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To cite this article: Renata Cristina da Silva, Raphael Rosa Fagundes, Klaas Nico Faber & Élida Geralda Campos (2022): Pro-Oxidant and Cytotoxic Effects of Tucum-Do-Cerrado (*Bactris setosa* Mart.) Extracts in Colorectal Adenocarcinoma Caco-2 Cells, Nutrition and Cancer, DOI: 10.1080/01635581.2022.2086704

To link to this article: https://doi.org/10.1080/01635581.2022.2086704

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Pro-Oxidant and Cytotoxic Effects of Tucum-Do-Cerrado (Bactris setosa Mart.) Extracts in Colorectal Adenocarcinoma Caco-2 Cells

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ABSTRACT
Colorectal cancer is one of the most common types of cancer. Bioactive natural compounds can act in cancer chemoprevention as tumor growth inhibitors. Tucum-do-cerrado (Bactris setosa Mart.) is a Brazilian fruit that contains several phenolic compounds. This study investigated the effect of tucum aqueous extract in Caco-2 cells in comparison to primary human intestinal organoids and fibroblasts. Cells were exposed to 0.5 and 1 mg/ml of tucum aqueous extract for 24 h. ROS production, mRNA levels for SOD1 and SOD2, CAT, GPX1, NFE2L2, HIF1A and NOS2 were evaluated in Caco-2 cells exposed to tucum extract. Cell viability of Caco-2 cells was decreased upon tucum extract exposure. Mitochondrial ROS levels increased in Caco-2 cells exposed to tucum extract. The mRNA levels of SOD1, SOD2, CAT, GPX, NFE2L2 and HIF1A were downregulated in Caco-2 cells exposed to tucum extract, while NOS2 mRNA levels remained unchanged. Protein levels of SOD2, CAT and NRF2 remained unchanged in Caco-2 cells treated with tucum extract, indicating that catalase and SOD2 cellular functions may be unaffected by the tucum extract at 24 h, of exposure. Aqueous extract of tucum-do-cerrado may induce cellular toxicity in a cancer cell-specific manner, possibly through increased mitochondrial ROS production and gene expression regulation.

Introduction
According to the World Health Organization (WHO), cancer is the second leading cause of death in the world and resulted in 9.6 million deaths in 2018. Colorectal cancer is one of the most common types of cancer, in both men and women, and dietary factors are key risk factors in the development and prevention of this disease (1). Multiple studies have shown that diets with a high content of fruits and vegetables prevent and reduce the risk of gastrointestinal cancer (2–5). For instance, vitamin D, calcium and antioxidants are associated with a relative risk reduction for the development of colorectal cancer when compared to low intake of these nutrients (6). Moreover, bioactive compounds found in a variety of plants, such as coffee, soy, green tea, tomato and curcuma, are widely studied for their anticancer effects (7). The antioxidant activity of polyphenols is well known, however, they can also act as prooxidants and induce oxidative DNA damage in the presence of metal ions such as copper and iron (8). Caffeic acid, for example, induces DNA damage in human peripheral lymphocytes in a copper-dependent reaction. This effect has also been observed with gallic acid, a structural constituent of tannins, and epigallocatechin-3-gallate (EGCG), an apoptosis inducer (9). The copper concentration in cancer cells is higher than in normal cells, which would explain a selective cytotoxic effect on these cells. The selective genotoxic effect of polyphenols led to the hypothesis that their prooxidant property is involved in the anticancer action of these compounds (9). Colon cancer cells show defective DNA repair (10) and, therefore, compounds that induce the DNA repair machinery are relevant to be considered as anticancer agents. Polyphenols have been shown to induce DNA repair genes. For example, a major polyphenol from green tea,
(-)-epigallocatechin-3-gallate (EGCG), inhibits, in cancer cell lines, the activity of the enzyme 5-cytosine DNA methyltransferase, involved in the methylation of newly synthesized DNA strand (11). As a consequence of this inhibition, reactivation of methylation-silenced genes coding DNA repair enzymes occurs. In addition, naringenin, a citrus flavonoid, can stimulate, in a beyond antioxidation manner, the induction of base excision repair gene expression in LNCap prostate cancer cells following oxidative stress (12). Modulation of the cellular redox status is one of the main target of polyphenols in their effect on cancer development and progression. Polyphenols may act either as pro-oxidants and induce cell cytotoxicity, or act as antioxidants and inhibit metabolism and cellular activity (7). The dual effects of polyphenols on cancer cells seems to depend on dose and treatment time. For example, treatment of MCF-7 breast cancer cells with low doses of flavonoids (1 to 8 µM) for 60 h slightly increases cell growth while high doses of flavonoids (20 to 120 µM) for 48 h inhibits cell proliferation and induces cell death due to excessive oxidative stress (13).

