

Coix Seed Extract Prevents Inflammation-mediated Skin Dryness Induced by Sodium Dodecyl Sulfate Exposure in HR-1 Hairless Mice



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ABSTRACT

Skin inflammation and dryness are the features of surfactant-induced irritant contact dermatitis, a common skin disorder. In Japan, Coix seed (CS, *Coix lacryma-jobi* L. var. *ma-yuen* Stapf) is widely used as a traditional medicine and functional supplement to treat skin inflammation and dry skin. However, the efficacy of CS against surfactant-induced skin disorders has not been reported. Here, we investigated the effect of CS on inflammatory dry skin disorders induced by multiple topical applications of sodium dodecyl sulfate (SDS), a representative anionic surfactant. Male HR-1 hairless mice received a water extract of CS for four weeks. Three weeks after CS administration, the dorsal skin of the mice was exposed once daily to 10% SDS for five days. CS efficacy was then evaluated by measuring epidermal water content; erythema index; severity of skin scaling; epidermal thickness; inflammatory cell infiltration; production of pro-inflammatory mediators, such as interleukin-1 α (IL-1 α) and prostaglandin E2 (PGE2); and protein expression of cyclooxygenase 2 (COX-2), in the dorsal skin. Administration of CS markedly attenuated the SDS-induced reduction in epidermal water content, elevated erythema index, and severity of skin scaling. Histological analysis demonstrated that CS suppressed epidermal hyperplasia and macrophage infiltration in SDS-exposed skin. Furthermore, CS significantly prevented SDS-induced production of IL-1 α and PGE2, as well as COX-2 upregulation. These results indicate that CS prevents SDS-induced inflammation-mediated skin dryness by inhibiting the production of pro-inflammatory mediators.

ABBREVIATIONS

CS	Coix seed
PGE2	prostaglandin E2
COX-2	cyclooxygenase 2
ICD	irritant contact dermatitis
SDS	sodium dodecyl sulfate
GluCer	glucosylceramide
H&E	hematoxylin, and eosin
HRP	horseradish peroxidase
Iba-1	ionized calcium-binding adaptor protein-1
IL-1 α	interleukin-1 α
CMC-Na	sodium carboxymethyl cellulose
MRM	multiple reaction monitoring
SIM	selected ion monitoring

Introduction

Coix seed (CS, seeds of *Coix lacryma-jobi* L. var. *ma-yuen* Stapf, *Gramineae*) is a traditional Chinese medicine used for invigorating spleen function, alleviating arthritis, arresting diarrhea, and treating diabetes [1]. CS contains fatty acids [2], saccharides [3], phenolics [4], and lactams [5]. Pharmacological research has demonstrated a wide spectrum of biological activities of CS, including anti-inflammatory [6–8], anti-allergic [9], antioxidant [10–12], and antitumor activities [1]. Several studies using animal models have demonstrated that CS exerts anti-inflammatory and anti-allergic effects by regulating pro-inflammatory cytokine expression and suppressing cyclooxygenase 2 (COX-2) and prostaglandin E₂ (PGE2) production [13, 14]. CS is widely used as a therapeutic agent for inflammatory skin disorders, such as atopic dermatitis [15, 16] and acute radiation dermatitis [17]. Surfactant-induced irritant contact dermatitis (ICD) is a common skin disorder. However, to the best of our knowledge, the efficacy and mechanism of action of CS against surfactant-induced skin disorder have not been reported to date.

Sodium dodecyl sulfate (SDS), a representative anionic surfactant, is widely used in domestic cleaning, cosmetic, and pharmaceutical products [18]. Prolonged exposure to SDS induces skin disorders characterized by skin dryness, scaling, erythema formation, epidermal hyperplasia, and inflammatory cell infiltration [19–21]. Although the exact mechanism underlying SDS-induced skin damage is not fully understood, removal of intercellular lipids or alteration of lipid profiles in the stratum corneum [22, 23] and the direct toxicity of SDS on epidermal cells due to the release of inflammatory mediators, such as interleukin-1 α (IL-1 α) [24, 25] and PGE2 [26–28], are recognized as the major causes of SDS-induced ICD progression. This study aimed to investigate the preventive effects of CS on skin inflammation and dryness induced by multiple topical applications of SDS on the dorsal skin of hairless mice and elucidate the possible mechanism involved.

