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Cytotoxic effect of *Kalanchoe flammea* and induction of intrinsic mitochondrial apoptotic signaling in prostate cancer cells

Iván Arias-González[a,1], Alejandro M. García-Carrancá[b], Jorge Cornejo-Garrido[a], Cynthia Ordaz-Pichardo[a,*]

[a] Laboratorio de Biología Celular y Productos Naturales, Escuela Nacional de Medicina y Homeopatía (ENMH), Instituto Politécnico Nacional, Guillermo Massieu Helguera 239, Col. La Escalera, Del. Gustavo A. Madero, 07320 CDMX, Mexico
[b] Laboratorio de Virus y Cáncer, Instituto Nacional de Cancerología, Secretaría de Salud, Av. San Fernando 22, Col. Sección XVI, Del. Tlalpan, 14080 CDMX, Mexico

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ABSTRACT

Ethnopharmacological importance: Kalanchoe flammea Stapf *(Crassulaceae)* is a medicinal plant grown in the South of Mexico (State of Tabasco), which is commonly used in traditional medicine for the treatment of fever, wounds, inflammation, and cancer.

Aim of the study: To establish the potential of *K. flammea* for the treatment of prostate cancer, evaluating its cytotoxic activity, its probable mechanism of action, and carrying out some toxicological safety studies.

Materials and methods: The cytotoxic activity of the ethyl acetate extract of *K. flammea* (Kf-EtOAc) was evaluated in several cell lines of prostate cancer by MTT viability assay. The cellular death mechanism was studied by evaluating the translocation of phosphatidylserine (Annexin V); overproduction of reactive oxygen species [2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay]; release of Cytochrome C; activation of caspase-3 and -9, and regulation of Bcl-2, XIAP, and PKCε proteins by Western Blot analysis. For the evaluation of the safety of Kf-EtOAc, the Ames test, Micronucleus assay, and acute toxicity study were determined.

Results: Kf-EtOAc exhibited selective cytotoxic activity against prostate cell lines as follows: PC-3, LNCaP, and PrEC (IC_{50} = 1.36 ± 0.05; 2.06 ± 0.02, and 127.05 ± 0.07 μg/mL, respectively). The F82-P2 fraction (rich in coumaric acid and palmitic acid) obtained by bioassay-guided fractionation of Kf-EtOAc also demonstrated selective cytotoxic activity against PC-3 cells (IC_{50} = 1.05 ± 0.06 μg/mL).

Kf-EtOAc induces apoptosis by the intrinsic pathway; this mechanism of cell death was confirmed after observing that the extract produces phosphatidylserine translocation, overproduction of reactive oxygen species, release of Cytochrome C at mitochondrial level, and activation of caspase-3 and -9. It was also observed that Kf-EtOAc produces significant downregulation of apoptosis-related proteins Bcl-2, XIAP, and PKCε and induces DNA fragmentation and cell cycle arrest.

In addition, Kf-EtOAc is non-genotoxic *in vitro* by Ames test and non-genotoxic *in vivo* by Micronucleus assay, and no signs of toxicity or death were reported after the administration of a single acute exposure of 2000 mg/kg.

Conclusion: K. flammea is a potential candidate for the development of new drugs for the treatment of prostate

List of abbreviations: 2AA, 2-aminoanthracene; 4NQO, 4-nitroquinoline; BCA, bicinchoninic acid; Bcl-2, B-cell lymphoma 2; CA, coumaric acid; CAD, caspase-activated dnase; DAPI, 4',6-diamidino-2-phenylIndole; DCF, oxidize 2'-7'-dichlorodihydrofluorescein; DCFH, reduced 2'-7'-dichlorodihydrofluorescein; DCFH-DA, 2'-7'-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EGTA, egtazic acid; EIS, electrospray ionization source; F82-P2, fraction rich in coumaric acid and palmitic acid; FBS, fetal bovine serum; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; GHS, Globally Harmonised Classification System; HDF, human dermal fibroblast; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; i.g., intragastric; i.p., intraperitoneal; IAP, inhibitor of apoptosis proteins; IC_{50}, 50% inhibition viability; Kf-EtOAc, ethyl acetate extract of *Kalanchoe flammea*; LNCaP, androgen-sensitive human prostate adenocarninoma cells; MN, micronuclei; MN-NCE, micronucleated – normochromatic erythrocytes; MN-RET, micronucleated – reticulocytes; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MOLT-4, human T lymphoblasts; MOMP, mitochondrial outer membrane permeabilization; MPTP, mitochondrial permeability transition pore; MS-EI, mass spectrometry by electronic impact; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NC, no cytotoxic; NCE, normo chromatic mature erythrocytes; ND, no determined; O.D., optical density; OECD, Organisation for Economic Co-operation and Development; PA, palmitic acid; PA, picrolonic acid; PARP-1, poly ADP-ribose polymerase-1; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PC-3, human prostate androgen-independent cells; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PKCε, protein kinase C epsilon type; PLC, preparative layer chromatography; PMSF, phenylmethylsulfonyl fluoride; PrEC, non-tumoral prostate epithelial cells; PrEGM, prostate epithelial cell growth medium; RET, reticulocytes; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute Medium; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, standard error; TdT, terminal deoxynucleotidyl transferase; TLC, thin layer chromatography; TUNEL, dUTP nick-end labeling; UV, ultraviolet; XIAP, X-linked inhibitor of apoptosis protein

* Corresponding author.
E-mail addresses: xeroarmstrong@hotmail.com (I. Arias-González), carranca@biomedicas.unam.mx (A.M. García-Carrancá), jcornejog@ipn.mx (J. Cornejo-Garrido), cordaz@ipn.mx, dra_cynthia@hotmail.com (C. Ordaz-Pichardo).

[1] Student in the program of "Doctorate of Science in Biotechnology of the National Polytechnic Institute".

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cancer. However, to propose their use in clinical trials, additional studies are required to understand their pharmacokinetic behavior, as well as the development of a suitable pharmaceutical form.

1. Introduction

Cancer is a major public health problem throughout the world and is the leading cause of death in both more and less economically developed countries (Torre et al., 2015). Prostate cancer is the second most frequently diagnosed cancer in men worldwide and is the third leading cause of cancer death in the male sex (Howlader et al., 2017).

In early stages of the disease, treatment is usually prostatectomy and/or radiation; however, the effectiveness of this therapy is reduced and in many cases, the disease is recurrent; moreover, a large number of patients are diagnosed after the cancer had spread (Dayyani et al., 2011). Early-stage prostate cancers can be treated with androgen ablation therapy; however, cancers that are not cured by surgery eventually become androgen-independent, highly aggressive, and metastatic (Feldman and Feldman, 2001). At this stage, the disease remains incurable despite current taxane-based drugs, which are the only chemotherapies approved for this condition by the United States Food and Drug Administration (FDA) in that survival benefits are modest (Dayyani et al., 2011).

Cancer has been classified into a set of common properties that allow it to grow uncontrollably; it has been proposed that eliminating some of these features will prevent the formation of tumors, their growth, and metastasis. These “hallmarks” include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, along with two emerging hallmarks including reprogramming of energy metabolism and immune system evasion (Hanahan and Weinberg, 2011).

Treatment of multifactorial diseases, such as cancer with agents targeting a single mark, could provide partial treatment and, in many cases, disappointing cure rates. Natural products are important for drug development as pharmaceutical agents and/or as precursors of bioactive molecules against cancer because they could possess inhibitory effects through targeting multiple cellular signaling pathways; thus, they could be classified as multi-targeted agents. Additionally, natural compounds may inhibit specific processes involved in carcinogenesis, playing an important role in cancer chemoprevention (Sarkar et al., 2009).

Apoptosis is a programmed cell death and a highly organized physiological mechanism to destroy injured or abnormal cells. It is characterized by biochemical and morphological changes that include cellular and nuclear shrinkage, chromatin condensation, formation of apoptotic bodies, and DNA fragmentation (Elmore, 2007). Deregulated cell death is a common feature of many human diseases, including cancer. A successful anticancer drug should be able to kill or inhibit the proliferation of cancer cells without causing excessive damage to normal cells. This potential is achievable by inducing apoptosis in cancer cells (Taraphdar et al., 2001).

Kalanchoe flammea Stapf (*Crassulaceae*) is a medicinal plant grown in the South of Mexico and commonly used in traditional medicine. It is mainly employed in the state of Tabasco for the treatment of fever, wounds, inflammation, and cancer (Maldonado Mares, 2002). Other species of the genus *Kalanchoe* are utilized for the treatment of cancer; extracts from some of these species have been studied, finding that they exert cytotoxic activity on tumor cell lines of cervix, breast, lung, liver, colon, and leukemia (Hsieh et al., 2016, 2012; Kuete et al., 2017; Lai et al., 2011; Mahata et al., 2012). In the vast majority of reported studies, steroids (sterols and bufadienolides), flavonoids, glycosides, coumarins, triterpenoids, and fatty acids have been found responsible for biological activity (Hsieh et al., 2016, 2012; Huang et al., 2013; Kuo et al., 2008; Lai et al., 2011; Supratman et al., 2001; Wu et al., 2006; Yamagishi et al., 1989). In addition, several important pharmacological activities have been attributed to different *Kalanchoe* species, such as antioxidant (Ahmed et al., 2013; Bopda et al., 2014; Fondjo et al., 2012; Iqbal et al., 2016; Kolodziejczyk-Czepas et al., 2016; Lai et al., 2011; Nascimento et al., 2013), anti-inflammatory (Lai et al., 2011; Mourão et al., 1999), hepatoprotective (Asiedu-Gyekye et al., 2014; Yadav and Dixit, 2003), antibacterial (Iqbal et al., 2016), gastroprotective (Sobreira et al., 2015), antihypertensive (Bopda et al., 2014), immunomodulatory, and antimutagenic (Umbuzeiro-Valent et al., 1999).

In this paper, we report the cytotoxic effects of an ethyl acetate extract of *K. flammea* (Kf-EtOAc) against human prostate androgen-independent cells (PC-3), androgen-sensitive human prostate adenocarninoma cells (LNCaP), and non-tumoral Prostate Epithelial Cells (PrEC). The ability of the extract to induce cell cycle arrest and several tests to evaluate its ability to induce apoptosis were performed. Finally, the genotoxic capacity of the extract, as well as its acute toxicity were determined, in order to ensure the true potential of Kf-EtOAc for the development of new drugs for the treatment of prostate cancer.

2. Materials and methods

2.1. Plant material and preparation of extracts

Leaves of *K. flammea* were collected from Jalpa de Méndez, Tabasco, in the southeast of Mexico, during May and June of 2010, 2011, and 2012. Edith López Villafranco, M.Sc., identified the species and voucher specimen no. 2374 was deposited at the Herbarium of Faculty of Higher Studies Iztacala-UNAM.

The plant material was washed with distilled water and then cut into 1-cm sections. The extracts were prepared by maceration for 5 days at room temperature (AR-grade solvents were purchased from J. T. Baker Chemical Co., TN, USA). The organic extracts were filtered and concentrated to dryness under low pressure at 40 °C in a rotary evaporator (Büchi, Sankt Gallen, Switzerland), repeating the extraction process three times. Extracts were placed in amber-colored glass bottles at 4 °C until their use.

