



Original Article

Euphorbia umbellata bark extracts – an *in vitro* cytotoxic study



Carla C. Kanunfre^a, Talissa Leffers^a, Luiza S. Cruz^b, Lívia E.C. Luz^b, Amanda R. Crisma^c, Mei Wang^d, Bharathi Avula^d, Ikhlas A. Khan^{d,e}, Flávio L. Beltrame^{b,*}

^a Departamento de Biologia Geral, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil

^b Departamento de Ciências Farmacêuticas, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil

^c Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil

^d National Centre for Natural Products Research, University of Mississippi, Oxford, MI, United States

^e Department of Biomolecular Sciences, School of Pharmacy, University of Mississippi, Oxford, MI, United States

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ABSTRACT

Euphorbia umbellata (Pax) Bruyns, Euphorbiaceae, is commonly used in folk medicine of southern Brazil to treat several kinds of cancer. The latex (part of the plant used for this purpose) is mixed with water and taken as treatment; but this matrix contains toxic potential related to the presence of some phorbol type diterpenes. So the aim of this study was to evaluate the cytotoxicity of the crude extract of the bark of *E. umbellata* and its fractions (Hex, CHCl₃, EtOAc and MeOH) using *in vitro* assay (applying Jurkat cells line). A preliminary cytotoxic study (MTT reduction, trypan blue exclusion and DNA quantification assays) was executed to identify the most active material. The CHCl₃ fraction displayed the highest activity and was selected for further investigation of any cytotoxic mechanism and evaluation of chemical composition; flow cytometry, Acridine orange and Hoechst 33342 staining experiments and Gas chromatography–mass spectrometry analysis were applied to achieve these results. This fraction demonstrated the best cytotoxic results against Jurkat cells line with IC₅₀ of 29.00 ± 1.49, 10.06 ± 1.48 and 4.83 ± 2.25 µg/ml for 24, 48 and 72 h of experiment, respectively (trypan blue exclusion). The mechanism responsible for this action can be associated with the promotion of cell cycle arrest and apoptosis. The two main classes of compounds present in the CHCl₃ fraction are steroids and triterpenes. Further, phytochemical studies with this fraction need to be evaluated, to try isolating these substances and establishing a more detailed cytotoxic study against Jurkat cells.

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Introduction

Cancer is one of the main causes of death in the world and the number of cases increases every year. According the World Health Organization, by 2035, the mortality caused by different types of cancer will increase by 70%, to more than 22 million new cases; most of them (60% approximately), occurring in non-developed countries of the African, Asian and American (mainly Central and South America) continents (WHO, 2015). In many of the countries, the use of medicinal plants to treat diseases, are very common and can be ascribed to two main factors: ease of access and low cost (Borges et al., 2013; Orlanda and Vale, 2015). These observations are reinforced when one considers that medicinal plants are often the first source for new drugs to treat cancer, inasmuch as the use of the material is based on long popular traditions and also because

more than half of the anti-cancer drugs present in the pharmaceutical market are derivate from natural sources (Bhanot et al., 2011; Manosroi et al., 2012).

The scientific literature describes many important drugs for cancer treatment that were obtained from plants. Some examples are vincristine and vinblastine that were isolated from *Catharanthus roseus*, Apocynaceae, paclitaxel obtained from some species of *Taxus* sp., Taxaceae, podophyllum toxin, a lignin from the genus *Podophyllum*, Berberidaceae and the alkaloid camptothecin identified in *Camptotheca acuminata*, Nyssaceae (Costa-Lotufo et al., 2010). These data corroborate the above statements, confirming that plants used for medicinal purposes can represent a rich matrix for the discovery of new molecules to treat different kinds of diseases, mainly cancer.

Euphorbia umbellata (Pax) Bruyns, Euphorbiaceae (Luz et al., 2015) is popularly known in Brazil as “janauba” and “cola-nota” and the latex has been used in folk medicine as anti-inflammatory, anti-ulcer, homeostatic and angiogenic; however, the main popular indication is as an anti-cancer agent (Melo-Reis et al., 2010; Costa

* Corresponding author.

E-mail: flaviobeltra@uepg.br (F.L. Beltrame).

et al., 2012; Oliveira et al., 2013; Badgujar, 2014; Munhoz et al., 2014; Luz et al., 2016).

The latex (18 drops) dissolved in one liter of water is often used to treat several types of cancer, and Costa et al. (2012), point out that *Synadenium grantii* (*E. umbellata*) latex contains a wide diversity of substances, and also describe the toxic potential of this matrix (Ortêncio, 1997).

