

Tomato juice saponin, esculeoside B ameliorates mice experimental dermatitis

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ABSTRACT

Background: Allergic diseases like atopic dermatitis have recently increased. A naturally occurring glycoside, Esculeoside B, has been identified as a major component in tomato juice from the can. Accordingly, the present study investigated the effects of esculeoside B on experimental dermatitis mice.

Results: Oral treatment with 10 mg/kg of esculeoside B on the experimental dermatitis mice for 4 weeks significantly decreased the skin clinical score of 2.0 compared to the control score of 5.0. Furthermore, the scratch frequency of mice treated with esculeoside B was lower compared to the

control group. Overall, the administration of esculeoside B significantly inhibited T lymphocyte proliferation and demonstrated a tendency to decrease in IL-4 production. For example, the 121.2 pg/ml in the control group decreased to 96.1 pg/ml. There was also a decrease in serum IgE levels from 928.0 ng/ml in the control group to 687.8 ng/ml.

Conclusion: Our study is the first to demonstrate how tomato juice saponin or esculeoside B may ameliorate mice experimental dermatitis by the inhibition of T cell proliferation.

Keywords: tomato juice; experimental atopic dermatitis; IgE; cytokine; tomato saponin; esculeoside B

INTRODUCTION

There has been a shift towards considering the type-2 helper T (Th2) response as the predominant concept as a background mechanism for the pathogenesis of atopic dermatitis [1, 2, 3]. Activated Th2 cells produce effector cytokines such as interleukin IL-4 and IL-10. These effector cytokines are important for switching from antibody production from B cells to IgE production against the allergen and promoting humoral immunity. Hyper IgE production is a mark of atopic dermatitis in humans and well-documented in transgenic mice or NC/Nga mice suffering from atopic dermatitis-like skin lesions [4, 5, 6]. Therefore, the analysis of the T cell, IL-4 production, and IgE level may reflect the alteration of homeostasis between type-1 and type-2 immune responses.

Our previous study demonstrated how esculeoside A, which contains 4-fold the amount of lycopene content in a ripe tomato, ameliorated experimental atopic dermatitis of mice [7]. However, the underlying immunologic mechanisms are unknown. Moreover, we identified esculeosides B as a major component in tomato juice from the can [8]. In contrast to the chemical structure of esculeoside A, which is a spirosolane-type glycoside, esculeoside B is a rare and naturally occurring compound which is also a solanocapsine-type glycoside. However, it has not been proven whether esculeoside B is effective against atopic dermatitis.

Therefore, the present study tested the effects of esculeoside B on BALB/c mice demonstrating atopic dermatitis-like immune alteration and skin lesions which were induced by 2, 4-dinitrochlorobenzene (DNCB). The results demonstrated how esculeoside B ameliorated experimental dermatitis by modulation of T cell proliferation *in vivo*.

