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Research Paper

Alternative therapeutic advantages of catfish bile on atopic dermatitis: protection of T cell-mediated skin disease via antioxidant activities

Do Ik Lee[a], Yoon Joo Huh[a], Kwang Woo Hwang[a], YoungWook Choi[a], Jae-Seok Choi[b], Sam-Yong Han[c], Young-Soo Gyoung[c] and Seong Soo Joo[d]

[a]College of Pharmacy, Chung-Ang University, Seoul, [b]Institute of Environmental Research, Kangwon National University, [c]Department of Chemistry, College of Natural Science and [d]Department of Marine Molecular Biotechnology, College of Life Science, Gangneung-Wonju National University, Gangwon, Republic of Korea

Abstract

Objectives In the present study, we aimed to examine the anti-atopic properties of bile from the cat fish, *Silurus asotus*, to determine its possible use as a pharmaceutical product.
Methods The anti-atopic activities of cat fish bile were examined in a non-cell antioxidant, in-vitro assay (splenocytes and mast cells) and a 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis-like mouse model.
Results The results of these experiments revealed that *Silurus asotus* bile (SAB) scavenges radicals and protects proteins from superoxide attacks, suggesting that SAB suppresses the T helper (Th) type 2-skewed immune response. Th1/Th2 mRNA cytokines (interleukin (IL)-2, interferon (IFN)-γ and IL-4) from mouse splenocytes were effectively inhibited, and the release of β-hexosaminidase in RBL-2H3 mast cells was significantly suppressed by SAB. These results were supported by screening the Th1/Th2 cytokine mRNAs (IL-2, IFN-γ and IL-4) from lymph nodes in DNCB-treated mice. More dramatic results were observed in the histological changes at higher SAB concentrations (5%) compared to the therapeutic control, visualized using hematoxylin–eosin (H&E) staining.
Conclusions The results presented in this study suggest that SAB may provide functional advantages with regard to treating atopic dermatitis because of its antioxidant and immune-suppressive effects.
Keywords 2,4-dinitrochlorobenzene; antioxidant; atopic dermatitis; immunosuppressant; *Silurus asotus*; T helper 1/2 cytokines

Introduction

An allergic reaction is a complex immune response to substances present in the body. Although a healthy immune system is crucial for fighting infections and viruses, an allergic reaction is actually the result of a hypersensitive response of our immune system to harmless substances. In addition, the skin is one of the largest immunologic organs in the human body and is often the target of allergic and immunologic responses. Among allergic skin disorders, including urticaria, angioedema, contact dermatitis and atopic dermatitis (AD), the last one is the only condition that involves complex interactions between skin barrier dysfunction and environmental factors such as allergens and microbes.[1] All types of skin cell, including melanocytes and keratinocytes, produce reactive oxygen species (ROS) and reactive nitrogen species (RNS).[2,3] In addition, ROS from skin cells can also produce reactive lipid species, such as lipid radicals, peroxides, hydroperoxide or aldehydes by reacting with lipid molecules or redox-sensitive lipid-metabolizing enzymes, such as lipoxygenases and cyclooxygenases. AD, which is a chronic inflammatory disease, can disrupt the redox balance, resulting in the overproduction of ROS and RNS, and high levels of lipid peroxidation products. This exacerbates the disease state and shifts the response toward a Th2-skewed immune response.[4,5]

A previous study demonstrated that antioxidants (i.e. thiol N-acetyl-L-cysteine or glutathione) downregulated Th2 cytokines, IL-4 and IL-5[6] in human T cells. Supplementation

Correspondence: Seong Soo Joo, Department of Marine Molecular Biotechnology, College of Life Science, Gangneung-Wonju National University, Gangwon 210-702, Republic of Korea.
E-mail: larryjoo@hanmail.net

with antioxidants may therefore be a good approach to the treatment of AD and other skin disorders. Previously, we demonstrated that the bile from catfish, *Silurus asotus*, which contains high amounts of taurocholic acid (TCA) and ursodeoxycholic acid (UDCA) compared to other representative zoophagous fish in domestic fresh-water habitats, has immunosuppressive effects similar to the calcineurin inhibitor cyclosporine A (CsA).[7] In other studies, we have also reported that UDCA inhibits the expression of pro-inflammatory compounds (IL-1β, tumour necrosis factor α and nitric oxide) and signal transduction of nuclear factor kappa B in microglial cells.[8,9] These preliminary findings strongly imply that catfish bile containing UDCA and TCA may have antioxidant and T cell-based immunosuppressive effects, which could be utilized for the treatment of AD. Thus, we hypothesized that treatment with catfish bile will inactivate immune cells (i.e. T lymphocytes, mast cells) that are highly associated with the pathogenesis of AD.

To verify this hypothesis and to demonstrate the potential efficacy of catfish bile as an AD therapeutic, the following three effects of catfish bile were examined:[1] the effect of catfish bile on the inactivation of Th cells in secondary lymphoid organs (i.e. spleen and lymph node),[2] the antioxidant properties of catfish bile (examined in 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and hydroxyl radical-mediated oxidation and radical scavenging assays)[3] and the anti-atopic effect of catfish bile in an animal model. Thus, in the present study, we determined whether the bile from catfish has therapeutic potential for the treatment of AD.

Materials and Methods

Sample preparation

Fresh fish bile was isolated from a fully grown domestic *Silurus asotus* (Gangwon-do, Korea) and lyophilized under aseptic conditions. *Silurus asotus* bile (SAB) was weighed and then diluted with distilled water at a stock concentration of 0.1 g/ml and stored at −70°C until further use.

Chemicals and experimental animals

L-ascorbic acid, hydrogen peroxide (H_2O_2), DPPH and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). BALB/c mice (7 weeks old) were purchased from Samtaco (Osan, Kyunggi-do, Republic of Korea), and were allowed to adapt to laboratory conditions (temperature: 20 ± 2°C, relative humidity: 50%, light/dark cycle: 12 h) over a week. The animal experiments were approved by the Chungbuk National University Animal Care and Use Committee and all procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health.