Researchers have shown that the latex contains steroids, terpenes (diterpenes, sesquiterpenes and triterpenes), phenolic compounds, proteins and other chemical substances that have already had their activity measured for anti-cancer activity (Premaratna et al., 1984; Rajesh et al., 2006; Dawidar et al., 2011; Oliveira et al., 2013; Luz et al., 2015). But, the presence of phorbol type diterpenes in this matrix gives the latex undesirable irritant and toxic properties that discourage the use (Docampo et al., 2010; Hassan et al., 2012).

Other species are been studied against different cancer models. The mutagenic potential of the ethanolic extract from the leaves of *Synadenium umbellatum* (*E. umbellata*) against bone marrow cells of mice using *in vitro* and *in vivo* models has already been reported describing a dose-dependent cytotoxic effect. Also, an acute and sub-acute toxicity study with the leaves extract describes that in the doses evaluated (50, 100 and 200 mg/kg) the materials did not presented histological modifications in the organs evaluated and the physiological parameters also did not show alterations (Valadares et al., 2007; Cunha et al., 2009).

However, no cytotoxic study using the bark of *E. umbellata* has appeared in the scientific literature. For this reason, the present research has the goal to evaluate the effect of bark crude extract of *E. umbellata* and the different fractions obtained from this material against Jurkat cells (leukemia).

Materials and methods

Reagents and equipment

Ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), chloroform (CHCl₃) and hexane (Hex) were purchased from Biotec[®] and were of analytical grade. Water was purified using distiller system (Quilms[®]) and was used for prepare all the solutions. RPMI 1640 medium and fetal bovine serum and phosphate both saline (PBS) were purchased from Cripion[®]. Histopaque reagent, diphenylamine (DPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, lanosterol, lupeol, penicillin and streptomycin were purchased from Sigma-Aldrich[®]. Euphol was obtained from Hex fraction and friedelin-3 β -ol from CHCl₃ fraction after several columns procedure, according describes by Oliveira et al. (2013) and Munhoz et al. (2014). The extracts were prepared by maceration and concentrated on a Fisatom[®] 558 evaporator coupled to a Marconi[®] MA053 vacuum pump. The cells count was determined on a fluorescence optical microscope (Olympus[®] BX 41) and an ELISA plate reader (Biotek[®], μ Quant). Flow cytometry experiments were performed on a FACS Calibur (Becton & Dickinson – BD[®]).

Botanical material

Stem bark of *Euphorbia umbellata* (Pax) Bruyns, Euphorbiaceae, was collected in Ponta Grossa, Paraná, Brazil (altitude: 975 m, coordinates: 25°05'38" S and 50°09'30" W) in August 2013. One voucher specimen was deposited on the Maria Eneida P. Kauffmann Fidalgo Herbarium (#453920).

Extraction and isolation

The powdered and dried material (stem bark, 334 g) was used to prepare the crude extract. In order to obtain this material, a fractional maceration procedure (at room temperature and in the dark) was applied using ethanol:water (70:30, v/v, 1 l); the solvent was changed daily and the procedure was repeated for seven days. The different solutions were pooled (7 l), filtered and the solvent removed under reduce pressure (40 °C). The viscous material (yielding 70 g, 21%, w/w) was freeze-dried and identified with the code CBE and after stored under refrigeration (4 °C). An aliquot of CBE (8 g) was subjected to a chromatographic column process and eluted applying an increasing gradient of solvents (Hex, CHCl₃, EtOAc and MeOH, 3 l each solvent). Friedelin-3 β -ol (1.2 mg) was isolated from CHCl₃ fraction as described by Munhoz et al. (2014).

Cell culture

The leukemic cells (Jurkat clone E6-1) were obtained from commercial cell bank (BCRJ code 0125, Rio de Janeiro) as a suspension culture and maintained under controlled humidified atmosphere and temperature (5% CO₂ and 37 °C, respectively). The cells growth occurred in RPMI 1640 both (pH 7.4) supplemented with 10% fetal bovine serum (FBS), 24 mmol l⁻¹ of sodium bicarbonate, 2 mmol l⁻¹ of glutamine and 1% penicillin and streptomycin. This material was used to obtain subcultures, also in suspension, in order to maintain cells in log growth phase.

Cytotoxicity assays

MTT reduction assay

Aliquots of the crude extract in several concentrations (50–800 μ g/ml solubilized in DMSO <0.5%) and fractions (6.25–100 μ g/ml solubilized in DMSO <0.5%) were added in a 24-well plate containing Jurkat cells in logarithmic growth phase (1 \times 10⁵ cells/well, seeded under culture conditions) at different times (24, 48 and 72 h). Before the reading procedure, the cells related to each time of experiment were centrifuged, the supernatant was discarded and the medium was replaced with 200 μ l of MTT solution (0.5 mg/ml). The 24-well plates were incubated at 37 °C for 30 min. After this period the resulting formazan crystals were solubilized in 200 μ l of dimethyl sulfoxide (DMSO) and the optical density was read at 570 nm using an ELISA plate reader (Mosmann, 1983). The negative control was prepared in the same way as described above, but the cells were incubated only with RPMI medium and DMSO (<0.5%). Using the same procedure, friedelin-3 β -ol was tested at 5 and 50 μ g/ml at 72 h (these concentrations were based on IC₅₀ value from the results obtained to the chloroform fraction and 10 fold this concentration).

