When nanomedicines meet tropical diseases

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Nanostructured lipid carriers containing benznidazole: physicochemical, biopharmaceutical and cellular in vitro studies

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Abstract

Chagas disease is a neglected endemic disease prevalent in Latin American countries, affecting around 8 million people. The first-line treatment, benznidazole (BNZ), is effective in the acute stage of the disease but has limited efficacy in the chronic stage, possibly because current treatment regimens do not eradicate transiently dormant Trypanosoma cruzi amastigotes. Nanostructured lipid carriers (NLC) appear to be a promising approach for delivering pharmaceutical active ingredients as they can have a positive impact on bioavailability by modifying the absorption, distribution, and elimination of the drug. In this study, BNZ was successfully loaded into nanocarriers composed of myristyl myristate/Crodamol oil/poloxamer 188 prepared by ultrasonication. A stable NLC formulation was obtained, with ≈80% encapsulation efficiency (%EE) and a biphasic drug release profile with an initial burst release followed by a prolonged phase. The hydrodynamic average diameter and zeta potential of NLC obtained by dynamic light scattering were approximately 150 nm and ~13 mV, respectively, while spherical and well-distributed nanoparticles were observed by transmission electron microscopy. Fourier-transform infrared spectroscopy, differential scanning calorimetry, thermogravimetric analysis, and small-angle X-ray scattering analyses of the nanoparticles indicated that BNZ might be dispersed in the nanoparticle matrix in an amorphous state. The mean size, zeta potential, polydispersity index, and %EE of the formulation remained stable for at least six months. The hemolytic effect of the nanoparticles was insignificant compared to that of the positive lysis control. The nanoparticle formulation exhibited similar performance in vitro against T. cruzi compared to free BNZ. No formulation-related cytotoxic effects were observed on either Vero or CHO cells. Moreover, BNZ showed a 50% reduction in CHO cell
viability at 125 µg/mL, whereas NLC-BNZ and non-loaded NLC did not exert a significant effect on cell viability at the same concentration. These results show potential for the development of new nanomedicines against *T. cruzi*.

**Introduction**

Chagas disease is a neglected disease endemic to Latin America, affecting around 8 million people and causing 2000 deaths per year, according to the World Health Organization [1]. Currently, this health problem is not restricted to Latin American countries, as it has spread to non-endemic regions such as the United States and Europe [2,3]. It is caused by the hemoflagellate protozoan *Trypanosoma cruzi*, whose life cycle involves transitioning from non-flagellated multiplicative intracellular forms (amastigotes) to blood-circulating non-multiplicative forms (trypomastigotes). It is mainly transmitted by an insect vector of the Triatominae subfamily, although other modes of transmission (blood transfusion, organ transplant, and congenital transmission) have gained importance over the last decades. It is characterized by two stages: acute, and chronic. During the acute stage, which lasts up to two months after infection, the patients might present or mild, nonspecific, or no symptoms. This phase is followed by a chronic stage where parasites can be primarily found inside specific tissues. Decades after infection, signs and symptoms of damage to target organs, mainly the heart, gastrointestinal tract, and brain appear in 20–30% of chronically infected individuals [1,4].

Currently, two drugs have been approved for the treatment of Chagas disease: benznidazole (BNZ) and nifurtimox. The first-line treatment, BNZ, is a nitroimidazole that generates radical intermediates via the reduction of its nitro group, which covalently bind to macromolecules under aerobic and anaerobic conditions [5]. Cure rates are high when BNZ is administered during the acute phase [6]; however, in the chronic stage the cure rate is estimated to be less than 10% [7]. Some authors differ about this percentage owing to the variability in sensitivity of the tests that are used to establish cure criteria [8,9]. BNZ is associated with a variety of adverse reactions including allergic dermatitis, hypersensitivity syndrome, gastric pain, anorexia, insomnia, vomiting, which ultimately lead to withdrawal in 12–18% of the patients [10]. Additionally, the BENEFIT (Benznidazole Evaluation for Interrupting Trypanosomiasis) trial could not prove that the standard treatment with BNZ can prevent disease progression [11].

BNZ has been classified as a class IV drug (low solubility, low permeability) in the Biopharmaceutics Classification System (BCS) [12]. It has an apparent volume of distribution (*V*ₐ) of 0.56 L/kg, and reactive products of its metabolism [13]. Such *V*ₐ and low permeability values across biological barriers could result in difficulties for BNZ to reach intracellular amastigotes.

The encapsulation of BNZ within nanoscale pharmaceutical carriers has been proposed as a strategy to reduce toxicity and improve efficacy [13]. Incorporation of drugs into nanoscale vehicles could result in changes in its absorption, distribution, metabolism, and excretion, which in turn could translate into improved efficacy and diminished BNZ toxicity. For example, BNZ-loaded nanoparticles could accumulate in the site of inflammation delivering the drug in the surroundings of their molecular target. In addition, nanocarriers may pass through the cell membrane via endocytosis to avoid BNZ efflux via the P-glycoprotein efflux pump [14-16], thus delivering the drug more efficiently. Many developments have been made in the past years resulting in lipid formulations such as liposomes, solid lipid nanoparticles (SLNs), and nanoemulsions, which increased the apparent solubility of BNZ and its efficacy against parasites [17]. Remarkably, oil-in-water nanoemulsions improved the trypansomoidal activity against trypomastigotes compared to that of the free drug [18]. Among the aforementioned nanosystems, SLNs have recently gained special attention owing to their biocompatibility properties, biodegradability, relatively easy surface and composition modification, and efficacy in loading and delivering active principles [19]. SLNs comprise a lipid core, solid at 25 °C, stabilized by steric effects with a surfactant. The addition of small amounts of a liquid lipid at 25 °C leads to the improvement of SLNs in terms of sustained drug release and encapsulation efficiency (EE%), enabling the development of nanostructured lipid carriers (NLC) [20].

Here, we resort to NLC encapsulating BNZ, describing the preparation, physicochemical and biopharmaceutical characterization, and in vitro evaluation against *T. cruzi* intracellular and blood circulating forms. Interestingly, our formulation achieves a higher cumulative release and considerable higher activity against amastigotes compared to previously reported BNZ-loaded NLCs. Moreover, we report the dose-response intrinsic activity of myristyl myristate, a relatively common constituent of NLCs, against *T. cruzi*, which might be of future interest to other researchers working in the field.

**Results and Discussion**

**Formulation and physicochemical characterization of NLC-BNZ**

Nanoparticle formulations were prepared by the ultrasonication method and named as NLC-BNZ or NLC-VEHICLE, in that
order, depending on whether they contained BNZ or not. Stable homogeneous formulations were prepared. The encapsulation efficiency of NLC-BNZ was considerably high for the lipid formulations, reaching approx. 80%. The theoretical drug loading was 2.5%. Our results were in concordance with the encapsulation results of a previous study by Vinuesa et al., involving different types of nanoparticles and BNZ, including SLN and NLC [21]. The NLC-BNZ formulation was analyzed using transmission electron microscopy (TEM) to confirm the presence of nanoparticles showing a spherical morphology and a narrow distribution of sizes (Figure 1). Image analysis through ImageJ [22] software showed a mean particle size of 150 ± 13 nm.

Accordingly, the hydrodynamic diameter of the nanoparticles measured by dynamic light scattering (DLS) was in the 100–200 nm range (≈150 nm), with a moderate distribution of sizes as indicated by a polydispersity index (Pdi) of 0.204. The zeta potential (ζ) was measured by Doppler anemometry, and it was found to be around −13 mV.

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed to determine the thermal stability and melting/recrystallization processes of the components after drug encapsulation. Overlaid DSC thermograms are shown in Figure 2, whereas the melting temperature (Tm), the enthalpy of fusion (∆Hf), and crystallinity index (CI) are presented in Table 1. Whereas BNZ showed an endothermic peak at its melting point (191.2 °C) [23], the formulation showed two endothermic peaks in the range of 40–50 °C, which could be referred to the melting points of the lipid and the surfactant, respectively. This suggests that no other endothermic changes occur to the formulation constituents or its load during the high-energy sonication procedure. A peak matching the phase transition peak of BNZ did not appear in the nanoparticle thermogram, indicating that BNZ was dispersed within the lipid matrix [24]. Correlating with the lower enthalpy of fusion, the CI (%) value of the nanoparticles was lower than that of the bulk myristyl myristate. Lipid molecules could be less ordered in the nanoparticles than in the bulk material, considering the disarrangement caused by the incorporation of the drug and the surfactant. For that reason, it might require less energy to melt in comparison to the pure crystalline substance [25].

Thermogravimetric curves of myristyl myristate, poloxamer 188, and BNZ showed one thermal degradation process, whereas NLC-BNZ and NLC-VEHICLE presented two events (Figure 3). That was also observed in the derivative curves. The weight loss process for the lipid started at 180 °C and finished at 320 °C. The poloxamer 188 thermogram showed a decomposition process starting at 300 °C and ending at 410 °C, and BNZ degradation occurred in the 190–300 °C range. Considering these processes, nanoparticle thermal behavior might be attributed first to lipid degradation, and second to poloxamer weight loss.

The attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) technique was used to analyze the nano-
Figure 3: Thermogravimetric (A) and derivative thermogravimetric (B) curves of BNZ, myristyl myristate, poloxamer 188, NLC-VEHICLE, and NLC-BNZ.

Figure 4: ATR-FTIR spectra of BNZ, myristyl myristate, poloxamer 188, NLC VEHICLE, and NLC-BNZ.

Table 1: Thermal properties of benznidazole (BNZ), myristyl myristate, poloxamer 188, and nanoparticles (NLC-VEHICLE and NLC-BNZ). Abbreviations: $T_m$, melting temperature; $\Delta H_f$, fusion enthalpy; CI (%), crystallinity index.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_f$ (J/g)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNZ</td>
<td>191.2</td>
<td>142.3</td>
<td>100</td>
</tr>
<tr>
<td>myristyl myristate</td>
<td>42.9</td>
<td>239.4</td>
<td>100</td>
</tr>
<tr>
<td>poloxamer 188</td>
<td>54.2</td>
<td>148.8</td>
<td>100</td>
</tr>
<tr>
<td>NLC-VEHICLE</td>
<td>41.4–51.7</td>
<td>61.1</td>
<td>12.7</td>
</tr>
<tr>
<td>NLC-BNZ</td>
<td>40.9–50.0</td>
<td>59.4</td>
<td>12.4</td>
</tr>
</tbody>
</table>

particle surface composition and determine the possible interactions among the formulation components (Figure 4). The BNZ spectrum presented its characteristic peaks at 3264 cm$^{-1}$ corresponding to N–H in the secondary amide bond, 1652 cm$^{-1}$ to C=O in the amide, 1523–1400 cm$^{-1}$ to N–H flexion in the amide (1500–1400 cm$^{-1}$ is also the absorption range of the C=C in the benzyl group), 1357 cm$^{-1}$ to the symmetric vibration of R–NO$_2$, and 1141 cm$^{-1}$ to C–N in the imidazole ring [26]. Myristyl myristate displayed peaks at 2913 and 2848 cm$^{-1}$ corresponding to C–H of alkane, 1731–1184 cm$^{-1}$ to C=O and C–O stretching of ester groups, respectively. The peak at 1467 cm$^{-1}$ was associated with the deforming vibrations of the C–H of alkanes [27]. The characteristic peaks of poloxamer 188 were at 3600 cm$^{-1}$ relative to the O–H stretching, the intense peak at 2873 cm$^{-1}$ corresponding to C–H stretching of alkanes, another intense peak at 1105 cm$^{-1}$ to the symmetric stretching of C–O–C, and 964–833 cm$^{-1}$ to asymmetrical and symmetrical stretching of C–C–O, respectively [28]. The NLC-BNZ spectra showed myristyl myristate and poloxamer characteristic peaks (overlapping of the most intense peaks in the 3000 cm$^{-1}$ region – 2910 cm$^{-1}$, 2883 cm$^{-1}$, and 2854 cm$^{-1}$ – due to the presence of the lipid and surfactant). In contrast, the spectra did
not show peaks that could be linked to BNZ, suggesting that drug molecules were not on the nanoparticle surface but rather dispersed into the lipid matrix [24].

Structural analysis was performed by selecting different angular regions from the small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS) patterns. The WAXS patterns (Figure 5) showed contributions of diffraction peaks from BNZ, myristyl myristate, and NLC. The nanostructured lipid carriers showed contributions from both the isolated myristyl myristate and additional Bragg peaks at 19.1° and 23.3° corresponding to the copolymer. This indicates that there was phase segregation, most likely a core–shell structure with the lipidic phase inside and the hydrophilic part of the copolymer in the outer part of the NLC. Myristyl myristate major peak positions expressed in terms of \( d \)-spacing were 4.1 and 3.8 Å, corresponding to a family of the \( \beta' \) polymorph [29] and did not change after NLC synthesis or BNZ addition. Furthermore, there were no contributions from the crystalline phase of BNZ within NLC because of its small quantity or to dissolution inside the NLC.

Figure 5: WAXS patterns for BNZ, myristyl myristate, poloxamer 188, NLC-VEHICLE, and NLC-BNZ.

The long period Bragg diffraction peaks for Myristyl myristate could be observed in SAXS patterns at the \( q \) range between 0.15 and 0.2 Å\(^{-1} \) (Figure 6). Bare myristyl myristate confirmed the presence of a \( \beta' \) polymorph with long period \( d \)-spacing of 3.99 (001) and 3.47 nm (002), while in the NLC only the 3.47 nm of \( d \)-spacing peak remains. Also, in the NLC systems, the main Bragg peak was wider, attributed to a nanosized crystal effect where the estimated crystallite average sizes were 94 ± 5 nm and 101 ± 5 nm for NLC-VEHICLE and NLC-BNZ, respectively, using the Scherrer approximation. However, a broadening of the lower part of the main peak in the NLC-BNZ samples suggests defects in the structure, probably due to the inclusion of BNZ in the formulation. At smaller angles, the copolymer on the surface exhibited a lamellar-like structure [30]. The Lorentz/Kratky plot (\( q^2 I(q) \) vs \( q \)) is shown in Figure 7, where the peaks of NLC and NLC-BNZ remained at the same position, independently of the presence of the BNZ load. The linear correlation function was obtained by using the following transformation (Equation 1) [31,32]:

\[
K(z) = \frac{\int_0^{\infty} I_{\text{norm}}(q) q^2 \cos(qz) dq}{\int_0^{\infty} I_{\text{norm}}(q) q^2 dq}
\]

where \( I_{\text{norm}} \) is the normalized intensity after removing the myristyl myristate (i.e., MM) contribution: \( I_{\text{norm}} = I_{\text{NLC}}(q) - I_{\text{background}} - c(I_{\text{MM}}(q) - I_{\text{background}}) \), \( c \) being a constant or weighted proportionality between phases. From this transformation the lamellar period obtained from the first maximum of the oscillation was 12.6 nm for both systems (Figure 7). In contrast with amphiphilic low-weight loading, BNZ is a lipophilic molecule that did not change the structure of the copolymer. Thus, it is proposed to be dissolved in the core of the lipidic nanoparticle.

Drug release and physical stability

The release profiles (Figure 8) showed that 78% of the free drug was dissolved in the first 15 min of the experiment. In contrast, during the first 15 min only about 12% of the drug was released from the NLC formulation. An initial burst release was observed, followed by a sustained release. This phenomenon could be explained in part by considering the presence of free drug molecules in the formulation (around 20% of the initial drug load) and in part by the release of drug molecules located...
near the surface of the NLC, which rapidly diffuse out of the vehicle. The slow increase of the drug concentration in the release medium observed after the initial stage could be attributed to the gradual release of drug molecules from the matrix core, where the drug is mainly located according to X-ray diffraction (XRD) results [33]. Remarkably, although our NLC possess a comparatively lower drug load, the maximal accumulated drug release is higher than that of similar systems previously reported [21].

The in vitro release data were fitted to different mathematical models. The model that best adjusted the data was the Korsmeyer–Peppas model followed by a first-order model (Supporting Information File 1, Table S1). The Korsmeyer–Peppas model, also called power law, was initially used to describe drug release in polymeric systems where the two predominant mechanisms were relaxation of the polymer chains and diffusion. In this model (Equation 2), \( M_t/M_\infty \) is the fraction dissolved, \( K \) is a constant that incorporates structural and geometrical information, and the exponent \( n \) is the diffusional or transport exponent, that provides information about the release mechanism. However, it can also be viewed as a generalization for the explanation of two different drug release mechanisms that could coexist [34]. The mechanism that dominates the release can be inferred through the value of the release exponent \( n \). For spherical systems, \( n \) will take a value of 0.43 for drug release governed by Fickian diffusion, a value of 1 for zero-order release, and intermediate values for intermediate behavior, often regarded as anomalous transport. In our case, the estimated value of \( n \) was 0.56, suggesting mixed release mechanisms at play with a strong contribution of diffusion. As in our case there is no polymer relaxation involved, it may be hypothesized that the burst effect could be slightly affecting the global kinetics of the process [35]. Although this description has its limitations, it has been widely used to describe drug release from similar lipidic formulations [35-38].

\[
\frac{M_t}{M_\infty} = Kt^n
\]

The mean particle size, PdI, zeta potential, and encapsulation efficiency were selected as parameters to follow the physical stability of the nanoparticle dispersion for six months under the selected storage conditions (refrigerator at 4 °C) (Figure 9). Based on these results, the formulation could be stored at 4 °C for at least three months without losing its initial properties in terms of size; polydispersity and encapsulation efficiency values remained unaltered during the storage period, and the zeta potential parameter started at \(-10\) mV and ended up at \(-15\) mV after six months. Dynamic light scattering analysis of the formulations revealed nanoparticles with a hydrodynamic diameter in the 100–200 nm range (\( \approx 50 \) nm) starting at 146 nm and slightly increasing in the third month up to 155 nm. The Z-average parameter was chosen to report the nanoparticles size. The size values were consistent with the TEM image analysis. The surface charges required to achieve a good dispersion of nanoparticles stabilized by electrostatic repulsion are around \( \pm 30 \) mV [39]. The zeta potential (\( \zeta \)) value of our formulation was \( \approx 14 \) mV. Although this value is not optimal for stabilization by electrostatic repulsion, it still contributes with a positive aspect, as high-negative \( \zeta \) values may impede cellular uptake [40]. On the other hand, it was observed that the nanoparticulated systems remained stable after six months with no precipitation. This suggests that in this case the stabilization is not achieved...
by means of surface charge alone, but also by the steric repulsion after adding a non-ionic surfactant [41].

Cytotoxicity and hemolytic activity

Cytotoxicity assays using the tetrazolium 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide salt method (MTT) showed that Chinese hamster ovary cells (CHO) viability was affected by BNZ concentration in a dose-dependent manner (Figure 10). Interestingly, the cell viability for NLC-VEHICLE or NLC-BNZ at the same tested concentrations of free BNZ resulted in values above 80% in all cases, suggesting a decreased cytotoxic effect. That decrease in toxicity generated by NLC-BNZ, in comparison with free BNZ, could be attributed to the release profile of BNZ from NLC, exposing cells to lower doses of BNZ during the first stages of cellular division. This is a remarkable result, as toxic effects of BZN are a major cause of treatment discontinuation in the clinical setting [42]. Additionally, cytotoxicity was evaluated in the Vero cell line by flow cytometry, where the percentage of dead cells labeled with propidium iodide (PI, Supporting Information File 1, Figure S2) was measured. Neither the drug-loaded or unloaded NLCs elicited significant toxicity in Vero cells.
papers evaluated the hemolytic activity (HA) of nanoparticles after 2, 3, or 5 h of incubation [43–45]. The standard methods to test hemolytic activity of nanoparticles (ISO/TR 7406 or ASTM E2524-08 standard) established that biomaterials that induce a critical hemolytic ratio of <5% can be considered safe for biological applications [46]. In our study, it was observed no hemolytic effects for BNZ, NLC-VEHICLE, and NLC-BNZ at different concentrations after 3 and 24 h of incubation (data not shown). However, some hemolytic activity was observed for NLC-VEHICLE and NLC-BNZ after 48 h of incubation (Figure 11). Despite NLC-BNZ showed 4.8% HA at the highest concentration, the formulation could still be considered safe according to the regulations. In fact, hemolytic activity could be caused by several reasons, including the ageing of the blood sample after 48 h of incubation with the concomitant release of hemoglobin, but also by the presence of surfactants that could destabilized the erythrocyte membrane [47]. On the other hand, the differences between NLC-VEHICLE and NLC-BNZ, the latter exhibiting a higher HA, could be explained by adding the HA of the free drug to the effect of the vehicle on erythrocytes. More studies would be necessary to investigate the effect of the composition, size, or porosity of these nanoparticles after a long term exposure to blood samples as was described for other type of nanoparticles [45]. Our results suggest that the reported NLC-BNZ formulations are hemocompatible [43].

