REVISTA BOLIVIANA DE QUÍMICA
ISSN 0250-5460 Rev. Bol. Quim. *Paper edition*
ISSN 2078-3949 Rev. boliv. quim. *Electronic edition*
Iturri et al. RBQ Vol. 35, No.1, pp. 6-14, 2018

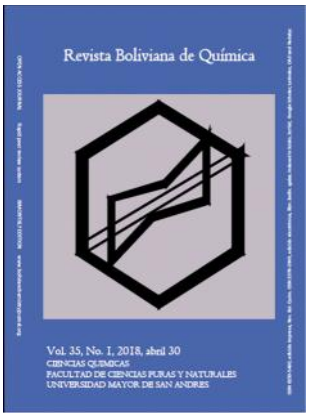
DAMSIN, CORONOPILIN AND SANTAMARIN, SESQUITERPENE-LACTONES THAT MANIFEST A CYTOTOXIC ACTIVITY, DNA DAMAGE CAPABILITY AND APOPTOSIS CAPABILITY ON CANCER CELLS LINES A549, HELA AND PANC-1

DAMSINA, CORONOPILINA Y SANTAMARINA, SESQUITERPEN-LACTONAS QUE MANIFIESTAN ACTIVIDAD CITOTÓXICA, CAPACIDAD DE DAÑO DEL ADN Y CAPACIDAD DE APÓPTOSIS EN CÉLULAS CANCEROSAS LINEAS A549, HELA Y PANC-1

Received 12 14 2017
Accepted 03 11 2018
Published 04 30 2018

Vol. 35, No.1, pp. 6-14, Ene./Abr. 2018
Revista Boliviana de Química

***35*(1), 6-14, Jan./Apr. 2018**
Bolivian Journal of Chemistry



Full original article Peer-reviewed

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Keywords: Sesquiterpene-lactones, Cancer, Damsin, Coronopilin, Santamarin, DNA damage, Apoptosis.

ABSTRACT

Cancer represents a group of diseases characterized by abnormal cell proliferation, currently represents major health problem. It is in this sense that derivatives of natural products are an important source of investigation as possible treatments. In the present research antiproliferative capacity, DNA damage and apoptotic effects of three sesquiterpene lactones (damsin, coronopilin and santamarin) were assayed in the following cancer cell lines: A549 (non-small cell lung cancer), HeLa (cervical cancer) and Panc-1 (pancreatic cancer).

In A549 cell line all molecules showed antiproliferative effect at every concentration without presenting DNA damage, santamarin and coronopilin increased the amount of apoptotic cells at high concentrations. Damsin and santamarin showed antiproliferative effect on HeLa cells at all concentration but coronopilin only at high, similarly DNA damage and apoptotic effect was observed for coronopilin and santamarin. For Panc-1 cell line, damsin and santamarin showed antiproliferative effect, DNA damage was observed at high concentrations of the three molecules, without effect on apoptosis induction.

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RESUMEN

El cáncer representa un grupo de enfermedades caracterizadas por la proliferación celular anormal, actualmente se trata de un problema de salud importante. Es en este sentido que los derivados de productos naturales son una importante fuente de investigación como posibles tratamientos. En la presente investigación, la capacidad antiproliferativa, el daño en el ADN y los efectos apoptóticos de tres lactonas sesquiterpénicas (damsin, coronopilina y santamarina) se analizaron en las siguientes líneas celulares de cáncer: A549 (cáncer de pulmón de células no pequeñas), HeLa (cáncer de cuello uterino) y Panc 1 (cáncer de páncreas).

En la línea celular A549, todas las moléculas mostraron un efecto antiproliferativo en cada concentración sin presentar daño en el ADN, la santamarina y la coronopilina aumentaron la cantidad de células apoptóticas a altas concentraciones. Damsina y santamarina mostraron un efecto antiproliferativo sobre las células HeLa en todas las concentraciones, pero la coronopilina solo a dosis elevadas, de forma similar, se observó daño al ADN y efecto apoptótico para la coronopilina y la santamarina. Para la línea celular Panc-1, damsina y santamarina mostraron un efecto antiproliferativo, se observó daño en el ADN a altas concentraciones de las tres moléculas, sin efecto sobre la inducción de la apoptosis.

