Antileishmaniasis Activity of Flavonoids from *Consolida oliveriana*

Clotilde Marín,[†] Samira Boutaleb-Charki,[†] Jesús G. Díaz,[‡] Oscar Huertas,[†] María J. Rosales,[†] Gregorio Pérez-Cordon,[†] Ramon Guitierrez-Sánchez,[§] and Manuel Sánchez-Moreno*[,†]

Department of Parasitology, University of Granada, Severo Ochoa s/n, E-18071 Granada, Spain, Department of Chemistry, University of La Laguna, Instituto Universitario de BioOrgánica "Antonio González", E-38206 La Laguna, Tenerife, Spain, and Department of Statistics, University of Granada, Severo Ochoa s/n, E-18071 Granada, Spain

Received December 23, 2008

A set of flavonoids from *Consolida oliveriana*, kaempferol (**1**), quercetin (**2**), trifolin (**3**), and acetyl hyperoside (**5**) and their *O*-acetyl derivatives (**1a**, **2a**, **3a**), and octa-*O*-acetylhyperoside (**4**) showed leishmanicidal activity against promastigote as well as amastigote forms of *Leishmania* spp. The cellular proliferation, metabolic, and ultrastructural studies showed that the acetylated compounds **2a**, **3a**, and **4** were highly active against *Leishmania (V.) peruviana,* while **2a** as well as **4** were effective against *Leishmania (V.) braziliensis*. These compounds were not cytotoxic and are effective at similar concentrations up to or lower than the reference drugs (pentostam and glucantim).

In humans, *Leishmania* spp. cause a variety of clinical diseases due to the ability of the organism to proliferate in deep tissue or close to the skin surface at low temperatures. The varied manifestations of the disease have been used by the World Health Organization as the basis for classifying leishmaniasis into four clinical forms: (a) visceral, (b) mucocutaneous, (c) cutaneous diffuse or disseminated, and (d) cutaneous. Certain species of the parasite have been associated with the different clinical forms of the disease; that is, the *Leishmania donovani* complex causes visceral leishmaniasis, while the *Leishmania tropica* complex is known to induce cutaneous lesions in the Old World and *Leishmania mexicana L. (V.) peruviana* does so in the New World. Some 25% of the mucous cutaneous forms cause mucocutaneous and cutaneous diffuse leishmaniasis in several countries of Latin America, the pathogen being *Leishmania (V.) braziliensis*.[1]

Historically, the chemotherapy of leishmaniasis has been based on the use of toxic heavy metals, particularly antimony compounds. Whenever these kinds of drugs are no longer effective, some others are used, including pentamidine and amphotericin B. These chemicals have to be injected, and clinical care or hospitalization during treatment may be necessary due to possible side effects;[2,3] thus other treatments are needed. Folk medicine is very often a valid source for researchers looking for bioactive substances potentially useful against many diseases. Extracts from medicinal plants or compounds derived from them are a valuable source of new medicinal agents for treating Leishmaniasis[4] and other diseases.[5] The range of families and species from which potentially active leishmanicidal substances can be extracted is very broad,[6] for example, *Bixa orellana* (Achiote), *Polypodium calaguala* (Calaguala), *Sida rhombifolia* (Escobilla), *Psidium guajava* (Guayaba), *Plantago major* (Llantén), *Ficus dendrocida* (Matapalo), *Piper angustifolium* (Matico), *Musa paradisiacal* (Plátano), *Lupinus tauris* (Tauri), *Solanum nigra* (Yerba mora), and *Lepidium peruvianum* (Maca).[7−12] The leishmanicidal effect may reside in its phytochemical component such as flavonoids and, specifically, quercetin, which is a strong candidate in the combination therapy against the infection and the anemia associated with VL.[13]

Part of our group (Tenerife group) has been working on flavonoids derived from the aerial parts of *Consolida oliveriana* (DC) Schrod, a species used medicinally in parts of Anatolia (Turkey). A large number of publications have dealt with the diterpenoid alkaloids of other *Consolida* species, but reports on the flavonoid content of members of this genus are scarce.[14−19]

Most of the studies directed toward the detection of secondary plant metabolites with leishmanicidal activity have used the promastigote form of the parasite because it is easier to maintain under *in vitro* conditions. However, since the promastigote is not the developed form of the parasite in vertebrate hosts, evaluations made with promastigotes have only an indicative value of the possible leishmanicidal activity of the metabolite tested. As a result, a preliminary evaluation using promastigotes needs to be complemented with an evaluation using intracellular amastigotes in macrophages. At the same time, an evaluation of the possible cytotoxicity of the metabolite should be carried out using nonparasitized macrophages, in order to establish whether the *in vitro* activity of the metabolite is due to its general cytotoxic activity or whether it is selectively active against the *Leishmania* parasite.[20]

We investigated the inhibitory effects of flavonoid compounds derived from aerial parts of *C. oliveriana* (DC) Schrod, on the extracellular promastigote and the intracellular amastigote stages of *L. (V.) peruviana* and *L. (V.) braziliensis*, causal agents of both cutaneous and mucocutaneous leishmaniasis. In addition, we studied the cytotoxic effects of these compounds against a cell line of macrophages, analyzing the mechanism by which these molecules act.

Results and Discussion

The importance of phytotherapy as a guide in the search for new antileishmanial drugs becomes evident in a single search of specialized literature. Thus, the strategy for discovering new drugs is to investigate natural products from medicina1 plants.[21−23] Dozens of potential new compounds from nature have been found in the past 5 years, especially in the rainforests of South America and Africa, only concerning Leishmaniasis.[24] Plant-derived active principles and their semisynthetic and synthetic analogues have served as a major path to new chemotherapeutic compounds.[23,25,26] Furthermore, the leads obtained from the search for natural products with antileshmanial activity give new impetus for developing valuable synthetic compounds.[27]

Flavonoids are found in abundance in diets rich in fruits, vegetables, and plant-derived beverages and appear to have anticancer, antimicrobial, and antiparasitic properties.[28−30] Our Tenerife group is searching for bioactive substances potentially useful against many diseases, and recently they have shown that flavonoid acetates derived from flavonoids extracted from *C. oliveriana* exhibited significant impact on the growth of three human cell lines: HL-60, U937, and SK-MEL-1.[19]

* To whom correspondence should be addressed. Tel: +34 958 242369. Fax: +34 958, 243174. E-mail: msanchem@ugr.es.
[†] Department of Parasitology, University of Granada.
[‡] University of La Laguna.
[§] Department of Statistics, University of Granada.

