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Antiproliferative response induced *Euphorbia umbellata* fractions and subfractions in melanoma and hepatocellular carcinoma tumor cells

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Abstract
Resistance to the therapies currently offered for melanoma and hepatocellular carcinoma requires new research that seeks more effective therapeutic agents with less toxicity. The present study aimed to evaluate the antiproliferative, antitumor effects and the modulation of the mitochondrial electrical potential of the Acetate and Chloroform fractions and the sub-fractions Methanol, Ethanol, Dichloromethane and Ether, isolated from *E. umbellata* latex sap in murine melanoma cells (B16 -F10), hepatocellular carcinoma tumor cells (Hepa1c1c7) and normal fibroblast cells (FN1). The Acetate and Chloroform fractions showed significantly cytotoxic potential for tumor cells B16-F10 and Hepa1c1c7, in addition to the fractions being sensitive to the ether and methanol compounds. Such compounds promoted the reduction of cell confluence, the modulation of mitochondrial electrical potential, the depolarization of the mitochondrial membrane, and the release of pro-apoptotic mechanisms that resulted in the death of both tumor lines.

Keywords: *E. umbellata*, breast cancer, apoptosis, mitochondrial, cytotoxicity

1. Introduction
Cancer is the second leading cause of death worldwide. Overall, about 1 in 6 deaths is due to cancer. According to the International Agency for Research on Cancer, the incidence of cases for the year 2040 will reach 29.5 million with an estimated 16.4 million deaths. Estimating for melanoma and hepatocarcinoma skin cancer in the year 2020, 301.6 thousand, and 883.9 thousand cases, with a forecast for 2040 of 466.9 thousand and 1.3 million cases [1].
The mutational burden of melanoma is one of the highest among all malignancies, this high frequency of mutation can be attributed to wide exposure to well-known carcinogens, such as ultraviolet radiation. In addition to this association, melanoma is notable for its metastatic propensity and lethality [2, 3]. Its incidence rate (number of cases) is about1/10 of the certification application as non-melanoma skin cancer (NMSC), butits death toll is ~ 8 times higher than the NMSC [4].
Although considered chemo resistant, Dacarbazine has long been the only option for treatment for melanoma, with 20% response, being a chemotherapy combined with the one that produced better responses, although associated with greater toxicity and not prolong survival. Currently, immunotherapy has gained space for the treatment of melanoma, producing more lasting responses, however in a small percentage of patients [5, 6].
In turn, hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide [7]. However, ineffective treatment and poor prognosis are common due to a lack of knowledge about the mechanism of hepatocarcinogenesis [8]. Its early diagnosis, correlated with a good prognosis, allows a more effective treatment through resection, transplantation or ablation, for those who are in an intermediate stage, the use of chemotherapy [9, 10]. However, in more advanced stages, the treatment used is Sorafenib, which allows only an increase in survival of 2.3 to 2.8 months [11].
Because the resistance to the therapies currently offered for both strains, research that seeks effective therapeutic agents that present less systemic and tissue toxicity is necessary. Studies carried out with bioactive extracts have recently been described in the literature as therapeutic alternatives in traditional c types of cancer [12].

Among these studies, the cytoxity of the Euphorbiaceae family against different cancers is widely studied. *Cnidoscuolus quercifolius* showed relevant cytotoxic activity, showing a high index of flavonoids is one of the fractions extracted against prostate tumor cells (PC3 and PC3-M) and breast tumor cells (MCF-7) [13]. *Euphorbia bicolor* reduced from its extract and phytochemicals the growth of breast tumor cells MCF-7, MDA-MB-231, and MDA-MB-468 [14].

With the same characteristic, *Euphorbia umbellata* has been shown from fractions extracted from its latex cytotoxic effects, in ileocecal colorectal adenocarcinoma cells (HCT-8), acute T cell leukemia (Jukart), human cervical tumor cells (HeLa), breast cancer cells (MDA-MB-231) and breast cancer cells murine (4T1) [15-19]. Tests were also carried out with the hexane fraction and its ether, methanol, and dichloromethane subfractions on murine melanoma tumor cells B16-F10 that showed a high cytotoxic potential, effects probably associated with terpenes, which are the main substances present in their latex [18, 20-23].

Therefore, the present work aims to evaluate the antiproliferative, antitumor effects and the modulation of the mitochondrial electrical potential of the Acetate and Chloroform fractions and the Methanol, Ethanol, Dichloromethane, and Ether subfractions isolated from *E. umbellata* sap in murine melanoma tumor cells B16-F10, Hepa1c1c7 hepatocellular carcinoma tumor cells and normal FN1 fibroblast cells.

