RESEARCH ARTICLE

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Antiproliferative and Apoptotic Effects of Murici (*Byrsonima crassifolia* (L.) *Kunth* and *verbascifolia* (L.) DC) and Taperebá (*Spondias mombin* L.) Extracts in Human Prostate Cell Line (PC-3)

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Abstract

Objective: The present study aimed to evaluate the antiproliferative and apoptotic effects of extracts obtained from the murici (*Byrsonima crassifolia* (L.) *Kunth and verbascifolia* (L.) DC) and taperebá (*Spondias mombin* L.) pulps, on cell proliferation, cell cycle and apoptosis on human prostate cell line (PC-3). **Methods:** Four extract was produced from the pulps: murici aqueous extract (MA), taperebá aqueous extract (TA), murici ethanolic extract (ME) and taperebá ethanolic extract (TE). In the present study, the analysis of cell viability, cell cycle and apoptosis analyze were performed using the MTT method and flow cytometry. **Results:** The results showed that murici and taperebá extracts proved to be inhibitors of cell growth, modulation of cell cycle promoters and capable of enhancing the death in prostate carcinoma cells PC-3; suggesting a regulatory effect in prostate cell line, depending on type of extract and dosage used. **Conclusion:** These results open a series of perspectives on the use of these bioactive extracts in the prevention and treatment of prostate cancer.

Keywords: Amazon fruits- Murici- Taperebá- cancer- prostate

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Introduction

Prostate cancer is the first most common cancer and the second leading cause of cancer death among men [1]. Once diagnosed, treatment is defined according to type, staging, location and risk-benefit, but in cases where prostate cancer metastasizes cure is discredited and patients have a 5-year survival rate of only 30%. Furthermore, the incidence of metastatic prostate cancer appears to have increased in all races and age groups over the past decade [2, 1]. This scenario shows the need to develop new alternative therapeutic approaches.

Among the main preventive health strategies, chemoprevention appears as a therapeutic option, which consists of the use of natural or synthetic chemical agents to prevent, interrupt, stabilize or reverse the genesis of cancer. Therefore, there is a growing interest in research involving phytochemicals and bioactive compounds present in fruits, vegetables and other plants, as agents that act against cancer [3, 4].

The Amazon region contains a range of fruit varieties with different aromas and exotic flavors, which have economic potential and an important prospect of appreciation for the region [5, 6]. Exotic fruits, such as murici (*Byrsonima crassifolia* (L.) *Kunth* and *verbascifolia* (L.) DC) and taperebá (Spondias mombin L.) have unique sensorial characteristics and high concentration of nutrients and bioactive compounds, with high antioxidant potential expressed in the scientific community that relate to health benefits [7].

There are several fields of research in the health area. Studies with cell cultures began in the 20th century with the aim of analyzing the behavior of these cells or animal tissues in specific and controlled situations outside the body [8]. Assays carried out with cell lines of prostate cancer make it possible to investigate not only the mechanisms of the pathogenesis of this cancer, but also the effects of treatments. The PC-3 cell line was established in 1979 from grade IV bone metastasis of prostate cancer in a 62-year-old Caucasian male. Due to the high capacity

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for migration and tissue invasion, this strain has a highly aggressive behavior [9, 10].

Studies involving prostatic cell lines are still scarce in the scientific literature, especially among the more aggressive ones, of bone metastases. In addition, different phytochemicals have already been described for murici and taperebá species, but their anticancer effects are still poorly understood. The present study aimed to evaluate the antiproliferative and apoptotic effects of murici and taperebá extracts on the human prostate cell line (PC-3). Our hypothesis was that Amazonian fruits extracts selected according to phenolic compouds profile contain multiple molecules with antitumor activities that could be very effective in killing human prostate cancer cells.

Materials and Methods

Samples

Pulps of murici (MU) and taperebá (TAP) packaged in sealed and labeled plastic bags (1 kg) were supplied by a company from Pará (PF, Castanhal, PA, Brazil) and stored at a controlled temperature (−18ºC). The frozen pulp was transported in an ice chest containing dry ice to the Laboratory for Analysis of Functional Foods (LAAF-UNIRIO), Rio de Janeiro (Brazil), where they remained frozen (−18ºC) until the moment of analysis.

Extraction of Samples

Aqueous extract

Approximately 250 g of pulp of MU and TAP was extracted with 80 mL of distilled water and then shaken for 2 h. After the pulp maceration period, the aqueous murici (MA) and aqueous taperebá (TA) extracts were filtered through Whatman #1 filter paper. The extracts were then frozen at −86 ºC in an ultra-freezer (Indrel® Ultrafreezer) and lyophilized (Terroni® LD 300, São Carlos, SP, Brazil) for 24 h. After this process, extracts were frozen at −18ºC until use in the experiments [11].

Ethanolic extract

The process employed to develop the lyophilized ethanolic extracts of MU and TAP included the same extraction steps as the lyophilized aqueous extract. After the pulp maceration period, the ethanolic murici (ME) and ethanolic taperebá (TE) extracts were filtered through Whatman #1 filter paper and the secondary ethanol residue was evaporated under low pressure at 45°C. The extracts were then frozen at −86ºC in an ultra-freezer (Indrel® Ultrafreezer) and lyophilized (Terroni® LD 300, São Carlos, SP, Brazil) for 24-hours. After this process, extracts were frozen at −18ºC until usage in the experiments [12].

