

Phytochemicals Antitumor activity of an aqueous from *Vaccinium meridionale* in a three-dimensional colon adenocarcinoma model

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Research Article

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Abstract

Vaccinium meridionale is an Andean Berry with proven antioxidant and anti-carcinogenic activities in two-dimensional models of colon cancer cells. In the present study, an aqueous extract from Andean berry induces apoptosis in a three-dimensional cell model of colon adenocarcinoma. The aqueous extract's total phenol and anthocyanin contents and antioxidant activity were determined; its Half-maximal inhibitory concentration (IC₅₀) value was obtained; and its effect on the morphology, cell cycle, apoptosis, and senescence of SW480 cell spheroids was evaluated. The most quantitatively significant phenolic compounds were total tannins and total anthocyanin. The ORAC value was the most active mechanism, followed by ABTS and DPPH values. The IC₅₀ (33.62 ± 1.3 mg/mL and 5.91 ± 1.0 mg/mL) was obtained at 24 and 48 hours, respectively. The extract decreased the size of spheroids, induced accumulation of hypodiploid cells in the SubG1 phase, reduced the percentage of senescent cells, and increased annexin-V positive cells involving caspases activation compared to non-treated spheroids. These results showed that *V. meridionale* aqueous extract exerts its antitumoral effect on colon adenocarcinoma spheroid cells by blocking the cell cycle, inducing apoptosis, and inhibiting senescence under the same treatment conditions.

Introduction

According to the World Health Organization, colon cancer is one of the most frequent diseases worldwide [1]. The consumption of fruits and vegetables is considered a protective factor because bioactive compounds can induce antioxidant mechanisms, arrest the cell cycle, and promote cell death among others [2, 3].

Berries of the *Vaccinium* genus, such as Andean Berry (*Vaccinium meridionale*), are a source of bioactive compounds like phenolic acids, flavonoids, flavonols, and anthocyanins [4]. Chlorogenic acid and cyanidin-3-galactoside have been reported in Andean Berry, which have antioxidant activity [4,5], as well as their antiproliferative capacity on colon adenocarcinoma cells SW480 and its derived metastatic line SW620 [6-8]. The antiproliferative activity of Andean Berry on colon cancer cells has been studied in monolayer (2D) models [6-9] that do not represent the microenvironment of a solid tumor, consequently, the effective doses determined may have little or no efficacy *in vivo* [10]. To counteract the disadvantages of the 2D model, three-dimensional (3D) cell cultures have been developed, including spheroids that can be obtained from cancer cells of established cell lines or disaggregated fragments of tumors distributed in several layers. In the outer layer, it is possible to find cells with the highest proliferation rate; in the middle layer, the senescent cells; and in the inner layer, the necrotic cells [11]. In addition, spheroids do not require support for adhesion or a scaffold base due to the creation of their cellular matrix that is formed as the cells attach, mimicking the environment of the tissue of origin, and thus preserving their cellular phenotype [11]. Therefore, this study aimed to evaluate the antitumor activity of an aqueous extract from Andean Berry (AEM) in a three-dimensional cell model of colon adenocarcinoma from the SW480 cell line.

Materials and methods

2.1 Plant material and preparation of the aqueous extract

Ripe berries were collected in the municipality of El Retiro, Antioquia, Colombia, at an altitude of 2,175 MASL and an average temperature of 16 °C. This material was identified in the Herbarium of the Universidad de Antioquia (National Register of Biological Collections # 027). The berries were processed for 2 min at 2500 rpm, and the pulp obtained was freeze-dried in a vacuum chamber at a pressure of 0.427 ± 0.5 mm Hg and a temperature of 50 °C. The freeze-dried powder was stored at room temperature (RT) and protected from light. A stock dilution of AEM was prepared with 1000 mg of lyophilized berry pulp in 1 mL of distilled water.

2.2 Determination of phytochemical compounds

The total phenol content was analyzed by the Folin-Ciocalteu colorimetric method [31]. The results were expressed as mg gallic acid/100 g of extract. A standard curve was constructed using gallic acid as the acid standard. Optical Density (OD) was determined at 760 nm.

The total tannin content was determined according to Deshpande's vanillin assay [32], 10 mL of absolute methanol were added to 500 mg of lyophilized extract and incubated for 24 h. The mixture was shaken and centrifuged for 10 min at 3000 rpm, 1 mL aliquot of the supernatant was taken and 5 mL of freshly prepared vanillin reagent (1% vanillin in methanol and 8% HCl in methanol in 1:1 ratio, v/v) were added. A blank was prepared with 1 mL of the sample extract plus 5 mL of 4% HCl in methanol. The reaction was carried out at 30 °C for 20 min and OD was obtained at 500 nm. The concentration of condensed total tannins was calculated using a calibration curve performed with (+)-catechin (0.02-0.20 mg/mL) dissolved in methanol. Condensed total tannins were expressed as grams of (+)-catechin equivalents per 100 g of dry extract.