2. Materials and Methods

2.1. Cell culture

B16-F10 murine melanoma tumor cells (ATCC ® CRL-6475 ™), hepa1c1c7 hepatocellular carcinoma tumor cells (ATCC ® CRL-2026 ™), and normal human FN1 fibroblast cells were used and maintained and stored in the cell bank by Dr. Durvanei Augusto Maria (Development and Innovation Laboratory, Butantan Instituto). The cells were transferred to a cell culture flask (25 cm^2), containing the RPMI 1640 culture medium (Cultilab, Campinas-SP) supplemented with 10% fetal bovine serum, 200mM sodium bicarbonate, pH 7,4 in an oven at 5% CO_2 at 37 ºC. The cells arranged in a monolayer were subjected to enzymatic dissociation with 0.2% trypsin solution+EDTA (ethylenediaminetetraacetic acid) 0.02% so that the detachment of the cells occurs. Enzymatic neutralization was performed in an 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% 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 24h treated with acetate and chloroform fractions and the methanol, ethanol, dichloromethane and ether subfractions, isolated from *E. umbellata* sap, 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 100µL 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 540nm. The concentration that induces toxicity in 50% of the cells ($IC_{50\%}$) was determined in the treatment after 24h to assess the dose-response effect.

2.3. Analysis of mitochondrial electrical potential by laser confocal microscopy

The normal and tumor 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 37 ºC 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, 10µL of rhodamine -123 (Sigma-Aldrich, USA) was added for 30min in the dark at 37 ºC. After incubation with rhodamine-123 for fixation, 100µL of 2% paraformaldehyde was added for 30min, them 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. The analysis of the total occupied area and the fluorescence intensity was performed using the ImageJ software (National Institutes of Health).

2.4. 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 7.0.

3. Results and Discussion

3.1. Cytotoxic potential

The tumor cell B16-F10 showed greater resistance to most treatments, showing selective cytotoxicity for the Acetate and Chloroform fractions during the 24h period (Figure 1A). The Hepa1c1c7 tumor cells showed greater sensitivity to the Acetate, Chloroform fractions, and Methanol and Ether subfractions respectively (Figure 1B). Both tumor cell lines were compared to normal FN1 cells for the evaluation of specific compounds cytotoxicity (Figure 1C). The Acetate fraction was more effective with the same $IC_{50\%}$ 2 µg/mL value for both tumor cell lines of melanoma B16-F10 and hepatocellular carcinoma Hepa1c1c7 (Table 1). Followed by the Chloroform fraction, which also showed cytotoxicity in both strains specifically when compared to normal FN1 cells, with $IC_{50\%}$ of 3 µg/mL and 6 µg/mL respectively for tumor cells B16-F10 and Hepa1c1c7 (Table 1).

Among the subfractions, methanol obtained greater cytotoxicity for Hepa1c1c7 tumor cells with an $IC_{50\%}$ of 7 µg/mL very close to the value of the Chloroform fraction for the same cell line (Figure 1B), as well as the Ether that obtained the value of 8 µg / ml. The dichloromethane subfraction was the most cytotoxic for normal human fibroblast cells FN1, with an $IC_{50\%}$ of 4 µg/mL.

After cardiovascular diseases, melanoma tumor corresponds to the second leading cause of death in the world, probably related to the growth and aging of the population and epigenetic susceptibility factors [24]. Therefore, from the data analyzed, the possibility of the Acetate and Chloroform fractions being involved in some process of regulated cell death modulation, whether involved as a therapeutic agent in the prevention or as an adjuvant in the treatment of some types of cancer, above all melanoma, allows for a promising outlook.

Studies carried out with bioactive extracts from other species of the *E. umbellata,* Euphorbiaceae family, indicate that such popular medicinal plants may be a source of bioactive compounds with important therapeutic potential not only for cancer but also for other diseases. Fractions of *E. helioscopia* were evaluated in five different human cancer cell lines, with the treatment of the Ethyl Acetate fraction being the most effective in inhibiting the proliferation of human hepatocellular carcinoma cells SMMC-7721with interruption of the cell cycle in the G0/G1 phase at concentrations of 100-200 µg/mL [25].

In turn, *E. umbellata* latex also exhibited cytotoxicity to several tumor cell lines, human cervical cancer (HeLa), acute T cell leukemia Jukart (E6-1), Acute promyelocytic leukemia (HL-60), colorectal ileocecal adenocarcinoma (HCT- 8), Triple-negative breast cancer (MDA-MB-231) [26-29, 18].

