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In vitro cytotoxic potential of extracts from *Aristolochia foetida* Kunth against MCF-7 and bMECs cell lines

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ABSTRACT

The aim of this study was to evaluate the cytotoxic potential of *Aristolochia foetida* Kunth. Stems and leaves of *A. foetida* Kunth (Aristolochiaceae) have never been investigated pharmacologically. Recent studies of species of the Aristolochiaceae family found significant cytotoxic activities. Hexane, dichloromethane, ethyl acetate and methanol extracts were analyzed by ¹H NMR and GC–MS to know the metabolites in each extract. In GC–MS analysis, the main compounds were methyl hexadecanoate (**3**); hexadecanoic acid (**4**); 2-butoxyethyl dodecanoate (**9**); ethyl hexadecanoate (**20**); methyl octadeca-9,12,15-trienoate (**28**) and (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (**40**). The results showed a significant reduction in cell viability of the MCF-7 (breast cancer) cell line caused by organic extracts in a dose-dependent manner. The cytotoxicity activity of the dichloromethane extract from the stems (DSE) showed IC₅₀ values of 45.9 µg/mL and the dichloromethane extract of the leaves (DLE) showed IC₅₀ values of 47.3 µg/mL. DSE and DLE had the highest cytotoxic potential in an *in vitro* study against the MCF-7 cell line and non-tumor cells obtained from the bovine mammary epithelial (bMECs). DSE and DLE induced a loss in mitochondrial membrane potential ($\Delta\Psi_m$) and can cause cell death by apoptosis through the intrinsic pathway in the MCF-7 cell line. DSE and DLE are cytotoxic in cancer cells and cause late apoptosis. Higher concentrations of DSE and DLE are required to induce a cytotoxic effect in healthy mammary epithelial cells. This is the first report of the dichloromethane extract of *A. foetida* Kunth that induces late apoptosis in MCF-7 cancer cells and may be a candidate for pharmacological study against breast cancer.

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Abbreviations: Act-D, Actinomycin D; 7AAD, 7-Aminoactinomycin D; ANOVA, Analysis of variance; bMECs, Bovine mammary epithelial cells; DEL, Dichloromethane extract from leaves; DMEM, Medium/nutrient mixture F-12 Ham; DSE, Dichloromethane extract from stems; EtOH, Ethanol; FBS, Fetal bovine serum; GC–MS, Gas chromatography-mass spectrometry; HLE, Hexane extract from leaves; HSE, Hexane extract from stems; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanineiodide; IM, Incomplete medium; NMR, Nuclear magnetic resonance; SE, Standard error; TMS, Tetramethylsilane.

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1. Introduction

Cancer is one of the world's leading health problems with around 9.5 million deaths. Breast cancer is the most frequently diagnosed cancer with mortality of 626,679 deaths per year (Bray et al., 2018). Breast cancer is an uncontrolled malignant proliferation of epithelial cells in the lobes or the mammary ducts, the latter being the most prevalent according to the World Health Organization (Arpino et al., 2004; Burstein et al., 2004).

Approximately 67% of the drugs used against cancer have their origin and design from a natural product (Newman and Cragg, 2016). It is common for cancer patients to be attracted to the use of plants, plants are more accessible, cost less, and can improve quality of life (Fregene and Newman, 2005). In the constant effort to obtain products that can treat cancer, it is necessary to look for alternatives in different types of plants (Dias et al., 2012). Cancer is a serious public health problem, so the Aristolochiaceae family has

the potential to solve it. Numerous studies report that plant extracts from the Aristolochiaceae family to have biologically potent agents with anticancer properties (Akindele et al., 2015; Ian et al., 2014; Yu et al., 2013). The main components of the Aristolochiaceae family are aristolochic acids and their derivatives, alkaloids, phenolic compounds, steroids, anthraquinones, terpenes (Kuo et al., 2012; Wu et al., 2004). In the chloroformic extract of aerial parts of *Aristolochia baetica*, aristolochic acid I gave an important *in vitro* cytotoxic effect in MCF-7 cells with an IC₅₀ value of 216 µg/mL (Chaouki et al., 2010). The alkaloids, flavonoids, steroids and anthraquinones present in the chloroform extract of aerial parts of *A. ringens* favored a significant cytotoxic effect in MCF-7 with IC₅₀ values of 81.6 µg/mL (Owolabi et al., 2017). Chloroformic extract was obtained from the leaves of *A. indica* and the phenolic compounds had a considerable cytotoxic effect on MCF-7 with an IC₅₀ value of 347.0 µg/mL (Subramaniyan et al., 2015).

To obtain new antitumor agents, we focused on the species *A. foetida* Kunth (Aristolochiaceae). This plant is native to the state of Michoacán, Mexico, and is known as “cigarette” due to its attractive pipe-shaped flowers. In traditional medicine *A. foetida* Kunth is often used to treat colds, chills, fevers, in the treatment of asthma, animal and insect bites, among others (Alonso-Castro et al., 2017; Heinrich et al., 2009; Santana-Michel and Cuevas-Guzmán, 2013). Here, is the first study that investigated the potential for cytotoxic activity of *A. foetida* Kunth stems and leaves analyzed *in vitro* and by ¹H NMR and GC-MS.