Results

Eight commercially available compounds (Listed in ► **Table 1**), previously reported as the bioactive components in CS extract or its

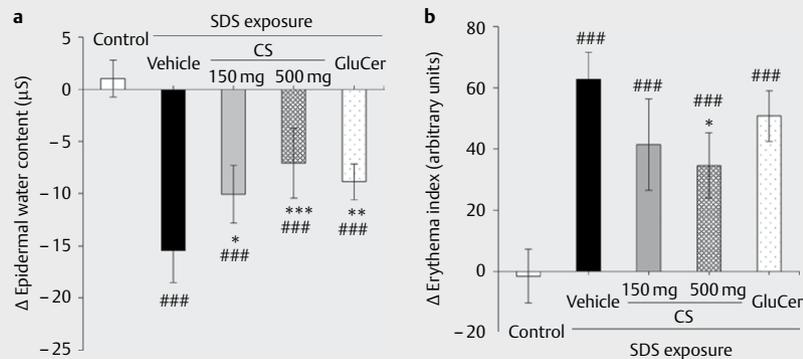
► **Table 1** Contents of the test compounds in Coix seed extract

Compounds	Contents ($\mu\text{g/g}$) *	RSD (%)
Protocatechuic acid	3.61	0.8
4-Hydroxybenzaldehyde	3.24	0.8
Caffeic acid	5.78	1.6
<i>p</i> -Coumaric acid	29.85	0.9
5,7-Dihydroxychromone	4.49	5.8
Naringenin	ND	ND
Luteolin	ND	ND
Coixol	9.10	4.9

* The content of each compound is presented as $\mu\text{g/g}$ of CS extract.; RSD: Relative standard deviation (n = 3); ND: not detected, when the signal-to-noise ratio (S/N) was below 3.

related parts [7, 8, 29–31], were selected to quantify their contents in the CS extract via LC MS/MS. The calibration curves for each standard were found to be linear with correlation coefficients (R^2) greater than 0.999 for the indicated concentration range (Table 3S, Supporting Information). This quantification was performed in triplicate, and the results are shown in ► **Table 1** and ► **Fig. 1S** (Supporting Information). Among the evaluated compounds, the content of *p*-coumaric acid was the highest, followed by coixol, a characteristic CS alkaloid. Naringenin and luteolin were not detected in the CS extract. We further analyzed the saccharide and fatty acid contents in the primary components of CS extract using HPLC and GC-MS, respectively. Three saccharides (glucose, fructose, and sucrose) and four fatty acids (palmitic acid, stearic acid, oleic acid, and linoleic acid) were detected, as shown in ► **Fig. 2S** (Supporting Information). The presence of these components was validated by comparison with authentic samples.

In our preliminary study, we found that daily exposure of mouse dorsal skin to SDS caused a gradual decrease in epidermal water content, an indicator of skin dryness, and an increase in erythema index, an indicator of skin inflammation. During the 5-day treatment period, both skin parameters were significantly altered after 5 days of SDS exposure (► **Fig. 3S**, Supporting Information); therefore, this time point was selected for evaluating CS extract efficacy in this study. The effects of CS against SDS-induced skin dryness and inflammation were evaluated by measuring the epidermal water content and skin erythema index. Both skin parameters were measured before the start of SDS treatment and on the 5th day of the treatment, and the ΔE values are shown in ► **Fig. 1**. CS significantly prevented the SDS-induced reduction in epidermal water content in a dose-dependent manner compared to that in the SDS-treated group (vehicle group) (► **Fig. 1a**). Glucosylceramide (GluCer), a ceramide precursor, was used as the positive control and showed an effect similar to that of CS (► **Fig. 1a**). In addition, oral administration of 500 mg/kg CS significantly prevented the increased erythema index caused by SDS exposure (► **Fig. 1b**). The change in the erythema index was not significantly different among the low CS (150 mg/kg), GluCer, and vehicle groups (► **Fig. 1b**). By day 5, SDS exposure caused an increase in the severity of skin scaling (► **Fig. 2a**), and the score was significantly increased compared to that of untreated skin (control group) (► **Fig. 2b**). Administration of 500 mg/kg CS significantly alleviated the severity of skin



► **Fig. 1** Effect of CS on SDS-induced reduction of epidermal water content and elevation of skin erythema index. Epidermal water content and erythema index of the dorsal skin were measured in each mouse before the topical application of SDS on day 5. The data are expressed as Δ epidermal water content (**a**) and Δ erythema index (**b**), calculated by subtracting the values obtained before SDS exposure from the values obtained on day 5 after SDS exposure. Bars represent means \pm standard deviations ($n = 5$ per group); ### $p < 0.001$ vs. control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle group; Tukey's test.

scaling compared with that in the vehicle group (► **Fig. 2b**). A similar effect was observed in the GluCer-administered group (► **Fig. 2**).

