

In Vitro Cytotoxic Potential and Apoptotic Activity of Bufadienolide-rich Fraction from Leaves of *Kalanchoe mortagei* against Human HeLa Cancer Cells

Research Article

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Abstract

Objective: The aim of this study was to evaluate the chemical composition as well as to assess the cytotoxicity of *Kalanchoe mortagei*. **Materials and Methods:** The plant chemical composition was determined by HPLC coupled to mass spectrometer. The cytotoxicity of methanol extract from leaves of *Kalanchoe mortagei* (KB) was carried out against human cervical cancer cells (HeLa), breast cancer (MCF-7), brain cancer (U373), breast adenocarcinoma (MDA-MB-231) and umbilical vein endothelial cells (HUVEC) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. KB was added at various concentrations of 6.25, 12.5, 25, 50, 100 and 200 µg/ml. In addition, cell morphological changes and apoptosis were also evaluated. **Results:** A bufadienolides-rich fraction (BRF) containing nine bufadienolides was identified by HPLC/MS which to significantly reduce proliferation of HeLa cells. Bufadienolides identified included 3β-Acetate Hellebrigenin, Bufotalinin, 19-Oxo-bufalin, Hellebrigenin, Monohydroxy-bufotalin, 19-oxo-desacetylcinobufagin, 1β-Hidroxybufalin, Bufalin, and 3-Dehydroxy cinobufagin. **Discussion & Conclusion:** *K. mortagei* contains bufadienolides with specific cytotoxic activity on cancer cells without affect to normal cells, suggesting that its consumption could be considered as a potential therapy against cancer.

Keywords: Bufadienolides, *Kalanchoe mortagei*, apoptosis, cytotoxic .

Introduction

After cardiovascular diseases, cancer represents the second cause of death for morbidity and mortality worldwide. In 2015, 8.8 million of people died from cancer according to World Health Organization and predicted to increase to over 24 million in 2035 (1). The most common types of cancer in men in order of incidence of major to minor are prostate, lung, colorectal, stomach and liver cancer; and in woman, breast, lung, cervix, thyroid, colon and stomach cancer (2). The type of treatment depends on the type of cancer and its status. The types most common are a combination of surgery with chemotherapy and/or radiation, immunotherapy, hormone or targeted therapy

(3); however, these therapies are not always successful, besides they are not specific against tumor cells damaging to healthy tissues and have strong adverse side effects reducing the quality of life for patients (4). Thus, it is very important to consider plants as a source to discover new potent anticancer drugs with side effects. Recently, extracts and phytochemicals from plants such as medicinal herbs, vegetables and fruits have had important attention due to their ability to modulate apoptosis signaling, may be potential anticancer drugs (5). Several secondary metabolites content in the medicinal plants are known to have anticancer effect inducing apoptosis in several cancer cell lines. Antitumor research is focused to find new treatments, especially from natural origin, which have no negative effects on normal cells. Actually in the chemotherapy, around 75% of prescribed drugs worldwide derive from plants (6).

The genus *Kalanchoe* (K) belongs to the family of the Crassulaceae, also known as *Cotyledon* or *Bryophyllum* (7). This plant is a perennial succulent herb native of Madagascar, that grows wild and are widely used in traditional medicinal in tropical America,

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Australia, Africa and China mainly (8). Previous studies reported that extracts from some *Brophyllum* exhibit anti-inflammatory (9), cardiovascular (10), antihyperglycemic (11), analgesic (12), antiulcer (13), antimicrobial and anticancer effects (14). A cytotoxic activity on human tumor cell lines was attributed to bufadienolides from the aerial parts of *K. gracilis* (6). Other species such as *K. tubiflora* and *K. daigremontiana* have shown also anti-tumor activity caused by bufadienolides (15, 16). *K. mortagei* is commonly cultivated as an ornamental plant in Mexico and is widely used in cancer therapy. It is our knowledge, no literature regarding the cytotoxic activity or phytochemical composition of *K. mortagei*. In this study we focused to evaluate the cytotoxic effect of a bufadienolides-rich fraction from *K. mortagei* on cancer cell line.

