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In Vitro and in Vivo Trypanocidal Activity of Flavonoids from *Delphinium staphisagria* against Chagas Disease

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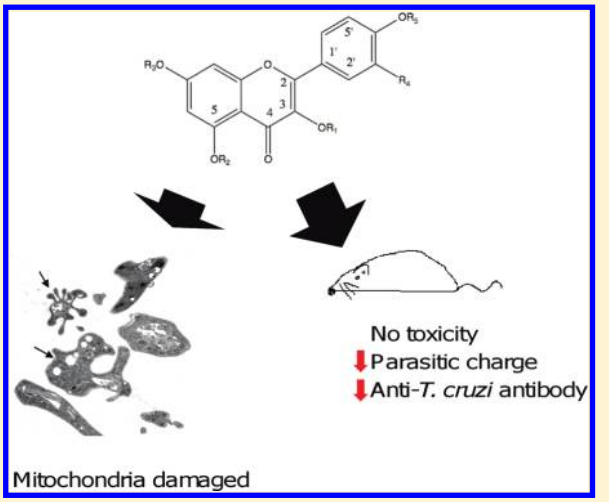
Supporting Information

ABSTRACT: The in vitro and in vivo trypanocidal activities of nine flavonoids (**1**−**9**) isolated from the aerial parts of *Delphinium staphisagria* have been studied in both the acute and chronic phases of Chagas disease. The antiproliferative activity of these substances against *Trypanosoma cruzi* (epimastigote, amastigote, and trypomastigote forms) in some cases exhibited more potent antitrypanosomatid activity and lower toxicity than the reference drug, benznidazole. Studies in vitro using ultrastructural analysis together with metabolism−excretion studies were also performed in order to identify the possible action mechanism of the compounds tested. Alterations mainly at the level of the mitochondria may explain metabolic changes in succinate and acetate production, perhaps due to the disturbance of the enzymes involved in sugar metabolism within the mitochondrion. In vivo studies provided results consistent with those observed in vitro. No signs of toxicity were detected in mice treated with the flavonoids tested, and the parasitic charge was significantly lower than in the control assay with benznidazole. The effects of these compounds were also demonstrated with the change in the anti-*T. cruzi* antibody levels during the chronic stage.



American trypanosomiasis, also known as Chagas disease, is one of the most devastating diseases from parasites of the family Trypanosomatidae. It is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, which is transmitted by an insect vector depositing feces on the skin surface and subsequently biting. Other routes of contamination are infected blood transfusions, organ transplants, and from mother to child during pregnancy or breastfeeding.[1] Chagas disease arises in the form of an acute infection, during which most patients are not aware that they are infected, with further development to chronic and systemic stages, which affect severely the heart, esophagus, and colon. This disease is endemic throughout Latin America and is classified by the World Health Organization as the third most widely spread tropical disease after malaria and schistosomiasis.[2] It is estimated that about 100 million people are at risk of infection and from 15 to 20 million are infected, with some 50 000 persons dying yearly from this disease.

Drugs currently used to treat Chagas disease are the nitro-. heterocyclic compounds nifurtimox (Lampit) and benznidazole (Rochagan, Radanil), a nitroimidazole derivative, for which the anti-*T. cruzi* activity was discovered empirically about 30 years ago. However, neither of these drugs can be regarded as ideal due to (i) their low efficacy in the chronic stage of the illness; (ii) regional variations of effectiveness due to drug-induced resistance; (iii) a high percentage of treatment being discontinued due to side effects: (iv) the long treatment period (30−60 days) required; and (v) the necessity for close medical supervision. Certain drugs used for other diseases, such as allopurinol and itraconazole, have not yet been evaluated for appropriate clinical traits.[3] The goals for specific treatment of Chagas disease should (i) address the removal of the parasite from the infected person in order to reduce the probability of cardiological and digestive pathologies; (ii) stop the transmission chain of *T. cruzi*; (iii) decrease the number of newborns contracting the protozoa; and (iv) increase the number of people able to act as organ and blood donors.[4] Therefore, all these desired properties make the development of new drugs for the treatment of Chagas disease a much needed and challenging research goal.[3,5,6]

One way to discover new drugs is to investigate natural products from plants used medicinally.[7] Folk medicine is very often a valid

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Table 1. In Vitro Activity of Flavonoids Evaluated against the Epimastigote Form of *Trypanosoma cruzi*

compound	*T. cruzi* IC_{50} (μM)	toxicity in Vero cells (IC_{50}, μM)[a]	SI[b]
benznidazole	15.8	13.6	0.9
1	15.7	174.3	11.1
2	6.5	294.1	45.2
3	0.8	164.0	205.0
4	20.5	>1000	48.8
5	21.7	>1000	46.1
6	17.2	>1000	58.1
7	30.3	>1000	33.0
8	27.3	>1000	36.6
9	27.0	362.3	30.0