2. Materials and methods

2.1. Plant material and preparation of organic extracts

A. foetida Kunth was collected in September 2018 in the locality of Apatzingán in the state of Michoacán, Mexico. The stems and leaves were macerated (3 times, 3 days each extraction), hexane, dichloromethane, ethyl acetate, and methanol were used as solvents successively to obtain the extracts. The organic extracts were filtered and the solvent was removed by evaporation under reduced pressure at 40 °C using a rotary evaporator. The hexane and dichloromethane extracts were dissolved in methanol at 50 °C, kept at 4 °C for 12 h, and filtered to remove fatty materials. The hexane, dichloromethane, ethyl acetate, and methanol extracts were analyzed by ¹H Nuclear Magnetic Resonance (¹H NMR) at 400 MHz. Deuterated chloroform (CDCl₃), deuterated methanol (CD₃OD), and deuterated dimethyl sulfoxide (DMSO-*d*₆) were used as solvents, and tetramethylsilane (TMS) was used as the internal standard. Varian Mercury Plus 400 spectrometer was used for the analysis. The hexane and dichloromethane extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis of the sample was performed using a Thermo Scientific Trace-1310 gas chromatograph-mass spectrometer interfaced with a simple quadrupole mass spectrometer, equipped with a TG-SQC fused silica capillary column (15 X 0.25 mm, with 0.25 µm film thickness). The identification of compounds was based on a comparison of their mass spectra with those of the NIST library.

2.2. Isolated compounds

Hexane, dichloromethane, ethyl acetate, and methanol extracts were purified by open column chromatography with silica gel 60 (230–400 mesh) and mixtures of hexane-ethyl acetate and ethyl acetate-methanol. Eluted fractions were analyzed by thin-layer chromatography, observed under UV light, and dyed with ceric ammonium sulfate.

2.3. Cell lines and primary culture

The MCF-7 human breast tumor cell line was obtained from the American Type Culture Collection. Cells were routinely cultured in DMEM medium/nutrient mixture F-12 Ham (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Corning) and 100 U/mL penicillin and streptomycin (Gibco) and were grown in a 5% CO₂ atmosphere at 37 °C. For maintenance of the MCF-7 cell line, the cells were seeded in 70 mm diameter Petri culture dishes in complete medium. The cells were incubated in a sterile atmosphere at 37 °C and 5% in CO₂. The cells were trypsinized and counted using a hemacytometer once the culture was at 80–100% confluence. The images were obtained under an inverted microscope (Primo Vert, Zeiss). Cells were transferred to the culture medium for proliferation and seeded in 96-well plates for cytotoxicity assays. The cryopreservation of the cells were carried out in liquid nitrogen, with dimethyl sulfoxide (DMSO) at 10% (v/v) and fetal bovine serum (1 × 10⁶ cells/mL).

bMECs were isolated from the alveolar udder tissue of a healthy lactating cow. The bMECs were cultured in medium consisting of DMEM, 10% fetal bovine serum (Corning), 10 µg/mL insulin, 5 mg/mL hydrocortisone, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 µg/mL of amphotericin B (Sigma). Cells from passage 7 were used in all experiments. The cells were cultured in a 5% CO₂ atmosphere at a temperature of 37 °C (Báez-Magaña et al., 2019).

2.4. MTT viability assay

The MTT (tetrazolium salt) test was performed according to the methodology described by Guzmán-Rodríguez et al. (2016). The MCF-7 cell line, as well as bMECs, were cultured at a density of 1 × 10⁴ cells in 96-well plates. The cell count was carried out in an automatic counter (Bio-Rad TC20™) using trypan blue and growth medium for 24 h. After that, it was changed to an incomplete medium (MI) and incubated again for 24 h. The treatments were performed in triplicate using a concentration scan, 0 µg/mL to 509.7 µg/mL for the extracts of stems and leaves of the different macerates. Subsequently, the treatments were placed in MI and left to act for 24 h. Afterward, the treatments were withdrawn and the MI was placed with 10% of MTT (5 mg/mL, Sigma) in phosphate buffer saline (PBS) allowing incubation for 4 h at 37 °C in an atmosphere of 5% CO₂. Once the incubation reached the established time, the medium was removed, the formazan crystals were dissolved in a mixture of isopropanol:HCl 1 M (19:1). The absorbance of the reduced MTT was measured at 595 nm on a spectrophotometer (Bio-Rad iMark™). Cell viability was determined as a percentage of viable cells relative to the ethanol vehicle (EtOH 2%).

2.5. Trypan blue dye exclusion viability assay

The trypan blue dye exclusion viability assay was used to assess cell death accompanied by the MTT assay. The test consisted of applying (1:1) the 0.4% trypan blue dye to cells previously cultured and treated 24 h in 96-well plates. The light microscope analysis allowed us to visualize the damaged cells with their blue membrane, unlike the viable ones, which remained colorless. The cells were counted using a hemocytometer (Guzmán-Rodríguez et al., 2016).

