Potential therapeutic use of herbal extracts in trypanosomiasis

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The aim of the present study was to evaluate the effects of crude extracts from *Handroanthus impetiginosa*, *Ageratum conyzoides*, and *Ruta graveolens* on *Leishmania amazonensis* and *Trypanosoma cruzi* infection *in vitro*. The results showed that the extracts caused significant toxicity in promastigotes and trypomastigotes. A significant decrease in the rate of cell invasion by pretreated trypomastigotes and promastigotes was also observed. The extracts caused a significant reduction of the multiplication of intracellular amastigotes of both parasites. Therefore, these herbal extracts may be potential candidates for the development of drugs for the treatment of leishmaniasis and Chagas disease.

Keywords: Chagas disease, Leishmaniasis, Treatment, Plant extracts

Introduction

Herbal medicine has its origins in ancient cultures, including those of Egyptians, American Indians, and Chinese. The isolation and purification of the active ingredients of medicinal plants were some of the major forces that led to the birth of the pharmaceutical industry in the 19th century. After a long period of neglect, there is renewed interest in the analysis of natural products. Many international studies have shown that plants are capable of treating disease and improving health, often without significant side effects.[1,2]

There are approximately 250 000 plant species worldwide, of which only a few have been studied and shown to have potent chemotherapeutic properties.[1,2] The native tropical America species *Ageratum conyzoides* L. and *Handroanthus impetiginosa* have shown important therapeutic results. Ingestion of *A. conyzoides* can cause liver lesions and tumors.[3,4] There was a mass poisoning incident in Ethiopia as a result of contamination of grain with *A. conyzoides*.[5] On the other hand, researchers have found that *A. conyzoides* L. has antimicrobial activity against Gram-positive and Gram-negative bacteria and wound healing properties.[6,7] Additionally, a flavonoid was isolated from this specie that showed activity against *Trypanosoma brucei rhodesiense* and *Leishmania donovani*.[8] Moreover, *H. impetiginosa* has shown anti-inflammatory, antibacterial, and antifungal properties.[9] The inner bark of *H. impetiginosa* is used in traditional medicine.[10] It is dried, shredded, and then boiled, making a bitter brownish-colored tea known as Lapacho or Taheebo. In ethnomedicine, Lapacho plays an important role for several South American indigenous peoples. In the past decades it has been used by herbalists as a general tonic, immunostimulant,[11] and adaptogen. It is used in herbal medicine for intestinal candidiasis.[12]

Ruta graveolens, a plant that is typically found in southeastern Europe, grown throughout the world as an ornamental plant, popularly known as a menstruation and abortion inducer, and used as a cough syrup, has shown important therapeutic usage. Alkaloids, such as dictamnine and methoxy dictamine, are present in this plant and have antimicrobial properties, and 2-quinoline alkaloids and lignan methyl pluviatolide have trypanocidal activity[13–15]

Notwithstanding the existence of rich flora with pharmacological potential, there is a lack of efficient drugs with low toxicity for the treatment of neglected tropical diseases, such as leishmaniasis and Chagas disease. Therefore, leishmaniasis treatment modalities are limited. Parasites have also developed resistance to pentavalent antimonials, the mainstay therapeutic agentes.[16] Only two drugs, benznidazole and nifurtimox, are recommended for the treatment of Chagas disease. Benznidazole, a nitroimidazole derivative, has been more extensively investigated in clinical studies and has a better safety and efficacy profile. Therefore, it is usually used for first-line treatment. Nifurtimox, a

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nitrofurane, has also been used since the 1970s. It acts by causing DNA toxicity in the parasite by generating free radicals and causing superoxide accumulation. Diverse side effects and host toxicity have been reported for both medications.[17]

Considering the present scenario, the development and introduction of new anti-trypanosomatid compounds are needed. The search for new drugs must consider drugs that are associated with low cost, low toxicity, and high availability. Plant-derived products are gaining ground and are easily available and relatively inexpensive. The purpose of the present study was to evaluate the effect of crude extracts from *R. graveolens*, *H. impetiginosa* (formerly known as *Tabebuia impetiginosa*), and *A. conyzoides* against the infective forms of *Leishmania amazonensis* and *Trypanosoma cruzi*.

