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Protective effects of spray-dried açaí (*Euterpe oleracea* Mart) fruit pulp against initiation step of colon carcinogenesis

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ABSTRACT

The present study aims to evaluate whether açaí (*Euterpe oleracea* Mart) fruit pulp powder (AP) feeding attenuates the initiation step of chemically-induced mouse colon carcinogenesis. Male Swiss mice were fed low fat diet containing 2.5% or 5.0% of AP (weeks 1 to 4) produced by a spray-drying system. All groups received an intraperitoneal (i.p.) injection of colon carcinogen azoxymethane (AOM, 15 mg/kg of body weight) at week 3. After the first administration of carcinogen, blood samples were collected to perform Single Cell Gel Electrophoresis Assay. Some mice were euthanized at week 3 ($n = 5$ mice/group) and liver samples were collected for immunohistochemical and glutathione analysis. Other mice received a second i.p. injection of AOM at week 4 and were fed a high-fat diet to accelerate the development of preneoplastic aberrant crypt foci (ACF) until week 14 ($n = 10$ mice/group). Cyanidin 3-glucoside and cyanidin 3-rutinoside, lutein, α-carotene and β-carotene were identified as the main anthocyanins and carotenoids in AP, respectively. At week 3, both dietary AP interventions (2.5% or 5.0%) reduced ($p < 0.001$) peripheral blood cell DNA damage induced by AOM. Moreover, dietary 5.0% AP increased ($p = 0.002$) hepatic total glutathione. At week 14, 5.0% AP intake reduced ($p < 0.05$) ACF multiplicity. The findings indicate that AP feeding attenuates chemically-induced mouse colon carcinogenesis by increasing total GSH and attenuating DNA damage and preneoplastic lesion development.

1. Introduction

Colorectal cancer (CRC) is an important disease that affects millions of people around the world every year and it is considered the second and the third most commonly diagnosed cancer in women and men, respectively (Torre et al., 2015). This malignancy develops through a multistep process involving multiple genetic and epigenetic alterations (Cappell, 2008; Watson & Collins, 2011). The main risk factors involved in the development of sporadic CRC are related to lifestyle and dietary habits, such as smoking, alcohol abuse, high consumption of red and processed meat, low intake of fruits and vegetables, obesity and lack of physical activity (Cappell, 2008; Williams et al., 2011).

Several rodent models of colon carcinogenesis are adequate for the study of risk factors, development and treatment of CRC, including genetically engineered, inflammatory and chemically-induced bioassays (Rosenberg, Giardina, & Tanaka, 2009). In chemically-induced carcinogenesis mouse models, the azoxymethane (AOM) agent is one of the most used carcinogens to induce initiation of and post-initiation steps of CRC carcinogenesis in mice (Rosenberg et al., 2009). Single or multiple doses of AOM induce preneoplastic lesions and tumors mainly in middle and distal colon and rectum (Chen & Huang, 2009; Rosenberg et al., 2009). Aberrant crypt foci (ACF) are putative preneoplastic lesions observed during the development of CRC that can be easily evaluated in the colon/rectum of mice receiving AOM (Bird, 1987; Mori, Hata, Yamada, Kuno, & Hara, 2005). Thus, ACF detection and quantification have been proposed for identification of potential preventive agents in rodent short- and medium-term CRC bioassays (Bird, 1987; Femia & Caderni, 2008).

Epidemiological evidences consistently show that the consumption of a diet rich in fruits and vegetables may offer protection against several human diseases, including CRC development (Van Duijnhoven et al., 2009). Açaí (*Euterpe oleracea* Mart.) is a native palm tree from the Central and South America, grown in the food plains of Amazon region. Recently, the fruit of this palm tree has emerged as a "functional food" (Schauss et al., 2006; Yamaguchi, Pereira, Lamarão, Lima, & da Veiga-Junior, 2015). When mature, it turns from green to dark purple

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because of a high concentration of anthocyanins with antioxidant and anti-inflammatory properties (Schauss et al., 2006; Yamaguchi et al., 2015). Because of beneficial for improved human health, açaí fruit pulp can be consumed *in natura* or in a variety of other products, including energetic beverages, ice cream, sweets and jelly (Menezes, Deliza, Chan, & Guinard, 2011; Vidigal, Minim, Carvalho, Milagres, & Gonçalves, 2011). The main anthocyanins present in açaí fruit are cyanidin 3-glucoside and cyanide 3-rutinoside (Schauss et al., 2006; Yamaguchi et al., 2015). Some studies have already shown different properties of crude açaí fruit pulp or its polyphenolic fractions in a variety of in vivo and in vitro bioassays (Del Pozo-Insfran, Percival, & Talcott, 2006; Kang et al., 2010; Ribeiro et al., 2010; Yamaguchi et al., 2015). However, little is known about anticarcinogenic potential of dietary açaí fruit against rodent chemically-induced cancers models (Fragoso, Prado, Barbosa, Rocha, & Barbisan, 2012; Fragoso, Romualdo, Ribeiro, & Barbisan, 2013).

Considering the increased popularity of açaí fruit pulp consumption in Brazil, the United States and Europe (Menezes et al., 2011; Vidigal et al., 2011; Yamaguchi et al., 2015), the present study evaluated the protective effects of açaí (*Euterpe oleracea* Mart.) fruit pulp powder (AP) intake against the initiation stage of mouse colon carcinogenesis. Since carcinogenesis is a complex multistep process initiated by DNA mutation, AP intake, before and during carcinogen exposure, was established trying to attenuate or inhibit the early induction of DNA damage and ACF development induced by AOM. As humans are exposed to hydrazines, heterocyclic amines and other mutagens/carcinogens, possible strategies for cancer prevention could be achieved using potential functional food such as açaí to reduce or eliminate the deleterious effects of human exposure to potential carcinogens.

