REVIEW ARTICLE

Natural products and Chagas' disease: the action of triterpenes acids isolated from *Miconia* species

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Abstract

The protozoan *Trypanosoma cruzi* causes Chagas' disease, a neglected illness that remains a relevant public health concern in Latin America. In Brazil, Benznidazole is available for its treatment. This compound is effective against circulating forms of the parasite in the acute phase of the disease, but its efficacy during the chronic stage is debatable. The search for new medications that can treat Chagas' disease is therefore mandatory. Natural sources display a wide range of secondary metabolites and may play an important role in the discovery of new potential drugs. *Miconia* is one of the largest genus of the family Melastomataceae and includes approximately 1,000 plant species; Brazil alone is home to approximately 250 of these species, which exist in forests and savannas. Studies on the various biological activities of the *Miconia* species have reported promising results. Several researchers have screened these plants as well as their extracts *in vitro* against trypomastigote forms of *T. cruzi*, which displayed significant trypanocidal activity. It has been demonstrated that the presence of ursolic and oleanolic determines this biological activity.

Keywords: Chagas' disease; *Trypanosoma cruzi*; *Miconia*.

Introduction

Neglected tropical diseases (NTDs) are life-threatening or disabling infections affecting more than a billion people worldwide. Underprivileged populations living in remote rural areas, urban slums, or conflict zones, often in underdeveloped and developing countries, are at the highest risk of acquiring NTDs. Consequently, people suffering from these diseases constitute an unattractive market to private-sector research. This situation is a matter of significant concern–NTDs not only affect health directly, but they also represent an impasse of socioeconomic impact that perpetuates poverty in a sizable number of the population (Schmidt et al. 2012a).

An estimated 1.2 billion people are currently infected with NTDs; about one billion of these people live on less than $1 per day. In some areas, women and children with NTDs are considered as neglected populations, because they have limited access to health and social support services. Other vulnerable groups that face poverty and live under the impact of NTDs include peri-urban slum dwellers (Herrera et al. 2011). The major effects of NTDs on public health stem from chronic conditions that result in disfigurement as well as lifelong disability and morbidity (Hotez et al. 2009).

The drugs that are currently available to treat NTDs have long been in the market and exhibit many toxic effects (Lemke 2012); this makes the search for more effective and safer medications to fight these conditions mandatory. However, a fundamental problem regarding NTDs is to convince pharmaceutical companies to invest resources and develop effective treatments: the population affected by these diseases cannot afford expensive drugs (Dimitri 2012).

An evaluation of the drugs approved by the FDA (Food and Drug Administration) between 2007 and 2011 shows that only five of the 119 approved medications belong to the class of antimicrobials (antibiotic, antimycobacterial, antiprotozoal, and antifungal agents) (Mullard 2011).

Seventeen NTDs exist, including three major protozoan diseases: human African trypanosomiasis (HAT or "sleeping sickness"), Chagas' disease, and leishmaniasis, which affect many millions of people worldwide. In 2009, an estimated 10 million people were infected with Chagas' disease, and the estimated number of annual deaths was 10,000. In turn, leishmaniases cause approximately 50,000 deaths annually. As for HAT, the total number of infected people in 2006 was 50,000-70,000 (WHO 2010), although the number of new infections appears to be dwindling (Schmidt et al. 2012a).

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease. It is estimated that there are eight million inhabitants infected with this disease in Latin America; 30-40% has or will develop cardiomyopathy or mega digestive syndrome (Parker & Sethi 2011). The life cycle of *T. cruzi* implicates hematophagous triatomine insect (vectors), a wide range of vertebrate mammalian hosts and different developmental forms of the parasite. Briefly, after insect vectors ingest bloodstream trypomastigotes from an infected mammalian host, *T. cruzi* forms convert into epimastigotes that proliferate and then differentiate into metacyclic forms inside the posterior intestine of the triatomine. These infective parasite forms are discharged in the feces of the triatomine and can invade new vertebrate cells, in which they undergo another round of differentiation into the intracellular amastigote forms. The latter proliferate and transform back to trypomastigotes, the form that disseminates the infection (Soeiro & Castro 2011).

Although effective efforts to control vectorial and blood transmission have significantly reduced the number of new cases, there are still many challenges that need to be confronted regarding Chagas' disease. The disease has a peculiar epidemiology, characterized by a variety of risk factors, several potential vectors and reservoirs, different forms of transmission, and a number of parasite isolates in domiciliary, peridomiciliary, and sylvatic environments; furthermore, prophylactic therapies and effective chemotherapeutic schemes are virtually inexistent (Coura 2007).