Materials and Methods

Materials

Acetonitrile (MeCN) (gradient-grade for LC), formic acid of p.a. grade and methyl-thiazolyl-tetrazolium dye (MTT), DMSO, annexin-V FITC apoptosis kit, propidium iodide, cycloheximide (CHX, >99% purity) and etoposide were purchased in (Sigma-Aldrich, St. Louis, MO, USA). Milli-Q water was prepared in-house. RPMI 1640, McCoy's medium base and streptomycin/penicillin stock solution were purchased from Gibco, Life Technologies (Carlsbad, CA, USA). Heat inactivated foetal calf serum from Biochrom, Berlin, Germany. Nucleosil C18 column (4.0 mm × 250 mm, 5 μm) from Macherey-Nagel, Duren, Germany. Cytotoxicity Detection Kit Plus (Roche, Grenzach-Wyhlen, Germany). Sephadex LH-20, and silica gel (200–400 mesh) were used for the column chromatography, and pre-coated silica gel (60 F-254) in plates were used for the TLC analyses which were purchased in Merck (Mexico).

Plant material

The leaves of *K. mortagei* were collected from Curungueo, in the municipality of Zitácuaro Michoacan State in Mexico in August 2015. These plants were identified and authenticated by the Department of Botany of the National School of Biological Sciences-IPN, a voucher specimen was deposited in the Department of plant taxonomy.

Plant extract

The dried leaves of *K. mortagei* (100g) were extracted with hexane (KAH), chloroform (KAC) and methanol (KAM) two times (2l × 2) under reflux. After filtration and evaporation of the solvent at reduced pressure extracts were tested.

In another experiment the leaves were air-dried in shade at room temperature and grinded into powder (800g). Extraction was performed in Soxhlet apparatus with methanol for 3hrs and then the solvent was evaporated until half volume. An equal volume of water was added while heating on 60°C and placed on refrigerator for 24hrs. The precipitated material was discarded and supernatant was used for this study.

Obtaining a bufadienolide-rich fraction

Supernatant was concentrated to dryness under reduced pressure to obtain slurry (48g). The slurry was dissolved in minimum amount of methanol and was adsorbed on silica gel (120–430 mesh). The slurry was subjected to a silica gel column eluted with chloroform/methylene chloride 2:1 gradient system to yield 11 fractions (F-1 to F-11). The elutes were examined by TLC and each one of them were determined cytotoxic activity. When comparing the cytotoxic activity of the fractions F-1 to F-11 found that F10 possess a potent effect which was bufadienolides-rich (BRF).

HPLC/MS assay

The analyses were performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10A VP diode array detector, LC-10AD VP binary pump, an autosampler, a DGU-14A degasser and SCL-10A VP system controller. The samples were separated on a Nucleosil C18 column. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), with the gradient elution as follows: 0–5 min, 20% to 35% B; 5–20 min, 35% to 50% B; 20–30 min, 50% to 95% B; 30–35 min, held at 95% B; 35–40 min, balanced to 20% B. The flow rate was 0.3 ml min⁻¹ and the temperature of the column was maintained at 30°C and an injection volume of 4 μl was used. All the samples were monitored at 296 nm. In addition, mass detection was performed in positive ion mode in a range of 150–1000 *m/z*. The parameters for LC-MS analysis were as follows: Curtain gas, 12 units; nebulizer gas, 8 units; entrance potential, 5 V, focusing potential, 200 V; declustering potential, 60 V and ionization voltage, 4000 V. [M+H]⁺ ions were selected and subjected to collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) analysis using nitrogen as the collision gas. The optimized parameters for MS/MS analysis were as follows: Collision cell exit potential, 15V, collision energy, 30 eV and collision gas, 2 units. Data processing was performed using Xcalibur 3.0, Networks 1.3 and Mass Frontier 7.0 software packages.

Cell culture

Cell lines were obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA). HeLa (cervical cancer cells), MCF-7 (human breast cancer), U373, (brain cancer), MDA-MB-231 (breast adenocarcinoma) and HUVEC (human umbilical vein endothelial cells) were cultured in U-2OS in McCoy's medium and RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum, 100 lg/ml streptomycin and 100 U/ml penicillin and 5% CO₂ at 37 °C. All cell lines were transferred to six-well plates at 4X10⁵/well 18 h before assay set-up. Cells were treated at different duration and concentrations of samples and then subjected to analysis.

