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Original Article

Cytotoxicity of extracts and compounds isolated from *Croton echioides* in animal tumor cell (HTC)

Citotoxicidade de extratos e compostos isolados de *Croton echioides* em célula tumoral animal (HTC)

I. Vendruscolo[a], S. R. T. Venturella[a], P. A. Bressiani[a], I. G. Marco[a], C. R. Novello[b], I. V. Almeida[c], V. E. P. Vicentini[d], J. C. P. Mello[e] and E. Düsman[f*]

[a]Universidade Tecnológica Federal do Paraná, Departamento de Engenharia Química, Francisco Beltrão, PR, Brasil
[b]Universidade Tecnológica Federal do Paraná, Departamento de Química e Biologia, Francisco Beltrão, PR, Brasil
[c]Universidade Federal Rural da Amazônia, Coordenação de Ciências Biológicas, Capitão Poço, PA, Brasil
[d]Universidade Estadual de Maringá, Departamento de Biotecnologia, Genética e Biologia Celular, Maringá, PR, Brasil
[e]Universidade Estadual de Maringá, Departamento de Farmácia, Maringá, PR, Brasil
[f]Universidade Tecnológica Federal do Paraná, Departamento de Química e Biologia, Francisco Beltrão, PR, Brasil

Abstract

The search for compounds with anticancer effects is of paramount importance today due to the high incidence of the disease. The Euphorbiaceae family is known for having compounds with therapeutic properties, one of its genera being *Croton*. It has several species, which contain compounds already known for their biological activities, presenting anti-inflammatory, antimicrobial and anticancer properties. Thus, the cytotoxicity/antiproliferative activity of semi-purified fractions and compounds isolated from *Croton echioides* in liver tumor cells of *Rattus norvegicus* (HTC) was evaluated by the MTT test. The semi-purified fractions showed cytotoxicity at concentrations above 200 µg/mL, at 24, 48 and 72 hours, reaching cell viability of 24.78% [400 µg/mL] at 24 hours, 12.79% [500 µg/mL] at 48 hours and 10.57% [300 µg/mL] at 72 hours. For the isolated compounds, lupeol had a cytotoxic effect in all concentrations (1, 5, 10, 15, 20, 40, 60, 80 and 100 µg/mL) and tested times (24, 48 and 72 hours), reaching minimum viability of 4.37% [100 µg/mL], within 72 hours. The clerodan diterpenes CEH-1 and CEH-4 also showed antiproliferative activity, with minimum viability of 36.19% [100 µg/mL] over 72 hours and 21.33% [100 µg/mL] over 48 hours, respectively. However, the clerodan diterpenes CEH-2 and CEH-3 did not shows a cytotoxic effect for HTC cells. Thus, there is a cytotoxic/antiproliferative potential of *C. echioides* against tumor cells, with targeted to mitochondrial enzymes, associated with cell proliferation, indicating that this species deserves prominence in the search for new molecules for the treatment of cancer.

Keywords: antiproliferative activity, *Rattus norvegicus* tumor cell, *Croton echioides*, clerodan diterpenes, MTT assay.