Cell Culture and Treatment Protocol

Human cancer prostate cell line (PC-3) were obtained from the Federal University of Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). The cells were plated in 25 cm^2 tissue culture flasks, 5.0×10^6 cells/flask, and maintained routinely in Dulbecco's medium supplemented (DMEM) with 10% fetal bovine serum and 2 g/L HEPES buffer, pH = 7.4, under 5% CO_2 atmosphere. Cells were passaged at 70–80% confluence, about twice a week by trypsinization. Cells were seeded at 2.0×10^4 cells/cm^2 in 6-multiwell plates (2 mL of standard culture medium) for cell cycle progression and apoptosis analyses and in 96-multiwell plates (200 mL of standard culture medium) for cell viability analyses. A control group was included for all analyzed samples, being treated only with the culture maintenance medium, free from samples. All experiments were performed in triplicate.

Cytotoxic Analysis

MTT Assay

The status of cancer cell line viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Sigma, New York, NY, USA), in which the substance is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product. This requires active mitochondria, and even recently dead cells do not reduce significant amounts of MTT. Exponentially growing cells were adjusted to 2.0×10^4/cm^2 with DMEM, plated in 96-well plates (Corning, Tewksbury, MA, USA) at 200 µL/well and incubated for 24 hours according to the routine procedure. The cells were then incubated with MA, ME, TA and TE (0,05mg/mL to 20mg/mL) for another day. Each of the substances was also incubated with MTT (10 µL/well; 5 g/mL) for 4 hours. After 85 µL/well the liquid was removed and 50 µL/well sodium dodecyl sulfate was added to dissolve the solid residue. Finally, the absorbance was measured using a microplate reader (POLARIS-CELER®, Celer Biotecnologia, Minas Gerais, BH, Brazil) at 570 nm. The cell proliferation inhibition rate was calculated using the following formula: Cell viability (relative% of control) = (1 − average value of experimental group/average value of control group) × 100%.

Cell Cycle Analysis

Human prostate cancer cell line (PC3) received treatment with MA, ME, TA and TE extracts at two concentrations (10 and 20 mg/mL). After incubation for 24 hours, the cells were briefly washed with PBS solution and resuspended in 500 µL of Vindelov's solution containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNAse, and 50 mg/mL propionate iodide (Sigma Chemical Co., St. Louis, MO, USA) and left for 15 min at room temperature [13]. After 15 min of incubation, the cell suspension was analyzed for DNA content by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The relative proportions of cells with DNA content diploid G0–G1 (2n), S phase (> 2n, but < 4n), and G2/M phase (4n) were acquired and analyzed with FlowJo software following the acquisition of 20,000 events. The percentage of cell population at a specific stage was estimated with FlowJo analysis software version 1.2.

Apoptosis

The human prostate cell line was seeded in 6-well plates at concentrations similar to those used for cell cycle analysis. After 24 hours of treatment with the extracts (MA, ME, TA and TE) at concentrations of 10 and 20 mg/mL, the cells were washed with buffered

saline solution (PBS), resuspended in a binding buffer with 5 µL annexin V-fluorescein isothiocyanate (FITC) and 5 µL of propidium iodide (PI) (Apoptosis Detection Kit II, BD Pharmingen, New Jersey, USA). FITC and PI staining were analyzed to determine the apoptotic rate. The percentage of total apoptotic cells was calculated by adding the percentages of early apoptotic gated cells (annexin V+/PI−) and late apoptotic gated cells (annexin V+/PI+). The reading was held on the flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), following the acquisition of 20,000 events on CellQuest, and the data analyzed using the FlowJo software (FlowJo v. 1.2).

Statistical Analysis

The results presented are the mean and the corresponding standard deviation of three independent experiments performed in triplicate (n=6). Data was analyzed using GraphPad Prism statistical software (version 5.04, GraphPad Software Inc., San Diego, CA, USA). The univariate analysis of variance (ANOVA) with the Tukey post-test at a 95% confidence level was used to test cell viability, cell cycle and apoptosis rate.

Results

Cytotoxic Analysis - MTT

In the present work, the antiproliferative effect of MA, ME, TA and TE on the viability of human prostate adenocarcinoma cells (PC-3) was investigated. The MTT results showed a reduction in cell viability with decrease of 39.19% when incubated with MA (20mg / mL), mean reduction of 70.41% with ME (5 and 10mg/mL) and 38.17% for TA (10 and 20mg/ mL). The highest concentration of TE (20mg/mL) was able to promote the maximum decreasing (17.49%), compared to control ($p<0.001$).

After 24 hours MA and TA, cell viability inhbition with lower doses studied (0.05mg/mL). As for the ME and TE, it was demonstrated a decrease in cell viability at concentrations of 5 and 20mg/mL (Figure 1).