10.1021/np8008122 CCC: $40.75
Published on Web 06/02/2009

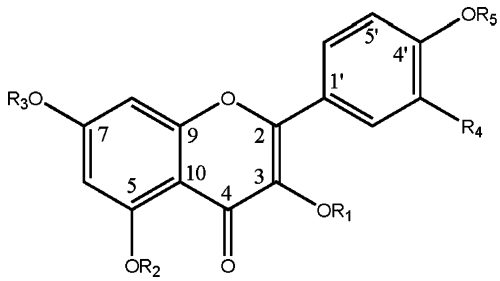

Figure 1. Chemical structure of flavonoid compounds.

Table 1. In Vitro Activity of Flavonoids on Promastigotes of *L. (V.) peruviana* and *L. (V.) braziliensis*[a]

compound	IC_{50} (μM) *L. (V.) peruviana*	IC_{50} (μM) *L. (V.) braziliensis*	toxicity IC_{50} on J774.2 macrophages cells (μM)[b]
pentostam	11.32	9.56	12.44
glucatim	15.33	25.61	15.20
1	71.29	53.65	53.67
1a	53.32	68.56	15.56
2	60.04	30.49	125.44
2a	11.18	46.78	109.23
3	53.34	52.46	161.32
3a	10.53	8.72	148.71
4	7.35	6.21	122.31
5	86.95	51.60	61.32

[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the concentrations used (0.1, 1, 10, 25, 50, and 100 μM) at 72 h culture. Note: Average of three separate determinations. [b] J774.2 macrophages cells at 72 h of culture.

Here, we evaluate whether the inhibitory effect on cell growth of four flavonoids (**1**, **2**, **3**, and **5**) obtained by natural means from *C. oliveriana* and their acetylated products (**1a**, **2a**, **3a**, and **4**)[19] (Figure 1) is active against intra- and extracellular forms of *L. (V.) peruviana* and *L. (V.) braziliensis*. The results are displayed in Table 1, using pentostam and glucatim as the reference drugs and including toxicity values against the J774.2 macrophage cells. After 72 h of exposure against *L. (V.) peruviana*, three of the acetylated compounds tested (**2a**, **3a**, and **4**) registered IC_{50} values lower than the reference drugs' IC_{50} (pentostam 11.32 and glucantim 15.33 μM), i.e., an IC_{50} of 7.35 μM for **4**, 11.18 μM for **2a**, and 10.53 μM for **3a**. Therefore, the toxicity values were very low when tested on J774.2 macrophage cells. In addition to being slightly toxic, these three compounds gave IC_{50} values on J774.2 macrophage cells of about 10- to 15-fold higher than those for the reference drugs. Against *L. (V.) braziliensis*, two of these acetylated compounds (**4** and **3a**) inhibited cell growth at a IC_{50} of 6.21 and 8.72 μM, respectively, this being significantly lower than the IC_{50} of the reference drugs. The rest of the compounds had an IC_{50} higher than the reference drugs and in some cases were toxic for the macrophages. These results indicate that the acetylated compounds performed better than the phenolic analogues. It appears that the kaempferol derivatives possessing a monosubstituted B-ring are more active than the quercetin analogues.

It has been shown that acetylation of certain flavonoids increases the antiproliferative activities of the parent compounds against HL-60 and other cell lines. For example, **3a** induced cell death in human leukemia cells.[26] This greater efficiency may possibly be due to acetylation, which facilitates the compound absorption, leading to greater effectiveness.[31] Little information is available on the leishmanicidal activity of flavonoids for comparison with our results; however, recently it has been possible to establish the activity of some of these compounds, showing them to be effective against promastigote development of both *L. (L.) mexicana*[32] and *L. (L.) donovani*,[33] with IC_{50s} values similar to those found by us. In a study by Tasdemir et al.,[34] quercetin was demonstrated to be highly effective against *L. (L.) donovani* and *T. cruzi*, at doses slightly lower than ours. These discrepancies presumably derive from the use of different methods and different life-cycle stages of the parasites.[35]

The products **2a**, **3a**, and **4** were selected because they had the greatest inhibitory effect on the *in vitro* growth of *L. (V.) peruviana* and *L. (V.) braziliensis* and had less toxic effects on macrophage cells, using the IC_{25} of each product as the test dosage.

When 1×10^5 J774.2 macrophage cells were incubated for 2 days and then infected with 1×10^6 promastigote forms of *L. (V.) peruviana* and *L. (V.) braziliensis* (Figure 2), the parasites invaded the cells and underwent morphological conversion to amastigotes from the first 3 h postinfection with a steadily increasing number of cells invaded until the last day of the assay (day 10). The values for infected cells (control experiment) reached 95% in the case of *L. (V.) peruviana* and 97% in the case of *L. (V.) braziliensis*. Nevertheless, in the amastigote-cell assay with the flavonoid compounds added simultaneously to the infection of macrophages cells with promastigote forms, the treatment significantly reduced the infection rate with respect to the control. That is, in the case of *L. (V.) peruviana* the infection rate reached 55%, 40%, and 59% for the compounds **4**, **2a**, and **3a**, respectively, on day 10 (Figure 2A). Meanwhile, in the infected *in vitro* cultures of *L. (V.) braziliensis* the results were 46% and 67% for compounds **4** and **3a** (Figure 2B). The addition of **4**, **2a**, and **3a** had a similar effect with a markedly lower amastigote number of *L.(V.) peruviana* per infected cell with respect to the control at day 10, i.e., reaching a value of roughly 72% (Figure 2C). When the cultured parasite was *L. (V.) braziliensis*, compound **4** decreased the amastigote number by as much as 60%, while compound **3a** reached 90% (Figure 2D).

