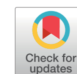
European Journal of Pharmaceutical Sciences 151 (2020) 105407

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Antileishmanial activity of the chalcone derivative LQFM064 associated with reduced fluidity in the parasite membrane as assessed by EPR spectroscopy



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ARTICLE INFO

Keywords:
Leishmania
Macrophages
Erythrocyte
Membrane fluidity
Electron paramagnetic resonance

ABSTRACT

A novel chalcone derivative, LQFM064, demonstrated antileishmanial activity against *Leishmania (L.) amazonensis*, with an IC_{50} value of ~10 μM for the promastigote form. Electron paramagnetic resonance (EPR) spectroscopy of a spin-labeled stearic acid incorporated in the plasma membrane of *L. amazonensis* promastigotes revealed that after 2 h of treatment with LQFM064, the parasite showed remarkable reductions in membrane fluidity. The features of the altered EPR spectra were similar to those reported for the erythrocyte membrane, which was suggested to be due to the cross-linking of oxidized hemoglobin with the cytoskeleton spectrin. In comparison to miltefosine (MIL), LQFM064 demonstrated a much lower hemolytic potential against both erythrocytes in PBS and whole blood, less cytotoxicity in J774.A1 macrophages and equivalent ability to kill parasites internalized in J774.A1 macrophages. Measurements of the IC_{50} values for assays with different cell concentrations enabled the estimation of the membrane-water partition coefficient ($K_{M/W}$), as well as the concentrations of LQFM064 in membrane (c_{m50}) and aqueous phase (c_{w50}) that reduces the cell population by 50%. From the $K_{M/W}$ and c_{m50} values it was deduced that LQFM064 has a greater affinity than MIL for the parasite membrane, but the antiproliferative activity of both substances is exerted at a similar concentration in the plasma membrane.

1. Introduction

There are more than 20 *Leishmania* species cause disease in humans, which are collectively termed the leishmaniases. These vector-borne neglected tropical diseases can manifest as either visceral leishmaniasis (the most serious form of the disease), cutaneous leishmaniasis (the most common) and mucocutaneous leishmaniasis [1]. According to the World Health Organization (WHO) [2], the disease mainly affects poor people in Africa, Asia and Latin America, with 97 countries and territories considered endemic for leishmaniasis in 2018. With 1.5 to 2 million new cases each year worldwide, leishmaniasis also causes 70,000 deaths annually [3]. In Brazil, *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*, are considered the most epidemiologically relevant species [4]. *L. amazonensis* typically causes cutaneous disease but can sometimes manifest as diffuse cutaneous leishmaniasis, a rare form of the disease in which parasites undergo uncontrolled growth leading to non-ulcerative lesions to form across the skin [4], or as disseminated cutaneous leishmaniasis, which presents as extensive, numerous nodular or ulcerated skin lesions [5]. The *Leishmania* parasite has two main developmental forms, the

Abbreviations: CC_{50}, half-maximal cytotoxic concentration; c_{m50}, compound concentration in membrane that reduces population by 50%; c_{w50}, compound concentration in aqueous phase that reduces population by 50%; EPR, electron paramagnetic resonance; FCS, fetal calf serum; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; HC_{50}, concentration for 50% hemolysis; IC_{50}, half-maximal inhibitory concentration; $K_{M/W}$, membrane-water partition coefficient; LQFM064, (*E*)-3-(3,5-di-*ter*-butyl-4-hydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one; BHT, butylated hydroxytoluene; MFI, mean fluorescence intensity; MIL, miltefosine; MTT, methylthiazolyldiphenyl-tetrazolium bromide; RBC, red blood cell; 5-DSA, 5-doxyl-stearic acid; SI, selectivity index

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https://doi.org/10.1016/j.ejps.2020.105407
Received 9 April 2020; Received in revised form 18 May 2020; Accepted 30 May 2020
Available online 03 June 2020
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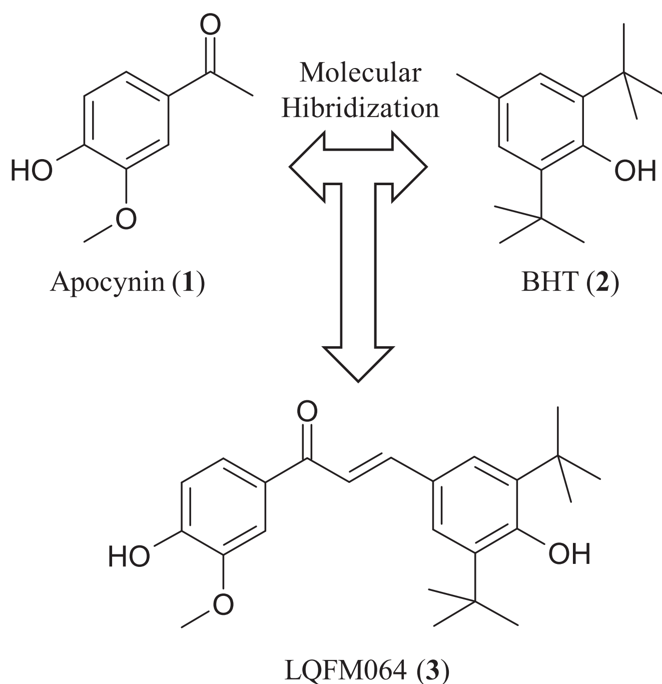

Fig. 1. Molecular structure of the compound LQFM064 (**3**) and the structural design of the molecular hybridization of apocynin (1) and butylated hydroxytoluene (BHT) (**2**) using a chalcone scaffold.

promastigote, which is found in the vector, and the amastigote, which is found inside immune cells of the human host. Leishmaniasis is currently treated with pentavalent antimonials, amphotericin B, miltefosine (MIL) and paromomycin [1, 6]. However, the emergence of drug resistance, toxicity of the available drugs, and lack of new antileishmanial agents highlight the need to search for new compounds with antileishmanial activities [6].