Effect of murici and taperebá bioactive extracts on cell cycle progression

Based on the huge percentage of reduction in the MTT analysis the concentrations of 10mg/mL and 20mg/mL were chosen to assess the different phases of cell cycle

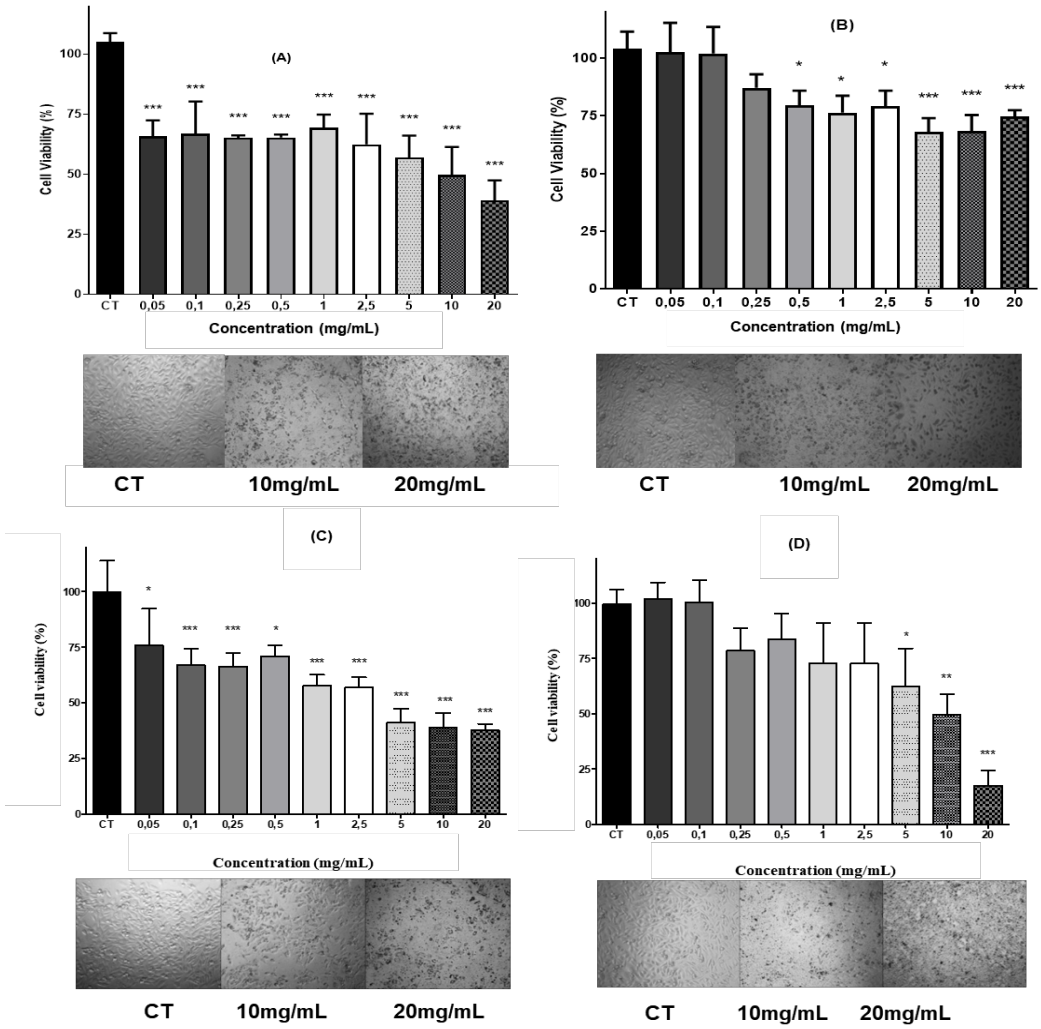

Figure 1. Effect of MA, ME, TA and TE on PC-3 Cell Viability. MA (A), ME (B), TA (C) and TE (D) were tested for their effect on PC-3 cell viability after 24h of treatmente using MTT assays. Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$; ** $p < 0.01$,***p <0,001). Bar 100 µm.

progression. Cycle analysis revealed that MA (10mg/mL) modulating cell cycle progression by promoting a reduction in the percentage of cells in the G0/G1 phase and an increase in the G2/M and S phases after the 24-hour incubation (Figure 2). During the incubation period with ME, an increase in the percentage of cells in the G0/G1 phase and a reduction in the percentage of cells in the G2/M phase were observed in both concentrations (10 and 20mg/mL) (Figure 3).

As for the TA (Figure 4), different patterns of modification in the phases of the cell cycle were proven to be dependent on the treatment dose, where the highest concentration studied (20mg/mL) led to an increase in cells in the G0/G1 and G2/M phases, while the lowest concentration (10mg/mL) led to a reduction in the percentage of cells in the G0/G1 phase.

No significant changes in the cell cycle pattern could be observed ($p<0.05$) after treatment with TE, with the exception of the treatment with 20mg/mL of TE in the S phase, in which there was a significant increase when compared to the control group ($p<0.05$) (Figure 5).

