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Evaluation of the cytotoxicity of products obtained from *Calotropis procera* (Apocynaceae)

Avaliação da citotoxicidade dos produtos obtidos de *Calotropis procera* (Apocynaceae)

Evaluación de la citotoxicidad de productos obtenidos de *Calotropis procera* (Apocynaceae)

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Abstract

Calotropis procera belongs to the Apocynaceae family and is found in the Northeast of Brazil. Several activities are attributed to this species, such as anti-inflammatory, antibacterial and anti-cancer activities. This research aimed to conduct a bioguided study of *C. procera* in order to isolate and identify triterpenes of the species and evaluate the cytotoxic activity of products of the plant species. The crude ethanolic extract (CEE) was obtained and partitioned. The hexanic phase was chromatographed giving the compound Cp-1, which was identified using spectroscopic techniques and thermal analysis. It was possible to identify Cp-1 as a triterpene called calotropenyl acetate, already reported in the literature. The cytotoxicity test was performed using the MTT technique and it was observed a good activity of CEE and hexanic phase against HL-60 and KS62 lines with an IC_{50} ranging between 26.8 ± 0.4 µg mL^{-1} and 47.0 ± 0.5 µg mL^{-1}, besides no toxicity in normal lines, while Cp-1 showed no relevant activity against the tumor cells tested. The results indicated that the species contains in its chemical composition products with pharmacological interest, in addition to cytotoxic for leukemic cells, which can be further explored in complementary studies.

Keywords: “Algodão de seda”; Titerpene; Cytotoxicity; Calotropenyl acetate.

Resumo

Calotropis procera é um vegetal arbustivo pertencente à família Apocynaceae, sendo amplamente encontrado na região Nordeste do Brasil. Diversas atividades são atribuídas a

espécie, tais como as atividades anti-inflamatórias, antibacteriana e anticancerígena e o gênero Calotropis caracteriza-se por apresentar flavonoides, terpenos, taninos e compostos fenólicos. Esta pesquisa teve como objetivo realizar um estudo bioguiado de C. procera com o intuito de isolar e identificar triterpenos da espécie e avaliar a atividade citotóxica dos produtos da espécie vegetal. O extrato etanólico bruto (EEB) foi obtido por maceração em etanol a 96% e particionado com solventes orgânicos, utilizando um gradiente de polaridade. A fase hexânica foi cromatografada em coluna com gel de sílica e eluída com solventes orgânicos puros ou em misturas binárias dando origem ao composto codificado por Cp-1, que foi identificado utilizando técnicas espectroscópicas (RMN e IV) e de análise térmica. A avaliação dos dados espectroscópicos e de análise térmica permitiu identificar Cp-1 como sendo um triterpeno denominado acetato de calotropenil, já relatado na literatura. Também foi realizado o ensaio de citotoxicidade dos produtos obtidos da espécie utilizando a técnica de MTT. No ensaio de citotoxicidade observou-se uma boa atividade do EEB e da fase hexânica frente as linhagens HL-60 e KS62 com um IC50 variando entre 26.8 ± 0.4 µg mL-1 e 47.0 ± 0.5 µg mL-1, além de nenhuma toxicidade em linhagens normais, enquanto que Cp-1 não mostrou atividade relevante contra as células tumorais testadas. Os resultados indicaram que a espécie contém na sua composição química produtos com potencial fitoquímico para isolamento e identificação de compostos por técnicas espectroscópicas e termoanalíticas, além de citotóxico para células leucêmicas, que podem ser mais explorados em estudos complementares.

Palavras-chave: “Algodão de seda”; Triterpeno; Citotoxicidade; Acetato de calotropenila.