Tucum-do-cerrado (Bactris setosa Mart.) is a fruit found in the Brazilian savanna, which has high levels of different bioactive compounds, including vitamin C, as well as at least 46 phenolic compounds, such as anthocyanins, catechins, rutins, cyanidin,peonidin, caffeic acid, resveratrol and quercetin (14–16). These compounds are mainly found in the peel of the fruit, which was shown to have the major antioxidant activity, in vitro and in vivo, when compared to the pulp (15–19). In the present study, the effects of the aqueous extract of the tucum-do-cerrado peel were evaluated in colorectal cancer cells (Caco-2) and normal intestinal mucosal cells, e.g., primary human intestinal organoids and fibroblasts.

**Material and Methods**

**Extract of Tucum-Do-Cerrado**

Fruits were obtained from a local merchant and were from “Fazenda Grama,” Teresópolis de Goiás - GO/Brazil. Fruits were washed with distilled water and the peel was manually removed and lyophilized. For preparation of the crude aqueous extract, 1 g of pulverized peel was mixed with 10 ml of distilled water. The contents were mixed for 16 h at 4°C and filtered through a 0.22 µm filter. The aqueous extract was stored at −20°C until use. The extract concentrations in mg/ml corresponded to the pulverized peel dissolved in water.

**Cell Culture**

Caco-2 cells (human epithelial colon adenocarcinoma; ATCC®, HTB-37TM, Manassas, USA) and primary human intestinal fibroblasts cells were maintained in DMEM medium (Dulbecco's Modified Eagle's Medium, ThermoFisher Scientific Inc, Massachusetts, EUA) supplemented with 10% fetal calf serum (FCS; Invitrogen), 1% antibiotic cocktail [penicillin (10 U/ml), streptomycin sulfate (100 µg/ml) and fungizone; Lonza, Basel, Switzerland] and 1% w/v non-essential amino acids (NEAA, Gibco). Cells were cultured at 37°C and 5% CO₂.

Human gut (jejunum) organoids were obtained from surgical resection material at University Medical Center Groningen and maintained on Matrigel® (Life science 354262) and hGF medium (DMEM/F12 medium, Glutamax 1%, HEPES 1 M, PSF 1%, Gentamycin 50 µg/ml). Organoids were cultured in expansion medium (EM), which consisted on hGF medium added with Wnt3-conditioned medium 1:1, Rspo-1 100 ng/ml (R&D Systems, Mineápolis, Minnesota, EUA, 4645-RS), 100 ng/ml Noggin (R&D Systems 6057-ng-100), 1x B27 (Invitrogen 17504-044), 1.25 mM NAC (Sigma-Aldrich, San Luis, Missouri, EUA, A9165), 50 ng/ml EGF (Invitrogen, Carlsbad, Califórnia, EUA, PMG8041), 10 mM nicotinamide (Sigma N0636-100g), 10 µM SB202190 (Sigma S7067-5 mg), 10 µM Y-27632 (Sigma Y0503), 0.5 µM A83 (Tocris Bioscience, Bristol, Reino Unido, 2939). Differentiation medium were the same as expansion medium, but without nt3, nicotinamide, SB202190 and Y-26632.

**Cell Viability**

Cells were plated at 5 × 10⁵ cells/well in a 24-well plate in supplemented DMEM medium. When cells reached 80% confluence, aqueous extract of tucum-do-cerrado (0.5 and 1 mg/ml) in DMEM medium was added and the cells were incubated for another 24 h. Cell viability after 24 h treatment was measured using the WST-1 reagent (Roche, Basel, Switzerland, 11644807001) according to the manufacturer’s instructions. Cells were incubated for 45 min with WST-1 reagent diluted on HBSS buffer at 1:10 and the absorbance of the samples was measured at 450 nm on Epoch2 microplate reader (Biotek Instruments). For IC₅₀ results, the following concentrations of the aqueous tucum extract were tested: 0.01, 0.025, 0.50, 0.100, 0.250, 0.500, 1.0, 1.25 and 1.5 mg/ml. The IC₅₀ was calculated using the GraphPad prism 6 software.
**ROS Production**