The novel chalcone-like compound (*E*)-3-(3,5-di-*ter*-butyl-4-hydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one, LQFM064 (**3**), was synthesized from molecular hybridization of apocynin (1) and butylated hydroxytoluene (BHT) (**2**) using a chalcone scaffold (Fig. 1). LQFM064 demonstrated activity against *L. amazonensis* promastigotes with IC_{50} values comparable to that reported for MIL [[7–9]. Thus, in this study, a more comprehensive assessment of LQGM064 was performed through *in vitro* studies to determine not only its cytotoxic effect against the parasite but also any potential affect on host cells. We evaluated its effect on two different host cells, J774.A1 macrophages and red blood cells (RBCs). In addition, the ability of the compound to kill the parasite internalized in the J774.A1 macrophage was ascertained. In addition, the mechanisms of action of the compound were investigated using spin-label electron paramagnetic resonance (EPR) spectroscopy, which probes membrane dynamics. Through this method it was determined that LQFM064 causes notable reductions in the plasma membrane fluidity of *L. amazonensis* parasites and that these were the result of oxidative stresses induced by the compound leading to the peroxidation of membrane proteins.

2. Materials and methods

2.1. Chemicals

Spin label 5-doxyl-stearic acid (5-DSA), methylthiazolyldiphenyltetrazolium bromide (MTT), Grace's insect medium, RPMI 1640 medium, L-glutamine, penicillin G, streptomycin, sodium bicarbonate ($NaHCO_3$), dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), hygromycin B and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was purchased from Corning Life Sciences, Corning, NY, USA). All other reagents were of analytical grade.

2.2. Compound

The compound LQFM064 (**3**) (MW = 382.5 g/mol) (Fig. 1) was synthesized according to Cabral et al. (2017) [10]. A 100 mM solution of the compound in DMSO has a reddish color.

2.3. Cells

Promastigotes of the *Leishmania (L.) amazonensis* (MHOM/BR/75/Josefa) reference strain were cultivated at 26°C in 24-well plates containing 2 mL Grace's insect medium supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin as previously described [8, 9]. Experimental tests were initiated when the parasites reached the stationary phase of growth (6th day of growth). Better standardization of the EPR spectroscopy and antiproliferative activity experiments was achieved using assays with different cell concentrations of parasites in this growth stage.

The J774.A1 murine macrophage cell line was acquired from the cell bank of Rio de Janeiro (NCE/UFRJ). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 11 mM sodium bicarbonate and 100 µg/mL streptomycin at 36.5°C in a humidified atmosphere (RH ~95%) and with a mixture of air/CO_2 (95/5%).

2.4. *In vitro* assays of antiproliferative activity and cytotoxicity

Promastigotes at several cell concentrations (5×10^6, 2×10^7, 1×10^8 and 1×10^9 cells/mL) were treated with increasing concentrations of LQFM064 in 96-well plates containing Grace's insect medium supplemented with 10% FCS. After incubation for 24 h at 26°C, the cell viability was assessed by measuring the reduction of MTT to formazan by mitochondrial reductases. The percentage of viable cells relative to the control was determined for each concentration of LQFM064, and the IC_{50} was obtained by adjusting the concentration response data to a sigmoid curve.

J774.A1 macrophages at 1×10^6 cells/mL treated with different concentrations of LQFM064 (0, 25, 50, 100, 200, 400, 800 and 1600 µM) were incubated in 24-well plates containing RPMI 1640 medium supplemented with 10% FCS. After 24 h at 36.5°C and 5% CO_2, the cell viability was assessed using the MTT method and the half-maximal cytotoxic concentration (CC_{50}) value was determined as described above.

2.5. J774.A1 macrophage infection with GFP-labeled parasites

Green fluorescent protein (GFP)-labeled *L. amazonensis* promastigotes (IFLA/BR/67/PH8) were cultivated in Grace's insect medium supplemented with 10% FCS and selected by using 30 µg/mL hygromycin B [11]. J774.A1 macrophages, at 4×10^6 cells/mL, were infected for 24 h with *L. amazonensis*-GFP parasites at a ratio of 5 parasites to 1 macrophage. After infection, the cells were washed with 1x PBS to remove non-internalized parasites, and were then cultured for a further 24 h in the presence of LQFM064 at 0, 7.5, 15, 30, 60, 120 and 240 µM.

The cells were then collected on ice by mechanical harvesting of the adherent macrophages using a cell scraper, washed twice with PBS, incubated with 1% paraformaldehyde and analyzed by flow cytometry on a C6 Flow Cytometry instrument (BD Bioscience, San Jose, CA, USA). Macrophages were selected by forward *versus* side scatter (FSC *vs* SSC). The data were analyzed using FCS Express software (De Novo Software™, Glendale, CA, USA). The percentage of cells expressing GFP (% infected cells) and mean fluorescence intensity (MFI, estimated amount of internalized parasites) were evaluated.

