

Biological activity of P2Et extract from *Caesalpinia spinosa* on colorectal cancer cell lines

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Abstract

Background: Colorectal cancer is the third most diagnosed malignancy and the second leading cause of cancer-related mortality worldwide, with approximately 1.9 million new cases and 0.9 million deaths reported in 2022. Although 5-fluorouracil is widely used in treatment, its clinical benefit is limited by systemic toxicity, low selectivity, and adverse effects that negatively impact patients' quality of life. In recent years, phytochemicals have gained increasing interest due to their ability to modulate multiple pathways involved in tumor progression. In this context, this study aimed to evaluate the antitumor potential of the P2Et extract derived from *Caesalpinia spinosa* in colorectal cancer cell lines.

Methods: The selective biological activity of P2Et was evaluated in human (SW480 and SW620) and murine (CT26) colorectal adenocarcinoma cell lines using a colorimetric viability assay. Cell viability data were normalized to untreated controls and analyzed by nonlinear regression to estimate half-maximal inhibitory concentration values. The selectivity index was calculated using a non-malignant cell line. Antiproliferative activity and intracellular reactive oxygen species levels were assessed using fluorescence-based assays and analyzed using two-way analysis of variance followed by Dunnett's post hoc test. Apoptosis was evaluated by caspase-3/7 activation and nuclear staining using fluorescence microscopy; a semi-quantitative approach was applied by analyzing three independent images per condition to estimate the proportion of apoptotic cells relative to total cells. Differences were assessed using the Mann–Whitney U test. Mitochondrial permeability transition pore opening was examined using a fluorescence-based assay and analyzed by microscopy. A p value ≤ 0.05 was considered statistically significant.

Results: P2Et did not induce significant cytotoxic effects in human colorectal cancer cell lines. In contrast, a decrease in cell viability was observed in the murine CT26 cell line. This effect was accompanied by increased reactive oxygen species production and activation of caspase-3/7. No changes were detected in mitochondrial permeability transition pore opening.

Conclusions: P2Et exhibits selective biological activity in murine colorectal cancer cells, associated with oxidative stress and caspase activation. These findings support the potential of phytochemicals as alternative or adjuvant therapeutic strategies and highlight the need for further studies to better understand their mechanisms of action.

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed malignancy and the second leading cause of cancer related mortality worldwide. According to GLOBOCAN, in 2022 CRC accounted for approximately 1.9 million new cases and 0.9 million deaths globally (Ferlay, 2022). In high-income countries, both incidence and mortality rates have declined, largely due to early detection through organized screening programs. In contrast, in low- and middle-income countries such as Colombia, the increasing burden of CRC underscores that it remains as a public health concern. Barriers to timely access to healthcare often result in diagnosis at advanced stages and limited therapeutic success (Simiao Chen et al., 2023),

allowing for more aggressive disease progression. This situation is reflected in data reported by the Cuenta de Alto Costo (CAC), the Colombian national registry for high-cost diseases, which indicated that 98.13% of CRC cases reported in 2024 were diagnosed at invasive stages (Cuenta de Alto Costo (CAC), 2025; Márquez Ustáriz et al., 2023).

Beyond the impact of social inequities and disease incidence, CRC poses significant clinical challenges. Although several therapeutic modalities are currently available including radiotherapy, immunotherapy, targeted therapies, and surgery, which remains the cornerstone of treatment and is frequently combined with chemotherapy to eradicate residual tumor cells and reduce the risk of recurrence-treatment outcomes are often compromised by emerging resistance mechanisms (Instituto Nacional del Cancer, 2025; Mayo Clinic, 2024). One of the most relevant challenges is multidrug resistance (MDR), defined as the ability of cancer cells to acquire resistance to multiple chemotherapeutic agents, ultimately contributing to treatment failure and disease relapse (Ashique et al., 2024)

Among the chemotherapeutic agents commonly used in CRC management is 5-fluorouracil (5-FU), an aromatic heterocyclic compound structurally related to pyrimidines and incorporated into DNA and RNA through analogous biochemical pathways (Blondy et al., 2020). Despite its widespread clinical use, 5-FU is associated with several limitations, including systemic toxicity, limited selectivity, and adverse effects such as diarrhea, nausea, vomiting, and mucositis, which significantly impair patients' quality of life (Alzahrani et al., 2023).

Given these limitations, there is a growing interest in identifying alternative or complementary therapeutic strategies. In this context, polymolecular agents of natural origin, commonly referred to as phytochemicals, have emerged as promising candidates.

Phytochemicals are bioactive compounds derived from natural sources and have gained increasing attention in oncology due to their ability to modulate multiple signaling pathways involved in programmed cell death, cell migration, and senescence (Zheng et al., 2021). In addition, they have been shown to act as adjuvants to conventional therapies by enhancing drug efficacy, reducing MDR, promoting cellular repair, and increasing overall anticancer activity (Gielecińska et al., 2023). For instance, curcumin, the principal bioactive compound of turmeric, has been reported to enhance the effectiveness of platinum-based chemotherapeutic agents such as cisplatin, carboplatin, and oxaliplatin by potentiating DNA damage and modulating protein pathways associated with chemoresistance in CRC (Bali et al., 2020). Moreover, specific classes of phytochemicals, particularly polyphenols, have been shown to regulate the gut microbiota and contribute to a reduced risk of CRC development (Ding et al., 2020).

Among the phytochemicals that have attracted considerable interest is the standardized P2Et extract, which is rich in gallotannins and derived from the pods of *Caesalpinia spinosa*, a Fabaceae species native to the South American Andes. In traditional medicine, this plant has been widely used for its antioxidant, anti-inflammatory, and antitumor properties, which are largely attributed to its high content of tannins, polyphenols, and flavonoids (Ken, 2014; Robledo Restrepo et al., 2020)

Previous studies have demonstrated that P2Et exhibits antitumor activity in murine models of breast cancer and melanoma, reducing both tumor growth and metastatic incidence while also modulating antitumor immune responses (Gomez-Cadena et al., 2016; Urueña et al., 2015). *In vitro*, P2Et has shown antitumor effects in murine breast cancer cell lines (4T1, TS/A, and the 4T1-H17 subpopulation enriched in tumor stem cell-like features) as well as in murine melanoma cells (B16F10). In these experimental models, P2Et treatment induced cell death through apoptosis and autophagy and triggered hallmarks of immunogenic cell death, including calreticulin exposure at the cell surface and the translocation of high-mobility group box 1 (HMGB1) (Prieto et al., 2019; Urueña et al., 2013). Antioxidant activity has also been reported for P2Et in the leukemia cell lines K562 and Reh (Arévalo-Ferrin et al., 2023). More recently, studies using three-dimensional co-culture models incorporating 4T1 cells and NIH-3T3 fibroblasts have shown that P2Et can modulate the tumor microenvironment by affecting cell proliferation, migration, and intercellular interactions (Arévalo-Ferrin et al., 2023). Collectively, these findings support the potential of P2Et as a broad-spectrum antitumor agent.

