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Fraction of latex of *Euphorbia umbellata* induced inhibitory proliferation in triple negative breast tumor cell

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Abstract
The objective of this study was to evaluate the antitumor potential of the acetate and chloroform fractions extracted from *Euphorbia umbellata*lat ex. The cytotoxicity of fractions was evaluated by the MTT colorimetric assay after 24 and 48h at concentrations ranging from 2-30 µg/mL, also the mitochondrial electrical potential by confocal microscopy. The cell cycle, the percentage of fragmented DNA, and the mitochondrial electrical potential were quantified by flow cytometry. Our results suggest that the cytotoxicity of fractions occurs due to the modulation of the apoptotic pathway, a fact that corroborates with compounds already identified from chemical alkaloids groups, with the fractions isolated by acetate and chloroform being those that presented better results in this and previous results antitumor potential new drugs developed by our group.

Keywords: Euphorbiaceae, euphorbia umbellata, breast cancer, apoptosis

1. Introduction
Cancer is one of the main public health problems in the world, being among the four main causes of premature death in most countries. The incidence and mortality from cancer have been increasing and are due to numerous factors, partly due to the aging of the population, changes in the distribution and prevalence of risk factors, especially those associated with socioeconomic development [1]. The latest worldwide estimate points to 18 million new cases of cancer and 9.6 million deaths. According to the International Agency for Research on Cancer by the year 2040, that number will reach 29.5 million. Breast cancer is more common among women worldwide, with an estimated 2 million diagnosed cases, corresponding to 25.2% of all female cancers, being composed of distinct subtypes associated with different clinical outcomes due to its heterogeneity, being of fundamental understanding for the development of preventive and therapeutic interventions [2].
The *Euphorbia umbellata* (Pax) Bruyns, Euphorbiaceae plant, popularly known as "janauba" and "cola-nota" in Brazil, produces a latex that has been used in folk medicine as an anti-inflammatory, antiulcer, homeostatic and antiangiogenic agent, and main as an antitumor agent, also, Euphorbia-derived products have great potential as a source of bioactive extracts and pure compounds, which can be used to promote longevity with more health [5-9]. In the southern region of Brazil, *E. umbellata* bottled latex is popularly used as a treatment for all types of cancer [9-13].
The chemical composition of essential oils of Euphorbia species revealed the presence of more than 80 phytochemicals, mainly oxygenated sesquiterpenes and hydrocarbons were identified in other secondary metabolite extracts, such as diterpenes, sterols, flavonoids and other polyphenols [14].
Recent studies with *E. umbellata* latex fractions have demonstrated cytotoxic potential in breast cancer cells (MDA MB-231) and hepatocellular tumor cells (Hepa1c1c7) [11-13], similar results have been found for murine melanoma cells (B16F10), adenocarcinoma rectal ileocecal (HCT-8), human cervical cancer (HeLa) and lymphoma Jurkat cells [10-13].
Taking into account the need to seek new therapies for the treatment of triple-negative breast cancer, this study aims to analyze the cytotoxic and antiproliferative potential of acetate and chloroform fractions in 4T1 triple-negative breast cancer cells.

2. Materials and Methods

2.1. Cell culture

Murine breast tumor line 4T1 (ATCC HTB-26TM) was used and maintained and stored in the cell bank by Dr. Durvanei Augusto Maria (Development and innovation laboratory, Instituto Butantan). After thawing, the cells were transferred to a cell culture bottle (25 cm^2), containing the RPMI 1640 culture medium (Cultilab, Campinas-SP) supplemented with 10% fetal bovine serum, 200 mM sodium bicarbonate, pH 7.4 in an oven 5% CO_2 at 37ºC. The cells arranged in monolayer were subjected to enzymatic dissociation with 0.2% trypsin solution + EDTA (Ethylenediaminetetraacetic acid) 0.02%so that the detachment of the cells occurs. The enzymatic neutralization was done using the RPMI culture medium containing 10% SFB. After neutralization, the cells in suspension were counted in a Neubauer chamber and the concentration adjusted to 10^5 cells/mL. Cell viability was determined by the Trypan blue exclusion test, with viability greater than 94% being considered ideal for carrying out the experiments.