2.6. Hemolytic assay

To analyze the hemolytic activity of DSE and DEL, the method of Rico-Mata et al. (2013) was used. For the present assay, the concentrations of 45.9 µg/mL for DSE and 47.3 µg/mL for DLE were used. Fresh whole human blood was obtained in tubes with

ethylenediaminetetraacetic acid (EDTA, 0.2 mg/mL) which was centrifuged at 1500 rpm for 5 min and washed in triplicate with PBS, later it was resuspended until the initial volume to be considered as 100% of erythrocytes. From the previous solution, a 2% dilution was prepared and the treatments were added in a reaction volume of 200 μ L and incubated at 37 °C for 1 h. After incubation, the samples were centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was measured at 540 nm in a spectrophotometer (Bio-Rad iMark™). The negative control was erythrocytes treated with PBS and the positive control was Triton 1% (Sigma).

2.7. Measurement of the transmembrane potential

In a 96-well plate with black-wall, 1×10^4 cells were seeded per well and incubated at 24 h. Previously incubated, cells were washed twice with Hanks-Hepes buffer and (200 μ M) DiSC₃(5) (3,3'-dipropylthiadiazocarbocyanine iodide, Sigma) per well and incubated for 30 min at 37 °C in CO₂ as described (Guzmán-Rodríguez et al., 2016). Subsequently, 5 baseline fluorescence points were read at 625–670 nm and after reading, the treatments were added and reading was monitored for 2 h using a Varioskan spectrophotometer (Thermo Scientific). Valinomycin (444.52 μ g/mL, Sigma) was used as a positive control for the change in membrane potential.

2.8. Calcium flux assessment

The calcium flux assessment was performed on a black-wall plate previously treated with rat collagen and seeded 1×10^4 cells per well in a 96-well plate. The 1X Signal Enhancer solution was prepared and from this, the 1X Dye solution was made. The cells were incubated for 1 h at 37 °C in CO₂ with a 1X Dye solution. Phorbol 12-myristate 13-acetate (PMA, Sigma) was used as a positive control at a concentration of 1.8 μ g/mL. Treatments and controls were added after the initial reading and read for 5 min at an excitation wavelength of 485 nm and an emission of 525 nm on a fluorometer. The methodology was modified from Flores-Alvarez et al. (2018).

2.9. Apoptosis rate determined by flow cytometry

The apoptosis and necrosis test were performed in a similar way to that reported by Guerra et al. (2019). In the apoptosis and necrosis assay, MCF-7 cells (4×10^4) were cultured per well in 24-wells incubated for 24 h. After the cells were trypsinized and washed with PBS, they were treated with Annexin V and 7AAD according to the manufacturer's instructions (Annexin V, Alexa Fluor® 488 conjugate, Invitrogen). For this test, a buffer for the fluorophores and treatments (Hepes 1 M, NaCl 1 M, and CaCl₂ 1 M) was prepared and the pH was adjusted to 7.4. After preparing the buffer, the fluorophores were prepared. The apoptosis and necrosis assay was analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.). Actinomycin D (Cayman Chemical Company, 627 710 μ g/mL) was used as a positive control for apoptosis. EDTA (0.14 μ g/mL) was the necrosis control. 10,000 events were counted in the analysis.

2.10. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)

To analyze the mitochondrial membrane potential, the method of Lara-Márquez et al. (2020) was used. The effect of DSE and DEL on the $\Delta\Psi_m$ of MCF-7 cells (4×10^4 cells/well) was evaluated. A 10X buffer was prepared in deionized water with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanineiodide) (BD Biosciences). The working solution (1:100) of the JC-1 stock in 10X buffer was previously tempered at 37 °C. Concerning the

above, JC-1 working solution was added to the Eppendorf tubes with the cells, and treatments were incubated at 37 °C for 15 min. Then 1X buffer was added and centrifuged at 2500 rpm for 10 min. Afterward, the samples were resuspended in 1X buffer, this was repeated twice and then read on the flow cytometer.

2.11. Statistical analysis

The data were obtained independently in triplicate each, an analysis of variance (ANOVA) was performed using a Tukey test with a significance level of $p < 0.05$. The concentration required to inhibit 50% of cell viability (IC₅₀) and the mean \pm standard error (SE) were calculated using a linear regression curve. Statistical analysis was performed using GraphPad Prism software (version 8.0.2) to determine significant differences.

3. Results

3.1. Organic extracts and isolated compounds

Before *in vitro* cytotoxic evaluations, the organic extracts were subjected to ¹H NMR and GC-MS. In DSE and DLE, β -sitosterol and stigmasterol were identified in a ratio of 7:1 in ¹H NMR analysis, and their identification was corroborated by comparison with published reference data (Luhata and Munkombwe, 2015). From the hexane extract from leaves (HLE), hexane extract from stems (HSE), DSE and DLE, have been identified after the comparison of mass spectra with the NIST library. From the results, it was observed that methyl hexadecanoate (**3**), hexadecanoic acid (**4**), 2-butoxyethyl dodecanoate (**9**), ethyl hexadecanoate (**20**), methyl octadeca-9,12,15-trienoate (**28**) and (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (**40**) were the main components (Supplementary Material).

