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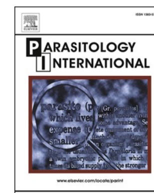

Amentoflavone isolated from *Selaginella sellowii* Hieron induces mitochondrial dysfunction in *Leishmania amazonensis* promastigotes

Yasmin Silva Rizk [a], Daiana de Jesus Hardoim [a], Kaique Bertrand Almeida Santos [a], Tânia Zaverucha-do-Valle [a,*], Noemi Nosomi Taniwaki [b], Fernando Almeida-Souza [a,c], Carlos Alexandre Carollo [d], Marcos André Vannier-Santos [e], Carla Cardozo Pinto de Arruda [f], Kátia da Silva Calabrese [a]

[a] *Laboratório de Imunomodulação e Protozoologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21045-900 Rio de Janeiro, Brazil*
[b] *Núcleo de Microscopia Eletrônica, Instituto Adolfo Lutz, 01246-000 São Paulo, Brazil*
[c] *Pós-graduação em Ciência Animal, Universidade Estadual do Maranhão, 65055-310 São Luís, Maranhão, Brazil*
[d] *Laboratório de Produtos Naturais e Espectrometria de Massas, Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, 79090-900 Campo Grande-MS, Brazil*
[e] *Laboratório de Inovações em Terapias, Ensino e Bioprodutos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21045-900 Rio de Janeiro, Brazil*
[f] *Laboratório de Parasitologia Humana, Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, 79090-900 Campo Grande-MS, Brazil*

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ABSTRACT

Leishmaniasis chemotherapy is a bottleneck in disease treatment. Although available, chemotherapy is limited, toxic, painful, and does not lead to parasite clearance, with parasite resistance also being reported. Therefore, new therapeutic options are being investigated, such as plant-derived anti-parasitic compounds. Amentoflavone is the most common biflavonoid in the *Selaginella* genus, and its antileishmanial activity has already been described on *Leishmania amazonensis* intracellular amastigotes but its direct action on the parasite is controversial. In this work we demonstrate that amentoflavone is active on *L. amazonensis* promastigotes (IC_{50} = 28.5 ± 2.0 μM) and amastigotes. Transmission electron microscopy of amentoflavone-treated promastigotes showed myelin-like figures, autophagosomes as well as enlarged mitochondria. Treated parasites also presented multiple lipid droplets and altered basal body organization. Similarly, intracellular amastigotes presented swollen mitochondria, membrane fragments in the lumen of the flagellar pocket as well as autophagic vacuoles. Flow cytometric analysis after TMRE staining showed that amentoflavone strongly decreased mitochondrial membrane potential. *In silico* analysis shows that amentoflavone physic-chemical, drug-likeness and bioavailability characteristics suggest it might be suitable for oral administration. We concluded that amentoflavone presents a direct effect on *L. amazonensis* parasites, causing mitochondrial dysfunction and parasite killing. Therefore, all results point for the potential of amentoflavone as a promising candidate for conducting advanced studies for the development of drugs against leishmaniasis.

1. Introduction

Leishmaniasis comprise a complex of diseases with a broad clinical spectrum caused by over 20 species of flagellate protozoan parasites from the genus *Leishmania* (Kinetoplastida, Trypanosomatidae), which are spread by sand fly insects [1]. *Leishmania* parasites develop in two main forms: the promastigote form occurs in the vector where it reproduces in the midgut of sand flies and then evolves to a metacyclic form, which is highly infective to the vertebrate host. The amastigote form occurs in the vertebrate, where it is phagocytized by cells, mainly macrophages, subverts its defenses and reproduces inside the parasitophorous vacuole [2–4].

Leishmaniases are neglected diseases widely distributed and considered a serious health problem around the globe with no available

* Corresponding author.
E-mail addresses: yasminrizk@gmail.com (Y.S. Rizk), hardoim@ioc.fiocruz.br (D.J. Hardoim), kaique.bertrand.biomed@gmail.com (K.B.A. Santos), taniazv@ioc.fiocruz.br (T. Zaverucha-do-Valle), ntaniwak@hotmail.com (N.N. Taniwaki), fernandoalsouza@gmail.com (F. Almeida-Souza), carlos.carollo@ufms.br (C.A. Carollo), marcos.vannier@ioc.fiocruz.br (M.A. Vannier-Santos), carla.arruda@ufms.br (C.C.P. Arruda), calabrese@ioc.fiocruz.br (K.S. Calabrese).

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human prophylactic immunization [1]. Among a few therapeutic options, pentavalent antimonials and the liposomal formulation of amphotericin B are the main effective drugs in the clinical routine. Nevertheless, these therapies are limited by the increasing parasite resistance [5] and their toxic, parenteral and long-lasting therapy [6,7]. Since treatment is a challenging problem, novel, cheaper, potent and safer antileishmanial compounds are needed.

Flavonoids are secondary plant metabolites whose antileishmanial activity has been widely studied [8–10]. Among them, the biflavonoid amentoflavone stands out as a polyphenolic apigenin dimer with several other biological activities including anti-inflammatory [11,12], antioxidant [13,14], antitumor [15–17] and antifungal [18]. Recently, amentoflavone has attracted some attention as a possible compound for COVID-19 treatment, as a strong inhibitor of the methyltransferase of SARS-CoV-2 [19].