Effect of murici and taperebá bioactive extracts on cell apoptosis rate

After incubation with MA, TA and ME (10mg/mL and 20 mg/mL), a significant decrease ($p<0.05$) of viable cells in relation to untreated cells was observed. Furthermore, MA (20 mg/mL) and ME extracts demonstrated an increase on the percentage of cell on late apoptosis. MA extracts showed a significant advance on early apoptosis at the concentration at 10 mg/mL ($p<0.05$) in comparison on non-treated cells. In relation TA, there was a significant increase on early apoptosis at both concentrations. The proportion of non-apoptotic cells increased significantly ($p<0.05$) when ME was used in the highest concentration (20mg/mL). No change was observed in the percentage of non-apoptotic cells ($p>0.05$) when compared to untreated cells (control) after MA, TA and TE treatment (Table 1).

The relative increase rate of apoptosis in the PC-3 cells treated with murici and taperebá extracts at concentrations of 10mg/mL and 20mg/mL are illustrated in the graphs (Figure 6). TE (20mg/mL) had a profile of modification in apoptotic cells and decreased viable cells. A significant increase ($p<0.05$) was noticed in later apoptosis cells at both concentrations (10mg/mL and 20mg/mL) and an higher in early apoptosis cells at the concentration of 10mg/mL when compared to the untreated cells (control). A growth in the rate of apoptotic cells was perceived in all extracts studied, with a maximum expansion of 10.25 times in the rate of apoptotic cells for TE (20mg/mL).

Discussion

Murici and taperebá are exotic Amazonian fruits with high nutritional value, and present an elevated source of antioxidant activity. Murici is great a source of iron and vitamin C, also being rich in vitamin A and E [14]. Furthermore, Murici is an excelent source of antioxidants, with high amounts of phenolic compounds (159.9 mg gallic acid equivalent (GAE)/100g) [15].

The composition of taperebá reveals that lutein is present in higher amounts than zeaxanthins, in addition to containing cryptoxanthin β and α, β carotene [16]. De Souza et al. [17] found six carotenoids in the pulps of murici and taperebá: β-cryptoxanthin, lutein, zeinoxanthin, α- and β-carotene; and zeaxanthin. β-cryptoxanthin (89.81 ± 4.58 μg/g) and lutein (23.39 ± 1.41 μg/g) were the major components among the carotenoids identified in taperabá and murici, respectively. The nutritional and physicochemical composition of murici and taperebá was

Table 1. Effect of Murici (MA and ME) and Tapereba (TA and TE) Extracts on Different Stages of the PC-3 Cell Death Process for 24 h. PC-3 Cell Line was Treated with MA, ME, TA or TE for 24 h at 10 and 20 mg/mL. The results were espressed in percentages of viable, early apoptotic, late apoptotic, and non-apoptotic cells after treatment. Different letters (a,b,c) in the same row indicate statistically significant differences ($p < 0.05$).

Extract	Phases	Control	10mg/mL	20mg/mL
MA	Viable cells (Annexin V- PI-)	99.08±0.35^{a}	95.88±0.32^{b}	82.53±2.97^{c}
	Early apoptotic (Annexin V+ PI-)	0.27±0.09^{a}	1.28±0.65^{b}	0.35±0.23^{a}
	Late apoptotic (Annexin V+ PI+)	1.10±0.15^{b}	1.07±0.49^{b,c}	11.96±4.39^{a,c}
	Non-apoptotic cells (Annexin V- PI+)	0.32±0.17^{a}	0.90±0.42^{a}	1.86±1.46^{a}
ME	Viable cells (Annexin V- PI-)	98.20±0.42^{a}	95.40±0.56^{b}	94.31±1.28^{b}
	Early apoptotic (Annexin V+ PI-)	0.21±0.04^{a}	0.20±0.03^{a}	0.20±0.06^{a}
	Late apoptotic (Annexin V+ PI+)	1.07±0.49^{c}	3.64±0.45^{b,d}	4.47±1.16^{a,d}
	Non-apoptotic cells (Annexin V- PI+)	0.52±0.13^{b}	0.76±0.19^{b}	0.99±0.19^{a}
TA	Viable cells (Annexin V- PI-)	98.43±0.40^{a}	96.06±1.00^{a}	91.98±3.20^{b}
	Early apoptotic (Annexin V+ PI-)	0.10±0.04^{b}	0.93±0.31^{a}	0.64±0.34^{a}
	Late apoptotic (Annexin V+ PI+)	1.19±0.40^{b}	2.61±0.71^{b}	6,91±2,83^{a}
	Non-apoptotic cells (Annexin V- PI+)	0.35±0.16^{a}	0.37±0.18^{a}	0.51±0.15^{a}
TE	Viable cells (Annexin V- PI-)	98.40±0.57^{a}	89.05±1.01^{a}	85.98±6.10^{b}
	Early apoptotic (Annexin V+ PI-)	0.23±0.16^{b}	2.68±1.50^{a}	0.41±0.15^{b}
	Late apoptotic (Annexin V+ PI+)	1.01±0.64^{b}	7.83±1.27^{a}	12.56±5.62^{a}
	Non-apoptotic cells (Annexin V- PI+)	0.34±0.25^{a}	0.43±0.21^{a}	1.05±0.63^{a}

