



Contact sensitizing potential of annatto extract and its two primary color components, *cis*-bixin and norbixin, in female BALB/c mice[☆]

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ABSTRACT

The present studies were performed to examine the contact allergenic effects of an annatto extract (ANT) in female BALB/c mice. ANT at 5–10% induced a greater than threefold increase in lymph node cell proliferation when compared to the control in the LLNA. Moreover, a significant increase in the percent ear swelling at 24 h after ANT challenge was observed in the MEST. A significant increase in the percentage of B cells was also observed. To determine which of the two predominant coloring components (norbixin and bixin) in ANT was responsible for the sensitizing effects of ANT, norbixin was subsequently examined, with negative results being observed in both the LLNA and MEST following treatment with norbixin (1–20%). These findings suggested that perhaps bixin was responsible for the positive responses in both the LLNA and MEST following exposure to ANT. Therefore, further studies using a partially purified *cis*-bixin extract were conducted. Positive responses in both the LLNA and MEST were observed in mice treated with *cis*-bixin at the concentrations as low as 0.1–0.5%. These results have demonstrated that *cis*-bixin, but not norbixin, is likely a contact sensitizer and contributes to the contact hypersensitivity effects observed following dermal exposure to ANT in mice.

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1. Introduction

Annatto seeds, also known as ‘achiote seeds’, are commonly used in South American cooking (Lucas et al., 2001). Annatto dye is an orange–yellow food coloring extracted from the outer coatings of annatto seeds (Collins, 1992; Scotter et al., 2000; Evans, 2000). It has been used in cosmetics and to color dairy products (butter, margarine, cheese, etc.), smoked fish, snack foods, sausage, coffee creamers, ice cream, seasoning, beverages, cereals and body care products (Paumgartten et al., 2002). The seeds, leaves, and roots of the annatto tree (*Bixa orellana* L.) have also been used in Brazilian folk medicine to treat fevers, inflammatory conditions, and parasitic diseases (Paumgartten et al., 2002). Additionally, annatto seeds have been used to make body paints and lipsticks in Central and South American natives (Ebo et al., 2009). Two main

coloring components in annatto extracts are oil-soluble bixin (C₂₅H₃₀O₄) and alkaline-water soluble norbixin (C₂₄H₂₈O₄), which are classified as xanthophylls, a type of carotenoid. However, unlike β-carotene, they do not have the correct chemical structures to be vitamin A precursors (Preston and Rickard, 1980; Mercadante et al., 1996).

Reported adverse reactions associated with annatto dye ingestion include urticaria, angioedema and possibly atopic dermatitis (Mikkelsen et al., 1978; Juhlin, 1981; Nish et al., 1991; Fuglsang et al., 1994; Lucas et al., 2001). In a study performed by Mikkelsen et al. (1978), 56 patients suffering from chronic urticaria and/or angioneurotic edema were orally provoked with a dose of annatto extract equivalent to the amount found in 25 g of butter, and 26% of these patients reacted to the challenge at 4 h following ingestion. In contrast, annatto extracts are not reported to induce any genotoxicity, reproductive toxicity or carcinogenicity in animals (Havenland-Smith, 1981; Harllagan et al., 1995; Paumgartten et al., 2002). Although the immunological mechanism underlying the development of urticaria is believed to be primarily a type I hypersensitivity involving IgE, a number of urticants have been identified as capable of producing clinical manifestations through both immediate and delayed-type mechanisms (Harvell et al., 1994). Moreover, urticaria and contact dermatitis may have some common innate immune components, e.g. the inflammasome (Kambe et al., 2010). Annatto oil is used increasingly in body care

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products, where it adds a rich sunny color to creams, lotions, and shampoos (The Raintree Group, 1997). Cosmetic-induced contact dermatitis is common, and it has been suggested that annatto might cause contact dermatitis (Winter, 2009). However, no reported case studies involving the contact hypersensitivity of annatto were located in literature.

We hypothesized that annatto and possibly one or more of its coloring components could cause contact sensitization in female BALB/c mice, a strain that is susceptible to chemical-induced hypersensitivity reactions (Woolhiser et al., 1998). The objective of the present study was to evaluate the potential sensitizing effects of an annatto extract (containing both bixin and norbixin), and if positive, to determine which of the carotenoid pigments (i.e. bixin and norbixin) are sensitizers. Thus, both the irritancy effects and the sensitizing potential of an annatto extract (containing 84.10% bixin) and norbixin (purity > 98%) were examined using the local lymph node assay (LLNA), the irritancy assay (IRR) and the mouse ear swelling test (MEST). It has been shown that the combination of the LLNA and the MEST are capable of reliably detecting moderate and strong sensitizers (Gad et al., 1986; Botham, 1992; Kimber et al., 1989, 1991). Additional studies using a partially purified *cis*-bixin extract (90.52% purity) were performed to determine if bixin was responsible for the sensitizing effect. Moreover, there is evidence that mice exposed to contact allergens show increases in the percentage of B lymphocytes (B220⁺ or IgG/IgM⁺) in the draining lymph nodes (Sikorski et al., 1996; Gerberick et al., 2002; Manetz and Meade, 1999). Therefore, flow cytometric analysis, using a B cell marker, was also employed to further confirm the findings of the IRR, LLNA and MEST assays.

