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Sinergystic effects of steroidal glycosides of Solanum upon Human A375 Melanoma cells. A comparison with Ketoconazole

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Abstract

Solanum glycosides such as α-solamargine, α-chaconine, α-solanine and α-tomatine were found to be highly cytotoxic towards human melanoma cells. In contrast, the aglycones solanidine, solasodine, demissidine, tomatidine and solanocapsine showed lesser activity. Mixtures of α-solamargine and α-chaconine; α-solamargine and α-solasonine; α-chaconine and α-solanine; as well as α-solasonine and α-solanine showed synergistic effects upon these cells. On the other hand, α-solamargine and α-solanine; α-chaconine and α-solasonine as well as α-chaconine and α-tomatine showed additive or antagonic effects.

Key words: Steroidal alkaloids, α-chaconine, α-solamargine, α-tomatine, synergism, antagonism, nitrogen- containing steroidal compounds, human A375 melanoma, glycosides.

Efectos sinergísticos de glicósidos esteroidales de Solanum sobre células del melanoma humano A375. Una comparación con Ketoconazol

Resumen

Glicósidos de *Solanum* tales como α-solamargina, α-chaconina, α-solanina and α-tomatina mostraron ser altamente citotóxicos sobre células del melanoma humano. En contraste, las agliconas solanidina, solasodina, demissidina, tomatidina y solanocapsina mostraron una menor actividad. Mezclas de α-solamargina y α-chaconina; α-solamargina y α-solasonina; α-chaconina y α-solanina, así como α-solasonina y α-solanina mostraron efectos sinergísticos sobre dichas células. Por otra parte, α-solamargina y α-solanina; α-chaconina y α-solasonina así como α-chaconina y α-tomatina mostraron efectos aditivos o antagónicos.

Palabras clave: alcaloides esteroidales, α-chaconina, α-solamargina, α-tomatina, sinergismo, antagonismo, compuestos esteroidales conteniendo nitrógeno, melanoma humano A375, glicósidos.

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Introduction

The Solanum family includes many species of plants, between them potatoes and tomatoes, important human food sources. The main secondary metabolites of these plants are nitrogen-containing steroids of the spirosolane or solanidane type, which generally occur as glycosides (Fig 1). The Solanum´s nitrogen-containing steroidal compounds spectrum of action upon different biological systems is broad. In this context, several Solanum species have been used in folk medicine for hundreds of years. De Grousourdy (1) described its use as sedative or narcotic in inflammations and cutaneous diseases. Fonnegra & Jiménez (2) described their use as anti-inflammatory. Among other activities of Solanum glycoalkaloids it is worth mentioning their activity against Hepatitis C virus (3) and their antimalarial activity against Plasmodium yoelii 17XL in mice (4). On the other hand it has been reported that they inhibit the growth of *Trypanosoma cruzi in vitro* (5) and that α-solamargine and α-solanine have antifungal properties (6). Other properties attributed to the glycoalkaloids of Solanum nigrum are their use as diuretic and an antipyretic agents (7). Also, berries of Solanum nigrum Linn (Solanaceae) are used for the treatment of asthma in folk medicine (8) and their isolated glycoalkaloids in herpes virus in humans (9). Solanum americanum Miller (black nightshade) has been known to have important properties of possible medicinal use in the treatment of cancer diseases, such as hepatic (10) and human prostate cancer (11), while extracts of Solanum nigrum Linn have shown cytotoxic activity upon mouse melanoma cells in vivo and α-solamargine, α-chaconine, α-tomatine and other glycoalkaloids showed activity (12) upon these cells and other different cancer cell lines when tested in vitro (13). In this study we report the cytotoxic activity of Solanum glycoalkaloids and aglycones, and their combined interaction upon human A375 melanoma cells.