Total anthocyanin was determined by the pH differential method [33]. The OD or absorbance (A) was measured at 530 nm and 700 nm in buffers of pH 1.0 and 4.5 and calculated with the following equation: $A = [(A_{530} - A_{700})_{pH1.0} - (A_{530} - A_{700})_{pH4.5}]$. A calibration curve with cyanidin-3-glucoside was used and results were expressed as milligram equivalents of cyanidin-3-glucoside per 100 g of dry extract.

2.3 Determination of Antioxidant Capacity

The antioxidant capacity of aqueous extract was analyzed by different methods: ORAC, ABTS•+, and DPPH. To determine the ORAC value, a solution was prepared with 21 µL 10 µM fluorescein, 2899 µL of 75 mM phosphate buffer (pH 7.4), 50 µL of 600 mM AAPH, and 30 µL of extract. Fluorescence was measured in a spectrofluorometer. ORAC values were calculated by a calibration curve using Trolox as standard and expressed as µmol Trolox/100g [34].

The ABTS assay 10 µL of extract and 990 µL of ABTS•+ stock solution was mixed and the resulting solution was incubated at RT for 30 min in darkness, subsequently, the absorbance was measured at

734 nm. Trolox was used as a standard for the calibration curve and the results were expressed as μmol Trolox/100 g dry extract.

The method of Brand-Williams and co-workers [35] was used to perform the DPPH assay, a stable free radical that shows a purple coloration in a methanolic medium and loses its color when it receives an electron or a proton from an antioxidant. Ten mL of the extract and 990 mL of methanolic DPPH solution were analyzed at 490 nm in darkness after 30 min of incubation at RT. The same amount of DPPH and 10 mL of the sample solvent were used as reference. The calibration curve was constructed using Trolox as standard. The results were expressed as Trolox equivalents (TEAC)/100 g of dry extract.

2.4 SW480 cell culture

Human colon adenocarcinoma SW480 cells were obtained from the European Animal Cell Culture Collection (ECACC, Salisbury, UK). Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich), Fetal Bovine Serum (FBS, Sigma-Aldrich), 100 IU/mL penicillin (Gibco), 100 $\mu\text{g/mL}$ streptomycin (Gibco), non-essential amino acids (Gibco), and 1X Trypsin-EDTA (Gibco) were used to prepare the culture medium. Phosphate buffered saline (PBS, Fuller Laboratories) at pH 7.5 and Insulin-Transferrin-Selenium Solution (ITS, Gibco) containing 10 $\mu\text{g/mL}$ Insulin, 5 $\mu\text{g/mL}$ Transferrin, and 5 ng/mL Selenium were used. SW480 cells were maintained in a growth medium (DMEM supplemented with 25mM glucose and 2mM L-glutamine, 10% SFB, 1% penicillin/streptomycin solution, and 1% amino acids-nonessential) at 37 °C and 5% CO₂. After cells reached 90% confluence, they were detached with 1X trypsin-EDTA solution to seed the spheroids with a viability of over 90%.

2.5 Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol 3-(4,5-dimethylthiazole-Protein Primers 2-yl)-2,5-diphenyltetrazolium bromide) assay was performed using the MTT Cell Growth kit following the manufacturer's instructions. Briefly, SW480 cells were seeded at a density of 20,000 cells/well in 96-well plates for 24h. Then, cells were treated with different concentrations of AEM (25, 50, 75, 100, 125, and 150 mg/mL) for 24 and 48 h; the control group was untreated cells. Subsequently, 20 μL of MTT was added to each well and incubated for 4 h at 37 °C and 5% CO₂; the medium was discarded and 200 μL /well of DMSO was added to dissolve formazan crystals and OD at 570 nm in a spectrophotometer (Thermo Fisher Scientific). All assays were performed in triplicate. The IC₅₀ was calculated by linear regression analysis using the Probit method.

2.6 Three-dimension cell model of colon adenocarcinoma

Spheroids were obtained from a monolayer culture of SW480 cells following the Casting, Equilibrating, and Seeding the 3D Petri Dish® protocol. Microgels were prepared with 20 mg/mL ultrapure agarose and a sterile NaCl solution (0.9% w/v), 340 μL of agarose solution were deposited in the micro mold and allowed to cool for 1 min at RT. The microgels were unmolded and transferred to 24-well plates, to which 500 μL of culture medium were added, after 10 min the medium was removed and 75 μL of cell

suspension were deposited at 3,733,000 cells/mL. For subsequent assays, spheroids were treated after 3 days of growth with different concentrations of AEM (7, 15, and 30 mg/mL) and incubated at 37 °C and 5% CO₂ for 24h. Spheroids treated with 5-FU at 5 µM were used as positive control and untreated spheroids as negative control.

2.7 Analysis of spheroid morphology

A morphological characterization was performed using circumferences; after 24 and 48 h of treatment, 20 micrographs were taken to a fluorescence inverted microscope (OPTIKA, IM3FL Ponteranica (BG), Italy), with 100x objective, at a scale of 200 µm and a representative image of the spheroids was chosen for each condition. The images were processed with OPTIKA Pro-5 Pro-View software.

