Ferulate Anion Intercalated into Zn/Al Layered Double Hydroxide: A Promising Intercalation Compound for Inhibition of Leishmania (L.) amazonensis

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Ferulic acid (FERH) is known to exert leishmanicidal action against Leishmania (Leishmania) amazonensis. In this study, the ferulate anion (FER−) was intercalated into a layered double hydroxide (LDH) for FER− delivery during an antileishmania in vitro test. The LDH-FER− intercalation compound was prepared by coprecipitation and was characterized by X-ray powder diffraction, thermogravimetry, UV-Vis, infrared, and Raman spectroscopy analyses. Leishmania (L.) amazonensis in vitro assays were conducted to assess the LDH-FER− potential. LDH-FER− was found to promote a dose-dependent reduction of L. (L.) amazonensis amastigotes (intracellular form, half maximal inhibitory concentration (IC50) = 1.78 µg mL−1) without exerting a deleterious effect on the host macrophages (medium cytotoxic concentration (CC50) = 212.61 µg mL−1 and selectivity index = 119.4). LDH-FER− was found to be an effective inhibitor of the amastigote form of L. (L.) amazonensis, suggesting that LDH-FER− was able to deliver FER− inside the parasitophorous vacuoles where the pH is more acidic.

Keywords: ferulic acid (FERH), intercalation, LDH-FER− intercalation compound, antileishmanial activity

Introduction

Leishmaniasis is a parasitic disease endemic to American, African, Asian and southern European countries.1 More than 350 million individuals in 98 countries are at risk of being infected with this neglected tropical disease, especially in developing countries, such as in Latin America, where 11 different dermotropic species are known that can affect humans.2 Leishmania (Leishmania) amazonensis is responsible for causing the most severe form of leishmaniasis, the diffuse (anergic) cutaneous leishmaniasis (DCL). This parasite induces Th1/Th2 CD4+ cell profiles, which silence the microbicidal responses of macrophages (host cell) creating a favorable environment for infection.1 Furthermore, even after receiving treatment, this cell profile cannot be reversed in patients.4,5

The World Health Organization (WHO) recommends the use of chemotherapy for this disease as it has proven to be one of the most effective treatments. Meglumine antimoniate (glucantime®), miltefosine, and amphotericin B (AMP-B) are examples of chemotherapeutics used to treat leishmaniasis.6 Nevertheless, toxic effects are reported for all of these compounds and they presented limited efficacy as parasite resistance becomes more prevalent. Therefore, the development of new antileishmanial inhibitors with lowest toxicity is needed. Compounds isolated from microorganisms7 and medicinal plants have also demonstrated leishmanicidal activity.6,14 Recently, the antileishmanial and cytotoxic activities of five natural compounds from Pluchea carolinensis were evaluated in vitro and in vivo. Among these natural products, ferulic acid (FERH; 4-hydroxy-3-methoxycinnamic acid) has been reported to be a potential inhibitor of Leishmania tropica promastigotes.15 FERH was one of the most active

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compounds against intracellular amastigotes form of
L. (L.) amazonensis. However, FERH has presented
cytotoxicity on noninfected peritoneal macrophages
and its small cellular uptake limits clinical applications.

Layered double hydroxides (LDH) are inorganic
nanoparticles with excellent features as nanocarriers in drug
delivery systems. These nanoparticles have the potential
to play an important role in healthcare. LDH also known
as anionic clay have become one of the most promising
candidates for various biological applications due to their
high cellular uptake, non-immunogenic response, and low
toxicity. LDH comprise a large family of anionic clays with
great potential in materials science and technology due
to their versatile structures, intercalation properties, wide
range of applications, good biocompatibility, improved drug
solubility, and potential for target delivery.

