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In Silico Study of Structural and Geometrical Requirements of Natural Sesquiterpene Lactones with Trypanocidal Activity

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Abstract: Chagas' disease, caused by the intracellular protozoan *Trypanosoma cruzi*, is one of the most serious health problems throughout South America. Despite the progress that has been made in the study of its biochemistry and physiology, more efficient chemotherapies to control this parasitic infection are still lacking. In this paper we report the trypanocidal and cytotoxic activities of a series of sesquiterpene lactones, isolated from Asteraceae medicinal plants. The significant trypanocidal activity and high selectivity indexes found for many of the compounds evaluated, prompted us to undertake a quantitative structure-activity relationship study. A model using 3D molecular descriptors allowed us to set up a high correlation of the observed activity and the atomic spatial arrangement of these sesquiterpene lactones closely related to steric parameters.

Keywords: QSAR study, sesquiterpene lactones, trypanocidal activity.

INTRODUCTION

Chagas´ disease or American trypanosomiasis is a vector-transmitted parasitic disease which is caused by the flagellated protozoan microorganism *Trypanosoma cruzi* (*T. cruzi*). It represents a major public health problem, mainly in Latin America, where approximately 10 million people are affected and around 100 million are at risk of infection [1]. The disease is being expanded by population migrations to metropolitan areas of the American continent and to developed countries mainly via blood transfusion, organ transplantation and congenitally [2]. However, an effective treatment has not yet been found. The nitroheterocyclic compounds nifurtimox and benznidazole are the only drugs currently available in clinical practice. Both drugs produce strong side effects, do not eliminate the parasite during short-term therapies and resistant strains are emerging. Moreover, no effective vaccines are available and their development in the near future seems to be out of reach [3]. Consequently, more efficient drugs are needed.

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The development of new drugs from nature is one possible approach to achieve this goal. Over the last century, natural products have provided molecules with drug-like properties and high structural diversity used either as direct agents or as templates for synthetic modifications [4]. Among natural products, sesquiterpene lactones (SLs) are regarded as promising molecules for the development of new drugs.

SLs belong to the terpenoids, the largest group of natural compounds. They constitute an interesting class of plant chemicals due to their high diversity and biological activities. Mainly found in the Asteraceae (Compositae) family, they are biosynthesized by condensation of three five-carbon building blocks (isoprene units) and subsequent cyclization and oxidative transformation to produce a *cis* or *trans*-fused lactone. Around 5000 SLs structures have been identified so far and they are primarily classified on the basis of their carbocyclic skeletons as germacranolides, guaianolides, pseudoguaianolides and eudesmanolides. Biogenetically, the germacranolides represent the most primitive class with the other SLs having evolved from them [5]. In nature, SLs play a vital role in plant defense as antifungals, antibacterials and insecticides [6].

These compounds have a wide-range of biological activities such as antitumoral, antiinflammatory, analgesic, antibacterial, antifungal, cardiotonic and antiparasitic [7-9].

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Several SLs have been reported as potential leads for new antiprotozoal drugs [10-12].

The biological activities of the SLs are most likely attributed to the presence of an α,β-unsaturated carbonyl group that is able to alkylate functional biological macromolecules [10]. Nevertheless, the geometry of the whole molecule is also important for the biological activity [13].

Based on these premises, our research group has focused on the investigation of SLs with trypanocidal activity. We have previously reported the isolation of psilostachyin, psilostachyin C and peruvin, from *Ambrosia* species [14,15] and enhydrin from *Smallanthus sonchifolius* [16], with significant activity against *T. cruzi.* In this work, we report the anti-*T.cruzi* and cytotoxic activity of a series of fifteen selected natural SLs. The significant results of trypanocidal activity prompted us to carry out a quantitative structure-activity relationship (QSAR) study of the selected compounds isolated from Asteraceae plants.

SOURCE OF THE SELECTED SLs

All compounds were previously isolated and identified by our group [14-27], with the exception of parthenolide (**11**) which is a commercial sample (Sigma) (Fig. **1**).