Resumen

Calotropis procera es una planta arbustiva perteneciente a la familia Apocynaceae, que se encuentra ampliamente en la región noreste de Brasil. A la especie se le atribuyen diversas actividades, como antiinflamatorias, antibacterianas y anticancerígenas, y el género Calotropis se caracteriza por presentar flavonoides, terpenos, taninos y compuestos fenólicos. Esta investigación tuvo como objetivo realizar un estudio bioguiado de C. procera con el fin de aislar e identificar triterpenos de la especie y evaluar la actividad citotóxica de productos de la especie vegetal. El extracto etanólico crudo (EEB) se obtuvo mediante maceración en etanol al 96% y se repartió con disolventes orgánicos, utilizando un gradiente de polaridad. La fase de hexano se cromatografió en columna con gel de sílice y se eluyó con disolventes orgánicos puros o en mezclas binarias dando lugar al compuesto codificado por Cp-1, que se identificó mediante técnicas espectroscópicas (RMN y IV) y análisis térmico. La evaluación de los datos

del análisis espectroscópico y térmico permitió identificar al Cp-1 como un triterpeno denominado acetato de calotropenilo, ya reportado en la literatura. La prueba de citotoxicidad de productos obtenidos de la especie también se realizó mediante la técnica MTT. En la prueba de citotoxicidad, se observó una buena actividad de la EEB y de la fase hexano contra las cepas HL-60 y KS62 con un IC50 que varió de 26,8 ± 0,4 µg mL-1 a 47,0 ± 0,5 µg mL-1, además de ausencia de toxicidad en cepas normales, mientras que Cp-1 no mostró actividad relevante contra las células tumorales ensayadas. Los resultados indicaron que la especie contiene en su composición química productos con potencial fitoquímico para el aislamiento e identificación de compuestos por técnicas espectroscópicas y termoanalíticas, así como citotóxicos para células leucémicas, que pueden ser explorados con mayor profundidad en estudios complementarios.

Palabras clave: “Algodão de seda”; Triterpeno; Citotoxicidad; Acetate de calotropenilo.

1. Introduction

The Apocynaceae family has about 300 genera and 2000 species, distributed in tropical and subtropical regions of the world. In South America 52 genera and 377 species are mentioned. It is characterized by a great morphological variability in its floral organs and by the diversity of secondary metabolites (Koch, 1994; Lima, Scareli-Santos, 2016). Due to the large number of secondary metabolites isolated from leaves and bark of the family representatives, it presents itself as an important source of bioactive compounds. Its constituents include: alkaloids, cardioactive and cyanogenic glycosides, saponins, tannins, coumarins, triterpenoids and phenolic acids (Oliveira, 2015).

The species *Calotropis procera* is a small tree (or shrub), erect, highly branched, with about 2.5 m in height, and can reach up to 6 m. It is popularly known as “algodão de seda”, “leiteira”, “saco de velho”, “queimadeira” e “ciúmeira”, varying according to its location (Costa et al, 2015; Quazi, Mathur, Arora, 2013; Silva, 2015; Tavares, 2017; Veloso, 2015).

Several biological activities have been described for *Calotropis procera* (Magalhães et al., 2010), such as antibacterial (Almeida et al., 2018), anti-inflammatory (Jucá et al.., 2013), analgesic (Barros et al., 2004), anticancer (Ibrahim et al., 2014), antioxidant (Yao et al., 2015), purgative, anthelmintic, expectorant and sedative (Ranjit et al., 2012).

Some pentacyclic triterpenes, such as lupeol and ursolic acid, have been previously isolated from the species. This class of metabolites arouses great interest due to the various biological activities presented, thus serving as candidates for new drugs (Moreira, 2013;

Nunes, 2013). The objective of this work was to carry out a bioguided study through the isolation and chemical characterization of calotropenyl acetate, as well as to evaluate its cytotoxic activity.

2. Materials and Methods

2.1 Collection and obtaining of CEE

C. procera, belonging to the Apocynaceae family, was collected in the coastal zone in the municipality of Cabedelo - PB (S 7 ° 02'29.9 "/ W 34 ° 50'23.6") on April 30, 2014. The plant material was processed in the city of Campina Grande - PB at the Phytochemistry Laboratory at the State University of Paraíba. An exsiccate is deposited at the Herbarium Lauro Pires Xavier of the Federal University of Paraíba under the inscription JPB 58031.