Fibroblasts and Caco-2 cells were plated in 96-well plates at 5×10^4 cells/well (100 µl/well) and after 24 h the aqueous extract of tucum-do-cerrado was added (0.5 and 1 mg/ml). The cells were then incubated for 24 h. ROS production assay was carried out according to the manufacturer’s instructions. Briefly, the stock solution (one vial dissolved in 13 µl DMSO) of Reagent MitoSox red (Roche) was diluted in culture medium (1:1,000 dilution) and 100 µl was added to each well. Cells were incubated for 30 min and after that the absorbance was measured at 510 nm on a Synergy H4 Hybrid Reader (Biotek Instruments) microplate reader.

**RNA Isolation and Gene Expression**

For total RNA isolation cells were washed twice with HBSS buffer (Gibco) and 500 µl of TRIzol reagent (Sigma-Aldrich) was added in each well, and RNA isolation was carried out according to manufacturer’s instructions. RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo-Scientific). Complementary DNA (cDNA) synthesis was made with RNA and mix with RT-buffer (50 mM Tris-HCl, 50 mM KCl, 3 mM MgCl₂, 5 mM DTT), 10% dNTP mix (1 mM dATP, dGTP, dTTP, dCTP, Sigma-Aldrich), 2% random primers (0.05 μg/μl, Sigma-Aldrich), 1.5% RNAse OUT and 2% M-MLV RT (respectively, 4 U/μl and 20 U/μl; Invitrogen) to a final volume of 50 µl. The reaction for cDNA synthesis was incubated for 10 min at 25 °C followed by 60 min at 37 °C and 5 min at 95 °C, using a thermal cycler (BioRad, CA, USA, T100). Annealing temperature for primers was 60 °C.

For gene expression analysis, cDNA was mixed with oligonucleotides (probe, sense and antisense) for different target genes. The gene expression levels of Superoxide Dismutase [Cu/Zn] (SOD1), Superoxide Dismutase [Mn] (SOD2), Catalase (CAT), Glutathione Peroxidase (GPX), Nuclear Factor Erythroid 2-Related Factor 2 (NFE2L2), Hypoxia-inducible factor 1-alpha (HIF1A), Nitric Oxide Synthase 2 (NOS2) and 18S were analyzed by quantitative real-time PCR (RT-qPCR). The cycle threshold (Ct) was calculated using QuantStudio software. For qPCR running, sample mix contained 0.4 µl cDNA, 0.8 µl probe, 0.86 µl sense primer, 0.36 µl anti-sense primer, 10 µl x2 RT buffer and 4.48 µl water in each well. The Primer sequences are described in Supplementary Table S1. The comparative Ct method was used for quantification of mRNA expression levels using the amplification efficiency of each gene. All results were normalized using 18S as the housekeeping gene.

**Cell Lysates, Protein Quantification and Western Blot Analysis**

Caco-2 cells were washed 2x with HBSS buffer and 200 µl of lysis buffer (25 mM HEPES, 150 mM KAc, 2 mM EDTA pH 8.0, 0.1% NP-40) containing protease inhibitor (10 mM NaF, 50 mM PMSF, 1 µg/µl a-protrine, 1 µg/µl pepstatine, 1 µg/µl leupeptin, 1 mM DTT; 1:25 dilution) were added to samples. Lysed cells were scraped and transferred to 1.5 ml conical tubes. Protein was quantified by BCA protein assay (Biorad). Electrophoresis was performed using 40 µg of total protein and the proteins were electro-transferred to nitrocellulose membranes. Membranes were stained with Ponceau-S to check for protein transfer and washed once with PBS before blocked with 0.5% BSA in 0.1% PBS Tween (PBS Gibco, 10019-015; Tween 20 Gibco, P2281) for 1 h. Samples were incubated overnight at 4 °C with rabbit polyclonal antibodies against MnSOD (manganese superoxide dismutase), pNRF2 (nuclear factor, erythroid 2-like 2) or β-actin, or mouse polyclonal antibodies against Catalase. All antibodies (Sigma-Aldrich) were used at a 1:1,000 dilution. Membranes were washed with 0.1% PBS Tween and incubated with secondary rabbit or goat IgG antibodies (1:2,000, Sigma Aldrich) for 1 h and washed again using 1x PBS. Enhanced chemiluminescence (ECL), advanced Western blotting detection kit and ECL Hyperfilm (GE Healthcare, Madison, WI) were used to detect protein expression. The signals were visualized by ChemiDoc Resolutions System (Biorad) and bands were quantified using Image Pro Plus software v 7.01 (Media Cybernetics, Silver Spring, MD, USA).

