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Stability in candy products of neocandenatone, a non-genotoxic purple pigment from *Dalbergia congestiflora* heartwood

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ABSTRACT

Neocandenatone is a newly identified purple pigment that is present in the heartwood of *Dalbergia congestiflora*. Three pigment samples with different neocandenatone concentrations were prepared: fraction A (crude extract), fraction B (degreased extract), and fraction C (pure pigment), containing 6.95%, 70.55%, and 98.00% neocandenatone, respectively. None of the three fractions showed mutagenicity using the Ames test with *Salmonella typhimurium* strains TA98, TA100, and TA102, and these fractions were not genotoxic according to the micronucleus test. Fraction B was selected to pigment gelatin gummies at 0.007, 0.014, 0.028, and 0.031% and hard candies at 0.025, 0.05 and 0.075% w/w. As a comparison, a commercial anthocyanin (3% enocyanin) was used to prepare the gummies (0.025, 0.05, 0.062, and 0.1%) and hard candies (025, 0.05, and 0.1%). The H° values for gummies ranged from 1.78 to 65.64° and 7.19 to 45.54° for neocandenatone and anthocyanin, respectively, while those for hard candies ranged from 33.64 to 62.52° and 25.31 to 68.16° for neocandenatone and anthocyanin, respectively. The concentration of neocandenatone in selected samples of gummies (63.3% sugars w/w) and hard candies (80% sugars w/w) showed no significant differences ($p > 0.05$) over 2 months. In contrast, the enocyanin concentration decreased following first-order kinetics in both gummies ($k = 0.104\ w^{-1}$) and hard candies ($k = 0.084\ w^{-1}$).

1. Introduction

Dalbergia species (Leguminosae) are known for their deeply pigmented heartwoods of varying colors. Often valued for their use in wooden crafts, the heartwoods are also used in traditional medicine and have antibiotic, antioxidant, and cytotoxic activities (Hamburger, Cordell, Tantivana & Ruangrungsi,1987; Zhao, Guo, & Zhang, 2000; Rana, Kumar, & Soni, 2012). The *Dalbergia congestiflora* Pittier tree, known in Mexico as campincerán, is a species from the tropical and mountainous deciduous forests of western Mexico. The wood is known for its hardness and resistance to insect attack and is used by native people to manufacture musical instruments. The purple pigment from the *D. congestiflora* heartwood is also extracted to dye fabrics and, when combined with resins, to paint handcrafted and wooden appliances. Neocandenatone is a newly identified isoflavan cinnamylphenol quinone methide (Fig. 1) that shows high stability and exhibits red and purple colors with pH changes in a manner similar to anthocyanin (Barragán, Peralta, González, & Karchesy, 2004). Neocandenatone has been shown to have antioxidant activity and *in vitro* cytotoxic activity against HeLa cervical cancer cells (Ramón-Gallegos, Vega-Barrita, & Barragán-Huerta, 2006; Pérez-Gutiérrez & García-Báez, 2013). Because of its color and biological properties, this new pigment may have applications as a colorant for foods.

There is a high demand for pigments, both to restore their natural levels in processed foods and to give color to foods that are not naturally pigmented, such as candies and soft drinks. Therefore, synthetic and extracted pigments are incorporated into the final food products. There is evidence that synthetic colorants, such as red and yellow azo-dyes, may form aromatic amines with carcinogenic effects when they are metabolized in the gut (Drumond et al., 2011). Because consumers are currently demanding products that contain natural ingredients, food scientists and manufacturers have begun to investigate the use of natural pigments, which may possess antioxidant properties

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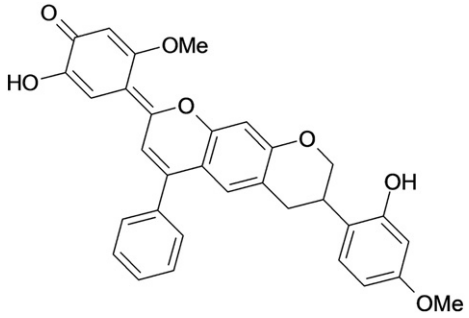

Fig. 1. Structure of neocandenatone.

and contribute to prevention of many degenerative diseases (Attila & Deli, 2011).

The application of new pigments requires chemical and technical assessments based on knowledge of their chemical composition, purity, toxicological evaluation, stability during storage, and reactions in model food systems (FAO, 2006).

Additionally, to protect human health, all new chemicals must be subjected to screening for genotoxic activity. The Ames test (Ames, Lee, & Durston, 1973b) is widely recommended by international organizations such as the Environmental Protection Agency (EPA), U.S. Food and Drug Administration (FDA), and International Agency for Research on Cancer (IARC) and is typically the first step in genotoxic evaluation for *in vitro* risk assessment studies (Arriaga-Alba, Montero-Montoya, & Espinoza Aguirre, 2012). After the Ames test, potential genotoxic agents are generally evaluated using the *in vivo* micronucleus test (OECD, 2007).

The successful application of natural colorants in foods primarily depends on their stability in the matrix where they will be applied. Therefore, many studies have investigated the impact of anthocyanin structure, pH, temperature, light, oxygen, and interactions with the food matrix (*i.e.*, copigments, sugars, proteins, ascorbic acid, and enzymes) on pigment stability (Cavalcanti, Santos, & Meireles, 2011; Sadilova, Stintzing, Kammerer, & Carle, 2009).

In this work, three neocandenatone samples of different purities were obtained from the heartwood of *D. congestiflora*, and the genotoxicities of these samples were analyzed using Ames and micronucleus tests. A selected pigment preparation was applied to model systems of candy products (gelatin gummies and hard candies), and its stability in those products was evaluated during storage and compared with candies pigmented using a commercial anthocyanin.

2. Materials and methods

2.1. Materials

The heartwood of *D. congestiflora* was collected in Michoacán, Mexico. A voucher specimen (EBUM-10194) was deposited in the herbarium of the University of San Nicolas de Hidalgo, Morelia, Michoacán, Mexico. Enocyanin powder (3%) was a kind gift from Jobari Colors and Flavors, México. All chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade.

