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Apoptotic activities of cardenolide glycosides from *Asclepias subulata*

L.A. Rascón-Valenzuela [a], C. Velázquez [b,*], A. Garibay-Escobar [b], W. Vilegas [c], L.A. Medina-Juárez [a], N. Gámez-Meza [a], R.E. Robles-Zepeda [b,*]

[a] Departamento de Investigaciones Científicas y Tecnológicas de la Universidad de Sonora, División de Ciencias Biológicas y de la Salud, Universidad de Sonora, Blvd. Colosio s/n, entre Sahuaripa y Reforma Colonia Centro, C.P. 83000 Hermosillo, Sonora, México
[b] Departamento de Ciencias Químico Biológicas, División de Ciencias Biológicas y de la Salud, Universidad de Sonora, Encinas y Rosales Hermosillo, Sonora, México
[c] UNESP - São Paulo State University – Institute of Biosciences, Coastal Campus of São Vicente, Brasil

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ABSTRACT

Ethnopharmacological relevance: *Asclepias subulata* Decne. (Apocynaceae) is a shrub occurring in Sonora-Arizona desert. The ethnic groups of Sonora, Mexico, Seris and Pimas, use this plant for the treatment of sore eyes, gastrointestinal disorders and cancer.
Aim of the study: To determine the cell death pathways that the cardenolide glycosides with anti-proliferative activity found in the methanol extract of *A. subulata* are able to activate.
Materials and methods: The effect of cardenolide glycosides isolated of *A. subulata* on induction of apoptosis in cancer cells was evaluated through the measuring of several key events of apoptosis. A549 cells were treated for 12 h with doses of 3.0, 0.2, 3.0 and 1.0 μM of 12, 16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, respectively. Apoptotic and necrotic cell levels were measured by double staining with annexin V-FITC/PI. Mitochondrial membrane depolarization was examined through JC-1 staining. Apoptosis cell death and the apoptosis pathways activated by cardenolide glycosides isolated of *A. subulata* were further characterized by the measurement of caspase-3, caspase-8 and caspase-9 activity.
Results: Apoptotic assays showed that the four cardenolide glycosides isolated of *A. subulata* induced apoptosis in A549 cells, which was evidencing by phosphatidylserine externalization in 18.2%, 17.0%, 23.9% and 22.0% for 12, 16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, respectively, compared with 4.6% of control cells. Cell death was also associated with a decrease in mitochondrial membrane potential, which was more than 75% in the treated cultures respect to control. The activation of caspase-3 was observed in all cardenolide glycosides-treated cancer cells indicating the caspase-dependent apoptosis of A549 cells. Extrinsic and intrinsic apoptosis pathways were activated by cardenolide glycosides treatment at the doses tested.
Conclusion: In this study was found that cardenolide glycosides, 12, 16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, isolated from *A. subulata* induced the cell death trough caspase-dependent apoptosis, which was activated, preferably, by extrinsic pathway.

1. Introduction

Cancer remains a major health problem in the world, accounting for more than 15% of human death. WHO estimated that about 27 million of new cases of cancer are expected in the next 20 years (Nath et al., 2014).

Cancer is a multifactorial process which is the result of a succession of genetic changes during which a normal cell is transformed into a malignant one; a loss of balance between cell division and cell death is characteristic in the development of cancer cell; evasion of cell death is one of essential changes that cause the malignant transformation; since cells that should have died did not do so (Wong, 2011).

Apoptosis program is complex and involve many pathways. Defects can occur at any point along these pathways, resulting in the development of cancer cells (Huerta et al., 2007). Thus, reduced apoptosis or its resistance, are crucial in carcinogenesis (Reed, 1999). However, apoptosis is very important in a cancer treatment. Since apoptotic programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential drugs targets (Wong, 2011).

* Corresponding authors.
E-mail addresses: velaz@guayacan.uson.mx (C. Velázquez), rrobles@guayacan.uson.mx (R.E. Robles-Zepeda).

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Therefore, apoptosis is both part of the problem and the solution.

Recently, compounds derived of plants have attracted a growing attention as anticancer agents due to their ability to modulate apoptosis signaling pathways (Millimouno et al., 2014). In this perspective, Mexico has about 4000 species of plants used in traditional medicine which represent a new field for the pharmaceutical research focused on to discover potential compounds with anticancer activity, since different herbal formulations from Mexican ethnopharmacopeia have been applied empirically for the treatment of cancer for many years (Ocegueda et al., 2005 Jachak and Saklani, 2007; Millimouno et al., 2014).

Asclepias subulata Decne. (1844) (Apocynaceae) is a shrub occurring in west of Arizona, in the United States of America, and northern of Baja California and Sonora, in Mexico. Since ancient times Sonoran ethnic groups, Seris and Pimas, have used different parts of this plant to treat cancer (Peinado et al., 1995; Wilder et al., 2008; CONABIO, 2009).

In order to provide scientific validation of traditional medicinal use of *A. subulata*, prior study evaluated the antiproliferative activity of methanol extract of the plant and its solvent fractions (hexane, ethyl acetate, ethanol and residual) demonstrating that methanol extract of *A. subulata* and its polar solvent fractions have a strong antiproliferative activity in a number of human cancer cell lines (A549, PC-3, HeLa and LS 180) with IC_{50} values in the range of 0.31–18 μg/mL. In addition, that study revealed that chromatographic fractions of methanol extract of the plant induced the cell death by apoptosis (Rascón-Valenzuela et al., 2015a).