Resumo

A busca por compostos com efeitos anticâncer é de suma importância nos dias atuais devido à alta incidência desta doença. A família Euphorbiaceae é conhecida por possuir compostos com propriedades terapêuticas, sendo um de seus gêneros o *Croton*. Este possui diversas espécies, que contêm compostos já conhecidos por suas atividades biológicas, apresentando propriedades anti-inflamatórias, antimicrobianas e anticancerígenas. Assim, a citotoxicidade/atividade antiproliferativa de frações semipurificadas e compostos isolados de *Croton echioides* em células tumorais hepáticas de *Rattus norvegicus* (HTC) foi avaliada pelo teste MTT. As frações semipurificadas apresentaram citotoxicidade em concentrações acima de 200 µg/mL, em 24, 48 e 72 horas, atingindo viabilidade celular de 24,78% [400 µg/mL] em 24 horas, 12,79% [500 µg/mL] em 48 horas e 10,57% [300 µg/mL] às 72 horas. Para os compostos isolados, o lupeol teve efeito citotóxico em todas as concentrações (1, 5, 10, 15, 20, 40, 60, 80 e 100 µg/mL) e tempos testados (24, 48 e 72 horas), atingindo a viabilidade mínima de 4,37% [100 µg/mL], em 72 horas. Os diterpenos clerodan CEH-1 e CEH-4 também apresentaram atividade antiproliferativa, com viabilidade mínima de 36,19% [100 µg/mL] em 72 horas e 21,33% [100 µg/mL] em 48 horas, respectivamente. No entanto, os diterpenos clerodanos CEH-2 e CEH-3 não apresentaram efeito citotóxico para células HTC. Assim, existe um potencial citotóxico/antiproliferativo de *C. echioides* contra células tumorais, com enzimas direcionadas a enzimas mitocondriais, associadas à proliferação celular, indicando que esta espécie merece destaque na busca de novas moléculas para o tratamento do câncer.

Palavras-chave: atividade antiproliferativa, célula tumoral de *Rattus norvegicus*, *Croton echioides*, diterpenos clerodanos, ensaio MTT.

*edusman@utfpr.edu.br; lisdusman28@gmail.com
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1. Introduction

Due to the wide botanical diversity worldwide, flora is a source for research and development of innovative medicines (Hao and Xiao, 2020). Within this, the Euphorbiaceae family stands out for having compounds with therapeutic properties, which can be extracted for use in medicine (Amorzo, 2002). One of the genera of Euphorbiaceae is Croton, which has about 1,200 species worldwide (Souza et al., 2020). This genus presents phenolic compounds, such as flavonoids, lignoids and protoanthocyanidins (Salatino et al., 2007), besides being abundant in several classes of alkaloids (Torres, 2008) and secondary metabolites, mainly clerodan diterpenes (Salatino et al., 2007), which are bicyclic diterpenes known for their biological activities, such as anticancer, anti-inflammatory and antioxidants (Alencar et al., 2017; Neri et al., 2021).

Croton genus plants have several proven pharmacological properties. Antimicrobial and antibacterial properties have been demonstrated for *Croton bonplandianum* (Vadlapudi, 2010), *C. campestris* (Almeida et al., 2013) and *C. zehntneri* (Agra et al., 2008). Antioxidant activity was shown by *C. celtidifolius* (Coutinho et al., 2011), *C. lechleri* (Marino et al., 2008) and *C. zehntneri* (Morais et al., 2006). Anti-inflammatory property was found in *C. celtidifolius* (Nardi et al., 2003), *C. crassifolius* (Zhao et al., 2012), *C. lechleri* (Risco et al., 2003) and *C. campestris* (El Babili et al., 2006). Antinociceptive attributes have been identified in C. *crassifolius* (Risco et al., 2003), *C. nepetaefolius* (Abdon et al., 2002), *C. celtidifolius* (Nardi et al., 2006) and *C. zehntneri* (Oliveira et al., 2001). Antiviral by *C. lechleri* (Ubillas et al., 1994) and antileishmaniasis by *C. echioides* Baill (Novello et al., 2022).

In addition, plants of this genus have been studied for their anti-cancer action. The extract of *C. campestris* had its antitumor activity confirmed, inhibiting the growth of lung tumor cells (Monteiro, 2012), and some indole alkaloids of *C. echioides* showed in vitro action to combat human colon carcinoma (Mello et al., 2010 and Novello et al., 2012).

The search for compounds with anti-cancer effect is of paramount importance due to the high incidence of the disease, in addition, many patients seek alternative treatments, such as the use of medicinal plants (Abubakar et al., 2020; Dhruva et al., 2012; Wang et al., 2021). This search occurs, mainly, due to the many side effects of the medical treatments used, such as chemo and radiotherapies (Reis et al., 2008). An example is hepatocellular carcinoma, an epithelial neoplasm derived from hepatocytes, the most frequent in the liver, characterized by the formation of a concentrated mass tumor, or nodules distributed in the liver (Hasan et al., 2018), it's considered a highly aggressive malignancy disease (Yang et al., 2017; Sharif et al., 2022). Chronic liver diseases have a high rate of morbidity and mortality, which is why the need to identify more effective therapeutic forms, given that current therapies still cause many adverse effects (Novello et al., 2016; Oh et al., 2016).

