctly. The average skin response scores in the DNCB-treated group were 0.6 and 0.4 at 24 and 48 h, respectively. Meanwhile, WSBE treatment was not associated with any changes on the skin surface including erythema and edema at 24 and 48 h following patch removal, and the reaction was graded as “0” based on the Buehler scoring system. This suggested that WSBE was not sensitizing in guinea pigs.

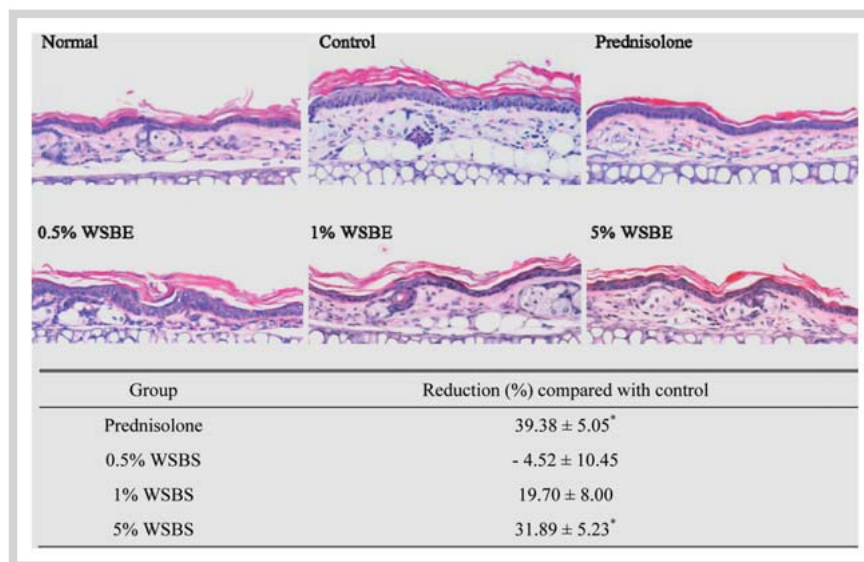


Fig. 4 Effects of WSBE on cutaneous reaction in the BALB/c mice ear (n = 7, each group). Mice were topically given 20 μ L of control (D. W.), 1% prednisolone (positive control), and WSBE in D.W (0.5%, 1.0%, and 5.0% WSBE) on each mouse ear lobe, for three consecutive days. The control group shows increased epidermal thickness in comparison with the normal group. Meanwhile, the 5% WSBE-treated group shows significantly decreased epidermal thickness compared to the control group.

* P < 0.05, a significant difference compared with the control group. (Color figure available online only.)

Table 1 Skin reaction scores in rabbit.

Rabbit No.	WSBE Erythema and eschar				Edema				D. W. Erythema and eschar				Edema			
	1	24	48	72	1	24	48	72	1	24	48	72	1	24	48	72
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Primary irritation index (PII) = (mean score at 24 h + mean score at 48 h + mean score at 72 h)/3

In conclusion, topical application of standardized extract of the root of *S. baicalensis* showed an antiallergic effect and did not cause skin irritation or sensitization. These preliminary study results provide a rationale for further studies on the topical application of *S. baicalensis*.

Materials and Methods

The radix of *S. baicalensis* was obtained from a local herbal market in Jangheung, Korea and authenticated by Dr. Kim of Jeollanamdo Development Institute for Traditional Korean Medicine. A voucher specimen (No. 2011-SB-07) was deposited at the herbarium of the Jeollanamdo Development Institute for Traditional Korean Medicine. The radix of *S. baicalensis* was extracted by refluxing with boiling water for 1 h (100 g of material in 1 L of water), and the resulting extract was spray-dried (31% of yield). The dried powder was stored at 4°C before use. To calculate the administration dose, the concentrations of baicalin, woogonoside, baicalein, and wogonin in the WSBE were quantitatively determined using high-performance liquid chromatography according to Gao et al. (2008) with some modifications [17]. The contents of baicalin, woogonoside, baicalein, and wogonin in WSBE were 6.45%, 3.37%, 2.07%, and 0.48%, respectively (Fig. 1).