Recently, a bioguided fractionation was performed for purposes of obtaining the metabolites with antiproliferative activity present in the methanol extract of *A. subulata*, resulting in the isolation of one new cardenolide glycoside, 12,16-dihydroxycalotropin, and three known cardenolide glycosides, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin (Rascón-Valenzuela et al., 2015b; Fig. 1). These compounds showed significant antiproliferative activity on human cancer cell lines (IC_{50} values in nM range), and they also were selective to cancer cell lines (Rascón-Valenzuela et al., 2015b). However, a limited number of scientific reports have been focused to elucidate the cell death mechanism induced by the cardenolide glycosides with antiproliferative activity founded in the methanol extract of *A. subulata* (Park et al., 2014). Therefore, in the present study, we determined the cell death pathways induced by the cardenolide glycosides with antiproliferative activity founded in the methanol extract of *A. subulata*.

2. Materials and methods

2.1. Chemicals

Water was purified by Milli-Q instrument (Millipore, Bedford, MA, USA). Dulbecco's Modified Eagle's Medium High Glucose, L-glutamine solution 200 mM (PubChem CID:24895310), L-arginine monohydrochloride (PubChem CID:87640969), L-asparagine (PubChem CID:24890831), sodium pyruvate solution100 mM (PubChem CID:24899804), penicillin-streptomycin solution (PubChem CID:86591708), doxorubicin hydrochloride (PubChem CID:31703), dimethylsulfoxide (DMSO) (PubChem CID:679), trypsin-EDTA solution 0.25% (PubChem CID:64965), JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (PubChem CID: 5492929), propidium iodide (PubChem CID: 4939) and annexin V-FITC, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco Life Technologies (Grand Island, NY, USA). Commercial kits for caspase activation measurement, Fluorescein Active Caspase 3, Fluorescein Active Caspase 8 and Fluorescein Active Caspase 9, were obtained of Abcam (Cambridge, MA, USA).

2.2. Purification of cardenolide glycosides from the methanol extract of A. subulata

The stems and flowers of *Asclepias subulata* Decne. (Apocynaceae) were collected at Hermosillo, Sonora, Mexico (29°8′43.25″N, 110°57′10.15″O) in September 2011. The plant was authenticated by Professor José Jesús Sánchez Escalante. A voucher specimen (No 17,403) has been deposited in the Herbarium of the University of Sonora. The scientific plant name was checked with The Plant List database (www.theplantlist.org). All plant materials were air dried in the shade at room temperature. The methanol extract was obtained by maceration of dried powder of aerial parts of *A. subulata* with methanol. Cardenolide glycosides 12, 16-

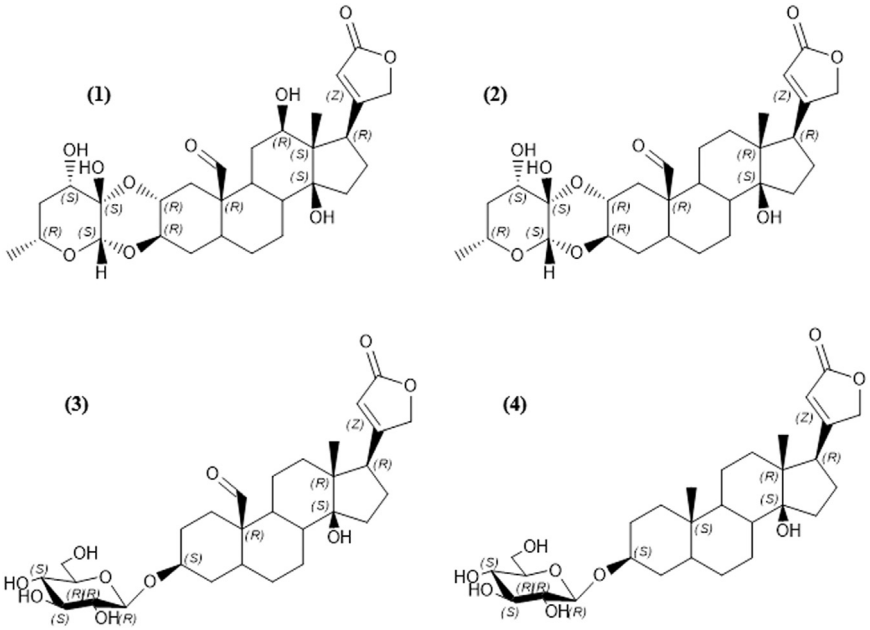

Fig. 1. Cardenolide glycosides isolated of *A. subulata*. **1**. 12,16- dihydroxycalotropin, **2**. Calotropin, **3**.Corotoxigenin 3-*O*-glucopyranoside, **4**. Desglucouzarin.

dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin were purified as previously described from aerial parts of *A. subulata* (Rascón-Valenzuela et al., 2015b). ^{1}H-NMR, ^{13}C-NMR, correlation experiments and ESI-IT-MS/MS spectral data analyses established the structures of the compounds.

2.3. Cell culture

Human alveolar adenocarcinoma A549 cells (ATCC number: CCL-185) were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cell line was maintained using DMEM high glucose growth medium supplemented with 5% of fetal bovine serum, 1% (v/v) penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin/mL), 0.75% (v/v) L-glutamine 200 mM solution and 1% (v/v) sodium pyruvate 100 mM solution.

Cells were grown in 25 cm^2 flask and were incubated at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged with fresh medium when they reached confluence and trypsin-EDTA solution (0.25%) was used for detach the cells.

2.4. Flow cytometric detection of phosphatidylserine externalization

For assaying the effect of cardenolide glycosides isolated of *A. subulata* on induction of apoptosis in A549 cells the externalization of phosphatidylserine in the cell membrane was analyzed by double staining with annexin V-FITC and PI using flow cytometry. Briefly, A549 cells (4.5×10^5) were seeded in 12 well plates and allowed to attach 24 h. The cells were treated with 12,16-dihydroxycalotropin (3 μM), calotropin (0.2 μM), corotoxigenin 3-*O*-glucopyranoside (3 μM) and desglucouzarin (1 μM) solutions for 12 h. Caffeic acid phenethyl ester (CAPE, 120 μM) was used as positive control. After an incubation period, cells were removed from the plates by trypsinisation and were transferred into 5 mL round-bottom polystyrene tubes. Cells were washed twice in cold PBS (200 × *g*, 4 °C, 7 min) and the pellet was resuspended in 50 μL of annexin V-FITC- binding buffer (1 μg/mL final concentration) allowing to incubate for 10 min at room temperature in the dark. Subsequently, propidium iodide staining solution (0.5 μg/mL final concentration) was added and the cells were incubated under the mentioned conditions. After of this period of time, the cells were washed twice and resuspended in cold PBS. The samples were immediately measured by FACS Canto II flow cytometer (BD Systems, San Jose, CA, USA).

