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TUCUMÃ EXTRACTS DECREASES PML/RARA GENE EXPRESSION IN NB4/APL CELL LINE

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Abstract: Acute promyelocytic leukemia (APL) is a cancer pharmacologically treated with all-trans retinoic acid (ATRA), although well tolerated by most patients, some develop toxicity to ATRA, Differentiation Syndrome. The Amazon Biome has several fruits and oil plants rich in micronutrients, particularly carotenoids as the fruit tucumã (*Astrocaryum aculeatum*). This study analyzed the antitumor and cytoprotective activity of tucumã with and without concomitant exposure of ATRA in high concentration mimicking the toxicity of differentiation syndrome, as the potential cytotoxic effect of chemotherapeutic in an APL cell line. The cultured NB4 cells were exposed to ethanolic extracts of tucumã and to synergism with extracts and ATRA. Determination of proliferation, cell viability, caspases 1, 3, 8 and cell differentiation by nested RT-qPCR. The ATRA control had a strong inhibitory effect and toxicity as expected. The extracts also reduced cell proliferation by triggering apoptosis in concentration-dependent and reversing chromosome translocation, especially the lowest tested concentration of tucumã pulp extract. In the synergism, extracts act to maintain the levels of viability and apoptosis equal to the ATRA control but in contrast to drug that causes death and destruction of the genetic material, tucumã demonstrated a reduction of the gene expression indicating a possible protection against the toxicity of high concentrations of ATRA. These results suggest that fruits rich in retinoid molecules may have a cytotoxic effect against APL cells and reduced concentrations of carotenoids

may act as cytoprotectors in APL cells treated with high concentrations of ATRA promoting cellular/molecular differentiation.
Keywords: *Astrocaryum aculeatum*. ATRA. Carotenoids. Leukemia. Differentiation syndrome.

1 INTRODUCTION

Acute promyelocytic leukemia (APL) is a cancer type with distinct subset of acute myeloid leukemia (AML) characterized by a unique chromosome translocation t(15;17) involving the retinoic acid receptor alpha (*RARα*). The translocation causes a *PML-RARα* gene fusion and formation of a chimeric protein.[1,2] For this reason, APL patients are pharmacologically treated with all-*trans* retinoic acid (ATRA) resulting in the degradation of PML-RARα.[3,4] There is many evidences that ATRA can induce disease remission in APL patients by triggering terminal differentiation of leukemic promyelocytes.[4] An important fact is the need for high doses of ATRA and treatments for long periods of time. Even ATRA being well tolerated by most patients, some may develop toxicity called differentiation syndrome, presenting clinical symptoms such as fever, respiratory insufficiency and pulmonary infiltrate. The differentiation syndrome is currently the main adverse effect in the treatment of APL affecting approximately 14-16% and presenting lethality between 2-10% of patients treated.[5]

The β-carotene molecule is considered the major dietary source of provitamin A, a constituent of many varieties of vegetables and fruit such as carrots, tomatoes, spinach and others.[6] The β-carotene central enzymatic cleavage and subsequent oxidation produces ATRA, which functions as a ligand for a family of nuclear transcription factors, the retinoic acid receptors (RARα).[7]

The Amazon basin presents several fruits and oleaginous plants rich in micronutrients, particularly carotenoids. Thus, it is the case of tucumã fruit (*Astrocaryum aculeatum*) that presents several carotenoids molecules including an important concentration of all-trans-·-carotene and others two carotenoid molecules.[8] Tucumã is a fruit produced by a palm tree native in tropical South America and has a range extending northwards towards Central America, Trinidad and the West Indies.[9] This fruit is a popular traditional component of regional breakfasts10 presents some biological properties including antioxidant,[11] anti-

inflammatory[12,13] and antimicrobial effect.[14] Yet, tucumã extract may present genotoxic action depending on concentration and time of exposure.[15]

However, until moment the potential cytotoxic tucumã effect against tumoral cells was not evaluated, as well as its cytoprotective action against high concentrations of ATRA, mainly considering acute promyelocytic leukemia cells. Being that tucumã also presents other bioactive molecules in their composition such as quercetin and caffeic acid that have some effect against leukemic cells[16-18] and cytotoxic capacity reversal effects caused by hydrogen peroxide.[11]

Therefore, we used an acute promyelocytic leukemia cells line cytogenetically characterized by a t(15;17)(q22;q11-12) translocation isolated by Lanotte, Martin-Thouvenin, Najman, Balerini, Valensi, and Berger[19] to evaluate the cytotoxic effect of tucumã extracts and the potential effect cytoprotective of this fruit against ATRA toxicity in high concentration (40 μM) mimicking the differentiation syndrome.

2 MATERIAL AND METHODS

2.1 CHEMICALS AND EQUIPMENT

Cell culture products as well a media was obtained from Gibco (BRL, Gaithersburg, MD, USA); double-strand DNA quantification was evaluated using quantitTM- Picogreen® ds DNA Kit Assay obtained from Invitrogen; caspases quantification was performed using Quantikine Human Caspases Immunoassays R&D systems (Minneapolis, MN). Chemical reagents were purchased from Sigma-Aldrich (Spain). RA (purity ≥99%). The analysis was performed using SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation equipment (Sunnyvale, CA, USA). For the analysis of gene expression were used equipment StepOne Real-time PCR Systems (Applied Biosystems®) and Picodrop® (United Kingdom) well as PureLink® kits RNA Mini Kit (Life Technologies®), SuperScript® III First-Strand Synthesis SuperMix (Invitrogen®) and Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen®) and some other separate reagents of Invitrogen®.