Most studies on activity assays of new compounds use parasite forms that develop in vectors (promastigote forms in the case of *Leishmania* spp.),[20] for the ease of working with these forms *in vitro*. However, in the present study we have included the effect of the flavonoid compounds on the forms that are developed in the host (amastigotes) to determine the effects in humans. For this objective, we selected the products that had the greatest inhibitory effect on the *in vitro* growth of *L. (V.) peruviana* (**2a**, **3a**, and **4**) and *L. (V.) braziliensis* (**3a** and **4**) and had less toxic effects on J774.2 macrophage cells, using the IC_{25} of each product at the test dosage. The infective capacity on macrophage cells by *L. (V.) peruviana* and *L. (V.) braziliensis* significantly decreased when acetylated flavonoids were added. This infective capacity declined from the early hours of culture, these compounds altering the invasive capacity of the parasites. Until now, the mechanism by which the parasite's invasive capacity is lost was not known. It was found that these compounds decrease the parasite's multiplication rate into the macrophages. The acetylated compounds (**2a**, **3a**, and **4**) are highly effective not only against the extracellular forms of the parasite (promastigote forms) but also against intracellular forms (amastigote forms).

Despite the good results achieved in some cases, only a few compounds of natural origin are under clinical evaluation, as most of them have been disapproved because of their high toxicity.[36] It should be noted that these antiprotozoal agents are basically cytotoxic and act selectively against the parasites. Currently, publications on antiparasitic agents include activity evaluations against mammals and/or human cell lines.[33,37] Following this pattern, we have determined the toxicity of our compounds against a human cell line, noting that compounds **2a**, **3a**, and **4** showed little toxicity for J774.2 macrophage cells, with IC_{50}'s of around 10 to 15 times higher than those of the reference drugs, confirming a selective activity of these compounds against *L. (V.) peruviana*. These data are consistent with findings in the case of *L. (V.) braziliensis*, given that compounds **3a** and **4** proved extremely effective and with very low toxicity, opening the possibility of using

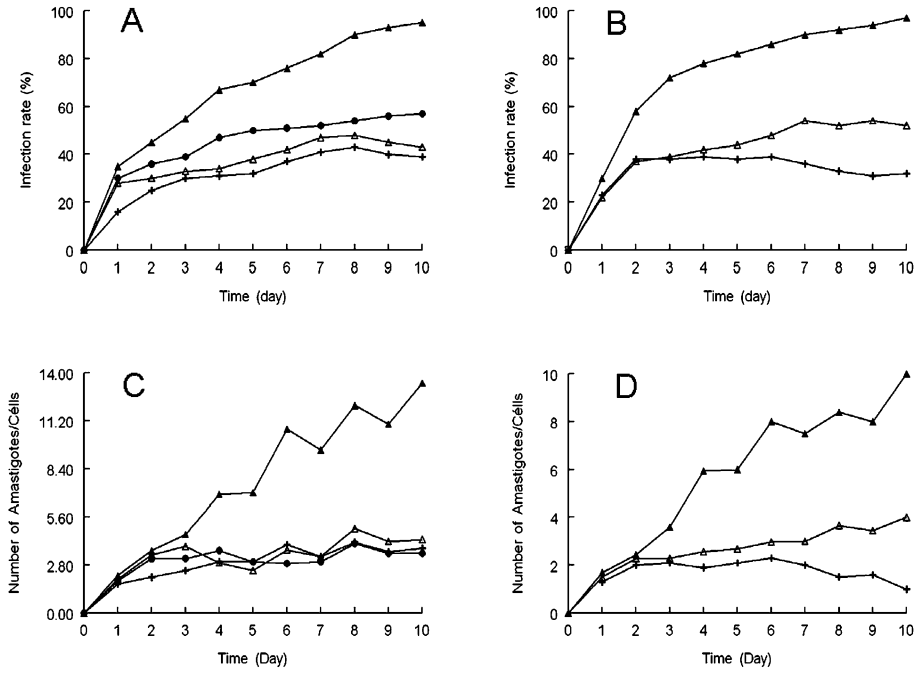

Figure 2. Effect of flavonoid compounds on the infection rate and growth of *Leishmania (V.) peruviana* (A) and *L. (V.) braziliensis* (B). (C) Mean number of amastigotes of *L. (V.) peruviana* and (D) for *L. (V.) braziliensis* per infected J774.2 macrophage cells. −▲−, control; −●−, **2a**; −+−, **3a**; and −△−, **4** (at IC_{25} conc). The values are means of three separate experiments.

these compounds in the treatment of both cutaneous and mucocutaneous leishmaniasis.[38]

The *in vitro* culture of trypanosomatids depends on the available carbon sources (mainly glucose) present in their culture medium for their energy metabolism.[39] None of the trypanosomatids studied are capable of completely degrading glucose to CO_2 under aerobic conditions, excreting a great part of their carbon skeleton into the medium as fermented metabolites, this difference depending on the species considered.[39] *L. (V.) peruviana* and *L. (V.) braziliensis* consume glucose at a high rate, thereby acidifying the culture medium due to incomplete acid oxidation. Using ^{1}H NMR spectra, we determined the fermented metabolites excreted by the parasites during their *in vitro* culture, and we identified and evaluated the inhibiting effect caused by the flavonoid compounds over final metabolite excretion in promastigote forms of *L. (V.) peruviana* and *L. (V.) braziliensis* cultured *in vitro* in MTL medium for 96 h (Figures 3 and 4). Figure 3B corresponds to the spectrum given by cell-free medium 96 h after inoculation with the promastigote forms of *L. (V.) peruviana*. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when the last spectrum (Figure 3B) was compared with that found with fresh medium (Figure 3A). *L. (V.) peruviana* excreted acetate and succinate as majority metabolites and L-alanine in a lower proportion. When the trypanosomatids were treated with the flavonoid compounds (**2a**, **3a**, and **4**; Figure 3C, D, and E), the excretion of some of these catabolites was clearly inhibited at the dosage tested (IC_{25}). Acetate and succinate were decreased by around 30 to 40%, while with compound **4** the acetate decreased up to 67%. L-Alanine also increased, and new excreted metabolites such as pyruvate and glycerol appeared. In the case of *L. (V.) braziliensis,* when the cell-free medium spectrum was compared (Figure 4B) to that of the parasite-free medium (Figure 4A), the excretion of acetate and succinate as major metabolites was found together with L-alanine in a lower proportion. When compounds **3a** (Figure 4C) and **4** (Figure 4D) were added to the cultures, a clear inhibition was detected in the peaks corresponding to acetate and succinate, while the peaks corresponding to L-alanine and D-lactate were higher, and peaks corresponding to pyruvate and glycerol appeared.

