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CYTOTOXIC ACTIVITY OF ACNISTINS UPON HUMAN CANCER CELLS

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Summary

The cytotoxicity activity against human A375 melanoma, MCF7 breast cancer and K652 leukemia cells of acnistins A, E and L, isolated from the leaves of A*cnistus arborescens* (L) Schletcht (Solanaceae) and the derivatives, 2,3-5,6-diepoxi-acnistin A and acnistin F, obtained in the laboratory by hemisynthesis, were examined using a microculturetetrazoliun (MTT) assay. The objectives were to determine if these compounds have cytotoxic activity against cancer cells; to determine the type of interaction between acnistin A with ketoconazole and a-solamargine as well as the type of interaction between acnistin E with a-solamargine and a-chaconine, two compounds which interfere with neutral lipid metabolism. On the other hand this study was addressed to obtain evidence about acnistin moieties that are important for activity. Results indicated that acnistins A, E and L were cytotoxic to human A375 melanoma cells with IC_{50} values ranging between 0.19 to 80.5mM, depending on the time of exposition to the steroidal lactone. Also, Acnistins A and E were cytotoxic toward MCF7 breast cancer and K652 leukemia cells, with IC_{50} values ranging from 10.2 to 134.4mM. The effectiveness of acnistins A and E against the human A375 melanoma cells was greater than against the other cell lines. Combinations of acnistin A with ketoconazole and a-solamargine; and acnistin E with a-solamargine and a-chaconine tested against human A375 melanoma cells showed antagonism between these compounds, a fact which could give some insight into the mechanism of action of the acnistins.Tthe results suggested that presence of an a,b-unsaturated ketone, an epoxy moiety between C-5 and C-6, and an hydroxyl group at C-17 are required for activity.

Key words: acnistins, withanolides, melanoma, cytotoxic effects, antagonism

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Introduction

Whitanolides are a group of natural steroidal lactones, isolated from several species of *Solanaceae* (1), characterized by a 28 carbon skeleton with a 9 carbon side chain and a 6-member lactone ring. Using the approach of testing different natural antitumoral compounds, the analysis of the effects of acnistins, withanolide-type lactones, was propose because some withanolides (steroidal lactones derived of ergostane) show biological activities of medicinal value (2). There are evidences that with anolides show antitumoral (3-5), antiinflamatory (6), cytotoxic (7), immunosuppresive (8) and antifungal (10) activity. Also, it has been demonstrated that some of them elicit humoral and cell-mediated immune response (11), inhibit cell proliferation; induce apoptois (12) and exhibit antileishmanial and trypanocidal activity (13,14). Acnistin A (1), E (**2**), and L (**3**), are three with anolide - type lactones, with a bicyclic side-chain at C17, isolated from *Acnistus arborescens* (L) Schlecht that grows in the Venezuelan Andes, and *Dunalia solanacea* Kunth (15-17). The cytotoxicity of acnistin A,E,L and two of their derivatives on human A375 melanoma, breast MCF7 cancer, and leukemia K652 cells is reported. The activity of acnistins upon these cell lines was compared to those of ketoconazole, an antimycotic agent which has been used in the treatment of prostate cancer (18); the steroidal glycosidesa-solamargine and α-chaconine, two Solanum glycoalkaloids, which show antitumoral activities against different cancer cell lines (19) and actinomycin D, an inhibitor of RNA synthesis anda known anticancer agent (20).

Materials and Methods

General Experimental Procedures

Melting points were measured on a Fisher Johns hot stage, and they are uncorrected. Specific rotations $[a]_D$ were measured at the sodium-D line using a Jasco electro polarimeter model DIP-370; the concentration c, is given in g/100 mL. IR spectra were recorded on a Perkin- Elmer 1720X FT-IR spectrometer as KBr discs. UV spectra were recorded on a Shimadzu 1606 spectrometer. ^1H and ^{13}C-NMR measurements were performed on a Bruker Avance DRX-400. For TLC Merck 60 F254 plates were used and as solvent a mixture of C_6H_6:EtOAc (1:1) was employed. Columns for vacuum chromatography were packed with TLC grade Merck Silica gel 60 H and eluted with C_6H_6 and C_6H_6/EtOAc mixtures. All the chemical and reagents used in the present studies were of an analytical grade. Ketoconazole and a-chaconine were obtained from Sigma Biochemical Company. a-Solamargine was isolated as described by Chataing et al. (21) and actinomycin D from *streptomyces sp.* was obtained from Calbiochem. Organic solvents were freshly distilled before use.