2.2 TUCUMÃ EXTRACTS PREPARATION AND BIOACTIVE MOLECULES CONTENTS

Tucumã extract were produced from fresh matured fruits obtained from a native forest near to Manaus City, Amazonas States, Brazil, (3.08°S, 60.01°W). The main bioactive molecules were quantified from previous studies performed by Souza Filho *et al*.,[15] Jobim *et al*.[14] and Sagrillo *et al*.[11]

Briefly, the tucumã pulp and peel extracts were manually removed, producing 800 g peel and 400 g pulp, and keep frozen at -18 °C until extraction procedures to be performed. This material was used to obtain an ethanolic tucumã extracts with absolute ethanol solution at a ratio of 1:5 (w/v). After four days the extraction was obtained, the pulp and peel extracts were then lyophilized and stored at -20 °C to be used and chemically characterized. A total of 3359 g and 6091 g of tucumã peel and pulp extract were obtained, respectively. The chemical composition determined by spectrophotometric assays and HPLC chromatography identified is in table 1.

Table 1 – Chemical composition determined by spectrophotometric assays and HPLC chromatography identified

Components	Extract Peel	Extract Pulp
Total polyphenol (mg/GAE g)	941.8	872.1
Flavonoids (quercetin mg/g)	92.8	53.3
Tannin (mg/g)	31.4	0,24
Alkaloids (mg/g)	1.5	0.93
β-Carotene (mg/g)	62.91	27.55
Rutin (mg/g)	30.54	19.06
Quercetin (mg/g)	12.72	6.53
Gallic acid (mg/g)	3.79	14.25
Caffeic acid (mg/g)	8.33	0.87
Chlorogenic acid (mg/g)	3.04	1.119

2.3 NB4-LPA CELLS CULTURES AND TUCUMÃ EXTRACT TREATMENTS

Human acute promyelocytic leukemia (APL) derived NB4 cells were cultured in RPMI-1640 medium and supplemented with 10% FCS, 100 units/ml penicillin and 100 µg/mL streptomycin at 37 °C in a 5% CO2 humidified environment as previously described in Falanga, Marchetti, Giovanelli, and Barbui.[20]

To perform the experimental assays, the extracts were firstly diluted in 40% ethanol to improve their solubilization. The solution was then diluted in cellular medium culture to obtain <0.5% ethanol concentration in all tucumã concentrations tested here. The ethanol was also added in all control treatments to eliminate the potential influence of this molecule in the results obtained. The concentrations of tucumã extracts tested in this study were 100, 300, 600, 900, 1200 e 1500 µg/mL. ATRA at 40 µM concentration dissolved in 4% of dimethylsufoxide (DMSO) was also used to treat NB4-APL cells. This higher the concentration of ATRA used, the lower the number of cells.[21]

2.4 CYTOTOXIC ASSAYS

Viability of tucumã extracts on NB4 cells with and without ATRA was evaluated using the MTT assay, a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase.[22]

To perform the MTT assay, this reagent (Sigma-Aldrich, St. Louis, MO) was dissolved in a 5 mg/mL phosphate buffer (PBS 0,01 M; pH 7.4), added into a 96-well microplate containing the sample treatments and incubated for 4 hours. In addition, the supernatant was removed from the wells and the cells were resuspended in dimethyl sulfoxide (DMSO). The absorbance at 560 nm was read in a plate reader TP-Reader (Thermoplate, China). This assay was performed in octuplicate for each treatment. Cell viability was expressed as a percentage of the untreated control value.

An additional assay was performed from quantification of double-strand DNA concentration present in supernatant culture using Picogreen® dye according manufactures

instructions. The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm recorded at room temperature Results were expressed as fluorescence values that indicate the DNA integrity as well as considering the DNA integrity percent in relation of negative control group treatment. Fluorescence values lower than untreated NB4 control group indicated cytotoxic effect of tucumã and ATRA treatments. Therefore, the results were expressed as a percentage of dsDNA calculated to each treatment in relation to untreated control samples.

2.5 APOPTOSIS BIOMARKERS ANALYSIS

ATRA is a morphogenetic signaling molecule inducing differentiation of immature blood cells resulting in a massive cell death characterized by apoptosis.[23] Considering that caspases are essential molecules in apoptosis pathway the quantification of caspases 1, 3 and 8 were performed in NB4 cells exposed to tucumã extracts with and without ATRA. To perform this analysis cells were incubated under the conditions described above for 24 hours. The analyses were performed using the Quantikine Human Caspases Immunoassays to measure caspase 1, 3 and 8 in cell culture supernants using a microplate with 96 wells, according to the manufacturer. Briefly, all reagents and working standards were prepared and the excess microplate strips were removed. The assay diluent RD1W was added (50 µL) to each well. Furthermore, 100 µL of the standard control for our sample was added per well, covered with an adhesive strip and incubated for 1.5 hours at room temperature. Each well was aspirated and washed twice, for a total of three washes. Caspase 1 antiserum was added to each well and covered, given a new adhesive strip and incubated for 30 minutes at room temperature. The aspiration/wash step was repeated and the caspase 1 conjugate (100 µL) was added to each well and incubated for 30 minutes at room temperature. The aspiration/wash step was repeated and 200 µL of substrate solution was added to each well and incubated for 20 minutes at room temperature. Finally, the 50 µL stop solution was added to each well and the optical density was determined within 30 minutes using a microplate reader set to 450 nm. Experiments were performed in triplicate.

