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# Anthocyanins-loaded Eudragit® L100 nanoparticles: in vitro cytotoxic and genotoxic analysis

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**ABSTRACT.** Anthocyanins are flavonoids compounds that have a great therapeutic potential acting as antioxidants, anti-inflammatory and anti-carcinogenic agents. They are consumed through food, pharmaceutical and cosmetics industries as a natural alternative to synthetic dyes. However, anthocyanins are unstable depending on pH, temperature, light and oxygen variations, which led to the

production of its encapsulation in nanoparticles as an alternative to increase its stability and protect them from chemical degradation for human consumption. Due to the fact that many of the beneficial properties of nanoparticles could also be harmful to human health, and the need of prior toxicological evaluation of any medicinal product, this research aims to evaluate the cytotoxic and genotoxic effects of anthocyanins-loaded Eudragit® L100 nanoparticles (AN). A range of AN doses were tested with a preliminary MTT assay, which allowed selecting five concentrations for comet and micronucleus assays: 0.2, 2, 20, 125 and 250 µg/mL, respectively, on human peripheral blood mononuclear cells (PBMC) (cells without liver metabolizing enzymes) and human hepatoma cell line HepG2 (with liver metabolizing enzymes). Results showed absence of cytotoxic effect in MTT test on both cell types after 24 h of exposure. However, the nuclear division index in PBMC cells assessed by micronucleus test indicates a decrease in the cell division, after 28 h of exposure. Genotoxicity analysis showed that AN did not produce significant genotoxic effects detected in comet assay, in either cell type. However, the micronucleus test on HepG2 cells showed that at concentration of 2 µg/mL and higher, AN produced clastogenic/aneugenic effects. Under our experimental conditions and limitations, the observed cytotoxic and genotoxic effects, not previously reported in the literature, may be due to anthocyanin metabolization and related to its concentrations and time of exposure.

**KEY WORDS**: Anthocyanin; Genotoxicity; Comet Assay; Micronucleus Test; MTT Assay.

## INTRODUCTION

Belonging to the flavonoids class ($C_6$-$C_3$-$C_6$), the anthocyanins represent the largest group of water-soluble plant pigments (Ghosh and Konishi, 2007), thereby being the main responsible for red, blue and purple colors present in fruits – apple, red and black currants, blackberry, blueberry, strawberry, raspberry, grape, jabuticaba, among others –, and vegetables, such as sweet potato, radish, red onion, red cabbage, eggplant and black carrot (Ghosh and Konishi, 2007; Teixeira et al., 2008; Bishayee et al., 2010). They act on plants as a defense mechanism, protecting them against both environmental stress – i.e. ultraviolet light action, low temperatures and drought (Wallace, 2011) – and pathogens; but also as an attractive agent, e.g. on flowers, attracting pollinators (Sert et al., 2007). Anthocyanins are the glycosylated products of anthocyanidins, which, because their poor stability, are rarely found in nature (Feng et al., 2007; Wallace, 2011). In human diet, cyanidin is an anthocyanidin type widely found, and cyanidin-3-rutinoside is a common glycosylated form (anthocyanin) of this (Feng et al., 2007). Recently, in the United States, was estimated the human individual daily intake of 12.5 mg anthocyanins (He and Giusti, 2010).

Numerous reports in literature collected the great therapeutic potential of both anthocyanins isolates and anthocyanin-rich extracts and combination of bioflavonoids in both *in vitro* and *in vivo* systems (Lila, 2004; Teixeira et al., 2008; He and Giusti, 2010; Wallace, 2011). They are pointed by their as strong antioxidant properties, anti-inflammatory and anticarcinogenic activity (Feng et al., 2007; He and Giusti, 2010; Ha et al., 2015); reduction of the cardiovascular disease risk (Lopes et al., 2007; Cardoso et al., 2011), capacity to modulate the cognitive and motor function, to improve memory and to act as preventive of neural function declines related to age (Lila, 2004), among others.

Food, pharmaceutical and cosmetic industries consider the anthocyanins as important alternative to the synthetic dyes. However, its low stability as well as its reduced capacity of color compared to synthetic dyes, along with the difficulty and high cost to obtain, are the limiting factors for this. For example, pH changes may reduce half the anthocyanins content during food preparation and processing (Lopes et al., 2007; Teixeira et al., 2008; Cardoso et al., 2011). Temperature, presence of oxygen and light, structure and concentrations of pigments are the other factors besides pH that may influence the anthocyanins stability, leading to their degradation (Lopes et al., 2007).

The encapsulation of medicinal products might increase the active substances stability and to protect the sensitive substances of chemical degradation induced by pH or ultraviolet light. Additionally, this enhances drug efficacy, specificity and tolerability (Cetin et al., 2010). Eudragit® is a versatile polymer for drug delivery used to improve the stability and bioavailability of several substances. The solid powder Eudragit® L100 is an anionic copolymer constituted by methacrylic acid and methyl methacrylate and widely employed for the several formulations such as nanoparticles (NPs) that are small particles ranging from 1 to 100 nm (Cetin et al., 2010; Patra et al., 2017). Eudragit® L100 is capable to protect the drug being degraded at acid pH (stomach), allowing its release only at pH above 6.0, with its absorption in the intestine. Study performed by Danay et al. (2017) proves the anthocyanins-loaded Eudragit® L100 resistance to acid pH (pH = 1.2) degradation, with 70% of its release occurring at intestinal pH (pH = 6.8) after 120 min. In the course of time, the percentage of dissolution increases gradually (Danay et al., 2017). Therefore, this polymer would be effective in preserving the properties of anthocyanin, allowing it intact to reach the site of its absorption. This polymer has been successfully used for increasing the therapeutic effects and bioavailability of different substances, such as curcumin, insulin and pantoprazole, among others (Patra et al (2017). So, would be also effective in improving the low bioavailability of anthocyanins.