The stems were submitted to the drying process in an air circulation oven at 50 ° C for three days. After being removed, the dry stems were ground in a knife mill. Obtaining the powder that weighed 500 g, used in the preparation of the crude ethanolic extract (CEE).

2.2 Preparation of CEE

The methodology used to obtain CEE was the classic maceration methodology known and used in phytochemistry. The dry powder obtained (500 g), was subjected to the maceration process in 70% ethanol. The macerate was filtered every three days, and the extractive solution was stored under refrigeration. This procedure was repeated for 15 days, totaling six extractions. Subsequently, the filtration and evaporation of the solvent was carried out in a rotary evaporator under reduced pressure, obtaining a dry weight of 146.17g of CEE.

2.3 CEE Fractionation

Part of the CEE (120.0 g) was solubilized in a solution of MeOH : H2O (7:3 – V:V) and partitioned with the organic solvent hexane, providing for this study the hexanic phase with weight of 4.07 g.

2.4 Obtaining the hexane phase from the crude ethanolic extract

The BSE of the stem was subjected to a liquid-liquid partition process, with solvents of increasing polarity. In this study the solvents used were: hexane, chloroform and ethyl acetate. Initially, we weighed about 120.0 g of BSE from the stem of C. procera, which was dissolved in 840 mL of hydroalcoholic solution, methanol-water in a 3: 1 ratio. The mixture was then transferred to a separating funnel, in which hexane was first added, the hexane phase was removed and then subjected to evaporation, obtaining the hexane phase of the stem of Calotropis procera, which weighed approximately 4.07 g.

2.5 Chromatographic procedure for triterpene isolation

The hexanic phase (4.07 g) was used for column chromatographic fractionation (CC), using silica gel as stationary phase and pure organic solvents or in binary mixtures (hexane - dichloromethane, ethyl acetate - methanol) as mobile phase.

156 fractions were obtained, among them, fraction 21 obtained directly from the column, presented as a white solid with weight of (69.4 mg) soluble in hexane-dichloromethane (1:1), called Cp-1.

2.6 Cp-1 structural identification

The infrared spectrum was obtained in a Bomem Michelson spectrometer, using KBr tablets, with a scan of 4000 to 650 cm^{-1}. The ^{1}H and ^{13}C NMR spectra, using uni and bidimensional techniques, were recorded in a BRUKER ASCEND^{TM} spectrometer operating at 400 MHz. $CDCl_3$ solvent was used, the chemical displacements (δ) being expressed in parts per million (ppm), using the solvent itself as internal reference and the coupling constants (*J*) in Hz.

2.7 Characterization of Cp-1 by thermoanalytical techniques

For thermogravimetric analysis (TG) the simultaneous thermal analyzer SDT Q600 (TA® - Instruments, New Castle, Delaware, EUA) was used. 3.00 ± 0.05 mg of the sample were used, with a heating ratio of 10 °C min^{-1}, in the temperature range of 30 °C to 900 °C, under a nitrogen atmosphere with a flow rate of 100 $mL.min^{-1}$. The differential scanning

calorimeter (DSC) were obtained in calorimetric module DSC Q20 (TA® - Instruments, New Castle, Delaware, EUA). 1.00 ± 0.05 mg of sample was used, packaged in aluminum crucible, analyzed at 10 °C min^{-1} heating ratio, with a temperature of 30 °C to 300 °C, under a nitrogen atmosphere with a flow rate of 50 mL min^{-1}.