In vitro Assay for Trypanocidal Activity and Cytotoxicity of SLs

The effect of the SLs (**1**-**15**) on *T. cruzi* epimastigotes was evaluated by a [^{3}H] thymidine uptake assay according to Sülsen *et al.* [15]. The pure compounds were tested at final concentrations ranging from 0.3 to 100 μg/ml. Stock solutions of the samples were prepared in ethanol:water (1:1). Epimastigotes in exponential growth phase were adjusted to 1.5×10^6 parasites/ml and seeded on 96 well-plates in the presence of different concentrations of the SLs. Parasites were cultured in triplicate for 72 h or 120 h. Control parasites were cultured in absence and presence of benznidazole (20 μM; Roche- Rio de Janeiro, Brazil). The percentage of growth inhibition was calculated as 100 – {[(cpm of treated parasites)/(cpm of untreated parasites)] x 100} and was expressed as the concentration (μM) that inhibited 50% of epimastigotes growth (IC_{50}).

The cytotoxic effect of the SLs on Vero cells was evaluated by using the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) colorimetric assay. Cells (5×10^4 cells/well) were seeded at a final volume of 150 μl in a flat-bottom 96-well microplate and cultured at 37°C in a 5% CO_2 atmosphere in the absence or presence of increasing concentrations of the compounds (1-50 μg/ml). After 24 h MTT was added at a final concentration of 1.5 mg/ml and plates were incubated for 2 h at 37°C. The purple formazan crystals were completely dissolved by adding 150 μl of ethanol and the absorbance was detected at 570 nm in a microplate reader. Results were calculated as the ratio between optical density in the presence and absence of the compound multiplied by 100. The 50% cytotoxic concentration (CC_{50}) was calculated for each SL. All experiments were made in duplicate. The selectivity index (SI) of each compound was calculated as the CC_{50} obtained with Vero cells divided by the IC_{50} obtained against *T. cruzi* epimastigotes. Results are shown in Table **1**.

Compunds **5**, **6**, **7** and **14** were the most active SLs with IC_{50} values between 0.24 and 0.84 μM. Recently, in the course of a bioassay-guided fractionation study, enhydrin (**5**) has shown a significant inhibition of *T. cruzi* amastigote replication [16]. Compounds **8**, **10**, **11** and **15** displayed moderate activities with IC_{50} values between 3.02 and 4.69 μM, which also resulted more active than the reference drug benznidazole.

None of the SLs studied showed remarkable unspecific mammalian cytotoxicity (except for compounds **3** and **9**). SLs **1**, **2**, **3**, **4**, and **9** exhibited values of SI below that of benznidazole. Estafietin (**6**) was the most selective SL (SI = 1789.25), being an excellent lead compound for further investigations.

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS

In order to rationalize the anti-*T.cruzi* activity of the fifteen SLs in terms of their three-dimensional arrangement, we employed the data shown in Table **1** to perform quantitative structure-activity relationships (QSAR) studies using molecular modeling and multivariate data analysis tools.

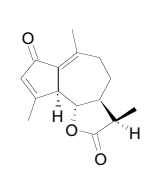
Geometry Optimization

With the aim of obtaining relationships between chemical structures and biological activities using computational approaches, it is necessary to find appropriate representations of the molecular structure of the compounds. To this end, the initial conformations (IC) of the compounds were drawn by means of the "Model Build" modulus of the HyperChem 7.0. We pre-optimized the molecular structures with the Molecular Mechanics Force Field (MM+) procedure included in the HyperChem software. The resulting geometries were refined by means of the AM1 semiempirical Method from the Molecular Orbitals Theory, setting the calculation of the Self Consistent Field (SCF) with a convergence limit equal to $1x10^{-7}$ and iteration limit equal to 1000, using the Polak-Ribiere algorithm and a RMS gradient norm limit of 0.001 kcal x $Å^{-1}$ x mol. The lowest energy conformer of each compound was corroborated by vibrational analysis.

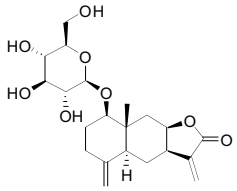
The study of the structural conformers was performed using Balloon 1.2.1 [28]. This software generates conformational ensembles (CEs) employing the multi-objective genetic algorithm (GA) together with the MMFF94 force field and was configured with the following options: *fullforce* (optimization of the found post-GA conformations), *nconfs*=90 (initial population size), *nGenerations*=500 (maximum number of generations) and *keepinitial* (output file including the IC).

