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SUSCEPTIBILITY

In Vitro Susceptibility of Kinetoplastids to Celastroloids from *Maytenus chiapensis*

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ABSTRACT Leishmaniasis and Chagas are among the most significant neglected tropical diseases. Due to several drawbacks with the current chemotherapy, developing new anti-kinetoplastid drugs has become an urgent issue. In the present work, a bioassay-guided investigation of the root bark of *Maytenus chiapensis* on *Leishmania amazonensis* and *Trypanosoma cruzi* led to the identification of two *D:A*-friedo-nor-oleanane triterpenoids (celastroloids), 20β-hydroxy-tingenone (celastroloid 5) and 3-*O*-methyl-6-oxo-tingenol (celastroloid 8), as promising antikinetoplastid leads. They displayed higher potency on *L. amazonensis* promastigotes (50% inhibitory concentrations [IC_{50}s], 0.44 and 1.12 μM, respectively), intracellular amastigotes (IC_{50}s, 0.83 and 1.91 μM, respectively), and *T. cruzi* epimastigote stage (IC_{50}s, 2.61 and 3.41 μM, respectively) than reference drugs miltefosine and benznidazole. This potency was coupled with an excellent selectivity index on murine macrophages. Mechanism of action studies, including mitochondrial membrane potential ($\Delta\psi$m) and ATP-level analysis, revealed that celastroloids could induce apoptotic cell death in *L. amazonensis* triggered by the mitochondria. In addition, the structure-activity relationship is discussed. These findings strongly underline the potential of celastroloids as lead compounds to develop novel antikinetoplastid drugs.

KEYWORDS Chagas disease, bioactive natural products, leishmaniasis

The term leishmaniasis comprises a group of diseases caused by the species *Leishmania* protozoans, transmitted by female sandflies of the genera *Phlebotomus* and *Lutzomyia*. Leishmaniasis, considered one of the most significant neglected tropical diseases, has diverse clinical manifestations, including visceral leishmaniasis, characterized by recurrent episodes of fever, weight loss, hepatosplenomegaly, and anemia; mucosal leishmaniasis leading to partial or complete destruction of the mucous membranes in nose and mouth, causing disabilities; and cutaneous leishmaniasis, the most common form of this infection, causing mostly ulcerative lesions that leave scars for life (1; https://www.who.int/news-room/fact-sheets/detail/leishmaniasis). The incidence of leishmaniasis as an opportunistic disease has increased in recent years because of growing immune depression resulting from chronic illnesses, such as neoplasms, immunosuppressive treatments, and HIV infection (2). Globally, leishmaniasis is among the top 10 neglected tropical diseases, with more than 12 million infected people and between 0.9 and 1.6 million new cases each year. Furthermore, leishmaniasis causes between 20,000 and 30,000 deaths per year and 350 million people at risk of infection (https://www.who.int/leishmaniasis/burden/en/).

Chagas disease is caused by infection with the flagellated protozoan *Trypanosoma cruzi*, which circulates among insect vectors of the subfamily Triatominae. Transmission in humans is mainly through contact between the urine and feces of the infected vector and mucous membranes or nonintact skin (3). Chagas disease has a substantial impact in Latin America,

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but there are also congenital and transfusion transmissions in other continents, altering the epidemiology of the disease. There are two clinical forms, the acute and the chronic phases. Around 75% of infected patients are asymptomatic during their lifetimes, while 25% of infections evolve into the chronic phase, leading to heart, digestive system, and/or nerve system damage. Its annual incidence is 28,000 cases in the region of the Americas, affecting between 6 and 8 million people and causing about 12,000 deaths per year (https://www.who.int/en/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis)).

The current treatment of leishmaniasis is pentavalent antimonials as the first-line treatment. Second-line treatments include amphotericin B and its liposomal formulation, miltefosine, pentamidine, azole drugs, and paromomycin in resistant cases to pentavalent antimonials (4). The available treatments for Chagas disease include two drugs, benznidazole and nifurtimox, both near to 100% efficacy against the acute phase of the disease, as well as effective against congenital transmission cases. However, after infection with the parasite, the effectiveness of the treatment decreases over the years (5).

Cutaneous leishmaniasis (CL) is endemic in El Salvador. Between 2012 and 2018, six cases of visceral leishmaniasis were reported, and in 2018, there were 50 new cases of CL (6), with 48% of the infected patients being children under 10 years old. The last official data regarding *T. cruzi* conclude that, in 2008, 67 acute cases were reported, and in 2009, 1,600 blood samples resulted positive for *T. cruzi*, with a seroprevalence of 1.93% (7). However, in 2014, the country was considered free of the main vector-borne infection (https://www.paho.org/els/index.php?option=com_content&view=article&id=317:presentacion-libro-enfermedad-chagas-salvador-evolucion-historica&Itemid=291).

Owing to numerous drawbacks of the current antikinetoplastid chemotherapy, which include widespread drug resistance, toxicity, long courses of administration, and cost, together with lack of an effective human vaccine, the development of efficacious, safe, and more accessible new drugs is becoming an urgent issue (8,9). In this regard, natural products can be understood as a population of "privileged structures" selected by evolutionary pressure to interact with various biological targets and represent a source of potent and structurally diverse scaffolds characterized by unique chemical architectures, pharmacophores, and inherent drug-like properties (10). Thus, natural products constitute a valuable source of therapeutic agents that hold promise for improvements in drugs to treat these diseases (11).

Species of the genus *Maytenus* (Celastraceae) have been intensely investigated for bioactive compounds since they are widely used in traditional medicine and agriculture in North Africa, South and Central America, and Central and East Asia (12). The therapeutic potential of *Maytenus* species has been mainly attributed to quinonemethide triterpenoids (QMTs) and their structurally related phenolic triterpenoids, a group of *D:A*-friedo-nor-oleanane triterpenoids with an unsaturated system involving A/B rings and an oxidized ring E. This particular class of naturally occurring products, which are exclusively accumulated in the root bark of the plants, is given the general name celastroloids since they are chemotaxonomic markers of the Celastraceae family. In particular, pristimerin and tingenone are the most frequently reported celastroloids and have long been used for the treatment of a variety of ailments (13). Previous phytochemical studies on *Maytenus chiapensis*, a species native to El Salvador and commonly named "escobo blanco," have reported sesquiterpenoids (14) and tetracyclic (15) and pentacyclic triterpenoids (16) from the areal parts of this plant. Moreover, a validated high-performance liquid chromatography (HPLC) method was developed for the qualification and quantification of pristimerin and tingenone in root bark extracts of *M. chiapensis* (17).