Chagas' disease constitutes a complex condition in which *T. cruzi* effectively invades human cells. All transmission routes involve infective but non-proliferative trypomastigotes entering the bloodstream and eluding the immune system by permeating a variety of cell types, including muscle and nerve cells of the heart and the gastrointestinal tract, as well as cells of the reticuloendothelial system. Tissue damage in the mammalian host results from both direct parasite action and an inflammatory process. The initial acute phase has low (<10%) mortality and generally presents mild and unspecific symptoms. Macrophages, IFN-gamma, and CD4+ and CD8+ Th1 lymphocytes are the key elements controlling parasite replication (Urbina 2010).

A life-long chronic condition follows the acute phase of Chagas' disease. In the chronic stage, the cellular immune response limits parasite proliferation but cannot eradicate the infection (Albareda et al. 2006). The most severe of these manifestations is chronic Chagas cardiomyopathy (CCC), which typically appears decades after the initial infection, and may result in cardiac arrhythmia, ventricular aneurysm, congestive heart failure, thromboembolism, and sudden cardiac death. This condition is the leading cause of cardiac disease and cardiac death in underprivileged, rural, and rural-originated urban populations, in Latin America (Rassi et al. 2009).

Nifurtimox and Benznidazole are the two drugs currently available to treat chagasic patients; however, their toxicity is well known as well as their limited effect on different parasite isolates and disease phases making the discovery of novel medications extremely urgent. Nifurtimox, which is produced and used mainly in Central America but not Brazil, has a high incidence of side effects (reported by 40% of patients), which include nausea, vomiting, abdominal pain, weight loss, severe anorexia, and adverse neurological effects such as restlessness, paresthesias, twitching, insomnia, and seizures (Marin-Neto et al. 2009).

Available in Brazil, nitroheterocyclic Benznidazole is used to treat Chagas' disease. Benznidazole is effective against the circulating form of the parasite (trypomastigotes) in the acute phase of the disease, but its efficacy during the chronic stage is debatable (Silva et al. 2013). Compared to Nifurtimox, Benznidazole has fewer side effects such as hypersensitivity (dermatitis, generalized edema, ganglionic infarction, and joint and muscle pains), bone marrow depletion, and peripheral polyneuropathy (Coura 2009). Benznidazole is produced by LAFEPE (Laboratório Farmacêutico do Estado de Pernambuco, Recife, Brazil); supported by recent DNDi (Drugs for Neglected Diseases initiative) efforts, it will begin to manufacture a pediatric formulation (Soeiro & Castro 2011). Because of the limited efficacy of the currently available drug treatments and the lack of a vaccine, controlling the transmission of the etiological agent in endemic areas necessarily depends on reducing the vector home population through insecticide spraying programs, improved housing conditions, and campaigns to raise awareness in vulnerable communities. Infection by *T. cruzi*, in particular, is likely to remain endemic in sylvatic hosts despite spraying and insect control efforts. Contemporary drug treatments fall short in treating this disease because of the long life span of infected human hosts, its resistance to triatomine insecticides, and the ease with which protozoans develop resistance to the drugs (Diotaiuti et al. 1995, Murta & Romanha 1998, Vassena et al. 2000).

Given the significant limitations of the medications currently available, particularly their inability to treat chronic patients, new steps have been taken toward specific chemotherapeutic approaches for the management of Chagas' disease (Urbina 2010). Natural sources such as plants exhibit a wide diversity of secondary metabolites and may play an important role as new drug leads (Schmidt et al. 2012b).

Research into natural products

In developing and less developed countries, underprivileged communities have used traditional plant-based curative applications and medicinal products since ancient times. Chemical substances derived not only from plants, but also from animals and microorganisms have long been employed to treat a number of human diseases. In fact, drug discovery studies based on natural toxins and their derivatives have culminated in the development of many therapeutic agents (Tempone et al. 2007).

The research, development, and use of natural products as therapeutic agents, especially those derived from higher plants, have increased significantly (Gurib-Fakim 2006). Commonly, the plant part subjected to the extraction procedure is the same as the one used in traditional medicine; however, other parts should be assessed, to determine which section contains the highest concentration of the active compounds. Because it is not certain whether only the most polar compounds will display the best action, activity

screening should use solvents of different polarities to extract the substances (Izumi et al. 2010).

Scientists have isolated several primary metabolites from plants, for example, vincristine, vinblastine, taxol, podopyllotoxin, and morphine. Many of these compounds have been modified, to yield better analogues in terms of activity, toxicity, and solubility (Hostettmann et al. 1997).

A preliminary investigation of chemical constituents is motivating; it provides information about the extracts and indicates the nature of its constituents, facilitating the selection of chromatographic techniques. The organic compounds should be identified by infrared spectroscopy (IR), which determines the presence or absence of functional groups; by ultraviolet spectroscopy (UV), which shows whether the compounds bear combined unsaturated bonds; by hydrogen and carbon-13 nuclear magnetic resonance spectroscopy (^{1}H-NMR and ^{13}C-NMR, respectively), which determines the nature and chemical environment of hydrogens and carbons, respectively, and represents the most important technique when investigating molecular structure; and by mass spectrometry (MS), which furnishes data on the weight and molecular formula, and helps identify the fragments typical of the molecule (Maciel et al. 2002).