2. Materials and methods

2.1. Animals and treatment

This study was carried out in strict accordance with the recommendations of the Ethical Principles for Animals Research adopted by the Brazilian College of Animal Experimentation (COBEA). The experimental protocols used herein were approved by Biosciences Institute/UNESP Ethics Committee on Use of Animals (CEUA) (Permit number 302/2011). All animals were euthanized by cervical dislocation under sodium pentobarbital anesthesia (45 mg/kg body weight) and all efforts were made to minimize suffering. Four-week-old male Swiss albino mice were randomly allocated into four groups ($n = 15$ mice/group [G1 to G3] and $n = 5$ mice/group [G4]) (Fig. 1). These groups were fed low fat diet (LFD) (G1) or LFD containing 2.5% or 5.0% açaí fruit pulp powder (AP) (G2 and G3, respectively) during weeks 1–4 or LFD containing 0.1% indole-3-carbinol (I3C, Sigma-Aldrich, Co, USA) (G4, positive control) (Plate & Gallaher, 2006) during weeks 1–3. All groups (G1 to G4) received a first i.p. injection of AOM (15 mg/kg, Sigma-Aldrich, Co, USA) at week 3. Samples of peripheral blood were collected from the venous orbital plexus 4 and 18 h after the first injection of AOM to perform Single Cell Gel Electrophoresis (SCGE) Assay. Some mice (G1 to G3 and all mice from G4) ($n = 5$ mice/group) were euthanized 24 h after the first injection of AOM at week 3. At necropsy, the colon was removed and processed for immunohistochemical analyses and liver was removed for glutathione analyses. Other mice (G1 to G3, $n = 10$ mice/group) received a second i.p. injection of AOM (15 mg/kg) at week 4 and were fed a high-fat diet (HFD) to accelerate the development of ACF induced by colon carcinogen (Padidar et al., 2012) during 10 weeks. At necropsy, colon was removed and processed for ACF analyses. Individual body weight, food and water consumption were recorded twice a week during the experimental period. Low-fat diet [LFD; 6% fat, 51% carbohydrate, 26% protein and 3.64 kcal g^{-1} with calories from saturated fat (2.5%) and unsaturated fat (9.5%)] or high-fat diet [HFD; 21% fat; 42% carbohydrate, 24% protein and 4.54 kcal g^{-1} with calories from saturated fat (9.95%) and unsaturated fat (39.1%)] were obtained from the Agroceres (Rio Claro-SP, Brazil).

2.2. Açaí fruit pulp powder

Açaí fruit was harvested from Belém-PA/Brazil, açaí fruit pulp was frozen (−3 to 5 °C) and samples of spray-dried açaí pulp were obtained using an industrial scale spray dryer system and manionic maltodextrin DE10 as a carrier agent (Fig. 2) (Fragoso et al., 2013). AP was produced and generously donated by Centroflora Group Brazil (Botucatu, SP, Brazil).

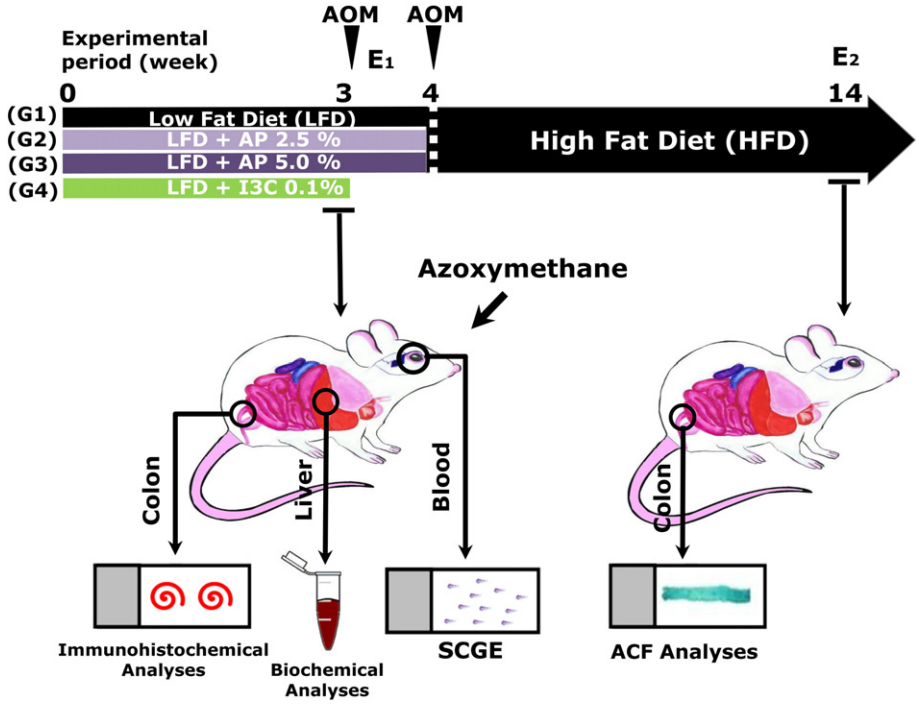

Fig. 1. Experimental design. For detailed information, see Materials and methods. E_1, E_2 = euthanasia at weeks 3 and 14, respectively.

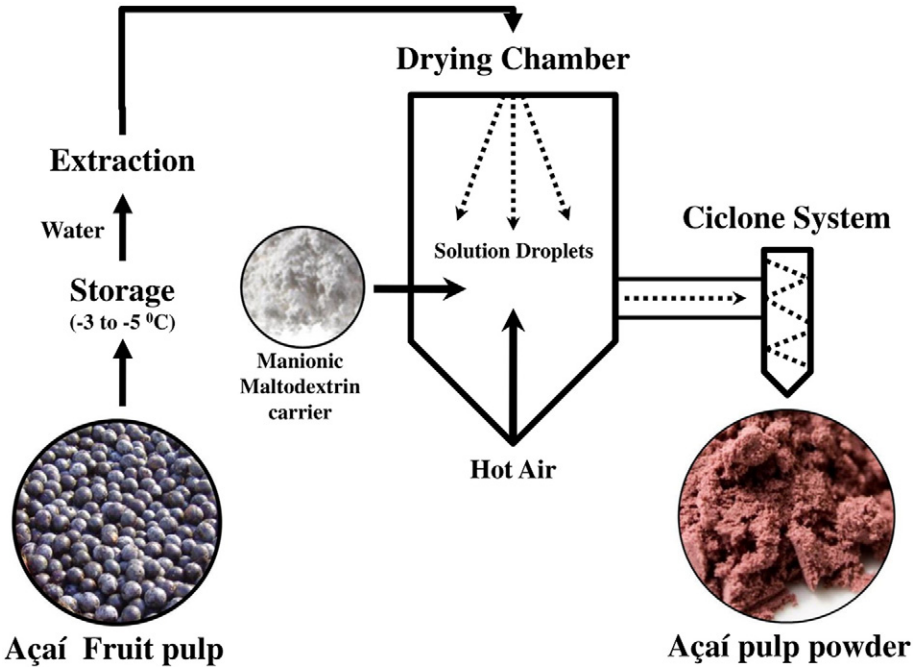

Fig. 2. Spray-drying process. Açaí fruit was harvested, frozen and samples of spray-dried açaí fruit pulp were obtained using an industrial scale spray dryer system and manionic maltodextrin DE10 as a carrier agent.

