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Antitumor potential of *Euphorbia umbellata* latex fractions and subfractions

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Abstract
The objective of this work was to evaluate the antitumor potential of the acetate and chloroform fraction and its subfractions in methanol, dichloro, ether and ethanol extracted from *Euphorbia umbellata*. The cytotoxicity of these fractions and subfractions was evaluated by the MTT colorimetric assay after 24h at different concentrations ranging from 2-30 µg/mL, also the mitochondrial electrical potential by confocal microscopy. In addition, the cell cycle, the percentage of DNA fragments and the mitochondrial electrical potential, was quantified by flow cytometry. In this context, our results suggest that the cytotoxicity of fractions and subfractions occurs due to modulation of the apoptotic pathway, a fact that corroborates with already identified compounds of chemical alkaloid groups, the fractions being separated by acetate and chloroform as well as the ether and methanol subfractions cytotoxic effects for the tumor cells studied.

Keywords: Euphorbiaceae; *Euphorbia umbellata*; cancer; cytotoxicity

1. Introduction
The development of new specific drugs for the treatment of cancer had priority of survival. Despite advances, cancer is responsible for the highest death rate in the world today [1]. The organization is global health - Who (2018), cancer is responsible for cases of death in the world being responsible for 9.6 million deaths in 2018.
According to the article published in the Economist Intelligence Unit (2017), which had as main control measures on Latin America, the current weight of deaths in Latin America. As already 2012 and 2035, the number of cases is 91% and mortality of 106% during this period [2].
In Brazil, the National Cancer Institute (INCA) for the 2018-2019 biennium, an occurrence of 600 thousand new cases of neoplasia for each year. With the exception of 170 thousand cases of diagnosed cases, there was an episode of 420 thousand new forms of cancer. The most well recorded men of the prostate (68,000) in men and the mother (60,000) in women [3]. Thus, a diagnosis is paramount for an adequate and efficient treatment. Bioactive plants or their extracts are emerging rapidly as an alternative source of new anticancer medicines. Plant products may have high antitumor efficiency with or without collateral [4]. An example is resveratrol (3,4,5-trihydroxy-transstilbene) is a polyphenolic phytoalexin together in plants [5]. Cellular cytotoxic cells have been shown to inhibit tumor cell proliferation in several cell types and tumor cell models [5, 6]. The plant *E. umbellata* (Pax) Bruyns, Euphorbiaceae [7], popularly known as "janaúba" and "cola-nota" in Brazil, produces a latex that has been used in folk medicine as an anti-inflammatory agent, antiulcer, homeostatic and antiangiogenic, and main as an antitumor agent [8, 9, 10]. In Southern Brazil, the bottled latex of *E. umbellata* is popularly used as a treatment for all types of cancer [11]. Recent studies with fractions from *E. umbellata* latex have demonstrated their antitumor potential in murine melanoma cells (B16F10), ileocecal colorectal adenocarcinoma (HCT-8), human cervical cancer (HeLa) and Jurkat cells [11]. The latex fractions from this plant were isolated for pharmacological evaluation: hexane, chloroform, ethyl acetate and methanol. Studies show that all fractions evaluated have cytotoxic potential alone, depending on the concentration and period of exposure of the cell to the compound. However, among those fractions with the highest cytotoxicity was the Hex fraction, with the possibility that other substances present in the hexane fraction promote anticancer activity [9]. Therefore, to identify which compound (s) present in the latex of *E. umbellata*, which may promote antitumor activity in B16-F10, hepa1c1c7 and MDA MB-231 is of great importance.

In this context, the present study aimed to evaluate the antitumor potential of acetate and chloroform fraction and its subfractions in methanol, dichloro, ether and ethanol.

2. Methodology

2.1 In vitro assays

2.1.1. Cell culture

Hepatocellular tumor cells [Hepa1c1c7](ATCC® CRL-1730™), melanoma [B16-F10](ATCC® CRL-6475™), breast cancer [MDA-MB231](ATCC® CRM-HTB-26™), and normal human fibroblast (FN-1), normal vascular endothelium (HUVEC)[ATCC® CRL-1730™] and smooth muscle cells [HASMC](ATCC® PCS-100-012™] used in this study belong to the cell bank of the Laboratory of Molecular Biology of the Butantan Institute, under the responsibility of Professor Dr. Durvanei Augusto Maria. Cells were cultured in RPMI-1640 (FN-1) culture or in αMEM (Hepa1c1c7), pH 7.2, supplemented with 10% inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% antibiotics (10,000 IU/mL penicillin and 10 mg/mL streptomycin). The 75 cm^2 culture flasks were incubated for incubation of cells containing 5% CO_2 humid atmosphere at 37ºC. After reaching approximately 90% confluency. Prior to the execution of the experiments, the cells were counted in Neubauer's chamber using the blue dye of Tripan (1%), which does not penetrate viable cells that have intact cell membrane. Only cells with viability equal to or greater than 90% were used in the assays.