Extraction and purification of acnistin A, E and L.

Acnistin A(**1**), E(**2**) and L(**3**) were isolated from the leaves of *Acnistus arborescens* according to the method described by Usubillaga *et al*. (15). The identity of acnistin A, E , and L was established by direct comparison with authentic **control** samples (TLC, mp and NMR spectra) (15, 16).

*Acnistin F(**4**): (17R,20R,22R,24R,25R)-5α,6β,17β,28-tetrahidroxy-1-oxowitha-2-enolide*

Acnistin A (100 mg) was treated with 1.0 M methanolic NaOH. The solution was left to dry at room temperature. The solid mass was treated with MeOH, filtered to separate the Na_2CO_3 that had formed by atmospheric CO_2 absorption, and **4** was obtained from the filtrate as fine white needles (70 mg) from MeOH, mp > 300° ; $[\alpha]_D$ –136° (c 0.020, MeOH); UV (MeOH) λ_{max} (log ε) 314 (3.20) nm; IR (KBr) ν_{max} 3480, 2940, 1716, 1675, 1130, 1128,1080, 956, 880 cm^{-1}; ^1H- NMR (CD_3Cl+ 2 drops of CD_3OD): δ 5.80 (1H, dd, *J* = 2.4 , 10.1 Hz, H-2); 6.58 (1H, ddd, *J* = 2.4, 4.9, 10.1 Hz, H-3); 4.78 (1H, d, J= 2.3 Hz, H-22); 3.60 (1H, brS, H-6); 3.23 (1H, dd, J= 2.4, 9.7, $H4_{ax}$); 2.54 (1H, t, J= 12.5 Hz, H-21b); 2.33 (1H, t, J=8.8 Hz, H-20); 2.08 (1H, dd, *J* = 4.9, 9.7,Hz, H-4_{eq}); 2.02 ((1H, d, J= 11.6 Hz, H-23b); 1.89 (1H, m, H-12b); 1.81 (1H,dd, J=

2.3; 11.6 Hz, H-23a); 1.65 (1H, m, , H-7a); 1.62 (1H, m, H-15-b); 1.53 (1H, m, H-7b); 1.49 (1H, m, H 21a); 1.47 (3H, S, H-27); 1.44 (1H, m, H-16a); 1.39 (1H, m, H-15a); 1.29 (3H, S, H-19); 1.26 (1H, m, H-14); 1.17 (3H, S, H-28); 0.92 (3H, S, H-18); ^{13}C-NMR (100.62 MHz, CD_3Cl+ 2 drops CD_3OD): δ 205.4(C-1), 179.8 (C-26), 142.3 (C-3), 128.8 (C-2), 85.8 (C-17), 84.6 (C-22), 77.4 (C-5), 77.0 (C-25), 74.4 (C-6), 52.4 (C-10), 52.0 (C-14), 50.8 (C-20), 47.9 (C-13), 45.8 (C-24), 41.6 (C-23), 41.2 (C-9), 37.5 (C-21), 37.1 (C-16), 35.8 (C-4), 34.2 (C-7), 33.4 (C-12), 31.3 (C-8), 25.7 (C-27), 24 (C-11 and C-15), 20.3 (C-28), 16.2 (C-19), 15.1(C-18) . This compound had the same IR, ^{1}H, and ^{13}C-NMR signals as acnistin F, described by Luis *et al.* (17).

2,3-5,6-diepoxy-acnistin A(**5**)