INTRODUCTION

Cancer is a major health problem all over the world, is the second leading cause of death in developing countries [1]. It is a large class of very different diseases where the cells are defected in their mechanism controlling cell division and cell death. Being lung and breast cancer the most common types disease worldwide [1].

Apoptosis is a normal cellular death mechanism and cancer is one consequence of impaired apoptotic mechanism [2] and cancer cells avoid apoptosis and continue to proliferative. It is characterized by different changes including plasma membrane blebbing, cell shrinkage, depolarization of the mitochondria, chromatin condensation, and DNA fragmentation [3].

Sesquiterpene lactones (SLs), are natural metabolites mainly found in the Compositae family, they possess a wide range of biological effects, some of them could be allergenic, poisonous and insect feeding deterrents [4]. Particularly the anti-tumor, anti-inflammatory and cytotoxic activities are of special interest, specifically affecting cancer cells without having an adverse effect on normal cells [5]. All of these activities are referred to their α -CH=C-C=O moiety as part of an ester or ketone or lactone, this functional group reacts this with the sulphydryl groups of enzymes and other proteins by Michael-type addition reaction [6];[7]. It has been hypotethized that these metabolites alkylate DNA causing single strand breaks and interfering with the DNA template [6]. They also inhibit cell proliferation, produce cell cycle arrest, promote cell differentiation and induce apoptosis in cancer cells [8].

Damsin and coronopilin showed slight antiproliferative activity against colon cancer cells Caco-2, but they inhibited DNA synthesis significantly, suggesting that they promote cesing of the cell cycle in the s-phase [9] in addition coronopilin inhibited of leukemia cell growth causing arrest and apoptosis of the cell cycle [3] and santamarin was cytotoxic in some breast cancer cell lines [12].

In this study we showed for the first time that damsin, coronopilin and santamarin possess a cytotoxic effect on A549, HeLa and Panc-1 cell lines inducing apoptosis and producing DNA damage.

RESULTS

Damsin, coronopilin and santamarin possess an antiproliferative effect on A549 and HeLa cancer cell lines

Figure 2a illustrates the antiproliferative activity on A549 cells treated with damsin, coronopilin and santamarin. Damsin promoted a significant reduction ($P < 0.001$) in cell proliferation at concentrations ranging from 12.5 to 100 μM), having an EC_{50} value of 10.72 μM. When cells were treated with coronopilin a significant cell proliferation reduction was observed at 50 and 100 μM, the EC_{50} value was 40.8 μM. Santamarin reduced cell proliferation in a dose-response manner for every concentration assayed, with an EC_{50} of 9.4 μM.

After treatment of A549 cells with coronopilin, a significant increase of apoptotic cells ($p<0,05$) was observed at certain concentrations (25-100 μM). Santamarin significantly increased the number of cells that had undergone apoptosis at certain concentrations (100 and 12.5 μM) ($p < 0.05$); in contrast, at 25 μM a significant reduction ($p < 0,05$) of the number of apoptotic cells compared to negative control was observed. Damsin did not exert any effect in this cell line (Figure 2 b).

The number of apoptotic cells in HeLa cell line after the exposure to damsin showed no effect at all concentrations (Figure 3 b). Coronopilin at 100 μM significantly increased apoptotic cells, as well as significant DNA

damage was observed (Table 1). This could mean that the antiproliferative effect of this molecule is related to DNA damage that finally derives on apoptosis. A similar effect was observed for Santamarin at 50 µM.

As figure 4a illustrates, damsin and coronopilin have a dose-manner effect on the pancreatic cancer cell line Panc-1, but the antiproliferative effect of santamarin is independent of dose. The number of apoptotic cells was significantly lower at every concentration after the exposure to damsin and santamarin. Coronopilin had only effect at 100 µM (Figure 4b).