Despite this evidence, the effects of P2Et on colon adenocarcinoma cells remain largely unexplored. Therefore, the present study evaluated the cytotoxic effects of P2Et in human (SW480 and SW620) and murine (CT26) CRC cell lines, as well as in non-tumorigenic murine fibroblasts (NIH-3T3), using the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). In addition, the CT26 cell line was selected to further investigate the antiproliferative and pro-apoptotic effects of P2Et, as well as its impact on mitochondrial membrane integrity. Overall, this study aims to provide new insights into the therapeutic potential of P2Et as an alternative strategy for CRC treatment.

2. Materials and methods

Plant material

Pods of *Caesalpinia spinosa* were collected in Villa de Leyva, Boyacá, Colombia. The plant material was taxonomically identified by Luis Carlos Jiménez at the Colombian National Herbarium (voucher specimen No. COL 523714). Collection and use of the plant material were conducted under Agreement No. 220/2018 issued by the Colombian Ministry of Environment, in accordance with national regulations governing access to genetic resources and their derived products. The standardized P2Et extract was produced under Good Manufacturing Practice (GMP) conditions and chemically characterized as previously described by Sandoval et al. (Sandoval et al., 2016)

Cell lines and culture conditions

The human colorectal adenocarcinoma cell lines SW480 and SW620 were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The murine colon carcinoma cell line CT26.WT was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Additionally, the murine fibroblast cell line NIH/3T3 was included as a non-malignant control cell to determine the selectivity index of the extract and was kindly provided by the Systems Biology Research Group at Universidad Pontificia Bolivariana (Medellín, Colombia). For routine maintenance and all

experimental procedures, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4500 mg/L glucose and L-glutamine (Sigma-Aldrich, Burlington, MA, USA), supplemented with 5% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, Burlington, MA, USA). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cell cultures were routinely screened for Mycoplasma spp. contamination by PCR (Sigma-Aldrich, Burlington, MA, USA), as previously described (Puerta & Usme, 2013).

Antitumor activity

The antitumor effect of the P2Et extract was assessed using the MTT assay (Sigma-Aldrich, Saint Louis, MO, USA). This colorimetric assay is based on the ability of mitochondrial dehydrogenases in metabolically active cells to reduce tetrazolium salt to insoluble formazan crystals, with the amount of formazan produced being proportional to the number of viable cells (Morgan, 2000). Cells were seeded at a final density of 1x10⁴ cells/well in 96-well culture plates and incubated for 24 h at 37°C with 5% CO₂ to allow cell attachment. Cells were then treated for 24 or 48 h with P2Et (1.9–250 µg/mL) diluted in 1% ethanol. Ethanol at the same concentration was used as the negative control. All conditions were tested in quintuplicate.

After treatment, the culture medium was removed and 50 µL of MTT solution (2.41 mM prepared in unsupplemented culture medium) was added to each well. Plates were incubated for 2 h at 37°C. Subsequently, the formed formazan crystals were dissolved in isopropanol, and plates were gently agitated for 30 min at room temperature. Absorbance was measured using a Varioskan LUX multimode microplate reader (Thermo Scientific, Thermo Fisher Scientific, USA) at 570 and 650 nm. Optical interference was corrected by subtracting absorbance at 650 nm (reference wavelength) from that at 570 nm (maximum formazan absorbance). Results were normalized to the negative control, which was defined as 100% cell viability. Data were fitted using nonlinear regression to calculate the half-maximal inhibitory concentration (IC₅₀), defined as the extract concentration that reduced cell viability by 50% relative to the control. The selectivity index (SI) was calculated as the ratio between IC₅₀ values obtained in the non-malignant cell line and those obtained in malignant cell lines. Data are expressed as mean ± standard deviation from at least three independent experiments.

Antiproliferative activity

Antiproliferative activity was evaluated using the CyQUANT NF Cell Proliferation Assay (Invitrogen, Thermo Fisher Scientific, USA). This assay employs a fluorescent dye and a dye-delivery reagent that facilitates dye entry into live cells, allowing cell proliferation to be quantified based on fluorescence intensity, which is directly proportional to the number of viable cells.

CT26 cells were seeded at a density of 1x10⁴ cells/well in 96-well plates and incubated under the same conditions described for the MTT assay. After 24 h, cells were treated with the IC₅₀ concentration of P2Et (18 µg/mL), the IC₁₀ concentration (3.6 µg/mL), or 1% ethanol as a negative control, for 6, 12, 24, or 48 h. Following treatment, staining and dye-delivery reagents (1X, 100 µL/well) were added, and plates were

incubated for 1 h at 37°C. Fluorescence was measured using a Varioskan LUX microplate reader (Thermo Scientific, Thermo Fisher Scientific, USA) at excitation/emission wavelengths of 485/530 nm.

Reactive oxygen species (ROS) levels

To assess the effect of P2Et on ROS production in CT26 cells, the fluorescent probe 2',7'-dichlorofluorescein diacetate (2',7'-DCFDA) was used according to the manufacturer's instructions (Calbiochem, San Diego, CA, USA). Cells were seeded at a density of 2×10^4 cells/well in 24-well plates and incubated under the same conditions described for the MTT assay. After 24 h, cells were treated with the IC_{50} concentration of P2Et (18 μ g/mL) or 1% ethanol as a negative control for 6, 12, 24, or 48 h. Following treatment, cells were incubated with 2',7'-DCFDA at a final concentration of 10 μ M for 30 min at 37°C. Fluorescence images were acquired using blue light on an EVOS M5000 microscope (Thermo Fisher Scientific, Waltham, MA, USA).