In a previous study, Rizk et al. [20] have shown a potential activity of amentoflavone on *Leishmania amazonensis* intracellular amastigotes, suggesting a role in parasite killing by the host cell, in a probably nitric oxide independent mechanism. More recently, it was demonstrated that intralesional treatment of mice with amentoflavone was able to reduce the parasite load at the lesion site [21]. In order to clarify if the biflavonoid would also have a direct action on the parasite, we studied the effect of amentoflavone on *L. amazonensis* promastigote forms and investigated the mechanism of action involved in amentoflavone-induced *L. amazonensis* cell death. To the best of our knowledge, this is the first report of amentoflavone-induced *Leishmania* mitochondrial dysfunction. The results suggest the involvement of mitochondria depolarization in parasite killing.

2. Material and methods

2.1. Amentoflavone

The biflavonoid amentoflavone was obtained from the hydro-ethanolic extract of *Selaginella sellowii* Hieron (Selaginellales: Selaginellaceae) described elsewhere, reaching 93% of purity [20]. The compound was diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to obtain a stock solution (1 mg/mL), which was further diluted in culture medium for use, to a maximum concentration of 1%.

2.2. Parasites

L. amazonensis amastigotes (IFLA/BR/1967/PH8) were isolated from BALB/c mice cutaneous lesions and maintained, for no more than eight *in vitro* passages, as promastigote forms at 26 °C in Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO-BRL, Grand Island, NY, USA), 10.000 U/L penicillin and 10 mg/L streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Ethics statemen

Female BALB/c mice (4–6 weeks) were obtained from the Institute of Science and Technology in Biomodels (ICTB, Fiocruz) and kept in an experimental laboratory with controlled room temperature, water and food *ad libitum*. The use of animals was previously evaluated and approved by the Animal Use Ethics Committee of the Oswaldo Cruz Institute (CEUA/IOC), under license No. L-053/2016.

2.4. In vitro antileishmanial activity of amentoflavone against L. amazonensis promastigote forms

L. amazonensis promastigotes in logarithmic growth phase cultured in complete Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) were distributed (1×10^5/well) in sterile 96-well flat bottom culture plates and incubated with amentoflavone (0.8–50.0 μg/mL) or the reference drug, amphotericin B. Amentoflavone and amphotericin B concentrations were obtained after dilution of DMSO-stock solution to the highest analysed concentration and posterior serial dilution 1:2 in medium. Wells with media without parasites, and DMSO-treated parasites were also tested. After 72 h incubation at 26 °C, parasite viability was evaluated by colorimetric method with tetrazolium-dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA). Ten microliters of MTT (5 mg/mL) were added to each well, and the parasites were incubated for 4 h at 26 °C. For the dissolution of formazan crystals, 80 μL of DMSO were added. The optical density (OD) of each well was measured at 570 nm wavelength using a spectrophotometer plate reader (EZ Read 400, Biochrom, Cambridge, UK). The OD were normalized as a percentage of the viability of treated cells as compared to the control and a concentration-response curve was plotted to calculate the half-maximal inhibitory concentration (IC_{50}) using GraphPad Prism 7.0 Software (San Diego, CA, USA) [22]. Each assay was carried out in quintuplicate in three independent experiments.

2.5. Transmission electron microscopy

L. amazonensis promastigotes (2×10^7) in the logarithmic growth phase were incubated in the presence of DMSO at 0.5% or amentoflavone at the calculated IC_{50}. After 72 h-incubation at 26 °C, parasites were washed twice with phosphate-buffered saline (PBS), and then fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, St. Louis, MO, USA) overnight. After centrifugation for 10 min at 2885 ×*g* at 4 °C, cells were washed three times with 0.1 M sodium cacodylate buffer for complete removal of the glutaraldehyde. Parasites were post-fixed in 1% osmium tetroxide solution in 0.1 M sodium cacodylate for 1 h, which was also removed after three washes in the same buffer. Parasites were dehydrated in increasing concentrations of acetone:water solution (30% to 100%) and embedded in EMBed-812® resin (Electron Microscopy Sciences, Hatfield, PA, USA). After ultramicrotomy (Marca Sorvall, model MT2-B), ultrathin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope Jeol 1011 (JEOL, Tokyo, Japan) [23].

For intracellular amastigotes, BALB/c peritoneal macrophages were infected with *L. amazonensis* at a ratio of 10 promastigotes/cell. After 6 h, cultures were washed with PBS and treated with 0.2 μM of amentoflavone, the IC_{50} calculated in a previous work [20]. Seventy-two hours later, cells were washed, fixed, scraped out from the plate and processed as described above.

