Trypanocidal and Leishmanicidal Activities of Flavonoids from Argentine Medicinal Plants

Valeria P. Sülsen, Silvia I. Cazorla, Fernanda M. Frank, Flavia C. Redko, Claudia A. Anesini, Jorge D. Coussio, Emilio L. Malchiodi, Virginia S. Martino, and Liliana V. Muschietti*
Cátedra de Farmacognosia, (IQUIMEFA)-(UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina; Cátedra de Inmunología, (IQUIMEFA)-(UBA-CONICET), Facultad de Farmacia y Bioquímica; Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

INTRODUCTION

American trypanosomiasis (Chagas disease) and leishmaniasis are a major health problem over the world. It is estimated that 18–20 million people are infected with Trypanosoma cruzi in Latin America and that 12 million people are infected with Leishmania spp. worldwide. In previous studies, we reported the existence of patients co-infected with T. cruzi and Leishmania spp., in which the two parasitoses were determined unequivocally by direct parasitological methods and later confirmed by PCR. Mixed infections are an important problem in several regions of Latin America where the endemic areas for these two parasitoses frequently overlap. The available treatment of Chagas disease includes nifurtimox and benznidazole, which are effective in acute and congenital diseases but which have lower efficacy in treatment of chronic infection. On the other hand, pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) and amphoterin B are used in the treatment of different leishmaniasises.

Although great advances have been made in the fields of molecular biology and pathophysiology, these advances are not aimed at the development of new products that fulfill the needs of patients suffering from parasitic infections. The scarcity of compounds, the high incidence of side effects, and the emerging resistance to available drugs have led to the urgent need for new therapeutic agents against these diseases.

Natural products are important sources of new drugs because their derivatives are extremely useful as lead structures for synthetic modification and optimization of bioactivity. As part of our ongoing search of antiparasitic compounds from natural sources, the trypanocidal activity of selected Argentine medicinal plants has been reported by our group. Among the active species, the organic extracts of Ambrosia tenuifolia and Eupatorium buniifolium were 2 of the most promising, with percentages of growth inhibition of T. cruzi epimastigotes > 70%. These results prompted us to select these species for further studies on trypanocidal activity of the purified compounds.

Ambrosia tenuifolia Sprengel (Asteraceae) is a shrub commonly known as “ajeno del campo,” “ultamisa,” “saltamisa,” or “artemisia,” widely distributed in the north and central regions of Argentina. The decoction of the aerial parts and roots is traditionally used against intermittent fevers, as stimulant, antihelmintic, antineuralgic, and for gastrointestinal pain. Previous chemical studies carried out on this species have led to identification of flavonoids and several sesquiterpene lactones.

Eupatorium buniifolium H. et Arn. (Asteraceae) is a medium-sized shrub known as “romerillo,” “romerillo colorado,” or “chica,” commonly found in the northeastern and central regions of Argentina. The decoction of the aerial parts has been used against rheumatic pains and as desinfec tant. Previous phytochemical studies on this species showed the presence of flavonoids, hydroxycinnamic acid derivatives, and ent-labdanes. Pharmacological activities—such as antioxidant, antiviral, analgesic—and identification of 3 anti-inflammatory compounds have been reported for this species.

Screening of candidate trypanocidal compounds is hindered by the lack of simple, reliable, and rapid drug-evaluation tests; instead, such studies have only been based on the microscopic counting of parasites. The microscopy method is time-consuming and incompatible with high throughput. Although improved techniques in which parasites have been transfected with β-galactosidase, β-lactamase, or luciferase reporter genes have been developed, these methods have limitations. They are restricted to the transfected parasite strain and species, making screening more difficult when testing different parasites. Transfection may render parasites with an altered metabolism or infectivity, and the transfected enzymes may interfere with the compounds under study. Thus, in the course of this study, a sensitive technique that takes advantage of the (H) thymidine (H)T uptake by dividing trypanosomatids has been developed for quantification of the parasiticial effect of natural products.

* Address correspondence to Liliana Muschietti, Cátedra de Farmacognosia, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires 1113, Argentina. E-mail: lmusch@ffyb.uba.ar

Copyright © 2007 by The American Society of Tropical Medicine and Hygiene


654
In this context, we undertook an investigation to identify trypanocidal and leishmanicidal lead compounds from *A. tenuifolia* and *E. buniifolium* by means of an easy, sensitive, and reproducible screening method.