Material and Methods

Plant material

Ageratum conyzoides L. (Asteraceae), *Handroanthus impetiginosus* (Bignoniaceae), and *R. graveolens* L. (Rutaceae) were collected in Uberlândia, Minas Gerais, Brazil, and identified by Diana Salles Sampaio, PhD, Jimi Naoki Nakajima, PhD, and Glein Monteiro de Araújo, PhD, respectively. Voucher specimens of *A. conyzoides* and *H. impetiginosus* were deposited in the Herbárium Uberlandense, HUFU (no. 64.464 and 64.463, respectively). A voucher of *R. graveolens* (49.455 – 2007) was consulted to confirm this specie.

The crude extracts were prepared using 40 g of dried and powdered aerial parts that were macerated in hydroalcoholic solution for 7 days. The extracts were left in a rotary evaporator for 2 hours at 35°C. The residues were resuspended in distilled water, frozen, lyophilized (LioTop Model L-108), and maintained at −20°C.[18]

Parasites

Trypanosoma cruzi trypomastigotes (CL strain; Brener)[19] were cultured in Dulbecco's Modified Eagle Medium (DMEM; HiMedia) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml gentamycin and maintained at 37°C in a 5% CO_2 atmosphere. Vero cells were used to maintain the *in vitro* life cycle of the parasites.

Leishmania amazonensis (IFLA/BR/67/PH8) promastigotes were cultured in brain heart infusion (BHI) medium (HiMedia Laboratories, India) that contained 10% FBS, 100 μg/ml gentamycin, and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) at 28°C. Parasites in the stationary phase were used for all of the experiments.

Cell culture

Murine J774.G8 macrophages (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) were cultured in DMEM and 10% fetal calf serum (FCS) and maintained at 37°C in a 5% CO_2 humidified incubator.

Inflammatory peritoneal macrophages were obtained by intraperitoneally injecting 1 ml of thioglycolate medium (Difco Fluid Thioglycolate Medium; Becton-Dickinson, USA) in BALB/c mice. After 72 hours, the mice were euthanized, and macrophages were extracted using 5 ml of cold DMEM.

Crude plant extract cytotoxicity against axenic cultured parasites and macrophages

Crude plant extract cytotoxicity was measured using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Applied Science, Mannheim, Germany) as previously described.[20] The measurements were performed in triplicate. Extract concentrations ranged from 1.0 to 0.03125 mg/ml.

Determination of the 50% inhibitory concentration for the parasites

The concentration that inhibited the viability of the parasites by 50% (IC50) was calculated using Prism 6.01 software (GraphPad, La Jolla, CA, USA) using a non-linear regression logarithm (Table 1).

Cell invasion induced by the crude extracts in pretreated parasites

Parasites from both species were pretreated for 1 hour with the following concentrations: *L. amazonensis* promastigotes (*A. conyzoides*, 0.0625 mg/ml; *H. impetiginosus*, 0.5 mg/ml; *R. graveolens*, 0.25 mg/ml) and *T. cruzi* trypomastigotes (*A. conyzoides*, 0.0625 mg/ml; *H. impetiginosus*, 0.25 mg/ml; *R. graveolens*, 0.5 mg/ml). Amphotericin B (1 μg/ml) and Benznidazole (50 μM) were used as control drugs.

The parasites were then plated into wells that contained adherent peritoneal macrophages for 4 hours Non-internalized parasites were removed by washing with phosphate-buffered saline (PBS). Coverslips were removed and fixed with 4% formaldehyde after invasion and immunofluorescently analyzed.[21–23]

Table 1 Calculation of the IC50 values for *Trypanosoma cruzi* and *Leishmania amazonensis*

	Ageratum conyzoides		*Handroanthus Impetiginosus*		*Ruta graveolens*	
IC50 (μg/ml)	*La*	*Tc*	*La*	*Tc*	*La*	*Tc*
IC_{50} (mean±SD)	107 ± 6	104.7 ± 3.78	651.3 ± 30.01	206.7 ± 21.73	294.3 ± 20.55	207.7 ± 18.6