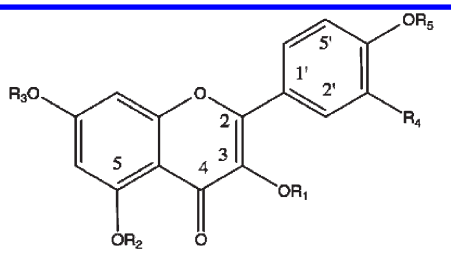
[a] Determined after 72 h of culturing. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50, and 100 μM). [b] Selectivity index = IC_{50} Vero cells/IC_{50} epimastigote, based on an average of three separate determinations.

source for researchers looking for bioactive substances that are potentially useful against many diseases, as demonstrated by the search for new medicinal agents for treating trypanosomiasis, leishmaniasis, and other diseases.[7] Many potentially trypanocidal and leishmanicidal substances have been isolated from a variety of plants.[8−14] Several trypanocidal flavonoids are strong candidates for use in combination therapy against infections. It has been reported that some substances from plants have antitrypanosomal properties, but unfortunately their side effects are still unknown.[14,15]

Active principles from plants as well as their synthetic and semisynthetic analogues have served as one of the main ways to obtain new chemotherapeutic compounds.[14,16,17] The search for natural products with antitrypanosomal activity has given fresh impetus to the development of new synthetic compounds.[18] Flavonoids have been found in abundance in the fruits, vegetables, and sap of plants and have been demonstrated to have anticarcinogenic, antimicrobial, and antiparasitic activity.[19,20] In a previous study, it was determined that certain flavonoid derivatives exerted significant effects on the in vitro growth of *Leishmania* spp. and *T. cruzi*.[21]

Most studies directed toward the detection of secondary plant metabolites with trypanocidal activity have used the extracellular forms (epimastigote for *T. cruzi*) for easier maintenance under in vitro conditions. However, since the extracellular forms are not the developed form of the parasite in vertebrate hosts, preliminary evaluations need to be complemented using intracellular forms (amastigotes in host cells), and for *T. cruzi* using trypomastigote forms. At the same time, an assessment of the possible cytotoxicity of the metabolite should be made using nonparasitized host cells, in order to establish whether the in vitro activity of the metabolite is due to its general cytotoxic activity or if it is selectively active against the *Trypanosoma* parasite.[22]

In the present work, the inhibitory effects of nine flavonoids (**1**−**9**) from aerial parts of *Delphinium staphisagria* L. (Ranunculaceae) were investigated in relation to the extracellular and intracellular stages of *T. cruzi*. In addition, the cytotoxic effects of these compounds against a host cell line were assessed. A detailed examination of their in vivo antiparasitic activity and toxicity was made, in both the acute and chronic phases. The ^{1}H NMR spectroscopic analysis concerning the nature and percentage of the excretion metabolites was carried out in order to gain information concerning the inhibitory effect of the listed compounds on the parasite metabolism, since the glycolytic pathway represents the prime source of energy for the parasite. Finally, the effect of flavonoids on the ultrastructure of *T. cruzi* was studied on the basis of transmission electronic microscopy experiments.



1 astragalin (R_1 = Glc, R_2 = R_3 = R_4 = R_5 = H)
2 2″-acetylastragalin (R_1 = Glc 2″-Ac, R_2 = R_3 = R_4 = R_5 = H)
3 astragalin heptaacetate (R_1 = Glc-Ac, R_2 = R_3 = R_5 = Ac, R_4 = H)
4 paeonoside (R_1 = R_3 = Glc, R_2 = R_4 = R_5 = H)
5 2″-acetylpaeonoside (R_1 = Glc 2″-Ac, R_3 = Glc, R_2 = R_4 = R_5 = H)
6 paeonoside decaacetate (R_1 = R_3 = Glc-Ac, R_2 = R_5 = Ac, R_4 = H)
7 petiolaroside (R_1 = Glc, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
8 2″-acetylpetiolaroside (R_1 = Glc 2″-Ac, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
9 petiolaroside decaacetate (R_1 = Glc-Ac, R_3 = Rha-Ac, R_2 = R_5 = Ac, R_4 = OAc)
(Glc = β-glucopyranosyl; Rha = α- rhamnopyranosyl)

