The α-Tomatine Exhibits Antiproliferative Activity, Rupture of Cell Membranes and Induces the Expression of APC Gene in the Human Colorectal Adenocarcinoma Cell Line (Ht-29)

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HIGHLIGHTS

• The α-tomatine was cytotoxic at the 10 µg/mL concentration.
• The α-tomatine inhibits cell proliferation after the first 24 hours of treatment.
• The α-tomatine does not induce apoptosis up to the concentration of 10 µg/mL.
• The α-tomatine induces increased APC gene expression.

Abstract: The α-tomatine is a steroidal glycoalkaloid found in immature tomatoes (Lycopersicon esculentum) that has important biological functions including the inhibition of cancer cell growth and preventing metastasis. This study aimed to evaluate the effects of α-tomatine on cytotoxicity, cellular proliferation, apoptosis, and mRNA expression of APC, CCNA2, β-catenin, CASP9, BAK, BAX and BCL-XL in colorectal adenocarcinoma cell line HT-29. HT29 cells were treated with three concentrations of α-tomatine (0.1, 1 and 10 µg/mL), although only the 1 µg/mL concentration of α-tomatine was used to evaluate genetic expression patterns by real time-PCR. Results showed that α-tomatine was cytotoxic only at the 10 µg/mL concentration. Cell proliferation was significantly inhibited after the first 24 hours of treatment only with concentrations of 10 µg/mL. In contrast, there were no significant differences in apoptosis for any treatment. In the gene expression studies, only APC expression was significantly altered by α-tomatine treatment. In conclusion, α-
tomatine has antiproliferative activity in the first 24h of treatment, does not induce apoptosis in this cell line and causes disruption of cell membranes, thereby increasing the expression of APC gene related to cell cycle.

**Keywords:** alpha-tomatine; gene expression; cytotoxicity; cell cycle; DNA damage.

**INTRODUCTION**

Cancer is one of the most important public health issues around the world and many studies have been given attention to its development, control and treatment strategies. In 2018, 9.5 million of people worldwide died from cancer and the colorectal cancer is among the top fourth types of cancer killing men and women. Even though, 30-50% of cancers could be prevented by healthy lifestyle choices such as avoiding tobacco products, reducing alcohol consumption, maintaining a healthy body weight and exercising regularly. Others can be detected early, treated and also cured [1].

Nutrition is an important factor in an individual's development from infancy to adulthood and likely affects vulnerability to chronic diseases, including cancer. It is estimated that diet affects 30 to 40% of all cancer cases. Bioactive components of foods can simultaneously alter more than one cancer process, including various events such as carcinogen metabolism, hormonal balance, cell signaling, cell cycle control, apoptosis and angiogenesis. These bioactive components found in food include essential nutrients (calcium, zinc, selenium, folate, and vitamins C, D and E) as well as non-essential components (carotenoids, flavonoids, indoles, conjugated linoleic acids, and omega-3 fatty acids) [2-4].

Glycoalkaloids are composed of six steroid rings (aglycone) with a sugar molecule attached to position three of the first ring and a nitrogen atom at the end of the sixth ring. Among the best-known glycoalkaloids are α-solanine and α-chaconine in potatoes and α-tomatine in tomatoes. The glycoalkaloid found in tomatoes is the steroidal glycoalkaloid α-tomatine, which consists of a branched tetrasaccharide, β-D-glucopyranosyl-(1→2)-[β-D-xilopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-D-galactose, linked to O-3 of the steroidal aglycone, tomatidine. This glycoalkaloid is primarily found in immature tomatoes, which have an average of 4-7 mg α-tomatine per 100 g of fresh fruit that is partially degraded as the fruit ripens. The α-tomatine content in mature tomatoes varies from 0.03-0.06 mg α-tomatine/100 g of fresh fruit [5-9]. α-tomatine possesses an important antifungal, antibacterial, antiviral, antimitastatic, cell membrane lysis, LDL cholesterol reducing, immunostimulatory, apoptosis and, most importantly, antiproliferative activity [7,10-19].