2.8 Cell cycle analysis

This analysis was made by DNA staining with PI [36]. The cells of the spheroids were mechanically disaggregated by gentle pipetting and washed with PBS at RT. Cells were fixed for 1 h with cold 70% ethanol and subsequently washed with PBS. Fixed cells were resuspended in 200 µL of PBS, stained (250 µg/mL RNaseA, 10 µg/mL PI), incubated at RT for 30 min in darkness, and 10,000 events were analyzed on a flow cytometer (LSR Fortessa™, BD Biosciences, USA) at an excitation and emission length of 488 nm and 610/15 nm, respectively.

2.9 Apoptosis analysis

Apoptotic cells were identified by using Annexin-V as a marker of apoptosis in treated and untreated spheroid cells [37]. Spheroids were mechanically disaggregated, mixed with 1 mL of Annexin-V binding buffer (10 mM Hepes; 0.14 M NaCl; 2.5 mM CaCl₂; pH 7.4), 4 µL of Annexin-V-FITC and 10 µL of PI at 1 mg/mL and incubated for 15 min at 4 °C in darkness. Then, 10,000 events were analyzed on a flow cytometer (LSR Fortessa™, BD Biosciences, USA) at 488 nm excitation, and emission was detected at 610/15 nm for PI and 518 nm for Annexin V-FITC. The activation of caspases (1, -3, -4, -5, -6, -7, -8, and -9) was determined using the commercial Vybrant® FAM Poly Caspases Assay kit (Invitrogen) containing a fluorescent inhibitor (FLIKA) that binds to any of the activated caspases. However, is not possible to discriminate which of them were activated. The treated and untreated spheroids were disaggregated and resuspended at a density of 1x10⁶ cells/mL in culture medium; 300 µL of cell suspension were incubated with 10 µL of FLIKA working solution (30X) for 60 min at 37 °C and 5% CO₂ in darkness. Subsequently, 2 mL of 1X/ wash buffer were added to the cell suspension, centrifuged at 8000 g for 1 min, and the supernatant was discarded, and the pellet was resuspended in 400 µL of wash buffer. Thereafter, 10,000 events were analyzed by flow cytometer (LSR Fortessa™, BD Biosciences, USA) at an excitation length of 488 nm and an emission length of 518 nm.

2.10 Detection of senescent cells

Senescent cells were detected following the manufacturer's instructions for Cell Event Senescence Green Flow Cytometry Assay commercial kit (Invitrogen). This assay measures the activity of the

enzyme β -galactosidase (β -gal) that resides in lysosomes and converts β -galactosides to monosaccharides under acidic pH conditions. Treated spheroids were resuspended at a density of 1×10^6 cells/mL in 100 μ L PBS and centrifuged at 8000 rpm for 1 min; the medium was then discarded, and the pellet was resuspended in 100 μ L of fixation solution for 10 min at RT in darkness. Subsequently, cells were washed with 1% BSA (Bovine Serum Albumin), resuspended in 100 μ L of working solution for 1 h at 37 °C, and then washed with 1% BSA. Finally, cells were resuspended in 1% BSA and 10,000 events were analyzed on a flow cytometer (LSR Fortessa™, BD Biosciences, USA) at 488 nm excitation and 518 nm emission length.

2.11 Statistical analysis

Data were presented as mean $\pm$ Standard Deviation (SD). Comparison between groups was performed using one-way or two-way analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's post hoc test. Differences were considered statistically significant if p-value was <0.05 . GraphPad Prism statistical software version 9.0.0 was used to process data (GraphPad Software Inc., La Jolla, CA, USA).

Results and discussion

The aqueous extract contains phenolic compounds, total tannins and total anthocyanin being the most significant quantitatively (Table 1). Regarding the antioxidant capacity, the ORAC (Oxygen Radical Absorbance Capacity) value corresponds to the most active mechanism, followed by the ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) value and DPPH (2,2-diphenyl-1 picrylhydrazyl) value (Table 1).

Table 1 Phytochemicals and antioxidant activity of aqueous extract from Andean Berry.

Phenols	Tannins ²	Anthocyanins ³	ORAC ⁴	ABTS ⁴	DPPH ⁴
2186 $\pm$ 51.92	918.22 $\pm$ 79,5	25219 $\pm$ 0.07	32927,37 $\pm$ 1334,9	1102,55 $\pm$ 38.3	129.25 $\pm$ 4.7

The results are expressed as the mean $\pm$ SD of three independent experiments in triplicates. ¹mg Gallic Acid Equivalents /100 g. ²mg Catechin Equivalents/100 g. ³mg Cyanidin-3-Glycoside Equivalents /100 g. ⁵ μ moles Trolox Equivalents/g.

Subsequently, SW480 cell viability decreased with longer exposure to the extract, because the IC50 value obtained was 33.62 ± 1.3 mg/mL and 5.91 ± 1.0 mg/mL at 24 and 48 hours, respectively. The extract produced a detachment of the spheroid cells that increased with exposure time to the extract and concentration. It is also observed that the center of the spheroid is becoming opaquer (darker) with increasing extract concentration and exposure time (Fig. 1).