**In vitro antiparasitic activity**

As shown in Figure 12A, free BNZ displayed a clear dose-dependent effect on *T. cruzi* trypomastigotes (with an EC$_{50}$ of 6.07 µM), whereas the NLC-BNZ and NLC-VEHICLE also exhibited a dose-response behavior despite comparatively large variability across replicates. While for free BNZ the estimated EC$_{50}$ value was 6.07 µM with similar reported values (6.04 µM [48]) for the same parasite classification (TcI), the NLC-BNZ presented a full trypanocidal effect at concentrations higher than 5 µM (10 µM). A similar observation was found for the empty particles (NLC-VEHICLE) suggesting that the formulation itself possesses intrinsic toxicity on *T. cruzi* trypomastigotes. A separate assay of the individual constituents of the formulations was thus performed, demonstrating that myristyl myristate, at a
relatively low concentration, has a negative effect on parasite viability (dose-response studies for myristyl myristate against amastigotes are shown in Supporting Information File 1). This may imply that myristyl myristate cannot be considered, in our case, as a pharmacologically inert constituent in our formulation. Instead, it should be considered as a pharmaceutical active ingredient based on its intrinsic effects against *T. cruzi*.

The dose-response effects of BNZ, NLC-VEHICLE, and NLC-BNZ on *T. cruzi* amastigotes were also evaluated (Figure 12B), and the corresponding EC<sub>50</sub> were calculated. Benznidazole and NLC-BNZ presented inhibition of the intracellular growth of the parasites even at the lowest concentration, with no significant differences observed between the treatments. Benznidazole and NLC-BNZ EC<sub>50</sub> values were 3.15 and 3.33 µM, respectively. In agreement with the in vitro trypanocidal assay, NLC-VEHICLE also displayed intrinsic anti-amastigote activity with an EC<sub>50</sub> value of 10.29 µM. This was unexpected, although not necessarily a negative outcome, having in mind that our formulation displayed reduced cytotoxicity against mammal cells. Isolated myristyl myristate lipid was also tested against amastigotes, showing a reduced amastigote density at effective concentrations (Supporting Information File 1, Figure S1). Neither P188 nor GTCC-LQ displayed any effect against trypomastigotes or amastigotes up to 50 µM. Of note, the more efficacious NLC encapsulating BNZ previously reported in the literature [21] had an EC<sub>50</sub> against amastigotes of 17.6 µM. The higher efficacy of our system may be explained by a higher maximal drug release and/or by the intrinsic activity of myristyl myristate, which adds to that of BNZ.

A hypothesis about the intrinsic toxicity of our nanoscale vehicle on *T. cruzi* may be linked to a modification of glycosylphosphatidylinositols (GPIs). Glycosylphosphatidylinositols are the main anchor complexes used by protozoans to bind to cell surface proteins. It covalently attaches to the C terminus of a protein connecting it to the outer leaflet of the lipid bilayer [49]. *Trypanosoma brucei* predominant membrane protein variant surface glycoprotein (VSG), which is involved in parasite host immune system evasion, is anchored by a GPI that requires myristate for its synthesis. Analogs of myristate have shown toxicity towards the parasite [50]. *T. cruzi* trypomastigotes connect mucin (a surface molecule implicated in parasite virulence) to the membrane through a GPI which is synthesized exclusively with a C16 fatty acid [51], though a C14 fatty acid incorporation could be toxic to the parasite. Experiments in *T. brucei* indicated that the specificity of fatty acid incorporation depends on chain length [52]. The lipid in our formulation is an ester of fatty acids that could hypothetically interrupt the anchoring of mucin to the lipid bilayer in *T. cruzi*, thus rendering the parasites non-viable. However, further studies are required to test this hypothesis.

**Conclusion**

Among the spectra of nanoformulations encapsulating BNZ that exist to date, the nanoparticles presented in this work might be considered a novelty in terms of the lipid and manufacturing technique of choice. We achieve physical stability for at least six months with acceptable particle size, PdI, and EE%. Complementary to these results, TEM images showed a spherical configuration. Thermal and crystallographic experiments indi-
cated that BNZ was dispersed into the lipid matrix. The formulations showed a sustained drug release profile for 24 h, achieving a maximal accumulated release above 74% during 24 h. The release profile was adequately fitted to the Korsmeyer–Peppas model with an estimated release exponent of 0.56, suggesting a mixed mechanism of release with a dominant Fickian behavior. In vitro experiments on T. cruzi trypomastigotes and amastigotes showed similar performances against the intracellular form of the parasite when comparing encapsulated and free BNZ. Surprisingly, the empty nanoparticles exhibited activity against the parasite, which was later attributed to one of the constituents of the formulation, myristyl myristate. This may explain why our formulations exhibited increased performance against T. cruzi compared with other previously reported BNZ-loaded NLC. It would be interesting to study the effect of other lipids on the parasite to optimize the efficacy of the formulations based on a potential additive or synergistic effect of BNZ and the formulation itself. Remarkably, the cytotoxicity effect on host cells was lower for the BNZ-loaded nanoparticles compared to that of the free drug, showing a possible benefit for the use of our formulation.

**Experimental Materials**

Benznidazole (Lot #MKCD5602, purity ≥ 97%) and Kolliphor®P188 (poloxamer 188) were purchased from Sigma-Aldrich. Myristyl myristate (Crodamol™ MM, melting range = 36–40 °C), and the oil (Crodamol™ GTCC-LQ, a mixture of fully saturated triglycerides, melting point = −5 °C) were kindly donated by Croda Argentina. All reagents used in the preparation and analysis of the formulations were of analytical grade and were obtained from different commercially available sources.

**Formulation of benznidazole-loaded nanostructured lipid carriers**

BNZ-loaded NLCs were obtained via ultrasonication as previously described in Scioli-Montoto et al. (2022) [53]. Solid myristyl myristate (2% w/v, 400 mg) was melted in a water bath at 60–70 °C. The oil (40 μL) was added to the melted lipid phase simultaneously with BNZ (10 mg). The aqueous phase was prepared by dissolving 600 mg (3% w/v) of poloxamer 188 (poly(ethylene glycol)-block-polypoly(polyethylene glycol)) in 20 mL of ultrapure water (Milli-Q®, Millipore, MA, USA) and was preheated at the same temperature as the melted lipid in the water bath. After 30 min of thermostatization, the aqueous solution was poured over the lipid phase, and ultrasonication was carried out for 20 min at an 80% amplitude using an ultrasonic processor (130 Watts, Cole-Parmer, USA) equipped with a 6 mm titanium tip. After the sonication process, NLC-BNZ were obtained by leaving the hot suspension to cool to room temperature. The remaining volume was then measured.

**Measurement of the encapsulation efficiency**

Concentration of the free drug in the dispersion medium was measured to calculate the encapsulation efficiency (EE%). For this, 500 μL of the formulation was placed in Microcon® centrifugation filters (MWCO = 10000, Merck Millipore, Billerica, MA, USA) and centrifuged at 10000 rpm for 15 min. The amount of BNZ was estimated by performing a high-performance liquid chromatography (HPLC) analysis of the filtrate. Considering the initial amount of BNZ added to the formulation, the EE% was calculated as follows:

\[
EE(\%) = \frac{M_0 - (C_{\text{free}} \cdot V_f)}{M_0} \times 100
\]

where \(M_0\) is the initial amount of BNZ added to the formulation, \(C_{\text{free}}\) is the drug concentration of the filtrate (i.e., the free drug concentration) in μg/mL, and \(V_f\) is the volume after ultrasonication (mL).

The theoretical drug loading (DL%) was calculated as follows:

\[
DL(\%) = \frac{\text{Mass of drug incorporated (mg)}}{\text{Lipid mass (mg)}} \times 100
\]

**HPLC analysis of benznidazole**

Chromatographic separation was achieved by HPLC (Gilson SAS, Villiers-Le-Bel, France) via UV detection. A Platinum EPS C8 (150 mm × 4.6 mm, 5 μm, Grace™, Columbia, MD, USA) column was used; the mobile phase consisted of a mixture of methanol and 0.02% phosphoric acid solution (60:40) for a final pH of 2.5. The system was operated isocratically at a 1.0 mL/min flow rate and the detection was performed at 324 nm. The volume of injection was 20 μL.

**In vitro benznidazole release assay**

The release of BNZ from the nanoparticles was performed in a rotating paddle apparatus (Vision Classic 6, Hanson Research, Chatsworth, CA, USA) at 75 rpm using 500 mL of KH₂PO₄ buffer (pH 6.8) as the dissolution medium. The bath temperature was set at 37.0 ± 0.5 °C. A volume of 5 mL of each formulation was placed in a pre-hydrated dialysis membrane (MWCO 10 kDa) and submerged into the dissolution vessels. A solution of free BNZ at the same concentration was used as control. At 0, 5, 10, 15, and 30 min, and at 1, 2, 3, 4, 5, 6, 7, and 24 h, 1 mL
of the dissolution medium was taken from the vessel. Samples were analyzed by HPLC as described above. Experiments were performed in triplicate and the mean values were used for data analysis. The data were fitted to mathematical models of drug release (i.e., First order, Hopfenberg, Baker–Lonsdale, Korsmeyer–Peppas, and Hixon Crowell) via the DDSolver complement developed by Zhang et al. and available for Excel® [54]. The model that best fitted the data according to the goodness-of-fit measures ($R^2$, $R^2$-adj, MSE, and AIC) was chosen.

**Particle size, zeta potential and polydispersion index**

Nano ZS Zetasizer (Malvern Instruments Corp, Worcestershire, UK) was used to measure particle size distribution and mean diameter by DLS at 25 °C in polystyrene cuvettes with a thickness of 10 mm. The zeta potential was determined by Doppler anemometry using the previously described equipment. As an estimation of the distribution of particle sizes, the polydispersion index was determined. All experiments were carried out in triplicate, except for the particle size estimation, which was measured six times.

**Physical stability**

The mean particle size, PdI, zeta potential, and encapsulation efficiency were measured to assess the physical stability of the nanoparticle dispersion during storage at 4 °C protected from light. Physical parameters (e.g., particle size, PdI, zeta potential) were measured by DLS and EE% was measured by HPLC, once a month, during a six-month period.

**Differential scanning calorimetry analysis**

Thermal analysis of BNZ, myristyl myristate, poloxamer 188, and NLC-BNZ was performed by differential scanning calorimetry (DSC Q2000, TA Instruments, New Castle, DE, USA) under an inert atmosphere of dry nitrogen (50 mL·min⁻¹). A standard aluminum pan containing approximately 5 mg of the dry sample after freeze drying the formulations was used. Scans were run in the range from 0 to 250 °C at a heating rate of 10 °C/min.

The degree of crystallinity (% crystallinity index, CI) was calculated using the following equation [55]:

$$\text{CI(%) = } \frac{\Delta H_{\text{NLC aqueous dispersion}}}{\Delta H_{\text{bulk material}} \times \text{Concentration}_{\text{l lipid phase}}} \times 100 \quad (5)$$

where $\Delta H_{\text{NLC}}$ and $\Delta H_{\text{bulk material}}$ are the melting enthalpies (J·g⁻¹) of the NLC dispersion and the bulk lipid, respectively. The concentration of the lipid phase was 2%.

**Thermogravimetric analysis**

Thermogravimetric analysis was performed to assess the thermal stability of BNZ, myristyl myristate, poloxamer 188, and NLC-BNZ on a TGA Q500 apparatus (TA Instruments, New Castle, DE, USA). Freeze dried formulations of approximately 10 mg were accurately weighed in a platinum pan. Measurements were performed from room temperature to 600 °C at a heating rate of 10 °C/min under nitrogen atmosphere to avoid thermo-oxidative degradation.

**Attenuated total reflection Fourier-transform infrared spectroscopy**

Fourier-transform infrared spectroscopy spectra were obtained. The attenuated total reflection mode was used to record the spectra over the range of 400–4000 cm⁻¹ at a resolution of 2 cm⁻¹.

**Transmission electron microscopy**

Transmission electron microscopy images were captured using a Jeol-1200 EX II-TEM microscope (Jeol, MA, USA). A drop (10 µL) of the nanoparticle dispersion previously diluted (1:10) with ultrapure water was spread onto a collodion-coated Cu grid (400 mesh). Excess liquid was drained with filter paper. A drop of phosphotungstic acid was added to the dispersion for contrast enhancement.

**X-ray diffraction structural analysis**

Small angle X-ray scattering/wide angle X-ray scattering measurements were performed using a XEUSS 2.0 equipment (XENOCS, France). Patterns were registered with two synchronous 2D photon-counting pixel X-ray detectors for SAXS Pilatus 200k (DECTRIS, Switzerland), and a Pilatus 100k (DECTRIS, Switzerland) placed 159 mm from the sample with a tilted angle of 36 °C for WAXS. The SAXS measurements were performed using two samples to detect distances, 1194 and 337 nm, to obtain a wide angular range. The scattering intensity, $I(q)$, was recorded by means of the scattering momentum transfer $q$, where $q = 4\pi\lambda \sin(\theta)/\lambda$, 2θ is the scattering angle and $\lambda = 0.15419$ nm is the weighted average of the X-ray wavelength of the Cu Kα1,2 emission lines. Owing to the small beam size pointed at the sample (< 1 mm × 1 mm) smearing effects were not considered. The NLC samples were placed under vacuum between Kapton® tapes. The measurements were done in transmission mode. The SAXS/WAXS patterns were taken for 10 min each.

**Cell toxicity assay on CHO cells**

The viability of CHO cells was analyzed by the reduction of the tetrazolium salt to a formazan product (i.e., the MTT method). A 96-well polystyrene microplate containing 1 × 10⁴ cells per well of CHO cells (obtained from the American Type Culture
Collection, Manassas, VA, USA) were cultured in Ham’s F12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Notocor Laboratories, Cordoba, Argentina) and antibiotics (50 IU penicillin and 50 µg/mL streptomycin) (Bagó Laboratories, Buenos Aires, Argentina) in a humidified atmosphere with 5% CO₂. After 24 h, the cells were incubated with increasing concentrations of RPMI as control, BNZ, NLC-VEHICLE, and NLC-BNZ (0, 15, 31, 62, and 125 µg·mL⁻¹). The MTT reagent (5 mg·mL⁻¹ in phosphate-buffered saline (PBS)) was then added for 3 h. Dimethyl sulfoxide (DMSO,100 µL per well) was added under agitation for 10 min to dissolve the MTT. The color was measured in a microplate reader (MultiskanTM GO spectrophotometer, Thermo Fisher Scientific) at 550 nm. The assays were performed in triplicate.

Cell toxicity assay on Vero cells
Cell viability was analyzed by flow cytometry as described in the “In vitro anti-amastigote effect” section after adding PI to obtain the percentage of dead cells following the incubation with the formulation of nanoparticles or the free drug.

Hemolytic effect
Hemolysis was assessed on 3 mL of a freshly drawn heparinized suspension of fresh human blood placed on a 6-well cell culture plate. Increasing concentrations of freshly prepared diluted solutions of the free drug and NLC-BNZ (1, 5, and 50 µg·mL⁻¹) were added to each well and incubated for 3, 24, and 48 h at 37 °C. Samples were then centrifuged for 5 minutes at 2500 rpm, and the absorbance of the supernatant was determined at 540 nm in a microplate reader (MultiskanTM GO spectrophotometer, Thermo Fisher Scientific). Triton X 100 (10%), saline solution, NLC-VEHICLE, and BNZ were used as the positive, negative, vehicle, and reference drug controls, respectively. The hemolytic activity was calculated as [56]:

\[
\text{HA} (\%) = \frac{A_{540 \text{ nm sample}} - A_{540 \text{ nm saline}}}{A_{540 \text{ nm Triton}} - A_{540 \text{ nm saline}}} \times 100
\]  

(6)

where \( A_{540 \text{ nm sample}} \) represents the absorbance value of the sample, \( A_{540 \text{ nm Triton}} \) the absorbance value of the positive control, and \( A_{540 \text{ nm saline}} \) the absorbance value of the negative control.

The blood was obtained from the “Institute of Hemotherapy” in La Plata, Buenos Aires, Argentina as part of a formal agreement between the “Instituto de Genética Veterinaria (IGEVET, UNLP-CONICET La Plata)” and this institution. Also, this assay protocol was approved by the National University of La Plata Ethics Committee and it was developed in accordance with the principles proclaimed in the Universal Declaration of Human Rights of 1948, the ethical norms established by the Nuremberg Code of 1947, and the Declaration of Helsinki of 1964 and its successive amendments and clarifications. Special attention was paid to Patient Rights in their relationship with health professionals and institutions and the National Law 25326 on the Protection of Personal Data.

Parasites
The \( T. \ cruzi \) strain K98 (TcI, low virulence) was used. Tissue culture trypomastigotes were obtained from the supernatants of 2- to 3-day-old infected Vero cells (African green monkey kidney epithelial cells) maintained in RPMI-1640 medium supplemented with 10% FBS (Internegocios S.A, Argentina) at 37 °C in a 5% humidified CO₂ atmosphere. Amastigotes were obtained after infecting Vero cells at a multiplicity of infection (MOI) of 1:2.

In vitro anti-trypomastigote effect
A trypomastigote suspension (1 × 10⁵ trypomastigotes per well) was co-cultured in a 96 well-plate with dilutions of both a solution of the free drug and of the nanoparticle formulations (concentration range = 1, 2.5, 5, and 10 µM) in RPMI-1640 supplemented with 5% FBS at 37 °C in 5% CO₂ atmosphere. The NLC-VEHICLE sample was tested using the same dilutions as the NLC-BNZ formulation. After 24 h of incubation, motile parasites were counted in a hemocytometer chamber under a light microscope. Controls consisted of RPMI-1640 supplemented with 5% FBS as well as RPMI-1640 with 0.1% of DMSO.

Results were expressed as mean viability of trypomastigotes (%) (regarding to RPMI-1640 + DMSO control). Experiments were performed in triplicate. The half maximal effective concentration (EC₅₀) against the trypomastigote form was determined from concentration-response curves fitted through a non-linear regression on GraphPad Prism version 8.0.1 software (San Diego, CA, USA).

In vitro anti-amastigote effect
Vero cells were infected with the trypomastigote form of GFP-expressing \( T. \ cruzi \) (K98 strain) [57] at a multiplicity of infection (MOI) 1:2. After 24 h the cells were washed with PBS, trypsinized for 10 min, and seeded onto 96-well plates (5 × 10⁴ cells/well). After the cells attached to the microplate (i.e., 2–3 h), increasing concentrations of freshly prepared dilutions of the formulations (1, 5, 10, 25, and 50 µM) or the free drug were added. After 72 h of treatment, the cells were harvested with a trypsin/EDTA solution and processed for flow cytometry analysis using a BD Biosciences FACSCANTO II Flow Cytometer (Franklin Lakes, NJ, USA). Propidium iodide (Sigma, St. Louis, USA) was added to the cell suspensions.
(50 µg/mL) for 10 min, prior to analysis. In total, 20000 events were acquired for each sample. Data analysis was performed using the FlowJo™ software (FlowJo, LCC). The EC$_{50}$ values were determined from dose-response curves fitted through a non-linear regression using GraphPad Prism version 8.0.1 software (San Diego, CA, USA). The experiments were performed in duplicates.

**Statistical analysis**

The normality of the variable distribution was assessed using the Shapiro–Wilk normality test. Comparisons of the means were performed by analysis of variance (ANOVA) followed by Tukey or Dunnet comparison tests. Statistical significance was set at $p < 0.05$.

