Regular Article

Anti-inflammatory Effects of Novel Polysaccharide Sacran Extracted from Cyanobacterium *Aphanothece sacrum* in Various Inflammatory Animal Models

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Received February 29, 2016; accepted April 26, 2016; advance publication released online May 10, 2016

The goal of this study was to investigate the topical anti-inflammatory effects of the megamolecular polysaccharide sacran extracted from cyanobacterium *Aphanothece sacrum* using various inflammatory animal models. Sacran showed potent anti-inflammatory effects with optimum effective concentrations at 0.01 and 0.05% (w/v). Sacran markedly inhibited paw swelling and neutrophil infiltration in carrageenan-induced rat paw edema. Additionally, 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionyl-carboxylic acid (DMEQ)-labeled sacran had the ability to penetrate carrageenan-induced rat paw skin rather than normal skin. Also, sacran significantly suppressed kaolin-induced and dextran-induced rat paw edema throughout the duration of the study. Furthermore, sacran significantly suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema and mRNA expression levels of cyclooxygenase (COX)-2 as well as pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6. Safety of sacran solution was verified by negligible cytotoxicity in HaCaT cells. These results suggest that sacran may be useful as a therapeutic agent against inflammatory skin diseases with no life-threatening adverse effects.

Key words sacran; polysaccharide; anti-inflammatory effect; phlogistic agent

Inflammatory responses are complex events of the body as it delivers the appropriate defense against harmful stimuli. Inflammation is partially regulated by carbohydrates, especially sulfated glycosaminoglycans, expressed on the surface of endothelial cells and leukocyte cells. Exogenous sulfated glycans, such as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, fucosylated chondroitin sulfate and fucoidans can induce anti-inflammatory effects. The anti-inflammatory effects of these sulfated glycans are the competitive inhibition of the molecular interactions between sulfated glycans and P-selectin or L-selectin, while other mechanisms of action, such as down-regulation of chemokine and transcription factor activities can also occur.

Skin operates not only as a protective physical barrier, but also as a dynamic organ that has other recognized functions, such as endogenous homeostasis, metabolism, and sensory input. Moreover, skin actively contributes in immunological regulatory processes and inflammatory responses.¹⁾ There is a wide range of dermatological conditions that include inflammatory skin disorders ranging in severity from mild skin rash to severe dermatitis. Nowadays, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly administered to reduce the inflammation. However, they can frequently cause a number of serious adverse effects.^{2,3)} Hence, great efforts have been devoted towards the discovery of new and safe anti-inflammatory natural products of plant origin as alternatives.^{4,5)} Importantly, numerous beneficial effects of plant polysaccharides have been demonstrated on human health to exhibit a spectrum of biological activities

such as anti-inflammatory effects.^{6,7)} Meanwhile, plant polysaccharides may have the potential to induce the allergic response. Therefore, plant polysaccharides with high safety are necessary.

Recently, a novel sulfated polysaccharide sacran attracts a considerable amount of attention. It is extracted from the Japanese indigenous cyanobacterium *Aphanothece sacrum*, which possesses a jelly-like extracellular matrix (ECM) with high water content (97.5–98.3%).^{8–12)} Sacran is a heteropolysaccharide composed of various sugar residues such as glucose, galactose, mannose, xylose, rhamnose, fucose, galacturonic acid and glucuronic acid, and contains traces of alanine, galactosamine and muramic acid; 11% of monosaccharides contain a sulfate group and 22% of them contain a carboxyl group. In addition, sacran was reported to be a supergiant molecule with extremely high molecular weight ranging over 10⁷ g/mol and micrometer-scaled (more than 8 μ m). Previously, Ngatu *et al.* revealed that sacran has anti-inflammatory effects on 2,4,6-trinitrochlorobenzene-induced allergic dermatitis model mice.¹³⁾ So far, however, other inflammatory models have not yet been examined. Therefore, in the present study, we focused on the investigation of the anti-inflammatory effects of sacran on various experimental animal models of inflammation.