Trypan blue exclusion assay

The samples were prepared as described above. After, 24, 48 and 72 h, an aliquot (100 μ l) of these materials (cell suspension with aliquots of samples) was removed and homogenized in 10 ml of 0.5% trypan blue. The cellular viability, for each time, was determined by the ratio of viable cells and total number of cells, counted in a Neubauer chamber (Freshney, 1994). Using the same procedure friedelin-3 β -ol was also tested at 5 and 50 μ g/ml with at 72 h.

DNA quantification assay

Quantification of deoxyribonucleic acid (DNA) was evaluated using diphenylamine (DPA) solution (Plummer, 2004). For this 3 \times 10⁵ cells/well of Jurkat cells in their logarithmic growth phase were seeded in a 24-well plate and incubated with aliquots of the fractions at same concentrations used in MTT reduction assay. After 48 h of incubation (time of cell proliferation/cell number

that allowed the good performance of DPA method), an aliquot of cells/well were transferred to tubes (2 ml) and centrifuged. After the supernatant was discarded, 500 μ l of DNA-DPA reagent was added in the tubes. The results were read 24 h later at 578 nm.

Assays with CHCl_3 fraction

Morphological study – Acridine orange and Hoechst 33342 staining

The morphological study was performed according to procedures standardized in the Cell Culture Laboratory at the State University of Ponta Grossa. The seed of Jurkat cells (1×10^5 cells/well) occurred as described above for the other methods. In sequence the test fraction (CHCl_3 , 12.5 $\mu\text{g/ml}$) and the positive controls: vincristine (0.033 $\mu\text{g/ml}$) and chlorambucil (6.06 $\mu\text{g/ml}$) were added and incubated for 24 h. After this time the material was transferred to tubes, homogenized and centrifuged. The cells were washed with 500 μ l of ice-cold PBS and centrifuged one more time. After the supernatant was discarded, 25 μ l of PBS and 2 μ l of Acridine orange solution and ethidium bromide (200 $\mu\text{g/ml}$) were added. The staining was observed with a fluorescence optical microscope using excitation 480/30 nm and emission 535/40 nm and the fields were recorded on a camera. For another aliquot of the washed cells, 20 μ l of Hoescht 33342 solution (0.5 $\mu\text{g/ml}$) was added to the material. The color development was observed with a fluorescence optical microscope using ultra-violet light (excitation) and blue fluorescence at 460–490 nm (for emission). The live, apoptotic and necrotic cells determination was performed as described by Ribble et al. (2005) and was compared against the positive controls (vincristine and chlorambucil).

Analysis of cellular cycle

The 2.5×10^5 cells of Jurkat cells per well were treated with the CHCl_3 fraction at different concentrations (4–16 $\mu\text{g/ml}$) for 24 h. After this cells were homogenized, transferred to eppendorf and centrifuged (this procedure was executed three times). Each time, the cells were washed with PBS and supernatant discarded. The cells were resuspended in 150 μ l of PBS, fixed in 70% ethanol (1.35 ml) and stained with propidium iodide (PI). The red fluorescence of the material was determinate through a 585/42 nm filter and the signals were measured on a linear scale of 1024 channels. Each analysis were acquired 10,000 events and the data analyzed (CellQuest[®], San Jose, CA, USA; WinMDI[®] 2.8).

GC-MS analysis

Gas chromatographic analyses were conducted on an Agilent Technologies 7890 GC system (Agilent Technologies[®], Santa Clara, CA, USA) coupled with a 5975C single quadrupole MS (MSD; Agilent Technologies[®]) and an Agilent 7693 auto-sampler with split/splitless inlet. The chromatographic separation was performed using a J&W DB-5MS (5%-phenyl-methylpolysiloxane) fused silica capillary column of dimensions 30 m \times 0.25 mm i.d. \times 0.25 μm film thickness (d_f) supplied by Agilent. Data acquisition was performed with Agilent Enhanced MDS Productivity ChemStation software (version E.02.02). The optimized chromatographic conditions were: oven temperature program 250–300 $^\circ\text{C}$ at 5.0 $^\circ\text{C}/\text{min}$ (hold 15 min); injector temperature 280 $^\circ\text{C}$; carrier gas helium at a constant flow rate of 1 ml/min; injection volume 2 μ l with a split ratio of 20:1. The EI spectra were recorded at 70 eV from m/z 40 to 600 in a scan mode. The MS source was set to 230 $^\circ\text{C}$, the quadrupole temperature was 150 $^\circ\text{C}$, and the transfer line temperature was set to 280 $^\circ\text{C}$. Wiley and NIST library (version 2.0) was used to assist in compound identification. Further identification was based on the relative retention times compared with the reference standards and the literature values. The fraction

(1 mg) were accurately weighed and diluted with 1 ml of CHCl_3 using calibrated micropipettes.