**Supporting Information**

This file includes a summary of the goodness-of-fit measures that indicate how different mathematical models of drug release fit our experimental data.

**Supplementary Information File 1**

Supplementary material.

[https://www.beilstein-journals.org/bijnano/content-supplementary/2190-4286-14-66-S1.pdf]

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**References**


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Green SPIONs as a novel highly selective treatment for leishmaniasis: an in vitro study against *Leishmania amazonensis* intracellular amastigotes

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**Abstract**

The main goal of this work was to evaluate the therapeutic potential of green superparamagnetic iron oxide nanoparticles (SPIONs) produced with coconut water for treating cutaneous leishmaniasis caused by *Leishmania amazonensis*. Optical and electron microscopy techniques were used to evaluate the effects on cell proliferation, infectivity percentage, and ultrastructure. SPIONs were internalized by both parasite stages, randomly distributed in the cytosol and located mainly in membrane-bound compartments. The selectivity index for intracellular amastigotes was more than 240 times higher compared to current drugs used to treat the disease. The synthesized SPIONs showed promising activity against *Leishmania* and can be considered a strong candidate for a new therapeutic approach for treating leishmaniasis.

**Introduction**

Leishmaniasis is one of the most important neglected diseases of chronic nature and remains a serious global health problem. A worrying increase has been observed in the number of leishmaniasis cases worldwide in recent decades. It is estimated that about 600 million people live in risk areas, and 0.6–1.2 million new leishmaniasis cases appear annually [1]. The treatment for
this disease involves pentavalent antimonials, miltefosine, amphotericin B, paromomycin, or pentamidine. However, side effects of these drugs and an increased number of drug-resistant parasites have been reported [2-5]. These facts demonstrate the need to develop new treatments or alternatives that are safer, more effective, and more accessible to patients.

In this context, nanomedicine is one of the most promising branches of contemporary medicine, currently concentrating a large part of the scientific effort on the search for new treatments for different diseases. Its main objective is to develop therapies with higher specificity, effectiveness, and safety, as well as less toxicity [6]. One interesting class of nanomaterials in medicine are superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs exhibit theranostic properties, that is, they can be used simultaneously for diagnosis and therapy. Thus, SPIONs have emerged as one of the best options for the development of new therapeutic methods. SPIONs offer several features such as good biocompatibility, degradability under moderate acid conditions, the ability for magnetic manipulation, the possibility of being used in magnetic resonance imaging, and the ability to generate controlled heat non-invasively when exposed to an alternating magnetic field [7,8]. In 2019, our group published an article describing a low-cost green synthesis of SPIONs using coconut water [9]. In this article, the ability of macrophages to uptake these SPIONs was evaluated, together with some physical and chemical characterizations. The synthesized green SPIONs are around 4 nm in diameter, are composed of pure nonstoichiometric magnetite, exhibit superparamagnetic behavior at room temperature, and are taken up by macrophages without being toxic for these mammalian cells [9].

The application of SPIONs in treating leishmaniasis has been studied by different groups over the past few years, showing promising and satisfactory results [10-13]; thus, using SPIONs to develop new topical treatments can mean a revolution. SPIONs could be used for topical application, associated with drugs and combined or not with thermotherapy by magnetic hyperthermia. Furthermore, the treatment can be applied to the localized cutaneous lesion, making the treatment more specific and less toxic to the patient. Thus, the main goal of this study is to evaluate the effects of green SPIONs against Leishmania amazonensis (L. amazonensis) in vitro.

Results

Uptake of SPIONs by L. amazonensis promastigotes and intracellular amastigotes

Bright-field optical microscopy of L. amazonensis promastigotes and intracellular amastigotes incubated with Prussian blue revealed that both parasite stages can uptake the SPIONs (Figure 1). The arrows and arrowheads in Figure 1 show the characteristic blue stain that indicates the positive reaction between potassium ferrocyanide and ferrous compounds. In promastigotes (Figure 1A,B), the SPIONs are distributed throughout the cytosol. In contrast, in the intracellular amastigotes cultivated in macrophages, the SPIONs appear in the mammalian cytosol, inside the parasitophorous vacuole, and in the parasite cytosol (Figure 1C,D).

After the first microscopic analysis, scanning electron microscopy and chemical element mapping analysis were carried out to confirm the uptake of the SPIONs by L. amazonensis intracellular amastigotes after removing the plasma membrane to expose the cytoplasmic environment (Figure 2). Secondary electron imaging revealed intracellular amastigotes inside the parasitophorous vacuoles (Figure 2A). Backscattered electron imaging showed several small electron-lucent structures randomly distributed throughout the macrophage cytosol, inside the parasitophorous vacuoles (Figure 2B, arrows), and in the intracellular amastigotes (Figure 2B, arrowheads). The ferrous nature of the observed structures was assessed by chemical element mapping analysis using energy-dispersive X-ray spectroscopy (Figure 2C), confirming that the electron-lucent structures contain iron atoms (Figure 2D).

Transmission electron microscopy (TEM) was used to confirm the internalization of the SPIONs. First, promastigotes were treated with 100 µg/mL of SPIONs for 24 h (Figure 3A–C). TEM images confirmed the presence of SPION aggregates randomly distributed throughout the cytoplasm of the promastigotes (Figure 3A–C, arrowheads). The images suggest that these aggregates have different sizes. Furthermore, at high magnification, it is possible to observe that the SPIONS are frequently surrounded by membranes (Figure 3B, arrows). In addition, SPIONs were also observed inside the flagellar pocket (Figure 3C, arrowheads) and closely associated with the membrane.

The uptake of SPIONs was also observed in macrophages infected with L. amazonensis intracellular amastigotes after treatment with 100 µg/mL of SPIONs for 24 h (Figure 3D–F). The images confirmed the presence of SPION aggregates inside the macrophage cytosol, the parasitophorous vacuoles, and the intracellular amastigotes (Figure 3C,D, arrowheads). SPIONs were also observed inside the macrophages close to the parasitophorous vacuole membrane (Figure 3D, large arrow), sometimes appearing inside membrane-bound structures and exhibiting different sizes (Figure 3E, arrowheads). Some alterations in amastigote ultrastructure can also be observed, namely electron-lucent lipid bodies, a multivesicular body close to the Golgi.
complex, and endoplasmic reticulum profiles very close to organelles such as mitochondrion and glycosome. Higher magnification revealed that the SPION aggregates are constituted of small nanoparticles that appear associated with tiny filaments (Figure 3F, thin arrow).

Antiproliferative effects of SPIONs in *L. amazonensis* promastigotes and intracellular amastigotes

The analysis of the antiproliferative effects of SPIONs in *L. amazonensis* promastigotes showed that they could not alter the growth for any of the concentrations evaluated (Figure 4A). In contrast, the SPIONs were very active against intracellular amastigotes (Figure 4B). Furthermore, analysis of the growth curve shows a statistically significant reduction in the percentage of infection for all tested concentrations of SPIONs (1, 5, 10, 25, and 50 µg/mL) and treatment times (24, 48, and 72 h) when compared with the control of infected macrophages. After the first 24 h of treatment, it was possible to observe a reduction in the percentage of infection of about 50% for a concentration of 1 µg/mL and of about 90% for 50 µg/mL of SPIONs. The data revealed a concentration-dependent effect, which increased within 48 and 72 h of treatment. The percentage of infection significantly reduces over time, indicating a time-dependent effect. The IC$_{50}$ values were calculated for each treatment time and confirmed the results obtained (Figure 4B), that is, 1.206, 0.848, and 0.668 µg/mL for treatment times of 24, 48, and 72 h, respectively.

Evaluation of possible effects on the ultrastructure of *L. amazonensis* intracellular amastigotes

Transmission electron microscopy allowed us to analyze ultrastructural alterations induced by treating *L. amazonensis* intracellular amastigotes with 100 µg/mL of SPIONs for 24 h (Figure 5). The images revealed several alterations, namely...
Figure 2: Scanning electron microscopy of macrophages infected with L. amazonensis intracellular amastigotes after treatment with 100 µg/mL SPIONs for 24 h. The plasma membrane was gently removed to observe the presence of nanoparticles inside the cells. Panel A shows infected macrophages with some amastigotes (arrowheads) inside the parasitophorous vacuoles (thin arrows). Panel B shows the same macrophage; however, the image was obtained by detecting backscattered electrons, revealing several electron-lucent aggregates (arrows). Digital magnification (highlighted rectangular area) showed electron-lucent aggregates even inside intracellular amastigotes (arrowheads). Panels C and D show the X-ray microanalysis mapping of infected macrophages, indicating the presence of iron in the cytosol (red color in Figure 2D).

Discussion

SPIONs represent a new approach to diagnosing and treating diseases, particularly when associated with magnetic hyperthermia, an emerging form of active treatment [14-18]. However, despite all their potential, the synthesis processes of the SPIONs are characterized by being expensive and toxic to humans and the environment [6]. In this scenario, our group demonstrated the therapeutic potential of low-cost biocompatible SPIONs produced by green synthesis [9]. The present study

(1) lipid bodies (Figure 5A–C, thin arrows), (2) cytoplasmic disorganization with many vacuoles, which may indicate activation of autophagic processes (Figure 5A–C, arrows), (3) myelin-like figures (Figure 5A, arrowhead), and (4) mitochondrial swelling (Figure 5C, star). Furthermore, in the intracellular amastigotes, there are membrane-bound compartments containing SPION aggregates and parasitophorous vacuoles containing cellular debris and dead amastigotes (Figure 5D, triangle).
Figure 3: Transmission electron microscopy of *L. amazonensis* promastigotes and intracellular amastigotes treated with 100 µg/mL of SPIONs for 24 h. Electron-dense aggregates of SPIONs (arrowheads) are randomly distributed in both developmental stages. (A) SPIONs (arrowheads) were observed in the promastigote cytosol, closely associated with endoplasmic reticulum profiles and lipid bodies. (B) High-magnification image with SPION aggregates (arrowheads) inside membrane-bound compartments (arrows). (C) SPIONs (arrowheads) are associated with thin filaments inside the flagellar pocket and in the cytosol closely associated with the flagellar pocket membrane. (D) In the macrophages infected with intracellular amastigotes, the SPIONs appear inside the parasitophorous vacuole and in the macrophage and parasite cytosol (arrowheads). In this image, it is also possible to observe the SPIONs surrounded by a membrane (arrows) and an aggregate close to the membrane of the parasitophorous vacuole (large arrow). (E, F) High-magnification images of intracellular amastigotes revealing SPIONs (arrowheads) inside membrane-bound compartments (arrows). The aggregates are formed by smaller individual nanoparticles (small arrow). Figure 3E also shows many lipid bodies, vacuoles, and a multivesicular structure, which are features typically found in treated parasites. F, flagellum; FP, flagellar pocket; LB, lipid body; M, mitochondrion; N, nucleus; and PV, parasitophorous vacuole.
aimed to evaluate in vitro the therapeutic potential of SPIONs produced with coconut water to treat cutaneous leishmaniasis caused by *L. amazonensis*.

Microscopy techniques efficiently revealed the uptake and distribution of SPIONs in *L. amazonensis* promastigotes and intracellular amastigotes. The first analysis confirmed the uptake of SPIONs by macrophages, which was published previously by our group [9]. Furthermore, in the article here, the images revealed SPIONs inside the parasitophorous vacuole and in the cytosol of intracellular amastigotes. In addition, SPIONs were also observed randomly distributed throughout the cytosol of promastigotes, in the flagellar pocket, and inside membrane-bound structures. It is the first time that superparamagnetic iron oxide nanoparticles SPIONs are observed inside the *Leishmania* spp and the parasitophorous vacuole. Chemical element mapping analysis by scanning electron microscopy confirmed the ferrous nature of the nanoparticle aggregates. These results prove the ability of both promastigotes and intracellular amastigotes to uptake SPIONs from the culture medium.

The acquisition of iron by *Leishmania* intracellular amastigotes that live inside mammalian host cells is important for cell differentiation and the pathogenesis of the disease [19-21]. Thus, it is possible to speculate that SPIONs use iron transport mechanisms to reach the parasitophorous vacuole and amastigote...
We evaluated the antiproliferative effects of SPIONs in *L. amazonensis* promastigotes and intracellular amastigotes. Despite being internalized by promastigotes, SPIONs did not affect the cell proliferation of the parasites (Figure 4A). A completely different result was observed for intracellular amastigotes, where the reduction in the percentage of infection was very significant already with the lowest concentration of SPIONs used [1 µg/mL] (Figure 4B). The IC_{50} values found for intracellular amastigotes during the treatment were 1.206, 0.848, and 0.668 µg/mL for treatment times of 24, 48 and 72 h, respectively. In a previous study published by our group, we analyzed the cytotoxicity of SPIONs against macrophages [9]. The results revealed no toxic effects up to a concentration of 300 µg/mL, indicating that SPIONs are well tolerated by cytosol [21]. However, further studies need to be carried out to confirm this hypothesis and to elucidate the mechanisms of SPION uptake in promastigotes and amastigotes.
Table 1: Estimated CC50 and SI obtained after the analysis of the macrophage cytotoxicity assay previously published in [9] using the GraphPad Prism software.

<table>
<thead>
<tr>
<th>Time</th>
<th>Estimated cytotoxic concentration of 50% (CC50) for macrophages</th>
<th>Estimated selective index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1271.5 µg/mL</td>
<td>1054</td>
</tr>
<tr>
<td>48 h</td>
<td>2250.6 µg/mL</td>
<td>2654</td>
</tr>
<tr>
<td>72 h</td>
<td>3420.0 µg/mL</td>
<td>5119</td>
</tr>
</tbody>
</table>

Table 2: Selectivity index values for different compounds and drugs studied and used for treating leishmaniasis.

<table>
<thead>
<tr>
<th>Time</th>
<th>Compound</th>
<th>SI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>amphotericin B</td>
<td>16</td>
<td>[26]</td>
</tr>
<tr>
<td>48 h</td>
<td>TC95</td>
<td>24</td>
<td>[23]</td>
</tr>
<tr>
<td>48 h</td>
<td>KH-TFMDI</td>
<td>81</td>
<td>[22]</td>
</tr>
<tr>
<td>72 h</td>
<td>itraconazole</td>
<td>103.17</td>
<td>[25]</td>
</tr>
<tr>
<td>72 h</td>
<td>ravuconazole</td>
<td>28.9</td>
<td>[24]</td>
</tr>
<tr>
<td>72 h</td>
<td>miltefosine</td>
<td>34.2</td>
<td>[27]</td>
</tr>
</tbody>
</table>

Iron homeostasis has been extensively studied because of its essential role in maintaining the cellular functions of several cell types. It is well established that, in mammalian cells, iron in its free state can participate in the Haber–Weiss reaction, catalyzing the formation of highly reactive hydroxyl radicals that lead to oxidative stress [28,29]. Thus, one of the possibilities for the observed antiproliferative effects could be the result of an imbalance in iron homeostasis with the consequent induction of oxidative stress and death of the parasites. However, further studies need to be carried out to confirm this hypothesis. In *Leishmania*, it is well known that available iron has an important influence on the homeostasis of reactive oxygen species [30]. Studies have already shown that iron excess in the diet of mice causes a decrease in the replication of *Leishmania* spp. in different tissues of infected animals due to the interaction with reactive oxygen and nitrogen species [31,32]. Several studies have shown the potential of using nanoparticles as a new method for treating leishmaniasis. However, only a few studies report the effects of using iron oxide nanoparticles [11,12,15,33-35]. Recently, the effects of magnetic iron oxide nanoparticles were demonstrated in *L. mexicana* axenic amastigotes. First, the amastigotes were treated with 200 µg/mL of magnetic nanoparticles. Subsequently, magnetic hyperthermia was applied using an alternating field of 30 mT with a frequency of 452 kHz for 40 min. The results showed that magnetic hyperthermia was efficient in killing *L. mexicana* axenic amastigotes [12]. Another study demonstrated the anti-*Leishmania* effect of magnetic nanoparticles synthesized by green chemistry in *L. major* promastigotes [35]. Finally, a study showed the effect in vitro and in vivo of amphotericin B encapsulated in magnetic iron oxide nanoparticles coated with glycine-rich peptides for treating visceral leishmaniasis caused by *L. donovani* [12]. All these studies demonstrated the potential gain of drug conjugation with magnetic nanoparticles for treating leishmaniasis.
Conclusion
The use of SPIONs synthesized with coconut water to treat macrophages infected with *Leishmania amazonensis* intracellular amastigotes revealed a significant anti-*Leishmania* effect with a selectivity index more than 240 times higher than those of other currently used drugs. Furthermore, it was also observed that the SPIONs could be directed into the parasitophorous vacuoles of infected cells and parasites. Thus, this new nanomaterial is a promising new therapeutic alternative as (1) an active treatment agent because of its intrinsic properties, (2) a treatment agent associated with heating through alternating current magnetic fields, and (3) a drug carrier.

Finally, SPIONs can be considered a strong candidate for a new therapeutic approach to treating cutaneous leishmaniasis, that is, an accessible and low-cost topical treatment.

Experimental

**SPIONs**

The SPIONs used in the present study were synthesized as described in [9] (patent application registration BR 10 2020 015814 [36]). For assays, after synthesis and purification, the SPIONs were dispersed in a 70% ethanol solution (Merck, Germany). The maximum ethanol concentration in cultures did not exceed 0.5%, which did not interfere with cell growth. The nanoparticles used in the biological tests were stored at −20 °C.

**Ethics committee for the use of laboratory animals**

The assays that used mammalian macrophages and parasites from animal models were approved by the Ethics Committee for the Use of Laboratory Animals (CEUA) of the Centro de Ciências da Saúde from the Universidade Federal do Rio de Janeiro according to the Brazilian Federal Law (11794/2008, Decreto No. 6,899/2009). For the use of peritoneal macrophages resident in mice and the maintenance of *Leishmania amazonensis* species in Balb/C mice, the protocol number was UFRJ/CCS-142/21. Furthermore, all animals received human care according to the guide published by the Brazilian Society of Zootechnics of Laboratory and Council National Control of Animal Experimentation.

**Cell culture**

The immortalized murine macrophages RAW 264.7 were grown in 25 cm² bottles in RPMI 1640 medium (Cultilab, Brasil) supplemented with 2% sodium bicarbonate, 10% fetal bovine serum, and 100 U/mL penicillin. Cells were cultured at 37 °C in 5% CO₂ atmosphere, and the medium was changed three times a week; cells were passed when they reached confluence in the bottles. In addition, primary cultures of murine macrophages were obtained from the peritoneal cavity of C57Bl/6 mice by washing with Hanks’ balanced solution. Then, they were plated on coverslips in a 24-well culture plate and placed to adhere for 24 h at 37 °C in an atmosphere of 5% CO₂. For the microscopic analyses, macrophages were grown in 25 cm² bottles or on glass coverslips in 24-well plates; after 24 h of culture, they were treated for 24 h with different SPION concentrations. This study used the WHOM/BR/75/JOSEFA *Leishmania amazonensis* strain as a standard model for cutaneous leishmaniasis. The parasites were maintained according to previously published protocols [22].

**Prussian blue staining**

For staining with Prussian blue (Sigma-Aldrich, Germany), promastigote and intracellular amastigotes were treated with 100 µg/mL of SPIONs for 24 h. The promastigotes (control and treated cells) were washed in phosphate-buffered saline (PBS) pH 7.2 and adhered for 10 min on glass coverslips previously coated with poly-L-lysine (Sigma-Aldrich, Germany). The intracellular amastigotes were obtained after infection of RAW 264.7 macrophages at a ratio of ten parasites to one macrophage. After treatment, cells were washed in PBS pH 7.2, fixed, and dehydrated, as described in [9]. Finally, cells were observed using a DM2500 optical microscope (Leica Microsystems, Germany) in bright-field mode.