To a solution of acnistin A (**1**) [100 mg] in acetamide($HCONMe_2$, 5 mL),4 mL of H_2O_2 (30%) and a catalytic amount of KOH were added and the reaction mixture kept at room temperature for 5 minutes.The mixture was diluted with water (20 mL), extracted with diethyl ether (3 x 100mL), and the ether extract was chromatographed over silica gel, yielding the2,3-5,6-diepoxide derivative **(5),** which crystallized from $CHCl_3$ as fine needles [83 mg, mp 294.5 °C]. ^{1}H- NMR (400.13 MHz, $CDCl_3$): δ 4.74 (1H, d, J= 3.0 Hz, H-22), 3.62 (1H, dd, J=3.9, 5.0 Hz, H-3), 3.55 (1H, d, J=5.0 Hz, H-2), 3.07 (1H, brS, H-6), 2.58 (1H, ddd, J=2.0; 8 10 Hz, H-4e**),** 2.41 (1H, d, J= 14 Hz, H-16a), 2.29 (1H, t, J=8.1 Hz, H-20), 2.14, (1H, dd, J= 1.8, 9.7 Hz, H-7a), 2.02 (1H, d, J=13.0 Hz, H-23b),1.96 (1H, dd, J=3.9, 9.8 Hz, H-4a), 1.82 (1H, dd J=3.9, 9.8 Hz, H-4a, 1.78 (1H, dd, J=3.9; 10.7 Hz, H-12a), 1.78 (1H, dd, J= 3.9; 10.7 Hz, H-12a), 1.67 (1H, br.s, H-12b),1.62 (1H, m, H-16b), 1.54 (1H, m, H-°15b), 1.48 (1H, m, H-21a),1.47 (3H, s, H-27), 1.43 (1H, m, H-11a), 1.35 (1H, m, H-8),1.30 (1H, m, H-21e),1.19 (1H, m, H-9),1.19 (3H, s, H-28), 1.07 (3H, s, H-19), 0.82 (3H, s, H-18). ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ 207.6 (C-1), 179.6 (C-26), 85.3 (C-17), 84.0 (C-22), 76.7 (C-25), 61.8 (C-6), 60.4 (C-5), 55.9 (C-2), 53.5 (C- 3), 51.4 (C-20), 50.6 (C-10), 50.1 (C-14), 47.0 (C-13), 45,5 (C-24), 41.2 (C-23), 39.9 (C-9), 37.2 (C-16 and C-21),36.9 (C-4), 32.6 (C-12), 30.9 (C-7), 30.1 (C-8), 25.6 (C-27), 23.5 (C-15), 20.3 (C-11), 19.9 (C-28), 14.0 (C-18), 12.0 (C-19). The chemical structure of acnistins A,E,L,F and the diepoxi-derivative are shown in Figure 1.

Biological assays in vitro

1. Microculture tetrazolium (MTT) assay for growth inhibition of cells and IC_{50} determination.

Growth inhibition was estimated by measuring the quantity of formazan produced at 490 nm by the number of living cells in a culture using the Cell Titer 96 aqueous one solution (MTS/PMS) as recommended by the manufacturer (Promega, Charbonnieres, France). The assay was carried out as follows: human cancer cells, after counting in a haemocytometer, at a concentration of 20,000 cells/well were seeded into a 96-well microplate (2x 10^4 cells/well in a total volume of 200 µL) added RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Acnistins**1-5**, steroidal glycosydes, ketonazole, or actinomycin D were added at varying concentrations in the range between 100 – 0.01 µg/ml. After being incubated at 37 °C in a humidified atsmophere of 5% CO_2 for 24, 48 or 72 hours, 40 µL MTT/PMS was then added to each well. The plates were incubated at 37 °C in a 5% CO_2, 95% air for 4 hours and then read on a micro-plate reader using a wavelength (O.D.) of 490 nm. Absorbances taken from cells grown in the absence of drugs were taken as 100% cell survival (control).Actinomycin D,was used as a positive control (20). For each compound and standard drug, the decrease in the absorbance values were analyzed by Dunnett´s one-tailed test (19). The dose-response lines were converted to probit and fitted using least-squares linear regression and IC_{50}(concentration required to reduce viability by 50%) against the cancer cell lines and its 95% confidence intervals were calculated. In those experiments concerning the interaction between acnistins and steroidal glycosides or ketoconazole, it was determined simultaneously in each experience the IC_{50} value of each interacting compound alone (IC_{50} compound alone) and the IC_{50} of each compound in the presence of a con-

stant amount of the other interacting compounds (IC_{50} combined). These measurements were performed after 24 h of incubation with the mixture of steroidal compounds or ketoconazole. The assay was performed in a bidimensional simultaneously design in a 96-well microplate, mixing the two drugs in fractions of their IC_{50} and serially diluted. Acnistin disposed in each column was added in increasing concentrations to the cells in RPMI-1640 medium (10% fetal bovine serum and 1% penicillin-streptomycin) either alone or in presence of a fixed suboptimal concentration of the other compound (ketoconazole, a-solamargine ora-chaconine). In each row, Ketoconazole, a-solamargine ora-chaconine, either alone or in presence of a fix suboptimal concentration of acnistin A or E was added. In this way the IC_{50} of each compound alone and the IC_{50} of each compound in the presence of a constant amount of the other interacting compound [IC_{50} combined) was determined. The experiment was performed in triplicate plates in three replicate wells.The fractional (percentage) IC_{50} foreach combined mixture was determined as: (IC_{50} compound combined/IC_{50} compound alone) x 100%. These data were plotted as isobolograms, constructed by plotting the IC_{50} values of the single agents on the X and Y axis, respectively, as described by Rayburn, Friedman and Bantle (22). The fractional inhibitory concentration (FIC) was defined as FIC= $IC_{50\ XY}$/ $IC_{50\ X}$ + $IC_{50\ YX}$/$IC_{50\ Y}$, 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 FIC = 1.0, then the effect was ascribed as additive; if the value FIC > 1.0, the effect was considered antagonist; and if FIC < 1.0 was considered that a synergetic combined effect was present (22,23).