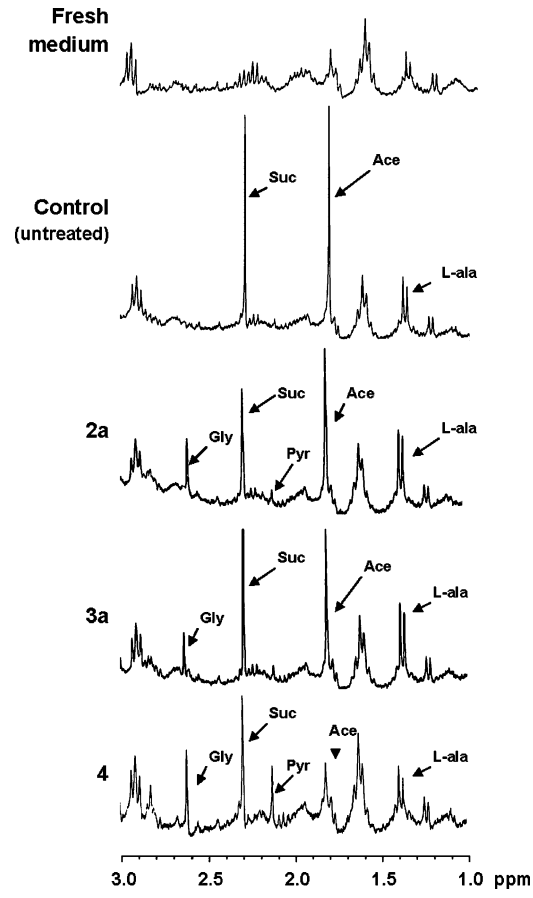

Figure 3. ^{1}H NMR study of the production of metabolites excreted by the promastigote forms of *L. (V.) peruviana* treated against flavonoid compounds (at a concentration of IC_{25}). (Ace) acetate; (Suc) succinate; (L-ala) L-alanine; (Pyr) pyruvate, and (Gly) glycerol.

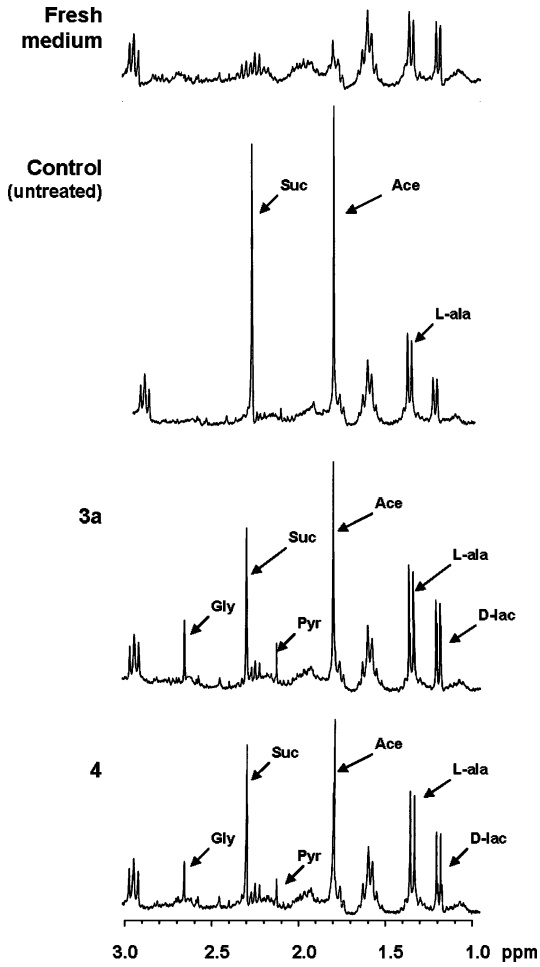

Figure 4. ^{1}H NMR study of the production of metabolites excreted by the promastigote forms of *L. (V.) braziliensis* treated against flavonoid compounds (at a concentration of IC_{25}). (Ace) acetate; (Suc) succinate; (L-ala) L-alanine; (Pyr) pyruvate (Gly) glycerol, and (D-lac) D-lactate.

The excretion of some of these catabolites (acetate and succinate) was clearly inhibited at the dosages assayed (IC_{25}). In the catabolism of glucose in *Leishmania* spp., pyruvate is located at metabolic branching points, leading to several excreted end products, such as acetate, L-alanine, ethanol, and L-lactate.[40] Acetate is a major end product formed in the mitochondrion and excreted by simple diffusion across the mitochondrial and cytoplasmic membranes. The inhibition of acetate excretion may be a direct consequence of the action of these flavonoids on the enzymes involved in their production (pyruvate dehydrogenase complex or acetate-succinate CoA-transferase), or else these compounds act on the mitochondrion and even on the cytoplasmic membrane, causing a loss of functionality. Succinate is another major end product excreted from glucose metabolism, but the pathway leading to its production has been the topic of a long-standing debate.[41] The controversy concerns the relevance of the NADH-dependent fumarate reductase activity detected in most trypanosomatids, for which the contribution in succinate production has not been clearly demonstrated. The main role of the succinate (succinic fermentation) is probably to maintain the glycosomal redox balance, by providing two glycosomal oxidoreductase enzymes that allow reoxidation of NADH, produced by glyceraldehydes-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenol pyruvate (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. The inhibition of acetate and succinate excretion explains the observed increase in L-alanine and D-lactate production and the appearance of two new peaks identified as glycerol and pyruvate.