RESULTS AND DISCUSSION

Nine flavonoids (**1**−**9**) derived from the aerial parts of *D. staphisagria* were isolated and identified, as described earlier.[23]

The inhibitory effects on the in vitro growth of *T. cruzi* epimastigote forms of flavonoids **1**−**9** were measured at different times, following established procedures (see Experimental Section). The ability of these compounds to inhibit the growth of the epimastigote forms was evaluated at 1 to 100 μM, and the IC_{50} values were determined for the most active compounds, with benznidazole used as a positive control. The results, including cytotoxicity values against Vero cells, are presented in Table1. After 72 h of exposure, three of the flavonoid compounds, the glycoside astragalin (**1**) and two acetylated derivatives (**2** and **3**), showed significantly lower IC_{50} when the values were compared to benznidazole. The acetylated derivatives were the most effective (**2** and **3**, with IC_{50} values of 6.5 and 0.8 μM, respectively), while **1** showed an IC_{50} value similar to that of benznidazole (15.7 and 15.8 μM, respectively). Of the six remaining compounds, paenoside (**4**), petiolaroside (**7**), and their acetylated compounds (**5**, **6**, **8**, and **9**) showed IC_{50} values close to or slightly greater than those of benznidazole.

The cytotoxicity of these compounds against mammalian cells was also evaluated in vitro at 1 to 100 μM, using Vero cells[14] (see Table 1). Benznidazole was again included as the reference drug. The heptaacetylated compound of astragalin (**3**) showed the best selectivity index (SI = IC_{50} Vero cells/IC_{50} epimastigote). In general, the rest of the eight compounds consistently presented SI values higher (6.7- to 52-fold) than those of benznidazole.

However, also included in this study were the effects of these compounds on the parasitic forms that develop in the host (amastigote and trypomastigote forms), the study of which is of great importance, given that the final aim is to determine the effects in the definitive host.[22] An experimental model designed in our laboratory has been used to predict the effect of those flavonoids that showed promising inhibition in vitro and a higher SI over the growth of extracellular forms, on the capacity of infection and growth of the intracellular forms and subsequent

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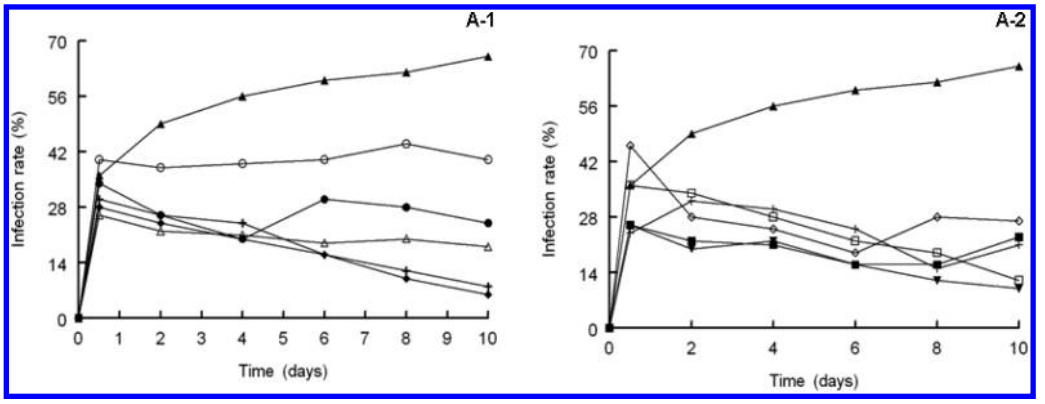

Figure 1. Effect of flavonoids **1**−**9** on the infection rate and *T. cruzi* growth. Rate of infection. A-1: (-▲-), control; (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**;(-♦-), **3**; (-Δ-), **4**. A-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (- ◇-), **7**; (- ■ -), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