In parallel, cell lysates were obtained from a separate plate, and relative fluorescence units (RFU) were measured at excitation/emission wavelengths of 485/525 nm using a Varioskan LUX microplate reader (Thermo Scientific, Thermo Fisher Scientific, USA). Total protein concentration was determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) to normalize RFU values.

Evaluation of apoptotic effects

To evaluate whether P2Et induced apoptosis in CT26 cells, the CellEvent Caspase-3/7 Green Detection Reagent kit was used following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated under the same conditions described for the MTT assay. After 24 h, cells were treated with the IC_{50} concentration of P2Et (18 μ g/mL), 1% ethanol as a negative control, or doxorubicin (5 μ M) as a positive control. After 48 h of exposure, nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min. The Hoechst solution was removed and replaced with PBS supplemented with 5% fetal bovine serum (Gibco, Waltham, MA, USA) containing 10 μ M CellEvent Caspase-3/7 Green Detection Reagent. Cells were incubated for 30 min at 37°C, and images were acquired using blue and violet light on the EVOS M5000 microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Although this assay was designed as a qualitative analysis, a semi-quantitative approach was applied by analyzing three images per experimental group. The proportion of apoptotic cells was calculated as the ratio between the total number of Hoechst-stained nuclei and the number of cells positive for caspase-3/7 activation.

Evaluation of mitochondrial permeability transition pore opening

Mitochondrial permeability transition pore (mPTP) opening was evaluated using the Image-iT LIVE Mitochondrial Transition Pore Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. CT26 cells were seeded in 24-well plates at a density of 2×10^4 cells/well

and incubated under the same conditions described for the MTT assay. After 24 h, cells were treated with the IC₅₀ concentration of P2Et (18 µg/mL), 1% ethanol as a negative control, or ionomycin (1 µM) as a positive control.

A labeling solution was prepared by combining 1.0 µL of each of the following reagents: calcein AM (1.0 mM), MitoTracker Red CMXRos (200 µM), Hoechst 33342 (1.0 mM), and CoCl₂ (1.0 mM). This mixture was diluted in 2996 µL of Hank's Balanced Salt Solution (HBSS) pre-warmed to 37°C. Cells were incubated with the labeling solution for 15 min at 37°C, protected from light. Excess dye was removed by washing with pre-warmed HBSS. Images were acquired using blue, violet, and green light on the EVOS M5000 microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Antitumor and antiproliferative assays were performed in at least three independent experiments. IC₅₀ values were calculated using nonlinear regression analysis. Statistical differences between the control group (cells treated with 1% ethanol) and treated groups at different time points were analyzed using two-way ANOVA followed by Dunnett's post hoc test. Differences in the percentage of apoptotic cells between control and P2Et-treated groups were evaluated using the Mann–Whitney U test. A p value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software, version 8 for Windows.

3. Results

Antitumor effect of P2Et extract on murine (CT26) and human (SW480 and SW620) colorectal cancer cell lines

To assess the effect of P2Et extract on cell viability, a range of concentrations was evaluated in malignant cell lines (SW480, SW620, and CT26) and in a non-malignant cell line (NIH-3T3) after 24 and 48 h of exposure. Results were expressed as IC₅₀ values derived from dose–response curves, as summarized in Table 1.

After 24 h of exposure to P2Et, the non-malignant cell line exhibited an IC₅₀ of 100.3 µg/mL, whereas the human colorectal cancer cell lines SW480 and SW620 showed IC₅₀ values of 169.5 and 140.8 µg/mL, respectively. The IC₅₀ is one of the most widely used parameters to assess drug efficacy (Aykul & Martinez-Hackert, 2016) and these results suggest a higher susceptibility of non-malignant cells to the extract, resulting in SI below 1. From a therapeutic standpoint, this finding is unfavorable, as an increased cytotoxic effect on tumor cells with minimal impact on healthy cells would be expected.

In contrast, the murine CT26 cell line displayed a different response. At 24 h, an IC₅₀ of 60.45 µg/mL and an SI of 1.65 were observed, indicating a moderate inhibitory effect of P2Et on this murine colorectal cancer model.

Following 48 h of treatment, SW480 and SW620 cells exhibited lower IC₅₀ values (60.48 µg/mL and 94.59 µg/mL, respectively) compared to those observed at 24 h. However, a decrease in IC₅₀ was also observed in the non-malignant NIH-3T3 cells (42.57 µg/mL), resulting in SI values of 0.7 for SW480 and 0.45 for SW620. For the CT26 cell line, an IC₅₀ of 18 µg/mL and an SI of 2.36 were obtained, further supporting the selective inhibitory effect of P2Et on this cell line.

Given that CT26 cells exhibited greater sensitivity to P2Et compared to the other malignant cell lines evaluated, this model was selected for subsequent biological assays. For these experiments, the IC₅₀ value determined after 48 h of treatment was used as the reference concentration.

Table 1. Half-maximal inhibitory concentration (IC₅₀) of P2Et extract in SW480, SW620, CT26, and NIH-3T3 cell lines at 24 and 48 h post-treatment.

P2Et	NIH-3T3	SW480	SI	SW620	SI	CT26	SI
	IC50 (µg/mL)	IC50 (µg/mL)		IC50 (µg/mL)		IC50 (µg/mL)	
	24 hours						
	100,3±5,47	169,5 ±15,8	0,59	140,80±12,79	0,71	60,45±4,16	1,65
	48 hours						
	42,57±2,69	60,48±4,49	0,7	94,59±7,74	0,45	18,00±0,87	2.36

IC₅₀ values were obtained from P2Et dose–response curves. The selectivity index (SI) was calculated as the ratio between the IC₅₀ determined in non-malignant cells (NIH-3T3) and the IC₅₀ corresponding to malignant cells (CT26). Data are presented as mean ± standard deviation (SD) from at least three independent experiments.

Antiproliferative effect of the P2Et extract on CT26 cells

As shown in Fig. 1, the P2Et extract exhibited a concentration- and time-dependent antiproliferative activity in malignant CT26 cells.

Treatment with the IC₁₀ (3.6 µg/mL) resulted in an approximate 20% reduction in cell viability during the first 12 h of exposure, followed by a recovery to near-baseline levels at 24 h, and a subsequent decrease of about 10% at 48 h. This pattern suggests a transient effect of the extract on cell viability at low concentrations.