Descriptors

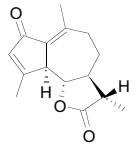
Molecular descriptors represent the way chemical information, contained in the molecular structure, is transformed and coded to deal with chemical, pharmacological and toxicological problems in QSAR studies. In order to establish appropriate descriptors, an investigation of the



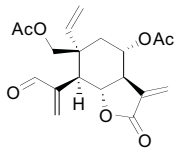
Achillin (**1**)



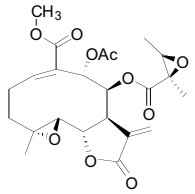
1-*O*-*β*-D-glucopyranosyl-asperilin (**2**)



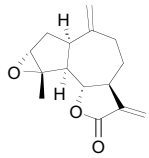
Desacetoxymatricarin (**3**)



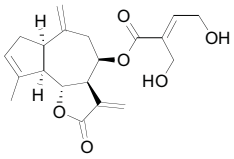
(5*R*,6*R*,7*R*,8*S*,10*S*)-8,14-diacetoxyelema-1,3,11(13)-trien-15-al-6,12-olide (**4**)



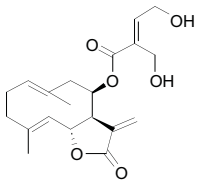
Enhydrin (**5**)



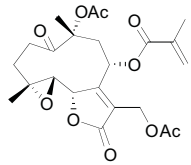
Estafietin (**6**)



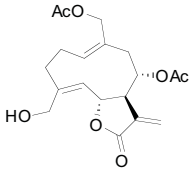
Eupahakonenin B (**7**)



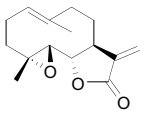
Eupatoriopicrin (**8**)



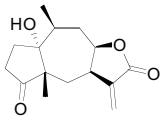
Glaucolide A (**9**)



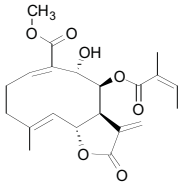
Minimolide (**10**)



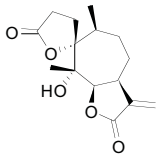
Parthenolide (**11**)



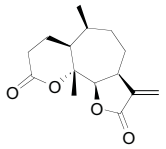
Peruvin (**12**)



Polymatin A (**13**)



Psilostachyin (**14**)



Psilostachyin C (**15**)

Fig. (1). Structures of the selected SLs.

similarity degree of SLs was performed. The CEs previously obtained were analyzed in terms of the topological superposition and electrostatic potential associated with the form of these molecules using the ShaEP 1.1.0 software [29]. The program was configured by default modifying the following options: *noOptimization*, *spotoffset*=0.33, *flatnesslimit*=0.50, *distancetoring*=2.5. The alignments obtained are given as field-graphs.

A series of 1D, 2D and 3D molecular descriptors were used to characterize each CEs in terms of their lipophilicity, connectivity indexes, topology, mass distribution and polar surface. Weighted Holistic Invariant Molecular (WHIM) descriptors, which contain information about whole molecular structure in terms of size, shape, symmetry and atom distribution, were also included in this characterization. The latter descriptors were calculated with the PaDEL 2.11 program [30]. Furthermore, we included molecular descriptors which encoded the presence of a particular sub-structure. A previous structure-activity relationship (SAR) analysis, has concluded that compounds possessing at least one potentially reactive α,β-unsaturated group as a pharmacophore, are more likely to display significant antiprotozoal activity [31]. To determine whether these parameters provide a significant contribution to the QSAR study, we focused on structural and electronic elements as detailed below. In order to explain the observed differences in anti-*T. cruzi* activity for this series of SLs, other molecular properties and structural features were considered. The molecular descriptors employed in the QSAR analysis are listed in Table **2**.

