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HEALTH SCIENCES

Antiproliferative effect of guava fruit extracts in MDA-MB-435 and MCF-7 human breast cancer cell lines

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Abstract: Breast cancer is the most frequent and lethal neoplastic disease among women worldwide. *Psidium Guajava* is a promising functional food against cancer, owing to a variety of bioactive compounds. This study aimed to evaluate the anticarcinogenic potential of Pedro Sato (PS), Hitigio (HI) and Tsumori (TS) guava cultivars fruit pulp extracts in MDA-MB-435 and MCF-7 human breast cancer cells. The antioxidant capacity of the extracts and their effect on cell viability, cell cycle and apoptosis were assessed. Additionally, the concentration of carotenoids, total phenolics, ascorbic acid and other physicochemical parameters were evaluated. PS pulp extract showed the highest *in vitro* antioxidative activity by all tested methods, as well as the highest content of lycopene and total phenolics, while TS pulp extract presented the highest concentration of β-carotene. After 48 hours treatment, all guava cultivars' extracts caused reduction of MDA-MB-435 and MCF-7 cells viability, with PS and HI being the most effective extracts. All guava extracts caused MDA-MB-435 and MCF-7 cell count reduction in G0/G1 and G2/M phases and increased apoptosis. The present results strongly suggest that guava pulp exerts antiproliferative effect on breast adenocarcinoma cells.

Key words: breast adenocarcinoma, cancer, carotenoids, guava fruit, MDA-MB-435 cell line, MCF-7 cell line.

INTRODUCTION

Cancer is a complex disease characterized by an increase in cell proliferation or a reduction in cell death or both (Lee & Park 2010, Toyokuni 2016). It is caused by endogenous and exogenous factors related to the overproduction of free reactive radicals or reactive oxygen species (ROS). Oxidative stress induced by free radicals can lead to base mutation, single- and double-strand breaks, DNA cross-linking, chromosomal breakage and rearrangement, disruption of function through lipid peroxidation of cell membranes, and degradation of nucleic acids, causing progressive mutations (Liu 2004, Chahal et al. 2018, Gonzalez et al. 2018).

According to reports from the International Agency for Research on Cancer (IARC), the incidence of cancer is increasing worldwide. Ten million cancer cases and 6 million deaths occurred in 2000, while in 2018 18.1 million new cases have been estimated, with 9.6 million deaths worldwide (IARC/WHO 2018). Together with lung cancer, breast cancer is the most frequently diagnosed type of cancer around the world, the 5th type of cancer to cause deaths in general and the 1st to cause deaths in women in 2018 (Bray et al. 2018, INCA 2019).

Once breast cancer is installed, most complications, including death, are due to metastasis. Mortality has been reduced with surgery, radiotherapy, chemotherapy and other therapies. However, such treatments have often caused serious adverse effects. Thus, the possibility of prevention and co-treatment through natural approaches are increasingly becoming the focus of interest and research in recent years (Brisken & O'Malley 2010, WHO 2013, Al-Mahmood et al. 2018).

Potentially, cancer-inducing oxidative damage might be prevented or limited by enzymatic and non-enzymatic antioxidants which are located in the intracellular and extracellular environments to detoxify free radicals, acting as biological response modifiers supporting immune system function and protecting living cells against damage from ROS (Liu 2004, Chahal et al. 2018). Dietary antioxidants include components of some fruits and vegetables, which act as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, synergists, and metal-chelating agents, modulating biochemical processes related to cell proliferation, differentiation, apoptosis, inflammation, angiogenesis, and metastasis (Niedzwiecki et al. 2016) .Among the main dietary antioxidants are phenolic compounds, carotenoid pigments and ascorbic acid (Ramos 2008, Roomi et al. 2016, Fiedor & Burda 2014).

From the tree of the *Myrtaceae* family, guava is a native fruit of tropical America that has spread to all tropical and subtropical regions of the world. In traditional South American, Asian and African medicine, guava root, bark and leaf teas and extracts have been used to treat a wide range of diseases (Levy & Carley 2012), but, along time, the fruit has gained great importance in the world market due to its sweet flavor and exotic aroma and colour (Gould & Raga 2002).

Currently, Brazil is the third world producer of guava fruit, with *Psidium guajava L.* being the main commercial species. In 2017, about 461 thousand tonnes of this fruits of this species were produced in the country (IBGE 2019). Most of the country's production is designated to the food industry as a source of a variety of products for exports and internal market, such as natural and concentrated juices, frozen pulps, jams, jellies and deserts (Ramos 2008, Joseph & Priya 2011, Manica et al. 2000).

Guava fruit is a source of a number of nutrients and bioactive compounds. Considering variations among cultivars, crops and edaphoclimatic conditions, on average, each 100 g contains: protein (0.1-2.5 g), carbohydrates (9.1-14.3 g), fat (0.4-1.0 g), calcium (15-30 mg), iron (0.2-1 mg), phosphorus (26 mg), β- and α-carotenes (pro-vitamin A) (2,32 -2,84 mg), thiamine (0.03-0.06 mg) and ascorbic acid (70-300 mg) (USDA 2006). According to USDA, such ascorbic acid content can be up to six times the content in oranges in general (50-100 mg) (Correa et al. 2016). Additionally, guava contains soluble fibres (0.9-5.4 g) (Chiari et al. 2012). Among the main bioactive compounds in guava are carotenoid pigments, usually, but not always, related to the characteristic red colour of the fruit. In addition to β- and α-carotenes, guava is an important source of lycopene, with average concentrations (5.4g/100g) two times higher than in tomatoes (2.54g/100g) (USDA 2006). The fruit also contains a number of phenolic compounds, including flavonoids (anthocyanins and quercetin) and tannins, triterpenes, saponins, and lectins, which contribute to guava's pulp bioactivity as a whole (Vindeloy 1977). Such variety of bioactive phytochemicals of different structures and types can be much more effective in disease

prevention than when isolated as a result of synergistic or additive effects (Khoo et al. 2017).

Among the reported *in vitro* biopharmacological effects of guava fruit derived from the action of bioactive compounds is the antioxidative capacity, particularly as free-radical scavenger and as metal chelator, the capability of catalyzing the peroxidation of lipids (Rodriguez-Amaya 2001, Barreiros et al. 2006, Nascimento 2006). Despite the high antioxidative capacity of the fruit pulp, mainly the anti-carcinogenic effects of *P. guajava* leaves have been widely investigated in several studies and in a large number of cancer cell types (cervical, metastatic prostate, human mouth, murine leukemia and fibrosarcoma, human breast and human colon), while only a few studies have evaluated the anticarcinogenic effect of the pulp, especially in the case of breast cancer, with only one study which evaluated one type of guava (Correa et al. 2016). In addition to being considerably more palatable than the leaf tea, guava pulp can be used daily in tropical countries for cold juices preparation, which is important in countries where hot teas are not often consumed owning to the warm climate (Dantaset 2010).

One of the most important carotenoids with health beneficial effects present in guava fruits is lycopene. Numerous studies investigating the effects of lycopene on cancer cells have been recently published. Together, these studies highlight different mechanisms of action: modulation of intracellular communication of the gap junction; induction of apoptosis; modulation of cell cycle proteins; increase in BRCA1 and BRCA2 mRNA (onco-suppressor genes in breast cancer); alterations in the gene expression profile of various other molecular pathways such as apoptosis (p53 and Bax), cell communication, MAPK, cell cycle, xenobiotic metabolism, and fatty acid biosynthesis (Chalabi et al. 2006, Chalabi et al. 2007, King-Batoon et al. 2008, Takeshima et al. 2014, Peng et al. 2017, Dos Santos et al. 2018). Lycopene inhibits oxidative damage to DNA and suppresses cell growth by downregulating redox-sensitive signaling pathways, including MAPK and NF-kB pathways in human prostate cancer, breast cancer, and hepatocellular carcinoma cell lines (Park et al. 2005, Palozza et al. 2010, Assar et al. 2016). Previously, it has been showed that lycopene decreased the formation of 8-OHdG, and suppressed the ROS-activated Jak1/Stat3 and Wnt/beta-catenin pathways in H. pylori-infected gastric epithelial cells (Jang et al. 2012, Park et al. 2019).