**Immunofluorescence**

Caco-2 cells were plated at 5 × 10⁵ cell/well in 12-well plates with coverslips and after they reached 80% confluence, the aqueous tucum extract (0.5 and 1 mg/ml) was added and the cells incubated for 24 h. Cells exposed to 1 mM hydrogen peroxide (H₂O₂) were used as positive control and coverslips incubated without the secondary antibody were used as negative control. Cells were washed twice with PBS (500 µl) and fixed with 4% paraformaldehyde for 10 min. After fixation, cells were washed 2x with PBS and 500 µl permeabilizing buffer (Triton 0.01% in PBS) was added. The samples were then incubated for 30 min at 37 °C. The coverslips were washed twice with PBS and were incubated 30 min in a blocking buffer (2% BSA in PBS). Rabbit polyclonal immunoglobulin G raised against MnSOD.
(1:400) was added and the coverslips were incubated for 1 h at room temperature. Cells were washed with a blocking buffer (2% BSA in PBS) and probed with an Alexa Fluor™488-conjugated anti-rabbit antibody (1:500) for 30 min at room temperature. Slides were mounted using a Vectashield antifade reagent with DAPI (nuclear stain) (Vector Laboratories, CA, USA) and images were taken using a fluorescent microscope (Leica DMI6000B) with software Leica Application Suite Advanced Fluorescence 3,1,0 build 8587.

Statistical Analysis

The results are expressed as mean ± SEM. Samples were compared using one-way analysis of variance (ANOVA) and the post hoc test of Bonferroni. For statistics significance, p-value < 0.05 was used.

Results

The cell viability of Caco-2 cells was dose-dependently reduced after 24 h exposure to the tucum aqueous extract with an IC₅₀ of 0.59 mg/ml (Fig. 1A). Thus,

![Figure 1](image-url)

**Figure 1.** IC₅₀ of tucum aqueous extract for Caco-2 cells (A) after a 24 h exposure of different concentrations (25, 50, 100, 250, 500, 1,000, 1,250 µg/ml). n = 3. Results expressed in mean ± SEM. WST-1 cell viability assay to evaluate proliferation of Caco-2 cells (B) n = 3. Human intestinal organoids (C) n = 2 and Primary human intestinal fibroblasts (D) n = 1 exposed to aqueous extract of tucum-do-cerrado (0.5 and 1 mg/mL) for 24 h. Results expressed in mean ± SEM, p < 0.05.
cells were exposed for 24 h to either 0.5 and 1.0 mg/ml tucum aqueous extract in subsequent experiments. Cell viability decreased with increasing extract concentration from 0.01 to 1.5 mg/ml and was 20.1% at the lowest concentration and 91.6% at the highest concentration. Caco-2 cell viability was significantly reduced to 57.8% and 33.8% after 24 h exposure to 0.5 and 1.0 mg/ml tucum aqueous extract, respectively, when compared to untreated control cells (Fig. 1B). In sharp contrast, cell viability of primary human intestinal organoids and primary human intestinal fibroblasts was not affected by exposure to tucum aqueous extract (Fig. 1C and D, respectively). Further analysis showed that mitochondrial ROS production in Caco-2 increased 99.1% in the cells exposed to 0.5 mg/ml and 84.3% in the cells exposed to 1.0 mg/ml of the tucum aqueous extract compared to untreated control cells \((p < 0.05, \text{ Fig. 2A})\). In contrast, tucum exposure did not show significant changes on mitochondrial ROS production in primary human intestinal fibroblasts (Fig. 2B).

Protein and mRNA levels were analyzed for antioxidant superoxide dismutases. Tucum aqueous extract dose-dependently reduced \(SOD1\) (Cu/ZnSOD) and \(SOD2\) (MnSOD) mRNA levels (Fig. 3A and B). However, MnSOD protein levels remained unchanged in Caco-2 cells exposed for 24 h to tucum aqueous extract, when compared to untreated control cells (Fig. 3C). Immunofluorescence microscopy analysis revealed that tucum aqueous extract did not evidently affect MnSOD-specific staining in Caco-2 cells, while it was clearly induced in cells exposed to 1 mM \(\text{H}_2\text{O}_2\) (Fig. 3D).