Thus, the objective of this study was to evaluate the cytotoxic/antiproliferative activity of the semi-purified fractions hexane (HF) and ethyl acetate (EAF), obtained by the partitioning of the crude extract from *C. echioides*, and of isolated compounds from the HF, the clerodan diterpenes 15,16-epoxy-3,13 (Nardi et al., 2003), 14-neo-clerodatrien-17,18-dicarboxylate, nasimalun B (CEH-1), 15,16-epoxy-3,13 (Nardi et al., 2003), methyl 14-neo-clerodatrien-17-carboxy-18-carboxylate (CEH-2), equilibrium mixture of ptychonal and ptychonal hemiacetal (CEH-3) and methyl ester of hardwickic acid (CEH-4) and lupeol, by the MTT test, in liver tumor cells of *Rattus norvegicus* (HTC), in order to assist in the search for new molecules effective in the treatment of cancer, and add value to this Brazilian species, with pharmaceutical potential.

2. Methodology

2.1. Treatment solution

For the extraction process of compounds and components of the plant, the dry stems of *C. echioides* were ground in a hammer mill and underwent extraction in Ultra turrax (Ika Works UTC 115 / KT) with 70% hydroalcoholic solvent (w/w) for a period of 48 hours. Afterwards, the extract was filtered, concentrated on a rotary evaporator and lyophilized. Part of the crude extract (300 g) was suspended in water and partitioned with organic solvents in an increasing order of polarity (Novello et al., 2016) from which the hexane (HF) and ethyl acetate (EAF) fractions were obtained. The HF was purified by chromatographic methods and the lupeol and the clerodan diterpenes CEH-1, CEH-2, CEH-3, and CEH-4 were characterized and previously reported (Novello et al., 2022).

Initially, 10 mg of EAF were diluted in 100 µL of DMSO and 900 µL of culture medium. Afterwards, diluted again in culture medium supplemented with fetal bovine serum until treatment solutions were obtained at treatment concentrations of 5, 10, 50, 100, 200, 300, 400, 500 and 1,000 µg/mL. Then, 27 mg of HF were diluted with 260 µL of DMSO and 740 µL of culture medium, and diluted again, within the same conditions as the EAF, obtaining the concentrations of 2, 5, 10, 50, 100, 200, 300, 400 and 500 µg/mL. For lupeol, 7.0 mg were diluted in 500 µL of DMSO and 500 µL of culture medium, the CEH-1, 6.0 mg was diluted with 410 µL of DMSO and 590 µL of culture medium, the CEH-2, 5.2 mg was diluted with 250 µL of DMSO and 750 µL of culture medium, the CEH-3, 5.2 mg was diluted with 410 µL of DMSO and 590 µL of culture medium and the CEH-4, 10.0 mg was diluted with 1,000 µL of culture medium. From these solutions, the treatment concentrations of 1, 5, 10, 15, 20, 40, 60, 80 and 100 µg mL of culture medium supplemented with fetal bovine serum were prepared.

2.2. Cytotoxic/Antitumor activity

Rattus norvegicus liver tumor cells (HTC) were grown in 25 cm^2 culture flasks containing 10 mL of DMEM culture medium (Invitrogen - Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Invitrogen - Carlsbad, CA, USA) and incubated in a BOD oven at 37 ° C.