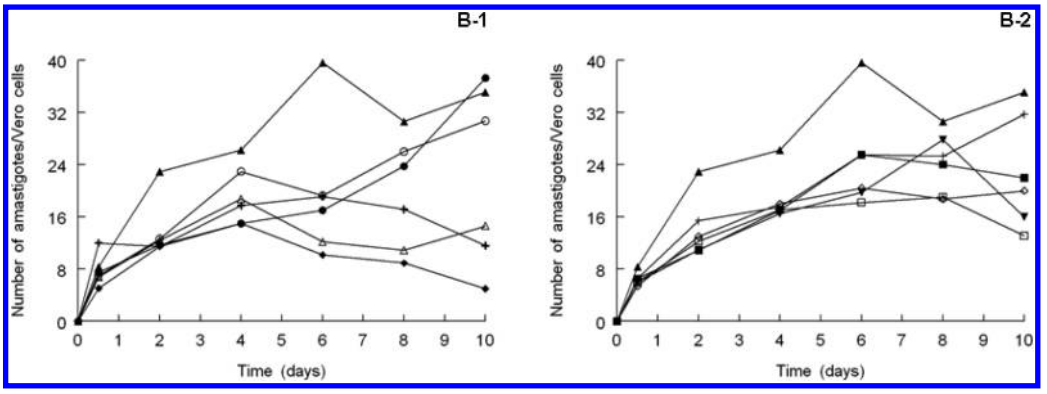

Figure 2. Effect of flavonoids **1**−**9** on the mean number of amastigotes per infected Vero cell. B-1: (-▲-), control; (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**; (-♦-), **3**; (-Δ-), **4**. B-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

transformation to bloodstream forms. Adherent Vero cells (1×10^5) were incubated for two days and then were infected with 1×10^3 metacyclic forms for 12 h (control experiment, Figure 1).

Then, the nonphagocytosed parasites were removed and the culture was kept in fresh medium for 10 days. The parasites invaded the cells and underwent morphological conversion to amastigotes within one day after infection. On day 10, the rate of host-cell infection reached its maximum. When the complexes (IC_{25} concentration) were added simultaneously to Vero cells with *T. cruzi* metacyclic forms that had been in the exponential growth phase for 12 h, the infection rate significantly decreased with respect to the control in the order **3** > **2** > **5** > **6** > **4** > **7** > **8** > **1** > **9**, with percentages of infection inhibition within a range of 91, 88, 85, 82, 73, 68, 65, 64, and 59%, respectively (Figure 1, A1 and A2). These values are considerably higher than the inhibition achieved by benznidazole (39%). In the control experiment, the average number of amastigotes per infected cell increased to 39.6 amastigotes/cell on day 6, decreasing to 30.6 amastigotes/cell on day 8, and increasing again to 35 amastigotes/cell on day 10 (see Figure 2, B1and B2); these fluctuations, in the number of amastigote forms per cell, are due to the parasite life cycle. When a cell is completely invaded by amastigote forms, the parasites break the cell, and the breakage of the Vero cells involves the release of amastigotes and their subsequent transformation into trypomastigotes. Afterward, the trypomastigote form can infect new cells, in which they multiply, increasing the number of amastigotes per cell. Flavonoids **1**−**9** inhibited *T. cruzi* amastigote replication in Vero cells in vitro, in a similar manner to how they inhibited the infection rate and again more efficiently than the reference drug, in the order **3** > **2** > **6** > **4** > **7** > **5** > **8** > benznidazole. Compounds **9** and **1** exhibited an inhibition percentage lower than that of benznidazole. In the control experiment, the number of trypomastigotes in culture medium was 4.4×10^3 on day 10 postinfection (Figure 3, C1 and C2). All the compounds tested showed a highly significant decline in the number of trypomastigotes in media (95, 85, 78, 77, 72, 68, 65, 58, and 44% for **3**, **6**, **2**, **5**, **7**, **4**, **1**, **8**, and **9**, respectively), also higher than for the reference drug (benznidazole caused a 37% decrease).

Due to the trypanocidal effect in vitro of flavonoids **1**−**9**, in vivo studies were performed to evaluate their activity against *T. cruzi* infection in mice. Previous results have shown that the intravenous doping route resulted in high mortality rates;[24] therefore, in this study the intraperitoneal route was chosen, using a concentration of 5 mg/kg, which did not result in any animal mortality (data not shown). Female Swiss mice were inoculated intraperitoneally with 1×10^3 blood trypomastigotes, and treatment began seven days postinfection, with an ip route of 1 mg/kg per day of each test compound for five days. Administration was conducted using saline solution. A control group

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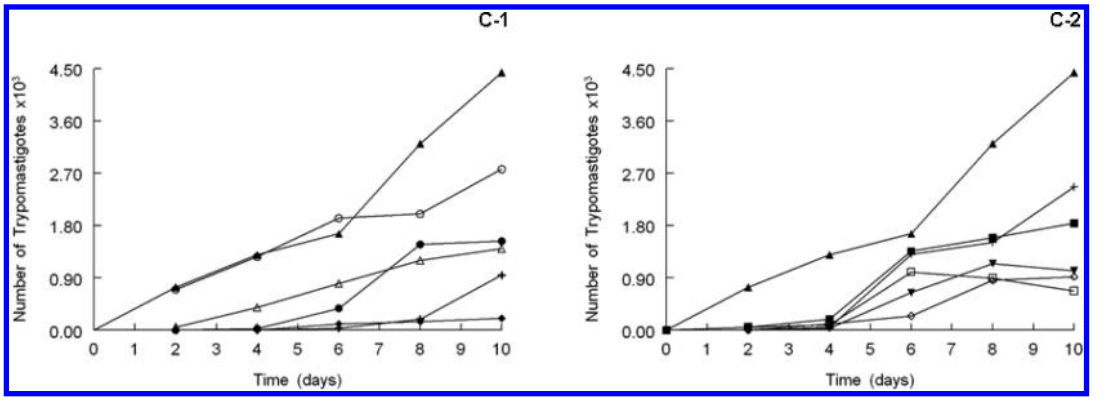