In our continuing research program for new antikinetoplastid agents from nature (18, 19), the root bark of *Maytenus chiapensis* is being investigated. A bioassay-guided fractionation based on leishmanicidal and tripanocidal activity has revealed that the quinonemethide triterpenoid 20β-hydroxy-tingenone and the triterpene phenolic and 3-*O*-methyl-6-oxo-tingenol are promising antiparasitic components in this plant. Analysis of the

structure-activity relationship (SAR) and mechanism induced in the parasites shed light on the potential of celastroloids as natural product scaffolds for kinetoplastid diseases drug development.

RESULTS AND DISCUSSION

***Maytenus chiapensis* root bark-bioguided fractionation.** The hexanes:Et_2O extract of the root bark of *M. chiapensis* showed potent activity against the two tested species, with 50% inhibitory concentrations (IC_{50}) values of 1.41 μg/ml and 2.93 μg/ml against *Leishmania amazonensis* promastigote and *T. cruzi* epimastigote stage, respectively. Cytotoxicity on murine macrophages was also assessed searching for selectivity, and the selectivity indexes (SI) (32.4 and 15.6, respectively) were a promising sign to continue with a bioassay-guided fractionation. Miltefosine and benznidazole were used as reference drugs against leishmaniasis and Chagas disease, respectively (Table 1). Miltefosine shows an IC_{50} value of 2.64 μg/ml against *L. amazonensis* and a 50% cytotoxic concentration (CC_{50}) of 29.42 μg/ml on murine macrophages, whereas benznidazole showed an IC_{50} of 1.81 μg/ml against *T. cruzi* and a CC_{50} of 104.1 μg/ml on murine macrophages.

Subsequently, the organic extract was submitted to a liquid chromatography on silica gel affording eight fractions (F1 to F8), which were assayed against both parasitic strains (Table 1; Fig. 1). Fractions F6 (IC_{50} value, 5.75 μg/ml), F7 (IC_{50} value, 5.95 μg/ml), and F8 (IC_{50} value, 5.72 μg/ml) exhibited remarkable activity on *L. amazonensis* and a moderated effect on *T. cruzi* (IC_{50} values ranging from 12.42 to 15.20 μg/ml). The results highlight these three fractions as the most promising ones to continue with a bioguided fractionation.

The active F6 fraction was chromatographed on a silica gel column and the fractions combined on the basis of their thin-layer chromatographic (TLC) analyses (F6A to F6E), which were assayed against the two parasites species (Table 2). Subfractions F6B, F6C, and F6E exhibited a potent antikinetoplastid effect on *L. amazonensis* (IC_{50} values ranging from 1.12 to 5.67 μg/ml), with subfraction F6B being 2.4-fold more potent than miltefosine (IC_{50} 2.64 μg/ml), the reference drug. Subfractions F6B and F6E exhibited a moderate antiprotozoal effect on *T. cruzi*, both lower than benznidazole, the reference drug. Therefore, subfraction F6B was submitted to purification steps by column chromatography (CC) over Sephadex LH-20 and preparative TLC to yield celastroloids 1, 2, 4, and 8. Following the same procedure, subfractions F6C and F6E were subjected to subsequent chromatographies to afford compounds 3, 6, 8, and 5, respectively (Fig. 1 and 2).

TABLE 1 Leishmanicidal and trypanocidal activity of crude extract and fractions from *Maytenus chiapensis* root bark[a]

Extract, fraction, or drug	*L. amazonensis* IC_{50} (μg/ml)	*T. cruzi* IC_{50} (μg/ml)	Murine macrophages CC_{50} (μg/ml)	Selectivity index calculated for *L. amazonensis*/*T. cruzi*
Hexane:Et_2O extract	1.41 ± 0.12	2.93 ± 0.02	45.68 ± 6.07	32.4/15.6
Fraction				
F1	>200	>200	ND	ND
F2	100–50	>200	ND	ND
F3	200–100	100–50	ND	ND
F4	100–50	100–50	ND	ND
F5	50–25	100–50	ND	ND
F6	5.75 ± 0.60	12.42 ± 2.66	26.22 ± 3.80	4.6/2.1
F7	5.95 ± 0.61	14.55 ± 3.05	28.62 ± 4.22	4.8/2.0
F8	5.72 ± 0.61	15.20 ± 1.81	71.05 ± 3.62	12.4/4.7
Drug[b]				
Miltefosine	2.64	ND	29.42	11.1
Benznidazol	ND	1.81	104.1	57.5

[a]± represents the standard deviation. ND, not determined.
[b]Miltefosine and benznidazole were used as positive controls against *L. amazonensis* and *T. cruzi*, respectively.

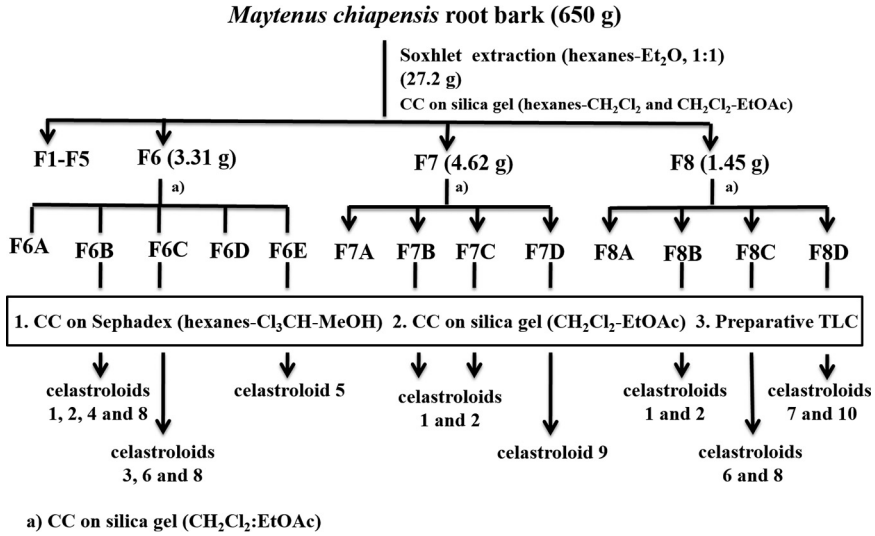

FIG 1 Flowchart of antikinetoplastid-bioguided fractionation of *Maytenus chiapensis* root bark.