For the cytotoxicity/antiproliferative activity test, the MTT test was performed according to Mosmann (1983)

and Lopes et al. (2022), with modifications. 96-well culture plates were used where 2.0 x 10^4 cells of HTC were seeded in each well, with the exception of the control wells without cells (white). After stabilization, the culture medium was discarded and 100 µL of treatments were added: culture medium (negative control), cytotoxic agent methyl methanesulfonate (MMS - 500 µM) (positive control), dimethyl sulfoxide (DMSO) in the same concentration used for the dilution of each fraction or compound (solvent control), and treatments with different concentrations of fractions or compounds isolated from *C. echioides*.

After 24, 48 and 72 hours of incubation, the culture medium was replaced with culture medium plus MTT (0.167 mg/mL). The plates were incubated for another four hours before discarding the medium containing MTT, followed by the addition of 100 µL of DMSO to solubilize the formazan crystals. The absorbances were read in a microplate reader (Thermo Plate) at 560 nm. The experiments were carried out in three independent repetitions.

The results were presented as mean and standard deviation of the absorbances and submitted to analysis of variance (one way ANOVA), followed by the Dunnet test, by the Action Stat Program. The differences were considered statistically significant when $p \leq 0.05$.

The percentage values of cell viability (VC) were estimated by the Equation 1.

$$VC = \left(\frac{ABS_T}{ABS_{CO-}} \right) \times 100 \quad (1)$$

Where:
VC = Cell viability [%];
ABS_T = Absorbance of the treatment;
ABS_{CO-} = Absorbance of the negative control.

3. Results and Discussion

Figure 1A presents the results obtained with the HTC tumor cells treated with the semi-purified fraction EAF, obtained from the crude extract of *C. echioides*. At 24 hours, the highest concentrations (400, 500 and 1000 µg/mL) showed lower absorbances than the negative control and, thus, cytotoxic/antiproliferative effect. Even the cell viability of these concentrations (Table 1) was less than 76%. In 48 and 72 hours this cytotoxic effect for these cells occurred from the concentration of 200 µg/mL, with cell viability less than 66% (48 hours) and 60% (72 hours), reaching 40.9% (300 µg/mL - 72 hours). And, in 72 hours, the concentration of 5 µg/mL also showed action. In fact, the data (Table 1) show an indication of a decrease in cell viability with the increase in the assessed concentration, but with an even more evident effect depending on the exposure time (from the concentration of 100 µg/mL).

In the study by Monteiro (2012), with extracts obtained from *C. campestris*, it was identified that the crude ethyl acetate extract had the best profile of antitumor activity in vitro due to the decrease in cell viability, increase in cell

Table 1. Percentage of viability (according to Equation 1) of tumor cells (VC) of rat liver (HTC), treated with the different concentrations (µg/mL) of the semi-purified fraction EAF, of *Croton echioides*, for 24, 48 and 72 hours, at MTT test.

Groups	VC % of HTC cells		
	24 h	48 h	72 h
CO-	100.00	100.00	100.00
CO+	54.35	49.56	43.37
CS	81.34	108.83	88.12
5	101.45	102.29	75.17
10	102.33	109.66	112.73
50	102.13	106.71	100.82
100	106.56	92.81	88.69
200	95.46	60.37	59.56
300	90.30	57.72	40.90
400	67.83	65.53	43.07
500	75.52	65.27	41.99
1000	75.56	57.33	49.65

CO-: Negative Control; CO +: Positive Control; CS: DMSO Solvent Control.

