

Anthocyanins, but not Anthocyanidins, from Bilberry (*Vaccinium myrtillus* L.) Alleviate Pruritus via Inhibition of Mast Cell Degranulation

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Abstract: We have previously reported that bilberry anthocyanins exhibit an anti-pruritic effect in a mouse model of allergic contact dermatitis. It has been reported that anthocyanins are particularly sensitive to thermal treatment and are easily hydrolyzed to anthocyanidins when exposed to high temperatures. The objective of this study was to compare the anti-pruritic effect of anthocyanin-rich quality-controlled bilberry extract and anthocyanidin-rich degraded extract using a mouse model of allergic contact dermatitis. BALB/c mice with allergic contact dermatitis induced by 4 weeks of repeated application of 2,4,6-trinitro-1-chlorobenzene (TNCB) were administered Bilberon-25 orally for 4 weeks after sensitization with TNCB. The effect of Bilberon-25 on pruritus was evaluated by measurement of scratching behavior. RBL-2H3 mast cells were used to investigate the effect of Bilberon-25 on degranulation in 48/80-stimulated mast cells. Compared with nonheated Bilberon-25, the proportion of anthocyanins in heated Bilberon-25 decreased, and the proportion of anthocyanidins was increased in a heated-time dependent manner. Treatment with non-heated Bilberon-25 significantly attenuated the TNCB-induced increase in scratching behavior, whereas treatment with 2 h-heated Bilberon-25 did not. Moreover, 300 $\mu\text{g/mL}$ nonheated Bilberon-25 showed significant inhibition of degranulation in RBL-2H3 mast cells, whereas 2 h-heated Bilberon-25 had no effect at any concentration studied. It is assumed that the inhibitory effect of bilberry anthocyanins on pruritus might be mediated, at least in part, by its inhibitory effect on mast cell degranulation. In conclusion, the anthocyanin-rich but not anthocyanidin-rich bilberry extract may be a useful dietary supplement for skin diseases involving pruritic symptoms, such as chronic allergic contact dermatitis, atopic dermatitis, and rhinitis.

Keywords: Anthocyanidins, bilberry anthocyanins, degranulation, mast cells, pruritus, thermal degradation

Introduction

There is growing interest in the use of herbs and dietary supplements for maintaining health. In Japan, blueberries are very popular, and their sales ranked ninth in 2004 in the herb category. Blueberries have received much attention due to their protective health effects, which have been attributed mainly to the wide range of constituent polyphenols, such as anthocyanins.

Anthocyanins have a variety of physiological functions, including antioxidation, and may be involved in protecting against chronic and age-related diseases, such as coronary heart disease (Dohadwala and others 2011), decreased visual acuity (Nakaishi and others 2000), type II diabetes (Wilson and others 2008), dementia (Shih and others 2010), ulcers (Kim and others 2011), and several types of cancer (Wang and Stoner 2008; Hertog and

others 1993). Moreover, extract of bilberry, a blueberry cultivar, has already been approved as a pharmaceutical product in many countries. In addition, we were the first to report that bilberry anthocyanins exhibit an anti-pruritic effect in a mouse model of allergic contact dermatitis (Yamaura and others 2011).

The composition, amount, and distribution of anthocyanins in blueberry are dependent on the cultivar, cultivation condition, and area of production (Nakajima and others 2004). Blueberry cultivars are mainly highbush blueberry and rabbiteye blueberry in Japan, lowbush blueberry in North America, and bilberry in central and northern Europe. Among these cultivars, the anthocyanin content of bilberry (*Vaccinium myrtillus* L) fruit is in the range 0.3 to 0.4%, which is more than 2-fold higher than the 0.15% in the fruits of highbush, lowbush, and rabbiteye blueberries (Moze and others 2011). For this reason, anthocyanin-containing supplements are usually manufactured using bilberry extract.