Apoptosis is a component of many processes including tissue maintenance and response to xenobiotic agents and endogenous changes such as inflammation and blood supply disruption, but also has an important role in the cancer development, prevention and therapy [20]. The balance between cell proliferation and apoptosis is fundamentally important for normal cellular function. Perturbation of this balance is the primary cause of tumors [21].

Due to the known beneficial effects of α-tomatine, the aim of this study was to evaluate its antiproliferative and apoptotic effects as well as changes in expression of cell proliferation related (APC, β-catenin, CCNA2) and apoptosis-related (BAK, BAX, BCL-XL, and Caspase-9) genes in intestinal adenocarcinoma epithelial cell cultures treated with α-tomatine.

**MATERIAL AND METHODS**

**Cell lines and culture conditions**

The human colorectal adenocarcinoma cell line HT-29 was obtained from the Rio de Janeiro Cell Bank. The cells were cultured in 25 cm² cell culture flasks with DMEM (Dulbecco’s Modified Eagle Medium (Gibco)) culture media supplemented with 10% Fetal Bovine Serum (Gibco) in a humidified incubator with CO2 (5%) at 37 °C.

**Carcinogenic Agents**

The carcinogenic agent Doxorubicin (CAS: 23214928: Rubex®), dissolved in Dimethyl Sulfoxide (DMSO), was used to cause DNA damage. The concentration of Doxorubicin used for the treatment of HT29 cells was 2 µg/mL, as determined previously in pilot experiments.
α-tomatine

A synthetic form of α-tomatine (CAS 17406-45-0) was obtained from Sigma Chemical Co., St. Louis, MO, dissolved in Dimethyl Sulfoxide (DMSO), and used at three concentrations (0.1, 1, or 10 µg/mL) for treatment in each experiment, as previously determined in pilot experiments.

Cytotoxicity Test (MTT)

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-tetrazolium-bromide] cytotoxicity test was performed following the protocol described by Mosmann [22]. HT29 cells were seeded in a 96-well cell culture plate to a density of 2.0 x 10⁴ cells/well and exposed to treatment for 24 h with different concentrations of α-tomatine (0.1, 1 and 10 µg/mL), doxorubicin (2 µg/mL) or with PBS and DMSO as vehicle controls. At the end of the 24 h period, the treatments were removed and the cells were washed with PBS. Next, the cells were incubated at 37 °C for 4 h with MTT (CAS 298-93-1; Sigma Aldrich; 0.005 g MTT; 5 mL PBS; 10 mL culture media without serum). After this stage, the culture media was removed, DMSO was added and readings were made in an Elisa reader with a 550 nm filter. The experiment was performed in triplicate.

Kinetic Analysis of Cell Proliferation

For this test, 2.0 x 10⁵ cells were seeded into culture flasks. The cells were treated with a DNA-damage promoting agent (2 µg/mL Doxorubicin), control (1% DMSO), or α-tomatine (0.1, 1 or 10 µg/mL). This procedure was performed for 24, 48, 72, or 96 h, after which the cells were collected and counted in a Neubauer chamber. Following analysis, a proliferation curve was generated. These experiments were performed in triplicate.

Cell viability analysis by the Trypan Blue Exclusion technique

After collection of the cells in culture flasks, 20 µl of cell suspension was collected and combined with 20 µl of Trypan Blue. The mixture was pipetted into a Neubauer chamber and analyzed under a microscope to obtain the percentage of viable cells by finding the ratio of living to dead cells. The study was performed in triplicate concurrent to the cell proliferation kinetics analysis.

In situ test for detection of apoptosis

Identification of apoptotic cells was performed by analyzing the DNA fragmentation pattern of nuclear DNA after staining with acridine orange. Cell treatments with the three tested concentrations (0.1, 1 and 2 µg/mL) were performed after culture stabilization (24 h). The concentration of 2 µg/mL was biologically accepted after further testing with lower concentrations, since the concentration of 10 µg/mL was considered lethal for this test. The collection was performed after 24h of treatment [23].

After washing with PBS, the cover slip was removed from the culture tube and fixed in Carnoy fixative for 5 min, quickly dipped in plates containing decreasing concentrations of ethanol (95% to 25%), followed by a wash in McIlvaine’s buffer for 5 min., staining with orange acridine (0.01%, 5 min) and another wash in the buffer.