2.2. Culture of cell lines

The strains were primary cultures (Lonza, Chemicals Biotechnology, Basel, Switzerland) that were kindly donated by José Luis Cruz-Colín, M.Sc.(Head Chemist, Oncogen Section, National Institute of Genomic Medicine, Mexico City). Human prostate cell lines PC-3, LNCaP, and PrEC and primary culture of Human Peripheral Blood Mononuclear Cells (PBMC) were used. PC-3, LNCaP and PBMC cells were maintained in Roswell Park Memorial Institute Medium (RPMI; Gibco, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, Thermo Fisher Scientific, UT, USA) and 1% antibiotic-antimycotic (Gibco, CA, USA), while PrEC cells were maintained in Prostate Epithelial Cell Growth Medium (PrEGM™, Lonza, MD, USA) supplied with SingleQuots™ (growth factors, cytokines, and supplements, Lonza, MD, USA). All cell cultures were grown at 37 °C in a 5% CO_2 and 95% humidity.

Confluent cells were removed with 0.1% trypsin (Sigma-Aldrich, MO, USA) and resuspended in RPMI or Prostate Epithelial Basal Medium (PrEBM; Lonza, MD, USA). LNCaP, PC-3, and PrEC cells were deposited in 96-well microplates at 8×10^3, and PBMC, at 1.4×10^4 cell/well. The microplates were incubated for 24 h at 37 °C in 5% CO_2 and 95% humidity before additional treatments.

2.3. Cell treatment and MTT viability assay

Kf-EtOAc extract, fractions, or isolated compounds were dissolved in DiMethyl SulfOxide (DMSO; Sigma-Aldrich, MO, USA), to form stock solutions at 2 mg/mL that were stored at −20 °C. The stock solutions were dissolved in RPMI or PrEBM and sterilized by filtration before the treatment.

The MTT viability assay was performed as described previously (Mosmann, 1983). This assay is based on the reduction of yellow MTT (3-(4,5-diMethyl-2-Thiazolyl)-2,5-diphenyl-2H-Tetrazolium bromide, Sigma-Aldrich, MO, USA) to formazan by mitochondrial succinate dehydrogenase, which is present only in living cells. The absorbance value of formazan is directly proportional to the number of viable cells (Eisenbrand et al., 2002).

Cells in 96-well microplates were treated for 24 h with concentrations between 1.56 and 200.00 µg/mL of Kf-EtOAc extract; between 1.00 and 90.00 µg/mL of its primary fractions, and between 0.03 µg/mL and 9.00 µg/mL for the isolated compounds. DMSO at a final concentration of 0.1% was used as vehicle control.

After treatment, the media was replaced with medium containing MTT (0.5 mg/mL) and then, cells were incubated for an additional 4 h. Medium was removed and formazan crystals were dissolved in DMSO. Finally, the plates were read at 570 nm in a microplate spectrophotometer (Epoch; BioTek, VT, USA).

The assays were performed in triplicate in three independent studies and the percentage of viability was calculated according to the formula: % viability = [mean Optical Density (O.D.) treated cells × 100]/(mean O.D. control cells). The concentration leading to 50% inhibition viability (IC_{50}) was calculated by non-linear regression analysis (percentage of viability *vs.* log concentration) with GraphPad Prism ver. 5.0 software (GraphPad, CA, USA).

2.4. Phosphatidylserine translocation (Annexin V-FITC)

The Annexin V–FITC Apoptosis Detection Kit (BioVision, CA, USA) was utilized to assess apoptotic activity as the externalization of phosphatidylserine residues, according to the manufacturer's instructions. Briefly, 2.5×10^4 cells were deposited in 6-well microplates. After 24 h of growth, IC_{50} of Kf-EtOAc extract was added to cells during 3, 6, 12, and 24 h. Subsequently, the cells were harvested, washed with Phosphate-Buffered Saline (PBS) solution, and suspended in 500 µl of 1× binding buffer. Then, the cells were stained with 5 µl Annexin V–FITC (Fluorescein IsoThioCyanate) and 5 µl Propidium Iodide (PI) for 5 min in the dark at room temperature. Finally, 1×10^4 cells were counted with a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) and analyzed with Summit Ver. 4.3 software (Beckman Coulter, Inc., CA, USA).

2.5. Determination of intracellular levels of Reactive Oxygen Species (ROS)

For ROS analysis, the 2'-7'-DiChlorodihydroFluorescein DiAcetate (Molecular Probes, Thermo Fisher Scientific, OR, USA) assay was used as previously described (Alonso-Castro et al., 2013). DCFH-DA is a lipophilic molecule that diffuses through cell membranes. At the intracellular level, DCFH-DA can be deacetylated by esterase enzymes producing DCFH, which is able to oxidize on contact with hydrogen peroxide or other ROS, forming the highly fluorescent DCF (Kalyanaraman et al., 2012).

Briefly, 2.5×10^5 PC-3 cells were treated with the IC_{50} of Kf-EtOAc extract in RPMI complete medium and incubated for 3, 6, 12, and 24 h. At the end of each treatment, cells were incubated with 5 µM DCFH-DA in serum-free medium for 20 min at 37 °C in the dark. Cells were washed and a total of 3×10^4 events were analyzed using a FACScan cytometer (Becton Dickinson, San Jose, CA, USA). The oxidation of DCFH was detected by the increase in fluorescence, which is proportional to the amount of intracellular ROS generated. Mean fluorescence was analyzed using CellQuest software (Becton–Dickinson, San Jose, CA, USA).

2.6. Preparation of mitochondrial, cytosolic, and total protein extracts

First, to prepare the whole-cell extract, PC-3 cells were treated with IC_{50} of Kf-EtOAc extract for 3, 6, 12, 24, and 48 h. At the end of each treatment time, supernatants were collected, the cells harvested, and centrifuged together at $200g \times 5$ min. The pellet was washed once with PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF (PhenylMethylSulfonyl Fluoride), and protease inhibitor cocktail) for 10 min over ice. The lysate was collected in a microcentrifuge tube and passed through a 21-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at $1.8 \times 10^4 g$ for 15 min at 4 °C and supernatant was transferred into a new microcentrifuge tube.

The cytosolic fraction was carried out as described previously (Krysko et al., 2008). Briefly, after treatment, PC-3 cells were lysed in 100 µl of CFS buffer (10 mM HEPES NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM $MgCl_2$, 2 mM NaCl, 2.5 mM H_2KPO_4, 0.5 mM EGTA, 0.5 mM sodium pyruvate, and 0.5 mM L-Glutamine) with 0.02% of digitonin and left in ice for 1 min. Next, the lysate was centrifuged for 10 min at 20,800 g at 4 °C and the supernatant was transferred to a microcentrifuge tube. The pellet obtained was lysed as a whole-cell extract to obtain the mitochondrial proteins.

The concentration of proteins in all supernatants was quantified by the BiCinchoninic Acid method (BCA; Thermo Fisher Scientific, MS, USA).

For immunoblot analyses, 30–70 µg protein per sample was denatured in Laemmli buffer, separated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) on 10%, 12%, or 15% polyacrylamide gels and transferred onto nitrocellulose membranes, which were blocked with 5% (w/v) non-fat milk in PBS-Tween 0.05% for 1 h at room temperature. Membranes were revealed with the respective primary antibodies: β-actin (1:2000, SC-1615); Bcl2 (B-cell lymphoma 2; 1:500, SC-65392); Caspase-3 (1:500, SC-56053); Caspase-8 (1:1000, SC-7890); Caspase-9 (1:1000, SC-56076); Caspase-10 (1:800, SC-6186); Cytochrome C (1:1000, SC-7159); PKCε (Protein kinase C epsilon type; 1:10, SC-56944); α-tubulin (1:1000, SC-5286); XIAP (X-linked inhibitor of apoptosis protein; 1:500, SC-55552) (Santa Cruz Biotechnology, CA, USA), and PARP-1 (Poly [ADP-ribose] polymerase 1; 1:1000, #9532) (Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C followed by peroxidase conjugated secondary antibodies for 1 h at room temperature and chemiluminescence detection. Immunoblotting with tubulin or actin antibody was performed to confirm equal protein loading.

2.7. Caspase inhibition assay

Briefly, 2.5×10^5 PC3-cells were deposited in 6-well microplates in 1 mL of complete medium. Cells were treated with 1 µl of general caspase inhibitor Z-VAD-FMK (BD Pharmigen, CA, USA) at a final concentration of 10 mM and were incubated for 15 min at 37 °C. Subsequently they were treated with Kf-EtOAc extract $IC_{50} = 1.36 \pm 0.05$ µg/mL for 24 h, using DMSO (0.1%) as a negative control, and paclitaxel (5 µg/mL) as a positive control. Subsequently, the cells were harvested, washed twice with PBS solution, and suspended in 300 µl of 1× calcium buffer. Then, the cells were stained with 1 µl Annexin V–APC (Allophycocyanin) and 7-aminoactinomycin D (7-AAD) (BD Pharmigen, CA, USA), and incubated for 15 min in the dark at room temperature. Finally, 1×10^4 cells were counted with an Accury flow cytometer (Becton Dickinson, CA, USA) and analyzed with FlowJo Ver. 10.4.2 software (LLC, OR, USA).

2.8. DNA fragmentation detection by TUNEL assay

Detection of nuclear fragmentation of apoptotic cells was carried out using the *in situ* Cell Death Detection Kit, AP (Roche Diagnostics Corporation, Indianapolis, IN, USA). This method is based on Terminal deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL), which identifies apoptotic cells *in situ* utilizing TdT to transfer fluorescein-dUTP to the free 3'-OH of cleaved DNA. Briefly, PC-3 cells were cultured in chamber slides systems (Thermo Fisher Scientific, MA, USA) and treated with the IC_{50} of Kf-EtOAc extract for 12 and 24 h. After treatments, cells were washed once with PBS and fixed with 4% paraformaldehyde for 1 h, washed with PBS, permeabilized with 0.1% Triton X-100 in a 0.1% sodium citrate solution, and incubated with the TUNEL reaction mixture (consisting of deoxynucleotidyl transferase and nucleotide mixture in a reaction buffer) for 60 min at 37 °C. After incubation, these were washed three times with PBS and finally, a coverslip was lowered onto a drop of Vectashield mounting medium with 4',6-DiAmidino-2-PhenylIndole (DAPI) (Vector Labs, CA, USA). Finally, images were captured using ZEN 2.3 Blue Edition software with an Axio Imager Z.1 fluorescence microscope (Carl Zeiss, Göttingen, Germany).

