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Oleanonic acid from *Lippia lupulina* (Verbenaceae) shows strong *in vitro* antileishmanial and antitrypanosomal activity

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ABSTRACT

Leishmaniasis and Chagas disease affect millions of people in tropical and subtropical regions. Drugs used currently to treat such diseases often present undesirable side effects and low efficiency. The aim of this work was to identify extracts and isolated compounds from the genus *Lippia* with leishmanicidal and trypanocidal activity. Fifteen extracts from different plant parts of *Lippia* species with partially known chemical compositions, four partition fractions, six compounds and a mixture of four interconverting flavanones previously isolated from *Lippia salviaefolia* and *Lippia lupulina* were assayed *in vitro* towards epimastigote forms of *Trypanosoma cruzi* and promastigote forms of *Leishmania amazonensis*. The root extract of *L. lupulina* had potent activity against *T. cruzi* and *L. amazonensis* (IC_{50} of 20.0 and 54.5 μg mL^{-1}, respectively). The triterpenoid oleanonic acid showed the strongest activity against these protozoans (IC_{50} of 18.5 and 29.9 μM, respectively). Our results indicate that *Lippia* plants and their derivatives deserve further investigation in the search for new antiprotozoal drugs, particularly for the treatment of leishmaniasis and Chagas disease.

KEYWORDS: Leishmanicidal, trypanocidal, triterpenoids, flavonoids, *T. cruzi*

Ácido oleanônico isolado de *Lippia lupulina* (Verbenaceae) apresenta potentes atividades leishmanicida e tripanocida

Leishmaniose e doença de Chagas afetam milhões de pessoas em regiões tropicais e subtropicais. As drogas atualmente usadas para tratar estas doenças frequentemente apresentam efeitos colaterais indesejáveis e baixa eficiência. Este trabalho teve como objetivo encontrar extratos, frações e compostos isolados de espécies do gênero *Lippia* com atividades leishmanicida e tripanocida. Quinze extratos de diferentes partes de plantas do gênero *Lippia*, com composições químicas parcialmente conhecidas, quatro frações de partição, seis substâncias e uma mistura de quatro flavanonas interconversíveis isolados de *Lippia salviaefolia* e *Lippia lupulina* foram testados, *in vitro*, frente a formas epimastigotas de *Trypanosoma cruzi* e promastigotas de *Leishmania amazonensis*. O extrato etanólico das raízes de *L. lupulina* apresentou atividade potente contra *T. cruzi* e *L. amazonensis* (IC_{50} de 20,0 e 54,5 μg mL^{-1}, respectivamente), enquanto que o ácido oleanônico mostrou as atividades mais fortes contra estes protozoários, com IC_{50} de 18,5 e 29,9 μM, respectivamente. Estes resultados indicam que espécies do gênero *Lippia* e seus derivados merecem investigações adicionais na busca por novas terapias antiprotozoárias, especialmente para o tratamento de leishmaniose e doença de Chagas.

PALAVRAS-CHAVE: leishmanicida, tripanocida, ácido oleanônico, flavonoides, *T. cruzi*

INTRODUCTION

Leishmaniasis and Chagas disease are classified as neglected diseases (Morel 2003). They occur in tropical and subtropical regions causing substantial morbidity among poor people living in low socioeconomic environment. These diseases have not been a priority in the search for drug development because of limited financial incentives from the private sector (Kappagoda and Ioannidis 2012).

Leishmaniasis is caused by the parasitic protozoa of the genus *Leishmania*. It affects about 12 million people worldwide in 98 countries (Barrett and Croft 2012; WHO 2016). This disease is characterized by high diversity and complexity; it is caused by more than 20 species of the genus *Leishmania* and is transmitted to humans by about 30 different species of sandflies (Chappuis *et al.* 2007). In most cases, leishmaniasis is divided into three primary clinical forms: cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis or Kala-azar (Van Assche *et al*. 2011). One of the etiologic agents of cutaneous leishmaniasis in South American countries is *Leishmania amazonensis*, a member of the *Leishmania mexicana* complex. *Leishmania amazonensis* can cause mild cutaneous leishmaniasis and diffuse cutaneous leishmaniasis (Soong *et al*. 2012). The leishmaniasis treatment includes pentavalent antimonials (Pentostam® and Glucantime®), the polyene antibiotic amphotericin B (including the lipid preparation Ambisome®) and pentamidine. However, such drugs are costly, show undesirable side effects and resistant strains have emerged (Croft and Olliaro 2011; Tempone *et al*. 2011) which reinforces the need for novel therapeutic agents.