Figure 2 shows a statistically significant increase in the percentage of spheroid cells in SubG1 (dead or in apoptosis) after treatment for 48 h at different concentrations of the extract (AEM) compared to

untreated cells (negative control). Spheroid cells in G1 decreased significantly in all treatments compared to the negative control. In addition, S-phase (proliferative) cells increased significantly in the spheroids after treatment with the extract at different concentrations compared to the untreated cells. The G2 phase (proliferative) cells also decreased significantly after treatment compared to the negative control. Finally, the results obtained with the extract are comparable to the effect observed with 5-Fluorouracile (5-FU).

Figure 3A shows the distribution of cells after double staining with Propidium Iodide (PI) and Annexin-V as follows: necrotic cells (Q1: annexin V-FITC- / IP+), cells in late apoptosis (Q2: annexin V-FITC+ / IP+), cells in early apoptosis (Q3: annexin V-FITC+ / IP-), and viable cells (annexin V-FITC/IP-). The percentage of necrotic cells decreased significantly in spheroids exposed to different concentrations of AEM. In contrast, the rate of apoptotic cells increased significantly in treated spheroids at different AEM concentrations concerning the negative control (Fig. 3b).

Considering the importance of caspases in the process of cell death by apoptosis, the detection or not of active caspases in spheroid cells after treatment was performed. Figure 4 shows that the extract (AEM) induced an increase in the percentage of cells positive for activated caspases. It is noteworthy that a concentration of 7 mg/mL of the extract produced the highest percentage of cells with active caspases, and induced the highest percentage of apoptotic cells (Fig. 4). This result is comparable with the effect of 5-FU induced apoptotic cells, although also a higher percentage of necrotic cells was observed (Fig. 4) when comparing these results with untreated cells.

Since the percentage of cells in the G1 phase of the cell cycle decreased and is associated with senescent cells [12], we verified if the extract induces senescence because this mechanism of death does not occur due to apoptosis. Figure 5 shows that the extract and 5-FU (positive control) compared to untreated cells (negative control) significantly decreased the percentage of senescent cells from spheroids after 24 h of treatment under the same conditions that induce apoptosis.

Monolayer or 2D cell models present several limitations because they do not mimic the *in vivo* tumor development, in which cell-cell or cell-cell plus extracellular matrix interactions occur; these interactions are related to cell proliferation events, gene expression, and drug response, among others [8, 13-15]. Another disadvantage of 2D cultures is that cells lose their polarity, which has an impact on cell response to apoptosis [16]. By contrast, 3D cultures allow us to obtain results that approximate the results of *in vivo* assays.

In our study, the aqueous extract from Andean Berry (*V. meridionale*) presented a higher content of total phenols such as anthocyanins compared to other studies employing the same extract (2546 mg gallic acid and 150.7 mg cyanidin-3-glucoside/100 g) [16]. A similar situation was presented for the antioxidant activity analyzed by three methods based on different antioxidant mechanisms that could be attributed to their phenolic content. these findings agree with the results of Maldonado-Celis et al, 2014 [8], who reported an ORAC value of an aqueous extract from *V. meridionale* berry corresponding to 41775.2 μmol Trolox/100 g, a higher value than the one obtained in our analysis. Factors that may affect the variation

of the antioxidant activity of the aqueous extract from Andean berries among different studies include the method of extraction, fruit maturity, and storage conditions of the fruit and/or extract [17].

The aqueous extract decreased the growth of SW480 (2D) cells at lower concentrations compared to SW480 spheroids. This could be due to differences in metabolism that make them sensitive to test compounds, and to the cell proliferation rate of monolayer culture that is higher compared to cells in 3D cultures. This suggests a higher efficacy of test compounds or extract; thus, data collected from 3D cell cultures concerning resistance to antitumor agents are generally more accurate than in 2D cultures [18, 19].

Regarding the cell cycle, it was found that spheroids with three days of growth treated with aqueous extract from Andean Berry at different concentrations for 24h, showed an accumulation of cells in sub G1. This result differs from the findings reported by Arango-Varela et al. [20], where SW480 cells in 2D culture exposed for 24 h with *V. meridionale* berry juice 18 mg/mL (30% v/v) presented a cell cycle arrest in the S and G2/M phases (SW480). This difference in cell distribution could be attributed to the fact that SW480 cell culture in monolayer was not synchronized, and the cell population was segregated in different phases of the cycle. Therefore, exposition to bioactive berry compounds such as ellagic acid, gallic acid, and methyl gallate present in *Vaccinium meridionale* might activate p53 and thus regulate the cell cycle [21, 22]. Moreover, SW480 spheroid cells have a more complex environment than monolayer culture with the same cell line, and bioactive compounds may interact differently; furthermore, spheroid cells do not have the same exposure to oxygen and nutrients, which may favor the increased percentage of dead or dying hypodiploid cells [23].