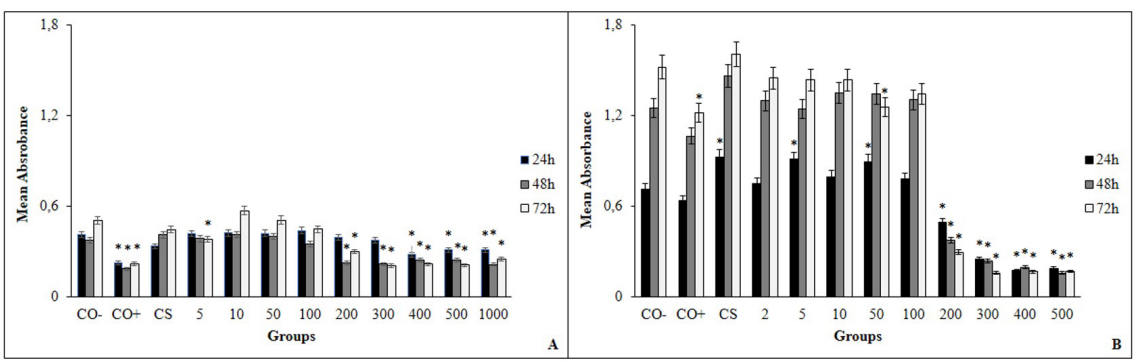

Figure 1. Mean absorbance and standard deviation of tumor cells from rat liver (HTC) treated for 24, 48 and 72 hours with the concentrations (µg/mL) of the semi-purified fraction with ethyl acetate (A) and hexane (B), of *Croton echioides*. CO-: Negative Control; CO +: Positive Control; CS: DMSO Solvent Control; 2.0x10^4 cells per well. * Result statistically different from the negative control (Dunnet test, $p < 0.05$).

population in sub-G1 and necrotic cell death. The essential oils of *C. doctoris* leaf also showed cytotoxic effect for melanoma cells (UACC62) and ovarian cancer (OVCAR), which according to the authors must be the presence of biologically active sesquiterpenes that acted by inhibiting the growth of cancer cells (Cândido et al., 2021). These results also may be due to the antioxidant activity of the *Croton* extract, as identified by Morais et al. (2006), with *C. zenhtneri*, *C. nepetaefolius* and *C. argyrophylloides* and the EAF fraction of the present study, as demonstrated by Novello et al. (2016). Antioxidants have antitumor activity because they generate metabolic changes in tumor cells, decreasing their cell proliferation, preventing the process of cell duplication and repair, reducing protein production or glucose metabolism, leading tumor cells to cell apoptosis (Rebello, 2005; Sanchéz, 2006).

The data in Figure 1B shows the results of the average absorbances obtained after the treatment of the tumor cells with the HF hexane fraction of *C. echioides*. The fraction showed cytotoxic potential for *Rattus norvegicus* hepatoma cells in concentrations above 200 µg/mL, at 24, 48 and 72 hours, with cell viability (Table 2) reaching 24.78% [400 µg/mL] at 24 hours, 12.79% [500 µg/mL] at 48 hours, and 10.57% [300 µg/mL] at 72 hours. It is worth noting that the data (Table 1) show an indication of a decrease in cell viability with the increase of the assessed concentration, but, like that observed for the EAF, with an even more evident effect depending on the exposure time (for all the assessed concentrations). According to Martins (2018), the hexanic extract of *Euphorbia tirucalli*, also from the Euphorbiaceae family, as well as *C. echioides*, presented itself as promising for the development of new anticancer drugs. Possibly this activity is again associated with the antioxidant activity of the extract, which was confirmed by Novello et al. (2012).

However, the concentrations of 5 and 50 µg/mL of the HF extract, within 24 hours, showed mean absorbances statistically higher than that of the negative control, indicating a stimulus of tumor cell proliferation. This effect was reversed, since, in 72 hours, the concentration of 50 µg/mL showed a mean absorbance that was statistically lower and different from the negative control.

Figure 2A shows the results of the average absorbances obtained with the liver tumor cells of *R. norvegicus* treated with the compound lupeol. The statistical analysis shows that all concentrations (1-100 µg/mL), at all times of evaluation (24, 48 and 72 hours), presented mean absorbances lower than their respective negative controls, indicating an evident cytotoxic effect of this compound for the HTC tumor cells. In fact, cell viability (Table 3) was less

Table 2. Percentage of viability (according to Equation 1) of tumor cells (VC) of rat liver (HTC), treated with the different concentrations (µg/mL) of the hexanic fraction (HF), of *Croton echioides*, for 24, 48 and 72 hours, for at least MTT test.