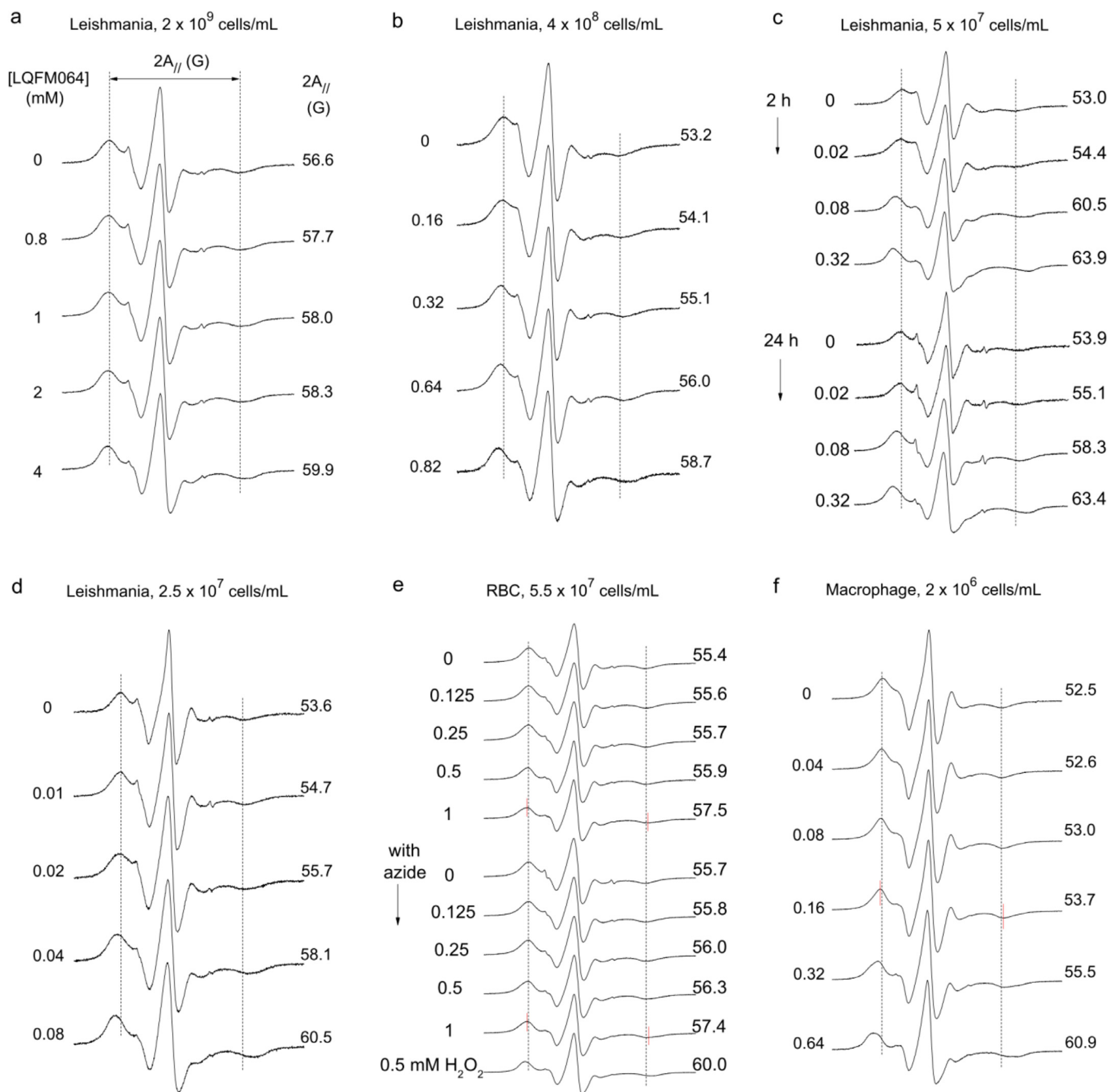

Fig. 2. EPR spectra of 5-DSA incorporated in the plasma membrane of *Leishmania amazonensis* promastigotes (a-d), RBCs (e) and J774.A1 macrophages (f) after 2 h of treatment with different concentrations of LQFM064 (**3**). The cell concentrations used are indicated on each panel. The LQFM064 concentration is given to the left of each spectrum, and the values of the EPR parameter $2A_{//}$ (outer hyperfine splitting), which is the magnetic field separation between the first peak and the last inverted peak of the spectrum, are indicated to the right of each spectrum. An experimental error of 0.5 G was estimated for the parameter $2A_{//}$. Panel (c) shows EPR spectra for treatments of 2 and 24 h. Panel (e) shows EPR spectra for treatment of RBCs in PBS and PBS with azide. The total scan range of the magnetic field in each EPR spectrum was of 100 G (X axis) and the intensity (Y axis) is in arbitrary units.

2.6. EPR spectroscopy

L. amazonensis promastigotes at several cell concentrations (2.5×10^7, 5×10^7, 4×10^8 and 2×10^9 parasites/mL) in PBS were incubated for 2 h at 26°C in the absence (control) and presence of different concentrations of LQFM064 (10 μM to 4 mM). In some EPR experiments, PBS containing sodium azide, a catalase inhibitor, was used. After incubation, the samples were centrifuged at 1800xg for 10 min to increase the cell concentration to 1×10^8 parasites in each 50 μL sample. Alternatively, promastigotes at a concentration of 5×10^7 parasites/mL were treated with different concentrations of LQFM064 in Grace's insect medium supplemented with 10% FCS and incubated for 24 h. After this incubation period, the samples were washed twice with PBS to remove the medium and avoid unwanted interactions with the spin label. After LQFM064 treatment, each sample was spin-labeled with the 5-DSA. To incorporate the spin label into the parasite membranes, firstly a film of spin label was prepared on the bottom of a glass tube using a 1 μL aliquot of a 5-DSA ethanolic solution (2 mg/mL) and, after evaporation of the solvent, each 50 μL cell sample was added on the spin label film and gently stirred. A similar procedure was used for