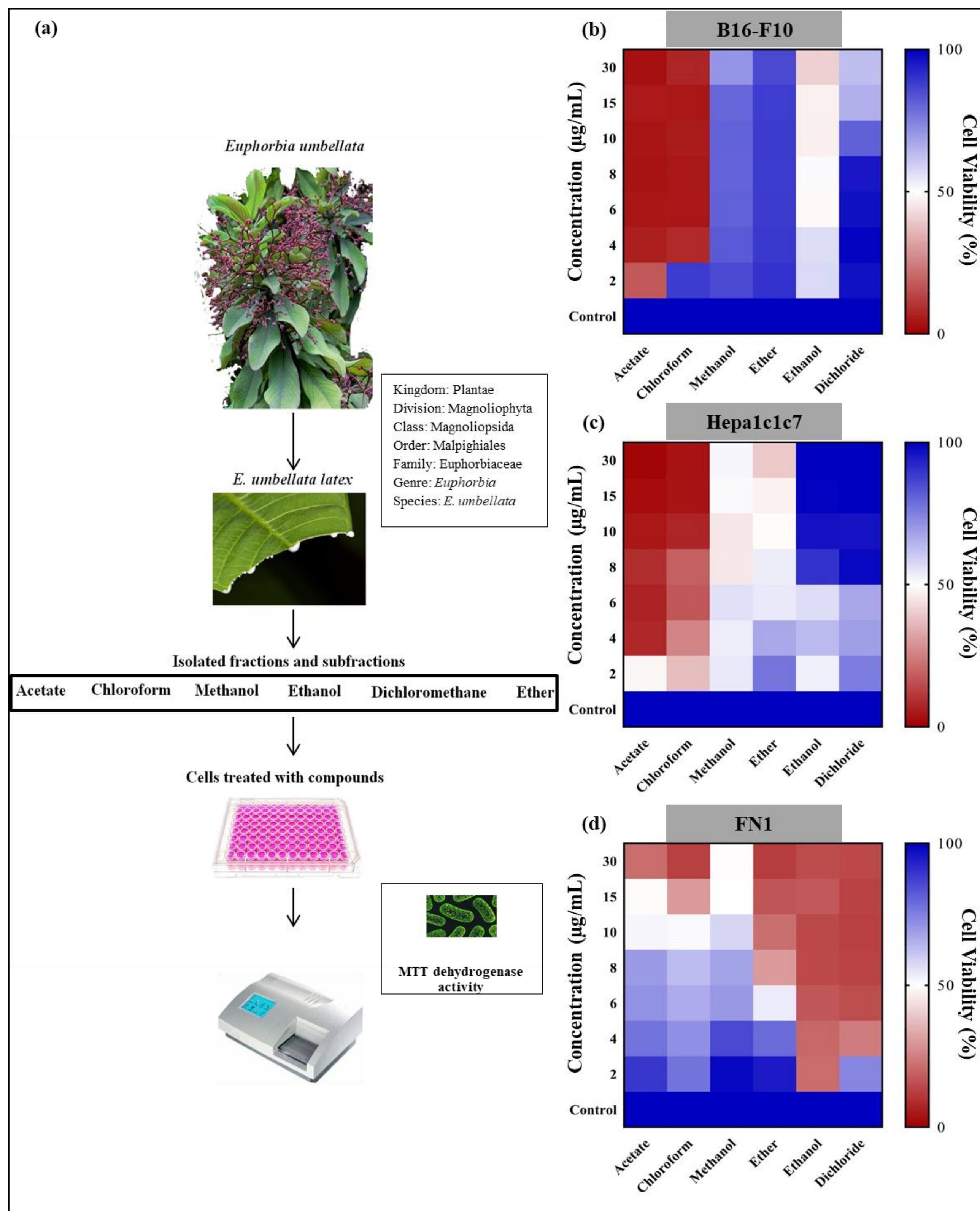


Fig 1: Determination of cytotoxicity by the MTT colorimetric assay. Heatmap of values of mean±SD of the viability of the normal cells and tumor cells after 24h of treatment with the fractions and subfractions. (a)Representative scheme of obtaining the fractions, subfractions and treatments carried out; (b) B16-F10;(c)Hepa1c1c7; (d)FN1. Graphs obtained by the GraphPad Prism 7 software. (n=3).

Table 1: $IC_{50\%}$ values obtained after 24h treatment with fractions and subfractions for tumor and normal cells.

Compounds	Cells	$IC_{50\%}$ Value
Acetate	FN1 (Fibroblast normal)	13 µg/mL
Chloroform		12 µg/mL
Methanol		30 µg/mL
Ethanol		4 µg/mL
Ether		19 µg/mL
Dichloromethane		3 µg/mL
Acetate	Hepa1c1c7 (*Hepatocellular carcinoma*)	2 µg/mL
Chloroform		6 µg/mL
Methanol		7 µg/mL
Ethanol		12 µg/mL
Ether		8 µg/mL
Dichloromethane		6 µg/mL
Acetate	B16-F10 (Melanoma)	2 µg/mL
Chloroform		3 µg/mL
Methanol		n/s
Ethanol		39 µg/mL
Ether		n/s
Dichloromethane		n/s

* n/s: Not significant.

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

The tumor cells of melanoma B16-F10 and hepatocellular carcinoma Hepa1c1c7 and normal cells of human fibroblast FN1 were treated for 24h with fractions and subfractions in the concentration of $IC_{50\%}$. Tumor cells B16-F10 and Hepa1c1c7 exhibited a considerable reduction in mitochondrial electrical potential, observed by the rhodamina-123 probe, with changes in cell compliance (Figures 3 and 4), showing cytoplasmic retraction, when compared to the control (Figures 3A and 4A), in addition to the presence of vacuoles, mainly in the Hepa1c1c7 tumor cell line. The Chloroform fraction was the least affected by Hepa1c1c7 cells (Figure 3C).

For normal FN1 cells, using the same concentrations that were used for tumor cells, no decrease in mitochondrial electrical potential, or any change in cell structure was observed through the rhodamina-123 probe (Figure 5).

Fluorescence and the total area occupied by the cell in the observed field, confluence, were quantified and analyzed using the ImageJ software. For normal human fibroblast cells FN1 (Figure 5) there was no significant reduction in cell confluence and mitochondrial electrical potential.