Chagas disease is caused by *Trypanosoma cruzi*, an intracellular parasite commonly transmitted to humans and other mammals by a Triatominae (Reduviidae). The disease may also be spread through blood transfusion, vertical transmission, and less frequently, through organ transplantation (Márquez *et al*. 2013). The World Health Organization (WHO) estimates that approximately 10 million individuals are currently infected with *T. cruzi* with potential for developing cardiac or gut pathology associated with chronic Chagas disease (Afonso *et al*. 2012). The two available drugs for treatment of Chagas disease, nifurtimox and benznidazole, have potential toxic side effects and variable efficacy. These drugs are unable to eradicate the infection during the chronic phase when most patients are diagnosed, which consequently contributes to their low prescription and rate of use (Coura 2009).

Based on these considerations, the search for new therapeutic agents for the treatment of leishmaniasis and Chagas disease is urgently needed. From 1981 to 2010, among the 1355 new chemical entities approved by the Food and Drug Administration (USA), only 29% were synthetic in origin, thus demonstrating the influence of "other than formal synthetics" on drug discovery and approval (Newman and Cragg 2012). Brazilian flora might also be regarded as one important source of new active compounds against parasitic protozoa (Funari and Ferro 2005, Mishra *et al*. 2009). The aim of this work was to identify extracts and isolated compounds from *Lippia* spp. with leishmanicidal and trypanocidal activity.

MATERIALS AND METHODS

Plant material

Parts of *Lippia salviaefolia* Cham. (Verbenaceae) and *Lippia velutina* Schauer were collected on April 1st, 2006 and February 12th, 2006, respectively, in Mogi-Guaçu city (Fazenda Campininha), São Paulo State, Brazil. Voucher specimens were deposited in "Herbarium Maria Eneida P. Kaufmann" of "Instituto Botânico de São Paulo", under the subscriptions Lima 90 and Brumati TI73, respectively. Parts of *Lippia balansae* Briq. and *Lippia lasiocalycina* Cham. were collected on March 7th, 2008 and May 15th, 2008 in Santa Cruz do Rio Pardo (Rodovia Castelo Branco, km 03) and Pratânia cities, respectively, São Paulo State, Brazil. Voucher specimens were deposited in the "Herbarium Coleção Botânica da Floresta Estadual de Assis", under subscriptions FEA 402, FEA 3556, respectively. Parts of *Lippia sidoides* Cham. and *Lippia lupulina* Cham. were collected on January 19th, 2009 and January 22th, 2009, respectively, in Iaras city, São Paulo State, Brazil. Voucher specimens were also deposited in the "Herbarium Coleção Botânica da Floresta Estadual de Assis", under subscriptions FEA 3639 and FEA 3638, respectively (Funari *et al*. 2012a).