Quantitative analysis of UDCA and TCA in SAB

Stock solutions were prepared by dissolving TCA and UDCA in water or methanol (corresponding to 20 mg/ml). The standard solutions were prepared at concentrations ranging from 2 to 0.5 mg/ml by diluting the stock solutions with each solvent. Samples were prepared by dissolving SAB in water (corresponding to 1 mg/ml). Standard solutions were injected into an HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a XDB-C18 column (4.6 mm i.d. × 150 mm) and mass detector (Agilent LC/Ms VL, Agilent Technologies). The column was maintained at 40°C, and a flow rate of 0.8 ml/min was used. The conditions used for HPLC purification were as follows: 0–5 min, isocratic of acetonitrile/water (5 : 95); 5–30 min, gradient from acetonitrile/water (5 : 95) to acetonitrile/water (50 : 50); 30–35 min, isocratic (acetonitrile/water 5 : 95). HPLC detection was performed in the single ion monitoring mode (negative charge, MW: 514).

Induction of AD-like skin lesions in 2,4-dinitrochlorobenzene-treated mice

DNCB (sigma) dissolved in acetone was used to induce dermatitis in BALB/c mice. Briefly, hair was removed from a 2 × 4 cm region spanning the neck to the pelvis and the dorsal skin was sensitized with 100 µl of 1% DNCB daily for 7 days. After the first challenge, 100 µl of 0.5% DNCB was repeatedly applied to the dorsal skin for an additional 5 weeks at 2-day intervals. At the same time, the entire dorsal skin of the mice was treated with the vehicle control (acetone : olive oil = 4 : 1 mixture) (untreated control), or SAB (1 or 5% cream) daily for 4 weeks. No substances were applied to the skin surface on the last day of the experiment. The mice were then euthanized, and the skin and lymph nodes were collected for further analysis.

Measurement of DPPH radical-scavenging activity

The DPPH radical, which has a deep-purple colour, is one of the few stable organic nitrogen radicals. Thus, SAB was reacted with the DPPH solution to evaluate its free radical-scavenging activity. Briefly, lyophilized SAB was dissolved in distilled water at a stock concentration of 100 mg/ml and various different concentrations of SAB (10–100 µg/ml) were reacted with 0.3 mM DPPH, which was dissolved in methanol, for 20 min at room temperature. The absorbance was then measured at 517 nm, and the DPPH free radical-scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity } (\%) = [\text{Ac}-(\text{A}-(\text{As})]/\text{Ac} \times 100 \quad (1)$$

where Ac is the absorbance of the control DPPH solution, A is the absorbance of the sample with the DPPH solution, and As is the absorbance of the sample.

Hydroxyl radical-mediated oxidation assay

Hydroxyl radical-mediated oxidation experiments were performed using a metal-catalysed reaction as described previously,[10] with some modifications. The target protein BSA was dissolved in a 150 mM phosphate buffer (pH 7.3) at a final concentration of 0.5 mg/ml. The BSA solution was incubated with and without 100 µmol copper (Cu^{2+}) and 2.5 mM H_2O_2 in the presence and absence of SAB. The control antioxidant consisted of 50 µmol of ascorbate, which was directly dissolved in phosphate buffered saline (PBS). The reactions

were carried out in open tubes and placed in a shaking water bath that was maintained at 37°C. After the reaction was completed, each mixture was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with a 0.1% Coomassie blue solution.

Cytotoxicity (lactate dehydrogenase) assay

The cytotoxicity induced by SAB was quantified by measuring lactate dehydrogenase (LDH) release in mouse splenocytes and RBL-2H3 mast cells. The LDH content was determined using a commercial non-radioactive LDH assay kit (Promega, Madison, WI, USA), which is based on a coupled enzymatic reaction that converts a tetrazolium salt into a red formazan product. The increase in the amount of formazan produced in the culture supernatant directly correlates with the increase in the number of lysed cells. The formazan was quantified spectrophotometrically by measuring its absorbance at 490 nm (Molecular Devices, Sunnyvale, CA, USA). Cytotoxicity in experimental samples was determined as the percentage of LDH released compared with cells treated with 1% Triton X-100.

RBL-2H3 cell culture and preparation of lymphocytes from spleen and lymph nodes

RBL-2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's Modified Eagle's Medium containing heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cultures were maintained in 5% CO_2 at 37°C. Cytotoxicity was confirmed using Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan). Immediately after the mice were euthanized, the spleens from the BALB/c mice were homogenized between frosted glass slides and washed in complete medium consisting of RPMI 1640 (Hyclone, UT, USA), 10% FBS (Gibco, Gaithersburg, MD, USA) and 1% penicillin/streptomycin (Cellgro, Herndon, VA USA). Mouse spleens were aseptically isolated from the BALB/c mice immediately after they were sacrificed and single primary splenocytes were prepared by mechanical dissociation in cold PBS at pH 7.2. Erythrocytes were depleted using a red blood cell lysis buffer (eBioscience, San Diego, CA, USA) containing ammonium chloride, which lyses red blood cells while only minimally affecting lymphocytes. Mouse lymph nodes were isolated from DNCB-treated or 5% DNCB/SAB-treated mice, lysed with a tissue lyser (Qiagen, Valencia, CA, USA) in trizol (Invitrogen), and used for mRNA quantification.