2.6. Flow cytometric assay

Promastigotes of *L. amazonensis* (1×10^6 parasites/mL, 1 mL) in the logarithmic growth phase were incubated at 26 °C with amentoflavone for 24 h at the calculated IC_{50}. In order to assess the integrity of the cell membrane, parasites were incubated for 15 min at room temperature with 10 μM propidium iodide (BD Pharmingen, San Diego, CA, USA), washed twice and resuspended in PBS. To detect changes in the mitochondrial membrane potential ($\Delta\Psi m$), after treatment with amentoflavone as mentioned above promastigotes were incubated with 50 nM Tetramethylrhodamine Ethyl Ester (TMRE) (Molecular Probes, Carlsbad, CA, USA) for 15 min at room temperature. For both analyses, heat-killed parasites (60 °C bath/10 min.) were used as positive control and DMSO-treated parasites (mock-treated) were used as negative control. All samples were submitted to flow cytometric analysis (BD FACSCalibur™, BD Biosciences, San Jose, CA, USA). A total of 10,000 events per sample were acquired. Data analyses were performed using Summit 4.3 software (Beckman Coulter Inc., Fullerton, CA, USA). Cells were gated using control-viable non-marked cells [24].

2.7. Statistical analysis

The IC_{50} value was obtained from the non-linear regression fit curve of the normalized response corresponding to the viable parasites *versus* log of the concentration treatment. Data were plotted in a graph as mean ± standard deviation. Analysis was carried out using GraphPad Prism 7.0 Software (San Diego, CA, USA).

2.8. In silico analysis of anti-Leishmania drugs

The simplified molecular input line entry system (SMILES) database file of the structures of amentoflavone and antileishmanial reference drugs meglumine antimoniate, amphotericin B, miltefosine and paromomycin were obtained from ChemDraw software (version Ultra 12.0, PerkinElmer Informatics, Waltham, MA, USA). *In silico* prediction for physicochemical, drug-likeness, medicinal chemistry and pharmacokinetics properties were performed with the pkCSM [25] and SwissADME [26] web tools.

3. Results

3.1. Amentoflavone treatment kills L. amazonensis promastigotes

After 72 h of incubation with amentoflavone (0.8–50 μg/mL), the MTT colorimetric assay demonstrated a concentration-dependent activity of amentoflavone on L. *amazonensis* promastigotes (Fig. 1). The calculated IC_{50} after the amentoflavone treatment was 28.5 ± 2.0 μM (15.6 ± 1.1 μg/mL). The reference drug amphotericin B presented an IC_{50} of 0.029 ± 0.005 μM (0.027 ± 0.004 μg/mL), which is lower than some findings in the literature with other strains of *L. amazonensis*, where it can vary from 0,078 to 0,5 μM [27,28].

Plasma membrane integrity of promastigotes was evaluated after incubation with the calculated IC_{50} of amentoflavone. After incubation, PI stained 66.0% of amentoflavone-treated promastigotes, a considerably higher number compared to 3.2% labelled parasites in the negative control, confirming the direct effect of amentoflavone on *L. amazonensis* parasites. Heat-treated parasites, used as PI-staining positive control, displayed a high percentage of stained parasites (96.36%) as expected (Fig. 2).

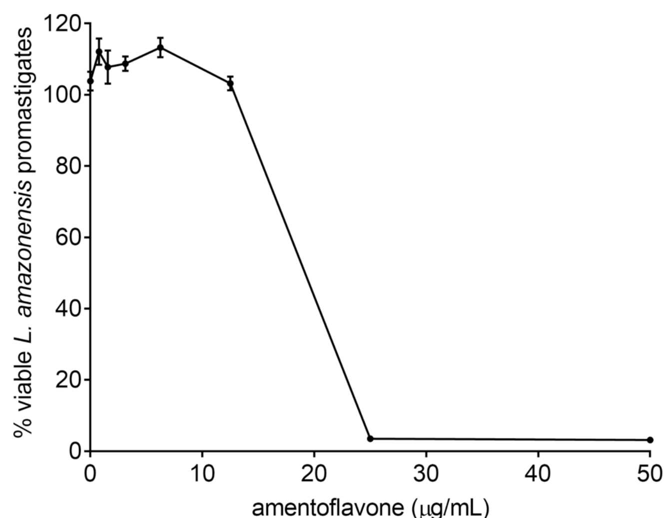


Fig. 1. Amentoflavone presents antileishmanial activity against *L. amazonensis* promastigotes. *L. amazonensis* promastigotes were treated for 72 h with different doses of amentoflavone (0.8–50.0 μg/mL) and the viability was determined at 570 nm using MTT assay. A dose-response curve was used to calculate IC_{50} using a regression analysis (mean ± standard, $n = 5$).