Cell viability

Cell viability was evaluated mediating the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to water-insoluble formazan. For this,

cells were exposed to diverse concentrations of the different crude extracts (6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/ml}$) for 48 and 72 h; after treatment, cells were incubated with 20 μl of a stock solution of MTT (5 mg/ml) for 4 h and optical density was measured at 570 nm in a microplate spectrophotometer (Benchmark Plus, BIO-RAD).

Crystal violet staining

HeLa cells were exposed to 6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/ml}$ of KAM for 72 h. After, cells were washed three times with de-ionized water and stained for 20 min 0.1 % crystal violet solution (in 200 mM phosphoric acid buffer at pH 6). Cells were washed exhaustively to eliminate the color non-incorporated and after, cells were analyzed in an inverted microscope (Olympus, model CKX41, BioRad, Hercules, CA, USA) and photographs were taken at a 20X magnification.

Cell death determination

Phosphatidylserine translocation was used to determine apoptosis. This was evaluated using the annexin-V-fluos staining kit. Cells were treated with all crude extracts for 48 h, then washed with PBS and centrifuged at 200 rpm for 5 min. Each cell pellet was resuspended in 100 μl of labeling solution (20 μl annexin-V-fluos labeling reagent in 1 ml Hepes buffer [10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl_2], 1 $\mu\text{g/ml}$ propidium iodide) and incubated for 15 min. After, samples were washed with Hepes buffer, pellets were suspended in 400 μl of Hepes buffer and the samples were analyzed in a flow cytometer (FACSCalibur, Becton Dickinson) at 488 nm (excitation) and 515 nm bandpass filter for fluorescein detection, and a filter >560 nm for propidium iodide detection. Cell analysis was performed using the Cell

Quest Software Program. Cells stained with annexin-V-fluos alone were considered apoptotic, whereas double stained cells (annexin-V-fluos + propidium iodide) were considered late apoptotic or necrotic.

Statistical analysis

Three independent experiments was performed for each condition. The results were expressed as the mean \pm standard deviation (SD). Comparisons between groups were tested by one-way analysis of variance (ANOVA) and post hoc Tukey's pairwise comparison using GraphPad Prism version 6 and Minitab version 17. Differences were considered statistically significant when $p \leq 0.05$.

Results

Analysis of cell viability

The three extracts obtained from *K. mortagei* were hexane (KAH), chloroform (KAC) and methanol (KAM) their cytotoxic effect was evaluated on four cancer cell lines HeLa, MCF-7, U373 and MDA-MB-231 by MTT assay. The inhibitory concentrations 50% of viability (IC_{50}) were calculated from concentration-response curve. KAM shown a growth inhibitory effect toward HeLa and U373 cells with an IC_{50} of <6.25 and 55 $\mu\text{g/ml}$, respectively (Table 1). KAH and KAC did not show effect on the viability at lower concentrations than 200 $\mu\text{g/ml}$ in all cell lines used. The criteria of cytotoxicity for the samples, was established by the U.S. National Cancer Institute, which is $\text{IC}_{50} < 20$ $\mu\text{g/ml}$ in the preliminary assay (17). Among the extracts the most cytotoxic was KAM and the less cytotoxic was KAH. It has been shown that extracts obtained from *Kalanchoe* with polar solvents are more efficient to decrease viability compared with the obtained with non-polar solvents.

Table 1. Sensitivity (IC_{50}) of different cancer cell lines to extracts obtained from *Kalanchoe mortagei*

Extract	IC_{50} ($\mu\text{g/ml}$)			
	HeLa	MCF-7	U373	MDA-MB-231
KAH	>200	>200	>200	>200
KAC	>200	>200	>200	>200
KAM	<6.25	>200	55	>200

Sensitivity (IC_{50}) of different cancer cell lines to extracts obtained from *K. mortagei*. Cancer cells were exposed to 6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/mL}$ of the hexane (KAH), chloroform (KAC) and methanol (KAM) leaves extracts and cell viability was evaluated by MTT reduction after 72 h.

Effect of *K. mortagei* extracts on HUVEC cells viability

To test of viability of normal cells was also evaluated on HUVEC cells. The results indicated that all extracts did not affect cell viability; in contrast, an increase of proliferation over 100% when the cells were treated at concentration of 6.25–50 $\mu\text{g/ml}$ with KAH, KAC and KAM was observed (Figure 1). KAM increased the cell viability in 50% compared with control cells ($p \leq 0.05$) at the concentration of 12.5 $\mu\text{g/ml}$, which was much stronger than the other extracts. To taking in consideration these results in the next experiments only HeLa cells and KAM were used.