Statistical analysis

The experiments were performed at least twice, and the results are representative of two experiments with leastwise three replicates/experiment for each concentration tested. The experimental values were expressed as the mean \pm standard error of the mean. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. The level of $p < 0.05$, $p < 0.001$ and $p < 0.0001$ was used to determine statistical significance. The data were determined using Graph Pad Prism 5.0 software (Graph Pad Software Inc.). The cytotoxicity was expressed as the concentration of sample that inhibited 50% of the cell growth (IC_{50}) and was calculated by *Probit* analysis, Finney method, using StatPlus v. 5.9.8.

Results and discussion

Several lines of cancer cells are used in *E. umbellata* cytotoxic studies to evaluate the possible activity, but recently we found that extracts of *E. umbellata* present a promising result against Jurkat cells (Luz et al., 2016). Thus, to evaluate the cytotoxic activity of *E. umbellata* barks extracts, Jurkat cells were promptly selected for the present study. Once the best conditions of cells growth were determined, the cytotoxic action of crude extract (CBE) and fractions (Hex, CHCl_3 , EtOAc and MeOH) of *E. umbellata* barks extract were tested using MTT reduction and trypan blue exclusion methodologies.

The cell metabolism assay assessed by MTT reduction expressed a decrease in cell viability with all the tested samples. The test showed that the fractions proved to be more active than the crude extract. Also, between the fractions, the CHCl_3 sample presented the higher cytotoxic effect compared to the Hex, EtOAc and MeOH fractions.

The trypan blue exclusion assay presented a confirmatory result for the MTT reduction assay. The experiment demonstrated a small number of viable cells, showing the cytotoxic effects as concentration and time dependent way. According the values obtained in these two experiments, the IC_{50} were calculated for each of the fractions evaluated (Table 1).

Cytotoxic effects of various species of *Euphorbia* genus have been tested against different cell lines. The results obtained in this study corroborate the effects observed in other studies. *Euphorbia helioscopia* apolar extracts demonstrated a cytotoxic effects (using MTT reduction assay) against five different human cancer cell lines (SMMC-7721; BEL-7402; HepG2; SGC-7901; SW-480), in a dose dependent way showing an antiproliferative effect at 200 $\mu\text{g/ml}$ for the CHCl_3 fraction (Wang et al., 2012); also the study of the latex of *S. grantii* (*E. umbellata*) against B16F10 melanoma cells using MTT and trypan blue methods demonstrated results of 64% and 98% inhibition, respectively (Oliveira et al., 2013). In the same way, a study with *S. umbellatum* (*E. umbellata*) aerial parts demonstrated cytotoxicity of the material against K-562 and leukemic Jurkat cells, in a concentration dependent manner (Mota et al., 2012a), and also, four terpenes (non polar substances) isolated from *Euphorbia sogdiana* presented a dose dependent cytotoxic effect against Jurkat and EJ-138 cell lines that can be related with the substitutions in the structure of the terpenes that can improve the activity against these cells (Yazdiniapour et al., 2016). Finally, values of reduction in the cell viability were already observed to CHCl_3 and Hex fractions of *S. umbellatum* (*E. umbellata*, aerial parts) against K-562 line cells when used the trypan blue exclusion assay (58% and 64%, respectively) (Nogueira et al., 2008).

Table 1
IC₅₀ (μg/ml) values of *Euphorbia umbellata* bark crude extract and fractions.

Samples	Assay	24 h	48 h	72 h
Crude extract	MTT reduction	465.53 ± 80.13	103.05 ± 38.16	51.96 ± 17.29
	Trypan blue exclusion	354.45 ± 113.76	92.96 ± 28.89	41.95 ± 10.06
Hexane fraction	MTT reduction	22.77 ± 2.06	11.58 ± 1.77	14.76 ± 4.98
	Trypan blue exclusion	21.96 ± 4.07	12.01 ± 1.88	10.79 ± 1.66
Chloroform fraction	MTT reduction	21.76 ± 1.40	7.72 ± 1.90	7.59 ± 1.69
	Trypan blue exclusion	29.00 ± 1.49	10.06 ± 1.48	4.83 ± 2.25
Ethyl acetate fraction	MTT reduction	49.19 ± 2.67	21.62 ± 3.24	27.75 ± 2.17
	Trypan blue exclusion	40.64 ± 2.43	20.39 ± 2.64	15.41 ± 3.08
Methanol fraction	MTT reduction	ND ^a	9.93 ± 9.26	18.48 ± 4.93
	Trypan blue exclusion	ND ^a	27.81 ± 6.01	15.58 ± 3.83

The results are expressed as mean ± standard error of the mean of two experiments with at least 3 replicates/experiments for each concentration tested. IC₅₀ was calculated by *Probit* analysis, Finney method, using StatPlus v.5.9.8. As negative control were used cells incubated in RPMI medium and DMSO (<0.5%).