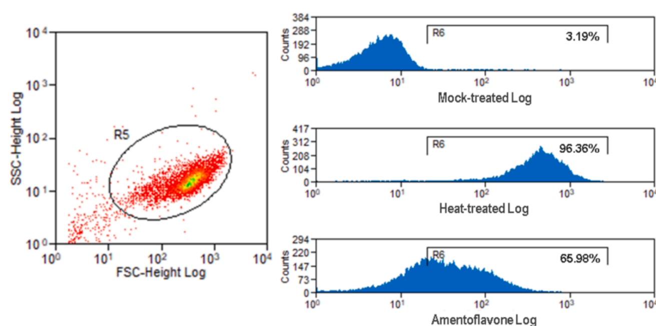


Fig. 2. Amentoflavone treatment perturbs the plasma membrane in *L. amazonensis* promastigotes. Flow cytometry of *L. amazonensis* promastigotes (1 × 10^6 cells) incubated with 28.5 μM of amentoflavone for 24 h at 26 °C. Parasites were stained with Propidium iodide (PI) and analysed in a total of 10,000 events. Percentages correspond to stained parasites in each experimental group. Non-treated promastigotes and heat-killed parasites (60 °C bath/10 min) were used as negative and positive controls, respectively.

3.2. Amentoflavone treatment leads to ultrastructural alterations in L. amazonensis

In order to investigate amentoflavone induction of ultrastructural alterations, treated and non-treated promastigotes were observed by transmission electron microscopy (TEM). Microscopy of non-treated or DMSO-treated promastigotes evidenced the classical ultrastructure of the parasites, with intact organelles. On the other hand, promastigotes treated for 72 h with the IC_{50} of amentoflavone (28.5 μM) showed several alterations, such as myelin-like figures eventually associated with the mitochondrial compartment. Autophagosomes formation by long ER cisternae secluding large portions of cytoplasm and several juxtaposed autophagosomes were observed, and these compartments appear to fuse. Treated parasites presented multiple and large lipid droplets, enlarged mitochondria with elongated cristae and altered basal body organization (Fig. 3).

Similarly, when intracellular amastigotes were analysed by TEM, the same type of alterations were observed. Amentoflavone-treated parasites displayed swollen mitochondria, membrane fragments in the lumen of the flagellar pocket, autophagic vacuoles and myelin-like figures (Fig. 4).

3.3. Amentoflavone decreases mitochondrial membrane potential (ΔΨm) in L. amazonensis promastigote forms

As mitochondria damage was observed in the ultrastructural analysis, flow cytometry analysis was performed to confirm amentoflavone-induced damage to the mitochondria. After incubation with the IC_{50} of amentoflavone (28.5 μM), parasites were labelled with TMRE, a permeable positively charged dye used to evaluate the mitochondrial membrane potential (ΔΨm). Amentoflavone strongly decreased mitochondrial membrane potential of promastigotes. TMRE labelling occurred in only 5.23% of amentoflavone-treated cells, the same pattern of heat-killed parasites, with 5.01% of cells labelled, whereas mock-treated parasites presented 86.38% of cells labelled with TMRE (Fig. 5).

3.4. In silico analysis predicts low solubility to amentoflavone

In silico predictions of physical-chemical, drug-like and medicinal chemical properties were carried out for amentoflavone, as well as for the antileishmanial reference drugs meglumine antimoniate, amphotericin B, miltefosine and paromomycin, through SwissADME tool (Supplementary material, S1–S3).

The analysis of physical-chemical properties showed that the molar refractivity of the compounds had different values, however amentoflavone showed similar values to miltefosine and paromomycin.