In contrast, treatment with the IC₅₀ (18 µg/mL) led to a marked reduction in viability as early as 6 h, with an approximate 40% decrease at this time point. However, similarly to the IC₁₀ condition, cells treated with the IC₅₀ showed a partial recovery at 24 h, reaching viability levels close to 80%, followed by a renewed decline to approximately 60% at 48 h.

For the IC₅₀ treatment (18 µg/mL), statistically significant differences (****, $p < 0,0001$) were maintained across all four evaluated time points, indicating a sustained cytotoxic effect of the P2Et extract. In contrast, the IC₁₀ condition showed significant differences (****, $p < 0,0001$) only at 12 and 48 h, consistent with a milder impact on cell viability.

Data are presented as the mean $\pm$ standard deviation (SD) of at least three independent replicates. P2Et: cells treated with 18 µg/mL (IC₅₀) and 3.6 µg/mL (IC₁₀). CV: cells treated with the vehicle (1% ethanol); CV was considered as 100% cell viability. Statistical analysis was performed using a two-way ANOVA followed by Dunnett's multiple comparisons test (**** $p < 0,0001$ vs. vehicle control).

Effect of P2Et on ROS levels in CT26 cells

After 6 and 12 h of exposure of CT26 cells to the IC₅₀ concentration of P2Et (18 µg/mL), a reduction in ROS levels was observed compared with both the vehicle control and the growth control, as shown in Fig. 2, suggesting a potential antioxidant effect.

In contrast, at 24 and 48 h, the reduction was reversed, with a marked increase in ROS production in P2Et treated cells relative to controls. This increase was statistically significant at 48 h (**** $p < 0,0001$), indicating a time dependent shift in the cellular response to the extract.

This biphasic behavior has been previously reported for polyphenol rich extracts, which, despite their well-known antioxidant properties, may also exert pro-oxidant effects under specific conditions, such as prolonged exposure or higher concentrations of the compound (Fukumoto & Mazza, 2000)

Data are presented as the mean $\pm$ standard deviation (SD) of two independent experiments.

Growth control (GC): untreated cells. Vehicle control (CV): cells treated with the vehicle (1% ethanol). P2Et: cells treated with 18 µg/mL of the extract. Statistical analysis was performed using a two-way ANOVA followed by Dunnett's multiple comparisons test (**** $p < 0,0001$ vs. vehicle control). RFU: relative fluorescence units.

Effect of P2Et on apoptosis induction in the CT26 cell line

To assess whether P2Et induces apoptosis in CT26 cells, a marked increase in the number of apoptotic cells (green fluorescence) was observed in cells treated with the IC₅₀ concentration (18 µg/mL) compared with the vehicle control (Fig. 3). These results indicate that P2Et exerts a pro-apoptotic effect in CT26 cells.

To obtain a semi-quantitative assessment of apoptosis, three representative images from each experimental group: vehicle-treated cells (1% ethanol) and cells treated with the IC₅₀ (18 µg/mL) of the P2Et extract were analyzed. In this analysis, the proportion of apoptotic cells was calculated as the ratio between the total number of cells, identified by Hoechst 33342 nuclear staining, and apoptotic cells labeled with CellEvent Caspase-3/7 Green.

The mean percentage of apoptotic cells was 0.56 ± 0.11 in vehicle-treated CT26 cells and 38.36 ± 11.05 in P2Et-treated CT26 cells. Although an increase in the average number of apoptotic cells was observed following treatment with the extract, this difference did not reach statistical significance, likely due to the limited number of replicates included in the analysis.

CV: cells treated with the vehicle (1% ethanol); P2Et: CT26 cells treated with P2Et (18 $\mu\text{g/mL}$). For each condition, three independent images were analyzed by quantifying the total number of cells stained with Hoechst 33342 and the number of apoptotic cells labeled with CellEvent Caspase-3/7 Green. Statistical analysis using the Mann–Whitney test revealed no significant differences between groups ($p > 0.05$). Images were acquired at 10X magnification.

Effect of P2Et on mPTP opening in CT26 cells

To evaluate the effect of the IC_{50} concentration of P2Et (18 $\mu\text{g/mL}$) on mitochondrial integrity in CT26 cells, mPTP opening was assessed and compared with vehicle treated cells (1% ethanol). No differences in mPTP opening were observed between P2Et treated and vehicle treated cells, indicating that, under the experimental conditions tested, P2Et does not compromise mitochondrial functional integrity. In contrast, treatment with ionomycin (1 μM), used as a positive control, effectively induced mPTP opening (Fig. 4).

Positive control: cells treated with ionomycin (1 μM); CV: cells treated with the vehicle (1% ethanol); P2Et: CT26 cells treated with P2Et (18 $\mu\text{g/mL}$). Representative images showing nuclear and mitochondrial staining in CT26 cells exposed for 48 h to ionomycin (A1–A4), 1% ethanol (B1–B4), or the IC_{50} of the P2Et extract (C1–C4). (A1, B1, C1) Mitochondria with closed mPTP, labeled with calcein AM (1 mM). (A2, B2, C2) Functional mitochondria labeled with MitoTracker Red (200 μM). (A3, B3, C3) Nuclei stained with Hoechst 33342 (1 mM). (A4, B4, C4) Merged images of A1–A3, B1–B3, and C1–C3. The experiment was performed in duplicate. Images were acquired at 40X magnification.

Discussion

Our study demonstrates that P2Et extract exerts a non-selective effect on CRC cell lines SW480 and SW620. In contrast, in the murine CT26 cell line, P2Et treatment resulted in increased ROS levels and the induction of apoptosis at 48 h, without evidence of mitochondrial permeability transition pore opening. These findings suggest that P2Et induced cell death may involve mechanisms that are independent of the canonical mitochondrial pathway. The complexity of this response highlights the need for further studies to elucidate the molecular processes underlying the antitumoral activity of P2Et. Although previous studies have reported antitumor effects of P2Et in other cancer models, available evidence regarding its activity in CRC remains limited, underscoring the relevance of the present work.