2.1.2. Evaluation of cytotoxicity by the colorimetric method of MTT

Cell viability was assessed by the colorimetric reduction test of MTT [3- (4,5-dimethylthiazol-2-1) 2,5-diphenyltetrazolium bromide]. Cells were seeded in 96-well plates at $2x10^4$ and quadruplicate density and cultured for 24h in a greenhouse containing 5% CO^2 at 37 ºC. After the incubation period, the acetate and chloroform fraction and its subfractions in methanol, dichloro, ether and ethanol, extracted from the latex of *E. umbellata* were treated for 24h at different concentrations (2–30μg/mL). After the treatment, 10 μL of MTT reagent (5 mg/mL) was added. After 3h of incubation, the supernatant was removed and 100 μL of DMSO added for dissolution of the formazan crystals. Absorbance quantification was performed in ELISA reader (Thermo Plate), at wavelength of 540 nm, for determination of inhibitory concentration ($IC_{50\%}$).

2.1.3. Analysis of mitochondrial electrical potential by laser confocal microscopy

For analysis of the mitochondrial potential using the confocal microscopy technique, Hepa1c1c7, B16-F10 and MDA-MB231 cells were plated on round coverslips in 24-well plates at the concentration of 1 x 10^5/well. The next day, the cells were $IC_{50\%}$ obtained over the 6h period. After treatment, cells were washed three times with αMEM culture medium (Cultilab, Campinas, SP, Brazil) at 37 °C and incubated with 15 μg/mL rhodamine-123 (Assay Designs Inc., Ann Arbor, MI, USA) for Hepa1c1c7 and MDA MB-231, for B16-F10 Rhodamine 6G, for 10 min in the dark at 37 °C. The excess rhodamine was removed by washing with culture medium at 4°C. Assembly of the slides was performed with a clamp, with which the coverslips were transferred to the glass slides, being fixed with ProLong® and stored at -20 °C in the dark until the time of reading in the confocal microscope a laser (Carl Zeiss LSM 700; Leica, Mannheim, Germany).

2.1.5. Analysis of the cell cycle phases by flow cytometry

Hepa1c1c7, B16-F10, MDA-MB231 and FN-1 cells were plated in 6-well plates at the concentration of $1x10^5$. The next day the cells were treated with the concentration obtained by $IC_{50\%}$ for 12h. After the incubation period, the cells were counted and the concentration adjusted to 100,000 cells/mL. The cells were then centrifuged for 10 min (1400 rpm) and the pellet resuspended in 1 mL of "Cold GM" (6.1 mM glucose, 137 mM NaCl, 4.4 mM KCl, 14 1.5M Na_2 HPO_4, KH_2 PO_4, 9 mM EDTA, 0.5 mM EDTA). Thereafter, absolute ethanol (4 °C) was slowly added, stirring slowly. The cells were fixed for 30 minutes and then centrifuged, washed with PBS containing 5 mM EDTA (Sigma Aldrich, USA) and resuspended in a solution containing PBS (5 mM EDTA), 18 μg/mLpropidium iodide (Sigma Aldrich, USA) and 0.3 mg/mL RNase-A. Cells were kept at room temperature for 1h in the dark. The reading was performed by cytometry (FASC Calibur BD, USA) on the FL2-H channel. The obtained results demonstrated in which period of the cell cycle the cells were found and the data were presented in percentage software analysis Modfit.

2.2 Statistical analysis

Numerical values were expressed as mean ± standard deviation (SD). The Kruskal-Wallis test (one-way non-parametric ANOVA) and multiple Dun comparisons were performed to identify the statistical differences between the measures of the groups studied. The graphs were obtained through the Prism program version 5.0.