For the β -hexosaminidase assay, RBL-2H3 were sensitized overnight with an anti-DNP IgE (0.2 μ g/mL) in 96-well plates (2×10^5 cells/well in MEM containing 10% FCS, 100 units/mL penicillin, 100 units/mL streptomycin; Gibco-BRL). The cells were washed with incubation buffer (119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂,

25 mM PIPES, 40 mM NaOH, 5.6 mM glucose, 1 mM CaCl₂, and 0.1% bovine serum albumin) to remove excess IgE and incubated in 160 μ L of the incubation buffer for 10 min at 37°C. After that, 20 μ L of WSBS (125, 250, and 500 μ g/mL) and azelastine hydrochloride (positive control; 50 μ g/mL; 98%, Sigma-Aldrich) solution (diluted with 0.5% DMSO) were added to each well and incubated for 10 min, followed by an addition of 20 μ L of antigen (DNP-BSA, final conc. 10 μ g/mL; Sigma-Aldrich) at 37°C for 10 min to stimulate the cells. After having stopped the reaction with ice bath (10 min), the supernatant (50 μ L) was transferred into a 96-well plate and incubated with 50 μ L of substrate (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide; Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 μ L of stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader at 405 nm.

For animal experiments, the protocols were approved (CNU-00156; 06. 2012) by the Institutional Animal Care and Use Committee of Chungnam National University (Daejeon, Korea), and the method details are included in Supporting Information.

The IgE-dependent cutaneous reaction on female BALB/c mice ears was performed as described in Supporting Information according to previous reports, with some modifications [18].

The dermal irritation/corrosion effect of WSBE was assessed in accordance with the OECD guideline for the testing of chemicals (OECD TG 404, 2002). The skin irritation/corrosion test was scored according to the Draize scale. The primary irritation index (PII) was calculated using the mean score at 24, 48, and 72 h.

Table 2 Skin sensitization study of guinea pig.

Group	Substance for induction	Substance for challenge	No. of guinea pigs	Hours after the patch removal	Mean score ^a	Sensitization rate ^b
Negative	D.W.	D.W.	20	24	0	0/20
				48	0	0/20
WSBE			10	24	0	0/10
				48	0	0/10
Positive	1% DNCB	0.1% DNCB	10	24	1.2	8/10
				48	0.9	8/10

^a Mean score = skin reaction score (according to Magnusson and Kligman)/no. of guinea pigs in group. ^b Sensitization rate = no. of guinea pigs with positive response/no. of guinea pigs in group

Evaluation of the skin sensitization effect of WSBE was carried out using Buehler's method, in accordance with the OECD guidelines for the testing of chemicals (OECD TG 406, 1992). The skin reactions are observed and scored at 24 and 48 h after the patch removal according to the Magnusson and Kligman grading scale. Results were expressed as mean $\pm$ standard error of the mean (SEM). The significance of differences among experimental groups was determined using one-way analysis of variance (ANOVA). The post hoc analysis using Tukey's multiple comparison was performed, and $p < 0.05$ was considered to be statistically significant.

Supporting information

Detailed protocols for animal experiments including cutaneous reaction, dermal irritation/corrosion, and the skin sensitization test are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

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Experimental animals

Female BALB/c mice (16 to 18 g), male New Zealand white rabbits (2 to 3 kg), and male Hartley guinea pigs (250 to 300 g) were purchased from Orient Bio Korea and were acclimated for 1 week before beginning the experiments. They were housed at 20 ± 2 °C temperature, $55\% \pm 3\%$ relative humidity, and 12-h light/dark cycle. Animals received tap water and a standard lab chow *ad libitum* throughout the study. The experimental protocols were approved (CNU-00156; 06. 2012) by the Institutional Animal Care and Use Committee of Chungnam National University (Daejeon, Korea).