In CT26 cells, P2Et induced cytotoxicity at relatively low concentrations, indicating a selective effect toward this murine CRC cell line. In contrast, cytotoxic activity in the human SW480 cell line and its metastatic derivative SW620 was observed only at higher concentrations. These findings are consistent

with those reported by Herrera-R. et al., who evaluated an ethanolic extract of *Bomarea setacea* in SW480 and SW620 cells and observed limited cytotoxic activity, requiring concentrations above 10 mM to achieve a significant inhibitory effect (Herrera-R et al., 2021). Similarly, Bou Malhab et al. assessed the cytotoxicity of a methanolic extract of *Artemisia herba-alba*, characterized by a high content of phenolic compounds and flavonoids, comparable to the phytochemical profile of P2Et. In their study, when evaluated across a panel of CRC derived cell lines (Caco-2, RKO, and HCT116), SW480 and SW620 cells displayed resistance to the extract (Bou Malhab et al., 2025).

This differential sensitivity may be attributed, at least in part, to the genetic and molecular profiles of SW480 and SW620 cells, which influence their response to natural extracts. Notably, both cell lines have been reported to overexpress the chemokine receptor CXCR4, a key component of the CXCL12/CXCR4 chemokine axis, which is known to support mechanisms of therapeutic resistance (Schimanski et al., 2005). CXCR4 is overexpressed in more than 23 different types of human cancers, including kidney, lung, brain, prostate, and breast cancers, where it contributes to tumor growth, angiogenesis, metastasis, and resistance to therapy. Moreover, in CRC patients, CXCR4 expression in primary tumor cells has been correlated with reduced survival, increased metastatic potential, and disease recurrence (Chatterjee et al., 2014; Vandercappellen et al., 2008).

Additionally, studies evaluating the response of SW480 and SW620 cells to chemotherapeutic agents have shown that resistant variants of these cell lines overexpress P-glycoprotein (P-gp), multidrug resistance associated protein 2 (MRP2), and the multidrug efflux transporter ABCG2 (BCRP), resulting in reduced intracellular accumulation of oxaliplatin and 5-fluorouracil (5-FU). Moreover, increased efflux rates and upregulation of ABC transporters have been reported in SW620 cells. Following 5-FU treatment, the reduction in the expression levels of prominin-1 (CD133) and telomerase reverse transcriptase (TERT), markers associated with tumor stem cell like subpopulations, was less pronounced in SW620 cells compared with SW480 cells (Luo et al., 2016; Park et al., 2022). These findings are consistent with the observed response of SW480 and SW620 cells to P2Et, suggesting that their genetic and molecular characteristics may play a decisive role in modulating the activity of the extract.

Taken together, these data indicate that P2Et exhibits a non-selective antitumoral effect when used as a single agent in SW480 and SW620 cells, likely due to the intrinsic resistance profiles of these cell lines. Nevertheless, P2Et may represent a promising candidate for combination therapy with conventional chemotherapeutic agents, such as 5-FU, to enhance therapeutic efficacy. In this regard, Sandoval et al. reported that the combination of P2Et with doxorubicin exerted a synergistic effect in cell lines displaying multidrug resistance phenotypes and tumor stem cell like characteristics, including MES-SA/Dx5, 4T1, and 4T1 H17. These observations suggest that P2Et may potentiate the effectiveness of standard anticancer drugs, improving responses in cells with established resistance mechanisms. Therefore, evaluating the combined treatment of P2Et and 5-FU in this model would be of particular interest (Sandoval et al., 2016).

Based on the selective activity of the P2Et extract toward the CT26 cell line, these cells were selected for subsequent biological assays aimed at further exploring its potential mechanisms of action. In this context, the antiproliferative effect of P2Et on CT26 cells exhibited a biphasic pattern. Following an early reduction in cell viability of approximately 20% at the IC₁₀ (3.6 µg/mL) and 40% at the IC₅₀ (18 µg/mL), cells showed a recovery over time. At the IC₁₀, this recovery reached values close to baseline levels. However, after 48 h of exposure to the extract, a second decrease in cell viability was observed, with reductions of approximately 10% at the IC₁₀ (3.6 µg/mL) and 40% at the IC₅₀ (18 µg/mL) relative to untreated cells. At this time point, a significant increase in the proportion of apoptotic cells was also detected compared with baseline controls.

This biphasic response is consistent with previous reports describing dual effects of phenolic compounds. In this regard, Chen et al. demonstrated that ferulic acid exerts a dual effect on CT26 cells, acting as a mildly cytoprotective agent at low concentrations, while inducing a marked reduction in cell viability at higher doses, thereby acquiring a cytotoxic role. This functional switch was associated with autophagy, which may initially serve as a protective cellular mechanism but, upon prolonged exposure to the compound, ultimately promotes cell death (Shanbin Chen et al., 2023).

Autophagy is an evolutionarily conserved process through which cells maintain metabolic homeostasis. This mechanism is triggered by cellular stress conditions such as hypoxia or nutrient deprivation, activating an intracellular degradation system that delivers cytoplasmic components to the lysosome for recycling (Mizushima, 2007). Several phytochemicals have been reported to exert antitumoral effects by modulating autophagy. For instance, curcumin increases ROS production in human colorectal cancer HCT116 cells, thereby inducing LC3-II accumulation and promoting autophagosome formation, a hallmark event in the autophagic process (Mosieniak et al., 2012). Similarly, increased LC3-II levels and subsequent autophagy induction have been observed in CT26 cells treated with trans-scirpusin A (TSA), a polyphenol found in various foods such as grapes and peanuts (Du et al., 2023).

Based on the antiproliferative behavior observed in the present study, it can be hypothesized that the recovery in cell viability detected after 12 h of P2Et exposure reflects the activation of autophagy as a survival mechanism in response to extract induced cellular stress. Through this adaptive response, a subset of cells may have temporarily restored their functionality. However, this process appears to have been insufficient to counteract the sustained damage, ultimately leading to cell death at 48 h. Cell death at this time point was mediated by the activation of effector caspases 3 and 7 and look like to be influenced by ROS, as a significant increase in ROS levels was detected after 48 h of P2Et exposure. It has been reported that under metabolic stress conditions, excessive ROS production can trigger cell death. This observation is consistent with the findings of Kim et al., who demonstrated that blocking ROS production reversed both apoptotic and autophagic effects induced by a *Chrysanthemum zawadskii* extract in CT26 cells, thereby confirming the central role of ROS in regulating these processes (Kim et al., 2019).

Although ROS are classically associated with mitochondria cell death pathways, our results indicate that P2Et does not directly compromise mitochondrial integrity, as no opening of the mitochondrial permeability transition pore was detected. Nevertheless, emerging evidence suggests that ROS may also promote cell death through extrinsic pathways. For example, in hepatocytes treated with matrine, cytochrome c release that is typically associated with the mitochondrial pathway, was accompanied by increased Fas expression and caspase 8 activation, both indicative of extrinsic apoptotic signaling (You et al., 2019).