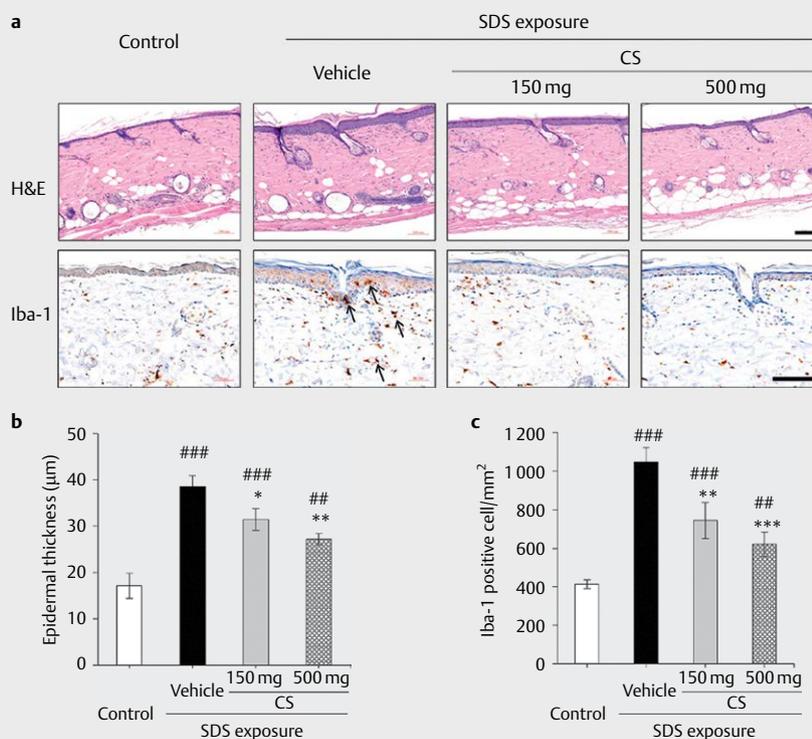
To evaluate the effect of CS on epidermal hyperplasia following SDS exposure, we stained skin tissue sections with hematoxylin and eosin (H&E) and measured epidermal thickness. By day 5, SDS exposure caused approximately 2-fold higher epidermal thickening in the vehicle group than in the control group (► **Fig. 3a** and **b**). CS significantly attenuated the increased epidermal thickness in a dose-dependent manner (► **Fig. 3b**). Infiltration of macrophages and neutrophils is reportedly involved in SDS-induced skin inflammatory reactions [21]. To evaluate the effect of CS extract on SDS-induced leukocyte infiltration in mouse skin, immunohistochemical staining of the macrophage marker ionized calcium-binding adaptor protein-1 (Iba-1) and neutrophil marker Ly-6G/Ly-6C was performed. By day 5, SDS exposure resulted in macrophage infiltration into the epidermis (► **Fig. 3a, Iba-1**). The number of Iba-1-positive cells was significantly higher in the SDS-treated group than in the control group. CS significantly reduced the number of Iba-1-positive cells in a dose-dependent manner (► **Fig. 3c**). However, no significant difference was observed in the number of neutrophils among the groups (data not shown).

Irritant-induced skin inflammatory reactions involve increased production of inflammatory mediators [32, 33]. To elucidate how CS attenuates the SDS-induced skin inflammation, we examined the expression of IL-1 α and PGE2, previously reported to be associated with the SDS-induced inflammatory responses [24, 26]. The concentrations of IL-1 α and PGE2 in the skin tissue in the vehicle group were significantly higher than those in the control group. The increased levels of IL-1 α and PGE2 were significantly attenuated by the administration of 500 mg/kg CS (► **Fig. 4a, b**). COX-2 regulates the synthesis of PGE2 from arachidonic acid. Therefore, we investigated the effect of CS on COX-2 expression using western blotting. SDS exposure significantly increased the expression of COX-2, and administration of 500 mg/kg CS markedly attenuated this upregulation (► **Fig. 4c**).