2.6 EVALUATION OF INDICATORS OF CELLULAR DIFFERENTIATION

Evaluation of indicators of cellular differentiation in the treatments was held, through the PML gene expression with the technique of Quantitative Polymerase Chain Reaction (qPCR). Initially, the total RNA was extracted with PureLink® RNA Mini Kit (Life Technologies®) according to the manufacturer's protocol, after quantification in Picodrop®. Extraction product was added in reverse transcriptase reaction (RT-PCR) to produce a complementary DNA (cDNA) with the SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen®) as recommended by the manufacturer.

Evaluation of indicators of cellular differentiation required optimization and modifications in the methodology described by Van Dongen, Macintyre, Gabert, Delabesse, Rossi, and Saglio.24 First a conventional PCR, as follows: reaction buffer 1 x, 1.5 mM MgCl2, 200 µM dNTP, 0.2 µM each primer PML-A1 and RARα-B (Table 2), 1 U/ µL of Taq DNA polymerase, 1 µL of cDNA and water in 25 µL reaction total. The condition of the amplification reaction was of 95 °C for 5 minutes, 35 cycles (94 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute) 72 °C for 5 minutes and 4 °C to stop. After the product has been subjected to a comparative CT method with the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen®) with the primers PML-C1 and RARα-D (table 2) at a concentration of 400 mM and 0.24 µL of template with final volume of 12 µL reaction the other components of the reaction were added as recommended by the manufacturer. As internal control gene GAPDH (Table 2) was used. The reaction occurred in the thermal cycler StepOne Real-Time PCR Systems, Applied Biosystems®. The program for amplification followed according to the kit used.

Table 2 – Primers used in molecular evaluation

Primers	Sequences (5' – 3') Forward/Reverse	Sizes of PCR products (bp)
PML-A1	CAGTGTACGCCTTCTCCATCA	381
RARα-B	GCTTGTAGATGCGGGGTAGA	
PML-C1	TCAAGATGGAGTCTGAGGAGG	214
RARα-D	CTGCTGCTCTGGGTCTCAAT	
GAPDH	AGAAGGCTGGGGCTCATTTG	258
	GAAGACTGTGGATGGCCCCT	

2.7 STATISTICAL ANALYSIS

The data were expressed as mean ± standard deviation (SD). The results of viability and antiproliferative effect of tucumã and ATRA were expressed as percent (%) of control group. Therefore, the data were submitted to one-way analysis of variance (ANOVA) followed by the Tukey or Dunnett Test. The statistical tests were performed using GraphPad Prism Software considering significant the comparisons with $p<0.05$. Gene expression results were expressed as mean ± standard deviation (SD) by calculating the relative concentration of the values ($\Delta\Delta CT$) using the program StepOne™ Real-Time PCR System (Applied Biosystems®). The experiments were conducted in triplicate.

3 **RESULTS**

3.1 CELLULAR PROLIFERATION

The Figure 1A presents the NB4 cell proliferation (% of control) when exposed to 72 h pulp and peel tucumã extract at different concentrations. The result of ATRA effects in these cells with and without association with tucumã extracts area also presented. Cells exposed to extracts higher than 600 µg/mL presented a significant decreasing on cellular proliferation after 72 h culture ($p=0.001$).

NB4 treated just with ATRA presented a strong inhibitory effect as expected (14.5 ± 2.7% in relation to untreated control group, $p=0.001$). Despite tucumã extracts also present inhibitory proliferative effect on NB4 cells, this effect was more moderate or equal to ATRA control.

In the presence of ATRA (figure 1B) all peel extract concentrations decreased cellular proliferation when compared to untreated cells. However, in relation to ATRA control, synergism increased cell viability, indicating that the tucumã extract could influence the chemotherapeutic response ($p=0.001$).