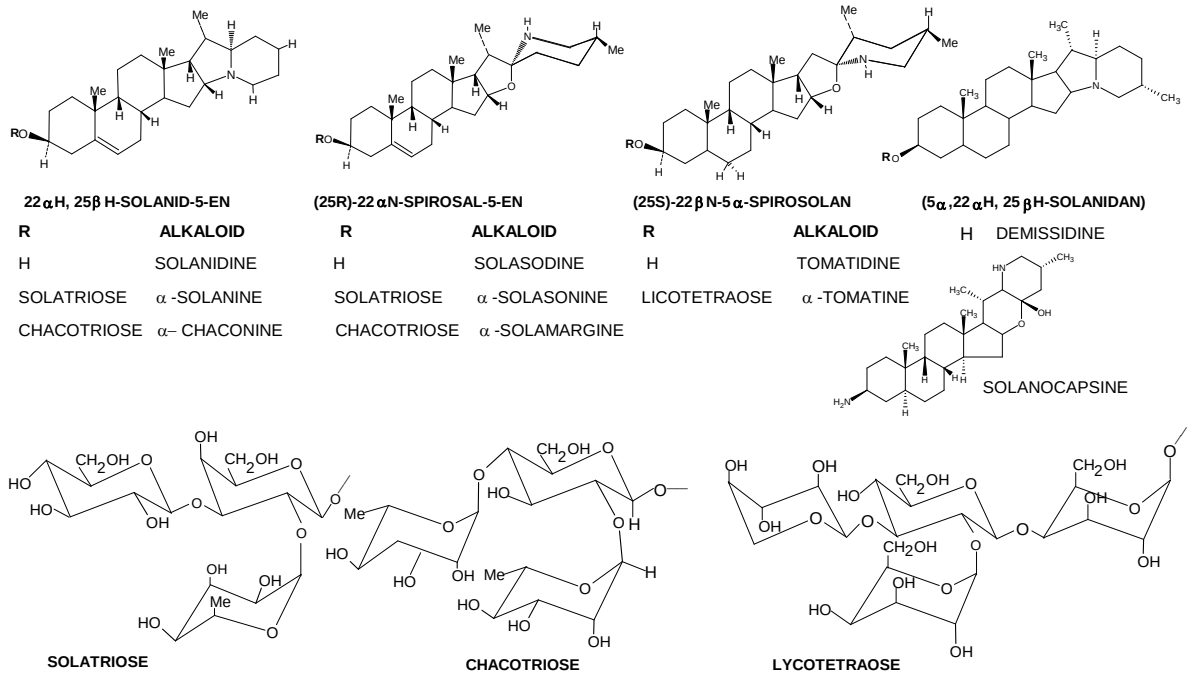


Figure 1. Structure of nitrogen-containing steroidal compounds of Solanum type

Materials and Methods

Solamargine isolation

Two-week old Solanum americanum Miller was harvested at the garden of the Faculty of Pharmacy, University of Los Andes, A voucher specimen (No.704) was deposited at the MERF Herbarium. Green berries (770 g) were treated as described before (9). The identity of the glycoalkaloids was confirmed by 13C-NMR spectra by comparison with values reported by Mahato et al.(14) and a value [α] 105º (c 0.05,EtOH) was measured for solamargine which was isolated 99% pure.

Other solanum alkaloids

Solanidine (98 % pure), solasodine (99% pure), tomatidine (85 % pure), α-tomatine (98 % pure), α-chaconine (95 % pure), α-solanine (95 % pure), and α-solasonine (95 % pure) were obtained from Sigma Biochemical Company, and Demissidine and solanocapsine (95 % pure each one) were purchased from Roth Laboratories. Other reagents were analytical grade.

Cell lines and cell cultures

The human A375 melanoma cells were a gift of Dr. Evrard from Université de Montpellier, France, and Dr. Peter Taylor of the IVIC, Venezuela. The cells were maintained in vitro in RPMI-1640 medium (Biomedia) supplemented with 10% fetal bovine serum, 1 % penicillin and streptomycin at 37 o C in a 5% CO2 incubator and diluted after trypsinization.