2.9. Cell cycle analysis

A total of 5×10^5 PC-3 cells were treated with Kf-EtOAc extract at IC_{50} in RPMI complete medium at 6, 12, and 24 h. The cell culture treatment were washed twice with ice-cold PBS, trypsinized, and centrifuged. The pellet was suspended to be fixed with cold 70% (v/v) ethanol and stored overnight at −20 °C. Fixed cells were centrifuged for 5 min at 200*g*, and the pellet obtained was washed with cold PBS. The cells were incubated with a solution containing Triton X-100 (1.1%, v/v) PI (50 µg/mL) and RNase A (100 µg/mL) in PBS for 30 min at room temperature. Analysis was performed with a FACS-Calibur flow cytometer (Becton Dickinson, NJ, USA). About 3×10^4 cells per sample were collected and DNA histograms were analyzed with ModFitLT software (Verily Software House, ME, USA).

2.10. In vitro genotoxic activity – Ames test

Incorporation method of *Salmonella* mutagenicity test was performed as previously described by Maron and Ames (Maron and Ames, 1983) employing the following histidine auxotrophic *Salmonella typhimurium* strains: TA98 (*hisD3502, rfa uvrB* pKm101); TA100 (*hisG46, rfa uvrB* pKm101), and TA102 (*hisG428 rfa pQ1*, pKm101) in the presence or absence of *the* S9 fraction (Arochlor1254-induced rat liver homogenate) as the metabolic activation system. Briefly, the concentrations employed to conduct the Ames test were between 50 and 200 µg per plate for the Kf-EtOAc extract and between 5 and 20 µg per plate for its primary fractions. After incubation for 48 h at 37 °C, the revertant colonies (His^+) were quantified. PA, Picrolonic Acid (50 µg/plate); 2AA, 2-AminoAnthracene (10 µg/plate); MNNG, N-Methyl-N'-Nitro-N-NitrosoGuanidine (10 µg /plate), and 4NQO, 4-NitroQuinoline 1-Oxide (10 µg/plate) were used as positive controls and DMSO 0.1% was used as vehicle control. Duplication or greater increases in the number of spontaneous revertant colonies are considered as a positive test.

2.11. Ethical statement for animal studies

Mice were purchased from the Bioterium of the FES Iztacala-UNAM, México. All animal experiments were conducted according to Mexican Official Norms (NOM-033-ZOO-1995; NOM-062-ZOO-1999) and this investigation was approved by the Laboratory Animal Ethics Committee of the National School of Medicine and Homeopathy Act (ENMH-CB-0094–2014), which comply with International Regulations and Policies concerning the use, care, and humane killing of laboratory animals.

2.12. In vivo genotoxic activity – micronucleus assay

Fasted CD-1 male mice weighing 22 ± 2 g were treated with 1000 mg/kg of Kf-EtOAc extract in a volume of 100 µl by intragastric route (i.g.). Each group was evaluated in triplicate and DMSO and water was used as negative control and cyclophosphamide (50 mg/kg) as a positive control intraperitoneally (i. p.). After 48 h, the animals were sacrificed and 120 µl of blood was obtained. Samples were processed according to the MicroFlow® PLUS kit for use with mouse peripheral blood (Litron Laboratories, NY, USA) and according to the manufacturer's instructions(LeBaron et al., 2013).

The number of NormoChromatic mature Erythrocytes (NCE) and RETiculocytes (RET), with and without MicroNuclei (MN), were determined, this providing a way to calculate the percentage of MicroNucleated- NormoChromatic Erythrocytes (% MN-NCE), the percentage of MicroNucleated-RETiculocytes (% MN-RET), and the total percentage of RETiculocytes (% RET) (LeBaron et al., 2013).

2.13. Acute oral toxicity study

An acute oral toxicity study was performed according to OECD-423 test guidelines (Organisation for Economic Co-operation and Development; OECD, 2002). This procedure is reproducible, uses few animals, and is able to rank substances in a similar manner to other acute-toxicity testing methods. For this method, we employed three male CD-1 mice weighing 22 ± 2 g, which were administered Kf-EtOAc extract by i.g. route at a single dose of 5000 mg/kg. Mice were maintained under ambient conditions and had free access to food (Rodent Diet™) and water with 12-h light/dark cycles. The rodents were observed for 14 days for behavioral changes and mortality. The lethal dose value was determined according to the Globally Harmonised Classification System (GHS) (OECD, 2002).

2.14. Bioassay-guided fractionation of Kf-EtOAc extract, and isolation of cytotoxic compounds

Kf-EtOAc extract (20 g) was fractionated by open column chromatography using Silica gel 60; 70–230 mesh (Merck Millipore, MA, USA) as stationary phase and n-hexane, ethyl acetate, and ethanol (AR-grade solvents were purchased from J. T. Baker Chemical Co., TN, USA), as well as a mixture of these solvents at increasing degrees of polarity as mobile phase. From this column, nine Primary Fractions were pooled according to their profile shown on Thin Layer Chromatography (TLC) utilizing 0.2-mm Silica gel 60, F_{254} aluminum plates coated with fluorescent indicator (Merck Millipore, MA, USA). Primary fractions were concentrated under low pressure in a rotary evaporator (Büchi, Switzerland), and the cytotoxic activity of each fraction was evaluated by MTT.

Active fractions F4 and F8 were subjected to Preparative Layer Chromatography (PLC) employing 2 mm Silica gel 60, F_{254} glass plates coated with fluorescent indicator (Merck Millipore, Billerica, MA, USA). PLC plates were eluted with Ethyl Acetate 100% and the cytotoxic activity of each Secondary Fraction was evaluated by MTT.

Active Secondary Fraction F82 was fractionated by High Performance Liquid Chromatography (HPLC) with an Agilent 1220 Infinity LC System Controller attached to an UltraViolet (UV) light detector at 220 nm. Equipment control, data acquisition, and information processing were performed using EZChrom Elite Compact software (Agilent), utilizing a Zorbax RX-C18 column (250 mm, 5-µm particle size; Agilent) coupled to a Zorbax RX-C18 guard column (12.5 mm, 5-µm particle size; Agilent) the stationary phase, and isocratic methanol 100% as mobile phase system (HPLC grade; J. T. Baker Chemical Co. Jackson, TN, USA).

Secondary Fraction F82 presented three main peaks; the most active was F82-P2. By analysis of Mass Spectrometry by Electronic Impact (MS-EI), two main compounds were found: Coumaric Acid (CA) and

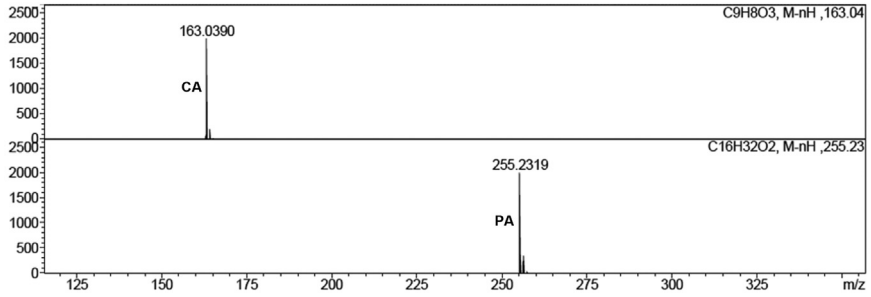

Fig. 1. Electrospray ionization mass spectrum (ESI-MS) of coumaric acid (CA) and palmitic acid (PA).

Palmitic Acid (PA) constituted this peak (Fig. 1). CA and PA concentrations were determined by HPLC using the isocratic methanol: acetonitrile (70:30) system as mobile phase with flow rate of 0.5 mL/min (Fig. 2) (HPLC grade; J. T. Baker Chemical Co. Jackson, TN, USA). A standard curve of PA and CA was performed (Sigma-Aldrich, St. Louis, MO, USA).

2.15. Structural elucidation by ESI-MS

Spectra were obtained utilizing a micrOTOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Electrospray Ionization Soource (ESI). Parameters were set as follows: capillary 500 V; nebulizer pressure 2.5 bar; dry gas flow 8 L/min, and dry gas temperature, 180 °C. The sample was run in the negative ion mode. Scan range was from 50 to 3000 *m/z*.

Accurate mass data of the molecular ions were processed by means of Data Analysis ver. 4.0 software (Bruker Daltonics), which provided a list of possible elemental formulas using Generate Molecular Formula Editor.

2.16. Statistical analysis

All statistical analyses were performed with the Prisma ver. 3.02 statistical software program (GraphPad Software, CA, USA). The data are expressed as mean ± SD or SEM, setting the significance level at $p < 0.05$.

3. Results

3.1. Cytotoxic activity by MTT viability assay

Mitochondrial reduction activity is indicative of decreased cell viability; this was measured by the MTT assay. The concentration of Kf-EtOAc extract leading to a viability of 50% (IC_{50}) was calculated by dose-response non-linear regression analysis, showing a concentration-dependent relationship in prostate cell lines after 24 h of treatment as follows: PC-3 (IC_{50} = 1.36 ± 0.05 µg/mL); LNCaP (IC_{50} = 2.06 ± 0.02 µg/mL), and PrEC (IC_{50} = 127.05 ± 0.07 µg/mL). This loss of cellular viability was not observed in the case of PBCM (Table 1).

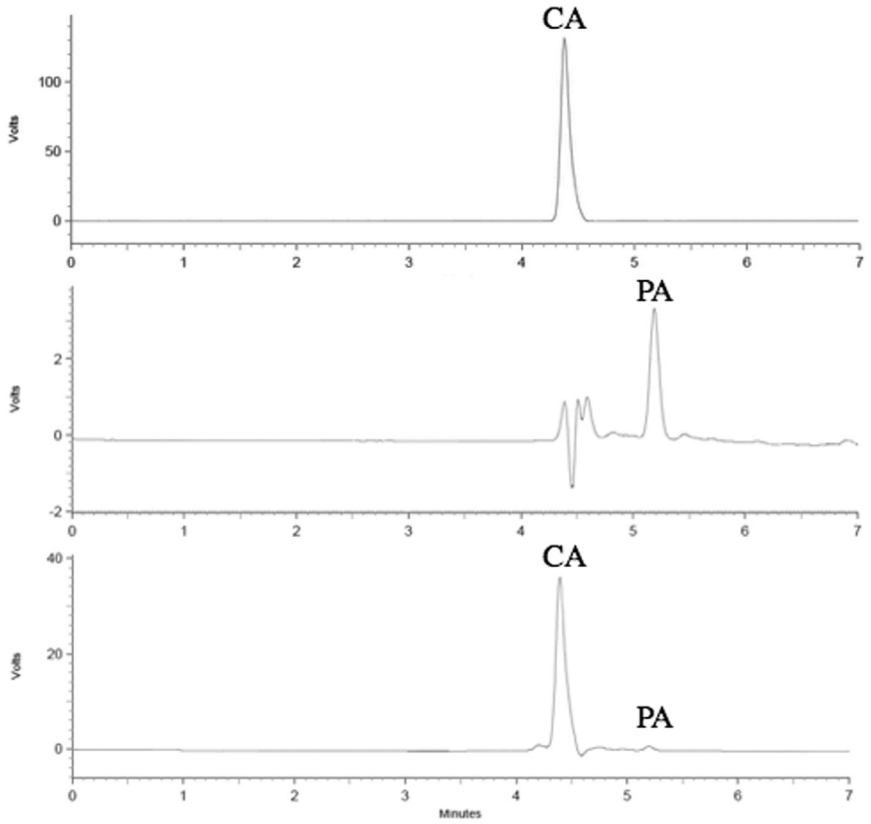

Fig. 2. Representative HPLC chromatograms showing standards of coumaric acid (CA) and palmitic acid (PA), and secondary fraction F82-P2 containing mainly these two compounds.