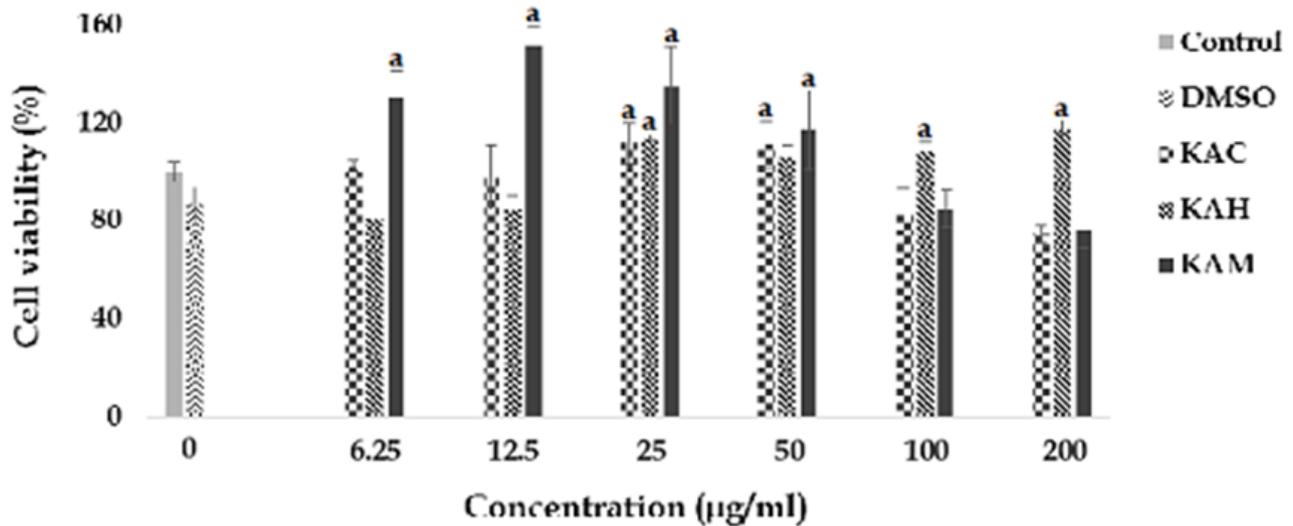


Figure 1. Effect of the extracts hexane (KAH), chloroform (KAC) and methanol (KAM) on the viability of HUVEC. Cells were cultured with different concentrations of extracts (6.25, 12.5, 25, 50, 100 and 200 µg/ml) for 72 h and cell viability was determined by MTT reduction. DMSO was used as a vehicle. The results are expressed as percentages with respect to the control without treatment (100%). Data represent the mean ± SD of a representative experiment of three performed in an independent way (n = 3). Compared with control group a = p≤0.05.

Effect of KAM treatment on cell morphology

The change in cell morphology is the first effect of toxic compounds exposure to the cells, which is used for measuring toxicity levels providing preliminary data about the mechanism of cytotoxicity; therefore, cell morphology was also evaluated in HeLa cells, which were more sensitive to the effect induced by KAM, mediating crystal violet staining. The results showed a decrease of the cell number observed from 6.25 µg/ml and with a maximum effect obtained at 200 µg/ml (Figure 2). Alterations on cell morphology such as detachment of cells from the well surface, rounding of cells, nuclear disintegration (50 µg/ml), augmented density of the cytoplasm (from 12.5 µg/ml), fragmentation and shrinkage of the cytoplasm and breaking of the cytoplasmic membrane (from 100 µg/ml) were observed in exposed cells, compared with control cells (Figure 2). These morphological changes are characteristic of severe cell damage.

