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Estudios de susceptibilidad de cepas de *Leishmania aethiopica* frente a alcaloides de *Galipea longiflora* (*Evanta*)

Susceptibility studies on *Leishmania aethiopica* strains against total alkaloids from *Galipea longiflora* (*Evanta*)

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Resumen

El Instituto de Investigaciones Fármaco Bioquímicas (IIFB), de la Facultad de Ciencias Farmacéuticas y Bioquímicas, de la UMSA, desarrolla trabajos sobre la actividad leishmanicida, de los alcaloides totales (CAT) obtenidos de la corteza de la especie medicinal amazónica conocida como Evanta (*Galipea longiflora*) por los Pueblos Tacana, Tsimane y Mosetene.

Como parte de las actividades del Proyecto UMSA-ASDI "*Biomoleculas de interés medicinal* e *industrial (antiparasitarios)*" hemos podido contar con la estadía, en el IIFB, de un investigador del Armauer Hansen Research Institute (AHRI) de Etiopia, lo que nos ha permitido desarrollar evaluaciones de CAT, Miltefocine y Amfotericina B, frente a cepas de *Leishmania aethiopica*, agente causante de las diversa formas de *Leishmania*is cutánea en Etiopía.

Abstract

The Instituto de Investigaciones Fármaco Bioquímicas (IIFB), at the Faculty of Pharmaceutical and Biochemical Sciences, from UMSA, carry out work related to the leishmanicidal activity of the total alkaloids (CAT) obtained from the bark of the Amazonian medicinal species known as Evanta (*Galipea longiflora*) by the Tacana, Tsimane y Mosetene people.

As part of the activities develop by the UMSA-ASDI Project "*Biomolecules of medicinal and industrial Interest (antiparasitic)*" we had a visit, in our laboratories at IIFB, of a researcher from The Armauer Hansen Research Institute (AHRI) from Ethiopia, during his stay we were able to carry out evaluations of CAT, Miltefocine and Anphotericin B, against strains of *L. aethiopica*, causative agent of the different manifestations of cutaneous leishmaniasis in Ethiopia.

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Un total de seis cepas, de *L. aethiopica*, fueron adaptadas a condiciones *in vitro* y mostraron un comportamiento homogéneo frente a CAT, cinco de estas cepas mostraron un valor promedio de IC50 = 8,68 ± 1,56 mg/mL, valor algo inferior a los calculados para nuestras cepas de referencia, *L. amazonensis* y *L. braziliensis* con IC50 = 11,73 ± 4,32 mg/mL y IC50 = 12,28 ±- 2,95 mg/mL, respectivamente. Excepto por una cepa de *L. aethiopica* que mostro valores consistentemente más elevados que el resto con IC50 = 14,37 ± 3,58 mg/mL.

Como consecuencia de esta interacción científica, la Universidad Mayor de San Andrés (UMSA) ha firmado un Memorandum de Entendimiento para el desarrollo de investigaciones conjuntas, con el Armauer Hansen Research Institute (AHRI), dependiente del Ministerio de Salud de Etiopia y explorar la posibilidad de que nuestra experiencia de validación clínica con Evanta en el tratamiento de leishmaiasis cutánea, en Bolivia, podría ser replicada en Etiopía, donde se reportan entre 20,000 a 30,000 nuevos casos de Leismaniasis por año.

PALABRAS CLAVE
Bolivia, Etiopia, *L. aetiopica*, *L.amazonensis*, *L.braziliensis*, susceptibilidad Alcaloides, *Galipea longiflora*

A total of six strains of *L. aethiopica*, were adapted to *in vitro* a conditions, at IIFB; and did show homogenous behavior against CAT. Five of the strains, showed an average calculated value for IC_{50} = 8.68 ± 1.56 mg/mL, a value somewhat lower to the calculated for the reference strains *L. amazonensis* and *L. braziliensis* with IC_{50} = 11.73 ± 4.32 mg/mL and IC_{50} = 12.28 +/- 2.95 mg/mL, respectively. Except for one strain that showed values somewhat higher, to the other strains, consistently through our studies, with IC_{50} = 14.37 ± 3.58 mg/mL.