The stability of anthocyanins has been previously investigated (Brownmiller and others 2008; Patras and others 2010; Fan and others 2008). It has been reported that anthocyanins are affected by environmental factors and are particularly sensitive to thermal treatment (Kechinski and others 2010). In natural bilberry, almost all anthocyanins exist in the glycoside form, with an anthocyanidin component of only a few percent. However, anthocyanins are easily hydrolyzed to anthocyanidins by exposure to high temperature. Spectrophotometry is generally used in the dietary supplement industry to determine total anthocyanidin content as delphinidin.

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However, the heating procedure as part of this method can alter the original composition of supplements by degrading anthocyanin to anthocyanidin. For this reason, there will be no difference in the results of the total contents of proper products and anthocyanidin-rich degraded products. Therefore, there is a risk of distributing degraded products to consumers. However, the pharmacological effects of anthocyanin-rich quality-controlled and anthocyanidin-rich degraded bilberry extracts have not been previously compared.

The objective of the present study was to compare the anti-pruritic effects of anthocyanin-rich quality controlled bilberry extract and anthocyanidin-rich degraded extract using a mouse model of allergic contact dermatitis. A further aim of this study was to clarify whether anthocyanins or anthocyanidins are more important for the anti-pruritic effect in allergic contact dermatitis.

Materials and Methods

Materials

Bilberon-25, a concentrated extract of bilberry (*V. myrtillus* L.), was generously donated by Tokiwa Phytochemical Co. Ltd. (Japan). Dexamethasone was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and 2,4,6-trinitro-1-chlorobenzene (TNCB) was obtained from Tokyo Chemical (Tokyo, Japan). Bilberon-25 and dexamethasone were dissolved in 1% hydrochloric acid (HCl) aqueous solution, and TNCB was dissolved in acetone. To prepare anthocyanidin-rich Bilberon-25, Bilberon-25 was dissolved in 1% HCl and heated to 100 °C within a boiling water bath for 0.5, 1, or 2 h.

Eagle's minimal essential medium (EMEM) was obtained from Nissui Seiyaku (Tokyo, Japan). Heat-inactivated fetal bovine serum (FBS) was obtained from Equitech-Bio (Kerrville, Tex., U.S.A.). Penicillin-streptomycin was obtained from Invitrogen (Carlsbad, Calif., U.S.A.). Compound 48/80 and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide were purchased from Sigma Chemical Co. Triton X-100 was obtained from Wako Pure Chemical Institute (Osaka, Japan).

High performance liquid chromatography (HPLC) analysis

A sample of 1 mg/mL Bilberon-25 (dissolved in 1% HCl aqueous solution) was subjected to HPLC analysis. The HPLC system employed to analyze Bilberon-25 was a SHISEIDO NANOSPACE SI-2 (Tokyo, Japan). The column, YMC-Peak Pro C18 RS (2.0 × 250 mm, 5 μ m, YMC Corporation, Kyoto, Japan) was used at 30 °C with a flow rate of 200 μ L/min. Bilberon-25 was detected at a wavelength of 535 nm, and the injection volume

was 20 μ L. Solvent A contained 90% water/10% formic acid (v/v); solvent B contained 22.5% acetonitrile/22.5% methanol/40% water/10% formic acid (v/v/v/v). The elution gradients used were as follows: between 0 and 35 min, 10 to 35% B; 35 to 45 min increased to 65% B; 45 to 46 min increased to 100% B; 46 to 50 min held at 100% B.

Animals

Female BALB/c mice, 6 weeks of age, were obtained from Japan SLC Inc. (Hamamatsu, Japan) and housed under controlled light (07:00 to 19:00 h) and temperature (24 °C) conditions with food and water available *ad libitum*. All experiments and procedures were approved by the Chiba University Institutional Animal Care and Use Committee.

