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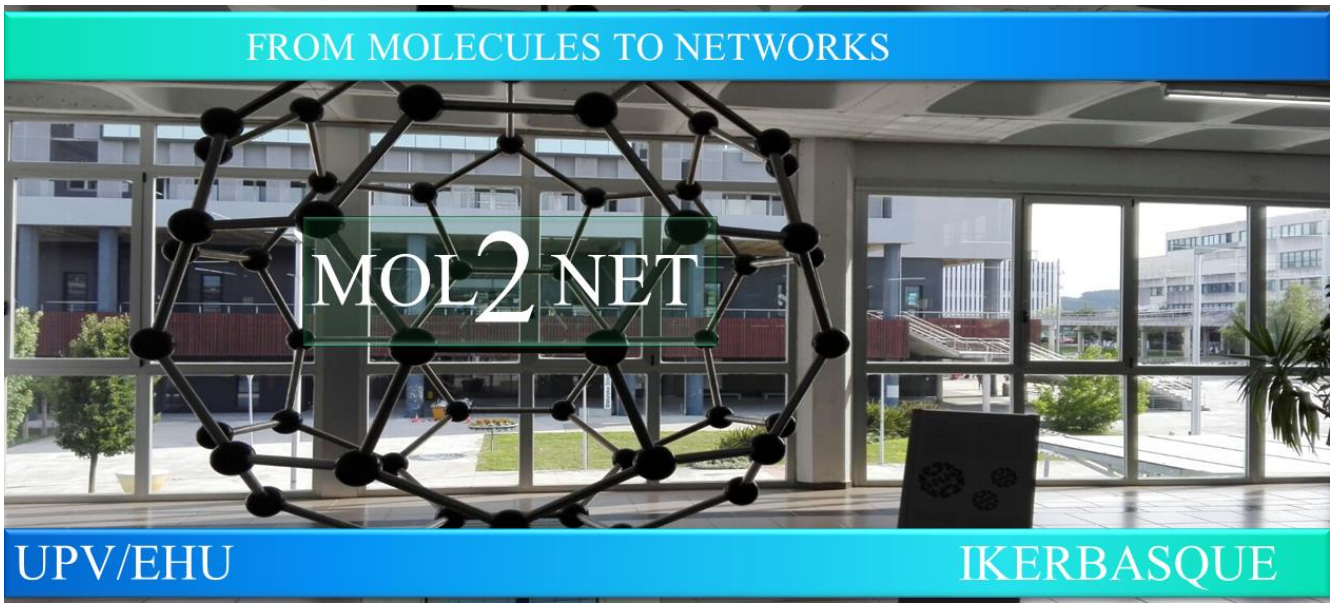

Identification of Natural Products with Potential Activity against *Leishmania amazonensis* using computational models and experimental corroboration

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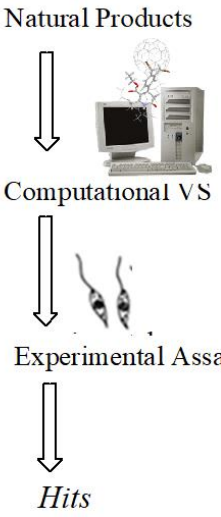
Graphical Abstract	**Abstract.**
	Leishmaniasis is one of the most important neglected tropical diseases according to the World Health Organization. The available drugs are expensive, not sufficiently effective, have serious cytotoxic effects and parasitic resistance has increased in the last years. In the present



work, a virtual screening protocol was used to identify new natural compounds potentially active against Leishmania spp. using machine learning-based models. Three vegetable origin compounds were selected by using a multiclassifier composed by models developed with k-nearest neighbor, classification tree, Multilayer perceptron and Support Vector Machine; all these models for *Leishmania amazonensis* promastigote form were developed with WEKA software. The selected compounds showed in vitro activity against *L. amazonensis* (MHOM/BR/77/LTB0016) promastigotes with CI_{50} lower than 1 µg/mL using 96-well plates and resazurine fluorescence method.