As a consequence of our scientific interaction, the Universidad Mayor de San Andrés (UMSA) has signed a Memorandum of Understanding for the development of joint research with the Armauer Hansen Research Institute (AHRI) that belongs to the Ministry of Health in Ethiopia, and explore the possibilities to replicate the Bolivian clinical validation experience of Evanta in the treatment of cutaneous leishmaniasis, in Ethiopia where the annual incidence is estimated to be between 20, 000 to 30, 0000.

KEY WORDS
Bolivia, Ethiopia, *L. aetiopica*, *L.amazonensis*, *L.braziliensis*, suceptibility Alkaloids *Galipea longiflora*

INTRODUCTION

*Leishmania*sis is part of the group of forgotten or neglected tropical diseases and one of the most important vector-borne diseases in humans. This disease could be caused by various species of *Leishmania* parasites, most of which are zoonotic. Different parasite species are associated with different clinical forms of the disease. Many species of *Leishmania* cause skin ulcers and nodules, leading to cutaneous leishmaniasis (CL) which may be local (LCL) or diffuse (DCL). Some species of these organisms can also affect mucous membranes and may cause injury and disfigure the nose, causing mucocutaneous leishmaniasis (MCL). Other species damage internal organs and cause visceral leishmaniasis (VL), the latter is fatal if not treated in time (Blackwell JM et al. 2009). *Leishmania*sis predominantly affectspoor people in Africa, Latin Amerca and Asia. Globally estimated It is estimated to be endemic in 98 countries with annual incidence of 0.9 to 1.6 million cases per year

There are about 20 species of the *Leishmania* cause leishmaniasis in human (Table 1). These species belong to the Trypanosomatidae family that includes at least eight different Genera. The different clinical manifestations associated with

different *Leishmania* species. . Currently, the genus *Leishmania* is divided into two groups: the sub-Genus *Leishmania* and sub-Genus Viannia (Ardaya 2015).

In the Old World, leishmaniasis could be grouped into two classes, cutaneous (**CL**), associated with the *L. major*, *L. tropica* and *L. aethiopica* complexes, and visceral (**VL**), involves up to 4 different species all associated with *L. donovani* complex (Ayele & Ali 1984). **VL** is endemic in many tropical and subtropical areas of the world, is the most severe form of leishmaniasis, almost always fatal if left untreated (Lyons et al. 2003). It is estimated that between 200,000 and 400,000 new cases of **VL** occur in the world each year (WHO, 2013) and of these, East Africa has the second highest number of cases, after the Indian subcontinent (Alvar et al. 2007 and Alvar et al 2012). In the New World, **CL** infections (**LCL** and **DCL**) are associated with *L. mexicana* and *L. braziliensis* complexes. **MCL**, is most often associated with *L. braziliensis* and *L. panamensis*, although other species may be involved. The **DCL** occurs with some species of the parasite and due to host immune factors, while **VL** is associated with *L. chagasi*.

In Bolivia, an estimated 0.8 millones of individuals are at high risk of becoming infected with leishmaniasis, the disease is spread in 7 of the 9 departments (except Oruro and Potosi). It is estimated that there are approximately 2,500 new cases per year based on published data, *L braziliensis* species (**CL** and **MCL**) and *L. amazonensis* (**CL**) have a clear dominance in national epidemiological indices. *L. lainsoni* (**CL** and **MCL**) and *L. chagasi* (**VL**) are presented in rare and few reports (Rojas et al. 2009; Martinez et al. 2002 and Mollinedo et al. 2000). In Bolivia, cases of leishmaniasis tend to increase every year, as well as other vector transmitted diseases like Dengue, Malaria, Chagas Disease (Garcia et al. 2009 and INLASA 2012).