A nanoparticulated substance has a surface area with reactivity increases due the reduction of the particles size, and this may lead to a number of side biological effects. Tests revealed that the toxic effects of NPs employed in same dose are size-dependent: the lower size, the more toxic (Chen et al., 2007). Because their tiny size, allows them to easily cross the cell membrane and other biological barriers (Fu et al., 2014). If toxic, NPs can cause direct or indirect DNA damage: the first, by crossing the plasma membrane and reach cell nucleus; and the last, by triggering oxidative and inflammatory responses. Likewise, the accumulation of these materials within the cell allows its interaction with both DNA and all components of the mitotic spindle during cell division when the nuclear membrane is broken, which may affect the chromosomal segregation in the daughter cells, giving rise to chromosomal aberrations. In the long run, DNA damage may initiate cancer development or impact future generations if it affects reproductive cells (Singh et al., 2009). However, not all NPs have adverse health effects. Their toxicity will depend of various other factors such as: aggregation, composition and crystallinity of substances, increase of the reactivity surface, physico-chemical properties change, and mainly for the genetic constitution of each person that makes it to adapt or combat toxic substances (Buzea et al., 2007; Savolainen et al., 2010). Eudragit® polymers are considered as non-irritant and non-toxic products, being present in the Canadian list of acceptable non-medicinal substances and in the FDA Inactive Ingredients Guide (oral capsules and tablets). In humans, daily intake of 2 mg/kg body-weight of Eudragit® is regarded as safe (Patra et al., 2017). The encapsulation is effective in maintaining the properties of anthocyanins, conferring their stability. But, the question is whether it would be safe to administer anthocyanins-loaded Eudragit® L100 nanoparticles in humans or not, bearing in mind the absence of studies investigating the genotoxic effects of anthocyanins after it has been encapsulated. Therefore, the present study aims to assess, *in vitro*, by using peripheral blood mononuclear cells (PBMC) and HepG2 human cells, the cytotoxic, genotoxic and mutagenic effects of anthocyanins-loaded Eudragit® L100 nanoparticles with and without its metabolism by liver enzymes, through of MTT assay and two of the most frequently used methods in genotoxicity investigation of nanomaterials: comet assay and micronucleus test (Landsiedel et al., 2009).

## MATERIAL AND METHODS

### Chemical compounds

Methyl methanesulfonate (MMS) (Sigma-Aldrich®, CAS number 66-27-3) and Benzo(a)pyrene (BaP) (Sigma-Aldrich®, Lot: LC04528V) were used as the positive control. The other main chemicals were obtained from the following suppliers: 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Invitrogen™, Lot: 1178042), Antibiotic-antimycotic (Gibco®, Ref. 15240-062), Cytocalasin-B (Sigma-Aldrich®), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich®), Ethidium bromide (Sigma-Aldrich®), Ethylenediamine tetraacetic acid (EDTA) (Synth), Fetal Bovine Serum (FBS) (Gibco®), Giemsa (Synth), Histopaque®-1077 (Sigma®), Low-Melting Point (LMP) agarose (Invitrogen™), Minimum Essential Medium (MEM) (Gibco®), Phytohemaglutinin-A (Sigma-Aldrich®), RPMI 1640 Medium (Gibco®), Sodium Pyruvate (Gibco®), Triton™ X-100 (Sigma-Aldrich®), Trypsin (Sigma®, Lot# 039K7013), and UltraPure™ agarose (Invitrogen™).

### Acquisition and preparation of nanoencapsulated anthocyanins

Anthocyanins-loaded Eudragit® L100 nanoparticles (AN) were kindly sent by Dr. José Carlos Tavares Carvalho from Drug Research Laboratory at Federal University of Amapá (Macapá, AP, Brazil). Total anthocyanins were obtained from freeze-dried açaí pulp (Euterpe oleracea Mart.) by a technique frequently used to extract active principles from vegetable products based of solid-liquid extraction as described by Dupeyrón et al. (2017).

According to Ribeiro et al. (2010), the major anthocyanins present in açaí pulp are cyaniding 3-glucoside (11.1 mg/100g) and cyaniding 3-rutinoside (241.8 mg/100g). All other major compounds found in the açaí pulp are described in Ribeiro et al. (2010). The AN used in this research were prepared using Eudragit® L100 anionic copolymers constituted by methacrylic acid and methyl methacrylate, with 1:10 ratio of polymer: anthocyanins (sample A6). All process of encapsulation, sedimentation, agglomeration and other physicochemical and morphological characterization of these nanoparticles were detailed in Dupeyrón et al. (2017). To be tested with human cells, AN was diluted in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco®, pH 7.4).

## Cell types

Human Peripheral Blood Mononuclear Cells (PBMC) – which do not have biotransformation liver enzymes –, were isolated from two healthy (one male and one female), non-smoking volunteers, aged from 18 to 35 years; and the human Hepatoma cell line HepG2, which is known to contain a variety of liver metabolizing phase 1 and phase 2 enzymes (Hewitt and Hewitt, 2004), were obtained from the Rio de Janeiro Cell Bank, located in the Federal University of Rio de Janeiro, Brazil. Peripheral blood samples donors provided a written consent at the time of donation. The present study was approved at 2015, under the number CAAE: 42783415.2.0000.5406, by the Human Ethical Committee of the Paulista State University "Júlio de Mesquita Filho" (UNESP), in Marília, Brazil.