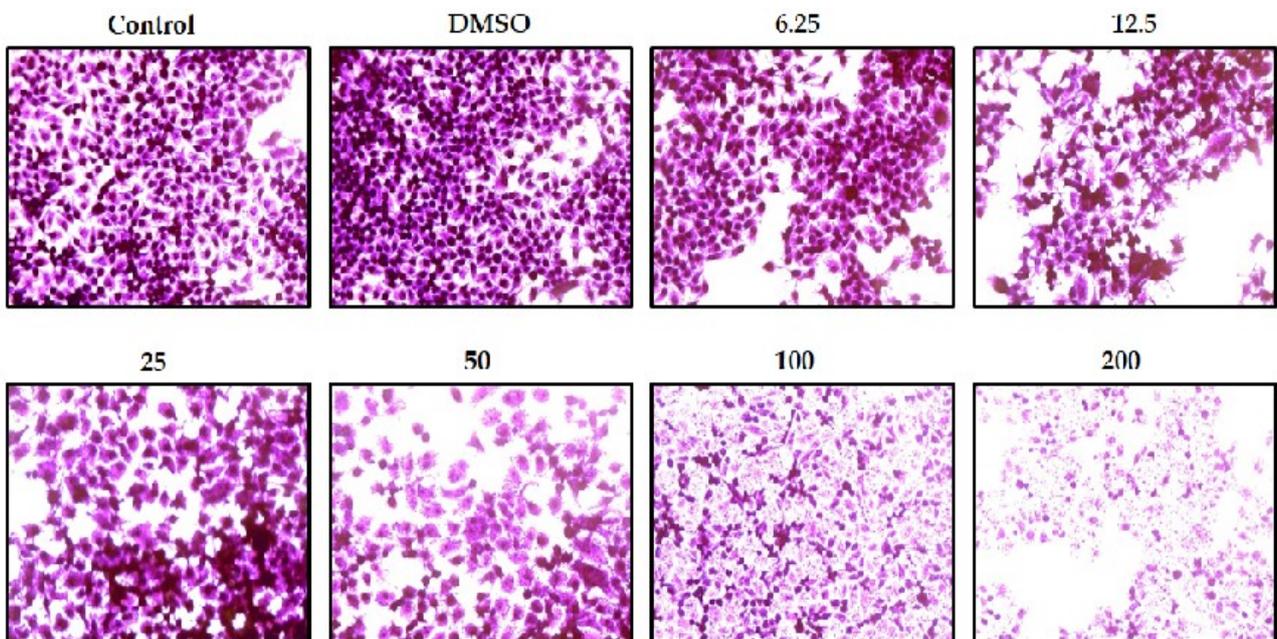


Figure 2. Effect of methanol extract (KAM) on the morphology of HeLa cells. Cell were cultured with 6.25, 12.5, 25, 50, 100 and 200 µg/ml of KAM for 72 h and cells were stained with crystal violet to observe morphology. Light microscopy micrographs were taken at a magnification of 20X.

Effect of KAM and F10 on HeLa cell death

Since damage in cells exposed to KAM was observed, the two best characterized types of cell death were evaluated by annexin-V assay and iodide propidium staining. Annexin-V protein is commonly used to detect in apoptotic cell surfaces phosphatidylserine molecules. However, the absorption and binding of nuclear DNA to propidium iodide can be used for differentiating necrosis of late stage apoptosis from early apoptosis. The results showed that concentrations of KAM from 25 µg/ml induced apoptosis (late and early) after 48 h of exposure and this effect was concentration dependent, reaching a 25% in comparison with control cells (Fig 3). No significant necrosis was observed with KAM, but the high apoptosis suggest a retarded cell proliferation in early stages of toxicity and that the major cytotoxicity mechanism of KAM is the apoptosis (Table 2).

Table 2. Effect of methanol extract (KAM) on the death of HeLa cells.

Treatment	Percent (%)			
	Alive	Early apoptosis	Late apoptosis	Necrosis
Control	99.0	0.2	0.4	0.4
Boiled cells	10.9	0.1	2.5	86.5
DMSO	97.7	1.1	0.8	0.5
6.25 µg/mL	98.8	0.4	0.3	0.5
12.5	96.6	1.3	1.3	0.7
25	86.6	4.5 ^a	5.2 ^a	3.7 ^a
50	81.5	6.8 ^a	9.1 ^a	2.6 ^a
100	75.5	7.4 ^a	14.7 ^a	2.4 ^a
200	72.2	10.8 ^a	15.0 ^a	2.0 ^a

The results are expressed as percentages of a representative experiment of three performed in an independent way. Compared with control group a = $p \leq 0.05$