MATERIALS AND METHODS

Materials Sacran was extracted from *Aphanothece sacrum* by Green Science Material (Kumamoto, Japan), as

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reported previously.^{8,11)} λ -Carrageenan and 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionyl-carboxylic acid (DMEQ)-hydrazide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Kaolin, dextran, 4-biphenyl acetic acid (BPAA) and prednisolone (PD) were obtained from Nacalai Tesque (Kyoto, Japan). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma-Aldrich (Tokyo, Japan). HaCaT cells, a human epidermal keratinocyte line, were purchased from Deutsches Krebsforschungszentrum (DKFZ; German Cancer Research Center, Cell Lines Service, Eppelheim, Germany). All other chemicals and solvents were of analytical reagent grade.

Animals Male Wistar rats (8 weeks old) and female BALB/c mice (5 weeks old) were purchased from SLC (Shizuoka, Japan). They were maintained under controlled conditions (22°C, 55% humidity and 12 h day/night rhythm) and fed a standard laboratory chow. Animal studies were approved by the Ethics Committee for Animal Care and Use of Kumamoto University.

In Vivo Anti-inflammatory Activity

Carrageenan-Induced Rat Paw Edema

The rats received a subplantar injection of λ -carrageenan (100 μ L, 1% (w/v) in normal saline) in the right hind paw. One hour later, sacran solutions with various concentrations (0.01, 0.05, 0.1% (w/v)) were topically administered every 1 h after carrageenan injection. The rats in the negative control group were treated with normal saline solution.¹⁴⁾ The animals of positive control group were treated with BPAA as a reference anti-inflammatory drug. The paw volume of each animal was determined before carrageenan injection, and then hourly intervals until 6 h after induction of inflammation, with a plethysmometer. The increase in the paw volume was calculated by subtracting the initial paw volume to the paw volume measured at each time interval.

Kaolin-Induced Rat Paw Edema

The animals were treated in a similar manner to that of carrageenan-induced rat paw edema model; kaolin (100 μ L, 20% (w/v) in normal saline) was used instead of λ -carrageenan.

Dextran-Induced Rat Paw Edema

The treatment of animals and measurements of the paw volume were performed as described above. Dextran (100 μ L, 1% (w/v) in normal saline) was used as an inducer of rat paw edema.

TPA-Induced Mouse Ear Edema

Ear edema was induced in mice by topical application of 3 μ L of TPA (1 mg/mL) and then 40 μ L of sacran solutions (0.01, 0.05, 0.1, 1.0% (w/v)) were administered to the opposite site of TPA-treated ear. Edema was expressed as the difference between the basal ear thickness and the ear thickness after 6 h of TPA application using dial thickness gauge (Teclon Corporation, Nagano, Japan).

Hematoxylin–Eosin (H&E) Staining of Carrageenan-Induced Acute Edema in Rat Paws For histological analysis, a paw tissue was resected 3 h after subplantar injection of λ -carrageenan solution (100 μ L, 1% (w/v) in normal saline). The samples were fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, sectioned at thickness of 8 μ m and stained with H&E for light microscopy analysis.

Skin Permeability of DMEQ-Labeled Sacran in Carrageenan-Induced Rat Paw Edema Model DMEQ-labeled sacran solution (0.05% (w/v)) was topically administered

every 1 h after subplantar injection of λ -carrageenan to a rat paw. Three hours later, resected segments were observed by a fluorescent microscope (KEYENCE Biozero BZ-8000, Osaka, Japan).