**Electron microscopy analysis**

Control and treated cells were washed in PBS pH 7.2, fixed, and post-fixed according to previously published protocols [23]. Then, cells were processed for scanning electron microscopy and chemical element mapping analysis as described in [9]. The micrographs were obtained using a TESCAN VEGA 3 LMU scanning electron microscope operating at 20 kV equipped with an OXFORD X-MaxN 20 mm² detector (Oxford Instruments, United Kingdom) for energy-dispersive X-ray spectroscopy. For transmission electron microscopy, after fixation, samples were dehydrated in increasing acetone concentrations and embedded in Epon. Ultrathin sections were obtained using a PT-PC PowerTome ultramicrotome (RMC Boeckeler, USA) stained with uranyl acetate and lead citrate and observed using a FEI TECNAI SPIRIT transmission electron microscope operating at 120 kV.

**Antiproliferative effects of SPIONs in *Leishmania amazonensis* promastigotes and intracellular amastigotes**

To evaluate the effect of the SPIONs on the growth of *L. amazonensis* promastigotes, cell density experiments were initiated with an inoculum of 1.0 × 10⁶ parasites/mL in M199 culture medium supplemented with 10% fetal bovine serum and cultivated at 25 °C. After 24 h of growth, different concentra-
tions of SPIONs (1, 5, 10, 50, and 100 μg/mL) were added, and cells were cultured for 96 h. The cell density was calculated every 24 h by counting the number of cells in a Neubauer chamber using contrast-phase light microscopy. Besides, SPIONs were also evaluated against intracellular amastigotes, the clinically relevant stage of leishmaniasis. For this analysis, murine macrophages and parasites were obtained as previously published [23]. After 24 h of the initial infection, different concentrations of SPIONs (1, 5, 10, 25, and 50 μg/mL) were added, and the medium with the nanoparticles was changed every day for three days. The IC$_{50}$ was calculated using the linear regression method defined in [37].

Statistical analysis
Statistical analysis was conducted using GraphPad Prism with one-way analysis of variance (ANOVA). The results were considered statistically significant for cases of $p \leq 0.05$ (*).

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Nanotechnological approaches in the treatment of schistosomiasis: an overview

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Review

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Abstract

Schistosomiasis causes over 200,000 deaths annually. The current treatment option, praziquantel, presents limitations, including low bioavailability and resistance. In this context, nanoparticles have emerged as a promising option for improving schistosomiasis treatment. Several narrative reviews have been published on this topic. Unfortunately, the lack of clear methodologies presented in these reviews leads to the exclusion of many important studies without apparent justification. This integrative review aims to examine works published in this area with a precise and reproducible method. To achieve this, three databases (i.e., Pubmed, Web of Science, and Scopus) were searched from March 31, 2022, to March 31, 2023. The search results included only original research articles that used nanoparticles smaller than 1 µm in the treatment context. Additionally, a search was conducted in the references of the identified articles to retrieve works that could not be found solely using the original search formula. As a result, 65 articles that met the established criteria were identified. Inorganic and polymeric nanoparticles were the most prevalent nanosystems used. Gold was the primary material used to produce inorganic nanoparticles, while poly(lactic-co-glycolic acid) and chitosan were commonly used to produce polymeric nanoparticles. None of these identified works presented results in the clinical phase. Finally, based on our findings, the outlook appears favorable, as there is a significant diversity of new substances with schistosomicidal potential. However, financial efforts are required to advance these nanoformulations.

Introduction

Schistosomiasis is a disease common in tropical countries caused by trematodes from the genus Schistosoma. More than 220 million people are affected by this disease, in addition to 800 million at risk of infection [1,2]. Every year, 200 thousand deaths are caused by schistosomiasis, making it the third most devastating tropical disease in the world after malaria and...
intestinal parasitosis [3]. After penetration of the skin by the larval form (cercariae), the schistosomes mature and migrate through the lung to the liver, gut, or bladder, depending on the species, where they elicit a marked immune response. The adult *Schistosoma mansoni* worms mate in the liver and lay eggs in the mesenteric venules of the intestine [4]. Nowadays, the only treatment available for this disease consists of praziquantel (PZQ) [5].

Praziquantel is a class II compound according to the biopharmaceutical classification system (BCS), so it has low solubility and high permeability in the gastrointestinal tract [6]. This drug is affected by the first-pass effect on the liver, which also impacts its bioavailability [6]. Unfortunately, this makes PZQ ineffective against young forms of *Schistosoma mansoni*, leading to concerns about the emergence of resistant strains. Indeed, reports of resistance have been documented worldwide, prompting research for alternative treatments or new approaches to improve the characteristics of PZQ [7]. Additionally, due to the first passage effect, high doses of PZQ are required, resulting in large tablet sizes, making its administration challenging for children, as no PZQ pediatric formulation is distributed by the World Health Organization (WHO). Consequently, people split adult PZQ tablets to treat children, but the bitter taste of PZQ makes it difficult for them to adhere to treatment [8]. Moreover, high dosages of PZQ have been associated with side effects such as abdominal pain, nausea, and allergy [9]. In this context, nanotechnological tools are being investigated as potential solutions to address all these issues related to PZQ and bring new treatment alternatives [10].

Nanotechnology involves the creation and use of materials and technologies at the nanoscale, while nanomedicine focuses on the application of nanotechnology to treat, monitor, and prevent diseases [11]. Nanomedicine uses nanocarriers to enhance drug delivery by ensuring that drugs are delivered in appropriate amounts to specific target areas and remains in the body for the necessary duration [12]. As a result, nanoparticles have been utilized mainly as drug delivery systems in various parasitic diseases, including schistosomiasis, to improve bioavailability, therapeutic efficacy, and decrease adverse effect profiles of the drugs used to treat such illnesses [13].

A few recent reviews provide a general overview of how nanotechnological tools are used in schistosomiasis treatment. However, most of these published works are narrative reviews limited to a specific drug or nanoparticle categories. For instance, some reviews only focus on PZQ [14], while others solely showcase nanosystems for drug delivery [15]. Nonetheless, recent literature reveals several works that employ various drugs and utilize nanoparticles not only as delivery systems but also with intrinsic action. Moreover, some of the previous works were not so clear about the methodology followed to include and exclude articles in their narrative review.

That said, the purpose of this work is to produce an integrative review of the theme with a well-defined research methodology, highlighting the main nanoparticles and drugs used in the literature to treat schistosomiasis.

### Results and Discussion

We found 65 available articles that met the requirements, 75% \((n = 49)\) were found in databases, while the remaining 25% (16 articles) were found through reference scanning. Table S1 (Supporting Information File 1) summarizes all the articles found regarding the use of nanosystems and encapsulated drugs. In Figure 1, it is possible to observe that only 59% of the publications show effectiveness data solely in vivo. Also, most articles use nanoparticles as drug delivery systems (82%), and most of them encapsulated PZQ (Figure 2). Polymeric (23%) and inorganic (20%) nanoparticles were used in the majority of the studies (Figure 3). Most of the papers (78%) have not done toxicity tests, and the main route of administration was the oral route (Figure 4).

![Figure 1: Percentage of articles that conducted effectiveness tests. The graph represents the number of articles out of the 65 found that conducted efficacy testing: solely in vitro, solely in vivo, both in vitro and in vivo tests, or not done.](image-url)
Nanosystems

Polymeric nanoparticles are nanoparticles composed of polymeric materials which may be natural or synthetic [16]. They are generally produced by two strategies: the dispersion of preformed polymers or the polymerization of monomers [17]. The first one is more commonly describe in the literature, and the techniques usually employed to produce them include nanoprecipitation, solvent evaporation, emulsification/solvent diffusion, and emulsification/reverse salting out [18,19]. The main advantages of using this type of nanoparticles as nanocarriers are their potential use for drug controlled release, the ability to
protect drugs and other molecules with biological activity against the environment, improvement of their bioavailability and therapeutic index [17].

These nanocarriers are divided into two types: nanocapsules and nanospheres [18]. Nanocapsules consist of reservoir systems with an oil or water core and an external polymeric shell. They are, overall, used to increase drug solubility [20]. Usually, once in the body, the encapsulated drug diffuses through the polymeric wall in a zero-order kinetic, that means, it constantly releases the encapsulated drug [17]. In opposition, nanospheres are matrix systems formed by polymers without a central core. During the administration, the matrix erodes and the drug diffuses, resulting in a first-order kinetic drug release, that is, an exponential drug release [17,20].

Our research found that many articles utilized poly(lactic-co-glycolic acid) (PLGA) and chitosan nanoparticles, especially because they are biocompatible polymers and present great biodegradability. The polymer PLGA is approved for clinical use by Food and Drug Administration since 1989 [21] and, although no human data attests to chitosan safety, many animal tests prove its safety [22]. Eudragit L 100 is another polymer commonly used in literature because of its biocompatibility [8]. Overall, it is used when a delayed release is required. It is derived from polyacid, and because of that, it is resistant to low pH values [23]. So, when nanoparticles with this material are orally administered, they resist against gastric secretions and release the drug in the intestine. This protects many drugs and increases their bioavailability [24].

Inorganic nanoparticles (IN) are derived from metals or silica [25]. The most common metal used in the production of IN found in this work was gold, even though it was also possible to observe a great amount of works using silver and zinc (Figure 5). While silica-derived nanoparticles are used in the treatment against schistosomiasis due to their characteristics as drug carriers [10], metal nanoparticles usually present intrinsic action even when not loaded with drugs. Works with other parasites suggest that metallic nanoparticles may affect enzyme activity necessary to the physiology and production of the tegument [26]. Therefore, metallic nanoparticles also have a curative role against schistosomiasis. A possible explanation for this action was suggested by Dkhil et al. in their work with gold nanoparticles [27]. They suggested that their curative effects are due to antioxidant properties which confer the ability to scavenge free radicals [27]. After that, many authors related a reduction in oxidative stress markers in vivo after metallic nanoparticle administration and/or amelioration in histopathological characteristics after infection, which corroborates the first hypothesis [27-34].

Solid lipid nanoparticles (SLN) are solid lipid matrices at room and body temperature [35]. Their advantages are similar to classic nanocarriers, such as protection of labile drugs from biodegradation process, excellent excipient tolerability, and prolonged release. In addition, some disadvantages of the classic nanocarriers are not present in SLN, such as lack of biocompatibility, difficulty to produce on a large scale, and high raw material cost [36]. Many methods are used to prepare SLN, and they are divided into (1) high-energy methods, for dispersion of the lipid phase (such as high-pressure homogenization); (2) low-energy methods, which requires the precipitation of nanoparticles from homogeneous systems (such as microemulsions); and (3) methods based on organic solvents (emulsification–diffusion method) [35].

Liposomes are vesicles composed of a phospholipid and cholesterol with an aqueous core. It can have one or multiple layers. Due to that, their size can range from 30 nm to the micrometer range [37]. As drug vehicles, they exhibit unique properties, such as protection of encapsulated compounds from physiological degradation, extended drug half-life, controlled release of the drug molecule, and excellent biocompatibility and safety [38]. Liposomes can also be modified to selectively deliver a drug to a specific site. This is very valuable because it can reduce potential side effects and increase the maximum tolerated dose, which improves therapeutic benefits [39]. For example, Adekiya et al. [40] produced PZQ encapsulated in nanoliposomes whose surface was modified with an antibody against calpain, a protein found in the tegument of the parasite and is upregulated in the regions where host–parasite interaction occurs [41]. The modified nanoparticles orally administered two or four weeks postinfection altered the drug release pattern in vitro, were more efficient in reducing worm burden and the
amount of eggs in the gut than PZQ alone, and altered the 
orgram pattern with the predominant presence of dead eggs. In 
addition, the nanoformulation showed no relevant toxicity in in 
vitro and in vivo models. Finally, the author discusses the possi-
bility that the nanoformulation could be used to treat cases of 
Schistosomiasis in the brain due to its smaller size [40]. Never-
theless, it is noteworthy that oral administration of biodegrad-
able nanoparticles, such as conventional liposomes, exposes 
them to degradation by stomach acid, bile salts, and enzymes. 
Consequently, in in vivo models, intact liposomes may encoun-
ter challenges to reach the bloodstream owing to the adverse 
conditions of the stomach [42]. This elucidates why, in in vitro 
tests, the author exclusively assessed the release pattern of praz-
iquantel by liposomes, omitting an examination of the impact of 
intact nanoparticles on the parasite, a facet explored by other 
researchers. Importantly, it is well known in the literature that 
modifications can be made to conventional liposomes to render 
these nanoparticles resistant to gastrointestinal barriers, rep-
resenting another avenue of opportunity for the presented 
nanoformulation [42].

Niosomes are nanosystems similar to liposomes but formed 
using non-ionic surfactants like Span 60 [43]. They also could 
incorporate cholesterol in their structure beyond other lipids 
such as liposomes [44]. Therefore, they are able to be used as a 
carrier of amphiphilic or lipophilic drugs [45]. The main advan-
tages of using this type of system are that they are osmotically 
active and stable and increase the stability of the entrapped 
drug. They could be used in oral, parenteral, and topical routes, 
and they are biodegradable, biocompatible, and non-immuno-
genic [45]. Moreover, they improve the therapeutic perform-
ance of the drug by protecting it from the biological environ-
ment and restricting effects to target cells, thereby reducing the 
clearance of the drug [45].

Drugs for treatment of schistosomiasis

Praziquantel

It is not surprising that praziquantel is the most encapsulated 
drug. It remains the only effective frontline medicine to treat the 
disease, and is currently characterized by its exclusive and ex-
tensive use as an important antischistosomal drug [46]. Howev-
er, PZQ also brings disadvantages, such as occurrence of resis-
tant strains, low bioavailability [47], and organoleptic character-
istics such as bitter taste [29,48]. In the literature, it is de- 
scribed that nanoformulations approaches can overcome these 
drawbacks.

Most publications used nanotechnology to alter pharmacoKI-
netics parameters. The nanoformulations were evaluated 
through efficacy criteria (e.g., parasite burden, egg counts, and 
granuloma diameter) or using traditional pharmacokinetics pa-
rameters (e.g., absorption rate or bioavailability). For example, 
Labib El Gendy et al. [49] showed that PZQ encapsulated in 
liposomes (500 mg/kg) could be more efficient than free PZQ 
treatment. Similar results have been shown in other works that 
also used liposome with PZQ in different concentrations [50-
53]. In addition, Xie et al. [54] studied the pharmacokinetics of 
solid lipid nanoparticles composed of castor oil encapsulating 
PZQ. They observed that the drug took more than one week in 
vitro to be released. A pharmacokinetic study in vivo also 
showed that the PZQ concentration in the plasma was sustained 
for longer times when the nanoformulation was studied in mice. 
Thus, the results show that solid lipid nanoparticles increase 
bioavailability in all administration routes tested (oral, subcuta-
neous, and intramuscular). However, results showed that subcu-
taneous delivery was superior to oral and intramuscular, 
promoting the longest therapeutic concentration in the circula-
tion (264h) and the highest bioavailability.

There is just one formulation of PZQ developed for pediatric 
use which is commercially available: Epiquantel (40 mg/kg), a 
liquid formulation produced by Eipico, an Egyptian pharmacy 
industry. However, this medicine is not distributed by WHO 
and, thus, few works tried to use nanotechnology to change the 
organoleptic properties of PZQ [8,48]. da Fonseca et al. [8] 
used poly(methyl methacrylate) nanoparticles loaded with PZQ 
produced by in situ mini emulsion polymerizations to mask the 
drug taste and develop an oral formulation. Although the taste 
was masked, the authors reported a gritty tongue sensation 
caused by the high solid content of the formulation. In vitro 
results were satisfactory and showed that the nanoformulation 
was effective against parasites, but in vivo results were inade-
quate due to fluctuations in the administered dose. Despite that, 
the work showed that this nanoformulation could be used in the 
future [8]. In another work, Gonzalez et al. [48] increased the 
dissolution of PZQ by producing nanocrystals through high-
pressure homogenization, followed by drying through spray-
drying. After that, they resuspended the powder in Oral plus® 
and Oral Sweet®, which are suspension vehicles known for 
their sweet taste and suitability for pediatric formulations [48].

Finally, few works tried to combine PZQ nanoformulations 
with other drugs/treatments [52,55]. Frezza et al. [52] tested 
PZQ-liposomes (oral route, 100 mg/kg) with hyperbaric oxygen 
and observed that it reduced the number of worms in mice. 
The combination also reduced the oviposition, changed the 
orgram pattern, and caused alteration in parasite tegument [52]. 
Eissa et al. [55] proved that a nanoformulation combining PZQ 
(250 mg/kg) and miltefosine (20 mg/kg) was efficient against 
all stages of the parasites, including juvenile forms. It was also 
noted alterations in parasite tegument and a reduction in granu-
ломatos reactions in murine liver.
Although finding new forms to improve PZQ characteristics is essential, it would not solve the problem once only this drug is available to treat the disease and resistant strains are described. Therefore, finding new approaches with new drugs is crucial to raise treatment possibilities. Despite the fact that PZQ is the most encapsulated drug and most of the reviews about schistosomiasis only focus on it, we would like to bring to this review other drugs that are being studied.

**Plant-derived drugs**

After PZQ, most of the works in the literature involved plant-derived compounds. Guimarães et al. [56] tested the efficiency of episipiloturine in vitro and the best way to extract this molecule from leaves. Episipiloturine is an imidazole alkaloid found in jaborandi leaves (*Pilocarpus microphyllus*), which has known activity against adult, young, and egg forms of *Schistosoma mansoni* [57]. Since this is an apolar molecule with poor solubility, the author proposed a nanosystem using liposomes to make this molecule more useful in schistosomiasis therapy. The results showed that episipiloturine (300 µg/mL) has an effect in vitro, but it is not superior to PZQ. However, other nanotechnological approaches can potentize the effect of the drug. Therefore, further studies should be made. Furthermore, the results showed that episipiloturine was not toxic to mice peritoneal cells, which is an encouraging prognosis for the development of future products [56].

Curcumin is a naturally yellow pigment obtained from the rhizomes of *Curcuma longa*. In the literature, many articles explore anti-inflammatory, antioxidant, antiviral, anti-infectious, and antitumoral properties of curcumin [58]. Mokbel et al. [59] showed that curcumin associated with a half-dose of PZQ and gold nanoparticles reduced the worm load in infected mice more than PZQ alone. This information is crucial since most side effects presented by patients who use PZQ could be avoided if there was a way to reduce the drug dose. Despite that, the combination could not reduce egg count more than that with PZQ alone. Nonetheless, the author affirms in the presentation of their results that the combination is more effective than the use of PZQ alone in this aspect [59].

Luz et al. [58] showed that curcumin encapsulated by polymeric nanoparticles could kill 100% of adult worms in vitro at a concentration of 100 µM. Lower concentrations reduced motility and caused tegumental alterations and couple separation [58]. However, curcumin has low bioavailability and poor water solubility. Thus, Aly et al. [60] tried to increase its solubility and permeability through the cellular membrane by making a nanoemulsion of *Curcuma longa* extract (i.e., the curcumin plant source). The nanoemulsion showed an effect against adults of *S. mansoni* in vitro (especially males). This is an interesting finding because data from the literature reports that females are usually more susceptible to drug action than males. However, in this work, the death of females was only possible in a high concentration of the nanoformulation (100 µg/mL). Every dosage tested was also effective against young forms (esquistosomules) [60].

El-Menawy et al. [61] used thymoquinone, a bioactive compound isolated from *Nigella sativa*, encapsulated in chitosan nanoparticles. The nanoformulation reduces the worm load in mice by 60% (predominantly female) and the number of couples found in vivo. Although the results showed a clear difference between control groups and the groups treated with nanoparticles, the author considered the results not good enough since other works showed a more prominent reduction [62].

Regarding egg counts in the liver and intestine, the nanoformulation was more efficient than blank particles which shows the relevance of nanoparticle for drug delivery. The histopathological exam also showed that nanoparticles could reduce the number and size of granulomas and diminish changes caused by infection. Although these results are very promising and interesting, the author does not mention the way in which the formulation was administered, which prevents a more critical analysis by the reader [61].

Elawamy et al. [63] also used *N. sativa* in their work, but instead of using one specific compound, they used the whole plant extract from this plant and encapsulated it in chitosan nanoparticles. The results showed that it is possible to diminish the worm load and change the oogram pattern in mice using the oral nanof ormulation alone or with PZQ. Furthermore, the nanoparticle alone had a more significant effect than that for when the extract was administered with PZQ regarding granuloma formation, reducing the number and diameter of granulomas. Thus, *N. sativa* extract associated with chitosan nanoparticles may be a pharmacological strategy to replace PZQ or to help lower its dosage. However, the author admits in this work that no data proves the biological safety of using chitosan in a nanoformulation [63].