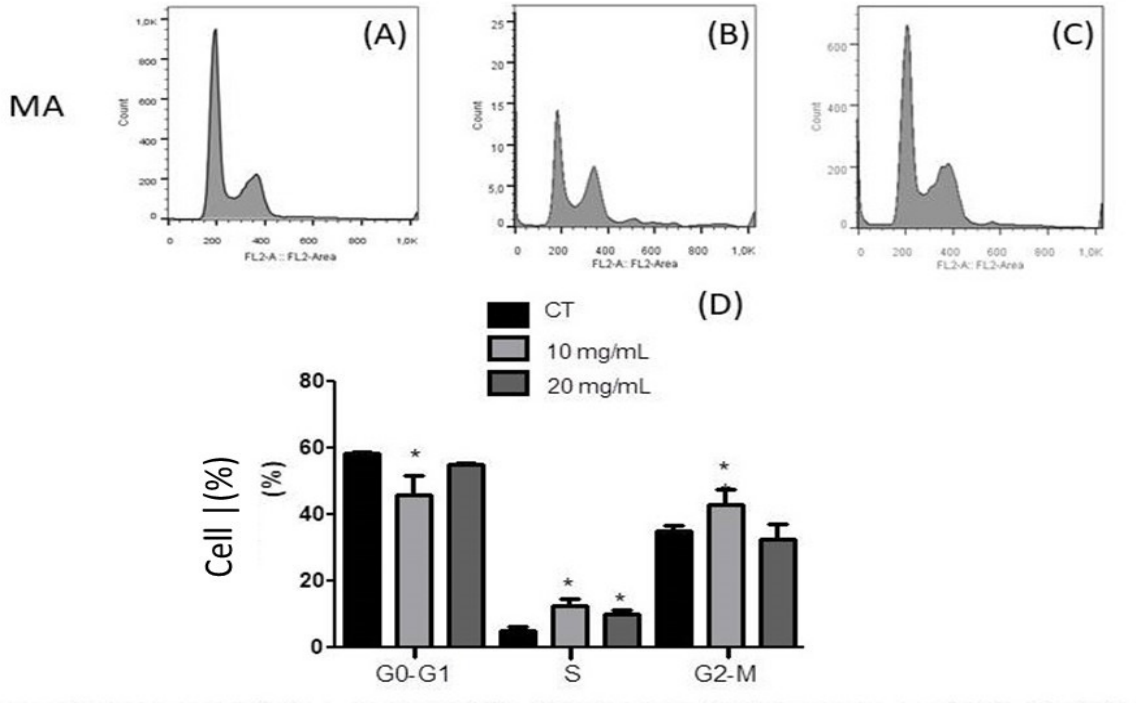

Figure 2. MA inhibit PC-3 cell cycle progression. PC-3 cells were tested for cell cycle progression in response to 24 h treatment with MA extracts. Flow cytometric analysis results are shown after treatment for 24 h with control (A), 10 mg/mL (B) and 20mg/mL (C) and bar graphs represent the percentage of PC-3 cells (D) in each cell cycle phase. The results are expressed as % of cells in G0/G1, S, and G2/M phases after cell treatment with CT, 10mg/m L and 20mg/mL. Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$). Bar 100 µm.