Table 1
Cytotoxic activity of *K. flammea* extract (Kf-EtOAc) and fraction rich in cumaric acid and palmitic acid (F82-P2).

	IC_{50} (μg/mL)			
	PC-3	LNCaP	PrEC	PBMC
Kf-EtOAc	1.36 ± 0.05	2.06 ± 0.02	127.05 ± 0.07	NC
F82-P2	1.05 ± 0.06	ND	NC	ND

PC-3: androgen independent prostate cancer cells; LNCaP: androgen dependent prostate cancer cells; PrEC: prostate epithelial cells; PBMC: human peripheral blood mononuclear cells; NC = no cytotoxic; ND = no determined. Data represent the mean ± SD of three independent experimental series performed in triplicate.

It was evident that a major cytotoxic effect presented in prostate cancer cells rather than normal epithelial prostate cells.

After bioassay-guided fractionation of the Kf-EtOAc extract, we obtained the F82-P2 fraction, which is rich in Coumaric Acid (CA) and Palmitic Acid (PA). F82-P2 also exhibited a cytotoxic effect in prostate cancer cell line PC-3 (IC_{50} = 1.05 ± 0.06 μg/mL) and a lower effect in normal epithelial prostate cells, PrEC (IC_{50} = not calculable).

To assess whether a Kf-EtOAc induced decrease of viability in PC-3 cells is mediated through induction of apoptosis, we later evaluated the effect of Kf-EtOAc extract in terms of its ability to induce some biochemical characteristics of apoptosis.

3.2. Kf-EtOAc extract induces phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane

Another classical probe for the biochemical distinctive of apoptotic programmed cell death is phosphatidylserine translocation, in that this event occurs during early apoptosis and late apoptosis. Annexin V-FITC and PI were used to distinguish apoptotic cells from necrotic cells after treatment with IC_{50} of Kf-EtOAc for 3, 6, 12, and 24 h. As depicted in Fig. 3, incidence of both early (Annexin V+/PI-) and late apoptosis (Annexin V+/PI+) were increased in Kf-EtOAc-treated PC-3 cells and no signal for necrotic cell death (Annexin V-/PI+) was demonstrated.

3.3. Effect of Kf-EtOAc extract on ROS production

Overproduction of Reactive Oxygen Species (ROS) is an early biochemical characteristic of apoptosis. The involvement of ROS in Kf-EtOAc-induced cytotoxicity in PC-3 cells was examined in this study. DCF fluorescence intensity is proportional to the presence of hydrogen peroxide. After treatment with IC_{50} of Kf-EtOAc extract, flow cytometry histograms revealed overproduction of ROS in PC-3 cells after 6 h of treatment, an effect that is sustained over 12 h and 24 h of treatment (Fig. 4A and B).

3.4. The effector phase of Kf-EtOAc-induced apoptosis comprises mitochondrial signals

It is thought that Cytochrome C released from mitochondria is one of the ways in which the caspase cascade is initiated. This event was monitored by fractionation of the mitochondria-enriched and cytosolic proteins of PC-3 cells treated with IC_{50} of Kf-EtOAc extract by Western Blotting. Fig. 5 illustrates that Kf-EtOAc treatment caused a time-dependent release of cytosolic Cytochrome C and a concomitant decrease in mitochondrial-enriched Cytochrome C. In this context, it is known that mitochondrial dysfunction results in the release of Cytochrome C and subsequent activation of caspase-9 that, as mentioned previously was activated. We showed representative immunoblots of three different experiments.

3.5. Kf-EtOAc extract induces activation of proteins associated with the intrinsic pathway of apoptosis

Caspase-mediated apoptosis is the most important program of cell death. Therefore, the proteins of PC-3 cells treated with the IC_{50} of Kf-EtOAc were extracted for 3, 6, 12, 24, and 48 h, and Western Blot analysis was performed to determine the proteins involved in the extrinsic and intrinsic pathway of apoptosis. We utilized specific antibodies that detected both the proenzyme and the active form of caspase-3, -8, -9, and -10, and we could not detect any activation of caspase-8 or -10 (Fig. 6). However, a reduction in the signal of proenzyme caspase-9 was observed at 24 and 48 h of treatment, and the caspase-3 antibody detected two bands of 20 and 17 kDa after 12, 24, and 48 h of treatment with the Kf-EtOAc extract. This suggests a time-dependent processing of procaspases in the intrinsic pathway.

3.6. Caspase inhibition assay

The caspase inhibition assay was performed to corroborate death by apoptosis. In Fig. 7 we can see that PC-3 cells treated with Kf-EtOAc extract had an induction of apoptosis of 41.5%, whereas the cells that were previously treated with general caspase inhibitor (Z-VAD-FMK), lost this capacity considerably, since only 7.0% of cells are observed.

3.7. Down-regulation of apoptosis-related proteins Bcl2, XIAP, and PKCε

Many proteins are involved in triggering apoptosis and maintenance apoptosis. Bcl2 family proteins regulate the permeabilization of the mitochondrial outer membrane, therefore Cytochrome C release. Then, we determined the levels of proteins such as anti-apoptotic Bcl2, and

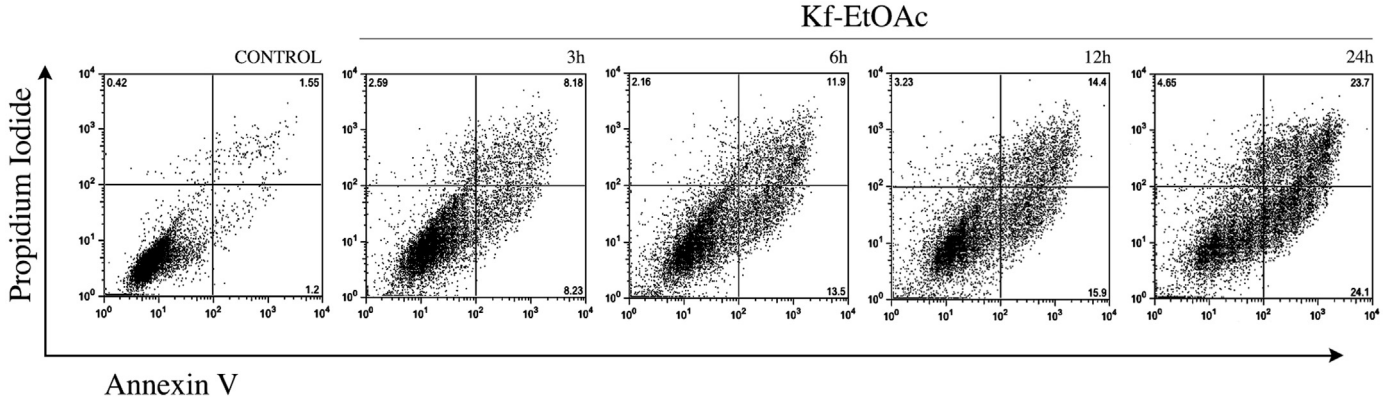

Fig. 3. Translocation of phosphatidylserine by flow cytometry (Annexin V-FITC/IP staining). Representative plots of PC-3 cells cultured in the presence *K. flammea* extract (Kf-EtOAc; IC50 = 1.36 μg/mL) are shown. Data show early apoptotic cells (Annexin V+/PI-) (lower right quadrant) and late apoptotic cells (Annexin V+/PI+) (upper right quadrant). Control (DMSO 0.1%).

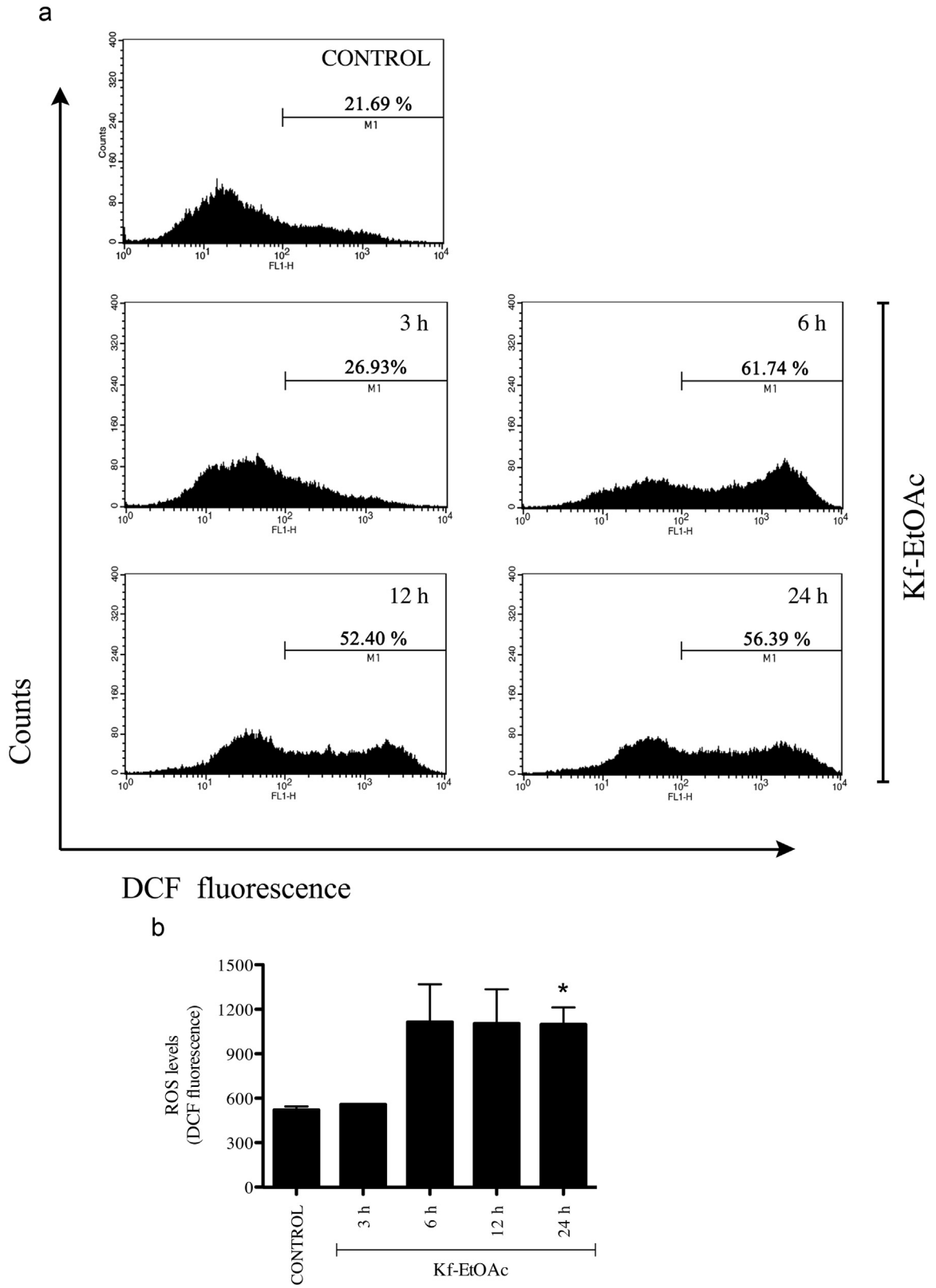

Fig. 4. Effect of *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 µg/mL) on the production of reactive oxygen species. A) Representative histograms show the indicated time courses for PC-3 cells incubated with IC_{50} of Kf-EtOAc. B) DCF fluorescence intensity. * Statistically significative difference *versus* negative control (DMSO 0.1%) by t test. (mean ± SEM of three independent experiments; $p \leq 0.05$).