2. Materials and methods

2.1. Chemicals

Crude annatto extract (ANT), a yellow or orange-red pigment containing 84.10% bixin, was obtained from Kalsec Inc. (Kalamazoo, MI) through the Midwest Research Institute (Kansas City, MO). Norbixin (NOR), a reddish purple crystal pigment with a purity of 98.18% containing 88.56% *cis*-norbixin and 9.62% *trans*-norbixin, was purified from ANT using the solvent purification method following conversion of bixin to NOR (Midwest Research Institute, 2005). Briefly, bixin was refluxed in methanolic NaOH to produce an initial norbixin salt, which was further purified to the free acid using tetrahydrofuran. The chemical structures of *cis*- and *trans*-norbixins are shown in Fig. 1A (JECFA, 2003). Partially purified *cis*-bixin extract (BIX), a

reddish purple crystal containing 90.52% *cis*-bixin, was obtained using a solvent purification method. The purification process included washing annatto extracts twice with hexane and once with ethyl acetate (Midwest Research Institute, 2005a). The structure of *cis*-bixin (9'-*cis*-6,6'-diapocarotene-6,6'-dioic acid, monomethyl ester; C₂₅H₃₀O₄) is shown in Fig. 1B.

2.2. Vehicles

Solubility tests were performed to determine the suitable vehicle to be used for each chemical being evaluated, with the goal being to achieve the highest solubility. Following the recommendation of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), acetone-olive oil (4:1; AOO) and acetone, the top two preferred vehicles to be used in the hypersensitivity studies, were evaluated as potential vehicles for the studies (ICCVAM, 1999). ANT was soluble at concentrations of 25% (w/v) and below in acetone. NOR was soluble at concentrations of 20% (w/v) and below in acetone. BIX was soluble at concentrations of 12.5% (w/v) and below in AOO. Acetone (99% HPLC grade) was obtained from Sigma-Aldrich (St. Louis, MO). Olive oil (Extra Virgin Olive Oil, Bentonville, AR, Italy) was purchased locally. 1-fluoro-2,4-dinitrobenzene (2,4-dinitrofluorobenzene; DNFB; purity > 99.4%; Sigma), a known contact sensitizer, was used as the positive control for these studies.

2.3. Animals

Female BALB/c mice, 6–8 weeks old, were obtained from Charles River Laboratories (Raleigh, NC). The mice were determined to be hepatitis and Sendai virus free by serology testing. The animals were quarantined for at least 7 days prior to being placed on studies, and they were housed and maintained at 21–24 °C and 40–70% relative humidity, with a 12-h light/dark cycle. Food (Harlan Teklad laboratory diets NIH-07) and tap water were given *ad libitum* in all experiments. The mice were randomized prior to each study, and eight mice were assigned to each dose group. The animals were weighed and observed for signs of moribundity during the study. All animal procedures were conducted under an animal protocol approved by the VCU Institutional Animal Care and Use Committee (IACUC) in an AAALAC-accredited facility.

2.4. Combined Local Lymph Node Assay (LLNA) and Irritancy Assay (IRR)

The LLNA measures the sensitization or induction phase of the hypersensitivity response, with the endpoint being a measurement of ³H-thymidine incorporation into proliferating lymphocytes within the auricular draining lymph nodes. Historically, non-specific proliferation due to irritation has confounded LLNA interpretation. In order to conduct an irritancy assay without having to use additional animals, a combination of LLNA and IRR was conducted following a modification of previously published procedures (Woolhiser et al., 1998; Anderson et al., 2003). Prior to exposure, mice were weighed, randomized and tattooed for identification. Before chemical application on the first day of the study, measurements of ear thickness were made (pre-measurement) using a modified Mitutoyo micrometer (Mitutoyo, Japan) and reported as mm × 10⁻². On day 1, mice were treated