Among the cell lines used as models for the study of human breast cancer are MDA-MB-435 and MCF-7. These cells have different characteristics. MDA-MB-435 cells do not express hormonal receptors (estrogen receptor – ER) and present high metastatic potential and high tumorigenicity. However, malignant tumors of patients with positive ER, that use estrogen as the main growth stimulant, correspond to about 80% of breast cancer cases (Alkhalaf & El-mowafy 2003). Considering that blockage of ER may inhibit cell growth in the treatment of a malignant tumor sensitive to hormones, ER is the direct target of hormonal therapies for this type of cancer, and the occurrence or not of its expression is a decisive point in treatment. MCF-7 epithelial cells present ER and progesterone receptor (PR), although they present low metastatic potential. Therefore, both cell lines are complementary in studies investigating the activity of extracts and compounds against human breast cancer (Weigel & Dowsett 2010). Considering all the points aforementioned, this study aimed to evaluate the anticarcinogenic potential of guava cultivars pulp extracts in MDA-MB-435 and MCF-7 human breast cancer cells.

MATERIALS AND METHODS

Samples

Ripe samples of guava (*Psidium guajava L.*) cultivars Pedro Sato (PS), Hitigio (HI) and Tsumori (TS) were obtained from the Fruit Project of the State Department of Agriculture and Livestock – SEAPEC-RJ in Cachoeira de Macacu, Rio de Janeiro, Brazil.

Physicochemical characterization of guava cultivars extracts

Physicochemical characterization, including determination of total sugars and ascorbic acid content, titratable acidity, and soluble solids (Brix), was performed according to the official methodologies of Adolfo Lutz Institute, Brazil (2005). The color of peels and pulps was measured by a Konica colorimeter (Minolta CM-5), using the CIELAB scale (L *, a *, b *). Fruits weight and size were also recorded.

Pulp extract preparation

Guava pulp extracts (including seeds) were prepared according to Rocha Ribeiro et al. (2007), adapted. After testing five solvents and solvent mixtures, a mixture of acetone and water (70:30) was chosen to prepare the extract used in all assays. Fifty grams of guava pulp and seeds were mixed with 50 mL of the hydro-acetone mixture and the volume was made up to 100 mL, with distilled water. The mixture was vortexed for approximately 3 mins, followed by water bath agitation for 60 mins at 25°C. The solution was vacuum-filtered through a Whatman n°1 quantitative paper filter. Extracts were used immediately after they were prepared, except in the case of cell assays, for which they were lyophilized. Prior to lyophilisation, extracts were placed in a hot air balloon for solvent evaporation in a rotary evaporator (Terroni, Brazil) at 60 °C, for 2 hours. Following, the concentrated extracts were frozen at -20 °C for 24 hours and transferred to a lyophilizer (LD3000, Terroni, Brazil), where they remained for 72 hours to reach 3% moisture. The final powdered pulp extract was packaged in amber vial and frozen at -20 °C, until cell assays.

DPPH assay

The evaluation of the antioxidant activity of guava extract by DPPH (2,2-diphenyl-1-picrilidrazil) radical assay was performed according to Brand-Williams et al. (1995). Aliquots of 0.5 mL of the fresh extracts were mixed with 2.5 mL DPPH methanolic solution (0.06 mM) and allowed to react for 1 hour in the dark. Measurements were performed at 515 nm, using a spectrophotometer (Shimadzu UV-2700, Japan).; the reduction in DPPH radical absorbance concentration caused by the extracts was compared to a trolox standard curve. Results were expressed as µmol trolox equivalents/g dry basis. Analyses were performed in triplicate.

Ferric reducing antioxidant power (FRAP) assay

The measurement of the antioxidant activity of the extracts by FRAP was performed according to Benzie & Strain (1996). Aliquots of 2.7 mL of TPTZ reagent (ferric 2,4,6-tripyridyl-s-triazine) were mixed with 0.5 mL of sample extract (aliquots 5, 10, and 20 µL). After 30 min at 37 °C, the absorbance was read at 595 nm. The antioxidant capacity (FRAP) was expressed as Fe3+ equivalents (µmol Fe3+/g dry basis).

Trolox equivalent antioxidant capacity (TEAC/ABTS) assay

The complex TEAC·+ cation was prepared by mixing a TEAC stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was allowed to rest for 16 hours at room temperature, until the reaction was completed and the absorbance was stable. The antioxidant capacity assay was carried out following the improved

TEAC method as described by Re et al. (1999). The TEAC solution (2.5 mL) was added to the extract or the commercial reference antioxidant (trolox) and mixed thoroughly. Absorbance was recorded at 734 nm, during 6 min. Aliquots of 5, 10, and 20 mL of the extracts were tested and their volumes were completed to 0.5 mL with water. Results were expressed as µmol trolox/g dry basis.

Carotenoids extraction and analysis

Carotenoids profiles were determined by liquid chromatography according to Pacheco et al. (2014), using a Waters TM HPLC system, controlled by the Empower software program with photodiode array detector (PDA). Carotenoid separation was obtained in a C30 column (S-3 Carotenoid, 4.6 mm × 250 mm, YCMTM) at 33 °C, by a gradient elution of methanol and methyl tert-butyl ether. The elution started with a mix of 80% methanol and 20% methyl tert-butyl ether. At 0.5 min the concentration of ether was increased to 25%, at 15.00 min to 85% and at 15.05 to 90% ether. The concentration of ether was maintained at 90% until 16.50 min and then, at 16.55 min, it returned to the initial condition (20%), remaining constant up to 28 min point. Flow rate was 0.8 mL/min and running time was 28 min. Sample injection volume was 15 µL. Carotenoids were identified based on their retention times and UV/Vis absorption spectra, compared to the standard retention times and UV/Vis absorption spectra of each carotenoid.

Total phenolics assay

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu method, as described by Singleton and Rossi (1965), with minor modifications. Aliquots of 0.5 mL of the extracts were added to 2.5 mL of Folin-Ciocalteu reagent and 2.0 mL of 4% sodium carbonate solution, and the mixture was put to rest for 2 hours in the dark. Measurements were performed at 760 nm in triplicate, applying a Turner 340 spectrophotometer. Gallic acid (range of 0–100 mg/mL) was used to produce a calibration curve. The concentration of total phenolic compounds in the extract was expressed as gallic acid equivalents, reflecting the total phenolic content in mg/100 g of sample material.

Cell culture and treatment protocol

Cell lines certified for identity and quality (INMETRO Rio de Janeiro, RJ, Brazil) were obtained from the Rio de Janeiro Cell Bank. Human breast carcinoma cell lines (MDA-MB-435 and MCF-7) were plated separately in 25 cm2 tissue culture flasks (5.0 × 106 cells/flask) and maintained routinely in Dulbecco's Modified eagle's medium high glucose (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin (PS), pH 7.4, under 5% CO2 atmosphere. Stock flasks were grown to 70% confluence and subcultured routinely. Medium renewal was executed 3 times per week. For each experiment, cells were seeded at 3.5 ×105 cells/cm2 and 2 × 104 cells/cm2 densities in 6-well plates and 96-well plates for cell cycle and cell proliferation analyses, respectively. After 24 h, the medium was removed and cells were treated with nine increasing concentrations of guava extracts from 15 to 5000 µg/mL, dissolved in DMEM. The controls, DMEM and DMEM + negative controls (hydro acetone solutions) were included on each plate. Cells were then incubated for 24 and 48 hours.