Microculture tetrazolium (MTT) assay for growth inhibition of cells and IC50 determination

The inhibitory concentration (IC50) of different glycosides and aglycones was obtained from human A375 melanoma cells. Cells were maintained in vitro in 10% fetal bovine serum supplemented RPMI (Biomedia) and diluted after trypsinization.

To perform in vitro activity tests, cells were adjusted with RPMI /10% fetal bovine serum, after counting in a haemocytometer, to 20,000 cells/well concentration. Drugs were then added at various concentrations to each well.

Growth inhibition was estimated measuring the quantity of formazan produced at 490 nm by the living cells in a culture using the Cell Titer 96 aqueous one solution (MTS/PMS) as recommended by Promega (Charbonnieres, France). The assay was carried out as follows: human A375 melanoma cells, at a concentration of 20,000 cells/well were seeded into a 96-well microplate (2x 104 cells/well in a total volume of 200 μL) and it was added RPMI-1640 medium containing 10 % fetal bovine serum and 1 % penicillin-streptomycin. The glycoalkaloids or aglycones initially dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI-1640 medium containing 10 % fetal bovine serum and antibiotics, so that the final DMSO concentration was less than 0.5 %, were added, by triplicate, at different concentrations to each hole in the plate. After being incubated at 37 o C in a 5% CO2 humidified atmosphere for 24, 48 or 72 hours, 40 μL MTT/PMS was then added to each well. The plates were incubated at 37 o C in a 5 % CO2, 95 % air atmosphere for 4 hours. The plate was read at 490 nm on a micro-plate reader. The assay procedure included the presence of the same amount of cells without drugs. Ketoconazole and Actimomycin D were used as a positive control (15,16,17). IC50 (concentration required to reduce viability by 50 %) values of each steroidal compound, ketoconazole or actinomycin D were estimated at 24, 48 and 72 h, respectively, after incubation of the cells with the compounds under study.

Interaction between glycoalkaloids

In those experiments concerning the interaction between glycoalkaloids or glycolkaloids with ketoconazole or actinomycin D, it was determined simultaneously in each experience the IC_{50}

value of each interacting compound alone (IC_{50} compound alone) and the IC50 of each compound in the presence of a constant amount of the other interacting compounds (IC_{50} combined). These measurements were performed after 24 h of incubation with the mixture of steroidal compounds. The fractional (percentage) IC_{50} for each combined mixture was determined as: (IC_{50} compound combined/IC_{50} compound alone) x 100%. These data were plotted as isobolograms as described by Rayburn, Friedman and Bantle (18). The fractional inhibitory concentration (FIC) was defined as FIC= IC_{50XY}/ IC_{50X} + IC_{50YX}/IC_{50Y}, where (IC_{50})X is the value for drug X acting alone, and (IC_{50}) $_{XY}$ represents the value observed for the same drug in the presence of a sub-optimal concentration of drug Y. If 1.2 ≥ FIC ≥ 0.8, then the effect was ascribed as additive; if the value FIC>1.20, the effect was considered antagonist; and if 0.80 > FIC ≤ 0.5, it was considered that a synergetic combined effect was present.

Trypan blue staining of cells

In order to prove that the MTT/PMS assay does not produce artifacts, this method was compared with results obtained by trypan blue staining to determine total cell counts and viable cell number. Briefly, melanoma A375 human cells were mixed with an equal volume of 4% Trypan blue and counted in a haemocytometer at different periods of time. This experiment indicated that there was no chemical interference between the alkaloids tested in the MTT/PMS assays.

Statistical treatment of data

PROBIT analysis was used to generate IC50. In this analysis, the numbers were converted to PROBIT values and fitted linearly at the 95 % confidence intervals. Application of a Student's t-test was used to determine statistical significance. p less than 0,01 was considered statistically significant. Data are shown as mean α standard deviation (s.d).