Recent evidence indicates that many natural compounds exert their biological effects through modulation of the endoplasmic reticulum (ER) (Liu et al., 2016). The ER is an extensive membrane network responsible for protein translocation, folding, and post translational modifications. Disruption of ER function triggers the unfolded protein response (UPR), a cellular adaptive mechanism aimed at restoring homeostasis (Sano & Reed, 2013). In melanoma cells, P2Et has been shown to activate PERK, one of the three major UPR sensor proteins, leading to calcium release from the ER, loss of mitochondrial membrane potential, and subsequent induction of apoptosis (Prieto et al., 2019).

However, given that no overt mitochondrial alterations were detected in our microscopic analyses, it is plausible that in murine colorectal cancer cells, P2Et exerts its effects through mechanisms in which mitochondria do not play a predominant role. In line with this notion, it has been reported that co-treatment with epigallocatechin-3-gallate (EGCG), a polyphenol abundant in green tea, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a proapoptotic molecule with anticancer therapeutic potential, promotes caspase 8 activation and poly ADP-ribose polymerase (PARP) cleavage, hallmark events of death receptor mediated apoptosis. Although the intrinsic mitochondrial pathway may contribute in a complementary manner, these findings demonstrated that the extrinsic pathway represented the predominant mechanism in this model (Kwon et al., 2020).

Taken together, these observations suggest that P2Et-induced cell death may be mediated by mechanisms in which mitochondria are not central effectors, but rather involve pathways associated with ER stress. This interpretation is supported by studies highlighting the role of caspase 12 as a direct ER stress sensor capable of activating caspase 9 independently of cytochrome c release (Morishima et al., 2002). Collectively, these findings support the hypothesis that P2Et may induce apoptosis through an alternative distinct from the classical mitochondrial pathway, potentially involving extrinsic signaling pathways and ER dependent mechanisms.

Conclusions

- P2Et extract exhibited an antitumor effect in the murine colon adenocarcinoma cell line (CT26), but not in the human cell lines (SW480 and SW620), suggesting differences in cellular sensitivity related to their genetic background.
- The antitumor activity observed in CT26 cells was associated with increased production of reactive oxygen species and activation of effector caspases, indicating apoptosis as the primary mechanism

of cell death.

- The absence of changes in the mitochondrial permeability transition pore suggests that P2Et induces apoptosis without significantly compromising mitochondrial membrane integrity.

Abbreviations

5-FU	5-Fluorouracilo
CRC	Colorectal cancer
CAC	Cuenta de Alto Costo
CSC	Cancer Stem Cells
CXCR4	Chemokine Receptor Type 4
EGCG	epigallocatechin-3-gallate
ER	Endoplasmic Reticulum
HMG-1	High Mobility Group Box 1 Protein
IC₅₀	Half-Maximal Inhibitory concentration
GMP	Good Manufacturing Practice
MRP2	Multidrug Resistance-Associated Protein 2
PARP	Poli ADP-ribosa polimerasa
Pgp	P-Glycoprotein
mPTP	Mitochondrial Permeability Transition Pore
RFU	Relative Fluorescence Units
ROS	Reactive Oxygen Species
MDR	Multidrug resistance
SI	Selectivity Index
TERT	Telomerase Reverse Transcriptase
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UPR	Unfolded Protein Response
CD133	Prominin-1
2',7'-DCFDA	2',7'-dichlorofluorescein diacetate

Declarations

Ethics approval and consent to participate

This study did not involve human participants or animal experimentation. The plant material (*Caesalpinia spinosa*) used to obtain the P2Et extract was collected in Colombia and taxonomically identified at the Colombian National Herbarium. Collection and use were conducted in accordance with national regulations governing access to genetic resources and were authorized under Agreement No. 220/2018 issued by the Colombian Ministry of Environment.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author contributions

LC, EC, PL, SF, and TN contributed to the design of the experiments.

LC performed data acquisition, analysis, and interpretation, and drafted the manuscript.

TN led the project and critically revised the manuscript.

EC, SF, and PL contributed to the revision of the manuscript.

SF provided the plant material and relevant information.