In vitro assay for trypanocidal activity

Epimastigote forms of *T. cruzi* Y strain were grown axenically at 28 °C in Liver-Infusion Tryptose (LIT) medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). LIT medium was prepared by mixing 10 mg mL^{-1} hemin (bovine, type I) (1 mL) with a solution containing NaCl (4.0 g), KCl (0.4 g), Na_2PO_4 (8.0 g), glucose (2.0 g), liver infusion broth (5.0 g), and tryptose (5.0 g) at pH 7.0 (900 mL). The epimastigotes were harvested during the exponential growth phase (7-day-old culture forms). An aliquot of 50 µL of a 1×10^7 parasites mL^{-1} suspension was added to each well of a 96 multi-well microplate. Extracts or compounds previously prepared or isolated by our group (Funari *et al*. 2011; Funari *et al*. 2012a; Funari *et al*. 2012b) were dissolved in dimethylsulfoxide (DMSO) and further added to each well to reach final concentrations from 1 to 100 µg mL^{-1}. Aliquots of 50 µL of LIT medium, with and without parasites, were added to the control wells in the absence of test compounds. Microplates were incubated at 28 °C for 72 h. After this period, an aliquot of 10 µL of a 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide-phenazine methosulfate (MTT-PMS) solution (2.5 mg mL^{-1}) was added to each well, and the plates were incubated in the dark, at 28 °C, for 75 min. Subsequently, 100 µL of a solution of 10% sodium dodecyl sulfate (SDS) were added to each well and maintained at room temperature, in the dark, for 30 min. Absorbance of the solutions contained in the wells were read at 595 nm (Readwell Touch, Robonik PVT LTD, Thane, India). All the assays were performed in triplicate. Test samples concentrations corresponding to 50% of parasite growth inhibition (IC_{50}) were determined from non-linear regression (Muelas-Serrano *et al*. 2000; Santos *et al*. 2012).

In vitro assay for leishmanicidal activity

Promastigote forms of *L. amazonensis* (MPRO/BR/1972/M1841-LV-79) were grown at 28 °C in LIT medium supplemented with 10% (v/v) heat-inactivated FCS. The parasites were harvested at the end of the exponential growth phase (4-day-old culture forms). An aliquot of 97 µL of a 8 × 10^6 parasites mL^{-1} suspension was added to each well of a 96 multi-well microplate. Extracts or compounds previously prepared or isolated by our group (Funari *et al*. 2011; Funari *et al*. 2012a; Funari *et al*. 2012b) were dissolved in DMSO and an aliquot of 3 µL was further added to each well to reach final concentrations from 1.6 to 100 µg mL^{-1}. Microplates were incubated at 28 °C for 72 h. After this period, a 10 µL aliquot of 2.5 mg mL^{-1} MTT-PMS solution was added to each well, and the plates were further incubated for 75 min, in the dark, at 28 °C. Subsequently, 100 µL of 10% SDS were added to each well and maintained at room temperature, in the dark, for 30 min. Absorbances of the solutions in the wells were read at 490 nm (Readwell Touch, Robonik PVT LTD, Thane, India). Pentamidine isethionate (Sigma-Aldrich, St Louis, MO, USA) was used as reference drug. The assays were carried out in triplicate. Test samples concentrations corresponding to 50% of parasite growth inhibition (IC_{50}) were determined from non-linear regression (Muelas-Serrano *et al*. 2000; Santos *et al*. 2012).

RESULTS

Among the fifteen ethanol extracts tested in this work against epimastigote forms of *T. cruzi*, the extract of *L. lupulina* roots was the most active with IC_{50} 20.0 µg mL^{-1}. An ethyl acetate fraction (FAc) obtained from the extract of *L. salviaefolia* leaves was the second most effective against this protozoa (IC_{50} 71.9 µg mL^{-1}), followed by the extract of *L. velutina* leaves and an extract of *L. lupulina* stems and leaves combined (both with IC_{50} 85.5 µg mL^{-1}). Regarding activity against promastigote forms of *L. amazonensis*, the extract of *L. lupulina* roots was again the most active with IC_{50} 54.5 µg mL^{-1}. It was followed by the extract of *L. lupulina* leaves (IC_{50} 95.4 µg mL^{-1}) and by FAc (IC_{50} 134 µg mL^{-1}). The results for all extracts are summarized in Table 1. Among the pure compounds, a triterpenoid (oleanonic acid, **1;** Figure 1) showed best inhibitory activity against both *T. cruzi* and *L. amazonensis,* with IC_{50} 8.4 and 13.6 µg mL^{-1}, respectively. The results obtained for pure compounds are summarized in Table 2.

Table 1. Trypanocidal and leishmanicidal activity of fifteen ethanol extracts from *Lippia* species and of four partition fractions obtained from the ethanol extract of leaves of *Lippia salviaefolia* (EELLsal). Benznidazole and pentamidine were used as reference drugs for *Trypanosoma cruzi* and *Leishmania amazonensis*, respectively.