Real-Time PCR Total RNA was isolated from ear biopsy samples, in the TPA-induced mouse ear edema model, using TRIzol[®] Reagent according to the manufacturer's protocol. The RNA (0.5 μ g) was subsequently reverse-transcribed using ReverTra Ace[®] qPCR RT Master Mix (TOYOBO, Osaka, Japan). The expression levels of interleukin (IL)-6, tumor necrosis factor (TNF)- α , cyclooxygenase (COX)-2 and IL-1 β in the ear of the tested animals were determined using a real time PCR assay. Real-time PCR was performed on a CFX96[™] Real-Time PCR (Bio-Rad, Tokyo, Japan) using 1 μ L of cDNA for each sample. THUNDERBIRD[™] SYBR[®] qPCR Mix (TOYOBO) was used to detect products, and 10 μ M concentrations of the following primers were used: mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5'-GGT GAA GGT CGG TGT GAA CGG ATT-3', mouse GAPDH reverse: 5'-AAT GCC AAA GTT GTC ATG GAT GACC-3', mouse IL-6 forward: 5'-TGG AGT CAC AGA AGG AGT GGCT AAG-3', mouse IL-6 reverse: 5'-TCT GACCAC AGT GAG GAA TGT CCA C-3', mouse COX-2 forward: 5'-AGC ACT GCA TCC TGCC CAG CTC-3', mouse COX-2 reverse: 5'-AGA GGA CTG TGG CTC TGA CC-3', mouse TNF- α forward: 5'-AAC ATCCA ACCT TCCCAA ACG-3', mouse TNF- α reverse: 5'-CTCTTA ACCCCC GAA CTCCCA G-3', mouse IL-1 β forward: 5'-AAA CCT CTT CGAG GCA CA AG-3', mouse IL-1 β reverse: 5'-GTT TAG GGCC ATC AG CTT CA-3'. The relative amount of cDNA in each sample was normalized using GAPDH, and the melting curve was used to verify specificity. PCR was set at 95°C initially for 30 s, followed by 40 cycles of 95°C×15 s, 55°C×15 s, 72°C×45 s. The relative amount of cDNA in each sample was normalized using GAPDH, and the melting curve was used to verify specificity.

Cytotoxicity of Sacran Solutions in HaCaT Human Keratinocyte Cell Line HaCaT cells were seeded at 5×10^4 cells onto 96-well microplate (Iwaki, Tokyo, Japan), and incubated for 24 h. Cells were washed once with phosphate-buffered saline (PBS, pH 7.4), and then incubated for 48 h with 200 μ L of Dulbecco's modified Eagle's medium (DMEM) containing sacran (0.001, 0.01, 0.1 or 0.25% (w/v)) or Tween 20 at 37°C. After washing with PBS, 100 μ L of fresh Hanks' balanced salt solution (HBSS, pH 7.4) and 10 μ L of WST-1 reagent were added. After incubation for 30 min, the absorbance at 450 nm against a reference wavelength of 655 nm was measured with a microplate reader (Bio-Rad Model 550).

Statistical Analysis Data are presented as the mean $\pm$ standard error (S.E.). Significant differences between experimental values were determined using Scheffe's test, with $p < 0.05$ considered significance.

RESULTS

Effects of Sacran on Carrageenan-Induced Acute Edema in Rat Paws In order to examine the anti-inflammatory activity of sacran in acute-phase inflammation *in vivo*, a carrageenan-induced paw edema experiment was conducted. The results revealed that sacran solutions showed a remarkable inhibitory effect in carrageenan-induced paw edema after carra-

geenan injection (Fig. 1). The time dependent curve illustrated a continuous rise in paw swelling (%) till 6 h after carrageenan injection. On the other hand, sacran solutions exhibited the bell shaped effect of down-regulating carrageenan-induced paw swelling, compared with the negative control group which was treated with normal saline only. Additionally, 0.05% (w/v) sacran solution was the most effective concentration which was superior to BPAA, as a reference NSAID in edema inhibition.

Effects of Sacran on Histological Changes Associated with Carrageenan-Induced Rat Paw Edema Figure 2 shows the representative photos from the histological changes in the paw tissues following the subplantar injection of carrageenan and the anti-inflammatory effects of sacran solution (0.05% (w/v)). The carrageenan inflammatory response was found to be associated with a notable decrease in the epidermal layer thickness along with invasive infiltration of neutrophils in the dermis. In contrast, the treatment of animals with 0.05% (w/v) sacran solution exhibited a conspicuous preservation of epidermis thickness as well as a decrease in neutrophils infiltration.

Skin Permeability of DMEQ-Labeled Sacran in Car-

Fig. 1. Effects of Sacran on Carrageenan-Induced Acute Edema in Rat Paws

Various sacran solutions were administered every 1 h after subcutaneous injection of λ -carrageenan solution to rat paws. Each point represents the mean $\pm$ S.E. of 5–20 experiments. * $p < 0.05$, compared with saline. † $p < 0.05$, compared with dextran.