Results

Citotoxicity of Solanum N-containing steroids

The citotoxicity towards melanoma cells of the different steroidal compounds is shown in Table 1. α-Chaconine showed the highest activity followed by α-tomatine (a spirosolane), α-solamargine, α-solanine and α-solasonine after 24 h of incubation. However, after 72 hours, α-tomatine appears to be the most cytotoxic glycoalkaloids against these cells. This could indicate that the strength of each steroidal compound as a cytotoxic agent depends upon the steroidal type and upon the sugar moiety that is attached to it. Thus, solanidanes could be to be more cytotoxic than spirosolanes in a short period of time (24 h) and glycosides containing a chacotriose sugar moiety appears to be more effective than those containing solatriose. Absence of sugar moieties drastically reduce the cytotoxic effect of Solanum alkaloids. This indicated that the presence of the sugar moiety is essential for activity. Interestingly, solanocapsine, which possess at C-3 an amino group instead of an hydroxyl and a nitrogen in the side ring structure, presented activity close to that of the other glycoalkaloids under study.

Interactions between α-solamargine and other alkaloids

The interaction between α-solamargine and α-chaconine is shown in figure 2. A strong synergistic effect between these two glycoalkaloids was observed in terms of cytotoxicity (FIC= 0.65 ± 0.03) (Fig. 2A). Similarly, the interaction of α-solamargine and α-solasonine appears to be synergistic with a FIC= 0.68 ± 0.06 (Fig. 2B). A comparison of the joint action of α-solamargine and α-chaconine or α-solamargine and α-solasonine indicated they were similar. In contrast, the effect of α-solamargine and α-solanine was almost additive as indicated by the value of FIC=

0.90 ± 0.06 (Fig. 2C). This led us to assume that the combined effect of spirosolane and a solanidane alkaloids attached to the same carbohydrate moiety produced a synergistic effect but that synergism also occurs if the same steroidal aglycone moieties associated to chacotriose and solatriosa are combined with different sugar moieties. This kind of behavior could be a characteristic of the interaction of these compounds in different systems as revealed by the synergic effect of α-solamargine and α-solasonine on fungal growth inhibition reported by Fewell, Roddick and Weissenberg (6). On the other hand, this effect could be low or absent in mixtures of glycoalkaloids with different structural rings attached to different sugar moieties.