The PBMC was cultivated in RPMI 1640 medium with 25 mM HEPES, supplemented with 15-20% FBS, and 1% antibiotic-antimycotic. The HepG2 was cultivated in Eagle's Minimum Essential Medium (MEM) with 2.2 mg/mL Sodium Bicarbonate ($NaHCO_3$), and 1% Sodium Pyruvate, supplemented with 10% FBS and 1% antibiotic-antimycotic, and prior to the start of each experiment, it grew to reach 80-90% confluence (5 days). For both cell types and all experiments, cells were incubated in a $CO_2$ incubator at 37ºC, and 95% relative humidity.

## Cytotoxicity analysis

The cytotoxicity test was performed according to Mosmann (1983), with some modifications, using 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, in triplicate for controls and treatments on both cell types. The mitochondrial dehydrogenase enzymes of viable cells reduce the MTT tetrazolium salt ring, generating the formazan crystals, a purple product that accumulates inside the cells. This can be quantified by spectrophotometer, and the number of viable cells is directly proportional to the optical densities (Mosmann, 1983).

PBMC isolated with histopaque®-1077, and HepG2 cells were seeded in 24- and 96-well plates, respectively, at a concentration of 2.5 x $10^5$ and 2.5 x $10^4$ cells/well for 24 hat 37 °C. After this time, the medium was discarded, and cells exposed to anthocyanins-loaded Eudragit® L100 nanoparticles for another 24 h (37 °C) at the following concentrations: 0.2, 2, 20, 125 and 250µg/mL, completing with cell-specific supplemented culture medium the final volume of 2000 µL/well (PBMC) and 250 µL/well (HepG2 cells). Limited dilution of AN in the solvent used (RPMI medium) made the test impossible at concentrations higher than 250 µg/mL. Blank wells containing medium without FBS was prepared in triplicate too. The negative control was RPMI 1640 medium, and 1% Triton X-100 (Sigma-Aldrich) solution was the positive control.

Next, the medium of the all wells was removed, and MTT solution (0.5 mg/mL) was added –300 µL/well (for PBMC) and 150 µL/well (for HepG2) –, and the plates were incubated for 4 h at 37 °C. MTT solution was bound from the dilution of the stock solution of MTT (obtained by an initial dilution in phosphate-buffered saline (PBS) (5 mg/mL)) in serum free medium, in the proportion of 1:9 (v/v). At the end, after MTT discarded, 100 µL/well of DMSO was added to dilution of the formazan crystals. Plates were shaken for 15 min, and then read with microplate spectrophotometer (Epoch-BioTek®) at a wavelength of 570 nm (PBMC) and 620 nm (HepG2). To obtain the percentage of viable cells, first the mean absorbance of each treatment was subtracted of the mean absorbance of the blank wells, and then, the mean absorbance of each treatment was divided by the mean absorbance of the negative control.

## Single-cell gel electrophoresis (comet assay)

Experiments were performed according to the method described by Singh et al. (1988) and Klaude et al. (1996), with some modifications. PBMC isolated with histopaque®-1077 and HepG2 cells were cultivated for 24 h (37 °C), respectively at a concentration of 1.5 and 0.5 x $10^5$ cells/well in 24-well plates in triplicate to controls and treatments. After this time, cells were exposed for 4 h to the five concentrations of anthocyanins-loaded Eudragit®

L100 nanoparticles selected by the MTT assay. The positive control was MMS (75 μM) (OECD 489, 2014), and the negative control was RPMI-1640 medium (20 μL).

The PBMC and the HepG2 cells were centrifuged (252 g), 10 and 5 min, respectively), and the supernatant was discarded until 100 μL to resuspend pellets, which were mixed at 100 μL of 0.5% LMP agarose (37 °C). Same volume was deposited on a previously prepared with a thin layer 1.5% UltraP ure™ agarose slide, and then covered with a cover glass. The slides were refrigerated at 4 °C for at least 20 min for agarose hardening. Next, the cover glasses were removed, and the slides were maintained in cold lysis solution [2.5 M NaCl, 100 mm EDTA, 10 metrics, 1% Triton x-100 and 10% DMSO (pH 10)] for 1 h at 4°C, protected from the light. Still in absence of light, to protect the additional DNA damage, the slides were deposited in electrophoresis box (in ice bath), where remained for 20 min for DNA denaturation in alkaline buffer [300 mm NaOH – 1 mMEDTA, prepared from a stock solution of 10 M NaOH and 200 mm, pH 10.0, EDTA at 4 °C (pH > 13)]. Next, the electrophoresis was started at 25 V (1 V/cm, 300 mA) for 20 min. Slides were neutralized in 0.4 M Tris solution (pH 7.5) three times, in a total of 15 min, and dried at room temperature and fixed in absolute ethanol for 10 min. Now of analysis and in the absence of light, slides were staining with 200 μg/mL ethidium bromide solution and analysis immediately in fluorescence microscope (Olympus – excitation filter: 515-560 nm; emission filter: 590 nm) with 400x magnification.

The extent and distribution of DNA damage were determined by examining 100 randomly selected and non-overlapping cells per culture well. These cells were scored visually, according to tail size, into the following 4 classes: class 0- no tail; class 1- tail shorter than the diameter of the head (nucleus); class 2- tail length one or two times greater than the diameter of the head; and class 3- tail length more than twice the diameter of the head. The total score for 100 comets, which ranged from 0 (no damage) to 300 (severe damage), was obtained from the sum of multiplying the number of cells in each class with the damage class. Apoptotic and necrotic cells were not considered.