The tumor cell Hepa1c1c7, in all treatments, showed a reduction in cell confluence, with the sub-fraction Methanol showing a higher value 87±0.8%, followed by the ether fraction 66.7±3.1%, Acetate fraction 69.04±4.8% and the chloroform fraction 61.24±1.1%. There was a reduction in the mitochondrial electrical potential for all treatments, with the Acetate and Chloroform fractions and the Methanol and Ether subfractions showed a percentage reduction of 69.26±2.4%, 91.8±0.9%, 97.25±0.7%, 92.9±1.4 (Figure 6).

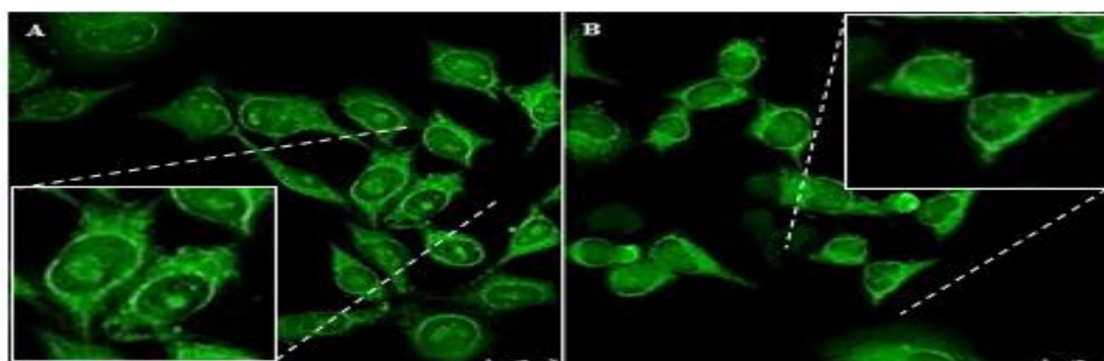
Among both tumor cell lines, murine melanoma tumor cells B16-F10 showed the greatest reduction in total area confluence for the Acetate 90.1±2.3% and Chloroform 92.9 ± 1.8% fractions. Likewise, for the mitochondrial electrical potential with percentage values of 87.5±1.2% and 92.7±1.0 respectively for the acetate and chloroform fractions (Figure 7).

The opening of the transition pore, involving increased permeability of the mitochondrial membrane, with the release of pro-apoptotic factors into the cytoplasm, is the main event of the intrinsic pathway of apoptosis, resulting from various stimuli such as deprivation of growth factors or DNA damage caused by cytotoxicity [30].

Recent studies with *E. umbellata* in melanoma tumor cells B16-F10, MDA-MB-231 and Hepa1c1c7 indicated a significant increase in fragmented DNA when treated with the Acetate and Chloroform fractions and with the ether and methanol subfractions in Hepa1c1c7 tumor cells [18]. In another study, the same compounds were evaluated for breast cancer tumor cells MDA-MB-231 and 4T1, a reduction in cell viability was observed, with cytotoxicity in most treatments with a marked reduction in mitochondrial electrical potential [19]. Such data, in agreement with those presented in this manuscript, indicate a possible modulation of the mitochondrial electrical potential and induction of the intrinsic pathway of cellular apoptosis.

Concomitantly, the formation of cytoplasmic vacuoles observed in both tumor strains also indicates a consequence of mitochondrial destabilization induced by cytotoxicity to the compounds. The destabilization of the lysosomal membrane, dependent on the destabilization of mitochondria, can also initiate the lysosomal apoptotic pathway, a consequence of the release of its content into the cytosol [30].

The capacity of latex from plants of the Euphorbiaceae family has already been demonstrated in several other studies, mainly regarding the modulation of the mitochondrial electrical potential [31-33]. *Euphorbia antiquorum* latex promoted the loss of mitochondrial electrical potential in HeLa cells, showing increased levels of reactive oxygen species (ROS), associated with changes in the integrity of the mitochondrial membrane, the electrical potential, and signs of initiation of processes apoptotic, also this study observed an increase in proteins such as p38 and caspases 8,9 and 3 [34-36]. Another study also carried out with *E. umbellata* showed that its extracts were able to decrease the viability of Ehrlich's ascitic tumor, with a time-dependent increase in the number of ROS and intracellular Ca^{2+}levels, resulting from the opening of the transition pore mitochondrial permeability [37, 38].