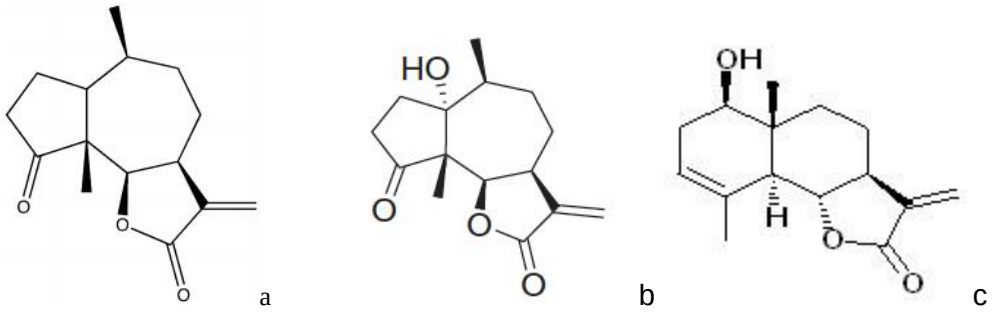

Figure 1. *Chemical structures. a) damsin, b) coronopilin and c) santamarin.*

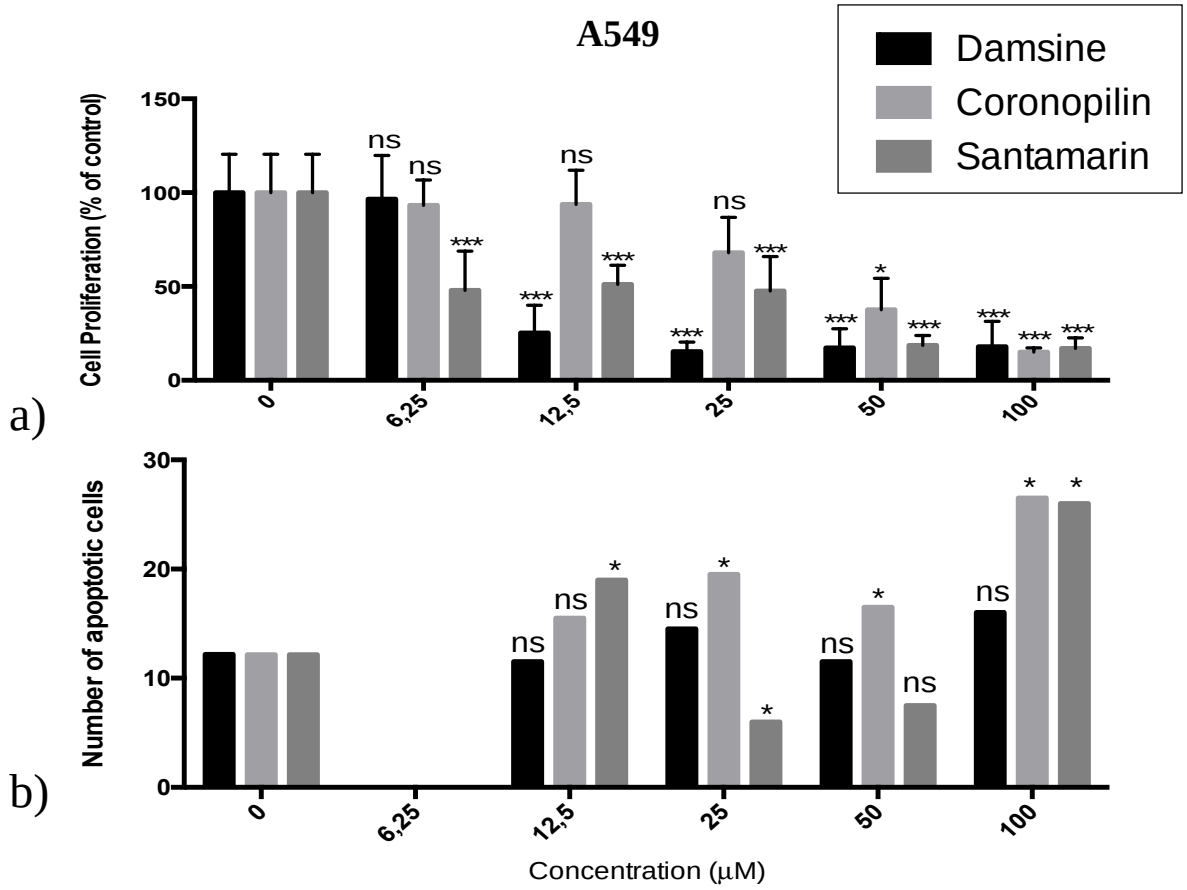

Figure 2. *Effect of damsin, coronopilin and santamarin in A549 cells. (a) Antiproliferative effect of the assayed molecules measured by the WST-1 reagent is shown after 24h of exposition, all values are expressed as the average ± SD from at least two experiments performed in triplicate. (b) Number of cells undergoing through apoptosis. * P < 0,05; **P < 0,01; *** P < 0,001 compared with negative control; 6,25 µM was not assayed.*