β-hexosaminidase secretion assay

Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase. RBL-2H3 cells were grown on 48-well plates (2.5×10^5 cells/well) and experiments were carried out for 3 h after plating. Cells were then treated overnight with 1 μg/ml IgE (sigma). Cells were washed four times with extracellular buffer (125 mM NaCl, 5 mmol KCL, 1.5 mmol $CaCl_2$, 1.5 mmol $MgCl_2$, 20 mmol HEPES, pH 7.3) to remove excess IgE before stimulation. The cells were then stimulated with 400 ng/ml dinitrophenylated human serum albumin, DNP-HSA (sigma), which was suspended in 500 μl extracellular buffer with 0.1% BSA and incubated at 37°C for 1 h. Following incubation, 50 μl of the supernatant was incubated with 200 μl of 1 mM *p*-nitrophenyl N-acetyl-β-D-glucosamine (sigma) in 0.05 M citrate buffer (pH 4.5) for 3 h at 37°C. The enzyme reaction was terminated through the addition of 500 μl of 0.05 M sodium carbonate buffer (pH 10.0) and the optical density of each reaction was read at 405 nm (SupraMax340, Molecular Devices, Sunnyvale, CA, USA). The effects of each treatment on β-hexosaminidase release were characterized as a percentage of IgE control.

Quantitative real-time PCR

Total RNA extracts from mouse splenocytes stimulated with concanavaline A (ConA) or total cells from mouse lymph nodes sensitized with 1% DCNB were prepared using the Trizol method (Invitrogen). cDNA was synthesized from RNA by reverse transcription of 1 μg of total RNA using the Improm-II reverse transcription system (Promega) and oligo dT primers in a total volume of 20 μl. PCR amplification was performed using the primers described in Table 1 (Bioneer, Deajeon, Korea). Quantitative real-time PCR reactions were run on a Rotor-Gene 6000 (Corbett Research, Mortlake, Sydney, Australia) using the SYBR Green PCR Master Mix (Qiagen) in 20 μl reaction mixtures. Each real-time-PCR master mix contained 10 μl 2X enzyme mastermix, 7.0 μl RNase-free water, 1 μl of each primer (10 pM each) and 1 μl diluted template. PCR was conducted using an initial pre-incubation step of 10 min at 95°C, followed by 45 cycles at 95°C for 15 s, annealing at 52°C for 15 s and extension at 72°C for 10 s. Melting curve analysis was used to confirm the

Table 1 Primer sequences used in real-time RT-PCR

Gene	Primer	Amino acid sequences	Product size (bp)	Accession no.
IL-2	5′	5′- GCTCTACAGCGGAAGCACAG	235	NM_008366
	3′	5′- GTCAAATCCAGAACATGCCG		
IFN-γ	5′	5′- GTTCTGGGCTTCTCCTCCTG	245	NM_008337
	3′	5′- CTGGCTCTGCAGGATTTTCA		
IL-4	5′	5′- ATATCCACGGATGCGACAAA	252	NM_021283
	3′	5′- AAGCCCGAAAGAGTCTCTGC		
IL-5	5′	5′- GGGGGTACTGTGGAAATGCT	247	NM_010558
	3′	5′- TTGCACAGTTTTGTGGGGTT		
β-actin	5′	5′- CTAGGCACCAGGGTGTGATG	291	NM_007393
	3′	5′- CTACGTACATGGCTGGGGTG		

formation of the expected PCR product, and products from all assays were additionally subjected to 1.2% agarose gel electrophoresis to confirm that the lengths were correct. An inter-run calibrator was used and a standard curve was created for each gene to obtain PCR efficiencies. Relative sample expression levels were calculated using Rotor-Gene 6000 Series Software 1.7 and were expressed relative to β-actin. β-actin expression was also used to correct for between-run variability. Data for the experimental samples were expressed as the percentage of the internal control gene.

Histopathologcal analysis

The dermis tissue was fixed by inflating the tissue with 10% formalin. The tissues were then embedded in paraffin, cut into sections (5 microns) and stained with hematoxylin–eosin (H&E) solution. All tissue samples were examined and photographed in a blinded fashion. Images were captured using an OLYMPUS DP controller and manager at ×100 magnification.

Statistical analysis

The statistical analyses used to determine the differences between the groups was the Kruskal–Wallis one-way analysis of variance with a Dunnet's post-hoc test, which was performed using SPSS software (v. 13). Statistical significance was set *a priori* at $P < 0.05$.

Results

In HPLC–mass spectra analysis, we found that SAB contained two active ingredients (TCA and UDCA) (Figure 1). The amounts of UDCA and TCA in SAB were 0.696 and 0.0625 mg/ml, respectively (calibration curve not shown). DPPH has been widely used to evaluate the free radical-scavenging activity of various antioxidant substances. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Thus, these radicals were used to initially assess the antioxidant potency of the different fractions.

In the DPPH assay, SAB was shown to significantly scavenge free radicals within 20 min at a concentration of 100 µg/ml. However, UDCA and TCA alone at 100 µm did not effectively scavenge free radicals when compared to 100 µg/ml of SAB (Figure 2a). To determine whether SAB displayed protein-level antioxidant properties, degradation of BSA by Cu^{2+} and H_2O_2 was monitored in the presence of 10–100 µg/ml SAB, as described in the Materials and Methods section. As shown in Figure 2b, SAB protected against the breakdown of BSA as did 50 µm ascorbic acid. SAB efficiently inhibited the degradation of BSA by free radicals in a dose-dependent manner with maximum inhibition at a concentration of 100 µg/ml. To investigate the cytotoxicity of SAB, mouse splenocytes were incubated with various concentrations of SAB (10–500 µg/ml). Figure 3