2.3. Anthocyanin and carotenoid analysis in AP

Anthocyanin extraction was conducted using 10 mg of sample and 10 mL of methanol solution (1:9 v/v) acidified with formic acid in the ultrasonic bath with subsequent centrifugation until discoloration of the solution (Brito et al., 2007). Then, an aliquot of 400 μL of the extract was dried and diluted with 100 μL of 5% formic acid solution in water: methanol (90:10). Analyses were performed in triplicate and were carried out on a Waters™ Alliance 2695 system, with a Waters™ 2996 photodiode array detector, with a Thermo™ Scientific C_{18} BDS (100 mm × 4.6 mm; 2.4 μm) column, flow 1.0 mL min^{-1}, column temperature of 40 °C, injection volume of 20 μL and gradient elution method with acetonitrile and formic acid (Gouvêa et al., 2015). The quantification of majority anthocyanins was performed by external standardization.

The amount and characterization of carotenoids were performed according to Rodriguez-Amaya (2001). Approximately 2.0 to 5.0 g of matrix was weighed and then macerated in a porcelain grail with 3 g of celite and 50 mL of acetone. The mixture was vacuum filtered on a glass funnel with sintered plate. The procedure was repeated three or four times until the array did not exhibit the characteristic color of carotenoids. Acetone extract was transferred quantitatively to a separatory funnel containing 50 mL of petroleum ether and washed, at least three times, with 300 mL ultrapure water. The ether extract was filtered through anhydrous sodium sulfate, collected in 100 mL volumetric flask and completed with petroleum ether. The level of total carotenoids in the sample extracts was determined by spectrophotometry at 450 nm (Shimadzu UV-1800, Shimadzu, Japan). Carotenoid profile was determined by taking a 1 mL aliquot and transferred to an amber vial, the sample was dried under a N_2 stream and then dissolved with 100 μL of acetone, the solution was vortex during 10 s and taken for HPLC analysis.

Carotenoid profile was determined in acetone extract by HPLC (Pacheco et al., 2014), using a Waters™ HPLC system controlled by Empower™ software with column oven at 33 °C and photodiode array detector (Waters™ 996). Carotenoid separation was obtained in a C30 column (S-3 Carotenoid, 4.6 mm × 250 mm, YCM) by a gradient elution of methanol and methyl tert-butyl ether, using a flow rate of 0.8 mL min^{-1} and running time of 28 min. The injection volume of the samples was 15 μL. Carotenoids were identified based on their retention times and UV/Vis absorption spectra, compared to the retention times of the carotenoid standards.

2.4. Single Cell Gel Electrophoresis (SCGE) Assay at week 3

Samples of peripheral blood were collected from the venous orbital plexus 4 and 18 h after the first administration of AOM. A volume of 5 μL of whole peripheral blood was mixed with 100 μL low melting point agarose (Invitrogen, USA) dissolved in phosphate buffer. The mixture was spread onto microscope slides precoated with normal melting point agarose (Invitrogen, USA). The slides were covered with a coverslip and maintained at 4 °C for 5 min. The coverslip was removed and slides were immersed in freshly cold lysis solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Co, USA), 10 mM Tris(Sigma-Aldrich, Co, USA), 1% N-lauroyl-sarcosine (Sigma-Aldrich, Co, USA), 1% Triton X-100 (Sigma-Aldrich, Co, USA) and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich, Co, USA)], pH 10 for about 60 min, at 4 °C. After lysis, prior to electrophoresis, the slides were placed in a horizontal electrophoresis unit and left in alkaline buffer containing 0.3 mM NaOH and 1 mM EDTA (pH > 13) for 20 min and electrophoresed for another 20 min at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol and stored at room temperature until blind analysis in a fluorescence microscope (Olympus; Optical Co. Ltd., Japan) at 100× magnification. Each slide was stained with 50 μL ethidium bromide (20 μL/mL, Sigma-Aldrich, Co, USA) and immediately analyzed. To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination. Automated analytical software (Comet Assay 2.0; Perceptive Instruments, UK) was used to determine DNA damage in peripheral blood nucleated cells. Two parameters were estimated: tail intensity (percentage of DNA in the tail) and tail moment (the product of the percentage of DNA in the tail and the mean distance of migration in the tail) (Tice et al., 2000) from 50 cells per each of two replicate slides.

2.5. Colon processing and immunohistochemical analyses at week 3

At necropsy, the colon was removed, opened longitudinally and gently rinsed with saline to remove residual bowel contents, fixed flat in 10% buffered formalin for 24 h at room temperature. The colons were Swiss-rolled (2 to 3.2-mm width segments) and embedded in paraffin. Tissue sections (5 μm) were obtained for immunohistochemical procedures. Immunoreactivity for Ki-67 (i.e., cell proliferation marker) and cleaved caspase-3 (i.e., apoptosis marker) was detected using a polymer system (MACH 4 Universal HRP polymer Detection, Biocare, CA, USA). Rabbit monoclonal anti-Ki-67 (clone SP6, 1:100 dilution, Abcam, UK) and rabbit polyclonal anti-cleaved caspase-3 (clone 5A1, 1:100 dilution, Cell Signaling Technology, USA) antibodies were used. Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Co, USA) and counterstaining with Harris's hematoxylin. Ki-67 labeling index (LI%) was evaluated by counting each epithelial cell along a single side of the intestinal crypt column, taking the base of the crypt as cell position one (Renehan, O'Dwyer, Haboubi, & Potten, 2002). Then, crypts were divided in two equal compartments, upper and lower, according to the half number of epithelial cells counted (Fig. 3). The cell proliferation indexes (%) (upper, lower and total) were determined by calculating the ratio of the labeled epithelial cells to the total number of epithelial cells along a single side of the crypt column. Cleaved Caspase-3 labeling index (LI%) was evaluated by placing a graticule in the eyepiece to limit the crypt bottom (Fig. 3), where the stem cells are located (Renehan et al., 2002). The apoptosis index (%) was determined by calculating the ratio of labeled cells in the crypt bottom to the total number of epithelial cells in it. Crypts were considered suitable for counting when sectioned axially from surface to crypt base, with a distinct crypt lumen and single column of epithelial cells on either side. Cell proliferation and apoptosis LI% were measured in 40 randomly chosen crypts/animal under light microscopy (400× magnification).