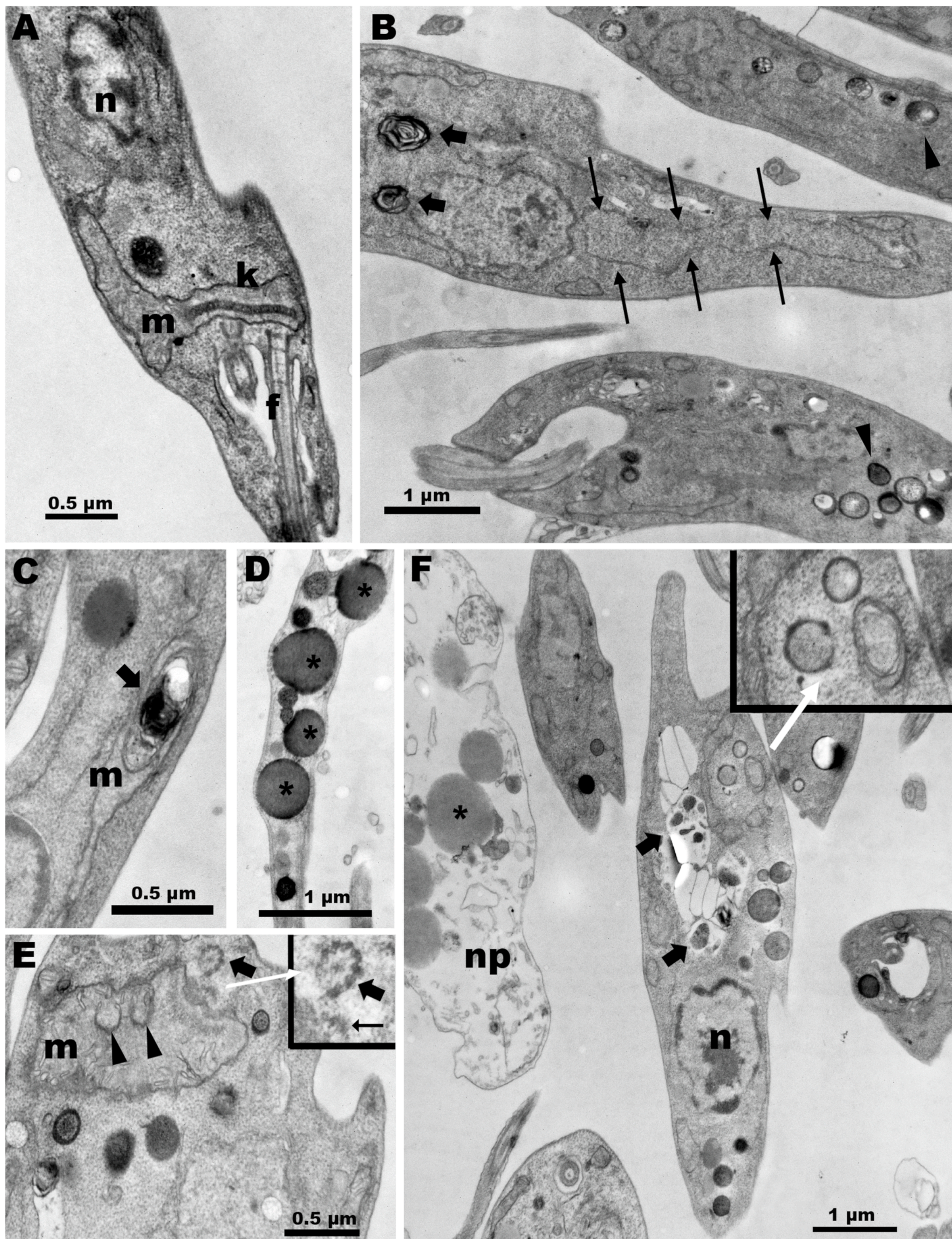


Fig. 3. Amentoflavone treatment leads to ultrastructural alterations in *Leishmania amazonensis* promastigotes. Transmission electron microscopy of *L. amazonensis* promastigote forms treated for 72 h with amentoflavone (28.5 μM). Non-treated (A) or DMSO-treated parasites showed characteristic ultrastructure of kinetoplast (k), mitochondrion (m), nucleus (n) and flagella (f). (B–F) Amentoflavone-treated promastigotes. (B) Parasites displayed numerous *circa* 500 nm myelin-like figures (thick arrows) and long endoplasmic reticulum cisternae circumscribing large cytoplasmic portion (thin arrows) and small autophagosomes (arrowheads). (C) myelin-like figure associated to mitochondrion (arrow). (D) Numerous parasites presented multiple lipid droplets (*). (E) Some promastigotes showed enlarged mitochondria with elongated and circular cristae (arrowheads), as well as altered basal body organization (arrows, inset). (F) Amentoflavone induced the formation of numerous and juxtaposed autophagosomes (thick arrows), compromising a significant portion of the parasite cells. Eventually necrotic parasites (np) were observed. f-flagella, k-kinetoplast, m-mitochondria, n-nucleus, np-necrotic parasites.

Fig. 4. Amentoflavone treatment leads to ultrastructural alterations in *Leishmania amazonensis* intracellular amastigotes. Transmission electron microscopy of BALB/c peritoneal macrophages infected with 10 promastigotes/cell *L. amazonensis* and treated for 72 h with amentoflavone (0.2 μM). Control *L. amazonensis* amastigote (A) showing normal mitochondrion at the kinetoplast region (K). Amentoflavone-treated parasites (B–F) displayed swollen mitochondria (B, m), small membrane profile containing vesicles (C, arrow) as well as membranes fragments in the flagellar pocket lumen (C and inset arrowheads). Treated amastigotes also presents typical doubled-membrane autophagic compartments (D, arrows) as well as large autophagic vacuoles (E, arrows) and myelin-like figures (F, arrowheads) within electron dense compartments (arrows). Flagellar pocket - fp; flagellum – f.