2.2. Determination of cytotoxic activity by the MTT method

Tumor cells were incubated in 96-well plates at 10^5 cells/mL concentration for 24 and 48h and treated with *E. umbellata* acetate and chloroform latex fractions, in concentrations of 2-30 µg/mL. After 24h of treatment, the supernatant was collected in another plate and 100 µL of MTT (Calbiochem - Darmstadt, Germany) was added at a concentration of 5 mg/mL, the cells were incubated for 3h in an oven containing 5% CO_2 at 37 °C. After this period, the contents were removed and 100 µL of methyl alcohol was added to dissolve the formed and precipitated formazan crystals. The absorbance was quantified in an ELISA reader at a wavelength of 540 nm. The concentration that induces toxicity in 50% of the cells ($IC_{50\%}$) was determined in the treatment after 24 and 48h to assess the dose-response effect.

2.3. Analysis of mitochondrial electrical potential by laser confocal microscopy

The 4T1 triple-negative breast cancer cells were cultured in 24-well plates containing coverslips with RPMI culture medium at 10% SFB kept in the greenhouse at 5% CO_2 at 37ºC for 24h. The samples from the control and treated groups underwent a removal process from the culture medium and were washed with RPMI culture medium. Then, 10 µL of Rodamina123 (Sigma-Aldrich, USA) was added for 30 min in the dark at 37º C. After incubation with Rodamina123 for fixation, 100 µL of 4% paraformaldehyde was added for 30 min, then washed with PBS. Non-binding Rhodamine123 was removed and the cells washed with culture medium. The coverslips were placed on slides for observation in the Confocal Laser fluorescence microscope (Fluoview™ 300) and the images were documented and analyzed.

2.4. Analysis of mitochondrial electrical potential by flow cytometry

The cells were subjected to treatments for a period of 24h. The treated cells and the control group were trypsinized and centrifuged at 1400 rpm for 5 min, the supernatant was discarded and 5 µL of Rhodamine 123 (5mg/mL-1 in ethanol) was added (Molecular Probes, USA). Then, the samples were kept in a 5% CO_2 oven at 37ºC for 30 min. After this period, the tubes were centrifuged, the supernatant discarded and the pellet resuspended in 100 µL of FAC's Flow buffer. The reading and analysis were performed on a FACS canto flow cytometer (BD) at FLH-1 fluorescence intensity and the histograms acquired and analyzed by the Cell-Quest program (BD).

2.5. Analysis of cell cycle phases by Flow Cytometry

The cells were subjected to treatments for 12 and 24h. The treated cells and the control group were trypsinized and centrifuged at 1400 rpm for 5 min. Then, the pellet was resuspended in a 70º alcohol solution and RNAse alcohol and stored in the -20ºC freezer for 24h. The samples were centrifuged at 3000 rpm for 10 min and resuspended in 200 µL of Fac´s buffer, 20 µL of Triton X-100 (Sigma-Aldrich) and 50 µg/mL of propidium iodide (Sigma-Aldrich), maintained by 30 min at room temperature, protected from light. After this period, the samples were transferred to cytometry tubes and taken for analysis in a FACScalibur (BD) flow cytometer at FLH-1 fluorescence intensity and the histograms acquired and analyzed by the Cell-Quest-BD program.

2.6. Statistical analysis

The data were expressed as mean ± deviation (SD). The Kruskal-Wallis test (one-way non-parametric ANOVA) and Dun's multiple comparisons were performed to identify the statistical differences between the measurements of the groups studied. The graphics were obtained using the software Prism version 5.0.

3. Results and discussion

3.1. Cytotoxic potential

The cytotoxic potential of the acetate and chloroform fractions were tested at different concentrations in 4T1 triple-negative breast tumor cells. Cell viability, after 24 and 48h, was analyzed by the MTT assay. Both fractions promoted cytotoxicity in various concentrations, triggering changes such as the formation of a precipitate of the acetate compound and the formation of cellular debris.

In the treatment with the acetate fraction in the periods of 24 and 48h, the $IC_{50\%}$ value was 4.2 and 2.2 µg/mL, respectively (Figure 1). Morphological changes were observed, such as cytoplasmic retraction and a significant reduction in population density at higher concentrations, as well as deposition of precipitate in the treatment (Figure 3). For the treatment with the chloroform fraction in the 24 and 48h, the $IC_{50\%}$ value was 5.9 and 3 µg/mL respectively (Figure 2). Morphological changes can also be observed, such as cytoplasmic retraction and loss of cell adhesion at lower concentrations, and a significant decrease in high treatment concentrations (Figure 4).