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Figures

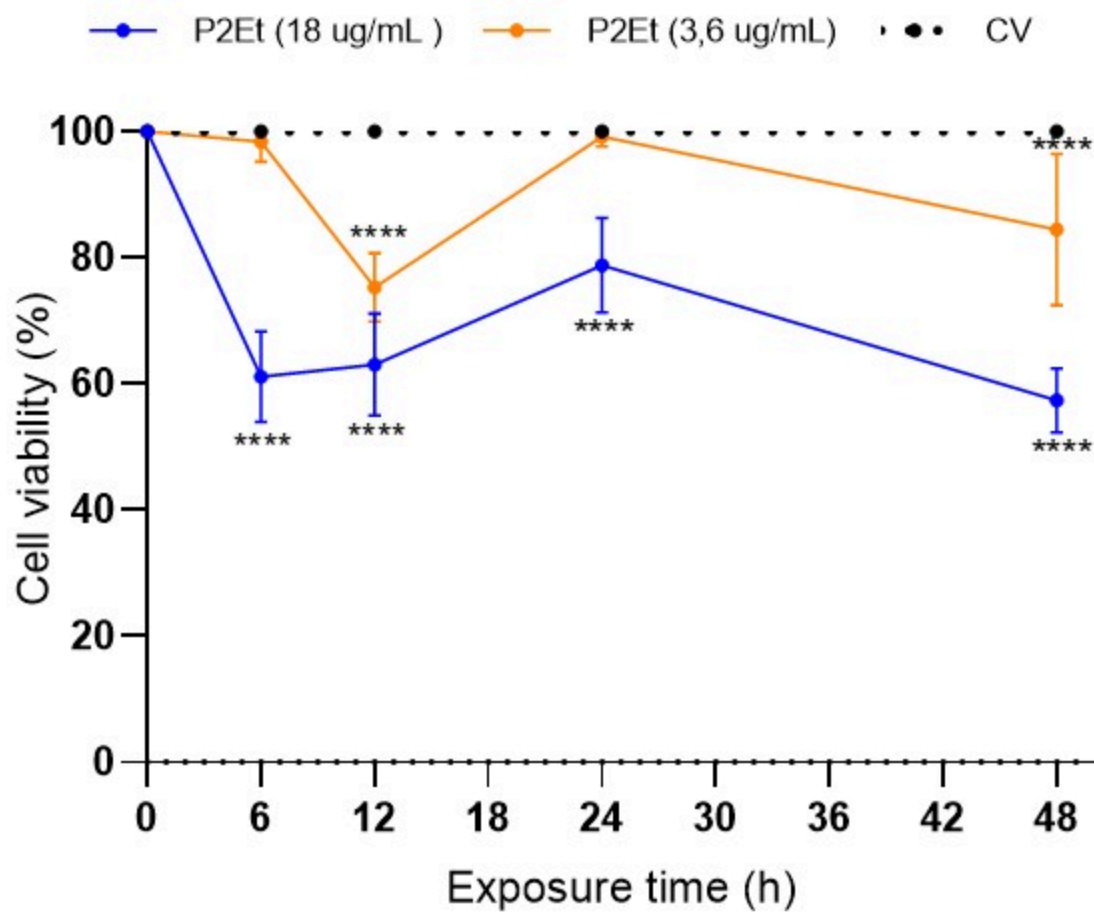


Figure 1

Antiproliferative effect of P2Et extract on CT26 cells at 6, 12, 24, and 48 h of exposure.

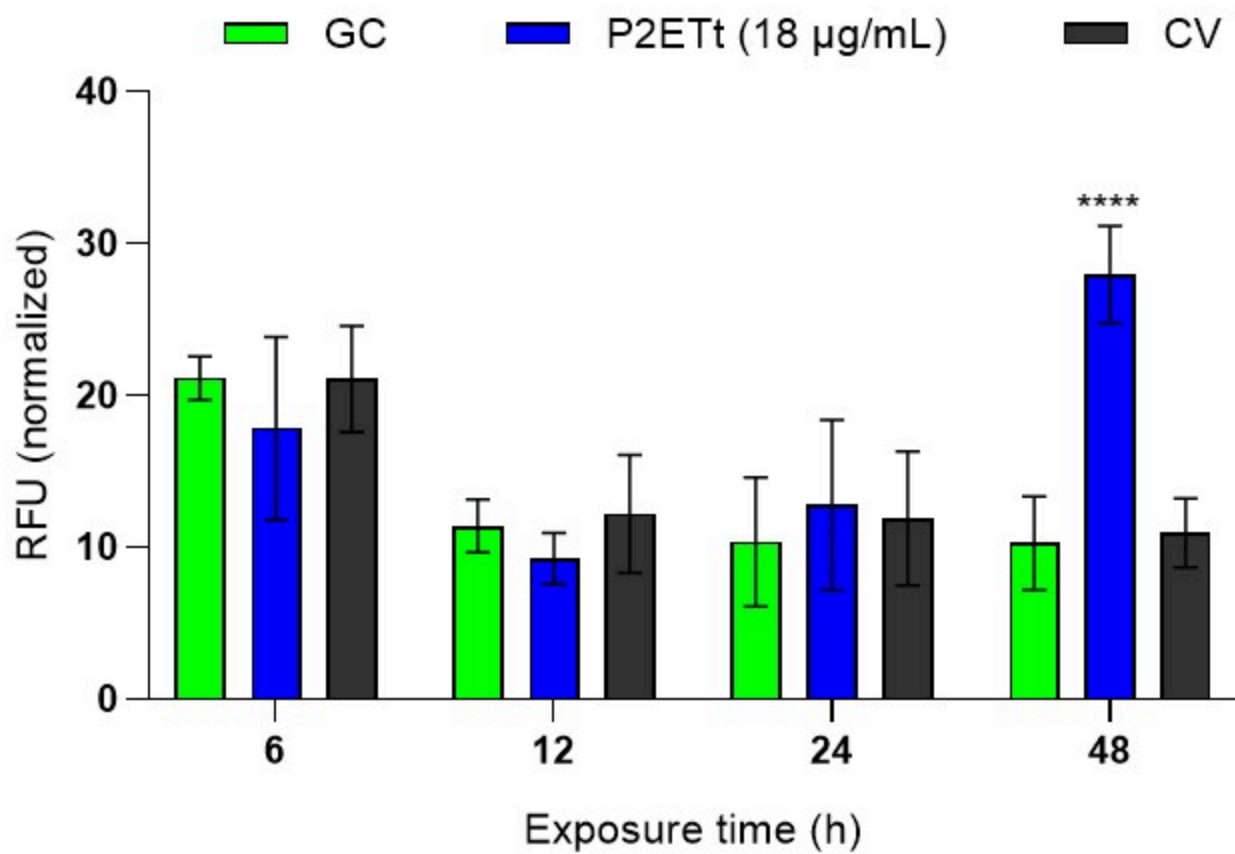


Figure 2

Effect of the P2Et extract (IC_{50}) on ROS generation in CT26 cells.