Novel technologies that include recombinant pathogens and rapid detection methods are contributing to the development of drugs for the treatment of neglected diseases. About ten years ago, the advent of high-throughput screening (HTS) technology revolutionized the process of early drug development, enabling researchers to rapidly collect enormous amounts of data and explore compound libraries with unprecedented thoroughness (Phatak et al. 2009). One of the fastest HTS systems is Lucio Freitas-Junior's at the Institute Pasteur in Korea: a 384-well cell-culture system that combines an automated confocal microscope with an image analyzer that can screen up to 30,000 compounds a week (Clayton 2010). In 2011, Andriani et al. (2011) also reported the advantages of using HTS for the discovery of drugs to treat neglected diseases.

The use of antiparasitic drugs is common worldwide; fittingly, incidences of parasite resistance to some of these drugs are frequent. As a result, alternative treatments, especially those involving natural products, have emerged over the last years (Santos et al. 2012). In the past decades, several research groups have described the effect of plant-derived compounds on *T. cruzi* in the particular case of trypanosomiasis (Grael et al. 2000).

More recent studies have reported on compounds isolated from natural products that are active against *T. cruzi*. Veiga-Santos et al. (2013) demonstrated the antitrypanosomal activity of the amides piperovatine and piperlonguminine isolated from *Piper ovatum* against epimastigotes (IC_{50} of 41.5 ± 0.7 and 53.8 ± 6.2 μM, respectively) and intracellular amastigotes (IC_{50} of 35 ± 6.9 and 33.9 ± 5.4 μM, respectively).

Similar to other high incidence diseases, basic research in Chagas' disease is characterized by a large number of studies conducted on animal models infected by *Trypanosoma cruzi*. Different aspects of the human disease are reproduced after infection with *Trypanosoma cruzi* in mice (Sato et al. 1992), hamsters (Santos-Buch & Teixeira 1974), rabbits (Ramirez et al. 1994), *Rhesus* monkeys (Galvão-Castro et al. 1984) and dogs (Andrade et al. 1984).

Several studies have also analyzed the *in vivo* trypanocidal activity of substances isolated from pepper. Esperandim et al. (2013), for example, evaluated the *in vivo* therapeutic properties of (-)-cubebin, a compound isolated from the dry seeds of *Piper cubeba,* and (-)-Hinokinin, prepared by (-)-cubebin oxidation. Results revealed increased cell nuclei in untreated infected animals as compared to uninfected mice. The values obtained for infected animals treated with (-)-cubebin and (-)-hinokinin were close to those observed for uninfected mice. For the spleen, perimeter values of 10.85 μm (p <0.01) and 10.90 μm (p<0.05) were obtained for mice treated with (-)-cubebin 50 mg/kg and (-)-hinokinin 20 mg/kg, respectively, whereas untreated infected animals provided a perimeter of 11.76 μm. Saraiva et al. (2010) loaded

(-)-hinokinin onto poly (D, L-lactide-co-glycolide) microparticles and revealed that the treatment of infected mice with 40 mg/kg of (-)-hinokinin-loaded microparticles, every 2 days could bring about a significant decrease in parasitemia levels in comparison with those recorded in untreated controls (P<0.05 at days 12, 14,16, 19, and 21 post-infection with *T. cruzi*).

Laurella et al. (2012) evaluated the trypanocidal activity of the Argentinean *Mikania* species. The organic extracts of *M. micrantha*, *M. periplocifolia*, *M. parodii*, and *M. cordifolia* displayed significant antiprotozoal activity against *T. cruzi* epimastigotes and *L. braziliensis* promastigotes *in vitro*. The authors suggested that terpenoids, mainly sesquiterpene lactones and diterpenes, present in the genus *Mikania* accounted for the antitrypanosomal activity.

Escobar et al. (2010) have reported that essential oils obtained from different species of *Lippia* spp, a widely distributed genus of Colombian plants, exhibited important activity against *Leishmania chagasi* and *Trypanosoma cruzi*.

Despite all the efforts by researchers from different countries, the treatment of Chagas' disease has seen little progress. Experiments with active compounds isolated from plants have shown that modifying the molecule increases activity significantly; however, scientists rarely perform such alterations to the original compound, perhaps overlooking opportunities to find new drugs. There are many advantages to studying the medicinal benefits of natural products. In the case of Chagas' disease, the prospect of developing new drugs has led to the screening of the antitrypanosomal activity of almost 400 species belonging to more than 100 plant families over the last 15 years (Izumi et al. 2010).