La: *Leishmania amazonensis*; Tc: *Trypanosoma cruzi*; SD: Standard deviation

Multiplication assay

Peritoneal macrophages were plated (10^5/well). After cell adhesion, the parasites were added (10^6/well). After 4 hours of invasion, coverslips were washed with 1 × PBS, and plant extracts were added at the same concentrations as mentioned above. The treatment was performed daily until day 3. After 72 hours, coverslips were fixed and immunofluorescently analyzed.[21–23]

Immunofluorescence reaction

The coverslips were incubated with anti-leishmanial antibody (1 : 100) or chagasic serum (1 : 500), diluted in PGN-saponin (0.2% PBS, 0.1% gelatin, 0.1% azide, and 0.1% saponin), and kept in a dark and humid chamber for 2 hours The coverslips were then washed with PBS and incubated with secondary antibodies (1 : 100) and 4'-6-diamidino-2-phenylindole (DAPI; 1 : 500). After 2 hours, the coverslips were washed in PBS and mounted in glycerol buffered with 0.1 M Tris (pH 8.6) and 0.1% paraphenylenediamine (PPD) as an anti-fading agent.[23]

Images were acquired at 63 × magnification using a confocal microscope (Zeiss LSM 510 Meta, Germany). The fluorescence of the images was analyzed using the LSM Image Browser program.

Dosage of nitrite

Macrophage nitrite production was examined using the Griess method.[24] The Griess protocol is based on the chemical reaction that uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. To determine the concentration of the sample, generating the standard curve is necessary. The intense purple color of the product allows the nitrite assay to have high sensitivity. Absorbance at 540 nm was measured and linearly proportional to the nitrite concentration in the sample.

Statistical analysis

The experiments were performed in triplicate in at least three independent experiments. The results are expressed as mean and standard deviation. Differences were considered statistically significant at $P<0.05$. The data were analyzed using analysis of variance (ANOVA) and Prism 6.01 software (GraphPad, La Jolla, CA, USA).

Results and Discussion

Crude extract toxicity against L. amazonensis *promastigotes and* T. cruzi *trypomastigotes*

To verify the toxicity of the crude plant extracts against promastigotes and trypomastigotes, these parasitic forms were plated and incubated with serial dilutions of crude extracts of *A. conyzoides*, *H. impetiginosus*, and *R. graveolens* (1.0, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml). After 72 hours, the toxicity of the plant extracts was measured using the MTT assay. The results showed that the three extracts exerted significant toxicity against the parasites compared with the untreated control (Fig. 1A–F).

The *A. conyzoides* extract induced high trypomastigote mortality at all of the concentrations tested (Fig. 1A). *Trypanosoma cruzi* trypomastigotes showed significant mortality when treated with 0.5, 0.25, and 0.125 mg/ml of the *H. impetiginosus* extract (Fig. 1B). Trypomastigotes showed slightly higher mortality rates when treated with the *R. graveolens* extract at the same concentrations as the *H. impetiginosa* extract (Fig. 1C).

Conversely, *L. amazonensis* promastigotes were less sensitive than *T. cruzi* to the extracts. All the concentrations of the *A. conyzoides* extract caused an increase in *L. amazonensis* promastigote mortality (Fig. 1D). However, treatment with *H. impetiginosus* at 1, 0.5, and 0.25 mg/ml showed to be less significant than the other extracts considering parasite mortality (Fig. 1E). Treatment with *R. graveolens* caused a significant increase in the mortality rate at concentrations of 1.0–0.125 mg/ml (Fig. 1F). Similar results were found in a previous study that tested the effects of five plant species (*Cassia sieberiana*, *Hymenocardia acida*, *Pericopsis laxiflora*, *Strychnos spinosa*, and *Trichilia emética*) against *T. brucei* and *Leishmania mexicana*. The authors also reported that *Trypanosome* was more sensitive to the extracts than *Leishmania*.[25]

Crude extract toxicity in J774.G8 macrophages

Considering the possibility of the future clinical use of these extracts or their active principal constituents for the treatment of leishmaniasis and Chagas disease, we performed toxicity assays in lineage macrophages. The concentrations of the extracts that were toxic to macrophage led to changes in cell morphology compared with untreated controls and were consequently unable to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, resulting in no formazan formation.