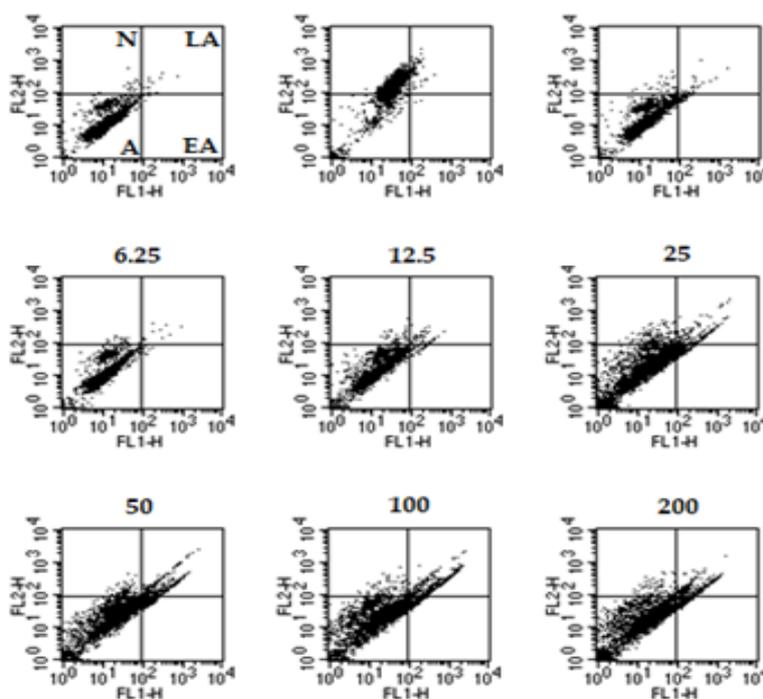


Figure 3. Effect of methanol extract (KAM) on the death of HeLa cells. Cells were cultured with different concentrations of extracts (6.25, 12.5, 25, 50, 100 and 200 µg/ml) for 48 h and cell death was determined by annexin-V assay and iodide propidium staining mediating flow cytometry. DMSO was used as a vehicle and boiled cells as a positive control of necrosis. In histograms are showed, where A = alive cells, EA = early apoptosis, LA = late apoptosis and N = necrosis. Compared with control group a = $p \leq 0.05$.