Groups	VC% of HTC cells		
	24 h	48 h	72 h
CO-	100.00	100.00	100.00
CO+	89.08	85.37	80.22
CS	129.80	117.21	105.66
2	105.49	104.04	95.25
5	127.76	99.90	94.36
10	111.77	108.12	94.37
50	125.68	107.79	82.70
100	109.33	104.54	88.43
200	69.24	30.01	19.52
300	35.40	19.17	10.57
400	24.78	15.76	11.07
500	26.53	12.79	11.24

CO-: Negative Control; CO +: Positive Control; CS: DMSO Solvent Control.

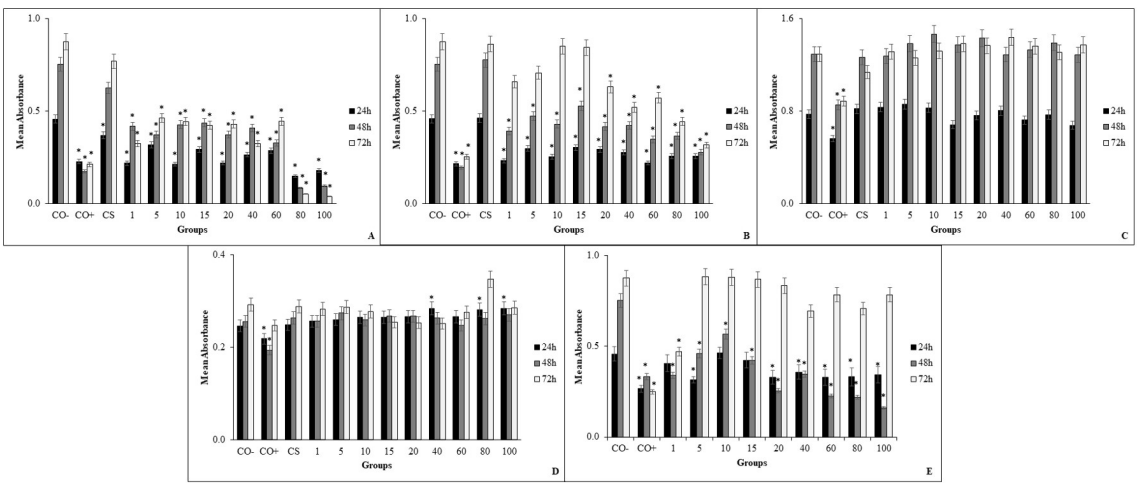

Figure 2. Mean absorbance and standard deviation of tumor cells from rat liver (HTC) treated for 24, 48 and 72 hours with the concentrations (µg/mL) of the compounds extracted and isolated from *Croton echioides*: Lupeol (A), CEH -1 (B), CEH-2 (C), CEH-3 (D) and CEH-4 (E). CO-: Negative Control; CO +: Positive Control; CS: DMSO Solvent Control; $2.0x10^4$ cells per well. * Result statistically different from the negative control (Dunnet test, $p < 0.05$).

Table 3. Percentage of viability (according to Equation 1) of tumor cells (VC) of rat liver (HTC), treated with different concentrations (µg/mL) of the compounds extracted and isolated from *Croton echioides*, for 24, 48 and 72 hours, by the MTT.