Three repetitions were performed and 1,500 cells were analyzed per treatment with a fluorescent microscope (420-490 nm excitation filter and a 520 nm barrier filter).

Real-time PCR

Cells were seeded in 25 cm² culture flasks to a density of 2.0 x 10⁵ cells/flask and incubated to allow cell growth for 24 h. They were then treated for twelve hours in the following experimental groups: 1 - negative control and 2 - α-tomatine (1 µg/mL).

Next, total RNA was extracted with Trizol LS (Invitrogen®) following the manufacturer’s instructions. Quantification and integrity analysis of the RNA was then performed by electrophoresis on a 0.8% agarose gel. cDNA synthesis was performed by reverse transcription using an oligo (dT) primer (10 pmol/µL Prodimol®) and reverse transcriptase (M-MLV reverse transcriptase – Invitrogen) following the manufacturer’s instructions.

The cDNA was stored at -80 °C. The cDNA samples were quantified using a spectrophotometer (260 nm and 280 nm). One microliter of the sample was diluted in 99 mL of TE buffer for measurement (0.01 M TrishCl, 1 mM EDTA, pH = 8.0). Following quantification, triplicate samples were mixed in one tube and then redistributed into three new microtubes and stored at -80 °C until real-time PCR was performed.
Real-time reactions were set up in a 96-well propylene plate. The PCR program was performed according to the following protocol: preheating at 50 °C for 1 min, cDNA denaturation at 95 °C for 3 min followed by 35 cycles consisting of 95 °C for 20 seconds, annealing of primers at 60 °C for 30 seconds and extension at 72 °C for 20 seconds. The dissociation curves were created as follows: 95 °C for 15 seconds, 60 °C for 20 seconds and 95 °C for 15 seconds. Melting curve analysis was performed at the end of the reaction with temperatures ranging from 50 °C to 95 °C at 0.5 °C intervals for 5 seconds.

The treatments were evaluated in duplicate and the reactions were performed in a PTC 200 DNA Engine Cycler thermocycler using the detection system Chromo4 (MJ Research). Primer sets (APC, CCNA2, Catenin, CASP9, BAK, BAX and BCL-XL) and normalization gene primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified as described in Table 1.

| Gene | Primers |
|------|---------|
| GAPDH | F: 5' GAAGGTGAAGGTCGGAGTC 3'  
R: 5' GGAAGATGGTGATGGGATT 3' |
| APC | F: 5' AAAGCGCCATGATATTGCACGGCT 3'  
R: 5' TGTTTGCTGTGCTCACGTTTCCAG 3' |
| CAT | F: 5' CCTATGCAGGGGTGGTCAAC 3'  
R: 5' CGACCTGGAAAACGCCATCA 3' |
| BAK | F: 5' CAAGATTGCCACCAGCTGTGA3'  
R: 5' ATGCGAGTGATGCAGCATGAATCG 3' |
| BAX | F: 5' TTCTGACGGCACTTCAACTGGG 3'  
R: 5' TGTCAGCCCATGCTCTGTGAT 3' |
| BCL-XL | F: 5' TGGGCTCACTCCTCAGTGAAAT 3'  
R: 5' ATGTAAGTGGTCTCTGTTGCAAT 3' |
| CASP9 | F: 5' GCTTTCTTTGCTCATTCC 3'  
R: 5' GTTTCTAGGTTGGCTTCCG 3' |
| CCNA2 | F: 5' GACCCCTGATTTGGCCTTG 3'  
R: 5' ACAACTCTGCTACTTCTGG 3' |

Statistical Analysis

Statistical analysis of the MTT test was performed by analysis of variance (ANOVA) followed by the Tukey test (α<0.05) using the Graphpad Instat program. Data obtained from the cellular proliferation kinetics, cell viability, and induction of apoptosis tests were statistically analyzed by analysis of variance (ANOVA) followed by the Dunnet test (α<0.05) using the Graphpad Instat program. Gene expression levels of the genes in this study were analyzed using Rest Software to estimate the calculation based on the delta Ct method [24,25].