LDH are structurally composed of layers with positive
charges that are electrically neutral thanks to the presence
of anions that, together with water molecules, promote
the characteristic stacking of layers observed in these
structures. The general chemical formula of this class of
inorganic materials is:

\[ \left[ \text{M}^{2+}_{x} \text{M}^{3+}_{y} (\text{OH})_{z} \right]^z+ (\text{A}^{n-})_{x} \text{zH}_2\text{O} \]

where A\textsuperscript{n-} is an exchangeable inorganic or organic
anion, often a carbonate and nitrate, that compensates
for the positively charged hydroxide brucite-type layers;
x refers to the molar fraction M\textsuperscript{2+} / (M\textsuperscript{2+} + M\textsuperscript{3+}), usually
(0.2 \leq x \leq 0.4), and z is the amount of hydration water
molecules.

Previous reports have focused on the synthesis of
LDH-FER\textsuperscript{-} hybrids, as well as on the study of their
drug release properties, using various methods. Also, the
coprecipitation method was used recently to intercalate
ibuprofen into layered double hydroxides (LDH-IBU) and
performed in vivo assays of the analgesic activity using male
Swiss mice. In this work, the ferulate anion (negatively
charged; FER\textsuperscript{-}) was intercalated by coprecipitation
method into a biocompatible Zn/Al layered double hydroxide
LDH (Zn/Al–NO\textsubscript{3}–). In addition, the effect by which the
FER\textsuperscript{-} released from LDH-FER\textsuperscript{-} intercalation compound
acts as a leishmanicidal agent against L. (L.) amazonensis
was investigated.

**Experimental**

**Chemicals and reagents**

All reagents were purchased from Sigma-Aldrich: ferulic
acid (99%), zinc nitrate hexahydrate (Zn(NO\textsubscript{3})\textsubscript{2},6H\textsubscript{2}O;
98%), aluminum nitrate nonahydrate (Al(NO\textsubscript{3})\textsubscript{3},9H\textsubscript{2}O;
98%), metallic sodium (Na(s); 99.9%), ethyl alcohol
(CH\textsubscript{3}CH\textsubscript{2}OH; 99.5%), dimethyl sulfoxide (DMSO, (CH\textsubscript{3})\textsubscript{2}SO; \geq 99.7%), and phosphate buffer (pH 7.40 ± 0.02;
98%). Deionized water was decarbonated prior to synthesis.

**Preparation of pristine LDH (Zn/Al–NO\textsubscript{3}–)**

The coprecipitation method was used in the synthesis of
the pure LDH (Zn/Al–NO\textsubscript{3}–). A mixed aqueous solution (50.0 mL) containing 13.8507 g (46.56 mmol)
of Zn(NO\textsubscript{3})\textsubscript{2},6H\textsubscript{2}O and 8.6901 g (23.16 mmol) of Al(NO\textsubscript{3})\textsubscript{3},9H\textsubscript{2}O
was added dropwise to 200.0 mL of deionized and
decarbonated water, of which the pH was adjusted to 9.57,
under stirring and in a nitrogen atmosphere. During the
procedure, the value of the pH was maintained approximately
constant (pH 9.47) by the continuous addition of 2.0 mol L\textsuperscript{-1}
NaOH solution. The resulting precipitate was kept under N\textsubscript{2}
gas flow at room temperature with stirring for 72 h. The solid
was then isolated by centrifugation and washed thoroughly
with water (50 °C) and dried in a vacuum desiccator.