While on the subject, other extracts of vegetal sources were also studied to treat schistosomiasis by using a nanotechnological approach. A method using ultracentrifugation and ultrasonic dispersion produced ginger (*Zingiber officinale*) extract-derived nanoparticles which an average size of 238.3 nm [64]. The author justified his choice to use this kind of nanoparticles, claiming that they are less expensive than conventional ones, and in the literature, they were already used to treat inflammatory diseases [65,66]. Data proved that these nanoparticles orally administered in mice reduced the worm load, but not more than PZQ or mefloquine. However, when ginger-derived nanoparti-
icles were combined with a half-dose of mefloquine, the reduction in worm load was 100% even in a short time of infection (6 weeks). This combination also causes a reduction of 100% in hepatic and intestinal egg counts in the same period, in addition to showing a hepatoprotective effect conserving the typical tissue structure. Regarding granuloma formation, the combination was also efficient, although another combination using ginger-derived nanoparticles and PZQ caused a more significant effect than the total dose of PZQ. In addition to this, ginger-derived nanoparticles alone or combined with other drugs were able to cause alterations in parasite tegument [64].

Another work used carvacrol, a monoterpen present in essential oils derived from plants such as Origanum vulgare. Besides being commonly used as a flavoring agent in food and cosmetics, it shows antimicrobial activity. Xavier et al. [67] reported that nanoemulsions with carvacrol orally administered were able to reduce worm burden and eggs in feces more than PZQ in the prepatent period (21 days post-infection). This impressive result shows that this nanoformulation is more efficient in juvenile forms. The author also suggests that the mechanism by which the nanoemulsion could reduce the worm burden is its antimicrobial activity, connecting changes in microbiota with the response to parasites. However, the mechanism of action of carvacrol remains unknown [67].

Repositioned drugs

Works utilizing compounds repositioned from other diseases have also been found in our search. Miltefosine, for example, is a drug created to treat cutaneous metastasis from mammary carcinomas [68]. After that, it was also approved to treat leishmaniasis [69,70], and in 2011, Eissa et al. [71] verified that the drug has activity against different forms of S. mansoni in vivo. After that, the same group, in 2015, developed lipid nanoparticles positively charged (cationic) and tested them with and without oleic acid as a membrane permeabilizer in the composition. Both nanoformulations were able to reduce the whole treatment of schistosomiasis in mice to one single oral dose (20 mg/kg) [72]. In 2016, it was shown that despite both nanoformulations being effective, the formulation without oleic acid was more effective when administered on the first day of infection. On the other hand, oleic acid nanoparticles were more effective when administered 21 days after infection [73]. Late in 2020, while cationic lipid nanoparticles were hemolytic [72], the same group tested lipid nanoparticles with oleic acid on the membrane and miltefosine (20 mg/kg) alone or combined with PZQ. They reported that nanosystems containing miltefosine with or without PZQ were potent (when orally administered in mice) against all forms of S. mansoni, including juvenile forms. These nanosystems caused alterations in parasite tegument and reduced granulomatous reaction in the liver [55].

When administered by oral route in mice, celecoxib, a traditional non-steroidal inhibitor of cyclo-oxygenase used as an anti-inflammatory, analgesic, and antipyretic drug, was also effective against juvenile forms of S. mansoni when associated with solid lipid nanoparticles causing damage to parasite tegument [74].

Spironolactone is a diuretic drug mainly used to treat hypertension. Abd El Hady showed in vitro that polymeric nanoparticles were able to confer a sustained biphasic release pattern in comparison with that of spironolactone alone. Moreover, they proved in mice that orally administered nanoformulation was efficient against S. mansoni infection and induced significant reduction in spleen, liver indices, and total worm count, and it induced decline in the hepatic and small intestinal egg load. Finally, it also caused extensive damage to adult worms on tegument and suckers, leading to the death of the parasites in less time compared to that for the drug alone, and improve liver pathology [75].

Other compounds

Some of the selected works used new synthetic compounds in their formulation for schistosomiasis treatment. For example, 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (BphEA) is a compound with poor solubility in water, which has demonstrated potential to be used in schistosomiasis treatment. Araújo et al. [76] developed a cationic nanoemulsion to increase solubility. This nanoemulsion increases efficiency in vitro, causing the death of female worms within three hours, alterations in tegument within 48 hours, and reduced male worm motility. A hypothesis suggested by the author is that the charge of nanoemulsion interacts with a negatively charged group in the tegument of parasites, facilitating drug delivery [76].

Articles utilizing synthetic drugs that were once used to treat schistosomiasis but, for safety reasons, were discontinued have also been found. Tartar emetic, for example, was part of the first class of compounds used to treat schistosomiasis [77]. However, due to their low therapeutic index and the rise of less toxic new drugs, it was discontinued. de Melo et al. [78] proved that pegylated liposomes could reduce toxicity and mortality of tartar emetic in mice even in high concentrations (27 mg Sb/kg). Although the mortality was reduced, drug efficiency remains unaltered, especially when the nanoformulation was intraperitoneally administered [78]. However, it is known that oral route adhesion is better than the others tested in this work (intraperitoneal and subcutaneous). Therefore, drug dosage forms with these characteristics may present compliance issues and problems with commercialization. Thus, an interesting pathway could be testing the same nanoformulations but using the oral route. After antimonials such as tartar emetic,
oxamnique was released in the market, and with PZQ they remain as the drugs that can be used to treat schistosomiasis. However, signs of rising resistance to the drug slowed down the demand [78-82]. In 1997, Frézard and Melo [83] showed that liposomes with oxamnique (10 mg/kg) subcutaneously applied efficiently reduce the worm load three to 14 days after infection (with a maximum reduction of 60%) in mice. These reports indicate that nanotechnological approaches may be a hope not only for PZQ or new compounds but also for bringing back improved versions of old medicines.

Amer et al. [43] used ubiquinol, a natural inhibitor of neutral magnesium-dependent sphingomyelinase, a key enzyme in sphingomyelin breakdown. This enzyme is essential because sphingomyelin is crucial in forming the outer leaflet of the tegumental lipid bilayer membrane in *Schistosoma mansoni* [43].

Araújo et al. [84] verified the activity of the sulfated polysaccharide α-ol-glucan, a polysaccharide naturally found in extracts of lichen from *Ramalina celastri*. This work shows that liposomes with this carbohydrate could eliminate adult worms from infected mice 56 days post-infection when it was administered by the intraperitoneal route. The results also show that the nanoformulation reduced the number of eggs in feces of infected mice and hepatic granuloma in the liver. However, no difference between the nanoformulation and the controls was observed (sulfated polysaccharide α-ol-glucan administered alone and empty liposomes). Furthermore, mice treated with sulfated polysaccharide α-ol-glucan presented granulomas with histochemical markers, which could mean that this molecule stimulates the immunological system causing changes in membrane carbohydrates. Moreover, it raises the hypothesis that this change in the membrane molecule pattern is related to the reduction in granulomas. Finally, the author suggests that sulfated polysaccharide α-ol-glucan could be used with other drugs with significantly higher effects against schistosomiasis, such as PZQ and oxamnique, to stimulate the immunological system [83].

Oleic acid, a common unsaturated free fatty acid in the outer layer of human skin, is commonly used as a permeation promoter, inducing the disruption of the lipid structure of the membrane. de Oliveira et al. [85] showed in vitro that oleic acid encapsulated in polymeric nanoparticles could potentially be used in schistosomiasis treatment. Cytotoxicity assays confirm the compatibility of this fatty acid with biosystems, and in vitro results showed that nanoparticles reduced the time of action of free oleic acid in four to six hours. Oleic acid nanoparticles (50 µg/mL) caused 100% of mortality of adult worms in 24 hours, while neither empty nanoparticles nor raw oleic acid were able to yield the same mortality rate at the same time in vitro. Doses lower than 50 µg/mL were able to cause worm separation and reduce motility. Doses higher than 25 µg/mL reduced oviposition when incubated for 24 hours. The results also show that even sublethal doses can cause alterations in parasite tegument [85].

Bee venom comprises various pharmacologically active components, including melittin (constituting more than 50% of total proteins) and a mixture of enzymes, cell-lytic peptides, proteases, and bioactive amines [86]. This mixture has antioxidant, anticoagulant, anti-bacterial, immunostimulatory, and hepatotoxic protection properties [87-89]. Because of that, it has been used in traditional medicine to treat inflammation and pain [90]. Mohamed et al. [91] reported that bee venom administered in infected mice reduces worm burden, ova count/liver, and granuloma diameter [91]. However, high concentrations of bee venom increase hepatic granuloma diameter. Thus, Badr et al. [92] tried to develop a nanoformulation approach to minimize the side effects of bee venom treatment. Polymeric nanoparticles created in their work allowed a sustained release and caused extreme changes in parasite tegument. In vivo, nanoparticles could reduce worm load and granuloma diameter and induce new biliary ducts. Nanoformulation was more effective in adult females than in juvenile forms and adult males [92].

Following the aforementioned studies, the majority of them (92%) utilized nanoformulations administered via the oral route. This outcome is unsurprising, as despite potential drawbacks such as first-pass metabolism, reduced bioavailability, and drug degradation throughout the digestive tract, the oral route is widely accepted and minimally invasive [93]. Consequently, releasing a new alternative to PZQ via a different route may not be the most advisable option, as it may not be well-received by patients, leading to potential commercialization challenges associated with a less familiar or less convenient delivery method.

**Effectiveness tests**

Effectiveness tests are important to demonstrate how powerful a drug is against the parasite. In previous sections it was detailed how certain studies demonstrated the effect of tested formulations. Overall, the parameters used to measure the in vitro efficacy of the treatment are reduction in mortality and mobility, couple separation, and tegument alterations. In vivo, the main criteria used is reducing worm burden, quantity and diameter of granuloma, eggs in feces, and oxidative stress markers (e.g., glutathione, nitrite/nitrate, and malondialdehyde).

Generally, articles that do not show effectiveness data use known drugs which have its effectivity attested, and aim to increase the dissolution of the drug in vitro [48,94]. Yang et al. [94] verified that PZQ nanocrystals had a more significant
dissolution rate than that of the conventional drug due to the particle size and, consequently, it also showed a bioavailability improvement. That is because bioavailability of orally administered drugs depends on their ability to be absorbed in the gastrointestinal tract. For class II drugs (e.g., PZQ) the absorption process is limited by drug dissolution rate in gastrointestinal media. Therefore, enhancement of the dissolution rate of these drugs will present improved bioavailability [95].

Other works do not show effectiveness tests because they are focused on evaluating pharmacokinetics. Cong et al. [96] showed that PZQ nanoemulsion has sustained drug release for a long time, both in vitro and in vivo. Mishra et al. [97] demonstrated similar conclusions using PZQ associated with solid lipid nanoparticles. Malhado et al. [98] concluded that PZQ associated with PMMA nanoparticles could not improve the pharmacokinetic curve. In fact, the absorption of the encapsulated drug was three times lower than that for conventional PZQ.

Other works do not address effectiveness tests because they evaluate the impact of nanosystems in physiological/pathological changes caused by S. mansoni. Dhlil et al. [32] showed that metallic nanoparticles could decrease all intestinal changes caused by the parasite. The nanoparticles avoided weight gain in infected mice, increased glutathione levels, and reduced the levels of oxidative stress markers. This work showed that selenium nanoparticles were even more effective than PZQ, reducing inflammation signs in jejunal tissue and tissue injury levels similarly to PZQ. El-Shorbagy et al. [34] showed that the treatment with gold nanoparticles decreased the granuloma index, but with less effectiveness in comparison to PZQ at concentrations of 2.5, and 1.25 µM/mL. Overall, the nanoparticles exhibited antioxidant effects in vitro.

Toxicity tests

Toxicity testing is essential to guarantee the safety of the treatment. Most articles have dealt with cytotoxicity testing in vitro or acute toxicity testing in vivo. Others deviated from traditional methods and used genotoxicity testing and mitochondrial metabolism evaluation to assess this. However, toxicity data were not reported in most of the articles. Although no explanation has been given in the articles regarding the absence of safety tests, there are possible reasons to explain why some tests are missing. Many articles use compounds that already have their safety established (e.g., PZQ or repurposed drugs) which have been approved before and their side effects are known. This was also the case in the Amer et al. [99] article in which ubiquinol, a natural compound approved as a dietary supplement, was used. Therefore, the safety tests were deemed unnecessary. Many papers that did not provide toxicity data concerning nanosystems referred to previous articles in which safety testing was performed. However, it is important to highlight that even nanosystems that were tested before must be tested again if the study uses a different experimentation design (different drug concentrations, different methods to produce nanoparticles, or a different therapeutic scheme). Numerous articles in our research have substantiated this information. For instance, the study conducted by Amara et al. [100] in 2018 demonstrated that diverse compositions of lipid nanocapsules resulted in varying IC₅₀ values. Additionally, this research revealed that the IC₅₀ value of encapsulated PZQ was considerably higher than that of PZQ administered alone, underscoring the significance of conducting toxicity testing even for well-known drugs. That means that part of the articles selected in this review still must prove the safety of their nanoformulations. This is the only way for the product to advance to the next stages, such as clinical phase.

In fact, none of the papers in this work was in clinical trials, reflecting the small number of nanosystems that enter the clinical phase. This probably happens not only because many of these works do not present safety data, but also because of the high costs of clinical trials [101]. As a neglected disease, schistosomiasis does not have the investment necessary by the private sector. Nevertheless, schistosomiasis remains a disease with a big economic impact, especially in underdeveloped and developing nations. For example, in 2015, its impact costs US$ 41.7 million to Brazil [102].

Moreover, it is imperative to address the additional complexities associated with nanoparticle formulations. While it is evident that manufacturing nanoparticles incurs high costs, it is essential to highlight other intricacies related to these formulations. Despite none of the authors explicitly mentioning stability challenges as a concern in the nanoparticle manufacturing process, especially in tropical regions characterized by elevated temperatures and humidity, it is a critical aspect to consider. Such environmental conditions pose formidable obstacles to the effective deployment of these formulations [103]. Furthermore, upscaling presents a significant issue. As demonstrated in previous discussions, many of the articles employed production techniques that are challenging to scale up, with batch-to-batch variations further complicating the manufacturing process [104]. As a result, achieving a consistent and reproducible manufacturing process becomes a daunting task in the realm of nanoparticle formulations.

Thus, regardless of the reasons for the challenges in bringing nanoformulations to the market, the responsibility falls on the government to make concerted efforts and provide the necessary support to overcome economic and other barriers. This support is crucial for aiding research institutions in introducing
new products to the market, which can effectively mitigate the impact of the disease in those countries.

Conclusion

In this review, we selected 65 papers using three databases: Pubmed, Scopus, and Web of Science; and the reference within the selected papers. This is a great number since none of the recent reviews have brought this amount of articles on this topic [13,15,105] together. This is due to the methodology used in this paper, which included a reference scanning stage, responsible for 25% of the articles found. Moreover, our strategy allowed us to include articles not included in any of the previous reviews, proving that our method is more inclusive.

Inorganic and polymeric nanoparticles are among the most widely utilized nanotechnological systems. Most research articles utilized gold nanoparticle as inorganic nanoparticles, while PLGA and chitosan are commonly utilized to produce polymeric nanoparticles due to its biocompatibility reported in various animal studies. However, there is currently a lack of data to support the safety of chitosan formulations for human use.

Most of the articles reported superior results to PZQ in preclinical tests; however, no article was found in clinical phase. One of the reasons for that is the low financial support to treat schistosomiasis since it is a neglected disease. Nonetheless, there is big diversity of solutions with great potential to be superior to PZQ using nanotechnological resources. However, governmental investment is necessary for these nanomedicines to achieve full potential.

Experimental

Searches were done in Pubmed, Scopus, and Web of Science databases. These searches were conducted from March 31st, 2022, to March 31st, 2023, using the following search keywords: (nano* OR encapsul*) AND (treatment OR therap* OR activity OR chemotherapy) AND schistosomiasis. After obtaining the list of papers, a filter by type of article was applied, selecting only original research and excluding reviews. After that, the titles and abstracts were read, and articles unrelated to the theme were excluded. Afterward, it was checked if there was access to the remaining work. For those that could not be accessed, attempts were made to contact the authors and ask for a copy. The available articles were read entirely, and those unrelated to the theme were excluded. For exclusion, the criteria used were: (1) particle size over 999 nm; (2) articles that approach only prophylactic nanoformulations. After that, a search in the references of the selected papers was done to guarantee the maximal articles related to the theme in this review (Figure 6).

Supporting Information

As supporting information we provide Table S1 cited in the results. This table shows the articles found using our methodology.

Supporting Information File 1
Nanosystems with their encapsulated drugs found in open access articles.
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-15-2-S1.pdf]

ORCID® iDs
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References

4. Crosby, A.; Jones, F. M.; Kolosn...
Curcumin-loaded nanostructured systems for treatment of leishmaniasis: a review

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Abstract
Leishmaniasis is a neglected tropical disease that has affected more than 350 million people worldwide and can manifest itself in three different forms: cutaneous, mucocutaneous, or visceral. Furthermore, the current treatment options have drawbacks which compromise efficacy and patient compliance. To face this global health concern, new alternatives for the treatment of leishmaniasis have been explored. Curcumin, a polyphenol obtained from the rhizome of turmeric, exhibits leishmanicidal activity against different species of Leishmania spp. Although its mechanism of action has not yet been fully elucidated, its leishmanicidal potential may be associated with its antioxidant and anti-inflammatory properties. However, it has limitations that compromise its clinical use. Conversely, nanotechnology has been used as a tool for solving biopharmaceutical challenges associated with drugs, such as curcumin. From a drug delivery standpoint, nanocarriers (1–1000 nm) can improve stability, increase solubility, promote intracellular delivery, and increase biological activity. Thus, this review offers a deep look into curcumin-loaded nanocarriers intended for the treatment of leishmaniasis.
Introduction

Neglected tropical diseases (NTDs) comprise a group of 20 diseases that are caused, in most cases, by viruses, fungi, bacteria, or parasites, such as helminths and protozoa. They affect mainly women and children from impoverished communities. Leishmaniasis is a NTD that has affected more than 350 million people worldwide, with worrisome 700,000 to one million new cases annually [1,2].

This tropical disease is caused by a vector-borne protozoan parasite of the genus Leishmania which is transmitted by the bite of female sandflies. Different species of Leishmania spp. can cause specific clinical manifestations. These are (i) cutaneous leishmaniasis, which can be the localized type when the lesions are limited to certain areas of the skin, or diffuse when several lesions occur over an extensive area of skin tissue [3]; (ii) mucocutaneous leishmaniasis, which causes total or partial degeneration of the mucous membranes of the nose, mouth, and throat [4], and (iii) visceral leishmaniasis (also known as kala-azar), can cause systemic infection affecting the liver, spleen, hematogenous and lymphatic systems [5,6].

For the treatment of these infections, therapies based on pentavalent antimony (first-line drug treatment), amphotericin B, miltefosine, and paromomycin have been employed [7]. Despite being effective, these drugs cause cardiotoxicity, renal, pancreatic, and liver toxicity, and teratogenicity. Furthermore, cases of drug resistance are already well reported for antileishmanial drugs, such as the pentavalent antimonial salts [8].

Therefore, finding new therapeutic alternatives for this neglected tropical disease continues to be of utmost importance [9]. Current studies have highlighted curcumin as a promising antiparasitic alternative [10]. Curcumin (diferuloylmethane) is a yellow polyphenol extracted from the rhizome of Curcuma longa, popularly known as turmeric [11-13]. This molecule presents a high tolerance profile, and it has shown in vitro and in vivo leishmanicidal properties against different species of Leishmania spp. [11,14].

Despite its pharmacological potential, curcumin has some physicochemical and biopharmaceutical limitations that should be highlighted, such as: (i) low aqueous solubility, (ii) poor gastrointestinal absorption, (iii) high rates of metabolism, (iv) inactivity of metabolic products, and (v) rapid elimination and clearance [15,16].