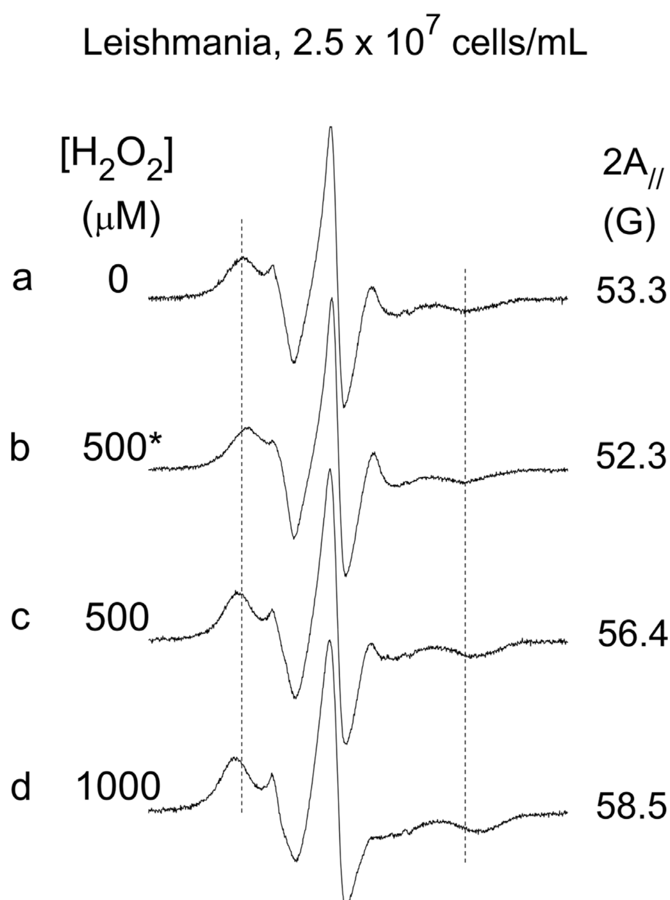

Fig. 3. (a) EPR spectra of 5-DSA incorporated in the plasma membrane of *Leishmania amazonensis* promastigotes after 24 h in Grace medium with 10% FCS and 3 mM azide. (b) Without azide and with 500 μM H_2O_2. (c) With azide and 500 μM H_2O_2. (c) With azide and 1000 μM H_2O_2. The values of parameter $2A_{//}$ are indicated for each spectrum.

the treatment and spin labeling of RBCs and J774.A1 macrophages. For the EPR measurements, each sample was transferred to a 1-mm-i.d. capillary tube, which was sealed using a flame.

The EPR spectra were recorded using an EPR EMX-Plus spectrometer of Bruker (Rheinstetten, Germany) operating with the following instrumental settings: microwave power, 2 mW; microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; magnetic field scan, 100 G; sweep time, 168 s; and sample temperature, 25 ± 1°C.

2.7. Hemolytic potential of RBCs in PBS and whole blood

Hemolysis assays of RBCs in PBS and whole blood were carried out according to previously described methods [12, 13]. Blood donated by laboratory researchers was collected into EDTA-coated blood tubes under vacuum. After diluted 3x in PBS, the blood was centrifuged at 800xg for 10 min at 4ºC. The supernatant as well as the white blood cells were removed and the pellet was resuspended in PBS; this washing procedure was repeated three times. Assuming a volume of 90 fL for the erythrocyte, the cell concentration in the suspension was adjusted to 5.5×10^7 cells/mL (0.5% hematocrit). LQFM064 initially solubilized in DMSO was diluted in 1.2 mL PBS before adding the RBCs. After incubation of 2 h at 36.5 ± 0.5°C, the samples were centrifuged and the percentage of hemolysis was determined based on the absorbance of the hemoglobin in the supernatant at 540 nm.

To measure the percentage of hemolysis in whole blood, the plasma was first separated from the blood cells by centrifugation at 1400xg for 10 min at 4ºC, and then LQFM064 was diluted in 58 μL of plasma. To reconstitute the total blood, 42 μL of the separated blood cells was added to each sample. The samples were incubated for 24 h at 7 ± 1°C. During this period, the samples were gently stirred several times. After incubation, 1.4 ml PBS was added to each sample and after centrifugation the percentages of hemolysis were determined based on the hemoglobin concentration in the supernatant as described above. The supernatant from the untreated sample was used as a reference for the spectrophotometric reading.

2.8. Statistical analysis

Data expressed as mean and standard deviation were obtained from at least three independent experiments. The means were compared through a one-way analysis of variance (ANOVA). Tukey's test was used to identify significant differences ($P < 0.05$) between means among the different treatments.

3. Results

3.1. LQFM064 reduces the fluidity of the parasite plasma membrane

Fig. 2 shows EPR spectra of the spin label 5-DSA in the plasma membrane of *L. amazonensis* promastigotes, RBCs and J774.A1 macrophages, either untreated or treated with LQFM064.

Large increases in the EPR spectral parameter $2A_{//}$ of 5-DSA were observed for cells treated with LQFM064, indicating remarkable reductions in membrane fluidity. For *L. amazonensis* (Fig. 2a–d) it is shown that with the reduction in parasite concentration used, there is a reduction in the required compound concentration to cause a change in $2A_{//}$ (> 0.5 G, the estimated experimental error). At a concentration of 2×10^9 parasites/mL in PBS the lowest concentration of LQFM064 to cause a change in the spectral parameter was approximately 800 μM; whilst for cell concentrations of 4×10^8, 5×10^7 and 2.5×10^7 parasites/mL, the minimum LQFM064 concentrations were much lower at ~160, ~20 and ~10 μM, respectively. Experiments performed with *L. amazonensis* in culture medium showed similar results for incubation periods of 2 and 24 h at 26°C (Fig. 2c).

While the minimum concentration required for a change in the EPR spectrum was only ~20 μM in *L. amazonensis* at a concentration of 5×10^7 parasites/mL (Fig. 2c), for the erythrocyte at 5.5×10^7 cells/mL it was ~1 mM (Fig. 2e). This indicates that LQFM064 has a good selectivity index for the parasite in relation to the erythrocyte. When the RBCs were treated in PBS containing 1 mM azide, a catalase inhibitor, the effects of LQFM064 did not change compared to treatment without the presence of azide. However, the addition of 500 μM hydrogen peroxide to untreated RBCs (containing azide) led to pronounced membrane rigidity. This effect was not observed without the catalase inhibitor for RBCs [14], and in the case of leishmania the presence of azide in the culture medium did not alter the effects of LQFM064 (**3**). Fig. 3 shows EPR spectra for *L. amazonensis* promastigotes incubated for 24 h in culture medium containing 3 mM sodium azide and treatment with hydrogen peroxide. For the sample in which 500 μM H_2O_2 was added to the medium, there was an increase in $2A_{//}$ of 3.1 G and for the sample with 1000 μM H_2O_2 the increase was 5.2 G. In one of the samples without the addition of azide and with 500 μM H_2O_2, only a small reduction in $2A_{//}$ was observed. In J774.A1 macrophages at a concentration of 2×10^6 cells/mL, the LQFM064 concentration required for a significant change in the spectrum was ~160 μM (Fig. 2f), which is much higher than that observed for promastigotes at low cell concentrations (10-20 μM).