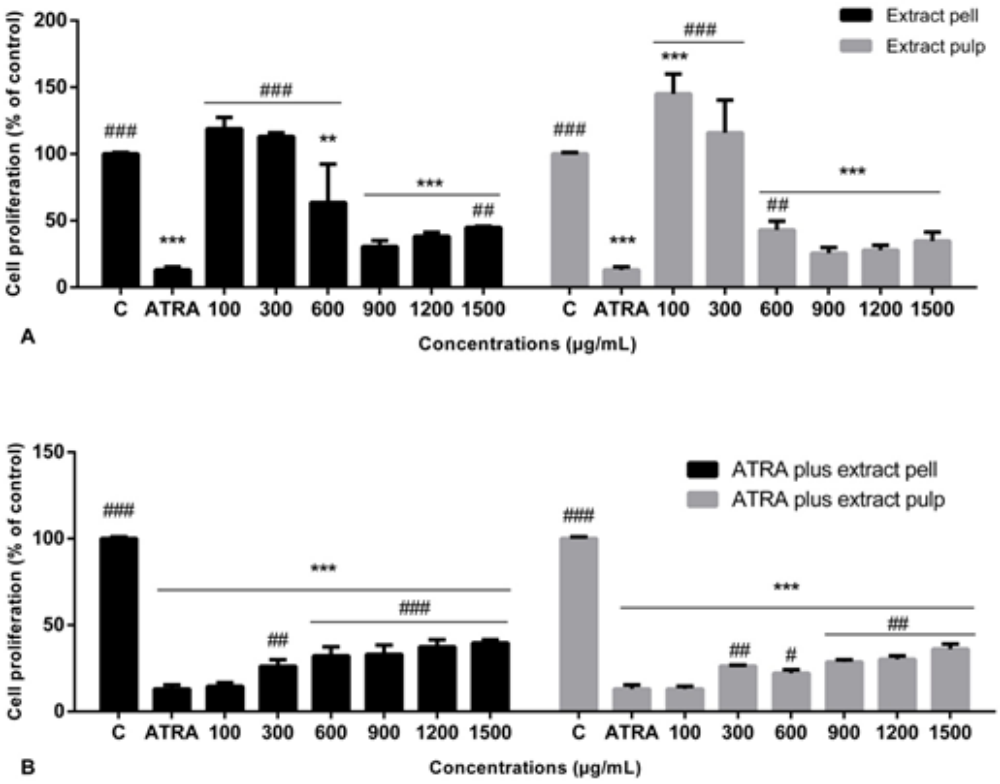

Figure 1 – Effect of peel and pulp tucumã extracts against proliferation of NB4 cells
Notes: (A) Differents tucumã extracts concentration; (B) effect of tucumã extracts on high doses ATRA. Data are shown as % of untreated control group. * indicate statistical differences among treatments and cellcontrol determined by analysis of variance followed by Tukey post hoc test ($p<0.05$). # indicate statistical differences among treatments and ATRA control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$).

Quantification of culture medium free dsDNA was also performed to evaluate if tucumã extracts presented some cytotoxic effect on NB4 cells after 72 h cultures. Both tucumã extracts increased significantly levels of dsDNA medium in culture levels in a dose-dependent way, with exception in the concentration of 100 µg/mL, in peel extract remained the same as control and in pulp extract the levels of dsDNA reduced as can be seen in figure 2A.

A second analysis that was compared the tucumã extracts effect in association with ATRA drug (figure 2B) showed that interaction between both tucumã extracts and ATRA caused a significantly increase of DNA damage higher than ATRA control. In this case a pattern similar to that described for the concentration of 100 µg/mL.

For this reason, the levels of caspases 1, 3 and 8 were performed to evaluate whether tucumã cytotoxic effect could involve triggering of apoptosis pathway.