Sample	IC_{50} values (µg mL^{-1})	
	T. cruzi	*L. amazonensis*
L. balansae Flowers	147.8	n.t.
L. balansae Leaves	145.8	n.t.
L. balansae Stems	374.8	n.t.
L. lasiocalycina Leaves and Stems	> 500	n.t.
L. sidoides Leaves	122.3	136.1
L. sidoides Stems	> 500	430.0
L. sidoides Roots	206.1	Inactive
L. lupulina Flowers	> 500	180.0
L. lupulina Leaves	> 500	95.4
L. lupulina Stems	85.5	390.1
L. lupulina Roots	20.0	54.5
L. velutina Stems	> 500	Inactive
L. velutina Leaves	85.5	n.t.
L. salviaefolia Stems	> 500	Inactive
L. salviaefolia Leaves	150.9	173.3
Hexane fraction of EELLsal	500	269.9
Ethyl acetate fraction of EELLsal	71.9	134.7
n-Butanol fraction of EELLsal	337.9	Inactive
Aqueous fraction of EELLsal	> 500	Inactive
Reference drug	9.7	4.0

n.t. not tested.

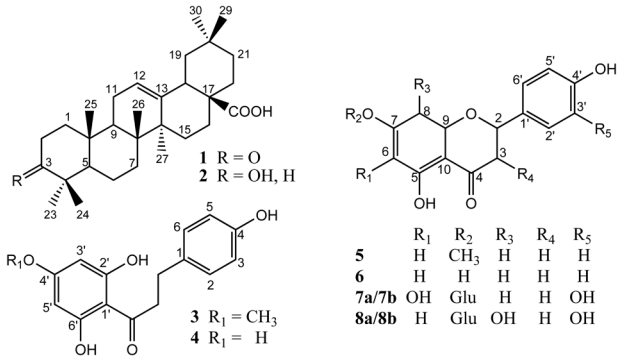

Figure 1. Compounds assayed against *Trypanosoma cruzi* and *Leishmania amazonensis*: oleanonic acid (**1**), oleanolic acid (**2**), asebogenin (**3**), phloretin (**4**), sakuranetin (**5**), naringenin (**6**) and the mixture of interconverting flavanones (2*R*)- and (2*S*)-3',4',5,6-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**7a**/**7b**); and (2*R*)- and (2*S*)-3',4',5,8-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**8a**/**8b**).

Table 2. Trypanocidal and leishmanicidal activity of compounds isolated from *Lippia* species.

Compound	*T. cruzi* IC_{50} values		*L. amazonensis* IC_{50} values	
	$\mu g\ mL^{-1}$	μM	$\mu g\ mL^{-1}$	μM
Oleanonic acid (**1**)	8.4	18.5	13.6	29.9
Oleanolic acid (**2**)	58.4	128.1	n.t.	n.t.
Asebogenin (**3**)	41.3	143.4	47.5	165.0
Phloretin (**4**)	>100	> 365.0	79.7	290.9
Sakuranetin (**5**)	47.5	166.1	39.4	137.8
Naringenin (**6**)	84.2	309.5	>100	> 367.6
Mixture (**7a**/**7b**/**8a**/**8b**)[1]	n.t.	n.t.	>100	> 214.6
Benznidazole	9.7	37.3	n.t.	n.t.
Pentamidine	n.t.	n.t.	4.0	11.8

[1]Mixture of interconverting flavanones (2*R*)- and (2*S*)-3',4',5,6-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**7a**/**7b**); and (2*R*)- and (2*S*)-3',4',5,8-tetrahydroxyflavanone-7-*O*-β-glucopyranoside (**8a**/**8b**).
n.t. not tested.