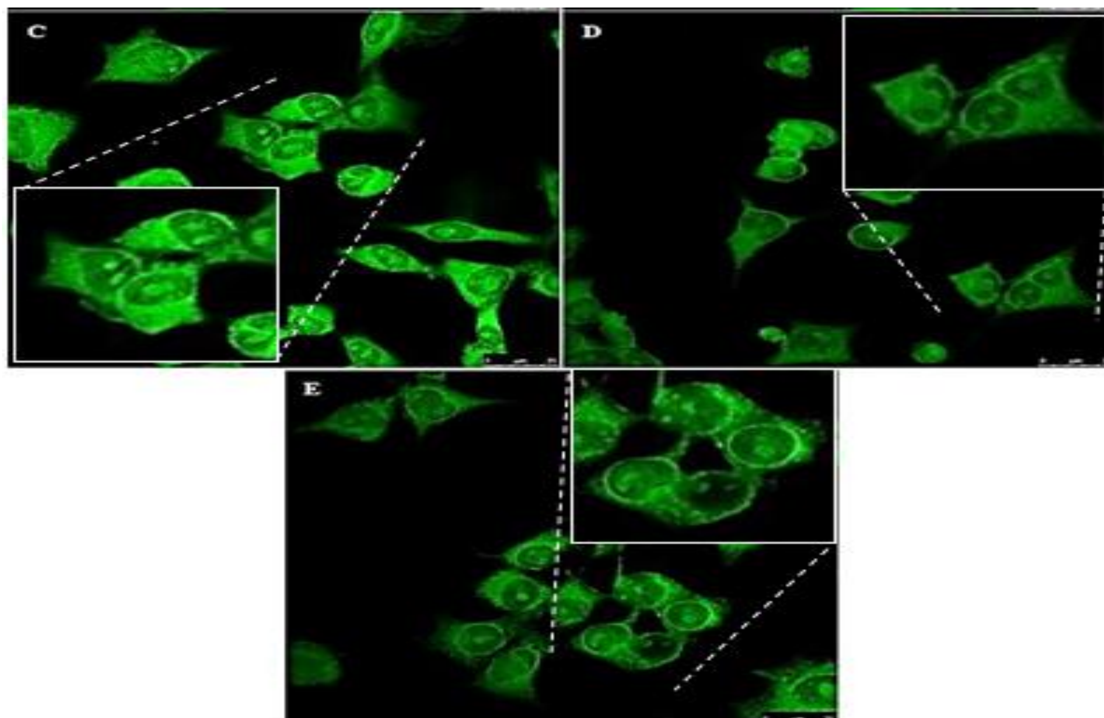


Fig 2: Photomicrographs of normal human FN1 fibroblast cells with mitochondria marked with Rhodamine 123 (green), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 2μg / mL; (c) Chloroform 6μg / mL; (d) Methanol 7μg / ml; (e) Ether 8μg / mL.

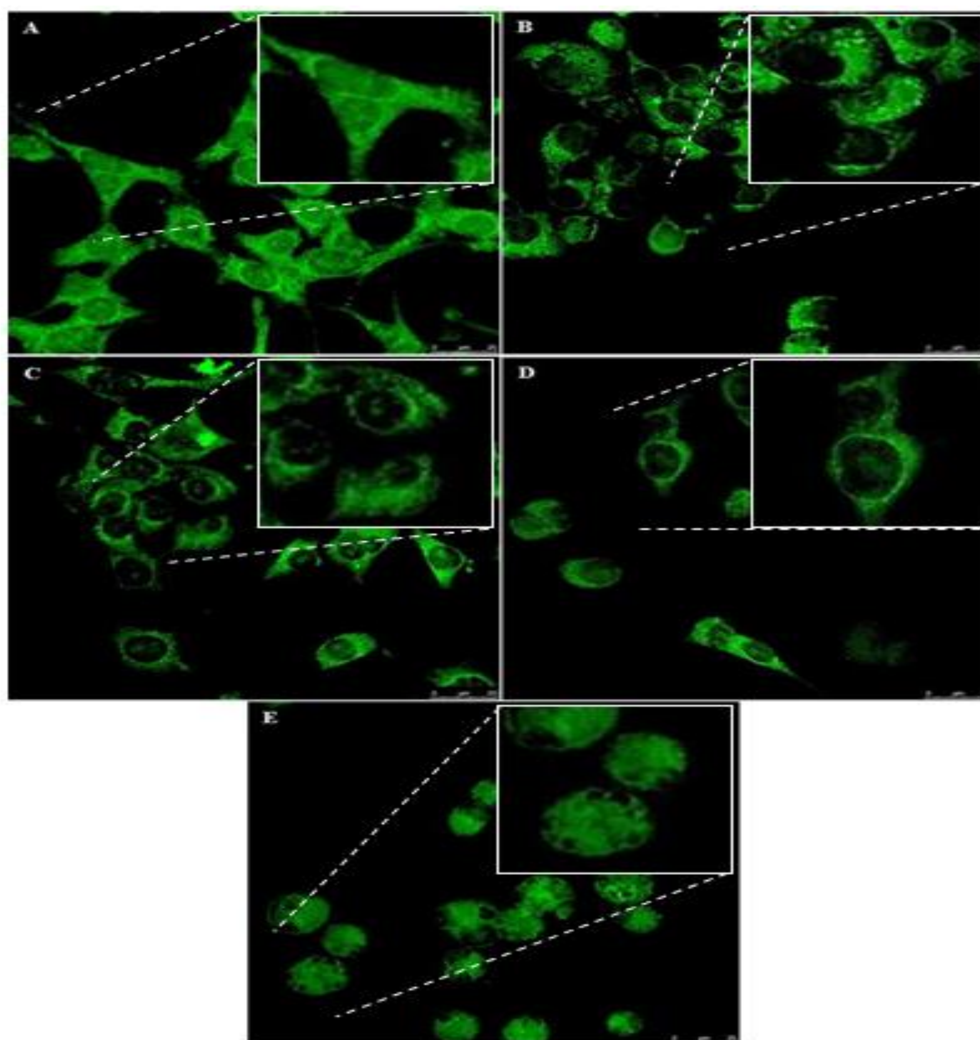


Fig 3: Photomicrographs of tumor cells Hepa1c1c7 with mitochondria marked with Rhodamine 123 (green), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 2μg / mL; (c) Chloroform 6μg / mL; (d) Methanol 7μg / ml; (e) Ether 8μg / mL.