2.2. Methods

2.2.1. Pigment extraction

Dried and ground heartwood of *D. congestiflora* (10 g) was added to 50 mL methanol, placed in a 150-mL flask, and refluxed at 76 °C for 30 min. Extraction of the heartwood was repeated two successive times under the same conditions to extract all the neocandenatone from the heartwood (Barragán, Hernández, & Peralta, 1999). The extracts were dried in a rotary evaporator (Büchi-Rotavapor R-210, Germany) at 40 °C to dryness, and their weights were recorded.

The methanolic dried extract, called fraction A, was placed in a Soxhlet apparatus and extracted under reflux with 250 mL diethyl ether for 3 h. The degreased powder obtained from this procedure (called fraction B) was dried in a fume extractor at 25 ± 1 °C.

One hundred milligrams of fraction B was dissolved in 0.5 mL methanol and purified by preparative thin-layer chromatography (silica gel $60F_{234}$, 20×20 cm, 2 mm, Merck, Mexico), using ethyl acetate–ethanol 4:1 as an eluent, to yield pure neocandenatone (fraction C) (Barragán et al., 2004). This pure pigment was used as the standard for subsequent HPLC analysis to quantify the pigment purities of fractions A and B.

2.2.2. Neocandenatone purity in fractions A and B

The neocandenatone concentrations in fractions A and B were determined by HPLC analysis using a 1260 Infinity Agilent Chromatograph equipped with a diode array detector and a quaternary pump system (Felisa, Germany). Fractions A and B (0.5 mg mL^{-1}) were dissolved in methanol, filtered through 0.45-μm nylon syringe filters (Fisher, USA), and then injected onto the chromatograph. The separation of samples was conducted using a C18 column ZORBAX Eclipse Plus (250 × 4.6 mm; 5 μm, Agilent, Mexico) at 25 °C with a gradient elution of 2.5% acetic acid (solvent A) and acetonitrile (solvent B). The following elution procedure was used: 100%:0% A:B (v/v) for 0 min; linear gradient at 50%:50% A:B (v/v) for 3 min; linear gradient at 0%:100% A:B (v/v) for 11 min, and maintained at 0%:100% A:B for 2 min. The mobile phase was pumped at a flow rate of 1 mL min^{-1}, and the response was detected at 260 and 550 nm.

A standard curve for neocandenatone was made using the pure pigment (fraction C) at 0.125, 0.25, 0.50, 0.75, or 1.0 mg mL^{-1} in duplicate using the same chromatographic conditions. Calculation of the neocandenatone concentrations in fractions A and B was performed by interpolating values from the standard curve.

2.2.3. Genotoxicity assays

Genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage by various mechanisms. A single test is not effective for detecting all genotoxic mechanisms relevant in tumorigenesis, therefore a standard test battery for assessment of mutagenicity should be conducted. This battery include a bacterial reverse gene mutation test (Ames assay) and an *in vivo* assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay. Negative results in appropriate *in vivo* assays (usually two) have been generally considered sufficient to demonstrate absence of significant genotoxic risk (ICH, 2012). In this research, preliminary studies on probably genotoxicity of neocandenatone using Ames and *in vivo* micronucleus assays have been conducted.

2.2.3.1. Ames test. Fractions A, B, and C from the heartwood extract were assayed as potential mutagens using the Ames tube-incorporation test. *Salmonella typhimurium* (His−) strains TA98 (his 03502, rfa uvrB pKm101), TA100 (his G46, rfa uvrB pKm101), and TA102 (his G428, rfa pQ1 pKm101), were grown in 25% nutrient broth (NB) liquid medium for 16 h at 37 °C in agitation at 90 rpm. A 100-μL suspension of strain TA98, TA100, or TA102 was transferred to a sterile screw-top tube with 2 mL of 0.6% soft agar, and the extracts were dissolved in DMSO and added at different concentrations (100, 50, and 25 μg/plate for fractions A and B or 50, 25, or 12.5 μg/plate for fraction C). The assay was performed with or without 500 μL of an enzymatic liver fraction (S-9 mix) obtained from male Wistar rats treated with 10% Aroclor-1254 (Ames, Durston, Yamasaki, & Frank, 1973a). The total tube content was spread immediately onto plates with Vogel-Bonner medium and incubated for 48 h at 37 °C. All experiments were performed three times in triplicate. The number of revertant colonies was determined using a Fisher colony counter. The reversion rate was compared to control plates with and without mutagen. Positive controls included 10 μg 2-aminoanthracene (AA)/plate as a mutagen for TA98, TA100,

Table 1
Formulations of gummies and hard candies.

Ingredients		g 100 g^{-1} product	
		Gelatin gummies	Hard candies
Gelatin		6.7	–
Sucrose		25.3	56
Glucose syrup		38.0	24
Glycerol		6.3	–
Water		22.8	19
Flavoring		0.1	0.1
Citric acid		0.3; 1.0	0.7; 1.0
Pigment	Neocandenatone	0.007; 0.014; 0.028; 0.031	0.025; 0.05; 0.075
	Anthocyanin	0.025; 0.050; 0.062; 0.1	025; 0.050; 0.1

and TA102 when enzymatic fraction S9 was added. In the absence of S9 mix, mutations for TA98, TA100, and TA102 were induced with picrolonic acid (50 μg/plate), N-methyl-N′-nitro-N-nitrosoguanidine (NMNG, 10 μg/plate), and oxide-4-nitroquinone (OQ, 10 μg/plate), respectively. The mutagenic index (MI) was calculated as the average number of revertants divided by the average number of revertants in the control. A sample was considered non-mutagenic when the MI was lower than 2 for at least one of the tested concentrations (Bernstein, Kaldor, McCann, & Pike, 1982; Margolin, Kaplan, & Zeigert, 1981).

For testing in candy products, one of the three fractions analyzed was selected based on sample purity and the absence of mutagenicity.