## Cytokinesis block micronucleus assay (CBMN assay)

The CBMN assay was developed according to Fenech (2000), in duplicate on human peripheral blood lymphocytes from two donors, and in triplicate on HepG2 cells, for controls and each anthocyanins-loaded Eudragit® L100 nanoparticles concentration. For human lymphocytes cultures, each 10 cm² flat face culture tubes (TPP®) was prepared with 4.5 mL supplemented RPMI 1640 medium, further 5 μg/mL Phytohemagglutinin-A to stimulate lymphocytes division, and 0.5 mL plasma followed by 6 to 8 drops erythrocyte concentrate. The tubes were incubated for 72 hours at 37 °C. The exposure to the five concentrations of anthocyanins-loaded Eudragit® L100 nanoparticles previously determined in the MTT assay was made 44 hours after incubation. According to OECD TG 487 (2014) guidelines, MMS (150 μM) was used as positive control; and the negative control was RPMI 1640 medium (50 μL). Cytocalasin-B (6 μg/mL) was added to each flask culture – to inhibit cytokinesis – 4 h after the addition of the test compound.

For HepG2 cells, were prepared 25 cm² culture flasks (TPP®) with 5 mL supplemented MEM medium and at a cell concentration of 2 x $10^5$, which were cultured at 37 °C, until complete a cell cycle (24 h). The exposure to anthocyanins-loaded Eudragit® L100 nanoparticles was made during 24 h with the five concentrations previously determined by the MTT test. Under recommendation of OECD TG 487 (2014) guidelines, the positive control was Benzo(a)pyrene (2 μM), and the negative control was the same above. Next, the cells were washed with 5 mL PBS, and the cytocalasin-B (6 μg/mL) was added in all flasks along with MEM medium, followed by incubation for more 28 h. Finally, cells were washed once again, and transferred to centrifuge tubes after trypsin (0.5% EDTA) treatment.

Both cell types were centrifuged for 5 min, and pellets suspended in 0.075 M KCl hypotonic solution and 10 mg/mL sodium citrate solution, both at 4 °C, for lymphocytes and HepG2 cells, respectively. Once again, cells were centrifuged and fixed in a cold solution 3:4 and 3:1 ratio of methanol and acetic acid to lymphocytes and HepG2, respectively, followed by 4% formaldehyde drops for the cytoplasm preservation. This step was repeated twice (without formaldehyde), being that was used 5:1 ratio fixative for lymphocytes. Slides were prepared by dripping and stained with 10% Giemsa for 10 min.

The analysis was made in light microscope (Zeiss, Primo Star) with 400x magnification, by counting of 1000 binucleated cells for exposure/repetition and controls, noting the presence of micronucleus (MN), nucleoplasmic bridges (NPB) and nuclear buds (NB) in cells. The Nuclear Division Index (NDI) was calculated too, as an additional cytotoxicity measure, following the formula: [M1 + 2(M2) + 3(M3) + 4(M4)/N, in which M1-M4

indicates the cell number containing 1-4 nucleus assessed in a total of 500 cells (N) to each exposure/repetition and controls.

## Statistical analysis

All data was analyzed statistically through GraphPad Prism® 5 (version 5.02) software, employing the one-way analysis of variance (ANOVA), followed by the Tukey's test to compare the experimental groups and controls. Results of this comparison were considered statistically significant with 95% confidence level ($p<0.05$).

## RESULTS

The analysis of the cytotoxic potential of anthocyanins-loaded Eudragit® L100 nanoparticles assessed by the MTT assay on PBMC (FIGURE 1.A) and HepG2 (FIGURE 1.B) cells showed that none of the concentration tested (0.2, 2, 20, 125 and 250 µg/mL) was significantly cytotoxic, with results of cell viability higher than 75%, which does not differ statistically ($p > 0.05$) from the negative control. However, is notorious that there was a substantial reduction in cell viability to the concentration of 250 µg/mL (76.77% viable cells) in PBMC compared to the lower concentrations (> 150% viable cells), although the concentration of 0.2 µg/mL has presented a mean of 90.91% viable cells. Already in HepG2 cells, the percentage of viable cells decrease gradually from the concentration of 125 µg/mL (96.32% viable cells). For this reason, since no significant cytotoxicity was observed, all five concentrations of anthocyanins-loaded Eudragit® L100 nanoparticles tested in the MTT assay were used in genotoxic assays for both cell types. Limited dilution of AN in the vehicle used (RPMI medium) made the test impossible at concentrations higher than 250 µg/mL.

According to findings from the comet assay presented in Tables 1 and 2, there was not statistically significant increase in the total number of damaged cells and their scores on both PBMC (TABLE 1) and HepG2 (TABLE 2) cells after 4 h of exposure to anthocyanins-loaded Eudragit® L100 nanoparticles concentrations, compared to the negative control, demonstrating the absence of genotoxic effects on both cell types.

**Table 1** DNA migration (mean ± SD) in the Comet Assay after 4 h of exposure on Peripheral Blood Mononuclear Cells (PBMC) to anthocyanins-loaded Eudragit® L100 nanoparticles (AN) concentrations.