Results were analyzed by FACS DIVA 6.0 software (BD Systems, San Jose, CA, USA). Quadrant dot plot was introduced to identify necrotic cells, cells in early apoptosis or cells in late apoptosis. Thus, necrotic cells expressed only PI positive, while cells in early apoptosis were identified as annexin V-FITC positive and cells in late apoptosis were recognized as double positive for annexin V-FITC and PI. Cells in each quadrant were expressed as percentage of the total number of stained cells counted.

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential was assessed using the fluorescent dye JC-1 (5, 5′,6, 6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide). For this assay, A549 cells (4.5×10^5 per well, 12 well plates) were treated with solutions of 12, 16-dihydroxycalotropin (3 μM), calotropin (0.2 μM), corotoxigenin 3-*O*-glucopyranoside (3 μM) and desglucouzarin (1 μM) for 12 h. Caffeic acid phenethyl ester (CAPE, 120 μM) and doxorubicin were used as positive controls. After trypsinisation and PBS washing, cells were incubated for 15 min in freshly prepared JC-1 solution (5 μg/mL in culture medium) at 37 °C in the dark. Cells were washed twice with PBS to remove the spare dye and the pellet was resuspended in fresh culture medium. Cells were, then, analyzed by flow cytometry as soon as possible. Flow cytometry was performed on FACS Canto II flow cytometer.

The red/green fluorescence intensity ratio was used as an indicator of membrane mitochondrial potential. In non-apoptotic cells, JC-1 exists as a monomer in the cytosol (green fluorescence) and also accumulates as aggregates in the mitochondria (red fluorescence). In apoptotic and necrotic cells, when the mitochondrial potential collapses, the JC-1 which cannot accumulate within the mitochondria remains in the cytoplasm as monomeric form increasing the green fluorescence intensity.

2.6. Caspase activity assay

Caspases play a principal role in the molecular mechanism of cell apoptosis. In order to investigate whether cardenolide glycosides isolated of *A. subulata* were able to activate the caspase dependent apoptosis and if so, to determinate the apoptotic pathway that the compounds can trigger, the activity of caspase-8 (initiator caspase of extrinsic pathway), caspase-9 (initiator caspase of intrinsic pathway) and caspase-3 (executioner caspase of both pathways) was measured using the commercial kits Fluorescein Active Caspase 3, Fluorescein Active Caspase 8 and Fluorescein Active Caspase 9. After treatment for 12 h with the cardenolide glycosides, the cells were detached and washed twice with cold PBS (200 × *g*, 4 °C, 7 min). Then, the pellet was resuspended in 300 μL of culture medium (DMEM 5% FBS) containing 1 μL of caspase inhibitor-FITC (FITC-DEVD-FMK) and was incubated for 1 h at 37 °C in the dark. Subsequently, the cells were washed twice and resuspended in wash buffer. The samples were immediately measured in FACS Canto II flow cytometer. The results were represented as the fold change in the activity of caspase-3, caspase-8 and caspase-9 compared to the cells treated with only vehicle (DMSO control).

2.7. Statistical analysis

All experiments were performed at least three times, in triplicate. The data were expressed as the mean ± standard deviation (GraphPad Prism 5, GraphPad Software, Inc., CA, USA). Statistical differences between groups of data were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test (Sigma Stat 3; Systat Software Inc., CA, USA).

3. Results

3.1. Effect of cardenolide glycosides isolated of A. subulata on apoptosis in A549 cells

Apoptosis has become a fundamental factor in the research of new compounds with antiproliferative activity due to apoptosis causes an organized cell death, avoiding the release of the intracellular content into surrounding tissue.

Previously, a study showed the cardenolide glycosides isolated of *A. subulata*, 12, 16-dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin had a strong antiproliferative activity and selectivity to human cancer cell lines A549, LS 180 and PC-3. The antiproliferative effects of 12,16-dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin were stronger in A549 cells with respect to the other human cancer cell tested, showed IC_{50} values of 2.48 ± 1.13, 0.013 ± 0.002, 2.64 ± 0.3 and 0.90 ± 0.02 μM, respectively (Rascón-Valenzuela et al., 2015b).

Thus, to determine whether the decrease in the cell viability was due to apoptosis, A549 cells were stained using annexin V-FITC/PI and analyzed by flow cytometry. As demonstrated the