The *A. conyzoides* extract presented toxicity at most of the concentrations tested, with the exception of 0.0625 mg/ml (Fig. 1G). The *H. impetiginosus* crude extract showed low toxicity only at concentrations of 1.0 and 0.5 mg/ml (Fig. 1H). The *R. graveolens* extract showed no toxicity at any concentration (Fig. 1I), thus revealing its potential use in clinical trials. Toxicity against inflammatory peritoneal macrophages from BALB/c mice showed similar results as those obtained for J774.G8 macrophages (data not shown).

Effect of crude extracts in pretreated promastigotes and trypomastigotes during peritoneal macrophage invasion

We observed morphological changes and reduced motility in parasites that survived the treatments. The

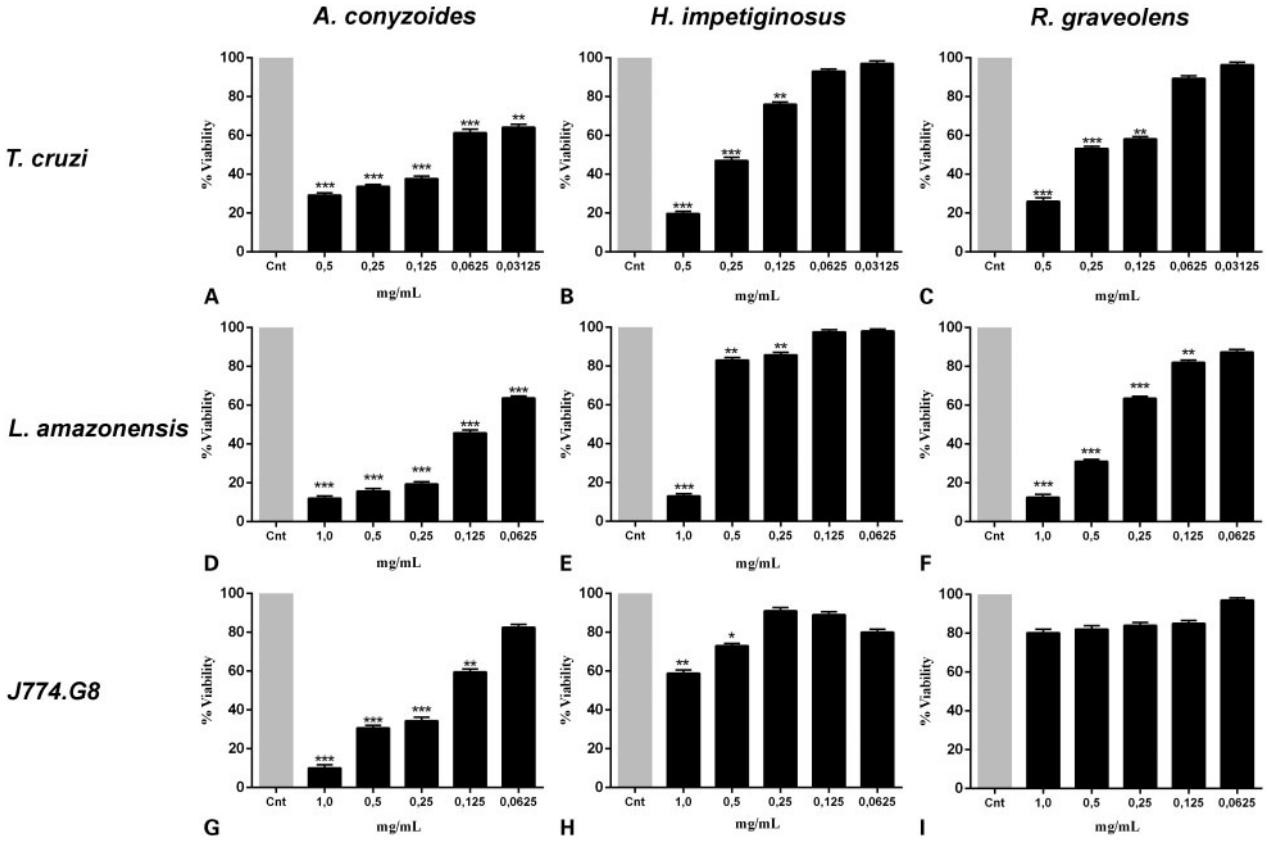

Figure 1 Parasite and macrophage viability after 72 hours of treatment with different concentrations of the plant extracts. *Trypanosoma cruzi* trypomastigotes treated with (A) *A. conyzoides*; (B) *Handroanthus impetiginosus*; and (C) *R. graveolens*. *Leishmania amazonensis* promastigotes treated with (D) *A. conyzoides*; (E) *H. impetiginosus*; and (F) *R. graveolens*. J774 macrophages treated with (G) *A. conyzoides*; (H) *H. impetiginosus*; and (I) *R. graveolens*. ***$P < 0.001$, **$P < 0.01$.

motility and integrity of membrane proteins and molecules are essential for the active entry and phagocytosis of parasites into cells, respectively. Thus, we tested whether pretreating the parasites with the extracts impacts cell invasion.