The active fractions F7 (Tables 1 and 3) and F8 (Tables 1 and 4) were subjected to CC over Sephadex LH-20 and silica gel and preparative TLC affording the known celastroloids 1, 2, and 9, and 1, 2, 6 to 8, and 10, respectively (Fig. 2). The structures of the isolated compounds were identified as pristimerin (compound 1) (20), tingenone (compound 2) (21), dispermoquinone (compound 3) (22), 22β-hydroxy-tingenone (compound 4) (23), 20β-hydroxy-tingenone (compound 5) (24), 6-oxo-pristimerol (compound 6) (25), 6-oxo-tingenol (compound 7) (25), 3-*O*-methyl-6-oxo-tingenol (compound 8) (25), 3-*O*-methyl-22β-hydroxy-6-oxo-tingenol (compound 9) (26), and 3-*O*-methyl-22β,23-dihydroxy-6-oxo-tingenol (compound 10) (25) by nuclear magnetic resonance (NMR) spectroscopy and comparison with data reported in the literature.

TABLE 2 Leishmanicidal and trypanocidal activity of F6 subfractions from *Maytenus chiapensis* root bark[a]

Subfraction	*L. amazonensis* IC_{50} (μg/ml)	*T. cruzi* IC_{50} (μg/ml)	Murine macrophages CC_{50} (μg/ml)	Selectivity index calculated for *L. amazonensis*/*T. cruzi*
F6A	25–50	>100	ND	ND
F6B	1.12 ± 0.17	4.20 ± 0.83	27.13 ± 3.29	24.2/6.5
F6B1	5.83 ± 0.87	>100	4.04 ± 0.61	0.7/ND
F6B2	0.28 ± 0.03	0.46 ± 0.07	2.26 ± 0.15	8.1/4.9
F6B3	0.15 ± 0.03	0.25 ± 0.04	6.86 ± 0.30	45.7/27.4
F6B4	0.22 ± 0.01	0.47 ± 0.10	3.74 ± 0.73	17.0/8.0
F6B5	0.39 ± 0.05	0.65 ± 0.06	46.65 ± 3.65	119.6/71.8
F6C	5.67 ± 0.55	>50	>100	>17.6/ND
F6C1	>50	>50	ND	ND
F6C2	5.15 ± 0.78	>50	>100	>19.4/ND
F6C3	2.11 ± 0.13	2.20 ± 0.38	5.55 ± 0.73	2.6/2.5
F6D	100–50	>200	ND	ND
F6E	2.42 ± 0.09	10.46 ± 0.21	53.78 ± 2.52	22.2/10.5
F6E1	>50	>50	ND	ND
F6E2	50–25	>50	ND	ND
F6E3	1.26 ± 0.17	25–12.5	29.40 ± 6.50	23.3/ND
F6E4	1.84 ± 0.09	25–12.5	21.74 ± 2.95	11.8/ND
F6E5	0.94 ± 0.11	1.06 ± 0.20	14.81 ± 2.55	15.7/14.0
F6E6	1.58 ± 0.02	25–12.5	>100	>63.3/ND
F6E7	25–12.5	50–25	ND	ND

[a]± represents the standard deviation. ND, not determined.

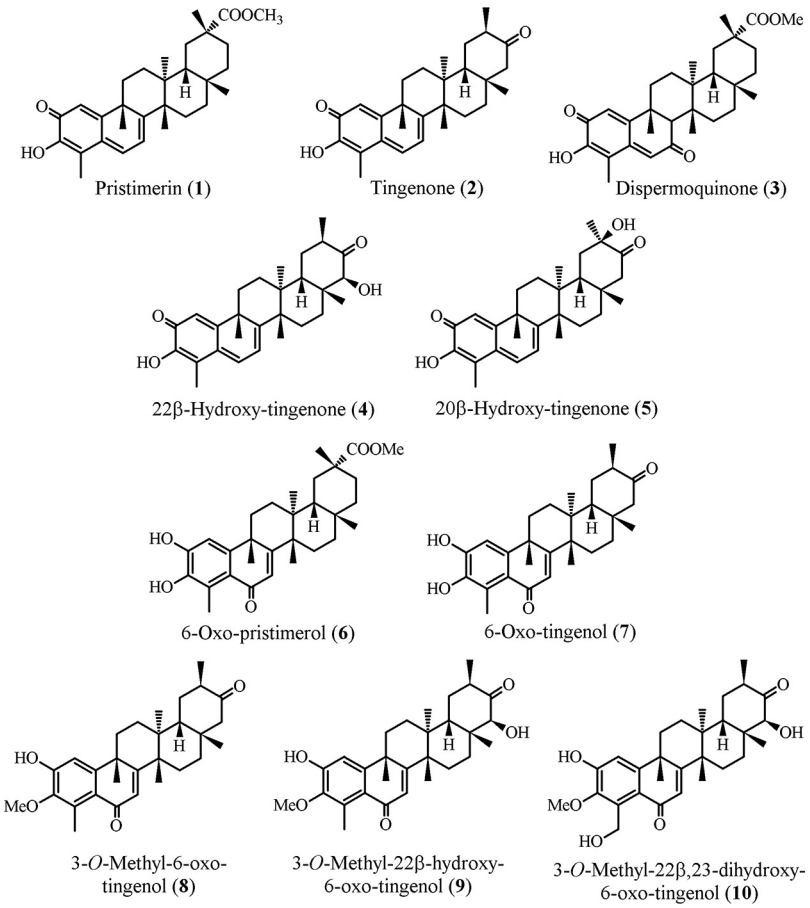

FIG 2 Chemical structures of celastroloids 1 to 10 isolated from *M. chiapensis* roots through bioguided fractionation.

The identified celastroloids 1 to 10 were evaluated against the two parasite species (Table 5). The results over the promastigote stage of *L. amazonensis* indicated that compounds 1, 2, 4, 5, 8, and 9 showed leishmanicidal activity (IC_{50}s ranging from 0.44 to 2.06 μM) higher than the reference drug, miltefosine (IC_{50}, 6.48 μM). In fact, compounds

TABLE 3 Leishmanicidal and trypanocidal activity of F7 subfractions from *Maytenus chiapensis* root bark[a]

Subfraction	*L. amazonensis* IC_{50} (μg/ml)	*T. cruzi* IC_{50} (μg/ml)	Murine macrophages CC_{50} (μg/ml)	Selectivity index calculated for *L. amazonensis*/*T. cruzi*
F7A	>100	>200		ND
F7B	2.34 ± 0.02	100–50	29.25 ± 5.19	12.3/ND
F7C	9.64 ± 0.36	100–50	>100	>10.4/ND
F7C1	25–12.5	>50	ND	ND
F7C2	1.86 ± 0.08	2.70 ± 0.24	10.39 ± 1.03	5.6/3.8
F7C3	4.39 ± 0.73	2.61 ± 0.26	5.53 ± 0.08	1.3/2.1
F7C4	4.12 ± 0.49	3.40 ± 0.45	4.81 ± 0.45	1.2 /1.4
F7C5	4.04 ± 0.69	3.92 ± 0.41	3.62 ± 0.04	0.9/0.9
F7D	2.47 ± 0.46	10.69 ± 3.16	53.72 ± 6.19	21.7/5.0
F7D1	11.91 ± 0.88	>50	>100	>8.4/ND
F7D2	1.56 ± 0.14	25–12.5	21.38 ± 4.69	13.7/ND
F7D3	1.30 ± 0.19	2.31 ± 0.40	5.21 ± 0.30	4.0/2.3
F7D4	3.52 ± 0.22	50–25	>100	>28.4/ND
F7D5	4.08 ± 0.35	50–25	>100	>24.5/ND

[a]± represents the standard deviation. ND, not determined.