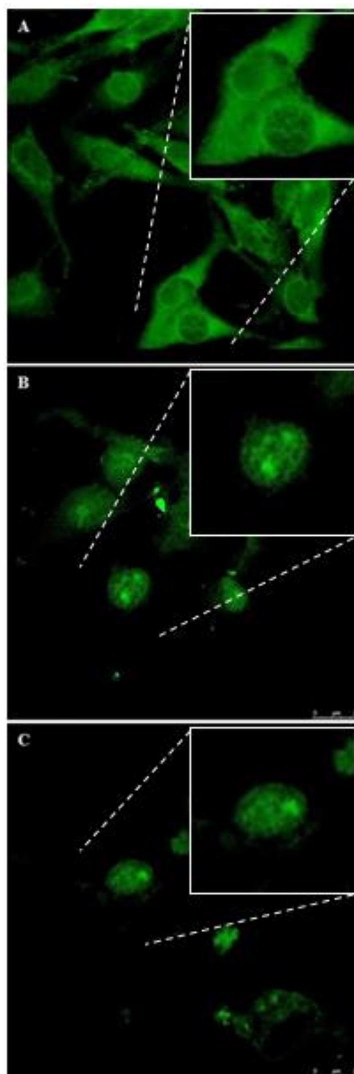
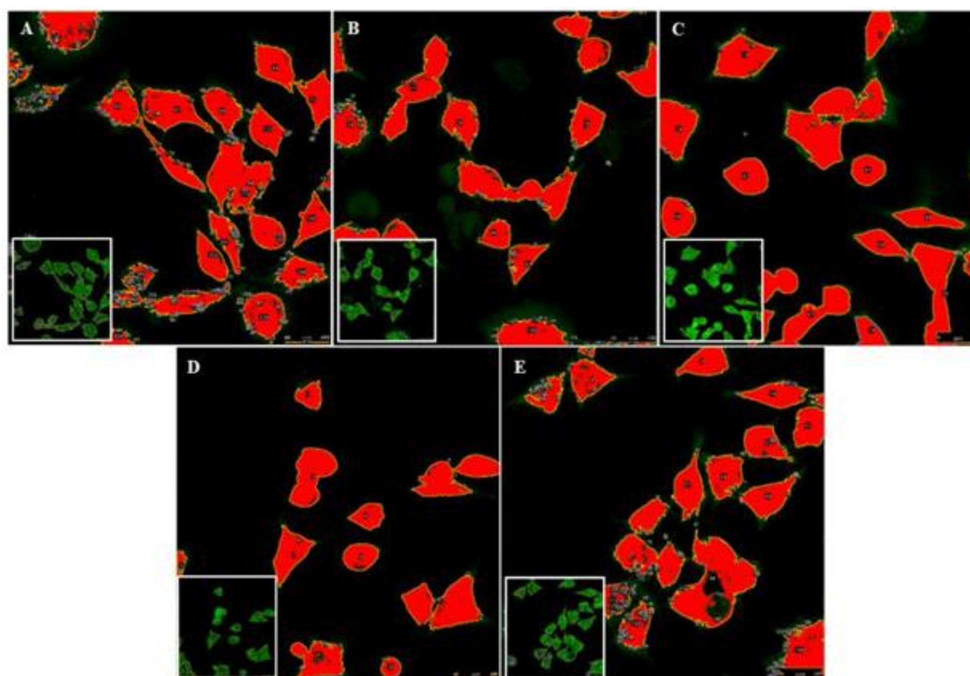


Fig 4: Photomicrographs of tumor cellsB16-F10 with mitochondria marked with Rhodamine 123 (green), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 2μg / mL; (c) Chloroform 6μg / mL;