WHIM descriptors are calculated by a principal component analysis (PCA) on the centered Cartesian coordinates of the atoms weighted according to different weighting schemes: atomic masses (mass), unitary weights (unity), van der Waals volumes (vol.), Mulliken atomic electronegativities (mull.), atomic polarizabilities (pol.) and electrotopological indexes (s). Directional and non-directional WHIM descriptors can be calculated. Directional WHIM descriptors are univariate statistical indexes which

Table 1. ***In vitro*** **Trypanocidal and Cytotoxic Activity of Selected SLs.**

SLs	Source	IC_{50} (μM)	CC_{50} (μM)	SI
1	*Artemisia copa* [17]	41.24	145.38	3.52
2	*Hyaloseris cinerea* [18]	43.81	254.52	5.81
3	*Artemisia copa* [17]	18.85	28.58	1.52
4	*Mikania minima* [19]	47.58	360.15	7.57
5	*Smallanthus sonchifolius* [16, 20, 21]	0.84	46.50	55.36
6	*Stevia alpina* [22]	0.24	429.42	1789.25
7	*Stevia gilliesi* [23]	0.78	363.72	466.31
8	*Stevia maimarensis* [24]	4.39	242.49	55.24
9	*Vernonanthura squamulosa* [25, 26]	7.11	20.69	2.91
10	*Mikania minima* [19]	3.13	257.22	82.18
11	Commercial Source (*)	3.02	370.89	122.81
12	*Ambrosia tenuifolia* [14]	6.06	344.09	56.78
13	*Smallanthus macroscyphus* [27]	6.71	239.48	35.69
14	*Ambrosia tenuifolia* [14]	0.75	290.95	387.93
15	*Ambrosia scabra* [15]	4.69	565.11	120.49
Benznidazole		5.39	82.79	15.35

IC_{50}: concentration that inhibits 50% of *T. cruzi* epimastigotes growth. CC_{50}: concentration that inhibits 50% of the cellular viability. SI: selectivity index. (*) Sigma.

Table 2. Molecular Descriptors Considered in QSAR Modeling.

Descriptor	Type	Comments
ACR	1D	ACR=1 α,β-insaturated ester present, ACR=0 α,β-insaturated ester absent
CAB	1D	CAB=1 α,β-insaturated ketone present, CAB=0 α,β-insaturated ketone absent
LAC	1D	LAC=1 α,β-insaturated lactone present, LAC=0 α,β-insaturated lactone absent
AlogP	2D	Lipophilicity (AlogP, AlogP2 y AMR)
VABC	2D	Van der Waals volume
Wiener Numbers	2D	Wiener path number
CPSA	3D	Charged partial surface area
Moment of Inertia	3D	Molecular geometry information
WHIM	3D	Weighted holistic invariant molecular descriptors
TPSA	2D	Topological polar surface area
AM1_LUMO	QM	Lowest unoccupied molecular orbital. Calculated by AM1 semiempiric quantum method

1D: monodimensional descriptors. 2D: bidimensional descriptor. 3D: tridimensional descriptor. QM: quantum mechanic descriptor.

Table 3. WHIM Descriptors Used in QSAR Models.

Descriptor	Comments
WD_{vol}	Non-directional WHIM, weighted by Van der Waals volumes
WD_{mass}	Non-directional WHIM, weighted by atomic masses
$W\eta 2_{pol}$	Directional WHIM, weighted by atomic polarizabilities

are calculated from the scores of each individual principal component (1, 2, 3), related to molecular size (λ1, λ2, λ3), shape (Θ1, Θ2), symmetry (γ1, γ2, γ3), and density of atom distribution (η1, η2, η3). The non-directional WHIM indexes, easily obtained from the directional ones, bear global information about molecular size (T, A, and V), shape (K), symmetry (G), and density (D). Table **3** shows the descriptors included in our QSAR models.

QSAR Analysis

The QSAR analysis was carried out with the McQSAR program [32], with which the correlation mathematical model was performed. The predictive quality of the correlation model was analyzed by the statistical parameters: R2 (squared correlation coefficient) and Q2 (squared correlation coefficient of leave-one-out cross validation). The statistical analysis was carried out with Microsoft Excel spreadsheet after the selection of the data set and the regression models employing these parameters.