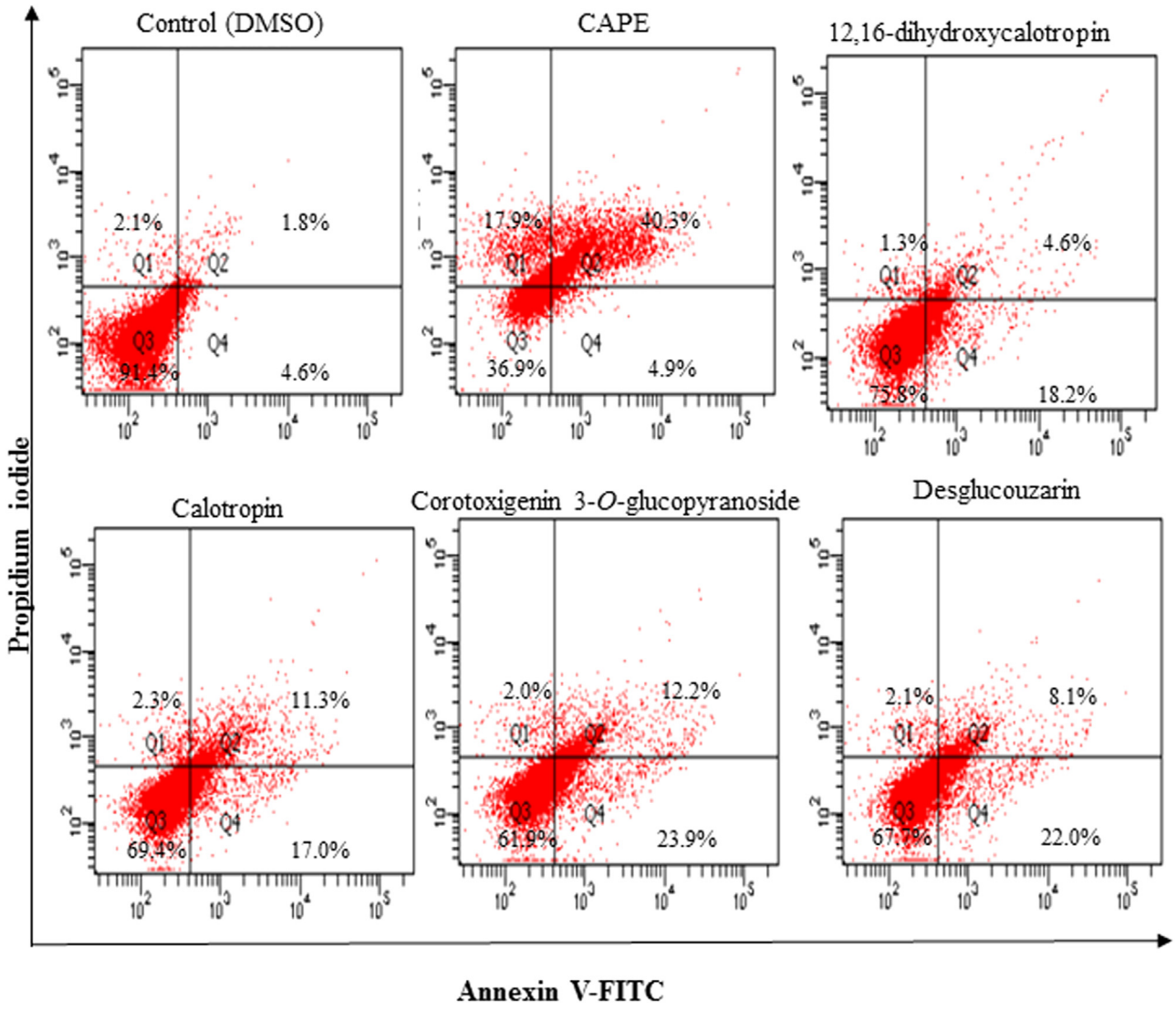

Fig. 2. Effect of compounds **1–4** isolated of *A. subulata* on phosphatidylserine externalization in A549 cells. Flow cytometric fluorescence patterns of annexin V-FITC/PI staining. Data are representative of least three independent experiments.

Figs. 2 and 3 the proportion of annexin V-stained cells increased significantly in all cell cultures treated with isolated compounds, from 4.6% from normal growth control to 18.2%, 17.0%, 23.9% and 22% for 12,16-dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, respectively. These data evidenced that early apoptosis events taking place in response to the treatment with the isolated cardenolide glycosides. Moreover, compared to control culture, the percent of double staining-cells (annexin V-FITC (+)/PI (+)) in treated cultures also increased from 1.8% to 4.6%, 11.3%, 12.2% and 8.1% for 12, 16-dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, respectively. The results indicated that late apoptosis events also occurred to the experimental conditions.

3.2. *Effect of cardenolide glycosides isolated of A. subulata on the disruption of mitochondrial membrane potential*

Disruption of mitochondrial membrane potential represents one of the early events occurring during the apoptosis process. To assess the effects of the cardenolide glycosides isolated of *A. subulata* on the disruption of mitochondrial membrane potential of A549 cells JC-1, a cationic lipophilic fluorescent probe, was used. This probe forms red-fluorescent dimers under high mitochondrial membrane potential, whereas in low mitochondrial membrane potential is found as monomeric form that emits a green-fluorescence. Thus, in non-apoptotic cells this probe accumulates in form of red-fluorescent dimers within the mitochondria; while in apoptotic cells, due to collapse of the mitochondrial membrane potential, JC-1 remains in the cytoplasm in its green-fluorescent monomeric form (Tannin-Spitz et al., 2007).

Fig. 4 shown a shift of fluorescence signal form the upper right quadrant to lower right quadrant leading lower red fluorescence intensity in cells treated with the cardenolide glycosides isolated of *A. subulata* than the vehicle treated cells, indicating disruption of mitochondrial membrane potential. As shown the Fig. 5, all cell cultures treated with the cardenolide glycosides had a significant decrease in the fluorescence intensity ratio (red/green) in more than 75% respect to cell culture control, suggesting that the treatment with 12,16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin induces the apoptosis of A549 cells through disruption of membrane mitochondrial potential.