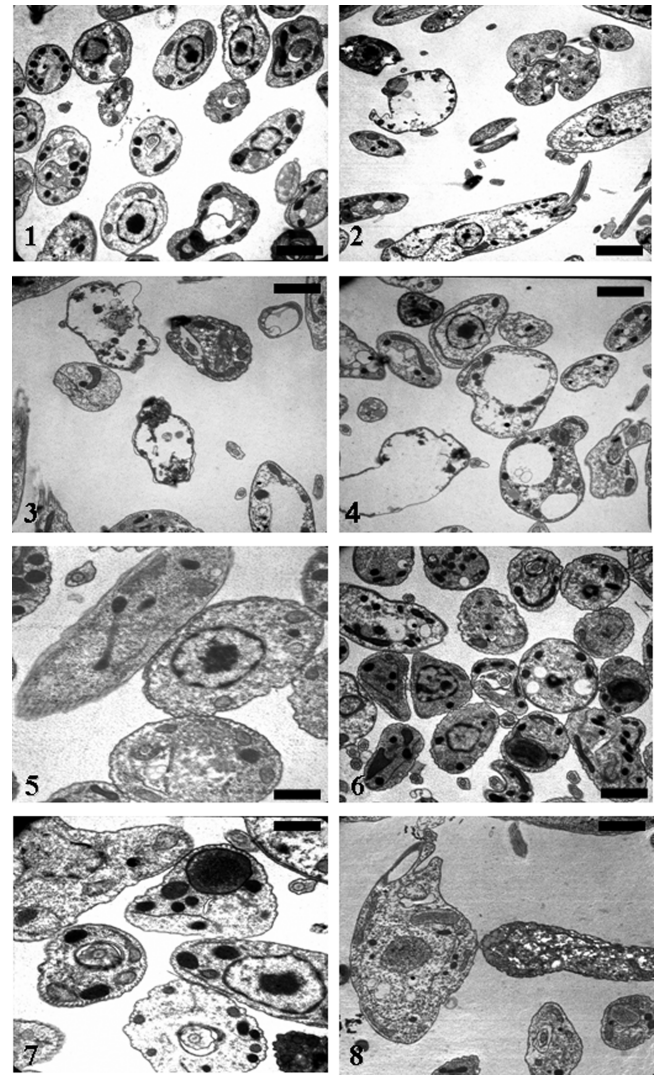

Figure 5. Ultrastructural alterations by TEM in *L. (V.) peruviana* and *L. (V.) braziliensis* treated with flavonoids compounds. (1) Control of *L. (V.) peruviana*, bar = 1.0 μm. (2 and 3) Promastigotes of *L. (V.) peruviana* treated with **2a**, bar = 1.0 μm. (4) Promastigotes of *L. (V.) peruviana* treated with **3a**, bar = 1.0 μm. (5) Control of *L. (V.) braziliensis*, bar = 1.59 μm. (6) Promastigotes of *L. (V.) braziliensis* treated with **3a**, bar = 1.0 μm. (7 and 8) Promastigotes of *L. (V.) braziliensis* treated with **4**, bar = 1.59 μm. (N) nucleus; (Nu) nucleolus; (CM) cytoplasmatic membrane; (MT) microtubules; (G) glycosomes; (R) reservosomes; (M) mitochondrion; (K) kinetoplast; (V) vacuole; and (LV) lipidic vacuoles.

Morphological alterations of *L. (V.) peruviana* treated with **2a** and **3a** were examined by transmission electron microscopy (TEM). The parasites were sensitive mainly to the treatment with these compounds at IC_{25}, and a considerable number of dead parasites were observed. Several alterations could be detected when compared with the images corresponding to control cells (Figure 5-1). Compound **2a** induced death with distortion of the parasite body and disruption of the cytoplasm, which appeared almost empty in some parasites but with many glycosomes throughout (Figure 5-2 and 3). Meanwhile, **3a** induced intense vacuolization in the parasites (Figure 5-4), and empty vacuoles and lipidic vacuoles were visible. Glycosomes were abundant in the altered parasites, while other parasites appeared to be dead.

The treatment with **3a** and **4** at IC_{25} for 96 h induced alterations in *L. (V.) braziliensis*, which can be observed when comparing the images made with untreated parasites (Figure 5-5). The treatment with **3a** (Figure 5-6) disrupted the cytoplasm, which in the majority of the parasites had little electrodensity and abundant vacuoles. The mitochondrion and kinetoplast were also affected, both organelles appearing swollen. Some parasites had abundant lipidic vacuoles. *L. (V.) braziliensis* treated with **4** (Figure 5-7 and 8) and showed marked alterations. Figure 5-7 shows an undulating cytoplasmic membrane and microtubules in disarray. The kinetoplasts were intensely swollen, glycosomes were abundant, and some parasites showed vacuolization. The rest of the compounds were highly toxic, but it was not possible to study them by TEM.

In general, several morphological alterations were noted in *L. (V.) peruviana* and *L. (V.) braziliensis* when additional compounds were added to the cultures. There was an intense vacuolization as well as disruption of the cytoplasmic, nuclear, and mitochondrial membranes, suggesting a possible effect on tubuline, a protein known to be affected by flavonoids.[42] This disruption of the membranes may in turn account for the strong inhibition of the acetate that is synthesized inside the mitochondria and then diffused to the cytoplasm and outside the cell.