Parasites from both species (promastigotes and trypomastigotes) were pretreated for 1 hour with the following extract concentrations: *L. amazonensis* promastigotes (*A. conyzoides*, 0.0625 mg/ml; *H. impetiginosus*, 0.5 mg/ml; and *R. graveolens*, 0.25 mg/ml) and *T. cruzi* trypomastigotes (*A. conyzoides*, 0.0625 mg/ml; *H. impetiginosus,* 0.25 mg/ml; and *R. graveolens*, 0.5 mg/ml).

Leishmania amazonensis pretreated with *A. conyzoides* and *R. graveolens* exhibited a decrease in entry into macrophages. These results were similar in parasites treated with 1 μg/ml amphotericin B

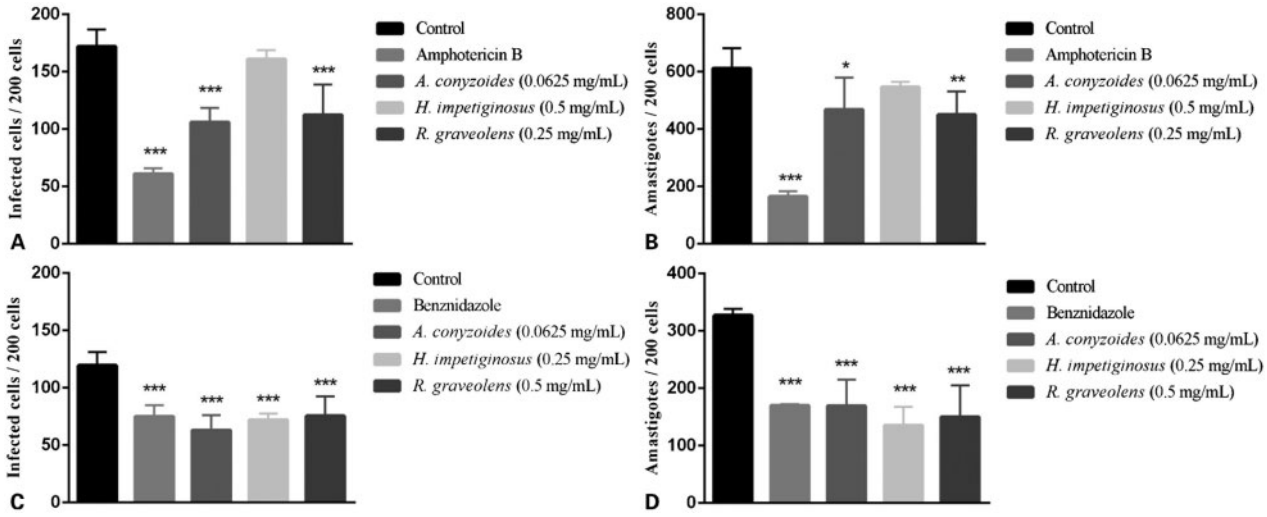

Figure 2 *Leishmania amazonensis* and *Trypanosoma cruzi* pretreated with herbal extracts exhibited a reduction of invasion in peritoneal macrophages from BALB/c mice: (A) number of *L. amazonensis*-infected cells in 200 cells; (B) number of *L. amazonensis* amastigotes in 200 cells; (C) number of *T. cruzi*-infected cells in 200 cells; and (D) number of *T. cruzi* amastigotes in 200 cells. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

(Bristol-Myers Squibb). *Leishmania amazonensis* pretreated with *H. impetiginosus* showed no impact on invasion or the number of infected cells (Fig. 2A). These results may be attributable to the fact that *Leishmania* invades macrophages principally through a phagocytotic process, and the parasites that did not die suffered fewer morphological changes compared with the other treatments. The amount of internalized amastigotes was also measured, indicating no reduction of the rate of amastigotes/cell only in the parasites pretreated with *H. impetiginosus* (Fig. 2B).