Ursolic acid (**1**) and oleanolic acid (**2**) (**Figure 1**) both compounds and triterpenoids are widely distributed in the plant kingdom and have been frequently isolated as isomeric mixtures. Several articles have exposed an abundant interest in these triterpenoids including their isolation, chemical modifications, and pharmacological and toxicological studies (Liu 2005).
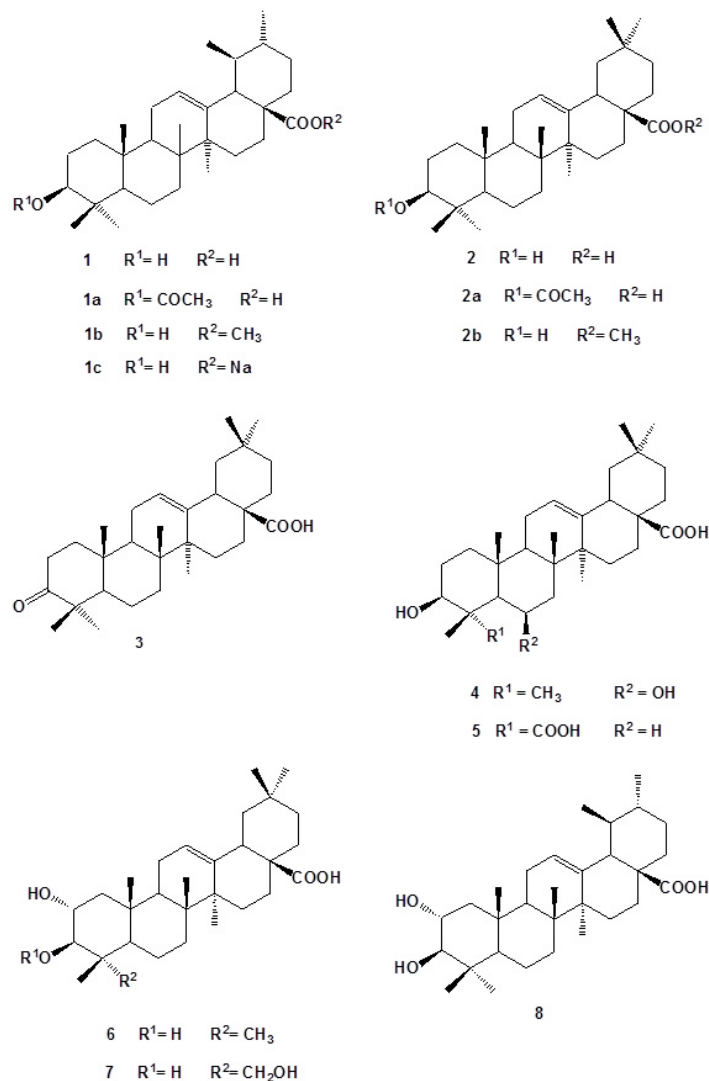
Fig. 1. Chemical structures of the triterpenes acids isolated from *Miconia* species and synthetic derivatives tested for trypanocidal activity. Figure from Cunha WR, *et al.*: In Vitro Trypanocidal Activity of Triterpenes from Miconia Species. Planta Med 2003; 69:470-472. Reproduced with kind permission from Georg Thieme Velag KG.

Compounds **1** and **2** effectively protect laboratory animals from chemically induced liver injury. In China, compound **2** has been marketed as an oral drug for human liver disorders. The hepatoprotective mechanism of these two compounds may inhibit toxic activation and enhance the body's defense systems (Liu 1995).

Triterpenoids have also been found to have antiparasitic activity against *Plasmodium falciparum* (Steele et al. 1999), *Trypanosoma* (Leite et al. 2006), and *Leishmania* species (Torres-Santos et al. 2004). They have also been reported to exert biological activity against *Mycobacterium tuberculosis* (Gua et al. 2004), and potential antiviral activity against HIV (Ovesna et al. 2004). Despite the biological activities displayed by these triterpenoids, further studies are necessary to explain their action mechanisms.

Miconia species contain triterpenes, coumarins, and benzoquinones (Gunatilaka et al. 2001). *Miconia* extracts and their isolated compounds display remarkable biological activities, such as antitumoral (Cunha et al. 2008), analgesic and anti-inflammatory actions. Ursolic acid and oleanolic acid are just two of the many biologically active substances that can be isolated from *Miconia* species. Fittingly, this genus may greatly contribute to the discovery of new drugs derived from natural products.