On the other hand, aristolochic acids and their derivatives, common compounds in species of the genus *Aristolochia*, have not been identified by ¹H NMR and GC-MS analysis (Supplementary Material).

3.2. Dichloromethane extract is cytotoxic to MCF-7 cells

The hexane, dichloromethane, ethyl acetate, and methanol extracts were evaluated *in vitro* for their cytotoxicity against MCF-7 and their selectivity against bMECs. DSE and DLE showed significant differences concerning the rest of the extracts evaluated (Table 1). DSE and DLE were used for additional *in vitro* assays.

The cytotoxicity of DSE and DLE was analyzed in the MCF-7 cell line 24 h after treatment. DSE with IC₅₀ values of 45.9 μ g/mL (Fig. 1A) and DLE with IC₅₀ values of 47.3 μ g/mL (Fig. 1B). The cytotoxic activity of DSE and DLE was similar to that reported by Mongelli et al. (2000) in an extract of the aerial parts of dichloromethane in *A. triangularis* evaluated *in vitro* in KB cells with IC₅₀ values of 47 μ g/mL.

Erythrocytes integrity was performed according to the methodology used by Rico-Mata et al. (2013). DSE and DLE showed less than 10% toxicity to human erythrocytes (Fig. 1C). In MCF-7 the morphology after the treatments showed the appearance of a death mode due to apoptosis with loss of adherence and rounded cells (Fig. 1D). In bMECs, toxicity was observed at higher concentrations than in MCF-7 cells, an IC₅₀ of 147.7 μ g/mL in DSE (Fig. 1E) followed by DLE (292.8 μ g/mL) (Fig. 1F).

Table 1
IC₅₀ values of the stems and leaves extracts of *A. foetida* Kunth at 24 h in MCF-7 cell line.

	µg/mL			
	Hexane	CH ₂ Cl ₂	AcOEt	Methanol
Stems	116.0 ± 1.6	45.9 ± 0.1*	>300.0 ± 2.0	162.1 ± 1.7
Leaves	60.0 ± 2.5	47.3 ± 0.2*	126.8 ± 0.5	>300.0 ± 1.5

*Shows significant differences between the stems and leaves extracts, Tukey (*p* < 0.05).