2. Trypan blue staining of cells

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a simple colorimetric assay to measure cell cytotoxicity, proliferation and viability (19) . On the other hand, trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. To distinguish between influences on proliferation and viability two assays were performed, MTT assay for viability and trypan blue staining to measure cell number. Briefly, human A375 melanoma cells were prepared by triplicate at 50,000 cells/well in 96-well culture plates in absence and presence of concentrations from 2 to 28 mg/ml of the substances to be tested and incubated by 24 hours. Viability was determined by MTT assay and viable cell count by trypan blue staining mixing the same number of cells with an equal volume of 4% trypan blue and counted in a haemocytometer.

Statistical analysis

All data are expressed as mean ± standard deviation (s.d.) of at least three independent experiments in which each tested compound concentration was determined in three replicate wells. Student' s t-test was used for the statistical significance of data when needed. Statistical significance was considered when p value was less than 0.05.

Drug interaction data were analyzed by a Kolmogorov –Smirnov non parametric analysis.

Results

Trypan blue staining of cells

It has been reported (24) that the MTT tetrazoliun assay leads to false positive results when testing natural compounds with intrinsic reductive potential. The assay with MTT and its comparison with trypan blue staining showed that there are not interference of the compounds tested (Figure 2) with the MTT assay as indicated by the lineal correlation obtained by both methods (correlation coefficient r 0.984) which discard false positive results.

see Fig. 2

Human A375 melanoma cells were prepared by triplicate at 50,000 cells/well in 96 well culture plates in absence and presence of concentrations of 2 to 28 µg/ml acnistin A and incubated during 24

hours, then 20 µL/well of MTT/PMS reagent was added. After 1 hour at 37 °C, the absorbance at 490 nm was recorded using an ELISA plate reader. The same protocol was performed adding to the cells an equal volume of trypan blue 0.4% in PBS Dulbecco buffer and counting the number of cells in a Neubauer chamber. The correlation coefficient of the line was 0.984, indicating that there was a linear response between cell number and absorbance at 490 nm. The background absorbance shown at zero cells/well was not sustracted from these data.

Cytotoxicity of acnistin A and E upon melanoma cells and other cancer cells.

The cytotoxicity of Acnistins A(**1**), E(**2**), L(**3**), F(**4**) and the diepoxy-derivative of acnistin A (**5**)on melanoma A375 human cells is shown in Table 1. Exposure of melanoma cells to each acnistin, ketoconazole, α-solamargine as well as α-chaconine and actinomycin D, resulted in a dose-dependent reduction in the viability of cells. IC_{50} values measured at 24, 48 and 72h obtained from separate experiments indicated that acnistin A,acnistin E and acnistin L were cytotoxic to this cell line. Acnistin L was more cytotoxic toward melanoma cells than acnistin A and acnistin E. The derivatives acnistin F (**4**) and diepoxy- acnistinA (**5**) showed a low cytotoxic effect. Ketoconazole showed lower IC_{50} than acnistin E at 24 hours but higher at 48 and 72 hours when compared with this compound. In contrast, acnistin L showed the highest cytotoxic effect of all acnistins. Actinomycin D, used here as a positive control, revealed nanomolar level activity upon these cells.

see Table 1.

The growth inhibitory rate (%) (IR) calculated according to the following formula: IR=(1- mean OD of tested cells/ mean OD of control cells) x 100%.The dose-response lines obtained were converted to probit and fitted using least-squares linear regression and IC_{50} (concentration required to reduce viability by 50%) against the cancer cell lines and its 95% confidence intervals were calculated. P values were calculated comparing mean OD of tested cells with mean OD of control cells.

Determinations were assayed by at least three measurements in triplicate wells. The number of cells was 50.000 cells/ml in the assay. Values are means ±SD of three independent experiments. $p< 0.05$.

To corroborate the effect of acnistins upon cancer cells, other cell lines were tested (Table 2). Acnistin A showed higher cytotoxicity than acnistin E to those cell lines.

see Table 2.