Cabral *et al.*, (2019) demonstrated that $IC_{50\%}$ value of fractions in normal cells such as human fibroblast cells (FN1) and human endothelium (HUVEC) is considerably high when compared to that obtained for tumor cells, reaching the $IC_{50\%}$ value of 13.1 and 12.2 µg/mL, respectively acetate and chloroform preparation in normal human fibroblast cells, for smooth muscle cells (HASMC), was obtained $IC_{50\%}$ 6.1 and 6.8 µg/mL and for vascular endothelial cells (HUVEC) the $IC_{50\%}$ was 7 and 8.2 µg/mL (Table 1) [13]. Selective cytotoxicity to muscle cells and endothelium represents a possible permeability to vascular structures, thus allowing the molecules to reach the tumor microenvironment.

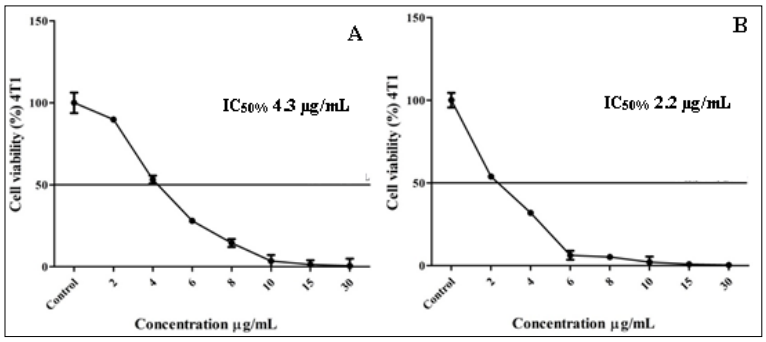


Fig 1: Determination of cytotoxicity by the MTT colorimetric assay. Dispersion plot of values of mean ± SD of the viability of the 4T1 triple negative-breast tumor cell after 24 and 48h of treatment with the acetate fraction. (A) 24h treatment (B) 48h treatment. Graphs obtained by the GraphPad Prism 5 software. (n=3).

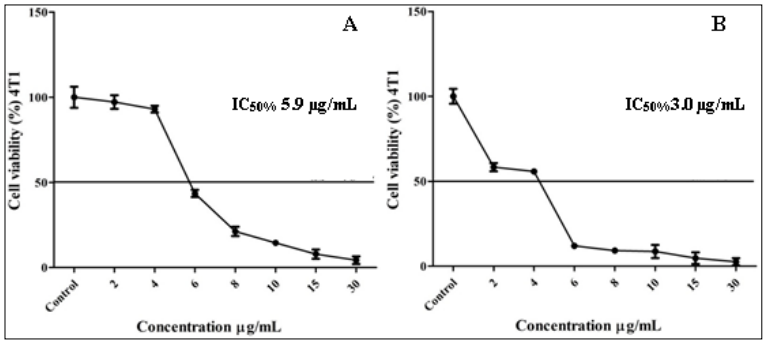


Fig 2: Determination of cytotoxicity by the MTT colorimetric assay. Dispersion graph of the values of mean ± SD of the viability of the 4T1 triple-negative breast tumor cell after 24 and 48h of treatment with the chloroform fraction. (A) 24h treatment (B) 48h treatment. Graphs obtained by the GraphPad Prism 5 software. (n=3).