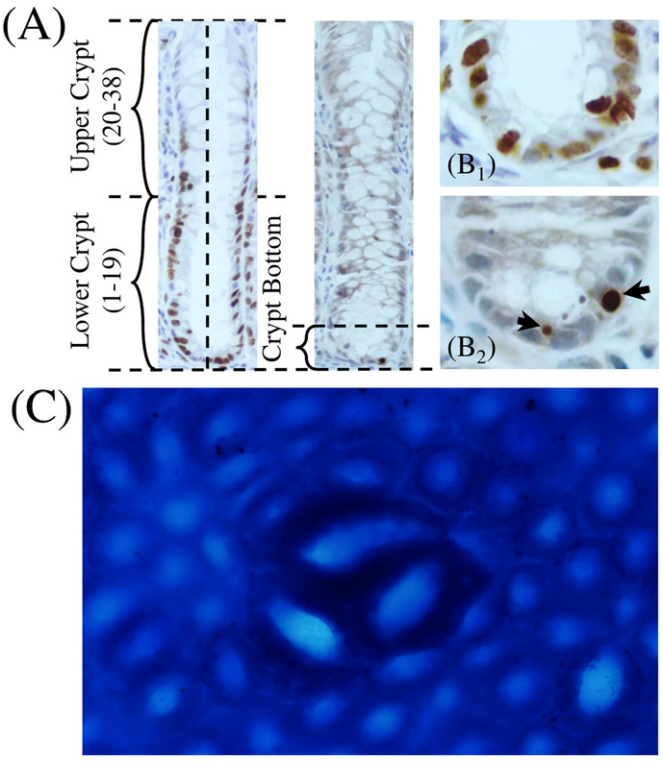

Fig. 3. Immunohistochemical and ACF analyses. (A) Representative microscopic views of immunostained colonic crypts limited in upper and lower compartments (Ki-67 LI%) and crypt bottom (cleaved caspase-3 LI%) (40× objective). Typical cell nuclei (brown) showing immunohistochemical expression for (B_1) Ki-67 and (B_2) cleaved caspase-3 (arrows) in colonic crypts (100× objective); (C) representative topographic view of classic ACF in a methylene blue-stained colon whole-mount containing three AC (40× objective).

2.6. Hepatic glutathione analyses at week 3

At necropsy, the liver was removed and samples (~200 mg) were homogenized in 5 mL chilled 0.01 M phosphate buffer (pH 7.4) with a homogenizer (Potter-Elvehjem, Pyrex, USA). The homogenates were centrifuged at 10,000×*g* for 15 min and, then, the supernatant fractions were collected to determine reduced (GSH) and total glutathione (Tietze, 1969). Reduced glutathione was measured in the liver by a kinetic assay in reaction medium containing 2 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB, Sigma-Aldrich, Co, USA), 0.2 mM NADPH, and 2 U of glutathione reductase in phosphate buffer (100 mM, pH 7.4) 5 mM EDTA. The total glutathione was assayed with 0.6 mM DTNB, and 1 U of glutathione reductase in buffer 0.1 M Tris–HCl, pH 8.0 containing 0.5 mM EDTA. The determinations were performed at 25 °C using a microplate reader (μQuant-MQX 200, Bio-Tec Instruments, USA).

2.7. Aberrant crypt foci analyses at week 14

At necropsy, the colon was removed, processed and fixed as above described. Then, colons were stained with 1.0% methylene blue dissolved in phosphate buffered salt solution (PBS) for analysis and quantification of ACF, using criteria previously described (Bird, 1987) (Fig. 3). The number of aberrant crypt (AC)/colon, number of ACF/colon and number of AC in each ACF (multiplicity)/colon was determined under light microscopy (400× magnification).

2.8. Statistical analysis

Data from body weight gain, food and water consumption, ACF analyses, SCGE, total and reduced glutathione and cell proliferation and apoptosis indexes were analyzed by one-way analysis of variance (ANOVA) and *post hoc* by Tukey test. The differences among groups were considered significant when $p < 0.05$.

3. Results

3.1. Anthocyanin and carotenoid analysis

The chemical analysis of AP revealed the presence of two main anthocyanins: cyanidin 3-glucoside and cyanidin 3-rutinoside (Fig. 4, Table 1). In addition, the analysis led to identification of lutein, α-carotene, β-carotene and 9-cis β-carotene as the main carotenoids present in AP (Fig. 4, Table 1).

3.2. General findings at weeks 1–4

No differences in body weight gain and food and water intakes were observed among groups (G1–G4) during AP and I3C feeding periods (Table 2). Also, no histopathology evidence of toxicity associated with AP feeding was observed after the macroscopic and microscopic examination of the stomach, intestines, kidneys and liver at the end of week 4(data not shown).

3.3. SCGE assay at week 3

No statistical differences were observed in both parameters measured (tail intensity and tail moment) in blood samples collected 4 h after the first AOM administration. On the other hand, both AP (G2 and G3) and 0.1% I3C (G4) intakes reduced ($p < 0.001$, for both parameters) DNA damage in peripheral blood cells when compared to the control group (G1) in blood samples collected 18 h after the first AOM administration (Fig. 5).