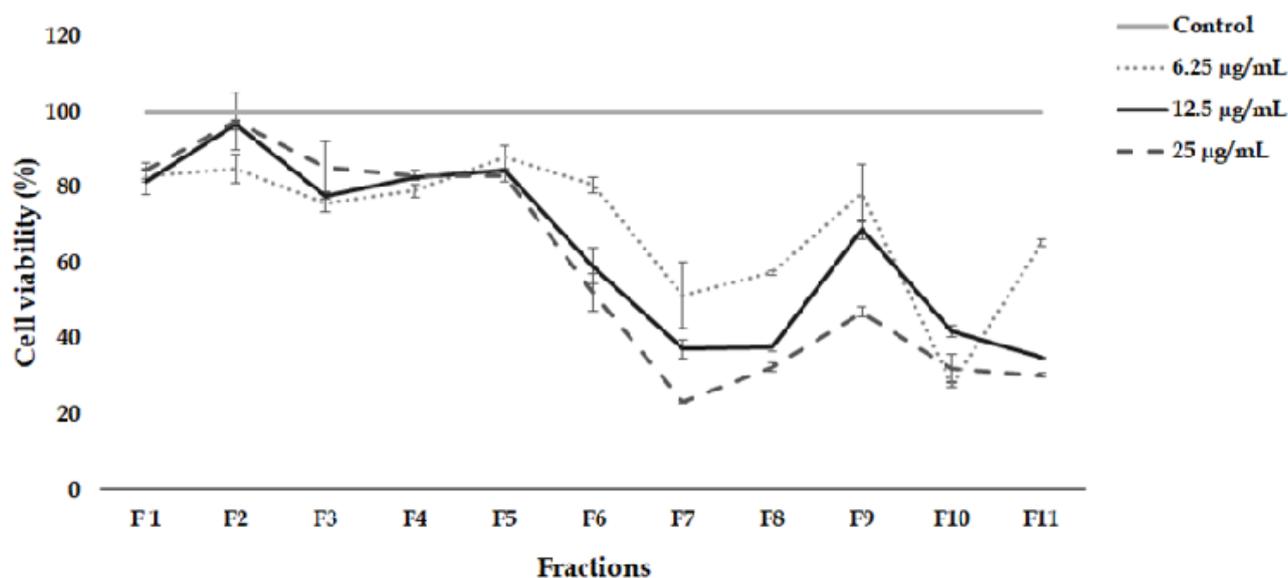


Figure 4. Effect of the 11 different fractions obtained from KAM on HeLa cells viability. KAM was fractionated by column chromatography and cells were cultured with 6.25, 12.5 and 25 µg/mL of each fraction for 72 h and cell viability was evaluated by MTT reduction. Data represent the mean ± SD of a representative experiment of three performed in an independent way (n = 3). Compared with control group a = p≤0.05.

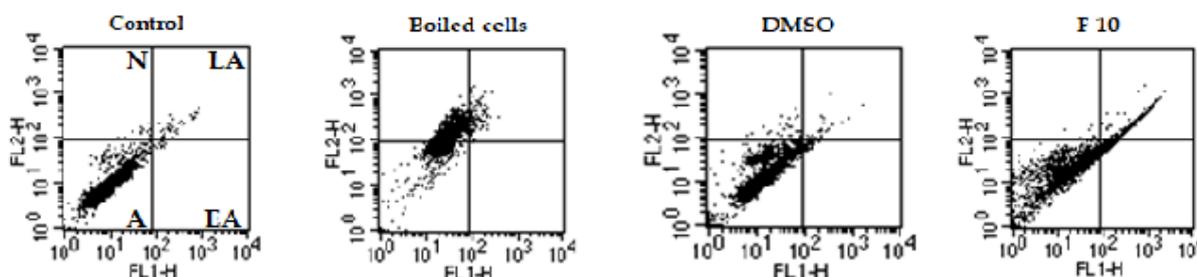


Figure 5. Effect of F10 on the death of HeLa cells. Cells were cultured with 25 µg/ml of F10 for 48 h and cell death was determined by annexin-V assay and iodide propidium staining mediating flow cytometry. DMSO was used as a vehicle and boiled cells as a positive control of necrosis. In histograms are showed, where A = alive cells, EA = early apoptosis, LA = late apoptosis and N = necrosis. Compared with control group a = p≤0.05.

Table 3. Effect of F10 from *Kalanchoe mortagei* on the death of HeLa cells.

Treatment	Percent (%)			
	Alive	Early Apoptosis	Late Apoptosis	Necrosis
Control	97.3	0.1	1.5	1.1
Boiled cells	4.0 ^a	0.0	4.3 ^a	91.8 ^a
DMSO	97.6	1.1	0.8	0.5
F10	73.6 ^a	2.3	22.4	1.8

The results are expressed as percentages of a representative experiment of three performed in an independent way. Compared with control group a = p≤0.05.

Identification of bufadienolides

The compounds responsible for the cytotoxic activity of KAM were chromatographed and were identified by HPLC/MS, obtaining 9 bufadienolides previously reported (Table 4). Other studies with plants of several species of *Kalanchoe* indicate that bufadienolides are the responsible of the anticancer activity (18, 19). Five bufadienolides isolated from *K. pinnata* y *K. daigremontiana* leaves inhibited the viability of Raji cells (15). Hellebrigenin, a bufadienolide isolated from *K. genera*, induced DNA damage, mitochondrial alterations, arrest of the cell cycle and apoptosis (14).

Table 4. Identification of bufadienolides in *Kalanchoe mortagei* by using HPLC/MS