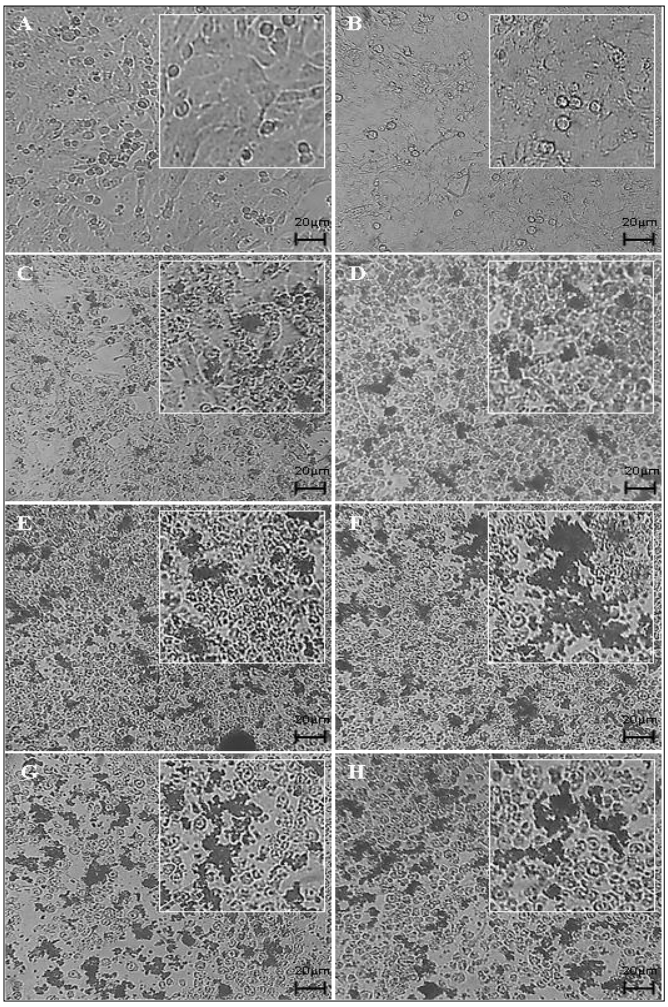


Fig 3: Photomicrographs of the morphological analysis of 4T1 triple negative breast tumor cells treated with the acetate fraction after 24h treatment. (A) Control (B) 2µg/mL (C) 4 µg/mL (D) 6 µg/mL (E) 8 µg/mL (F) 10 µg/mL (G) 15 µg/mL (H) 30 µg/mL.

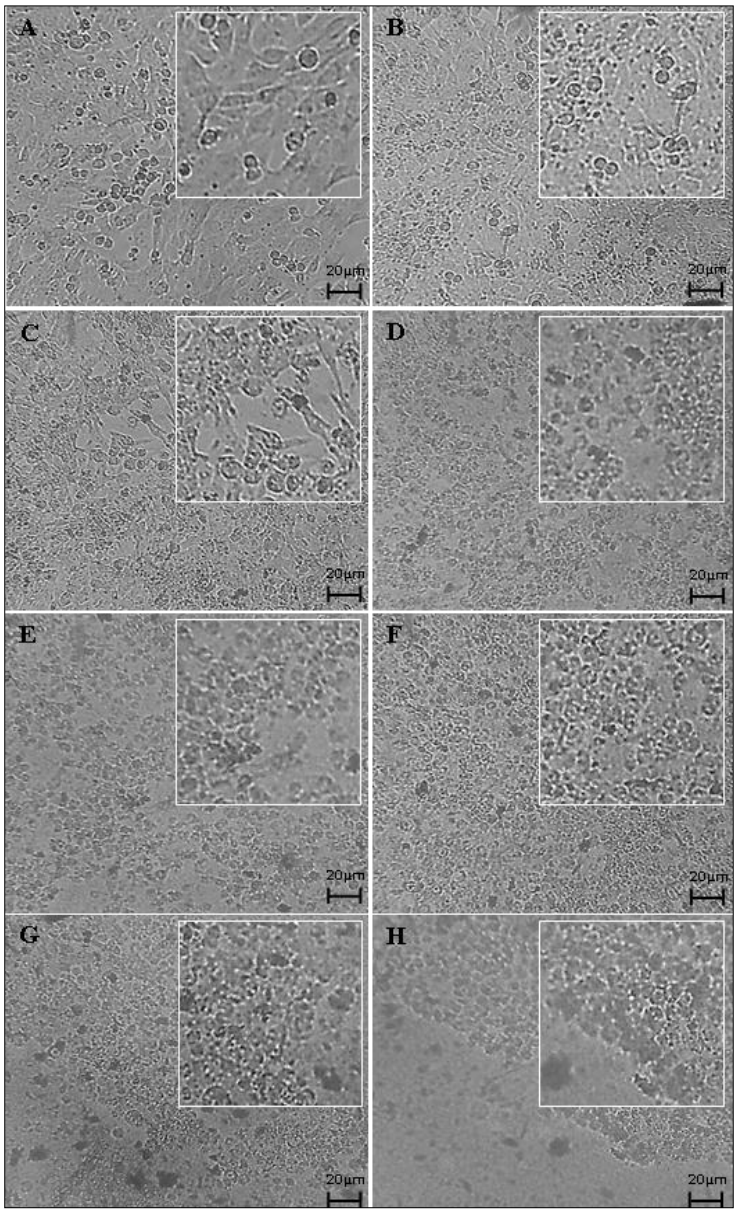


Fig 4: Photomicrographs of the morphological analysis of 4T1 triple negative breast tumor cells treated with the chloroform fraction after 24h treatment. (A) Control (B) 2µg/mL (C) 4 µg/mL (D) 6 µg/mL (E) 8 µg/mL (F) 10 µg/mL (G) 15 µg/mL (H) 30 µg/mL.