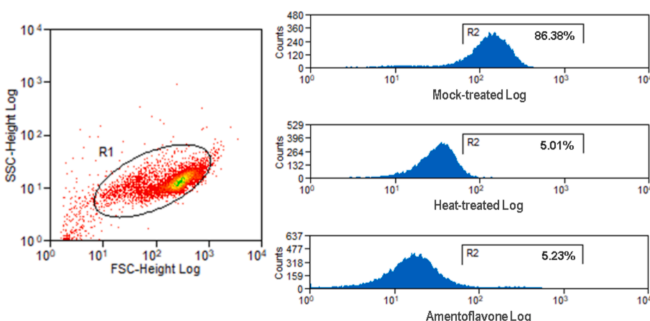


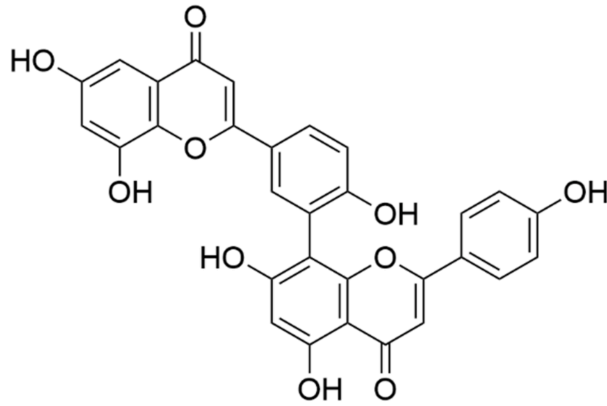
Fig. 5. Amentoflavone treatment decreases the mitochondrial membrane potential in *L. amazonensis* promastigotes. Flow cytometry of *L. amazonensis* promastigotes (1×10^6 cells) incubated with 28.5 μM of amentoflavone for 24 h at 26 °C. Parasites were stained with Tetramethylrhodamine Ethyl Ester (TMRE) and analysed in a total of 10,000 events. Percentages correspond to stained parasites in each experimental group. Non-treated promastigotes and heat-killed parasites (60 °C bath/10 min) were used as negative and positive controls, respectively.

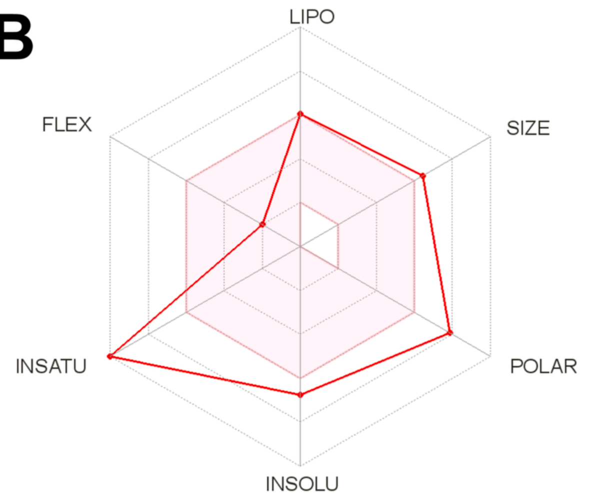
Compared to miltefosine, all compounds had a high topological surface area (TPSA) value and the lipophilicity values of amentoflavone and miltefosine were similar. The drug probability rules of Lipinski, Ghose, Veber, Egan and Muegge [29–34] were applied to the five compounds, where all presented at least one violation, except for miltefosine which had no violation of Lipinski's rule.

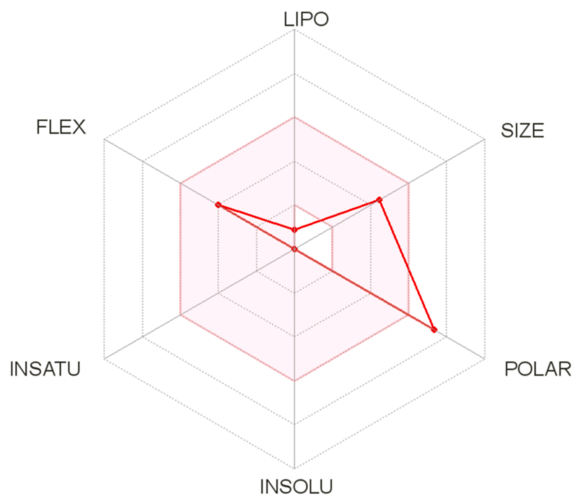
In medicinal chemistry properties, two pattern recognition methods were used to identify fragments that could be problematic: pan test interference compounds (PAINS); and Brenk filters. There was no PAINS alert for any evaluated compound. In Brenk prediction, meglumine antimoniate and miltefosine showed one and two alerts, respectively, while the other compounds showed absence of alerts. In the bioavailability radar of drug-likeness properties, amentoflavone exhibited prediction values inside the ideal range for flexibility and lipophilicity, but was slightly out of range for the size of molecule, and out of range for polarity, solubility and saturation parameters (Fig. 6). The pharmacokinetic properties were also predicted for the compounds amentoflavone, meglumine antimoniate, amphotericin B, miltefosine and paromomycin through the website pkCSM tool (Supplementary material, S4).