Table 1. Taxonomic Classification of Pathogenic Parasites of *Leishmania*

FAMILY	Genera	Sub-Gender	Complex	Species	Clinical Classification
TRYPANOSOMATIDAE	LEISHMANIA	LEISHMANIA	*L. donovani*	*L. donovani*	Visceral Leishmaniasis **VL**
				L. chagasi	
				L. infantum	
				L. archibaldi	
			L. mexicana	*L. mexicana*	*Cutaneous Leishmaniasis* **CL**
				L. amazonensis	
				L. garhani	
				L. pifanoi	
				L. venezuelensis	
			L. tropica	*L. tropica*	
				L. Killicki	
			L. major	*L. major*	
			L. aethiopica	*L. aethiopica*	
		VIANNIA	*L. braziliensis*	*L. braziliensis*	Mucocutaneous *Leishmaniasis* **MCL**
				L. peruviana	
			L. lainsoni	*L. lainsoni*	
			L. guyanensis	*L. guyanensis*	
				L. panamensis	

Adapted from Bañuls et al 2007

Although no official data is available, it is estimated that the population at high risk of contracting some form of leishmaniasis in Ethiopia, is about 30million inhabitants and the total number of cases diagnosed as positive, each year, is estimated at around 20,000 to 30,000, with 25% of cases of **VL** (*L. donovani*) and 75% of cases of **CL** (*L. aethiopica*), the latter comes in three different clinical forms (**LCL**, **MCL** and **DCL**). **LCL** injuries by L .aethiopica, do not form open sores, as with strains in the New World, (*L. mexicana* complex). The injuries caused by *L. aethiopica* are closed (nodules) and is often at the site of vector inoculation. Although, occasionally, severe and persistent **LCL**, **MCL** and **DCL** cause disfiguring and often require prolonged treatment schemes with very low success rate. In the case of **DCL**, definitive cure is almost never achieved, and relapse is common (Hailu et al. 2006; Lemma et al. 1969; Bryceson 1969 and Sarojini 1984).

In the area of Pharmaceutical Chemistry at the IIFB (AQF-IIFB), at the Faculty of Pharmaceutical and Biochemical Sciences, UMSA, within the "Project UMSA-SIDA Infections Diseases: Evanta in the Treatment of cutaneous leishmaniasis", we have produced Evanta based treatments (creams and syrups) (Tacana 1999 and Beatriz 2006), and the effectiveness assessed in a clinical trial (Phase II, treatment of **CL**), in a comparative treatment versus Glucantime. The study has been carried out in the Hospital of Palos Blancos, from June 2007 to December 2012, with patients diagnosed with **CL** caused by *L. amazonensis* and *L. braziliensis*. A total of 60 individuals (patients with primary infection, over 18 and under 50 years old, as inclusion criteria), were treated, the results of our studies of comparative efficacy for Evanta (29 patients, creams and syrups) are bordering the 68% cure of patients and over 90% cure of patients treated with Glucantime (31 patients, intramuscular) (Magariños 2013).

Based on the information gathered in the last 10 years, from field and clinic experiences developed in Bolivia, and adding research results from our laboratories, at IIFB, using Evanta (CAT), we can suggest that our experiences of validation of traditional medicines, for the treatment of cutaneous leishmaniasis, could be replicated in Ethiopia (OMS, 2013), where there are reported up to ten times more new cases of leishmaniasis per year, compared to the estimated 2,500 cases per year, in Bolivia.

MATERIALS Y METHODS

Leishmania aethiopica strains from AHRI

The *Leishmania* aethiopica, strains were donated by AHRI, these were obtained from patients ulcers, from a total of ten strains received, six were able to adapt to *in vitro* cultures at IIFB and were used in the present study.

Strain No	AHRI Code	Clinical Form	Place of lesion
1	396/04	**LCL**	Right Cheek
2	799/04	**LCL**	Left cheek
3	1116/03	**LCL**	Left cheek
5	1475/03	**LCL**	Left eye lid
7	1502/03	**LCL**	Nose
8	1540/03	**LCL**	Nose

Reference *Leishmania* strains at IIFB

Leishmania braziliensis (M2904 C192 RJA) and *Leishmania* amazonensis (Clon 1 NHOM-BR-76-LTB-012). *Leishmania* lainsoni (INL 125-11)

Drugs preparation.