2.8 Evaluation of CEE and Cp-1 cytotoxicity against several tumor and normal strains by MTT

The following tumor cells were used for the assay: JUKART (acute T-cell lymphoid leukemia), HL-60 (acute promyelocytic leukemia), K562 (chronic myeloid leukemia) and HCT 116 (colorectal carcinoma). The non-tumoral cells used were: L929 lineage (murine fibroblast) and PBMC (mononuclear cells of peripheral human blood). The cells were obtained from the Cell Bank of Rio de Janeiro, Brazil, and maintained in the cell culture sector of the Pharmacotoxicology Prospecting Laboratory of Bioactive Products of UFPE. For the test, the cells were plated in a 96-well plate in the following concentrations: HL-60, JUKART and K562 (0.3 × 106 cells/mL) using RPMI medium supplemented with 10% fetal bovine serum; PBMC (106 cells/mL), HCT-116 and L929 (105 cells/mL) using DMEM medium supplemented with 10% fetal bovine serum. The compounds previously dissolved in DMSO (Dimethylsulfoxide, Vetec, São Paulo, Brazil) were diluted in medium and added to each well at the desired concentrations. To calculate the concentration that inhibits 50% of cell growth (CI_{50}), the compounds were diluted in series to obtain the final concentrations of 1.56-100 µg mL^{-1}. Doxorubicin was used as standard in the concentration range between 0.0024-50 µg mL^{-1}. After 72 hours of compound incubation, 25 µL of MTT solution (5 mg mL^{-1}) were added to each well. The plates were left for another 3 hours in the incubator at 37 ºC and, at the end of this period, the supernatural was aspirated. To perform the reading, 100 µL of DMSO were added to each well for the dissolution of the formazan crystals. The amount of formazan was measured at 560 nm absorbance in the spectrophotometer (Thermo Plate, São Paulo, Brazil). CI_{50} was calculated from non-linear regression in the Prism 5.0 software (GraphPad, San Diego, CA, USA). Each sample was tested in triplicate in three independent experiments.

3. Results and Discussion

In the analysis of the Cp-1 IR spectrum absorption bands of 2943 cm^{-1} and 2854 cm^{-1} were observed suggestive of C-H sp^3 stretching. In 1728 cm^{-1} and 1639 cm^{-1} we observed

characteristic bands of ester carbonyl and C=C, respectively, besides bands in 1246 cm^{-1} and 883 cm^{-1} suggestive of C-O binding and C-H deformation of oleofins.

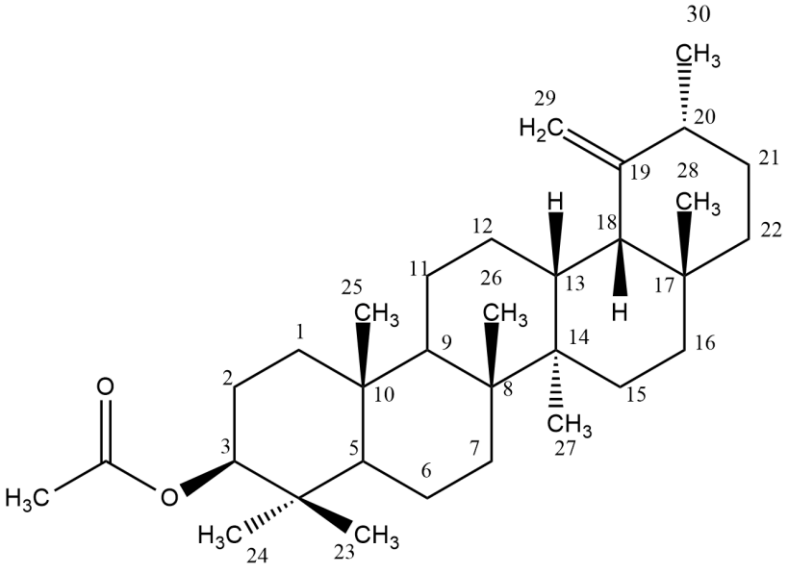
In the ^{1}H (400 MHz, $CDCl_3$) NMR spectrum a signal was observed between δ_H 2.5-0.7 which are characteristic of methylene and methylene hydrogen from triterpenic or steroidal nuclei. A sign at δ_H 4.50 (dd, $J_{ax,ax}$ = 9.70 Hz and $J_{ax,eq}$ = 6.70 Hz, 1H) suggests the presence of H-3 in carbon carbinolic. In addition, the two signs in δ_H 4.60 (d, J = 2.20 Hz, 1H) and δ_H 4.59 (d, J = 2.00 Hz, 1H) suggest the presence of olefinic hydrogen present in the C-29, characteristics of ursane nucleus triterpenes, already isolated from the same species in the works of Cavalcante (2015), Chundattu, Agrawal, Ganesh (2016), Kakkar et al. (2012) and Khan et al. (1988).