RESULTS AND DISCUSSION

Firstly, we investigated the degree of similarity of the SLs evaluated in terms of shape and electrostatic potentials. In this sense, multiple structures (CEs) were used for the template compounds as well, which causes ShaEP to superimpose each conformer of a database compound on each of the template structures and report the maximum similarity index obtained over all of the comparisons. The most active SL, estafietin, was taken as a reference compound and was given a relative value of 1. As seen in Table **4**, the similarity indexes considered do not allow a correlation between the determined activities.

The studied SLs were divided in two sets: one training set comprising twelve compounds (**1-11, 13**) and one external test set made up of psilostachyin, psilostachyin C and peruvin.

Table 4. Similarity Indexes Determined by the ShaEP Software.

Compound	pIC_{50}	Best similarity	Shape similarity	ESP similarity
1	4.38	0.853	0.855	0.852
2	4.36	0.744	0.704	0.784
3	4.72	0.902	0.858	0.945
4	4.32	0.764	0.739	0.789
5	5.84	0.745	0.679	0.812
6	6.61	1.000	1.000	1.000
7	6.11	0.761	0.731	0.790
8	5.36	0.751	0.744	0.758
9	5.15	0.737	0.688	0.786
10	5.50	0.806	0.754	0.857
11	5.52	0.840	0.866	0.815
12	5.22	0.743	0.833	0.654
13	5.17	0.717	0.679	0.754
14	6.13	0.787	0.841	0.732
15	5.33	0.860	0.887	0.833

ESP: electrostatic potential. pIC_{50}: - $logIC_{50}$.

Table 5. Equations for QSAR Models.

Model	Log ($1/CI_{50}$)	n [a]	r [b]	R^2 [c]	R^2_{adj} [d]	SD [e]	F [f]
1	$13.20(\pm0.26) - 7.76(\pm0.25)xWD_{vol}$	10	0.99	0.99	0.99	0.07	942.05
2	$8.81(\pm0.20) - 3.62(\pm2.00)xWD_{vol}$	12	0.49	0.24	0.17	0.66	3.26
3	$8.20(\pm0.72) - 7.76(\pm1.96)xW\eta2_{pol}$	12	0.81	0.66	0.62	0.43	15.68
4	$11.13(\pm0.41) - 6.13(\pm0.43)xWD_{mass} + 1.46(0.36)xLAC$	12	0.85	0.72	0.66	0.42	11.59
5	$11.26(\pm0.22) - 7.74(\pm0.24)xWD_{vol} + 1.92(\pm0.06)xLAC$	12	0.99	0.99	0.99	0.07	644.52

a: Number of observations. **b:** correlation coefficient. **c:** R square, **d:** adjusted R square. **e:** standard deviation. **f:** Fisher test.

Table 6. Correlation Analysis (training set).

Compound	LAC	WD_{mass}	WD_{vol}	$W\eta2_{pol}$	pIC_{50} exp. [a]	pIC_{50} calc. [b]	Error [c]
1	0	0.822	0.893	0.422	4.38	4.35	0.03
2	1	1.031	1.145	0.433	4.36	4.32	0.04
3	0	0.746	0.841	0.328	4.72	4.75	-0.03
4	1	1.018	1.143	0.210	4.32	4.33	-0.01
5	1	0.847	0.940	0.278	5.84	5.90	-0.06
6	1	0.752	0.866	0.408	6.61	6.48	0.13
7	1	0.821	0.911	0.387	6.11	6.13	-0.02
8	1	1.038	1.017	0.436	5.36	5.31	0.05
9	1	0.905	1.041	0.388	5.15	5.13	0.02
10	1	0.769	0.985	0.329	5.50	5.55	-0.05
11	1	0.865	0.979	0.330	5.52	5.60	-0.08
13	1	1.010	1.041	0.335	5.17	5.12	0.05

a: pIC_{50} exp.= $-\log(IC_{50})$ experimental. b: pIC_{50} calc.= $-\log(IC_{50})$ calculated from model 5. c: calculated as a-b.