3.3. *Effect of cardenolide glycosides isolated of A. subulata on the caspase-3, caspase-8 and caspase-9 activity*

As mentioned previously, the apoptosis is a process that

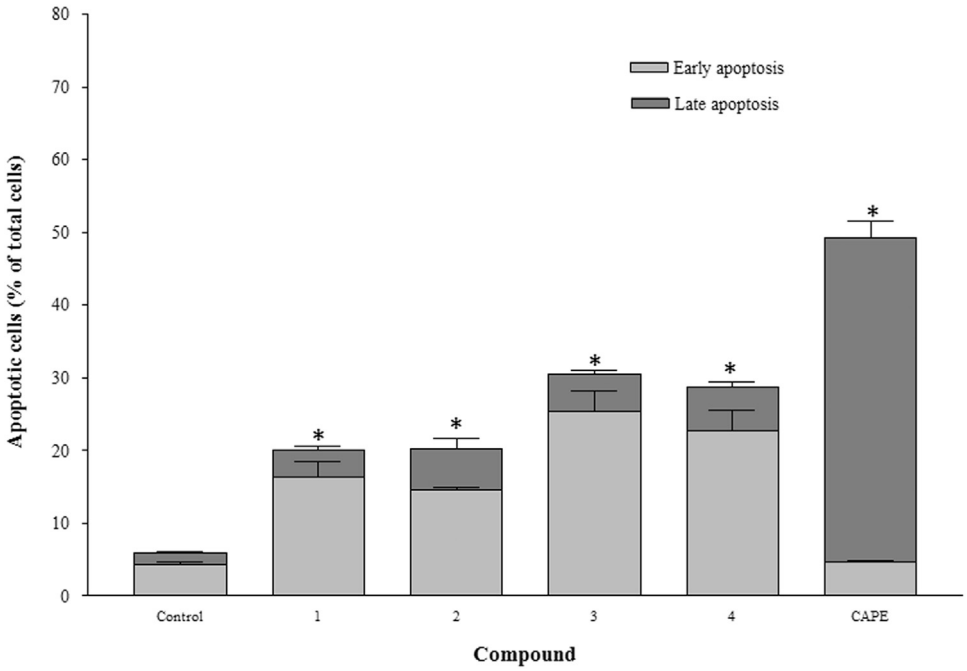

Fig. 3. Percentages of apoptotic cells induced for the treatment with compounds **1–4** isolated of *A. subulata*. Values represent the mean ± SD of at least three independent experiments. * Statistical significance based on the comparison with control cells (DMSO) for each condition, $p < 0.05$ (Tukey's test).

involves a high complex interaction of molecules, which conduct the cell to organized cell death without side effects to surrounding tissue. Key molecules in apoptosis program are the proteolytic enzymes called cysteine-aspartic specific proteases (caspases); the activation of these proteins occurs for two main pathways which mediate the morphological and biochemical changes of apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Fernald and Kurokawa, 2013).

To explore whether cell death induce for treatment with cardenolide glycosides was dependent of caspase activation, the activity of caspase-3 in A549 cells treated with cardenolide glycosides in study was examined. Fig. 6, showed that 12,16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin caused a significant activation of caspase-3 from 1, 0.5, 0.2 and 0.3 fold of control of untreated cell respectively, evidencing that cell death induced for cardenolide glycosides isolated of *A. subulata* is caspase-dependent, since caspase-3 is an executioner caspase which is activated through both caspase-dependent pathways.

These results directed the research to determine the caspase-dependent pathway that was activated by cardenolide glycoside treatment; thus, the activity of caspase-8, which is specific for extrinsic pathway, and caspase-9, which is representative of intrinsic pathway, was measured. Fig. 7 shown that caspase-8 was activated in 1.5, 1.3, 1.2 and 0.6 fold of control, respectively, for 12,16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, respect to vehicle treated cells. Whereas, caspase-9 was activated more than 1.1 fold of control for 12,16-dihydroxicalotropin treatment, 0.6 fold for calotropin, 0.7 fold of control for corotoxigenin 3-*O*-glucopyranoside and 0-fold for desglucouzarin (Fig. 8). Thus, caspase-8 was more activated than caspase-9 for the treatment with the isolated compounds; suggesting that cardenolide glycosides isolated were able of induce the cell death by activation of both caspase-dependent apoptosis pathways, but preferably by extrinsic apoptosis pathway.

4. Discussion

In a previous study four cardenolide glycosides, named 12,16-dihydroxicalotropin, calotropin, corotoxigenin 3-*O*-glucopyranoside and desglucouzarin, isolated of *A. subulata* demonstrated a strong antiproliferative effect on a lung adenocarcinoma cell line (A549), a prostate cancer cell line (PC-3), a colon cancer cell line (LS 180) and a lesser effect on a non-cancer cell line of retinal pigment epithelia (ARPE-19) (Rascón-Valenzuela et al., 2015b). Even though, the potent antiproliferative activity of the mentioned cardenolide glycosides on cancer cell lines, the pathways through which they induce the cell death are not clearly established. Therefore, this approach needs a better understanding of the mechanism of the anticancer effect of cardenolide glycosides, the signaling pathways involved and the basis of their selectivity against cancer cells. Thus, the present study was focused to characterize the possible mechanisms of action of the cardenolide glycosides isolated of *A. subulata*.

Cardenolide glycosides were originally prescribed as treatment for congestive heart failure due to their ability to inhibit the ubiquitous cell surface enzyme Na^+/K^+-ATPase and, subsequently, increase the cardiac muscle contractility (Li et al., 2014). However, recently, cardenolide glycosides have demonstrated to possess a strong anticancer activity (Rashan et al., 2011; Li et al., 2012; Cerella et al., 2013; Xue et al., 2013). The above notwithstanding, the mechanisms of action remain unclear, since the modes of action of cardenolide glycosides are very complex as several signaling pathways are targeted simultaneously (Xue et al., 2015).