To get around these several limitations, nanotechnological systems such as nanoemulsions [17], microemulsions [18], self-nanoemulsifying systems [19], nanoparticles [20], nanolipo-
somes [21], micelles [22], and nanocrystals [23] have been utilized. These systems can promote (i) protection of the drug against degradation in physiological media, (ii) increase in drug solubility, and (iii) modification/targeting of the drug enabling transport through biological membranes [13,24].

Therefore, this review focuses on mapping the nanotechnologies used to load curcumin and discussing the increase in the leishmanicidal properties of this drug according to its nanostructured vehicles.

Review

Leishmaniasis: general aspects

Leishmaniasis is a neglected tropical disease caused by a flagellated protozoa of the genus Leishmania. The genus belongs to the Trypanosomatidae family, and it is transmitted by insect vectors of the genus Phlebotomus (in the Old World) or Lutzomyia (in the New World) [25]. The disease is present in several countries and it has affected more than 350 million people worldwide. Its incidence has increased more than 40-fold in the last 20 years, making it the second most prevalent parasitic disease in the world after malaria [2,26,27].

The disease can manifest in three different forms: cutaneous leishmaniasis (CL), which is the most abundant form; mucocutaneous leishmaniasis (MCL); and visceral leishmaniasis (VL), which is the severe and lethal form of the disease, with a mortality rate above 95% [28]. The form in which the disease manifests itself in the patient is determined mainly by immunological aspects and general health conditions of the host and by the species of the parasite. In general, CL and MCL are caused by L. tropica, L. major, L. amazonensis, and L. brasiliensis. Meanwhile, VL is caused by L. donovani, L. infantum, and L. chagasi. However, there are reports of cutaneous leishmaniasis caused by the L. donovani and L. infantum complex. This is due to advances in the molecular detection of these species worldwide [29-31].

The Leishmania spp. life cycle is mainly divided into two evolutionary stages: (i) extracellular promastigote, which is the flagellate form and is in the intestine of the invertebrate host; (ii) intracellular amastigote, a spherical form that is found in cells of the vertebrate host. Infected sandflies inject blood with the parasite in promastigote form into the vertebrate host, which causes macrophages or other cells of the mononuclear phagocytic system to phagocytose the promastigotes. The Leishmania spp. cells then differentiate into amastigotes inside the phagocytic cells, multiply by binary fission until the host cell breaks down and releases the parasites to infect other cells and tissues [2,32,33].
The Food Drug Administration (FDA) recommends five drugs for the treatment of leishmaniasis: pentavalent antimonials, amphotericin B, pentamidine, paromomycin, and miltefosine. Among these drugs, only the pentavalent antimonials were designed for leishmaniasis, while the other four were initially approved to treat other diseases. Although used in different treatment protocols, most are only capable of controlling the infection and relieving symptoms, while displaying concerning toxicity and numerous therapeutic limitations [34-36].

Furthermore, treatment abandonment and failure due to drug resistance are two of the problems encountered with the usual treatments. Thus, seeking therapeutic alternatives to those currently available, many natural or synthetic molecules have been studied and evaluated for their antileishmanial potential, among which curcumin may be featured [4].

Curcumin and its antileishmanial properties
Curcumin (curc) is a polyphenol (Figure 1) obtained from the rhizome of \textit{Curcuma longa} and is the main curcuminoid present in this plant [11,12]. Due to its good tolerance profile and safety even at high doses (12 g/day), curc has been extensively studied as a therapeutic agent [11,14]. Numerous preclinical and clinical trials have concluded that curc has great potential for the treatment of various diseases in humans [37-39].

Among the diverse biological potential of this molecule, its antiparasitic properties against different diseases have attracted considerable attention in recent decades [10,40,41]. Among such, the potential of curc against cutaneous and visceral leishmaniasis has been explored [42]. \textit{In vitro} and \textit{in vivo} studies have revealed that curc displays leishmanicidal activity against amastigotes and promastigotes of the species \textit{Leishmania amazonensis} [43], \textit{Leishmania braziliensis} [44], \textit{Leishmania donavani} [45], \textit{Leishmania infantum} [46], \textit{Leishmania major} [46,47], and \textit{Leishmania tropica} [46,48].

Although proposed mechanisms of action are not fully elucidated, curc has been shown to have antileishmanial effects through its anti-inflammatory and antioxidant properties [42,47,49,50]. Figure 2 reveals the possible mechanisms of action of this drug against leishmaniasis.

Curc inhibits the activation of nuclear factor-κB (NFκB); the production of TNFα, IFNγ, and nitric oxide; and gene expression of inducible nitric oxide synthase (iNOS) [49,51]. These pro-inflammatory factors are related to the parasitic infection of leishmaniasis. Additionally, this molecule produces reactive oxygen species (ROS) and elevates cytosolic calcium. These occur in the exposure of phosphatidylserine to the outer plasma.
membrane leaflet and DNA fragmentation, causing the death of the leishmaniasis parasite [47,52].

Despite its promising antileishmanial potential, curc has several drawbacks, such as: (i) low aqueous solubility, (ii) rapid clearance, (iii) low tissue absorption, and (iv) notable chemical degradation (neutral and alkaline pH), which severely reduces its bioavailability and hinders its clinical use [30,65]. Given this scenario, approaches such as carrying curc in nanostructures have been used to overcome such drawbacks.

**Nanostructured systems for the treatment of leishmaniasis**

The existing treatments for leishmaniasis (cutaneous, mucocutaneous, or visceral) are still insufficient or frequently ineffective. This is due to several limitations of the drugs used, such as (i) high levels of toxicity and prolonged treatment time, which leads patients to discontinue treatment, (ii) high cost of treatment and parasite resistance to drugs, which is a major issue and occurs as a result of genetic mutations that reduce the response of the parasite towards a given drug through decreased uptake of the drug by macrophages [55-57].

Thus, nanotechnology-based systems are a promising alternative for drug delivery and vectorization in the treatment of leishmaniasis as they present several advantages. One could mention decreased side effects, modified drug release, prevention of rapid metabolism, protection of photosensitive molecules, the ability to deliver multiple antileishmanial drugs that can have a synergistic effect, and increased solubility, which results in increased bioavailability. These advantages are determined by the physicochemical properties of the systems, and by the release and targeting features of the loaded nanosystems, rather than by the drug properties themselves [58-62].

The intracellular amastigote form of *Leishmania* spp. allocates in macrophage phagolysosomes from infected individuals [63]. However, the intracellular uptake of bioactive molecules is especially hindered for hydrophobic molecules [64], making it difficult for the drug to reach the parasite. On the other hand, nanocarriers can target the interior of macrophages residing in the spleen, liver, and bone marrow, effectively delivering antileishmanial drugs to such sites. Overall, drug targeting results in increased treatment efficacy and reduced toxicity, mostly by reducing drug doses and preventing its interaction with unwanted receptors [30,65].

In this scenario, active targeting happens by the functionalization of nanocarriers, making drug delivery specific to macrophage targets, such as α-mannose, phosphatidylserine, or lactoferrin. This may reduce the drug resistance of the parasite in the long term. Furthermore, the surface charge of nanostructures may influence internalization since positive charges favor electrostatic interactions of these carriers with the macrophage membrane. As a result, the macrophages uptake the drug-loaded nanocarrier by phagocytosis, where they will directly act on the parasites [65-67].

Several types of nanosystems have been studied for carrying antileishmanial drugs, such as polymeric nanoparticles, lipid nanoparticles, nano- and microemulsions, liposomes, or metallic nanoparticles [68]. Costa-Lima and colleagues incorporated bisnaphthalimidopropylaminoctane (BNIPDaoct) into PLGA polymeric nanoparticles and obtained particles with sizes around 150 nm, with encapsulation efficiency around 90% [69]. BNIPDaoct is a bisnaphthalimidopropyl derivative, which acts on the life cycle of *Leishmania infantum*. Therefore, the authors evaluated the antileishmanial potential of the formulations and the free molecule in amastigote forms of *Leishmania infantum*. The study showed an eight- to ten-fold decrease in macrophage cytotoxicity of the nanoencapsulated molecule when compared to its free form. In addition, the uptake of these nanoparticles by macrophages was higher than that by fibroblasts, with an IC$_{50}$ approximately two times lower than that of the free drug, and a selectivity index 20 times higher. In *vivo* studies demonstrated that the nanoformulations were more effective in reducing parasitemia in the spleen, with results equivalent to the group treated with amphotericin B.

Das and collaborators, on the other hand, addressed the development of nanostructured lipid carriers (NLC) with ursolic acid (UA) functionalized with N-octyl chitosan. The NLC were approximately 143 nm of the hydrodynamic diameter. The authors also found encapsulation and drug-load efficiencies of 88.63 ± 2.70% and 12.05 ± 0.54%, respectively [70]. They evaluated the antileishmanial potential of the nanosystems against resistant strains of *Leishmania donovani*, which resulted in a 15-fold improvement in drug activity when into NLC, with a selectivity index for the intracellular model in macrophages almost three times higher than that of the free drug. In *vivo* studies showed that the suppression of parasite load in the spleen of mice was around 60% for free-UA and close to 90% for NLC-UA. Confocal microscopy images proved the cell uptake of NLC into macrophages.

Metal nanoparticles are also excellent alternatives for carrying antileishmanial drugs [71]. Almayouf et al. produced silver nanoparticles (Ag-NP) with sizes ~100 nm by green synthesis using extracts of *Ficus carica* Linn and *Olea europaea* L., which are rich in phenolic compounds. The authors also evaluated the antileishmanial potential of the nanosystems regarding concurrent treatment or pretreatment against *Leishmania major*.
cutaneous infection in female Balb/c mice [72]. The results of the study showed an improvement in skin lesions of groups treated with Ag-NP both before and after infection. The group treated after infection displayed a significant decrease in lesion size starting in the second week of treatment, with complete healing after 21 days, while the group treated with Pentostan healed after 28 days.

Moraes and colleagues prepared nanoemulsions (NE) of andiroba oil (Carapa guianensis Aublet, anoandi) and copaiba oil (Copaifera sp. Linnaeus, nanocopa) and tested their effects against L. infantum and L. amazonensis promastigotes and intracellular amastigotes, as well as the effects of oral administration of the formulations in infected mice [73]. The droplet size of the NEs was 76 and 88 nm for nanocopa and nanoandi, respectively. The authors observed a significant decrease in parasite load for both investigated species when treated by both nanoandi and nanocopa. Moreover, there was a decrease in lesion size and parasite load from the liver and spleen of mice treated with NE. In ultrastructural analysis performed by scanning electron microscopy, it was possible to observe morphological changes, oval aspect, and disappearance of the flagellum in the promastigote parasites treated with doses of NE above the IC50.

Peixoto and collaborators developed epoxy-α-lapachone-loaded microemulsions (ME). They assessed the ME in vivo performance against L. amazonensis in infected BALB/c mice. The ME droplet size was smaller than 120.4 ± 7.7 nm and displayed a good stability profile over 73 days. The in vivo studies demonstrated that after two weeks of treatment, BALB/c mice infected with L. amazonensis showed a decrease in paw lesions (about two-fold) in response to microemulsion, compared to the untreated group. Additionally, the amount of parasites in the lymph nodes (31.5%) and footpad (60.3%) decreased [57].

Another nanostructured platform explored for the delivery of leishmanicidal drugs is the liposomal platform [74]. Artemisinin-loaded nanoliposomes smaller than 100 nm were obtained and evaluated in a murine model infected with L. donovani. Artemisinin-NPs reduced the number of ex vivo infected macrophages and the intracellular infection of Leishmania donovani amastigotes (IC50 of 6.0 ± 1.4 µg/mL and 5.1 ± 0.9 µg/mL, respectively). Artemisinin-NPs showed better efficacy than free artemisinin after therapy in a mouse model of visceral leishmaniasis. Percentages of inhibition in the liver and spleen were 82.4 ± 3.8% and 77.6 ± 5.5%, respectively [75].

The results of the studies highlighted in this topic confirm the relevance of the use of nanostructured formulations for the delivery of bioactive compounds and uptake by macrophages, promoting an increase in antileishmanial efficacy, a decrease of toxicity, and, as a result, an increase in the selectivity index of these compounds. The same rationale is applied to the incorporation of curc in nanostructured systems, which will be discussed in the following topics.

**Nanostructured systems with curcumin for the treatment of leishmaniasis**

Different nanostructured systems with curc intended for the treatment of leishmaniasis have been developed (Figure 3). The articles included in this work approach the following nano-systems: (i) self-nanoemulsifying drug-delivery systems (SNEDDSs), (ii) nanoliposomes, (iii) nanostructured lipid carriers, (iv) polymeric, and (v) metallic nanoparticles. Different performances of nanostructured systems containing curc are discussed in the section below.

![Figure 3: Overall scheme demonstrating curcumin-loaded nanostructured systems that have been assayed in vitro and/or in vivo against different Leishmania species for the treatment of leishmaniasis. Created with BioRender.com (https://biorender.com). This content is not subject to CC BY 4.0.](https://biorender.com/)

**Self-nanoemulsifying drug-delivery systems**

The SNEDDSs are lipid-based drug-delivery systems made of an isotropic blend of oils, surfactants, and co-surfactants or co-solvents [76]. These spontaneously form O/W nanoemulsions (≤200 nm) in aqueous media (generally in a physiological media) [77,78]. The SNEDDSs have been successfully used to carry molecules with biopharmaceutical limitations, improving their physicochemical and leishmanicidal properties [79,80].

In this context, curcumin-loaded SNEDDSs intended for the treatment of leishmaniasis were developed. Khan and collabora-
tors developed curcumin-loaded self-emulsifying drug delivery systems (curc-SNEDDSs) for topical administration in cutaneous and mucocutaneous leishmaniasis [81]. The systems were produced by homogenization using a vortex mixer. The authors obtained two nanoformulations, which showed droplet size between 26–29 nm, polydispersity index (PdI) lower than 0.2, and zeta potential between −3.6 and −4.4 mV. The curc-SNEDDSs (1% of curc) promoted an increase in antileishmanial (in vitro) activity (IC\textsubscript{50}: 0.13–0.18 μg/mL and 0.25–0.27 μg/mL) when compared to curc in its free form (IC\textsubscript{50}: 22.50–24.60 μg/mL) against promastigotes and amastigotes of Leishmania tropica, respectively. The nanoformulations were able to kill the intracellular amastigotes in macrophages. Moreover, the authors developed a further study wherein seven nanoformulations with different mixtures of oils, surfactants, and co-surfactants were reported [48]. The obtained SNEDDSs showed droplet size between 30–80 nm and IC\textsubscript{50} values of 0.26–0.36 μg/mL and 0.23–0.37 μg/mL against promastigotes and amastigotes of Leishmania tropica, respectively. However, in this study, they did not perform intracellular studies on infected macrophages. Nevertheless, both studies containing curcumin-loaded SNEDDSs promoted an increase in leishmanicidal activity.

Differently, Khan and collaborators also developed SNEDDSs for the entrapment of amphotericin B against Leishmania tropica. The authors also observed low IC\textsubscript{50} values promoted by the nanosystems (two different SNEDDSs) against amastigote (IC\textsubscript{50} values for SNEDDSs A and B, 0.025 and 0.056 μg/mL, respectively) and promastigote forms (IC\textsubscript{40} values for SNEDDSs A and B, 0.017 and 0.031, respectively). These results evidenced the effectiveness of different SNEDDSs when compared to that of the control Ambisome, which demonstrated IC\textsubscript{50} values 10 times higher for amastigotes and promastigotes [79]. Although this nanostructured system is a promising carrier, further \textit{in vitro} and \textit{in vivo} studies are necessary to evaluate such improvements for curc or other leishmanicidal drugs.

**Nanoliposomes**

Nanoliposomes are nanoscale lipid bilayer vesicles mainly composed of phospholipids. These systems (small liposomes: 20–100 nm and large liposomes: >100 nm) are capable of loading both hydrophilic and lipophilic drugs [82,83]. Leishmanial drugs, such as miltefosine [84], buparvaquone [85], nita-zoxanide [86], artesominin [75], berberine [87], and paromomycin [88] have already been successfully loaded into nanoliposomes promoting increased antiparasitic activity.

Recently, Bafghi and collaborators produced nanoliposomes for curc delivery as a new alternative for leishmaniasis treatment [89]. Briefly, the nanoliposomes were prepared by thin-film hydration, resulting in spherical vesicles with 176.5 nm of diameter, PdI <0.2, zeta potential of +35 mV, and an entrapment efficiency of 92%. Curcumin-entrapped nanoliposomes showed leishmanicidal activity (\textit{in vitro}) against the promastigotes of \textit{Leishmania major}, whose IC\textsubscript{50} values were 6.41, 3.80, and 2.33 μg/mL for the incubation times of 24, 48, and 72 h, respectively. Additionally, this nanosystem proved to be biocompatible with skin fibroblasts (\textit{in vitro}). However, neither the cell viability of this system in healthy macrophages nor models of parasite infection in this cell type were evaluated. Considering that the nanoscale platform approved by the FDA and currently used in the treatment of leishmaniasis is liposomal amphotericin-B (IC\textsubscript{50}: 0.18 μg/mL) [90], this liposomal formulation containing curc should receive greater attention. In addition, more complex efficacy and safety studies (\textit{in vivo}) must be conducted so that they could be transferred to the market following the Ambisome\textsuperscript{®} path.

**Nanostructured lipid carriers**

Nanostructured lipid carriers (NLCs) are lipid-based formulations with a solid matrix at room temperature that differ from solid lipid nanoparticles when it comes to their matrix organizational level. Nanostructured lipid carriers offer advantages such as enhanced stability, low toxicity, increased shelf life, improved drug loading capacity, and biocompatibility over other conventional lipid-based nanocarriers, such as nanoemulsions and solid lipid nanoparticles [91]. Due to their properties, the use of NLCs has been a successful strategy for entrapping drugs with leishmanicidal activity [92-95]. Accordingly, Riaz and collaborators entrapped curc into NLCs to evaluate their activity in the treatment of cutaneous leishmaniasis (\textit{L. tropica}) [96]. The authors obtained the NLCs by hot homogenization, resulting in structures with a hydrodynamic mean particle size of 312 ± 1.89 nm, PdI of 0.305 ± 0.17 and zeta potential of −38 ± 0.93 mV. These particles were able to entrap 88% of curc due to the irregular lipid crystal structure of NLCs [53]. The NLCs proved to be safe for macrophages, which promptly internalized the nanostructures, as proven by the strong intracellular fluorescence levels. Furthermore, NLCs increased the \textit{in vitro} leishmanicidal activity of curc (IC\textsubscript{50}: 105.49 ± 3.71 μg/mL) when compared to curc-free NLCs (IC\textsubscript{50}: 165.06 ± 4.64 μg/mL). Similar performance occurred \textit{in vivo} in axenic amastigotes, (IC\textsubscript{50} of the curc-NLC: 190.3 ± 3.19 and IC\textsubscript{50} value of the curc-free: 243.56 ± 2.89 μg/mL) corroborating the improvements in the leishmanicidal response after entrapment of curc in the nanostructure.

This biological performance was also observed in artemether-NLC against \textit{L. infantum}. Nanostructured lipid carriers conferred an increase in the leishmanicidal activity of the drug. Artemether-NLCs presented IC\textsubscript{50} values of \textit{L. infantum}
promastigote and amastigotes of 16.43 and 15.42 µg/mL, respectively. On the other hand, free artemether IC50 values were 2-fold higher (i.e., 37.12 and 32.10 µg/mL). Finally, the nanostructured system revealed the lowest cytotoxicity against J774 macrophages [97].

Amphotericin B-loaded NLCs were also developed for the treatment of leishmaniasis. Free amphotericin B (Amb) and Amb-NLCs (250 nm) were evaluated for their leishmanicidal performance against the amastigote form and host cells. Unlike curc-NLCs, which revealed an increase in activity, Amb-NLCs obtained IC50 with efficacy equivalent to Amb-free against the amastigote form of L. braziliensis (11.7 ± 1.73 and 5.3 ± 0.55 ng/mL respectively). However, NLCs greatly increased drug selectivity (1046 versus 813), as macrophages revealed smaller toxicity indicators, without evidence of nitric oxide or TNFα production [95].