Inhibitor of Apoptosis (IAP) proteins, such as XIAP and PKCε by immunoblotting analysis. The levels of these proteins were significantly inhibited by Kf-EtOAc extract treatment in PC-3 cells in a time-dependent manner. XIAP and PKCε downregulation was observed at 3 h after treatment and at 12 and 24 h of treatment, respectively (Fig. 8). On the other hand, Blc-2 protein was slightly downregulated by the Kf-EtOAc extract, this downregulation supporting the downstream signaling leading to apoptosis.

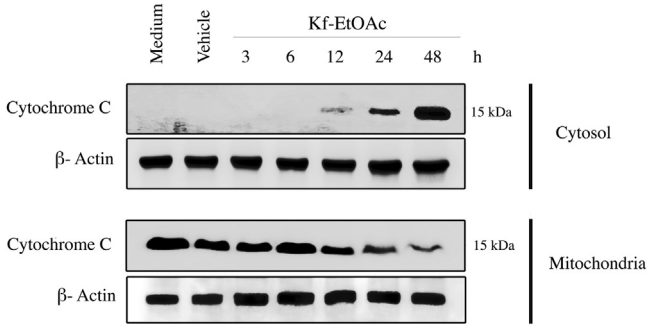

Fig. 5. Effect of *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 µg/mL) on cytochrome C release. PC-3 cells were permeabilized with digitonin and divided into enriched mitochondrial and cytosolic fractions.

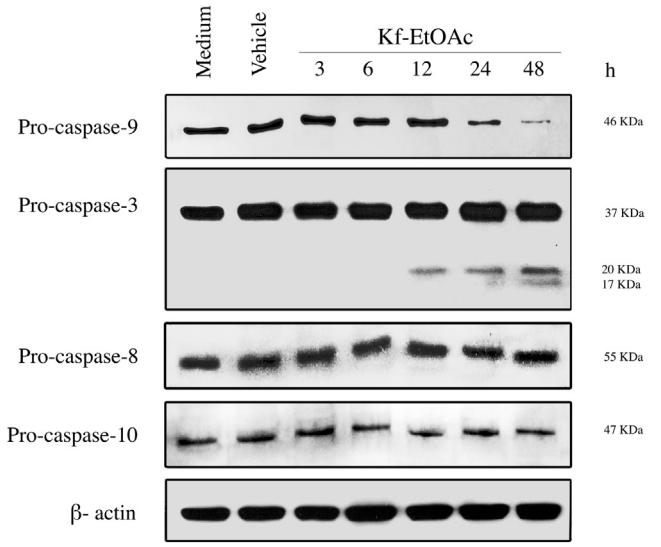

Fig. 6. *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 µg/mL) induces activation of proteins related to the intrinsic pathway of apoptosis. PC-3 cells were treated with vehicle (DMSO 0.1%) or with IC_{50} of Kf-EtOAc. Whole cell lysate were prepared, and subjected to Western Blotting. β-actin was used as the protein loading control.

3.8. Kf-EtOAc induces DNA fragmentation and morphological changes related to apoptosis in PC-3 cells

The terminal morphological and biochemical events of programmed cell death are characterized by specific changes in cell-surface and nuclear morphology. One of the most studied events comprises DNA fragmentation in an internucleosomal pattern, which is detectable in cultures of apoptotic cells. PC-3 cells treated with IC_{50} of the plant extract for 12 and 24 h were subjected to the TUNEL assay. The Kf-EtOAc extract induces DNA fragmentation at 12 and 24 h, which was evidenced by a significant increase in regions with high percentages of fluorescence TUNEL-positive nuclei cells (Fig. 9A). In that poly (ADP-Ribose) Polymerase-1 (PARP-1) is activated during DNA damage, PARP-1 cleavage during the course of apoptosis is a crucial event, allowing DNA degradation to occur. The Kf-EtOAc extract induces PARP-1 cleavage during the treatment of PC-3 cells; immunoblots revealed a significant time-dependent increase in the cleaved form of 89 kDa (Fig. 9B). PC-3 cells treated with the Kf-EtOAc extract exhibited the usual morphological changes in adherent cells, such as loss of cell attachment, cell rounding, and formation of blebbing and apoptotic bodies (Fig. 9C) where hypersegmented nuclear structures are shown.

3.9. Kf-EtOAc extract blocked PC-3 cells in S-phase

The evaluation of the cell cycle of PC-3 cells treated with Kf-EtOAc, showed that the mechanism of action of the extract is mediated by cellular blockade in the S-phase. As can be seen in Fig. 10 and Table 2, treatment with Kf-EtOAc showed an increase in cells in S-phase (58.3 ± 17.7% of cells) relative to the negative control cells (30.5 ± 1.4%) after 12 h of treatment. While fraction F82-P2 showed a greater increase after 24 h of treatment: 59.3 ± 1.9% of cells in S-phase compared to 29.5 ± 0.9% of cells in negative control.

3.10. Kf-EtOAc extract is non-mutagenic by Ames test

The Kf-EtOAc extract was tested for bacterial mutagenicity using *Salmonella typhimurium* strains TA98, TA100, and TA102 (which can detect frameshift mutations, base-pair substitutions, and reactive ROS-induced DNA damage, respectively), in the presence or absence of the S9 fraction as the metabolic activation system. Statistically significant increases of two or more spontaneous His^+ revertant colonies were obtained for the positive controls in all cases. On the other hand, our results showed that none of the extract concentrations evaluated increased the number of revertants, this indicating that the Kf-EtOAc extract is not mutagenic under both conditions evaluated, in the presence or absence of the metabolic activation system (Table 3).

3.11. Kf-EtOAc extract does not induce micronucleus formation

The Micronucleus assay is widely used to assess the potential for genotoxic damage due to drug exposure, as it may detect clastogenic or aneupleudogenic damage. The assay is based on quantifying the incidence of micronuclei in RET and NCE in peripheral blood. Administration of the Kf-EtOAc extract (1000 mg/kg) did not present a statistically significant increase with respect to animals administered with the control, while animals administered with cyclophosphamide (50 mg/kg) presented a remarkable increase with respect to control of water (Fig. 11).

3.12. Acute toxicity of the Kf-EtOAc extract

According to Protocol OECD 423, three male mice of the CD-1 strain were administered with the Kf-EtOAc extract by i.g. route at a single dose of 5000 mg/kg. Mice did not show a change in their physical activity or signs of toxicity or death after 14 days of observation. Therefore, according to the Globally Harmonised System (GSH), the LD_{50} of the Kf-EtOAc extract is > 5000 mg/kg. No macroscopic damage was observed in the vital organs of the treated animals, and there was no statistically significant difference in the weight gain of the treated animals compared to the non-treated control group (Table 4).

4. Discussion

Despite decades of research, interest in new drugs for the treatment of cancer has not decreased, since cancer remains as a major public health problem. Prostate cancer is one of the major cancers that threaten men's health and the first-line drugs used for its treatment produce severe side effects and also become ineffective after long-term use in early disease stages (Kallifatidis et al., 2016). Therefore, it is important to search for novel drugs that improve current treatments for prostate cancer and that are free of adverse effects. Natural products have been positioned as precursors of bioactive molecules and have been proposed as raw material for the development of new drugs, because they could exert inhibitory effects through targeting multiple cellular signaling pathways (Sarkar et al., 2009). During the period from the 1940s until the end of 2014, regulatory agencies have approved about 175 molecules for the treatment of cancer of which 85 (49%) are natural products or derived directly from them (Newman and