TNCB-induced chronic allergic contact dermatitis

The experimental protocols are illustrated in Figure 1. The abdominal region of BALB/c mice was shaved with a hair clipper 1 day before sensitization. On day -7, animals were sensitized by a single epicutaneous application of 50 μ L TNCB (1.1% w/v) solution to the shaved abdomen, and on day 0 the animals were challenged with the application of 10 μ L TNCB solution to each ear. TNCB solution was then applied repeatedly to each ear 3 times a week until day 24. Bilberon-25 (400 mg/kg) and dexamethasone (0.5 mg/kg) were administered orally every day from day -7 to day 24. Each reagent was administered on the day of each application of TNCB solution, 4 h beforehand. Ear thickness and scratching behavior were evaluated 24 h after the application of TNCB on days 15 and 22. Acetone and 1% HCl were applied as vehicle controls for TNCB and reagents (Bilberon-25 and dexamethasone), respectively; for TNCB nontreatment (Nil) mice were challenged with application of acetone, and 1% HCl was administered orally every day from day -7 to day 24, and animals treated with TNCB but not with reagents (vehicle) were challenged with the application of TNCB solution and received oral administration of 1% HCl.

Pruritus measurement

The number of bouts of scratching behavior was evaluated for 2 h after TNCB application for 24 h. Pruritus was evaluated by automatic counting of the scratching bouts using MicroAct (Neuroscience Inc., Tokyo, Japan), as reported previously (Inagaki and others 2003).

Measurement of ear thickness

Right and left ear thickness was measured with a micrometer (Mitsutoyo, Kanagawa, Japan) under light ether anesthesia 24 h

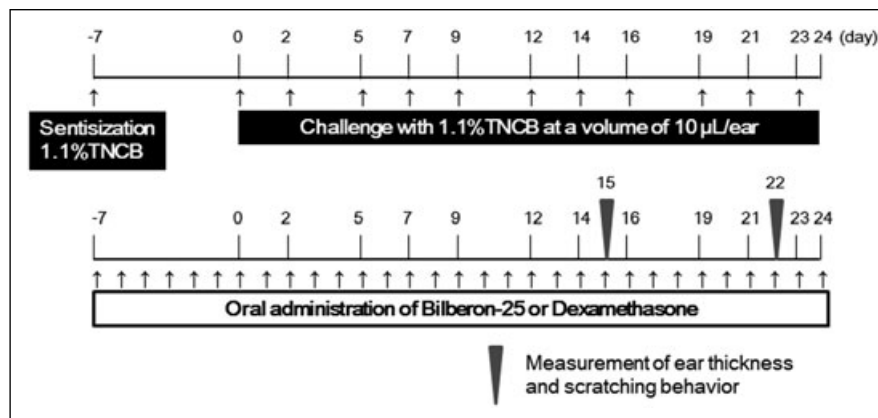


Figure 1—Experimental schedule: administration of Bilberon-25 and induction of allergic contact dermatitis with TNCB.

after each challenge. Ear swelling was calculated as the average thickness of both ears.

Cell culture

RBL-2H3 mast cells, from a rat basophilic leukemia cell line, were obtained from RIKEN Bio Resource Center (Tsukuba, Japan). In brief, RBL-2H3 cells were maintained in EMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in 5% CO₂.

Mast cell degranulation assay

RBL-2H3 mast cells in EMEM containing 10% FBS were seeded onto 24-well culture plates at a density of 1×10^5 cells/well and incubated for 24 h at 37 °C under a humidified atmosphere containing 5% CO₂. Bilberon-25 was added to the cells and incubated for 30 min at 37 °C. Cells were washed with EMEM and stimulated with 30 µg/mL compound 48/80 for 30 min at 37 °C. Degranulation was assessed by measuring β-hexosaminidase release. In brief, cells were lysed in 50 µL 0.1% Triton X-100. Next, 50 µL supernatant or cell lysates and 50 µL 2 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (in 0.4 M citrate and 0.2 M phosphate buffer, pH 4.5) were added to each well of a 96-well plate and incubated for 2 h at 37 °C. The enzyme reaction was terminated by adding 100 µL 0.25 M glycine buffer at pH 10.4. Absorbance at 405 nm was measured using a Multiscan JX microplate reader (Thermo Lab Systems, Yokohama, Japan).