Introduction

Leishmaniasis is a neglected parasitic disease of great global importance. It is endemic in more than 98 countries, with estimated million cases and deaths per year. It is caused by about twenty species of protozoa of the subgenera's *Leishmania* and *Vianna,* and transmitted by the bite of sandfly insects.[1, 2] Currently, there are no sufficiently effective drugs for the treatment of forms of leishmaniasis caused by *Leishmania spp*. Among the therapeutic agents used to treat it are pentavalent antimonials, amphotericin B, pentamidine and miltefosine,[3] they have a large number of secondary adverse effects, some of which may put the lives of treated patients at risk, they are also expensive and ineffective in several cases due, among other factors, to the emergence of an increase in the drug resistance of the parasite.[4]

Natural products, particularly those used to treat infectious diseases, constitute an important source of chemotherapeutic agents. These compounds have a huge structural richness, being an important source of new therapeutic alternatives and prototype molecules for the development of valuable active substances.[5] There is a growing interest in the employment of herbal remedies, however much remains to be explored regarding the use of plants as medicinal sources.

Taking into account the lack of structural diversity of antileishmanial active pharmaceutical ingredients used in current therapeutics, the search for new active compounds is an urgent need, and in this sense, plants have shown to have a wide potential to provide effective metabolites for the treatment of parasitic diseases. The QSAR studies are useful tools for screening chemicals, especially in early stages of the drug discovery process.[6-8] In this work, a virtual screening protocol is used to identify natural compounds isolated from plants with potential activity against *L. amazonensis* promastigotes, using previously obtained models and also carry out an experimental corroboration of the *in vitro* activity.

Materials and Methods

Computational models

The models used for virtual screening presented adequate statistical parameters and were previously published by some of the present authors.[9] The four QSAR models were obtained using the following classification techniques: k-nearest neighbors (IBK), Classification Trees (J48), Multilayer Perceptron (MLP) and Support Vector Machine (SVM). They were used to predict the activity of naturally occurring compounds against to *L amazonensis* promastigotes.

Virtual screening and assembled multiclassifier system based on QSAR models.

Each model separately captures a large fraction of the chemical information contained in the database through the MDs; this fraction of information needs complementarity from the rest of the models for its completeness[10]. So, in addition to the individual techniques, an assembled personalized majority voting system was used. In this technique each classifier has a vote that has equal value. In essence, the most popular classification is the one that is chosen by the group as a final decision. The most basic variant is the plurality vote, where the class with the most votes wins[11,12].

In vitro evaluation of potentially active compounds according to prediction models

The *in vitro* antileishmaniasic activity of compounds identified as potentially active according to the prediction models was evaluated. They were first experimentally tested in a parasiticide activity assay at different concentrations against *L. amazonensis* promastigotes. Subsequently, they were assayed in an appropriate concentration range to determine their growth inhibitory activity (IC_{50}) against promastigotes.

The concentrations of product that inhibited the growth of promastigotes by 50% with respect to the untreated control (Mean inhibitory concentration or IC_{50}) and the concentration of product that caused total inhibition of promastigotes motility (Minimal Parasiticidal Concentration, CPM) were used as indices of *in vitro* activity against promastigotes.

Tested compounds

Three compounds of natural origin from plants studied by researchers belonging to Pharmacy Department of the Central University of Las Villas, were experimentally evaluated. They were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to an initial concentration of 20 mg/mL, and then, serial dilutions were made to achieve the appropriate concentration range depending on the assay and the activity displayed by products.

Parasite and culture

L. amazonensis promastigotes (MHOM/BR/77/LTB0016) were used. Cultures were performed in Schneider's medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (Hyclone®, Logan, Utah), sodium penicillin (200 IU/mL) and streptomycin (200 µg/mL) and incubated at 26 ºC. To maintain the promastigotes in exponential multiplication, sowings were made in fresh medium every 3-4 days.