Evaluation of cutaneous reaction

The IgE-dependent cutaneous reaction on female BALB/c mice ear was performed according to previous reports, with some modifications [18]. In brief, the mice were randomly divided into 6 groups ($n = 7$) as follows: normal (no treatment), control (distilled water [D.W.]), prednisolone, and WSBE group (0.5%, 1.0%, and 5.0% WSBE). Mice were treated topically with 20 μ L of D.W. (control), 1% prednisolone (positive control; 99 %; Sigma-Aldrich), and 0.5%, 1.0%, or 5.0% WSBE in D.W. on each mouse ear lobe for 3 consecutive days. The 1% prednisolone was prepared by using 10% prednisolone in DMSO with dilution. At 3 h after the last treatment (except for the normal group), anti-DNP IgE was injected intravenously (250 ng/mouse, 250 μ L; Sigma-Aldrich) for sensitization. After 24 h, the cutaneous reaction was induced by painting the ear lobe with 25 μ L of 0.5% DNFB (Sigma-Aldrich) acetone-olive oil solution. Ear thickness was measured at 0 and 24 h after the DNFB challenge using a dial thickness gauge (Mitutoyo Corporation). Ears were then fixed immediately in a 10% buffered formalin phosphate solution, embedded in paraffin and cut into 5- μ m sections. Four random sections for each skin sample were stained with hematoxylin and eosin (H&E) to

evaluate epidermal hyperplasia and infiltration of immune cells in the dermis. Image analysis for the quantification was performed using Image J (Rasband WS; ImageJ,) in 10 randomly-selected microscopic fields per specimen.

Assessment of dermal irritation/corrosion

The dermal irritation/corrosion effect of WSBE was assessed in accordance with the OECD guideline for the testing of chemicals (OECD TG 404, 2002). Three healthy male New Zealand white rabbits were used. Approximately 24 h before the test, the fur on the dorsolumbar region of each rabbit was clipped (approximate size, 12 cm²). The exposed area of skin was divided into 2 equal parts: one for vehicle control area and one for WSBE application. WSBE powder (0.5 g) was suspended in D.W. and applied to a 2.5-cm² gauze patch. Another patch was prepared with D.W. to act as the vehicle control. After 4-h exposure of the patches to the rabbit skin, all patches were removed and any residue was wiped from the skin. The rabbits were observed at 1, 24, 48, and 72 h after patch removal to assess the degree of erythema, crust formation, and edema. If the reversibility still existed, observation was continued up to 14 days after removal of the patches. The skin irritation/corrosion test was scored according to the Draize scale. The primary irritation index (PII) was calculated using the mean score at 24, 48, and 72 h.

Determination of skin sensitization

Evaluation of the skin sensitization effect of WSBE was carried out using Buehler's method, in accordance with the OECD guidelines for the testing of chemicals (OECD TG 406, 1992). Forty healthy male guinea pigs were used and randomly allocated into 3 groups: Group 1, WSBE treatment group (n = 20); Group 2, DNCB (97%, Sigma-Aldrich) in ethanol positive control group (n = 10); Group 3, D.W. treatment group (n = 10). Dose levels for the induction

and challenge phases were determined based on a preliminary study (data not shown).

The day before induction exposure, the left flank of each guinea pig was closely clipped (2.5 cm²) to expose the induction site. On day 0, test materials (0.4 g of WSBE powder in D.W., DNCB, and D.W. for each group) were fully loaded onto a gauze patch and tightly placed on the left flank for 6 h. After exposure, the patch was removed and the skin was gently washed with water. This process was repeated on days 7 and 14.

On day 28, the hair-free right flank of each animal was treated with an occlusive patch for challenge exposure for 6 h. Approximately 21 h after removing the patch, the challenge area was closely clipped for observation. The skin reactions were observed and scored at 24 and 48 h after the patch removal according to the Magnusson and Kligman grading scale.