3.2. The antiproliferative activity of LQFM064 was dependent on cell concentration

The IC_{50} values of LQFM064 for *L. amazonensis* promastigotes,

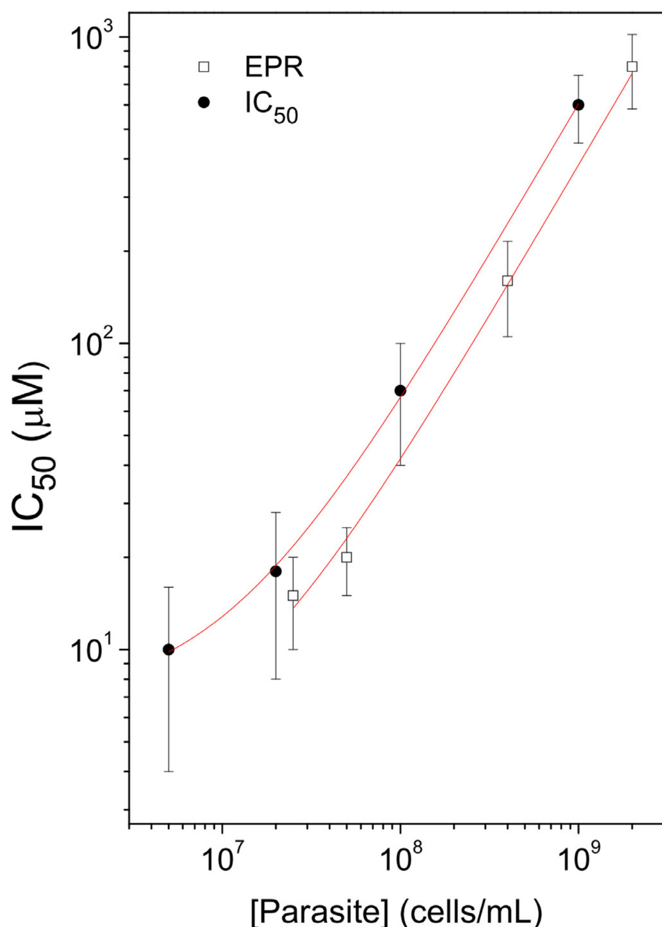

Fig. 4. IC_{50} values of LQFM064 (**3**) in *Leishmania amazonensis* promastigotes for different initial cell concentrations. IC_{50} values (solid circles) were determined using the MTT assay with different concentrations of parasites treated for 24 h with LQFM064. Based on the EPR spectra shown in Fig. 2, the minimum concentration of the compound that could cause a detectable change by EPR for each cell concentration was determined (open squares). The best-fit curves shown are based on Eq. 1.

determined using the MTT assay, were dependent on the concentration of cells used. Fig. 4 shows that with the increase in cell concentration from 5×10^6 to 1×10^9 parasites/mL (200x), the IC_{50} values of LQFM064 increased from ~10 to ~600 µM (60x). Hydrophobic molecules such as LQFM064 accumulate in the cell membrane, and thus the IC_{50} values are influenced by the amount of cell membrane present in the suspension. In previous works [8, 9], the equation that describes the variation of the IC_{50} with the cell concentration was determined to be as follows:

$$IC_{50} = \left[\frac{(V_{mc}\ .\ c_c)^{-1} + K_{M/W}}{(V_{mc}\ .\ c_c)^{-1} + 1}\right] c_{w50}. \quad (1)$$

V_{mc} is the estimated cell membrane volume for the *L. amazonensis* promastigote (8.17×10^{-13} mL) [8] and c_c is the number of cells per mL. Since the biophysical parameters $K_{M/W}$ and c_{w50} are covariant in Eq. (1), they can be determined by fitting the curve provided by this equation to experimental data of IC_{50} versus c_c. For very dilute cell samples, the plasma membrane content is negligible and the IC_{50} values tend towards the c_{w50} value; in Eq. (1), assuming that $(V_{mc}\ .\ c_c)^{-1} >> K_{M/W}$, the IC_{50} is equal to $\sim c_{w50}$. However, for high cell concentrations, a considerable fraction of the test compound goes to the membrane and its concentration in the aqueous phase is notably lower than in the suspension ($IC_{50} > c_{w50}$). As $K_{M/W} = c_{w50}/c_{m50}$, the c_{m50} value can also be determined.

Table 1
Biophysical parameters associated with interactions of **LQFM064 (3)** with plasma membranes of *L. amazonensis* promastigotes.

Compound	$K_{M/W}$ (10^4)[a]	log $K_{M/W}$	c_{w50} (µM)	c_{m50} (M)
LQFM064	10.2 ± 0.9 (A)[b]	5.01	6.8 ± 1.6 (A)	0.69 ± 0.19 (A)
Miltefosine[c]	6.8 ± 0.3 (B)	4.83	10.8 ± 3.0 (B)	0.73 ± 0.21 (A)

[a] Best-fit parameters obtained by fitting of Eq. 1 to the experimental data from Fig. 4; $K_{M/W}$, membrane-water partition coefficient; c_{w50} and c_{m50}, molecular concentrations in the aqueous phase and membrane, respectively, to reduce the cell population by half.
[b] Statistical significance: in each column, the data indicated with same capital letter are not significantly different at $P < 0.05$.
[c] Data from previous work with miltefosine [8] are shown for comparison.

As can be seen in Fig. 4, the membrane changes detected by EPR spectroscopy were observed at concentrations of LQFM064 apparently lower than their IC_{50} values, although the assays were not performed in parallel. The EPR data represents the lowest concentrations of the compound that can cause a membrane change detectable by EPR. The experiments were carried out with incubations of only 2 h at 26°C in PBS, however, measurements after 24 h of incubation in Grace's insect medium supplemented with 10% FCS showed equivalent results (Fig. 2).