Currently, an intensive search of new targets for the cancer treatment has been performed and the activation of apoptosis in cancer cells has proven to be one of the main targets of the new therapeutic strategies due to this type of programmed cell death avoid the damage of surrounding tissue. Failures in normal apoptosis pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations, allowing disobedience of cell cycle checkpoints that would induce cell death (Reed, 1999). On another hand, the cancer therapy evokes cell death by inducing apoptosis, thus,

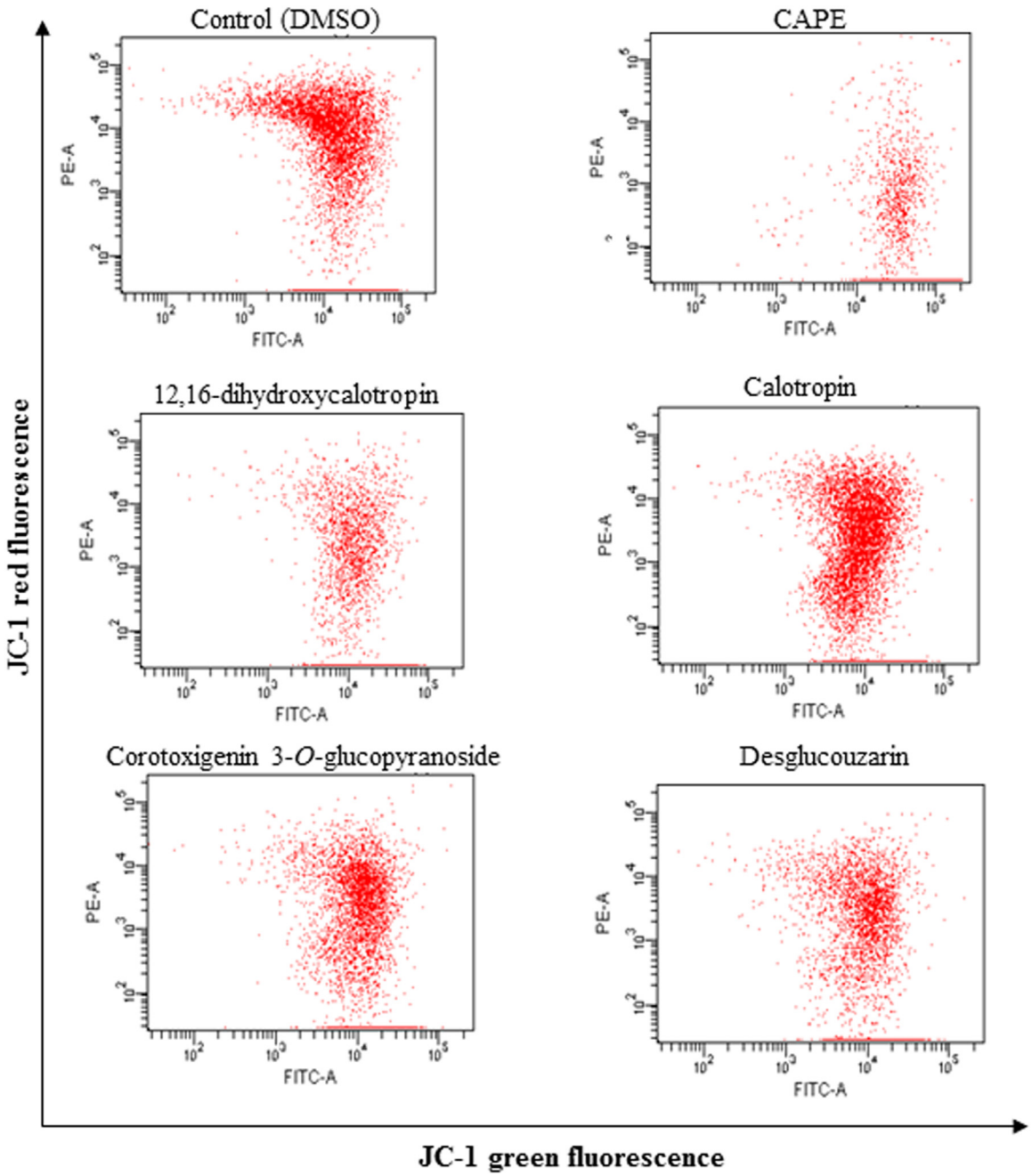

Fig. 4. Effect of compounds **1–4** isolated of *A. subulata* on depolarization of mitochondrial membrane potential in A549 cells. Flow cytometric fluorescence patterns of JC-1 staining. Data are representative of least three independent experiments.

precise identification of apoptosis events in cancer cells is pivotal for understanding cancer pathogenesis and designing effective therapeutic drugs (Coates et al., 2010).

The apoptosis program occurs in a sequential manner; first morphological changes appear at the cell membrane, then cell to cell adhesion decreases and cytosolic or mitochondrial proteins are altered. Finally, an orderly form of intranucleosomal DNA fragmentation is carried out as result of activation of caspase cascade or endonucleases (Huerta et al., 2007). Thus, in this study, three different elements involved in the apoptotic process were considered to determine whether the treatment with the cardenolide glycosides isolated of *A. subulata* can induce the apoptosis of A549 cells: phosphatidylserine externalization, changes in mitochondrial membrane potential and activity of caspase-3, caspase-8 and caspase-9.

Phosphatidylserine is localized in the inner leaflet of the cell membrane; however when the cell is committed to apoptosis phosphatidylserine is externalized to outer leaflet of plasmatic membrane. *In vivo*, the phosphatidylserine exposure is fundamental for apoptotic cells can be recognized by macrophages. In this way, this event is associated with early stages of the apoptosis process (Huigsloot et al., 2001). The four cardenolide glycosides isolated of *A. subulata* were potent inducers of phosphatidylserine externalization in A549 cells, statistically significant percentages of cells in early apoptosis were detected after 12 h of treatment with each compound (Fig. 3). Among the tested compound corotoxigenin 3-*O*-glucopyranoside caused the greatest percentage of early apoptotic cells. This suggested that four cardenolide glycosides isolated of *A. subulata* are able to induce the cell death through apoptosis process.