In the ^{13}C NMR spectrum (100 MHz, $CDCl_3$), using the APT technique, 32 signals were observed, 8 for methyl carbons, 11 for methyl groups, 6 for methyl carbons and 7 for non hydrogenated carbons, according to the study reported by Chundattu, Agrawal, Ganesh (2016). The shift in δ_C 171.11 suggests the presence of an ester carbonyl, corroborating with what was observed in the IV spectrum. In the HSQC heteronuclear correlation map a shift was seen at δ_H 4.50/δ_C 81.08. On the HMBC, the long-distance correlation between δH 4.50/δC 171.11, corroborated by the band in the IV in 1728 cm^{-1}, reinforced the presence of an ester grouping connected in the C-3. A signal was also seen at δ_C 154.70 suggestive of non-hydrogenated carbon (C-19), which together with the peak observed at δ_C 107.26 marked for C-29 indicates the presence of methylenic carbon.

The presence of shifts at δ_H 4.60 (d, J = 2.20 Hz) and δ_H 4.50 (d, J = 2.00 Hz) characterized a geminal coupling, suggestive of the presence of a methylenic terminal group in the C-29, reinforced by the couplings seen in the HSQC of δ_H 4.60 and 4.50/δ_C 107.26 and in the HMBC between δ_H 4.60 and 4.59/δ_C 50.51 and 39.49. According to Olea and Roque (1990) pentacyclic triterpenes contain at most a double bond, and reinforce the presence of the ursane-type triterpene.

The interpretation of IR and NMR data allowed identifying Cp-1 as calotropenyl acetate (Figure 1), a compound already isolated previously in *C. procera*, according to Cavalcante (2015), Chundattu, Agrawal, Ganesh (2016), Kakkar et al. (2012) and Khan et al. (1988).

Figure 1. Chemical structure of calotropenyl acetate.



Source: Research data.

Pentaciclic triterpenes are of great interest due to the diverse biological activities presented, thus serving as candidates for new drugs. One can mention the activities, anti-inflammatory, hepatoprotective and gastropotectors (MORERIRA, 2013; NUNES 2013).

With the help of NMRI and bi-dimensorial techniques it was possible to point out all direct (HSQC) and indirect (HMBC) correlations unequivocally for calotropenil acetate as shown in Table 1 below:

Table 1. ^{1}H and ^{13}C NMR data ($CDCl_3$, 400 MHz) and correlations between ^{1}H x ^{13}C signals (HSQC and HMBC) of calotropenyl acetate.