^a ND, not defined.

Table 2
IC₅₀ (μg/ml) values of *Euphorbia umbellata* fractions for Jurkat by DNA quantification assay, after 48 h.

Samples	IC ₅₀ (μg/ml)
Hexane fraction	42.19 ± 26.02
Chloroform fraction	15.52 ± 2.61
Ethyl acetate fraction	37.77 ± 4.82
Methanol fraction	>100 ^a

The results are expressed as mean ± standard error of the mean of two experiments with at least 3 replicates/experiments for each concentration tested. IC₅₀ was calculated by *Probit* analysis, Finney method, using StatPlus v.5.9.8. As negative control were used cells incubated in RPMI medium and DMSO (<0.5%).

^a The concentration 100 μg/ml correspond the highest dose tested and the fraction was not cytotoxic.

The results indicate that the fractions of *E. umbellata* bark extract have an interesting and promising action. The IC₅₀ obtained for the samples shows a highest cytotoxicity when compared with other results ascribed to species of this genus as: *Euphorbia antiquorum* latex was IC₅₀ 2 mg/ml for HeLa cells; *Euphorbia macrorrhiza* roots essential oil with IC₅₀ 11.86 mg/ml against CaCo₂ cells; *S. umbellatum* (*E. umbellata*) leaves extract with IC₅₀ 3.6 mg/ml against bone marrow cells of mouse, IC₅₀ 41 μg/ml to Jurkat cells and IC₅₀ 73 μg/ml to K562 cells (Valadares et al., 2007; Hsieh et al., 2011; Lin et al., 2012; Mota et al., 2012a).

The DNA quantification assay was performed to corroborate the cytotoxicity assays; however, this can only be considering an antiproliferative indirect test (Sellitti et al., 2001). It was possible to determine a decrease of the DNA content related to the concentration (concentration dependent) statistically significant when compared to the negative control (Table 2).

The CHCl₃ fraction presented effective results against Jurkat cells in the three preliminary experiments (MTT reduction – IC₅₀: 7.59 ± 1.69 μg/ml (72 h), trypan blue exclusion – IC₅₀: 4.83 ± 2.25 μg/ml (72 h) and DNA quantification – IC₅₀: 15.52 ± 2.61 μg/ml), implying that this fraction may enhance the number of chemical compounds that may be responsible for the observed cytotoxic action. Although vincristine can present cytotoxic activity against Jurkat cells with IC₅₀ lower than 0.0031 μg/ml (Schempp et al., 2002; Takashima et al., 2007), according to the standard National Cancer Institute criteria (Suffness and Pezzuto, 1990), plant extracts possessing an IC₅₀ lower than 30 μg/ml are considered active against tested cancer cells.

So, in an attempt to propose some mechanism of action associated with the toxicity and identify the compounds present in the CHCl₃ fraction, this fraction was studied further. The process of cell death induced by the CHCl₃ fraction was initially studied employing the fluorescence microscopy analysis. After 48 h of treatment, it was possible to observe a great number of viable

cells in the negative control (which have normal nuclei and green staining) while the chloroform fraction demonstrated a reduction of cells, presenting apoptotic (loss of membrane integrity in which it is observed chromatin condensation and orange areas in the core) and necrotic cells (which have orange core uniform) (Fig. 1).

The Hoechst 33342 staining methodology (Fig. 2) showed a complementary response, where it was observed that the DNA condensation (blue fluorescence), confirming the presence of apoptotic cells.

Mota et al. (2012b) observed cell death finding formation of blebs, chromatin condensation, nuclear fragmentation and apoptosis in ascites cells of Ehrlich tumor treated with *S. umbellatum* (*E. umbellata*) leaves extract. In addition, the researchers described that the results indicate increases in reactive oxygen species, the mitochondrial membrane potential, externalization of phosphatidylserine and activation of caspase 9, events that demonstrate the induction of programmed death. Luz et al. (2016) studying the latex of *E. umbellata* against Jurkat cancer cell line also observed apoptosis process; Kwan et al. (2015) found that *Euphorbia hirta* extracts exhibited growth inhibition of MCF-7 cells with marked morphological features characteristic of apoptosis; Amirghofran et al. (2011) showed that *Euphorbia cheiradenia* induces DNA fragmentation and apoptosis in leukemic cells and finally Yang et al. (2016) observed a similar result with crude extract of *Euphorbia formosana* on prostate cancer cells, which caused DNA condensation, fragmentation and damage, in a time dependent manner.