**Intercalation of FER\textsuperscript{-} into LDH (Zn/Al–NO\textsubscript{3}–)**

Similarly, the coprecipitation method was used for
intercalation of FER\textsuperscript{-} into LDH (Zn/Al–NO\textsubscript{3}–). Here, 25.0 mL
of ethoxide solution (EtO\textsuperscript{-}) obtained from a mixture of
0.2576 g (11.20 mmol L\textsuperscript{-1}) of the sodium metal, ethanol, and
DMSO (4:1 v v\textsuperscript{-1}) were added to a three-neck flask containing
6.00439 g (30.92 mmol) of ferulic acid (FERH), producing
sodium ferulate salt (FER\textsuperscript{-}Na\textsuperscript{+}) as an intense red solution.
After a few minutes, a mixed aqueous solution (25.0 mL)
containing 3.7061 g (12.458 mmol) of Zn(NO\textsubscript{3})\textsubscript{2},6H\textsubscript{2}O and
2.3294 g (6.209 mmol) of Al(NO\textsubscript{3})\textsubscript{3},9H\textsubscript{2}O (Zn / Al = 2.0)
was slowly added to the FER\textsuperscript{-}Na\textsuperscript{+} solution. To ensure the
dissolution of FERH, the initial pH was adjusted to 9.53 using
2.0 mol L\textsuperscript{-1} NaOH solution. The addition was performed in
the dark and under vigorous stirring in a nitrogen atmosphere
to protect the reagents from light and oxygen. During the
procedure, the pH was maintained approximately constant
(final pH 9.51) by the continuous addition of a 2.0 mol L\textsuperscript{-1}
NaOH solution. The resulting dispersion (final pH 9.51) was
kept in the dark and under N\textsubscript{2} gas flow and stirred at room
temperature for 72 h. The solid was isolated by centrifugation and washed thoroughly
at 50 °C and dried in a vacuum desiccator.

**Sample characterization**

Characterization of the samples was performed by X-ray
diffraction (XRD), and the composition of the samples was
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Antipromastigote assay

The cell line J774 of mouse macrophages (10^3 cells per plate) was infected with L. (L.) amazonensis promastigotes (ratio 1:10) in the stationary growth phase (7 days) for 3 h, in an atmosphere of 5% CO₂ at 34 °C. After 3 h of incubation, the infected cells were treated for 72 h with 0.9, 2.1 and 4.3 µg mL⁻¹ of LDH-FER⁻ for 96 h at 25 °C, which corresponds to 0.4, 0.9, 2.1, 4.3 and 8.5 µg mL⁻¹, taking into consideration only the amount of FER⁻ intercalated in LDH. After completing 96 h of treatment, aliquots were harvested, and promastigotes were incubated for 4 h with 20 µL of MTT (stock solution of 2 mg mL⁻¹). After incubation, 20 µL of DMSO were added for the solubilization of the formazan crystals. The resulting solution was read in a microplate reader (Bio-Rad Model 450 microplate reader) at a wavelength of 570 nm. Promastigotes treated with 0.5 µg mL⁻¹ of AMP-B were used as a positive control.

Antiamastigote assay

The cell line J774 of mouse macrophages (10^3 cells per plate) was infected with L. (L.) amazonensis promastigotes (ratio 1:10) in the stationary growth phase (7 days) for 3 h, in an atmosphere of 5% CO₂ at 34 °C. After 3 h of incubation, the infected cells were treated for 72 h with 0.9, 2.1 and 4.3 µg mL⁻¹ of LDH-FER⁻ for 96 h at 25 °C, which corresponds to 0.4, 0.9, 2.1, 4.3 and 8.5 µg mL⁻¹, taking into consideration only the amount of FER⁻ intercalated in LDH. After completing 96 h of treatment, aliquots were harvested, and promastigotes were incubated for 4 h with 20 µL of MTT (stock solution of 2 mg mL⁻¹). After incubation, 20 µL of DMSO were added for the solubilization of the formazan crystals. The resulting solution was read in a microplate reader (Bio-Rad Model 450 microplate reader) at a wavelength of 570 nm. Promastigotes treated with 0.5 µg mL⁻¹ of AMP-B were used as a positive control.