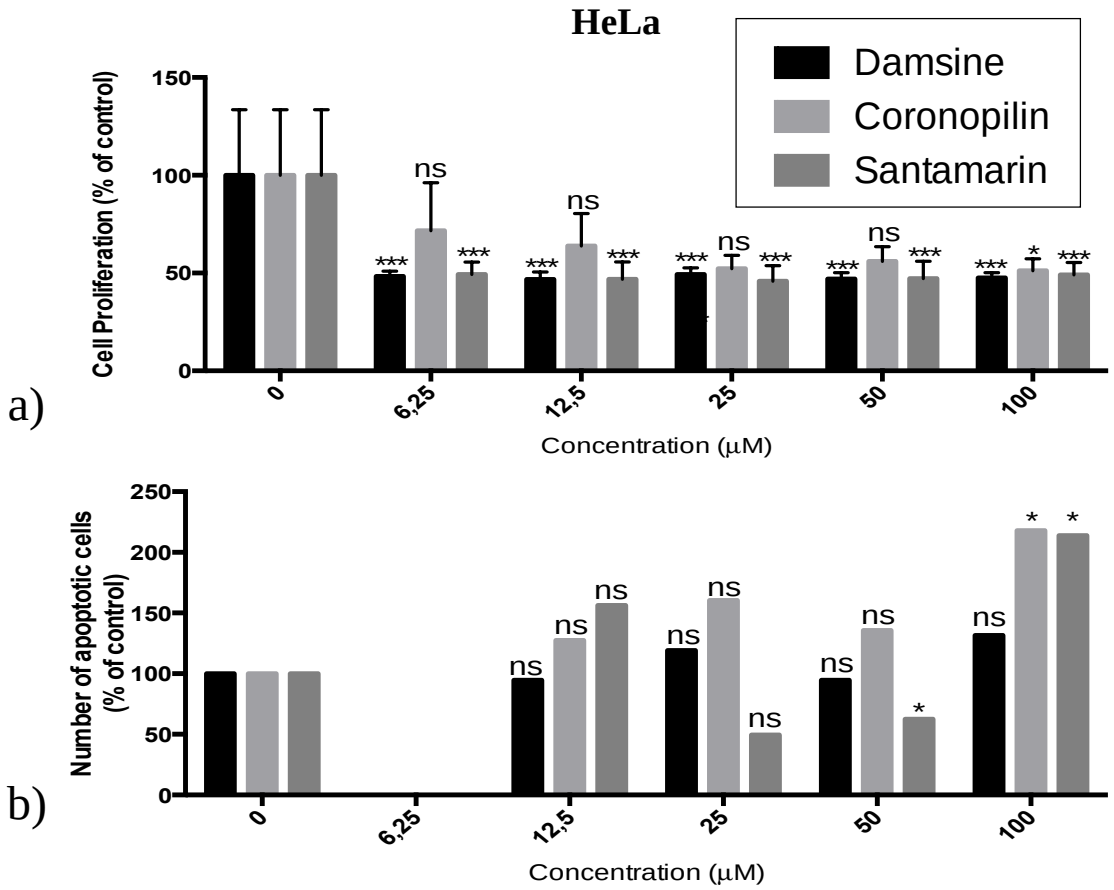

Figure 3. *Effect of damsin, coronopilin and santamarin in HeLa cells. (a) Antiproliferative effect of the assayed molecules measured by the WST-1 reagent is shown after 24h of exposition, all values are expressed as the average ±SD from at least two experiments performed in triplicate. (b) Number of cells undergoing through apoptosis. * P < 0,05; **P < 0,01; *** P < 0,001 compared with negative control; 6,25 µM was not assayed.*

Table 1. *Damage index results from Single-Cell Gel Electrophoresis. Results in bold are significantly different from negative control. Values are expressed as the average of three replicates * P < 0,05; *** P < 0,001 compared with control (0µM).*