TABLE 4 Leishmanicidal and trypanocidal activity of F8 subfractions from *Maytenus chiapensis* root bark[a]

Subfraction	*L. amazonensis* IC_{50} (μg/ml)	*T. cruzi* IC_{50} (μg/ml)	Murine macrophages CC_{50} (μg/ml)	Selectivity index calculated for *L. amazonensis*/*T. cruzi*
F8A	>100	>200	ND	ND
F8B	100–50	>200	ND	ND
F8C	7.69 ± 0.24	100–50	21.76 ± 5.50	2.8/ND
F8C1	15.27 ± 2.84	>50	>100	>6.5/ND
F8C2	>100	>100	ND	ND
F8C3	7.27 ± 0.85	50–25	15.86 ± 0.19	2.2/ND
F8C4	2.73 ± 0.13	25–12.5	31.07 ± 1.36	11.4/ND
F8D	14.51 ± 0.92	7.51 ± 0.78	28.15 ± 4.22	1.9/3.7
F8D1	11.83 ± 2.44	>100	9.98 ± 0.52	0.8/ND
F8D2	4.36 ± 0.83	6.89 ± 0.99	9.39 ± 1.71	2.2/1.4
F8D3	1.71 ± 0.09	4.54 ± 0.69	7.78 ± 0.27	4.5/1.7
F8D4	2.37 ± 0.46	3.65 ± 0.38	8.79 ± 1.72	3.7/2.4
F8D4.1	16.32 ± 1.31	>50	>100	>6.1/ND
F8D4.2	9.10 ± 1.68	1.72 ± 0.19	9.30 ± 0.97	1.0/5.4
F8D4.3	2.52 ± 0.13	25–12.5	>100	>48.1/ND
F8D4.4	2.08 ± 0.08	50–25	41.49 ± 5.46	19.9/ND
F8E	34.75 ± 3.68	7.38 ± 0.91	48.96 ± 5.63	1.4/6.6
F8E1	1.92 ± 0.03	3.08 ± 0.61	9.07 ± 0.95	4.7/2.9
F8E2	25–12.5	>50	ND	ND
F8E3	50–25	>50	ND	ND
F8E4	4.79 ± 0.18	25–12.5	19.58 ± 0.70	4.1/ND
F8E5	25–12.5	50–25	ND	ND

[a]± represents the standard deviation. ND, not determined.

4 and 5 (IC_{50s}, 0.55 and 0.44 μM, respectively) were 11.7- and 14.7-fold more potent than the reference drug. Moreover, compounds 5 and 8 (IC_{50s}, 2.61 and 3.41 μM, respectively) were 2.7- and 2.0-fold more potent than benznidazole (IC_{50}, 6.95 μM) against *T. cruzi* epimastigotes. Thus, the results indicated that *Leishmania* is much more sensitive to the assayed celastroloids than *Trypanosoma*. Regarding the SI on macrophages (Table 5), it is worth highlighting compound 5 on *L. amazonensis* (SI, 55.0 versus 11.1 for miltefosine) and *T. cruzi* (SI, 9.3 versus 57.5 for benznidazole). Furthermore, compounds 4, 5, 8, and 9

TABLE 5 Leishmanicidal, trypanocidal, and cytotoxic activity of compounds 1 to 10 isolated from *Maytenus chiapensis* root bark[c]

Compound or drug	*L. amazonensis* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	Murine macrophages CC_{50} (μM)	Selectivity index[a]
Compound				
1	0.71 ± 0.17	12.51 ± 2.50	5.26 ± 0.88	7.4
2	0.71 ± 0.17	44.42 ± 7.14	5.35 ± 0.02	7.5
3	50–25	>100	ND	ND
4	0.55 ± 0.07	18.48 ± 0.09	13.24 ± 2.21	24.0
5	0.44 ± 0.01	2.61 ± 0.05	24.18 ± 2.91	55.0
6	10.22 ± 2.23	100–50	72.46 ± 15.62	7.1
7	7.30 ± 1.26	50–25	>100	>32.6
8	1.12 ± 0.11	3.41 ± 0.45	20.80 ± 0.92	18.7
9	2.06 ± 0.28	50–25	>100	>104.0
10	25.03 ± 0.42	100–50	>100	>9.3
Drug[d]				
Miltefosine	6.48	ND	72.18	11.1
Benznidazole	ND	6.95	400	57.5[b]

[a]The selectivity index (SI) was calculated for *Leishmania amazonensis* promastigotes unless otherwise noted.
[b]The SI was calculated for *Trypanosoma cruzi* epimastigotes.
[c]± represents the standard deviation. ND, not determined.
[d]Miltefosine and benznidazole were used as positive controls against *L. amazonensis* and *T. cruzi*, respectively.

also showed excellent SIs on murine macrophages (SIs ranging from 18.7 to 104), higher than the control (SI, 11.1). Compound 7 exhibited lightly lower activity than the control on *L. amazonensis* (IC_{50}, 7.30 μM) but higher SI (>32.6). On the other hand, compounds 3, 6, and 10 were less active against *L. amazonensis* ($IC_{50} > 10\ \mu$M) than the reference drug and were inactive on *T. cruzi* ($IC_{50} > 50\ \mu$M) (Table 5).

Quinonemethide triterpenoids (QMTs; compounds 1, 2, 4, and 5) were found to be more potent on *L. amazonensis* promastigotes than the phenolic triterpenoids (compounds 6 to 10), suggesting that the conjugated carbonyl moiety system on A/B rings may undergo a Michael addition-type reaction with cellular target thiols (27) as has been reported for the QMT celastrol (28). Moreover, the expression of cellular thiols was found to be elevated in a number of parasites. In fact, trypanothione, the main low-molecular-weight thiol in trypanosomatids, is a key molecule playing an important role against oxidative and chemical stress (29). Therefore, QMTs could be considered thiol alkylators targeting a metabolic pathway in *Leishmania*, which are either absent or different from the mammalian host. This assumption is reinforced by the observed selectivity (Table 5).