Tucum aqueous extract also reduced, in a dose-dependent manner, the mRNA levels of \(GPX1\), \(CAT\) (Fig. 4A and B) and of the oxidative stress-related transcription factors \(HIF1A\) and \(NFE2L2\) (Fig. 5A and C). Just like observed for \(SOD2/MnSOD\), however, protein levels of catalase and Nrf2 were not overtly affected in the 24 h time frame of tucum exposure. In contrast to effects on mRNA levels on antioxidant genes, tucum aqueous extract did not change mRNA levels of the inflammation marker \(NOS2\), encoding inducible nitric oxide synthase iNOS (Fig. 5B).

**Discussion**

In this study, we investigated the oxidative and cytotoxic effects of aqueous extract of tucum-do-cerrado in human colon cancer cells and the likely underlying mechanisms. We choose the Caco-2 cell line because: 1) colorectal cancer ranks 3rd in terms of cancer incidence, 2) these cells are a model of the intestinal epithelium, 3) the intestinal epithelium enters in contact with tucum components after ingestion of the fruit or its products (20). The \(IC_{50}\) for aqueous extract of tucum’s peel was 590\(\mu\)g/ml. Studies with plant extracts have reported reduction on viability in Caco-2 cells (21–23). For example, Caco-2 cells showed to be sensitive to a colored fraction of sweet cherry fruits and had an \(IC_{50}\) value of 667.84 ± 2.46\(\mu\)g/mL (21). Three concentrations of juice (10, 1 and 0.1 mg/mL) and 50% hydroethanolic extract (1, 0.1 and 0.01 mg/
Figure 3. Tucum extract exposure downregulates Cu/Zn SOD (SOD1) and MnSOD (SOD2) genes in Caco-2 cells. (A and B) Cu/Zn SOD and MnSOD gene expression (qPCR-RT) and (C) MnSOD protein expression (Western Blot). Caco-2 cells were exposed to aqueous extract of tucum-do-cerrado (0.5 and 1 mg/mL) for 24 h in DMEM medium without fetal bovine serum. Control represents cells exposed to medium alone. Results expressed in mean ± SEM. (D) Immunofluorescence analysis for mitochondrial MnSOD expression (green staining) on Caco-2 cells exposed for 24 h with aqueous extract of tucum-do-cerrado (0.5 and 1 mg/mL). Negative control are cells without secondary antibody. Positive control are Caco-2 cells exposed to 1 mM H$_2$O$_2$. Scale bar = 25 µm. The qPCR-RT experiments were performed with two biological replicates, each with $N=4$. The western blot was performed with one gel containing samples from two experiments performed in different days.
mL) from *Saposhnikovia divaricata* root statistically significantly reduced Caco-2 cells viability (22). Extracts from *Arthrocnemum indicum* shoot inhibited Caco-2 cell growth in a dose-dependent manner at the concentrations of 10 to 100 μg/mL (23).

Our results indicate that tucum extract (0.5 and 1 mg/ml), had a toxic effect on Caco-2 cells, although it did not showed the same effect on organoids (on tested concentrations of 0.25, 0.5 and 1 mg/ml) and fibroblasts (on tested concentrations of 0.025 up to 1.5 mg/ml), which may indicate a selective action toward cancer cells. Further analysis may be important to determine if this effect is also antiproliferative. Caco-2 cells exposed to a grape seed proanthocyanidins extract (10–100 µg/ml) had survival pathway inactivated and activation of apoptosis, while the viability of normal colon cells was not altered by the same treatments (24), in line with our results. Resistance to multiple drugs remains one of the challenges in the area of cancer treatment and, therefore, the search for potential sources of new anticancer drugs can help this endeavor. The selective effect of tucum extract on Caco-2 cells, when compared to organoids or fibroblast, is relevant when considering its anticancer potential. Changes in the composition and metabolism of tumor cells, when compared to normal cells, may be related to this selective effect. Regarding the fact that mitochondrial ROS production was higher at 0.5 mg/mL than at 1.0 mg/mL, we speculate that the extract may be exerting some antioxidant effect at the concentration of 1.0 mg/mL, but this remains to be investigated. This same effect has been observed in a study with eugenol, a flavoring agent used in cosmetic and food products, showing that it caused biphasic ROS production in human submandibular cell line treated with H₂O₂ and horse-radish peroxidase characterized by enhanced at lower eugenol concentrations (5–10 μM) and decreased at higher concentrations (500 μM) (25).