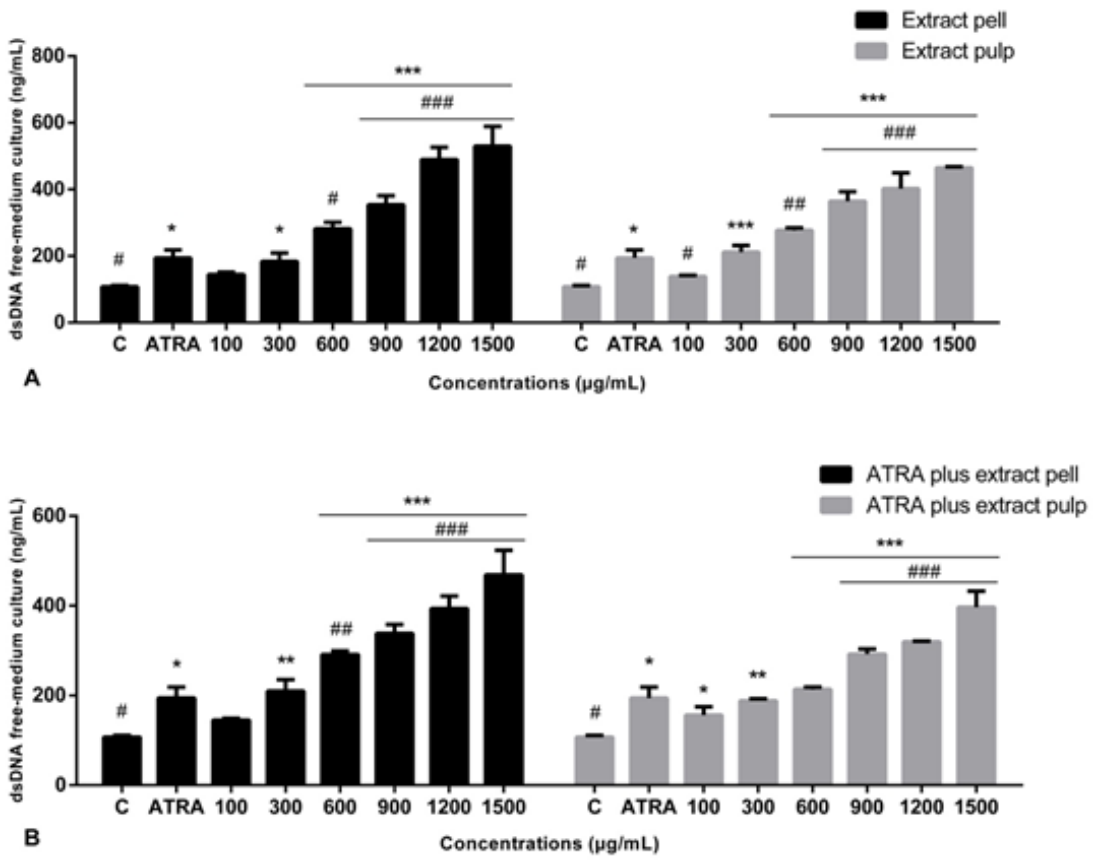

Figure 2 – Effect of peel and pulp tucumã extracts and extracts plus ATRA against viability of NB4 cells determined by dsDNA levels in the culture medium

Notes: (A) Different tucumã extracts concentration; (B) effect of tucumã extracts on high doses ATRA. Data are shown as % of untreated control group. * indicate statistical differences among treatments and cell control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$). # indicate statistical differences among treatments and ATRA control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$).

3.2 CASPASES ACTIVITY

In general, as can see in Figures 3, 4 and 5 caspase levels presented a significant increase when compared to untreated control cells depending of tucumã extracts ($p=0.0001$), except in the concentration of 100 µg/mL of pulp that remains equal to the cell control. In presence of ATRA the caspases levels increased further than cells were exposed to extracts only tucumã in concentration-dependent. These results also indicated that tucumã extracts can induce apoptosis of NB4 cells and present synergic effect when these cells were exposed concomitantly to ATRA plus tucumã extracts.

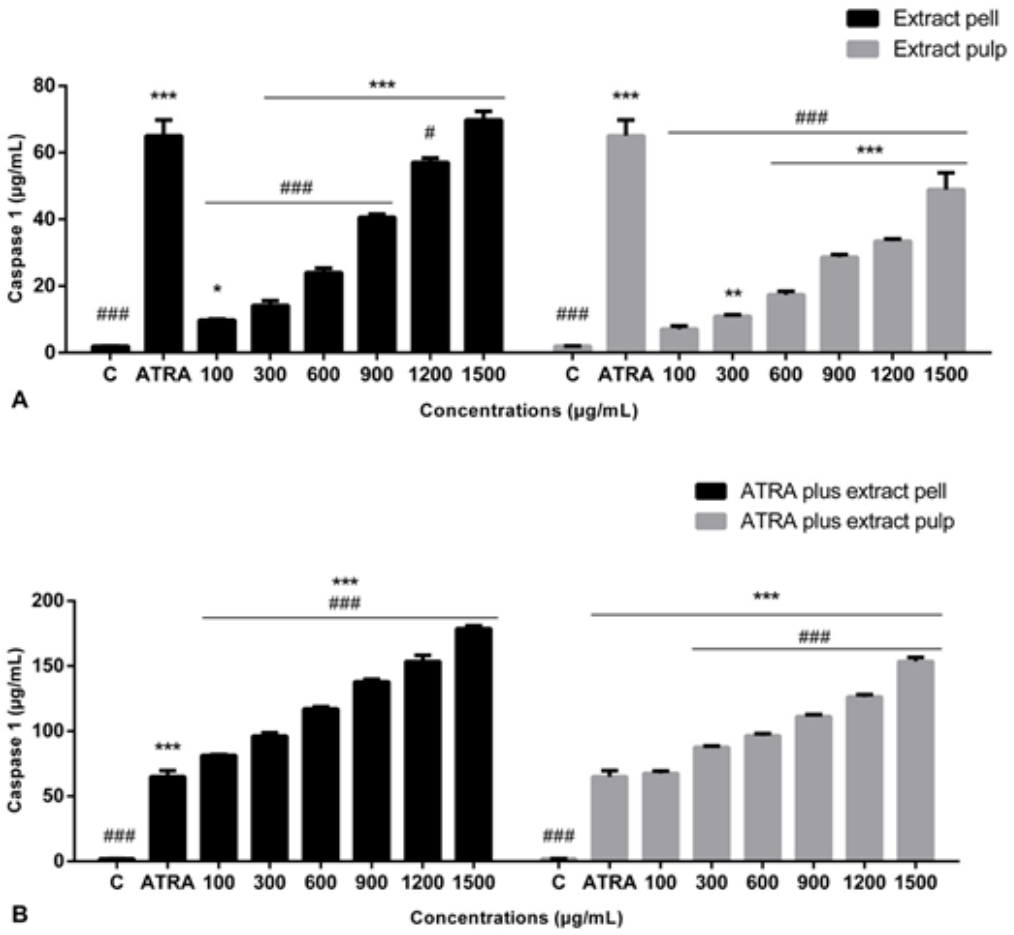

Figure 3 – Effect of peel and pulp tucumã extracts on caspase 1 levels (µg/mL) of NB4 cells with and without ATRA concomitant treatment

Notes: (A) Differents tucumã extracts concentration; (B) effect of tucumã extracts on high doses ATRA. * indicate statistical differences among treatments and cell control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$). # indicate statistical differences among treatments and ATRA control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$).