Materials and Methods

Preparation of Esculeoside B

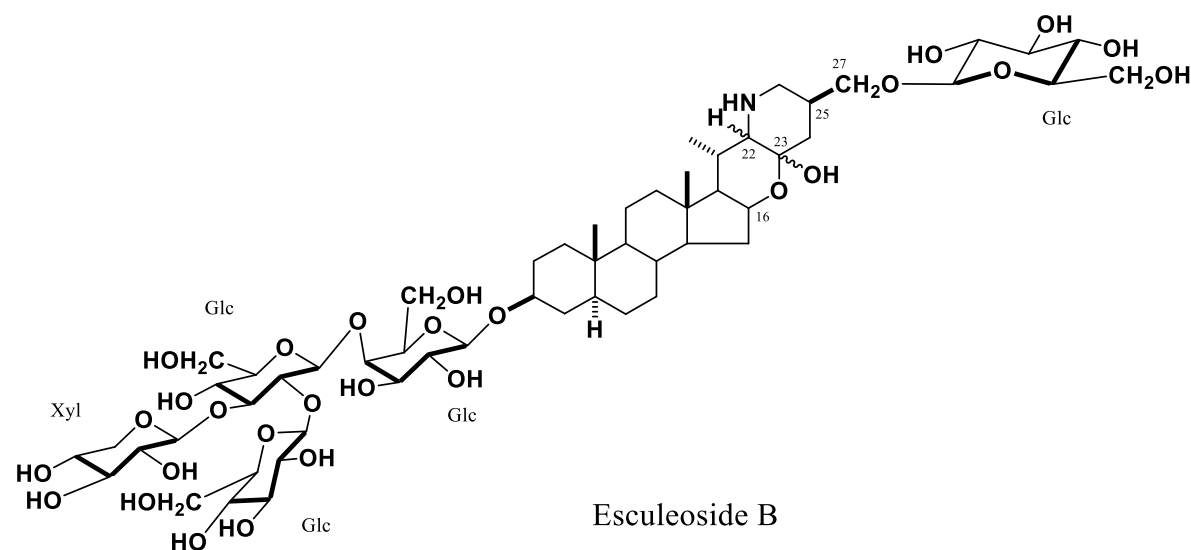
Esculeoside B was extracted [8]. 900 g of commercially processed tomato juice (*Solanum lycopersicum* L.) was centrifuged. The filtrate passed through a highly porous polystyrene gel. The resulting methanol residue was then subjected to dextran gel column chromatography and afterwards eluted with 90% methanol. The 90% methanol eluates lacked esculeoside A but contained esculeosides B on TLC with chloroform-methanol-water (6 : 4 : 1). Next, the obtained tomato saponin was identified by the NMR spectrum. The average of esculeoside B from commercially produced tomato juice was calculated to be about 0.041%. The chemical structure of esculeosides B was shown in Fig. 1A.

Animal Experimental Design

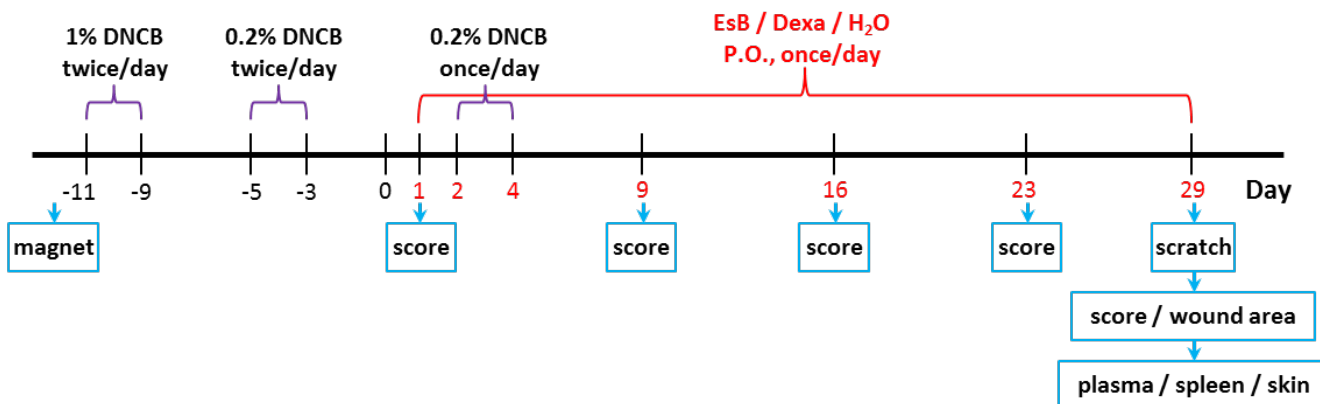
The animal experimental design was illustrated in Fig. 1B. The study was submitted to and approved by the Ethics Committee of Sojo University. All animal experiments were conducted in strict accordance with the Guidelines of the Japanese Pharmacological Society for the Care and Use of Laboratory Animals. 6 weeks old Female BALB/c mice were purchased from Kyudo Co., Ltd. (Fukuoka, Japan). The mice were housed under the following controlled conditions: temperature (24 ± 2 °C), humidity ($50\pm 10\%$), and a 12-hour light/dark cycle (7:00 a.m. to 7:00 p.m.). Food was available *ad libitum* and water was available from a drinking bottle. There was a one-week adaptation period after which the mice were divided into four groups.