Figure 3. Effect of flavonoids **1**−**9** on the number of trypomastigotes in the culture medium. C-1: (-▲-), control; (-○-), benznidazole; (-●-), **1**; (-+-), **2**; (-♦-), **3**; (-□-), **4**. C-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

was treated in the same way with the vehicle. The level of parasitemia was determined every two days (Figure 4), with mortality observed daily, and serological tests were performed 40 and 90 days postinfection (Table 2). None of the animals treated with the test compounds or the control died during the treatment, while in the groups treated with benznidazole the survival cohort was 80%. Flavonoids **1**−**9** decreased the trypomastigote forms on the day the experiment reached the maximum parasitic charge (day 22 postinfection), in comparison to the positive control. In the groups of animals treated with the test compounds (only one was tested in each group), a significant decrease in parasitemia was registered on day 40 postinfection with most of the substances tested.

The percentage decrease, with respect to the control, ranged from 59% to 23% for the flavonoids and 16% for benznidazole. In fact, the parasitemia levels observed for the compounds assayed agreed with their in vitro behavior, with the in vivo activity varying, in general, in the order **3** > **6** > **2** > **5** > **4** > **7**. The two acetylated derivatives of petiolaroside (**8** and **9**) exhibited an antiparasite potency greater than the untreated control group.

None of the animals treated with the flavonoids tested in this study or benznidazole, used as control in the antibody studies, showed negative anti-*T. cruzi* serology. However, all compounds lowered antibody levels between days 40 and 90 (Table 2), showing improved results compared with benznidazole in this assay. The differences in the level of anti-*T. cruzi* antibodies were consistent with the parasitemia findings. Although the test flavonoids did not completely eliminate the trypomastigote forms circulating in the bloodstream, no clear relationship was found between the number of remaining parasites and the chronic stage of the disease. Thus, there was no evidence of protection against heart-rate alterations characteristic of *T. cruzi* infection.

To date, none of the trypanosomatids studied is capable of completely degrading glucose to CO_2 under aerobic conditions, and they excrete metabolites into the medium that depend on the species considered.[25,26] *T. cruzi* consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H NMR spectroscopy has indicated the nature of the fermented metabolites excreted by this trypanosomatid during in vitro culture. One of the major metabolites excreted by *T. cruzi* is succinate, the probable main role of which is to maintain the glycosomal redox balance by providing two glycosomal oxidoreductase enzymes that allow reoxidation of