Statistical analysis

Data are presented as mean ± SEM values. Statistical significance was analyzed using Dunnett's method for multiple comparisons. Statistical differences between groups (Nil and Vehicle controls) were determined using the Student's *t*-test or Welch's test after an *F*-test. Differences at *P* < 0.05 were considered statistically significant. All statistical analyses were conducted using StatLight software (Yukms Co. Ltd., Tokyo, Japan).

Results

Five main anthocyanidins are common in bilberry: cyanidin, delphinidin, malvidin, peonidin, and petunidin. The anthocyanins in bilberry, the glycoside forms of these anthocyanidins, include 3-glucosides, 3-galactosides, and 3-arabinosides (Figure 2A). Therefore, there are a total of 15 types of glycosides and 5 anthocyanidins in bilberry. The chromatographic analysis of Bilberon-25 by HPLC shows the typical profile of bilberry extract (Figure 2B). Compared with nonheated Bilberon-25, the proportion of anthocyanins decreased from 98.3% to 23.5% following heat treatment for 2 h. By contrast, the proportion of anthocyanidins was increased by heat treatment for 2 h from 1.7% to 76.5% (Figure 3).

Repeated exposure of mouse ears to TNCB induced chronic allergic contact dermatitis characterized by skin swelling on days 15 and 22. Oral administration of dexamethasone, as a positive control, significantly reduced the amount of ear swelling, whereas there was no clear effect of non-, 0.5 h- and 2 h-heated Bilberon-25 (Figure 4).

Repeated application of TNCB evoked a significant increase in the number of scratching bouts on day 15. Oral administration of non-heated Bilberon-25 significantly suppressed this increase (Figure 5). On the other hand, administration of 0.5 h- and 2 h-heated Bilberon-25 did not show any obvious suppression of the number of scratching bouts. In particular, compared with the

number of scratching bouts on day 15, 2 h-heated Bilberon-25 had no effect on day 22.

We also examined the effect of Bilberon-25 on mast cell degranulation. Stimulation of RBL-2H3 mast cells with 48/80 led to the release of β-hexosaminidase. We found that 300 µg/mL non-heated Bilberon-25 showed significant inhibition of this degranulation in mast cells, whereas 2 h-heated Bilberon-25 had no effect on degranulation at any concentration studied (Figure 6).

Discussion

Twenty compounds, 15 anthocyanins and 5 anthocyanidins, in bilberry extracts are separable by HPLC analysis with gradient elution using formic acid and methanol-acetonitrile as the mobile phase (Ishikawa and others 2008). In the present study,