| | | Comet class | | | | |
|---|---|---|---|---|---|---|
| **Test substance** | **Total[1]** | **0** | **1** | **2** | **3** | **Scores** |
| Negative Control | 7.50 ± 3.72 | 92.50 ± 3.72 | 3.00 ± 2.19 | 2.16 ± 2.04 | 2.33 ± 0.51 | 14.33 ± 5.64 |
| 0.2 µg/mL AN | 10.00 ± 6.03 | 90.00 ± 6.03 | 6.33 ± 4.13 | 2.00 ± 1.89 | 1.66 ± 1.21 | 15.33 ± 9,26 |
| 2 µg/mL AN | 4.66 ± 3.67 | 95.33 ± 3.67 | 2.33 ± 1.86 | 0.83 ± 1.32 | 1.50 ± 2.34 | 8.50 ± 9,00 |
| 20 µg/mL AN | 12.00 ± 12.31 | 88.00 ± 12.31 | 8.16 ± 7.98 | 2.00 ± 2.68 | 1.83 ± 2.63 | 17.67 ± 19,66 |
| 125 µg/mL AN | 9.83 ± 4.26 | 90.17 ± 4.26 | 5.00 ± 3.34 | 2.50 ± 1.76 | 2.33 ± 2.06 | 17.00 ± 8,62 |
| 250 µg/mL AN | 17.17 ± 9.10 | 82.83 ± 9.10 | 9.16 ± 7.44 | 3.83 ± 1.94 | 4.16 ± 2.31 | 29.33 ± 13,43 |
| 75 µM MMS | 100.00 ± 0.00[b] | 0.00 ± 0.00[b] | 6.00 ± 3.57 | 23.50 ± 8.36[a] | 70.50 ± 11.22[b] | 264.50 ± 14,40[b] |

SD = Standard deviation. MMS, methyl methane sulfonate (positive control). Negative control: RPMI 1640 medium. One-way analysis of variance (ANOVA), and Tukey multiple comparison test: statistically different from the negative control: [a]($p<0.01$), [b]($p<0.001$). [1] Total number of damaged cells (class 1 + 2 + 3).

**Table 2.** DNA migration (mean ± SD) in the Comet Assay after 4 h of exposure on Human hepatoma cell line HepG2 to anthocyanins-loaded Eudragit® L100 nanoparticles (AN) concentrations.

| | | Comet class | | | | |
|---|---|---|---|---|---|---|
| **Test substance** | **Total[1]** | **0** | **1** | **2** | **3** | **Scores** |
| Negative Control | 11.67 ± 11.02 | 88.33 ± 11.02 | 9.66 ± 9.01 | 1.00 ± 1.00 | 1.00 ± 1.00 | 14.67 ± 14.01 |
| 0.2 µg/mL AN | 2.00 ± 2.00 | 98.00 ± 2.00 | 2.00 ± 2.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.00 ± 2.00 |
| 2 µg/mL AN | 1.00 ± 1.00 | 99.00 ± 1.00 | 1.00 ± 1.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00 ± 1.00 |
| 20 µg/mL AN | 7.33 ± 5.77 | 92.67 ± 5.77 | 7.33 ± 5.77 | 0.00 ± 0.00 | 0.00 ± 0.00 | 7.33 ± 5.77 |
| 125 µg/mL AN | 0.66 ± 1.15 | 99.33 ± 1.15 | 0.66 ± 1.15 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.66 ± 1.15 |
| 250 µg/mL AN | 5.00 ± 4.00 | 95.00 ± 4.00 | 3.33 ± 4.04 | 1.00 ± 1.00 | 0.66 ± 1.15 | 7.33 ± 5.50 |
| 75 µM MMS | 99.33 ± 0.57[b] | 0.66 ± 0.57[b] | 7.33 ± 3.78 | 34.00 ± 14.00[a] | 58.00 ± 16.37[b] | 249.30 ± 18.56[b] |

SD = Standard deviation. MMS, methyl methane sulfonate (positive control). Negative control: RPMI 1640 medium. One-way analysis of variance (ANOVA), and Tukey multiple comparison test: statistically different from the negative control: [a](p<0.01), [b](p<0,001). [1] Total number of damaged cells (class 1 + 2 + 3).

The CBMN assay showed that in human blood lymphocytes exposed to anthocyanins-loaded Eudragit® L100 nanoparticles there was no increase in the percentage (or number) of binucleated cells with MN, which was near to the result of the negative control (0.45%) for all concentrations, as are set out in Table 3. Unlike this, as show in Table 4, in HepG2 cells only the concentration of 0.2 µg/mL anthocyanins nanoparticles has not produced a statistically significant increase in the number of binucleated cells with MN, compared to the negative control. The other four tested concentrations showed a marked increase in the percentage of micronucleated cells, approaching the value of the positive control, which indicates a clastogenic and/or aneugenic effect of these anthocyanins nanoparticles after its metabolism. The incidence of nucleoplasmic bridge (NPB) and nuclear buds (NB) was higher in treated HepG2 cells (TABLE 4), but there was only statistical difference from the negative control at the three higher concentrations. In this assay, NDI values calculated for lymphocytes suggest a possible cytotoxicity of the AN (TABLE 3) after 28 h of exposure, because a statistical difference was found (p > 0.05) between its all five tested concentrations and the negative control, whereas this did not occur for NDI values in HepG2 cells (TABLE 4) after 24 h of exposure, which obtained similar results to the negative control.