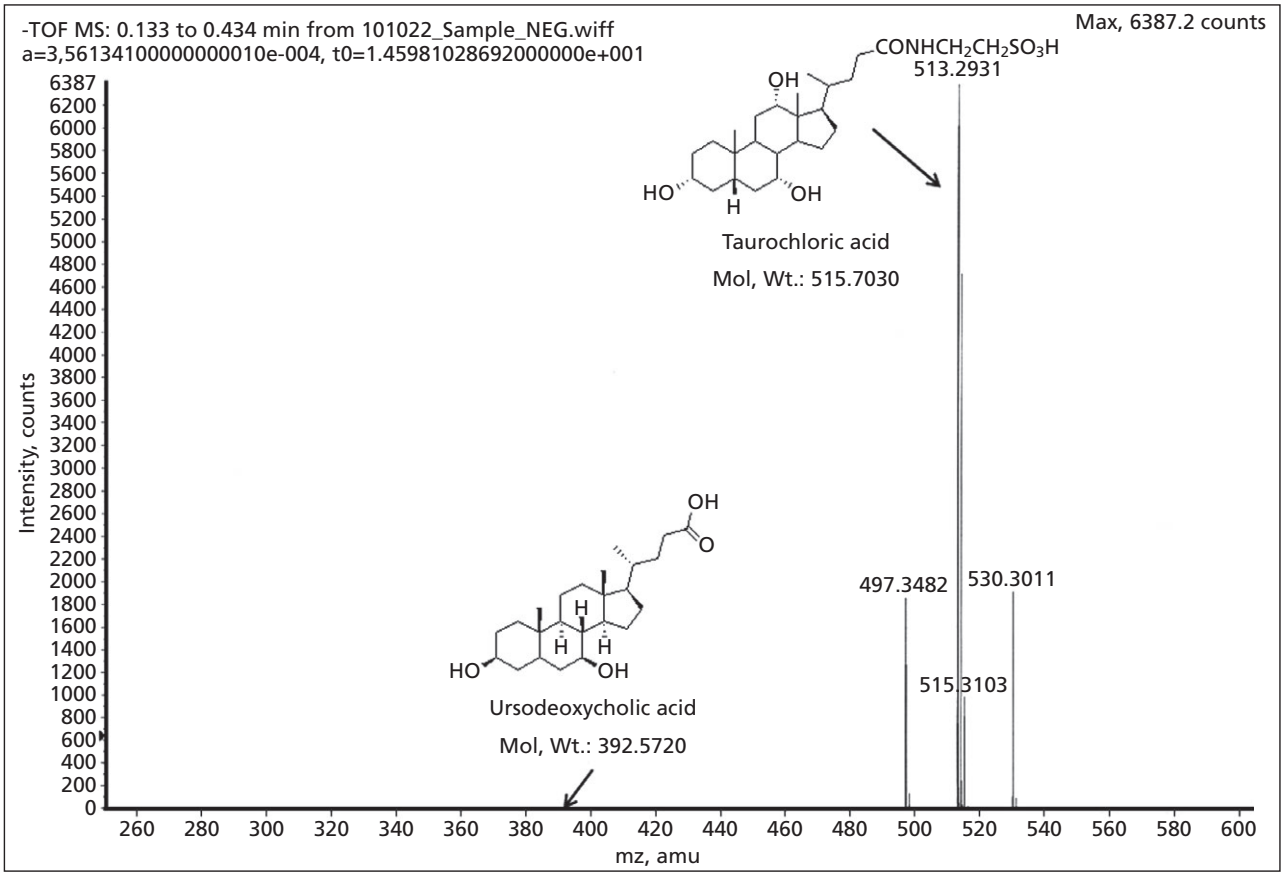

Figure 1 HPLC–mass spectra of SAB.

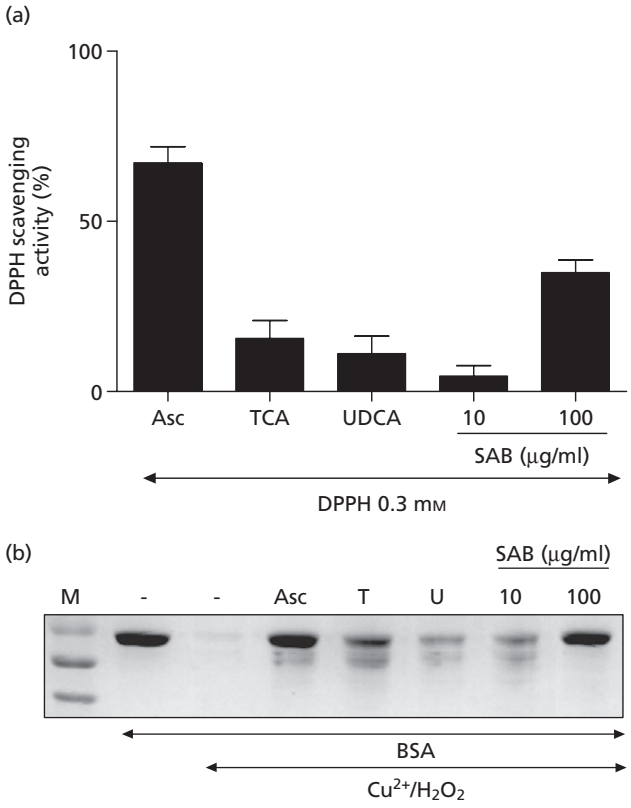

Figure 2 Radical scavenging activity (DPPH) and PAGE profiles of the BSA protein with Cu^{2+}/H_2O_2 in the presence of SAB. (a) DPPH free radical-scavenging activity of UDCA (100 μM), TCA (100 μM) and SAB (10, 100 μg/ml) was measured over a fixed time (20 min). The experiments were performed in quadruplicate and data are expressed as mean ± SD. (b) The gels show the protein obtained without treatment, with Cu^{2+}/H_2O_2 and at different SAB concentrations (10, 100 μg/ml). Ascorbic acid (0.5 mM) was used as a positive control. The final steps included incubation of all reactants, including BSA, for 2 h and electrophoresis in 10% SDS-PAGE. T, TCA; U, UDCA; Asc, ascorbic acid.

shows that SAB was not cytotoxic up to 24 h at concentrations ranging from 10 to 100 μg/ml. However, a significant increase in LDH release from cells was observed at higher concentrations (>500 μg/ml), indicating that these higher concentrations were cytotoxic. Because the cytotoxicity of SAB at concentrations >500 μg/ml was so large, higher concentrations were not tested.