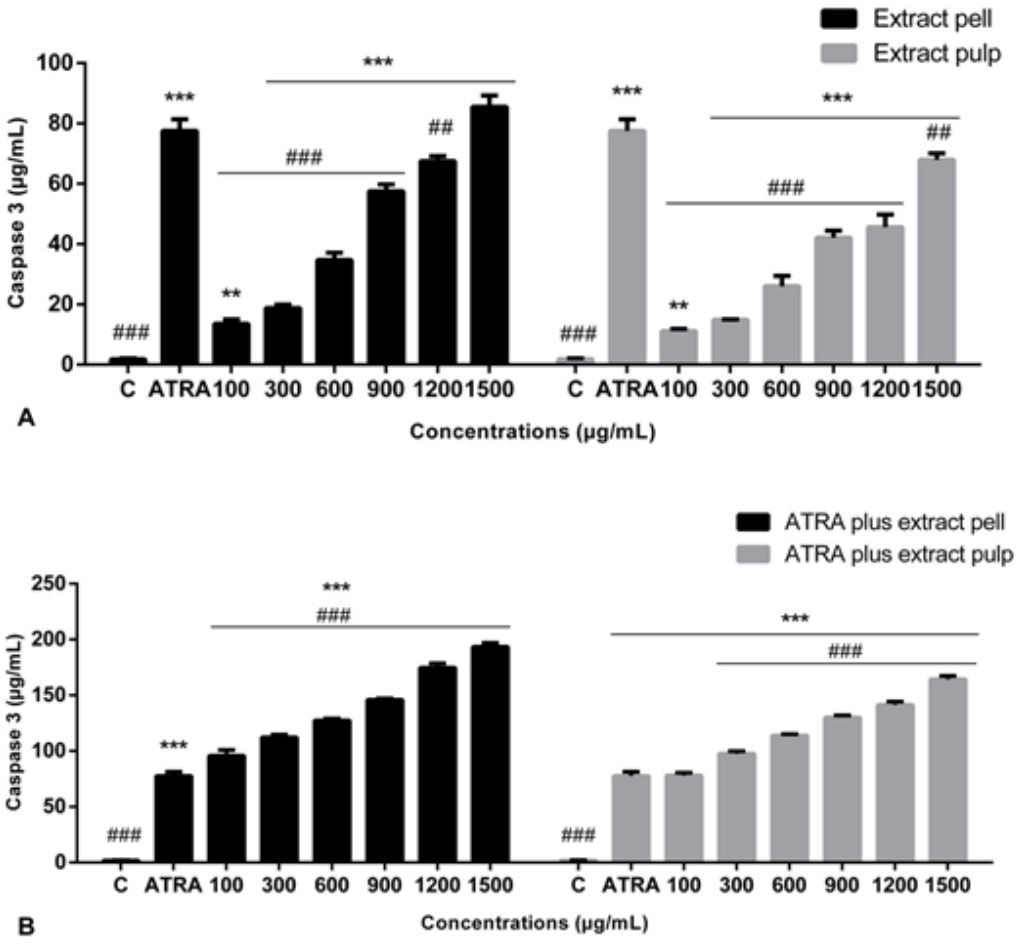

Figure 4 – Effect of peel and pulp tucumã extracts on caspase 3 levels (µg/mL) of NB4 cells with and without ATRA concomitant treatment

Notes: (A) Differents tucumã extracts concentration; (B) effect of tucumã extracts on high doses ATRA. * indicate statistical differences among treatments and cell control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$). # indicate statistical differences among treatments and ATRA control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$).