3. Results

3.1 Cytotoxicity study

The cytotoxic potential of the acetate and chloroform moieties and their subfractions in methanol, dichloro, ether and ethanol were tested at different concentrations in human fibroblast (FN-1), vascular endothelial (HUVEC) and smooth muscle cells (HASMC). Cell viability, after 24h of treatment, was analyzed by the MTT assay. Subfractions that promoted higher cytotoxicity in FN-1, HUVEC and HASMC cells were dichloro and ethanol (Table 1). These subfractions promoted morphological changes from the initial concentrations; in the higher concentrations they precipitate the formation and the total cellular invisibilization. For the other fractions and subfractions tested, it reached a considerably high value when compared to the $IC_{50\%}$ obtained for tumor cells. It was less cytotoxic for the ether and methanol subfractions, reaching $IC_{50\%}$ of 18.9 and 29.5 μg/mL and for acetate and chloroform fractions with $IC_{50\%}$ of 13.1 and 12.2 μg/mL in FN-1 cells, HASMC cells in the largest concentrations for treatments with the ether and methanol subfractions reduced cell viability by 40 and 29%, respectively, in the treatments with acetate and chloroform fractions, the $IC_{50\%}$ was 6.1 and 6.8 μg/mL (Table 1). The treatments with the ether and methanol subfractions only promoted reduction of 14 and 20% of cell viability in the highest concentrations and for the acetate and chloroform fractions $IC_{50\%}$ of 7 and 8.2 μg/mL were obtained for the HUVEC cells (Figure 1C). B16-F10 cells showed greater resistance to treatment with the fractions and subfractions tested, where only two of the tested compounds presented cytotoxic potential. In the 24-hour treatment period with the acetate and chloroform fractions, cytotoxicity was observed with $IC_{50\%}$ of 2.1 and 2.8 μg/mL, respectively (Table 1), showing marked changes in its morphology, cytoplasmic retraction and significant reduction population density. In addition, the precipitates formed at higher concentrations

(Figure 2). For other compounds such as ether and methanol, the $IC_{50\%}$ obtained for B16-F10 was 144 and 138.5 µg/mL, respectively (Figure 1D). Treatment with dichloro subfraction, even with treatment sensitivity in the same period, does not become feasible because it showed a high cytotoxicity for normal human fibroblast cells. Taking into account the tumor cells used in the tests, Hepa1c1c7 showed higher cytotoxicity being sensitive to four of the compounds, with acetate fractions with $IC_{50\%}$ 1.9µl/mL and chloroform with $IC_{50\%}$ 6.2 µg/mL, subfractions of ether with $IC_{50\%}$ 7.5 µg/mL and methanol with $IC_{50\%}$ 7.3 µg/mL (Table 1). Photomicrographs show morphological changes, such as cytoplasmic retraction after exposure to fractions and subfractions (Figure 3).