2.2.3.2. Micronucleus test. Animal studies were conducted in compliance with the Official Mexican Standard for the Care and Use of Laboratory Animals (NOM-062, 1999) and were approved by the appropriate Institutional Animal Care and Use Committee (Act ENMH-CB-0094-2014). A MicroFlowPLUS Kit (Mouse blood) from Litron Laboratories (Rochester, NY, USA), containing mouse anti-CD71 antibodies, mouse platelet antibody, diluent solution, RNase solution, propidium iodide (PI) solution, and biological samples (positive control samples, negative control samples, CD71-setup samples, and fixed malaria-infected biostandard blood samples), was purchased. Additional positive control (cyclophosphamide at 50 mg kg^{-1} I.P.) and negative control (propylene glycol as vehicle and water) samples were used. Male BALB/c mice (22 ± 2 g) were maintained at 23 °C ± 1 with a 12-h light/dark cycle and were fed *ad libitum* throughout the experimentation.

One hundred-microliter samples of fractions A and B were dissolved in propylene glycol and were administrated intragastrically in each mouse after fasting for 8 h. Fasting continued for 2 h after treatment, and blood samples were collected after 48 h for determination of micronuclei content. Each group (n = 3 mice per group) was tested using 1000 mg kg^{-1} fraction A or 100 mg kg^{-1} fraction B. Peripheral blood samples for analysis of micronucleated reticulocytes (MN-RETs) were collected by cutting a small part from the end of the tail of each mouse. Analysis of micronuclei in the mature normochromatic erythrocyte population (MN-NCEs) was performed using a MicroFlowPLUS Kit (mouse) according to the kit's instructional manual (Cammerer, Elhajouji, & Suter, 2007).

The presence of MN-RETs and MN-NCEs was analyzed in peripheral blood samples. The cells of interest (RETs and NCEs) were labeled with fluorescein isothiocyanate (FITC)-conjugated antibodies directed against a cell surface antigen (CD71 transferrin receptor). Micronucleated cells were detected by analyzing the fluorescent signal produced by propidium iodide staining and were quantified using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

The percentages of RETs, MN–RETs, and MN–NCEs were calculated according to Eqs. (1), (2), and (3):

$$\%\ \mathrm{RET} = \left[\frac{(\mathrm{MN{-}RET}) + (\mathrm{RET})}{(\mathrm{MN{-}RET}) + (\mathrm{RET}) + (\mathrm{MN{-}NCE}) + (\mathrm{NCE})}\right] * 100 \qquad (1)$$

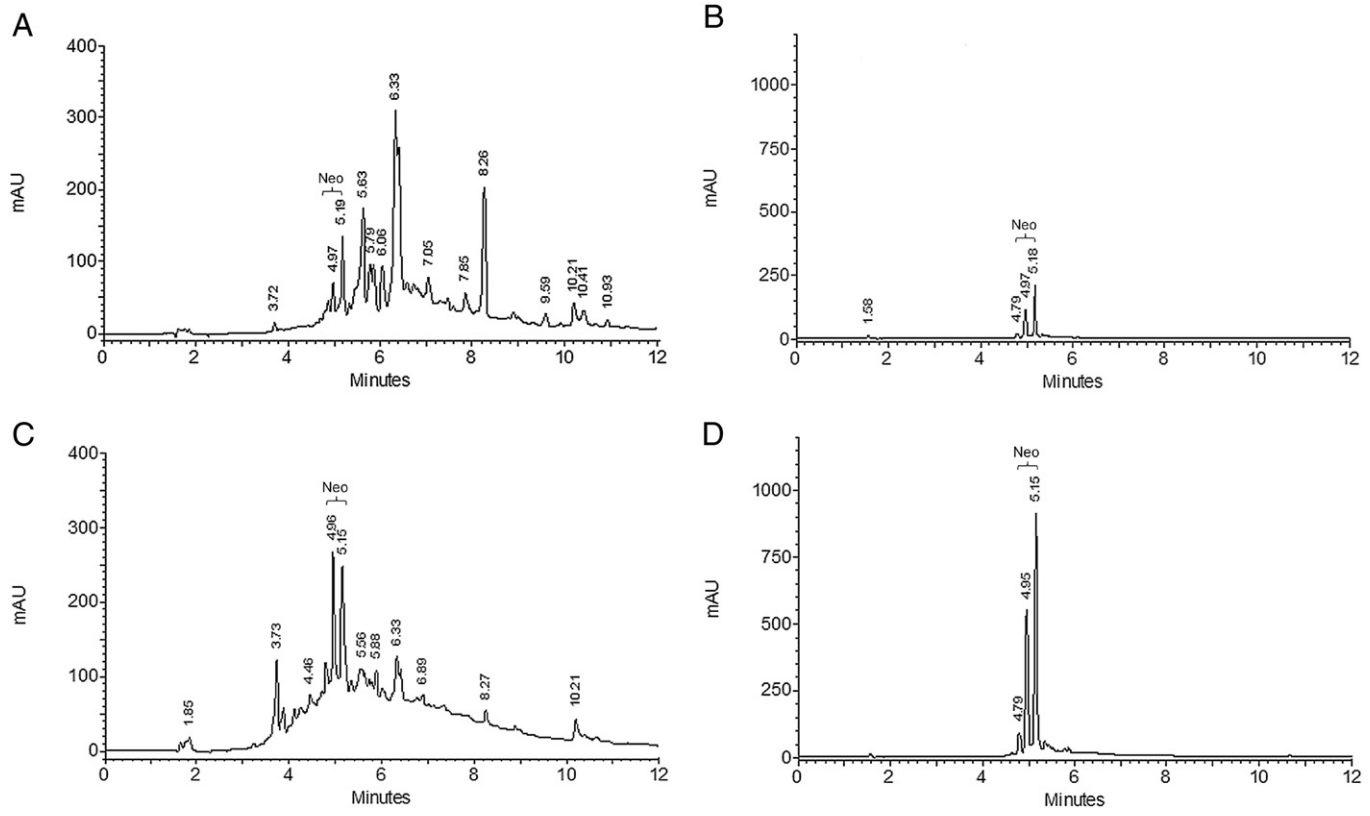

Fig. 2. Chromatogram of the neocandenatone fractions. a) Fraction A, 260 nm; b) Fraction A, 515 nm; c) Fraction B, 260 nm and d) Fraction B, 515 nm. Conditions: Gradient acetic acid 2.5%: acetonitrile; column C18 ZORBAX Eclipse Plus, 20 μL, 0.5 mg mL^{-1}, 1 mL/min.