The genus *Miconia*: a natural source of biologically active substances against *Trypanosoma cruzi*

Melastomataceae is one of the main families of neotropical flora, with about 5,000 species belonging to 185 genera and 11 tribes. The interest in these plants stems from its eye-catching flowers; in Brazil, *Miconia*, the largest genus of the *Melastomataceae* family, is represented by about 250 ornamental species (Martins et al. 1996, Marcon & Costa 2000). It is native to the forests of Central and South America extending from southern Mexico, in the north, toward the south through Guatemala, British Honduras, Nicaragua, Costa Rica, Panama, Colombia, Ecuador, Peru, Bolivia, Brazil, Paraguay, and northern Argentina.

The biological properties of some *Miconia* species have drawn researchers' attention; extracts and isolated compounds exhibit antibiotic, antitumoral, analgesic, and antimalarial activities (Hasrat et al. 1997, Cunha et al. 2003, Serpeloni et al. 2008). Our team has been working with *Miconia* species and has tested several of their biological

actions, including the trypanocidal activity of isolated compounds and structurally modified molecules. We have also screened *Miconia* plant extracts against trypomastigote blood forms of *T. cruzi in vitro* and have found that methylene chloride extracts of *M. fallax* and *M. stenostachya* showed significant antitrypanosomal activity.

In 2003, we conducted a study in our laboratory (Cunha et al. 2003), which demonstrated that some compounds isolated from methylene chloride extracts of *M. fallax* and *M. stenostachya* present a trypanocidal effect. Using spectroscopic methods–MS, and ^{1}H- and ^{13}C-NMR, this same study elucidated the structure of the isolated compounds and synthetic derivatives. To assay trypanocidal activity according to a previous report (Grael et al. 2000), we elicited lysis of bloodstream trypomastigotes of the Y strain of *T. cruzi* by adding the isolated compounds and their synthetic derivatives to the infected blood. Ursolic (**1**) and oleanolic (**2**) acids were the major components of the methylene chloride extract of *M. fallax*, which also contained oleanolic (**3**) and sumaresinolic (**4**) acids (Figure 1). A previous study on *M. stenostachya* revealed the presence of sumaresinolic and gypsogenic (**5**) acids (Figure 1). We treated the mixture of compounds **1** and **2** with excess acetic anhydride in pyridine, to obtain the C-3 acetoxy derivatives **1a** and **2a** which, in CH_2N_2 and Et_2O, yielded a mixture of C-28 methyl ester derivatives **1b** and **2b** (Figure 1). Table 1 shows lysis percentages of *T. cruzi* tripomastigotes forms induced by addition to the infected blood of the isolated compounds and their synthetic derivatives. The natural compounds **1**, **2**, and **5**, which contain OH and COOH groups, provided the lowest IC_{50} values (21.3, 80.4, and 56.6 μM, respectively); the synthetic derivatives afforded higher IC_{50} values (**Table 1**). The presence of polar groups appeared critical to the trypanocidal activity.

As part of our ongoing search for potential trypanocidal compounds, in our laboratory, we also fractionated the methylene chloride active extracts of *M. sellowiana* Naud. and *M. ligustroides* (DC.) Naudin. and identified other triterpenes acids active against the trypomastigote forms of *T. cruzi* (Cunha et al. 2006). This latter work, employed

Compounds	% lysis ± S.D./concentration (µM)[b]			Ic_{50} (µM)
	100	250	500	
1	91.1±1.4	95.5±0.7	99.6±0.7	21.3
2	69.2±7.4	90.7±2.5	99.2±0.7	80.4
Mixture 1 + 2	60.3±3.7	91.1±1.4	95.1±	109.4
Mixture 1a + 2a	2.8±4.8	15.8±11.0	16.9±6.7	4,238.9
Mixture 1b + 2b	42.9±4.4	44.1±0	47.4±5.1	610.5
3	19.8±8.3	76.4±11.5	97.2±1.4	294.9
4	46.6±4.8	47.4±5.1	70.4±1.8	402.3
5	73.1±8.4	97.4±2.9	100±0	56.6
Gentian violet[a]	100.0±0	100±0	100±0	76.0

a methodology different from that previously reported in Cunha et al. (2003); we tested other isolated triterpenes, and assessed their activity against *T. cruzi* trypomastigotes by using the MTT colorimetric method [MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] using low concentrations of the compounds. Vacuum liquid chromatography of the methylene chloride extract of *M. selloviana* over 400 g of silica gel 60 provided fractions F2 and F3. After filtration of over 60 g of a celite:norit mixture, the elution of the combined fractions F2 and F3 with methylene chloride produced 350 mg of compound **1**. The ^{1}H- and ^{13}C-NMR data of this solid revealed the presence of a mixture of maslic acid (**6**) and 2α-hydroxyursolic acid (**8**) (Figure 1).