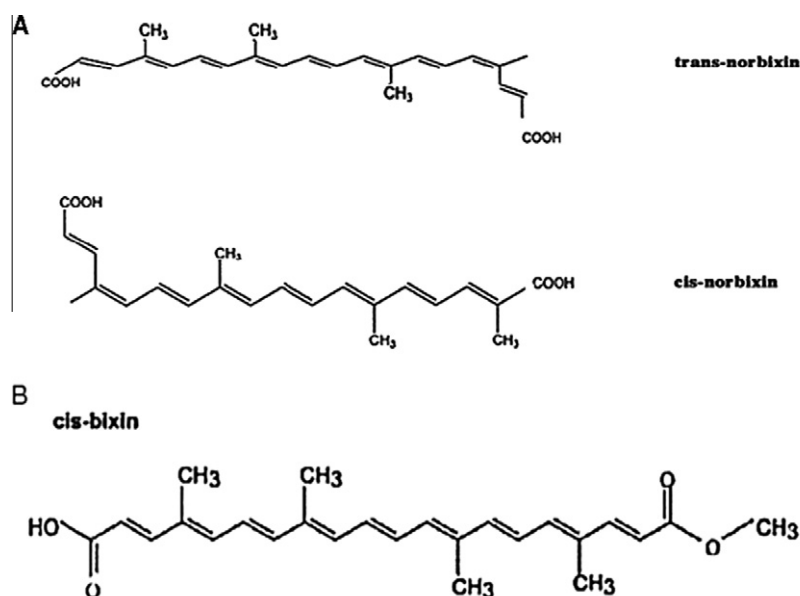


Fig. 1. (A) Chemical structures of norbixin; (B) Chemical structure of *cis*-bixin.

with the test substances (ANT, NOR, or BIX) at various concentrations, vehicle (acetone or AOO) or positive control (DNFB) by applying a total volume of 25 μ l to the dorsum of each ear. Identical treatments were repeated for the next two days. On days 4 and 5, the mice were rested. On day 4, 24 ($\pm$ 2) hours after the final treatment, measurements of ear thickness (post-measurement) were made in the same manner as the pre-treatment measurements for the IRR. The results of the IRR were expressed as percent ear swelling using the formula $\{[(\text{mean post-treatment ear thickness}/\text{mean pre-treatment ear thickness}) \times 100] - 100\}$. On day 6, all animals were injected with 0.25 ml ^3H -thymidine (20 μCi) by lateral tail vein (i.v.). Five hours later, mice were euthanized and both the right and left auricular lymph nodes from each mouse were excised and placed into 4 ml cold phosphate buffered saline (PBS). All lymph nodes were dissociated by mashing between the frosted ends of two microscope slides. Lymph node cells were washed twice and then resuspended in 3 ml of 5% trichloroacetic acid (TCA) (w/v, distilled H_2O), and allowed to set for 18–24 h at approximately 4 $^{\circ}\text{C}$. Cell suspensions were then centrifuged and resuspended in 1 ml of 5% TCA and subsequently transferred into 5 ml of scintillation cocktail (Beckman Coulter, Inc., Fullerton, CA). ^3H -thymidine incorporation (CPM) for each sample was determined using liquid scintillation and a beta counter (1214 Rackbeta LKB Wallac, Perkin Elmer Instruments, MA, USA). Disintegrations per minute (DPM) for each mouse were calculated as $(\text{CPM} - \text{background count})/(\text{Beta Counter efficiency})$. Results were expressed as lymph node DPM/mouse. Based on the ICCVAM-recommended criteria (ICCVAM, 1999), a chemical was classified as positive if at least one concentration reached or exceeded a stimulation index (SI) ≥ 3 , with the results being statistically significant and dose-responsive. The value representing three times the vehicle DPM is noted on the figure to indicate a SI of 3.

2.5. Mouse Ear Swelling Test (MEST)

The MEST evaluates the elicitation phase of the hypersensitivity response, with the endpoint being a measurement of ear thickness caused by an inflammatory reaction to a recall antigen (edema). Based on the results of the LLNA and IRR, various concentrations of annatto extracts (Table 1) were selected for the sensitization and elicitation phases in the MEST (Gad et al., 1986). On day 1, the dorsal lumbar surface of each animal was shaved. Exposure was accomplished by a direct application of 50 μ l of vehicle, ANT, NOR, BIX, or DNFB to the prepared site with a pipette (sensitization phase). The sensitization procedures were repeated on days 2 and 3. Mice were rested for days 4–7. On day 8, the thickness of the right ear of each mouse was measured (pre-measurement) prior to the challenge on the right ear using a modified Mitutoyo micrometer and reported as $\text{mm} \times 10^{-2}$. After pre-measurement, mice were challenged on the dorsal side of the right ear pinna with a total volume of 25 μ l of vehicle, ANT, NOR, BIX, or DNFB. Post-measurements of ear thickness were then made 24 ($\pm$ 2) hours and 48 ($\pm$ 2) hours after challenge in the same manner as the pre-measurements. The results were expressed in percent ear swelling for 24 and 48 h as described for the IRR.