RESULTS

MTT Test

The viability values (mean ± standard deviation) obtained from the MTT test after treatment with α-tomatine at various concentrations (0.1, 1 and 10 µg/mL) in HT29 cells for 24 h are shown in Figure 1. The results from the α-tomatine treatments using 0.1 µg/mL were not significantly different from the control. However, there was a significant difference compared to the control at the 1 µg/mL concentration. There was cell death when cells were treated with 10 µg/mL, indicating that α-tomatine is cytotoxic at this concentration. Therefore, increased concentrations of α-tomatine result in inhibitory effects on HT29 cells.
α-Tomatine is Antiproliferative

Cell proliferation study

The cell proliferation test was performed at 24, 48, 72, and 96 h, and the scores (mean ± standard deviation) are presented in Figure 2 and shows the kinetics of cell proliferation for each treatment at each tested time point. There was a significant difference at 24 h between treatment with α-tomatine at 0.1, 1, and 10 µg/mL and control using Doxorubicin. At the highest tested concentration (10 µg/mL), there was no increase in cell proliferation rate in the first 24 and 48 h of treatment. After 72 h of treatment, a decrease in cell proliferation was observed in cells treated with Doxorubicin when compared to controls. At 0.1 and 1 µg/mL of α-tomatine, there was an increase in cell proliferation without a statistically significant difference compared to the control. In the 96-h treatment, there was a significant difference in the rate of proliferation between control and treated cells with the largest dose of α-tomatine and Doxorubicin. At the 0.1 and 1 µg/mL concentrations, there was an increase in cell proliferation, but this was not significantly different from the control.
Cell viability

The viability of cells treated with 0.1 µg/mL or 1 µg/mL of α-tomatine was greater than 80% at all time points. However, the cells showed 0% viability in all treatment time points at 10 µg/mL. The cells treated with Doxorubicin had less than 80% cell viability only after 96 h. These data are shown in Table 2.

Table 2. Means of percentage of cell viability and standard deviation of HT29 cells treated with three different concentrations of α-tomatine at 24, 48, 72 and 96 h.

| Treatment          | Treatment time |
|--------------------|----------------|
|                    | 24 h           | 48 h           | 72 h           | 96 h           |
| Control            | 96.75±0.99     | 97.59±2.85     | 90.89±14.41    | 98.00±2.83     |
| α-tomatine 0.1 µg/mL | 99.20±1.38     | 97.11±2.67     | 100.00±0.00    | 100.00±0.00    |
| α-tomatine 1 µg/mL  | 100.00±0.00    | 97.13±3.43     | 98.43±2.21     | 97.56±0.00     |
| α-tomatine 10 µg/mL | 00.00±0.00*    | 00.00±0.00*    | 00.00±0.00*    | 00.00±0.00*    |
| Doxorubicin        | 88.53±4.68*    | 97.22±4.81     | 93.33±11.54    | 66.66±57.73    |

Control – 0.1% DMSO; Doxorubicin – 2µg/mL. * = P ≤ 0.05 - ANOVA followed by Dunnett post-test.

Apoptosis

The percentage of viable and apoptotic cells analyzed is shown in Figure 3. No change in the apoptosis rate was observed in treatments of α-tomatine (0.1, 1, and 2 µg/mL) compared to controls. Only Doxorubicin showed a significant increase compared to the control.

Figure 3. Average frequency of normal and apoptotic cells observed in HT29 cell lineage treated with α-tomatine at 0.1, 1 and 2 µg/mL. Control – 0.1% DMSO; Doxorubicin – 2µg/mL. 500 cells were analyzed per slide under the fluorescent microscope at a magnification of 400x. The cells were classified by morphological appearance and differential staining by orange acridine and ethidium bromide. Necrotic cells were not found. **p<0.01; significant difference compared to control (ANOVA/Dunnet).