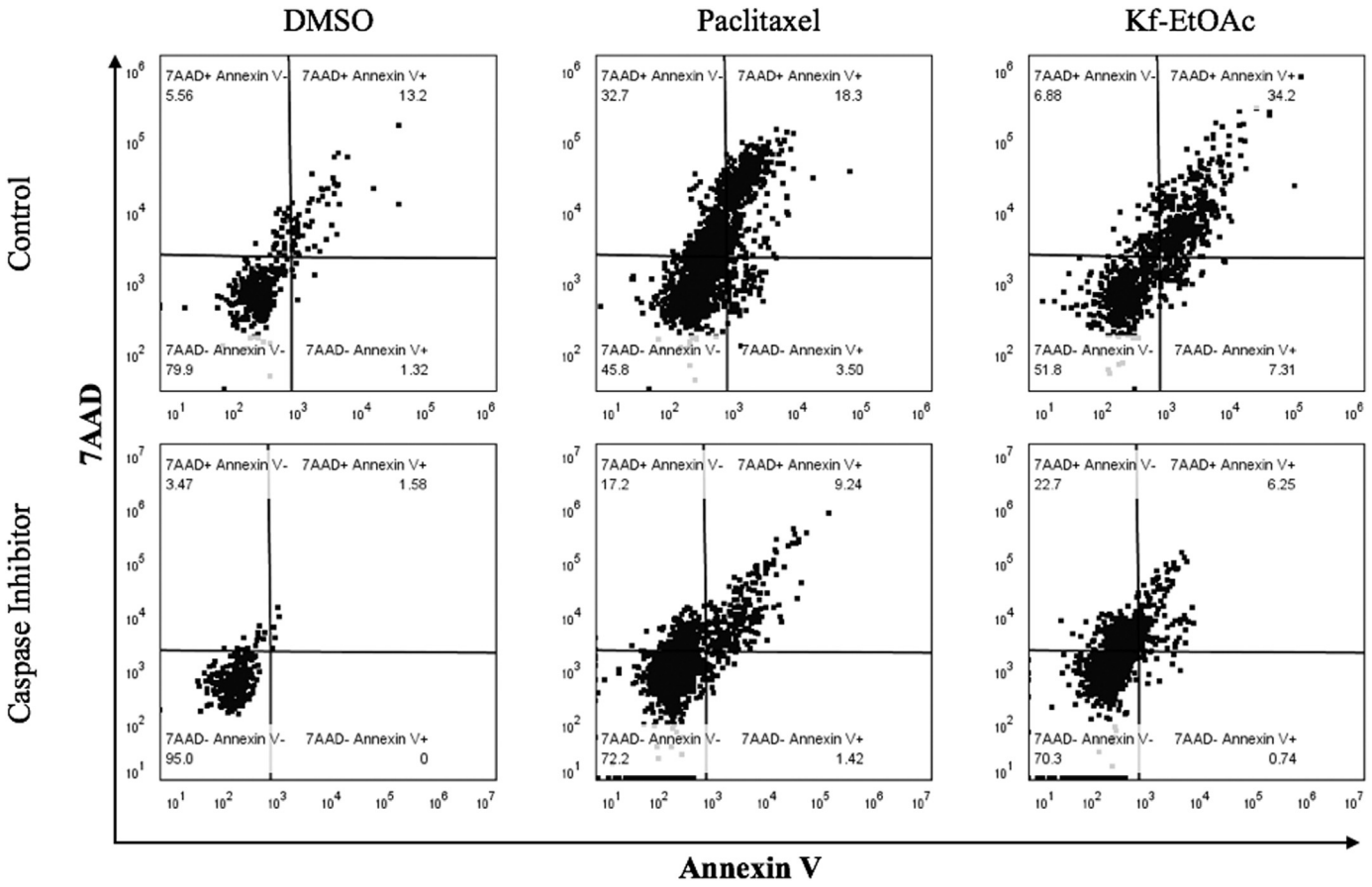

Fig. 7. Caspase inhibition assay by flow cytometry (Annexin V-APC/7-AAD staining). Representative plots of PC-3 cells cultured and treated for 24 h with Vehicle (DMSO 0.1%), Paclitaxel (5 μg/mL), and *K. flammea* extract (Kf-EtOAc IC_{50} = 1.36 μg/mL) are shown in the presence or absence of general Caspase Inhibitor (Z-VAD-FMK). Data show early apoptotic cells (Annexin V+/7-AAD-) (lower right quadrant) and late apoptotic cells (Annexin V+/7-AAD+) (upper right quadrant).

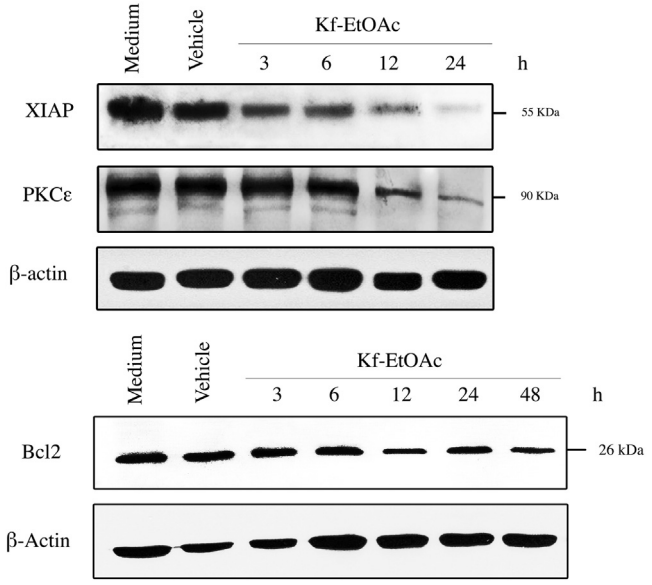

Fig. 8. Effect of *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 μg/mL) on the expression of anti-apoptotic Bcl2, and inhibitor of apoptosis XIAP and PKCε, proteins. PC-3 cells were treated with vehicle (DMSO 0.1%) or with IC_{50} of Kf-EtOAc. Whole cell lysate were prepared, and subjected to Western Blotting. β-actin was used as the protein loading control.

Cragg, 2016). In addition, there are many types of secondary metabolites in nature that possess a larger structural diversity than the compounds obtained by synthesis. Therefore, so this could be an important and effective way to find leading compounds with improved effects and low toxicity (Kinghorn et al., 2009).

Several studies have proven the cytotoxic effects of extracts and compounds isolated from various species of the *Kalanchoe* genus against a wide variety of cancer cell lines of cervix, breast, lung, liver, colon, and leukemia (Hsieh et al., 2016, 2012; Huang et al., 2013; Kuete et al., 2017; Kuo et al., 2008; Lai et al., 2011; Mahata et al., 2012; Supratman et al., 2001; Wu et al., 2006; Yamagishi et al., 1989). Previously in our working group the cytotoxic activity of the extracts obtained by maceration of *K. flammea* with hexane and ethanol on the PC-3 cell line was evaluated, finding that the IC_{50} of the hexane extract is not calculable, and for the ethanolic extract an activity 8 times lower was found with respect to ethyl acetate (IC50 = 10.54 ± 0.52 μg/mL). In this study, we show that the Kf-EtOAc extract significantly reduces the viability of androgen-independent PC-3 cells (IC_{50} = 1.36 ± 0.05 μg/mL), as well as of androgen-dependent LNCaP cells (IC_{50} = 2.06 ± 0.02 μg/mL) in a concentration-dependent relationship and exhibit low cytotoxicity in non-neoplastic human prostate epithelial cells, PrEC (IC_{50} = 127.05 ± 0.07 μg/mL).

To our knowledge, the cytotoxicity of any other species of the genus *Kalanchoe* has been tested on prostate cancer cells; however, similar IC_{50} values have been found when evaluating the activity of *Kalanchoe tubiflora* (Harv.) Raym.-Hameton lung A549, breast MCF-7, and cervix HeLa cells (IC_{50} = 6.75 μg/mL) (Hsieh et al., 2012) and *Kalanchoe crenata* (Andrews) Haw. on pleural cavity SPC-212 (IC_{50} = 2.33 ± 0.23 μg/mL) and lung A549 cells (IC_{50} = 8.23 ± 0.15 μg/mL)

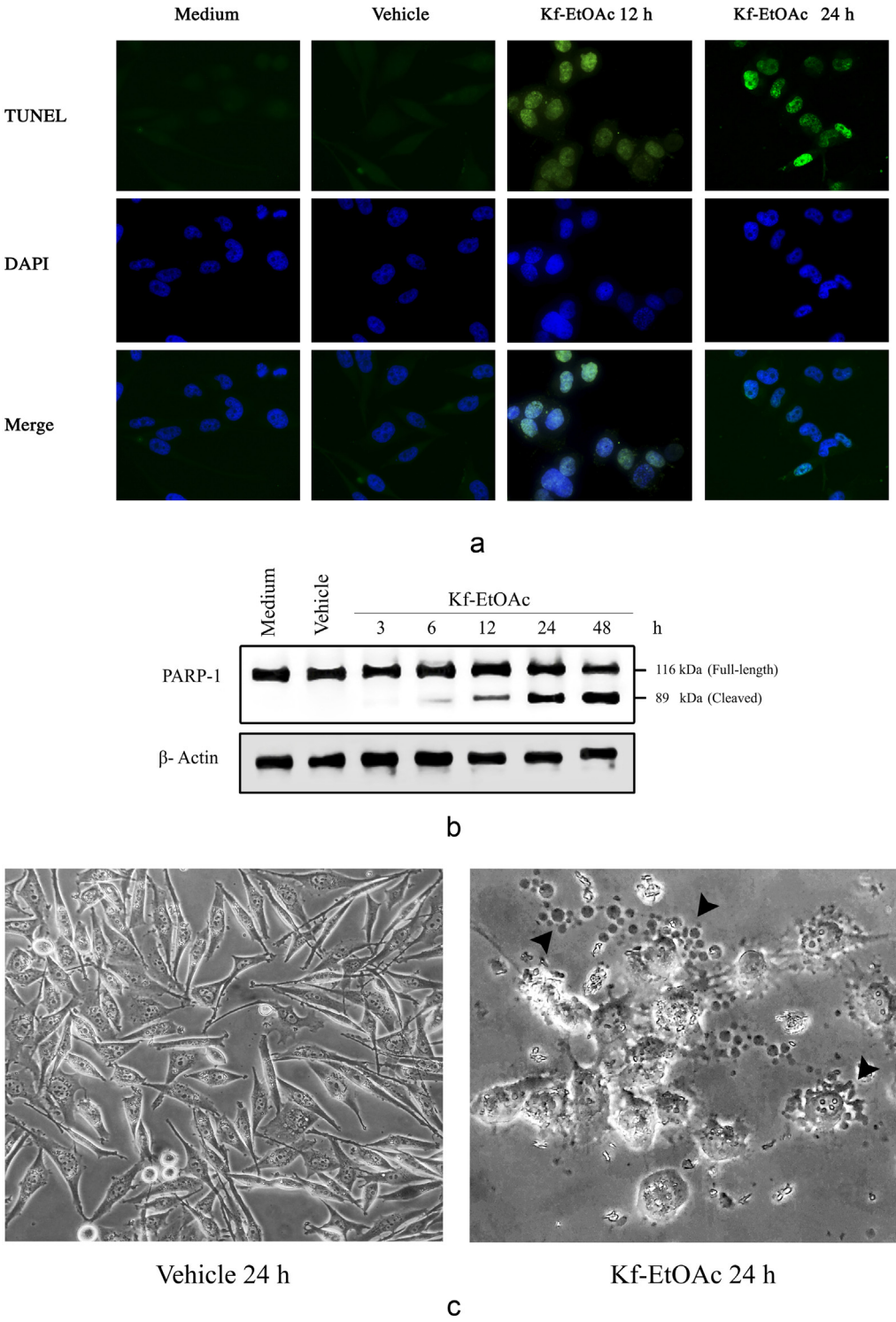

Fig. 9. DNA fragmentation and apoptotic morphological changes induced by *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 µg/mL) on PC-3 cells. A) TUNEL assay and DAPI staining were performed for DNA fragmentation detection related to apoptosis. B) PARP-1 cleavage analysis by Western Blotting in PC-3 cells treated with vehicle (DMSO 0.1%) or with IC_{50} of Kf-EtOAc. Whole cell lysate were prepared, and subjected to Western Blotting. β-actin was used as the protein loading control. C) PC-3 cells were treated with Kf-EtOAc or Vehicle (DMSO 0.1%) for 24 h, and consequently photographed under a phase-contrast microscope (magnification ×400) to determinate morphological changes and apoptotic bodies formation.

(Kuete et al., 2017), while extracts of other species, such as *Kalanchoe pinnata* (Lam.) Pers. and *Kalanchoe gracilis* Hance, have been reported to possess lower cytotoxic activities against HeLa and HepG2 cells (IC_{50} = 552 and 136.85 ± 2.32 µg/mL, respectively) (Lai et al., 2011; Mahata et al., 2012).

Apoptosis is a highly organized physiological mechanism to destroy injured or abnormal cells. Under apoptotic stimuli, these cells exhibit remarkable morphological features and characteristic molecular expression. This issue is important since the avoidance of apoptosis is one of the hallmarks of cancer cells, and its induction in neoplastic cells has become an ideal and attractive mechanism of action for anticancer drugs (Taraphdar et al., 2001). To assert the induction of apoptosis by the Kf-EtOAc extract, we evaluated different biochemical characteristics of this type of cell death according to the criteria and recommendations of the Nomenclature Committee on Cell Death (Galluzzi et al., 2012).