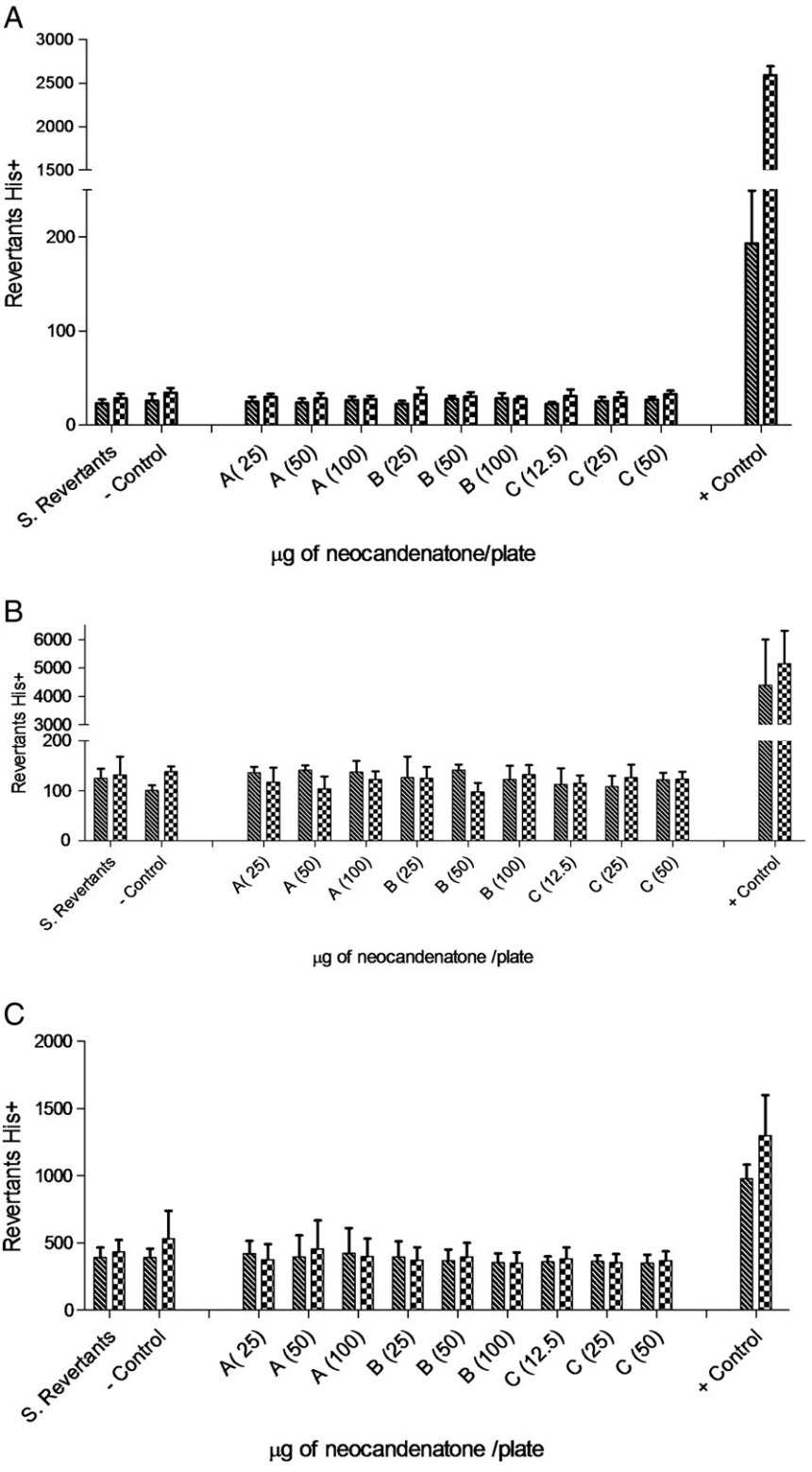

Fig. 3. The results of the Ames test of the neocandenatone fractions a) TA98 *S. typhimurium* tests b) TA102 *S. typhimurium* tests c) TA100 *S. typhimurium* tests. Without S9. With S9.

$$\%\mathrm{MNRET} = \left[\frac{(\mathrm{MN}-\mathrm{RET})}{(\mathrm{MN}-\mathrm{RET}) + \mathrm{RET}}\right] * 100 \quad (2)$$

$$\%\mathrm{MN}-\mathrm{NCE} = \left[\frac{(\mathrm{MN}-\mathrm{NCE})}{(\mathrm{MN}-\mathrm{NCE}) + (\mathrm{NCE})}\right] * 100 \quad (3)$$

where RET represents reticulocyte, NCE represents normochromatic mature erythrocyte, and MN represents micronucleus.

The values presented are the means ± standard errors of the replicates. The experimental data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test of significance. Differences with $p < 0.05$ were considered statistically significant.

Table 2
Percentages of RETs, MN–RETs, and MN–NCEs in mouse peripheral blood, as measured by flow cytometry (n = 3).

Treatment concentration	Concentration	%RET	%MN–RET	%MN–NCE
Fraction A (crude extract)	1000 mg kg^{-1}	6.08 ± 0.77	0.0024 ± 0.0012	0.0023 ± 0.0008
Fraction B (degreased extract)	100 mg kg^{-1}	5.83 ± 1.18	0.0018 ± 0.0004	0.0016 ± 0.0001
Vehicle control (propylene glycol)	100 μL per mouse	5.78 ± 0.31	0.0036 ± 0.0011	0.0016 ± 0.0001
Negative control (water)	100 μL per mouse	5.41 ± 0.44	0.0027 ± 0.0008	0.0018 ± 0.0003
Positive control (cyclophosphamide)	50 mg kg^{-1}	0.81 ± 0.16 *	0.0844 ± 0.0222 *	0.0069 ± 0.0023 *

*$p \leq 0.05$ by ANOVA followed by Dunnett's *post hoc* test.