Cell viability assay

Cell viability was monitored through MTT assay (Amresco,Solon,OH).MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product.

Exponentially growing cells were adjusted to 2.0 × 104/cm2 with DMEM, plated in 96-well plates (Corning, Tewksbury, MA) at 200 mL/well and incubated for 24 hours, according to the routine procedure. The cells were then incubated with guava extracts for 24 and 48 hours. Each well was also incubated with MTT (10 µL/well; 5 mg/mL) for 4 hours. Following, 85 mL/well of the liquid was removed and 50 µL/well of sodium dodecyl sulfate was added to dissolve the solid residue. Finally, the absorbance was measured using a microplate reader (POLARIS, CELER) at 570 nm. The cell proliferation inhibition rate (CPIR) was calculated as follows:

CPIR = (1 – average value of experimental group/average value of control group) × 100%.

Cell cycle analysis

Cells were briefly rinsed with calcium and magnesium-free phosphate-buffered saline and detached with trypsin at room temperature. After centrifugation, cells were washed twice with phosphate-buffered saline and 1 x 106 cells were resuspended in 1.0 mL of ice-cold Vindelov solution (Vindelov 1977), containing 0.1% Triton X-100, 0.1% citrate buffer and 0.1 mg/mL RNase, and 50 mg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 min incubation, the cell suspension was analyzed for DNA content by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells with DNA content diploid G_0–G_1 phase (2n), S phase (>2n but <4n) and G_2/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9, respectively. The percentage of cell population at a particular stage was estimated with EXPO32 V1.2 Analysis software. The cell dissociation procedure does not affect fluorescence under the experimental conditions that were used in this study or in any other studies as far as the authors know. Nuclei of viable cells were gated according to FL-2W x FL2-A relation (Vindeloy 1977).

Apoptosis assay

In order to measure the apoptosis rate, the studied cells were resuspended in 400 µL of binding buffer containing 5 µL of annexin V FITC and 5 µLpropidium iodide (Apoptosis Detection Kit II, BDBiosciences) for 15 min at room temperature. Annexin V binding was evaluated by flow cytometry (FACS calibur, BD Biosciences), and after acquisition of 30,000 events, data were analyzed in CellQuest and FlowJo software.

Statistical analysis

Results are presented as mean with the corresponding standard deviation of three independent experiments performed in triplicate (n = 9). Data from cell viability test, cell cycle and apoptosis essays were analyzed using the statistics softwares GraphPad Prism (version 5.04, San Diego, CA), Statistica (version 7.0, StatSoft Inc., Tulsa, OK), and Minitab 17 Statistical Software (version 17, State College, PA: Minitab, Inc.). One-way analysis of variance (ANOVA) test followed by Tukey's test were used to test cell viability, cell cycle, and apoptosis. Differences were considered at a significance level of 5% ($p \leq 0.05$).

RESULTS

Physicochemical characterization of guava cultivars extracts

The physicochemical parameters of peels and pulps of guava cultivars are presented in Table I. Regarding peel colorimetric results, PS and HI cultivars presented the highest mean values for brightness (L*), TS presented the least greenish shade (a*), and PS and HI the most intense yellow shade (b*). HI presented predominance of green (-a*). Regarding the pulps, there was

Table I. Physical and chemical characterization of guava cultivar extracts. Significant differences are indicated as a, b and c.

Physical parametres				Chemical and antioxidant parameters			
	PS	TS	HI		PS	TS	HI
Weight (g)	220.16±40.08[a]	297.52±79.06[c]	158.16±15.43[b]	**Total sugar (g%)**	12.33±0.45[a]	13.12±1.73[a]	10.95±.1.22[a]
Dimension (cm)	8.34±0.51[a]	8.78±1.01[c]	7.27±0.40[b]	**Soluble Solids (°Brix)**	10.11±0.02[a]	11.08±0.01[a]	9.47±0.01[a]
CIELAB scale (peel)				**Titratable Acidity (g%)**	0.36±0.01[a]	0.47±0.03[b]	0.39±0.01[a]
L*	65.56±1.72[a]	55.49±10.06[b]	59.90±3.71[a,b]	**Ascorbic acid (mg%)**	81.81±2.04[a]	80.36±1.87 [a]	79.35±3.30[a]
a*	-0.40±2.22[b]	0.90±4.59[b]	-5.18±2.32[a]	**Lycopene (mg/100g)**	5.43±0.32[a]	2.34±0.43[c]	3.21±0.85[b]
b*	42.30±3.16[a]	28.59±9.12[b]	39.59±3.08[a]	**β-carotene (mg/100g)**	0.13±0.08[a]	1.54±0.98[c]	0.50±0.12[b]
CIELAB scale (pulp)				**Total phenolics (mg GAE/100 g)**	200.88±1.44[a]	125.56±8.74[c]	156.64±1.14[b]
L*	55.21±1.68[a]	61.83±4.59[a]	58.53±1.72[a]	**DPPH (% reduction)**	76.96±2.35	56.84±1.04	55.91 ±0.80
a*	32.25±1.17[a]	22.45±1.97[b]	30.99±1.24[a]	**ABTS (µmol Trolox/g)**	208.05±9.69	151.07±1.93	129.91±3.04
b*	21.69±2.02[a]	21.01±1.16[a]	21.81±0.51[a]	**FRAP (µmol ferrous sulfate/g)**	41.84±0.84	30.41±0.46	23.73±0.20

no difference in brightness among cultivars. PS and HI presented more intense red shade, while TS pulp was paler. No significant difference was observed in b * scale, meaning that pulps presented similar shade of yellow.

The three cultivars presented similar soluble solids, sugar and ascorbic acid contents (Table I). The most acidic cultivar was TS, while the remaining two presented similar acidity values. The three cultivars pulp extracts presented different concentrations of carotenoids. PS presented the highest concentration of lycopene, which was about two times higher than in TS and one and a half time higher than in HI cultivar. For β-carotene, the highest concentration was observed in TS, which was three times higher than in HI and eleven times higher than in PS. On the other hand, PS contained the highest concentration of phenolic compounds (which includes anthocyanins), followed by HI and TS.

Antioxidant activity (AA)

PS cultivar presented the highest AA values when evaluated by all three *in vitro* methods (DPPH, FRAP and TEAC) (Table I). A positive correlation between ascorbic acid content and FRAP results ($r = 0.999$; $p = 0.03$) was observed. Other correlations were not relevant (data not shown).

Cell viability by MTT assay

In order to assess the antitumor effect of guava extracts, two breast carcinoma cell lines were used in this assay (MDA-MB-435 and MCF-7). Guava extracts changed MDA-MB-435 cells growth profile after 24 and 48 hours incubation (Figure 1). After 24 hours incubation with guava

pulp extracts, PS cultivar presented the highest percentage of reduction in cell viability (45%, compared to untreated cells), followed by HI. Cells treated with TS pulp extract did not grow differently from control (untreated) cells after 24 hours incubation. However, after 48 hours, all guava cultivars' extracts promoted reduction in MDA-MB-435 cells viability. TS extract showed the greatest reduction in cell viability, in concentrations from 15 to 5000 µg/mL, with average inhibition of 70% compared to negative control. Results from PS and HI cultivars showed no statistical difference in the concentrations tested (from 15 to 5000 µg/mL), with maximum reductions of 57% and 44% in these two concentrations for PS and HI, respectively.