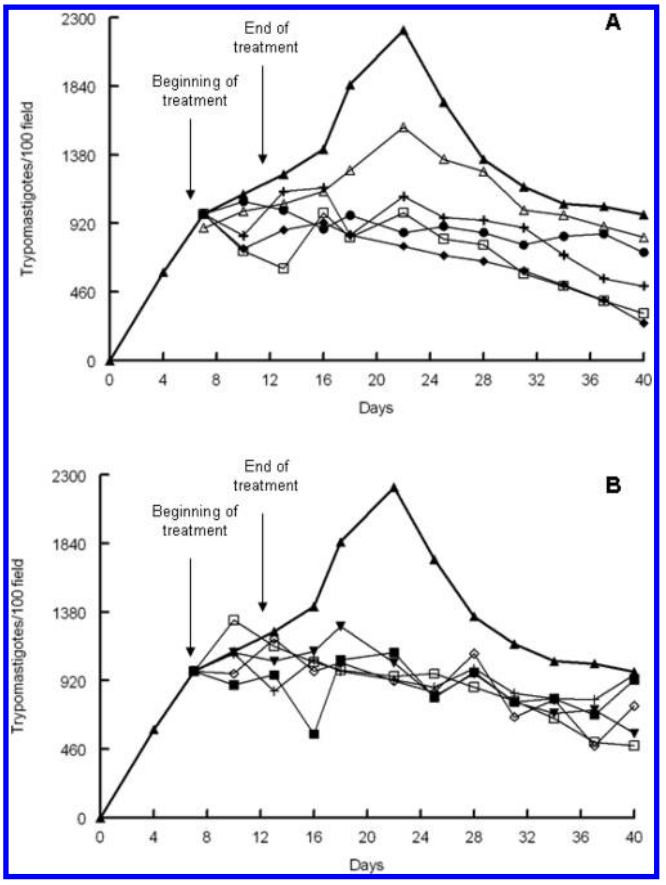

Figure 4. Parasitemia in the murine model of acute Chagas disease by flavonoids **1**−**9**: (-▲-), control and dose receiving 5 mg/kg of C-1: (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**;(-♦-), **3**; (-Δ-), **4**. C-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**.

NADH, produced by glyceraldehyde-3-phosphate dehydrogenase, in the glycolytic pathway. Succinic fermentation offers a significant advantage of requiring only half of the phosphoenolpyruvate (PEP) produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species considered.[25]

To gain information concerning the effect of the flavonoids **1**−**9** on metabolite excretion, ^{1}H NMR spectra were measured

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Table 2. Differences in the Level of anti-*T. cruzi* Antibodies between Days 40 and 90 Postinfection for Compounds 1−9 and Benznidazole, Expressed in Absorbance Units

compound[b]	ΔA[a]
control (untreated)	0.186
benznidazole	0.116
1	−0.035
2	0.056
3	−0.246
4	0.062
5	−0.104
6	−0.074
7	−0.291
8	0.330
9	0.327

[a] ΔA = Absorbance at 490 nm, day 90 pi − Absorbance at 490 nm, day 40 pi. [b] Dose 1 mg/kg/day, intraperitoneal route administered for five days (see Experimental Section).

from the trypanosomatids treated with these compounds and were compared with those from cell-free medium (control) four days after inoculation with the *T. cruzi* strain. In the control experiment, *T. cruzi* excreted acetate and succinate as the major metabolites and, in a lower percentage, L-alanine (Figure S1A, Supporting Information). These data agreed with those reported previously.[27] When trypanosomatids were treated with the test flavonoids, the excretion of some of these catabolites was slightly altered at the doses employed. Percentages of inhibition in the height of the peaks corresponding to the significant catabolites are shown in Table S1, Supporting Information. It was observed that the excretion of succinate and also of acetate was inhibited by acetylated flavonoid compounds **3**, **5**, **6**, **8**, and **9** (for these compounds only the spectrum shown corresponds to **2**, Figure S1C, Supporting Information). The inhibition of acetate and succinate excretion could explain the observed increase in L-lactate and ethanol production, considering that these compounds operate at the electronic chain level preventing reducing-power recharge, or they may act on the mitochondria and, consequently, oxidative phosphorylation. The acetylated flavonoid **3**, in addition to inhibiting the production of succinate and acetate, also inhibited the excretion of alanine (Figure S1D, Supporting Information). The three nonacetylated flavonoids **1**, **4**, and **7** behaved similarly, clearly inhibiting acetate and augmenting the production of lactate, alanine, and ethanol (Figure S1B shows only the spectrum of the derivative **1**). It should be noted that we found no significant alteration of the energetic metabolism in the presence of benznidazole (spectrum not shown).

The transmission electron microscope evaluation of flavonoids **1**−**9** against *T. cruzi* epimastigotes showed notable ultrastructural alterations, as reflected in Figure 5 (panels 2−6) with respect to the control (Figure 5, panel 1). All compounds induced ultrastructural changes in the parasites, with the most common being greater vacuolization and the shrinkage of the protozoa in the presence of more electrodense structures. The most effective compounds were **1**, **2**, **4**, **6**, and **9**, resulting in a distinct deformation of the parasites, which appeared to have burst and had an undulating cytoplasm membrane, as if extending pseudopodia. For compound **9** (Figure 5, panel 2) most of the epimastigotes had large, empty vacuoles, while the others had a