DISCUSSION

Trypanocidal activity

Osorio *et al*. (2007) tested plant extracts against epimastigote forms of *T. cruzi* and promastigote forms of *L. amazonensis*, and proposed that extracts should be classified as highly active (IC_{50} lower than 10 μg mL^{-1}), active (IC_{50} between 10 and 50 μg mL^{-1}), moderately active (IC_{50} higher than 50 and lower than 100 μg mL^{-1}) or non-active (IC_{50} higher than or equal to 100 μg mL^{-1}). According to such criteria, the extract of *L. lupulina* roots could be classified as active against *T. cruzi* (IC_{50} 20.0 μg mL^{-1}). FAc (IC_{50} 71.9 μg mL^{-1}) and the extracts of *L. velutina* leaves and of *L. lupulina* stems and leaves combined might be classified as moderately active (both with IC_{50} 85.5 μg mL^{-1}). The remaining extracts (Table 1) should be classified as non-active (Osorio *et al*. 2007).

Our findings corroborate previously reported data on trypanocidal activity of *Lippia* species polar extracts (Abe *et al*. 2005; Sülsen *et al*. 2006). Sülsen *et al*. (2006) reported *in vitro* activity of an organic and an aqueous extracts obtained from leaves of *Lippia integrifolia* against epimastigote forms of *T. cruzi*, with 34.5 and 71.2% inhibition, respectively, at 10 μg mL^{-1}. On the other hand, Abe *et al*. (2005) reported *in vitro* activity of a methanol extract of *Lippia dulcis* leaves against trypomastigote forms of *T. cruzi*, with MIC (minimum inhibitory concentration) between 125 and 250 μg mL^{-1}. Similar inhibitory activities have been observed in our experiments against epimastigote forms of *T. cruzi*, with IC_{50} between 20 and 375 μg mL^{-1} (Table 1).

Among the pure compounds isolated from *Lippia* species, the triterpenoid oleanonic acid (**1**) was the most active compound against both parasites tested in this work (Table 2). It exhibited 2.4-fold more potent trypanocidal activity than that from the original root extract of *L. lupulina* (IC_{50} 8.4 and 20.0 μg mL^{-1}, respectively). Considering the trypanocidal IC_{50} values in μM, compound **1** was 2-fold more potent than the reference drug benznidazole (Table 2). Oleanonic acid (**1**) was 6.9-fold more potent against *T. cruzi* than another triterpenoid, oleanolic acid (**2**) (IC_{50} 8.4 and 58.4 μg mL^{-1}, respectively, Table 2). This suggests that a keto group at C-3 might be more important for trypanocidal activity than a hydroxyl group at the same position (Figure 1).

On the other hand, Cunha *et al*. (2003) reported just the opposite. These authors found a 3.1-fold higher *in vitro* activity of oleanolic acid (**2**) against blood trypomastigote forms of *T. cruzi* Y strain when compared to oleanonic acid (**1**), with IC_{50} 80.4 and 294.9 μM, respectively. Such chemical groups could influence the molecules lipophilicity, interfering with their ability to penetrate through biological membranes and exert its activity. More recently, Ferreira *et al*. (2010) reported *in vitro* trypanocidal activity of oleanolic acid (**2**) against trypomastigote forms of *T. cruzi* H6 strain (Bolivian strain) with IC_{50} 45.2 μM, which is 2.2-fold more potent than the trypanocidal activity observed for compound **2** when tested against the epimastigote form of *T. cruzi* (Table 2).

Among the flavonoids, the dihydrochalcone asebogenin (**3**) and the flavanone sakuranetin (**5**) (IC_{50} 41.3 and 47.5 μg mL^{-1}, respectively, Table 2) exhibited higher trypanocidal activity than their original source sample (FAc, IC_{50} 71.9 μg mL^{-1}, Table 1). On the other hand, the flavanone naringenin (**6**) (IC_{50} 84.2 μg mL^{-1}) was slightly less effective than FAc. Compounds **3, 5** and **6** were also detected in the remaining active extracts against *T. cruzi* which presented similar chromatographic profiles by HPLC-PDA and UHPLC-PDA-TOF-MS experiments (Funari *et al*. 2012a, Funari *et al*. 2012b). Therefore, these findings suggest that such compounds might be partially associated to the trypanocidal activity of the crude extracts and FAc.