The mouse hind paws were implanted with magnets for the following scratch analysis. After the experimental dermatitis was induced by DNCB (Wako, Osaka, Japan) on the dorsal skin, esculeoside B 10 mg/kg of body weight (BW)), dexamethasone (Nichiiiko, Toyama, Japan) (0.1 mg/kg of BW), or water was given as an oral treatment daily for 4 weeks. Esculeoside B and dexamethasone were prepared with pure water. In the period of treatment, the skin disorders were evaluated. The scratch behavior was measured after 4 weeks. In the end, the blood, spleen, and dorsal skin of mice were collected for the further analysis of IgE production, cytokine level, and histopathological alteration respectively.

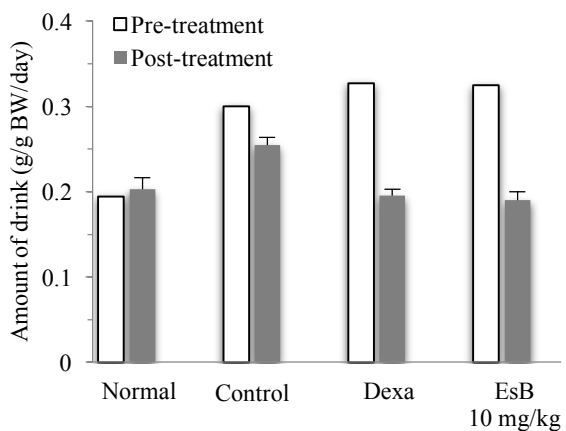
A.



B.



C.



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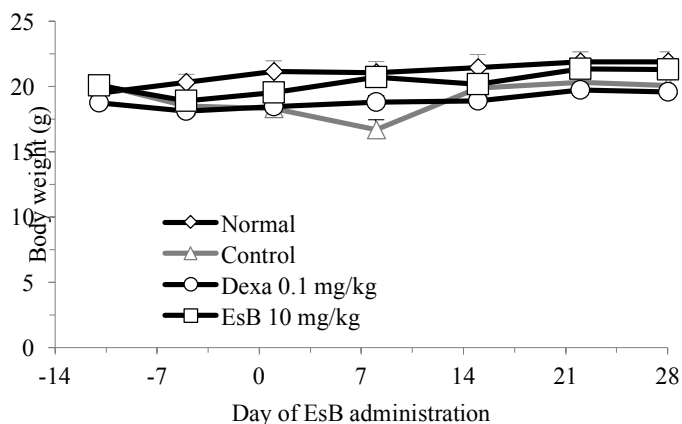


Figure 1. Chemical Structure of Esculeoside B, Experiment Design and Effect on Mice Water Intake by Esculeoside B Administration.

(A). Chemical structure of esculeoside B. Glc: glucose; Gal: galactose; Xyl: xylose. The molecular weight of esculeoside B is 1228.34 g/mol.

(B). Experiment design. The mice were divided into 4 groups (n=6 per group). The administration was performed through oral gavage in the morning. The volume was 0.2 mL/20 g body weight of mouse. Pure water application was normal. The control was pure water administration after atopic dermatitis induction. Dexamethasone (0.1 mg/kg) and esculeoside B (10 mg/kg) administration after atopic dermatitis induction were the treatment groups.

(C). Water-intake of mouse was assessed as the average water-intake per gram of mouse body weight per day. The water-intake just before esculeoside B administration (day 1) was pre-treatment. The water-intake after 4-weeks of administration (day 29) was post-treatment.

(D). Change in mouse body weight was measured during DNCB application and 4-week administration with esculeoside B. The results were expressed as means \pm S.E.M. (n=6). Dexa: dexamethasone; EsB: esculeoside B.

Induction of Experimental Dermatitis

For induction of experimental dermatitis, DNCB was treated on mice skin as described by Lee *et al* [4, 7]. After dorsal hairs of approximately 8 cm² were removed, 0.1 ml of 1% DNCB (prepared in acetone) was treated on their dorsal skin as a sensitizer twice (morning and afternoon) a day on day -11, -10, and -9. In the second week, 0.1 ml of 0.2% DNCB was treated on their dorsal skin twice a day on days -5, -4, and -3. In the third week, 0.1 ml of 0.2% DNCB was treated on their dorsal skin once per day in the morning on days 2, 3, and 4. Water, dexamethasone and esculeoside A were also applied.