Num	Compound	Rt (min)	%	m/z	Molecular Formula	UV(nm)
1	Hellibrigenin	0.6	25	417 [M+H], 476, 458, 399, 363, 335	C ₂₄ H ₃₂ O ₆	246
2	Bufotalin	2.4	7.2	415 [M+H], 474, 456, 397, 379, 351, 333	C ₂₄ H ₃₀ O ₆	249
3	19-Oxo-bufalin	2.7	11	401 [M+H], 460, 442, 418, 383, 365, 347, 319	C ₂₄ H ₃₂ O ₅	245
4	3β-Acetato-hellebrigenin	11.5	4.4	459 [M+H], 381, 363, 345, 335, 289, 255	C ₂₆ H ₃₄ O ₇	219, 276
5	Bufalin	12.1	1.7	387 [M+H], 446, 428, 404, 369, 351, 333	C ₂₄ H ₃₄ O ₄	244
6	Monohydroxybufotalin	16.2	3.7	461 [M+H], 365, 347, 267, 251	C ₂₆ H ₃₆ O ₇	214, 254
7	19-Oxo-desacetylcinobufagin	19.6	14.9	415 [M+H], 456, 474, 397, 379, 361, 351, 333	C ₂₄ H ₃₀ O ₆	236
8	1 β-Hydroxybufalin	21.3	17.5	403 [M+H], 462, 444, 385, 367, 349, 339, 253	C ₂₄ H ₃₅ O ₅	212, 249
9	3-Dehydroxycinobufagin	36.6	1.0	439 [M+H], 397, 379, 361, 319, 271	C ₂₆ H ₃₀ O ₆	212, 258

Discussion

In the treatment of cancer and its prevention chemotherapy plays an important role; but cell resistance to the drugs, necessitate a constant amelioration in the treatments. In the last decade novel classes of chemotherapeutic drugs have been continuously studied, among natural compounds have led to new drug development. Several species of the generous *Kalanchoe* have shown antitumoral properties; however, the effect of aerial part of *K. mortagei* against different cancer cell lines have not been studied as yet. Thus, in the present investigation we extracted, purified and characterized a fraction anti-cancer from leaves of *K. mortagei*. Cell viability assay is an important study to measure the cellular response to toxicant or drug providing information on metabolic activities, cell survival and death (20).

Selective toxicity and efficacy are important requirements of cancer therapeutics. The ability of a drug to sparing normal cells and induce apoptosis of cancer cells is an important requirement in cancer treatment. Many drugs act on the immune system to fight cancer, and they should not have an unfavorable affect immune system. Hereby, the antiproliferative of all extracts were studied in primary cultures of normal endothelial cells. It was interesting to observe that KAM increased HUVEC proliferation at lower concentrations, and that higher concentrations did not affect cell viability, indicating its high selectivity. One work have reported similar results, where different extracts from *K. pinnata*, *K. gastonis-bonnieri* and *K. flammaea* did not

affect the viability of primary cultures of lymphocytes (21).

HeLa cells exposed to KAM change to round shape and detached from surface, indicating a strong damage. Exposure of HeLa cells to KAM significantly increased apoptotic death from 25 µg/ml and did not induce necrosis. The results displayed that cytotoxicity mechanism of KAM is the apoptosis. Apoptosis and necrosis are two fundamental types of cell death. Necrosis is cell rupture produced by excessive exogenous damage which is characteristic to inflammation, while apoptosis cell death is an active process regulated physiological, where is programmed cell death leading to a sequence of morphological changes by modifications of the cellular surface (22), which not showing inflammatory response (23). Apoptosis is a natural cell death which is considered the most important requirements of cancer drugs in treatments that induce tumor cells death.

Cervical cancer cells (HeLa) were the most sensitive to the toxic effect induced by KAM and the less sensitive were breast cancer cells (MCF-7 and MDA-MB-231), indicating that the cytotoxic effect of KAM depends of the type and stage of the cancer cells. HeLa cells are classified in an IVB stadium, are infected with human papilloma virus and are metastatic. It will very important to compare the toxic effect of KAM in other cervical cancer cells.

1□-hidroxibufalin, obtained from F10, is a metabolite of the hydroxylation of the bufalin. This has anti-cancerigen properties in human leukemia and

melanoma cells (15). Monohydroxibufalin, another hydroxylated form of bufalin, promoted apoptosis of HeLa cells (24), in accordance with our results. Finally, 3-dehydrocinobufagin has a potent anti-neoplastic activity, decrease osteosarcoma proliferation and induces apoptosis (25). Therefore, it will be very interesting to isolate other compounds of KAM and to test their cytotoxic effect.

Conclusion

Leaves of *Km* are a rich source of bufadienolides. In the present study, nine known bufadienolides were identified from the methanol extract. Total bufadienolides from aerial part cause potent cytotoxic activity toward HeLa cell line. Our findings suggest for the first time that *K. mortagei* can act as an inducer of apoptosis of tumor cells.

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