Some species of the Celastraceae family have been investigated in order to identify antiprotozoal metabolites, and celastroloids have been identified as promising antiparasitic agents. Thus, the QMTs, pristimerin, tingenone, and 22β-hydroxy-tingenone (tingenone B), the main celastroloids from the root bark of Celastraceae species, have been reported to possess antiparasitic activity against trophozoites of *Giardia lamblia* (30) and various multidrug-resistant isolates of *Plasmodium falciparum*, although pristimerin was found to be less active than the conventional antimalarial drugs tested (31). In Kenya, species of the genus *Maytenus* are used in traditional medicine to treat malaria. Through activity-guided fractionation of *M. undata*, *M. putterlickioides*, *M. senegalensis*, and *M. heterophylla* extracts, pristimerin was isolated as the compound responsible for *in vitro* antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* strains (IC_{50}s, 3.63 and 3.95 μg/ml, respectively) (32). Similar results were reported for the active dichloromethane extract from the root bark of *Elaeodendron trichotomum*, containing pristimerin and tingenone, against *Entamoeba histolytica*, *G. lamblia*, *Trichomonas vaginalis*, and *T. cruzi* (IC_{50} below 3 μg/ml) (33). Furthermore, the QMTs 28-nor-isoiguesterin-17-carbaldehyde, 17-(methoxycarbonyl)-28-nor-isoiguesterin, 28-hydroxyisoiguesterin, celastrol, pristimerin, and isoiguesterol, isolated from *Salacia kraussii* by a bioassay-guided fractionation, were showed to be active *in vitro* on both chloroquine-sensitive and multidrug-resistant *P. falciparum* strains (34). Trypanocidal activity of tingenone and tingenol was reported on *T. cruzi*, exhibiting a lower activity on trypomastigotes (35), the infectious form of the parasite, than on epimastigotes (36). Tingenone A (20α-hydroxy-tingenone) has also been reported to be active against *Trypanosoma* sp. (37). Bioactivity-directed fractionation of the chloroform extract of the root bark of *M. senegalensis*, widely used in Sudan and other African countries in traditional medicine to treat malaria, led to the isolation of pristimerin, which exhibited promising activity *in vitro* against the chloroquine-resistant strain of *P. falciparum* and lower activity (IC_{50}, 6.8 μg/ml) on *Leishmania major* promastigotes (38).

Tingenin B (22β-hydroxytingenone), isolated from the root bark extract of *Elaeodendron schlechteranum*, showed antiparasitic activity against *T. cruzi* ($IC_{50} < 0.25\ \mu$g/ml), *Trypanosoma brucei* ($IC_{50} < 0.25\ \mu$g/ml), *Leishmania infantum* (IC_{50}, 0.51 μg/ml), and *P. falciparum* (IC_{50}, 0.36 μg/ml); however, it was highly cytotoxic to MRC-5 cells (CC_{50}, 0.45 μg/ml), indicating poor selectivity (39). In addition, tingenone B, isolated from *Maytenus guianensis*, which is used in traditional medicine as an antiparasitic, was found to produce an 80 to 90% inhibition of the promastigote form of *L. amazonensis* (40). Isoiguesterin and 20-epi-isoiguesterinol, isolated from *Salacia madagascariensis*, were an order of magnitude more active against *Leishmania donovani* than amphotericin B and had a comparable selectivity index (41). Tingenone (maytenin) and pristimerin, isolated from *Maytenus ilicifolia* root bark, were effective against *L. amazonensis*, *Leishmania chagasi*, and *T. cruzi*. These two quinonemethide triterpenes exhibited a marked *in vitro* leishmanicidal activity against promastigote and amastigote forms of *L. amazonensis* and *L. chagasi*, with IC_{50} values lower than 0.88 nM, and high

TABLE 6 Activity of selected celastroloids against the *L. amazonensis* amastigotes

Compound or drug	*L. amazonensis* IC_{50} (μM)	Selectivity index[a]
Compound		
20β-hydroxy-tingenone (compound 5)	0.83 $\pm$ 0.23	29.3
6-oxo-tingenol (compound 7)	2.19 $\pm$ 0.69	108.7
3-*O*-methyl-6-oxo-tingenol (compound 8)	1.91 $\pm$ 0.55	10.9
22β-hydroxy-3-*O*-methyl-6-oxo-tingenol (compound 9)	6.26 $\pm$ 1.37	>34.2
Drug		
Miltefosine[b]	3.12 $\pm$ 0.29	23.2

[a]The selectivity index was calculated for *L. amazonensis*.
[b]Miltefosine was used as a positive control.

selectivity for *Leishmania* sp. compared to BALB/c macrophages (42). Recently, Macedo et al. evaluated the antileishmanial activity of the hexanic eluate subfraction from *Maytenus guianensis* bark (HEMg) incorporated in microparticles of poly(lactic-co-glycolic acid) (PLGA), and the results suggested that HEMg was safe for administration *in vivo* (43).

In the present study, pristimerin (celastroloid 1), tingenone (celastroloid 2), and 22β-hydroxy-tingenone (tingenone B) (celastroloid 4) were also identified from active fractions of *M. chiapensis* roots, and although some studies (39, 40, 42) have reported them for their leishmanicidal and trypanocidal activity, we also evaluated them for comparative purposes and to broaden the structure-activity relationship studies. The results of inhibition obtained for celastroloid 1 (IC_{50}s, 0.71 μM for *L. amazonensis* and 12.51 μM for *T. cruzi*), celastroloid 2 (IC_{50}s, 0.71 μM and 44.42 μM, respectively), and celastroloid 4 (IC_{50}s, 0.55 μM and 18.47 μM, respectively) (Table 5) are in accordance with previous works. In addition, to our knowledge, the antiparasitic evaluation of celastroloids 3 and 5 to 10 are reported herein for the first time.