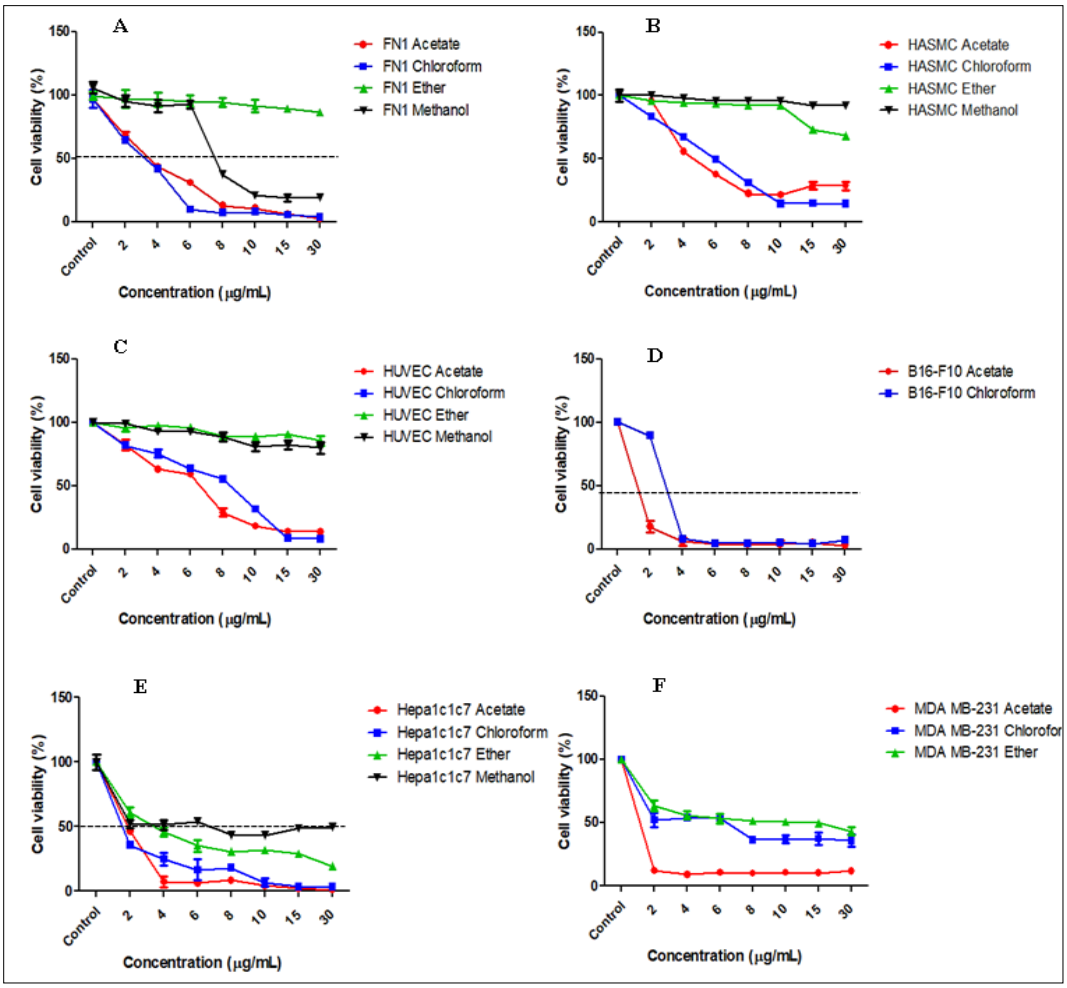

Fig 1: Determination of cytotoxicity by the MTT colorimetric method. The comparative plot shows the correlation of the cytotoxic effect expressed in mean ± SD of three independent experiments. Treatments in different concentrations in the 24-hour period. (A) FN1 (B) HASMC (C) HUVEC (D) B16-F10 (E) Hepa1c1c7 (F) MDA MB-231.

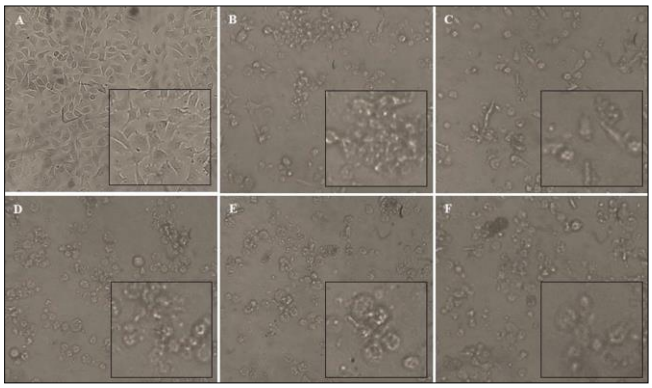

Fig 2: Photomicrographs of the morphological analyzes of the B16-F10 cells treated with the acetic fraction in the 24h period. (A) Control (B) 2 µg/mL(C) 4 µg/mL(D) 6 µg/mL(E) 15 µg/mL(F) 30 µg/mL.

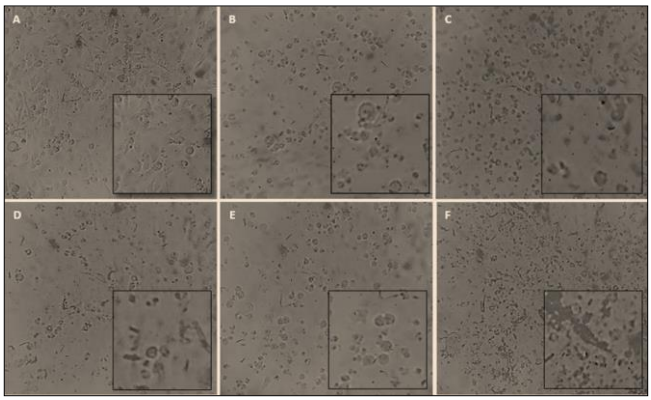

Fig 3: Photomicrographs of the morphological analyzes of the Hepa1c1c7 cells treated with the acetate fraction in the 24h period. (A) Control (B) 2 µg/mL(C) 4 µg/mL(D) 6 µg/mL(E) 15 µg/mL(F) 30 µg/mL.