Taking into account the statistics results and the relationship of the included descriptors, we selected the most significant five models. WHIM descriptors seem to allow a good correlation of the structural features of the SLs with the observed activity (Table **5**). The non-directional descriptors that appear in the selected equations demonstrated that the biological activity is highly related to the three dimensional structures of SLs (models 1, 2, 4 y 5: WD_{vol} and WD_{mass}). On the other hand, the inclusion of directional WHIM descriptors in the models suggested that the placement of certain atoms in the molecular skeleton influences the anti-*T. cruzi* activity (model 3). In particular, models 4 and 5 consider the presence or absence of a determined functional group (LAC). Model 2 showed that adding the two SLs (achillin and desacetoximatricarin), without LAC, caused a loss of regression fit found for model 1. The addition of the LAC variable in model 5 indicated that the presence of such moiety is an important structural feature for the studied activity. Analyzing the equation corresponding to model 5, it can be verified that the coefficient of internal prediction value R^2 is highly significant, which indicates that the model is fairly robust.

Tables **6** and **7** show a significant validation of the training set and test set, respectively. The equation that corresponds to model 5 was used for the activity calculation. In (Fig. **2**) a straight line of the points representing the adjustment for the calibration of the model can be observed.

CONCLUSIONS

The results reported herein on a series of fifteen SLs demonstrate that this type of compounds has a high potential as source of new leading trypanocidal molecules.

As stated by Schmidt *et al.* [32], some families of SLs show a dependence of the antiprotozoal activity with the AM1_LUMO descriptor. According to our results, a higher dependence of the anti-*T. cruzi* activity on steric factors was observed. The number of studied compounds did not allow us to determine the electronic influence as another factor that modifies the activity. However, it is likely that the electronic effects are included in the steric descriptors analyzed in this work, as the electronic cloud of the molecules can influence the molecular form.

Table 7. Correlation Analysis (test set).

Compound	LAC	WD_{mass}	WD_{vol}	$W\eta 2_{pol}$	pIC_{50} exp. [a]	pIC_{50} calc. [b]	Error [c]
Peruvin	1	0.933	1.033	0.300	5.22	5.18	0.04
Psilostachyin	1	0.825	0.906	0.307	6.13	6.17	-0.04
Psilostachyin C	1	1.104	1.004	0.265	5.33	5.41	-0.08

a: pIC_{50} exp.= -log(IC_{50}) experimental. b: pIC_{50} calc.= -log(IC_{50}) calculated from model 5. c: calculated as a-b.

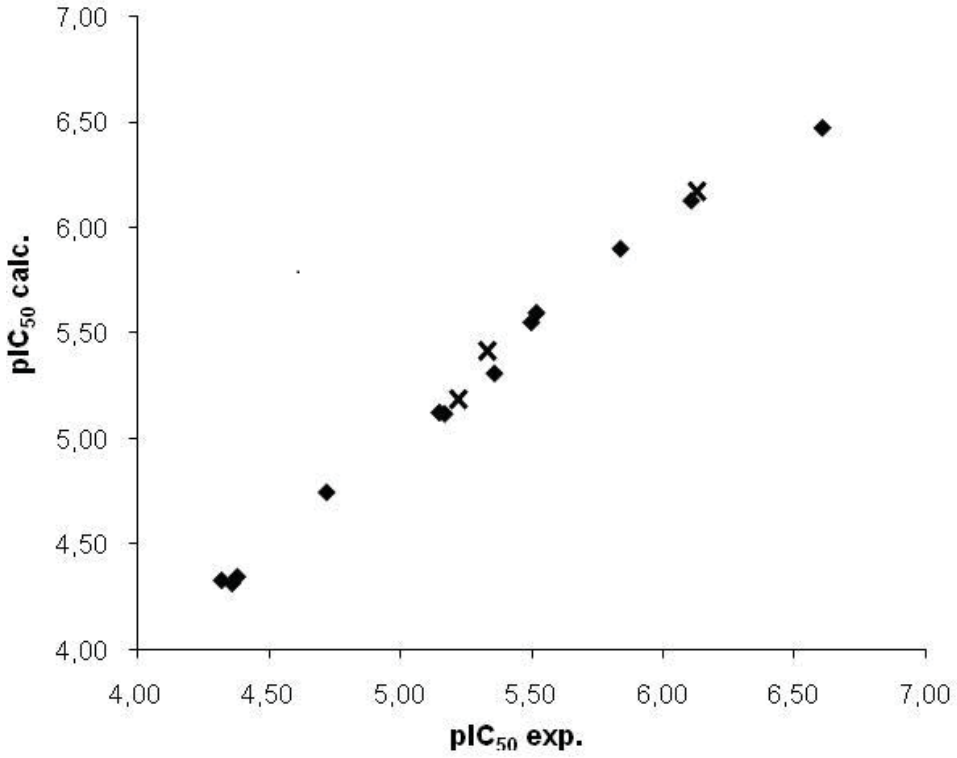


Fig. (2). Experimental activity values versus predicted activity values for the training set and test set. ♦: calibration of training set data. ×: calibration of test set data.