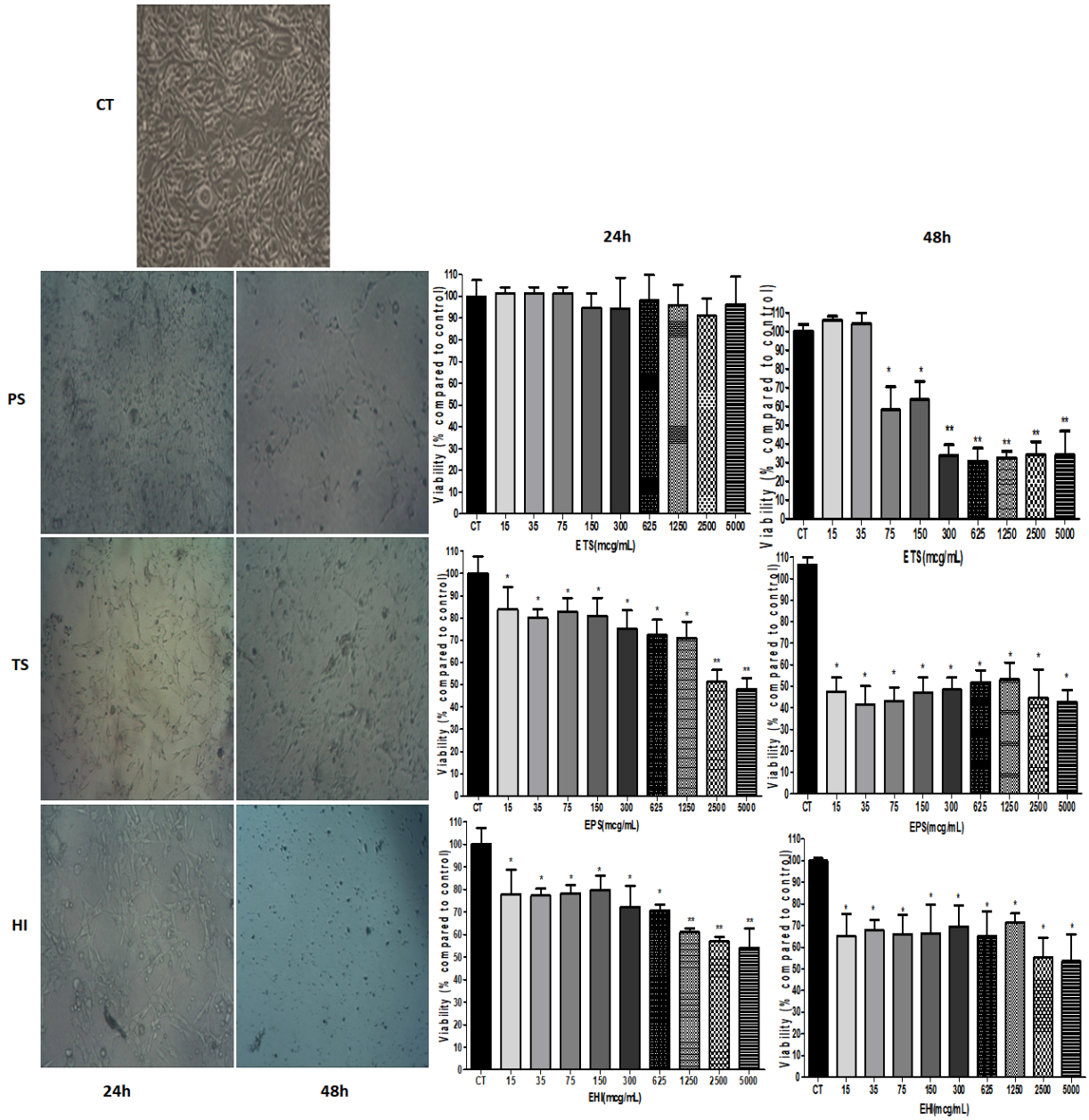

Figure 1. Effect of Tsumori (TS), Pedro Sato (PS), and Hitígio (HI) guava extracts (15-5000µg /mL) on MDA-MB -435 cells viability after 24 and 48 hrs treatment. Results were compared by One- way ANOVA test and Tukey test (* p<0.05; ** p <0.01). CT= Results of untreated (CT) and treated cells.

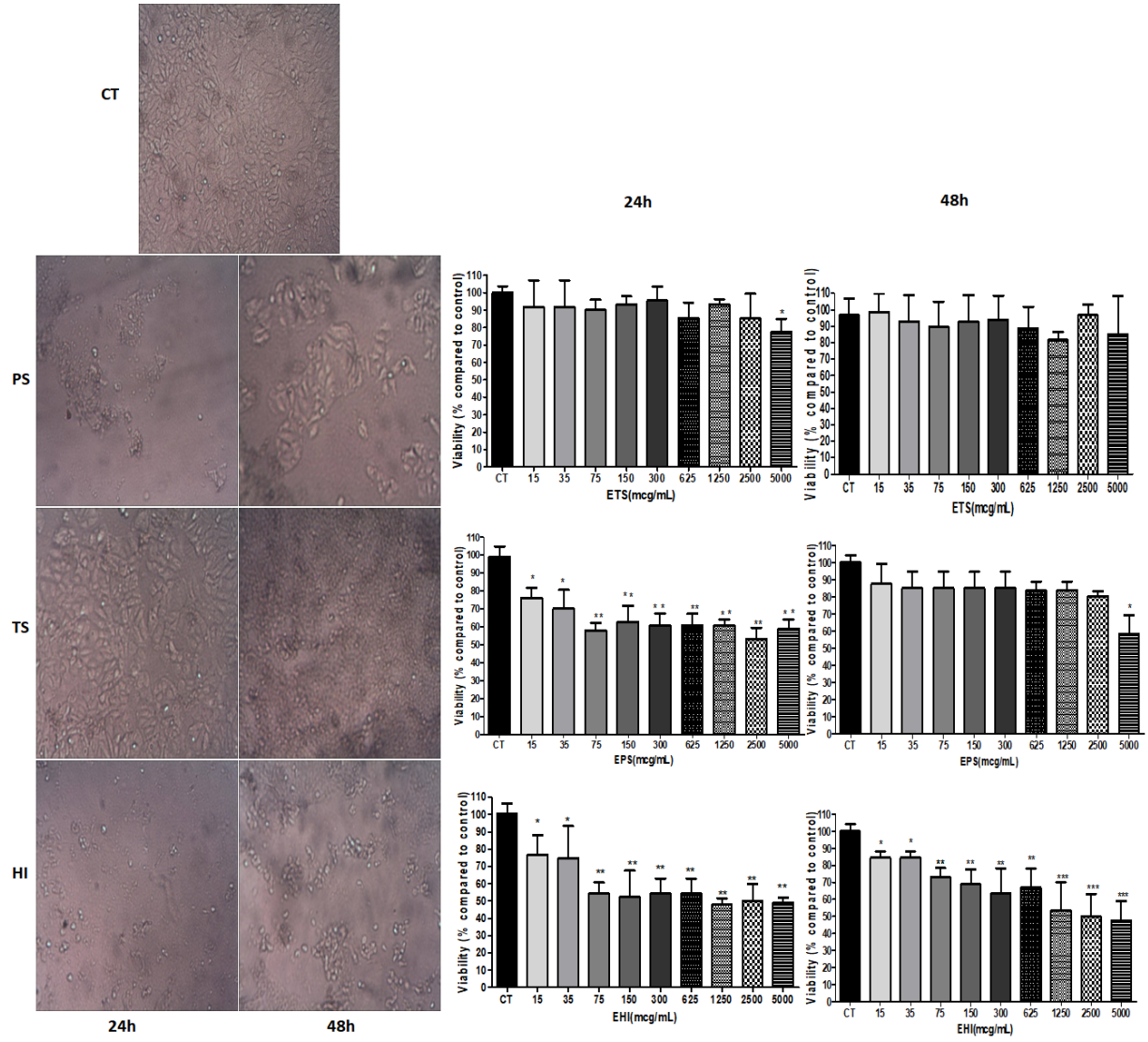

Figure 2. Effect of 24 and 48 hrs incubation of MCF-7 cells with Tsumori (TS), Pedro Sato (PS) and Hitígio (HI) guava cultivar extracts (15-5000µg /mL) on cells viability. Results were compared by One- way ANOVA followed by Tukey test Significant differences are indicated as * p<0.05 and ** p <0.01.