To assess if SAB inhibits the expression of T cell-mediated Th1/Th2 cytokines, splenocytes were first activated with ConA, which is a T cell mitogen. The expression of IL-2, IFN-γ and IL-4 mRNAs was then examined in the presence of varying concentrations of SAB (1–100 μg/ml). The immunosuppressant CsA was included for comparison. The mRNA levels of the selected Th1/Th2 cytokines were assessed using quantitative real-time PCR. As shown in Figure 4a–c, T cells were highly activated by ConA. However, the mRNA expression levels of IL-2, IFN-γ and IL-4 were significantly inhibited after treatment with SAB at concentrations higher than 50 μg/ml. Interestingly, SAB was particularly effective in inhibiting the expression of IL-4 (Th2 cytokine) mRNA at lower concentrations. Thus, these results clearly demonstrate

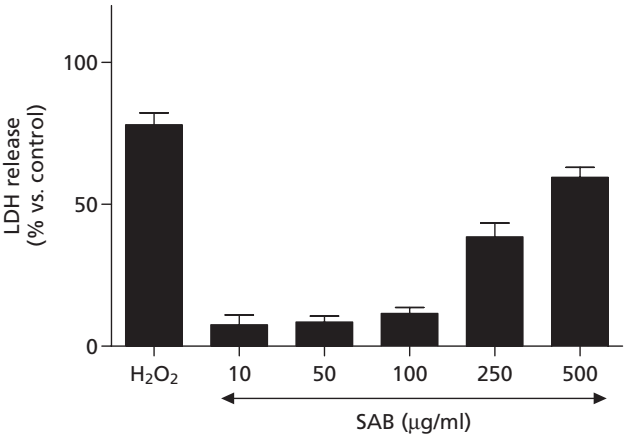

Figure 3 Cytotoxicity in mouse splenocytes. Splenocytes were exposed to different concentrations of SAB (10–500 μg/ml) for the indicated times. Concentration-dependent cytotoxicity was measured as percentage LDH released into the culture media. The positive control was 0.5 mM H_2O_2. The experiments were performed in triplicate and the results are expressed as the mean ± SD.

that SAB can effectively control the expression and release of T cell-derived cytokines, especially from Th2 cells, after stimulation with ConA.

As shown in Figure 5, the release of β-hexosaminidase, which is an indicator of mast cell degranulation, was measured in supernatants after treatment with DNP-HSA. Compared to the positive control (CsA), SAB effectively inhibited the release of β-hexosaminidase from RBL-2H3 at a concentration of 100 μg/ml (66.2%), which was comparable to the positive control (60.3%), whereas SAB alone at varying concentrations, without antigen stimulation, did not affect β-hexosaminidase secretion (Figure 5b).

In the histological analysis (H&E staining) of DNCB-treated mice, we observed that skin thickness was higher in DNCB-treated mice than in normal mice (control), but the thickness decreased when treated with SAB (Figure 6a). Higher concentrations of SAB (5%) were expected to result in an increase in the amount of recovered AD-like skin. These findings were confirmed in the analysis of the Th1 and Th2 cytokine mRNAs from the lymph nodes in DNCB-treated mice. As shown in Figure 6b–d, 5% SAB effectively inhibited the expression of IFN-γ and IL-4 mRNA, which suggests that SAB may play an important role in suppressing T cell activation in lymph nodes, where T cells first meet with and are activated by dendritic cells.

Discussion

Bile is a bitter-tasting, dark green to yellowish brown fluid that is produced in the liver of most vertebrates and aids in the process of digesting lipids in the small intestine. UDCA and TCA, which are believed to be the major active components of SAB, are bile acids found in bile. However, only small amounts of UDCA exist in the total bile acid pool.[11] In a previous study, we found that fish bile, especially in zoophagous fish, contained high amounts of UDCA and TCA, and that bile from *Silurus asotus* contained approximately ten