As part of the biochemical changes in apoptosis induction, treatment of the cells with Kf-EtOAc triggered a time-dependent translocation of phosphatidylserine detected by Annexin V. Although the exact reason for the appearance of phosphatidylserine on the external surface

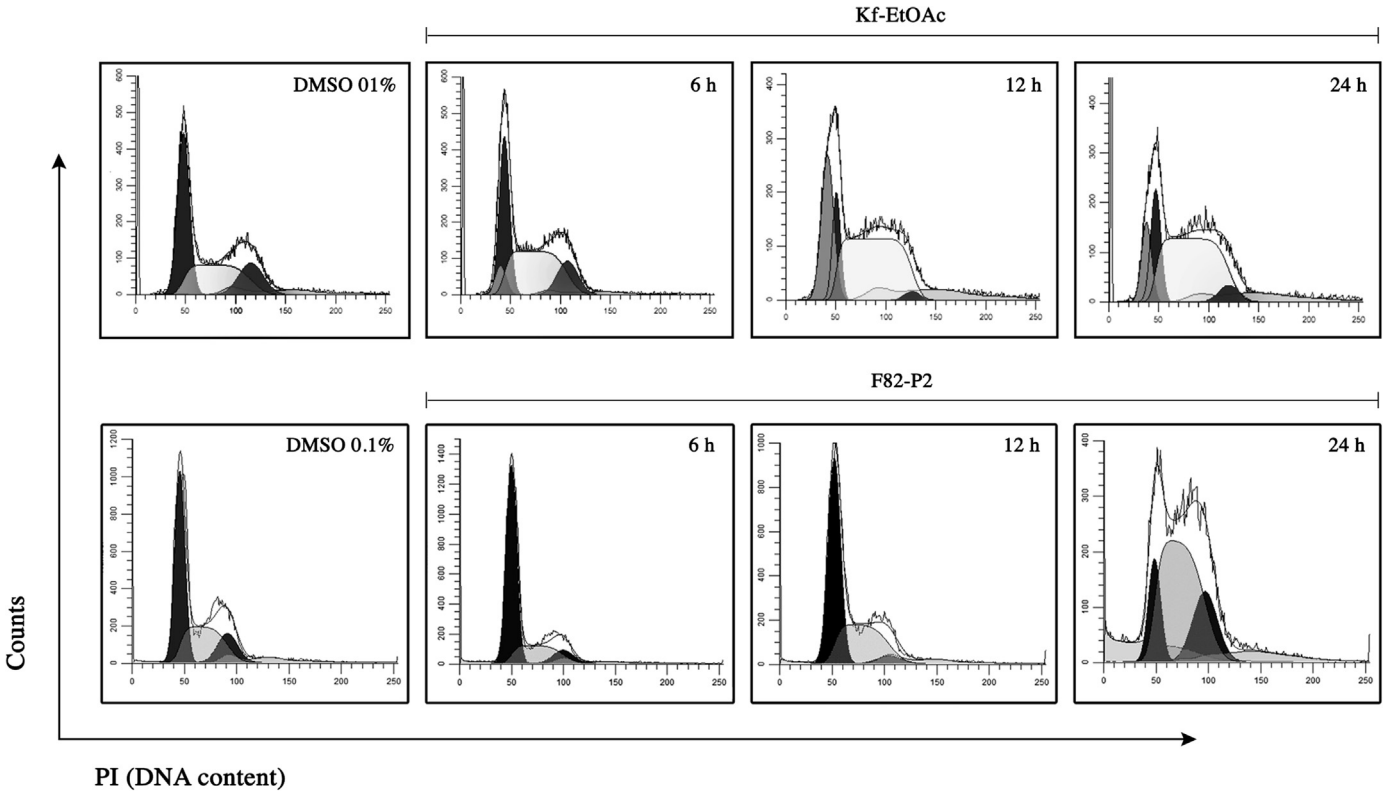

Fig. 10. Cell cycle phase distribution. DNA histograms of PC-3 cells treated with *K. flammea* extract (Kf-EtOAc; IC_{50} = 1.36 µg/mL) and F82-P2 (fraction rich in cumaric acid and palmitic acid; IC_{50} = 1.05 µg/mL) for 6, 12 and 24 h.

Table 2
Percentage of cells per phase of the cell cycle.

Phase	Treatment time 24 h	6 h	12 h	24 h
	DMSO 0.1%	Kf-EtOAc		
G0/G1	44.9 ± 6.4	36.2 ± 3.6	19.1 ± 14.3	38.0 ± 0.6
S	30.5 ± 1.4	37.5 ± 7.5	58.3 ± 17.7	35.2 ± 6.8
G2/M	24.5 ± 5.3	26.6 ± 4.1	22.5 ± 3.4	26.6 ± 6.2
	DMSO 0.1%	F82-P2		
G0/G1	48.5 ± 3.7	57.6 ± 13.2	47.8 ± 13.0	19.9 ± 4.9
S	29.5. ± 0.9	34.5 ± 14.7	48.6 ± 14.0	59.3 ± 1.9
G2/M	12.9 ± 8.0	7.7 ± 4.1	4.5 ± 0.5	19.6 ± 1.6

K. flammea extract (Kf-EtOAc); F82-P2 (fraction rich in cumaric acid and palmitic acid). Data represent the mean ± SD (n = 3).

is not clear, some experimental evidence suggests that this occur relatively early, immediately after segmentation of nuclei, and this is an early event, as is chromatin condensation (van Engeland et al., 1998). There are no studies, to our knowledge, that report the translocation of phosphatidylserine in other species of the genus *Kalanchoe*; it only has been reported that bufadienolide glycosides from *K. tubiflora* increase the number of apoptotic cells (9–18% of cells) (Huang et al., 2013), which is in agreement with this apoptotic mechanism found in our study. Subsequently, the caspase inhibition assay allowed to corroborate that cell death caused by the Kf-EtOAc extract is mediated by caspasas. As a positive control, paclitaxel was used, which has been reported as an inducer of apoptosis (Park et al., 2004).

Evidence in cancer research suggests that a number of apoptotic stimuli share a common mechanistic pathway characterized by the generation of ROS and mitochondrial-related events and culminating in

Table 3
Genotoxicity *in vitro* by the Ames test (plate incorporation method).

	Concentration / plate	Strain					
		TA98		TA100		TA102	
		−/S9	+/S9	−/S9	+/S9	−/S9	+/S9
Kf-EtOAc	50 µg	17.5 ± 9.9	20.0 ± 3.2	122.9 ± 7.7	115.8 ± 8.8	463.8 ± 35.4	483.8 ± 29.5
	100 µg	27.8 ± 4.0	18.3 ± 3.7	118.7 ± 21.8	102.5 ± 11.8	527.7 ± 60.8	501.8 ± 50.9
	200 µg	29.0 ± 5.1	42.5 ± 5.5	108 ± 8.5	138.8 ± 11.1	509.7 ± 62.3	367.3 ± 62.7
PA	50 µg	1258.3 ± 252.3					
2AA	10 µg		4817.3 ± 394.0		3527.6 ± 751.5		2466.5 ± 235.6
MNNG	10 µg			3253.7 ± 386.9			
4NQO	10 µg					2858.3 ± 188.2	
DMSO	0.1%	23.25 ± 5.3	28.83 ± 5.8	112.3 ± 20.4	114.6 ± 28.4	530.6 ± 54.5	479.2 ± 134.8

Data represent the mean ± SD of the number of histidine revertant colonies by plate in each treatment. Three independent experiments were performed by triplicate in the presence (+/S9) or absence (−/S9) of rat liver microsomal fraction induced with Arochlor 1254. PA, picrolonic acid; 2AA, 2-amino-anthracene; MNNG, methyl-N-nitro-N-nitrosoguanidine; 4NQO, 4-Nitroquinoline 1-oxide and DMSO, dimethyl sulfoxide.

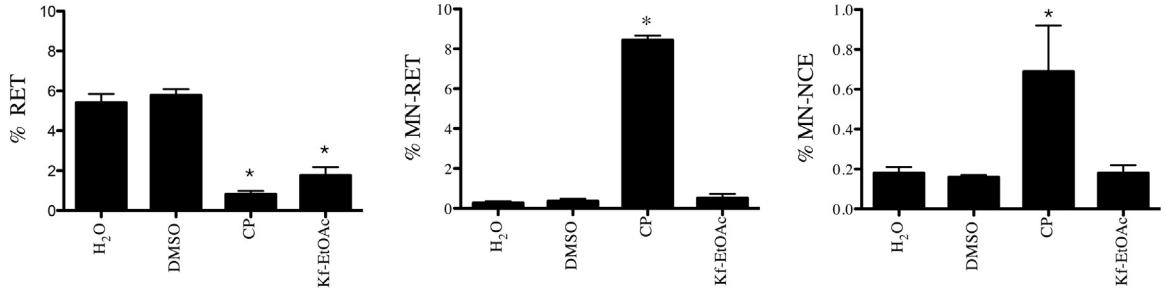

Fig. 11. Micronucleus assay. Reticulocyte percent (% RET), micronucleated reticulocyte percent (% MN-RET) and micronucleated normochromatic erythrocytes percent (% MN-NCE) after a 48 h of treatment. CP (Ciclophosphamide); DMSO (Dimetyl sulfoxide). * Statistically significative difference *versus* negative control (DMSO 0.1%) by multiple comparison Dunnett test. (n = 3, mean ± SEM; $p \leq 0.05$).

Table 4
Acute toxicity. Weight of animals treated with Kf-EtOAc.

	Day 1	Day 14	Weight gain to 14th day
Control	22.3 ± 1.8	35.7 ± 1.5	13.3 ± 0.3
Kf-EtOAc	21.7 ± 1.8	36.3 ± 0.3	14.7 ± 1.9

Control (untreated animals). No statistically significant difference was observed by *t test.* (n = 3, mean ± SEM).

the release of apoptotic factors (Ryter et al., 2007). Our findings indicate that the Kf-EtOAc extract at IC_{50} induces ROS generation when PC-3 cells are exposed from 6 to 24 h of treatment; this action could lead to the activation of an apoptotic stimulus of the extract by the mitochondrial pathway. This result is consistent with that recently reported by Kuete et al., who found that the methanolic extract of the leaves of *K. crenata* induces an increase in ROS levels of more than 3-fold compared to untreated cells (Kuete et al., 2017).

Some chemotherapeutic agents can modulate the Mitochondrial Permeability Transition Pore (MPTP). The opening of these pores occurs by detection of DNA damage at cell cycle checkpoints, releasing pro-apoptotic factors. Our results support evidence that the exposure of PC-3 cells to the Kf-EtOAc extract triggers the release of pro-apoptotic Cytochrome C from mitochondria to cytosol at 12 h; this event is key for the formation of the apoptosome, since this complex activates Caspase-9 and leads to the activation of Caspase-3, all of these events evidenced in our experiments.

Another mechanism during which Cytochrome C release is enhanced, involves a pro-apoptotic member of the Bcl2 family. Apoptosis regulator Bax, can directly cause the mitochondrial release of Cytochrome C by means of its ability to promote Mitochondrial Outer Membrane Permeabilization (MOMP) (Adams and Cory, 2007; Galluzzi et al., 2012). On the other hand, overexpression of Bcl2 in cells has been reported to prevent the release of Cytochrome C *via* inhibition of Bax (Adams and Cory, 2007). Our results indicate that downregulation of Bcl2 by Kf-EtOAc could benefit Bax expression and consequently, the release of Cytochrome C.