**Table 3 –** Micronucleus (MN) frequency, nucleoplasmic bridge (NPB), nuclear buds (NB) and nuclear division index (NDI) in human peripheral blood lymphocytes exposed to anthocyanins-loaded Eudragit® L100 nanoparticles (in duplicate for each sample).

| | Exposure (28 h) | Binucleated cells with MN | | NPB | NB | NDI |
|---|---|---|---|---|---|---|
| **Test substance** | **Concentrations** | **(4000 cells)** | | | | **(2000 cells)** |
| | **(µg/mL)** | **No.** | **%** | **No.** | **No.** | **(Mean ± SD)** |
| Negative control | 0 | 18 | 0.45 | 2 | 1 | 1.80 ± 0.10 |
| MMS (Positive control) | 150* | 245[c] | 6.12[c] | 20[b] | 40[c] | 1.12 ± 0.04[c] |
| Anthocyanins-loaded Eudragit® L100 nanoparticles | 0.2 | 26 | 0.65 | 0 | 3 | 1.56 ± 0.07[b] |
| | 2 | 13 | 0.32 | 2 | 3 | 1.55 ± 0.07[b] |
| | 20 | 17 | 0.42 | 1 | 4 | 1.59 ± 0.08[a] |
| | 125 | 10 | 0.25 | 0 | 4 | 1.58 ± 0.08[a] |
| | 250 | 28 | 0.70 | 3 | 3 | 1.61 ± 0.02[a] |

*Concentration = µM. SD = Standard deviation. Negative control: RPMI 1640 medium. One-way analysis of variance (ANOVA), and Tukey multiple comparison test: [a]Statistically different from the negative control: [a](p<0.05), [b](p<0.01), [c](p<0.001). Note: Presence of micronucleus, nuclear buds and nucleoplasmic bridge within the same cell.

**Table 4** – Micronucleus (MN) frequency, nucleoplasmic bridge (NPB), nuclear buds (NB) and nuclear division index (NDI) in human hepatoma cell line HepG2 exposed to anthocyanins-loaded Eudragit® L100 nanoparticles (in triplicate).

| Test substance | Exposure (24 h) Concentrations (µg/mL) | Binucleated cells with MN (3000 cells) No. | % | NPB No. | NB No. | NDI (1500 cells) (Mean ± SD) |
|---|---|---|---|---|---|---|
| Negative control | 0 | 43 | 1.43 | 27 | 41 | 1.25 ± 0.16 |
| Benzo(α)Pyrene (Positive control) | 150* | 179[b] | 5.96[b] | 82[a] | 115[b] | 1.25 ± 0.14 |
| Anthocyanins-loaded Eudragit® L100 nanoparticles | 0.2 | 97 | 3.23 | 47 | 93 | 1.25 ± 0.02 |
| | 2 | 152[b] | 5.06[b] | 43 | 82 | 1.24 ± 0.03 |
| | 20 | 136[a] | 4.53[a] | 30 | 109[a] | 1.25 ± 0.05 |
| | 125 | 147[b] | 4.90[b] | 39 | 170[c] | 1.21 ± 0.02 |
| | 250 | 162[b] | 5.40[b] | 41 | 149[c] | 1.18 ± 0.08 |

*Concentration = µM. SD = Standard deviation. Negative control: RPMI 1640 medium. One-way analysis of variance (ANOVA), and Tukey multiple comparison test: [a] Statistically different from the negative control: [a]($p<0.05$), [b]($p<0.01$), [c]($p<0.001$). Note: Presence of micronucleus, nuclear buds and nucleoplasmic bridge within the same cell.

# DISCUSSION

In recent years, the anthocyanins have attracted the interest of researchers and healthcare professionals because their great therapeutic and/or protector potential for the human health, in addition of an alternative to replace synthetic dyes in food, cosmetics and medicines. But, due to their instability and low bioavailability (< 1%) (McGhie and Walton, 2007; Wallace, 2011; Khandelwal and Abraham, 2014), an alternative for the maximum use of its benefits was the encapsulation of these substances. Novel pharmaceuticals invariably need to undergo preclinical safety testing before being made available for human use. The genotoxicity assays belong to this test battery (Singh et al., 2009). So, in the present study, to certify the safety in the use of anthocyanins nanoparticles by humans, anthocyanins-loaded with Eudragit® L100 nanoparticles were assessed using the MTT, comet and micronucleus assays on two distinct models of human cells: normal mononuclear blood cells, obtained from human peripheral blood, which do not have liver biotransformation enzymes; and a transformed adherent cell line, named HepG2, derived of human hepatoma, which is known to contain a variety of liver metabolizing enzymes (Hewitt and Hewitt, 2004).

We chose the açai (*Euterpe oleracea* Mart.) pulp to extract total anthocyanins because it is considered an anthocyanin-rich fruit and also it is commonly commercialized and consumed by the Brazilian population besides it is also found all over the world. Cyanidin 3-glucoside and cyanidin 3-rutinoside, the latter being found mainly in freeze-dried açaí pulp, were described as the predominant anthocyanins in this fruit (Carvalho et al., 2016). These two anthocyanins were also identified by Ribeiro et al. (2010) as major components of total anthocyanins in the açai pulp. The cytotoxic results obtained in the present study evaluating the nanoencapsulated anthocyanins are with agreement to the other studies investigating anthocyanins present in the açaí fruit, without nanoencapsulation. Our results for the MTT test showed that nanoencapsulated anthocyanins, after 24 h exposure, did not importantly influence the viability of both cell types, as compared to negative control. Although there was a considerable increase in the percentage of viable cells for the concentrations of 2, 20 and 125 µg/mL AN pointed by MTT test for human PBMC, there was no statistically significant difference ($p > 0.05$, ANOVA and Tukey) compared to negative control. The MTT test provide absorbance results (subsequently converted into percentage of viable cells) generated from the reduction of MTT tetrazolium salt by mitochondrial enzymes of living cells. Therefore, the percentage of viable cells corresponds directly to the amount of MTT metabolized for these cells. Thus, the high percentage of viable cells observed for the concentrations mentioned above indicates that cells exposed to them were capable to convert a greater amount of MTT than the negative control cells. Similarly to our results, Feng et al. (2007) also verified an increased reduction of MTT on human PBMC (stimulated or not with phytohemagglutinin) treated with cyanidin-3-rutinoside (C-3-R), the same anthocyanin present in the acai pulp, reaching absorbance two times higher than that for negative control. These authors found that there was a reduction in the levels of intracellular peroxide on C-3-R-treated human PBMC in the time interval of 15 min to 4 hours, which would have generated a less oxidative environment in these cells (Feng et al., 2007), improving the mitochondrial metabolism. It is possible that the same occurred in our study, with the effect in a concentration-dependent manner. Nevertheless, van Tonder et al. (2015) noted that the MTT test present a variability in their results. The amount of formazan crystals synthesized from MTT metabolism depends of the number and physiological conditions of mitochondria and its metabolic rate. Therefore, the variation of these indicators in the

cells can change the absorbance obtained and, consequently, the estimated number of cells (van Tonder et al., 2015).