In this perspective, NLCs have been a nanoscale platform with promising results aimed at encapsulating anti-leishmanial drugs. This encourages further studies to better understand how NLCs might affect the mechanisms of action of curc when delivered via these nanostructured systems.

Polymeric nanoparticles

Polymeric nanoparticles (PNPs) are colloidal systems made of natural or synthetic polymers [98]. These systems can encapsulate or adsorb active pharmaceutical ingredients (APIs) and macromolecules [99-101]. In addition, PNPs can impact specific drug release kinetics and increase biocompatibility [60]. Different biodegradable polymers have been used for the development of targeted PNPs for the treatment of leishmaniasis [102].

In this scenario, chitosan is an interesting polymer for NP synthesis due to its positive charge, which favors adsorption by negatively charged cell membranes [103]. Additionally, studies have revealed antileishmanial properties of this polymer against Leishmania parasites, making it attractive for the synthesis of NPs intended for this treatment [56,104].

Therefore, Chaubey and colleagues developed mannose-conjugated curcumin–chitosan nanoparticles (curc-MCNPs) intended for visceral leishmaniasis [50]. The selected nanoparticles (curc-MCNPs) presented spherical morphology, hydrodynamic mean particle size of 215 nm, PdI of 0.381, zeta potential of +24.37 mV, and entrapment efficiency of 82.12%. Curc-MCNPs showed a more effective uptake and pronounced in vitro leishmanicidal activity (curc-MCNPs, median effective dose (ED50): 0.518 ± 0.01 µg/mL) against L. donovani amastigotes than curc-chitosan nanoparticles (curc-CNPs, ED50: 1.87 ± 0.075 µg/mL) and free curc (ED50: 2.8 ± 0.03 µg/mL). Furthermore, the in vivo uptake study indicated that the endocytosis of the NPs effectively occurred within macrophages of the reticuloendothelial system. Moreover, in another work of the research group, the authors evaluated the in vitro and in vivo leishmanicidal efficacy and toxicity of these nanoparticles [105]. In vivo antileishmanial activity in hamsters demonstrated significantly greater suppression of parasite replication in the spleen with Cur-MCNPs compared to that of Cur-CNPs. In addition, no cytotoxic profile was observed in vitro in macrophages (J774.A1), which was also confirmed by the minimal cytotoxicity observed in in vivo studies.

Hence, chitosan-based nanoparticles are a good strategy for drug delivery intended to treat leishmaniasis. This polymer can stimulate macrophages to produce pro-inflammatory cytokines, as they bind to receptors present in the cells of the immune system [106]. In this way, there is an internalization of the NPs containing this polymer and delivery of the drug for leishmanicidal activity. Additionally, the conjugation of the nanoparticles with sugars, such as mannose, makes drug delivery targeted and specific to the macrophage receptor, increasing the uptake by the system and consequently the intracellular leishmanicidal activity [50,105].

Poly(lactic-co-glycolic) acid (PLGA) is another polymer used for the development of nanoparticles for the treatment of leishmaniasis [107,108]. PLGA is an FDA-approved polymer that is commonly used in the synthesis of nanoparticles due to its special features such as biocompatibility, biodegradability, low toxicity, and adjuvanticity [109,110]. Given these properties, Tiwari and co-workers produced curcumin-loaded Eudragit-PLGA-nanoparticles (curc-E-PLGA-NPs) and evaluated their leishmanicidal activity with miltefosine combination therapy. The authors functionalized the surface of PLGA-NPs with Eudragit L30D, a polymer that provides pH-dependent drug release and significantly improved targeted action, thus increasing the efficacy of the drug [45].

Curc-E-PLGA-NPs showed spherical morphology, with a hydrodynamic mean diameter of 182.3 ± 7.4 nm, PdI of 0.281 ± 0.015, zeta potential of −12.7 ± 0.141 mV, and entrapment efficiency of 93.2 ± 3.9 %. Curc-E-PLGA-NPs exhibited IC50 values in vitro of 1.34 ± 0.045 and 1.61 ± 0.032 µg/mL for promastigotes and amastigotes of L. donovani, respectively. Furthermore, the association of curc-E-PLGA-NPs with miltefosine revealed synergism in both promastigotes and amastigotes. In the in vivo hamster model, curc-E-PLGA-NPs also showed leishmanicidal activity, individually or associated with miltefosine. The synergy evidenced between these drugs (i) increased the production of toxic reactive oxygen/nitrogen metabolites, (ii) increased phagocytic activity, and (iii) in-

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Badirzadeh and collaborators synthesized curc-coated silver nanoparticles (curc-AgNPs) for the treatment of cutaneous leishmaniasis [124]. Curc-AgNPs presented a spherical shape, 32 nm of diameter, and a zeta potential of $-19.8$ mV. This nanoformulation prevented the in vitro growth of $L. \text{major}$ promastigotes and inhibited their viability (IC$_{50}$: 58.99 $\mu$g/mL).

In addition, it eliminated amastigotes inside macrophages (EC$_{50}$: 57.14 $\mu$g/mL), which remained viable above 50% at concentrations below 307.16 $\mu$g/mL. Further, the authors carried out in vivo assays with BALB/c mice containing lesions caused by $L. \text{major}$ infection. The treatment was carried out for 50 days with different concentrations of curc-AgNPs (20–60 $\mu$g/kg). The results indicated a decrease in lesions when compared to the negative control. Furthermore, a significant decline in $L. \text{major}$ load was observed in 4 weeks.

A different study evaluated the leishmanicidal performance of curc-coated gold nanoparticles (curc-AuNPs) against $L. \text{major}$ [125]. The NPs showed spherical morphology, particle size of 22 nm, and adequate polydispersity. Curc-AuNPs revealed IC$_{50}$ values against $L. \text{major}$ promastigotes of 64.79 $\mu$g/mL and 29.89 $\mu$g/mL in 24 h and 48 h, respectively. Additionally, they inhibited and eliminated amastigotes inside macrophages (IC$_{50}$: 63.29 $\mu$g/mL on 24 h and 54.04 $\mu$g/mL on 48 h). In vivo treatment (BALB/c mice for 50 days) with curc-AuNPs (20–60 $\mu$g/kg) reduced lesions caused by $L. \text{major}$ promastigotes after four weeks. The parasite burden of the paws and lymph nodes of mice infected with $L. \text{major}$ was also significantly reduced compared to the negative control.

Hence, it is noteworthy that both MNP's had an impact on cutaneous leishmaniasis infections. However, there was no influence of metal (Ag or Au) particles on the leishmanicidal activity of the systems. In these studies, the found activity may be mostly attributed to curc-coated NPs. The same behavior was observed in Miltefosine-AgNPs developed by Kalangi and collaborators. Silver nanoparticles alone (50 $\mu$m) did not demonstrate an antileishmanial effect on the promastigote stage of the Leishmania parasite. However, when AgNPs were combined with miltefosine (12.5 $\mu$M and 25 $\mu$m) the leishmanicidal effect of the drug doubled [122]. Therefore, combining leishmanicidal drugs, such as curc, with metallic particles can be an effective strategy against the leishmania parasite.

The formulations described in Section 1.4 are summarized in Table 1, which also describes their physicochemical characteristics and performance in vitro and in vivo.

General discussion and final considerations

This study discussed that curcumin is a polyphenol with diverse biological properties, including a potent leishmanicidal activity. Despite the promising activity, this molecule shows poor water
solubility, high metabolism, and fast elimination impair, which results in low systemic bioavailability and poor in vivo pharmacological effect. In this context, molecules with similar limitations have been combined with nanotechnology tools to allow their clinical use. In recent years, different nanostructured systems have been explored, such as lipid nanoparticles, metallic nanoparticles, polymeric nanoparticles, nanovesicles, and self-emulsifying nanosystems. These can promote increased solubility, permeability, protection against degradation in biological media, and a controlled release profile. Additionally, nanostructures, especially those smaller than 200 nanometers, are susceptible to uptake by the cells infected with the etiological agent of leishmaniasis. This ability allows an expressive increase in the leishmanicidal activity of curcumin against the parasite.

Despite its versatility, several studies stop at the development of nanostructures containing curc and fail to further assess their activity in the parasites, which slows down the path to a future product. Therefore, only a few studies have evidenced in vitro and in vivo proof-of-concept that curc nanostructured systems might be promising for the treatment of leishmaniasis. Overall, alternative therapeutic nanostructured systems have been presented. They are nanoliposomes, SNEDDSs, NLCs, poly-

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### Table 1: Summary of the curcumin-loaded nanoformulations intended for the treatment of leishmaniasis.

<table>
<thead>
<tr>
<th>Nanoformulation</th>
<th>Composition</th>
<th>Size/ PdI/ ZP/ EE/ DL</th>
<th>Parasite</th>
<th>In vitro and in vivo outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNEDDSs [61]</td>
<td>Captex® 355, Crem EL®, Crem RH® 40, Tween® 80, PEG 200, PEG 300, caprylic acid and curc</td>
<td>≈29 nm/ 0.2/ −3.6 to −4.4 mV/ −/ −</td>
<td>L. tropica</td>
<td>IC₅₀ decreased by 125 times and more than 90 times compared to free curcumin against promastigotes and amastigotes, respectively.</td>
</tr>
<tr>
<td>SNEDDSs [48]</td>
<td>Captex® 355, Crem EL®, Crem RH® 40, Tween® 80, PEG 200, PEG 300, caprylic acid and curc</td>
<td>30−80 nm/ ≈0.2/ −1.5 to −4.8 mV/ −/ −</td>
<td>L. tropica</td>
<td>IC₅₀ decreased by more than 86 times and more than 66 times compared to free curc against promastigotes and amastigotes, respectively.</td>
</tr>
<tr>
<td>nanoliposomes</td>
<td>phosphatidylicholine, cholesterol, curc</td>
<td>176.5 nm/ ≈0.2/ +35 mV/ 92%/ −</td>
<td>L. major</td>
<td>IC₅₀ values decreased as the incubation time increased, from 24 h to 72 h.</td>
</tr>
<tr>
<td>NLC [96]</td>
<td>glyceryl monostearate, soy phosphatidylicholine, curc</td>
<td>≈312 nm/ ≈0.3/ −38 mV/ 88%/ 1.07%/ −</td>
<td>L. tropica</td>
<td>NLCs increased the leishmanicidal activity of curc in 1.55 times in vitro and 1.28 times in vivo.</td>
</tr>
<tr>
<td>polymeric NPs</td>
<td>chitosan, mannose, curc</td>
<td>215 nm/ ≈0.3/ +24.73 mV/ 82.12%/ −</td>
<td>L. donovani</td>
<td>curc-CNPs and curc-MCNPs showed in vitro IC₅₀ values (1.5 and 4.5 times, respectively) lower than that of free curcumin. The in vivo uptake study showed the endocytosis of NPs by macrophages.</td>
</tr>
<tr>
<td>polymeric NPs</td>
<td>chitosan, mannose, curc</td>
<td>215 nm/ ≈0.3/ +24.73 mV/ 82%/ −</td>
<td>L. donovani</td>
<td>In vivo antileishmanial study showed greater suppression of parasite replication in the spleen by curc-MCNPs compared to curc-CNPs.</td>
</tr>
<tr>
<td>polymeric NPs</td>
<td>PLGA, Eudragit® L30D, curc, miltefosine</td>
<td>≈182 nm/ ≈0.2/ −12.7 mV/ ≈93%/ +18%/ −</td>
<td>L. donovani</td>
<td>Association of curc-E-PLGA-NPs with miltefosine revealed synergism in promastigote and amastigote forms.</td>
</tr>
<tr>
<td>metallic NPs</td>
<td>silver nitrate, curc</td>
<td>32 nm/ −/ −19.8 mV/ −/ −</td>
<td>L. major</td>
<td>curc-AgNPs (i) prevented the in vitro growth of L. major promastigotes, and (ii) inhibited their viability; (iii) eliminated amastigotes inside macrophages, and (iv) decreased cutaneous lesions in vivo.</td>
</tr>
<tr>
<td>metallic NPs</td>
<td>Au, curc</td>
<td>22 nm/ −/ −/ −/ −</td>
<td>L. major</td>
<td>curc-AuNPs IC₅₀ values decreased as the incubation time increased (24 to 48 h). In vivo treatment demonstrated curc-AuNPs reduced cutaneous lesions after four weeks.</td>
</tr>
</tbody>
</table>

Ag: silver; Au: gold; C: chitosan; curc: curcumin; DL: drug load; EE: encapsulation efficiency; M: mannose; MNPs: metallic nanoparticles; NPs: nanoparticles; NLC: nanostructured lipid carriers; SNEDDSs: self-nanoemulsifying drug-delivery systems; PdI: polydispersity index; PLGA: poly(lactic-co-glycolic acid); ZP: zeta potential.
meric and metallic nanoparticles. These nanostructured systems have different compositions, sizes, charges, and modified surfaces which improves the leishmanicidal activity of curc and other control drugs.

To date, only three in vivo studies in mice using nanostructured curc have been carried out. These were all based on polymers and metallic nanostructures. Despite the conducted investigations, there are still gaps regarding a better understanding of the mechanisms of action of curc against leishmaniasis parasites. This review demonstrated that the known aspects related to curc leishmanicidal activity involve the increased production of ROS and intra-amastigote release of Ca$^{2+}$. In this context, we could observe that the studies failed to correlate consecutive intramacrophage and intra-amastigote cellular uptake kinetics, once it appears to greatly interfere with the proposed biochemical triggers of Leishmania spp. cell death.

In addition, the work could also summarize that lipid-based nanostructures are great alternatives. However, the lack of in vivo studies on this matter limits their fair comparison to polymeric formulations. Overall, all assessed studies could prove that nanostructures improve curc dispersion in aqueous media (increase in apparent solubility). Altogether, we could also observe a general decrease in IC$_{50}$ when compared to free curc, which was mainly attributed to the cell uptake of these structures. Indeed, studies that functionalized nanostructures with mannose for an increase in macrophage phagocytosis evidenced that functionalized nanoparticles decreased IC$_{50}$ when compared to non-functionalized nanostructures and the free drug. These results take us in the direction of avoiding futile conditions when developing further nanostructures for leishmaniasis.

Hence, based on the biological potential of curcumin and known safety/tolerability, and based on the existing proof of concept that nanostructured systems are more effective than conventional medicines, reduce the duration of treatment and the frequency of administration, there is an urgent need for industrial innovation towards new treatments for leishmaniasis.

**Conclusion**

Given that leishmaniasis is a neglected tropical disease, treatment is still poorly funded. Although the disease is spread in countries with large populations, such as Brazil and India, there are insufficient worldwide investments and not enough priority to prevent this disease from spreading, which makes it deadly to a large part of the population. Due to resistance to many drugs, several research works have focused on optimizing the use of amphotericin B nanoformulations (e.g., AmBisome®, Abelcet®, and Anforicin B®), which is an antifungal and now widely used for leishmaniasis. However, this potent drug is not selective enough to be used in all cases. Present research and future works might still focus on this effective, traditional, and toxic molecule, once there are already different products on the market. However, due to the fast increase in drug resistance, alternatives must be taken into consideration and there is room for new medicines with drugs that are not only effective but less toxic.

This work showed that many nanostructures are being developed and assessed for leishmaniasis on a research level. However, policies and investments that fast-track the development of a nanostructured product from the bench to the market might be key in the future. Although promising, biopharmaceutical limitations still should be regarded and might limit the current studies on nanostructures containing curcumin. Therefore, efforts, time, and resources could be saved by optimizing a single nanostructure for different administration routes, which takes into consideration the biological barriers involved in the treatment of different forms of leishmaniasis (VL, CL, and MCL). This rationale is based on the lack of information observed in the studies regarding skin permeability on lesioned and healed skin, and gastric stability of nanostructured curc in vivo.

Also, clinical studies that prove the efficacy and safety of nanostructured curc must be conducted to encourage the transfer of these formulations to the therapeutic scenario. Based on the findings, polymeric nanoparticles reveal themselves to be a step ahead in the game, once more in vivo information is available and current medicines based on nanoparticles provide insights for fast-tracking this system from the technological and regulatory point of view.

**Abbreviations**

Table 2 lists the in this article used abbreviations and their explanations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTDs</td>
<td>neglected tropical diseases</td>
</tr>
<tr>
<td>Curc</td>
<td>curcumin</td>
</tr>
<tr>
<td>NLCs</td>
<td>nanostructured lipid carriers</td>
</tr>
<tr>
<td>Pdi</td>
<td>polydispersity index</td>
</tr>
<tr>
<td>SNEDDSs</td>
<td>self-nanoemulsifying drug-delivery systems</td>
</tr>
<tr>
<td>NE</td>
<td>nanoemulsions</td>
</tr>
<tr>
<td>ME</td>
<td>microemulsions</td>
</tr>
<tr>
<td>PNP</td>
<td>polymeric nanoparticles</td>
</tr>
</tbody>
</table>
Table 2: Explanation of abbreviations. (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APIs</td>
<td>active pharmaceutical ingredients</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median effective dose</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>inhibitory concentration</td>
</tr>
<tr>
<td>ZP</td>
<td>zeta potential</td>
</tr>
<tr>
<td>NPs</td>
<td>nanoparticles</td>
</tr>
<tr>
<td>SI</td>
<td>selectivity index</td>
</tr>
<tr>
<td>VL</td>
<td>visceral leishmaniasis</td>
</tr>
<tr>
<td>CL</td>
<td>cutaneous leishmaniasis</td>
</tr>
<tr>
<td>MCL</td>
<td>mucocutaneous leishmaniasis</td>
</tr>
<tr>
<td>DL</td>
<td>drug load</td>
</tr>
<tr>
<td>EE</td>
<td>encapsulation efficiency</td>
</tr>
<tr>
<td>FDA</td>
<td>Food Drug Administration</td>
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</table>

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Author Contributions

Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


44. Pereira, A. H. C.; Marcolino, L. M. P.; Pinto, J. G.; Ferreira-Strixino, J. Antibiotics (Basel, Switz.) 2021, 10, 634. doi:10.3390/antibiotics10050634


Development and characterization of potential larvicidal nanoemulsions against Aedes aegypti

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Full Research Paper

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Keywords:
colloidal stability; drug delivery system; hydrophile–lipophile balance; monoterpenes

Abstract

Plant-based insecticides offer advantages such as negligible residual effects, reduced risks to both humans and the environment, and immunity to resistance issues that plague conventional chemicals. However, the practical use of monoterpenes in insect control has been hampered by challenges including their poor solubility and stability in aqueous environments. In recent years, the application of nanotechnology-based formulations, specifically nanoemulsions, has emerged as a prospective strategy to surmount these obstacles. In this study, we developed and characterized nanoemulsions based on cymene and myrcene and assessed their toxicity both in vitro using human keratinocytes (HaCAT) cells and in an in vivo model involving Galleria mellonella larvae. Additionally, we investigated the insecticidal efficacy of monoterpenes against the mosquito Aedes aegypti, the primary dengue vector, via larval bioassay. Employing a low-energy approach, we successfully generated nanoemulsions. The cymene-based nanoemulsion exhibited a hydrodynamic diameter of approximately 98 nm and a zeta potential of −25 mV. The myrcene-based nanoemulsion displayed a hydrodynamic diameter of 118 nm and a zeta potential of −20 mV. Notably, both nanoemulsions demonstrated stability over
60 days, accompanied by controlled release properties and low toxicity towards HaCAT cells and Galleria mellonella larvae. Moreover, the nanoemulsions exhibited significant lethality against third-instar Aedes aegypti larvae at a concentration of 50 mg/L. In conclusion, the utilization of nanoemulsions encapsulating cymene and myrcene presents a promising avenue for overcoming the limitations associated with poor solubility and stability of monoterpenes. This study sheds light on the potential of the nanoemulsions as effective and environmentally friendly insecticides in the ongoing battle against mosquito-borne diseases.