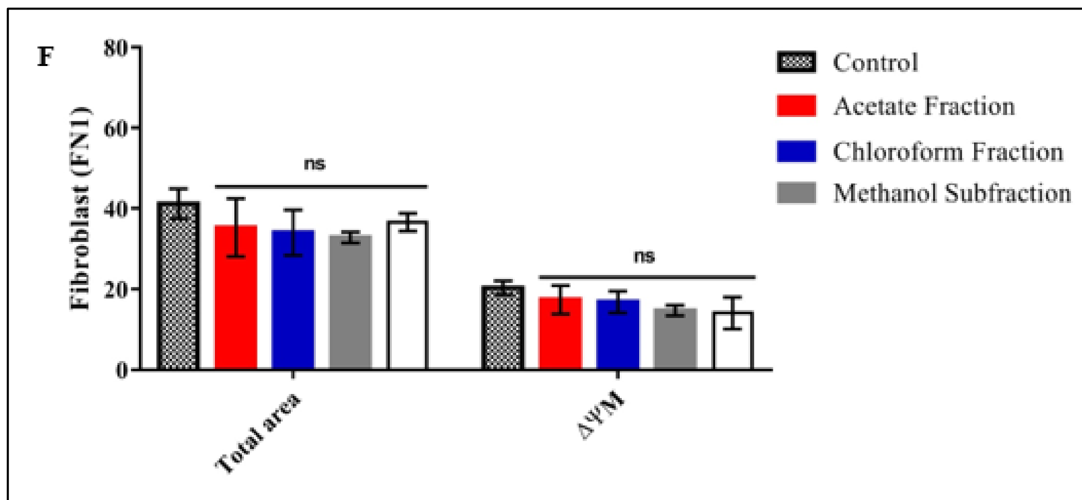
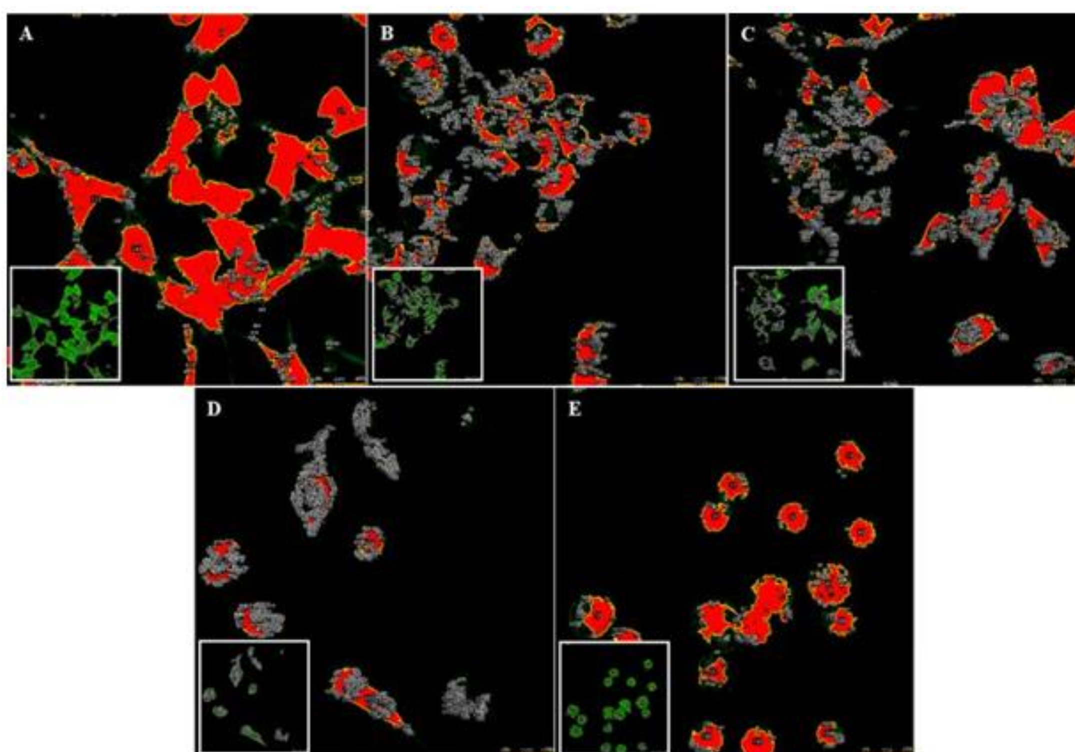


Fig 5: Photomicrographs of normal human FN1 fibroblast cells obtained by confocal microscopy and analyzed using ImageJ software. The red fluorescent area (Rhodamine 123) was selected by the software and the fluorescence and total cell area were quantified. (A) Control group; (B) Acetate; (C) Chloroform; (D) Methanol; (E) Ether; (F) Bar graph. ns: not significant.



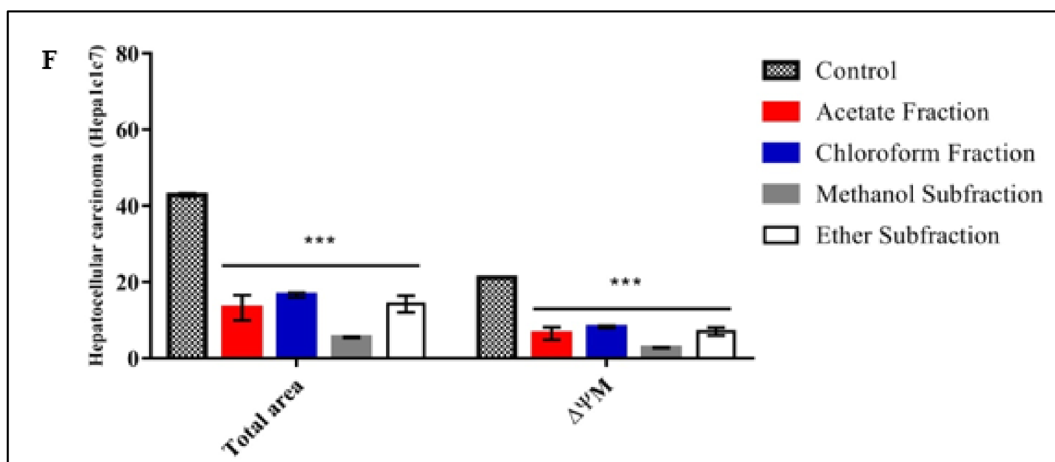


Fig 6: Photomicrographs of tumor cells Hepa1c1c7obtained by confocal microscopy and analyzed using ImageJ software. The red fluorescent area (Rhodamine 123) was selected by the software and the fluorescence and total cell area were quantified. (A) Controlgrupo; (B) Acetate; (C) Chloroform; (D) Methanol; (E) Ether; (F) Bar graph. Significance values with p *** <0.01, obtained by the ANOVA variation test followed by the multiple Turkey-Kremer tests. Experiments performed, n = 3.