Groups	VC% of HTC cells														
	Lupeol			CEH-1			CEH-2			CEH-3			CEH-4		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CO-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO+	49.82	23.09	24.20	46.93	25.84	28.94	72.91	66.13	68.71	88.59	75.85	84.63	58.12	44.12	28.54
CS	80.54	82.94	87.99	100.84	102.98	98.61	105.98	97.77	88.06	100.88	102.99	98.63	-	-	-
1	47.86	55.47	37.11	50.75	51.92	75.30	107.84	98.63	101.92	103.98	100.20	96.64	88.78	45.02	53.90
5	69.52	49.45	52.96	65.21	62.83	80.79	111.25	106.99	97.60	105.47	107.10	98.06	68.92	61.11	100.84
10	46.55	56.75	50.71	55.39	57.16	97.10	107.27	113.21	102.45	107.56	101.30	94.82	101.17	75.21	100.50
15	64.12	58.02	48.39	66.41	70.13	96.44	88.44	106.26	107.15	107.43	104.69	86.85	92.38	56.00	99.30
20	47.91	49.53	49.25	64.01	55.34	72.07	98.64	110.50	105.83	108.17	104.43	86.51	71.98	33.81	95.27
40	58.01	54.47	37.22	60.24	56.13	59.51	103.94	99.60	111.42	115.22	102.93	85.94	78.09	46.18	79.42
60	62.48	43.59	50.89	48.07	46.05	65.29	93.41	102.83	105.55	107.90	96.61	94.37	72.08	30.09	89.62
80	32.58	11.38	6.06	56.26	48.67	50.76	99.61	107.54	101.26	114.25	102.54	118.61	72.47	28.96	80.96
100	39.23	12.84	4.37	55.99	37.02	36.19	87.68	99.49	106.52	115.33	105.47	97.55	74.87	21.33	89.59

CO-: Negative Control; CO +: Positive Control; CS: DMSO Solvent Control.

than 70% (24 hours), 59% (48 hours) and 53% (72 hours), reaching a minimum viability of 32.58% (24 hours), 11.38% (48 hours), and 4.37% (72 hours). It is worth noting that for the highest concentrations (80 and 100 µg/mL) a dose and time dependent effect can be observed. Exposure-dependent effect was evidently observed for the two highest concentrations (80 and 100 µg/mL), reaching the lowest percentages of cell viability within 72 hours (80 µg/mL = 6.06% and 100 µg/mL = 4.37%). Saleem et al. (2005) and Wu et al. (2013) also proved that treatment with lupeol significantly decreased the viability of prostate cancer cells and gastric cancer, respectively, in a dose dependent manner. Lupeol has antioxidant, anti-inflammatory, antiarthritic and antimutagenic activities, both in vitro and in vivo (2013). And, Saleem et al. (2005) justifies that the antiproliferative activity of lupeol is due to the forwarding of cells to cell apoptosis, an event not evaluated in the present study, but which may have occurred with HTC cells treated with lupeol.

The results of the study with lupeol can also be justified by its antioxidant activity. Prasad et al. (2007) showed that lupeol has the ability to restore antioxidant enzymes and decrease the lipid peroxidation of liver cells, events that may have contributed to the triggering of tumor cell death. Again, according to Noldin et al. (2013) lupeol inhibited the growth of tumor cells of hepatocellular carcinoma (Hep-G2), B cell hybridoma (A-431) and hepatoma (H-4IIE). And, in the study by Pitchai et al. (2014), breast carcinoma (MCF-7) was also inhibited by the same compound.

The data in Figure 2B shows the results of the average absorbances obtained with the treatment of HTC cells with the compound CEH-1 (Figure 3). The data show that all concentrations (1-100 µg/mL), at the evaluation times of 24 and 48 hours, showed mean absorbances statistically lower than their respective negative controls, with cell viability (Table 3) less than 67% (24 hours) and 71% (48 hours), indicating cytotoxic/antiproliferative effect of this compound for the tumor cells evaluated. At 72 hours, only concentrations greater than 20 µg/mL showed this cytotoxic effect, with cell viability below 73% [20 µg/mL], reaching 36.19% [100 µg/mL]. This effect demonstrated that the long-term applied doses of this product were obtained in order to allow for the toxic/timer effect.

Grynberg et al. (1999) studying *C. cajucara*, identified in vitro and in vivo antitumor activity of substances of the clerodane-type diterpene class, such as CEH-1. Freire et al. (2003) identified a cytotoxic effect of clerodan-type diterpenes on human promyelocytic leukemia cells, justifying their action due to the formation of adducts with DNA and proteins and/or the induction of oxidative stress, events that may also have been stimulated by the diterpene type clerodane CEH-1 in the present study.