Contradictorily, the mean of NDI values obtained by the CBMN assay in PBMC showed that all five nanoencapsulated anthocyanins concentrations were able to affect cell division, indicating a cytotoxic activity. On the other hand, NDI values in HepG2 remained similar to the negative control after the same exposure time used in MTT test. Our cell count to determine NDI values in PBMC-treated pointed a greater number of mononuclear cells (interphase cells), showed a statistically significant difference compared to the NDI value for negative control that presented largest number of dividing cells (with 2 or more nuclei).

Fimognari et al. (2005) also obtained a reduction in a dose-dependent manner in NDI values of lymphocytes treated with the Cyanidin-3-O-β-glucopyranoside (Cy-g) anthocyanin. However, NDI decreased from 50 µg/mL (7.5%) to 200 µg/mL (>80%), whereas our results for all concentrations remained similar with each other. Unlike our study whereby none concentration tested by MTT assay presented statistically significant difference of viable cells compared to negative control, Cy-g induced apoptosis in both transformed cells (Jurkat T leukaemia cells) and normal cells (human T lymphocytes) modulated by a rise of p53 and bax proteins. The first cell type was more susceptible to effects at lower tested concentrations (12.5 µg/mL) with greater number of apoptotic cells compared to normal T cells, whose statistically significant apoptotic effect was observed at 100 µg/mL Cy-g (Fimognari et al., 2005).

Also for this anthocyanin (Cy-g, 3-200 µM), Sorrenti et al. (2015), unlike, that found in normal human epithelial cells – also assessed by MTT test, however, during 48 h (24 h longer than in our study) – Cy-g does not play cytotoxic effect. But, on human prostate cancer cell line (LnCap and DU145), Cy-g reduced significantly the number of viable cells, compared to negative control, from concentration of 6 µM, being that LnCap was more sensitive to treatment in a concentration-dependent manner. These results, according analysis for the concentrations of 25, 50 and 100 µM Cy-g, could be due to activation of caspase-3 in DU145 cells, and an increase in the p21 protein expression (cell cycle inhibitor), regulated by p53, in LnCap cells after Cy-g treatment.

Feng et al. (2007) showed that the Cyanidin-3-rutinoside (C-3-R) anthocyanin, the same found in açai pulp, lead to selective apoptosis in human leukemia cell line HL-60 in a time- and dose-dependent manner. There were around 50% apoptotic cells within 18 hours of treatment (50 µM C-3-R) and almost complete apoptosis with 120 µM C-3-R or more. However, according to these authors, hepatocellular carcinoma cell-line is resistant to treatment with C-3-R, which could explain the slight cytotoxicity to HepG2 cells observed in our present study in MTT test. Curiously, C-3-R (40 and 80 µM) maintained the apoptotic cell level equal to negative control for human PBMC, but the same was not showed by lymphocytes stimulated with phytohemagglutinin, which were more susceptible to the action of C-3-R (80 µM) after 18 h of exposure, presenting some toxicity in Hoeschst 33258 staining and MTT test (Feng et al., 2007). The above data corroborate our present results for NDI values and the percentage of viable cells for 250 µg/mL concentration of AN in human PBMC.

Our literature review showed that few previous study involving an evaluation of the genotoxic potential of Eudragit® L 100 nanoparticles or similar nanoparticles were performed. Onishi et al. (2008) studied the toxicity of Eudragit L100-coated chitosan–succinyl-prednisolone conjugate microspheres in rats with colitis. The group with only Eudragit-coated chitosan microspheres exhibited similar results to the control group that received saline alone, in the other words, not presented any toxicity. A study using Chinese hamster ovary CHO-K1 cells and MN tests in *in vitro* experiments, Shah et al. (2013) assessed the cyto- and genotoxicity of variously composed nanocarriers, including those of nanoparticulate polyethylene glycol polymers, as present in Eudragit L 100, and observed no significant micronucleus induction.

In another study, Hao et al. (2013) analyzed the *in vitro* cytotoxic activity of omeprazole (OME) nanoencapsulated with Eudragit L 100-55 (methacrylic acid:ethyl acrylate copolymer, 1:1, type A), an enteric nanomaterial very similar to Eudragit L 100, reporting the internalization of nanoparticles by Caco-2 cells after a 4 h incubation. Authors also observed that the viability of these cells decreased linearly with decreasing Eudragit L 100-55 concentration, indicating that the enteric nanoparticle's toxicity derived from OME. Empty nanoparticles exhibited insignificant levels of cytotoxicity, suggesting that the nanomaterial studied can be used as a safe drug delivery system. More recently, Froder et al. (2016) evaluated the cytotoxic and genotoxic effects of indomethacin-loaded Eudragit® L 100 nanocapsules also in leukocytes and HepG2 cells. The authors reported no significant DNA damage, by the comet assay, in either cell type for all the concentrations tested. The CBMN test confirmed these results, and the clastogenic/aneugenic effect observed in the highest concentration of 500 µg/mL was attributed by the authors to the high indomethacin concentration, after comparison with the literature data.