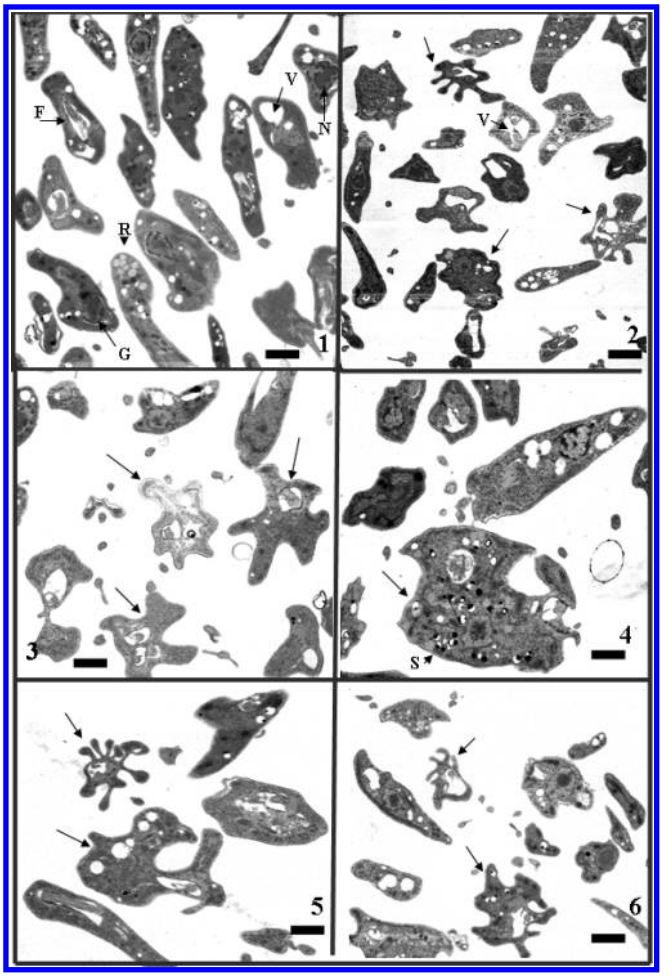

Figure 5. Ultrastructural alterations by TEM in epimastigotes of *Trypanosoma cruzi* treated with flavonoids **1**−**9**. (1) Control parasite of *T. cruzi* showing organelles with their characteristic aspect, such as nucleus (N), reservosomes (R), mitochondrion (M), glycosomes (G), vacuoles (V), and flagellum (F) (bar: 0.583 μm). (2) Epimastigotes of *T. cruzi* treated with **9** with star-shaped (arrow) and strongly vacuolated (V) (bar: 1.59 μm). (3) Epimastigotes of *T. cruzi* treated with **1** with star-shaped and waving cytoplasmatic membrane (arrow) (bar: 1.59 μm). (4) Epimastigotes of *T. cruzi* treated with **4** with strongly electrodense structures (S) and some very dilated (arrow) (bar: 1.59 μm). (5) Parasites treated with **6** with waving shape (arrow) (bar: 1.00 μm). (6) *T. cruzi* treated with **2** with start-shaped (arrow), electrodense structures (S), and dilated mitochondria (M) (bar: 1.00 μm).

strongly electrodense cytoplasm. On the other hand, with compound **1** (Figure 5, panel 3) the effect was reversed, since many of the protozoa showed cytoplasm with low electrodensity and had a dead appearance. Compound **2** (Figure 5, panel 6) also dilated the mitochondria and induced the production of certain small structures that were strongly electrodense, perhaps the products of refuse. These appeared in small quantities, in contrast to the data obtained for flavonoid **4** (Figure 5, panel 4), in which these structures appeared in high abundance, with some parasites appearing dilated and having sizes larger than normal.

EXPERIMENTAL SECTION

Parasite Strain and Culturing. The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health,

Maracay, Venezuela. Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy−Nicolle−McNeal), supplemented with minimal essential medium (MEM) and 20% inactivated fetal bovine serum, and reseeded afterward in a monophasic culture (MTL), following the method of Luque et al.[28]

Plant Material. Aerial parts of *Delphinium staphisagria* were collected and processed as described previously.[23] Nine flavonoids (**1**−**9**) were isolated, derivatized, and identified.[23] These substances were dissolved in dimethyl sulfoxide (Panreac, Barcelona, Spain) at a concentration of 0.1%. The effects of each compound against epimastigote forms, as well as the concentrations, were evaluated at 24, 48, and 72 h using a Neubauer hemocytometric chamber, and the trypanocidal effects were expressed as IC_{50} values (the concentration required to give 50% inhibition as calculated by linear regression analysis from the K_c values at the concentrations employed).

In Vitro Trypanocidal Activity Assay. To obtain the parasite suspension for the trypanocidal assay, the epimastigote culture (in the exponential growth phase) was concentrated by centrifugation at 1000*g* for 10 min, and the number of flagellates counted in an hemocytometric chamber and distributed into aliquots of 2×10^6 parasites/mL. Compounds were dissolved in DMSO at a concentration of 0.01%, after being evaluated as nontoxic and without inhibitory effects on parasite growth. The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM.