Guava extracts also caused reduction in MCF-7 cell viability after 24 and 48 hours treatment when compared to control group (Figure 2). After 24 hours treatment, PS and HI cultivars showed 50% reduction in cell viability, in concentrations ranging from 75 to 5000 µg/mL, while TS extract only reduced 10% in the highest concentration. After 48 hours treatment, TS cultivar did not affect cell viability, while PS at 5000 µg/mL reduced 40% compared to control. HI extract presented similar behaviour after 48 hours treatments compared to that observed after 24 hours treatment. Therefore for other assays, 24h incubation was considered.

Effect of guava cultivars extracts on cell cycle

In order to assess the effect of guava pulp extracts on cell cycle, based on MTT assay results for human breast carcinoma cell lines, MDA-MB-435 cells were incubated with the

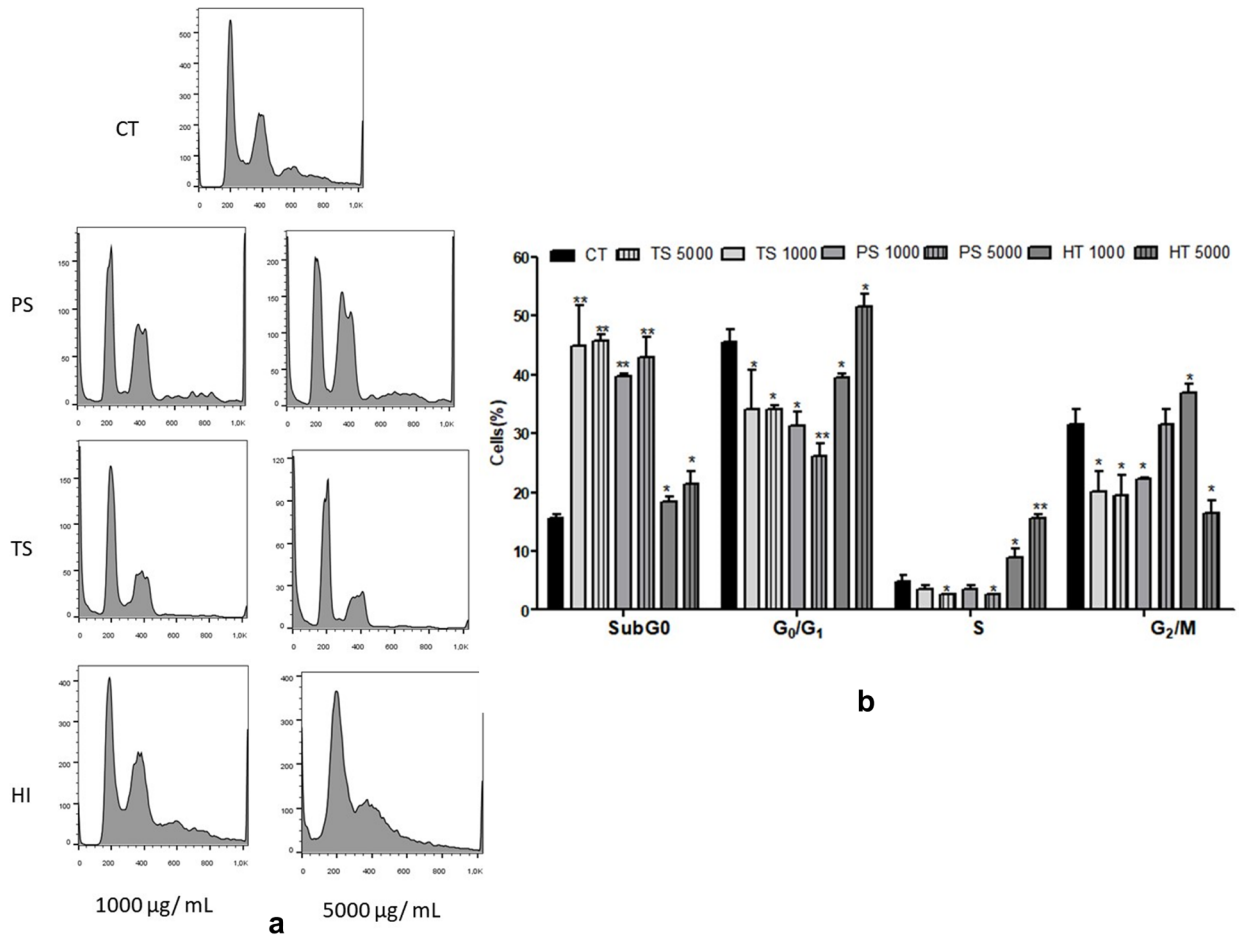

Figure 3. Effect of 48h incubation of MDA-MB 435 cells with 1000 and 5000 µg/ mL Pedro Sato (PS), Tsumori (TS) and Hitígio (HI) guava cultivar extracts on cell cycle progression. Cell cycle phases are illustrated in (a). Quantitative results are shown in graph (b). Results are expressed as mean ± standard deviation of two experiments. Significant differences between untreated (CT) and treated cells were compared by One- way ANOVA followed byTukey test Significant differences are indicated as * p<0.05 and ** p <0.01.

extracts for 48 hours (Figure 3) and MCF-7 cells for 24 hours (Figure 4), with two concentrations of each of the three guava pulp extracts (1000 µg/mL and 5000 µg/mL), followed by evaluation of the percentage of viable cells in the different cell cycle phases. Treatment of MDA-MB-435 cells with PS and TS extracts caused an increase in the percentage of cells in the G_0 subphase, followed by a reduction of cells in G_0/G_1 and G_2/M phases. There was also a reduction of cells in the S phase at the highest concentration (5000 µg/mL) for all cultivars. In MCF-7 cell line, after 24 hours treatment with PS, HI, and TS extracts, a reduction in proliferation was observed. All cultivars increased the percentage of cells in the G_0 sub-phase (p=0.01) and caused a reduction of cells in the G_0/G_1 phase, when compared to the untreated cells, except for HI cultivar in the lowest concentration (1000 µg/ mL). It was still possible to see a reduction of cells in the S phase after the treatment of guava extracts, except for TS and PS cultivars at 1000 µg/mL. Additionally, both concentrations of HI (1000 µg/mL and 5000 µg/mL) and the highest concentration of PS (5000 µg/mL) produced a decrease in cells count in the G_2/M phase (p

= 0.01). An increased number of cells in G_2/M phase was also observed when PS was tested at 1000 µg/mL.