Table 1: Values of 50% inhibitory concentration ($IC_{50\%}$) in normal and tumor cells.

Compounds	Cells	$IC_{50\%}$ µg/mL	Compounds	Cells	$IC_{50\%}$ µg/mL
Acetate	Fibroblast Normal	13.1	Acetate	B16-F10 Melanoma	2.1
Chloroform		12.2	Chloroform		2.8
Dichlore		2.7	Dichlore		N/S
Ethanol		3.7	Ethanol		38.3
Ether		18.9	Ether		N/S
Methanol		29.5	Methanol		N/S
Acetate	HASMC Normal	6.1	Acetate	Hepa1c1c7 Hepatocellular Tumor cells	1.9
Chloroform		6.8	Chloroform		6.2
Dichlore		7.8	Dichlore		5.9
Ethanol		4.3	Ethanol		11.7
Ether		N/S*	Ether		7.5
Methanol		N/S	Methanol		7.3
Acetate	HUVEC Normal	7	Acetate	MDA MB-231 Breast Cancer	2
Chloroform		8.2	Chloroform		5.8
Dichlore		10	Dichlore		N/S
Ethanol		4.4	Ethanol		16.7
Ether		N/S	Ether		7.8
Methanol		N/S	Methanol		N/S

*N/S: No significant
Fonte: Cabral *et al.* (2019)

The cytotoxic effect of components extracted from plants of the genus Euphorbia can be explained by the presence of some steroid molecules such as sitosterol, lanosterol, and the higher concentration of eupol. The results obtained here are consistent and corroborate with Awad *et al.* and Athmouni *et al.*, who showed that the cytotoxic effect is attributed to steroid molecules [15-16]. Gupta *et al.*, also found that steroid molecules are compatible with the nuclear receptor and can act as enzyme inhibitors and cytotoxic molecules [17]. Nascimento *et al.*, reported that Euphorbia species present cytotoxic activityarising fromterpenoids [18].

Alcoholic extracts from *Mercuria lisannua* L. showed a cytotoxic and inhibitory effect on Burkitt's lymphoma (BJAB) and multiple myeloma (U266) tumor cells, being widely used in Jordan country for the treatment of leukemia [19]. Other studies have verified the antitumor effects of Euphorbia latex in murine B16-F10 melanoma cells [12-13]. Studies by Luz *et al.*, (2019) also evaluated the cytotoxic effects of various fractions of *E. umbellate* latex in human rectal adenocarcinoma HRT-18, in human cervical carcinoma HeLa and acute lymphoblastic leukemia cells Jurkat [7].

3.2. Evaluation of mitochondrial electrical potential (ΔΨm) by confocal microscopy and flow cytometry

4T1 triple-negative breast tumor cells were treated for 24h with the acetate and chloroform fractions in the $IC_{50\%}$ concentration and showed a considerable reduction in the mitochondrial electrical potential, observed by the reduction of fluorescence emitted by the Rodamina 123 probe, as well as morphological changes by confocal microscopy. The treatment with the acetate fraction showed greater modulation of the electrical potential and promoted significant structural changes in the mitochondria (Figure 5). Both treatments provided nuclear changes. The analysis of electrical potential by flow cytometry also showed similar results for both treatments in the 24h, the control showed 90.4±2.2% of viable mitochondria, the treatments had a modulation of this potential, with the fraction reduction to 62.5 ± 1.8%, with chloroform the reduction was 72.7 ± 2.4% (Figure 6).

The ability of fractions extracted from latex from plants of the Euphorbiaceae family to modulate ΔΨm has already been reported in the literature [18-20]. *E. antiquorum* latex was able to promote the loss of ΔΨm in HeLa tumor cells, increasing the levels of reactive oxygen species. It was also observed that this extract increased the expression of active proteins and caspases, and proapoptotic proteinsFas, FasL, JNK, p38, and MAPK 8, 9, and 3 [18]. Other studies show that *E. antiquorum* latex was able to modulate the activation of the programmed cell death mechanism. Assaf *et al.*, (2013) showed that the cytotoxic effect of the methanol subfraction of the hexane fraction, extracted from the latex of *E. umbellata*, in hepatocellular carcinoma (HCC) cells [19]. In this study, Hepa1c1c1 tumor cells treated with methanol subfraction showed a reduction of ΔΨm due to the modulation of the apoptotic pathway. Other studies have shown that the dichloromethane subfraction is promising for the treatment of leukemia, possibly due to the synergistic action of the present terpenes, and the modulation of ΔΨm [20].