Fruits, vegetables and other plants with bioactive compounds, such as polyphenols, have been studied for their antiproliferative and potential anticancer effects (26–31). Polyphenols have been related to cancer chemoprevention and adaptive responses to stress, but they can also induce production of reactive oxygen species that can play a crucial role in cancer therapy through activation of apoptotic pathways (32). The results show that tucum aqueous extract induces mitochondrial ROS production in colorectal adenocarcinoma Caco-2 cells, but not in primary human intestinal epithelial cells (organoids) or primary human intestinal fibroblasts. This is accompanied by a reduction in viable cells after 24 h of tucum aqueous extract exposure. The increase in ROS production in Caco-2 cells exposed to tucum extract may be related to its higher copper and iron content. Colorectal cancer tissue from patients has been reported to have higher levels of copper and iron compared to normal tissue (33). Copper and iron are important catalysts in Fenton reactions, which generate the extremely reactive hydroxyl radical (34). Tucum aqueous extract contains gallic and caffeic acids (*Supplementary Table S2*), compounds that induce DNA damage in a copper-dependent reaction in human cells (35). It is reasonable to think that these polyphenols may be acting as pro-oxidants and causing DNA damage in Caco2-cells. This effect remains to be investigated.

The composition of the tucum peel aqueous extract has been described and is summarized in *Supplementary Table S2*. The total phenolics in the
tucum peel is 28,287.6 mg of gallic acid equivalent (GAE)/100 g fresh matter (16). This is a high value when compared with some common fruits such as apple (red delicious) 73.96 ± 3.52 mg GAE/100 g fruit (14). An study by Rosa et al. (16) identified major phenolic compounds of the tucum-do-cerrado (*Bactris setosa*) peel, as well as antioxidant activity and total phytochemical compound concentration of different extracts of the peel, including an aqueous extract. It will be relevant to repeat these analyses with the extract used in the present study. One characteristic of our extract is its purple color indicating the presence of anthocyanins. Tucum, as green tea (*Camellia sinensis*), has catechins, whose antioxidant mechanism of action is linked to a transition metal chelating property (36). However, tucum extract demonstrated a potent pro-oxidant effect in Caco-2 cells by increasing mitochondrial ROS production that was not observed in intestinal fibroblasts, thus indicating a cell type-specific action. The pro-oxidant effect of catechins is related to apoptosis induction in tumor cells, such as HL-60, HT-29 and RAW 264.7 due to interference with cellular signaling pathways or by increasing \( \text{H}_2\text{O}_2 \) and hydroxyl radical production under experimental conditions (36–39). ROS production is increased when *Rosa canina* is combined with a drug to treat colorectal cancer, but when Caco-2 cells treated with hydrogen peroxide are exposed to only *Rosa canina*’s extract, ROS production decreases, showing an antioxidant effect (40, 41).

Tucum extract reduced mRNA levels of the antioxidant enzymes SOD, GPx and catalase, but this reduction was not observed in protein levels of catalase and MnSOD. These results suggest that Caco-2 cells treated with aqueous tucum extract alone can develop a higher oxidative stress environment since

![Figure 5. HIF-1α, NOS2 and Nrf2 gene expression in Caco-2 cells exposed to tucum-do-cerrado extract. (A, B and C) HIF-1α, NOS2 and Nrf2 gene expression (qPCR-RT), respectively. (D) Nrf2 protein expression. Caco-2 cells were exposed to aqueous extract of tucum-do-cerrado (0.5 and 1 mg/mL) for 24 h in DMEM medium without fetal bovine serum. Control represents cells exposed to medium alone. Results expressed in mean ± SEM. The qPCR-RT experiments were performed with two biological replicates, each with \( N = 4 \). The western blot was performed with one gel containing samples from two experiments performed in different days.](image)
they produce more mitochondrial ROS whilst their antioxidant defense is not being strengthened. Aqueous extract of *Barringtonia racemosa* leaf on Caco-2 cells increased catalase and GPx activity, however ROS production was increased (42). Similar results were found with grape seed extract which increased SOD and GPx mRNA levels (43). Plant extracts such as sage, oregano, rosemary and echinacea rich in polyphenols, likewise tucum extract, do not induce a significant increase in antioxidant enzymes when exposed to H₂O₂ (44). A diet rich in fruits and vegetables is highly important to prevent chronic diseases such as diabetes and obesity, thence effective on preventing inflammatory environment and an increase on oxidative status. Although it was not observed in this study a significant effect of tucum extract on antioxidant response, the fruit is rich in bioactive compounds which may be important to maintain a healthy diet.