Interaction of acnistin A with ketoconazole (FIC= 1.28 ± 0.08) and a-solamargine (FIC= 1.30 ± 0.09) in melanoma cells [Figures 3 and 4] and acnistin E with α-solamargine (FIC= 1.38 ± 0.09) and α-chaconine (FIC= 1.44 ± 0.03) [Figures 5 and 6] respectively, showed an antagonic effects in both cases, as indicated by the isobolograms.

see Fig. 3

Data points with 95% confidence intervals for ketoconazole (horizontal) and acnistin A (vertical) are plotted in % IC_{50} values. Data points are in the antagonistic section of the graph. The line indicates the theoretical region of no-effect (concentration-addition). FIC were determined as a mean ±s.d. of the experimental points; $p< 0.02$ and $*p < 0.05$ when compared the IC_{50} value of each interacting compound alone (IC_{50} compound alone) and the IC_{50} of each compound in the presence of a constant amount of the other interacting compounds (IC_{50} combined).

see Fig. 4

see Fig. 5

Discussion

The literature contains numerous reports on the

biological activity of the withanolides. For instance, 20-deoxywithanolide D is active against gram-positive bacteria (25), whitanolides from *Tubocapsicum anomalum* are cytotoxic (7) and immunosuppressive (8) and withaferin A exhibits antimicrobial, cytotoxic and inmunostimulating activities (10,26). All these compounds present a steroidal skeleton, an α,β-unsaturated carbonyl group, a 17-hydroxyl and some of them an epoxy moiety, as it is the case for acnistins A, E and L. The cytotoxic properties of these compounds have been attributed to the presence of the epoxy moiety and the 17β-hydroxyl group (1, 8).On the other hand, the immunossupressive effect has been attributed to the lactone ring and to their structural similarity with cortisol (1). The inhibitory activity of acnistins A, E, and L against melanoma cells is dose-dependent. The effect appears to be more pronounced with acnistin L than with acnistin A. This could indicate that the presence of a hydroxyl group at C-4, which could increase the polar character of acnistin L, also increases its activity.In this study it was observed that the absence of the epoxy ring, as in acnistin F, caused a decrease in activity. Introduction of an additional epoxy ring α,β between C-2 and C-3 as in **5** also decreased the biological activity, which seems to indicate that the presence of an intact α β-unsaturated carbonyl moiety is required for activity.It was interesting to observe the difference in activity between acnistin L and acnistin E, since the only structural difference between these two compounds is their configuration at C-17. Acnistin L has a α oriented hydroxyl, while on Acnistin E it is β oriented, as in most with anolides. This fact could indicate that acnistin L interacted with a protein in the tumor cell in a correct configuration. The fact that acnistin A/ketoconazole, acnistin A/α-solamargine, and acnistin E/α-solamargine and acnistin E/α-chaconine presented antagonic effects upon each other in their activity on melanoma cells, and both α-solamargine and chaconine as well as ketoconazole act on lipid/sterol metabolism (28, 29), suggest that acnistins affects the membranes of these cells. Ketoconazole, a N-substituted imidazole, is an active antimicotic agent active against prostate cancer (18, 28), which blocks testosterone synthesis in humans and rats (28,30). At high doses ketoconazole decreases the serum adrenal androgens, androstenedione and dihydro-epiandrosterone.At high concentrations (>10^{-7} M) also affects some mammalian cytochrome P450- dependent enzymes inhibiting as such the enzymes 17,20-lyase and 17α-hydrolase (28, 29). Added to fungi and *Trypanosomacruzi*, ketoconazole acts upon 14α-demethylase, a cytochrome P450 dependent enzyme, inhibiting the hydroxylation step of lanosterol C-14 demethylation, and stopping the ergosterol biosynthesis by the accumulation of 14α-methyl sterols (31, 32). Moreover, some *solanum* glycoalkaloids affect sterol biosynthesis. Inhibition of synthesis of cholesterol from 24,25-dihydrolanosterol by solacongestidine, solafloridine and solasodine in rat liver homogenates has been reported by Kusano *et al* (27, 32). The observed effects suggested that the action of acnistins upon these cancer cells could be exerted either by affecting directly the lipids on the membrane or indirectly by affecting some enzymes of the sterol metabolic pathway. The cytotoxic activity of acnistins against cancerous cells under the present study appears to depend of the presence of the epoxy moiety between C- 5 and C-6; a hydroxil group on C-17; and a double bond between C-2 and C-3**.**