2.6. Flow cytometric analysis

The percentages of T and B lymphocyte subsets in the auricular lymph node cells were measured using flow cytometric analysis with modification (Karrow et al., 2001). Lymph node single-cell suspensions were prepared and incubated with anti-CD16/32 (Mouse FC block) for 5 min at 4 $^{\circ}\text{C}$ to block non-specific binding. The cells were then labeled with appropriate antibodies for 30 min on ice (4 $^{\circ}\text{C}$). Following the initial staining with antibody and washing twice with staining buffer (1% BSA and 0.1% sodium azide in PBS), enumeration was performed on a Becton Dickinson FACScan Flow Cytometer in which a forward scatter threshold was set high enough to eliminate red blood cells. The data were analyzed using the CellQuest software (Becton Dickinson, San Jose, CA). The antibodies utilized, which included goat anti-mouse Ig-FITC, anti-mouse CD3-PerCP, and anti-mouse B7.2-PE, were obtained from BD PharMingen (San Diego, CA, USA) and diluted 1:80 with staining buffer.

2.7. Statistical analysis

The data obtained in this study were first tested for homogeneity of variances using Bartlett's Chi Square Test. Homogeneous data were evaluated by a parametric one-way analysis of variance. When significant differences occurred, treatment groups were compared to the appropriate control using Dunnett's test. If data were non-homogeneous, they were evaluated using a non-parametric analysis of variance. When significant differences occurred, treatment groups were compared to the appropriate control using the Wilcoxon rank test. For the Irritancy and LLNA results, treatment groups were compared to vehicle control group; the positive control and naive animals were compared to the vehicle control using the Student's *t*-test. For the MEST, the vehicle control group was compared to the vehicle irritancy control (VHIC) using the Student's *t*-test. All treatment groups were compared to VHIC using Dunnett's test. The positive control was compared to the positive control challenge only (PCCO) using the Student's *t*-test.

3. Results

In all studies conducted, mice appeared clinically normal throughout the course of the experiments. Mouse body weight ranged between 18 and 20 g at the end of the studies, and there were no significant changes in body weight for any of the treatment groups when compared to the vehicle control group over the course of the studies (data not shown).

ANT was the first substance examined for its sensitizing potential, producing positive responses in both the LLNA and the MEST. In the LLNA, exposure to ANT at the 5% and 10% concentrations significantly increased the proliferation of the draining lymph node cells above the threefold threshold when compared to the vehicle control (Fig. 2A). At 25% ANT, the increase was also statistically significant when compared to the vehicle animals; however, it did not reach the threefold threshold. Exposure to ANT at lower concentrations (1% and 2.5%) did not produce any statistically significant effects.

In the MEST, ANT treatment concentrations were 5% and 10% for the sensitization phase, and 10% for the challenge phase. ANT-treated animals exhibited significant increases in percent ear swelling when compared to the VHIC group (background control) 24 h after ANT challenge (Fig. 2B). Increases in percent ear swelling were also observed at 48 h post-challenge, however, none of the increases reached the level of statistical significance when compared to the VHIC group (Fig. 2C).

A possible mechanism by which contact sensitization may occur is by altering the number of B cells in the auricular draining lymph nodes (Gerberick et al., 2002). As demonstrated in Fig. 3A, dermal exposure to ANT at 5%, but not at 2.5%, 10% and 25%, for 4 days on the ears induced a significant increase in the percentage of B cells (Ig^+) in the auricular draining lymph node cells at 24 h after the last ANT treatment. In the MEST study, a significant increase in the percentage of B cells was also observed 48 h after challenge with 10% ANT in the group that had been sensitized with 10% ANT (Fig. 3B). There were no significant changes in the percentage of T cells in mice treated with ANT (data not shown).

Table 1
Experimental design for MEST with annatto extracts.^a

Group	Sensitization phase	Challenge phase	Designation group
1	Acetone or AOO	Acetone or AOO	Vehicle (VH)
2	Acetone or AOO	ANT (10%) NOR BIX	Vehicle irritancy control (VHIC)
3	ANT (5% and 10%) NOR (10% and 20%) BIX (0.05% and 0.5%)	ANT (10%) NOR (20%) BIX (0.5%)	Treatment groups
4	Acetone or AOO	0.15% DNFB	Positive control challenge only (PCCO)
5	0.15% DNFB	0.15% DNFB	Positive control (PC)

^a Because the test compound might have the potential to induce an irritant effect, which could confound the measurement of mouse ear swelling, both VH and VHIC were included.