Clinical Skin Score

According to the criteria of Hanifin and Rajka [9], skin lesions were measured using four symptoms: erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. Each symptom was graded from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). Clinical skin score was calculated as the sum of the individual scores as previously reported [7, 10].

Evaluation of Scratching Behavior

In order to detect and evaluate the scratching behavior from the hind toes of mice, Microact® (Neuroscience, Tokyo, Japan) was used [7, 11, 12]. Under anesthesia, the dorsal side of both hind paws of the mice was implanted subcutaneously with a small Teflon-coated magnet (1 mm in diameter, 3 mm long, and 0.018 g weight) on the day before DNCB challenge. The magnet was checked to remain in situ throughout the whole experimental period. The mouse with magnets was put in an observation chamber of 11 cm in diameter and 18 cm high, surrounded by a round coil. An electric current in the coil, which was induced by the movement of hind paws with the implanted magnets, was amplified and recorded by the MicroAct® software. The recording time for a mouse was 40 minutes. The measurement was performed on day 29 after esculoside B administration. The following parameters were used to register scratch events: Threshold (V) 0.05, Event Gap (s) 0.05, Max Freq (Hz) 20, Min Freq (Hz) 5, and Min Duration (s) 0.25.

Measurement of IgE Levels

After the 4-week tomato saponin application period, the mice were sacrificed under anesthesia and their blood samples were collected from abdominal aorta with heparin. The blood samples were allowed to stand for 2 hours at room temperature then centrifuged at $12,000\times g$ for 15 minutes at 4°C to separate the plasma. The plasma IgE levels were determined using a sandwich ELISA method and Mouse IgE Quantitation Kit (Bethly, Montgomery TX, USA) which were outlined by the manufacturer. Optical densities were determined using a micro-plate reader (Infinite M200, Tecan, Switzerland) at 450 nm.

Measurement of Splenocyte Proliferation

Immediately after the blood collection, the spleens were removed and then minced. A single-cell suspension was obtained in a RPMI 1640 medium before being seeded into 24-well plates at a density of 10^6 cells per well. After the splenocyte were activated with concanavalin A ($5\ \mu\text{g}/\text{ml}$) at 37°C for 48 hours, the effect of splenocyte proliferation was examined by a MTT assay and the absorbance was recorded using a micro-plate reader at 570 nm.

Measurement of Cytokine production

After those splenocytes were stimulated with immobilized anti-CD3 mAb ($5\ \mu\text{g}$) at 37°C for 48 hours in a CO_2 incubator, their supernatants were collected and stored at -80°C . The cytokine production in culture supernatants were determined using a sandwich ELISA method. The Mouse

IL-4 ELISA Kit (eBioscience, San Diego CA, USA) was used in accordance with the manufacturer. Optical densities were determined using a micro-plate reader at 450 nm.

Histological Analysis

After the mice were killed, skin samples were collected from the dorsal area. The samples were fixed in 10% neutral formalin, embedded in paraffin, sectioned into 5 µm slices, stained with hematoxylin and eosin (H&E), and then observed by optical microscopy. A minimum of 2 sections per mouse were examined for the presence and degree of incrustation, epidermal and dermal hyperplasia, parakeratosis, hyperkeratosis, dermal edema, vesicular formation, and inflammation. Histological analysis for the epidermal and dermal thickness and the infiltration of inflammatory cell was evaluated respectively using the following 4-grade system; 0 (none), 1 (mild), 2 (moderate), and 3 (severe) [7].

Statistical Analysis

All data are the mean ± S.E.M. Comparisons between the two groups were performed with an unpaired Student's t-test. Multiple comparisons were carried out using the Dunnett-test. Probability (*p*) values less than 0.05 were considered to be statistically significant. All statistical analyses were performed with Prism 5.0 (GraphPad Software, San Diego, USA).