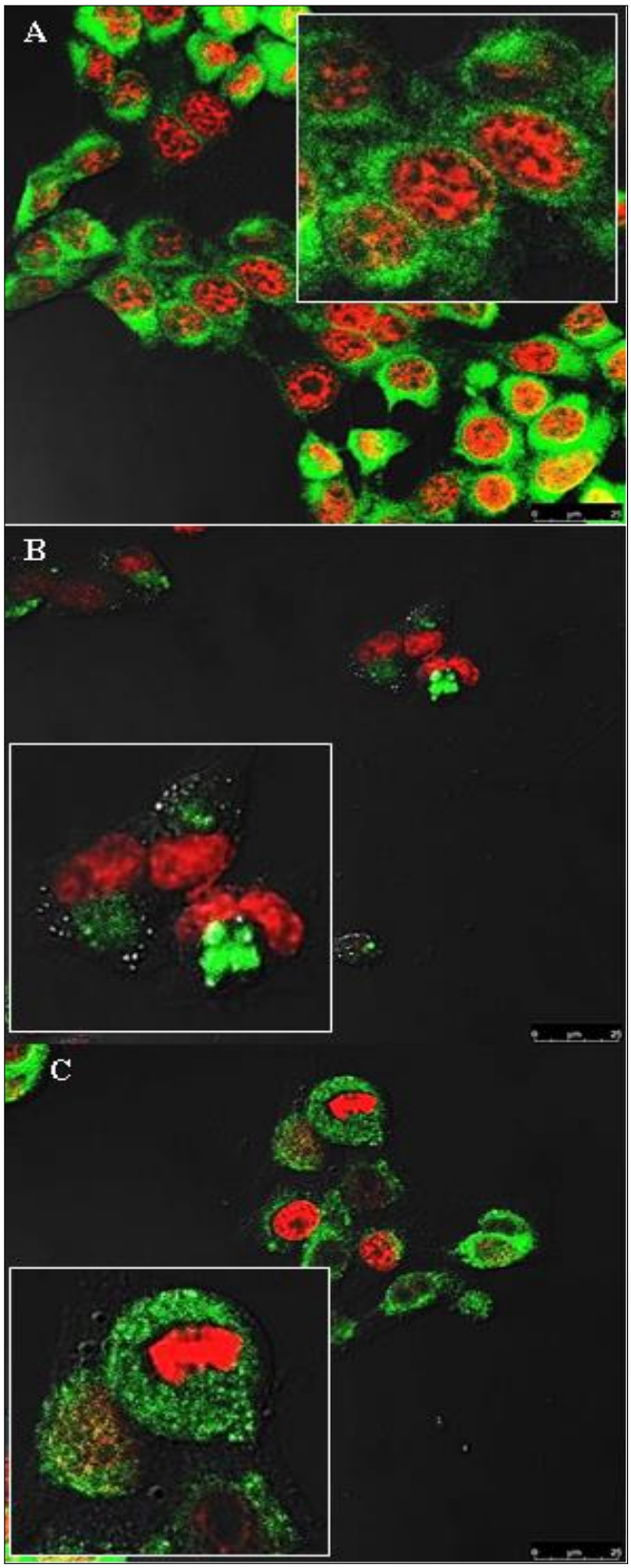
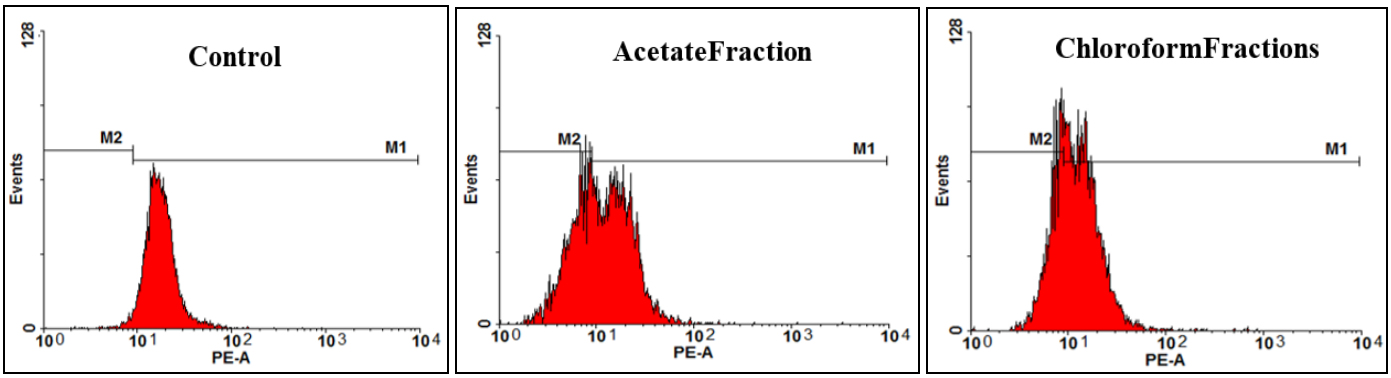
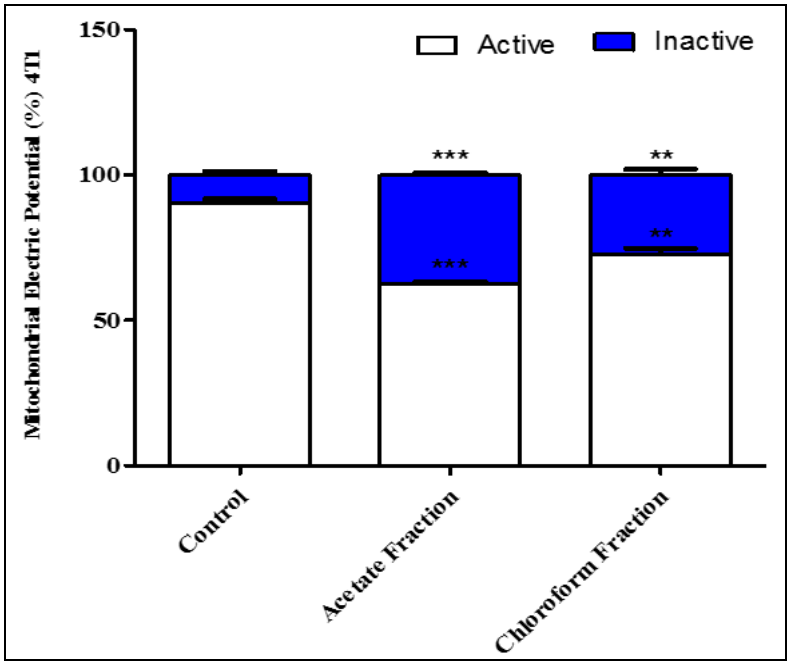