The ethanol subfraction induced cytotoxicity, but was not dose dependent, significant changes occurred in the morphology of Hepa1c1c7 cells, as cytoplasmic retraction, and also the formation of cellular precipitate in the treatments at the highest concentrations. Triple-negative breast cancer cells MDA-MB231 were sensitive to three compounds. For

treatment with the acetate fraction, the cells lost on average 90% viability in the first concentration, obtaining $IC_{50\%}$of 2 µg/mL. The chloroform fraction and the ether subfraction have considerable cytotoxicity with $IC_{50\%}$ of 5.8 and 7.8 µg/mL, respectively. In the photomicrographs of the cells treated with acetate it is possible to visualize in the first concentrations the unviability of more than 90% of the cells, the cytoplasmic retraction, the loss of cell adhesion and in the higher concentrations precipitate the formation (Figure 4). Similar results were observed for chloroform and ether.

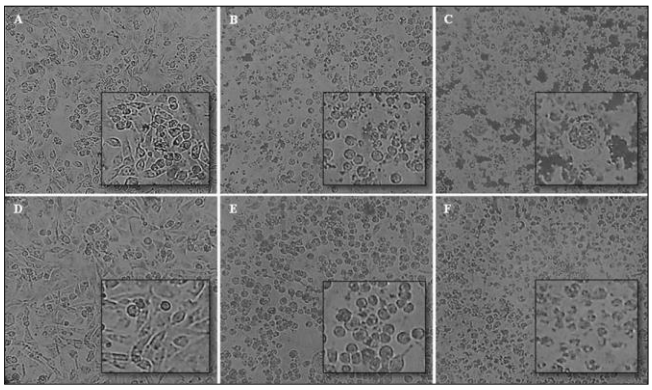

Fig 4: Photomicrographs of the morphological analyzes of the MDA MB-231 cells treated with the acetate fraction in the 24h period. (A) Control (B) 2 µg/mL(C) 4 µg/mL(D) 6 µg/mL(E) 15 µg/mL(F) 30 µg/mL

Plants of the genus Euphorbia are known to possess high levels of bioactive compounds, and it is probable that some species may present biological activity capable of promoting a clinical improvement in some pathological situations such as cancer. Several studies have evidenced the cytotoxic effects of the Euphorbiaceae family on different tumor types [12-15]. Studies have shown positive results for extracts from plants of the family Euphorbiaceae, in which extracts of *Mercurialis annua* L. showed cytotoxic and inhibitory effect for burkitt lymphoma (BJAB) and multiple myeloma (U266) cells. These extracts are commonly used in Jordan for the treatment of leukemia [12]. Showed that the methanol extract of *Viscumcruciatum* was selective against Burkitt BJAB lymphoma cells, presenting $IC_{50\%}$ of 14,21µg/mL [12]. Other studies have verified the antitumor effects of latex of *Synadeniumgrantii* Hook f. in B16-F10 murine melanoma cells and in mice bearing B16-F10 melanoma.

Recent studies have also evaluated the cytotoxic effects of various fractions of *E. umbellata* latex on HRT-18 human rectal adenocarcinoma, HeLa human cervical carcinoma and Jurkat acute lymphoblastic leukemia cells [9]. Further work with *E. umbellata* shows that for the B16-F10 melanoma cells the dichloro, ethanol, ether and methanol subfractions obtained high $IC_{50\%}$. However, fractions such as chloroform and acetate significantly [16].

Table 1: 50% inhibitory concentration values ($IC_{50\%}$) for normal and tumor cells.

Compounds	Cells	$IC_{50\%}$	Compounds	Cells	$IC_{50\%}$
Acetate	Fibroblast	13.1	Acetate	B16-F10	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	6.1	Acetate	Hepa1c1c7	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	7	Acetate	MDAMB-231	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: Not Significant

3.2. Evaluation of mitochondrial electrical potential (ΔΨm)

Normal cells FN1, HASMC and HUVEC treated with fractions of acetate, chloroform, ether and methanol for 6 h at 50% concentrations showed no significant modulation of mitochondrial electrical potential change in the treatment period. For the subfraction of ether there was a slight reduction of the fluorescence, in relation to the control group, for the other compounds there was modulation of ΔΨm. Positive labeling of the treated cells and the original conformation of the maintained cell demonstrates that the mitochondria are functional and therefore the cells are metabolically active.