Host cell

The mouse macrophage cell line J774 was cultured in 150-mm plates (10³ cells per plate) and treated with different concentrations (0.9-17 µg mL⁻¹) of FER⁻ released from LDH-FER⁻ for 72 h at 34 °C. Cells were then washed twice with PBS pH 7.2 and incubated with 0.5 mg mL⁻¹ MTT for 3 h at 37 °C, with 5% CO₂, as described in the literature. The samples were read in a microplate reader
In vitro ferulate anion release

The release of FER\(^{-}\) \textit{in vitro} was performed as follows: the LDH-FER\(^{-}\) sample (10 mg) was immersed in PBS at pH 5.0 and 7.4 (100 mL) with mild shaking at 150 rpm; the temperature was maintained at 37 ± 0.5 °C. An aliquot of 5 mL was taken from the suspension from time to time to measure the release of ferulate anion into the solution. Aliquots were immediately replaced by an equal volume of PBS at 37 ± 0.5 °C to keep the temperature and volume constant. The content of ferulate anion in each aliquot was filtered and measured at an absorbance of 310 nm using a Shimadzu UV-2600 UV-Vis spectrophotometer at \(\lambda_{\text{max}} = 310\) nm according to a previously determined calibration curve (\(y = 0.0735, x = 0.0902; r^2 = 0.9992\)). The percentage released at each time point was expressed as a fraction of the total amount of FER\(^{-}\). Drug release was monitored for 10 h, and the amount of FER\(^{-}\) was obtained by an average of the three determinations, where the data found was within the calibration curve obtained previously.

Results and Discussion

XRD powder analysis

Figure 1 shows the X-ray powder diffraction (XRPD) patterns of the pristine LDH (Zn/Al–NO\(_3\))\(^{-}\) and LDH-FER\(^{-}\) intercalation compound. The 003 reflection in the LDH-FER\(^{-}\) intercalation compound is at lower angle (ca. 5.10° 2\(\theta\)) compared with that of the pristine LDH sample, suggesting a lattice expansion along the \(c\)-axis induced by the intercalation of FER\(^{-}\), as has been previously reported.\(^{32,39-41}\) In Figure 1, the pattern of the LDH-FER\(^{-}\) intercalation compound corresponds to a single-phase compound with no evidence of crystallized impurities, for example, sodium nitrate, zinc oxide, or sodium ferulate salt. The new phase has a layer distance close to two times the nitrate phase. Consequently, both phases will have eclipsed basal peaks and both phases cannot be excluded from a mixture.

The 003 spacing value was 1.72 nm, close to the value reported in the literature for LDH-ferulic obtained by coprecipitation (1.69 nm).\(^{30}\) With a brucite layer thickness of 0.48 nm,\(^{42}\) the gallery height of the interlayer region of the LDH-FER\(^{-}\) intercalation compound can be estimated at about 1.24 nm, within the range previously reported for FA intercalated into [Zn/Al–NO\(_3\)]-LDH.\(^{30,32}\) This gallery height suggests that ferulate anions may be arranged in the interlayer region in a zig-zag pattern with alternating phenol-acrylate \(\pi\)-\(\pi\) interactions (see Scheme 1), as suggested in the literature.\(^{29,40}\) It is also worth noting that the 110 reflection appears at the same position for both the pristine LDH and LDH-FER\(^{-}\) intercalation compound, indicating that the structure of the brucite layer is barely affected by the intercalation of the ferulate anions. Moreover, the crystalline domains in the organic phase are smaller than in the nitrate phase due to the basal lines broadening.