Trypanosoma cruzi trypomastigotes pretreated with *A. conyzoides*, *H. impetiginosus*, and *R. graveolens* exhibited a significant reduction of the number of infected cells and amount of internalized amastigotes compared with control untreated parasites (Fig. 2C and D). Literature on the effects of *R. graveolens* compounds against trypanosomes is sparse. However, *A. conyzoides* has been shown to exert toxicity against *T. brucei*, *L. donovani*, and *Plasmodium falciparum*. This toxicity was attributed to flavonoids. Moreover, the crude extracts

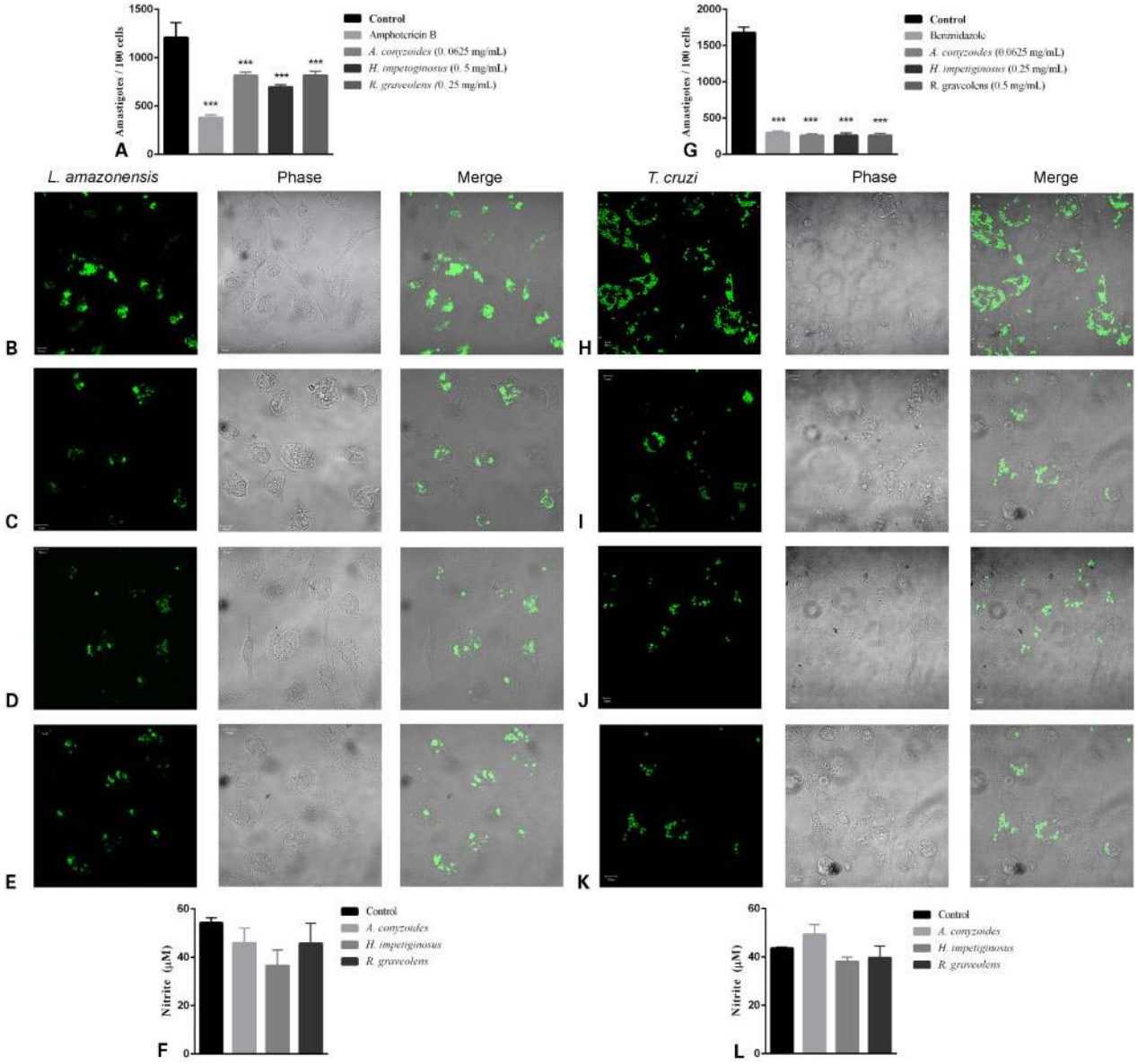

Figure 3 ***Leishmania amazonensis*** **and** ***Trypanosoma cruzi*** **amastigote multiplication after 72 hours of treatment and nitrite production. (A) Number of** ***L. amazonensis*** **amastigotes in 100 infected cells; (B) control peritoneal macrophages infected with** ***L. amazonensis***; **(C) peritoneal macrophages infected with** ***L. amazonensis*** **and treated with** ***A. conyzoides*** **(0.0625 mg/ml); (D) peritoneal macrophages infected with** ***L. amazonensis*** **and treated with** ***H. impetiginosus*** **(0.5 mg/ml); (E) peritoneal macrophages infected with** ***L. amazonensis*** **and treated with** ***R. graveolens*** **(0.250 mg/ml). Intracellular** ***L. amazonensis*** **amastigotes are shown in green. (F) Nitrite concentration in macrophages infected with** ***L. amazonensis*** **and treated or untreated with herbal extracts; (G)** ***T. cruzi*** **amastigotes in 100 infected cells; (H) control peritoneal macrophages infected with** ***T. cruzi***; **(I) peritoneal macrophages infected with** ***T. cruzi*** **and treated with** ***A. conyzoides*** **(0.0625 mg/ml); (J) peritoneal macrophages infected with** ***T. cruzi*** **and treated with** ***H. impetiginosus*** **(0.250 mg/ml); K) peritoneal macrophages infected with** ***T. cruzi*** **and treated with** ***R. graveolens*** **(0.5 mg/ml). Intracellular** ***T. cruzi*** **amastigotes are shown in green. (L) Nitrite concentration in macrophages infected with** ***T. cruzi*** **and treated or untreated with the herbal extracts.** ***$P < 0.001$.