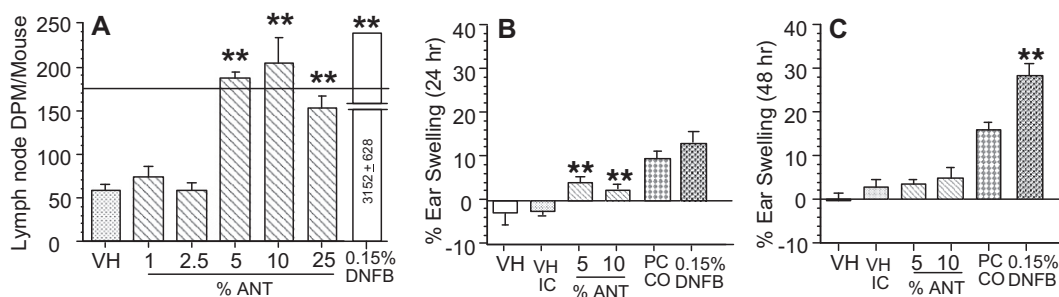


Fig. 2. Annatto (ANT) induced positive responses in both the LLNA (A) and MEST (B: 24 h after challenge, and C: 48 h after challenge) in female BALB/c mice. For the MEST, both 5% and 10% ANT-treated mice were challenged with ANT at 10% on day 8. Data were expressed as the means $\pm$ S.E. ($N = 8$ in each group). VH = acetone; VHIC = vehicle, irritancy control; PCCO = positive control, challenge only; positive control (PC) = 0.15% 2,4-Dinitrofluorobenzene (DNFB). ** $p \leq 0.01$ as compared to the corresponding control group. The solid horizontal line across the graph in panel A indicates that stimulation index (SI) = 3.

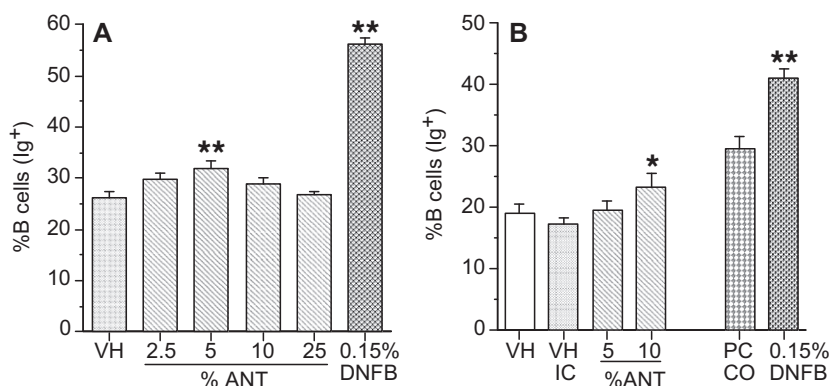


Fig. 3. Annatto (ANT) increased the percentage of B cell (Ig^+) in the auricular draining lymph nodes in female BALB/c mice. (A) Twenty-four hours after the last treatment of ANT with a total four days of dermal exposure on the ears. (B) Forty-eight hours after the challenge of ANT in the MEST. PC = 0.15% 2,4-dinitrofluorobenzene (DNFB). Data were expressed as the means $\pm$ S.E. ($N = 8$ in each group). * $p \leq 0.05$, ** $p \leq 0.01$ as compared to the corresponding control group.

In the irritancy assay, there were no statistically significant changes in percent ear swelling in animals exposed to ANT at any of the tested concentrations (1–25%) when compared to the vehicle controls (data not shown). In addition, there was no significant difference between the naïve group and the vehicle control group in percent ear swelling (data not shown). The positive control DNFB (0.15%) produced the expected increases in each of the assays when compared to the corresponding controls, except for the ear thickness at 24 h post-challenge in the MEST (Figs. 2A–C and 3A and B).

To determine which of the two coloring components was responsible for the sensitizing effects of ANT, NOR (with a purity of 98.12%) was subsequently examined for its hypersensitivity-inducing potential in the LLNA at concentrations ranging from 1% to 20% (w/v in acetone). Dermal exposure to NOR had no effect on lymph node cell proliferation (Fig. 4A).

Consistent with the negative results in the LLNA, NOR did not produce any changes in percent ear swelling at either 24 h (Fig. 4B) or 48 h (Fig. 4C) after challenge in the MEST. The treatment concentrations of NOR were 10% and 20% for the sensitization phase and 20% for the challenge phase. Additionally, NOR did not induce any changes in the percentages of B and T cells in the draining lymph nodes at 24 and 48 h after challenge with NOR (data not shown).

In the irritancy assay, there were no statistically significant changes in the percent ear swelling in groups of animals exposed to NOR (data not shown). As expected, the positive control DNFB (0.15%) produced significant increases in each of the above assays when compared to the appropriate control, with the

exception of ear thickness at 24 h post-challenge in the MEST (Fig. 4A–C).