In conclusion, our study demonstrates that several acetylated flavonoids derived from *C. oliveriana* (**2a**, **3a**, and **4**) were very active *in vitro* against both the extracellular as well as the intracellular forms of *L. (V.) peruviana* and *L. (V.) braziliensis* (**3a** and **4**). These compounds are not toxic to the host cells and are effective at concentrations similar to or lower than the reference drugs used in the present study. These flavonoids have promising leishmanicidal properties. The data from transmission electronic microscopy and nuclear magnetic resonance raise the possibility that the action (or part of the action) could be at the level of the parasite membranes. The potent leishmanicidal activities described here for flavonoids represent an exciting advance in the search for new antiprotozoal agents.

Experimental Section

General Experimental Procedures. The ^1H and ^{13}C NMR spectra were measured using a Bruker AMX-400 or a Bruker MAX-500 instrument. FAB5 and exact mass measurements were determined using a Micromass Autospec instrument at 70 eV. ESIITMS data were obtained by tandem electrospray ion trap mass spectrometry (LCQ Deca XP Plus; ThermoFinnigan; San Jose, CA). Column chromatography was performed over Sephadex LH-20 Pharmacia (ref 17-0090-01; Upsala, Sweden), silica gel 60 (Merck 230−400 mesh; Darmstadt, Germany, and analytical TLC, Merck Kieselgel 60 F 254. HPLC separations were performed on a JASCO Pu-980 series pumping system equipped with a JASCO UV-975 ultraviolet detector and with a Waters Kromasil 5 (5 mm × 250 mm) column. A Macherey-Nagel VP 250/10 nucleodur Sphinx RP 5 mm column (Düren, Germany) was used for HPLC-RP chromatography; chromatograms were visualized under UV light at 255 and 366 nm and/or sprayed with oleum followed by heating. All solvents were distilled before use; the purity of all compounds was 99.0% as judged by HPLC. Stock solutions of 10 mM flavonoids were made in DMSO, and aliquots were frozen at −20 °C. The metabolite excretion ^1H NMR spectra were obtained at 300 MHz on a Bruker AM-300 spectrometer, which was operated at pulse in Fourier transformation with quadrature detection. The temperature of the probe was maintained at 27 °C. The acquisition parameters were as follows: pulses of 90° in radius and a wavelength of 3287.5 Hz, 8 s recycle time, and 160 accumulations. The chemical displacements were expressed as parts per million (ppm) using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the reference signal. The ultrastructural alterations were examined with an EMCIO Zeiss transmission electron microscope.

Parasite Strain, Culture. *L. (V.) peruviana* (MHOM/PE/1984/LC26) and *L. (V.) brazilensis* (MHOM/BR /1975/M2904) were cultured *in vitro* in MTL medium plus 10% inactivated fetal bovine serum kept in an air atmosphere at 28 °C, in Roux flasks (Corning, USA) of 75 cm^2 in surface area, according to the methodology described by González et al.[22]

Plant Material. Aerial parts of *C. oliveriana* were collected and identified near Pazarkik in eastern Turkey at an altitude of 980 m by Prof. Julian Molero Briones, Department of Botany, Faculty of Pharmacy, University of Barcelona (Spain), where a voucher specimen (BCF-37810) has been deposited.

Extraction and Isolation. Dried and powdered aerial parts of *C. oliveriana* (2.23 kg) were defatted with hexanes (6 L) for one month and subsequently extracted repeatedly with 80% EtOH (7 L) at room temperature for two weeks. The extract was filtered and concentrated at reduced pressure. The remaining aqueous layer was exhaustively extracted with *n*-BuOH to give, after removal of the solvent, 36 g of a brown viscous residue. The aqueous layer was concentrated and filtered through a column of Amberlite XAD-2 resin (8 × 40 cm) to remove the polar compounds, while the flavonoids remaining on the column were eluted with MeOH (see below). The viscous *n*-BuOH extract (10 g) was fractionated on a 50 × 8 cm column packed with Sephadex LH-20 and eluted with hexanes−CH_2Cl_2−MeOH, 1:1:2, 15 500-mL fractions (S1−S15) being collected. Fractions S1−S15 contained mainly alkaloids contaminated by material exhibiting no UV absorption, and fractions S4−S8 contained mixtures of glycosides. Fractions S9 and S10 and fractions S11−S13, after recrystallization from MeOH−EtAc, gave quercetin (**2**, 556 mg) and kaempferol (**1**, 472 mg), respectively. Fractions S4−S8 were chromatographed over a 40 × 4 cm Sephadex LH-20 column using hexane−CH_2Cl_2−MeOH (1:1:1), the elution being monitored by TLC analysis. This resulted in three fractions: A (430 mg), B (70 mg), and C (55 mg). Fraction B afforded 90 mg of trifolin (**3**) after recrystallization from MeOH. Rechromatography of fraction A over silica gel using 40 mL of hexanes−EtOAc mixtures of increasing polarity rendered, from fractions 64−77 (hexanes−EtOAc, 2:8), 24 mg of 6″-*O*-acetylhyperoside after further purification over Sephadex LH-20 (hexanes−MeOH−CH_2Cl_2, 2:1:1). Fractions 86−90 (*n*-hexane−EtOHc, 1:9) yielded 21 mg of 6″-*O*-acetylhyperoside (**5**).

The material eluted from the Amberlite XAD-2 column with MeOH was further purified over a 50 × 8 cm column packed with Sephadex LH-20 and eluted with CH_2Cl_2−MeOH (1:1), six fractions (J1−J6) of 500 mL each being collected. Fractions J1−J3 containing mainly alkaloids and other components exhibiting no UV absorption were combined with fractions S1−S3. Fractions J4−J6 (2 g) were subjected to gel filtration on Sephadex LH-20 using 42 200-mL fractions of H_2O−MeOH (1:1). Fractions 28−31 yielded 45 mg of trifolin (**3**) and fractions 33−38 18 mg of octa-*O*-acetylhyperoside (**4**).

General Method for Acetylation. Dry phenolic material was dissolved in the minimum volume of pyridine. Twice the amount of acetic anhydride was added, and the solution was allowed to stand overnight at ambient temperature. The mixture was diluted with H_2O and extracted three times with EtOAc. The extract was evaporated under vacuum, and the residue containing the polyacetate was further purified by column chromatography over silica gel using hexanes−EtOAc as the eluent. Mass spectra of the polyacetylated compounds, all gums, are listed below.