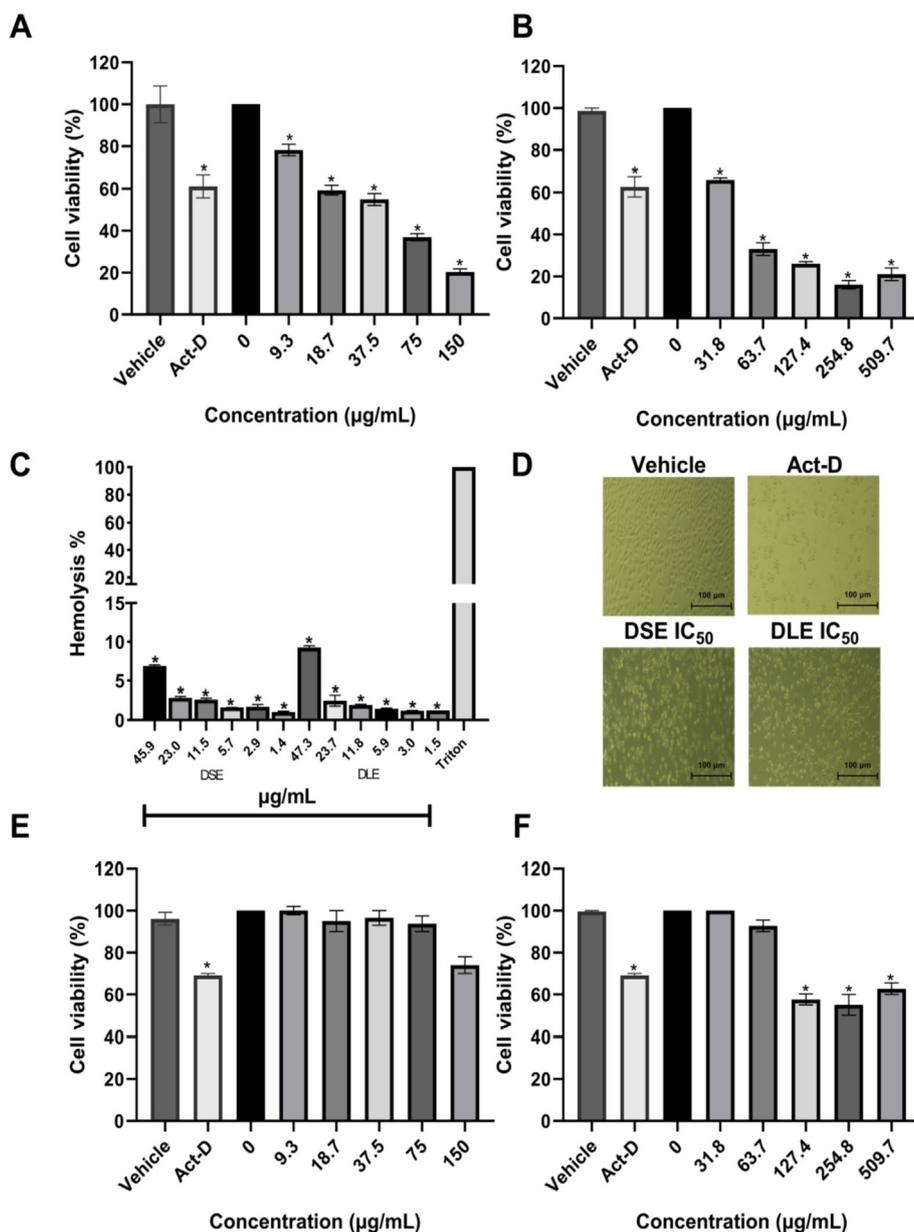


Fig. 1. Cytotoxicity induced in MCF-7 by DSE and DLE. (A) Cytotoxicity of DSE in MCF-7. The half-maximal inhibitory concentration (IC₅₀) of DSE on MCF-7 cells; IC₅₀ = 45.9 µg/mL; R² = 0.956. (B) Effect of DLE on the viability of MCF-7. The half-maximal inhibitory concentration of DEL on MCF-7 cells; 47.3 µg/mL; R² = 0.983. Data are shown as the percentage of viable cells concerning cells treated with vehicle (EtOH 2%). (C) Effect of IC₅₀ of DSE and DLE on the integrity of human erythrocytes after the interaction. The hemoglobin released was determined by measuring the absorbance at 540 nm. The erythrocyte integrity was expressed as the percentage of hemoglobin released concerning the total hemoglobin obtained by incubation of the sample with 1% Triton X-100. (D) MCF-7 cell morphology after different treatments. Representative photographs of at least three independent experiments and were taken by inverted microscope. Scale bars: 100 µm. (E) The half-maximal inhibitory concentration of DSE on bMECs; IC₅₀ = 147.7 µg/mL; R² = 0.753. (F) The half-maximal inhibitory concentration of DLE on bMECs; IC₅₀ = 292.8 µg/mL; R² = 0.624. Each bar shows the mean of triplicates ± SE of three independent experiments. *Indicates statistically significant differences concerning the vehicle. (One-way ANOVA and Tukey's pairwise comparison, *p* < 0.05).

3.3. Dichloromethane extract does not affect the membrane integrity and calcium efflux of MCF-7 cells

In the membrane potential test to determine calcium flux, valinomycin (444.5 $\mu\text{g}/\text{mL}$) was used as a positive control which showed a significant change in cell membrane potential. Meanwhile, vehicle, DSE, and DLE did not show an increase in fluorescence intensity in MCF-7 (Fig. 2A). In the intracellular calcium flux test, it was found that the MCF-7 cells in the presence of the treatments did not present significant differences with the cells treated with the vehicle. This result may explain that calcium release is not related to cell membrane damage. In particular, we observed specific differences concerning the positive control (PMA) for 3.7 min with the change in an increase in fluorescence (Fig. 2B).

3.4. Dichloromethane extract induces apoptosis in MCF-7 cells

Previously determined the cytotoxic effect of DSE and DLE from *A. foetida* Kunth, the type of cell death in MCF-7 cells was analyzed by flow cytometry. The test was performed after 24 h of treatment and actinomycin D was used as a positive control for apoptosis and EDTA as a control for necrosis. Annexin V and 7AAD fluorophores were also used in the assay. The IC_{50} of actinomycin D was 12554.2 $\mu\text{g}/\text{mL}$ and the majority of the population was determined in early apoptosis. EDTA (0.14 $\mu\text{g}/\text{mL}$) showed a part of the cell population in a state of necrosis. A higher concentration of the population treated with IC_{50} values of DSE showed late apoptosis 91.2% at 24 h, as well as the MCF-7 cells treated with DLE (77.0%) (Fig. 3).

3.5. Cytotoxicity of dichloromethane in MCF-7 cells implies intrinsic apoptosis

After determining the apoptotic effect of DSE and DEL in MCF-7 cells, a flow cytometric assay was performed to determine whether the functionality of the mitochondria was affected. In the mitochondrial membrane potential assay, the JC-1 fluorophore was used in such a way that the depolarization of the mitochondrial membrane was positive with the actinomycin D control (22.6%), as well as in DSE (79.0%) and DLE (87.2%). Therefore, cell death may be by the intrinsic pathway of apoptosis (Fig. 4).