Because NOR demonstrated no potential to induce contact hypersensitivity, studies were conducted to determine the sensitizing potential of BIX, the other primary carotenoid in ANT extract. Although BIX (purity 90.52%) used in the present study was only partially purified, it induced positive responses in both LLNA and MEST at lower concentration than ANT (an extract containing 84.1% bixin). In the first LLNA study, various concentrations of BIX ranging from 0.5% to 12.5% (w/v) were used. As demonstrated in Fig. 5A, there was a significant increase in auricular lymph node cell proliferation in mice treated with 0.5% BIX. Thus, a second experiment was conducted using lower concentrations of BIX. Dermal exposure to 0.1% BIX produced a similar increase (statistically significant but below the level of threefold increase over the control) in the proliferation of the draining lymph node cells (Fig. 5B).

Based on the LLNA results, BIX treatment levels selected for the MEST were 0.05% and 0.5% for the sensitization phase, and 0.5% for the challenge phase. Animals that were sensitized with 0.5% BIX and challenged with 0.5% BIX exhibited a significant increase in percent ear swelling when compared to the VHIC group at 48 h after challenge (Fig. 5D). None of the BIX test groups demonstrated significant differences in percent ear swelling from the VHIC group at 24 h after challenge (Fig. 5C).

There were no differences in percent ear swelling between BIX exposed animals and vehicle controls in the irritancy assay (data not shown). As expected, the positive control DNFB (0.15%) produced significant increases in each of the above assays when compared to the appropriate controls (Fig. 5A–D, and data not shown).

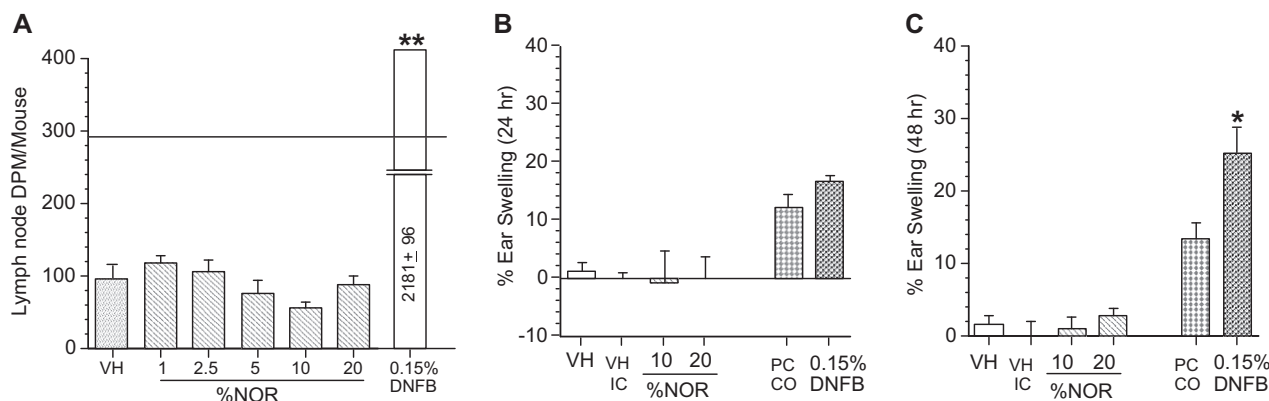


Fig. 4. Norbixin (NOR) had no effects in either the LLNA (A) or the MEST (B: 24 h after challenge, and C: 48 h after challenge) in female BALB/c mice. For the MEST, both 10% and 20% NOR-treated mice were challenged with NOR at 20% on day 8. Data were expressed as the means $\pm$ S.E. ($N = 8$ in each group). VH = acetone; VHIC = vehicle, irritancy control; PCCO = positive control, challenge only; positive control (PC) = 0.15% 2,4-Dinitrofluorobenzene (DNFB). * $p \leq 0.05$, ** $p \leq 0.01$ as compared to the corresponding control group. The solid horizontal line across the graph in panel A indicates that stimulation index (SI) = 3.