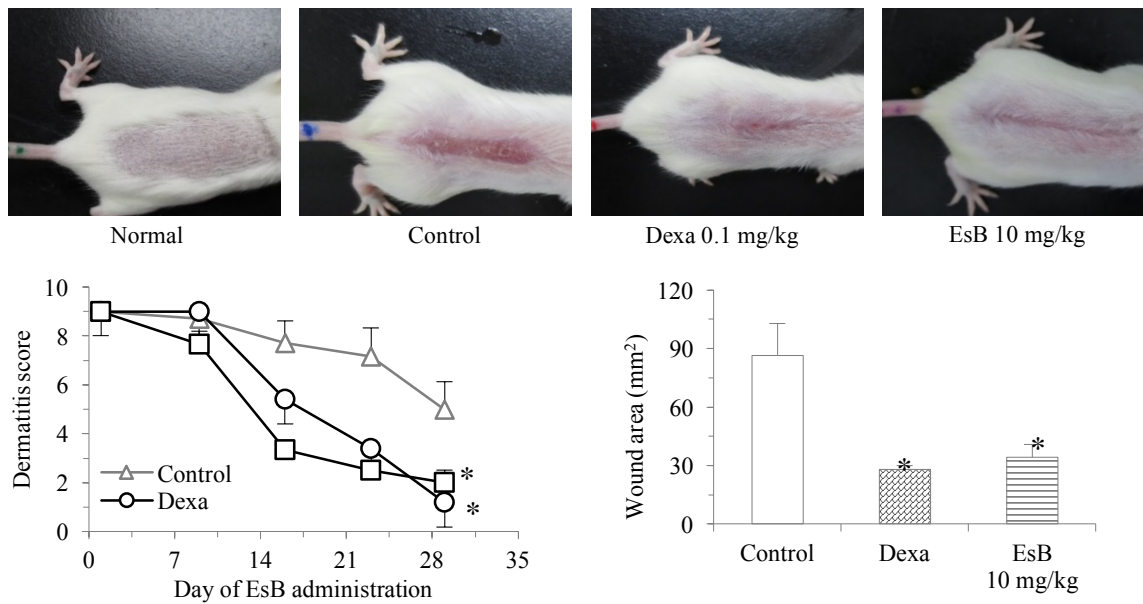
RESULTS

Effect on Increased Tendency Water-Intake in Experimental Dermatitis Mice by Esculeoside B Administration

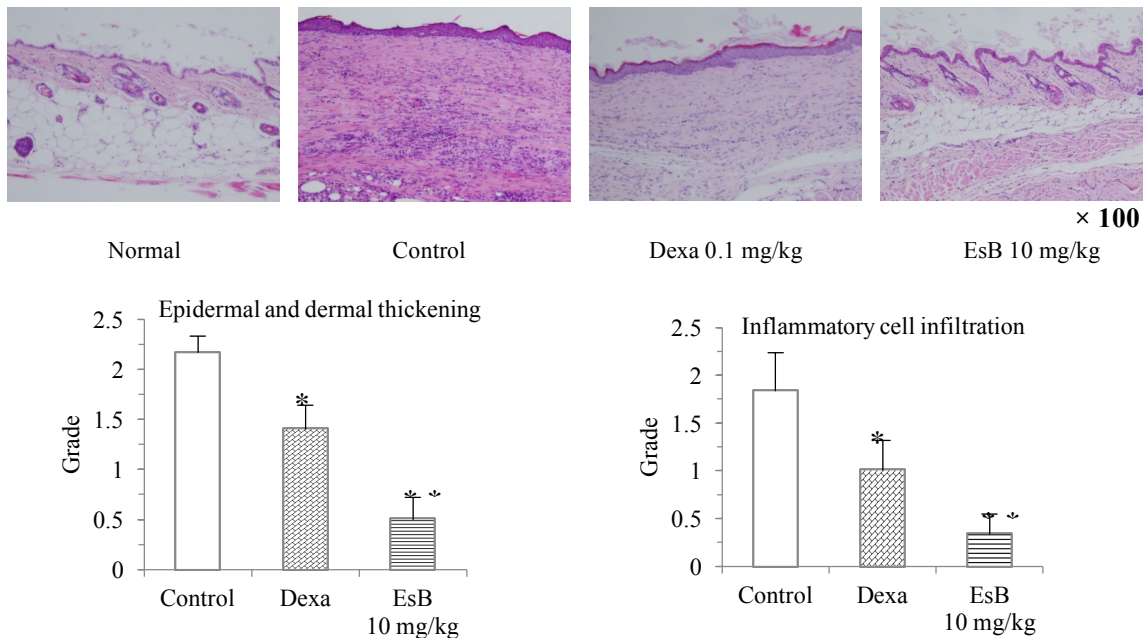
As shown in Fig. 1C, the water-intake of normal mice was about 0.19 g/g BW/day while the intake of dermatitis mice increased to 0.29 ~ 0.33 g/g BW/day after sensitivity and the first challenge were applied by DNCB. The increased water consumption was limited to 0.18 ± 0.01 g/g BW/day following administration of 10 mg/kg of esculeoside B 10 for 4 weeks. The body weights of mice were not affected during esculeoside B administration as shown in Fig. 1D.