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis (Cammerer et al., 2007).

The results of micronucleus assays were reported according to the following criteria: a compound was classified as genotoxic in mice if it induced a statistically significant increase in the frequency of micronuclei above the negative control.

2.2.4. Testing in candy products

2.2.4.1. Selection of commercial products for color matching. Hydrophilic pigments such as anthocyanins and betalains are alternatives to red azo dyes (Buchweitz, Brauch, Carle, & Kammerer, 2013) and are used in food industries for producing confectionery and soft drinks and in the pharmaceutical industry. In this study, commercial confectionery products rich in sugar (hard candy) or sugar and protein (gelatin gummies) with red-purple coloring were selected from a local market for analysis of their color and determination of the proper pigment concentrations required to match their coloration in the candy formulations.

2.2.4.2. Candy formulations. To match the colorations of different commercial samples, various pigments and acid concentrations were assessed. Eleven formulations for gummies (G) and 10 formulations for hard candies (C) were prepared in duplicate, as indicated in Table 1. For gelatin gummies, the mixture was placed in a crockpot and heated with stirring until it reached a final cooking temperature of 115 °C. For hard candies, the final cooking temperature was 137 °C.

Neocandenatone was applied as a dispersion in propylene glycol (1:1 w/w), and commercial anthocyanin was applied as an aqueous solution (1:1 w/w).

The pH and chromatic parameters for each batch were recorded and compared with those obtained for commercial candies. For the shelf-life study, some candy samples were selected based on color preference established by a panel of 20 untrained judges with product knowledge and by sensory evaluation techniques using simple selection criteria among the gummies and hard candies pigmented with neocandenatone or anthocyanin.

2.2.5. Color and pH measurements

Three grams of selected products were placed in a Hatch ® tubes, mixed with 2 mL distilled water at 60 °C, and homogenized at 3000 rpm with a vortexer for 10 min. The dispersion was centrifuged at 1010 ×*g* for 15 min. Cleared solutions were scanned from 400 to 800 nm (Spectrophotometer HACH, DR5000, Mexico) and used to measure color parameters (L*, a*, and b*) with a colorimeter (Minolta CR-10, Japan; measuring area, 8 mm), D65 illuminator, and 10° observer with a white background. The hue angle (H°) and *C* values were calculated with Eqs. (4) and (5). The ΔE values were calculated for equalization with a commercial product using Eq. (6).

$$H^{\circ} = \tan^{-1}\left[\frac{b^*}{a^*}\right] \quad (4)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (5)$$

$$\Delta E = \sqrt{(L^* - L^*_o)^2 + (a^* - a^*_o)^2 + (b^* - b^*_o)^2}. \quad (6)$$

In these equations, L*, a*, and b* or L^*_o, a^*_o, and b^*_o values correspond to formulated or commercial candies, respectively.

The pH for each candy solution was measured using a UB-10 pH meter (Denver Instrument, USA).

2.2.6. Pigment concentration

Because the neocandenatone and commercial anthocyanin used in this study exhibited pigment changes with changes in pH, standard curves were constructed for anthocyanin and neocandenatone at each pH values of the pigmented products using their λ_{max} values.

2.2.6.1. Determination of neocandenatone concentrations in candies. Standard curves for gelatin gummies pigmented with neocandenatone were constructed at 0.007%, 0.012%, 0.023%, and 0.045% pigment, adjusting the pH to 5.1, 3.5, and 2.9 for each percentage. In the same way, the standard curves for hard candies were constructed at 0.012%, 0.025%, 0.050%, and 0.075% pigment at pH values of 2.3 and 4.2. The pigment concentrations in the samples were calculated by interpolation using the standard curve.

2.2.6.2. Determination of anthocyanin concentrations in candies. Standard curves for gelatin gummies pigmented with neocandenatone were constructed at 0.025%, 0.050%, 0.062%, and 0.1% pigment at pH values of 5.1, 3.5, and 2.9. For the hard candies, standard curves were constructed at 0.025%, 0.050%, and 0.1% pigment at pH values of 2.32 and 4.23. The pigment concentrations in the samples were calculated by interpolation using the standard curve.

2.2.7. Antioxidant activity

One milliliter of gelatin gummy solution prepared as described in Color and pH measurements section was mixed with 750 μL of 5% trichloroacetic acid to break the protein network and obtain a clear

Table 3
Chromatic parameters of commercial products selected.

Pigmented product	Code	Color	H° value	Chroma value
Gelatin gummies	GA	Orange	42.99 ± 1.16	17.83 ± 1.11
	PN	Orange	52.77 ± 0.73	15.20 ± 0.68
	M	Purple	81.71 ± 7.22	1.98 ± 0.25
	S1	Red-orange	21.74 ± 1.94	30.36 ± 1.61
	S2	Red	18.82 ± 1.46	32.32 ± 2.01
	S3	Red	14.93 ± 1.98	26.06 ± 1.89
	S4	Red-brown	27.88 ± 0.72	16.50 ± 2.24
Hard candy	PMG	Purple	305.27 ± 5.73	2.60 ± 0.25
	PMR	Purple	348.85 ± 1.38	6.59 ± 0.55
	PMV	Purple	331.06 ± 4.11	0.61 ± 0.35
	PRR	Red	15.23 ± 0.72	18.52 ± 0.15
	CRG	Red	18.38 ± 2.22	20.82 ± 1.27
	CNG	Orange	49.30 ± 2.8	22.35 ± 0.33
	CNC	Orange	27.51 ± 2.84	26.81 ± 0.65

Table 4
Chromatic parameters of pigmented products.