In the current study, the analysis of cellular cycles demonstrates that the CHCl₃ fraction promotes cell cycle arrest at G₀/G₁ phase. As shown in Table 3, after 24 h of treatment, the G₁-phase population of control cells was 49.63%. Compared with control (negative control), the G₁-phase population of the CHCl₃-treated cells increased 12%. In addition, treatment with CHCl₃ also decreased the percentage of cells in S-phase; moreover, compared with control, CHCl₃ increased the sub-G₀ phase (Table 3).

Similar results were observed with ethyl acetate extract of *E. helioscopia* against hepatocellular carcinoma cells. According to the authors, the extracts inhibited the proliferation of human hepatocellular carcinoma cell line producing an arrest in the cell cycle in G-1 phase, however using higher concentrations (100–200 μg/ml) (Nogueira et al., 2008), was observed cycle arresting in G phases, not only G₀/G₁ phase, but also G₂/M for *S. umbellatum* (*E. umbellata*) (Wang et al., 2012). On the other hand, the crude extract of *E. formosana* in DU145 (prostate cancer cell) was analyzed and reported induction of S phase arrest, with a increase proteins levels of p21, p27 and Cdc 25; and decrease levels of CDK1, CDK2, CDK6 and cyclin a, all of them are associated with cell cycle regulation (Yang et al., 2016).

Chloroform extracts normally contain nonpolar compounds that are very common in the *Euphorbia* genus, also several researches

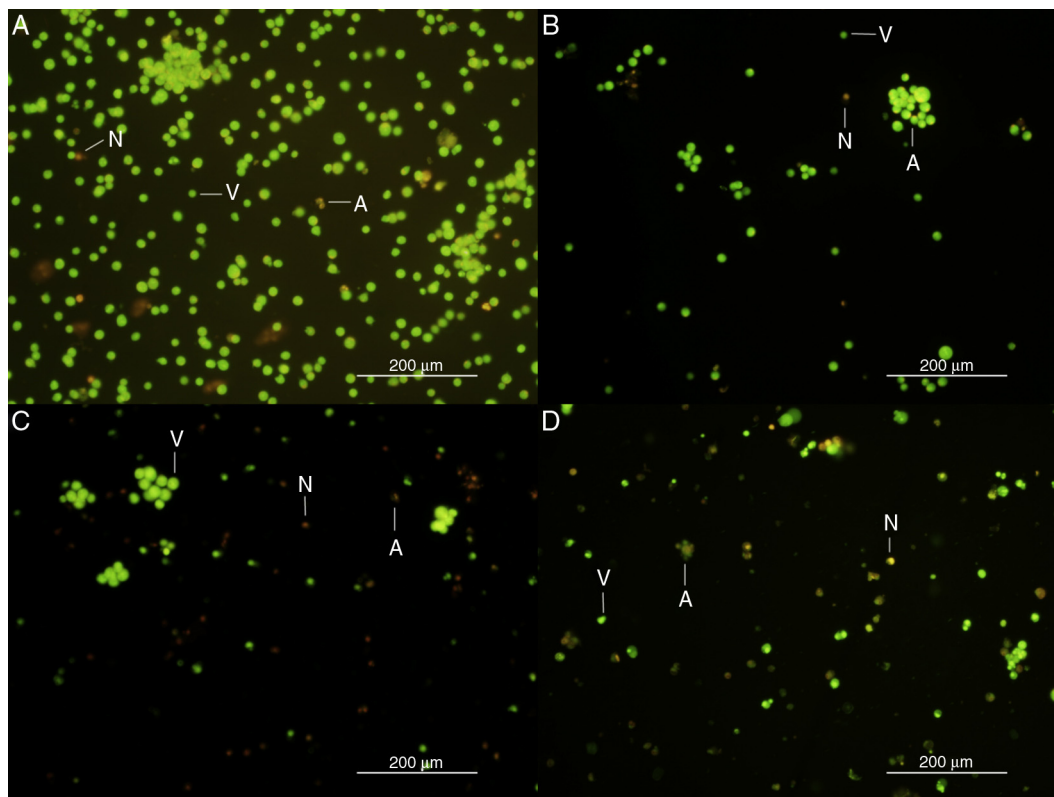


Fig. 1. Images of morphological evaluation of Jurkat cells after treatment (48 h). (A) Negative control (RPMI medium and DMSO (<0.5%)); (B) chloroform fraction (12.5 µg/ml); (C) chlorambucil (6.06 µg/ml); (D) vincristine (0.033 µg/ml). A, apoptotic cell; N, necrotic cell; V, viable cell.