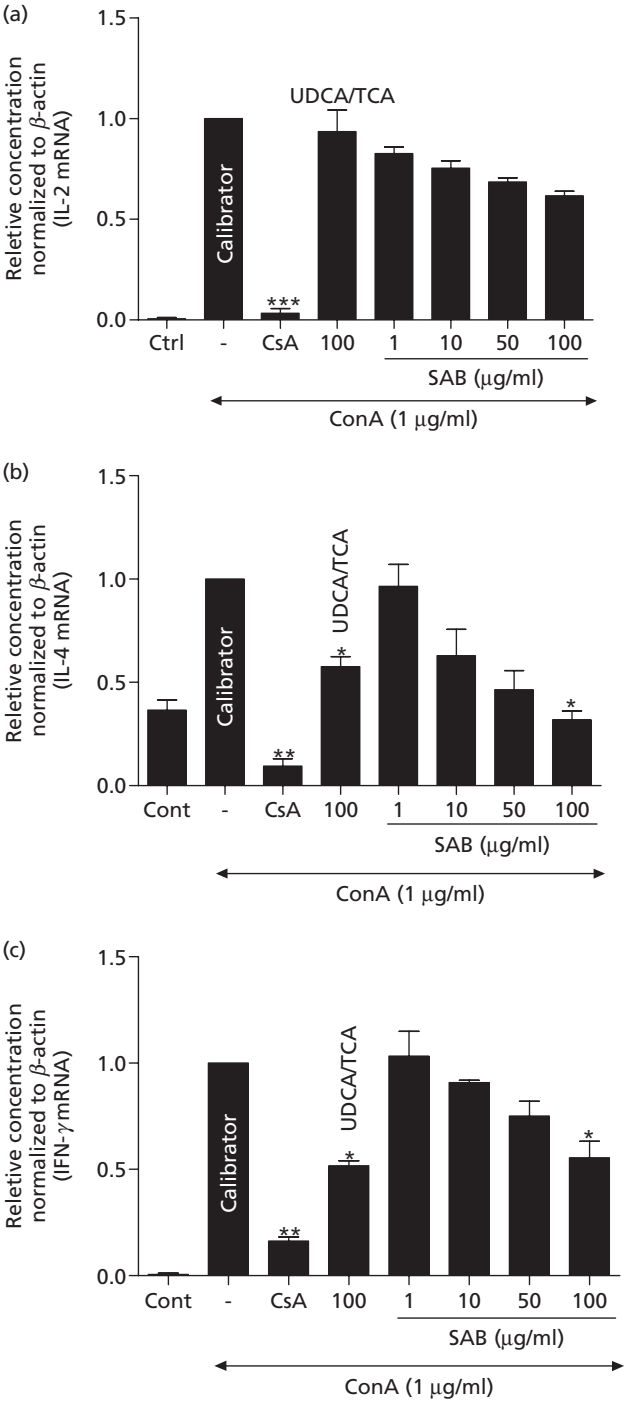

Figure 4 Th1/Th2 cytokine mRNA expression and cytotoxicity in splenocytes. Cells were cultured for 24 h as described in the Materials and Methods section and cyclosporine A (CsA) was used as the positive control for mRNA analysis. Isolated mRNA levels of IL-2, IFN-γ, IL-4 and β-actin were analysed by real-time RT-PCR. Results were internally confirmed by comparative cycle count (Ct, cycle number threshold) against β-actin, which was used as the standard gene. The calibrator was 1 μg/ml ConA (concanavalin A-activated group), to give a relative comparison of each group. The experiments were performed in triplicate and the results are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

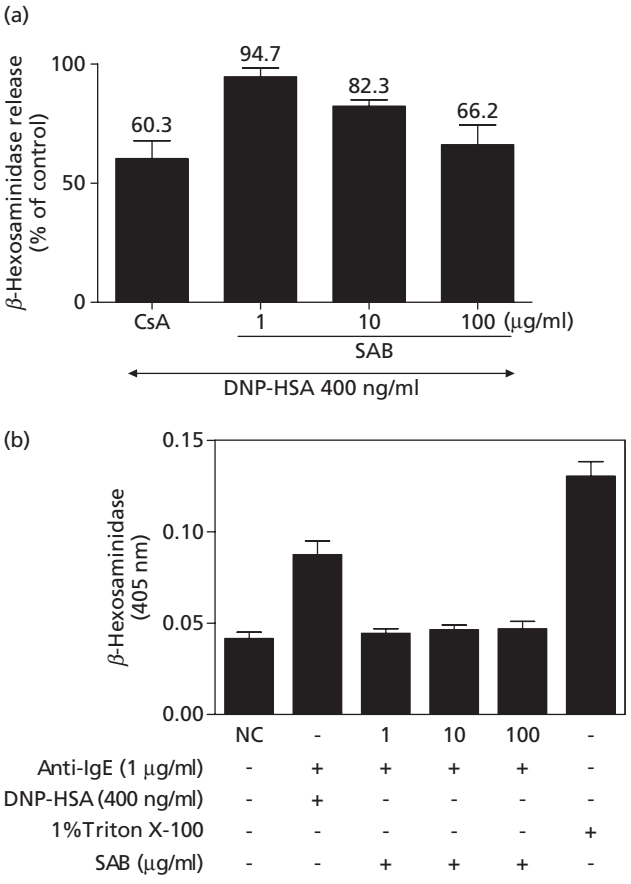

Figure 5 Inhibition of β-hexosaminidase release in RBL-2H3 mast cells. (a) Cells were cultured for 24 h as described in the Materials and Methods section, and CsA was used as a positive control. RBL-2H3 cells (2.5×10^5/well) were sensitized with 1 μg/ml of DNP-specific IgE overnight and pretreated with varying doses of SAB (1–100 μg/ml) for 30 min. Cells were stimulated with 400 ng/ml of DNP-HSA for 1 h. The absorbance was measured using a microplate reader at 405 nm. (b) Control RBL-2H3 cells were treated with or without DNP-HSA after sensitization with DNP-specific IgE overnight, while SAB-treated cell groups (1–100 μM) were sensitized with DNP-specific IgE alone. 0.1% Triton X-100 (sigma) was used to lyse whole cells. The experiments were performed in triplicate and the results are expressed as the mean ± SD.

times more of these compounds than other zoophagous fish (i.e. *Siniperca scherzeri* and *Micropterus salmodides*).[7]

Although UDCA has been widely used to treat cholestatic liver disease, it has also been used to suppress immune responses.[12] In addition, bile acids have been shown to decrease the degree of allograft rejection after liver transplantation by altering the expression of the major histocompatibility complex class molecules in the bile duct epithelium and central vein endothelium.[13] In other studies UDCA has been found to inhibit apoptosis by preventing cytochrome c release and modulating mitochondrial membrane perturbation.[14,15] In addition, UDCA has been used to treat putatively immune-mediated liver diseases, although the mechanism of this action is unknown.[16] Furthermore, several studies have demonstrated that UDCA may play an important role in preventing oxidative injury induced by several agents, through either a