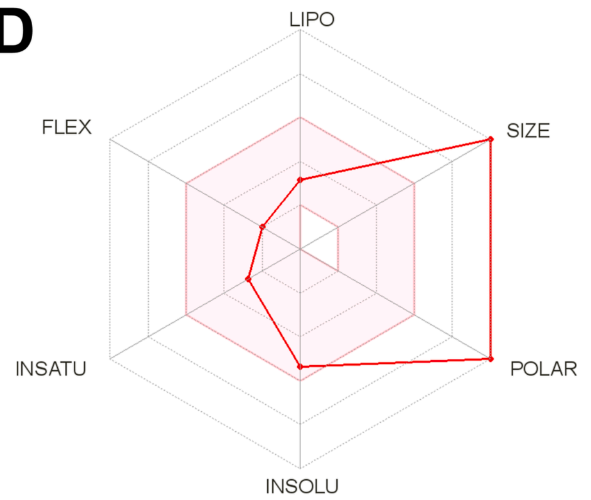
4. Discussion

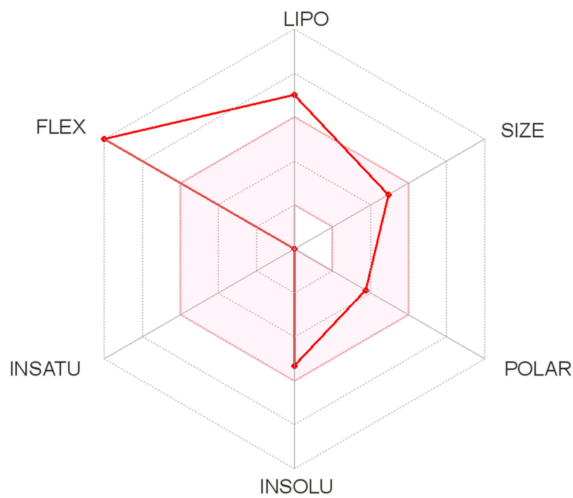
Amentoflavone was previously shown to have no effect on *Leishmania* axenic amastigotes [35–37]. However, previous work from our group had shown that amentoflavone was highly active on *L. amazonensis* intracellular amastigotes after 72 h of incubation ($IC_{50} = 0.2$ μM) [20]. Since this biflavonoid was only active on intracellular amastigote forms we questioned ourselves if amentoflavone had any effect on the parasite itself or if it acted on cell response against intracellular amastigotes. Therefore, we used the *L. amazonensis* promastigotes to clarify whether amentoflavone exerted a direct effect on the parasite. Ultrastructural approach and cytometric analysis were used to elucidate











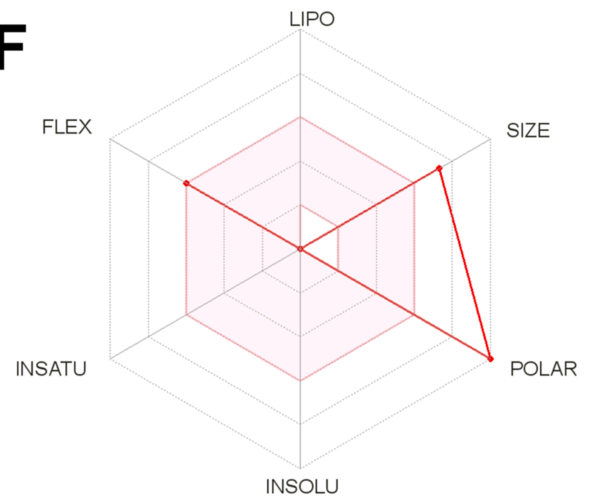


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Fig. 6. *In silico* analysis shows similarities between amentoflavone and miltefosine. Chemical structure of amentoflavone (A) and bioavailability radar of drug-likeness properties of amentoflavone (A) and antileishmanial reference drugs (B–E) obtained by SwissADME output. The pink area portrays the ideal range prediction. LIPO: lipophilicity; POLAR: polarity; INSOLU: solubility; INSATU: saturation; and FLEX: flexibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

its mechanism of action. Ultrastructure of intracellular amastigotes was also analysed to see whether structural changes caused on both forms were similar.

Although Camacho et al. [35] found low activity on *L. donovani* promastigotes (IC_{50} = 101.2 μM), our results are similar to the activity obtained by Njock et al. [38] on *L. infantum* promastigotes (IC_{50} = 19.6 μM). Possibly the compound actions observed are assay- and species-dependent. As discussed by Njock et al. [38], the expression of ABC transporters, proteins known to be involved in drug resistance, is different even among strains.

It is interesting to note that although promastigotes are susceptible to amentoflavone, the compound seems to be more effective to intracellular amastigotes, showing lower IC_{50}. Nevertheless, this higher activity is probably related to a NO-independent mechanism, since no increase in NO production was observed on treated cells [20]. In fact, Banerjee et al. [39] showed that amentoflavone inhibits inducible nitric oxide synthase in A549 human lung adenocarcinoma cell line. Moreover, our results showed that amentoflavone had a direct effect on parasites, even in an environment independent from the host cells. This result was observed by the viability assay based on MTT and by flow cytometry using PI staining. PI is an impermeable dye that crosses the membrane of non-intact cells, binding to cellular DNA, in the cytoplasm of promastigotes. As plasma membrane disruption is well-known as the most characteristic event of necrosis [40], we performed a transmission electron microscopic analysis of amentoflavone-treated parasites searching for ultrastructural alterations that are compatible with necroptic parasites. Indeed, the formation of autophagosomes by long endoplasmic reticulum (ER) cisternae secluding large portions of cytoplasm with some of these compartments appearing to fuse indicated intense autophagy triggering [41] in parasites treated with amentoflavone. In addition, the basal body alterations might be related to cell cycle arrest as trypanosomatid treatment with antiparasitic agents may disorganize this structure [42–44] that comprises a milestone in parasite mitosis [45].