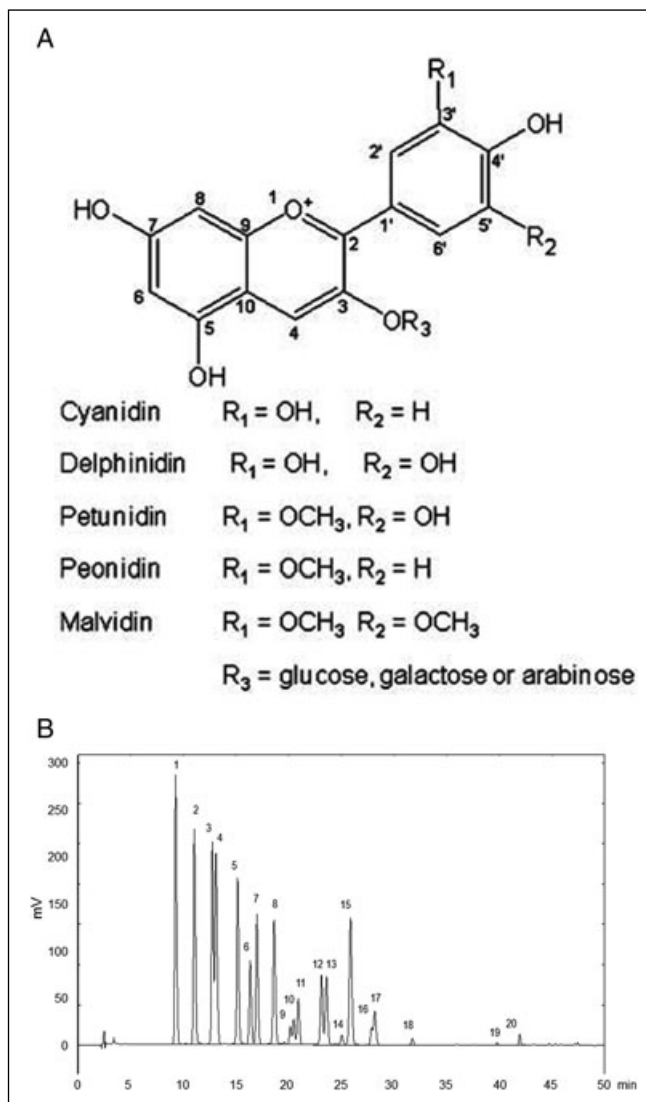


Figure 2—Chemical structure of anthocyanins and anthocyanidins (A), and HPLC profiles of Bilberon-25 at 535 nm (B). Peak identities: (1) delphinidin 3-O-galactoside, (2) delphinidin 3-O-glucoside, (3) cyanidin 3-O-galactoside, (4) delphinidin 3-O-arabinoside, (5) cyanidin 3-O-glucoside, (6) petunidin 3-O-galactoside, (7) cyanidin 3-O-arabinoside, (8) petunidin 3-O-glucoside, (9) delphinidin, (10) peonidin 3-O-galactoside, (11) petunidin 3-O-arabinoside, (12) peonidin 3-O-glucoside, (13) malvidin 3-O-galactoside, (14) peonidin 3-O-arabinoside, (15) malvidin 3-O-glucoside, (16) cyanidin, (17) malvidin 3-O-arabinoside, (18) petunidin, (19) peonidin, and (20) malvidin.

we detected 20 compounds in Bilberon-25 by HPLC and demonstrated that Bilberon-25, an anthocyanin-rich extract, was degraded in a time-dependent manner to an anthocyanidin-rich compound by heating.

Temperature is one of the most important factors affecting the stability of anthocyanins. Therefore, storage of commercial bilberry extract supplements at high temperature may result in thermal degradation of anthocyanins to anthocyanidins. Anthocyanidins are much more apt to avoid chemical decomposition than the glycosides, and they only survive for minutes in the bio-phase (Thomasset and others 2009). Therefore, in the light of their superior stability as compared to that of the aglycons, the anthocyanins seem much more suitable for dietary supplements than their anthocyanidin counterparts. We have previously shown the effectiveness of bilberry anthocyanins on chronic pruritus of experimental allergic contact dermatitis induced by repeated application of TNCB in mice (Yamaura and others 2011). However, no information has been available concerning the difference in anti-pruritic efficacy between anthocyanin- and anthocyanidin-rich bilberry extracts. In the present study, we compared the antipruritic effect of “anthocyanin-rich quality-controlled Bilberon-25” and “anthocyanidin-rich degraded Bilberon-25”, using the same experimental model described above. We demonstrated that anthocyanin-rich but not anthocyanidin-rich bilberry extract can alleviate pruritus. Furthermore, we found that repeated application of TNCB induced significant ear swelling, which was not inhibited by treatment with either type of Bilberon-25.

Controlling pruritus is important for the treatment of inflammatory skin diseases such as atopic dermatitis. However, pruritus does not necessarily correlate with skin inflammation (Kido and others 2010). In this study, we showed that Bilberon-25 did not decrease ear swelling, in contrast to the findings of our previous study. The difference in the intensity of experimental dermatitis might have affected the anti-inflammatory effect of Bilberon-25, but further study is needed to clarify this contradiction.