MATERIALS AND METHODS

**General experimental procedures.** The UV and IR spectra were recorded on Shimadzu UV2101PC (Shimadzu, Tokyo, Japan) and on Bruker FT-IR IFS25 (Bruker Optics, Inc., Billerica, MA) spectrophotometers, respectively. MS analysis was performed on a Shimadzu QP 5000-GC 17 A apparatus, and 1H-NMR data at 300 MHz were recorded on a Bruker MSL300 instrument. A semipreparative RP-18 [LiChrospher, 5 μm (125 × 4)] column was used for the HPLC analysis (Waters Corporation, Milford, MA).

**Parasite strains.** *T. cruzi* epimastigotes, República Argentina (RA) strain, were grown in biphasic medium as previously described.²⁶ *Leishmania mexicana* promastigotes strain MNYC/BZ/62/M were grown on liver infusion tryptose (LIT) medium. Both cultures were routinely maintained by means of weekly passages at 26 and 28°C, respectively. *T. cruzi* bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture, at the peak of parasitemia.²⁷

**Animals.** Inbred male CF1 mice, 21 days old, were nursed at the Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires. Inbred female BALB/c mice (22 ± 2 g) were purchased from the Instituto Nacional de Tecnología Agropecuaria (INTA). Sixty 100-day-old animals were kept according to the Guide for the Care and Use of Laboratory Animals, U.S. National Research Council.

**Plant material.** The aerial parts of *A. tenuifolia* were collected in the province of Buenos Aires, Argentina, in 2004. Plant material was identified by Dr. G. Giberti, and a voucher specimen (BAF 649) is deposited at the Herbarium of the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. The aerial parts of *E. buniifolium* were collected in the province of Entre Ríos, Argentina, in 2004 and identified by J. de D. Muñoz. A voucher specimen (ERA-MUNOZ 5067) is deposited at the Facultad de Ciencias Agropecuarias, Universidad Nacional de Entre Ríos.

**Extraction and purification.** The aerial parts of *A. tenuifolia* (1000 g) and *E. buniifolium* (300 g) were air-dried, ground to powder, and extracted with dichloromethane/methanol (1:1) as previously described.¹³ Fractionation of these extracts was performed as follows.

Twenty grams of an organic extract of *A. tenuifolia* was chromatographed on a silica gel 60 (500 g) column eluted with cyclohexane, cyclohexane/ethyl acetate (1:1), ethyl acetate, and methanol. Twenty-four fractions of 500 mL each were collected. According to their profile in thin layer chromatography (TLC), on silica gel 60 F₂₅₄, fractions were combined into 5 fractions, F₁₅₋₅₄. Each fraction was tested for growth inhibition on *T. cruzi* epimastigotes. Fraction F₁₅₋₅₄ (3 g) was then subjected to gel filtration on a Sephadex LH-20 (110 g) column eluted with an n-hexane/ethyl acetate gradient (from 100:0 to 0:100) and 100% methanol to yield 120 fractions of 10 mL each. Compound 1 was obtained from fractions 105–107 as an amorphous yellow powder.

Twenty grams of an organic extract of *E. buniifolium* was subjected to column chromatography (CC) on silica gel 60 (500 g) eluted with cyclohexane, cyclohexane/ethyl acetate (5:5), ethyl acetate, and methanol to afford 18 fractions of 500 mL each. Eluates were monitored by TLC on silica gel 60 F₂₅₄ using toluene/ethyl acetate (5:5) and combined into 4 fractions (F₁₋₄). Each fraction was subjected to a trypanocidal activity bioassay on *T. cruzi* epimastigotes. Fraction F₁₋₄ (5 g) was further fractionated using a Sephadex LH-20 column (100 g) and eluted with gradients of hexane/ethyl acetate (100:0 to 0:100) and ethyl acetate/methanol (100:0 to 0:100) obtaining 70 subfractions of 30 mL each that were combined into 13 groups (F₁₋₇,F₂₋₁₃) according to their TLC migration profile. The purification of F₁₋₇ was performed by a semipreparative reverse-phase HPLC (RP-18, water/methanol gradient, flow rate = 1 mL/min). The eluate, containing a single peak, was dried to obtain compound 2.