For B16-F10 melanoma cells, there is a considerable reduction of ΔΨm. Treatment with the acetate fraction showed a marked reduction in ΔΨm with the presence of condensed mitochondria in the cytoplasm of some cells and a slight reduction in the cell population. When analyzed in relation to chloroform, it showed a significant reduction in ΔΨm, with a greater reduction in the cell population. There were no significant changes in morphology (Figure 5).

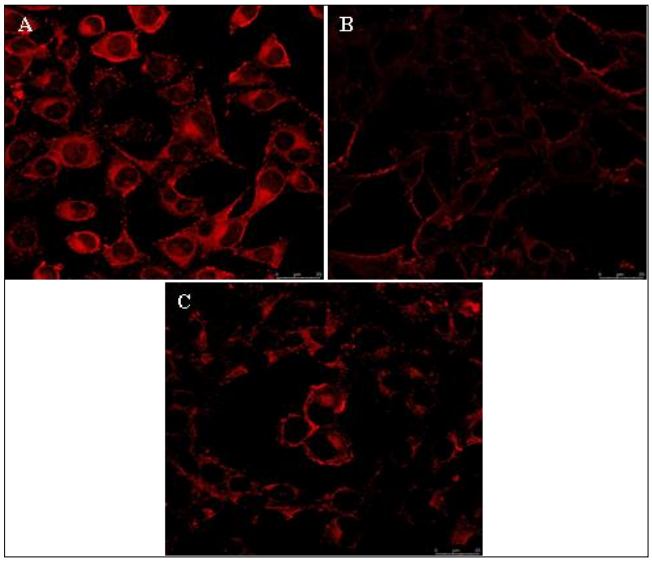

Fig 5: Photomicrographs of B16-F1 cells with 6G rhodamine-labeled mitochondria (red), analyzed by laser confocal microscopy over a period of 6 hours. (A) Control; (B) Acetate 2.1µg/mL; (C) Clofrom 2.8µg/mL.

Hepa1c1c7 cells treated with the acetate and chloroform fractions, as well as the ether and methanol subfractions, showed considerable modulation in mitochondrial electrical potential. For treatment with the acetate and chloroform fractions, a reduction of ΔΨm is observed when compared to the control group that showed high emission of fluorescence and mitochondria dispersed in the cytoplasm, with a redistribution of the mitochondria in the treated cells, with a considerable reduction in their viability (Figure 6). Another characteristic is the reduction of the cellular population followed by conformational change. The same is true for the subfractions, with a more drastic reduction in the cell population, and a marked conformational change, leading to the belief that the cell presented greater sensitivity for the subfractions.

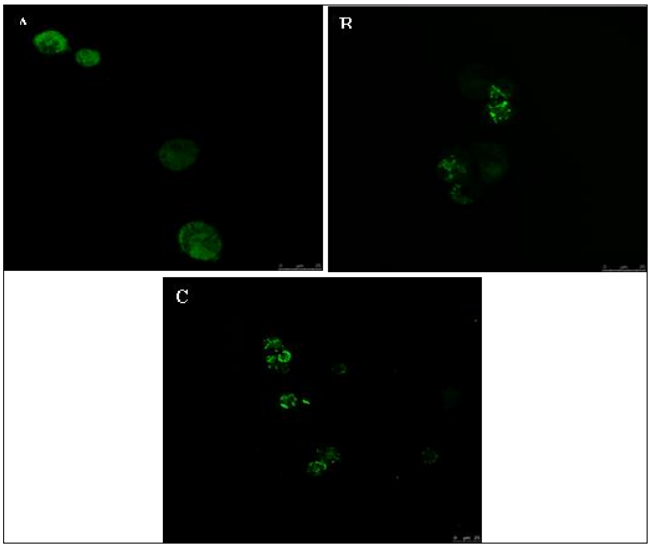

Fig 6: Photomicrographs of Hepa1c1c7 cells with 123 rhodamine-labeled mitochondria (green), analyzed by laser confocal microscopy over a period of 6 hours. (A) Control; (B) Ether7.5µg/mL; (C)Methanol7.3µg/mL.