Vacuum liquid chromatography of the methylene chloride extract of *M. ligustroides* (6.5 g) on silica gel with increasing proportions of the same organic solvents used to fractionate the *M. selloviana* extract, produced arjunolic acid (**7**) and a mixture of compounds **1** and **2**. Purification of the latter mixture by HPLC yielded compound **2** and the potassium salt derivative of 1 (1c) (Figure 1). The *in vitro* biological assay of compounds **1** and **2** on the trypomastigote forms of *T. cruzi* (obtained by culturing the $LLMCK_2$ cell lineage) using the MTT colorimetric (Muelas-Serrano et al. 2000) indicated that compounds **1** and **2** were the most active amongst the isolated compounds, with IC_{50} of 17.1 and 12.8 µM, respectively (**Table 2**). The IC_{50} value of the mixture of compounds 1 and 2 was lower than those achieved for the pure acids **1** and **2** in isolation, suggesting a synergistic effect. The potassium salt derivative of **1** (**1c**) was more potent (IC_{50} 8.9 µM) than its active parent acid **1**. In contrast, a mixture of compounds **6** and **8** was much less potent than a mixture of **1** and **2**. Finally, compound **7** (Figure 1) displayed weak trypanocidal activity (IC_{50} = 76.3 µM) when compared with the other triterpenes.

Table 2. Results of the *in vitro* trypanocidal activity of the isolated compounds and synthetic derivatives. Gentian violet (positive control) displayed $IC_{50} = 31$ µM. The negative control, RPMI-1640 medium plus 1% DMSO, displayed 0% lysis. Table 2 from Cunha WR, *et al.*: A study of trypanocidal activity of triterpene acids isolated from *Miconia* species. Phytother Res 2006; 20:474-478. Reproduced with kind permission from John Wiley & Sons, Ltd.

Compounds	% lysis ± S.D./concentration (µM)[b]			Ic_{50} (µM)
	8	32	128	
1	33.0±12.4	68.2±2.6	75.7±10.1	17.1
1a	40.4±5.9	68.9±3.4	68.2±3.4	12.8
Mixture 1 + 2	50.2±2.5	69.7±11.9	68.2±7.9	5.4
1c	49.4±2.2	59.9±14.6	71.1±7.9	8.9
Mixture 6 + 8	36.7±22.6	38.2±13.7	63.7±6.9	48.5
7	23.2±25.5	45.7±13.5	53.2±10.1	76.3

We tested the most active compounds *in vivo* using mice (swiss albino) infected with approximately 2×10^4 trypomastigote forms. The treatment group received a daily intraperitoneal injection of the tested compound at a concentration of 2 mg kg^{-1} for 13 days. The number of parasites was counted according to the technique described by Brener (1962); the *in vivo* trypanocidal activity was expressed as the percentage of *T. cruzi* trypomastigote lysis. We also evaluated the animals' survival time; compound **1** and its derivative salt **1c** reduced parasites in the parasitemic peak more significantly (75.7% and 70.4%, respectively), increasing the survival time for all the treated animals. The results indicated that the structural variable most consistently influencing the activity were the hydroxyl groups at C-2, C-23, and C-28. Taking into account that a mixture of compounds **6** and **8** was much less active than the mixture of compounds **1** and **2**, we inferred that hydroxylation at C-2 decreased the trypanocidal activity. In the same way, hydroxylation at C-23 (compound **4**) should also diminish the *in vitro* activity against *T. cruzi*. In principle, this reduction might be interpreted as the result of the increased polarity of compounds **6**, **7**, and **8** as compared to compounds **1** and **5**. Compound **1c** (much more polar than compound **1**) was most potent. Thus, introducing an additional hydroxyl at the A-ring (at C-2 or C-23) may affect trypanocidal activity, may develop an intramolecular hydrogen bond with the hydroxyl at C-3, or the additional hydroxyl may hinder binding to the enzymatic site. On the basis of these results, we concluded that the free hydroxyl at C-3 and the polarity of C-28 determines the *in vitro* trypanocidal activity of triterpenes, at least for the analyzed set of compounds.

Ferreira et al. (2010) examined the *in vitro* and *in vivo* trypanocidal activity of compounds **1** and **2** against the Bolivian strain of *T. cruzi*. They also evaluated the acute toxicity of these compounds by determining the median lethal dose (LD_{50}) and quantifying its biochemical parameters. The authors undertook the *in vitro* trypanocidal assay according to the protocol established by Cunha et al. (2006). For the *in vivo* assay, BALB/C albino mice received 20 mg kg^{-1} of the target compound orally, on a daily basis, for 20 days. The number of parasites was quantified according to the technique described by Brener (1962); the *in vivo* trypanocidal activity was expressed as the percentage of trypomastigote lysis.