**Biologic assays.** (³H)Thymidine uptake assay. Exponentially growing parasites were centrifuged at 1200g for 15 min, adjusted to a cell density of 10⁵–10⁷ parasites/mL in fresh LIT medium supplemented with 10% decomplemented fetal bovine serum (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Parasites were seeded in a 96-well microplate (150 μL per well). One microcurie of (³H)T was added to each well, and cultures were incubated for 16 hours. Counts per minute (cpm) of triplicate cultures were measured by an automated liquid scintillation counter (Packard, Downers Grove, IL). The correlation between cell counts (carried out in a Neubauer chamber) and incorporated cpm was evaluated. The susceptibility of parasites to each drug was assayed at different doses: benznidazole (Roche) was tested at 5, 10, 15, and 20 μM for *T. cruzi*, and amphotericin B (ICN) was tested at 25, 50, 100, and 200 ng/mL for *L. mexicana* during 72 and 120 hours, respectively. For the last 16 hours of culture, cells were pulsed with 1 μCi (³H)T per well.

**Trypanocidal and leishmanicidal activity of fractions and purified compounds.** Growth inhibition of *T. cruzi* epimastigotes was evaluated for fractions F₁₅₋₅₄, F₁₋₄, F₁₋₇ and purified compounds at final concentrations ranging from 0.3 to 100 μg/mL. Compounds 1 and 2 were also evaluated for leishmanicidal activity on *L. mexicana* promastigotes at the same concentrations. Exponentially growing parasites were harvested and adjusted to a cell density of 1.5 × 10⁶ parasites/mL in fresh medium. Parasites were allowed to grow for 72 and 120 hours in medium alone or in the presence of different concentrations of the compounds (in triplicate for each of the 6 concentrations analyzed) at 28°C. Percent of inhibition was calculated as [100 – [(cpm of treated parasites/cpm of untreated parasites) × 100]]. The compound concentration at which the parasite growth was inhibited by 50% (IC₅₀) was determined after 72 hours.

The trypanocidal effect of the purified compounds was also tested on bloodstream trypomastigotes according to a standard WHO protocol with slight modifications.²⁸ Briefly, mouse blood containing trypomastigotes was diluted in complete LIT medium to adjust the parasite concentration to 1.5 × 10⁶/mL. Parasites were seeded in duplicate in a 96-well microplate in the presence of each compound and controls and incubated at 4°C for 24 hours. The number of remaining living parasites in each sample was determined in 5 μL of cell suspension diluted 1:5 in lysis buffer (0.75% NH₄Cl, 0.2% Tris, pH 7.2) and counted in a Neubauer chamber. Positive controls were performed with either different concentrations
of benznidazole for *T. cruzi* or amphotericin B for *L. mexicana*.

**T-cell toxicity.** Lymphoid cell suspensions were obtained from the lymph nodes of BALB/c mice as previously reported.\(^2\) Briefly, T-cell–enriched populations were obtained by passage of the cell suspension through a nylon wool column.\(^3\) T-cell percentage was >97%, as checked by lysis with an anti-Thy antiserum plus complement and by indirect immunofluorescence. Cells were cultured at a concentration of 2 × 10⁶ cells/mL in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Cells were adjusted to a final volume of 0.2 mL per well in a 96-well flat-bottom microtiter plate and cultured at 37°C in a 5% CO₂ atmosphere for 3 and 24 hours. Cell viability was determined by the trypan blue exclusion method in the absence and presence of increasing concentrations of the purified compounds dissolved in 50% v/v EtOH (0.1, 1, 10, and 50 μg/mL).

## RESULTS

**Isolation and structure elucidation.** Compounds 1 and 2 were isolated and purified by bioassay-guided fractionation from fractions F₁₅ and F₂₇ from the organic extracts of *A. tenuifolia* and *E. buniifolium*, respectively. Both compounds were identified by comparing their spectroscopic data (IR, UV, MS, \(^1\)H-NMR) to those reported in the literature as the flavonoids hispidulin (compound 1) and santin (compound 2)\(^3,4,5\) (Figure 1).