Modulation at ΔΨm and conformational change with cytoplasmic retraction of MDA-MB231 cells are well accentuated in the treatments with the acetate and chloroform and ether subfraction fractions. The acetate and chloroform fractions promoted a greater reduction in the mitochondrial electrical potential than the ether subfraction when compared to the fluorescence emitted by the control group. Both presented cytoplasmic retraction, with circular cells, leading to believe that the cells were sensitive to the treatment, losing its viability (Figure 7).

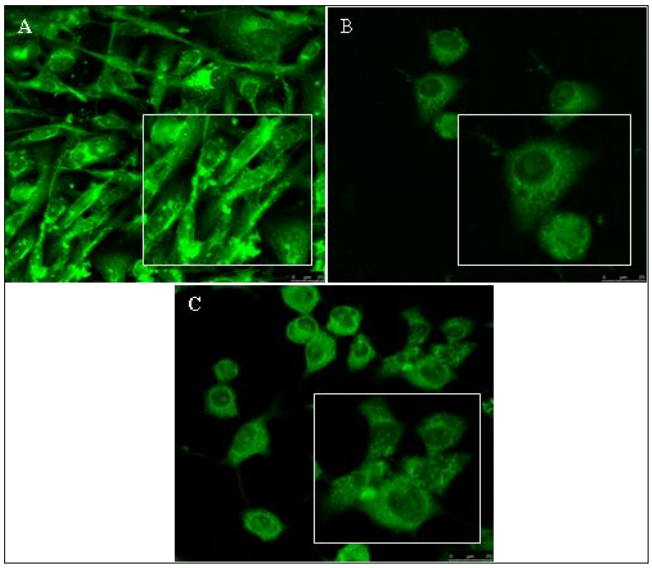

Fig 7: Photomicrographs of MDA MB-231 cells with 123 rhodamine-labeled mitochondria (green), analyzed by laser confocal microscopy over a period of 6 hours. (A) Control; (B)Acetate2µg/mL; (C)Chloroform5.8µg/mL.

An important parameter of mitochondrial functionality is ΔΨm [14, 17]. The ability of plant latex from the Euphorbiaceae family to modulate ΔΨm has already been reported in the literature. Studies have shown that *E. antiquorum* latex was able to promote ΔΨm loss in HeLa cells, increasing levels of reactive oxygen species. It was also observed that this extract induced the increase of Fas, FasL, JNK, p38 and MAPK proteins and caspases 8, 9 and 3 [18]. Thus, these results proved that *E. antiquorum* latex was able to modulate the activation of the programmed cell death mechanism. Showed the cytotoxic effect of the methanol subfraction from the Hexane fraction, extracted from the *E. umbellata* latex, in hepatocellular carcinoma cells (HCC) [19]. In this study, the Hepa1c1c1 line treated with the methanol subfraction showed a reduction of ΔΨm due to modulation of the apoptotic pathway. In another study [20], concluded that the dichloromethane subfraction is promising for the treatment of leukemia, possibly because of the synergistic action of the terpenes present in it, where there was also modulation of the ΔΨm of the cells tested.

3.3. Analysis of Cell Cycle Phases

The results showed that acetate and chloroform fractions significantly increased the population of cells with fragmented DNA after treatment for all test tumor cells (Figure 8A-C). There is also a considerable increase in cell paresis in the G2/M cycle cell phase (Figure 8A-C). Results were obtained for B16 cells, Hepa1c1c7 and MDA MB-231 when using the acetate and chloroform fractions, as well as the ether and methanol subfractions for Hepa1c1c7 cells (Figure 8A-C). Cells treated with subfraction of acetate and chloroform in relation to the other fractions and subfractions presented higher percentage of fragmented double-stranded DNA (Figure 8).

This effect may have occurred by stopping the cells at the control point between the S and G2/M phases. This control point is put into operation as a safety mechanism to ensure that changes in the molecular structure of DNA or its breakdown, which would cause harmful changes, do not proceed before being repaired, contributing to the maintenance of genomic stability and mutation transmissions to the daughter cells [21, 22].