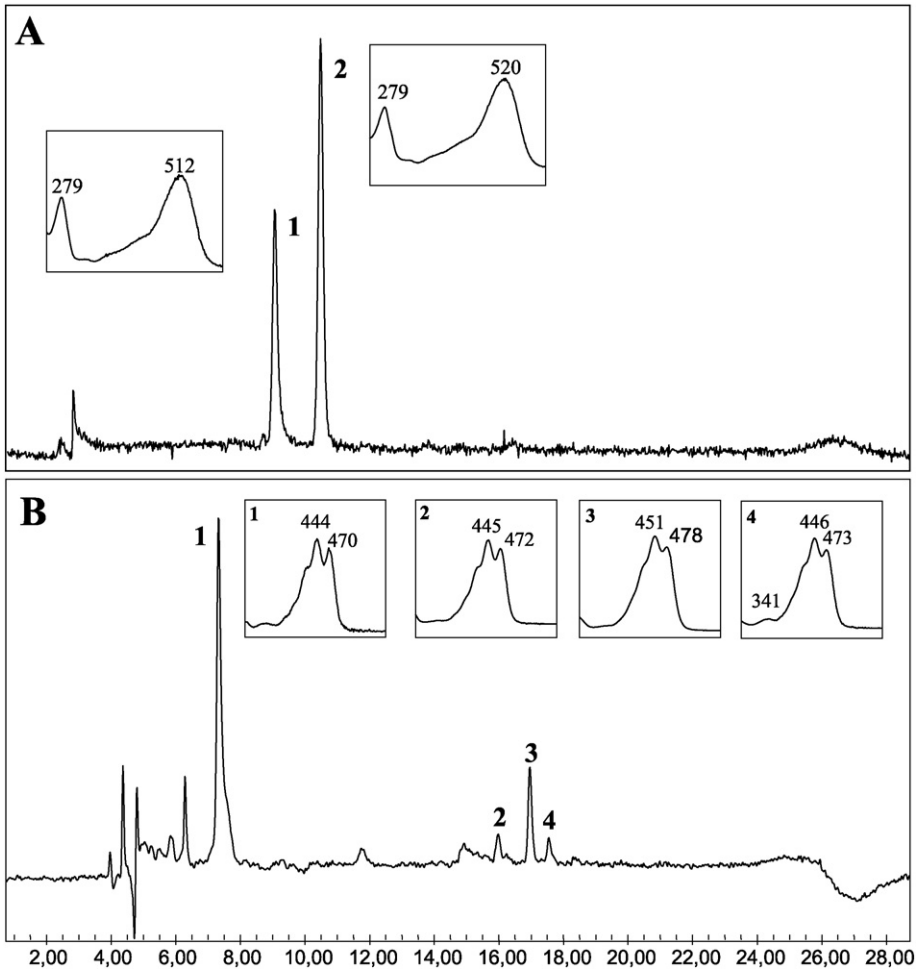

Fig. 4. Chromatograms and UV–Vis spectra of (A) anthocyanins [peak identification: (1) cyanidin-3-glucoside; (2) cyanidin-3-rutinoside] and (B) carotenoids [peak identification: (1) lutein, (2) α-carotene, 3) β-carotene, 4) 9-cis β-carotene] from AP.

3.4. Ki-67 and cleaved caspase-3 labeling indexes (LI%) at week 3

Twenty-four hours after first AOM administration, colonocytes labeled for Ki-67 were observed preferentially in the middle and bottom of the crypts (i.e. proliferative and stem cells zones, positions 1 to 25) (Renehan et al., 2002), while epithelial cells labeled for cleaved caspase-3 were observed in all crypt column but preferentially in the bottom of the crypts (stem cell zone) (Renehan et al., 2002) (Fig. 3).

Table 1
Anthocyanin and carotenoid content of açaí fruit pulp powder (AP).

Analytes[a]	Concentration
Anthocyanins[b] (mg/100 g AP)	
Cyanidin 3-glucoside	133.25 ± 13.51
Cyanidin 3-rutinoside	225.61 ± 17.21
Total	358.85 ± 30.49
Carotenoids (μg/100 g AP)	
Lutein	717.00 ± 15.00
α-Carotene	42.33 ± 7.76
β-Carotene	149.00 ± 37.64
9-cis β-carotene	35.66 ± 4.61
Total	1112.67 ± 34.96

Values are mean ± S.D.
[a] Analysis was performed in triplicate.
[b] Represented as cyanidin 3-glucoside equivalents.

Dietary 0.1% I3C (G4) reduced ($p < 0.001$) upper crypt cell proliferation levels when compared to control and 2.5% AP-treated groups(G1 and G2) (Table 3). Besides, both dietary AP concentration (G2 and G3) reduced upper, lower and total crypt proliferation levels when compared to control group (G1), without a statistically significant difference (Table 3). Also, no statistical difference in cleaved caspase-3 LI% was observed among the groups (G1–G4), but increases of 42% and 45% in apoptosis levels were observed in 5.0% AP and 0.1% I3C-treated groups(G3 and G4), respectively, when compared to the control group (G1) (Table 3).

3.5. Hepatic reduced and total glutathione at week 3

Dietary 5.0% AP and 0.1% I3C (G3 and G4) increased ($p = 0.002$) hepatic total glutathione levels when compared to the control group (G1) (Fig. 6). Moreover, 0.1% I3C (G4) feeding increased ($p = 0.018$) hepatic reduced glutathione levels when compared to the control group (G1) (Fig. 6).

3.6. ACF analyses at week 14

All mice receiving AOM developed ACF, mostly comprising of 2–3 AC/focus (Fig. 3). No statistical difference was observed among the groups (G1–G4) in the total number of AC and ACF. However, dietary 5.0% AP (G3) decreased ACF multiplicity (AC/ACF) ($p < 0.05$) when compared to other groups (G1 and G2) (Table 4).

Table 2
Effects of açaí pulp powder intake on body weight, food and water consumption until week 3 (G4) and 4 (G1 to G3).

Parameters	Groups/treatments[a]			
	(G1) AOM	(G2) AOM + 2.5% AP	(G3) AOM + 5.0% AP	(G4) AOM + 0.1% I3C
Number of mice	15	15	15	5
Final body weight (g)	40.81 ± 6.38	45.33 ± 4.47	45.73 ± 4.36	42.92 ± 5.20
Body weight gain (g)	8.03 ± 3.39	11.11 ± 4.01	11.72 ± 3.21	10.84 ± 4.29
Food consumption (g/mouse/day)	7.18 ± 1.32	8.07 ± 1.68	7.42 ± 1.68	7.61 ± 1.85
Water consumption (ml/mouse/day)	10.64 ± 4.03	11.24 ± 2.11	9.93 ± 1.76	10.59 ± 1.58
AP consumption (g/mouse/day)	–	0.21 ± 0.04	0.40 ± 0.08	–

Values are mean ± S.D.
[a] AOM = azoxymethane (15 mg/kg b. wt., i.p.); AP = Low fat diet containing 2.5% and 5.0% of açaí pulp powder for 4 weeks; I3C = Low fat diet containing 0.1% of Indole-3-Carbinol for 3 weeks.