**\(^{(3)}\)HThymidine uptake assay.** To determine the number of parasites measurable by the \(^{(3)}\)HT uptake assay, 3-fold dilutions of parasites were grown in triplicate and ranging from 10⁴ to 10⁷ epimastigotes/mL (Figure 2A). The linear correlation between number of epimastigotes/mL and cpm was optimum (r² > 0.99). Similar results were obtained when promastigotes of *L. mexicana* were used (data not shown). To evaluate the use of the \(^{(3)}\)HT uptake assay for parasite counting as a substitute for Neubauer chamber, we determined the viability of epimastigotes in the presence of benznidazole by both methods. Parasites were cultured with the drugs at different concentrations during 72 hours and allowed to grow with the addition of \(^{(3)}\)HT for the last 16 hours before counting in the Neubauer chamber and in the scintillation counter. There was a significant correlation (P < 0.03) between microscopic counting and cpm (Figure 2B). Similar results were obtained for incubation periods of 120 hours (data not shown). The dose-dependent proliferative inhibition was corroborated by culturing parasites in the presence of benznidazole or amphotericin B for *T. cruzi* or *L. mexicana* respectively, during 72 and 120 hours (Figure 3).

**Trypanocidal and leishmanicidal activities of fractions and purified compounds.** Fractions F₁₅ and F₂₇ from *A. tenuifolia* and *E. buniifolium*, respectively, showed the strongest trypanocidal activity against *T. cruzi* epimastigotes. At 100 μg/mL, F₁₅ inhibited the growth of epimastigotes by 87.09% ± 4.2% and F₂₇ by 64.1% ± 1.1% at 72 hours of incubation.

The results for the in vitro assay with hispidulin and santin, on *T. cruzi* epimastigotes are shown in Figure 4. At 100 μg/mL hispidulin induced a growth inhibition of 78.9% ± 4.6% at 72 hours and 98.0% ± 0.3% at 120 hours (P < 0.01), whereas with santin there were no differences between both times of incubation (95.2% ± 0.8% and 92.2% ± 4.2%, respectively). On *T. cruzi* epimastigotes, IC₅₀ values for hispidulin and santin were 46.7 and 47.4 μM, respectively, whereas for trypanostigmates IC₅₀ values were 62.3 μM for hispidulin and 42.1 μM for santin.

The activity of the compounds on *L. mexicana* promastigotes is shown in Figure 5. At 100 μg/mL, hispidulin induced percentages of growth inhibition of 78.0% ± 6.6% and 81.1% ± 6.6% and santin 85.7% ± 4.5% and 89.1% ± 4.1%, at 72 and 120 hours, respectively. No significant differences were found between both times of incubation. Hispidulin exhibited a significant leishmanicidal activity with an IC₅₀ value of 6.0 μM, while santin was less active (IC₅₀ = 32.5 μM).

**Cytotoxicity assay.** The results of the cytotoxic activity on lymphoid cell suspensions, expressed as cell viability, are...
shown in Figure 6. The 50% cytotoxic concentration (CC<sub>50</sub>) was >50 μg/mL when cell suspensions were treated with both compounds.

DISCUSSION AND CONCLUSION

The relevance of a specific antiparasitic treatment in Chagas disease has been the subject of intense debate for many years, particularly during the chronic stage of the disease. Despite many studies demonstrating the existence of autoimmune phenomena in Chagas disease, current knowledge suggests that parasite persistence, coupled with an unbalanced immune response, plays a pivotal role in the development of the characteristic pathology present in both acute and chronic stages of the disease. At present, there is a consensus on the elimination of <i>T. cruzi</i> from infected individuals as a requirement for the prevention of the chronic-stage characteristic lesions.

However, currently available chemotherapy, based on nifurtimox and benznidazole, is unsatisfactory because of their limited efficacy in the prevalent chronic stage of the disease and their toxic side effects. These effects, including anorexia, vomiting, peripheral polyneuropathy, and allergic dermatopathy, probably as a consequence of oxidative or reductive damage in the host’s tissues, can lead to discontinuation of treatment.

Mixed infections caused by <i>T. cruzi</i> and <i>Leishmania</i> spp. have recently been demonstrated in patients. Nevertheless, the use of a single drug to treat both parasitoses is still out of reach.

