Food and Chemical Toxicology 69 (2014) 237–243

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluations of the mutagenicity of a pigment extract from bulb culture of *Hippeastrum reticulatum*

Viriya Nitteranon [a,1], Chokchai Kittiwongwattana [b,1], Supachai Vuttipongchaikij [c,d,1], Jenjira Sakulkoo [c], Monthira Srijakkoat [c], Pakawieng Chokratin [c], Poontariga Harinasut [e], Saowanee Suputtitada [c], Somsak Apisitwanich [c,d,*]

[a] Department of Food Science and Technology, Faculty of Science and Technology, Rajamangala University of Technology Tawan-ok, Bangpra, Sriracha, Chonburi 20110, Thailand
[b] Department of Biology, Faculty of Science, King Mongkut's Insitute of Technology Ladkrabang, Chalongkrung Road, Ladkrabang, Bangkok 10520, Thailand
[c] Department of Genetics, Faculty of Science, Kasetsart University, 50 Ngarm Wong Wan Road, Chatuchak, Bangkok 10900, Thailand
[d] Center of Advanced Studies for Tropical Natural Resources, Kasetsart University, Ngam Wong Wan, Chatuchak, Bangkok 10900, Thailand
[e] Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Ngarm Wong Wan Road, Chatuchak, Bangkok 10900, Thailand

ARTICLE INFO

Article history:
Received 22 April 2013
Accepted 4 April 2014
Available online 19 April 2014

Keywords:
Hippeastrum reticulatum
Anthocyanin
pH stability
Heat stability
Mutagenicity
Tissue culture

ABSTRACT

The use of anthocyanins in food products as colorants has been limited because of their instability toward alkaline pH and high temperature. This study aimed to determine color stability and mutagenicity of the anthocyanin-based pigment extract from bulb cultures of *Hippeastrum* (*Hippeastrum reticulatum*). The pigment extract retained its reddish-orange color under alkaline conditions (⩽pH 11) and was stable up to 6 h at 95 °C. The mutagenicity of the extract was evaluated *in vitro* and *in vivo*. *Hippeastrum* pigment extract up to 1.25 mg plate^{-1} was found non-mutagenic in Ames test using *Salmonella typhimurium* strain TA98 and TA100. Chromosome aberrations were observed when human lymphocytes were treated with the extract up to 1.5 mg ml^{-1}. However, the extract up to 1.4 mg ml^{-1} was found to exhibit relatively low or no mutagenicity in *in vitro* comet assays with human lymphocytes. In *in vivo* micronucleated reticulocyte assay, mice were treated orally with the extract up to 1 g kg^{-1}. No significant increase of the percentage of micronucleated peripheral reticulocytes compared to the negative control groups was found. Taken together, our study indicates that *Hippeastrum* pigment extract is potentially applicable as an additive colorant in the diet and related products.

1. Introduction

Colorants play important roles in food industry by providing enhancement, imitation or masking of the natural color of food products. Synthetic colorants have been widely used in food and related industry. However, concerns regarding their harmfulness to human's health are rising because of their potential toxicity, (Amin et al., 2010; Ganesan et al., 2011). This has led to the search for alternative natural colorants that are derived from plants and microorganisms (Wrolstad and Culver, 2012). The main groups of natural food colorants are carotenoids, chlorophylls, betacyanins, curcuminoids, and anthocyanins (Bridle and Timberlake, 1997). They are generally regarded as safe and preferable for their potential nutritional and therapeutic benefits (Wallace and Giusti, 2008).

Anthocyanins are generally used as natural colorants especially for reddish to purplish hues. They are collectively the largest group of water-soluble pigments in the plant kingdom and have been used as food additives in various traditional cooking and food industry (Clifford, 2000). Anthocyanins have attracted considerable interest because of their biological properties as antioxidants, anti-tumor, anti-inflammatory and cardio-protective agents (Kong et al., 2003). In general, anthocyanins retain their color under strong acidic conditions (pH < 4) and become colorless at pH values higher than 5 (Castañeda-Ovando et al., 2009). Therefore,

Abbreviations: ADI, acceptable daily intake; AF2, 2-2(2-furyl)-3-(5-nitro-2-furyl) acrylamide; BAP, 6-benzylaminopurine; BP5, benzo(a)pyrene; CB, chromosome break; DEL, deletion; DBMA, dimethylbenz(a)anthracine; DMFA, dimethylformamide; FDA, food and drug administration; GAP, chromosome gap; JECFA, the Joint FAO/WHO Expert Committee on Food Additive; MMC, mitomycin C; MNRETs, micronucleated peripheral reticulocytes; NAA, 1-naphthalene acetic acid; NOEL, the no-observed-effect-level; OECD, the organisation for economic co-operation and development; SA, satellite joining; TLC, thin layer chromatography.