Tetra-*O*-acetylkaempferol (1a): EIMS $m/z = 412$ ($M^+ - C_2H_3O_2$, 23), 370 ($M^+ - 2\ C_2H_3O_2$, 57) 286 ($M^+ - 4\ C_2H_3O_2$, 100).

Penta-*O*-acetylquercetin (2a): HREIMS $m/z = 513.0997$, calcd for $C_{25}H_{20}O_2 + H^+$ 513.1033.

Hepta-*O*-acetyltrifolin (3a): HRFABMS $m/z = 743.1784$, calcd for $C_{35}H_{34}O_{18} + H^+$ 743.1823.

The compound purity of 99% was determined by HPLC.

Promastigote Assay. The compounds obtained (Figure 1) were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.1% and were afterward assayed as nontoxic and without inhibitory effects on the parasite growth, according to Luque et al.[43] The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM. The effect of each compound against promastigote forms, as well as the concentrations, was evaluated at 24, 48, and 72 h using a Neubauer hemocytometric chamber. The leishmanicidal effect is expressed as IC_{50} values, i.e., the concentration required to give 50% inhibition, calculated by linear-regression analysis from the K_c values at the concentrations employed.

Cell Culture and Cytotoxicity Tests. Macrophage line J774.2 (ECACC number 91051511) cells was obtained from a tumor in a female BALB/c rat in 1968. Macrophages were kept in the laboratory by cryopreservation in liquid N_2 and then by successive subcultures in RPMI medium. For the cytotoxicity test, macrophages were placed in 25 mL cone-based bottles (Sterling) and centrifuged at 1500 rpm for 5

min. The culture medium was removed, and minimal essential medium (MEM; Gibco) supplemented with l0% inactivated fetal bovine serum (adjusted to pH 7.2) was added to a final concentration of 10^5 cells/mL. This cell suspension was distributed in a culture tray (24 wells) at a rate of 100 μL/well and incubated for 2 days at 37 °C in a humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at a concentration of 100, 50, 25, 10, and 1 μM). The cultures were incubated for 72 h. The vital stain trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, the percentage of viability was calculated in comparison to that of the control culture, and the IC_{50} was calculated by linear-regression analysis from the K_c values at the concentrations employed.

Amastigote Assay. J774.2 macrophage cells were grown 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 cultivated for 2 days. Afterward the cells were infected *in vitro* with promastigote forms of *L. (V.) peruviana* and *L. (V.) brazilensis*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 6 h at 37 °C in 5% CO_2. The nonphagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell 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.

Metabolite Excretion. Cultures of *L. (V.) peruviana* and *L. (V.) braziliensis* promastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the drugs (except for control cultures). After incubation for 96 h at 28 °C, the cells were centrifuged at 1500 rpm for 10 min. The supernatants were collected to determine excreted metabolites by nuclear magnetic resonance spectroscopy (^{1}H NMR) as previously described by Sánchez-Moreno et al.[44] The chemical displacements were expressed in parts per million (ppm), using sodium 2,2 dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernandez-Becerra et al.[45]

Ultrastructural Alterations. *L. (V.) peruviana* and *L. (V.) brazilensis*, at a density of 5×10^6 cells/mL, were cultured in MTL medium plus 10% inactivated fetal bovine serum kept in an air atmosphere at 28 °C, in Roux flasks (Corning, USA) of 75 cm^2 in surface area, containing the drugs at IC_{25} concentration. After 96 h, the cultures were centrifuged at 1500 rpm for 10 min, and the pellets were 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. Pellets were prepared for transmission-electron microscopy following the technique of Luque et al.[43]

Acknowledgment. We wish to thank M. J. Martínez-Guerrero and E. Onorato-Gutiérrez for their expert technical help in transmission-electron microscopy and NMR spectroscopy, respectively. We also appreciate the help of L. F. Espigares-Hervías for his kind assistance in the preparation of electron microscopy photographs and D. Nesbitt for the English revision. This investigation received financial support from MEC (Spain), CGL2006-27889-E/BOS.