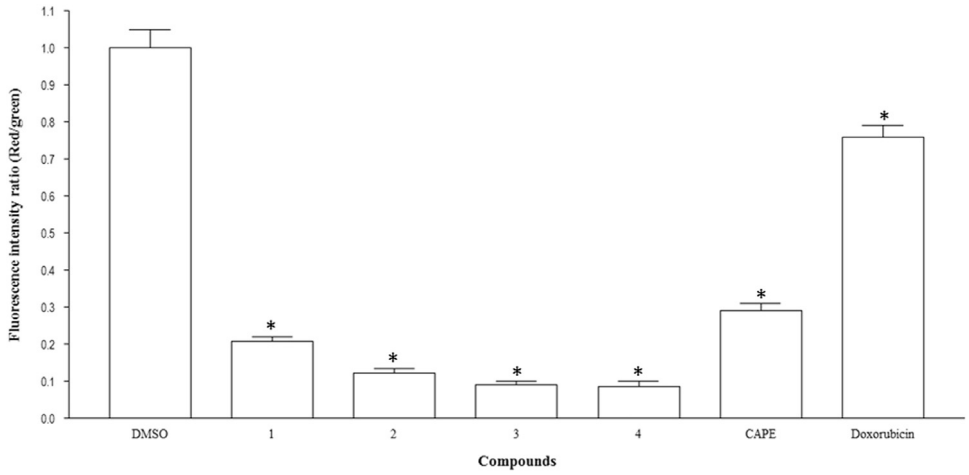

Fig. 5. Depolarization of mitochondrial membrane potential in A549 cells treated with compounds **1–4** isolated of *A. subulata*. Results are expressed as the fluorescence intensity ratio (red/green) for JC-1 ± SD of triplicate, and are representative of three independent experiments. ANOVA * significant differences respect to control cells (DMSO), $p < 0.05$ (Tukey's test). CAPE and doxorubicin were used as positive control of apoptosis.

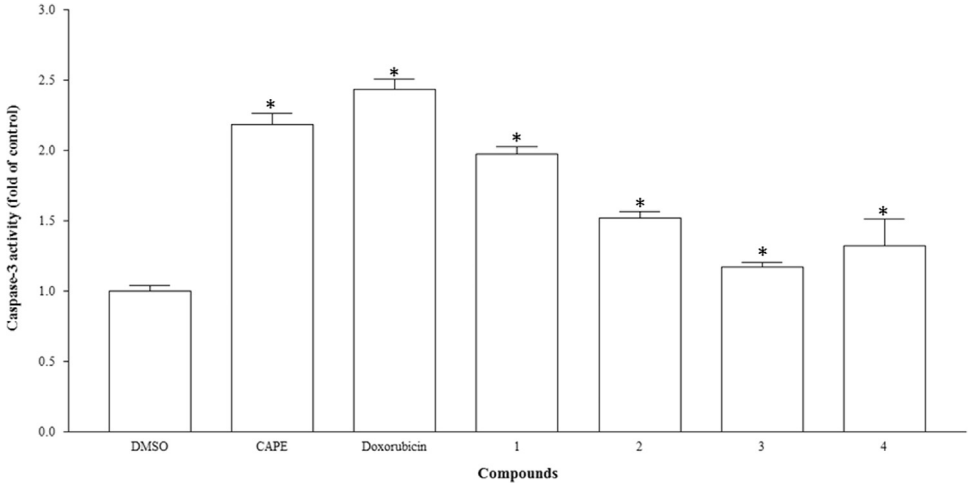

Fig. 6. Caspase-3 activity in A549 cells treated with compounds **1–4** isolated of methanol extract of *A. subulata*. Results are expressed as mean ± SD, $n > 3$. ANOVA * significant differences respect to control cells (DMSO), $p < 0.05$ (Tukey's test). CAPE and doxorubicin were used as positive control of apoptosis.

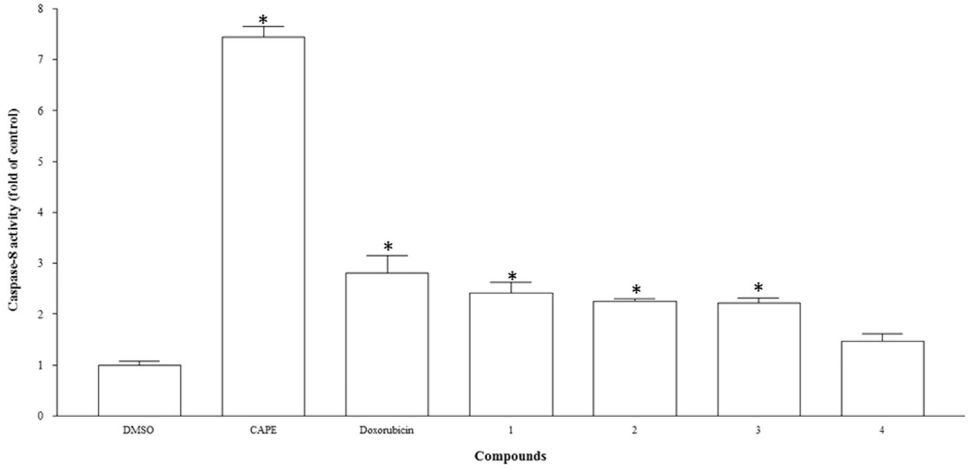

Fig. 7. Caspase-8 activity in A549 cells treated with compounds **1–4** isolated of methanol extract of *A. subulata*. Results are expressed as mean ± SD, $n > 3$. ANOVA * significant differences respect to control cells (DMSO), $p < 0.05$ (Tukey's test). CAPE and doxorubicin were used as positive control of apoptosis.