* Corresponding author at: Department of Genetics, Faculty of Science, Kasetsart University, 50 Ngarm Wong Wan Road, Chatuchak, Bangkok 10900, Thailand. Tel.: +66 2 562 5444x4220; fax: +66 2 579 5528.
E-mail address: fscissa@ku.ac.th (S. Apisitwanich).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fct.2014.04.007
0278-6915/

application of anthocyanins as coloring agents is limited to products that are in relatively strong acidic conditions (Patras et al., 2010). The use of high temperature required for pasteurization and sterilization during product processing also results in color loss especially in alkaline aqueous solutions (Patras et al., 2010). A number of studies have been dedicated to identification of novel anthocyanins with new colors or higher stability from various plant species (Bassa and Francis, 1987; Cevallos-Casals and Cisneros-Zevallos, 2004; de Pascual-Teresa et al., 2002; Goda et al., 1997). Additionally, anthocyanins are being considered a potential replacement for synthetic dyes (He and Giusti, 2010). Regarding the safety of anthocyanins, the Joint FAO/WHO Expert Committee on Food Additive (JECFA) approved that anthocyanins are of a very low order of toxicity based on a number of tests including mutagenicity, reproductive toxicity, teratogenicity and acute and short-term toxicity evaluations (WHO, 1982).

Hippeastrum (*Hippeastrum reticulatum*) is a bulbous monocotyledon belonging to the family Amaryllidaceae. They are widely used as ornamental plants because of their flowers whose petals are reticulated with colors ranging from light pink to dark purple. In this study, we demonstrated the extraction of anthocyanin-based color pigments from suspended cells that were released from axenic bulb cultures of *Hippeastrum*. pH and heat stability of *Hippeastrum* pigment extract was investigated at different pH values (1–11) and high temperature (95 °C). Mutagenicity of the extract was determined using Ames test, *in vitro* chromosome aberration assay, *in vitro* comet assay and *in vivo* micronucleated reticulocyte assay. To the best of our knowledge, this is the first study that investigated the potential of *Hippeastrum* bulb culture as a novel source for natural colorants.

2. Materials and methods

2.1. Plant materials and growth condition

Bulbs of *Hippeastrum* variety "Rang-Ngern" were axenically propagated in Murashige and Skoog (MS) medium pH 5.8 supplemented with 0.01 mg l^{-1} NAA (Sigma), 2 mg l^{-1} BAP (Sigma), 60 g l^{-1} sucrose and 1% (w/v) agar under 16 h/8 h (light/dark) photoperiod (100 μmol m^{-2} s^{-1}) and 25 °C conditions. To induce the production of color pigments, bulbs were transferred into MS liquid medium pH 6.8 supplemented with 0.01 mg l^{-1} NAA, 6 mg l^{-1} BAP and 60 g l^{-1} sucrose. Approximately 5–6 bulbs were cultured in 60 ml medium for 30 days at 25 °C under dark conditions with constant shaking at 100 rpm.

2.2. Pigment extraction and TLC analysis

Suspended cells containing color pigments were harvested by centrifugation at 1500×*g* for 15 min. The supernatant was discarded. Cells were washed with distilled water, centrifuged at 1500×*g* for 15 min, and incubated at 60 °C for 2–3 days. Cells were ground into fine powder using a mortar and a pestle before dissolving in acetone (1:50 w/v). Solvent was removed using a vacuum rotary evaporator to obtain the pigment extract. For TLC analysis, the extract was dissolved in 1% HCl in methanol at 0.5 g ml^{-1} concentration. Three microliters of the extract were spotted on cellulose coated glass plates and irrigated using HCOOH:H_2O:HCl (5:3:2) solvent. TLC was then visualized under visible and UV light. Additionally, the plates were sprayed with NH_4OH and observed under UV light. Pigments from various plants were extracted using the same method and used as standard. These included Petunia flowers, Begonia flowers, red grape skin, apple skin, beet root bulbs, purple cabbage and orchid flowers. Separated *Hippeastrum* pigment spots were scraped off from the TLC plates and dissolved in HCOOH:H_2O:HCl (5:3:2) solvent. Pigment suspensions were spotted on two TLC plates and irrigated once more with the same solvent. The pigment spots were scraped off from the first TLC plate, dissolved in the irrigated solvent and analyzed for maximum wavelength (λ_{MAX}) using absorption spectrophotometer. The second TLC plate was irrigated with the same solvent and sprayed with 5% $AlCl_3$ in methanol before the pigment spots were scraped off from the plate for the λ_{MAX} shift assay.

2.3. pH and heat stability tests

The pigment extract was dissolved in small volume of 95% ethanol and made up to 15 mg ml^{-1} concentration with Na_2HPO_4/citric-acid buffer (0.2 M Na_2HPO_4, 0.1 M citric acid and 2% SDS) with pH ranging from 1 to 11 (adjusted by 0.1 N NaOH and 0.1 N HCl). The extract was incubated at room temperature for 4 h. Changes in absorbance spectra were determined using Nanodrop (Thermo Scientific, USA). Heat stability of the extract was tested at pH 5 and 9. The pigment extract was prepared as described above and incubated at 95 °C for 0, 0.5, 1, 2, 3, 4, 5 and 6 h. The absorbance value at maximum wavelength according to the pH stability test was determined using the Nanodrop.