Structure-activity relationship studies. In order to identify the structural requirements of the celastroloids under study for leishmanicidal activity, the influence of the substitution pattern on the *D:A*-friedo-nor-oleanane skeleton was analyzed, revealing the following trends: (i) the quinonemethide triterpenoids (celastroloids 1, 2, 4, and 5), holding a methylene quinoid moiety in A/B rings, were found to be more effective than their congeners, the phenolic triterpenoids (celastroloids 6 to 10), characterized by an aromatic A ring and a ketone α,β-unsaturated B ring (celastroloids 1 and 2 versus 6 and 7). These results are in agreement with previous studies for anticancer (26) and antimicrobial (44,45) profiles, suggesting that the α,β-unsaturated carbonyl system on A/B rings could undergo a Michael addition-type reaction with cellular thiols (27–29); (ii) dispermoquinone (celastroloid 3), although containing the quinonemethide structural feature, showed a drastic decrease in activity (IC_{50}, 25 to 50 μM). A mechanistic explanation for the loss of activity is that the ability of dispermoquinone to give active interactions, for example, as thiol alkylators, is blocked by the reversal of natural occurring electrostatic polarization (46) at C-7 (celastroloids 1, 2, and 4 versus 3); and (iii) regarding the phenolic triterpenoids, it seems that a catechol monomethyl ether is an advantageous substituent over an ortho-catechol group for antikinetoplastid profile (e.g., celastroloids 8 and 9 versus 7). In contrast, hydroxylation of methyl-23 has a detrimental effect on the activity (celastroloids 9 versus 10; IC_{50s}, 2.06 μM and 26.03 μM, respectively), suggesting that the lipophilicity contributes to the activity. These results are in accordance with previous studies on structural requirements of phenolic triterpenoids for antimicrobial activity (47). Therefore, preliminary SAR studies revealed the structural type of the basic skeleton, type of functional group, and overall oxidation level of the triterpenoid skeleton contributing to the antikinetoplastid activity.

Evaluation of selected celastroloids on *Leishmania amazonensis* amastigotes. On the basis of previous works and results reported herein, celastroloids 5, 7, 8, and 9 were selected to be evaluated against the intracellular amastigote form of *L. amazonensis* (Table 6). The activities of the quinonemethide triterpene 5 (IC_{50}, 0.83 μM) and phenolic triterpenoids 7 (IC_{50}, 2.19 μM) and 8 (IC_{50}, 1.91 μM) were higher than that of

miltefosine (IC_{50}, 3.12 μM). Moreover, compounds 5 and 7 showed a higher selectivity index than the reference drug (SIs, 29.3 and 108.7, respectively, versus 23.2 for miltefosine).

Mechanism of induced cell death. Despite some studies having been conducted on the antikinetoplastid potential of celastroloids, only one work by Goijman and coworkers reported that tingenone (celastroloid 2) inhibited *T. cruzi* growth by DNA double-strand intercalation (36), though no studies on their leishmanicidal mechanism of action have been reported. Therefore, encouraged by previous works and the results reported herein, highlighting the antikinetoplastid potential of this type of natural product, the mechanism induced by selected compounds in *Leishmania* was investigated. The effect on the mitochondrial membrane potential when *L. amazonensis* promastigotes were treated with the selected celastroloids, 5, 7, and 8 (Fig. 3A) revealed decreases in mitochondrial membrane potential. However, no change in the ATP levels of *L. amazonensis* parasites after incubation with the IC_{90} of the three compounds were found (Fig. 3B). Furthermore, observed alteration levels of mitochondrial potential in celastroloids-treated parasites were not significantly lower than the control, indicating that the machinery of the mitochondria was still able to continue with its functions in accordance with the maintained ATP levels mentioned above. In addition, similar alteration levels of the mitochondrial potential have been reported in previous studies using the reference drug, miltefosine (48). These results indicate that celastroloids 5, 7, and 8 could induce an apoptotic cell death in *L. amazonensis* mediated by the mitochondria.

Taking into consideration the potency of selected celastroloids on *Leishmania* promastigote and amastigote forms and their selectivity index on murine macrophages, studies in an *in vivo* model will be undertaken in order to reinforce their therapeutic potential against neglected infectious diseases caused by parasites (49). Celastroloids are the main metabolites biosynthesized in the root barks of Celastraceae species, representing a first resource of such bioactive compounds. Moreover, despite the synthesis is not commercially viable because of their complex structures (50), tissue culture *in vitro* is an alternative to solve the shortage of natural resources for the production of these secondary metabolites (51). In particular, adventitious roots culture is an effective technique to produce secondary metabolites that usually accumulated in plant roots, and scale-up culture of *Maytenus* sp. adventitious roots could be a potential alternative source for celastroloids industrial production, as has been reported for celastrol, also a quinonemethide triterpenoid isolated from Celastraceae species (52).

Conclusions. The current study reports our efforts to find new drug candidates for kinetoplastid diseases as alternatives to current treatments. Therefore, a bioassay-guided investigation of the root bark of *Maytenus chiapensis* was developed to discover new

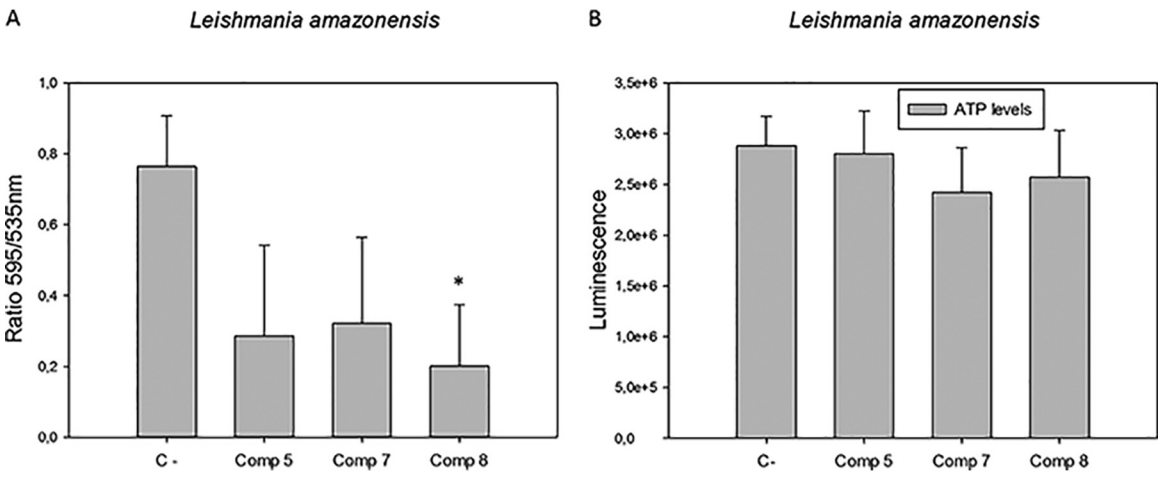

FIG 3 (A) Changes in the mitochondrial membrane potential ($\Delta\Psi$m) of *Leishmania amazonensis* promastigotes after 24 h of incubation with the IC_{90} of the celastroloids. (B) ATP levels in relative luminescence units (RLU) of promastigotes after 24 h of incubation with the IC_{90} of the celastroloids. Error bars represent the standard deviations (SD). C−, negative control.