According to Ribeiro *et al*. (1997), sakuranetin (**5**) isolated from leaves of *Trixis vauthieri* L. killed 100% of trypomastigote forms of *T. cruzi* Y strain *in vitro*, at 100 μg mL^{-1}. In another report, this flavanone isolated from *Baccharis retusa* showed *in vitro* activity against *T. cruzi* trypomastigotes (IC_{50} 20.2 μg mL^{-1}) (Grecco *et al*. 2012). Similar results were observed for sakuranetin (**5**) in our experiments against epimastigote forms of *T. cruzi* Y strain (IC_{50} 47.5 μg mL^{-1},Table 2).

Although the dihydrochalcone phloretin (**4**) did not show detectable trypanocidal activity in our work (IC_{50} higher than 365 μM, Table 2), it has been described to influence the glucose metabolism of *T. cruzi*, *T. brucei*, and *T. rangeli* (Bakker *et al*. 1999; Einicker-Lamas *et al*. 2000; Miletti *et al*. 2006).

Leishmanicidal activity

The extract of *L. lupulina* roots and the extract of *L. lupulina* leaves might be classified (Osorio *et al*. 2007) as moderately active against *L. amazonensis* (IC_{50} 54.5 and 95.4 μg mL^{-1}, respectively). All the remaining extracts should be considered non-active against this protozoan, since they exhibited IC_{50} higher than 100 μg mL^{-1} (Table 1).

Oleanonic acid (**1**) proved to be the most active pure compound isolated from *Lippia* spp. assayed in this work (IC_{50} 13.6 μg mL^{-1}, Table 2). It was 4-fold more potent than the extract of *L. lupulina* roots, which afforded compound **1** in a previous study (Funari *et al*. 2012a). Considering the leishmanicidal IC_{50} values in μM, oleanonic acid was 2.5-fold less potent than pentamidine (Table 2). On the other hand, Torres-Santos *et al*. (2004) reported 11-fold higher activity of oleanonic acid (**1**) isolated from leaves of *Pourouma guianensis* against promastigote forms of *L. amazonensis* (IC_{50} 10.0 μg mL^{-1}).

The flavonoids sakuranetin (**5**), asebogenin (**3**) and phloretin (**4**) exhibited higher leishmanicidal activities (IC_{50} 39.4, 47.5 and 79.5 μg mL^{-1}, respectively) than their original source sample FAc (IC_{50} 134.7 μg mL^{-1}). It is noteworthy that such flavonoids were previously detected in the ethanol extract from *L. sidoides* leaves and might therefore be related to its leishmanicidal activity (Funari *et al*. 2012a).

Grecco *et al*. (2012) reported IC_{50} 51.9 μg mL^{-1} for sakuranetin (**5**) isolated from *Baccharis retusa* against promastigotes of *L. amazonensis*, which is similar to the results from our experiments against the same type of protozoa (Table 2). Asebogenin (**3**) isolated from an extract of *Piper elongatum* aerial parts was described by Hermoso *et al*. (2003) as more active against promastigote forms of *L. braziliensis*, *L. tropica* and *L. infantum* (IC_{50} 28.5, 3.8 and 6.3 μg mL^{-1}, respectively) than the observed inhibition against *L. amazonensis* promastigotes (IC_{50} 47.5 μg mL^{-1}, Table 2) in our experiments. The leishmanicidal activity observed for phloretin (**4**) might be related to its influence on the glucose metabolism of *L. amazonensis*, since it was previously described to affect the glucose metabolism in *L. mexicana* (Burchmore and Hart 1995) and *L. donovani* (Ter Kuile and Opperdoes 1993).

CONCLUSIONS

The results presented in this work suggest that *Lippia* genus and its chemical constituents deserve further investigation in the search for novel antiprotozoal drugs. Special attention should be given to *L. lupulina* roots, since its ethanol extract exhibited the strongest trypanocidal and leishmanicidal activity among all extracts assayed in this work. Oleanonic acid also deserves further investigation since it showed leishmanicidal activity similar to that observed for the antileishmanial compound pentamidine, and trypanocidal activity higher than benznidazole, the only anti-Chagas therapeutic agent currently in medical use.

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