The drugs used in the study were Amphotericin-B (ANFOTERICIN-CRITÁLIA), Miltefosine (IMPA VIDO) and Total Alkaloids from *Galipea longiflora* (CAT-IIFB). Amphotericin-B was diluted in Schneider medium (1mg/mL) and dilutions with concentrations ranging from 0.62 to 0.01µg/mL were prepared. Miltefosine and CAT were diluted with DMSO (10mg/mL), and dilutions with concentrations ranging from 100 to 1.5µg/mL were prepared. Final concentration of DMSO was kept below 1%.

Leishmanicidal test

The activity was measured on *in vitro* cultures of the *Leishmania* parasite in promastigote forms, (modified from Salamanca et al 2008), cultivated at 26°C in Schneider medium (pH 6.8) supplemented with inactivated (56°C x 30min) calf bovine serum (10%). Parasites in logarithmic phase of growth, at a concentration of $1x10^6$parasites/mL, were distributed on a 96 micro well plates and the different concentration of the drugs were added. The micro well plates were incubated for 72hrs at 26°C. After incubation, a solution of XTT (1mg/mL) in PBS (pH 7.0 at 37°C) with PMS (Sigma-Aldrich, 0.06mg/mL), was added (50µL/well), and incubated again for 4hrs at 26°C. DMSO (1%) and Anphotericine B (0.5 mg/mL) were used as reference drugs during the evaluations, were done by sextuplicate. Optical density of each well was obtained with a Synergy HT microplate reader with I 200-450nm. The IC_{50} values were calculated using The Gen5 program (Biotek).

RESULTS

Because *Leishmania*sis is a neglected disease, lack of attention also happens with research and development of new diagnostic tools, drugs or vaccines, because there is no market interested in social groups suffering from the disease (WHO). The misuse and mismanagement of drugs can lead to the emergence of resistance forms (Gil et al. 2007) such is the case with Glucantime, which is well documented in countries like India (Osorio et al 2005), Brazil (Nascimento-Zauli et al. 2010) and Bolivia (10% of treatment failure, Bermudez et al. 2006) and such behavior has been observed within patients with visceral L. coinfected with HIV (Koert et al 2011). While in a clinical trial using Miltefosine, in Palos Blancos, Bolivia, only 50% of patients with MCL were cured (Soto et al. 2007).

The significant adverse effects reported, such as myalgia, arthralgia, anorexia, nausea and headache and the high costs, in the case of antimonials, stimulate that many patients do not conclude the treatment, favoring the reactivation of the injury, mucous damage and the emergence of drug resistance

(Clem A, 2010 and Bhandari et al. 2012). The disadvantages associated with the available drugs and the variability of response to those by *Leishmania*sis species, have evidenced the urgent need to develop alternative treatments and monitor treatment efficacy and emerging resistance to drugs in use

The medicinal plant *Galipea longiflora* (Evanta) is used in the treatment of cutaneous leishmaniasis by different Amazonian ethnic groups, in Bolivia, and we evaluated the response of six *Leishmania* aethiopica strains, being the species responsible of the different forms of cutaneous leishmaniasis in Ethiopia, and compared with the response observed for the reference strains at IIFB, when the different strains were exposed to the total alkaloids (CAT) of Evanta. The results are shown on Table 2.

Table 2. Susceptibility test of *Leishmania* strains against Amphotericin B, Miltefosine and CAT

Strain/Isolate	IC_{50} (Mean ± SD)		
	CAT	Amphotericin B	Miltefosine
L. aethiopica 1	8.23±2.16	0.08±0.01	2.43±1.1
L. aethiopica 2	8.68±1.59	0.19±0.20	2.57±1.41
L. aethiopica 3	6.9±2.16	0.44±0.12	1.57±0.06
L. aethiopica 5	14.37±3.58	0.22±0.11	3.7±2.39
L. aethiopica 7	8.33±0.90	0.11±0.11	1.6±0.05
L. aethiopica 8	11.27±0.97	0.27±0.04	1.67±0.06
L. braziliensis (M2904)	11.73±4.32	0.39±0.06	7.5±1.48
L. amazonensis (Lma)	12.28±2.95	0.33±0.04	6.7±0.6
L. lainsoni (INL 125-11)	9.70±1.41	0.22±0.02	3.5±0.35