3.3. Biophysical parameters $K_{M/W}$, c_{m50} and c_{w50}

Table 1 shows the biophysical parameters $K_{M/W}$, c_{m50} and c_{w50} obtained from the fit curves shown in Fig. 4, in comparison with MIL data reported in a previous work [8]. LQFM064 showed a greater affinity than MIL for the membrane of *L. amazonensis*, as deduced from the lower values of $K_{M/W}$, however, a membrane concentration equal to that of MIL was necessary to inhibit cell growth by 50%, as indicated by the c_{m50} values (Table 1).

3.4. Hemolytic potential and cytotoxicity in J774.A1 macrophages

The hemolytic activity of LQFM064 was assessed in whole blood and isolated erythrocytes in PBS. To assess the hemolytic potential in whole blood, the compound was first diluted in plasma separated from the blood cells then the cells were added back for blood reconstitution. LQFM064 showed very low hemolytic activity against isolated RBCs in PBS and in whole blood (Table 2). For MIL, the hemolytic potential in whole blood was much lower than against the erythrocytes in PBS [12], a result that can be explained by the fact that MIL binds to blood plasma

Table 2
Hemolytic activity against red blood cells (RBCs) in whole blood and in PBS (5.5×10^7 cells/mL), and cytotoxicity in J774.A1 macrophage (1×10^6 cell/mL), of the compound LQFM064 (3) compared to miltefosine (MIL).

[Compound] (mM)	Whole blood – %Hemolysis MIL	LQFM064
5	100[a]	1.0 ± 0.5
10	100	1.8 ± 0.8
	RBCs in PBS – %Hemolysis	
0.13	100[b]	1.1 ± 0.9
0.25	100	1.5 ± 0.6
0.50	100	5.3 ± 1.4
1.00	100	17.9 ± 2.5
	Macrophage – CC_{50} (µM)	
	64 ± 12[c]	179 ± 28

[a] Whole blood hemolysis data for MIL are from previous work, where 50% hemolysis was observed for 2.1 mM MIL [13].
[b] For the concentration of erythrocytes in PBS (0.5% hematocrit), total hemolysis was reported for MIL concentrations greater than 100 µM [12].
[c] Data from reference [9].

albumin [13]. It should be mentioned that when LQFM064 was diluted in plasma at a high concentration, the plasma acquired a whitish appearance forming an apparently homogeneous suspension, which was not the case for MIL. Even at a concentration of 10 mM LQFM064 and incubation for a period of 24 h, only ~1.8% hemolysis occurred in the whole blood. While for the RBCs in PBS, 1 mM of LQFM064 caused less than 20% hemolysis, which is in agreement with the EPR data that showed a significant change in the erythrocyte membrane only at compound concentrations close to 1 mM (Fig. 2e). These results suggest that the studied compound has greater affinity for the parasite plasma membrane than for that of erythrocyte.

In the case of J774.A1 macrophage, the LQFM064 also displayed a lower interaction capacity, with a CC_{50} value almost three times greater than that of MIL (Table 2). This indicates a good selectivity index (SI) of LQFM064 for *L. amazonensis* promastigotes in relation to J774.A1 macrophages (SI = CC_{50}/IC_{50}).

3.5. Action of LQFM064 against intracellular L. amazonensis amastigotes

Finally, LQFM064 was investigated as to whether it was capable of exerting a leishmanicidal effect against the *L. amazonensis* parasite whilst internalized by the host cell. For this, J774.A1 macrophages were infected with *L. amazonensis* expressing green fluorescent protein (GFP), then treated with different concentrations of LQFM064 for 24 h. Macrophages were then analyzed, in terms of the rates of infection, by flow cytometry. Both the percentage of infected cells, cells displaying GFP, and the estimated amount of internalized parasites, by mean fluorescence intensity, decreased significantly with LQFM064 treatment from the concentration of 15 μM onwards compared to the untreated control. This was the same for MIL. However, there was essentially no significant difference between LQFM064 and MIL treatment (Fig. 5). The data demonstrate that LQFM064 and MIL are capable of reducing *L. amazonensis* infection in murine macrophages and that LQFM064 has a leishmanicidal action similar to that of MIL.