Since in our present study the genotoxicity of Eudragit® L 100 nanocapsules without anthocyanins was not investigated, this vehicle must be investigated in further studies.

Comparing the above-mentioned studies with our present work, we can observe different results comparing PBMC and HepG2 cells. HepG2 cells evaluated by the CBMN assay presented a significant clastogenic and/or aneugenic effect at the four higher tested concentrations (2, 20, 125 and 250 µg/mL). The biotransformation's of the test compound by the liver enzymes could be responsible for this difference. Alternatively, or simultaneously, the short time of exposure to the test compound (4 h) may not have been sufficient time for the total release of anthocyanins into cells. This idea gains support with the work of Hao et al. (2013), that showed that the internalization of Eudragit L 100-55 nanoparticles on Caco-2 cells occur after a 4 h incubation. This idea must be deepened and tested in further studies.

It is important to mention that in our study, there was limitations in regard to the experimental controls, thereby it was not possible to include controls for both, anthocyanins without the Eudragit® nanoparticle and Eudragit® nanoparticle without anthocyanins. This same limitation occurred in the work of Froder et al. (2016). Despite the experimental limitations above mentioned, our findings corroborate the recent work of Patra et al. (2017), where Eudragit® polymers are considered as non-toxic products, being present in the Canadian list of acceptable non-medicinal substances and in the FDA Inactive Ingredients Guide (oral capsules and tablets).

There are several studies in the literature evaluating the genetic and cellular toxicity of anthocyanins. By the comet assay, Sorrenti et al. (2015) noted a sharp increase in the DNA strand breaks on human prostate cancer cell line in a dose-dependent manner, after cyanidin-3-O-β-glucopyranoside treatment (25, 50 and 100 µM) for 48 h, differently of our findings, but we employed 44 h less of treatment with nanoencapsulated anthocyanin, which may justify our negative results for HepG2 in this test. High concentrations (≥ 50 µM) of delphinidin, cyanidin (CY), malvidin, pelargonidin and paeonidin anthocyanidins induce DNA strand breaks on HT29 cells (human colon carcinoma) after 1 h of incubation in the comet assay. CY showed significant increase in DNA strand breaks to at concentration of 10 µM. But, low doses of all anthocyanidins don't affect DNA integrity. These compounds display great affinity with DNA, interacting with human topoisomerases, a group of enzymes that produces transient breaks in the DNA, and participate in events related to transcription, replication, chromosome condensation and segregation, and DNA repair (Habermeyer et al., 2005).

Human lymphocytes exposed to Red cabbage anthocyanin extract (25 µM, for 1 h) – majority cyanidin derived – showed mitotic index and number of micronucleus similar to negative control, therefore, presenting no cytotoxic and mutagenic potential. And, after a 2 h treatment, these anthocyanins protect DNA against copper-induced damage (Posmyk et al., 2009). Anthocyanins are the important components of bean (Azevedo et al., 2003). Therefore, in the study carried by Azevedo et al. (2003), investigating the effect of 20% black bean diet for 15 days in mice, signs of micronucleus induction were not found, as well as the absence of cytotoxicity in mouse bone marrow cells assessed by micronucleus test. The black bean showed ability to reduce MN frequency on polychromatic erythrocytes and leukocytes of cyclophosphamide-induced mice, independent of the concentration used in the diet (1-20%). Meanwhile, when commercial anthocyanins (50 mg/kg) were analyzed by the comet assay, an increase in DNA lesions was observed in polychromatic erythrocytes, although they did not use purified anthocyanins and the administered concentration was four times higher than the acceptable dietary intakes for anthocyanin (Azevedo et al., 2003). As well as in our research, Fimognari et al. (2005) we did not find an increase of MN in human lymphocytes exposed at Cy-g anthocyanin (3.1 to 200 µg/mL) assessed by CBMN assay, although both Jurkat T cells (a tumor cell line) and normal lymphocytes presented p-53-mediated apoptotic effect, whose activation occur by DNA damage. Probably, the variation in the exposure time to test-substance will determine whether these damages will be fixed as micronucleus or not. The genotoxic and antigenotoxic effects of açai pulp with anthocyanins (252.9 mg/100g) being one of the main compounds was investigated in mice by Ribeiro et al. (2010). The doses used (3.33, 10.0 and 16.67 g/kg) in acute (24 h) and subacute treatment (14 days) showed that açai pulp was not genotoxic in mouse bone marrow, liver or kidney cells in the comet assay and micronucleus test. A strong protective effect of açai pulp against doxorubicin-induced DNA damage was verified for all tested concentrations and the three cell types (Ribeiro et al., 2010). Toxicological studies including mutagenicity assay performed to Joint FAO/WHO Expert Committee on Food Additives (JECFA) pointed that anthocyanins-containing extracts had an extremely low toxicity (He and Giusti, 2010).

Considering the experimental conditions of the present study and its limitations, it can be concluded that Eudragit® L100 nanoparticles seems did not present an evident cytotoxic and genotoxic effect in cultured human cells. Comparing the general cytotoxicity and genotoxicity pattern of the anthocyanins reported in the literature with the results of the present study, it is very likely that the cellular and genetic toxicity observed in our present study probably is due to the anthocyanin type and concentration, metabolic activation or other cell-type

specificities, rather than the nanoparticles used in the nanoencapsulation of anthocyanins. Results obtained encourage future studies involving the nanoencapsulation of natural products, such as anthocyanins, as well as, shows the necessity of further studies evaluating their toxicity in mammalian cells *in vivo*.

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