Fig. 2. Hematoxylin & Eosin Staining of Carrageenan-Induced Acute Edema in Rat Paws

Sacran solution (0.05% (w/v)) was administered every 1 h after subcutaneous injection of λ -carrageenan solution to a rat paw. A paw tissue was resected 3 h after subcutaneous injection of λ -carrageenan solution. The experiments were performed independently three times, and the representative images are shown.

Carrageenan-Induced Rat Paw Edema Model To reveal the permeation of sacran into the skin, carrageenan-induced rat paw skin was cross sectioned after 3 h treatment with DMEQ-labeled sacran. As illustrated in Fig. 3, the fluorescence of labeled sacran was observed in the epidermis and dermis layers, indicating the penetration ability of sacran in carrageenan-induced rat paw edema. Meanwhile, in the case of normal rat skin, the fluorescence was noticed only in the stratum corneum layer, suggesting negligible penetration of sacran.

Effects of Sacran on Kaolin-Induced Rat Paw Edema Kaolin is one of the few phlogistic agents which mediate kinins and it does so mainly by prostaglandins.¹⁵⁾ The results of the kaolin-induced rat paw edema are shown in Fig. 4. In this experimental model, the inflammatory edema start to induce after 1 h and peaked at 5 h after kaolin injection. Topical application of sacran solutions at concentrations of 0.01 and 0.05% (w/v) significantly suppressed the edema formation, compared with that of 0.1% (w/v).

Effects of Sacran on Dextran-Induced Rat Paw Edema Dextran is a well-known phlogistic agent which induces paw edema as a result of liberation of histamine and serotonin from mast cells.¹⁶⁾ The anti-inflammatory effect of sacran against dextran-induced rat paw edema is illustrated in Fig. 5. The results demonstrated that the administration of sacran solutions significantly inhibited dextran-induced paw edema throughout the duration of the study in a concentration-dependent manner with the optimum effective concentration at 0.05% (w/v).

Effects of Sacran on TPA-Induced Mouse Ear Edema Figure 6 demonstrates the effects of the topical administration of sacran solutions on TPA-induced mouse ear edema. The data indicated that application of TPA promoted an increase in ear thickness. Contrarily, a sacran solution (0.05% (w/v)) showed the maximum inhibitory effect on ear swelling in the TPA-induced ear edema model.

Effects of Sacran on mRNA Expression Levels of COX-2 and Inflammatory Cytokines in TPA-Induced Mouse Ear Edema Topical application of TPA, a specific activator of protein kinase C (PKC), represents a suitable skin inflammation model for assessing the topical anti-inflammatory agents. Next, we investigated the effect of 0.05% (w/v) sacran solution on the mRNA expression levels of COX-2 and various cytokines such as TNF- α , IL-6, and IL-1 β after 6 h in TPA-induced mice model. These results indicated that 0.05% (w/v) sacran solution significantly suppressed those mRNA expressions, compared with TPA alone (Fig. 7).

Fig. 3. Photographs of Tissue Slices of Rat Paw after Treatment of DMEQ-Labeled Sacran

Labeled sacran solution (0.05% (w/v)) was administered every 1 h after subcutaneous injection of λ -carrageenan solution to a rat paw. After 3 h, resected segments were observed with a fluorescent microscope.

Fig. 4. Effects of Sacran on Kaolin-Induced Acute Edema in Rat Paws

The sacran solution (0.01, 0.05, 0.1% (w/v)) was administered every 1 h after subcutaneous injection of kaolin solution to rat paws. Each point represents the mean $\pm$ S.E. of 5–20 experiments. * p <0.05, compared with saline. † p <0.05, compared with sacran (0.1% (w/v)).

Fig. 5. Effects of Sacran on Dextran-Induced Acute Edema in Rat Paws

The sacran solution (0.01, 0.05, 0.1, 0.2% (w/v)) was administered every 1 h after subcutaneous injection of dextran solution to rat paws. Each point represents the mean $\pm$ S.E. of 5–6 experiments. * p <0.05, compared with saline. ‡ p <0.05, compared with sacran (0.1% (w/v)). † p <0.05, compared with sacran (0.2% (w/v)).