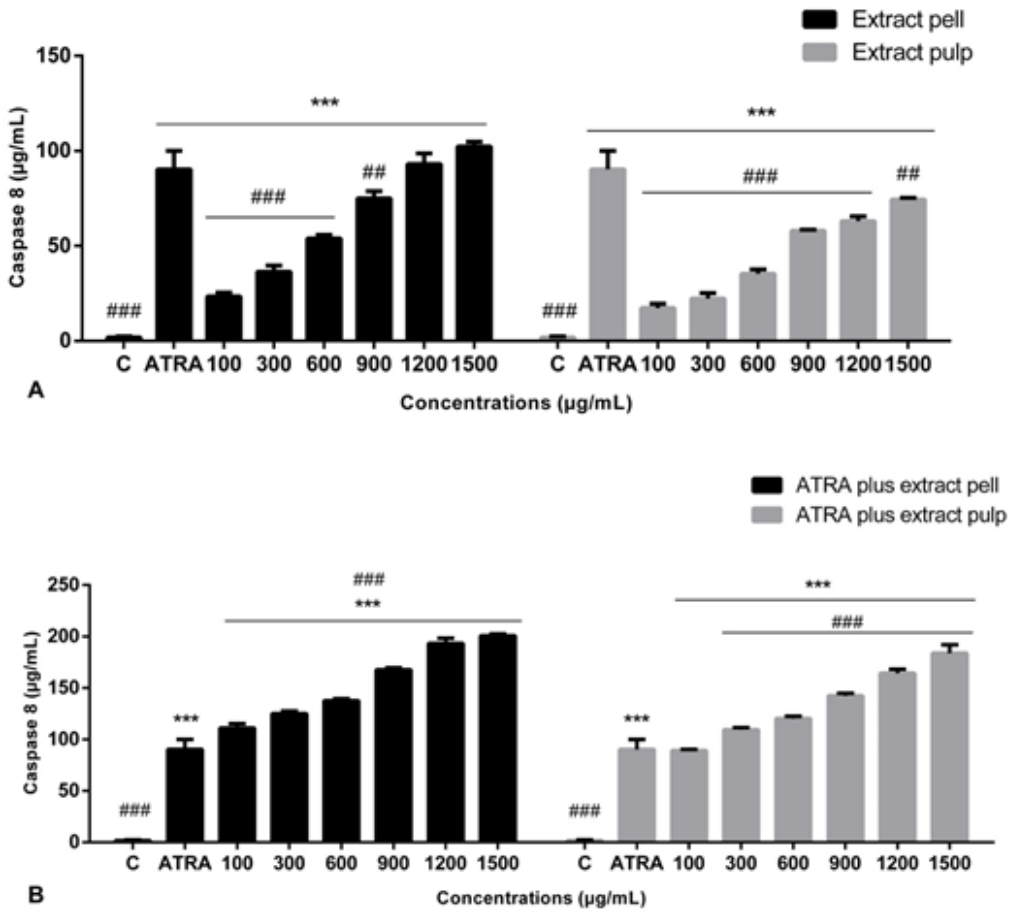

Figure 5 – Effect of peel and pulp tucumã extracts on caspase 8 levels (µg/mL) of NB4 cells with and without ATRA concomitant treatment

Notes: (A) Differents tucumã extracts concentration; (B) effect of tucumã extracts on high doses ATRA. * indicate statistical differences among treatments and cell control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$). # indicate statistical differences among treatments and ATRA control determined by analysis of variance followed by Tukey post hoc test ($p<0.05$).

3.3 GENE EXPRESSION

The results of the gene expression are presented below. In all graphs ATRA control and treatments with extracts in the two largest concentrations (1200 e 1500 µg/mL) were removed to avoid confusion in the analysis of results, since there was no amplification of genes interest demonstrating the possible toxicity of these treatments as it was also observed cellular alterations in the previous tests.

In Figure 6A are shown the results of peel that concentration of 100 µg/mL decreased the transcriptional amplification unlike other treatments. The results for the pulp (Figure 6B) showed that all concentrations reduced the number of transcribed except in the concentration of 300 µg/mL. However, unlike peel, the concentration of 100 µg/mL was more effective.

Considering the promising results obtained with tucumã extracts in concentration of 100 µg/mL, a final test was performed with synergism between extracts and ATRA (ATRA and peel extract; ATRA and pulp extract) (Figure 6C) where it was verified that in synergy revealed the potential cytoprotective effect of the extract in other words at a concentration of 100 µg/ml the treatments prevented toxicity and cell death by high doses of ATRA and further differentiated tumor cells into normal cells.

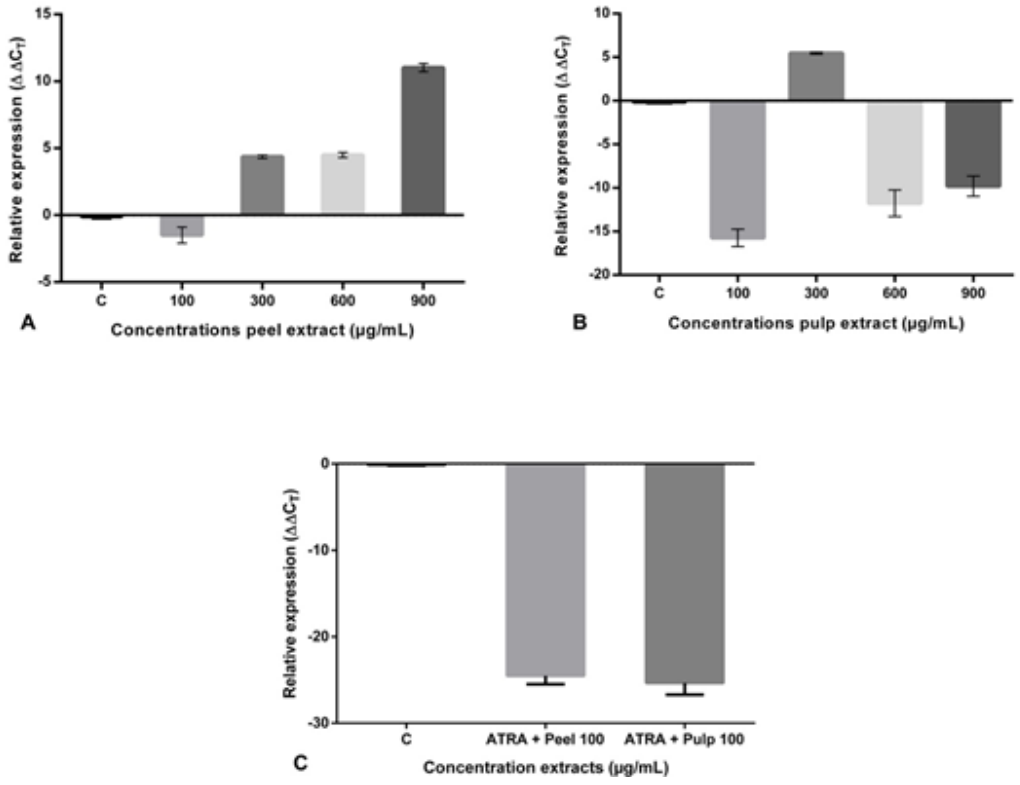

Figure 6 – Expression of the gene *PML-RARα*
Notes: (A) Differents tucumã extract peel concentration; (B) differents tucumã extract pulp concentration; (C) effect of tucumã extracts on high doses ATRA. Compared to control cells. Relative expression (Log10 RQ). Results expressed as mean ± standard deviation (SD) by calculating the relative concentration of values (ΔΔCT) using the program StepOne™ Real-Time PCR System (Applied Biosystems®).