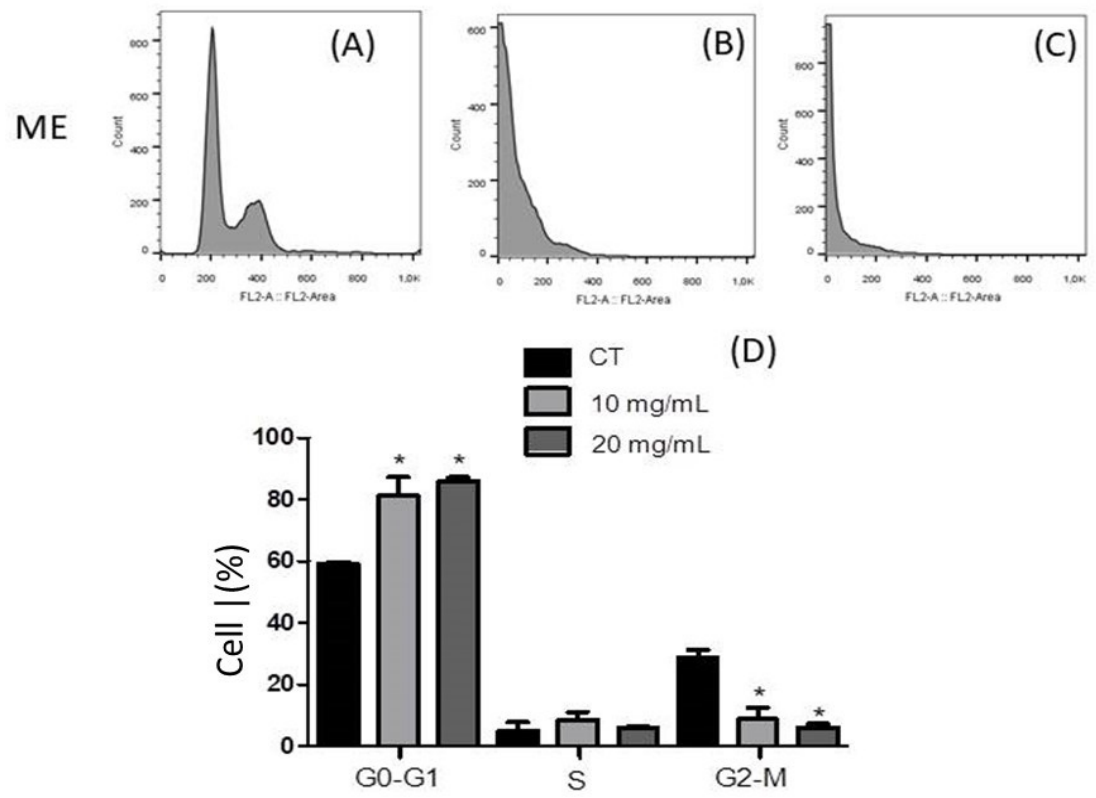

Figure 3. ME Inhibit PC-3 Cell Cycle Progression. PC-3 Cells were Tested for Cell Cycle Progression in Response to 24 h Treatment with ME Extracts. Flow cytometric analysis results are shown after treatment for 24 h with control (A), 10 mg/mL (B) and 20mg/mL (C) and bar graphs represent the percentage of PC-3 cells (D) in each cell cycle phase. The results are expressed as % of cells in G0/G1, S, and G2/M phases after cell treatment with CT, 10mg/m L and 20mg/mL. Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$). Bar 100 µm.

Table 2. Nutritional and Physicochemical Composition of Murici and Taperebá

	Murici (*)	Taperebá (**)
Moisture (g/100g)	76.18	86.87
Protein (g/100g)	1.33	0.71
Ask (g/100g)	0.81	0.58
Carbohydrate (g/100g)	12.98	13.90
Fat (g/100g)	4.27	0.44
Fiber (g/100g)	9.43	1.38
Sodium (g/100g)	45.43	4.28
Potassium (g/100g)	346.73	214.14
Calcium (g/100g)	83.38	20.88
Magnesium (g/100g)	43.70	13.55
Manganese (g/100 g)	0.08	0.02
Phosphorus (g/100g)	7.69	26.40
Zinc (g/100g)	0.37	0.17
Copper (g/100g)	0.09	0.07
Iron (g/100g)	1.00	0.76
Selenium (g/100g)	2.36	-
Cobalt (g/100g)	27.24	-
Nickel (g/100g)	26.41	-
Soluble solids (º Brix)	8.89	12.24
pH	3.93	2.91
Total sugar (g/100g)	-	5.07
Total Soluble (g/100g)	-	-
Acidity (%)	0.47	1.68
Reducing sugars (g/100g)	2.97	4.80

demonstrated in Table 2.

The bioactive compounds and antioxidative capacity of murici and taperebá fruits show the potential of their use in chemoprevention. The study of extracts from these fruits in cell lines is promising for evaluating the chemopreventive action, as growing evidence suggests that protective responses against cancer are not exclusively associated with a single factor, but with the presence of multiple factors acting synergistically [18, 19].

De Souza et al. [17] studied the effect of TA on human ovarian carcinoma cell lines (A2780 and ACRP). The concentration of 20 mg/mL promoted a significant decrease in cell viability of A2780 (69.40%) and ACRP (65.54%) lineages. Malta et al. [20] evaluated the antiproliferative activity of the extract (acetonic/methanolic) from Murici fruit on the growth of human liver cancer cells HepG2 and showed that during 72 hours with doses of 25.0 to 95.0 mg/mL, an amount higher than used in the present study. These results demonstrated that, murici and taperebá has different effects in inhibition of different cells lines viability, based in a concentration-dependent manner.

To explore the mechanism of action of murici and taperebá extracts to decrease cell viability, cell cycle assay was performed using flow cytometry. The transition from G1 to S, G2 to M phases are cell cycle checkpoints that function as surveillance mechanisms, wich can influence cell cycle arrest to prevent the defective DNA from replicating and buy some time for the DNA to be repaired [21].

Rafi et al. [22] showed that lutein, a carotenoid presents in murici and taperebá, altered the expression of biomarker genes associated with growth and apoptosis