To know if apoptosis is involved in the increase of SubG1 cells in the spheroids treated with the extract mentioned in this study, the cells were analyzed by double staining with Annexin-V/PI, as well as the activity of caspases. The aqueous extract from Andean Berry induced apoptosis and activation of caspases, although the strategy available for this research did not enable the identification of a differential activity of each enzyme. These results were comparable with 5-FU. The effect of the extract observed here on spheroids coincides with that described by Agudelo et al. in 2017 [6] and Arango-area et al., [20] in SW480 cells treated with Andean Berry juice that inhibited the proliferation of these cells by apoptotic mechanisms through perturbation of the intracellular oxidative state, activation of caspase-3 and p53.

The 5-FU was employed in this study as a positive control because is a cytotoxic agent, an antimetabolite that interferes with RNA and DNA synthesis by inhibiting the enzyme thymidylate synthase [24]. In cancer cells, 5-FU unbalances the autoregulatory system that controls and limits cell division leading to cell death. Thus, 5-FU is used as a chemotherapeutic agent in the treatment of various types of cancer, including colorectal [24]. SW480 cells have been reported to be resistant to 5-FU, which cytotoxic, antiproliferative, and apoptotic effect has been evaluated in 2D culture at 48 h incubation times at concentrations of 0.84 to 89 mg/mL [25], 2.0 µg/mL [26], 1 to 200 µM for 24 h to 72 h [27], 25 mg/mL for 48 h [28]. In our study, it was found that 5µM 5-FU induced apoptosis in spheroids obtained from

SW480 treated for 24 h; however, the mechanisms of action involved in this result were not analyzed. Further studies are required to do so and to determine whether this effect can be potentiated by combining 5-FU with plant extracts such as the one evaluated here.

Finally, our data suggest that extract and 5FU behaved as senolytic agents under the same conditions that induced apoptosis. Senescent cells are resistant to apoptosis [29]; however, it is necessary to analyze the status of anti-apoptotic proteins in the spheroids before and after treatments—as well as the selectivity of the extract concerning non-malignant colon cells—to know their selectivity and safety, since senescent cells also play an important role in tissue damage repair [30].

Conclusion

The use of an aqueous extract from *V. meridionale* berry is a natural agent with antitumor activity—by blocking the cell cycle, inducing apoptosis, and decreasing senescent cells—comparable to the effect observed with the positive control 5-FU. These results highlight the use of the 3D spheroid model to evaluate chemopreventive natural agents alone or combined with therapeutic agents.

Declarations

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Conflicts of interest/Competing interests

No potential competing interests were reported by the authors.

Data availability

All the data are available in the main text. All the data generated in this study can be obtained from the corresponding authors upon reasonable request.

Code availability

Not applicable

Authors' contributions

Susana M Ceballos-Duque: Conception and design of the study, data collection, article drafting. María Elena Madonado-Celis: design experiments, prepare manuscript content, review and final approval of

content and English language, analyze results, and obtain funding for research development.