4. Discussion

Aristolochia species are often used as herbal medicines in different parts of the world (Grollman and Marcus, 2016). Aristolochic acids are present in several species of the Aristolochiaceae family. The presence of aristolochic acids and their derivatives has caused controversy due to their nephrotoxic and carcinogenic effects (Zhou et al., 2019). However, these phenanthrenic compounds show significant cytotoxic effects with IC_{50} values of 216 $\mu\text{g}/\text{mL}$ in the chloroform extract of aerial parts of *A. baetica* (Chaouki et al., 2010). The hexane, dichloromethane, ethyl acetate, and methanol extracts of *A. foetida* Kunth analyzed by ^1H NMR in $\text{DMSO}-d_6$ have not been found in the presence of signals of aristolochic acids and their derivatives. In GC-MS, aristolochic acids or their derivatives were not identified in hexane and dichloromethane extracts from stems and leaves. However, by GC-MS compounds **3**, **4**, **9**, **20**, **28** and **40** were the main components. In the dichloromethane extract of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* it exhibited the strongest cytotoxicity on SW-620 colorectal cancer cells ($\text{IC}_{50} = 7.49 \mu\text{g}/\text{mL}$), MCF-7 cell line ($\text{IC}_{50} = 13.35 \mu\text{g}/\text{mL}$) and A2780 ovarian cancer cells ($\text{IC}_{50} = 15.58 \mu\text{g}/\text{mL}$), respectively. The gas chromatographic-mass spectrometry study of the dichloromethane extract of the aforementioned species identified major compounds including 1-heptatriacotanol and hexadecanoic acid (Faisal et al., 2021).

DSE and DLE showed the best cytotoxic effect against MCF-7, whose ^1H NMR spectra in $\text{DMSO}-d_6$ allowed the identification and isolated to the mixture of triterpenes β -sitosterol and stigmasterol. In the same way, in the *Synadenium glaucescens* species, euphol and β -sitosterol were identified in the dichloromethane extract of roots and leaves (Nyigo et al., 2016). Likewise, our study coincides with some components found in the dichloromethane extract of aerial parts of *Satureja khuzistanica* (Lamiaceae). The compounds were identified β -sitosterol, β -sitosterol-3-O- β -D-glucopyranoside, ursolic acid and 4',5,6-trihydroxy-3,7-dimethoxy flavone (Moghaddam et al., 2007). The presence of β -sitosterol and phytol could influence the decrease in IC_{50} values in the MCF-7 cell line (Rahman et al., 2018).

DSE and DLE showed cytotoxicity in MCF-7 with an IC_{50} values of 45.9 $\mu\text{g}/\text{mL}$ and IC_{50} values of 47.3 $\mu\text{g}/\text{mL}$, respectively. The data obtained correspond to IC_{50} values less than 100 $\mu\text{g}/\text{mL}$ suggested to have relevance and selectivity in cytotoxicity assays (Cos et al., 2006). DSE and DLE were lower than the IC_{50} values of 347 $\mu\text{g}/\text{mL}$ of the chloroform extract from *A. indica* leaves against MCF-7 cell line (Subramaniyan et al., 2015). Also, DSE and DLE showed low IC_{50} values compared to an *in vitro* study of chloroform extract from *A. baetica* roots, with IC_{50} values of 216.0 $\mu\text{g}/\text{mL}$ in MCF-7 cell line (Chaouki et al., 2010). In the current study, the anticancer activity may be due to the induction of late intrinsic apoptosis of DSE and DLE in the MCF-7 cell line.

The cytotoxicity of DSE and DLE does not affect the membrane integrity of MCF-7 cells because the membrane potential and intra-

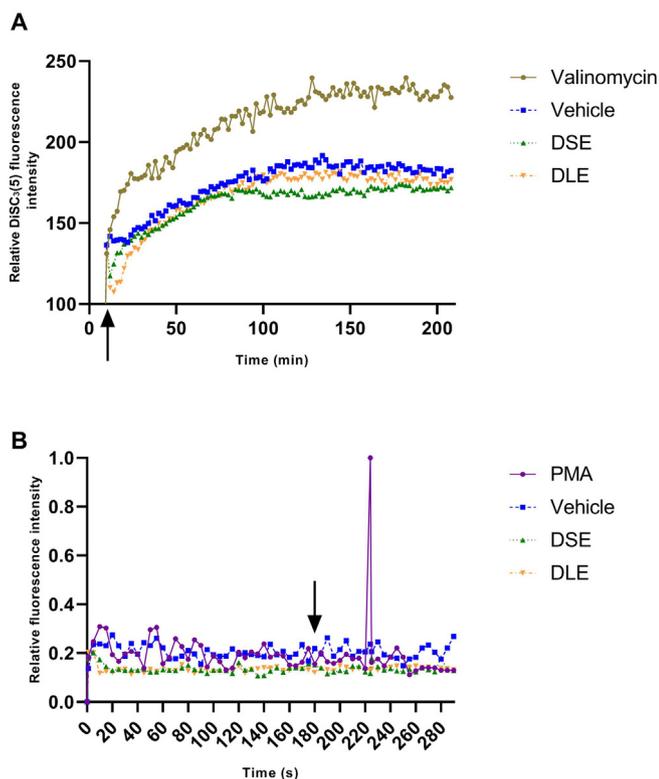


Fig. 2. DSE and DLE do not affect the membrane integrity of MCF-7 cells (A) Changes in the membrane potential of MCF-7 cells were measured using a membrane potential-sensitive dye. Cells were previously incubated with the dye DiSC₃(5) (200 μM) for 30 min at 37 °C and then treated with DSE and DLE IC_{50} . Valinomycin (444.5 $\mu\text{g}/\text{mL}$) was used as a positive control. (B) Relative fluorescence intensities for intracellular calcium release. Measurements were performed for 5 min. PMA (1.8 $\mu\text{g}/\text{mL}$) was used as a positive control. Arrows indicate the time at which the treatments were added.