Cell line	Molecule	Concentration (µM)				
		0	**12,5**	**25**	**50**	**100**
A549	Damsin	2,3 ± 1,21	2,5 ± 0,71	2,5 ± 0,71	3 ± 0	4,5 ± 0,71
	Coronopilin	2,3 ± 1,21	3,5 ± 0,71	3 ± 0	3,5 ± 0,71	**11,5 ± 0,71**
	Santamarin	2,3 ± 1,21	**6 ± 1,41**	**9 ± 0**	**7,5 ± 0,71**	**8 ± 2,83**
HeLa	Damsin	3,8 ± 1,47	3,5 ± 0,71	2,5 ± 0,71	6,5 ± 3,53	3,5 ± 0,71
	Coronopilin	3,8 ± 1,47	**8 ± 1,41**	**8,5 ± 0,71**	**20,5 ± 0,71**	**20,5 ± 0,71**
	Santamarin	3,8 ± 1,47	1 ± 0,71	4,5 ± 0,71	**10,5 ± 0,71**	6 ± 1,41
Panc-1	Damsin	1,6 ± 0,98	3 ± 0	4,5 ± 0,71	3,5 ± 0,71	**8,5 ± 0,71**
	Coronopilin	1,6 ± 0,98	4 ± 0	2,5 ± 0,71	**6,5 ± 0,71**	**8,5 ± 0,71**
	Santamarin	1,6 ± 0,98	2 ± 0	1,5 ± 0,71	**8 ± 1,41**	**8 ± 1,41**