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Figures

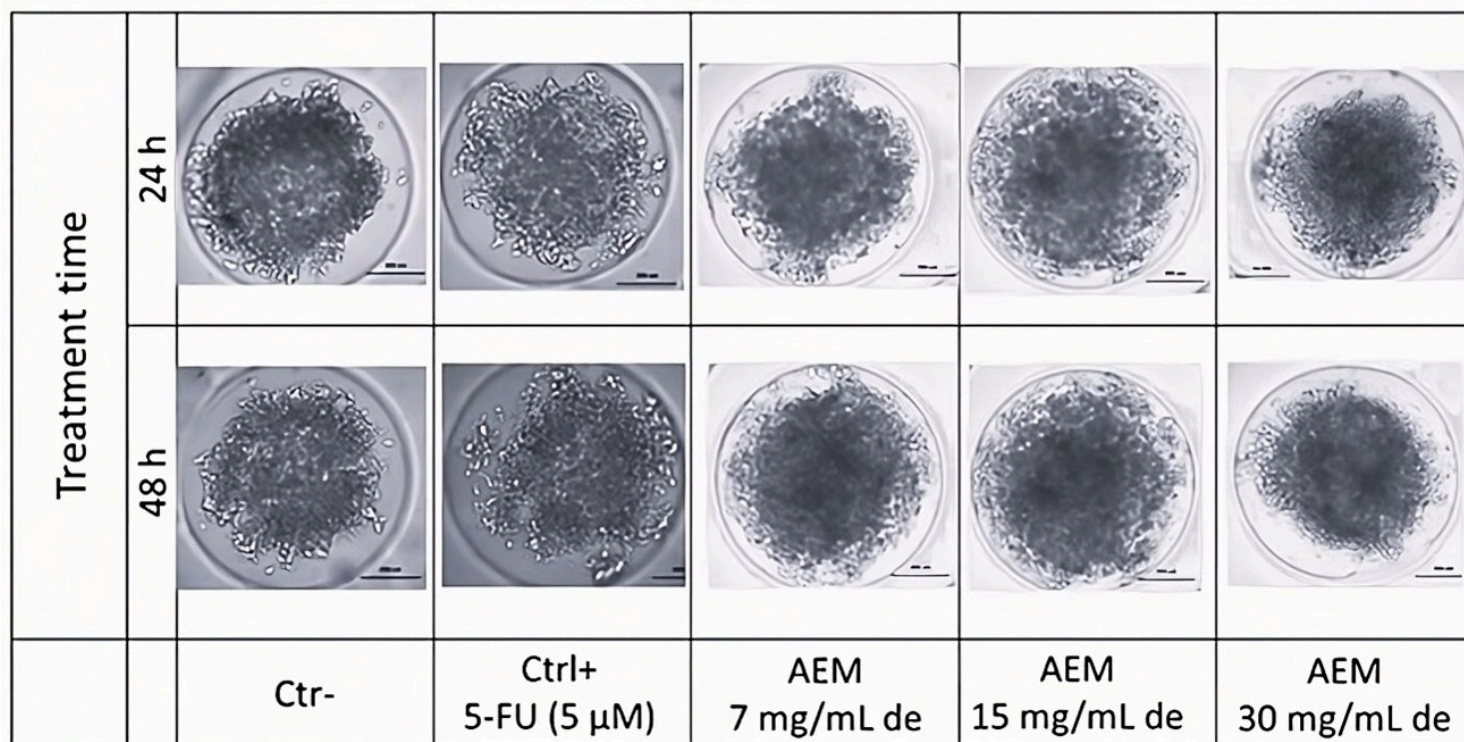
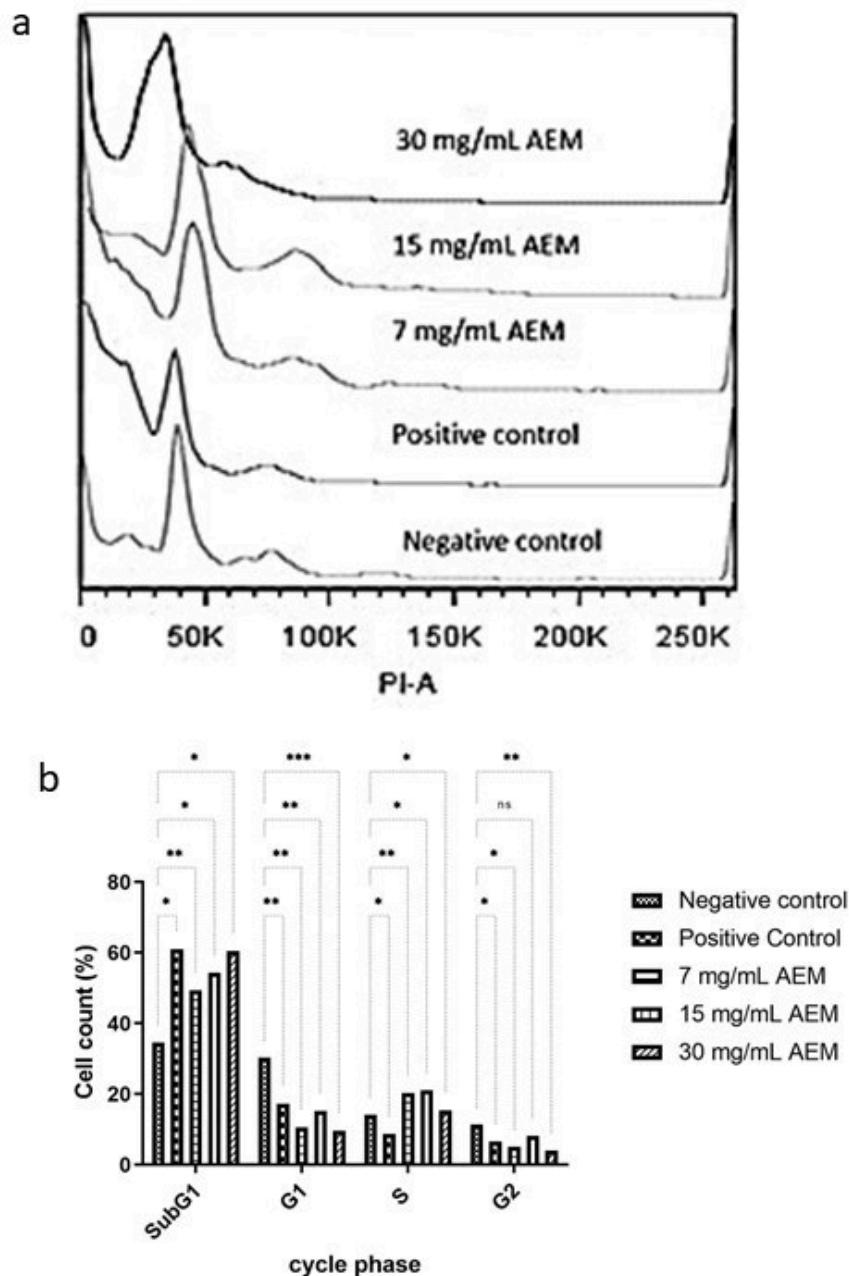


Figure 1

Micrographs of SW480 spheroids with 3 days of growth treated for 24 and 48 h with aqueous extract (AEM) at 7, 15, and 30 mg/mL.