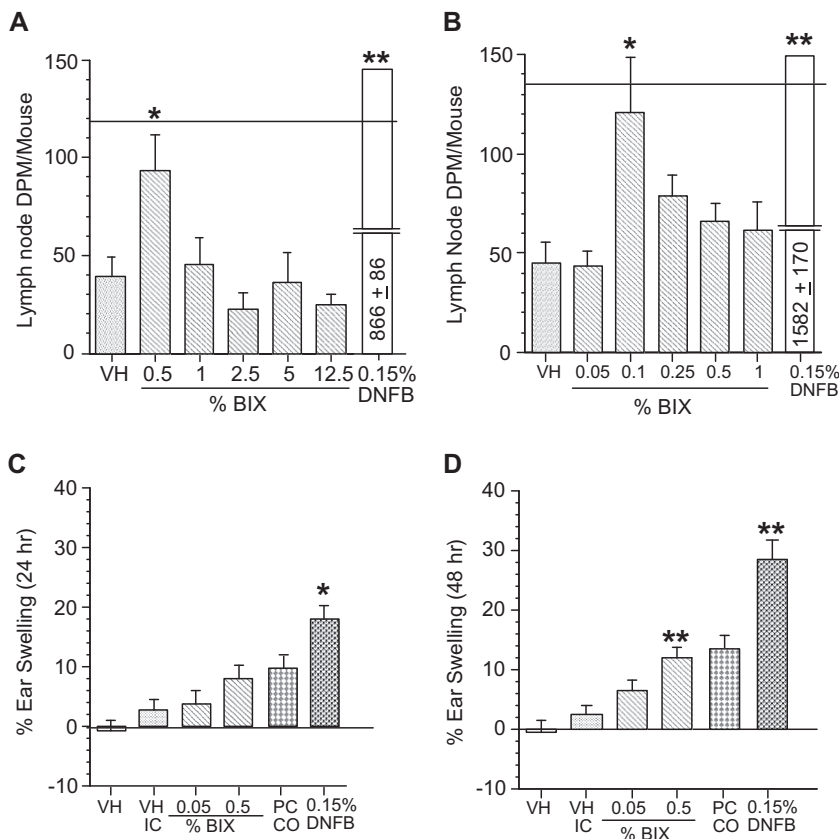


Fig. 5. The *cis*-Bixin (BIX) induced positive responses in both the LLNA (A and B) and MEST (C: 24 h after challenge, and D: 48 h after challenge) in female BALB/c mice. For the MEST, both 0.05% and 0.5% BIX-treated mice were challenged with BIX at 0.5% on day 8. Data were expressed as the means $\pm$ S.E. ($N = 8$ in each group). VH = acetone; VHIC = vehicle, irritancy control; PCCO = positive control, challenge only; positive control (PC) = 0.15% 2,4-dinitrofluorobenzene (DNFB). * $p \leq 0.05$, ** $p \leq 0.01$ as compared to the corresponding control group. The solid horizontal line across the graph in panel A indicates that stimulation index (SI) = 3.

4. Discussion

ANT extracts contain both *cis*-bixin and norbixin. Several lines of evidence from the present study suggest that *cis*-bixin is most likely a contact sensitizer. First, both ANT (an extract containing 84.1% bixin) and BIX (an extract containing 90.52% *cis*-bixin) were shown to be skin sensitizers as demonstrated by significantly enhanced proliferative responses in auricular draining lymph nodes during the induction phase of contact sensitization after topical treatment on the ears (the LLNA assay). Second, in the MEST, which assesses the

elicitation phase of contact sensitization, a significant increase of the percent ear swelling was also observed in both ANT and BIX-treated mice. Third, BIX, which contained more *cis*-bixin than ANT, elicited the sensitizing responses at much lower concentrations than ANT, and fourth, NOR (an extract containing 98.18% norbixin) did not induce significant changes in the LLNA, MEST, IRR, or lymph node lymphocyte populations. Therefore, results suggest that the *cis*-bixin component of ANT is most likely responsible for ANT-mediated contact hypersensitivity responses, or is, at a minimum, a contributor to these effects. Since *cis*-bixin has been reported to be the

predominant coloring compound in the annatto dye, *trans*-bixin was not evaluated in this study and therefore cannot be confirmed or eliminated as a contributor to the sensitizing effects of ANT.

It has been reported that some irritants induce proliferation in the draining lymph nodes, which are regarded as false positives of the LLNA (Gerberick et al., 1992; Montelius et al., 1994; Basketter et al., 1998). Thus, it is important to perform an irritation test to determine the potential for irritation if a positive response is obtained for a test compound in the LLNA. In our studies, no irritancy response was observed for any of the three extracts.

If dermal exposure to the compound produces positive responses in both the LLNA and MEST, the compound should be classified as a contact allergen. If dermal exposures to the compound produce no responses in either of the LLNA or MEST, the compound should be classified as a non-sensitizer. Using the above criteria, both ANT and BIX should be classified as a sensitizer, while NOR should be classified as a non-sensitizer. Although it has been accepted by several agencies as a stand-alone test for skin sensitization, the LLNA has not been accepted by regulatory agencies for investigations into the sensitization potential of mixtures, and the calculation of an EC3 value was formulated to determine the potency of individual chemicals, not mixtures (Gamer et al., 2008). In our studies, ANT was identified as a sensitizer, and subsequent studies suggested that bixin was most likely responsible for the dermal sensitizing effect of ANT. Although both ANT and BIX were identified as sensitizers in the MEST, the magnitudes of maximal response in the LLNA were different for ANT and BIX. Specifically, the response produced by 10% ANT was above the EC3 value, while that of BIX was below the EC3 value. If only the LLNA was performed, BIX may have been mistakenly classified as a non-sensitizer. Several mechanisms may be responsible for differences in the lymphocyte proliferation of mixtures as compared to the individual sensitizing constituents, including additive, synergistic or antagonistic reactions between the chemical components (Anderson et al., 2009). It would be interesting to determine if NOR and BIX interact synergistically to increase the lymphocyte proliferation in the LLNA.