Thermogravimetric and UV-Vis spectroscopy analyses

Weight loss as a function of temperature for ferulic acid (protonated neutral, FERH), pristine LDH (Zn/Al–NO\(_3\)), and LDH-FER\(^{-}\) intercalation compound were obtained...
using DTA-TGA curves (Figure 2). FERH exhibits a single-stage thermal degradation, with an endothermic peak at 176.2 °C followed by a complete thermal decomposition at temperatures above 300 °C due to the exothermic effect of decomposition phenomena in its DTA curve (Figure S1, Supplementary Information section). For pristine LDH, the thermal decomposition took place in two stages. The first weight loss was observed below 200 °C, because of the loss of physically adsorbed and partial interlayer water, with an endothermic peak at 76 °C. The second weight loss took place in temperatures between 200 and 600 °C and can be attributed to the decomposition of the brucite-like layer and the removal of interlayer anions, with evolution of NO₂ and NO gases from LDH (Zn/Al–NO₃) around 438 and 489 °C in two stages (Figure S2, Supplementary Information section). However, a different pattern was observed in the LDH-FER⁻ intercalation compound thermogravimetric curve, where the disappearance of the FERH molecules melting peak denoted a full intercalation of the compound into the matrix (Figure 2). The weight loss between 100 to 1000 °C is attributable to an intercalated FER⁻ decomposition, with nitrate anions (NO₃⁻), water (H₂O) loss, and dehydroxylation of the inorganic layers. The thermally transformed products at 800 °C could be ZnO and ZnAl₂O₄, as described in the literature. From the TGA analyses and the cationic ratio (Zn/Al) obtained from ICP, the composition of the positive charge of the brucite layer was calculated, where the amount of intercalated FER⁻ and water of hydration per formula unit were obtained (Table 1). These results are consistent with those found in the literature. Furthermore, Figure 3 shows the UV-Vis spectra of both pure FERH and FER⁻ release from the LDH-FER⁻ complex. These data were obtained using a phosphate buffer solution at pH 7.40 and also in the solid state, as described above in the “Sample characterization” sub-section. In solution, the absorbance peaks at 216 and 290 nm of the methoxyphenol part and 314 nm of the acrylate part, representing the n → π* and π → π* transition, respectively, show that intercalation compound was capable to deliver ferulate anion making it suitable for interaction with its biological targets. In addition, the UV-Vis solid state spectrum showed peaks at 383 and 387 nm of the energy transitions of the π–π to π → π* interactions, which correspond to FERH alone and to the ferulate anion into LDH layers, respectively. It is also important to note that the amount of FER⁻ intercalated into LDH gallery was calculated at 42.6 ± 0.8% (m m⁻¹) using UV-Vis spectroscopy analysis, which is in accordance with the range previously reported for ferulate anion intercalated into Mg/Al–NO₃ LDH by anion exchanger (34-48%).

![Figure 2](image2.png)  
**Figure 2.** DTA-TGA curves of the LDH-FER⁻ intercalation compound. Solid line: TGA; dotted line: DTA.

![Figure 3](image3.png)  
**Figure 3.** Solution UV-Vis spectra of (a) FERH, (b) released FER⁻ from the LDH-FER⁻, and (c) solid-state UV-Vis spectra of the neutral FERH and (d) LDH-FER⁻ intercalation compound.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average chemical composition</th>
<th>Total weight loss / %</th>
<th>Weight of FER⁻ / %</th>
<th>Cell parameter / nm</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>a</td>
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<tr>
<td>LDH (Zn/Al–NO₃⁻)</td>
<td>[Zn₉0₆₋₁₃₋₀(Hₙ)₉₋₁₄₋₀(NO₃₀₋₁₃₋₀,0₅₋₁₋₀H₂O₉₋₁₄₋₀]⁻₂⁻</td>
<td>38.5</td>
<td>0.0</td>
<td>0.306</td>
</tr>
<tr>
<td>LDH-FER⁻</td>
<td>[Zn₉0₆₋₁₃₋₀(Hₙ)₉₋₁₄₋₀(NO₃₀₋₁₃₋₀,FER⁻₉₋₁₃₋₀,0₂₋₁₋₀H₂O₉₋₁₄₋₀]⁻₂⁻</td>
<td>53.1</td>
<td>42.6</td>
<td>0.305</td>
</tr>
</tbody>
</table>

*Based on TG analyses; †based on UV-Vis titration. FER⁻: ferulate anion; a and c: lattice constants; LDH (Zn/Al–NO₃⁻): Zn/Al layered double hydroxide; LDH-FER⁻: ferulate anion intercalated into a layered double hydroxide.
Infrared and FT-Raman analysis

Infrared spectroscopy was used to analyze the vibrational behavior of FERH before and after their intercalation into the LDH (Zn/Al–NO$_3^-$) galleries. Figure 4 shows these results, which are in agreement with the data reported in the literature for these types of materials.$^{31,32,40}$