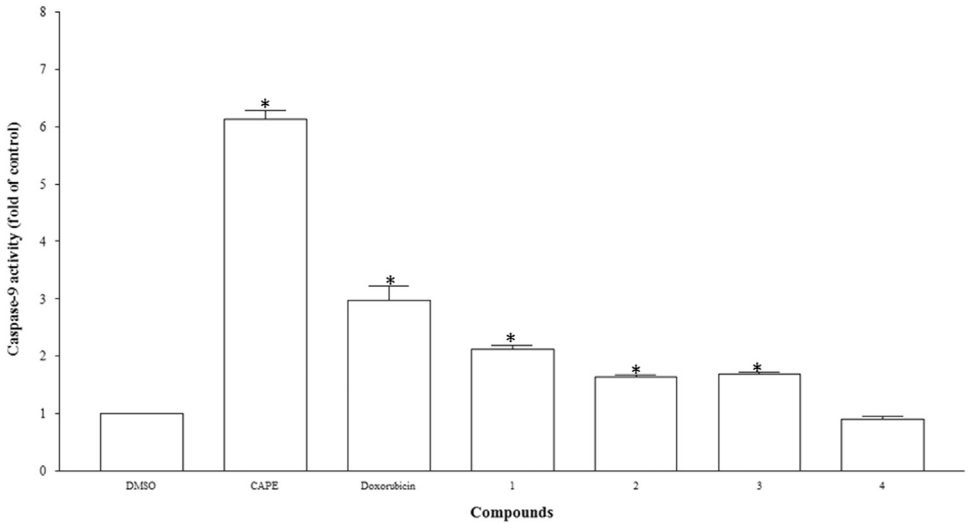

Fig. 8. Caspase-9 activity in A549 cells treated with compounds **1–4** isolated of methanol extract of *A. subulata*. Results are expressed as mean ± SD, $n > 3$. ANOVA * significant differences respect to control cells (DMSO), $p < 0.05$ (Tukey's test). CAPE and doxorubicin were used as positive control of apoptosis.

Mitochondria are fundamental to apoptotic signaling and act as integrating sensors for a variety of death stimuli. The mitochondrial membrane potential is critical for the maintenance of the physiologic function of electron transport chain, which results in the ATP production, therefore, a significant loss of mitochondrial membrane potential causes energy depletion and, subsequently, the death of the cell, since the loss of mitochondrial membrane potential leads the increase of proapototic proteins into cytosol which triggers the activation of mitochondrial death cascade (Liu et al., 2010). In the present study, the mitochondrial membrane potential decreased in more of 70% respect to control in A549 cells after to cardenolide glycosides exposure (Fig. 4), which further evidenced that cardenolide glycosides isolated induce the cell death by apoptosis.

Apoptosis process can be caspase-independent and caspase-dependent. The former involves translocation of apoptosis-inducing factor (AIF) or endonuclease G from the mitochondria to the nucleus, while the latter includes signaling through the death receptor (extrinsic) or the mitochondria (intrinsic) pathway. In caspase-dependent apoptosis, caspases are classified in initiator caspases (caspase-2,-8,-9 and -10) which are involved in early stages of the proteolytic cascade cleaving the inactive pro-form of the executioner caspases (caspase-3,-6, and -7) which trigger the cleavage of specific intracellular substrates resulting in the apoptosis (Sikdar et al., 2014).

Activation of caspase-3 is a central event in the execution of caspase-dependent apoptosis (Chan et al., 2010). Fig. 6 showed a significant increase in caspase-3 activity in A549 cells treated 12 h with each cardenolide glycoside in study; these results indicated that the mentioned compounds possessed the ability to induce the cell death through caspase-dependent apoptosis process. Based on the foregoing, the research was directed to determine the apoptosis pathway that is activated by cardenolide glycosides tested; thus caspase-9 activity (intrinsic pathway) and caspase-8 activity (extrinsic pathway) were measured in A549 cell treated with the cardenolide glycosides isolated. Figs. 7 and 8 shown that both caspases were activated; however the caspase-8 activity was higher than caspase-9 activity at 12 h of treatment in all cases. Intrinsic and extrinsic pathways are not exclusive one for another; several studies have reported a feedback between both caspase-dependent apoptosis pathways. Caspase-8 stimulates to Bid protein which leading the cytochrome c release and the apoptosome activation; while caspase-6 can feedback the caspase-8 activation (Huerta et al., 2007). These facts can explain the significant activity obtained for caspase-8 and caspase-9 in the present study.

Several studies have documented that intrinsic apoptotic pathway is ubiquitously activated in cancer cells by cardenolide glycosides treatment. Accordingly, release of cytochrome *c* and loss of the mitochondrial membrane potential (Ramirez-Ortega et al., 2006); in addition, the activation of the proapoptotic Bcl-2 family members has also been reported (Juncker et al., 2011). In other hand, it has been demonstrated that cardenolide glycosides activate the extrinsic pathway increases the expression of Fas ligand and death receptors four and five (Raghavendra et al., 2007; Kumar et al., 2013).

Finally, the experiments performed, clearly showed that the compounds induce caspase-dependent apoptosis, preferably, by extrinsic pathway. To respect, previous studies have reported that calotropin inhibits Wnt signaling due to increasing casein kinase 1 α in colon cancer cells (Park et al., 2014). It was also noted that calotropin inhibits the growth of K562 cells due to upregulation of the expression of p27 and dowregulation the G2/M proteins, cyclins A and B. Furthermore, it downregulating antiapoptotic signaling and survival pathways, leading caspase-3 activation which resulted in the induction of apoptosis (Wang et al., 2009). The mechanism of cell death induced for treatment with 12,16-dihydroxycalotropin, corotoxigenin 3-*O*-glycoside and desglucouzarin had not been investigated in other reports. However more studies are necessary to investigate the specific signaling pathways activated by 12,16-dihydroxycalotropin, corotoxigenin 3-*O*-glycoside and desglucouzarin treatment.

5. Conclusion

The results of this study revealed that cardenolide glycosides isolated from *A. subulata*, named 12,16-dihydroxycalotropin, calotropin, corotoxigenin 3-*O*-glycoside and desglucouzarin, induced the cell death through apoptosis process which is caspase-dependent and it is activated, preferably, by extrinsic pathway. This work constitutes one step to prove that these cardenolide glycosides, from *A. subulata* could be potential anticancer agents; however is necessary to design more studies to test the theory. This work provide rational basis for the use of *A. subulata* in the traditional medicine of ethnic groups of Sonora, Mexico, as a treatment to cancer.

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