Effect of guava cultivars extracts on apoptosis

Considering the cell cycle results, MDA-MB-435 cells and MCF-7 cells were treated with guava cultivars extracts (1000 µg/mL and 5000 µg/mL) for 48 hours and for 24 hours, respectively, for apoptosis rate evaluation. Results are presented in Table II and in Figure 5. In MDA-MB-435 cells, 48 hours-treatments with all cultivars (in both concentrations) caused a relative increase in apoptotic cells of 6 to 8 fold- compared to control. PS cultivar showed the highest reduction in viable cells (54.6%) at 1000 µg/mL, while the highest reduction of HI cultivar (61.2%) occurred at 5000 µg/mL (Table II). In MCF-7 cells, which offer greater resistance to chemotherapy, 24h-treatment with PS and HI cultivars caused relative decrease in viable cells, in a similar magnitude (4.1% and 21.4% at 1000 µg/mL, respectively, and 11.7% and 9.1% at 5000 µg/mL, respectively. Also, both cultivars showed a relative increase in the percentage of early apoptosis (80.4% at 1000 µg/mL and 90.2%

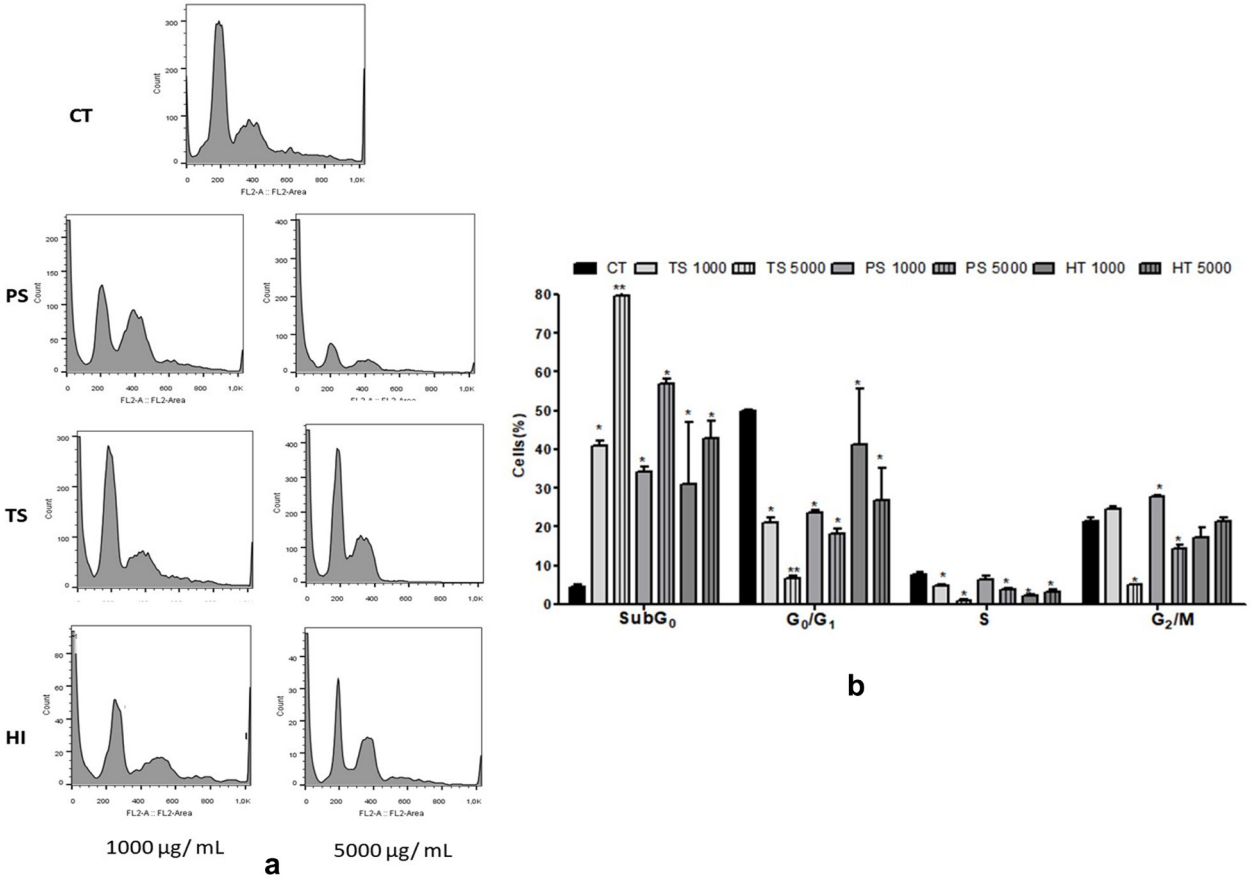

Figure 4. Effect of 24hrs-incubation of MCF-7 cells with 1000 and 5000 µg/ mL of Pedro Sato (PS), Tsumori (TS) and Hitígio (HI) guava cultivars extracts on cell cycle progression. Cell cycle phases are illustrated in (a). Quantitative results are shown in graph (b). Results are expressed as mean ± standard deviation of two experiments. Results of untreated (CT) and treated cells were compared by One- way ANOVA followed by Tukey test. Significant differences are indicated as * p<0.05 and ** p <0.01.

Table II. Effect of Pedro Sato(PS), Tsumori(TS) and Hitigio(HI) guava cultivars extracts on death process stages in MDA-MB-435 and MCF-7. Significant differences are indicated as *, **p<0.01, ***p<0.001.

		MDA-MB 435			MCF-7		
Guava Cultivars extract	**Stages of cell death**	**CT**	**1000µg/mL**	**5000µg/mL**	**CT**	**1000µg/mL**	**5000µg/mL**
PS	Viable cells (Annexin V- PI-)	86.03±3.21	39.10±4.10***	44.25±1.77**	93.60±1.34	89.75±1.64*	73.55±10.92**
	Early apoptosis (Annexin V+ PI-)	3.77±1.88	46.25±0.21***	17.85±0.96***	0.84±0.78	4.29±0.39**	8.58±3.69***
	Late apoptosis (Annexin V+ PI+)	3.81±0.99	16.75±0.35***	36.15±1.20***	0.25±0.57	2.93±0.11*	3.90±0.86*
	Non-apoptotic cells (Annexin V- PI+)	6.42±1.52	0.61±0.16***	2.25±1.06**	4.29±1.13	5.15±1.90	5.36±0.78
TS	Viablecells (Annexin V- PI-)	82.60±4.81	39.10±4.20**	44.25±1.77**	91.85±2.19	90.30±1.45	90.35±1.34
	Early apoptosis (Annexin V+ PI-)	3.27±0.76	17.58±2.39***	2.20±1.70	2.51±0.78	1.77±0.39	0.94±0.34*
	Late apoptosis (Annexin V+ PI+)	5.05±1.80	16.75±2.33***	36.15±1.20***	3.57±0.85	2.38±0.30	2.87±1.90
	Non-apoptotic cells (Annexin V- PI+)	9.06±3.73	1.03±0.63***	2.25±1.06**	4.04±1.06	7.15±1.90*	8.87±2.60*
HI	Viable cells (Anexin V- PI-)	80.28±1.35	47.45±9.00**	31.15±1.83***	92.10±4.12	81.35±4.74*	83.70±2.38*
	Early apoptosis (Annexin V+ PI-)	2.73±1.74	42.55±19.16**	1.30±1.00	3.78±1.47	7.62±2.52**	8.24±0.72**
	Late apoptosis (Anexin V+ PI+)	6.32±3.20	9.78±2.01	50.55±0.78***	2.42±2.62	7.98±0.57**	6.36±1.25**
	Non-apoptotic cells (Annexin V- PI+)	11.72±2.98	0.30±0.16***	13.55±3.64	3.71±1.21	2.08±1.64	2.94±0.98

at 5000 µg/mL for PS, and 50.4% and 54.1%, respectively, for HI) and late apoptosis (91.5% and 93.6%, respectively, for PS, and 69.7% and 62.0%, respectively, for HI). Twenty four hour-treatment with TS did not cause significant changes in apoptosis.