The presence of nitrate anions (NO$_3^-$) is identified by a characteristic peak at 1380 cm$^{-1}$, unambiguously assigned to its $\nu_3$ vibrational mode.$^{46}$ The disappearance of the NO$_3^-$ anion band is a result of the anionic exchange of the coprecipitation method. New vibrational modes for FER$^-$ (Figure 4) correspond to those previously proposed.$^{47}$ For example, the very strong band observed in the FTIR spectrum at 1508 cm$^{-1}$ corresponds to C–C stretching vibration for both aromatic and propanoic acids. Moreover, the appearance of antisymmetric $\nu_{\text{antisym}}$(COO$^-$) and symmetric $\nu_{\text{sym}}$(COO$^-$) stretching modes of carboxylate groups observed at 1592 and 1390 cm$^{-1}$, respectively, indicates that a FER$^-$ has been intercalated, as previously reported in the literature for ZnAl-FA.$^{32}$ The difference between $\nu_{\text{antisym}}$(COO$^-$) and $\nu_{\text{sym}}$(COO$^-$) stretching was ca. 202 cm$^{-1}$. This clearly indicates the deprotonated nature of the intercalated FER$^-$ compared to FERH. In addition, the broad absorption band at 3450 cm$^{-1}$ corresponds to the stretching frequency mode of O–H groups in the brucite-like layer and interlayer water. Similarly, absorption at 1645 cm$^{-1}$ originates from the O–H bending vibration of hydroxyl groups.$^{46}$ Bands at 425 and 602 cm$^{-1}$ correspond to the lattice vibration modes attributed to Al–OH and Zn–OH vibrations.$^{32,48}$ Therefore, our results suggest that FER$^-$ was stabilized between stacked LDH layers, most likely as a result of an electrostatic interaction, as previously proposed by Kang et al.$^{40}$

Figure 4. FTIR spectra of (a) pristine LDH (ZnAl–NO$_3^-$), (b) pure FERH, and (c) LDH-FER$^-$ intercalation compound.

Raman spectroscopy analysis was also used to complement the infrared study. The data obtained are shown in Figure S3 (Supplementary Information section) for free FERH, pristine LDH (Zn/Al–NO$_3^-$), and LDH-FER$^-$ intercalation compound. In the LDH sample, a band related to the nitrate (symmetry D$_{3h}$) can be observed at 1055 cm$^{-1}$, as reported by Kloprogge et al.$^{48}$ The measured FERH spectrum confirms that the drug is pure and corresponds well with the full indexation proposed in the literature.$^{47,49}$ The FT-Raman analysis confirms the intercalation of FER$^-$ in the hosts as evidenced by ring breathing at 673, 814, 1177 cm$^{-1}$ and $\delta$(=C–H) at 1240 and 1273 cm$^{-1}$, and by the asymmetric and symmetric vibrations of $\delta$(CH$_3$) and aromatic C=C stretching at 1601 and 1630 cm$^{-1}$. Moreover, two bands at 573 and 710 cm$^{-1}$ are attributed to M–OH of the LDH-FER$^-$ intercalation compound.

SEM

The scanning electron micrographs of the samples are shown in Figure 5 for both pristine LDH (Figure 5a) and the LDH-FER$^-$ intercalation compound (Figure 5b). The image suggests an aggregated sheet-like morphology, characteristic of hydrotalcite materials,$^{33,50}$ with a diameter smaller than 1 µm. This indicates that the particles are composed of a small number of layers, which is consistent with intercalated ferulate anion at room temperature. Moreover, the size of the particle is consistent with this material type as shown in previous studies.$^{29,32}$

Figure 5. Scanning electron micrographs of (a) pristine LDH and (b) the LDH-FER$^-$ intercalation compound.