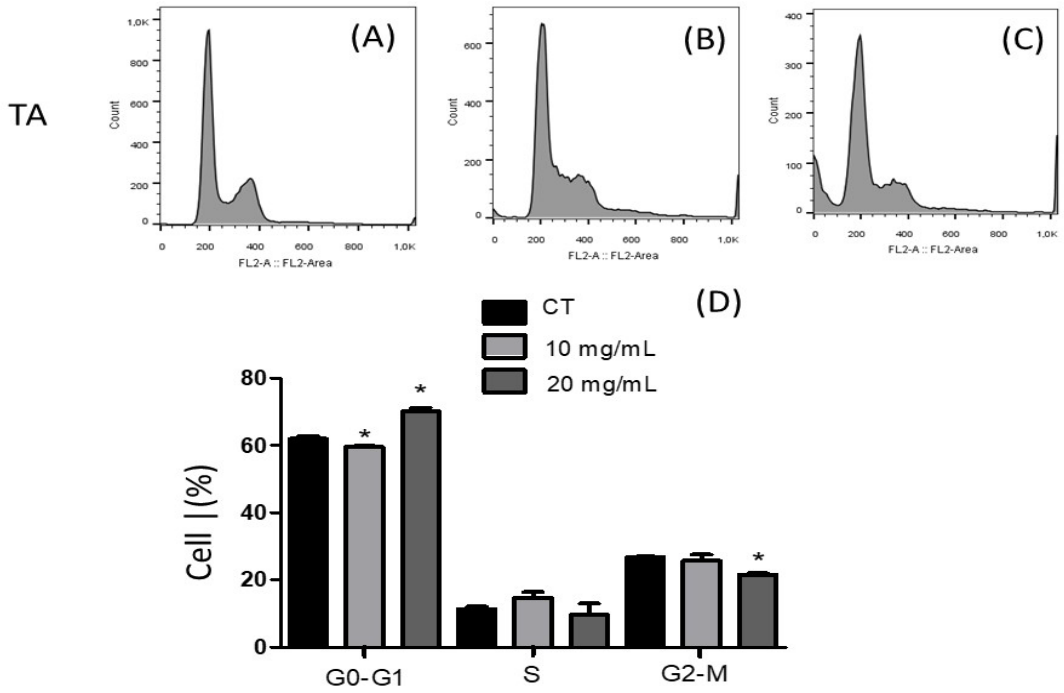

Figure 4. TA Inhibit PC-3 Cell Cycle Progression. PC-3 Cells were Tested for Cell Cycle Progression in Response to 24 h Treatment with TA Extracts. Flow cytometric analysis results are shown after treatment for 24 h at control (A), 10 mg/mL (B) and 20mg/mL (C) and bar graphs represent the percentage of PC-3 cells (D) in each cell cycle phase. The results are expressed as % of cells in G0/G1, S, and G2/M phases after cell treatment with CT, 10mg/m L and 20mg/mL. Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$). Bar 100 μm.

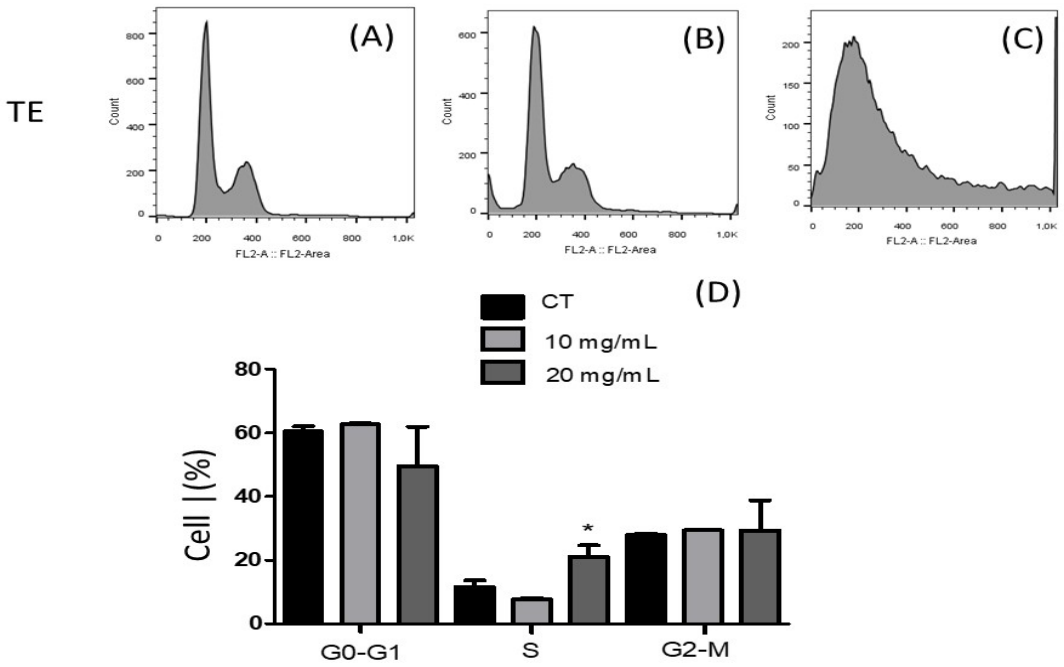

Figure 5. TE Inhibit PC-3 Cell Cycle Progression. PC-3 Cells were Tested for Cell Cycle Progression in Rsponse to 24 h Treatment with TE Extracts. Flow cytometric analysis results are shown after treatment for 24 h at control (A), 10 mg/mL (B) and 20mg/mL (C) and bar graphs represent the percentage of PC-3 cells (D) in each cell cycle phase. The results are expressed as % of cells in G0/G1, S, and G2/M phases after cell treatment. Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$). Bar 100 µm