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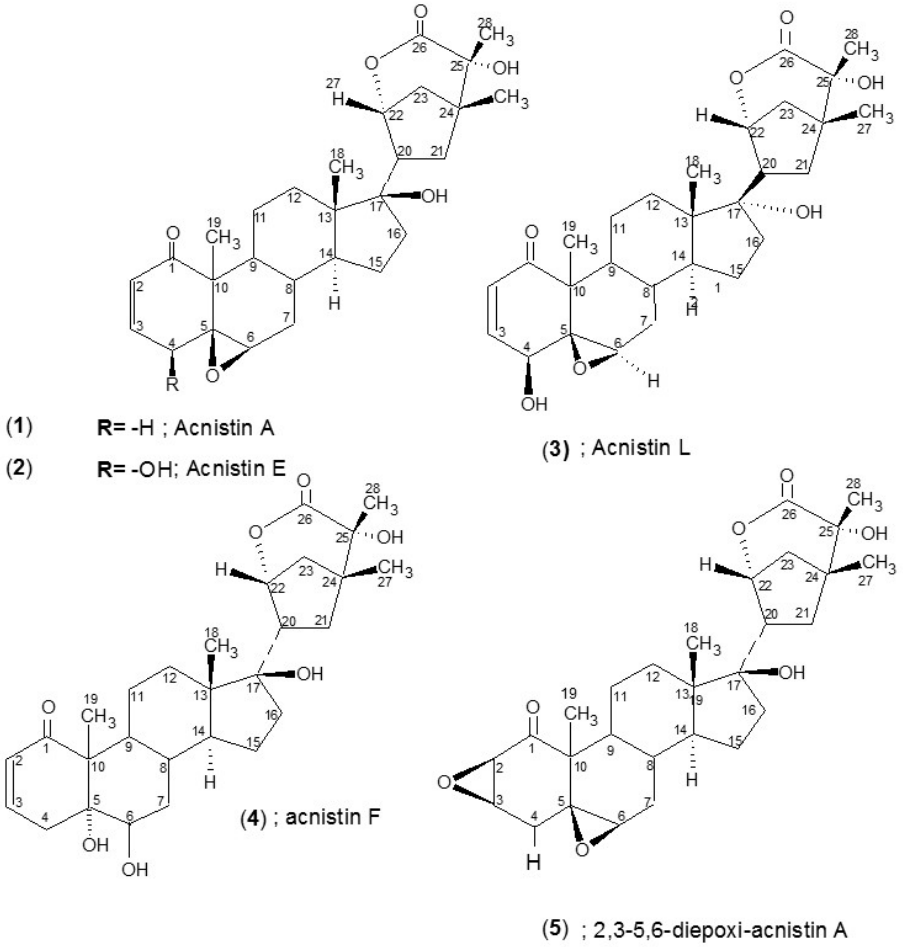

Figure 1.-Structure of acnistins A (1), E (2), L(3), K(4) and diepoxy-acnistin A (5)

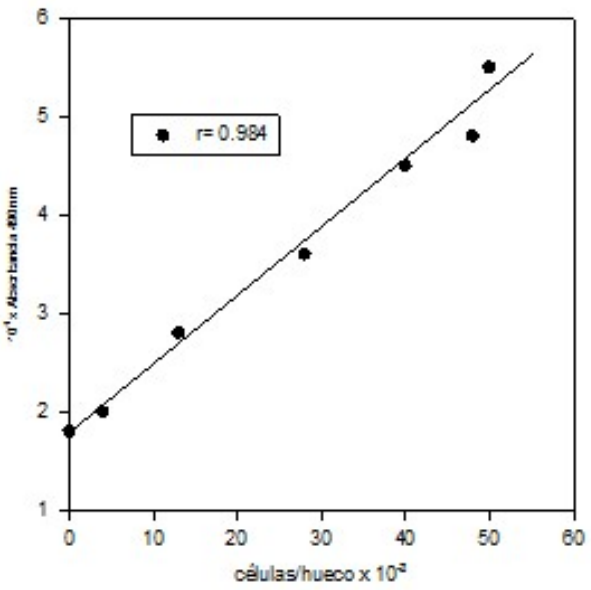

Figure 2. Correspondence between the number of cells counted by the method of Trypan. Blue and the absorbance determined at 490 nm using the MTT/PMS assay.