Fig 5: Photomicrographs of 4T1 tumor cells with mitochondria marked with Rhodamine 123 (green) and Propidium Iodide (Red) analyzed by laser confocal microscopy. Cells treated with acetate and chloroform after 24h treatment. (A) Control; (B) Acetate 4.2 µg/mL; (C) Chloroform 5.9 µg/mL.



(a)

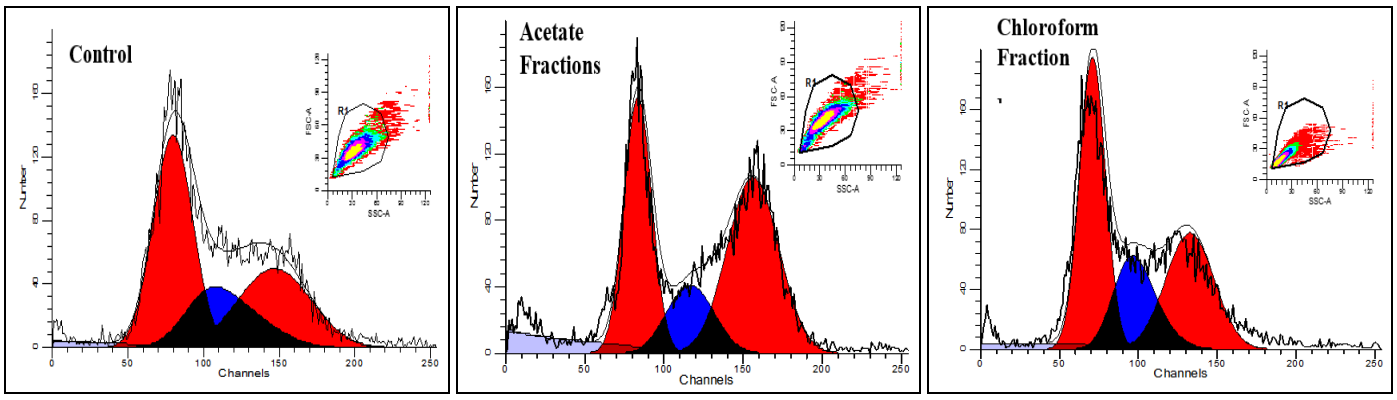


(b)