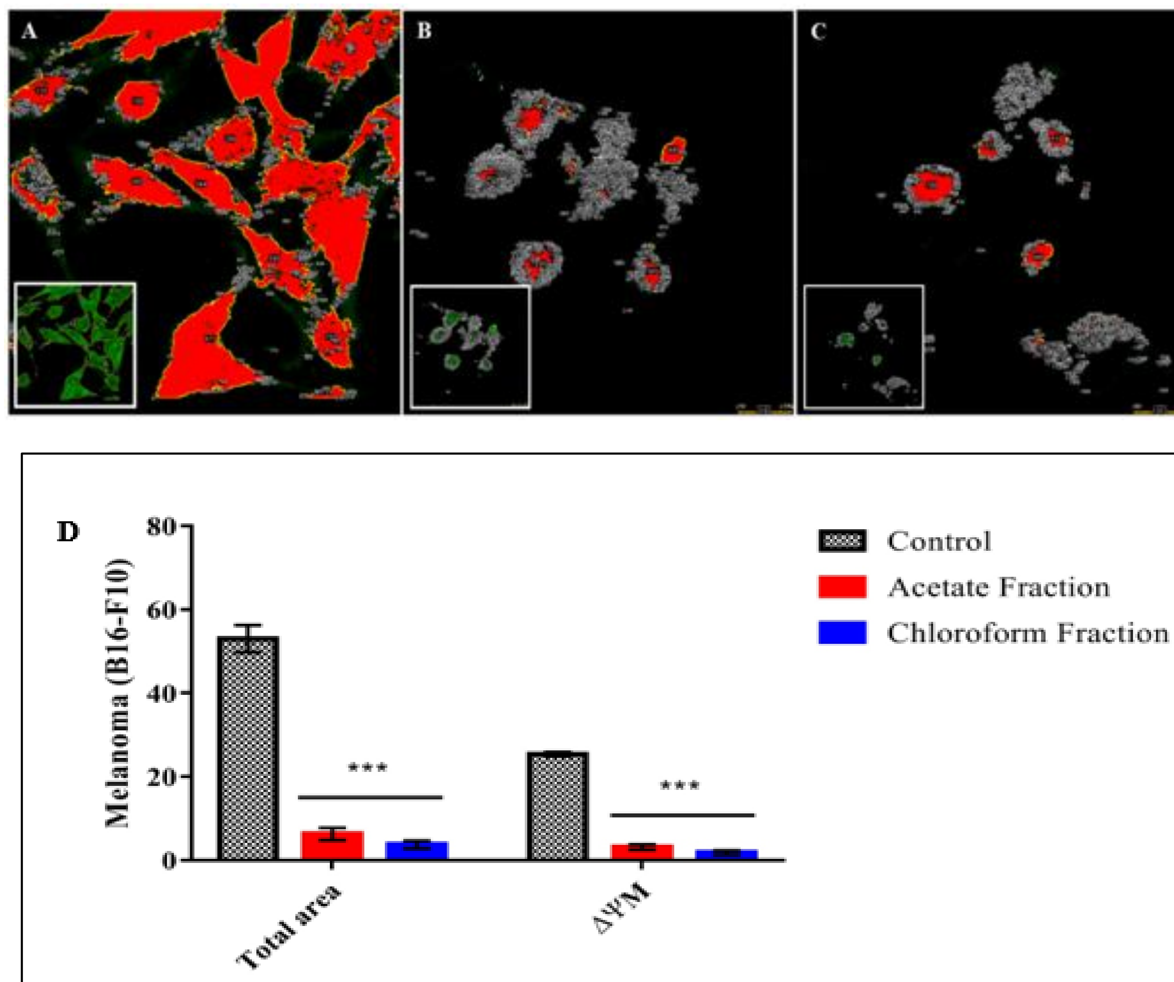


Fig 6: Photomicrographs of melanoma murine tumor cells B16-F10obtained by confocal microscopy and analyzed using ImageJ software. The red fluorescent area (Rhodamine 123) was selected by the software and the fluorescence and total cell area were quantified. (A) Control group; (B) Acetate; (C) Chloroform (D) Bar graph. Significance values with p *** <0.01, obtained by the ANOVA variation test followed by the multiple Turkey-Kremer tests. Experiments performed, n = 3.

4. Conclusion

The Acetate and Chloroform fractions are more promising for the induction of toxicity in tumor cells of murine melanoma B16-F10 and hepatocellular carcinoma Hepa1c1c7, as well as the ether and methanol subfractions for this cell line. The Dichloromethane subfraction is the most aggressive for normal human fibroblast cells FN1, as well as the Ethanol subfraction, showing itself as non-selective subfractions. According to what was observed in the photomicrographs and analyzes using the ImageJ Software, there was a reduction in cell confluence, a decrease in mitochondrial electrical potential, and cell viability, which indicates a regulated cell death, a consequence of the depolarization of the mitochondrial membrane and release of pro-mechanisms. apoptotic.

5. Acknowledgment

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