	Negative control		Positive Control		7 mg/mL AEM		15 mg/mL AEM		30 mg/mL AEM	
	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
SubG1	36.6	3.1	61.0	3.8	46.4	3.9	31.0	2.6	60.5	5.7
G1	30.3	1.5	17.3	1.6	10.6	0.1	15.3	1.9	9.6	1.8
S	13.6	1.0	8.7	0.9	21.4	2.2	21.1	1.5	13.1	0.9
G2	11.3	1.2	6.7	1.0	5.1	0.0	8.2	1.9	4.1	0.8

Figure 2

a. Cell cycle analysis of SW480 spheroids with 3 days of growth treated for 24h with AEM at 7, 15, and 30 mg/mL or positive control (5 μ M 5-FU). b. Representative histograms. The percentage of cells in each phase was presented as mean $\pm$ SD, n=3 independent experiments. *p $\leq$ 0,01. **p $\leq$ 0,001. ***p $\leq$ 0,0001

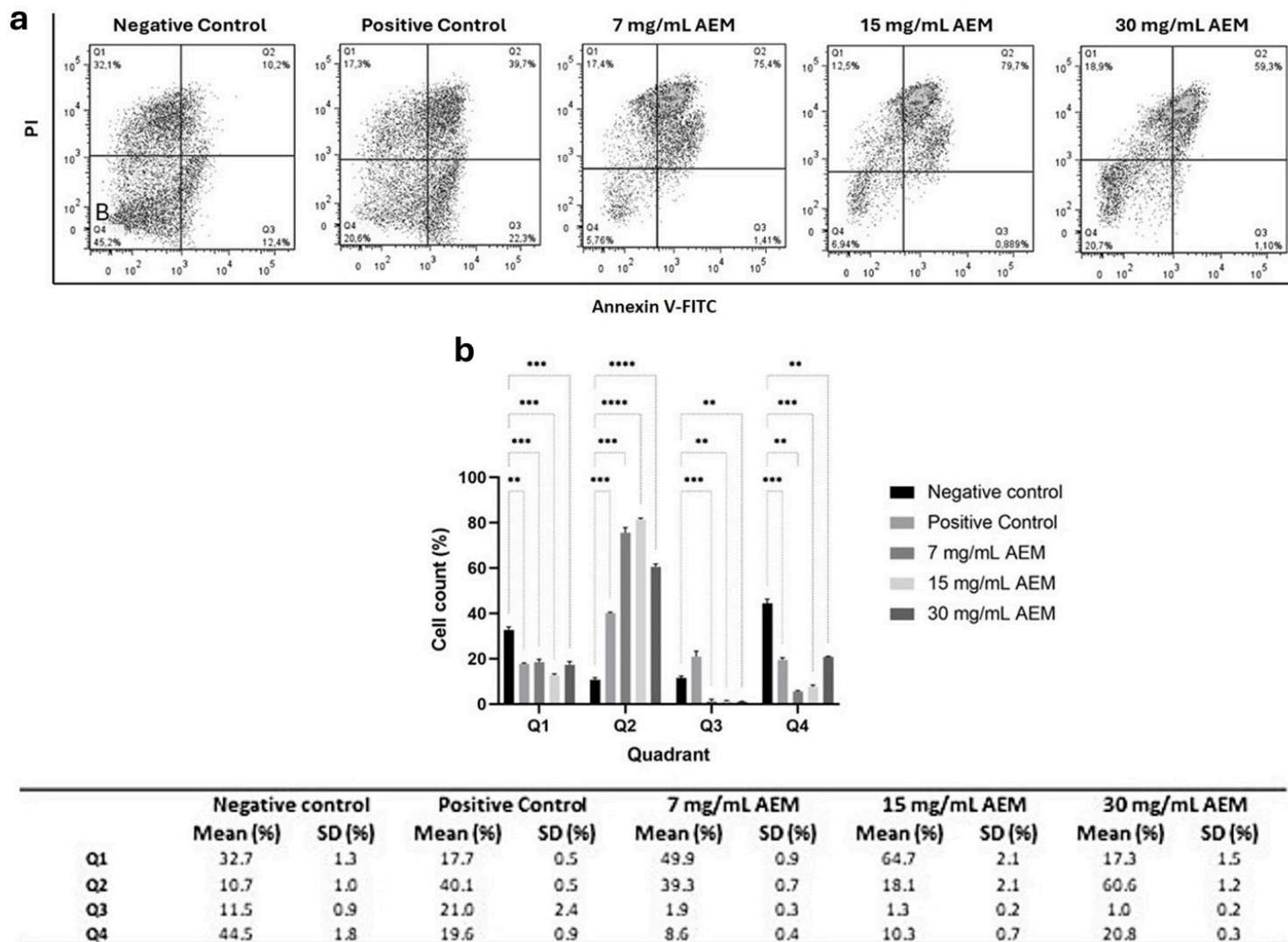
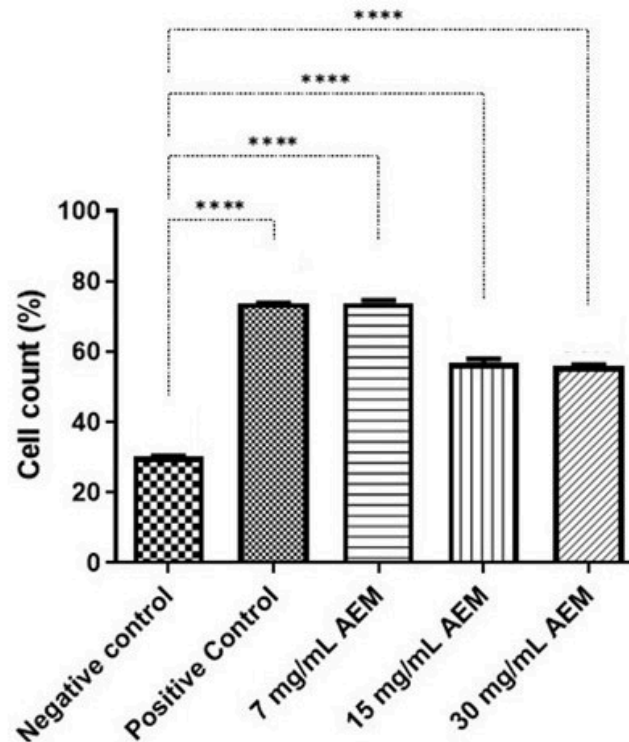