Real-time PCR (RT-PCR)

APC gene expression

HT-29 cells under treatment with α-tomatine (1 µg/mL) resulted in a 2.26-fold significant increase in APC gene relative expression (p<0.05). The other evaluated genes showed small variations (CAT - 1.64-fold increase, BAK - 1.71-fold increase, BAX - 1.11-fold increase, BCL-XL - 0.94-fold decrease, CASP9 - 1.42-fold increase and CCNA2 - 0.90-fold decrease) that were not statistically significant (p>0.05), results presented in Figure 4.
α-Tomatine is Antiproliferative

Glycoalkaloids are compounds consisting of a nitrogen-containing steroidal aglycone. α-tomatine, one of the main glycoalkaloids in tomato (*Lycopersicon esculentum*), has been reported to exert many beneficial effects, including biological, nutritional and pharmacological properties. Among the main activities of α-tomatine, its immunological adjuvant action, antifungal and antibacterial activity, anticholesteremic effects, steroid hormone precursor status, anticancer potential, antimetastatic activity and cytostatic effect in various cancer cell lines are significant [11-14,19,26-29].

However, even though α-tomatine shows great potential to lyse cell membranes, this glycoalkaloid was not toxic by oral consumption in rats [10]. This can be explained by its weak absorption in the digestive tract into the bloodstream, and the formation of an insoluble complex between α-tomatine and endogenous cholesterol, which is quickly eliminated in the stool. Roddick [30] demonstrated that both a mix of potato steroid glycoalkaloids and α-tomatine have the capacity to form complexes with steroids such as cholesterol, sitosterol, stigmasterol, campesterol, and ergosterol.

In this study, the cytotoxicity of α-tomatine was evaluated at three different concentrations (0.1, 1, and 10 µg/mL) in HT29 cells by the MTT test after 24 h of treatment. We observed that use of the DNA damage-inducing agent, Doxorubicin, led to cytotoxicity. The results, presented in Figure 2, show that α-tomatine treatment at a concentration of 0.1 µg/mL was not cytotoxic. However, treatments at 1 and 10 µg/mL resulted in statistically significant differences in toxicity when compared to controls. In contrast, cell proliferation assays showed that while the first 24 hours of treatment had an inhibitory effect on cell proliferation for the treatments with 0.1 and 1 µg/mL, the time points at 48, 72 and 96 h had increased cell proliferation, corroborating the cell viability results. At a concentration of 10 µg/mL, the seeded cells died and did not proliferate throughout the entire treatment period.

In the cell viability test, performed in conjunction with the test mentioned above, the results showed that more than 80% of the cells were alive for the concentrations of 0.1 and 1 µg/mL, confirming that there was no cell death but an inhibition of cell proliferation. In contrast, the cells were not viable after 24 h of treatment at a concentration of 10 µg/mL.

Lee, Kozukue [12] performed a study to evaluate the antiproliferative activity of the glycoalkaloids α-chaconine, α-solanine and α-tomatine on various cell lines at three different time points (4, 24 and 48 h). α-tomatine was tested at five different concentrations (0.1, 0.5, 1, 5, and 10 µg/mL) and the authors observed that α-tomatine inhibited between 60.6% and 86.9% of the cell proliferation of HT29 cells at 24 h of treatment. However, contrary to the study presented here, the authors observed between 65.6% and 86.5% inhibition of HepG2 cell proliferation at the 48 h time point, and with the HT29 cell line, the same used in this study, the authors observed a 38% inhibition of cell growth at a concentration of 0.1 µg/mL, 59.9% at a concentration of 1 µg/mL and 81.5% at a concentration of 10 µg/mL after 4 h of α-tomatine treatment.

In another study by Friedman, Levin [16], α-tomatine inhibited proliferation of mammary (MCF-7), colorectal (HT-29), gastric adenocarcinoma (AGS), and hepatoma (HepG2) cancer cell lines and was more effective than tomatidine, tomatidenol, and dehydrotomatine, even though α-tomatine is structurally similar to dehydrotomatine.
A study with lung adenocarcinoma cell line A549 showed that α-tomatine potentially inhibits the metastatic ability of these cells either by reducing the activity of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and urokinase type plasminogen activator (u-PA), suppressing phosphoinositol kinase/Akt (PI3K/Akt) or inhibiting NF-κB or AP1 through ERK1/2 signaling [17].