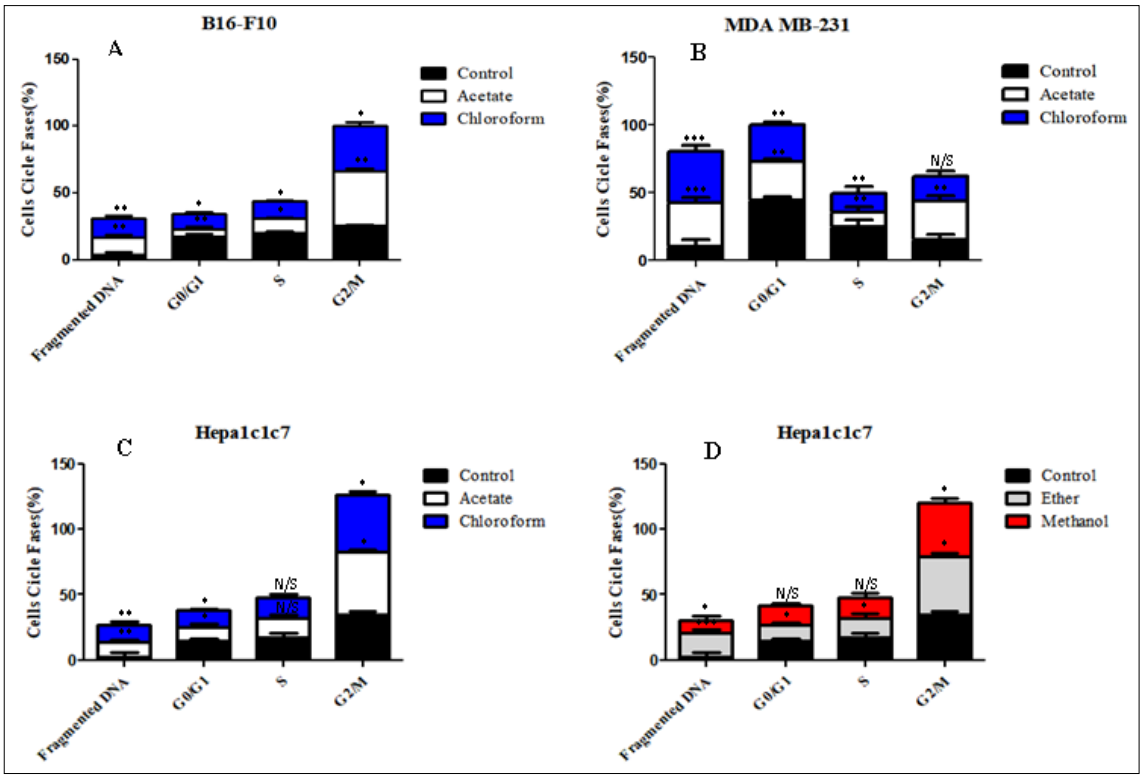

Fig 8: Determination of phases of the cell cycle. Cells treated with IC 50% values within 24 h.(A) B16-F10treated with acetate and chloroform; (B)MDA MB-231 treated with acetate and chloroform; (C)Hepa1c1c7 treated with acetate and chloroform (D) Hepa1c1c7 treated with ether and methanol. Statistical significance level n/s: not significant *p<0.05, **p<0.01 e ***p<0.001.

4. Conclusion

The results showed that the dichloroethanol subfractions did not show selectivity, since they induced cytotoxicity in similar proportions in Hepa1c1c7 and normal FN-1 cells, whereas B16-F10 and MDA MB231 cells did not present considerable cytotoxicity, taking into account the $IC_{50\%}$ higher than for normal FN-1 cells. In contrast, acetate and chloroform fractions showed selective cytotoxicity for B16-F10 cells. The same acetate and chloroform fractions, as well as the ether and methanol subfractions, promoted cytotoxic activity in Hepa1c1c7 cells, without triggering cytotoxicity in normal FN-1 cells at the same concentrations and treatment periods. For MDA MB231 cells the acetate, chloroform and ether subfraction fractions showed relevant cytotoxicity for the period tested. In addition, both compounds modulated the mitochondrial electrical potential of the cells to which they were applied, without inducing changes in the normal fibroblast cells FN-1. The compounds tested also caused a G2/M phase stop of the cell cycle in tumor cells with high concentration of fragmented DNA. In this context, our results suggest that the cytotoxicity of fractions and subfractions occurs due to the modulation of the apoptotic pathway, a fact that corroborates with already identified compounds of chemical alkaloid groups. Thus, the set of results demonstrated that the acetate and chloroform fractions; the ether and methanol subfractions present potential for the treatment of the tumor cells tested in the work.

5. Acknowledgements

Not applicable

6. Conflict of interest Statement

The authors declare that there are no conflicts of interest.

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Higher Education Personnel Improvement Coordination - CAPES

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