DISCUSSION

The colorimetric parameters were measured in order to correlate them with the content of bioactive compounds, mainly carotenoids and total phenolic compounds (used in this study to reflect the content of anthocyanins, the main polyphenols in guava), considering that the intense red colour of guava pulp has been reported to be associated with high contents of these compounds in addition to some carotenoids (Kuskoski et al. 2005). Additionally, we aimed to associate the content of such compounds with the bioactivity results for the different cultivars. The bright pinkish-red shade of PS and HI, and the white-yellowish shade of TS (known as “white” guava), were expected, since they are characteristic of the respective cultivars. Corroborating these findings, in the present study, PS pulp, which presented the

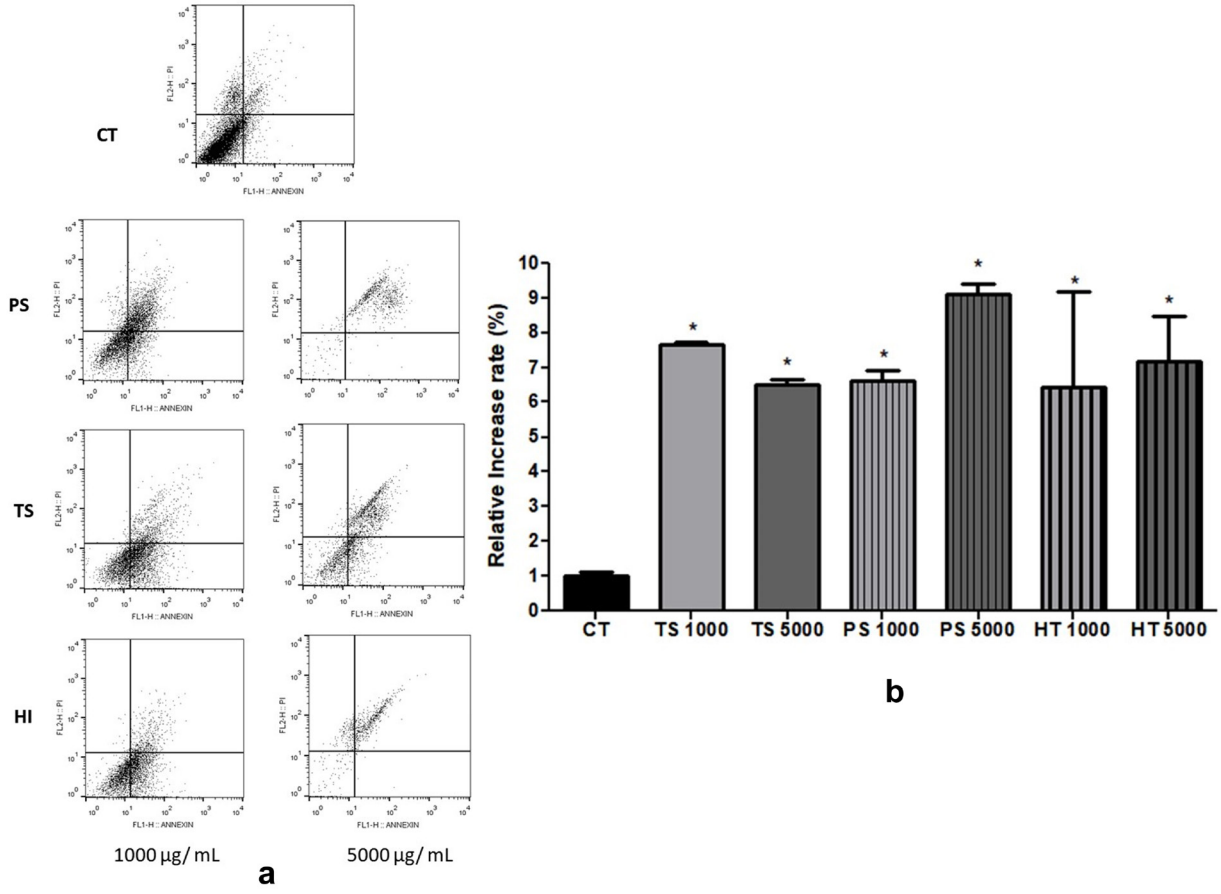

Figure 5. Increased relative rate of apoptosis in MDA-MB 435(a and b) treated for 48 hrs, with 1000 and 5000 µg/ mL of Pedro Sato (PS), Tsumori (TS) and Hitigio (HI) guava cultivars extracts. Results were compared by One- way ANOVA followed by Tukey test for significance (* p<0.05; p <0.01). CT= Results of untreated (CT) and treated cells.

best results for all performed essays, also presented the highest content of total phenolic compounds and lycopene. Among anthocyanins, cyanidin 3-glycoside has been reported to have great relevance for red guava, corresponding to about 50% of total anthocyanins (Kuskoski et al. 2006). Additional phenolic compounds identified in different types of guava are derivatives of kaempferol and quercetin, esculin, sinapoylquinic acid, among other minor compounds (Chiari-Andréo 2017). Amid carotenoids, β-Cryptoxanthin and lycopene are of great importance in guava pulp (Kuskoski et al. 2006, Nora et al. 2014). The highest content of β-carotene observed in the white-yellowish cultivar (TS) and lower in red cultivars indicate that this carotenoid pigment does not contribute importantly to guava red color, which was surprising, since its content in guava pulp is comparable to that in papaya pulp (Ribeiro el at. 2014). This occurs possibly either because in guava pulp β-carotene is stored and hidden in a cell compartment or because it is masked by the presence of other pigment such as chlorophyll (Chitarra & Chitarra 2005). On the other hand, considering the highest content of lycopene in PS cultivar, this carotenoid compound seemed to have contributed to its intense red color, together with anthocyanins, corroborating literature data (Kuskoski et al. 2005).

Regarding the antioxidative capacity, in view of the fact that no single assay can reflect

the exact "total antioxidative capacity" of a food matrix owing to the different nature of its bioactive compounds, we used methods based on different mechanisms of action (Hou et al. 2004). This is especially important for guava, which is rich in both lipophilic and hydrophilic bioactive compounds that act through different mechanisms to promote antioxidant activity (Huang et al. 2005, Martínez et al. 2012). As aforementioned, the fact that PS cultivar presented the highest antioxidant capacity measured by all tested methods is in line with its higher content of phenolic compounds, which present medium hydrophilicity, and carotenoid pigments, mainly lycopene, which is part of the lipid fraction (Dávalos et al. 2005, Stahl & Sies 2005). Some naturally occurring phenolic compounds in guava, as well as analogs, are known to display a wide variety of biological functions, including anti-inflammatory and antioxidant activities, which are closely related to modulation of carcinogenesis. (Stahl & Sies 2005). Phenolic compounds work as radical hijackers and sometimes as transition metal chelation, acting both at the initiation and propagation stages of the oxidative process reaction, by donating hydrogen atoms to these molecules and disrupting the chain reaction (Reische et al. 2002, Fu et al. 2011).

The antioxidant property of licopene and other carotenoids also stems from their ability to perform free radicals scavenging (Caetano et al. 2011). They are known to eliminate free radicals such as Reactive Oxygen Species (ROS) in damaged tissues (Lacroix et al. 2004). They can scavenge radicals by electron transfer, hydrogen abstraction and addition. Conjugated double bonds qualify these compounds to accept electrons from reactive species, and then neutralize free radicals (Sznarkowska et al. 2016, Milani et al. 2016). It is worth reminding that PS cultivar presented the lowest content of β-carotene and the highest content of total phenolics and lycopene. Therefore, considering that ascorbic acid content was similar in all tested extracts, phenolic compounds and lycopene seemed to have contributed more importantly to the highest antioxidative activity found in PS cultivar. The correlation between AA and FRAP indicates that FRAP was the best used method to evaluate the AA of ascorbic acid.