Discussion

In this study, we investigated the effect of CS on skin inflammation and dryness using the SDS-induced ICD model of HR-1 hairless mice and found that CS attenuated SDS-induced skin dryness, scaling, erythema, epidermal hyperplasia, and inflammatory cell infiltration. Furthermore, CS inhibited the SDS-induced production of pro-inflammatory mediators. Our study showed that oral administration of CS can protect against surfactant-induced inflammation-mediated skin dryness.

Enhanced production of pro-inflammatory mediators in the skin is a major characteristic of ICD [32, 33]. PGE2 is one of the most important mediators implicated in ICD [26–28]. It accelerates the blood flow and enhances vascular permeability, leading to erythema formation and inflammatory cell infiltration at the site of inflammation [34]. Additionally, PGE2 increases keratinocyte proliferation and inhibits keratinocyte differentiation in a fibroblast-keratinocyte co-culture system [35]. In this study, CS significantly prevented SDS-induced PGE2 production (► **Fig. 4b**). During PGE2 synthesis, COX-2 catalyzes the conversion of membrane-released arachidonic acid to PGE2. Our results also indicated that SDS-induced COX-2 expression is significantly attenuated by CS administration (► **Fig. 4c**). Therefore, suppressing PGE2 production by inhibiting the COX-2 expression may partially ameliorate SDS-induced erythema formation, epidermal hyperplasia, and macrophage infiltration by CS (► **Fig. 1b** and ► **3**). Previous studies have indicated that CS and its components, such as coixol, caffeic acid, and *p*-coumaric acid exert anti-inflammatory effects by suppressing COX-2 [7, 13, 36, 37]. These components were predominantly found in the CS extract (► **Fig. 1S** and ► **Table 1**). However, identification of the compounds responsible for the protective activity of CS against SDS-induced COX-2-mediated PGE2 production requires further investigation. IL-1 α , the most abundant cytokine present in the skin, is another key mediator involved in the initiation and maintenance of ICD [38]. Previous studies have shown that SDS induces the overexpression of IL-1 α both *in vitro* and *in vivo* [24, 25]. In response to irritants, IL-1 α could be released from keratinocytes as an initial step in the inflammatory cascade, subse-



► **Fig. 3** Effect of CS on SDS-induced epidermal hyperplasia and macrophage infiltration in mouse skin. Representative H&E and Iba-1 immunohistochemically stained dorsal skin sections (a) on day 5 after the start of SDS topical application. Arrow, Iba-1-positive macrophages; Scale bars, 100 µm. H&E and Iba-1 stained skin sections were used to quantify the epidermal thickness (b) and the density of Iba-1-positive macrophages in the skin (c), respectively. Both quantifications were performed as described in the Materials and Methods section. Data are expressed as means ± standard deviations (n = 5 per group). ^{##}p < 0.01, ^{###}p < 0.001 vs. control group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle group; Tukey's test.

ards of Kracie Pharmaceutical Ltd. A voucher specimen (No. 24211) was deposited in the herbarium of Kampo Research Laboratories, Kracie Pharmaceutical, Ltd. The CS water extract was prepared according to the Guidance published by the Japanese Pharmaceuticals and Medical Devices Agency [45]. Briefly, 30 g of CS, the daily dosage recommended for adults, was soaked in 600 mL distilled water (20 times the amount of CS, v/w) for 1 h in a Santo earthenware teapot and concentrated to a final volume of 390 mL (13 times the amount of CS, v/w) by boiling. The mixture was filtered through gauze and lyophilized to obtain 840 mg of the extract powder (yield 2.8%). This extraction was repeated to obtain sufficient extract; the extracts were homogenized and stored at -20 °C until further use.