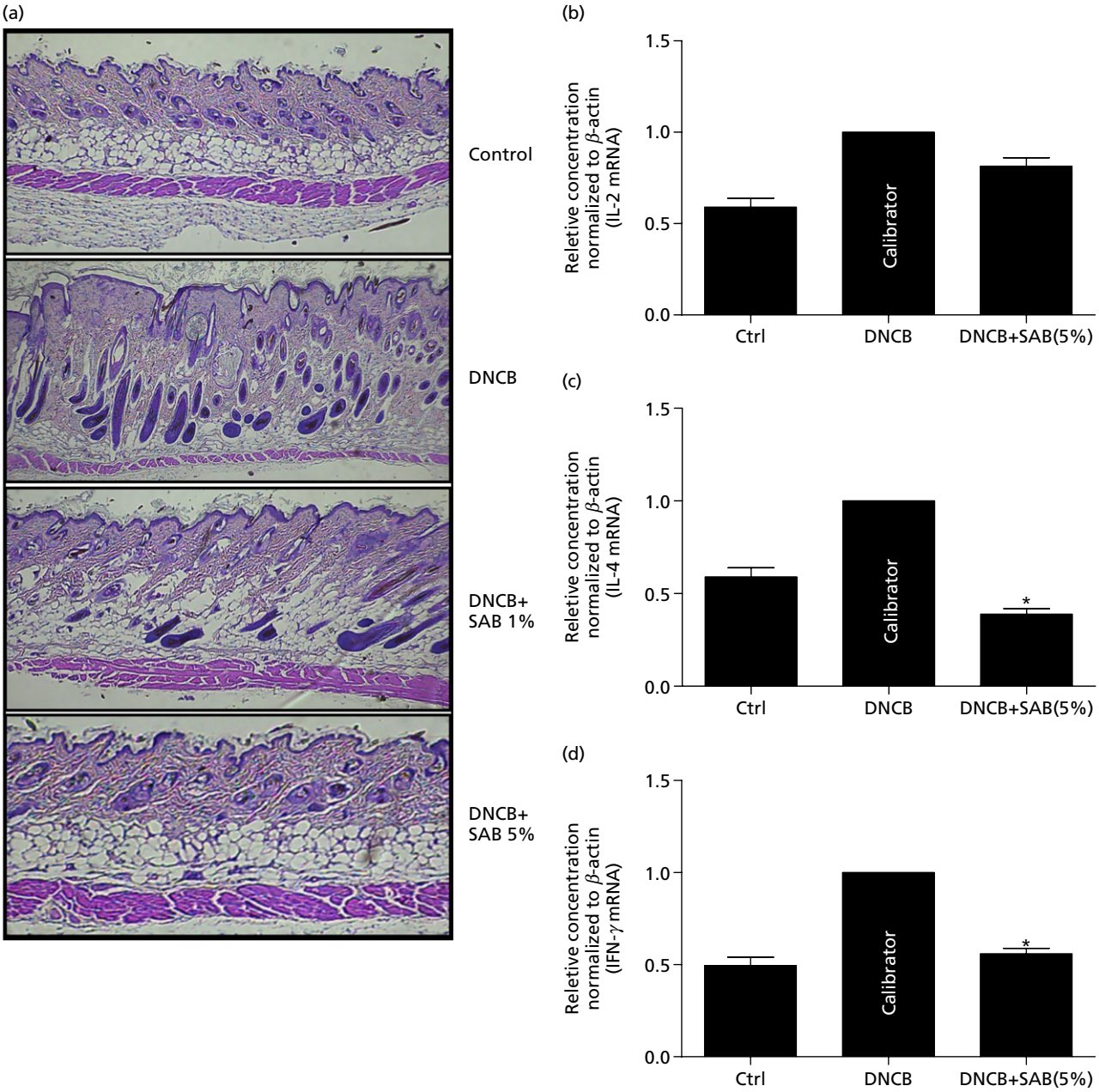

Figure 6 Histological recovery of the dorsal skin lesion after 4 weeks of treatment and the effect of SAB treatment on Th1/Th2 cytokine mRNA expression in the lymph nodes of DNCB-treated mice. (a) Tissues were fixed with formaldehyde, embedded in paraffin and stained with a H&E staining solution as described in the Materials and Methods section. (b–d) Expression of IL-2, IFN, IL-4 for skin lesions treated with either DNCB or DNCB + SAB and control. 5% SAB cream was evenly applied over the skin lesions for 4 weeks and real-time RT-PCR analysis on the lymph nodes was performed to assess the expression of IL-2, IFN-γ and IL-4. Values are expressed as mean ± SD of three animals per group and H&E staining captures were representative of each group. mRNAs in lymph nodes were prepared by pooling lymph nodes from each mouse. $^*P < 0.05$. Ctrl, control; DNCB, DNCB-treated positive control.

direct antioxidant effect or an increase in antioxidant defences.[17]

With these immunosuppressive and antioxidative properties, UDCA and, in part, TCA may play an active role in attenuating the progression of AD by suppressing T cell activation and scavenging free radicals. AD is a common inflammatory skin disease that involves various immune cells.

Typically, the existence of Th1/Th2 subsets in Th lymphocytes provides a framework for understanding the normal and pathological immune response in allergic responses. Many studies have suggested that Th1 and Th2 types of reactions can reciprocally regulate one another. Balancing the Th1/Th2 types of reaction may therefore be fundamental to the treatment of AD.[18]

In the present study we investigated the effects of SAB in a non-cell model, cell model and DNCB-treated AD-like mouse model, and demonstrated that SAB displays anti-AD properties. From the HPLC–mass spectra analysis, we determined that SAB contains high amounts of TCA (69.6%). However, UDCA was detected in only trace quantities (0.6%). SAB was shown to significantly scavenge free radicals and successfully prevented proteins from superoxide attack, which strongly implies that the antioxidative effects of SAB can prevent the immune response from shifting towards a Th2-skewed immune response. Furthermore, SAB effectively suppressed the expression of Th1/Th2 cytokines (IL-2, IFN-γIL-4) in splenocytes, which suggests that SAB may contribute to the suppression of T cells when activated with mitogens. SAB also effectively decreased β-hexosaminidase secretion from IgE-stimulated RBL-2H3 mast cells. In the histological analysis, 5% SAB cream successfully eliminated AD-like skin lesions induced by DNCB, and cytokine mRNAs (IFN-γ, IL-4) in the lymph nodes of DNCB-treated mice were significantly inhibited.