	Pigmented product	Composition**	H° value	Chroma value	pH
Gelatin gummies	1GA	0.025% A, 0.7% ca	45.54 ± 1.02	13.81 ± 1.33	3.5 ± 0.05
	2GA	0.050% A, 0.7% ca	15.61 ± 2.42	13.58 ± 0.62	3.4 ± 0.03
	3GA	0.062% A, 0.7% ca	53.75 ± 3.04	9.40 ± 0.89	3.39 ± 0.01
	4GA	0.075% A, 0.7% ca	14.45 ± 8.18	5.26 ± 1.05	3.47 ± 0.05
	5GA	0.10% A, 0.7% ca	7.19 ± 1.36	11.16 ± 2.24	3.49 ± 0.03
	1GN	0.007% A, 1.0% ca	65.64 ± 1.32	10.31 ± 0.36	2.90 ± 0.03
	2GN	0.007% N, 0.3% ca	41.18 ± 1.41	8.11 ± 0.73	3.7 ± 0.01
	3GN	0.014% N, 1.0% ca	44.21 ± 2.18	13.04 ± 0.53	2.90 ± 0.01
	4GN	0.028% N, 1.0% ca	34.24 ± 0.54	10.40 ± 1.18	2.8 ± 0.02
	5GN	0.31% N, 0.7% ca	15.25 ± 0.09	7.98 ± 0.58	3.5 ± 0.04
	6GN	0.014% N, 0.0% ca	1.78 ± 0.92	9.75 ± 0.49	5.1 ± 0.03
Hard candy	1CA	0.025% A, 0.7% ca	68.16 ± 1.35	11.58 ± 0.06	2.47 ± 0.08
	2CA	0.05% A, 0.7% ca	25.31 ± 2.37	14.41 ± 1.03	2.58 ± 0.10
	3CA	0.1% A, 1.0% ca	51.26 ± 2.67	11.52 ± 1.16	2.10 ± 0.05
	4CA	0.025% A 1.0% ca	25.47 ± 1.53	10.08 ± 0.91	2.15 ± 0.15
	5CA	0.1% A, 0.0% ca	31.61 ± 0.49	11.52 ± 1.10	4.40 ± 0.20
	1CN	0.025% N, 0.7% ca	41.56 ± 1.80	11.71 ± 0.30	2.50 ± 0.30
	2CN	0.05% N, 0.7% ca	33.64 ± 0.63	8.76 ± 1.29	2.32 ± 0.10
	3CN	0.025% N, 0.0% ca	34.53 ± 1.19	6.23 ± 0.14	4.29 ± 0.08
	4CN	0.05% N, 0.0% ca	62.52 ± 1.29	3.10 ± 1.55	4.23 ± 0.06
	5CN	0.075% N, 1.0% ca	44.04 ± 1.83	3.84 ± 0.33	2.05 ± 0.15

** A: Anthocyanin; N: Neocandenatone; ca: Citric Acid; C: Hard candy; G: Gummy.

solution. For hard candy, 1 mL of the solution prepared as described in Color and pH measurements section was diluted with 2.5 mL of water. In both cases, 30 μL of each dilution was added to 1.5 mL of ABTS stock solution and mixed for 6 min. The absorbance was measured at 734 nm (HACH, DR5000, Mexico). The ABTS percentage of radical scavenging (%RS) was calculated using Eq. (7) (Pérez-Jiménez & Saura, 2006):

$$\%\text{radical inhibition} = \left(\frac{A_{734}\ \text{initial} - A_{734}\ \text{sample}}{A_{734}\ \text{initial}}\right) * 100. \quad (7)$$

2.2.8. Storage stability of candies

Batches of 16 selected pigmented gummies and hard candies were maintained at 40 °C for 2 months. Sampling was performed in duplicate every week, with samples analyzed for pigment concentration, color, and antioxidant activity as described in Color and pH measurements, Pigment concentration, and Antioxidant activity sections.

2.2.9. Statistical analysis

The data are expressed as means ± standard deviations, calculated using one-way ANOVA in SAS statistical software (V 6.0, SAS Institute SA de CV, Mexico). Duncan's multiple range tests were used to compare

Fig. 4. Pigmented products with neocandenatone (N) and with anthocyanins (A) in gelatin gummies (G) and hard candies (C).