After long-term dexamethasone treatment, mice showed obvious and significant reductions in body weight and immune organ

weight, indicating possible adverse effects of glucocorticoid treatment. By contrast, non-, 0.5 h-, and 2 h-heat treated Bilberon-25 did not have any such effects (data not shown).

In a mouse model of TNCB-induced allergic dermatitis, it was demonstrated that specific immunoglobulin E (IgE) plays an essential role in the induction of persistent increases in scratching behavior and continuous degranulation of mast cells (Yamashita and others 2009). The total serum IgE level was significantly increased by repeated challenge with TNCB. Dexamethasone significantly ameliorated this increase. By contrast, Bilberon-25 did not have any obvious effect on IgE level (data not shown).

Therefore, we investigated the effect of Bilberon-25 on degranulation of mast cells. Anthocyanin-rich non-heated Bilberon-25 significantly decreased the degranulation of

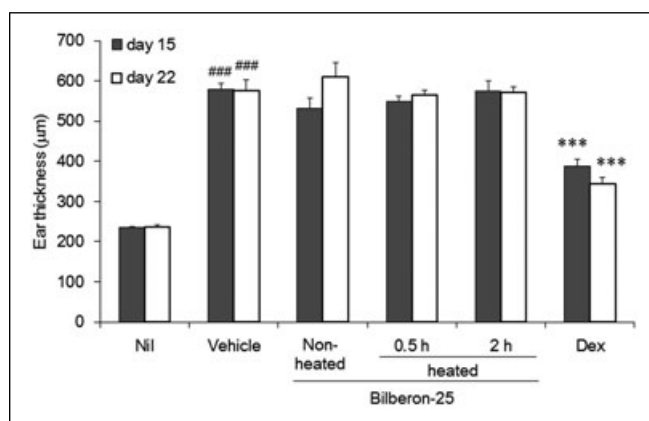


Figure 4—Effect of Bilberon-25 administration on ear thickness in mice repeatedly exposed to TNCB.

Ear thickness was measured 24 h after the application of TNCB on days 14 and 21.

Nil: non-treatment; Vehicle: TNCB-treated control; 0.5 h, and 2 h: Bilberon-25 heated for 0.5, and 2 h, respectively (400 mg/kg); Dex: dexamethasone (0.5 mg/kg). Results are expressed as mean $\pm$ SEM for $n = 7$ mice. ### $P < 0.001$ vs Nil (Student's t -test); *** $P < 0.001$ vs Vehicle (Dunnett's test).