showed better activity compared with the isolated compound.[26]

Effect of crude extracts on intracellular multiplication and nitrite production in amastigotes

Amastigotes are the replicative and predominant form in leishmaniasis and Chagas disease. Thus, testing the effect of the plant extracts on these parasite forms is crucial. Peritoneal macrophages from BALB/c mice were infected with *T. cruzi* trypomastigotes or *L. amazonensis* promastigotes. After 4 hours, the cells were washed and treated with the extracts. We found that the three plant extracts downregulated amastigote multiplication in both parasite species (Fig. 3A and G). Similar to the results shown above, *T. cruzi* amastigotes were more sensitive to the extracts than *L. amazonensis* amastigotes.

To verify whether the extracts are directly toxic to amastigotes or whether they modulate nitrite production, we conducted the supernatant multiplication assay and analyzed nitrite secretion. Our data showed that the extracts from the three plants had no modulatory effect on nitrite production (Fig. 3F and M). This was an interesting result because host immune response modulation can be variable, and such modulation could be different in each infected individual.

Concluding remarks

Studies on the effects of plant extracts and compounds derived from plants on Tripanosomatidae infection are rapidly increasing and providing new and promising plant crude extracts and plant compounds.[27–31] The present results demonstrated reductions of viability, potential for invasion, and multiplication rate of the parasites *L. amazonensis* and *T. cruzi*. However, the reduction of viability in mammalian cells was much reduced. We believe that the identification of the active compounds from *R. graveolens* may be valuable for single or combined therapy; hence, adding novel alternatives to control and treat infection by these important neglected parasites.

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Conflict of Interest

The authors declare no conflicts of interest.

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