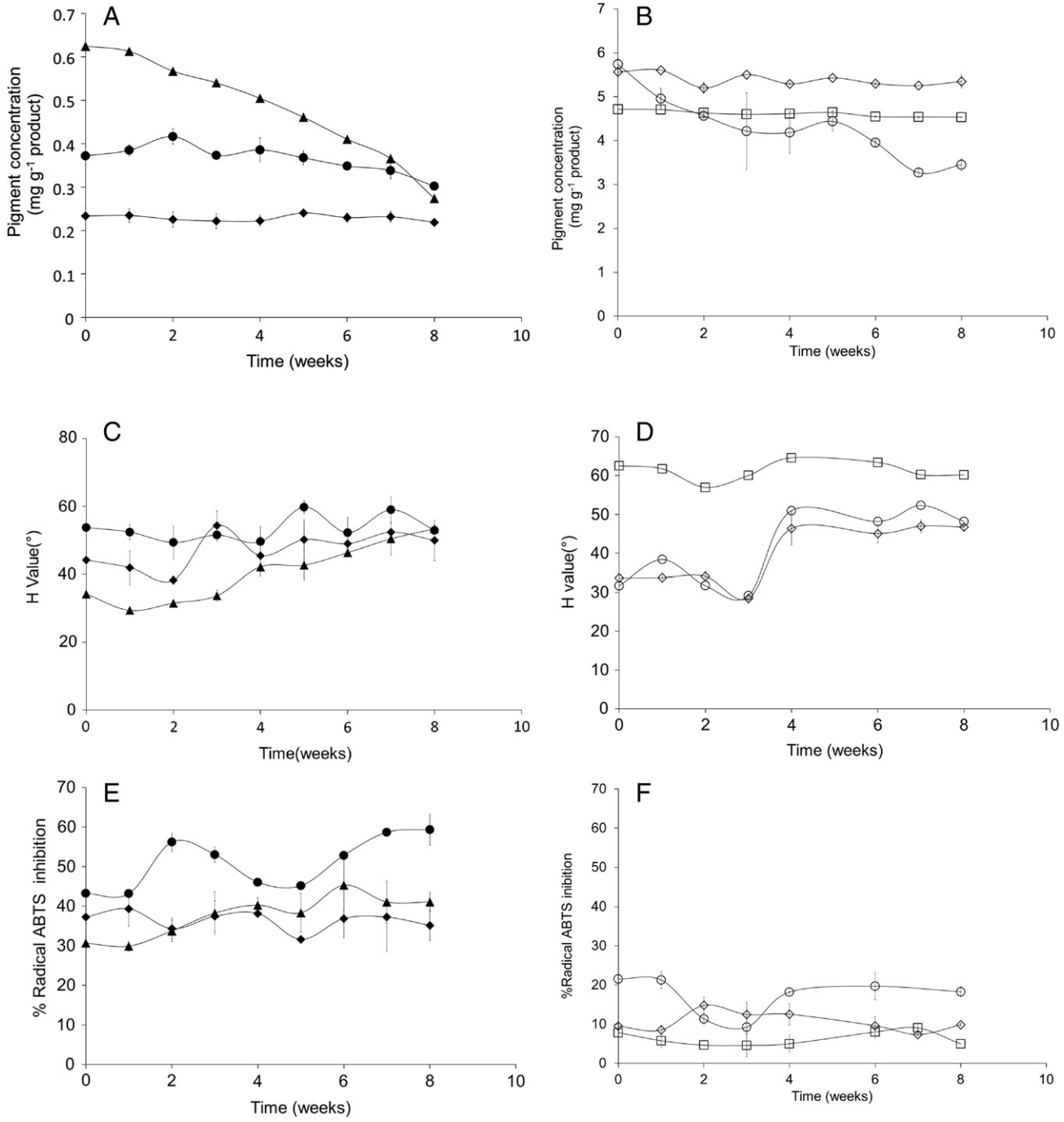

Fig. 5. Shelf life test at 40 °C. A–B) Changes in the pigment content. C–D) Changes in coloration. E–F) Changes in antioxidant activity. A, C, E) Gelatin gummies. B, D, F) Hard candies.

means. Differences between the means were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Neocandenatone purity in fractions A and B

The yields of the crude (fraction A) and degreased (fraction B) extracts in the wood were 37% and 4% dry matter, respectively. The homogeneity levels of fractions A and B are shown in Fig. 2. Because the fractions were pigment extracts of different purities, the chromatograms showed that both fractions contained several components including neocandenatone as a pair of tautomers.

The neocandenatone concentrations in fractions A and B were 6.95% and 70.55%, respectively, indicating that the pigment content in the wood was 2.72% ± 0.18% dry matter. The neocandenatone content in the wood was higher than the 2.41% and 1.37% anthocyanin content (dry matter) found in the grape skin Sousón cultivar (Río, Soto & Díaz, 2008) and skins of *Vaccinium ashei* var. Climax fruits (Lohachoompol, Mulholland, Srzednick, & Craske, 2008), respectively.

3.2. Genotoxicity assays

3.2.1. Ames test

The Ames test is useful for detection of mutagenic substances. This assay identifies whether the sample induces the mutation of genetically modified DNA from selected *S. typhimurium* strains. Because some

chemicals that are not mutagenic by themselves can become mutagens after being metabolized in the liver, the Ames test also evaluates mutagenicity in the presence of a mammalian mixture of liver enzymes, better known as the S9 microsomal fraction, to mimic this *in vivo* activation process (Ames et al., 1973a).

Fig. 3 shows the results of the Ames test for fractions A, B, and C. None of the fractions analyzed had revertant counts greater than or equal to 2-fold of the blanket control and solvent control, and no dose–response relationship was observed. The positive control mutagens induced increases in revertant colonies, confirming the validity of the assay. The MI values calculated for all fractions were 0.868–1.223 and 0.741–1.152 in the presence or absence of the S9 fraction, respectively. These results indicated that none of the neocandenatone preparations had mutagenic effects on the three strains of *S. typhimurium* tested under the different assay conditions (Arriaga-Alba et al., 2012).

3.2.2. Micronucleus test

Micronuclei were not induced in the peripheral blood by fractions A or B. The percentages of RETs, MN–RETs, and MN–NCEs induced by these extracts were similar to those of the negative control, but were significantly different from those of the positive control ($p < 0.05$). The obtained data are presented in Table 2.

To select a proper fraction for pigment application in food matrices, we considered the absence of genotoxicity and high neocandenatone content. Fraction A (not genotoxic and containing 6.95% neocandenatone) showed some problems when it was incorporated to aqueous matrices due to the presence of hydrophobic compounds in the sample. Fraction B (not genotoxic and containing 70.55% neocandenatone) was incorporated easily into the products and was therefore chosen for coloring gummies and hard candies. This fraction was also used for the storage stability study.

3.3. Testing in candy products

3.3.1. Selection of commercial products for color matching

The H° and *C* values for the selected commercial products are shown in Table 3. The chromatic parameters a and b were characteristic for the red–orange–purple colors (Tiwari, Patras, Brunton, Cullen, & O'Donnell, 2010). H° values from 14.93 ± 1.98° to 348.85 ± 1.38° and *C* values from 0.61 ± 0.35 to 32.32 ± 2.01 were obtained. Based on this information, several candy formulations were prepared to match the colors of some of the commercial preparations.

3.3.2. Candy formulations

Candies obtained with different concentrations of neocandenatone or enocyanin and citric acid are shown in Fig. 4. Different shades were obtained depending on the concentration of pigment and the amount of citric acid in the final product. Neocandenatone and enocyanin produced similar colors with and without acid. The pigment and acid concentrations tested in the candy formulations are shown in Table 4.