Release study of LDH-FER$^-$

Figure 6 shows the release profiles of FER$^-$ from its LDH-FER$^-$ intercalation compound at different pH values, indicating that the release rate of ferulate anion from the LDH-FER$^-$ is pH-dependent. The release rate at pH 7.4 is remarkably lower than that at pH 5.0. It is noteworthy that in this pH range there is predominance of the ionic form of
the ferulic acid. The percentage release of ferulate anion from LDH-FER is of approximately 90 and 71% after 10 h when exposed to pH 5.0 and 7.4, respectively. The release of ferulate anion from the LDH-FER intercalation compound follows different molecular mechanisms at pH 5.0 and 7.4. Tyner et al. found that Zn/Al-LDH is more stable at pH 7.4 than 4.8 due to electrostatic interactions between the sodium cholate anions and the positively charged Zn/Al-LDH layers. Moreover, they suggested that the release of sodium cholate at pH 7.4 occurs via an ion exchange with the ions in the buffer solution, while the release at pH 4.8 occurs through both dissolution of the LDH layers and ion exchange. This lower release rate of ferulate anion from the intercalation compound at pH 7.4 suggests that the LDH-FER could be a potential drug delivery system. According to the literature, the burst effect observed in the matrix during the first 2 h, followed by a slow release from the second to the tenth hour, could be due to the release of intercalated ions. The second slow-release stage can be attributed to the release of ferulate anion from deeper interlayer sites, where the effective distance to diffuse up to the edges reduces the release rate, together with a possible increase in the rigidity of the layers.

The antipromastigote activity assay (Figure 7a) showed that LDH-FER promoted a decrease of approximately 10% (0.4 and 0.9 µg mL⁻¹), 18% (2.1 µg mL⁻¹), 20% (4.3 µg mL⁻¹), and 30% (8.5 µg mL⁻¹) of L. (L.) amazonensis promastigotes (IC₅₀ = 16.6 µg mL⁻¹). LDH-FER demonstrated partially ineffective action against the promastigote forms of L. (L.) amazonensis. However, the leishmanicidal activity of a compound is not evaluated only by its action on the extracellular promastigote forms. It is important to also consider the action of the compound against the intracellular amastigote forms found in the lesions of patients with leishmaniasis. Then, the activity of LDH-FER against intracellular amastigote form was evaluated following the experimental procedure described in “Antiamastigote assay” sub-section. After the biological test, it was found that LDH-FER reduced the number of L. (L.) amazonensis amastigotes (IC₅₀ = 1.78 µg mL⁻¹) after 72 h of treatment (Figure 7b). Reductions of approximately 58% (0.9 µg mL⁻¹), 71% (2.1 µg mL⁻¹), and 86% (4.3 µg mL⁻¹) were observed. It is interesting to note that LDH-FER at a concentration of 4.3 µg mL⁻¹ presented an amastigote reduction rate close to that observed in cells treated with AMP-B (see Figure 7). It is important to clarify that IC₅₀, CC₅₀, survival index and % of viable cells were calculated considering the amount of ferulate anion present in LDH-FER used for antipromastigote and antiamastigote activity assay.

The literature reports that ferulic acid (FERH) isolated from Pluchea carolinensis presents leishmanicidal action against L. (L.) amazonensis. One study showed that FERH promoted a reduction in the number of promastigotes in Leishmania tropica after 3 days of cultivation. Another experimental work suggests that ferulic acid presented no inhibition of amastigote forms from L. (L.) amazonensis inside J774 macrophages. The present study shows for the first time that FER intercalated into pristine LDH (Zn/Al–NO₃) has leishmanicidal action. Our results show that the LDH-FER intercalation compound presented a more effective association against amastigote forms of L. (L.) amazonensis than against promastigotes. Leishmania amastigotes are obligatory intracellular forms that can be found in the parasitophorous vacuoles of phagocytic cells, where the pH is acidic and where there are numerous lysosomal enzymes. LDH nanoparticles are negatively charged which facilitates their interaction with cell membranes, favoring the process of endocytosis. It is interesting to note that in the presence of an acidic pH, LDH nanoparticles dissolve and release their contents. Thus, the action of the LDH-FER intercalation compound against amastigote forms seems to occur by the degradation of the LDH-FER in an acidic environment, causing the release of all intercalated FER eventually prompting the parasite to die. Obviously, this proposition awaits further experimental studies.