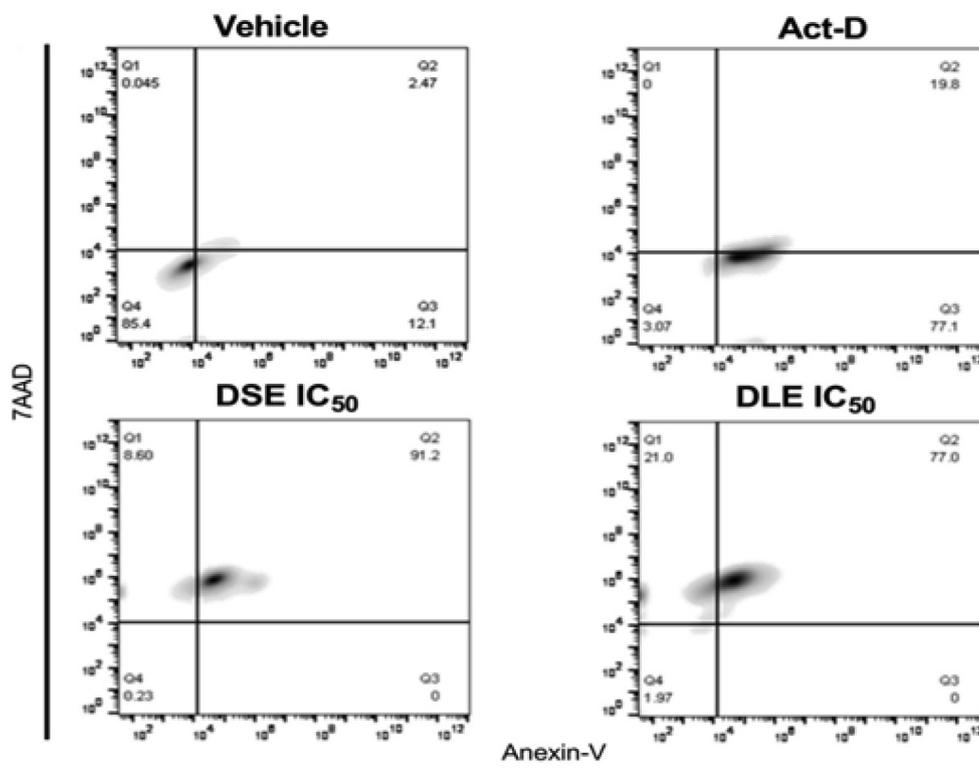


Fig. 3. DSE and DLE induce apoptosis in MCF-7 cells. Cells were treated with vehicle (EtOH 2%), Act-D (Actinomycin D, 12554.2 µg/mL), and DSE and DLE IC₅₀ for 24 h. Cells were analyzed by flow cytometry using Annexin V, 7AAD staining to establish the apoptotic rate. The quadrants indicate viable cells (lower left quadrant), early apoptosis (lower right quadrant), late apoptosis (upper right quadrant), and necrotic cells (upper left quadrant).

cellular calcium flux were not affected by the formation of pores that lyse the cells. These data coincide with those obtained with avocado defensins (*Persea americana* var. *drymifolia*) in MCF-7 cells (Guzmán-Rodríguez et al., 2016). It can be assumed that the cytotoxicity mechanism of the extracts in MCF-7 cells may be involved in another type of pathway.

Apoptosis in this type of extract is related to cytotoxic mechanisms induced by suppressor tumors (p53), transcription factors (c-myc) and the caspase pathway (Özdemir et al., 2017; Valiyari et al., 2013). Apoptosis by caspases is classified into the extrinsic pathway (mediated by transmembrane receptors and caspase 8 activity) and the intrinsic pathway (mediated in the mitochondria and activated by caspase 9) (Flores-Alvarez et al., 2018). Programmed cell death (apoptosis) is a necessary mechanism for cell growth. The relationship between Annexin V, 7AAD, or propidium iodide in cells allows differentiating states of late apoptosis and necrosis (Eboji et al., 2017). Our results show that DSE and DLE induced a higher proportion of late apoptosis in MCF-7 cells after 24 h. The aristolochic acid derivatives of the methanolic extract of *A. elegans* (root), *A. clematitis* (seed), and *A. acuminata* (root) were cytotoxic in human kidney cells (HK-2) and the mechanism of cell death was attributed to G2/M phase arrest and apoptosis (Michl et al., 2016). Induction of apoptosis has also been reported in dichloromethane extract of species such as *Scrophularia oxypsepala* and in the same way in the organic extract of *Cyclotrichium niveum*, the type of cell death was determined in apoptosis and to a lesser extent in necrosis. Therefore, the study obtained evidence of cytotoxicity in MCF-7 cells with DSE and DLE, for which it can promote apoptosis by the intrinsic pathway due to the change in the mitochondrial membrane potential ($\Delta\Psi_m$) that these cells presented. The evaluation of DSE and DLE of *A. foetida* Kunth against MCF-7 and bMECs shows agreement with the data obtained by Benarba et al. (2017) for aqueous extract of *A. longa*, the authors attribute

the type of cell death to the modification of the mitochondrial membrane potential and the activation of caspase 9 in BL41 lymphoma cells.