3.3.2.1. Gelatin gummies. Gelatin gummies displayed different shades depending on the citric acid concentration and the type and concentration of the pigment used (Fig. 4). Use of neocandenatone provided a wider range of colors, with H° values ranging from 1.78 ± 0.92 to 65.64 ± 1.326, than did anthocyanins, for which H° values ranged from 7.19 ± 1.36 to 45.54 ± 1.0 (Goncalves et al., 2007). Variations in the H° value have been reported to be related to the content of anthocyanins in fruits. In this case, at higher pigment concentrations, lower H° values were observed in the candies.

With the different formulations assayed, it was possible to produce pigmented gummies with purple, orange, and red colorations, similar to the commercial products with $\Delta E \leq 3$, which is indicative of acceptable color equalization (Choi, Kim, & Lee, 2002; Martínez, Melgosa, Pérez, Hita, & Negueruela, 2001). The presence of citric acid increased hue values. Purple color (H° = 1.78) was obtained in products with 0.014% pigment and 0.0% citric acid; in contrast, red color (H° = 41.3) was obtained with 0.014% pigment and 1.0% citric acid. Smaller amounts of neocandenatone than enocyanin produced similar colorations.

Although enocyanin displayed a purple color in solution, it was not possible to produce this coloration in gummies.

3.3.2.2. Hard candy products. Pigmented candies produced with anthocyanin and neocandenatone also exhibited different shades depending on the pigment concentration (Fig. 4). The H° values of neocandenatone products ranged from 21.69 ± 3.52 to 46.87 ± 0.06, and those for anthocyanin ranged from 26.63 ± 1.71 to 68.16 ± 1.35, indicating that both pigments gave an equally broad range of colors in the final product (Table 4 and Fig. 4).

The H° value reported for natural pigments depends on the pigment source (Mapari, Meyer, & Thrane, 2006). Thus, the H° values reported for anthocyanin from black carrot, purple corn, elderberry, and strawberry were 4.44, 8.87, 10.79, and 53.20, respectively (Yang & Zhai, 2010; Sadilova et al., 2009).

Moreover, anthocyanin coloration changes with pH and the presence of organic acids, such as citric and acetic acids, and it is not always possible to establish a correlation between acid concentration and color parameter values (Yang, Han, Gu, Fan, & Chen, 2008). Therefore, it is difficult to perform a reliable comparison of reported values of chromatic parameters in the literature for anthocyanins from various sources because these parameters have been determined under different conditions (concentration, pH, solvent, matrix, *etc.*). In this work, it was clear that the range of colors in the candy products depended heavily on the candy matrix.

As expected, the colorations in hard candies depended on the pigment and acid concentrations. In contrast to gummies, similar neocandenatone and enocyanin concentrations must be used to achieve similar colorations in hard candies. It was not possible to obtain purple color in hard candies using any of the tested formulations.

3.4. Stability test

Based on the results of sensorial evaluation, samples from batches 3GN, 3GA, and 4GN for gelatin gummies and batches 4CA, 5CA, 2CN, and 4CN for hard candies were selected for the assessment of pigment stability. Fig. 5 shows changes in the pigment content, color (as H° value), and antioxidant activity of the selected samples after 2 months at 40 °C.

Color is one of the most crucial sensory properties of food; therefore, pigment loss during processing and storage is a very important quality parameter. The concentration of neocandenatone in gummies of samples 4GN and 3GN (63.3% sugars w/w) showed no significant differences ($p > 0.05$) over 2 months. In contrast, the enocyanin concentration decreased following first-order kinetics ($k = -0.104\ w^{-1}$).

Similarly, the concentration of neocandenatone in hard candies (80% sugars w/w) did not show significant differences over time, but the enocyanin concentration decreased following first-order kinetics ($k = -0.084\ w^{-1}$). Although it has been demonstrated in some cases that the presence of saccharides or their degradation products decreases the stability of anthocyanins (Dyrby, Westergaard, & Stapelfeldt, 2001), there are many studies showing that increasing the sugar concentration stabilizes pigments because the water activity is decreased (Erlandson & Wrolstad, 1972). In other cases, the presence of sugars has been shown to have no effect on stability (Sadilova et al., 2009). Many studies have attempted to improve our understanding of the effects of several independent matrix components on pigment stability (Cavalcanti et al., 2011). However, our results showed that pigment stability was defined by the whole matrix. Comparatively, neocandenatone was more stable that enocyanin in gummies (6.7% gelatin w/w); therefore, application of neocandenatone is promising in these types of confectionery products.

There was a slight but not significant color change (H° value) in products pigmented with neocandenatone, while products pigmented with enocyanin (3GA) showed a light discoloration when the exposure time was increased (the H° increased from 32.6 to 51.5). When the H° value moves away from zero, approaching 90°, the color of the product will tend to become more of an orange-brown due to decreasing a* values (Fischer, Carle, & Kammerer, 2013).

The antioxidant activity was increased in sample 3GA; this may have been due to the presence of degradation products of anthocyanin, such as caffeic acid compounds, which do not have color, but possess antioxidant activity (Wang & Xu, 2007), and because of the formation of Maillard products (Laroque et al., 2008), as shown by the slight increase in absorbance at 400–420 nm in the tested samples.

4. Conclusions

Natural dye extracts are commonly used in the food industry. However, the crude extract of *D. congestiflora* was composed of approximately 93% hydrophobic compounds, thereby restricting its dispersion in aqueous matrices, such as candy products. Use of pure pigment obtained chromatographically is very expensive; therefore, the use of a semi-purified extract containing 71% neocandenatone is recommended. However, in fatty matrices where the crude extract can be dispersed, the crude extract may be used. *D. congestiflora* extracts were tested to assess their health safety, demonstrating that none of the three fractions studied (A, B or C) showed genotoxic activity when evaluated in the Ames and micronucleus tests. It was also shown that the degreased extract had good incorporation and stability in gummies and hard candies; therefore, the neocandenatone pigment has excellent potential for food coloring.

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