In vitro, compound **1** proved to be a more active trypanocidal agent than compound **2**; IC$_{50}$ values were 45.2 and 25.5 µM, respectively. Corroborating the *in vitro* assays, *in vivo* assays showed that compound **1** reduced the number of parasites at the parasitemic peak more significantly (60%) than compound **2** (40%). Additionally, all the animals treated with compound **1** survived longer as compared with the negative control. The presence of a hydroxyl group and a carboxyl group is important for the trypanocidal effect of both compounds **1** and **2** (Cunha et al. 2006, Leite et al. 2006). However, while compound **1** contains methyl groups at C19 and C20, compound **2** has two methyl groups at C20; this implies that the methyl group at C19 enhances trypanocidal activity against the Bolivian strain, which is more resistant to chemotherapy with Benznidazole (Ribeiro et al. 1988, Martinez-Díaz et al. 2001). The lethal dose (LD$_{50}$) in BALB/C albino mice receiving a single oral dose of the tested terpenoid at a concentration of 2,000 mg kg^{-1}, as determined by the Litchfield & Wilcoxon method (1949) after 72 h (Pires et al. 2004), did not evidence mortality or toxicity. Hence, it is totally safe to use these compounds in trials involving a much lower dose (20 mg/kg/day).

Biochemical parameters, were measured using male Wistar rats that received compounds **1** and **2** at concentrations of 20 and 50 mg kg^{-1} for 20 days; their diet was normal and drinking water was available to them *ad libitum*. At the end of the experimental period, the animals were sacrificed. Their blood was collected, to measure serum aspartate amino transferase (AST), serum alanine amino transferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltranspeptidase (GGT), bilirubin, glucose, urea, and creatinine levels, to obtain information about liver and kidney functions. Neither compound **1** nor compound **2** altered the levels of these biochemical parameters, deeming these substances safe for experimental tests. One of the most important properties of these compounds is their antioxidant capacity. Martin-Aragón et al. (2001) suggested that compound **1** may prevent the initiation and propagation of the lipid peroxidation process as it scavenges free radicals through conjugation with glutathione, controlling oxidative damage and tissue protection. Recent studies have also confirmed that compounds **1** and **2** offer antioxidative protection and exert hepatoprotective effects (Saravanan et al. 2006, Liu et al. 2012).

The most recent study by our group researched the effects of compounds **1** and **2** on interleukin levels by comparing treated and untreated chagasic mice (Ferreira et al. 2013). For the *in vivo* assay, we administered daily doses of 20 and 50 mg of the target drug kg^{-1} for 20 days to male BALB/C albino mice by oral and intraperitoneal means. We counted the number of trypomastigotes following the method described by Brener (1962) to evaluate parasitemia. For the cytokine assays, we treated mice with 20 mg kg^{-1} of the substances; collected plasma samples seven days post-infection, and measured γ-IFN and IL-10 concentrations using a specific two-site enzyme-linked immunosorbent assay (ELISA).

Intraperitoneal delivery of 20 mg kg^{-1} compounds **1** and **2** did not significantly affect the Y strain of *T. cruzi* as compared with control groups. Similarly, 50 mg kg^{-1} of these compounds did not reduce the number of circulating parasitic forms; treatment with these triterpenes did not enhance survival, either. On the other hand, orally administered compound **1** (20 mg kg^{-1}) decreased the number of parasites by approximately 30% as compared with the negative control group. However, 50 mg kg^{-1} oral compounds **1** and **2** exhibited significant biological activity against the Y strain of *T. cruzi*, reducing parasite burden by 79% and 76% at the parasitemic peak, respectively. Additionally, all the mice treated with these triterpene acids displayed longer survival time compared to the negative control group. Animals treated with these substances presented significantly lower γ-INF concentrations, which explains the exacerbated parasitemia detected in these mice; treated animals also displayed elevated IL-10 concentrations. The results from intraperitoneal administration were unexpected; earlier studies on animals infected with the same *T. cruzi* strain and treated with 2.0 mg/kg/day compounds **1** and **2** showed significantly reduced parasitemic levels and increased survival times (Cunha et al. 2006). However, the cytokine

assays performed by our group demonstrated significantly diminished γ-IFN levels. This effect could account for the exacerbated parasitemia because γ-IFN is associated with host resistance during the acute phase of infection. In contrast, treatment with compounds **1** and **2** elicited significantly higher IL-10. Various studies have reported that increased susceptibility of infected animals correlates with enhanced production of the anti-inflammatory cytokine IL-10 (Minoprio et al. 1993, Reed et al. 1994). Our results are consistent with previous works; they demonstrate that treatment with triterpenes suppresses the production of proinflammatory cytokines such as γ-IFN and generates IL-10 (Nataraju et al. 2009, Martín et al. 2010). Therefore, compounds **1** and **2** initiate an immunosuppressive effect that could interfere with host parasitemia control. The significantly reduced parasitism observed with oral administration of compound **1** may be due to its low absorption in the bowels, as verified in a recent study evaluating ursolic acid pharmacokinetics after oral administration (Jeong et al. 2007). In conclusion, orally administered compound 2 was more effective in our study, possibly because it is less absorbed by the intestines, which leads to fewer immune alterations and better control of the number of parasites.