Promising therapeutic targets against prostate cancer as a strategy for promoting apoptosis involve the downregulation of inhibitors of apoptosis such as XIAP and PKCε. The transcendence of the negative regulation of these two proteins by Kf-EtOAc could lead to clinical relevance, in that XIAP triggers resistance to drug treatment in prostate cancer (Devi, 2004), in addition to that PKCε activation transduces multiple signals involving the inhibition of apoptotic pathways and the promotion of cell survival pathways (Wu et al., 2004).

It has been described that two other species of the genus *Kalanchoe* can induce apoptosis; *K. crenata via* ROS production in MCF-7 cells (Kuete et al., 2017), and *K. pinnata* (a fraction rich in Bryophylin A) by an increase in Bax expression, suppression of Bcl2, activation of caspase-3, and cleavage of PARP-1 on HeLa cells (Mahata et al., 2012). However, this is the first time, to our knowledge, that the downregulation of XIAP or PKCε by extract from *Kalanchoe* family has been reported.

Cytochemical demonstration of free DNA strand ends induced by the Kf-EtOAc extract was observed by TUNEL assay as a result of a late event of apoptosis at 12 and 24 h of treatment. Major morphological changes in late apoptotic cells occur when hypersegmented nuclear structures bud from the blebbing cell surface to form apoptotic bodies (Collins et al., 1997). All of these apoptotic bodies were observed only at long exposure times with the Kf-EtOAc extract in PC-3 cells. Experimental evidence from the literature indicates that apoptotic DNA fragmentation is achieved by DNases such as CAD (Caspase-Activated DNase). Since this protein is one of the downstream effectors of Caspase-3, our evidence suggests a caspase-dependent pathway fragmentation (Susin et al., 2000). Inhibition of PARP-1 through proteolytic cleavage was observed by immunoblot from 12 to 24 h; this event clearly increases the susceptibility of cells to DNA damaging agents such as ROS overproduction. Finally, we must mention that inhibition of PARP-1 still remains a promising avenue that continue to explored in the treatment of cancer (Bouchard et al., 2003).

Control of cell cycle progression in cancer cells is considered a potentially effective strategy for control of tumor growth (Schwartz and Shah, 2005). Our *in vitro* data indicated that treatment of PC-3 cells with the Kf-EtOAc extract resulted in significant S-phase arrest of cell cycle progression. This suggests that one of the mechanisms by which the Kf-EtOAc extract may act is through the inhibition of the cell cycle. There is an intra S-phase checkpoint network that is activated during DNA damage and that is controlled by ATM/ATR signaling machinery; this gives rise to the slowdown of the ongoing DNA synthesis (Kastan and Bartek, 2004). Exploration of this pathway, as well as the involvement of proteins related to DNA repair mechanism pathways, would be convenient for further elucidation of the S-Phase cell cycle arrest caused by the Kf-EtOAc extract. It is known that DNA repairs pathways are coupled with apoptosis effectors to ensure that irreparable damage will not be passed down to daughter cells (Norbury and Zhivotovsky, 2004). In advanced hormone-independent prostate cancer, cells become resistant to apoptosis; hence, we found it interesting to investigate the molecular effect of the cell death related to the cytotoxic effect induced by the extract in PC-3 cells. Moreover, as mentioned previously, there is a correlation between molecular events in ROS overproduction and cell cycle progression. As we know, ROS cause DNA damage and it is known that this event could lead to the expression of p21, which contains a carboxyl terminal binding site for Proliferating Cell Nuclear Antigen (PCNA). Additionally, through this interaction, p21 is able to block DNA synthesis (Boonstra and Post, 2004). According to this, p21 evaluation need to be further evaluated to affirm this correlation among these events in Kf-EtOAc-treated PC-3 cells. Other species of the genus *Kalanchoe* have exhibited cell cycle arrest. *K. tubiflora* demonstrated cell cycle arrest in G2/M phase in HeLa and A549 cells (Hsieh et al., 2016, 2012). The extract of *K. crenata* gave rise to the progressive increase of sub-G0/G1 phase in MCF-7 cells (Kuete et al., 2017), and bufadienolides isolated from *K. tubiflora*

blocked the cells in G2/M phase when evaluated in Human promyelocytic Leukemia (HL-60) cells (Huang et al., 2013).

Analysis of the bioactive compounds of the Kf-EtOAc extract conducted by the different chromatography techniques described previously indicated the presence of the mixture of Palmitic Acid (PA) and Coumaric Acid (CA) in one of the most active fractions. This mixture maintained the cytotoxic effect in PC-3 cells, exerted no effect on PrEC cells, and maintained its effect on cell arrest in S phase synthesis. Plant extracts containing CA have exerted a cytotoxic effect by decreasing cell viability and inducing apoptosis through the activation of caspases-3-8-9/PARP-1 in multiple cell lines (N2a, HTC-15, MCF-7, HepG2, Caco2, HTC-116 and CT-26) without damaging healthy phenotype cells, and have also shown cell cycle arrest and anti-proliferative activity (Chen et al., 2016; Deepa et al., 2013; Ghanemi et al., 2017; Shailasree et al., 2014). It has also been observed that CA exerts an effect on the regulation of mRNA in colon cancer cells (Caco-2), where it has been found that the regulation of genes related to proliferation and the cell cycle, such as PCNA, CDC25A, Myc, Cyclin A2, decreases (Janicke et al., 2011). This provides evidence of the effect of the Kf-EtOAc extract and its fractions on cell cycle arrest in the S phase observed in PC-3 cells.

Long-chain fatty acids play an important role in many biological functions, in that they can serve as a source of metabolic energy, as substrates for the biogenesis of cell membranes, or as precursors of many intracellular signaling molecules (Menendez et al., 2004). PA is one of the fatty acids with a high plasma concentration due to diet, and this increase has been associated with a negative impact on metabolic health. However, some evidence suggests that consumption of saturated fat does not increase the risk of cardiovascular diseases (Agostoni et al., 2016). The majority of biological studies indicate that PA is a compound that induces cell death by apoptosis in pancreatic beta cells by inducing different pro-apoptotic stimuli, such as the ROS production (Gehrmann et al., 2010). PA has also demonstrated selective cytotoxicity in human T lymphoblasts (MOLT-4) compared to its effect on normal human dermal fibroblast (HDF) (Harada et al., 2002). The cell death induced by PA was apoptotic with activation of caspase-3–8 and-9 in HepG2 and PC-12 cells (Ulloth et al., 2003; Zhu et al., 2016). It was also found that the concomitant treatment of Cyclosporine A and PA induces synergized expression of caspase-3–7 activity and synergized cytotoxicity (Luo et al., 2012). Finally, It has been reported that PA has an inhibitory effect on topoisomerase I activity in adenocarcinomic human alveolar basal epithelial cells (A549) and causes cell death by autophagy (Karna et al., 2012). This inhibition causes death in cells that are under DNA replication or repair; therefore, this property could be used to inhibit the growth of cancer cells.

Although natural products have beneficial biological activities and they are considered safe, they could have negative effects on health. Therefore, knowledge of the genotoxic and toxicological effects of plants is very important. Thus, we evaluated the genotoxic *in vitro* and *in vivo* potential of the Kf -EtOAc extract by means of the Ames test and the Micronucleus assay. These techniques are those most frequently used and recommended by regulatory agencies such as the FDA. The Kf-EtOAc extract did not produce mutations by displacement in the frameshift, by substitution of base-pairs or by production of ROS and also showed no induction of micronuclei in RETs and NCEs in mouse peripheral blood. *K. pinnata*, another species of the same genus showed that it is not mutagenic when evaluated in strains TA98, TA100, TA1535 and TA1537 in the presence and absence of metabolic activator S9, and also, showed that on the contrary it has antimutagenic activity when evaluated by the method of incorporation against 2AA (Umbuzeiro-Valent et al., 1999); with respect to the Micronucleus assay, this is the first time that the evaluation of some *Kalanchoe* extract is reported in this model.

In addition, the OECD-423 test was conducted to evaluate the acute toxicity of Kf-EtOAc extract. No signs of toxicity or death were reported after 14 days of observation, then, according to the GSH system, the LD_{50} of Kf-EtOAc extract > 5000 mg/kg. The acute toxicity of other species of the genus has been evaluated, finding that *Kalanchoe brasiliensis* Cambess. LD_{50} > 5000 mg/kg, *Kalanchoe laciniata* (L.) DC. LD_{50} > 3000 mg/kg, and *K. crenata* LD_{50} > 2000 < 5000 mg/kg (Fondjo et al., 2012; Iqbal et al., 2016; Mourão et al., 1999). While studies of sub-acute toxicity showed that the extract of *K. pinnata* and *Kalanchoe integra* (Medik.) Kuntze do not produce alterations when administered daily for 14 days at the doses of 1000 mg/kg and 900 mg/kg respectively [26,57]. Therefore, with these toxicity tests, it was verified that it is very unlikely that Kf-EtOAc extract causes negative health effects.

On the other hand, an increase in the trend of the use of herbal remedies, as well as some type of complementary and alternative medicine (CAM) has been documented, especially in cancer patients (Cheng et al., 2010). It is estimated that in the United States 38% of adults and 12% of children use some form of CAM (17.7% are natural products) (Barnes et al., 2008). In a study conducted in 453 patients with cancer, it was found that 77% consume herbs or vitamins along with their conventional treatment (Richardson et al., 2000). Therefore, it is clear that there is a need to study and develop therapies for cancer treatment based on medicinal plants.

Therefore, these data provide evidence of the importance of emphasizing the relevance of the use of extracts, since thousands of phytochemicals are present and those differing in molecular size, polarity and solubility, which may affect the bioavailability and distribution of each phytochemical in different macromolecules, subcellular organelles, cells, organs and tissues to improve their therapeutic effect, besides, it is necessary to find a validation for the pharmacological and therapeutic superiority of many of them in comparison to isolated single constituents, the exploration of synergistic mechanism of herbal ingredients in deregulated pathways of cancer will help researchers to discover both new phytomedicines and drug combinations for the treatment of prostate cancer (Liu, 2004; Wagner and Ulrich-Merzenich, 2009; Yang et al., 2014).

To propose the use of the extract of *K. flammea* in clinical trials for the treatment of cancer, additional studies are required to understand their pharmacokinetic behavior, as well as the development of a suitable pharmaceutical form. Both objectives are being developed in our working group.

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Authors contributions

Arias-González Iván

Ph.D. student in Biotechnology Sciences, practical development of the project.

García-Carrancá Alejandro M.

Establishment of cytotoxic activity by MTT assay.

Cornejo-Garrido Jorge

Obtaining the extract and its fractionation and advice in toxicological tests.

Ordaz-Pichardo Cynthia

Establishment of molecular biology techniques to determine the mechanism of action and general director of the project.

Conflict of interest

Authors declare no conflict of interest.

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