The DSE showed IC₅₀ values of 292.8 µg/mL and DLE IC₅₀ values of 147.7 µg/mL higher for bMECs. Furthermore, the DSE and DLE had less than 10% toxicity to human erythrocytes. Cancer cells have been established to be more susceptible to oxidative stress conditions in mitochondria than normal cells (Liu and Wang, 2015; Marchi et al., 2012). Extracts of *A. foetida* Kunth are attractive for evaluating their effects on cancer cells; however, more studies are required to demonstrate this.

5. Conclusions

DSE and DLE from *A. foetida* Kunth are cytotoxic against MCF-7 causing late apoptosis by the intrinsic pathway. This effect was selective against cancer cells because higher concentrations are required to show a cytotoxic effect in primary non-tumor culture (bMECs). Regarding the results obtained in the MCF-7 cell line, the cytotoxicity of the plant extracts towards another cancer cell line requires investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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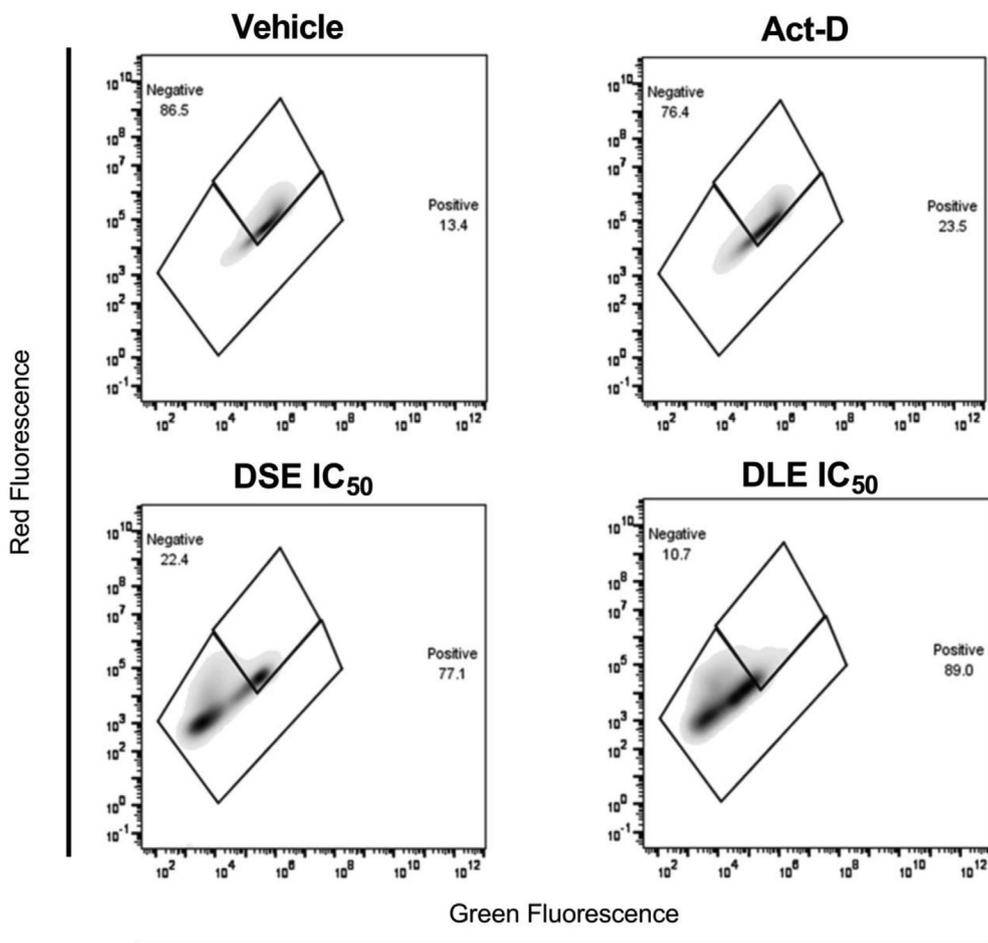


Fig. 4. DSE and DLE induce loss of mitochondrial membrane potential ($\Delta\Psi_m$) in MCF-7 cells. Cells were analyzed by flow cytometry using the JC-1 dye after adding the treatments: Vehicle (EtOH 2%), Act-D (Actinomycin D, 12554.2 $\mu\text{g}/\text{mL}$), and DSE and DLE IC₅₀ for 24 h.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2021.08.007>.

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