Figure 3

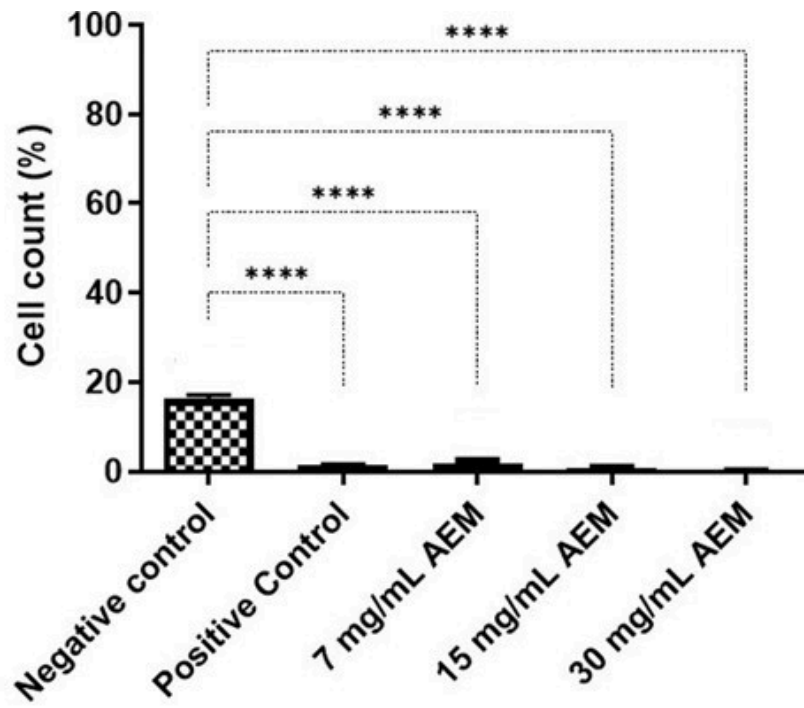
Apoptotic cells of SW480 spheroids with 3 days of growth treated with aqueous extract from Andean berry (AEM) at 7, 15, and 30 mg/mL for 24 h. a. Scatter plots of representative dot plots of the distribution of events (cells) in each quadrant (Q1: death with membrane damage; Q2: late apoptosis; Q3: early apoptosis; Q4: viability). b. Percentage of cells by quadrant. Data presented as mean $\pm$ SD, n=3 independent experiments, for each condition, * $p \leq 0.01$. ** $p \leq 0.001$. *** $p \leq 0.0001$. **** $p \leq 0.00001$.



Cell count	Negative control		Positive Control		7 mg/mL AEM		15 mg/mL AEM		30 mg/mL AEM	
	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
	30.1	0.5	73.7	0.4	73.9	0.7	56.7	1.2	55.9	0.3

Figure 4

Detection of active caspases in spheroid SW480 cells with 3 days of growth treated with aqueous extract from Andean Berry at 7, 15, and 30 mg/mL for 24 h. Data are presented as mean ± SD, n=3 independent experiments, ****p≤0,00001



	Negative control		Positive Control		7 mg/mL AEM		15 mg/mL AEM		30 mg/mL AEM	
	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
Cell count	16.2	0.9	1.5	0.2	1.8	0.7	0.8	0.2	0.1	0.1

Figure 5

Detection of senescent cells from spheroid with 3 days of growth treated with aqueous extract from Andean Berry (AEM) at 7, 15, and 30 mg/mL for 24 h. Data are presented as mean ± SD, n=3, ****p≤0,00001