The transcription factor Nrf2 (*Nuclear factor E2-related factor 2*) is involved in the antioxidant response (45–48). Tucum extract decreased mRNA, but not protein levels of Nrf2. While the difference between the mRNA and protein levels seems striking, it is not an unusual observation. For example, an analysis of matrix metalloproteinases and the tissue inhibitor of metalloproteinases 1 in prostate cancer cells showed no correlation between their levels of mRNA and proteins (45). Protein and mRNA levels within the same lung adenocarcinoma show significant correlation only in a subset of the proteins (49). Down regulation of Nrf2 is associated with cancer cells death as shown in lung cancer cells A549 in which up to 200 µM catechins from green tea suppressed this transcriptional factor and led to a lower antioxidative response and activated apoptosis (50).

Hypoxia-Inducible Factor-1 is a key transcription factor that induces angiogenesis and is important in mammalian cells survival under low oxygen concentrations (51,52). It is composed of two subunits, HIF-1α and HIF-1β, and HIF-1α is regulated in an oxygen dependent manner. Under normoxia, ROS are able to indirectly regulate HIF-1α by inhibiting proline hydroxylation, the event that triggers its degradation pathway (53). Our findings showed that tucum extract caused a decrease in HIF-1α gene expression in a dose-dependent way in Caco-2 cells, while superoxide radical increased under the same conditions. Superoxide radical is known to increase HIF-1α levels under hypoxia and H₂O₂ is able to stabilize it under normoxia (54,55).

Peroxynitrite (ONOO⁻) is formed by the reaction between nitric oxide (•NO) and the superoxide radical and is a potent oxidizer that can attack various biological targets (56). We then investigated whether tucum-do-cerrado has an effect on the gene expression of the inducible enzyme that catalyzes the synthesis of nitric oxide. The gene expression of NOS2 remained unchanged when the Caco-2 cells were exposed to the tucum-do-cerrado extract, indicating that the extract has no molecules with effect on the gene expression of this enzyme. Therefore, tucum might be responsible for increasing ROS production and suppresses antioxidant response while not affecting the inflammation pathway.

For two investigated antioxidant enzymes, MnSOD and CAT, and also for the transcription factor Nrf2, a decrease in mRNA levels, caused by tucum exposure, did not correlate with a decrease in protein levels. These data are interesting with regard to the importance of these proteins in colorectal cancer because it indicates that they are differently influenced at mRNA and protein levels and that post-transcriptional regulation of gene expression is a mechanism to maintain their function in Caco-2 cells. While the results seem promising, these are only primary experiments and, therefore, more cell lines should be investigated to conclude about tucum anticancer activity.

**Conclusion**

In conclusion, tucum-do-cerrado extract probably contains molecules with antioxidant action, and others with pro-oxidant action. The results of our study reflect the total sum of these opposite actions in Caco-2 cells and indicate that there is a prevalence of pro-oxidant action related to a decrease in cell viability. Our findings show the antiproliferative activity of tucum-do-cerrado extract on colon cancer cells possibly through ROS production. This observation is relevant from the point of view of searching for compounds that reduce tumor cell growth. We hope that the results described here pave the way to further studies on tucum-do-cerrado as a potential source of anticancer compounds.

**Author Contributions**

Study concept and design: Élida Geralda Campos, Renata Cristina da Silva and Klaas Nico Faber; acquisition of data: Renata Cristina da Silva and Raphael Fagundes; analysis and interpretation of data: Renata Cristina da Silva and Élida Geralda Campos; drafting of the manuscript: Renata Cristina da Silva, Raphael Fagundes, Élida Geralda Campos and Klaas Nico Faber; statistical analysis: Renata Cristina da Silva; obtained funding: Renata Cristina da Silva;
contributed reagents/materials/analysis tools: Klaas Nico Faber; study supervision: Klaas Nico Faber and Elida Geralda Campos. Authors that contributed equally to this work.

**Declaration of Interest Statement**

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials described in this manuscript.

**Funding**

Renata Cristina da Silva was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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