lead compounds. We have successfully identified two chemical entities, 20β-hydroxy-tingenone (celastroloid 5) and 3-*O*-methyl-6-oxo-tingenol (celastroloid 8), with enhanced potency and selectivity on *L. amazonensis* and *T. cruzi* compared to reference drugs currently in clinical use. Preliminary SAR studies revealed the structural type of the basic skeleton, substitution patterns, and overall oxidation level of triterpenoids contributing to the antikinetoplastid profile. Furthermore, mechanism of induced cell death assays in *L. amazonensis* revealed mitochondrial damage due to a loss of membrane potential without ATP-level alterations. This fact strongly suggests apoptosis as the physiological mechanism of cell death in the parasite. The current study provides new insights into the potential of celastroloids as promising candidates for the development of antikinetoplastid chemotherapy (53, 54). In the future, further studies will be conducted to delve deeper into the mechanism of action and pharmacokinetic profile of this series of metabolites, which are chemotaxonomic markers of the Celastraceae species. Moreover, studies in an *in vivo* model will be undertaken in order to reinforce their therapeutic potential, and the scale-up culture of *Maytenus* sp. adventitious roots could be a potential alternative source for industrial production of these valuable metabolites.

MATERIALS AND METHODS

General procedures. NMR spectra were recorded on a Bruker Avance 500 spectrometer; chemical shifts are given in parts per million and coupling constants in hertz. Solutions were typically prepared in $CDCl_3$ with chemical shifts referenced to deuterated solvent as an internal standard. Sephadex LH-20 used for column chromatography (CC) was obtained from Pharmacia Biotech. Silica gel 60 (particle sizes, 15 to 40 and 63 to 200 μm; Macherey-Nagel) used for CC and silica gel 60 F254 used for analytical and preparative thin-layer chromatography (TLC) were purchased from Panreac (Barcelona, Spain). The developed TLC plates were visualized by UV light and then sprayed with HOAc-H_2SO_4-H_2O (80:16:4), followed by heating at 100°C for 3 min. All the used solvents were purchased from Panreac. Reagents, deuterated solvents, and benznidazole, used as a reference drug for trypanocidal activity, were provided by Sigma-Aldrich (St. Louis, MO, USA), whereas miltefosine, used as a reference drug for leishmanicidal activity, was purchased from Æterna Zentaris (Charleston, SC, USA).

Plant material and extraction procedure. The root bark of *Maytenus chiapensis* Lundell (Celastraceae) was collected at the National Park Montecristo (latitude, 14°23′39″N; longitude, 89°23′10 W; elevation, 1,617 m above sea level [MASL]) in the municipality of Metapán, Santa Ana, El Salvador, in March 2018. The plant was identified by Jenny Elizabeth Menjívar Cruz, curator of the herbarium at the Museo de Historia Natural de El Salvador, and a voucher specimen (J. Menjívar 4255) was deposited in the cited herbarium.

The root bark of *M. chiapensis* was dried at room temperature for 5 days and grounded to small particle size (approximately 3 mm). The dried plant material (650 g) was subjected to exhaustive extraction procedure in a Soxhlet apparatus, using hexanes-Et_2O (1:1, 48 h) as solvent. The organic solvent (4 liters) was removed under vacuum to yield the crude organic extract (27.2 g).

Bioassay-guided fractionation and isolation. The hexanes-Et_2O extract of *M. chiapensis* root bark was assayed against *Leishmania amazonensis* promastigotes and the epimastigote stage of *Trypanosoma cruzi*. Cytotoxicity on the murine macrophages J774A.1 cell line was also assessed, searching for selectivity. After the preliminary screening, the active extract was subjected to a bioassay-guided fractionation procedure. In this way, the extract was fractioned by chromatography on silica gel, eluted with mixtures of increasing polarity of hexanes-CH_2Cl_2 and CH_2Cl_2-EtOAc, affording 66 fractions which were combined on the basis of their thin-layer chromatographic (TLC) profile in eight fractions (F1 to F8) (Fig. 1). Fractions F6, F7, and F8 showed potent antiprotozoal activity against both assayed parasite lines. The active fraction F6 (3.31 g) was chromatographed on a silica gel column (CH_2Cl_2-EtOAc, 10:0 to 7:3) to yield 15 subfractions, which were combined on the basis of their TLC analysis in 5 subfractions (F6A to F6E). Subfraction F6B (429.1 mg) was subjected to CC over Sephadex LH-20 (hexanes-$CHCl_3$-MeOH, 2:1:1) to give six subfractions (F6B1 to F6B6) based on TLC pattern. Subfraction F6B4 (18.8 mg) was further purified by preparative TLC (PTLC) with hexanes-EtOAc (1:1) as eluent to yield celastroloids 2, 4, and 8. Subfraction F6B5 (57.2 mg) was subjected to CC over silica gel (hexanes-EtOAc of increasing polarity, 30 to 70%) and TLC analysis, affording the known compounds, 1, 2, 4, and 8. Subfraction F6C (213.7 mg) was subjected to CC over Sephadex LH-20 (hexanes-$CHCl_3$-MeOH, 2:1:1) to give three subfractions (F6C1 to F6C3) based on TLC analysis. Subfraction F6C3 (15.3 mg) was further purified by PTLC with CH_2Cl_2-Me_2CO (9:1) to yield celastroloids 3, 6, and 8. Subfraction F6E (605.0 mg) was subjected to CC over Sephadex LH-20 (hexanes-$CHCl_3$-MeOH, 2:1:1) to give subfractions F6E1 to F6E7. Subfraction F6E4 (12.3 mg), after a final purification step by PTLC with CH_2Cl_2-Me_2CO (9:1), yielded compound 5. The active fraction F7 (4.62 g) was subjected to CC over Sephadex LH-20 (hexanes-$CHCl_3$-MeOH, 2:1:1) to afford four subfractions (F7A to F7D). Bioactive subfractions were subjected to a final purification step by CC and/or PTLC on silica gel, using mixtures of hexanes-EtOAc (8:2), hexanes-Et_2O (2:8), CH_2Cl_2-AcOEt (8:2), or CH_2Cl_2-Me_2CO (7:3) to afford celastroloids 1, 2, and 9. Celastroloids 1, 2, 6, 7, 8, and 10 were obtained from the active fraction F8 after several chromatographic methods, including CC and PTLC, using mixtures of hexanes-EtOAc (8:2), hexanes-Et_2O (2:8), CH_2Cl_2-AcOEt (8:2), or CH_2Cl_2-Me_2CO (7:3).