Although mouse skin is widely used for AD studies, it should be noted that mouse skin is much thinner and more permeable than human skin.[19]

These results also strongly suggest that SAB may inhibit T cell-based progression of AD. To date, topical corticosteroids have been the primary choice for the treatment of AD. However, side effects such as skin atrophy, striae and perioral dermatitis in sensitive areas (face or skin fold) have prevented long-term use of corticosteroids for the treatment of AD.[20] Recently, new topical calcineurin inhibitors, such as tacrolimus ointment and pimecrolimus cream, have been used as a monotherapy treatment for AD when conventional treatments were not possible or unsuccessful. However, topical calcineurin inhibitors are relatively new and the long-term side effects of this type of treatment are not fully known.[21]

Compounds from plants have recently been reported as a potential alternative method for anti-AD treatment. This type of treatment is expected to prevent the onset of allergic diseases and ameliorate allergic symptoms.[22] Based on the combined findings of this study, it is believed that SAB may be able to inhibit the progression of AD pathogenesis, and thus can be used as a functional compound or an alternative therapeutic approach for the treatment of AD, which is mainly induced by T cell-based hyperactivation of immune cells. More in-depth studies are necessary to clearly determine the mechanisms of SAB, but based on the results presented in this study, it appears that SAB may be a potent functional material for the treatment of AD.

Conclusion

UDCA and TCA are increasingly being considered for use in treating a variety of chronic diseases, and the antioxidant and immunosuppressant activity of these components could be exploited for the treatment of allergic diseases, such as AD. Our results clearly demonstrate that SAB has radical scavenging activities and protects against protein degradation, suggesting that SAB possesses multifunctional antioxidative activities. More importantly, SAB significantly suppresses the expression of Th cytokines (IL-2, IFN-$\gamma$and IL-4), which play a central role in AD progression. Inhibition of mast cell degranulation and histological recovery of DNCB-treated AD-like mouse model also strongly indicates that SAB holds much promise for use as functional supplement for the treatment of AD.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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References

1. Incovaria C *et al*. Allergy and the skin. *Clin Exp Immunol* 2003; 153: 27–29.
2. Pelle E *et al*. Keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide to melanocytes. *J Invest Dermatol* 2005; 124: 793–797.
3. Kornina L, Pastore S. The role of redox regulation in the normal physiology and inflammatory disease of skin. *Front Biosci* 2009; 1: 123–141.
4. Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. *J Eur Acad Dermatol* 2003; 17: 663–669.
5. Wu Z *et al*. Desferrioxamine modulates chemically induced T helper 2-mediated autoimmunity in the rat. *Clin Exp Immunol* 2004; 135: 194–199.
6. Bengtsson A *et al*. Thiols decrease cytokine levels and down-regulate the expression of CD30 on human allergen-specific T helper (Th) 0 and Th2 cells. *Clin Exp Immunol* 2001; 123: 350–360.
7. Joo SS. Immunosuppressive properties of catfish bile from *Silurus asotus*: inhibition of T cell activation in mouse splenocytes. *Food Sci Biotechnol* 2008; 17: 598–602.
8. Joo SS *et al*. Ursodeoxycholic acid inhibits proinflammatory repertoires, IL-1 beta and nitric oxide in rat microglia. *Arch Pharm Res* 2003; 26: 1067–1073.
9. Joo SS *et al*. Potential role of ursodeoxycholic acid in suppression of nuclear factor Kappa B in microglial cell line (BV-2). *Arch Pharm Res* 2004; 27: 954–960.
10. Mayo JC *et al*. Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. *Biochim Biophys Acta* 2003; 1620: 139–150.
11. Hofmann AF. Pharmacology of ursodexoycholic acid, an enterohepatic rug. *Scand J Gastroenterol Suppl* 1994; 204: 1–15.
12. Calmus Y *et al*. Immunosuppressive properties of chenodeoxycholic and ursodeoxycholic acids in the mouse. *Gastroenterology* 1992; 103: 617–621.
13. Chen W, Gluud C. Bile acids for liver-transplanted patients. *Cochrane Database Syst Rev* 2005; (20): CD005442.
14. Rodrigues CMP *et al*. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J Clin Invest* 1998; 101: 2790–2799.
15. Rodrigues CMP *et al*. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death Differ* 1999; 6: 842–854.

16. Lacaille F, Paradis K. The immunosuppressive effect of ursodeoxycholic acid: a comparative in vitro study on human peripheral blood mononuclear cells. *Hepatology* 1993; 18: 165–172.
17. Mitsuyoshi H *et al*. Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. *Biochem Biophys Res Commun* 1999; 263: 537–542.
18. Heinzel FP *et al*. Reciprocal expression of interferon-γ or IL-4 during the resolution or progression of murine leishmaniasis: evidence for expression of distinct helper T cell subset. *J Exp Med* 1989; 169: 59–72.
19. Reifenrath WG *et al*. Percutaneous penetration in the hairless dog, weanling pig and grafted athymic nude mouse: evaluation of models for predicting skin penetration in man. *Br J Dermatol* 1984; 111(Suppl. 27): 123–135.
20. Thaci D. Long term management of childhood atopic dermatitis with calcineurin inhibitors. *Hautarzt* 2003; 54: 418–423.
21. Remitz A, Reitamo S. Long-term safety of tacrolimus ointment in atopic dermatitis. *Expert Opin Drug Saf* 2009; 8: 501–506.
22. Kawai M *et al*. Flavonoids and related compounds as anti-allergic substances. *Allegol Int* 2007; 56: 113–123.