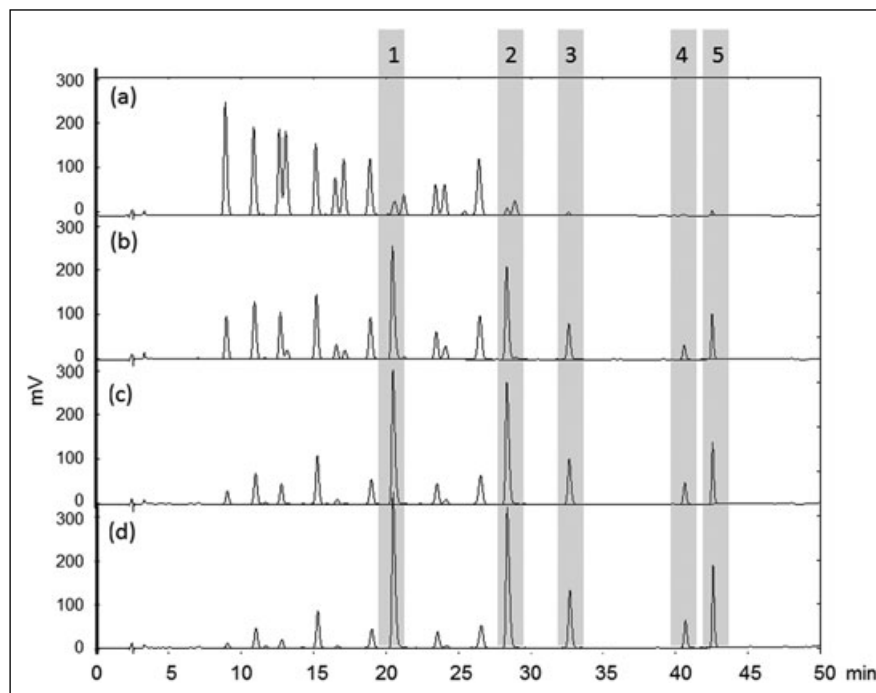


Figure 3—HPLC profiles of Bilberon-25 after thermal treatment.

Bilberon-25 was heated to 100 °C in a boiling water bath for (a) 0 h, (b) 0.5 h, (c) 1 h, and (d) 2 h.

Peak identities: (1) delphinidin, (2) cyanidin, (3) petunidin, (4) peonidin, and (5) malvidin. Other peaks represent glycosides of these anthocyanidins.

48/80-stimulated RBL-2H3 mast cells; however, anthocyanidin-rich 2 h-heated Bilberon-25 did not have any effect on degranulation. This suppressive effect was well correlated with the antipruritic effect. Therefore, it appears that the inhibitory effect of Bilberon-25 on scratching behavior might be mediated, at least in part, by its inhibitory effect on mast cell degranulation.

It was reported that delphinidin suppresses expression of the high-affinity IgE receptor FcεRI on mast cells which mediate mast cell degranulation (Tamura and others 2010). The most common anthocyanidin in 2 h-heated Bilberon-25 was delphinidin (27.2%). However, in the present study, 2 h-heated Bilberon-25 suppressed neither scratching behavior nor degranulation of 48/80-stimulated mast cells. These results indicate the possibility that the role of FcεRI on pruritus in our experimental dermatitis model is small, or that delphinidin may not exist under physiological conditions.

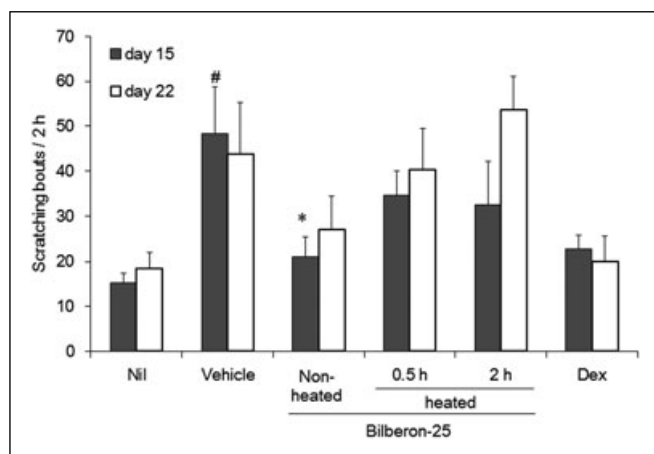


Figure 5—Effect of Bilberon-25 administration on scratching behavior in mice repeatedly exposed to TNCB.

Scratching bouts were counted for 2 h using MicroAct 24 h after the application of TNCB on days 14 and 21.

Nil: nontreatment; Vehicle: TNCB-treated control; 0.5 h, and 2 h: Bilberon-25 heated for 0.5, and 2 h, respectively (400 mg/kg); Dex: dexamethasone (0.5 mg/kg). Results are expressed as mean $\pm$ SEM for $n = 7$ mice. # $P < 0.05$ vs Nil (Student's t -test); * $P < 0.05$ vs Vehicle (Dunnett's test).