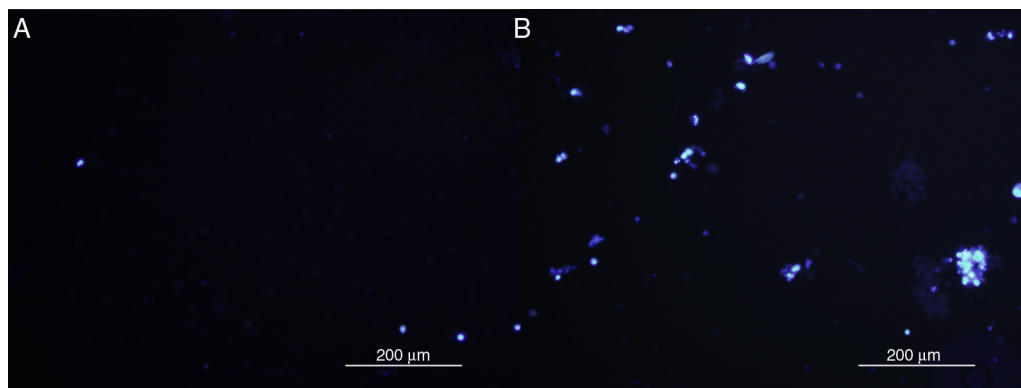


Fig. 2. Images of the DNA condensation by Hoechst 33342 staining. (A) Negative control (RPMI medium and DMSO (<0.5%)); (B) chloroform fraction (12.5 µg/ml).

Table 3

Values of means of cell populations in the phases of the cycle of Jurkat cells after 24 h of treatment with CHCl₃ fraction of *Euphorbia umbellata*.

Samples	Concentration (µg/ml)	Sub-G0	G0/G1	S	G2/M
Negative control	–	12.46 ± 0.37	45.80 ± 0.91	13.64 ± 0.27	28.10 ± 0.84
Vincristine	0.033	11.55 ± 0.40	48.08 ± 0.94	12.60 ± 0.48	27.78 ± 1.30
Chlorambucil	6.06	14.15 ± 0.90	31.41 ± 0.38	11.30 ± 0.38	43.14 ± 0.55
Chloroform fraction	4	15.61 ± 0.95	60.98 ± 1.43 ^{a,b,c}	5.94 ± 0.38 ^{a,b,c}	17.47 ± 1.73 ^{a,b,c}
	8	17.63 ± 0.55 ^{a,c}	60.74 ± 1.54 ^{a,b,c}	5.87 ± 0.26 ^{a,b,c}	15.76 ± 1.03 ^{a,b,c}
	16	19.03 ± 0.57 ^{a,b,c}	55.91 ± 1.34 ^{a,b,c}	6.19 ± 0.34 ^{a,b,c}	18.87 ± 0.56 ^{a,b,c}

Values represent the mean ± standard error of the mean of quintuplicates. * $p < 0.001$. Vincristine and chlorambucil were used as positive control, RPMI medium and DMSO (<0.5%) was used as negative control.

^a CHCl₃ fraction vs. vincristine.

^b CHCl₃ fraction vs. chlorambucil.

^c CHCl₃ fraction vs. negative control.

Table 4
Constituent identification of CHCl₃ fraction from *Euphorbia umbellata* barks.

No.	t _R (min)	Compound identified	Major fragment ions	Identification
1	12.439	Euphol	55, 69, 81, 95, 109, 393, 411 (100%), 426 (M ⁺)	t _R , MS
2	12.939	Sitosterol	55, 81, 95, 107, 145, 396, 414 (100%, M ⁺)	MS
3	13.052	Lanosterol	55, 69, 91, 95, 109, 393, 411 (100%), 426 (M ⁺)	MS
4	13.327	Lupeol	55, 68 (100%), 81, 95, 109, 426 (M ⁺)	t _R , MS
5	14.228	Cycloartenol	55, 69 (100%), 81, 107, 109, 121, 135, 426 (M ⁺)	MS
6	16.542	Friedelin-3β-ol	55, 69, 81, 96 (100%), 109, 125, 428 (M ⁺)	t _R , MS
7	16.905	Friedelin	55, 69 (100%), 81, 95, 109, 125, 426 (M ⁺)	MS

Compounds listed in order to their elution on the DB-5MS column. Identification based on the retention times (t_R) of genuine compounds; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and NIST libraries and comparison with literature data.