In vitro activity against promastigotes

From a culture of *L. amazonensis* promastigotes (MHOM/BR/77/LTB0016) in logarithmic growth phase, subcultures were prepared in fresh Schneider medium at a concentration of 8.3 x105 parasites/mL. The compounds to be tested, previously dissolved in dimethyl sulfoxide (DMSO) at a rate of 20 mg/mL, were transferred to a 96-well plate (2 µL in 198 µL of parasite-free Schneider medium), from which eight serial dilutions were obtained, following a geometric progression of 1:2.5. Thus, the maximum concentration tested was 120 µg/mL and the minimum 0.2 µg/mL. Each concentration was tested in duplicate and several replicates were carried out. The assay took into account a control with culture medium without parasite, a positive control (2 µL of furvin with a concentration of 10 µg/mL), a negative control treated with 1 µL of 0.5% DMSO, and control wells that received no treatment. To each well, 80 µL of fresh cultures of promastigotes of *L. amazonensis* in logarithmic growth phase were added at a concentration of 8.3x105 parasites/mL, except for the medium controls. The plates were sealed with Parafilm® (American National Can, Greenwich, England) and incubated at 26°C for 72 h. After this incubation time, the cultures were examined with the help of an inverted microscope (Olympus Tokyo CK, Japan) to evaluate the presence of mobile promastigotes at different concentrations and thus estimate the minimum concentration with parasiticide effect (CPM). Subsequently, 20 µL of 3 mM resazurin was added to each well and incubated for another 12 h under the same conditions. Then, the plates were read in a fluorescence reader (Plate Reader PR 621, Suma, País), the growth inhibition values associated with each concentration were calculated, and the mean inhibitory concentrations (IC_{50}) were estimated by nonlinear sigmoid curve fitting. Simultaneously, the activity of amphotericin B-sodium deoxycholate as the reference drug was tested. IC_{50} values for each product were expressed as the mean of at least three independent experiments.

Results and Discussion

Virtual screening, assembled multi-classifier system based on QSAR models

Molecules tested to predict antileishmaniasis activity included 12 natural origin compounds: cardiotonic glycosides, phenols, flavonoids, terpenes, coumarins and saponins. By performing the virtual screening of this compounds, a prediction of the activity was obtained for each individual model, and then, a simple majority vote assembled multiclassifier system was carried out to improve the results obtained in the modeling, since the fundamental principle of in silico methods is to select a small number of potentially active compounds with a consequent saving of resources of all kinds, which that would bring rationality to the process of identifying new leading compounds.[13] Three natural compounds, digitoxin, escine and hesperidine were identified as potencial antileishmaniasic agents using this ensamble.

In vitro evaluation of potentially active compounds according to prediction models

In the primary assay of activity against *L. amazonensis* promastigotes, three compounds of natural origin (secondary metabolites of plants), that are been studied in the Department of Pharmacy of the Central University "Marta Abreu" de Las Villas, were evaluated. The study of *in vitro* activity against *L. amazonensis* promastigotes showed an adequate performance taking into account the fluorescence values and colors exhibit by the different controls. In the colorimetric assay to measure cell viability, resazurin (blue, non-fluorescent) is reduced to resofurin (pink, highly fluorescent) by oxido-reductases found primarily in the mitochondria of viable cells. Thus, there is a direct correlation between the reduction of rezasurin and the rate of proliferation of living organisms[14,15]. Within the range of studied concentrations, only digitoxin showed CPM at 120 µg/mL and all three compounds presented mean inhibitory concentrations (IC_{50}) below 1 µg/mL. These results were compared with the criteria who stated that an antiprotozoal drug candidate must have an $IC_{50} \leq 1$ µg/mL. all present IC_{50} values lower or close to 1 µg/mL, indicative of potent activity (Digitoxin a cardiotonic glucoside, CI50 (µM)± DS (standard deviation)=0.40±0.19; Hesperidine, flavonoid, 1.15±0.22; Escine a saponin 0.57±0.06.

Conclusions

The three theoretically identified compounds evaluated using *in vitro* assays for activity against *Leishmania amazonensis* were found to be active against the promastigotes form with IC_{50} values lower than 1 µg/ml. The *in vitro* studies carried out on promastigotes forms of *Leishmania amazonensis* allowed the experimental corroboration of the activity of three new compounds identified as potentially active through QSAR studies. It demonstrates the predictive capacity of the models used in virtual screening.

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