It is also well known that the flavonoid apigenin promotes cell cycle arrest and apoptosis through the p53-related pathway and induction of autophagy in several human cancer cell lines [46]. Amentoflavone is also known to induce autophagy by the modulation of p53, preventing cell aging [47] and its anti-inflammatory effect on microglial cells was related to the cell cycle arrest at G2/M phase, apoptosis and autophagy [48]. In this regard, it is noteworthy that mitochondrial network disorganization is associated with the G2/M phase [49]. Mitochondrial fission in kinetoplastid parasites is triggered prior to cytokinesis, but the fission-fusion regulation remains to be elucidated. It is unknown if a thread-grain transition (ie. accumulation of small roundish mitochondria near the nucleus) may occur, as it is seen in the apoptotic process of pluricellular organisms [50]. Nevertheless, in the *Trypanosoma cruzi* autophagic process, part of the single mitochondrion may be engulfed in mitophagy [43]. Hwang et al. [18] showed that amentoflavone stimulated mitochondrial dysfunction and induced apoptotic cell death in *Candida albicans.* It is also well known that amentoflavone induces apoptosis in cancer cell lines [51–53]. All these literature descriptions highlight the direct effect of amentoflavone to induce molecular mechanisms that lead to cell death.

As we observed mitochondrial damage by TEM in parasites treated with amentoflavone, we carried out TMRE analysis to confirm this finding. We used an earlier time (24 h) so that we could see mitochondrial alteration before parasite death. We did not however include kinetics because previous work has shown no difference in mitochondrial alterations between 2 and 24 h of treatment [23]. In *Leishmania* parasites, the effect of amentoflavone on mitochondria has not yet been described; however, there are studies that show the impairment of this organelle after treatment of promastigote forms with the flavonoid apigenin in concentrations very close to the one tested here. Promastigotes treated with 45 μM of apigenin presented mitotracker dye (a cationic fluorescent dye that labels the mitochondria within live cells) accumulating in the cytoplasm and distorted mitochondria [54,55].

Mitochondria are involved in the regulation of several forms of cell death, such as apoptosis, necrosis and necroptosis. Loss of $\Delta\Psi m$ seen by TMRE have already been associated with necrosis in U266B1 cells treated with abrin, a protein isolated from the seeds of *Abrus. precatorius* [56]. Mitochondrial swelling and loss of $\Delta\Psi m$, which are found in necrotic cells [57], are also occurring in amentoflavone-treated promastigotes, as shown by electron microscopy and absence of TMRE labelling. The depolarization of mitochondria in 95% of cells points to the effect of amentoflavone on this organelle, a recognized target of antileishmanial drugs [58].

The depolarization and decrease of $\Delta\Psi m$ are often associated to increased levels of reactive oxygen species (ROS) in *Leishmania* parasites [24]. Apigenin increased ROS generation in *L. amazonensis* promastigotes followed by mitochondrial dysfunction observed by an extensive swelling in parasite mitochondria, leading to an alteration of the mitochondrial membrane potential [54]. In fact, we also already described the induction of ROS level increase by amentoflavone treatment *in vitro* in macrophages infected with *L. amazonensis* [21]. The involvement of ROS leading to mitochondrial collapse, observed by ultrastructural and $\Delta\Psi m$ alterations, are part of the mechanism of action of amentoflavone parasite death induction, verified here by growth inhibition and PI uptake.

In addition to the biological assays performed to elucidate the *in vitro* antileishmanial activity of amentoflavone, we carried out an *in silico* prediction of physical-chemical properties, drug-likeness, medicinal chemistry and ADME (absorption, distribution, metabolism and excretion) pharmacokinetics properties and compared to the main antileishmanial reference drugs, ie. meglumine antimoniate, amphotericin B, meglumine and paromomycin.

In general, amentoflavone showed physical-chemical, drug-likeness and bioavailability characteristics similar to miltefosine, a drug administered orally, indicating its good absorption by the intestinal tract and its potential to be administered in this *via*. However, some *in vivo* studies point to low bioavailability through this pathway due to high levels of conjugation [59,60]. Despite this, amentoflavone has been used in pharmacological studies on *in vivo* models, and interest in pharmacotechnical improvements that ameliorate its use is growing [61,62], given the potential of the biological properties of this biflavonoid. The *in vitro* antileishmanial activity described here and the *in silico* prediction results highlighted the potential of amentoflavone as a promising candidate for conducting advanced studies for the development of drugs against leishmaniasis. Changes in the molecular structure of amentoflavone could be essayed for enhancing its efficacy against *Leishmania* parasites and resolve issues such as the difficulty in solubilization.

5. Conclusions

Our results have shown that amentoflavone-treated promastigotes had a direct antileishmanial activity against promastigote forms of *L. amazonensis*, inducing plasma membrane disruption and mitochondrial membrane potential alteration. Ultrastructural analysis revealed the presence of lipid droplets, cytosolic vacuolization and mitochondrial alterations. New experiments are needed to investigate the molecular

mechanism of action of amentoflavone, but our data already point to the potential use of amentoflavone or its use as a scaffold for the synthesis of new antileishmanial agents. In addition, *in silico* predictions points out for its low solubility and suggests that it might be suitable for oral use.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102458.

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