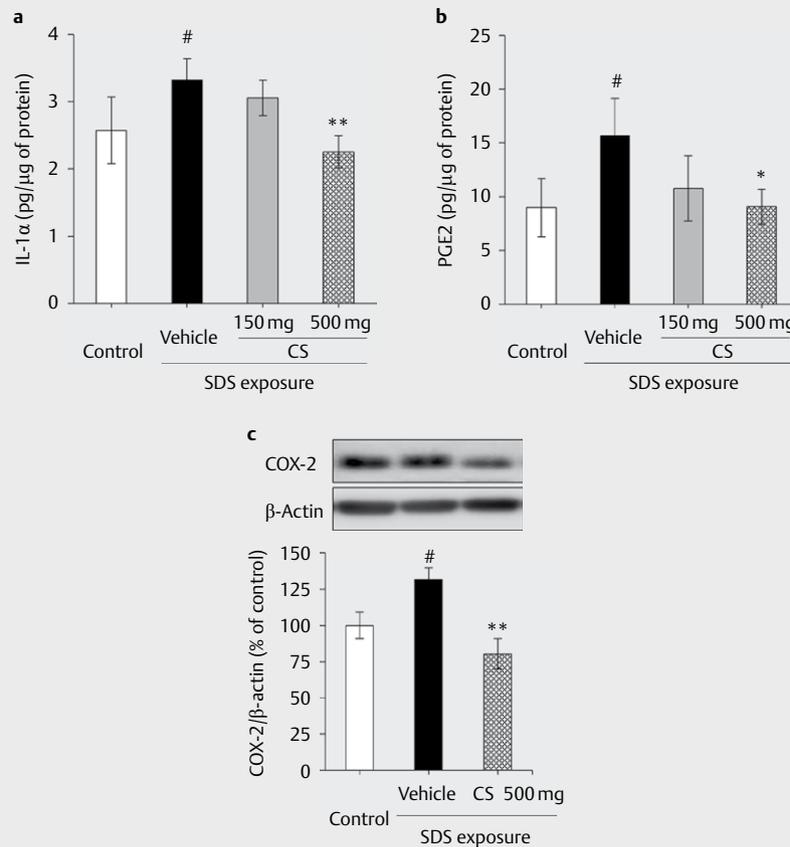
Preparation of the standard and sample solutions

The standards, coixol and 5,7-dihydroxychromone, were obtained from MedChemExpress Co., Ltd., and ChemFaces Biochemical Co., Ltd., respectively. Protocatechuic acid, caffeic acid, *p*-coumaric acid, and 4-hydroxybenzaldehyde were purchased from Sigma-Aldrich Co. LLC. Naringenin and luteolin were obtained from FUJIFILM Wako Pure Chemical Corporation. All the standards were of analytical grade, with purity above 98%. Each standard was accurately weighed and dissolved in HPLC-grade methanol (Wako) to prepare the respective stock solutions. Calibration standard solutions were prepared by appropriate dilutions of the mixed stock solution. The sample solution was prepared as described previously [46]. Briefly,

the CS extract (1.0 g) was weighed accurately and dissolved in 10 mL of ultrapure water (Wako). Next, 10 mL of HPLC-grade acetonitrile (Wako) was added, and the mixture was agitated using a shaker (Taitec Corporation) for 10 min. A premixed sachet of the QuEChERS Extraction salts (Agilent Technologies) was further added to the solution and shaken for 10 min. After centrifugation (3000 × g, 10 min), 1 mL of the upper layer solution was transferred into a 10 mL volumetric flask and diluted with water to obtain the sample solution.

Chemical analysis of the CS extract

The standard and sample solutions were analyzed via LC MS/MS on the Shimadzu UFLC HPLC system coupled to an LCMS-8030 triple quadrupole mass spectrometer. The MS/MS analyses of the standards, except for coixol, were performed in the multiple reaction monitoring (MRM) mode; for coixol, analysis was in the selected ion monitoring (SIM) mode because no ion pairs were detected in the MRM mode. The LC MS/MS conditions and the parameters for each analyte are described in the Supporting Information. The saccharides and fatty acids in the CS extract were analyzed by HPLC and GC-MS, respectively; the details are described in the Supporting Information.



► **Fig. 4** Effect of CS on SDS-induced IL-1 α , PGE2, and COX-2 levels in mouse dorsal skin. Dorsal skin was collected on day 5 after the start of SDS topical application. The production of IL-1 α (a) and PGE2 (b) in the dorsal skin was quantified using ELISA. The COX-2 protein levels (c) in the dorsal skin were analyzed by western blotting; β -actin was used as the internal standard; Representative immunoblots are shown above the plot in panel C. Data are expressed as means \pm standard deviations ($n=5$ per group). [#] $p<0.05$ vs. control group; * $p<0.05$, * $p<0.01$ vs. vehicle group; Tukey's test.