2.4. Ames test

Salmonella typhimurium strain TA98 and TA100 were employed for frameshift mutation and base substitution tests, respectively. In the preliminary test, tester bacteria were exposed to the pigment extract up to 10 mg $plate^{-1}$ with and without S-9 mix, and growth inhibition was observed at 5 mg $plate^{-1}$. The toxicity was assessed by the absence of bacterial lawn and the presence pin-point colonies. The bacterial strains were grown overnight in nutrient broth at 37 °C. Bacterial cells were mixed well with 0.3125–2.5 mg of the pigment extract dissolved in DMFA (Sigma), and 500 μl of S-9 mix or sodium phosphate buffer were subsequently added. 2.5 ml of melting top agar was added into the mixture, poured onto minimal glucose agar plates and incubated at 37 °C for 48 h. The number of revertant colonies on the plates was counted. For positive control treatments, 0.1 and 0.01 μg $plate^{-1}$ of AF-2 (Wako Pure Chemical Industries, Japan) were used for strain TA98 and TA100 strains, respectively under conditions without S-9 mix whereas 5 and 1.25 μg $plate^{-1}$ of BP5 (Sigma) were used for strain TA98 and TA100, respectively, under S-9 mix treated conditions. DMFA was used as the negative control. This experiment was performed in duplicate.

2.5. In vitro chromosome aberration assay

Human lymphocytes were obtained from two healthy females and two healthy males and cultured separately in RPMI medium-1640 (Gibco) supplemented with 25% fetal calf serum and 2.5% phytohaemagglutinin (Gibco) at 37 °C for 48 h. Doses used in this assay were in relative to the concentrations used in Ames test. The cultures from each subject were mixed with the extract that was dissolved in DMFA at 0.3 and 1.5 mg ml^{-1} concentrations and incubated under the same conditions for additional 22 h. 0.5 μg ml^{-1} of MMC (Sigma) was used instead of the pigment extract as the positive control, and DMFA was used as the negative control. After 70 h, 50 μl of 0.05% (w/v) colchicine was added, and the cultures were incubated for 2 h. Cells were harvested by centrifugation at 1200×*g* for 20 min and washed three times with 6 ml of Hank balance salt solution and washed twice with 6 ml of 0.075 M KCl. Cells were washed three times with fixative solution (3 methanol: 1 glacial acetic acid) before being dropped onto warmed glass slides. Chromosomes were stained with 4% Giemsa solution (Merck) for 10 min and washed thoroughly with distilled water. Chromosomes were examined under the light microscope. 100 well-spread metaphases per culture for each treatment were examined.

2.6. In vitro comet assay

Comet assay was performed according to Östling and Johanson (1984). Briefly, human blood samples were obtained from two healthy males and two healthy females and mixed with 0.3, 0.7 or 1.4 mg of pigment extract (dissolved in DMFA) per 1 ml of blood samples. These doses were selected in accordance with Ames test. Distilled H_2O and DMFA were used as the negative controls whereas 0.5 mg of MMC per 1 ml of blood samples was used as the positive control. Samples were incubated for 3 h at 37 °C. 20 μl of each treated blood samples was embedded in 0.6% low-temperature-melting agarose gel (Sigma) and layered onto separate glass slides pre-coated with 1% normal agarose. The slides were covered with cover slips and placed on an ice-cold tray for 2 min. Cover slips were removed and the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH10) at 4° for 20 min. Electrophoresis was performed with the slides placed in the chamber with cold alkaline buffer (pH 13) at 13 V, 300 mA for 30 min. The slides were then submerged in the neutralization buffer and stained by adding 35 μl of 75 μg ml^{-1} ethidium bromide (Sigma) directly onto the gel. The slides were examined under the fluorescence microscope and analyzed using Meta System ISS092 software. 100 lymphocytes were analyzed for each treatment.

2.7. In vivo micronucleated reticulocyte assay

The mouse micronucleated reticulocyte assay was performed according to the OECD Guidelines for testing Chemicals No. 474. All experimental mouse protocols were reviewed and approved by the institutional animal ethics committee at Mahidol University (Bangkok, Thailand). Healthy young adult (7-weeks old) Swiss albino mice were purchased from National Laboratory Animal Center (Mahidol University, Thailand). The animals were housed in stainless cages under 25 ± 2 °C, 12 h/12 h (light/dark) photoperiod and provided the standard pellet diet with free access to water. The animals were acclimated for at least 5 days before being placed on the study. Eight female and eight male mice were used in each treatment. The dose for the pigment extract was set at 1 g kg^{-1} b.w. This dose is 120 times higher than the estimated daily ingestion of proanthocyanin by human at approximately 0.5 g per 60 kg body weight (Deprez and Scalbert, 1999). Treatments included direct mutagen positive control (2 mg kg^{-1} MMC) via intraperitoneal injection, indirect