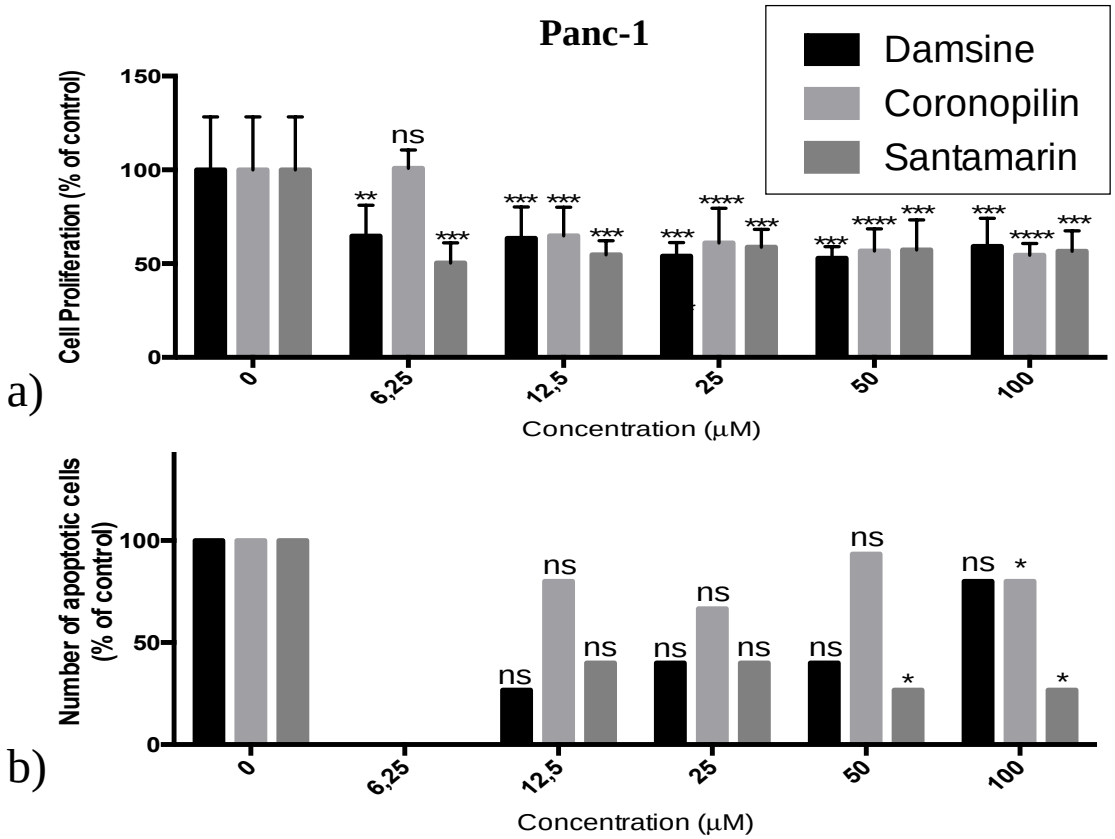

Figure 4. *Effect of damsin, coronopilin and santamarin in Panc-1 cells. (a) Antiproliferative effect of the assayed molecules measured by the WST-1 reagent is shown after 24h of exposition, all values are expressed as the average ± SD from at least two experiments performed in triplicate. (b) Number of cells undergoing through apoptosis. * P < 0,05; **P < 0,01; *** P < 0,001 compared with negative control; 6,25 µM was not assayed.*

Sesquiterpene lactones produce DNA damage on A549, HeLa and Panc-1 cell lines

In order to evaluate if DNA damage was somehow associated with the cytotoxic effect of SLs tested in the present research, a Single Cell Gel Electrophoresis assay was performed. In A-549 cell line, santamarin was the only molecule to damage DNA vs. negative control. Genetic material of HeLa cells was significantly damaged by coronopilin at every concentration tested, especially at 100 µM where the significant antiproliferative and apoptotic effects were also observed; santamarin at 50 µM also caused significant damage to genetic material. DNA of Panc-1 cells were damaged by coronopilin and santamarin at 50-100 µM, and by damsin at 100 µM (Table 1).

DISCUSSION

Antiproliferative and apoptosis effect

In the present research we showed the cytotoxic effect of SLs, two of them pseudoguaianolides (damsin and coronopilin) and one eudesmanolide (santamarin) in three cancer cell lines. Cytotoxic activity of SLs could be associated to the presence of α-methylene-γ-lactone residue, which participates in a Michael addition with compounds containing a -SH group [4]. Cotugno and coworkers [5] have demonstrated that a structural analogue of coronopilin, dihydrocoronopilin, lacking this residue, could not inhibit a leukemia derived cell line. Also, there is a clear relationship between chemical structure and cytotoxic activity, existing more reactive families, where the number of alkylating centers is related to the difference in their activity [10]. Scotti and coworkers [11], in a QSAR study, revealed that comparing 4 of the most representative skeletons of SLs (germacranolides, elemanolides, guaianolides, and pseudoguaianolides), where the molecules with higher activity were the ones with guaianolide, and pseudoguaianolide skeletons, because they are stereochemically more convenient for the cytotoxic activity. All the

tested molecules in our research possessed the α-methylene-γ-lactone residue, which probably conferred them their cytotoxic activity.. In the other hand santamarin (eudesmanolide), which possesses a hydroxyl at position 1, has the highest cytotoxicity, (i.e. presenting the lowest IC_{50} value at A-549 cell line), but also being the only molecule that has cytotoxic activity at every concentration in all the cell lines that we have tested in the present research. In HeLa and Panc-1 cell lines, antiproliferative activity of damsin and santamarin is similar, being the coronopilin, the one that possesses the lowest cytotoxic activity. Our research team has recently showed that damsin, coronopilin, ambrosin and dindol-01 (the latest two compounds were synthetized from the isolated damsin) possess cytotoxic activity in the breast cancer cell lines MCF-7, JIMT-1 and HCC1937; interestingly damsin had a significantly lower cytotoxic effect in the normal-like breast epithelial cell line MCF-10A [12]. Moreover, damsin and ambrosin were able to inhibit cell migration after 72 hours of treatment, showing its potential against cancer stem cells, which have been suggested to be responsible for cancer relapse.

When the antiproliferative effect is related to the increasing of apoptotic cells, it is possible to assume that apoptosis is the main mechanism of cell death; mainly by the intrinsic pathway, which is the main action mechanism of SLs [4]. One of the most studied SLs parthenolide (PTL) induced apoptosis associated to intracellular events, which include mitochondrial membrane potential depletion, resulting in the release of certain pro apoptotic proteins such as cytochrome c, Omi/HtrA2 and Smac. In different model systems, PTL has diminished the expression of the antiapoptotic protein Bcl2 and increased the expression of pro apoptotic proteins such as Bax, Bid, tBid, among other, liberating apoptotic factors and the subsequent caspase 3 and 9 activation [4, 13], leading us to think that the molecules tested in the present research may have a similar pathway. In the present research only morphological techniques was applied, but in future studies we recommend to use specific techniques (reagents, kits, etc.) that could not only detect but quantify, the different apoptosis pathways (among other kinds of cell death) involved in the cytotoxic effects of this molecules.