Amelioration of Experimental Dermatitis in DNCB-treated Mice by Esculeoside B Administration

The macroscopic photographs in Fig. 2A revealed how after 4-week oral administration the control mice experienced severe cutaneous inflammation in contrast to the normal mice. Both esculeoside B and dexamethasone ameliorated the experimental dermatitis.



A. Macroscopic photograph after oral administration for 4 weeks. Dermatitis scores were assessed macroscopically in a blinded fashion during a 4-week period. And cutaneous wound area were measured by caliper after 4-week administration.



B. Histopathological findings at week 4 after various oral administration following atopic dermatitis induction. H&E staining, ×100. Summarized the effect of esculeoside B on grades of epidermal and dermal thickness and on the grade of inflammatory cell infiltration in DNCB-treated mice. The results were expressed as means ± S.E.M. (n=4~6). *: $P < 0.05$, **: $P < 0.01$, which were significantly different from the control results. Dexa: dexamethasone; EsB: esculeoside B.

Figure 2. Amelioration of Experimental Dermatitis in Experimental Dermatitis Mice by Esculeoside B Administration.

Through the evaluation of skin severity score and cutaneous wound area, it is clear that the 4-week administration of 10 mg/kg of esculeoside B 10 and dexamethasone significantly decreased the skin severity score to 2.0 ± 0.2 and 1.2 ± 0.7 compared to the control score of 5.0 ± 1.1 (*: $P < 0.05$). The cutaneous wound area was also significantly reduced to 34.4 ± 6.4 and 28.1 ± 2.0 mm² compared to the area of 86.4 ± 16.4 mm² in the control (*: $P < 0.05$).

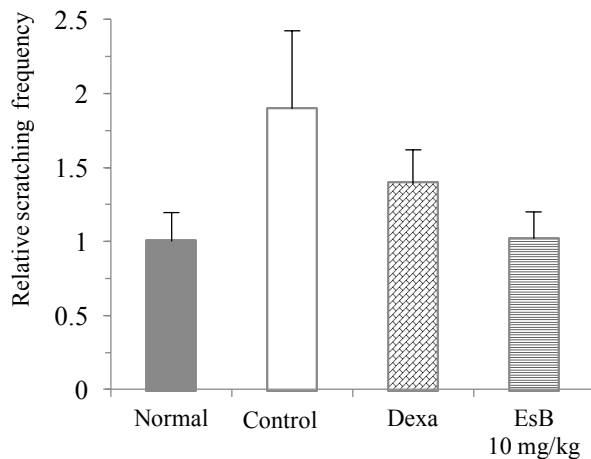
The dermatitis histological evaluation in Fig. 2B demonstrated hypertrophy, hyperkeratosis of the epidermis, and inflammatory cells infiltration (1.8 ± 0.4) in the control group. The treatment with 10 mg/kg of esculeoside B significantly decreased the grades of epidermal and dermal thickness to 0.5 ± 0.2 and inflammatory cell infiltration to 0.3 ± 0.2 (**: $P < 0.01$).