Cell type	IC_{50} (μM)		
Time (hours)	**IC_{50} 24 h**	**IC_{50} 48 h**	**IC_{50} 72 h**
Acnistin A (**1**)	28.6 ± 3.8	18.6 ± 2.1	8.3 ± 2.8
Acnistin E (**2**)	80.5 ± 4.0	43.7 ± 4.0	12.4 ± 4.3
Acnistin L (**3**)	9.6 ± 0.9	0.8 ± 0.1	0.19 ± 0.02
Acnistin F(**4**)	>205	>205	>205
Diepoxi-acnistin A (**5**)	92.5 ± 5.5	103 ± 5	84.0 ± 2.0
Ketoconazole	60.1 ± 0.6	54.3 ± 1.9	41.6 ± 1.9
α-solamargine	9.8 ± 0.1	8.1 ± 0.1	7.5 ± 0.1
α-chaconine	4.8 ± 2.1	4.7 ± 0.1	2.3 ± 0.1
Actinomycin D	0.018 ± 0.001	0.0088 ± 0.0032	0.0028 ± 0.0002

Table 1.-Cytotoxicity IC_{50} values (μM) of acnistins, ketoconazole and α- chaconine and α-solamargine upon human A375 melanoma cells. Actinomycin D was used as a positive control.

	IC_{50} (μM)					
Cell Type		**MCF 7**			**K562**	
Time (hours)	24	48	72	24	48	72
Acnistin A (1)	44.6 ± 1.1	18.6 ± 2.0	10.2 ± 2.5	80.8 ± 4.0	46.8 ± 4.2	21.7 ± 2.6
Acnistin E (2)	134.4 ± 4.4	130.0 ± 4.0	≥120	79.8 ± 4.2	70.8 ± 4.2	68.2 ± 4.2
Acnistin F (4)	ND	ND	ND	≥212	111 ± 8	119 ± 4
Diepoxi-acn A (5)	ND	ND	ND	166 ± 5	113 ± 12	109 ± 2

Table 2.-Cytotoxicity IC_{50} values (mM) of acnistins upon human breast MCF7 cancer

Determinations were assayed by at least three measurements in triplicate wells.
Values are means ±SD of three independent experiments. $p < 0.05$ calculated comparing mean OD of tested cells with mean OD of control cells.
ND: no detected values

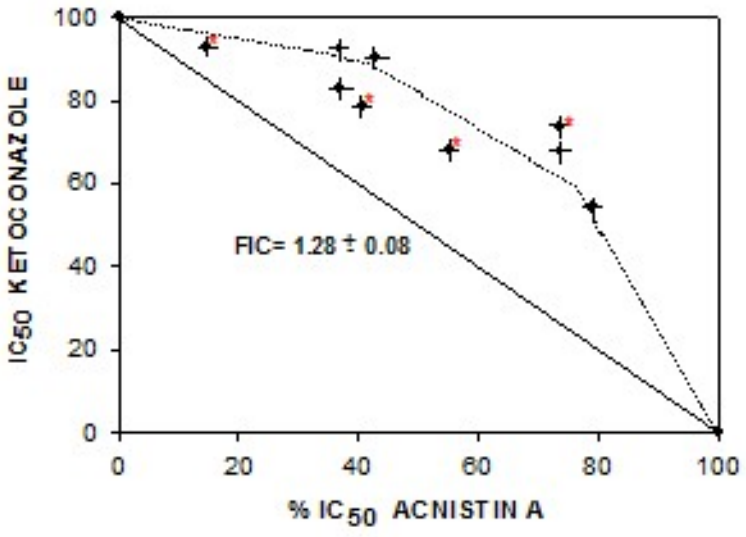

Figure 3.-Isobologram of the interaction between acnistin A and ketoconazole.