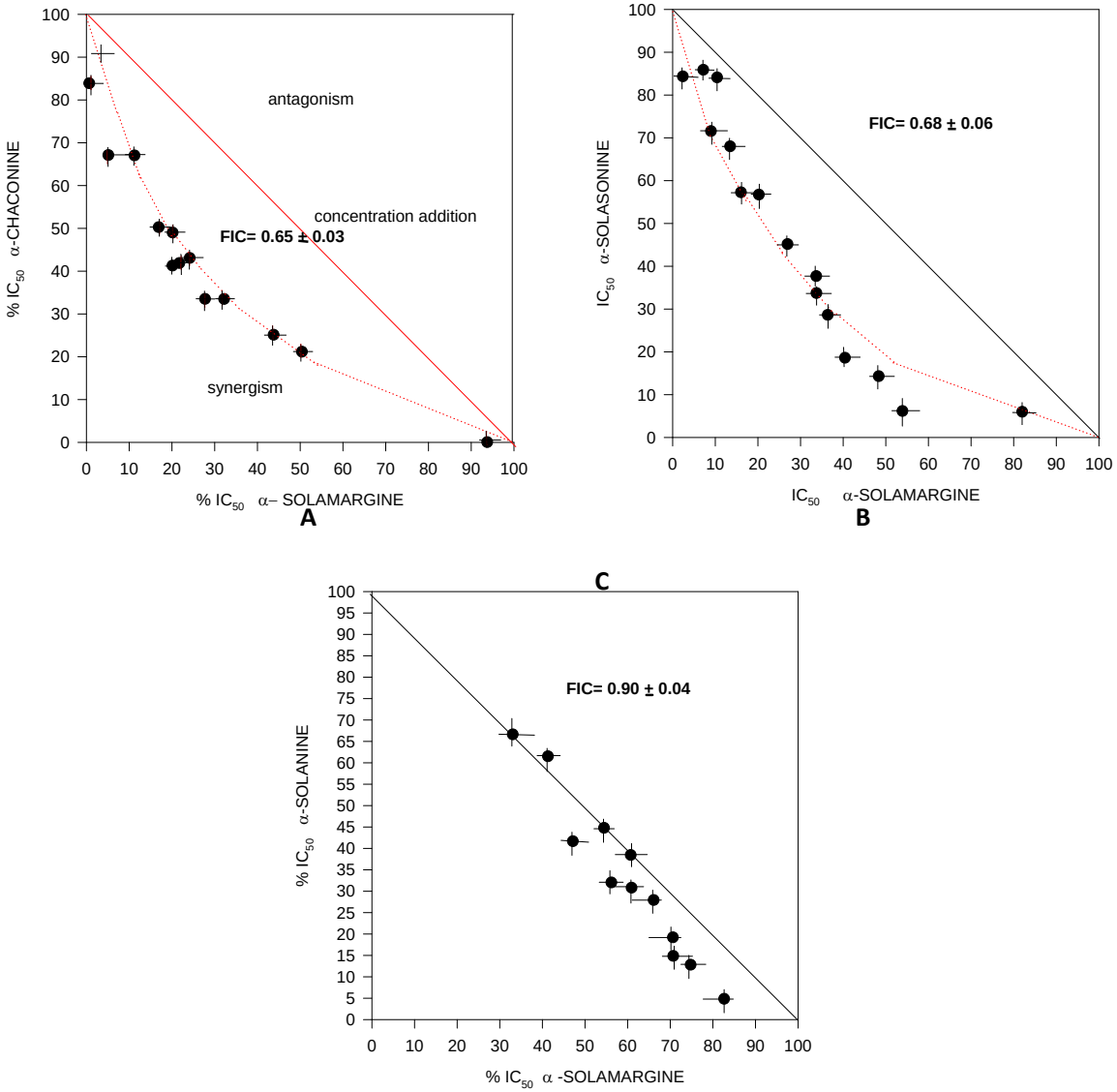


Figure 2. Isobole diagram for the joint action α-solamargine and α-chaconine (A); α-solamargine with α-solasonine (B), and α-solamargine and α-solanine (C) on citotoxicity of human A375 melanoma cells. Data points with 95 % confidence intervals for α-solamargine (horizontal) and α-chaconine, α-solasonine or α-solanine (vertical) are plotted in % IC_{50} values. Line indicates a theoretical region of no-effect (concentration-addition). FIC were determined as a mean ± s.d.of the experimental points. The assay was performed simultaneously in a 96-well microplates in a design bidimensional in which one of the glycoalkaloids disposed in ceach column was added in increased concentrations to the cells in RPMI-1640 medium containing 10 % fetal bovine serum and 1 % penicillin-streptomycin either alone or in presence of a fixed suboptimal concentration of the other glycoalkaloids. In each row was added the second glycoalkaloid alone and this glycoalkaloid in presence of a fix suboptimal concentration of the former glycoalkaloid. In this way was determined the IC_{50} of each glycoalkaloid alone and the IC_{50} of each compound in the presence of a constant amount of the other interacting compound (IC_{50} combined). The experiment was realized in plates by triplicate

Interactions between α-chaconine and other alkaloids

In the present study, α-chaconine and α-solanine acted synergistically with a FIC= 0.71 ± 0.02 (Fig 3A). This interaction appears to be a common feature as was shown by published data on the interaction of these glycoalkaloids on other systems, such as destabilization of cell membranes (19), lysis of phospholipid/sterol liposomes (20) as well as in antifungal activity (6). α-chaconine and α-solasonine presented an additive effect with a FIC = 0.82± 0.05 (Fig. 3B).