Cell Culture and Cytotoxicity Tests. Vero cells (Flow) were grown in MEM (Gibco), supplemented with 10% inactivated fetal calf serum, and adjusted to pH 7.2, in a humidified 95% air−5% CO_2 atmosphere at 37 °C for two days. For the cytotoxicity testing, cells were placed in 25 mL Colie-based bottles (Sterling) and centrifuged at 100*g* for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μL/well and incubated for two days at 37 °C in a humidified atmosphere enriched with 5% CO_2. The medium was removed, and fresh medium was added together with each test compound (at concentrations of 100, 50, 25, 10, and 1 μM). After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide (PI) solution (100 μg/mL) was added and incubated for 10 min at 28 °C in the dark. Afterward, 100 μL/well of fluorescein diacetate (100 μg/mL) was added and incubated under the same conditions as above. Finally, the cells were recovered by centrifugation at 400*g* for 10 min, and the precipitate was washed with PBS. Flow cytometric analysis was performed on a FACS Vantage flow cytometer (Becton Dickinson). The live cells with their plasma membrane intact were associated with a green fluorescence, due to the effect of sterases on fluorescein diacetate. On the other hand, cells that had lost membrane integrity or were dead allowed the penetration of PI by passive diffusion, which specifically bound to their DNA with fluorescence in the range of 580 nm. Percentages of viability were calculated in comparison to that of the control culture, and IC_{50} values were calculated by linear regression analysis from the K_c values at the concentrations employed.

Transformation of Epimastigote to Metacyclic Forms. Metacyclogenesis was induced by culturing the parasites at 28 °C in modified Grace's medium (Gibco) for 12 days, as described previously.[29] Twelve days after cultivation at 28 °C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

Cell Selectivity Assay. Vero cells were cultured in MEM medium in a humidified 95% air−5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and then cultivated for two days. Afterward, the cells were infected in vitro with metacyclic forms of *T. cruzi*, at a ratio of 10:1. The test compounds (IC_{25} concentrations) were added immediately after infection and incubated for 12 h at 37 °C in a 5% CO_2 atmosphere. The nonphagocytosed parasites and the test compounds were removed by washing, and the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

The activity of each test compound was determined from the percentage of infected cells and the number of amastigotes per cell infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations. The number of trypomastigotes in the medium was determined as described previously.[29]

In Vivo Trypanocidal Activity Assay. Groups of five BALB/c female mice (6 to 8 weeks old; 25−30 g) maintained under standard conditions were infected with 1×10^3 bloodstream *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (i) group 1: uninfected (not infected and not treated); (ii) group 2: untreated (infected with *T. cruzi* but not treated); (iii) group 3: uninfected (not infected and treated with 1 mg/kg body weight/day, for five consecutive days [7 to 12 days postinfection] by the intraperitoneal route);[23] and (iv) group 4: treated (infected and treated for five consecutive days [7 to 12 days postinfection] with the test compounds and benznidazole). This animal experiment was performed with the approval of an ethical committee of the University of Granada.

Five days after infection, the presence of circulating parasites was confirmed by the microhematocrit method. A blood sample (5 μL) drawn from the tail of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer and 20 μL of lysis buffer at pH 7.2), and this vehicle was also employed as a negative control. The parasites were counted in a Neubauer chamber. The number of deaths was recorded every two days.

One group of four animals treated with each compound and benznidazole was included for serological studies. Treatments were started seven days after animal infection. Compounds were administered in a similar way to that explained above and at the same concentrations.

Circulating anti-*T. cruzi* antibodies, at days 40 and 90 postinfection, were evaluated quantitatively by an enzyme-linked immunoassay. The sera, diluted to 1:100, were reacted with an antigen composed of an excreted Fe-SODe of *T. cruzi* epimastigotes. The results are expressed as the ratio of the absorbance of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

Metabolite Excretion. Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5cells/mL) received the IC_{25} dosage of the test compounds (except for control cultures). After incubation for 72 h at 28 °C, the cells were centrifuged at 400*g* for 10 min. The supernatants were collected to determine the excreted metabolites by ^{1}H NMR spectroscopy as previously described by Fernández-Becerra et al.[30] Chemical shifts were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the reference signal, and were used to identify the respective metabolites, with the results proving to be consistent with those described previously.[30]

Ultrastructural Alterations. The parasites, at a density of 5×10^6 cells/mL, were cultured in their corresponding medium, containing the test compounds at their IC_{25} concentration. After 72 h, the cultures were centrifuged at 400*g* for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. The pellets were prepared for transmission electron microscopy following the technique of Luque et al.[28]

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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