4. Discussion

The present study demonstrated a protective effect of dietary açaí fruit pulp powder (AP) against the initiation stage of mouse colon carcinogenesis induced by the carcinogen azoxymethane (AOM). Dietary 5.0% AP reduced DNA damage in peripheral blood cells and ACF multiplicity in colonic mucosa, while increased hepatic total GSH levels. Moreover, no signs of systemic or organ toxicity were observed in the animals based on their weight gain profile, food consumption and macroscopic or microscopic pathology, suggesting that AP produced by a spray drying system is a safe and a functional food ingredient for cancer chemoprevention studies, as previously described (Fragoso et al., 2012, 2013).

Indole-3-carbinol was used herein as a positive control to prevent the deleterious effects induced by acute AOM exposure. In fact, a significant reduction in DNA damage, colonic epithelial cell proliferation as well as a significant increase in hepatic reduced and total GSH were observed in 0.1% I3C-treated group after first AOM administration. This compound is a natural component of cruciferous vegetables such as cabbage, radishes, cauliflower and broccoli, showing potential chemopreventive effects against the exposure to different carcinogens (Plate & Gallaher, 2006; Weng, Tsai, Kulp, & Chen, 2008). I3C and their derived substances can induce mixed-function oxidases and phase II antioxidant enzymes, including induction of glutathione S-transferase (GST) and quinine reductase (QR) activity (Plate & Gallaher, 2006; Weng et al., 2008).

Since there are no studies on the safety levels of açaí pulp intake by humans, we chose a 5.0% maximum dose level in our feeding study due to the absence of toxicity observed in previous rodent studies and to avoid nutritional imbalances, as recommended by the European Food and Safety Authority Scientific Committee (European Food Safety Authority, 2011). AP samples were obtained by spray drying method, which is a well-established and widely used technique to turn food matrices into powder form, protecting them from deterioration by external agents (Tonon, Brabet, & Hubinger, 2008). It consists of entrapping an active agent (as solid particles) in a polymeric matrix, such as

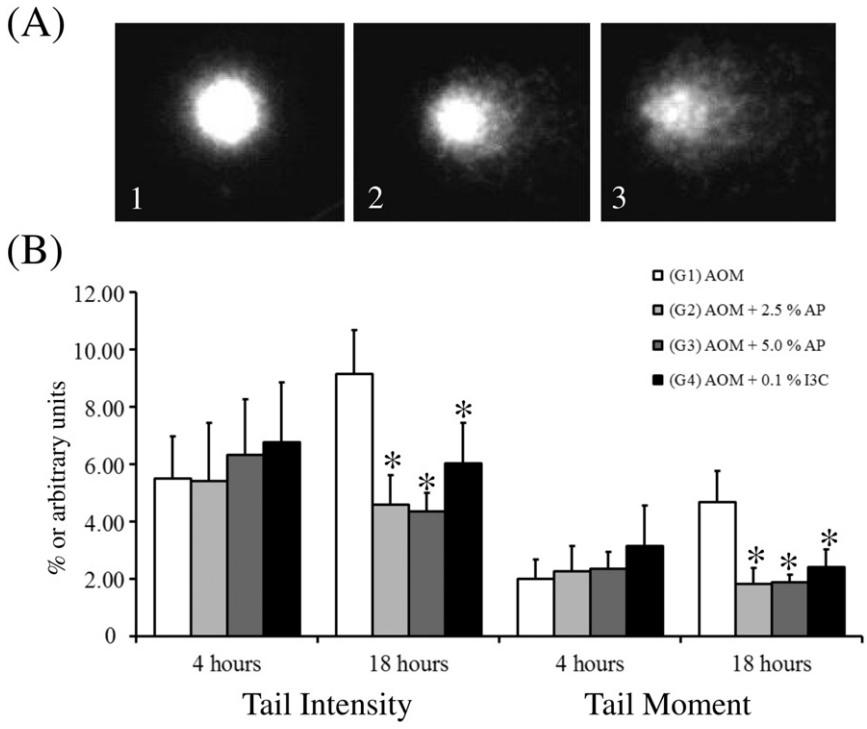

Fig. 5. Effects of AP intake on DNA damage at week 3. (A) Representative microscopic view of peripheral blood cell DNA migration patterns (1 to 3) produced by SCGE Assay (40× objective); (B) tail intensity (% of DNA in the tail) and tail moment (arbitrary units) results from SCGE Assay of peripheral blood collected 4 and 18 h after first AOM administration. AOM = azoxymethane (15 mg/kg b. wt., i.p.); AP = LFD containing 2.5% and 5.0% of açaí pulp powder for 4 weeks; I3C = LFD containing 0.1% of Indole-3-Carbinol for 3 weeks. Values are Mean ± S.D. *Different from the control (G1) group, $p < 0.001$.

Table 3
Effects of açaí pulp powder intake on cell proliferation (Ki-67) and apoptosis (cleaved caspase-3) in colonic crypt at week 3.

Parameters	Groups/treatments[a]			
	(G1) AOM	(G2) AOM + 2.5% AP	(G3) AOM + 5.0% AP	(G4) AOM + 0.1% I3C
Number of mice	5	5	5	5
Ki-67 LI%				
Upper crypt	20.79 ± 5.79	18.58 ± 8.49	14.84 ± 8.61	11.99 ± 2.74*
Lower crypt	72.57 ± 5.57	68.47 ± 6.58	70.27 ± 7.65	68.02 ± 3.73
Total crypt	43.73 ± 7.06	43.57 ± 7.50	42.53 ± 8.58	41.82 ± 2.43
Cleaved caspase-3 LI%				
Bottom crypt	3.29 ± 1.63	3.88 ± 1.02	4.68 ± 0.74	4.79 ± 1.00

Values are mean ± S.D.
[a] AOM = azoxymethane (15 mg/kg b. wt., i.p.); AP = low fat diet containing 2.5% and 5.0% of açaí pulp powder for 4 weeks; I3C = low fat diet containing 0.1% of Indole-3-Carbinol for 3 weeks.
* Different from G1 (control) and G2 ($p < 0.001$), using Tukey test.

maltodextrin D10 used in AP. The AP phytochemical evaluation indicates that, when compared to fresh and freeze-dried açaí fruit pulp, spray drying is a good alternative process for producing AP with anthocyanins and carotenoid content, since açaí is a highly perishable fruit and its nutrient content is very unstable (Krishnaiah, Nithyanandam, & Sarbatly, 2014; Pacheco-Palencia, Duncan, & Talcott, 2009; Ribeiro et al., 2010; Schauss et al., 2006; Tonon et al., 2008).