As mentioned in materials and methods, our criteria to detect apoptosis in the present research was only morphological and besides observing proper characteristics of apoptosis (pyknosis, karyorrhexis and apoptotic bodies), we have also observed a rapid cell membrane integrity loss, data not shown. Pozarowski and coworkers [14] have also observed this phenomenon and they propose that the capacity of SLs to inhibit the transcription factor NF-kB, accelerating plasmatic membrane integrity loss and finally inducing to a “necrotic phase“ of apoptosis; preventing NF-kB induce genes that promote plasmatic membrane integrity preservation [15]. Another possible explanation is that SLs act directly at membrane level, interacting with its components, affecting lipids oxidation; protons pump among other properties [16].

In certain concentrations, the difference of apoptotic cells between negative control and treatments were significantly lower, but an antiproliferative effect has been shown; at the beginning this result may seem contradictory, but actually means that the main cause of cell death is not the apoptotic one. Especially with Panc-1 cells, morphologic characteristics of other kinds of cell death have been observed, like aberrant mitosis, caryolisis and oncosis. This phenomenon has also been observed by some other research groups, for example Du and coworkers [17] noticed that in Panc-1 cells after 48 h of exposure with the SL artesunate (ART), the antiproliferative effect of this molecule did not show apoptotic characteristics, and in spite of adding the pan-caspase inhibitor z-VAD-fmk, cytotoxic activity of ART could not be inhibited, suggesting ART kills cells independently of apoptosis, inducing us to think that the molecules assayed in the present research could be acting in a similar way.

Cotugno and coworkers [5], after exposing U937 cells, pre-treated with a pan caspase inhibitor, to coronopilin, observed that cell death was induced in a caspase independent manner, in which prolonged mitotic suppression , necrosis and mitochondrial depolarization could be observed with a latter cell death.

Something interesting about coronopilin at 100 µM, is that a significant antiproliferative effect, a DNA damage and the increase of apoptotic cells was observed, suggesting that that these three events might be related in HeLa cell line.

Damsin, coronopilin and santamarin produce DNA damage

The three molecules tested by us proved to induce DNA damage, to asses this goal we have done SCGE assay in order to detect if DNA single and double strand brakes as well as alkali labile produced, the one that could be related to antiproliferative effects of SLs in cancer cell lines. DNA damage has proven to cause cell death, as DNA is a target molecule of SLs, its alkylation is the molecular basis of certain substances cytotoxicity [18], this could explain the antiproliferative effect of coronopilin (100 µM) and santamarin (50 µM) in HeLa cells. The presence of several nucleophilic sites makes DNA a suitable target for the α- methylene -γ-lactone group of the evaluated.

It has been observed that coronopilin induces DNA damage in Jurkat and U937 cell lines, increased levels of c H2AX, marker of DNA damage [19]. This finding suggests, that DNA damage may be one of the initiating events, following coronopilin exposure. In response to DNA damage, different pathways, ultimately resulting in early or mitotic arrest⁄delayed apoptotic cell death, may be activated in Jurkat and U937 cells [5].

In summary, our data suggest that damsin, coronopilin and santamarin can effectively inhibit proliferation, produce DNA damage and induce apoptosis in certain cancer cell lines .

EXPERIMENTAL

Molecules

Damsin and coronopilin were isolated from *Ambrosia arborescens* [20], and santamarin was isolated from *Kaunia lasiophthalma* [9]. Figure 1. Bleomicin was used for line A549 and cisplatin for PANC-1 and HeLa as positive controls.

Cell culture and proliferation assay

A549 (non-small lung cancer), HeLa (cervix cancer) and Panc-1 (pancreas cancer) cell lines were cultured in DMEM medium supplemented with 10% (v⁄v) FCS, L-glutamine and antibiotics at 37 °C in humidified atmosphere with 5% CO_2. Each cell line was cultivated to approximately 90% of confluence was reached. For all the assays performed cells were detached with 0.05% trypsin/0.02% EDTA.