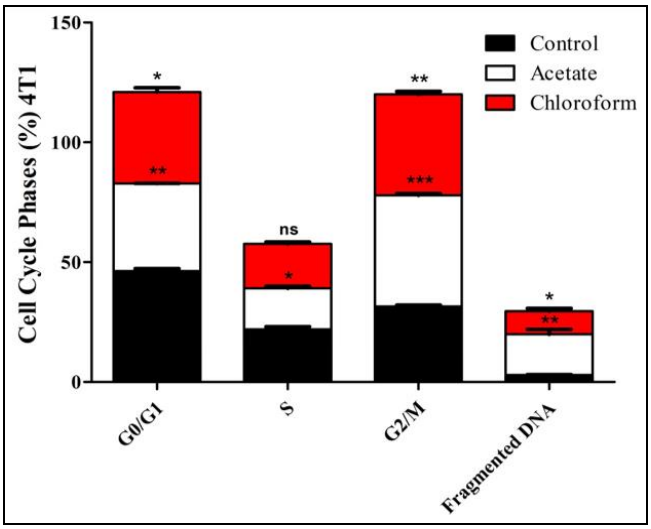
Fig 6: Determination of mitochondrial electrical potential (ΔΨm) by flow cytometry. Tumor cells 4T1 treated with acetate and chloroform after 24h treatment. **(**A) Histograms representative of the distribution of cell populations with active and inactive mitochondria. (B) Bar graphs of values of mean ± SD of the percentage of the mitochondrial potential of 4T1 tumor cells. Significance values with p* <0.05 and p*** <0.01, obtained by the ANOVA variation test followed by the Turkey-Kremer multiple tests. Experiments performed, n = 3

3.3. Analysis of the cell cycle phases

The results obtained showed after the acetate and chloroform fractions show significantly modulated inthe distribution of cell population (Figure 7A-B). Treatment with acetate promoted an increase in fragmented DNA when compared to the chloroform fraction. There is also a significant increase in cells in the G2/M phase of the cell cycle for both treatments and a reduction in the G0/G1 phase, only the group of cells treated with acetate showed a reduction in the synthesis phase (S) (Figure 7A-C).



(a)



(b)

Fig 7: Determination of the cell cycle phases by flow cytometry. Tumor cells 4T1 treated with acetate and chloroform after 24h treatment. **(A)** Histograms representative of the distribution of cell populations in the cell cycle phases. Obtained by the Mod Fit software. **(B)** Bar graphs of the mean ± SD values of the percentage of cells in the cell cycle phases. Significance values with p* <0.05 and p*** <0.01, obtained by the ANOVA variation test followed by the Turkey-Kremer multiple tests. Experiments performed n = 3

This effect may be due to the cell arrest at the G2/M phase checkpoint due to the chemical composition of *E. umbellata* fractions such as the presence of alkaloids [14, 20]. This checkpoint is put into operation as a safety mechanism to ensure that changes in the molecular structure of DNA or its decomposition, which would cause harmful changes, do not occur before being repaired, contributing to the maintenance of genomic stability and transmission of DNA mutation to daughter cells [21-22].

4. Conclusion

The results show that the acetate and chloroform fractions obtained from *E. umbellata* present cytotoxicity to the 4T1 triple-negative breast tumor cells, promote a cell death-inducing mechanism regulate by the reduction of the mitochondrial electrical potential and cell arrest in the cell cycle phases. The death cell mechanism results in the disruption of DNA/chromatin, cause instability, and regulate pathway cell death. Our results suggest that the cytotoxicity of the fractions occurs due to the modulation of the apoptotic pathway, a fact that corroborates with compounds already identified by the presence of alkaloids groups. Thus, the set of results demonstrated that the acetate and chloroform fractions have potential antiproliferative in breast tumor cells.

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