In relation to the cytotoxicity assay, no reduction in macrophages viability was observed after treatment with different concentrations of FER released from LDH-FER (0.9-17 µg mL⁻¹) with a CC₅₀ of 212.61 µg mL⁻¹ (Figure 8).
It is important to keep in mind that the comparison of different experiment with distinct protocols could lead to wrong interpretations. However, it is interesting to compare the biological activity of our LDH-FER$^-$ intercalation compound with the experimental data of other compounds from literature. In this work was started the comparison with miltefosine and AMP-B that are important drugs for the treatment of leishmaniasis$^6$ and are more effective than FERH.$^{58}$ The in vitro cytotoxicity study of amphotericin B and miltefosine presented CC$_{50}$ of 57.3 and 34.4 $\mu$g mL$^{-1}$, respectively.$^{58}$ In addition, the SI observed for both drugs correspond to 38.2 and 5.8, respectively,$^{58}$ which is lower than the SI of FER$^-$ released from LDH-FER$^-$ (see Table 2).

It is also worth mentioning that the inhibitory effect of the LDH-FER$^-$ intercalation compound is slightly lower than ferulic acid (FERH) but it is important to emphasize that the SI of the FER$^-$ released from LDH-FER$^-$ corresponds to 119.4, while the SI of FERH is 17 (Table 2). Therefore, LDH-FER$^-$ may be used as potential drug with low risk of toxicity.

The present study demonstrated that LDH-FER$^-$ presents selective action against $L. (L.)$ amazonensis. LDH-FER$^-$ eliminated amastigote forms of $Leishmania$ without causing damage to the host cell. Substances intercalated with LDH have advantages compared to non-intercalated substances. LDH has a low toxicity and a controlled release of the intercalated compounds which makes its use attractive in the treatment of various diseases.$^{59}$ Indeed, the LDH-FER$^-$ intercalation compound was selective against $L. (L.)$ amazonensis, presenting an SI of 119.4 for intracellular amastigote. Based on these results, LDH-FER$^-$ was shown to act against $L. (L.)$ amazonensis amastigotes with no negative effect on mammalian cells, suggesting that the intercalation compound could be used as start point for the development of a new leishmanicidal agent.
In this work, ferulate anion was intercalated into a LDH by a simple coprecipitation method. The anion incorporated within the LDH interlayer space (LDH-FER\(^{-}\)) was analyzed by XRD, FTIR, Raman, DTA-TGA, and UV-Vis spectroscopy. The analysis of the intercalated compound showed an intercalation percentage of 42.6% and an increase of the interlayer space from nitrate anion due to the presence of the ferulate anions. Moreover, it was also demonstrated that the LDH-FER\(^{-}\) could be used as an efficient leishmanicidal intercalation compound against amastigote forms of *Leishmania (Leishmania) amazonensis*. The release of FER\(^{-}\) from LDH-FER\(^{-}\) is of ca. 90% at pH 5 after 10 h, which is 20% more than the ferulate anion released at pH 7.4. This study demonstrates, for the first time, the action of LDH-FER\(^{-}\) on *L. (L.) amazonensis* in vitro.

Our results also highlight the importance of LDH as a potential drug delivery vehicle for potential treatment of cutaneous leishmaniasis without any side effect. Overall, LDH-FER\(^{-}\) could be useful for the selective treatment of cutaneous leishmaniasis and may be of great potential as an anti-leishmanial agent.

### Supplementary Information

Supplementary data (DTA-TGA curves for neutral FERH and pristine LDH (Zn/Al–NO\(_3\)) samples, and FT-Raman spectra of pristine LDH (Zn/Al–NO\(_3\)), neutral FERH, and LDH-FER\(^{-}\) intercalation compound) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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References

33. SigmaPlot, version 12.0 for Windows; Systat Software Inc., San Jose, California, USA, 2011.

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