DISCUSSIONS

In Bolivia leishmaniasis infection are caused mainly by *L braziliensis* (CL and MCL) and *L. amazonensis* (CL) both species have a clear dominance in national epidemiological indices. While *L. lainsoni* (CL and MCL) and *L. chagasi* (VL) are presented in rare and few reports (Rojas E et al. 2009).

From the six strains of *L. aethiopica* included in the present studies, five showed homogeneous behavior against all drugs, with an average calculated value for IC_{50}=8.68 ± 1.56µg/mL, when exposed to CAT, a value somewhat inferior to the calculated for the reference strains *L. amazonensis*, and *L. braziliensis* with IC_{50}=11.73 ± 4.32µg/mL and IC_{50}=12.28 ± 2.95µg/mL, respectively, but closer to the *L. lainsoni*, IC_{50}=9.7 ± 1.45 µg/mL. (except *L. aethiopica* 5, with values somewhat superior to the other strains consistently through our studies: IC_{50} = 14.37 ± 3.58µg/mL against CAT and Miltefosine IC_{50}=3.7 ± 2.39µg/mL).

All six *L. aethiopica* showed homogeneous behavior against Amphotericin B (average IC_{50}=0.22±0.02µg/mL) somewhat lower values when compared with the reference strains *L. braziliensis* (IC_{50}=0.39±0.06µg/mL) and *L. amazonensis* (IC_{50}=0.33±0.04µg/mL), a similar trend is observed when compared all *L. aethiopica* strains against Miltefosine (IC_{50}=2.26±0.76µg/mL) with *L. brazi-*

liensis and *L. amazonensis* (IC_{50}=7.5±1.48µg/mL, and IC_{50}=6.7±0.6µg/mL, respectively), Interesting to notice that susceptibility values for the *L. aethiopica* strains are much closer to the values calculated for the less common *L. lainsoni* strain in Bolivia (IC_{50}=0.22±0.02µg/mL and IC_{50}=3.5±0.35µg/mL, respectively).

The Instituto de Investigaciones Fármaco Bioquímicas (IIFB) belonging to the Faculty of Biochemical and Pharmaceutical Sciences at UMSA, has been working in the analytical (Herrera et al. 2008 and Limachi 2013) chemical (Llanos et al. 2009; Ticona 2005 and 2008; Espinoza 2012) biological (Salamanca 2008 and Herrera 2008) and pharmaceutical formulations (Rodriguez 2006) assessments of the total alkaloids (CAT) produced by Evanta (*Galipea longiflora*) since 2000 (Gimenez et al. 2005). Moreover, in collaboration with researchers from Brazil, through the CYTED Ibero-american research network, CAT gastro-protective et al. 2009) and anti-nociceptive (Campos-Buzzi et al 2010) effects were assessed, and results on G. longiflora shows significant inhibition of ulcer formation by decreasing gastric secretion and increase gastric mucus content. Therefore, the IIFB, has produced Evanta based treatments which were used in a clinical study on patients diagnosed with LC, caused by *L. amazonensis* and *L. braziliensis*, in Palos Blancos Hospital (2007-12), with efficacy bordering the 68% cure of patients.

CONCLUSIONS

Our *in vitro* studies developed at UMSA by researchers from IIFB and AHRI, using strains of *L. aethiopica* and different local leishmania spp strains circulating in Bolivia, against total alkaloids of Evanta (CAT), have shown that *L. aethiopica* parasites (media IC_{50}=6.93+/-1.89) and the Bolivian strains (media IC_{50}=11.24+/-2.89) are sensitive to Evanta alkaloids, suggesting that our experience in clinical validation, previously carried out in Bolivia, could be replicated in Ethiopia, where an estimated of 20,000 to 30,000 new cases of leishmaniasis are reported each year.

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