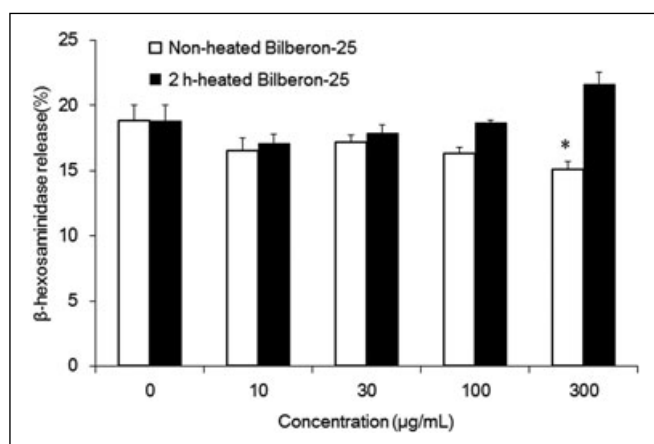


Figure 6—Effect of Bilberon-25 on degranulation in RBL-2H3 cells stimulated with compound 48/80.

RBL-2H3 cells were incubated with non- or 2 h-heat treated Bilberon-25 at the indicated concentrations for 30 min, and then stimulated with compound 48/80 (30 μ g/mL) for 30 min at 37 °C. Degranulation was assessed by measuring β -hexosaminidase activities as described in the Materials and Methods. Values represent the mean $\pm$ SE for $n = 4$ experiments. * $P < 0.05$ vs 0 μ g/mL Bilberon-25 (Dunnett's test).

Cyanidin 3-O-glucoside, but not cyanidin, was detected in rat plasma after oral administration of cyanidin 3-O-glucoside; cyanidin, which is the aglycon of cyanidin 3-O-glucoside, is unstable under physiological conditions and rapidly degraded to protocatechuic acid (Tsuda and others 1999).

Most flavanols and isoflavones are present in the diet as glycosides. In the intestinal lumen, they undergo de-glycosylation by lactase-phlorizin hydrolase, a disaccharidase located on the epithelial surface, and the released aglycones are transported into the intestinal cells where they are again conjugated, mainly to glucuro- and sulpho-derivatives (Passamonti and others 2009). However, it has been suggested that anthocyanins are absorbed and excreted intact across the intestine via glucose transporters in the mucosal epithelium (Wu and others 2005; Milbury and others 2002; Faria and others 2009).

Han reported that oral administration of cyanidin 3-O-glucoside, cyanidin, and protocatechuic acid inhibited histamine-induced acute pruritus, but these agents did not inhibit degranulation of RBL-2H3 mast cells, in contrast to our result (Han and others 2009). They used cyanidin 3-O-glucoside as the anthocyanin, and cyanidin as the anthocyanidin in their study; however, we used crude extract. The results of Han's investigation differ from ours possibly because they used a single compound whereas we used a crude extract. Therefore, it was thought that investigation of the influence of degradation of commercial dietary supplements containing bilberry extract should use a crude extract.

Our results demonstrated, for the first time, that the anthocyanin-rich bilberry extract alleviates pruritus, whereas the anthocyanidin-rich extract does not have this effect. In addition, the concentrations of both anthocyanins and anthocyanidins should be considered for quality assessment of dietary supplements that contain bilberry extract.

Conclusions

Anthocyanin-rich but not anthocyanidin-rich bilberry extract alleviates pruritus, suggesting that the anthocyanins are the main active components and that the effectiveness of bilberry extract may be lost by thermal degradation.

The suppressive effect of nonheated or heated Bilberon-25 on mast cell degranulation was well correlated with the antipruritic effect. Therefore, the inhibitory effect of bilberry anthocyanins on chronic pruritus of experimental dermatitis might be mediated, at least in part, by inhibition of mast cell degranulation.

The anthocyanin-rich but not anthocyanidin-rich bilberry extract may be a useful dietary supplement for skin diseases involving pruritic symptoms, such as chronic allergic contact dermatitis, atopic dermatitis, and rhinitis. To ensure that products are rich in anthocyanin, quality control of dietary supplements containing bilberry extract should be performed not by spectrophotometry, but by chromatographic analytical methods such as HPLC or ultra high performance liquid chromatography.

Acknowledgments

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