For a better understanding of the chemopreventive mechanisms of α-tomatine it is important to consider that several molecular events are responsible, including complex formation with cholesterol and direct destruction of tumor cells by cell membrane lysis [7,10].

In order to elucidate the effects of α-tomatine on cell cycle, we investigated the molecule’s functional mechanism by studying the relative expression levels of APC (Adenomatous polyposis coli), β-catenin and CCNA2. Somatic mutations are found in the majority of colorectal tumors, and the APC gene is frequently expressed in advanced colon cancer and has multiple cellular functions such as involvement in the WNT signaling pathway and intracellular adhesion. The APC protein is involved in cell cycle (acting as a negative regulator of β-catenin levels), in the transformation of colon epithelial cells, and in the progression of melanoma. The WNT signaling pathway regulates a complex consisting of axin, APC, β-catenin, and glycogen synthase kinase-3β (GSK3β). Axin forms a complex with APC, β-catenin and GSK3β to promote the phosphorylation of β-catenin and subsequent binding to β-TRCP which mediates the ubiquitination and degradation of β-catenin in the proteasome. GSK3β regulates this process by phosphorylating β-catenin, APC and the axin complex. The activation of the WNT signaling pathway inhibits GSK3β to stabilize β-catenin [31-35].

On the other hand, APC has also been reported to be involved in cytoskeletal integrity, intracellular adhesion and cell migration. Disruption of the integrity of the actin cytoskeleton could lead to perturbations in cellular junctions and cell migration. The role of the APC protein in actin cytoskeleton maintenance is related to its interaction with β-catenin, which creates a connection between APC and actin [36-38].

Narayan and Roy [33] have reported that the C-terminal domain of E-cadherin interacts with β and γ-catenin, which associates with α-catenin and forms a complex binding E-cadherin to actin in the cytoskeleton, maintaining stability in the intercellular adhesions [32]. If WNT pathway and epidermal growth factor receptor (EGFR) or c-Met pathways are activated at the same time, the degradation of β-catenin can be inhibited and translocated into the nucleus. This way, these interactions can result in a reduction of E-cadherin that mediates intercellular junctions and cell migration. The translocation of β-catenin to the nucleus occurs naturally, through external signals that promote proliferation. However, mutations in APC result in a loss of β-catenin regulation, leading to an exacerbated increase in its cytoplasmic concentration and increased translocation to the nucleus. In the majority of intestinal colon cancers, as in the case of HT29, APC is mutated rendering the protein product of this gene not functional, which causes a loss of cell proliferation regulation [39,40].

Based on our results, we observed that there was an increase in the expression of the APC gene, similar to the β-catenin gene, in cultures treated with α-tomatine (Figure 5). It is assumed that the role of glycoalkaloids may be involved in the disruption of cell membranes, causing changes in some genes’ expression involved in cell cycle leading to a decrease in the number of cells during this period of treatment (12 h). Thus, inhibition of cell proliferation in the first 24 hours corroborates the gene expression results. In addition, the small (non-significant) increase observed in β-catenin gene expression could be a result of an increase in demand due to increased APC expression.

Wang, Song [41] suggested that the deregulation of CCNA2 expression is closely linked to the early events of tumor transformation, and its suppression is considered typical in various types of human cancers when compared to normal tissues. However, our results show that α-tomatine does not statistically interfere with CCNA2 expression. As part of our goals, we also analyzed apoptosis by morphological analysis under a fluorescent microscope and by expression analysis of the BAK, BAX, BCL-XL and Caspase-9 genes. The experimental results obtained in this study demonstrate that there was not an induction of apoptosis, either by physiological changes found in apoptotic cells such as chromatin condensation, formation of apoptotic bodies, fragmentation of nuclear DNA, or by increased expression levels of the analyzed genes.

This research helps in the understanding of nutrigenomics and opens new perspectives that will allow better insights on the mechanisms of action of α-tomatine.
CONCLUSIONS

Based on our results, it is suggested that α-tomatine has antiproliferative activity in the first 24 h of treatment does not induce apoptosis in human colorectal adenocarcinoma cell line (HT-29) and causes disruption of cell membranes, thereby increasing the expression of APC gene related to cell cycle.

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