Interactions between α-solanine and α-solasonine

Combined mixtures of α-solanine and α-solasonine acting upon melanoma cells appeared to be synergistic as indicated by a FIC= 0.73± 0.01 (Fig. 3D) even though their effect was lower than observed in mixtures of α-solamargine and α-chaconine.

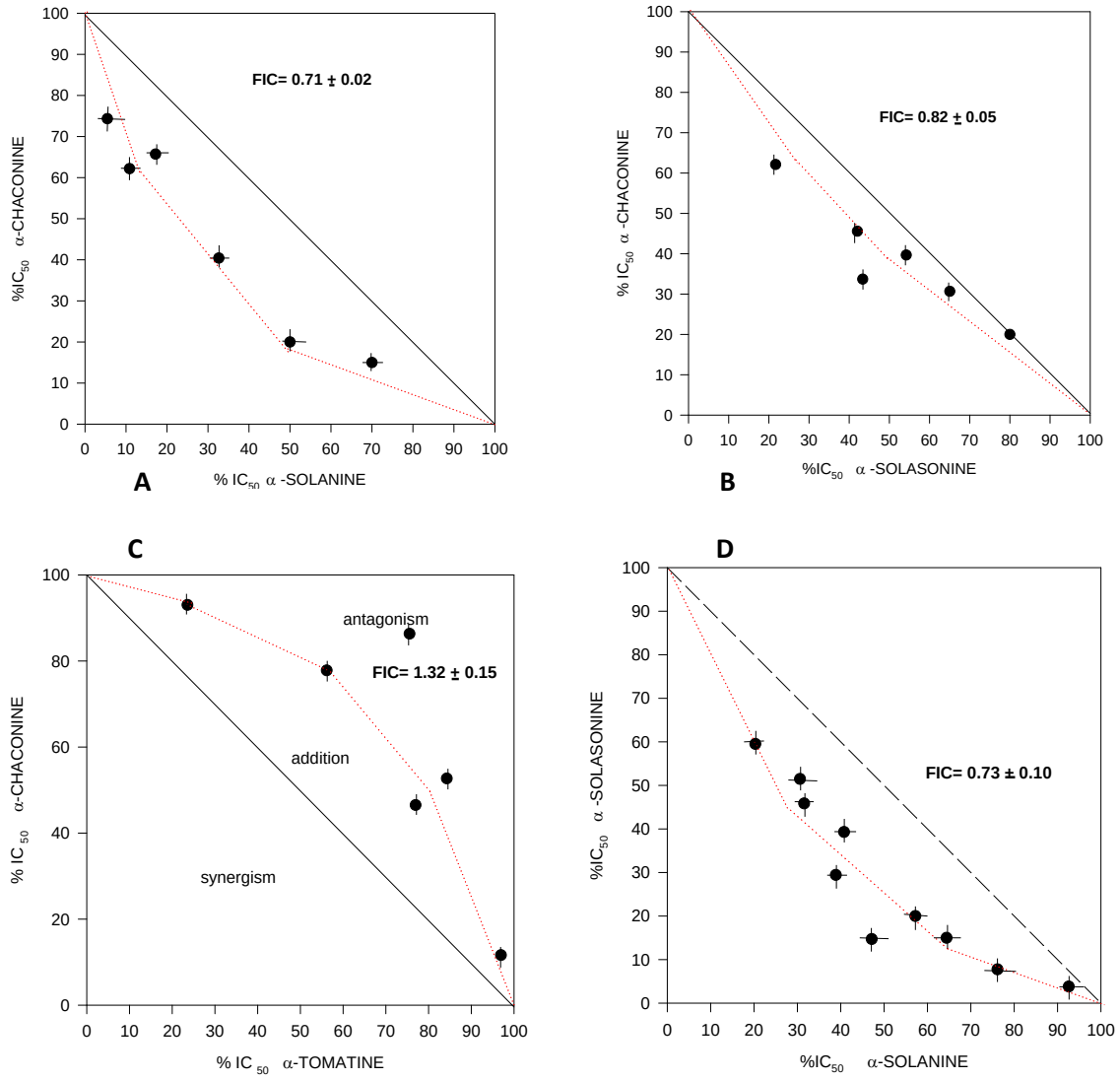


Figure 3. Isobolograms describing the cytotoxic effect of α-chaconine and α-solanine (A); α-chaconine with α-solasonine (B); α-chaconine with α-tomatine (C) and α-solasonine with α-solanine (D), respectively on citotoxicity of human A375 melanoma cells. The line indicates a theoretical region of no-effect (concentration-addition)