Conclusion

Controlling or eliminating NTDs could bring millions of people out of poverty; it could increase access to education, because NTDs currently infect more than 400 million school-aged children in the developing world. Treating these children's infections is the single most cost–effective way to boost school attendance, opening the door to growth and learning for the next generation of workers. Additionally, controlling and eliminating NTDs strengthens worker productivity, contributing hundreds of billions of dollars to developing economies through increased worker productivity (Franco–Paredes et al. 2007).

However, economic consideration by the pharmaceutical industry outweighs all other benefits. Returns on development costs are unsatisfactory; consequently, it is necessary to find cheaper alternatives to treat Chagas' disease. This could be achieved by increasing the activity of the presently used anti-Chagasic drugs, or by modifying the host's immune response, which would make current therapies more effective (Maya et al. 2007). Some research groups have focused their research on finding a safe and reliable therapeutic drug to eradicate the parasite as well as a prophylactic drug to enhance human blood prior to transfusion (Mckerrow et al. 2009).

Studying natural substances yields valuable results, especially against diseases for which a cure does not yet exist or for which a treatment is available but its administration is challenging for the patient. Determining the efficacy of a tested drug or its optimum concentration is very important; however, providing information on target drugs, parasite biology, or drug interaction with the host cell is a valuable additional stride (Maya et al. 2007).

Several studies of *Miconia* plant extracts against trypomastigotes blood forms of *T. cruzi* have demonstrated significant trypanocidal activity. The isolated triterpenes ursolic and oleanolic acids were the most active compounds against *T. cruzi*. Nevertheless, more studies on the derivatives of these two compounds and on other triterpenoid acids are vital, to elucidate structure-activity relationships and develop novel therapeutic agents against Chagas' disease.

Acknowledgment

Conflict of Interest

The authors have no conflict of interest.

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Productos naturales y mal de Chagas: la acción de los ácidos triterpenos aislados de especies de *Miconia*.

Resumen. El protozoario *Trypanosoma cruzi* causa mal de Chagas, enfermedad que aún es problema de salud pública relevante en América Latina. El fármaco disponible en Brasil para el tratamiento es benznidazol. Este compuesto es eficaz contra la forma de circulación del parásito en la fase aguda de la enfermedad, pero su eficacia durante la fase crónica es discutible. Por lo tanto, la búsqueda de nuevos medicamentos que pueden tratar el mal de Chagas es necesaria. Las fuentes naturales presentan amplia gama de metabolitos secundarios y pueden desempeñar papel importante en el descubrimiento de nuevas drogas potenciales. *Miconia* es un género de los más grandes perteneciente a la familia *Melatomateceae*, incluyendo cerca de 1.000 especies de plantas; sólo en Brasil hay aproximadamente 250 de estas especies en florestas y sabanas. Los estudios sobre las diversas actividades biológicas de las especies *Miconia* han presentando resultados promisorios. Varios autores han evaluado extractos de estas plantas contra *T. cruzi*, comprobando que estos exiben actividad tripanocida significativa. La presencia de ácidos ursólico y oleanólico determinan la actividad biológica.

Palabras clave: Mal de Chagas; *Trypanosoma cruzi*; *Miconia*.

Produtos naturais e doença de Chagas: a ação de triterpenos ácidos isolados de espécies de *Miconia*.

Resumo. A doença de Chagas é uma doença negligenciada causada pelo protozoário *Trypanosoma cruzi* e ainda permanece como um relevante problema de saúde pública na América Latina. O medicamento disponível para o tratamento clínico no Brasil é o benzonidazol, que é efetivo contra as formas circulantes do parasita na fase aguda da doença, entretanto, sua eficácia na fase crônica permanece discutível. A pesquisa por novas drogas que possam ser utilizadas no tratamento dessa doença é urgentemente necessária e as fontes naturais com os seus metabólitos secundários diversificados, podem desempenhar um papel extremamente importante. *Miconia* é um dos maiores gêneros pertencente à família *Melastomataceae* incluindo cerca de 1.000 espécies e, no Brasil existem cerca de 250 espécies em florestas e savanas. Estudos realizados com o objetivo de descrever as diferentes atividades biológicas de espécies de *Miconia* têm apresentado resultados promissores. Vários autores têm avaliado os extratos dessas plantas contra as formas tripomastigotas sanguíneas de *T. cruzi* (*in vitro*) e verificado que essas amostras exibem atvidade tripanocida significativa. A presença dos triterpenos ácidos ursólico oleanólico está relacionada com esta atividade biológica.

Palavras-chave: Doença de Chagas; *Trypanosoma cruzi*; *Miconia*.