Utilization of percent B cells in draining lymph nodes to differentiate contact sensitizers from irritants has been reported (Gerberick et al., 2002). Mice exposed to contact sensitizers, but not irritant compounds, exhibited increases in the percentage of B lymphocytes in the draining lymph nodes (Sikorski et al., 1996; Gerberick et al., 2002). In our studies, an increase in the percentage of B cells (Ig⁺) was indeed observed in mice treated with 5% ANT. In addition, significant increases in the percentage of B7.2 (CD86) positive cells were also observed in the draining lymph nodes of mice treated with BIX (data not shown).

There is evidence to suggest that free radicals such as nitric oxide (NO) are involved in the expression of contact hypersensitivity (Sikorski et al., 1996; Ross et al., 1998; Bruch-Gerharz et al., 1998). Both bixin and norbixin has been reported to possess antioxidant properties (Andressa et al., 2002; Silva et al., 2001; Zhao et al., 1998; Júnior et al., 2005). It is possible that the non-statistically significant increases in the percent ear swelling in the MEST at 48 h after ANT challenge may reflect the antioxidant nature of both bixin and norbixin. Silva et al. (2001) have reported that treatment with bixin at 5.0 mg/kg by gavage resulted in a statistically significant decrease in oxidative damage in rats as evidenced by reductions in cisplatin-induced abnormal metaphases and lipid peroxidation. Therefore, the antioxidant activity of *cis*-bixin may be responsible for the bell-shaped response induced by BIX in the LLNA and by ANT in the percentage of B cells in the draining lymph nodes following exposure on the ear.

It should be noted that BIX produced significant positive responses in both the LLNA and MEST at much lower concentrations than ANT. The optimal concentrations were 5–10% for ANT, and

0.1–0.5% for BIX. Several reasons may account for the differences. The choice of vehicle can influence skin sensitization (Marzulli and Maibach, 1976). As indicated previously, the choices of vehicle in these studies were based on the solubility tests and the ICCVAM recommendation, e.g. AOO was used for BIX, while acetone was used for ANT. Thus, the vehicles (AOO vs. acetone) may have contributed to the differing effective concentrations between ANT and BIX. Another explanation for the different optimal concentrations observed for ANT and BIX may be the purity of the compounds. BIX was a partially purified extract containing 90.52% *cis*-bixin, while ANT was an extract containing 84.1% bixin. The 6% bixin difference between ANT and BIX is critical, not just because bixin is a sensitizer that contributes directly to the potency of BIX, but also possibly because of the 6% norbixin difference when considering that norbixin has antioxidant effects but no sensitizing potential.

In our study, the No Observable Adverse Effect Level (NOAEL) was 2.5% for ANT and 0.05% for BIX, which was approximately equal to 62.5 mg ANT/kg body weight and 1.25 mg BIX/kg body weight, respectively. If the NOAEL is divided by the standard 10-fold inter- and 10-fold intra-species uncertainty factors, the allowed daily dermal exposure should be equivalent to levels of 0.625 and 0.0125 mg/kg for ANT and BIX, respectively. Currently, the allowed daily intake (ADI) in Europe is less than 1.25 mg/kg body weight/day for annatto (a preparation containing 2.6% carotenoids expressed as bixin) and less than 0.065 mg/kg body weight/day for the pure pigment bixin (JECFA, 1982, 2007). The ADI of 1.25 mg/kg body weight/day for annatto containing 2.6% bixin is well justified after taking into consideration the bixin content; however, it seems that the interactions among the components in the annatto preparation may not have been considered in the determination of the ADI for the pure pigment bixin (0.065 mg/kg body weight/day). Our data suggest that bixin in its purified form is a more potent sensitizer following dermal exposure. The absorption and metabolic kinetics underlying oral exposure for ADI determination and dermal exposure in our studies may be different; thus, future studies are necessary to determine if there exist any interactions among the components in the annatto that may result in adverse health effects when ingested.

In summary, the results from our studies suggest that the *cis*-bixin component in annatto extract was most likely responsible for the contact sensitization induced by ANT in female BALB/c mice. Further studies to determine the biochemical and immunological mechanisms underlying the sensitizing potential of bixin may prove useful in a more thorough evaluation of the adverse health effects following exposure to annatto dye.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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