Fig. 6. Effects of Sacran on TPA-Induced Skin Inflammation in Mice

Three microliters of TPA (1 mg/mL) was treated on mice ear, and then 40 μ L of sacran solutions (0.01, 0.05, 0.1, 1.0% (w/v)) were applied to the ear. Ear swelling was measured by using a thickness gauge. Each point represents the mean $\pm$ S.E. of 5–17 experiments. * p <0.05, compared with Non-treated. † p <0.05, compared with 0.01% (w/v) sacran.

Cytotoxicity of Sacran Solutions in HaCaT Human Keratinocyte Cell Line Keratinocyte is considered to be one of the major cell types involved in skin inflammatory responses.¹⁷⁾ To reveal the safety of sacran solutions, we examined the cytotoxicity of sacran solutions in HaCaT cells after incubation for 48 h by the WST-1 method (Fig. 8). As a result, sacran solutions showed negligible cytotoxicity up to a concentration of 0.25% (w/v).

DISCUSSION

This study highlights the topical anti-inflammatory effects of sacran polysaccharide on various experimental inflammatory models induced by different phlogistic agents as useful pharmacological tools for the investigation of new anti-inflammatory substances, particularly of plant origin, to treat inflammatory skin disorders.

Firstly, carrageenan-induced rat paw edema was used as a model of acute inflammation for assessing the anti-inflammatory effects of sacran. Subplantar injection of carrageenan

Fig. 7. Effects of Sacran on mRNA Levels on TPA-Induced Skin Inflammation in Mice

Three microliters of TPA (1 mg/mL) was treated on mice ear, and then 40 μ L of 0.05% (w/v) sacran solution was applied to the ear. After 6h, the ears were collected. Total RNA was extracted by TRIzol[®] reagent, and then the mRNA levels were determined by real-time PCR. Each value represents the mean $\pm$ S.E. of 3–4 experiments. * p < 0.05, compared with TPA alone.

Fig. 8. Cytotoxicity of Sacran Solutions in HaCaT Cells

Cells were incubated with sacran solutions for 48h. Cell viability was assayed by the WST-1 method. The concentrations of sacran were 0.001, 0.01, 0.1 and 0.25% (w/v). Each value represents the mean $\pm$ S.E. of 5–6 experiments.

produces a biphasic response. The initial phase results from release of histamine, serotonin, and kinins for the first 2h; meanwhile, the subsequent phase is attributed to the release of prostaglandins as well as neutrophil infiltration.¹⁸⁾ In the present study, topical application of 0.05% (w/v) sacran solution significantly reduced two critical events related to skin inflammatory response induced by carrageenan; both paw edema and neutrophils infiltration in rat paw tissues, as compared with the controls (Figs. 1, 2). This effect was similar to that of BPAA as a reference drug at the same concentration. Non-steroidal anti-inflammatory drugs such as BPAA, the active metabolite of fenbufen, act as potent inhibitors of prostaglandins synthesis which are important mediators of acute inflammation.^{19–22)} Based on these findings, the ability of sacran to reduce edema formation in carrageenan-induced rats might be attributed to its inhibitory effect on prostaglandin synthesis. A similar proposal was reported for the anti-inflammatory activity of fucan, a sulphated polysaccharide, against carrageenan-induced paw edema in rats.²³⁾ Therefore, the prostaglandins levels in the skin should be determined hereafter.

It is worth noting that the fluorescence of labeled sacran was observed in the epidermis and dermis layers in carrageenan-induced rat paw edema. Meanwhile, the fluorescence was noticed only in the stratum corneum layer in normal rat skin (Fig. 3). These results suggest that sacran can penetrate into the epidermis of skin in edema.

A second model of acute inflammation used in our study was induced by kaolin. Sacran solutions at concentrations of 0.01 and 0.05% (w/v) significantly elucidated a prominent inhibition of the kaolin-induced rat paw edema throughout the duration of the study (Fig. 4). It has been reported that the inflammatory response to kaolin induction was mediated by kinins and mainly by prostaglandins.¹⁵⁾ Therefore, our data further suggest the anti-inflammatory activity of sacran which could be at least partially through inhibition of prostaglandins biosynthesis.