		HSQC	HMBC	
C	**δC**	**δH**	**^{2}J**	**^{3}J**
4	37.90	-	-	-
8	41.02	-	-	-
10	37.15	-	-	-
14	42.14	-	-	-
17	34.63	-	-	-
19	154.70	-	-	-
CH				
3	81.08	4.50 (d, *J*ax,ax=9.7 Hz d, Jax,eq =	C-2	C-1, C-4, C=O
5	55.55	6.7 Hz)	C-6, C-10	C-7
9	48.75	1.35 (m)	C-11	C-12, C-25
13	39.27	1.35 (m)	C-12	C-17, C-19
18	39.49	1.40 (m)	C-17, C-19	C-20
20	50.51	1.37 (m)	C-19, C-30	-
		1.31 (m)		
CH_2				
1	3841	1.37 (m)	C-10	C-9
2	23.81	1.62 (m)	C-1, C-3	-
6	18.30	1.48 (m)	C-5, C-7	-
7	34.11	1.36 (m)	C-8	C-5, C-9
11	21.58	1.23 (m)	C-12	C-13
12	25.73	1.64 (m)	C-11, C-13	C-14
15	26.26	1.65 (m)	C-14	C-13
16	26.76	1.68 (m)	C-17	C-18, C-22
21	38.55	1.34 (m)	C-22	C-17, C-19, C-30
22	38.98	1.37 (m)	C-17	C-22
29	107.26	4.60 (*J*= 2.2 Hz) e 4.59 (*J*= 2.0 Hz)	C-19	C-18, C-20
CH_3				
23	28.05	0.83 (s)	C-4	C-3, C-5
24	16.62	1.01 (s)	-	-
25	14.84	1.00 (s)	-	C-1, C-9
26	16.45	0.83 (s)	C-8	C-7, C-9
27	25.61	0.91 (s)	C-14	C-13
28	16.01	0.84 (s)	C-17	-
30	19.60	1.00 (d) (*J* = 3.7 Hz)	C-20	C-19, C-21
$OCOCH_3$	171.11	-	-	-
$OCOCH_3$	21.42	2.03 (s)	C=O	-

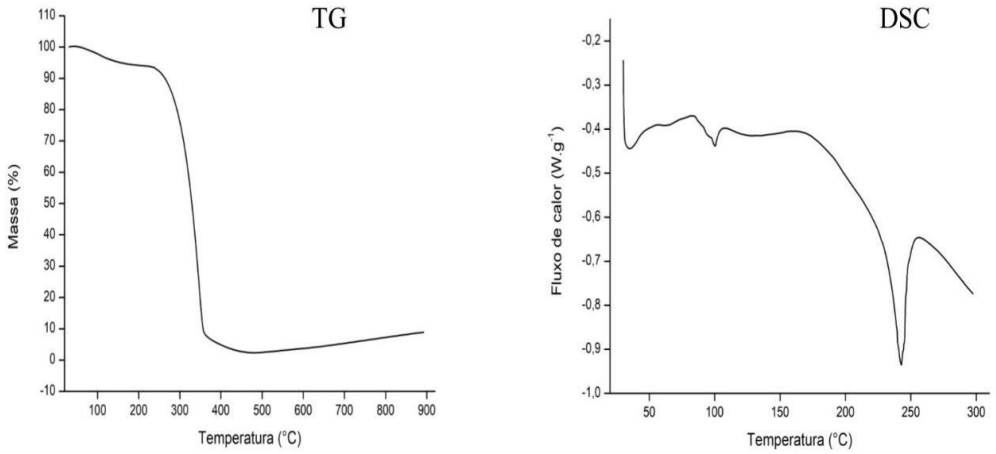
Fonte: Dados da pesquisa.

All data of chemical displacements of hydrogen and carbon plotted in table 1, with their coupling constants calculated, as well as all the correlations observed in the two-dimensional spectra HSQC and HMBC were essential for the complete clarification and identification of the ursane calotropenyl acetate type triterpene.

3.1 Cp-1 Characterization by Thermal Analysis

The Figure 2 below presents the thermogravimetric and calorimetric curves obtained from Cp-1.

Figure 2. Thermoanalytical curves of Cp-1.



Source: research data.

The thermogravimetric curve showed two stages of mass loss. The first occurred between 51.46 and 225.39 °C, with a mass loss of 6.339 %, and the second in the temperature range of 225.39 to 512.79 °C, with 91.19 % mass loss. The residue at 900 °C was 8,865 %, referring to the mineral residue of the sample. Two endothermic events were detected in the sample's calorimetric curve. The first had a peak temperature (T_{pico}) of 100.15 °C with a variation of enthalpy (ΔH) of 2.757 J g^{-3}, indicative of impurities in the sample. The second event had T_{pico} = 242.47 °C and ΔH = 18.30 J g^{-3}, suggestive of the melting point of the compound, since the amount of energy involved in this event was greater than that presented by the previous event. In the works of Chundattu, Agrawal, Ganesh (2016) and Khan et al. (1988), the fusion point of the compound presented was 198 °C, which shows a discrepancy with that obtained in this work, but the DSC is a technique with greater sensitivity in the detection of these events (Troni, 2017). It is important to highlight that the impurity present in Cp-1 may have interfered in the analysis, causing an increase in the melting value of the sample.