As aforementioned, for the evaluation of the antitumorigenic effect of guava pulp extracts we used two cell lines derived from breast carcinoma because of their complementary characteristics. The star-shaped MDA-MB-435 epithelial cells do not express hormonal receptors and present high metastatic potential and high tumorigenicity (Netzel et al. 2007). The MCF-7 epithelial mesenchymal cell line, on the other hand, presents estrogen and progesterone receptors and low metastatic potential. These cells have a fusiform morphology and are luminal. However, they have low invasion potential in Matrigel, which is a meshwork of extracellular matrix proteins and growth factors that mimics the basement membrane underlying epithelial cells and have been broadly used for assessing cancer cell invasion, *in vitro* (Williams et al. 2000). In this study, the cell proliferation was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which is one of the most commonly used methods to access the action and interaction of natural products, like fruits and plants, on cell proliferation, viability and cytotoxicity. This assay is based on the reduction of a tetrazolium salt to a purple insoluble formazan by metabolically active cells. The absorbance of the solubilized formazan is taken as a measure of the number of living cells. In this essay, PS cultivar also presented the best results, with up to 70% reduction in MDA-MB-435 proliferation after 48 hours and 50% of MCF-7 cells in 24 hours. No

further reduction in the proliferation of both cell lines was observed after these treatment times. Therefore, no evaluation was performed after these time points. It is noteworthy mentioning that we have evaluated the effect of *P. guajava* extract on normal breast cancer cell lines (MCF-10A) and found no increase in cell death (data not shown).

Considering the major bioactive compounds found in PS pulp, the present study results suggest that polyphenols and/or lycopene were/was capable of inhibiting the growth of human breast adenocarcinoma cells. It is more probable that lycopene and anthocyanins acted together as antiproliferative agents, being supported by other bioactive compounds, including as β-carotene.

Previous studies have reported proliferation reduction (in one case by more than 60% at 200 µg/ mL) of different cancer cells caused by strawberry and grape anthocyanins (Palozza et al. 2002, Pandeyv et al. 2009). Other *in vitro* studies have also established the antitumorigenic effect of lycopene in several tumor types, including prostate colon, liver and lung cancer (Hwang and Bowen 2004). In addition to ROS scavenging, lycopene is known to act by modulating redox sensitive cell targets, including protein kinases, protein tyrosine phosphatases (PTP), Mitogen Activated Protein Kinase (MAPKs) and transcription factors (Palozza 2012). Lycopene has also exerted anti-proliferative effect on breast cancer cell model, reducing the expression of cell cycle regulatory proteins, such as cyclins (D1 and E) and cyclin-dependent kinases (-2 and -4), as well as suppressing insulin-like growth factor (IGF-I) action (Karas et al. 2000, Burgess et al. 2008, Teodoro et al. 2012). The complexes of cyclin and Cdk play key roles in the control of cell cycle progression. Cyclins contain a well-conserved amino acid sequence known as the cyclin box, which is required for the binding to and activation of specific target Cdks in each cell cycle phase (Malumbres 2014). These results have been supported by epidemiological studies in which the consumption of tomato (one of the main sources of lycopene) was associated with decreased risk of several types of cancer, including lung, prostate and colon cancer. Higher circulating levels of carotenoids in women have also been reported to reduce the risk of breast cancer (Eliassen et al. 2012). The consumption of β-carotene has also been inversely associated with the incidence of colon (Diehl 2002) and prostate (Bertoli et al. 2013) cancers.

Regarding the cell viability and cell cycle results, the lack of regulation of cell cycle is a fundamental aspect in cancer development. The normal cells only proliferate in response to the cell development or to signals that occur during mitosis. The cell cycle consists of distinct phases of events that occur in a cell in preparation for cell division: G_0 phase is viewed as an extended G_1, (gap $_1$, or G1, stage), copies its DNA (synthesis, or S, stage), prepares to divide (gap 2, or G_2, stage), and divides (mitosis, or M, stage). The stages G_1, S, and G_2 makeup and interphase, which accounts for the span between cell division (Prakash et al. 2001). Each phase of the cell cycle is tightly regulated, and checkpoints exist to detect potential DNA damage and allow it to be repaired before a cell divide. If damage cannot be repaired, a cell becomes targeted for apoptosis, which is a programmed cell death process that occurs in multicellular organisms, under different circumstances and involves different stages (Obermuller-Jevic et al. 2003).

The fact that in the cell cycle assay PS and HI pulp extracts increased the proportion of cells in the sub G_0 and reduced the total number of cells present in G_2/M, after 24 hours treatment, suggest a cell cycle interruption in G_0/G_1 phase, which makes impossible the progression to mitosis and multiplication. Therefore, considering the fact

that guava extracts inhibited the proliferation and viability of adenocarcinoma breast cells, it is presumable that a smaller number of cells will be able to reach phases S and G_2. In this study, especially PS and HI extracts increased the percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in the G_2/M phase in both MCF-7 and MDA-MB-435 lines. The cell cycle analysis revealed similarities with other studies that have reported the effect of lycopene on breast cancer to be associated with the inhibition of cell cycle progress through G_0/G_1 phases. It has been stated that this carotenoid is able to act as an antitumor agent by arresting cell proliferation and inducing apoptosis (Palozza et al. 2007) It has also been stated that lycopene treatment increased the percentage of cells in the G_0/G_1 phases in additional types of cancers such as colon and prostate cancer (Vuolo et al. 2019, Grivicich et al. 2007). Concerning anthocyanins, there are no reports on the anticarcinogenic effect of guava anthocyanins but in an ethanolic extract of red-jambo peel, anthocyanins content was strongly correlated with the antioxidant capacity evaluated by cellular antioxidant activity and oxygen radical absorbance capacity, and showed significant effect on reducing the tumoral cell growth and proliferation. Anthocyanins rich-black chokeberry extracts were also able to inhibit cancer metastasis (Thi & Hwang 2018). An anthocyanin rich-Java plum extract has suppressed proliferation of HCT-116 cells and elevated apoptosis in both HCT-116 cells and colon CSCs (Charepalli et al. 2016). Finally, an anthocyanins-rich extract markedly decreased Caco-2 cell proliferation, induced apoptosis by activating caspase-3 cleavage, and upregulated cyclin-dependent kinase inhibitor 1 (p21Waf/Cif1) expression in a dose dependent manner. Furthermore, this extract was able to produce a dose-dependent increase of intracellular ROS in Caco-2 cells, together with an increase in the cell total antioxidant status (Prakashet et al. 2001).

In a normal situation, a programmed sequence of events leads to the elimination of cells without causing damage adjacent to tissues. The apoptosis process is responsible for keeping cells healthy, eliminating excess or abnormal cells. When abnormal cells fail to undergo apoptosis, the likelihood of mutations is increased, which can become carcinogenic cells. Apoptosis occurs by the action of a family of cysteine proteases, the caspases, which are activated in response to pro-apoptotic stimuli. Caspases promote apoptosis by: (a) inducing destructive enzymes such as DNases, (b) releasing cytochrome c via Bcl-2 family proteins, and (c) destroying major structural and regulatory proteins (Anwar et al. 2016). Among the several types of cancer, evidences show that the resistance to apoptosis is one of the most characteristic traits of the majority of malign tumors (Willers et al. 2013). In the present study, guava extracts, especially PS and HI extracts, promoted increased apoptotic death, both in MCF-7 and in MDA-MB-435 cell lines. The inhibition of the growth of many human colon adenocarcinoma cell lines by carotenoids has been reported. They induce cell-cycle arrest and apoptosis and are responsible for reducing cyclin A, an important regulator of cell-cycle progression (Gloria et al. 2014).

CONCLUSIONS

The data presented in this study demonstrate that guava cultivars pulp extracts, especially Pedro Sato, can play an important role in the reduction of cancer cells viability, regulation of cell cycle, and in the control of tumor progression, via the induction of apoptosis. Additional studies need to be performed to

confirm the higher anti-carcinogenic effect of Pedro Sato compared to other guava cultivars.

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