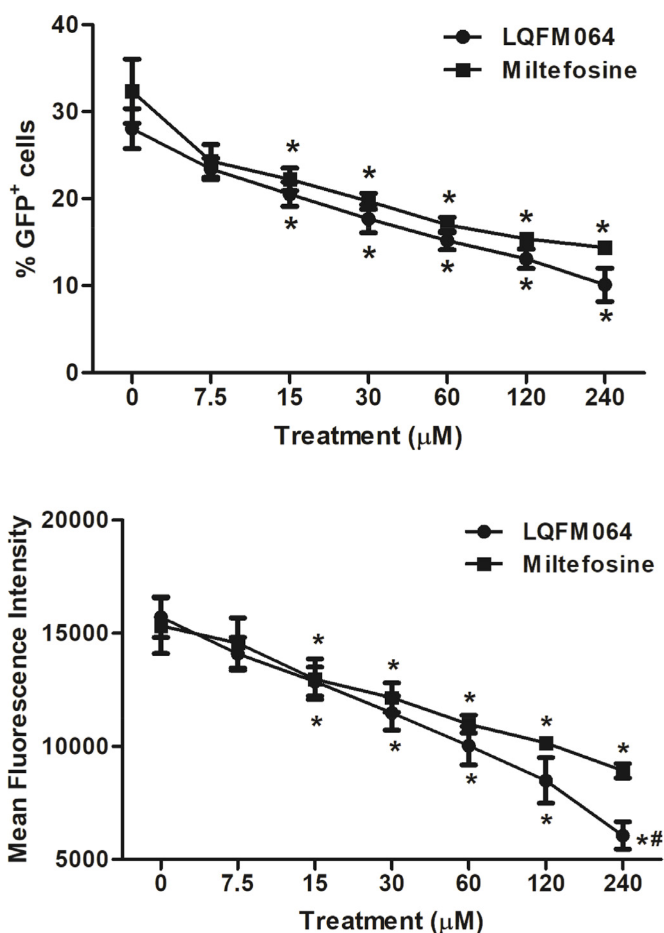

Fig. 5. Leishmanicidal action of LQFM064 (**3**) on *L. amazonensis*-infected J774.A1 macrophages. Macrophages were infected with *L. amazonensis*-GFP for 24 h then treated for an additional 24 h with different concentrations of LQFM064. Miltefosine was used as a positive control. % of GFP^+ cells and mean fluorescence intensity (MFI) were evaluated by flow cytometry. Statistical significance: * indicates a difference between the means for treated and untreated samples (control), and # indicates a difference between the means for LQFM064 (**3**) and MIL treated samples ($P < 0.05$).

4. Discussion

Spin label EPR data indicated that treatment of *L. amazonensis* promastigotes with the novel chalcone-like compound, LQFM064 (**3**), caused rigidity in the parasite plasma membrane. This is an atypical change in the molecular dynamics of the membrane that cannot be explained by the simple presence of the compound, at relatively low concentrations, but instead it must be the result of some oxidation process. Similar changes in the EPR spectra of spin label 5-DSA inserted in plasma membrane were found in a previous study of erythrocytes oxidized with hydrogen peroxide in a phosphate buffer with azide, a catalase inhibitor [14]. Those EPR spectra showed changes upon treatment with 200 μM H_2O_2, with the parameter $2A_{//}$ varying from 57.5 G for untreated RBCs to 60.5 G for RBCs oxidized with H_2O_2. Hydrogen peroxide has been shown to induce the formation of hemoglobin cross-linking with skeletal proteins of human erythrocytes in an azide phosphate buffer, which was associated with a progressive change in the cell shape to echinocyte morphology, decreased cell deformability and increased phagocytosis [15]. Heme proteins were crucial for the occurrence of these cellular changes, as they could be completely inhibited by pre-exposure of RBCs to carbon monoxide. Although lipid peroxidation was also observed, it did not appear to be important, as the antioxidant BHT decreased the fluorescent derivatives but did not prevent the formation of the hemoglobin-spectrin complex [15]. In addition, hemolysis and TBARS formation, observed for higher concentrations of H_2O_2, were inhibited by the antioxidants, ascorbic acid and α-tocopherol. However these antioxidants and others, such as L-carnosine and (+)-catechin, were unable to prevent membrane rigidity, which can be observed even for low concentrations of H_2O_2 and short incubation periods [14]. These observations suggest that the compound, LQFM064, of the present study is capable of inducing protein oxidation in the plasma membrane of *L. amazonensis* promastigotes.

It is well known that the treatment of RBCs with oxidizing agents, causing protein-protein cross-linking, markedly decreases the erythrocyte deformability due to the formation of oxidative cross-links between individual spectrin tetramers and between spectrin and hemoglobin [15–18]. Such cross-linking within the skeletal network could limit the cell deformation and increase membrane rigidity [19]. In addition, RBC aging has been associated with an increase in hemoglobin-spectrin complexes, which contribute to an increased rigidity of the senescent erythrocytes [17, 20]. However, while catalase inhibition in RBCs (using PBS with azide) markedly increased the oxidative stress caused by hydrogen peroxide, there was no further increase in the oxidative effects of LQFM064 when azide was used in the buffer (Fig. 2e). Furthermore, the use of PBS with azide in the experiments with *L. amazonensis* promastigotes did not alter the effects of the LQFM064 on the parasite membrane (data not shown). However, for 24 h experiments in culture medium containing azide and 500 μM H_2O_2, the changes in the EPR spectra of the *L. amazonensis* promastigotes were similar to those observed with the compound LQFM064 (Fig. 3).

EPR spectroscopy associated with the spin label method has been used to assess membrane rigidity as a parameter for lipid and protein peroxidation. In model membranes, as in the case of soy phospholipid

vesicles followed by lipoperoxidation with iron/ascorbate [21], the increases in membrane rigidity detected by the technique are generally small. Whereas in cell membranes, considerable increases in the parameter $2A_{//}$ of 5-DSA, associated with increased malondialdehyde (MDA) formation, have been found in apical segments of coffee seedling roots after chilling stress [22, 23], in mitochondria submitted to iron-induced lipoperoxidation [24], and in HepG2 cells exposed to lead [25]. When only lipid peroxidation occurs, the increase in $2A_{//}$ reaches a limit of ~2 G, which is four times greater than its estimated experimental error. However, when lipoperoxidation is accompanied by the oxidation of membrane proteins, the increases in $2A_{//}$ may exceed 5 G, as the observed in this work for cells treated with LQFM064 (Fig. 2). It is worth noting that the Fe^{++}/H_2O_2-induced lipoperoxidation in erythrocyte ghosts caused a maximum increase of ~2 G in $2A_{//}$, indicating the absence of cross-linking between membrane proteins [14]. The EPR technique has also been used to monitor the oxidative stress of integral proteins in cell membranes by assessing the reduction in mobility of the protein backbone with thiol-specific spin labels [26–28]. However, the fatty acid spin label 5-DSA incorporated into the cell membrane in annular or boundary lipid configuration can provide dynamic information on the hydrophobic surface of transmembrane proteins, and has shown greater sensitivity to detect membrane rigidity caused by peroxidation of membrane proteins [14].

In general, the insertion of small molecules into biological membranes results in an increase in membrane fluidity, as in the case of terpenes that act as spacers causing disruption of the lipid chain packing and the weakening of the hydrogen bonding network at the polar membrane interface [29–32]. The physical presence of molecules in the biological membrane rarely leads to membrane rigidity. But it can usually occur for molecules with the ability to dehydrate the polar region of the membrane. In model membranes, a reduction in the fluidity of the lipid bilayer has been demonstrated in the presence of sodium salt [33]. However, the observed levels of membrane rigidity are small compared to cases in which lipid or protein peroxidation occurs. The cell membrane is generally the first target of hydrophobic antiparasitic agents because they may bind to it in large amounts, altering its fluidity, which can lead to parasite death mainly through the leakage of electrolytes [7], which in turn reduces the resting potential of the cell and depolarizes the mitochondrial membrane.