Animals

Seven-week-old male hairless HR-1 mice were purchased from Japan SLC Inc., housed at $24 \pm 2^\circ\text{C}$ under a 12/12-h light/dark cycle, and provided laboratory pellet chow (CE-2, CLEA Japan Inc.) and water *ad libitum*. The animal experiments in this study were approved (approval #190023, August 30, 2019) by the Experimental Animal Care Committee of Kracie Pharmaceutical, Ltd. and were performed in accordance with the principles of the Basel Declaration and recommendations in the Guidelines for Proper Conduct of Animal Experiments.

Treatment

The mice were divided into five groups ($n=5$) according to their body weight. Over 4 weeks, the mice in the first and second groups were orally administered 150 and 500 mg/kg per day of CS extract, respectively. The dose conversion between animals and humans was performed using a previously described calculation [47]. The third group received pure GluCer (1 mg/kg per day) (purity $\geq 99\%$, Nagara Science) orally and served as a positive control [25]. Both CS extract and GluCer were dispersed in 1% (w/v) sodium carboxymethyl cellulose (CMC-Na) (Wako). The remaining two groups

were administered 1% CMC-Na alone. Topical application of SDS on the dorsal skin was performed using a previously described method [25]. Briefly, after 3 weeks of oral administration, medical absorbent cotton (\sim ca. 3×4 cm, Kawamoto Corporation) containing 3 mL of 10% (w/v) SDS (Wako) was placed in contact with the dorsal mouse skin for 10 min under isoflurane anesthesia (Wako). SDS exposure was performed once daily for 5 consecutive days. CS and GluCer were continuously administered 1 h before SDS exposure. As a control for intact skin, one group administered 1% CMC-Na alone was not exposed to SDS.

Measurement of skin parameters

Before SDS exposure and on day 5, the skin parameters epidermal water content and erythema index were measured using Corneometer[®] CM825 and Mexameter[®] MX18 (Courage + Khazaka Electronic), respectively. All measurements were performed at three sites (upper, middle, and lower) along the central line of each SDS-treated skin area under isoflurane anesthesia and were repeated five times on the same position. Changes in each parameter were calculated using the following formula: index change (ΔE) = (value after SDS exposure) – (value before SDS exposure).

Evaluation of the severity of skin scaling

The dorsal mouse skin was photographed on day 5 using a digital camera. To evaluate the severity of SDS-induced skin scaling using the digital images, a macroscopic scoring system ranging from 0 to 4 was developed and evaluated blindly by five investigators as follows: score 0, none (absence of scaling on the SDS-treated skin area); score 1, slight (appearance of scaling on <25% of the area); score 2, moderate (appearance of scaling on 25–50% of the area); score 3, severe (appearance of scaling on 50–75% of the area); score 4, very severe (appearance of scaling on >75% of the area).

Histological and Immunohistochemical analysis

For histologic analysis, the dorsal skin was fixed in Bouin's fluid (Wako), embedded in paraffin, sectioned (5 µm thickness), and stained with H&E. For immunohistochemical analysis, tissues sections (5 µm thick) were stained with anti-Iba-1 or anti-Ly-6G/Ly-6C antibody (Abcam) using the procedure described in our previous study [48]. Images of the stained skin sections were captured using a Zeiss Axio Observer Z1 microscope. Epidermal thickness was calculated by dividing the area of the epidermis by the length of the basal layer, and the densities of Iba-1- or Ly-6G/Ly-6C-positive cells were calculated by dividing the number of cells counted by the volume of the counted area in five randomly sites of each section using the ZEN2.3 software (Zeiss).

ELISA measurements and Western blotting analysis

The levels of IL-1α (R&D Systems) and PGE2 (ENZO) in the skin were quantified using ELISA kits according to the manufacturer's protocol. The expression levels of COX-2 protein in the skin were measured by western blotting according to the procedure described previously [48]. Immunoreactive bands were visualized using an Amersham Imager 680, and band intensities were quantified using ImageQuant TL8.2 (GE Healthcare).

Statistical analysis

All statistical analyses were performed using EZR (Version 3.5.2) [49]. Statistical comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's or Kruskal-Wallis test followed by Steel's post hoc tests. Differences were considered significant at $p < 0.05$.

Supporting Information

The LC MS/MS conditions and the parameters for each analyte, the procedures for HPLC analysis of saccharides and GC-MS analysis of fatty acids in the CS extract, and the time-dependent changes in epidermal water content and erythema index in both SDS-untreated and treated mouse dorsal skin, are available in the Supporting Information.

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Conflict of Interest

The authors declare that they have conflict of interest.

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