Natural bioactive from *Euphorbia umbellata* latex as a modulator of mitochondrial activity in breast cancer cells

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Abstract

The objective of this study was to evaluate the antiproliferative effect, antitumor and modulation of the mitochondrial electrical potential of the acetate and chloroform fractions and the methanol, ethanol, dichloromethane and ether subfractions extracted from *Euphorbia umbellata*. The cytotoxicity of fractions and subfractions was evaluated by the MTT colorimetric assay after 24h at concentrations ranging from 2-30 μg/mL, also the mitochondrial electrical potential by confocal microscopy. A reduction in cell viability was observed, with cytotoxicity in most treatments for all cells with a marked reduction in mitochondrial electrical potential. Possibly the treatments are modulating regulated cell death pathways. The acetate and chloroform fractions showed the highest cytotoxic potential with greater selectivity for tumor cells.

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

1. Introduction

Cancer is one of the main public health problems in the world, being among the top four causes of death in most countries [1]. The latest global estimate points to 18 million new cases of cancer and 9.6 million deaths. By the year 2040, that number will reach 29.5 million. Breast cancer is more common among women worldwide, corresponding to 25.2% of all woman cancers, with about 2 million diagnosed cases, being composed of different subtypes associated with different clinical outcomes due to its heterogeneity, being of fundamental understanding for the development of preventive methods and therapeutic interventions [2].

Natural products, obtained from various sources, have been used for the prevention and treatment of various chronic diseases for several centuries. The currently used chemotherapeutic agents of origin include taxol, obtained from the Pacific Yew (*Taxus brevifolia*) [3, 4], vincristine and vinblastine, present in Madagascar Periwinkle (*Vinca rosea*) [5, 6].

Seeking to find new drugs with greater efficiency for the treatment of different types of cancer, medicinal plants and ethnopharmacological knowledge have been evaluated and can lead to the discovery of new molecules and contribute as important tools in biomedical research and therapy [7, 8]. Therefore, biomonitoring studies are important to expand and improve cancer therapy.

In this sense, Euphorbiaceae species have high pharmacological potential. *Euphorbia umbellata* (Pax) Brunys latex is used in Brazilian folk medicine to treat patients with different types of cancer [8-15]. In the South region (mainly in Paraná) people use latex to treat bowel cancer, leukemia, skin cancer, etc. People prepare a mixture of 18 drops of latex in 1 liter of water (called a bottle) and drink a glass of it three times a day [8].

Some phytochemical tests have shown that terpenes are the main substances of latex and in several scientific works this class of substances is considered a promising source of cytotoxic agents [16-18]. Other studies have described the cytotoxic activity of latex against different cell types and some have attributed these effects to terpenes [19, 20].

The present work aims to evaluate the antiproliferative effect, antitumor and modulation of the mitochondrial electrical potential of the acetate and chloroform fractions and the methanol, ethanol, dichloromethane and ether subfractions isolated from *E. umbellata* latex in MDA MB-231 human triple-negative breast cancer cells, 4T1 murine triple-negative breast cancer cells, and HUVEC normal human endothelium cells.
2. Materials and Methods

2.1. Cell culture

Human triple-negative breast cancer cells MDA MB-231 (ATCC® HTB-26™), and Murine triple-negative breast cancer cells 4T1 (ATCC® CRL-2539), and normal human endothelium HUVEC (ATCC® CRL-1730) was used and maintained and stored in the cell bank by Dr. Durvanei Augusto Maria (Development and Innovation Laboratory, Instituto Butantan). The cells were defrost and transferred to culture bottle (25 cm²), containing the RPMI 1640 culture medium (CultiLAB, Campinas-SP) supplemented with 10% fetal bovine serum, 200mM sodium bicarbonate, pH 7.4 in an oven 5% CO₂ at 37°C. The cells arranged in monolayer were detached to enzymatic dissociation with 0.2% trypsin solution+EDTA (Ethylenediaminetetraacetic acid) 0.02%. 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 10⁵ 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 10⁵ 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 concentration 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% CO₂ 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 (IC₅₀) 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% CO₂ at 37 °C 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 6G (Sigma-Aldrich, USA) in concentration 0.02 g/mL, was added for 30min in the dark at 37 °C. After incubation with Rhodamine 6G, for fixation, were used 100 µL of 2% paraformaldehyde for 30min, 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. 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 cytotoxic potential of the acetate and chloroform fractions and of the sub-fractions methanol, ethanol, dichloromethane and ether were evaluated for MDA MB-231 and 4T1 triple-negative breast tumor cells and for the normal HUVEC cell within 24h of treatment. The fractions, acetate and chloroform showed cytotoxicity to tumor cells in the tested period, with the acetate fraction being more specific when compared to the normal HUVEC cell. For the subfraction, the most effective with greater specificity for tumor cells was the ether subfraction (Figure 1). The human triple-negative breast tumor cell MDA MB-231 was the most sensitive to the treatments presenting IC₅₀% for the acetate and chloroform fractions of 2 and 6 µg/mL, the ethanol and ether subfractions, the values obtained were 16 and 8 µg/mL respectively (Table 1). For the 4T1 murine triple-negative breast tumor cell treated with the acetate and chloroform subfractions, the IC₅₀% obtained was 4 and 6 µg/mL respectively, when treated with the ethanol and ether subfractions, the values of 2.5 and 2 µg/mL respectively (Table 1). The ethanol subfraction was the most cytotoxic for the normal human endothelial cell HUVEC, with an IC₅₀% of 5 µg/mL.