In a recent study, the treatment of *L. amazonensis* promastigotes with novel β-carboline-oxazoline derivatives caused changes in the EPR spectra of 5-DSA inserted in the parasite plasma membrane, indicating membrane rigidity at a level similar to that found here for the parasite treated with LQFM064 [34], which was associated with the antileishmanial activity of these compounds. In another study, the activity of a β-carboline compound against *L. amazonensis* was associated with increased production of mitochondrial superoxide anion and the accumulation of lipid storage bodies in promastigotes [35]. In *Trypanosoma cruzi*, a parasite in the same family as *Leishmania*, treatment with the sesquiterpene (-)-elatol has been shown to cause plasma membrane rigidity detected by EPR spectroscopy, associated with an effective trypanocidal action [36]. The authors found changes in the $2A_{//}$ parameter of the spin label 5-DSA from 56.8 G for untreated samples to 61.1 G for elatol-treated samples, indicating membrane rigidity as strong as those observed here for compound LQFM064 in *L. amazonensis*. Interestingly, the action of (-)-elatol on the trypomastigote form of *T. cruzi* was associated with depolarization of the mitochondrial membrane, increased formation of mitochondrial superoxide anion and loss of cell membrane and DNA integrity [36]. In a previous study of LQFM064, the cytotoxic activity against MCF7, a human breast cancer cell line, was associated with excessive production of intracellular ROS, externalization of phosphatidylserine, cytochrome c release, increased expression of caspases-7, −8 and −9 and reduced mitochondrial membrane potential [10].

It has been demonstrated that the aldehyde 4-hydroxy-2-nonenal, a Michael acceptor scaffold, can promote covalent modifications to phosphatidylethanolamine of the membrane through the formation of Michael adducts [37]. This effect increased the permeability to sodium ions and was consistent with the reduction in the dynamics of the membrane bilayer. In its chemical structure, the LQMF064 compound has a Michael acceptor scaffold and thus further studies would be needed to understand whether, at least in part, the effect observed for LQFM064 occurs through the formation of Michael adducts with the bilayer membrane like a molecular anchor. Furthermore, β-carboline-oxazoline derivatives, which have the ability to reduce membrane fluidity [34], have oxazoline and oxazine scaffolds in their chemical structures that have an electrophilic profile. These kinds of electrophiles can form adducts with nucleophiles, as well as Michael acceptors [37], and thereby could alter the dynamics of the bilayer membrane.

Recently, it has been shown that by measuring the IC_{50} values of hydrophobic drugs in assays with different cell concentrations, it was possible to assess the membrane-water partition coefficient of the drug, $K_{M/W}$, in addition to the drug concentrations in the membrane, c_{m50}, and in the aqueous phase, c_{w50}, of the suspension, that reduce cell viability by 50% [8, 10, 32]. This method has also been applied to obtain these same parameters based on the cytotoxicity (CC_{50}) [8, 9, 32] or the hemolytic potential (HC_{50}) of compounds [12, 13]. The observed $K_{M/W}$ value in this study showed that the LQFM064 has an affinity for the parasite membrane, even greater than that of the antileishmanial drug miltefosine. While the c_{m50} value was as high as that of miltefosine (Table 1), indicating that the growth inhibition only occurs when a large amount of the compound is accumulated in the parasite membrane. Miltefosine has been shown to have a high c_{m50} for *L. amazonensis* due to its strong interactions with membrane proteins [7, 8, 13]. However, in theory, LQFM064 should penetrate the interior of the parasite and partition itself between the aqueous medium and the internal membranes. Our work, however, does not present any information about the oxidative processes triggered by LQFM064.

5. Conclusions

EPR spectroscopy was used to demonstrate that the treatment of *L. amazonensis* promastigotes with the compound LQFM064 (**3**) causes strong rigidity in the parasite plasma membrane. Membrane rigidity was observed with only 2 h of incubation at 26°C in PBS or in complete culture medium. The alterations in the EPR spectra were atypical and consistent with the occurrence of peroxidation of parasite membrane proteins. Evaluations of biophysical parameters associated with the antiproliferative activity of LQFM064 indicated that, compared to miltefosine, the compound had greater affinity for the parasite membrane, but the antiproliferative effects were observed for similar concentrations in the membrane. The selectivity index of LQFM064 for *L. amazonensis* promastigotes in relation to J774.A1 macrophages ($SI = CC_{50}/IC_{50}$) was almost three times higher than that of miltefosine. In addition, the compound demonstrated very low hemolytic potential for assays with isolated erythrocytes in PBS and whole blood, denoting a high parasite/erythrocyte selectivity index. New studies are important to analyze whether the action of the compound is greater in the parasite than in the erythrocyte due to its greater affinity for the leishmania plasma membrane, as suggested by this study, or because its ability to induce protein peroxidation is higher in the parasite membrane than in the erythrocyte.

CRediT authorship contribution statement

Lais Alonso: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Ricardo Menegatti:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing. **Rodrigo Saar Gomes:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Miriam Leandro Dorta:** Conceptualization, Formal analysis, Funding acquisition,

Methodology. **Rangel Magalhaes Luzin:** Conceptualization, Investigation, Methodology. **Luciano Morais Liao:** Conceptualization, Investigation, Methodology. **Antonio Alonso:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - review & editing.

Acknowledgments

This study was financially supported by grants from the Brazilian research funding agencies CNPq (445666/2014-5), CAPES and FAPEG (201210267001110). Lais Alonso was a recipient of a postdoctoral fellowship from CNPq (150369/2018-2). Antonio Alonso received a research grant from CNPq (303829/2016-8).

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