$1x10^4$ cells were plated in 96-well plates. 24 hours after incubation with cell culture medium only, cells were treated for 24 h at five concentrations between 6,25 and 100 µM. Working solutions were prepared in culture medium immediately prior to use; final concentration of DMSO, was equal in samples and negative control. At the end of the exposure 10 µl of 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3- benzene disulfonate (WST-1) (Roche) was added. This reagent it is cleaved for mitochondrial dehydrogenases, as a result formazan is formed, a dark red dye. The formation of the formazan is proportional to the total mitochondrial dehydrogenase activity in the sample, which in turn correlates with the total number of viable cells. After incubation for 1 h at 37 °C, the plate was shaken thoroughly for 1 min and the optical density (OD) was read at 405 nm using 655 nm as background.

DNA damage assay

The Single Cell Gel Electrophoresis (SCGE) assay was used to measure DNA damage produced by the tested molecules. It measures single and double strand breaks. The protocol used was suggested by Singh and coworkers [21] for alkaline conditions with modifications.

Slides were covered with normal melting point agarose to which 7,5 µl of cell-containing layer of low-melting point agarose were added. One hour of cell lysis followed, with a solution containing 2,5 M of sodium chloride, 100mM EDTA, 10mM Trizma base, 0,34M NaOH, 1% N-laurylsarcosine, 1% triton X100 and 10% DMSO. After lysis, slides were covered for 20 minutes by buffer alkaline solution (10N NaOH and 200mM EDTA, pH 8) to unwind DNA. Slides were electrophored for 20 min (25 V and 300 mA,).

The slides were neutralized for 15 minutes with Trizma base 0,4 M (pH 7.5) and fixed. To stain the slides we used the silver nitrate protocol proposed by Nadin and coworkers (2001) [22] with some modifications. Fifty cells were counted by plate (three plates by treatment were prepared), DNA damage was determinate by the proportion of cells with altered migration categorized according the parameter suggested by Garcia and coworkers [23] by size of its tail,

Damage index (DI) was calculated by the following formula:

$$ID = (N_0x0) + (N_1x1) + (N_2x2) + (Nx_33) + (N_4x4)$$

N_x= Number of cells under at a certain damage degree
0,1,2,3 or 4 = Degree of cell damage.

Cell morphology assay

In order to measure if cells were undergoing apoptosis, we evaluated cell morphology, quantifying living cells and the ones that underwent through different cell death events. As well as DNA damage assay, after cells reached confluence, they were detached an seeded in 6 well plates, grown for 24 hours with medium only, then the medium was discarded and cells were exposed for 24 hours to damsin, coronopilin and santamarin. After exposure cells were detached mechanically and stored in Eppendorf tubes. HeLa and Panc -1 cell lines were fixed with Carnoy (Methanol- glacial acetic acid 3:1) and stained using the protocol proposed for Papanicolaou stain [24]. For A549 cell line, Maygrouwald-Giemsa stain was used.

Fifty cells per slide were evaluated in duplicate, searching for cell morphology apoptotic characteristics (i.e. nuclear condensation, fragmentation and apoptotic bodies) according the criteria suggested by Kerr [3].

Statistical analysis

Data reported for the cell proliferation and SCGE assays are mean values of at least three experiments, performed in triplicate. Differences between treatment groups were analysed by Dunnet's test. Differences were considered significant when $P < 0.05$, $P < 0.01$ or $P < 0.001$.

For the apoptotic morphology assay, a hypothesis test for two proportions was performed between control results and each concentration of every molecule. Results statistically significant ($p<0,05$) are marked with a star.

ACKNOWLEDGEMENTS

We thank the Institute of Biomedical Research of Universidad Mayor San Simon (Cochabamba-Bolivia), for providing experiment space and experimental instruments. We also wish to thank the Laboratory of Natural Products from the Chemistry Department of Universidad Mayor de San Andrés for providing us damsin and coronopilin especially to Giovana Almanza and Rodrigo Villagómez. We are grateful with Eliana Maldonado from the Department of Organic Chemistry of Lund University for providing us santamarin.

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