Rodent models of colorectal carcinogenesis induced by AOM are commonly used to mimic human sporadic CRC development due to their similar pathological features (Mori et al., 2005). The carcinogen AOM, when administered intraperitoneally, undergoes metabolic activation by cytochrome P450 enzymes, specifically by CYP2E1 in the liver (Megaraj et al., 2014; Rosenberg et al., 2009). The hepatic metabolism of AOM results in highly reactive methyldiazonium ions, which are transported to the colon primarily via blood system or, in small proportion, via bile (Megaraj et al., 2014; Rosenberg et al., 2009). These noxious ions alkylate specific DNA bases, resulting in DNA adduct formation, such as O^6-methylguanine (O^6-mG) and N^7-methylguanine (N^7-mG). If not repaired, these adducts can cause DNA damage and contribute to the initiation stage of colon carcinogenesis (Megaraj et al., 2014; Rosenberg et al., 2009). Despite the known role of hepatic enzymes in AOM activation, P450 enzymes in colonic mucosa are also capable of metabolizing AOM and contribute to AOM-induced DNA adduct formation and damage (Megaraj et al., 2014; Rosenberg et al., 2009).

Many studies have performed the phytochemical characterization of acai and most of them also identify cyanidin 3-glucoside and cyanidin 3-rutinoside as the main anthocyanins in acai fruit (Gordon et al., 2012; Lichtenthäler, Marx, & Kind, 2003; Schauss et al., 2006). Phenolic components found in açaí fruit pulp, such as both anthocyanins identified in AP, demonstrated high antioxidant capacity against highly reactive DNA-damaging species when compared to other fruits (Kang et al., 2010; Zielinski et al., 2014). In addition, lutein, a carotenoid found in AP, also showed to decrease oxidative stress and DNA damage (Serpeloni et al., 2014). In the present study, both AP feeding (2.5% and 5.0%) reduced blood cells DNA damage induced by acute exposure to AOM, suggesting an antigenotoxic activity, as also observed in other in vivo studies (Fragoso et al., 2012; Ribeiro et al., 2010). These results indicate that AP increased the resistance of genomic DNA to strand breaks, alkaline labile sites and oxidative lesions detected in SCGE assay and can contribute for the potential cancer-preventive activity of this exotic fruit. Although the evaluation of DNA damage in blood cells by SCGE may be used as a general biomarker of exposure, primary DNA damage in colonic epithelial cells by SCGE assay must be further investigated.

Considering the key role of the liver in AOM metabolism, with subsequent induction of DNA adducts and DNA damage, the modifying effects of AP feeding on hepatic glutathione levels were determined, since the liver plays important roles in endogen antioxidant defense, detoxification of carcinogens and their ultimate metabolites (Megaraj et al., 2014; Rosenberg et al., 2009). Glutathione is a tripeptide synthesized intracellularly that exists mainly in two forms: thiol-reduced (GSH) and disulfide-oxidized (GSSG) (Ballatori et al., 2009). GSH has potent electron-donating capacity and its oxidation to GSSG is carried out either by direct interaction with reactive electrophiles or when GSH acts as substrate for antioxidant enzymes, such as glutathione peroxidase (GSH-Px) and glutathione S-transferases (GST) (Ballatori et al., 2009). This endogen antioxidant can provide cell protection against electrophilic DNA-damaging agents. However, severe oxidative stress can deplete cellular GSH, leading to an increased susceptibility to DNA damage and, thus, development and progression of many diseases, including cancer (Ballatori et al., 2009). Thus, we found that 5.0% AP feeding increased total glutathione levels in the liver after acute exposure to AOM. These results indicate that AP increased this specific endogen antioxidant defense.

Different studies showed that low concentrations of polyphenols stimulated the expression of critical genes for cellular glutathione synthesis (Masella, Di Benedetto, Varì, Filesi, & Giovannini, 2005). Cyanidin 3-rutinoside, identified in AP used herein, as well as quercetin, resveratrol and other polyphenols present in açaí fruit pulp showed potential to increase the expression of subunits of glutathione cysteine ligase (GCL), an important enzyme for glutathione synthesis, and then, increase intracellular glutathione levels (Masella et al., 2005; Zhu, Jia, Wang, Zhang, & Xia, 2012). Some carotenoids, as β-carotene, also found in AP, also showed increased GSH concentrations by enhancing GCL expression in vitro (Akaboshi & Yamanishi, 2014), Thus, the anthocyanin and carotenoid contents identified in AP or açaí fruit pulp may be responsible for increasing hepatic total glutathione levels. When increased, this endogen antioxidant can protect cells from early AOM-induced DNA damage and also contribute for the potential cancer-preventive activity of AP.

In the colon, after acute exposure to AOM, adduct formation levels in the mucosa increase to a peak 6 h after AOM treatment, and these adducts are mainly located in lower and middle crypt compartments (positions 1 to 25) (Renehan et al., 2002). It has been proposed that apoptotic response is a suitable cell mechanism for protection against acute DNA damage (Chen & Huang, 2009; Renehan et al., 2002). Indeed, high apoptotic response is triggered in colonic mucosa 8 h after AOM treatment, mostly in lower crypt compartment, where the stem cells are located (Renehan et al., 2002). However, this apoptotic response showed to be a limited mechanism, allowing adduct-containing colonocytes to survive (Renehan et al., 2002). Since the colonic epithelium is a rapidly renewing tissue, supported by pluripotent progenitor stem cells located at the bottom of the crypt, a failure to delete such mutated cells by apoptosis may give rise to an abnormal clone with the potential to arise ACF and tumors (Renehan et al., 2002). In the present study, cell proliferation (Ki-67) and apoptosis (cleaved caspase-3) indexes were evaluated. Although an increase of 42% in apoptosis levels was observed in crypt stem cell zone from the 5.0% AP-treated group, the evaluation failed to detect a significant protective effect of dietary