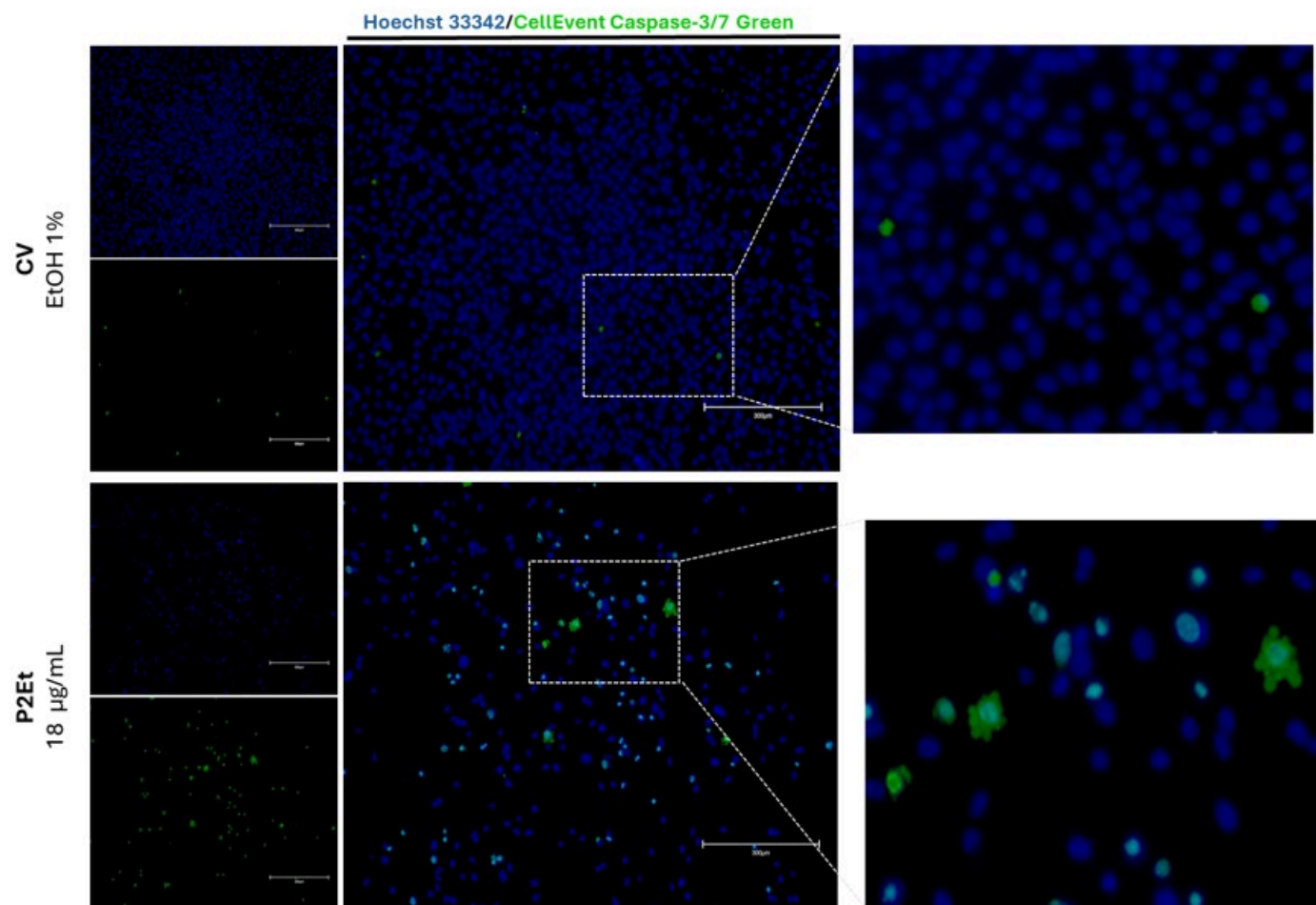


Figure 3

Pro-apoptotic effect of P2Et extract in CT26 cells compared with the control.

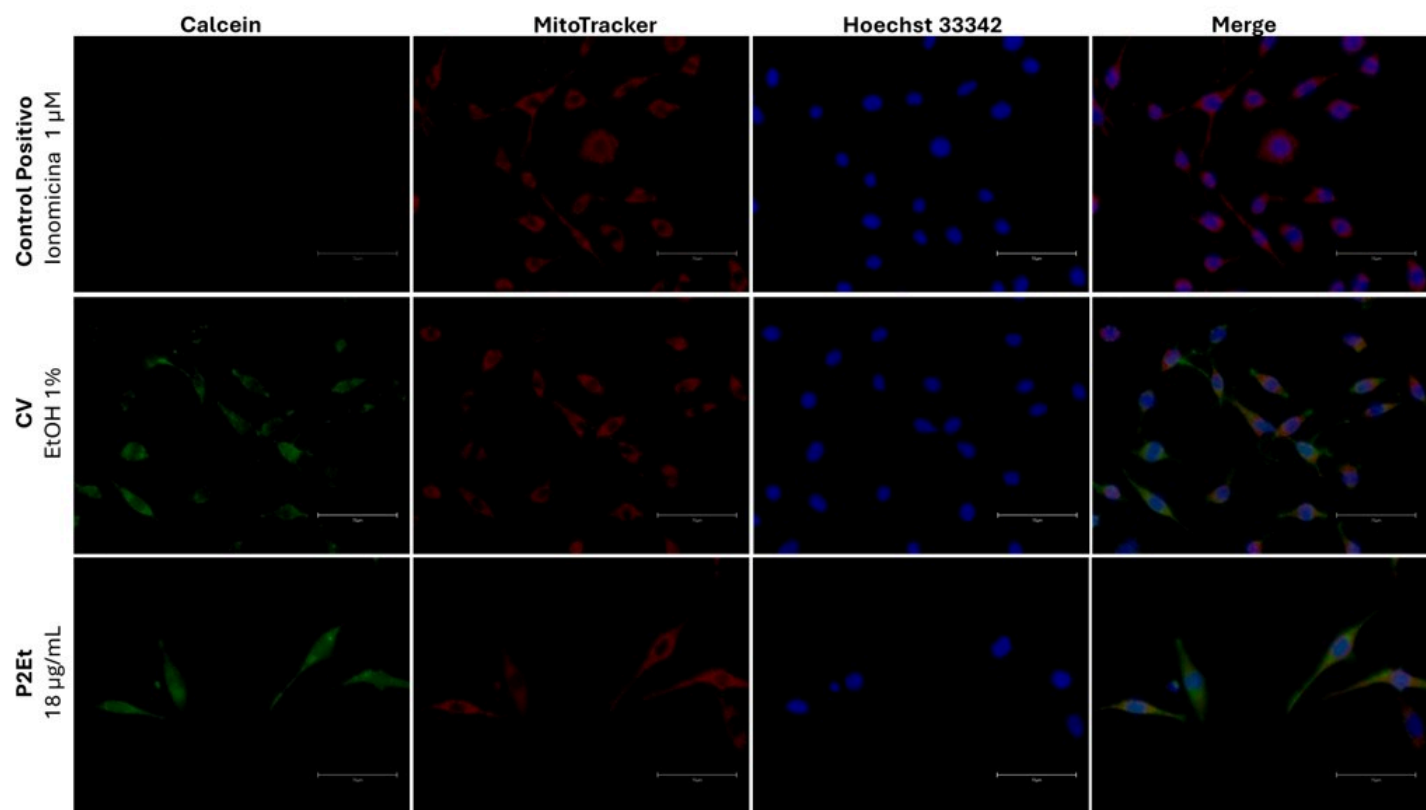


Figure 4

Effect of P2Et extract on mPTP opening in CT26 cells compared with vehicle and positive controls.