Interactions between α-tomatine and other alkaloids

Even through α-chaconine showed the highest activity followed by α-tomatine after 24 h of incubation, it was observed that α-tomatine, a spirosolane glycoalkaloid, appears to be the most cytotoxic glycoalkaloids against these cells after 72 hours as compared with the other glycoalkaloids tested (Table 1).

The interaction between α-chaconine and α-tomatine seemed to be antagonic as revealed by the FIC value of 1.32± 0.15 (Fig. 3C). Again, we observed that the interaction of two glycoalkaloids with the same steroidal structure and different sugar moieties or vice versa acted synergistically, while the observed effects on mixtures of glycoalkaloids, which differ both in steroidal structure and sugar composition, as in the case of α-chaconine and α-tomatine, could present either additive or antagonistic effects.

Table 1.-Cytotoxity IC_{50} (μM) values determined for each glycoalkaloid and aglycone of *Solanaceas* studied upon human A375 melanoma

	IC_{50} values (μM)		
N-Steroidal compound	24 hours	48 hours	72 hours
α-chaconine	4.80 ± 0.11	4.69 ± 0.12	2.34 ± 0.12
α-solamargine	9.80 ± 0.10	9.34 ± 0.11	8.65 ± 0.11
α-solanine	12.44 ± 0.80	8.67 ± 0.90	6.33 ± 0.42
α-tomatine	8.70 ± 0.10	4.83 ± 0.19	1.93 ± 0.19
α-solasonine	11.3 ± 0.10	10.18 ± 0.13	7.92 ± 0.56
solanidine	48.9 ± 2.2	26.6 ± 5.0	22.5 ± 3.7
solasodine	84.38 ± 3.45	64.6 ± 11.8	58.1 ± 9.2
demissidine	> 98	49-74	19.70 ± 1.97
tomatidine	> 120	82.1 ± 8.9	66.4 ± 2.9
solanocapsine	39.5 ± 4.65	20 .9 ± 1.2	11.63 ± 2.32
Actinomycin D*	0.018 ± 0.001	0.0088±0.0032	0.0028± 0.0002
Ketoconazole*	60.11 ± 0.57	54.3 ± 1.9	11.6 ± 1,9

Determinations were carried out on 20.000 cells/ hole in a total volume of 200 μL. Measurements are presented as mean ± standard deviation (s.d.). / *Ketoconazole, an antimycotic, and Actinomycin D, an inhibitor of RNAsynthesis, with a very broad anticancer spectrum was used as positive control

Interaction between solamargine with ketoconazole and Actinomycin D

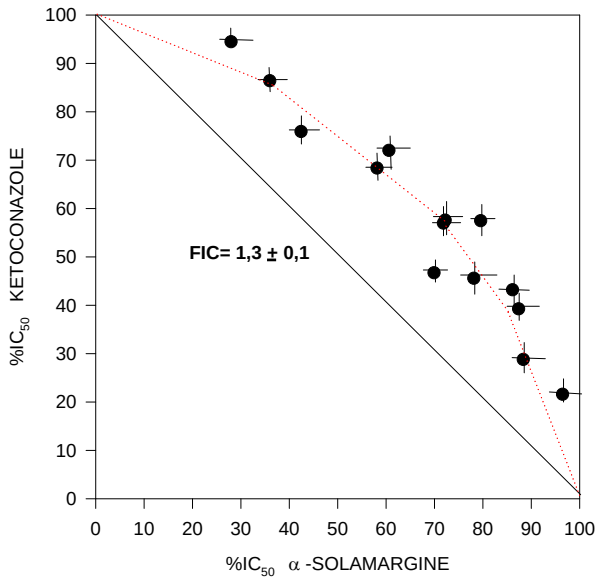
Ketoconazole, an antimicotic N-substituted imidazole derivative, has been studied in patients with prostate cancer (15). Also, It shows significant effects upon some fungal disease by inhibition of the ergosterol biosynthesis in fungal cells with the accumulation of 14 α-methyl sterols. In *Trypanosoma cruzi* inhibits the hydroxylation of C-14 demethylation of lanosterol, which stop the biosynthesis of ergosterol (16).