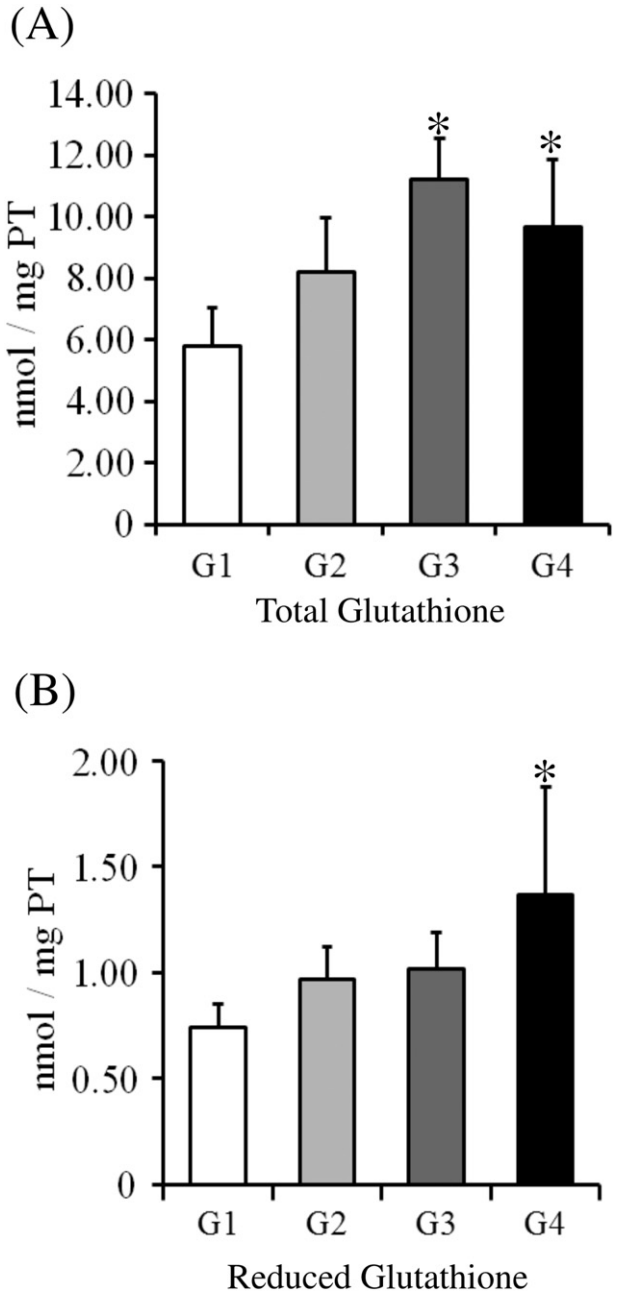

Fig. 6. Effects of AP intake on glutathione analyses at week 3. (A) Total and (B) reduced glutathione (both measured in nmol/mg of protein) determinations from liver samples. Group/treatment: AOM (G1), AOM + 2.5% AP (G2), 5.0% AP (G3) or 0.1% I3C (G4). AOM = azoxymethane (15 mg/kg b. wt., i.p.); AP = LFD containing 2.5% and 5.0% AP for 4 weeks; I3C = LFD containing 0.1% of I3C for 3 weeks. Values are Mean ± S.D. *Different from the control (G1) group, $p < 0.05$.

AP on colonic crypts analyzed 24 h after the first AOM administration. Earlier time analysis could elicit a significant apoptotic response by 5.0% AP treated group and characterize a potential cancer-preventive property of AP, since isolated anthocyanins and carotenoids present in açaí already showed to induce an apoptotic response through caspase-3 activation in vitro (Del Pozo-Insfran et al., 2006; Müller, Carpenter, Challis, Skepper, & Arends, 2002).

After the first AOM administration, mice received a second injection of AOM and were fed a high-fat diet until week 14. Recent studies provided data for a link between HFD feeding and higher risk of ACF development (Padidar et al., 2012). The early development of ACF is considered one of the hallmarks of chemical colon carcinogenesis (Bird, 1987; Mori et al., 2005). Aberrant crypts, which are larger, thicker and darker than normal surrounding colonic crypts in whole-mount methylene blue staining, are considered putative preneoplastic lesions in preclinical colon carcinogenesis models (Bird, 1987; Mori et al., 2005). Thus, ACF have been considered suitable biomarkers for screening chemopreventive agents and risk assessment of chemicals in the environment and food (Bird, 1987; Mori et al., 2005). One of the main advantages of using ACF as a biomarker in carcinogenic studies, besides the morphological similarities between AC in rodents and humans, is the brief time of experimentation required when compared to classic methods to analyze tumor development (Bird, 1987; Mori et al., 2005). In previous study, dietary 5.0% AP, when administered in the post-initiation period, attenuated dimethylhydrazine (DMH)-induction of ACF and colon tumors in male Wistar rats (Fragoso et al., 2013). The present study revealed that ACF multiplicity was decreased by 5.0% AP feeding when compared to the control group. ACF with increased number of AC have been associated with dysplastic features, presenting higher tendency to progress to malignancy (Mori et al., 2005). Thus, a decrease in ACF multiplicity indicates a direct modulation of AOM-induced initiation phase of colon carcinogenesis by AP feeding.

In summary, the present study shows that 5.0% AP feeding attenuates AOM-induced DNA damage and early mouse colon carcinogenesis. The potential antigenotoxic property, as well as the increase of glutathione antioxidant defense, can be key mechanisms for protecting genomic DNA from AOM-induced DNA damage, resulting in attenuation of chemically-induced initiation step of colon carcinogenesis. These protective effects by AP feeding could be associated to the presence of anthocyanins and carotenoids in their composition.

Table 4
Effects of açaí pulp powder intake on the development of colonic aberrant crypt foci at week 14.

Parameters[a]	Groups/treatments[b]		
	(G1) AOM	(G2) AOM + 2.5% AP	(G3) AOM + 5.0% AP
Number of mice	10	10	10
AC/colon	11.00 ± 2.92	14.00 ± 9.12	6.72 ± 4.67
ACF/colon	4.12 ± 1.15	4.75 ± 2.93	3.50 ± 2.00
AC/ACF (ACF multiplicity)	2.73 ± 0.59	3.80 ± 0.90	1.79 ± 0.54*

Values are mean ± S.D.
[a] AOM = azoxymethane (15 mg/kg b. wt., i.p.); AP = low fat diet containing 2.5% and 5.0% of açaí pulp powder for 4 weeks.
[b] AC = aberrant crypt; ACF = aberrant crypt foci.
* Different from G1 (control) and G2 ($p < 0.05$), using Tukey test.

Conflict of interest

All authors disclose any actual or potential conflict of interest.

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