3.2 Evaluation of CEE, hexanic phase and Cp-1 cytotoxicity

The CEE and hexanic phase presented the best cytotoxic activity for leukemia strains (HL-60 and K562), with IC_{50} between 26.8 ± 0.4 µg mL^{-1} and 47.0 ± 0.5 µg mL^{-1}. The Cp-1 compound did not show significant results for the 72 h treatment of the tested strains.

Similar results with the present work can be found in the studies of Magalhães et al. (2010) and Al-Taweel et al. (2017), which showed selective cytotoxic activity of hexic phases

of the raw stem extract and raw leaf, flower and fruit extracts, respectively, of the species *C. procera* for leukemia and human colon strains.

The data suggest that the cytotoxic activity is not related to the presence of the Cp-1 compound, but to other components present in the raw extract and in the hexanic phase. The values for crude extract were within the cut off point of the National Cancer Institute criteria for cytotoxicity (IC_{50} < 30 µg mL^{-1}) in the *screening* of crude plant extracts, which indicates that these extracts are promising for further purification and study (Aguiar et al., 2012).

The CEE and hexanic phase were selective for *in vitro* treatment since they did not show cytotoxic activity in the non-tumoral lineage tested and in human cells (L929 and PBMC). The results of cytotoxic activity in normal and tumor cells are described in Table 2.

Table 2. Cytotoxic activity of CEE, hexanic phase and Cp-1 in normal and tumor cell lines for a period of 72 h. The values are expressed in CI_{50} concentration ± standard deviation of the mean. The compounds were evaluated at concentrations of 1.56-100 µg mL^{-1}.

	CI 50 (µg mL^{-1})			
Cell lines	**CEE**	**Hexanic Phase**	**Cp-1**	**Doxorubicin**
JUKART	80.5 ± 0.5	> 100	> 100	-
HL-60	29.5 ± 0.5	47.0 ± 0.4	> 100	0.28 ± 0.002
K562	26.8 ± 0.4	45.5 ± 0.5	> 100	0.8 ± 0.02
HCT 116	95.9 ± 0.5	> 100	> 100	0.35 ± 0.02
L929	> 100	> 100	> 100	2.4 ± 0.4
PBMC	> 100	> 100	> 100	0.26 ± 0.01

Source: Research data.

Based on Table 2, it can be seen that the CEE of C. procera and the hexane phase inhibited cell proliferation in leukemic lines and did not show cytotoxicity in normal lines, while the isolated compound did not show cytotoxicity, indicating that the activity is related to other compounds present in the extract and in the hexane phase.

4. Final Considerations

The objective of the work was achieved with the isolation and identification of a ursane-type triterpene, called calotropenyl acetate, the compound was isolated in good quantity (69.4 mg) which made it possible to perform cytotoxicity tests, although the isolated compound did not has shown positive results, the crude extract and the hexane phase have shown promise.

The thermoanalytical characterization by TG and DSC performed of the compound allowed to obtain its profile of change in its physical-chemical properties as a function of temperature, an important result in relation to the stability of the compound, considerable, for example, for the development of a possible formulation with the substance.

C. Procera CEE and the hexane phase inhibited cell proliferation in leukemic lines and did not show cytotoxicity in normal lines, while the isolated compound did not show cytotoxicity, indicating that the activity is related to other compounds present in the extract and in the hexane phase.

In this way, the phytochemical contribution of the species under study makes us glimpse a relevance with regard to the search for new biological compounds with biological activities from plants, which may add up as alternatives to the problems currently faced in the health area. In view of the great and diversified biodiversity of Brazil that has not yet been explored.

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