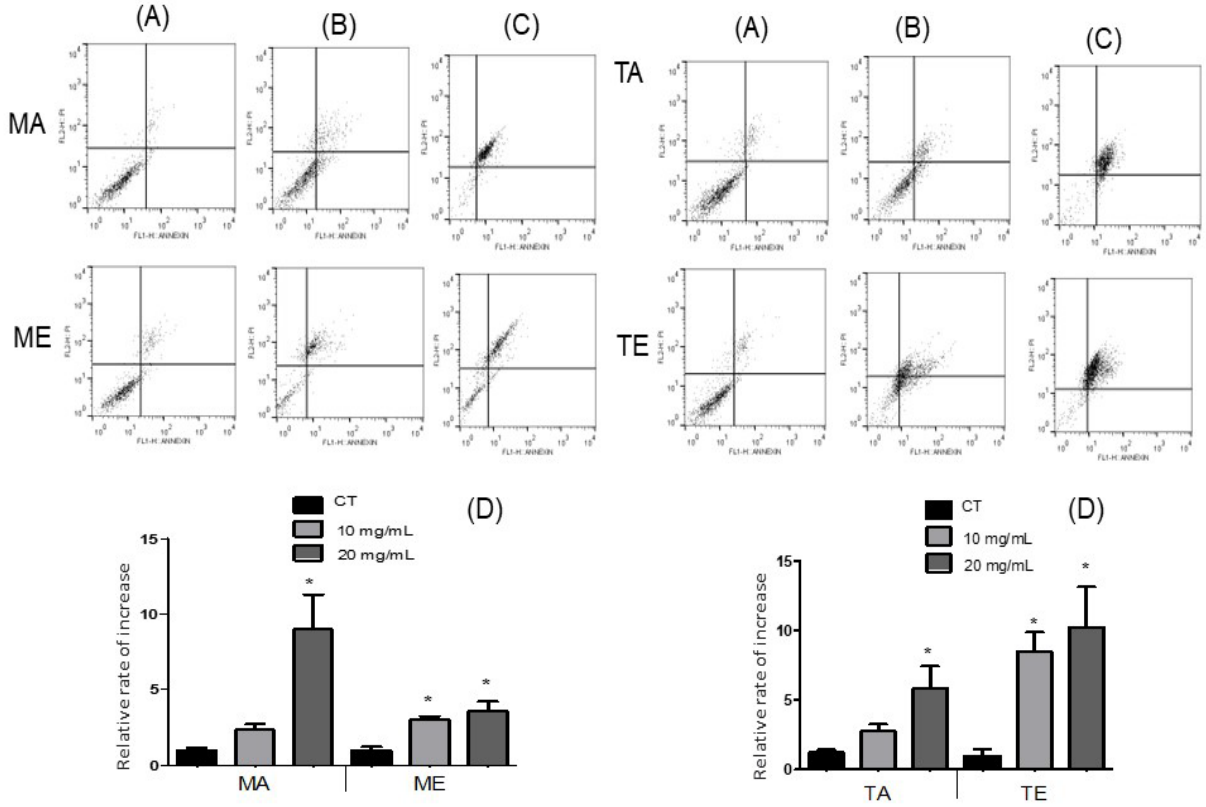

Figure 6. Effect of Murici (MA and ME) and Tapereba (TA and TE) Extracts on Apoptotic Rates in PC-3 Cell Line. Flow cytometric analysis results are shown after treatment for 24h with untreated cell (A) and MA, ME, TA or TE at 10mg/mL (B) and 20 mg/mL (C). Relative rate of PC-3 apoptosis treated after 24h with MA, ME, TA or TE at 10mg/mL and 20 mg/mL are shown in the graphs (D). Significant differences between the untreated cells and those incubated with the respective extracts (10 and 20 mg/mL) in relative rate were compared by one-way ANOVA, followed by Tukey's post-test (* $p < 0.05$).

in cells PC-3. Furthermore, analysis by flow cytometry showed that lutein improved drug-induced cell cycle arrest and also the induction of apoptosis in prostate cancer, confirming the potential for use in conjunction with chemotherapeutic agents capable of enhancing the death in prostate carcinoma cells PC-3.

The apoptotic death of cancer cells is considered a potential anticancer mechanism, which can control their proliferation [23]. Our apoptosis assays demonstrated an increase in the apoptosis rate in all extracts and these data reinforce the previously described cell viability results. De Souza et al. [17] tested aqueous extract of murici and taperebá in the A2780 parental cell line and showed an increase of apoptotic cells compared to untreated cells. Evidences reveals that carotenoids induce apoptosis in human cancer cells by regulating intrinsic pathways, such as Bcl-2 e and Bcl-xL family proteins by activating caspases and disrupting mitochondrial functions [24, 25].

This study demonstrated that the bioactive extracts of murici and taperebá were able to promote inhibition of cell growth, specific changes in the cell cycle and increase in the rate of apoptosis of human prostate adenocarcinoma (PC-3) cells. Further investigations are needed to identify the mechanisms of antiproliferative activity, the apoptosis induction pathways and changes in cell cycle patterns observed in the current study.

Author Contribution Statement

T.O.M.P.: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing - review & editing. N.F.S: Formal analysis, Validation. J.P.A,: Formal analysis. L.R., formal analysis. T.P.B.L: Formal analysis, Writing - review & editing. Antonio Palumbo Junior: Methodology, Conceptualization. A.J.T.: Methodology, Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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Approval

This study makes a lot of the Master Degree of Thuane Oliveira do Amaral Muxfeldt Paim.

Data Availability

The data used to support findings of this study are included within the article.

Conflict of Interest

The authors declare no conflict of interest.

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