Dextran is a well-known phlogistic agent which induces paw edema as a result of liberation of histamine and serotonin from mast cells.¹⁶⁾ Sacran solution (0.05% (w/v)) efficiently suppressed the dextran-induced paw edema (Fig. 5). Previously, we demonstrated that sacran solution suppressed the β -hexosaminidase release in RBL-2H3 cells, which are now known to be an analog of rat mucosal mast cells, indicating the inhibition of mast cell degranulation.²⁴⁾ These results suggest that the anti-inflammatory effects of sacran may contribute to inhibition of not only prostaglandins but also other inflammatory mediators.

The inflammatory features of TPA-induced mouse ear edema are characterized by edema, epidermal hyperplasia and infiltration of inflammatory cells as well as liberation of inflammatory mediators as COX-2, IL-6, TNF- α , and IL-1 β in mouse skins.^{25–29)} Our data demonstrated that topical application of 0.05% (w/v) sacran solution significantly suppressed TPA-induced ear edema and mRNA expression levels of COX-2 as well as pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Figs. 6, 7). These results suggest that the anti-inflammatory effects of sacran solution may be a consequence of down-regulation of TPA-induced expression of COX-2, IL-1 β , IL-6, and TNF- α . The detailed mechanism remains to be further elucidated.

Sacran is a unique cyanobacteria-derived glycosamino-

glycanoid. The anti-inflammatory effect displayed by sacran could be related to the similarity of its chemical structure to that of glycosaminoglycans, sulfated polysaccharides, which possess numerous bioactivities including an anti-inflammatory effect.^{30–34} The high sulfate and carboxyl group content of glycosaminoglycans permits them to interact with a wide range of proteins, enzymes, cytokines, chemokines, lipoproteins, and adhesion molecules.^{35–38} Also, Ngatu *et al.* reported that epicutaneous application of sacran to 2,4,6-trinitrochlorobenzene-induced NC/Nga mice significantly inhibited the development of allergic dermatitis skin lesions, and decreased the number of scratching behavior episodes by improving the stability, elasticity, and hydration of skin barrier as well as inhibiting the production of T-helper 2 (Th2) cytokines (IL-4, IL-5), Th1 cytokines (TNF- α , interferon- γ (IFN- γ)) and inflammatory chemokines such as monocyte chemoattractant protein-1 (MCP-1) and eotaxin; thus, inhibiting immunoglobulin E (IgE) and eosinophilic infiltration in mice.¹³ These unique properties exhibited by sacran are not common in most sulfated polysaccharides.

In the view of the data mentioned above, the anti-inflammatory activity of sacran solutions in this study was in a concentration-dependent manner, with the optimum effective concentrations at 0.01 and 0.05% (w/v). This effect is related to chain properties such as electric charges or the chain conformation. Recently, Mitsumata *et al.* demonstrated that, in diluted regime less than 0.09% (w/w), the single chain of sacran occupies a spherical free volume with a diameter of the fluctuation length. Meanwhile, at the concentrated regime the sacran chain loses its electric charges and forms double helixes at 0.1% (w/w) due to reduction in the electric repulsive force on the saccharide chain. In addition, it forms a weak gel at 0.2% (w/w). A macroscopic domain of liquid crystals observed at concentrations over 0.2% (w/w) is probably due to the hydrophobic cross-linking points between double helixes, indicating their extremely persistent length.³⁹ As a consequence, from a biological point of view, the gelation mechanism of sacran chain is so critical that it may affect its molecular structure, size and length and subsequently its cutaneous penetration through the inflamed tissue.

CONCLUSION

Currently, scientific research is focused on the discovery of new natural products with anti-inflammatory activity. In the present study, sacran solution provoked potent anti-inflammatory effects when applied topically to different acute and chronic inflammatory animal models. The probable anti-inflammatory mechanism of sacran involves numerous targets, resulting in suppression of essential inflammatory mediators in the cutaneous tissue. Nevertheless, further elaborate studies are required to determine the precise mechanism of inflammatory action of sacran and ascertain its clinical use.

Conflict of Interest The authors have no conflict of interest directly relevant to the content of this article. S. Kaneko is CEO of Green Science Material Inc.

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