The WHIM descriptors would be adequate to establish the relationship between these compounds and the tested activity. In particular, WD_{vol} and WD_{mass} represent a description of steric properties and are able to give better estimates than other classical descriptors and may be useful for the study of new SLs with this activity.

These findings could be considered an important tool for the identification or development of new trypanocidal lead compounds structurally related to the SL group.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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[18] SL **2** was isolated from *Hyaloseris cinerea* (Griseb.) Griseb. var. *cinerea* collected at Siján, Pomán Department, province of Catamarca, Argentina. Aerial parts (300 g) were extracted (2x) with chloroform (2 l) at room temperature for 7 days to give 26.0 g of crude extract which was suspended in EtOH (220 ml) at 55º C, diluted with H_2O (160 ml) and extracted (3x) with hexane (300 ml) and then (3x) with $CHCl_3$ (450 ml). Removal of solvent from the $CHCl_3$ fraction *in vacuo* furnished 6.5 g of residue which was chromatographed over silica gel using $CHCl_3$ with increasing amounts of EtOAc (0-100%); 90 fractions were collected. Fractions 74-86 showed a major polar spot on TLC and were combined; the residue obtained after solvent evaporation gave a solid residue (2.4 g) which after several washings using first a 1:1 mixture of ether-EtOAc and finally pure EtOAc afforded 931 mg of 1-*O*-*β*-D-glucopyranosyl-asperilin (**2**) as a white amorphous solid which was characterized by 1D and 2D NMR spectroscopy. The structure of the glycoside was confirmed by enzymatic hydrolysis. Treatment of **2** (30 mg) with β-glucosidase (68 mg) in deionized H_2O at 37º C for 48 h gave an equimolecular mixture of asperilin and glucose. Extraction with $CHCl_3$ and solvent evaporation gave a residue (16 mg) which after flash column chromatography on silica gel (230-400 mesh) yielded a crystalline solid, mp 149-151º C, which showed to be identical in every respect with asperilin isolated from *Iva asperifolia* [Herz, W. and Viswanathan, N. Constituents of *Iva* species. II. The structure of asperilin and ivasperin, two new sesquiterpene lactones. *J. Org. Chem.*, **1964**, *29*, 1022-1026] Spectroscopic data of SL **2**: ^{1}H NMR (DMSO-*d*6, 200 MHz): δ0.74 ppm (s, 3H, H-14), 1.0-2.6 (m, 9H, overlaped signals, H-2a, H-2b, H-3a, H-3b, H-5, H-6a, H-6b, H-9a, H-9b), 2.85-3.25 (m, 8H, overlapped signals, H-1, H-7 and sugar protons H-2', H-3', H-4', H-5', H-6'a, H-6'b), 4.23 (d, *J* = 7.5 Hz, anomeric proton H-1'), 4.49 (s br, H-15b), 4.57 (t br, *J* = 4 Hz, H-8), 4.80 (s br, H-15a), 5.69 (s, H-13b), 6.00 (s, H-13a). ^{13}C NMR (DMSO-*d*6, 50 MHz): δ 170.4 ppm (s, C-12), 147.9 (s, C-4), 142.5 (s, C-11), 120.4 (t, C-13), 107.5 (t, C-15), 100.4 (d, C-1'), 83.5 (d, C-1), 77.0 (d, C-8), 76.8 (d, C-2' and C-3'),73.7 (d, C-5'), 70.5 (d, C-4'), 61.6 (t, C-6'), 43.6 (d, C-5), 39.1 (d, C-7), 38.2 (s, C-10), 37.2 (t, C-9), 33.4 (t, C-3), 26.8 (t, C-2), 26.4 (t, C-6), 12.3 (q, C-14).

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