The data in Figure 2C shows the results of the average absorbances of the treatment with the compound CEH-2 (Figure 3). The statistical analysis showed no difference between the absorbances of the treatments with the different concentrations of this and the negative control, at all times of evaluation (24, 48 and 72 hours). In fact, cell viability (Table 3) was above 87% (24 hours), 98% (48 hours) and 97% (72 hours). Thus, this compound, extracted and isolated from *C. echoides*, did not show cytotoxic potential for liver tumor cells, at the concentrations and times evaluated.

Two diterpenes isolated from *C. blanchetianus* obtained a significant decrease in the viability of *Streptococci* biofilms, a bacterium that causes cavities Firmino et al. (2019). In addition, kaurenoic acid, a diterpenoid obtained from copaiba oil, increased the frequency of DNA damage in a bowel cancer strain in a dose-dependent manner Cardoso et al. (2017). These results indicate that the biological activities of diterpenes can vary according to their origin and isolated metabolite.

Figure 2D shows the results of the average absorbances obtained with the treatment of HTC cells with the mixture of ptychonal compounds CEH-3a and ptychonal hemiacetal CEH-3b (Figure 3). Differently from the results obtained with the other compounds, the data show, within 24 hours, the concentrations of 40, 80 and 100 µg/mL showed mean absorbances statistically higher than that of the negative control, indicating a stimulus of the proliferation of tumor cells exposed to this compound. Even cell viability (Table 3) reached 115.33% (concentration of 100 µg/mL - 24 hours). However, with the time of treatment (48 and 72 hours), the absorbances became similar to those of their negative controls.

Figure 2E, which presents the results of the average absorbances obtained with the treatment of HTC liver tumor cells with the compound CEH-4 (Figure 3), shows that within 24 hours the concentrations of 5 µg/mL and above 20 µg/mL showed mean absorbances statistically lower than that of the negative control, indicating a cytotoxic/antiproliferative effect. Within 48 hours, all concentrations (1-100 µg/mL) had a cytotoxic effect on HTC cells, obtaining the lowest cell viability (Table 3) (<76%), reaching 21.33% (100 µg / mL). However, at 72 hours, the cytotoxic effect was only maintained at the lowest concentration evaluated (1 µg/mL). These results suggest that for the continuation of cytotoxic activity in longer exposure times, probably, new doses of this compound should be applied over time.

According to Pittaluga et al. (2013) compounds isolated from hardwickic acid, such as CEH-4 (methyl ester of hardwickic acid) in the present study, deserve further studies to clarify their pharmacological and toxicological profiles in vitro, since hardwickic acid has an antiproliferative effect on tumor cells (Lama et al., 2014).

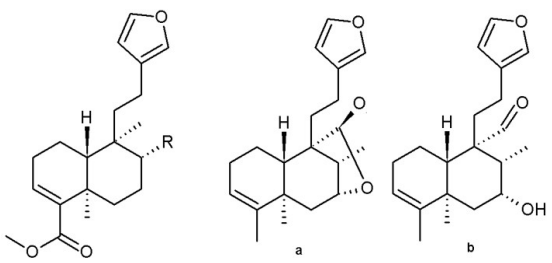

Figure 3. Clerodan diterpenes tested.

Furthermore, CEH-4 has a structure similar to CEH-1, differing mainly in the displacement of hydrogens and carbons in positions C-7 and C-8, a fact that occurs because in carbon 8 there is a carboxy group in the molecule of CEH-1, and a methyl group in CEH-4. Thus, the cytotoxic effect found in both molecules can be related to their similar structure.

4. Conclusions

In conclusion, the Euphorbiaceae family constitutes a group with high potential for the discovery of new molecules. In this work it was possible to confirm the cytotoxic/antiproliferative potential of *C. echioides* and its isolated compounds: lupeol, CEH-1 and CEH-4 to HTC cells, possibly due to its antioxidant activities that may have activated cellular apoptosis pathways and thus resulted in decreased cell viability. Thus, it is encouraged those other studies related to the cytotoxic/antiproliferative activity of this botanical group, especially of *C. echioides*, be carried out, in order to ascertain the anticancer effect in other cell lines and in vivo tests.

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