Several studies have shown the cytotoxic effects of fractions and subfractions isolated from E. umbellata latex on tumor cells. In previous studies developed by our group, a reduction in cell viability and marked cytotoxicity was observed for tumor cells of melanoma B16-F10 and breast tumor cells, with IC₅₀% higher values for normal cells, showing the selectivity of treatment. E. umbellata latex also showed cytotoxicity to tumor cells from ileocecal colorectal adenocarcinoma (HCT-8), human cervical cancer (HeLa), Jurkat cells (E6-1) and acute promyelocytic leukemia (HL-60) [10-12, 21].
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) MDA MB-231; (c) 4T1; (d) HUVEC. Graphs obtained by the GraphPad Prism 7 software. (n=3).

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

| Compounds  | Cells   | IC_{50}% Value |
|------------|---------|----------------|
| Acetate    | MDA MB-231 | 2 µg/mL        |
| Chloroform | MDA MB-231 | 6 µg/mL        |
| Methanol   | MDA MB-231 | n/s            |
| Ethanol    | MDA MB-231 | 16 µg/mL       |
| Dichloromethane | MDA MB-231 | 8 µg/mL       |
| Ether      | MDA MB-231 | n/s            |
| Acetate    | 4T1      | 4 µg/mL        |
| Chloroform | 4T1      | 6 µg/mL        |
| Methanol   | 4T1      | 25 µg/mL       |
| Ethanol    | 4T1      | 2.5 µg/mL      |
| Dichloromethane | 4T1    | 2 µg/mL        |
| Ether      | 4T1      | n/s            |
| Acetate    | HUVEC    | 7 µg/mL        |
| Chloroform | HUVEC    | 8 µg/mL        |
| Methanol   | HUVEC    | 10 µg/mL       |
| Ethanol    | HUVEC    | 5 µg/mL        |
| Dichloromethane | HUVEC | n/s            |
| Ether      | HUVEC    | n/s            |

n/s= no significant
3.2. Evaluation of mitochondrial electrical potential (ΔΨm) by confocal microscopy

The human MDA MB-231 and murine 4T1 triple-negative breast tumor cells, and the normal human endothelium cell HUVEC, were treated for 24h with fractions and subfractions in the IC50% concentration. The MDA MB-231 and 4T1 tumor cells showed a considerable reduction in the mitochondrial electrical potential, observed by the reduction of the fluorescence emitted by the Rhodamine 6G probe, important structural changes, and reduction of the cellular confluence (Figures 2 and 3). For the HUVEC cell, the treatments caused a low reduction in the mitochondrial electrical potential and a reduction in confluence (Figure 4).

The analyzes performed in the ImageJ software in order to quantify the fluorescence and the total area occupied by the cell in the observed field (confluence) also showed changes for all of the analyzed points. The MDA MB-231 tumor cell, when treated with the acetate and chloroform fractions, showed a mitochondrial electrical potential reduction of 69.8±2.1% and 67.4±3.8% respectively, whereas for the ethanol addition, the reduction value was 80.65%±4.1% and for subfraction ether the value was 77.9±0.8%. Regarding the reduction of confluence total area, when treated with the acetate fraction, there was a reduction in confluence by 37.3±3.5%, which is not statistically significant for the treatment with the chloroform fraction, when treated with the ethanol and ether there was a reduction of 73.4±2.8% and 49.8±3.1% respectively (Figure 5).

The 4T1 tumor cell, when treated with the acetate and chloroform fractions, showed a reduction in the mitochondrial electrical potential of 80.2±4.6% and 76.1±3.9% respectively, while for the treatment with the ethanol subfraction the reduction value was 74.3%±2.4%, not being statistically significant for the treatment with the ether subfraction. Regarding the reduction in confluence, when treated with the acetate fraction, there was a reduction in confluence by 69.7±5.8, for the chloroform fraction the value was 68.1±4.2%, when treated with the ethanol subfraction there was a reduction of 52.4±1.1%, not being significant for the ether subfraction (Figure 6). The HUVEC cell only showed a reduction in confluence for the treatment with the ether subfraction, with a value of 50.3±2.7%, with no statistically significant changes for any other treatment for both the total area and the mitochondrial electrical potential (Figure 7).