As a comparison, it was determined the interaction between solamargine and ketoconazole as indicated in the Figure 4.

(FIC= 1.30 ± 0.10). The isobologram shows antagonic effects between these two compounds.

Actinomycin D, a potent anticancer agent, even no selective for diverse studied cells blocks the synthesis of ARN (17). Even when the data is not conclusive, it was observed that the interaction between α-chaconine and actinomycin D seems to be synergic, as is shown by the fact of a FIC=0.64 ± 0.16. (data not shown). Also, the interaction between α-solamargine and this compound is apparently synergic. However, the data obtained do not show a confiability of this behavior.

Figure 4. Isobole diagram for the joint action α-solamargine and ketoconazole on citotoxicity of human A375 melanoma cells. Data points with 95 % confidence intervals for α-solamargine (horizontal) and ketoconazole (vertical) are plotted in % IC_{50} values. Data points are in the synergism section of the graph. Line indicates theoretical region of no-effect (concentration-addition)



Discussion

The literature contains several reports of the cytotoxic activity of nitrogen-containing steroids of the Solanum type against diverse cancer cell lines. Solanum americanum Miller (black nightshade) has been known to have important properties of possible medicinal use as in the treatment in cancer diseases. In fact, among the most investigated roles of Solanum nigrum and their glycoalkaloids are their properties upon cancer lines.

Data presented here indicated that the activity of Solanum alkaloids upon human melanoma cells depends on the steroidal ring structure and also on the sugar moiety that is attached to it. Thus, solanidanes according to the tests performed on this work, show to be more cytotoxic than spirosolanes. On the other hand, oligosaccharides as chacotrioside show to be more effective than solatrioside. Absence of sugar moieties drastically reduces the cytotoxic effect of Solanum alkaloids. In effect, aglycones showed lower activity against human melanoma cells, which indicates that the presence of the sugar moiety is essential. Steroidal glycosides with solanidane ring structure and a chacotriose sugar moiety appears to be the most cytotoxic compounds in terms of cytotoxic and synergistic activity against this type of cells. The effect appears to be more pronounced in α-chaconine than in α-solamargine, probably due to the rigid ring structure of solanidanes which have a tertiary nitrogen atom. The presence of rhamnose in the saccharide moiety appears to be an important feature in the structure of the active compounds. Interestingly, solanocapsine, which possess at C-3 an amino group instead of an hydroxyl presents an activity half way between glycoalkaloids and aglycones.

The activity of glycoalkaloids such as α-solamargine and α-chaconine in different biological systems indicates that such compounds could have lytic properties due to their intimate relation to the lipidic composition of the membranes.Perhaps, the association of glycoalkaloids with lipids would allow them to act upon sterols and phospholipids in the membranes or inhibit some enzymes of the sterols biosynthesis. Compounds which use the same mode of action should theoretically be strictly additive (concentration additive) when acting in combination .However, it is possible that compounds which act synergistically could act upon different stages on a biosynthetic pathway. The synergistic effect implicate a potentiation in activity of the drugs when used in combination. Thus, the combined effect of ketoconazole and α-solamargine upon the melanoma cells could be due to the combined effects of both compounds acting on sterol biosynthesis and/or membrane structure.

Acknowledgments

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Conflicts of interests

We declare no conflicts of interests.

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