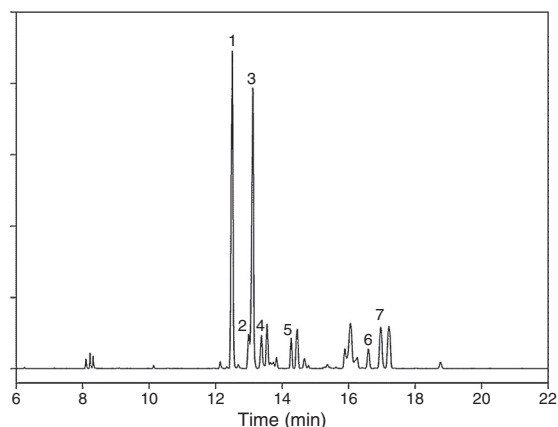


Fig. 3. Gas chromatogram of CHCl₃ fraction from the crude bark extract of *Euphorbia umbellata*. Peak assignment: 1. euphol; 2. sitosterol; 3. lanosterol; 4. lupeol; 5. cycloartenol; 6. friedelin-3β-ol; 7. friedelin.

describe the cytotoxic effects of these compounds, including the apoptosis by arresting the cell cycle (Gallo and Sarachine, 2009; Sadeghi-Aliabadi et al., 2009; Saleem et al., 2009; Bishayee et al., 2011; Prabhu et al., 2011; Liu et al., 2014; Lu et al., 2014; Yang et al., 2014; Lanzotti et al., 2015; Luz et al., 2016).

Trying to identify the possible compounds responsible for the determined effects presented in this fraction a GC/MS analysis was performed. This procedure demonstrated the phytochemical profile presented in Fig. 3.

Seven of these compounds, presents in the fraction were identified and listed in order of their elution on the DB-5MS column. The identification was based on the retention times (t_R) of standard compounds, the computer matching of the mass spectra with those of the Wiley and NIST libraries and comparison with literature data. The majors compounds identified were the isomers euphol and lanosterol (Table 4).

Furthermore, corroborating the information described above, some of these identified compounds as already described in the literature as compounds present in *Euphorbia* genus with cytotoxic activity (Dawidar et al., 2011; Oliveira et al., 2013; Wang et al., 2013; Munhoz et al., 2014; Luz et al., 2016). This result allows suggesting that the effect observed to the CHCl₃ fraction of *E. umbellata* against Jurkat cells, can be related with the activity of the triterpenes and steroids present in the mixture.

Mujoo et al. (2011) describes the action of triterpenoid saponins that induces apoptosis of Jurkat and MDA-MB-435 breast cancer cell lines, also Luz et al. (2016) describes that the euphol presents a cytotoxic effect when compared against the negative control, but lower than the action of vincristine that was used as a positive control in the study. According Petronelli et al. (2009), the triterpenes can develop the activity blocking the nuclear factor-kappa

B activation, inducing apoptosis, inhibiting signal transducer, activating transcription or interfering in the angiogenesis. Apoptosis is one of the mechanisms of action ascribed to the CHCl₃ fraction.

In the same way, some research describes the cytotoxic effects attributed to some steroids molecules (Awad et al., 2000). Gupta et al. (2013) report that the steroids have some compatibility with nuclear receptor; and can act as enzyme inhibitors, cytotoxic molecules or conjugates and for these reasons are important biodynamic agents against cancer disease.

The cytotoxic activity demonstrated in this work for the fractions corroborates the literature studies that report that cell apoptosis induction is the most cited mechanism (Paduch et al., 2007; Vasas, 2014).

The pentacyclic triterpene friedelin-3β-ol previously isolated and identified from CHCl₃ fraction (Munhoz et al., 2014) had the cytotoxicity against Jurkat cells studied, but a weak effect was observed as compared to controls in 72 h of cells treatment (data not shown).

In conclusion the crude bark extract of *E. umbellata* and fractions of that presented potential cytotoxic effects against Jurkat cells. The CHCl₃ fraction was the most active fraction and the mechanism of action related involves apoptosis and cycle arrest. The chromatographic evaluation of the CHCl₃ fraction demonstrated that these mechanisms can be related with the triterpenes and steroids present in the fraction. Phorbol-type diterpenes were not observed by GC of this matrix. Friedelin-3β-ol did not present cytotoxic activity but the CHCl₃ fraction can be studied for further evaluation trying to isolate and test other substances that can be responsible for the action and establish a more detailed cytotoxic study of these against Jurkat cells.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CCK analyzed the data and helped to write and revise the article; TL (undergraduate student) and LECL (MSc student) were performed the tests presented in the article; LSC (MSc student) corrected the text, prepared the data tables and revised the article; ARC was responsible for the analysis of cellular cycles; MW and BA were responsible for GC-MS analyses and helped to write the paper; IAK

was responsible to financial support and discussions concerning the outcomes; FLB was responsible for financial support, writing the paper, coordinating the research work and providing the material for the research.

Conflicts of interest

The authors declare no conflicts of interest.

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