4 DISCUSSION

The present study showed that peel and pulp extracts of tucumã, an Amazonian fruit that presents high levels of retinoids molecules has an important cytotoxic effect against APL cell line (NB4). For our best knowledge this is the first study showing leukemic cells mortality by tucumã.

In addition, a possible cytoprotection against the toxicity caused using high concentrations of ATRA is speculated due to retinoid resistance, which lead to differentiation syndrome in this pathology. The results described herein showed synergistic effect between tucumã and ATRA extracts.

The potent effect of ATRA against APL cells is because this leukemia type is unique among others myeloid leukemia presenting high sensitivity to ATRA that at 1 μM concentration induces both cell cycle arrest and the maturation of cells triggering caspase-dependent apoptosis.[25] But when used in high concentrations (5 to 50 μM), significantly decrease the cells viability.[21] As expected, when NB4 cells were exposed to ATRA was observed a significant increase on caspases 3, 8 and 1 level. Even though the effect on caspases levels was more moderate than ATRA, tucumã extracts also induced the increase of these proteins indicating its capacity to apoptosis induction.

Effect of tucumã extracts on NB4 cell proliferation was not so intense than ATRA treatment but induces cell death, a promising result since the ATRA control used was in high concentration. In addition, when cells were concomitantly exposed to extracts and ATRA, cell proliferation in all tested concentrations of the extracts did not cause cell death as ATRA control, perhaps indicating positive synergistic effect of the extracts against toxicity evidenced by ATRA in high concentrations. These results are not so surprisingly, considering that ATRA action is more concentrated in triggering APL cells death.

Toxicity can be proven with the result of MTT method, which has reduced the number of cells in a concentration greater than 600 μg/mL in dose-dependence, mainly in ATRA control. Yet, previous studies reported cytotoxicity, genotoxicity and high levels of caspase 1 concentrations greater than 1000 μg/mL in peripheral blood mononuclear cells.[15]

Still, in the lower concentration of the extract, 100 µg/mL, mainly the pulp, reverses the leukemic cell translocation and maintains the cellular levels presented by MTT method, it is believed that this is normal cell proliferation, also according to the coefficient of caspases, where these are in concentrations as the control used.

So, it should be noticed that low concentrations of carotenoids, such as tucumã pulp, modulate the activity of DNA repair, and some carotenoids, such as β-carotene, increased resistance to oxidative damage of DNA in relatively low concentrations.[26] This characteristic is lost with the increased concentration of carotenoids, the higher the concentration less effect, as presented by the ATRA control and higher extracts concentrations. Thus, the high occurrence of chemicals in tucumã, these in high concentrations may decrease the positive effect of other antioxidant compounds are also present in the extract.[27]

From results described here, two important aspects could be discussed: the role of habitual consume of fruits rich in retinoids as preventive strategy against APL development or by leukemic patients undergoing ATRA chemotherapy. Many evidences showed the occurrence of cancer-preventive constituents of fruits and vegetables that may inhibit carcinogen activation, enhance chemotherapeutic detoxification, prevent carcinogens from interacting with critical target sites, or impede tumor progression. Unfortunately, in APL disease these two important epidemiological points are scarcely investigated.

Investigations identified and described more than 4000 molecules that are chemically related to vitamin A, including retinoids present in some types of vegetables. These molecules have many biological functions, being retinoic acid the active metabolite of retinol.[28] Retinoic acid can suppress skin, lung, oral, bladder, ovarian and prostate cancer in tumorigenic animal models.[28] However, the potential preventive role of vitamin A in pediatric cancer should be done by moderate and habitual intake of foods rich in these compounds. This statement is based in recent dietary guidelines there are no evidences to support the use of antioxidant supplements such as β-carotene, vitamin A and vitamin E in the primary prevention of cancer, especially in well-nourished populations.[28,29]

Despite the lack of epidemiological data analyzing the potential preventive effect retinoids on APL carcinogenesis, the habitual consumption of foods rich in vitamin A can be considered the best way to help in general cancer prevention including leukemia. This presupposition is based in some studies that analyzed the role of other fruits and

bioactive molecules obtained from diet on APL, such as resveratrol from red grape fruit and wine,[30] lycopene from tomato and other vegetables,[31] pomegranate,[32] curcumin and tea,[33] hibiscus,[34] anthocyanidins present in several red darked-fruits.[35]

5 CONCLUSION

Although *in vitro* studies represent exploratory research on the potential effects of extracts and molecules against cancer, our results described here suggest that fruits rich in retinoids molecules may have cytotoxic effect against APL cells and cytoprotective against high concentrations of ATRA.

CONFLICTS OF INTEREST

There is no conflict of interest.

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