Important metabolic processes occur in mitochondria, which are essential for the survival of all eukaryotic cells, being important in mediating the signaling of regulated cell death [22]. The permeabilization of the mitochondrial outer membrane, due to the opening of the transition pore, releases numerous pro-apoptotic proteins in the cytosol, being the main event in the intrinsic apoptotic pathway [23, 24]. These potentially apoptogenic factors are found in the mitochondrial intermembrane fraction, including cytochrome c, pro-caspases 2, 3 and 9 and AIF, which forces isolated nuclei to adopt an apoptotic morphology [23, 25, 26].

The ability of fractions extracted from latex from plants of the Euphorbiaceae family to modulate the mitochondrial electrical potential in various tumor cells has been shown in other works [27–29]. Fractions and subfractions of E. umbellata latex was able to promote modulation of the mitochondrial electrical potential (ΔΨm) in tumor cells of murine melanoma B16-F10 and hepatocellular carcinoma (Hepa1c1c7) [14, 19]. Latex from Euphorbia antiquorum was able to reduce ΔΨm in HeLa tumor cells, increasing the levels of reactive oxygen species. Increased expression of caspases and pro-apoptotic proteins was also observed, such as, Fas, FasL, JNK, p38 and MAPK 8, 9 and 3 [29]. 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 ΔΨm modulation [27].

Fig 2: Photomicrographs of tumor cells of human triple-negative breast cancer MDA MB-231 with mitochondria marked with Rhodamine 6G (red), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 2 μg/mL; (c) Chloroform 6 μg/mL; (d) 16 μg/mL ethanol; (e) Ether 8 μg/mL.
Fig 3: Photomicrographs of tumor cells of murine triple-negative breast cancer 4T1 with mitochondria marked with Rhodamine 6G (red), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 4 μg/mL; (c) Chloroform 6 μg/mL; (d) 2.5 μg/mL ethanol; (e) Ether 2 μg/mL.

Fig 4: Photomicrographs of normal cells human endothelium HUVEC with mitochondria marked with Rhodamine 6G (red), analyzed by laser confocal microscopy. Cells treated with fractions and subfractions after 24h of treatment. (a) Control; (b) Acetate 7 μg/mL; (c) Chloroform 8 μg/mL; (d) 5 μg/mL ethanol.

Fig 5: Photomicrographs of tumor cells of human triple-negative breast cancer MDA MB-231 obtained by confocal microscopy and analyzed using the ImageJ software. The red fluorescent area (Rhodamine 6G) was selected by the software and quantified fluorescence and total cell area. (a) Control group; (b) Acetate; (c) Chloroform; (d) Ethanol; (e) Ether; (f) Bar graph. Significance values with $p^* < 0.05$ and $p^{***} < 0.01$, obtained by the ANOVA variation test followed by the Turkey-Kramer multiple tests. Experiments performed, $n = 3$ n/s= not significant

Fig 6: Photomicrographs of tumor cells of murine triple-negative breast cancer 4T1 obtained by confocal microscopy and analyzed using the ImageJ software. The red fluorescent area (Rhodamine 6G) was selected by the software and quantified fluorescence and total cell area. (a) Control group; (b) Acetate; (c) Chloroform; (d) Ethanol; (e) Ether; (f) Bar graph. Significance values with $p^* < 0.05$ and $p^{***} < 0.01$, obtained by the ANOVA variation test followed by the Turkey-Kramer multiple tests. Experiments performed, $n = 3$ n/s= not significant
Euphorbia umbellata latex. The acetate and chloroform fractions were effective in the treatment in vitro of triple negative breast tumor cells.

4. Conclusion

The fraction *E. umbellata* latex show cytotoxicity on tumor cells. The results show that the acetate and chloroform fractions are more effective in inducing toxicity for human MDA MB-231 and murine 4T1 triple-negative breast tumor cells, as well as the ethanol and ether subfractions, with the ethanol subfraction being very cytotoxic to normal endothelial cells HUVEC are not a selective compound. There was modulation of the mitochondrial electrical potential and reduced cell viability, evidenced by the reduction of the confluence observed in the photomicrographs obtained by confocal microscopy and analyzed in the ImageJ software. Our results indicate that there is an induction of regulated cell death, mediated by depolarization of the mitochondrial membrane and possible release of pro-apoptotic proteins in the cytosol. This fact corroborates published works relating this action to possible alkaloids and terpenes found in *E. umbellata* latex. The acetate and chloroform fractions were effective in the treatment in vitro of triple-negative breast tumor cells.

5. References

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