Natural products play important roles in the drug discovery and development process, particularly in the field of infectious diseases, where 75% of these drugs are of natural origin. We have previously found in vitro trypanocidal activity in <i>A. tenuifolia</i> and <i>E. bunifolium</i> organic extracts. In this
Both flavonoids inhibited the growth of *T. cruzi* epimastigotes and trypanostigote at IC₅₀ values ranging from 42.1 to 62.3 μM. Hispidulin showed a greater inhibitory effect than santin on *L. mexicana* promastigotes.

Flavonoids are natural polyphenolic compounds widely distributed in the plant kingdom. These compounds display a remarkable array of biochemical and pharmacological effects, suggesting that certain members of this group may affect the function of various mammalian cellular systems. Among the main properties that may account for the potential health benefits of flavonoids are their antioxidant, antiinflammatory, anticarcinogenic, hepatoprotective, antithrombotic, antiallergic, and antiviral activities.¹⁴

The antiprotozoal activity of several flavonoids, such as quercetin, quercetin, showing certain selectivity has been previously reported.³⁵ Tasdemir and others found that methylation of the OH groups present in flavones and flavonols, reduced the leishmanicidal activity significantly.³⁶ Our results are in agreement with this previous finding, as hispidulin was found to be more active than santin.

Hispidulin had already been reported as antioxidant, spasmyotic, and antiinflammatory.³⁷ The main biologic activities reported for santin are those related to its in vivo and in vitro anti-inflammatory properties.³⁸ Hispidulin had already been isolated from *A. tenuifolia*³⁹; however, the occurrence of santin in *E. buniifolium* and the antiprotozoal activity of these compounds is reported herein for the first time.

The in vitro cytotoxicity tests carried out on mammalian cells provide preliminary information about the selectivity of these compounds. When lymphoid mice cells were treated with hispidulin and santin, the CC₅₀ found were > 50 μg/mL, thus indicating that selectivity of these compounds for the parasites studied is greater than that for mammalian cells.

To evaluate the usefulness of the (³²H)T uptake assay as a substitute for the parasite counts employing the hemocytometer (Neubauer chamber), we determined the viability of epimastigotes in the presence of benzimidazole by both methods. Unlike other manual screening methods, in the (³²H)T uptake assay, subsequent manipulation steps are not required, thus increasing the reproducibility and reliability of this assay and the speed at which data can be collected. These results demonstrate that (³²H)T uptake assay is a more appropriate assay for the screening of natural products. Moreover, this assay is capable of discerning parasite concentrations of 2 × 10⁵ parasites/mL, a concentration that can hardly be handled when the microscopic counting is used.

In conclusion, the results obtained in this study showed that hispidulin and santin have significant trypanocidal and leishmanicidal activities. These flavonoids could serve as potential lead compounds for the development of more efficient drugs for the treatment of leishmaniasis and Chagas disease. Further in vivo studies and evaluation of the underlying mechanisms are required. Identification of other active compounds from these species is currently being undertaken in our laboratory.

Received January 8, 2007. Accepted for publication June 17, 2007.

Acknowledgments: The technical assistance of Mr. Jerónimo Ulloa is gratefully acknowledged.

Financial support: This investigation was supported in part by PICT 2002 05-11240 (Agencia Nacional de Promoción Científica y Tecnológica), PIP 02419 (Consejo Nacional de Investigaciones Científicas y Técnicas), UBA-SECYT B101 and PICT 12216 (Agencia Nacional de Promoción Científica y Tecnológica).

Authors’ addresses: V. Sülse, F. Redko, C. Anesini, J. Coussio, V. Martino, and L. Muschietti, Cátedra de Farmacognosia, (IQUIMEFA)-(UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires; S. Cazorla, F. Frank, and E. Malchiodi, Cátedra de Inmunología, (IDEHU)-(UBA-CONICET), Facultad de Farmacia y Bioquímica; Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina, Junín 956, Buenos Aires 1113, Argentina.

Reprint requests: Liliana Muschietti, Cátedra de Farmacognosia, (IQUIMEFA)-(UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 2ºP (113), Buenos...
REFERENCES