Effects on Scratching and Immunomodulation in Experimental Dermatitis Mice by Esculeoside B Administration

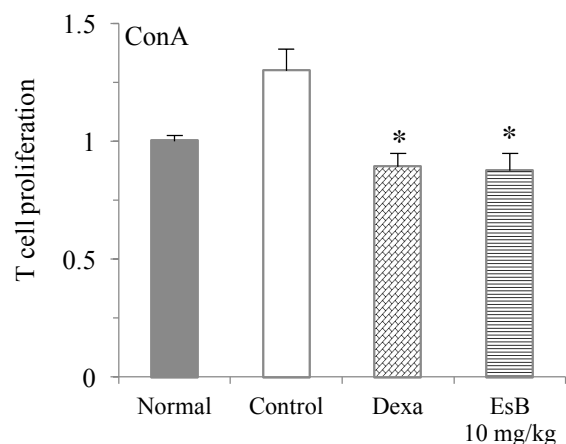
As shown in Fig. 3A, the relative scratching frequency was 1.89 times of the scratch frequency within the control mice. Immunodmodulation was increased 1.02 times by administration of 10 mg/kg.

The immunomodulation was analyzed after 4-week esculeoside B administration. The rate of ConA-activated spleenocyte proliferation was significantly suppressed as 0.87 ± 0.02 of esculeoside B group compared with 1.24 ± 0.09 of control (Fig. 3B). Furthermore, IL-4 production had a tendency to decrease as 96.1 ± 31.5 pg/ml by esculeoside B group compared with 121.2 ± 21.9 pg/ml of the control group (Fig. 3C). Furthermore, the plasma IgE production was demonstrated as 928.0 ± 102.8 ng/ml by the control mice. There was also a tendency to decrease demonstrated as 687.8 ± 165.4 ng/ml following esculeoside B administration (Fig. 3D).

A.



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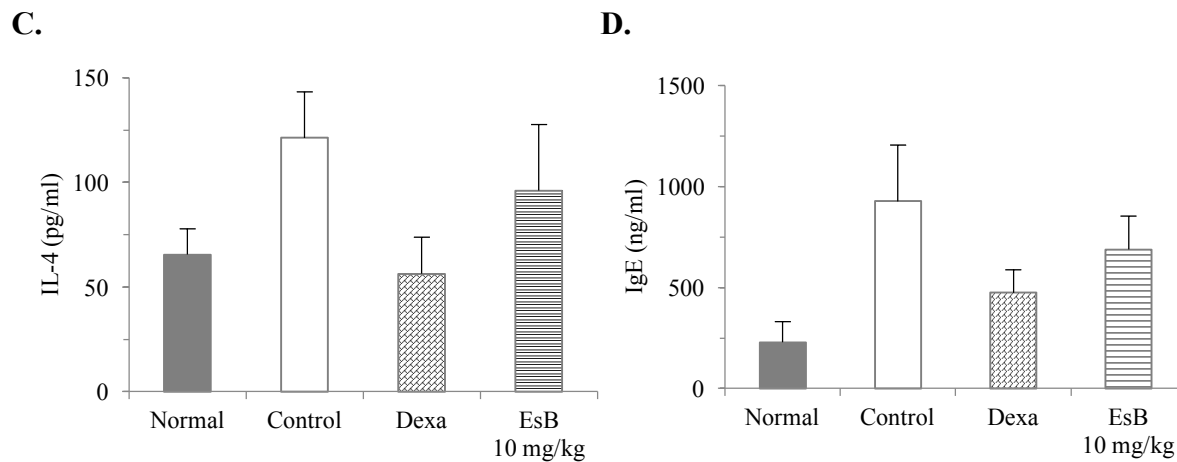


Figure 3. Effects on Scratching and Immunomodulation in Experimental Dermatitis Mice by Esculeoside B Administration. Mouse hind paws were implanted with magnets before DNCB application. Scratching behavior on the back skin within 40 minutes was recorded after 4-weeks of esculeoside B administration. **(A).** The computer panel demonstrated the primary view of changes in voltage in accordance with the mouse hind paws movement. One movement of the mouse hind paw was named as a scratch. Scratching frequency was analyzed as the scratching numbers during 40 min **(B).** Effect on the rate of ConA-activated T cell proliferation. **(C).** Effect on the production of Th2 cytokine IL-4 **(D).** Effect on plasma IgE level. The results were expressed as means \pm S.E.M. (n=4~6). *: $P < 0.05$, significantly different from the Control. Dexa: dexamethasone; EsB: esculeoside B.