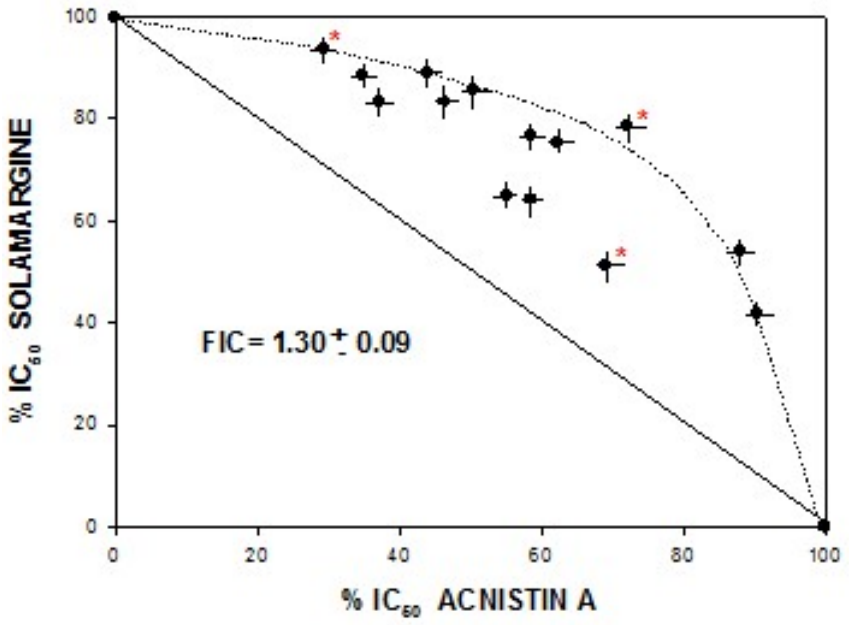

Figure 4.-Isobologram of the interaction between acnistin A and α-solamargine.

The experiment was performed in a similar way as experiment indicated in Fig. 3.

p< 0.02 and *p < 0.05 when compared the IC_{50} value of each interacting compound alone (IC_{50} compound alone) and the IC_{50} of each compound in the presence of a constant amount of the other interacting compounds (IC_{50} combined).

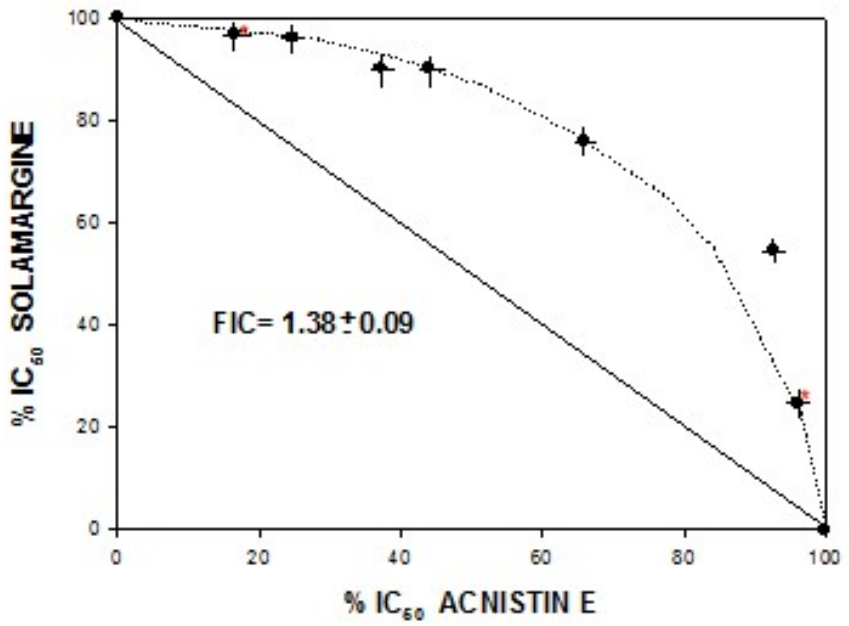

Figure 5.Isobologram of the interaction between acnistin E and α-solamargina.

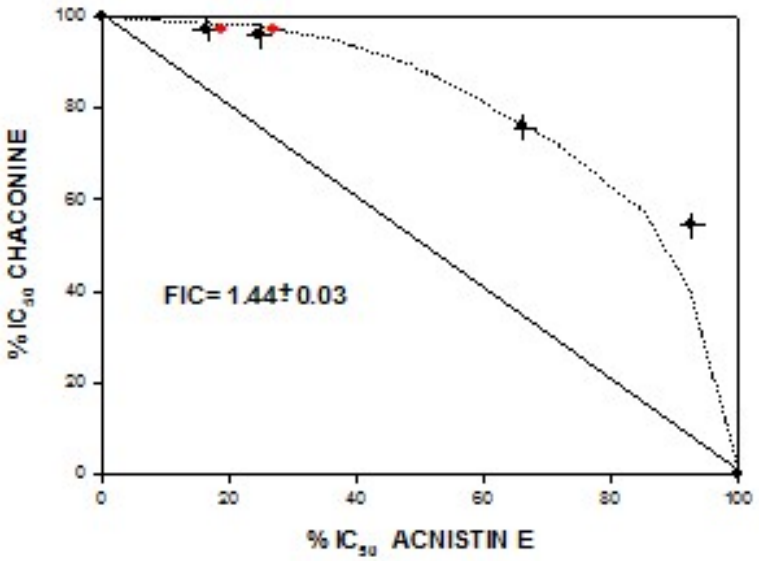

Figure 6.Isobologram of the interaction between acnistin E and α-chaconine