References and Notes

(1) Lezama-Dávila, C. M.; Isaac-Márquez, A. P. *Nat. Prod. Rep.* **2001**, *18*, 674–688.
(2) Croft, S. L.; Yardley, V. *Curr. Pharmacol.* **2002**, *8*, 319–342.
(3) Tempone, A. G.; Treiger, S. E.; Andrade, H. F.; Amorin, N. C.; Yogi, A.; Salerno, C.; Bachiega, D.; Lupo, F. N.; Bonotto, S. V.; Fischer, D. C. H. *Photochemistry* **2005**, *12*, 382–390.
(4) Rocha, L. G.; Almeida, J. R. G. S.; Macêdo, R. O.; Barbosa-Filho, J. M. *Phytomedicine* **2005**, *12*, 514–535.
(5) Carvalho, P. B.; Ferreira, E. *Fitoterapia* **2001**, *72*, 599–618.
(6) Chan-Bacab, M. J.; Peña-Rodríguez, L. M. *Nat. Prod. Rep.* **2001**, *18*, 674–688.
(7) Li, G.; Ammermann, U.; Quiroz, C. F. *Econ. Bot.* **2001**, *55*, 255–262.
(8) Ganzera, M.; Zhao, J.; Muhammad, I.; Khan, I. *Chem. Pharm. Bull.* **2002**, *50*, 988–991.
(9) Sandoval, M.; Okuhama, N.; Angles, F.; Melchor, V.; Condezo, L.; Lao, J.; Millar, J. *Food Chem.* **2002**, *79*, 207–213.
(10) Dini, A.; Migliuolo, G.; Rastrelli, L.; Saturnino, P.; Schettino, O. *Food Chem.* **1994**, *49*, 347–349.
(11) Muhammad, I.; Zhao, D.; Khan, I. *Phytochemistry* **2002**, *59*, 105–110.
(12) Cui, B.; Zheng, B.; He, K.; Zheng, Q. *J. Nat. Prod.* **2003**, *66*, 1101–1103.
(13) Sen, G.; Mandal, S.; Roy, S. S.; Mukhopadhyay, S.; Biswas, T. *Free Radical Biol. Med.* **2005**, *38*, 1257–1264.
(14) Kayser, O.; Kiderlen, A. F.; Croft, S. L. *Parasitol. Res.* **2003**, *90*, 55–62.
(15) Da Silva, F.; Bueno, P. C. P.; Gregório, L. E.; Silva, M. L. A.; Albuquerque, S.; Bastos, J. K. *J. Pharm. Pharmacol.* **2004**, *56*, 1195–1199.
(16) Bailey, M. S.; Lockwood, D. N. *Clin. Dermatol.* **2007**, *25*, 203–211.
(17) Ulubelen, A.; Desai, H.; Haridutt, K.; Hart, B. P.; Joshi, B. S.; Pelletier, S. W.; Meriçli, A. H.; Meriçli, F.; Ozen, H. C. *J. Nat. Prod.* **1996**, *59*, 907–910.
(18) Grandez, M.; Madinaveitia, A.; Gavin, J. A.; Alva, A.; De La Fuente, G. *J. Nat. Prod.* **2002**, *65*, 513–516.
(19) Díaz, J. G.; Carmona, A. J.; Torres, F.; Quintana, J.; Estévez, F.; Herz, W. *Planta Med.* **2008**, *74*, 171–174.
(20) Chen, M.; Lin, Z.; Soren, B.; Kharazmi, T. *Antimicrob. Agents Chemother.* **2001**, *45*, 2023–22029.
(21) Singh, S.; Sivakumar, R. *J. Infect. Dis.* **2004**, *10*, 307–315.
(22) González, P.; Marín, C.; Rodríguez-González, I.; Hitos, A. B.; Rosales, M. J.; Reina, M.; Díaz, J. G.; González-Coloma, A.; Sánchez-Moreno, M. *Int. J. Antimicrob. Agents* **2005**, *25*, 136–141.
(23) Urbina, J. A. *Curr. Pharm. Des.* **2002**, *8*, 287–295.
(24) de Carvalho, P. B.; Ferreira., E. I. *Fitoterapia* **2001**, *72*, 599–618.
(25) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461–477.
(26) Torres, F.; Quintana, J.; Díaz, J. G.; Carmona, A. J.; Estévez, J. F. *Apoptosis* **2008**, *13*, 716–728.
(27) Carvalho, P. B.; Arribas, M. A. G.; Ferreira, E. I. *Rev. Bras. Ci. Farm.* **2000**, *36* (Suppl. 1), 69–96.
(28) Taleb-Contini, S. H.; Salvador, M. J.; Balanco, J. M.; Albuquerque, S.; De Oliveira, D. C. R. *Phytother. Res.* **2004**, *18*, 250–254.
(29) Grael, C. F.; Albuquerque, S.; Lopes, J. L. *Fitoterapia* **2005**, *76*, 73–82.
(30) OPS/WHO/NTD/IDM. *Estimación cuantitativa de la enfermedad de Chagas en las Américas*; OPS/HDM/CD/425-06, 2006.
(31) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
(32) Suárez, A. I.; Diaz, B. M.; Delle Monache, F.; Compagnone, R. S. *Fitoterapia* **2003**, *74*, 473–475.
(33) Weniger, B.; Vonthron-Sénécheaua, C.; Kaiser, M.; Brun, R.; Anton, R. *Phytomedicine* **2001**, *13*, 176–180.
(34) Tasdemir, D.; Kaiser, M.; Brun, R.; Yardley, V.; Schmidt, T. J.; Tosun, F.; Rüedi, P. *Antimicrob. Agents Chem.* **2006**, *8*, 1352–1364.
(35) López-Posadas, R.; Ballester, I.; Abadía-Molina, A. C.; Suárez, M. D.; Zarzuelo, A.; Martínez-Augustin, O.; Sánchezde Medina, F. *Biochem. Pharmacol.* **2008**, *76*, 495–506.
(36) Osorio, E. J.; Robledo, S. M.; Bastida, J. *J. Acad. Press* **2008**, *66*, 113–190.
(37) Barrett, M. P.; Burchmore, R. J.; Stich, A.; Lazzari, J. O.; Frasch, A. C.; Cazzulo, J. J.; Krishna, S. *Lancet* **2003**, *362*, 1469–1480.
(38) Labraña, J.; Machocho, A. K.; KricsfaLusy, V.; Brun, R.; Codina, C.; Vi1adomat, F.; Bastida, J. *Phytochemistry* **2002**, *60*, 847–852.
(39) Bringaud, F.; Riviere, L.; Coustou, V. *Mol. Biochem. Parasitol.* **2006**, *149*, 1–9.
(40) Hannaert, V.; Bringaud, F.; Opperdoes, F. R.; Michels, P. A. *Kinetoplastid Biol. Dis.* **2003**, *2*, 1–30.
(41) Besteiro, S.; Biran, M.; Biteau, N.; Coustou, V.; Baltz, T.; Canioni, P.; Brimgaud, F. *J. Biol. Chem.* **2002**, *277*, 38001–38012.
(42) Quintín, J.; Roullier, C.; Thoretb, S.; Lewin, G. *Tetrahedron* **2006**, *62*, 4038–4045.
(43) Luque, F.; Fernandez-Ramos, C.; Entrala, E.; Sánchez-Moreno, M. *Comp. Biochem. Phys. C* **2000**, *126*, 39–44.
(44) Sánchez-Moreno, M.; Lasztity, D.; Coppens, I.; Opperdoes, F. R. *Mol. Biochem. Parasitol.* **1992**, *54*, 185–200.
(45) Fernandez-Becerra, C.; Sánchez-Moreno, M.; Osuna, A.; Opperdoes, F. R. *J. Eukaryotic Microbiol.* **1997**, *44*, 523–529.

NP8008122