DISCUSSION

The present results indicate that oral administration with esculeoside B or tomato juice saponin could ameliorate DNCB-induced experimental atopic dermatitis of mouse, with its effects being mediated by T lymphocyte proliferation. We previously demonstrated that esculeoside A, a tomato fruit saponin, was able to inhibit hyaluronidase activity *in vitro* and ameliorate mouse experimental dermatitis [7]. We also demonstrated how anti-hyaluronidase activity by esculeoside B was about 100 times weaker than that of esculeoside A (our unpublished data). The present results suggest that esculeoside B may improve experimental dermatitis by an immunological mechanism.

While there has been a reported increase in transepidermal water loss from the dorsal skin there has been a compensatory increase of water intake in the DNCB-induced atopic dermatitis like skin lesions [13, 14, 15]. As shown in Fig. 1C, the dermatitis mice were shown to be about 1.5 times likely to increase in water intake. 4-week administration with esculeoside B prevented this compensatory increase tendency without remarkable toxic effects such as reduction of body

weight, which also suggests a decrease in transepidermal water loss. Additionally, 10 mg/kg of esculeoside B and dexamethasone from the positive control significantly prevented the increase of the dermatitis score and wound area, the epidemic from thickening, and inflammatory cell infiltration (Fig. 2). Both demonstrated a recovery in impaired skin barrier function. Moreover, esculeoside B administration demonstrated a decreasing tendency in DNCB-induced scratching frequency (Fig. 3A). Since scratching behavior induces skin damage, promotes inflammatory response, and aggravates pruritus [17, 18, 19], esculeoside B may also prevent aggravation of skin damage related to pruritic disorders. Nonetheless, as esculeoside B is a plant steroid glycoalkaloid, it is unknown if such effects have a relation with the hormone homeostasis mechanism. Nohara *et al.* reported that the final metabolite was eliminated as androsterone analogues in men's urine after an orally administered ripe cherry tomato (the main content being esculeoside A). However, unlike an easy conversion into pregnane derivative by esculeoside A sapogenol (esculeogenin A), the sapogenol of esculeoside B (esculeogenin B) is difficult to change due to a trigger stage of elimination at 23-hydroxyl group being absent [20]. As a potential next step, it would be interesting to investigate the pharmacokinetics of esculeoside B.

Considering the results from Fig 3B, we can conclude that the oral administration of esculeoside B on DNCB-induced dermatitis mice may inhibit T cells proliferation activated by T cell mitogen ConA. The role of esculeoside B on CD4⁺ cells and differentiation into different subsets, such as, Th1, Th2, and Treg, still need to be studied. However, Fig 3C and 3D demonstrated that the esculeoside B administration for 4 weeks did not significantly suppress serum IgE and Th2 cytokine production. The final challenge with DNCB was performed on day 4 and evaluation of dermatitis phenotype were conducted on day 29. The period between challenge and evaluation is long. As a result, it may be difficult to detect the difference among the experimental groups, such as scratching, IgE, and cytokine levels. Future studies will be required to elucidate how Esculeoside B inhibited dermatitis phenotype and what the target mechanism of Esculeoside B is. Furthermore, tomato juice contains both esculeoside B and lycopene and the oral administration of lycopene prevents atopic dermatitis in association with a suppression of T-helper 2 chemokines in a murine model [21]. Accordingly, the synergistic efficacy on the atopic dermatitis through the intake of tomato juice can be expected.

CONCLUSION

Our study is the first to demonstrate tomato juice saponin's immunoregulatory potential for

alleviating atopic dermatitis through inhibition of T cell proliferation.

Conflict of Interest: The authors declare no competing interest.

Authors' contributions: J-R Zhou conceived of the study, participated in its design, and drafted the manuscript. J Urata carried out the animal treatment. T Shiraishi, C Tanaka carried out the ELISA experiment. T Nohara performed the extraction of esculeoside. B. K Yokomizo performed the statistical analysis. All authors read and approved the final manuscript.

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