Cell lines. *Leishmania amazonensis* (MHOM/BR/77/LTB0016) promastigotes were maintained in Schneider's medium supplemented with 10% fetal bovine serum at 26°C and were grown to the log phase. The parasites were cultured in RPMI 1640 medium, with or without phenol red. Epimastigotes of *Trypanosoma cruzi*, strain Y, was cultured in liver infusion tryptose (LIT) medium and supplemented with 10% fetal bovine serum at 26°C. The murine macrophage J774A.1 (ATCC TIB-67) cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

***In vitro* effect on promastigote form of *Leishmania amazonensis*.** The activity of crude extract, fractions, and pure compounds was determined by the modified alamarBlue reagent (Invitrogen, Life Technologies, Madrid, Spain) assay as previously described (55). This simple and rapid test is based on oxido-reduction reaction. Briefly, the oxidized, blue, nonfluorescent alamarBlue is reduced to a pink fluorescent dye in the medium by cell activity. Samples were dissolved in dimethyl sulfoxide (DMSO), and further dilutions were made in RPMI 1640 medium. The final DMSO concentration never exceeded 0.1% (vol/vol), with no effect on parasite proliferation or morphology. Promastigotes of *L. amazonensis* were grown at 26°C in RPMI 1640 modified medium and supplemented with 10% heat-inactivated fetal bovine serum (FBS). Logarithmic-phase cultures were used for experimental purposes, and the *in vitro* susceptibility assay was performed in sterilized 96-well microtiter plates (Corning). We added 10^6/well parasites and samples at the concentration to be tested to a final volume of 200 μl/well. After incubation for 72 h, analysis of the plates was carried out visually using an inverted microscope. Subsequently, in the case of the pure compounds, the plates were analyzed on an EnSpire multimode plate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Miltefosine was used as a reference drug. Percentage of inhibition, 50% inhibitory concentration (IC_{50}) for active samples, was calculated by linear regression analysis with a 95% confidence limit. All experiments were performed three times each in duplicate, and the mean values were calculated. A nonparametric regression, adjusting data to a four-parameter logistic curve, was used for analysis. Inhibition curve statistical analysis was undertaken using SigmaPlot 12.0 software program (Systat Software Inc.).

***In vitro* effect on *Leishmania amazonensis* amastigote stage.** Activity assay against intracellular amastigotes was performed according to Jain et al. (56). Macrophages (J774A.1 cell line) were placed in a 96-well flat-bottom plate at a density of 2×10^5/ml in RPMI 1640 medium supplemented with 10% FBS and incubated for 1 h at 37°C in a 5% CO_2 environment. Additionally, 100 μl of stationary-phase promastigotes (7-day-old culture) was added in a 10:1 ratio, and plates were reincubated at 37°C overnight to allow a maximum infection. After incubation, free promastigotes were washed off with the culture medium at least 3 times. We added 50 μl of culture medium into each well. Subsequently, a serial dilution of test compounds was made in a 96-deep-well plate with the culture medium, and then 50 μl of this serially diluted standard were added to each well. The plates were incubated at 37°C, 5% CO_2, for 24 h. After incubation, the medium was removed, and 30 μl of Schneider's medium (with 0.05% sodium dodecyl sulfate) was added to each well. Plates were shacked for 30 s, and 170 μl of medium were added to each well. alamarBlue at 10% was added into each well of the 96-well plates and incubated at 26°C for 72 h to allow transformation of rescued amastigotes to promastigotes. After incubation, the emitted fluorescence was measured in a PerkinElmer EnSpire spectrofluorometer at 585 nm.

***In vitro* assay on epimastigote stage of *Trypanosoma cruzi*.** The activity of the extract, fractions, and compounds was tested *in vitro* on the epimastigote stage of *T. cruzi*, and a colorimetric assay based on alamarBlue reagent was performed as previously described (55). Briefly, the extract and all fractions were serially diluted in 100 μl RPMI 1640 medium without phenol red and supplemented with 10% SBF in 96-well plates. After that, parasites in the log phase of growth were counted and diluted (10^5/well) and were added to these wells. Finally, 10% alamarBlue reagent was added to the plates and incubated at 26°C. After 72 h, the plates were analyzed using an EnSpire multimode plate reader by relative fluorescence units (RFU) measurement. Percentages of inhibition and IC_{50} were calculated by four-parameter logistic curve analysis with 95% confidence limit using SigmaPlot 12.0 statistical analysis software. All experiments were performed three times each in duplicate, and the mean values were also calculated. A paired two-tailed *t* test was used for analysis. Values of P of <0.05 were considered significant.

Cytotoxicity assays. Murine macrophages (J774A.1 cell line) cultured in RPMI 1640 medium were counted and seeded in 96-well plates (10^5 cells/ml), and the tested sample was diluted in the culture medium and added in a total volume of 100 μl in each well as previously described (57). As a negative control, cells were incubated with culture medium alone. Finally, 10 μl of alamarBlue was added into each well, and the plates were incubated for 24 h at 37°C and 5% CO_2 atmosphere. The plates were analyzed using EnSpire microplate reader. The cytotoxic concentration (CC_{50}) was calculated using SigmaPlot 12.0 statistical analysis software. The selectivity index was the ratio between the CC_{50} value on murine cells and the IC_{50} value on parasites.

Analysis of mitochondrial membrane potential. The collapse of an electrochemical gradient across the mitochondrial membrane during apoptosis was measured using a JC-1 mitochondrial membrane potential assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the instructions of the kit. Treated promastigotes with IC_{90} concentrations of the tested compound resuspended in JC-1 buffer were incubated with JC-1 reagent (1:10, vol/vol) at 26°C for 15 min. Green and red fluorescence intensity was measured with an EnSpire microplate reader. The EVOS FL cell imaging system (Invitrogen, Thermo Fisher, Carlsbad, CA, USA) was used to observe the cells, using the green fluorescent protein (GFP) and red fluorescent protein (RFP) light cubes.

Analysis of ATP level. ATP level was measured using a CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA), which generates a proportional signal to the ATP amount. Promastigotes were incubated with different concentrations of compounds for 24 h. Aliquots were taken and mixed

with the kit reagent following the manufacturer's instructions for posterior measurement of the luminescence on a PerkinElmer spectrophotometer.

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