Introduction

Aedes aegypti (Linnaeus, 1762) is a mosquito species that is cosmopolitan and well adapted to anthropized and peridomestic environments. It is an important vector of arboviruses, including dengue, chikungunya fever, zika, and urban yellow fever and can cause alarming socio-economic impacts in the affected regions [1]. The World Health Organization (WHO) considers dengue, zika, and chikungunya as neglected and emerging tropical diseases transmitted by mosquitoes and as one of the main concerns in developing countries, which may become a major public health problem worldwide. This problem is evidenced by recent cases of Zika virus infection in Brazil and their relationship with microcephaly in newborns [2]. In the case of dengue, the most prevalent viral infection transmitted by Aedes mosquitoes with clinical forms ranging from asymptomatic to fatal cases, around 3.9 billion people in more than 129 countries are at risk [3]. The continuous and indiscriminate use of synthetic insecticides for the control of the Aedes aegypti mosquito (Linnaeus) has been responsible for the emergence of insecticide-resistant mosquitoes [4,5]. Therefore, it becomes urgent to search for safer and more effective vector control agents to prevent vector-borne diseases [6].

Bioinsecticides from plant derivatives, which degrade rapidly in the environment and have less toxicity in non-target organisms, are a promising option for vector control [7]. Terpenes are the largest group of secondary plant metabolites and have shown promising health benefits as antioxidant and anti-inflammatory agents in many animal studies [8,9]. The compound p-cymene, also known as p-cymol or p-isopropyltoluene, is a monocyclic hydrocarbonated monoterpene naturally occurring in essential oils (EOs) of various aromatic plants, including the genera Artemisia, Protium, Origanum, and Thymus. Myrcene is an acyclic monoterpenes found in hops, lemongrass, basil, and mangos [10].

Some intrinsic characteristics of monoterpenes, mainly poor water solubility and high volatility, make their formulation a true challenge. In this regard, nanoemulsions (NEs), which are dispersions of two immiscible liquids with one of them dispersed as small droplets [11,12], stand out as new delivery vehicles for these bioactive compounds. They are especially important to enhance the water availability of poorly water-soluble compounds, which is achieved when the oil constitutes the internal phase. In this case, oil-in-water nanoemulsions or aqueous nanoemulsions are obtained. The main advantage of NEs is their better kinetic stability compared to macroemulsions. Also, the NEs protect the EO constituents from oxidation, in addition to promoting better sensorial properties [13]. Moreover, the development of aqueous nanoemulsions would enable a better dispersion of vector control agents, inducing a controlled release and a possibly higher effectiveness in eliminating immature stages of mosquitoes [14].

NEs can be obtained through two general approaches, that is, high-energy methods and low-energy methods. The high-energy methods are characterized by using equipment such as sonicators, high-speed homogenizers, and high-pressure homogenizers, which provide high energy input during processing, leading to the generation of dispersed material on a nanoscale [15]. The low-energy methods are characterized by the use and control of the chemical energy of the system in the formation of droplets on the nanoscale. A crucial point is that these systems can be obtained at low cost and with eco-friendly techniques [16,17].

Griffin established the hydrophilic–lipophilic balance (HLB) as a tool for classifying and selecting non-ionic emulsifiers [18]. The determination of the required HLB (rHLB) of essential oils appears as a critical step for the development of stable emulsions [19]. Determining the required HLB, one can obtain the nanoemulsion with the smallest droplet size, leading to more stable formulations [20]. The rHLB is usually determined by preparing NEs with different ratios of surfactant blends and choosing the most stable formulation to determine the rHLB of the oil phase [21].

Biocompatibility assessment is an essential aspect of the development of NEs, particularly for biomedical and cosmetic applications, as it determines the safety and efficacy of the formulations. The assessment involves evaluating the potential cytotoxicity and genotoxicity of the NEs on different cell types and determining the effect on the immune response in vivo. In vitro cytotoxicity assays are an important tool for evaluating the safety of NEs. HaCaT cells are a widely used human keratinocyte cell line that exhibits several characteristics of
normal human epidermal keratinocytes, making them an excellent model for evaluating cytotoxicity [22].

In vivo toxicity studies are also crucial for evaluating the safety of NEs. *Galleria mellonella* larvae have emerged as an alternative to mammalian models for in vivo studies of acute toxicity because of their low maintenance cost, easy handling, and high similarity in immune response with mammals. Furthermore, *G. mellonella* larvae have been successfully used to evaluate the acute toxicity of various nanoparticles and drugs [23]. The immune response of *G. mellonella* larvae can be evaluated by monitoring their survival rate and melanization response [24].

The aim of the present work was (i) to develop stable oil-in-water nanoemulsions containing myrcene or cymene as the dispersed phase, (ii) to determine the required rHLB values for emulsion stability, (iii) to assess the biocompatibility via in vitro and in vivo assays, and (iv) to evaluate the bioefficacy of the NE against *Aedes aegypti* mosquito larvae.

Results and Discussion
Preparation and characterization of the nanoemulsions

The determination of the required HLB (rHLB) is an important step in the development of NEs containing volatile oils [19,25]. From the determination of the rHLB, it is possible to determine the best ratio between two surfactants, one more lipophilic and one more hydrophilic, which will be necessary to obtain a stable NE [18]. The rHLB of myrcene and cymene was determined using a mixture of Span 80 (lipophilic) and Tween 20 (surfactant). At a time of 24 h after preparation, formulations containing cymene with HLB values of 10–13 showed a yellowish layer on the surface, which may be an indication of phase separation (Figure S1, Supporting Information File 1). Formulations with HLB values of 16 and 16.7 showed a milky appearance and slight creaming, which may be indicative of Ostwald maturation, a very important mechanism when it comes to the instability of NEs. It is related to the difference between the droplet sizes in the formulation, with the smaller droplets having a greater chemical potential and, thus, diffusing to the larger ones [26]. Formulations with HLB values of 14 and 15 were the ones that presented the best visual characteristics, in addition to a bluish appearance, a characteristic of NEs [27,28]. Thus, the formulations with HLB values of 14 and 15 were selected for analysis by DLS.

After 24 h (D1), the formulation with HLB 14 had a droplet size of 116 ± 0.40 nm, and after 21 days there was no significant change in particle size, nor in polydispersity index (PdI) and zeta potential. The formulation with HLB 15 exhibited smaller particle size and lower PdI and zeta potential than the HLB 14 formulation. Also, there was no significant variation in these parameters throughout the analyzed period (60 days) (Table 1). For this reason, the formulation with HLB 15 was the formulation chosen as the rHLB of cymene.

After 24 h, the myrcene formulations with lower HLB values (10–11), that is, a greater amount of the surfactant (Tween 20) plus lipophilic (Span 80), showed classic signs of instability (i.e., creaming) (Figure S2, Supporting Information File 1). Formulations with HLB values of 12–14 and 16.7 showed a milky appearance and a more viscous appearance, characteristic of emulsions with droplets on the micrometric scale. It is important to mention that these formulations also showed signs of instability after 21 days. The formulations with HLB 15 and 16 were the ones that presented the best visual appearance, such as a bluish appearance characteristic of nanoemulsions, and maintained these characteristics over time. Thus, these formulations were selected for DLS.

<table>
<thead>
<tr>
<th>Time</th>
<th>HLB 14</th>
<th>HLB 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (nm)</td>
<td>PdI ± standard deviation</td>
</tr>
<tr>
<td>D1</td>
<td>116.0 ± 0.40</td>
<td>0.322 ± 0.024</td>
</tr>
<tr>
<td>D7</td>
<td>111.2 ± 1.58</td>
<td>0.285 ± 0.007</td>
</tr>
<tr>
<td>D14</td>
<td>107.6 ± 1.59</td>
<td>0.331 ± 0.023</td>
</tr>
<tr>
<td>D21</td>
<td>106.5 ± 0.73</td>
<td>0.350 ± 0.003</td>
</tr>
<tr>
<td>D30</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D60</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*The data are expressed as mean ± standard deviation, n = 3.*
The droplet size and PdI of the formulation with HLB 16 were slightly smaller than those of the formulation with HLB 15 (Table 2). Over time, there was no significant variation in the size for both formulations. Unlike the formulation containing cymene, the best formulation with myrcene was the one with HLB 16, which has in its composition a greater amount of Tween 20, the more hydrophilic surfactant.

It has been shown that a significant difference of the headgroup size of the surfactants has a synergistic effect on emulsion stabilization. Furthermore, the use of mixed surfactants enhances the properties of the interfacial film, leading to improved adsorption between the oil and water phases and enhancing the stability of nanoemulsions [29].

Previous studies have reported similar findings regarding NEs containing terpenes. Polydispersity index values comparable to those observed in our study were obtained, indicating the formation of stable and suitable NEs for larvicidal applications [30]. It is noteworthy that several studies have utilized essential oils containing chemical components structurally analogous to terpenes, thus, achieving table formulations using the same surfactants and active ingredient concentration (5%) [26-28]. However, there is only a limited number of studies that focus specifically on the production and characterization of nanoemulsions incorporating cymene or myrcene. Nevertheless, it has been demonstrated that a high-energy method can yield a nanoemulsion comprising 5% p-cymene stabilized with 1% Tween 80, with droplet sizes measuring approximately 150 nm, which maintained its stability for 60 days [31].

The zeta potential is used to predict the stability of dispersions, and its value depends on the physicochemical properties of active ingredients, polymers, vehicles, and the presence of electrolytes and their adsorption [32]. The zeta potential values found for the NEs obtained remained stable in the analyzed period, which indicates the stability of the formulation to avoid Ostwald maturation and coalescence of the droplets. Similar zeta potential characteristics, between 20 and 30 mV, have been described in other studies about nanoemulsions containing terpenes suitable for larvicidal applications [30,33,34].

Regarding the physical characterization, the bluish reflex is characteristic of this type of colloid system, and it is attributed to the Tyndall effect, making it a valuable macroscopic indicator of nanodroplet generation [16]. In addition, Forgiarini et al. indicated that a suitable nanoemulsion should have small drops of the dispersed phase (average below 300 nm) [35]. Izquierdo et al. stated that polydispersity index values close to 0.2 are an indication of kinetic stability with an almost monomodal distribution [36]. Thus, considering that in this study the stable formulations had similar size distribution profiles and low polydispersity index, the present study on cymene and myrcene nanoemulsions may be considered promising.

### Nanoparticle tracking analysis

From the two results obtained above, the HLB 15 formulation containing cymene and the HLB 16 formulation containing myrcene underwent nanoparticle tracking analysis (NTA). NTA is a technique for direct and real-time visualization, sizing, and counting of nanometric materials suspended in aqueous media [37]. According to NTA measurements, the Cym-NE particle size was 145.7 ± 7.7 nm, while the Myr-NE particle size was 126.4 ± 5.6 nm, confirming the nanometric droplet size.

### Cryogenic transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) is one of the most useful techniques for the investigation of NEs, since it provides detailed information about the internal structure of colloidal systems observed in their native state [38]. In cryo-TEM, it was possible to observe spherical droplets (Figure 1).

<p>| Table 2: Hydrodynamic diameter, PdI and zeta potential of Myr-NEs.(^a) |
|----------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                      | HLB 15           |                  | HLB 16           |                  |                  |                  |</p>
<table>
<thead>
<tr>
<th>Time</th>
<th>Size (nm)</th>
<th>PdI</th>
<th>Zeta potential</th>
<th>Size (nm)</th>
<th>PdI</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>123.9 ± 1.15</td>
<td>0.369 ± 0.02</td>
<td>−17.4 ± 0.0</td>
<td>118.8 ± 1.2</td>
<td>0.241 ± 0.01</td>
<td>−21.1 ± 0.3</td>
</tr>
<tr>
<td>7 D</td>
<td>113.5 ± 1.45</td>
<td>0.352 ± 0.05</td>
<td>−20.7 ± 0.3</td>
<td>118.0 ± 3.7</td>
<td>0.227 ± 0.006</td>
<td>−21.5 ± 0.5</td>
</tr>
<tr>
<td>14 D</td>
<td>112.0 ± 0.51</td>
<td>0.364 ± 0.01</td>
<td>−26.8 ± 0.4</td>
<td>110.8 ± 3.4</td>
<td>0.235 ± 0.007</td>
<td>−22.6 ± 2.3</td>
</tr>
<tr>
<td>21 D</td>
<td>115.4 ± 0.45</td>
<td>0.240 ± 0.02</td>
<td>−24.3 ± 0.5</td>
<td>104.5 ± 0.4</td>
<td>0.255 ± 0.005</td>
<td>−25.5 ± 0.6</td>
</tr>
<tr>
<td>D30</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>105.5 ± 0.7</td>
<td>0.227 ± 0.009</td>
<td>−25.3 ± 1.63</td>
</tr>
<tr>
<td>D45</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>99.93 ± 1.45</td>
<td>0.246 ± 0.012</td>
<td>−21.0 ± 2.47</td>
</tr>
<tr>
<td>D60</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>84.50 ± 0.82</td>
<td>0.217 ± 0.008</td>
<td>−20.7 ± 0.95</td>
</tr>
</tbody>
</table>

\(^a\)The data are expressed as mean ± standard deviation, n = 3.
Similar results of spherical droplets smaller than 180 nm were observed with cryo-TEM [39]. This technique is widely used to characterize the morphology of nanoemulsions and faithfully confirms the results obtained with other techniques [40].

In vitro drug release

One potential advantage of using NEs is their ability to enhance drug solubility and bioavailability. NEs have been shown to increase the solubility of poorly soluble drugs, such as monoterpenes, which can improve drug delivery and efficacy. The cumulative release of both free terpenes was lower than the cumulative release of nanoemulsions (Figure 2).

![Figure 1: Cryogenic transmission electron microscopy of (A) Cym-NE and (B) Myr-NE.](image)

The observed differences in the release of terpenes can be attributed to their specific chemical characteristics. Cymene has a lower log $P$ value than myrcene, indicating higher water solubility. This greater solubility in water may contribute to its higher release rate when compared to myrcene. Additionally, cymene has a lower molar volume than myrcene, which could also enhance its release rate (Table 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>log $P$</th>
<th>Molar volume (cm$^3$)</th>
<th>Water solubility (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cymene</td>
<td>4.02</td>
<td>155.8 ± 3.0</td>
<td>27.88</td>
</tr>
<tr>
<td>myrcene</td>
<td>4.58</td>
<td>177.0 ± 3.0</td>
<td>6.923</td>
</tr>
</tbody>
</table>

Previous research supports the fact that monoterpenes exhibit slower release than other drugs from delivery nanosystems [41]. This phenomenon can be explained by the higher surfactant/oil solubility, which leads to a stronger affinity to the oil–surfactant core–shell structure within the micelles. Consequently, a lower amount of monoterpenes is released into the surrounding medium [42].

Among the mathematical models used to study drug kinetics, the Korsmeyer–Peppas release model proved to be the most suitable for our formulations (Table 4). Our results show that Cym-NE has a $k$ value of 10.4, while Myr-NE has a $k$ value of 3.3. A higher $k$ value indicates faster drug release, while a lower $k$ value indicates slower transport kinetics and, consequently, poor drug release from nanocarriers [43].

![Figure 2: The in vitro drug release of nanoemulsions (Cym-NE, Myr-NE) and free terpenes (Cym-Sol and Myr-Sol).](image)

Furthermore, both Cym-NE and Myr-NE demonstrated a transport exponent value ($n$) of 0.3, indicating a release mechanism primarily driven by Fickian diffusion [44]. The free terpenes...
Table 4: Mathematical release kinetics models for nanoemulsions (Cym-NE, Myr-NE) and free terpenes (Cym-Sol and Myr-Sol).

<table>
<thead>
<tr>
<th>Model</th>
<th>Zeroth order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Hixson–Crowel</th>
<th>Korsmeyer–Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cym-Sol</td>
<td>$k$ = 0.0001</td>
<td>0.0069</td>
<td>24.783</td>
<td>0.0023</td>
<td>1.5587</td>
</tr>
<tr>
<td></td>
<td>$R^2$ = 0.8946</td>
<td>0.881</td>
<td>0.8993</td>
<td>0.8729</td>
<td>0.9534</td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.6685</td>
</tr>
<tr>
<td>Cym-NE</td>
<td>$k$ = 0.0008</td>
<td>0.0191</td>
<td>6.5154</td>
<td>0.0059</td>
<td>10.465</td>
</tr>
<tr>
<td></td>
<td>$R^2$ = 0.6729</td>
<td>0</td>
<td>0.6161</td>
<td>0</td>
<td>0.8776</td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.3005</td>
</tr>
<tr>
<td>Myr-Sol</td>
<td>$k$ = 0</td>
<td>0.0017</td>
<td>0.6424</td>
<td>0.0006</td>
<td>0.4836</td>
</tr>
<tr>
<td></td>
<td>$R^2$ = 0.857</td>
<td>0.7223</td>
<td>0.8576</td>
<td>0.7195</td>
<td>0.8813</td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.6152</td>
</tr>
<tr>
<td>Myr-NE</td>
<td>$k$ = 0</td>
<td>0.0053</td>
<td>2.1477</td>
<td>0.0017</td>
<td>3.3949</td>
</tr>
<tr>
<td></td>
<td>$R^2$ = 0.6317</td>
<td>0</td>
<td>0.6145</td>
<td>0</td>
<td>0.8419</td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.3079</td>
</tr>
</tbody>
</table>

exhibited a value of 0.6, suggesting an anomalous transport mechanism for drug release. This mechanism involves a combination of diffusion and dissolution processes for drug release [45].

These results indicate that the use of NEs may be an effective strategy to improve the control of the release rate of terpenes for more durable and effective control of immature stages of pest vectors.

Larvicidal properties of NEs against Aedes aegypti

The potential larvicidal activity of free monoterpenes and nanoemulsions was assessed using third-instar Aedes aegypti larvae. The negative control group was treated with surfactant solutions (Span 80 and Tween 20) at the same concentrations as in the nanoemulsions. Mortality of mosquito larvae was recorded after 24 h of exposition according to the WHO protocol [55].

Free cymene exhibited a concentration-dependent larvicidal activity. At 5 mg/L, mortality was 20% ± 4%, rising to 83% ± 2.3% at 25 mg/L and peaking at 98.6% ± 2.3% at 50 mg/L. Surprisingly, the cymene NE displayed a slightly reduced efficacy at lower concentrations (5 mg/L and 25 mg/L) compared to free cymene. This suggests that the encapsulation influences the bioactivity, potentially because of improved dispersion and controlled release of cymene.

Similarly, free myrcene exhibited a concentration-dependent efficacy. Myrcene NEs consistently outperformed free myrcene at all concentrations, indicating a better dispersion of the nanoemulsions in aqueous media. This was most prominent at lower concentrations, resulting in mortality rates of 10.6% ± 2.3% at 5 mg/L and up to 100% at 50 mg/L (Table 5).

Cytotoxicity of NEs in human keratinocytes

The evaluation of the biocompatibility in human cells is an important step in the development and commercialization of any drug [46]. Here, the toxicity of the terpene-based formulations was evaluated in the HaCAT cell line (Table 6). The results show that the IC$_{50}$ values of the free terpenes were lower than those of the nanoemulsions, suggesting that the nanoemulsification reduces the cytotoxicity of terpenes. It is important to note that the surfactant solutions presented the highest IC$_{50}$ values, which indicates that the composition of the nanoemulsion may influence its ability to decrease terpene toxicity. These results are in line with the existing literature, which indicates that monoterpenes exert low cytotoxicity on keratinocyte cells, either free [47-49] or in nanoemulsions [50,51]. It is important to highlight that the excipients used in the formulation are within the maximum concentration recommended by the FDA (7% for Span 80 and 5% for Tween 20) [52].

Acute toxicity of LNCs in alternative in vivo model using Galleria mellonella

The in vivo acute toxicity of the NEs was assessed against G. mellonella larvae. No mortality was observed at concentr-
tions ranging from 250 to 1000 mg/kg, indicating that the NEs did not cause acute toxicity (Figure 3). However, irritation was observed on day 0, as the larvae exhibited abnormal movements, such as repetitive jumping, after injection of the NEs. This behavior was not constant and ceased after 10 min. On day 1, the larvae treated with both free drugs produced a web of oily/sticky nature, particularly at higher concentrations, which persisted up to day 2. The absence of acute toxicity of nanoparticles on *G. mellonella* larvae is consistent with previous observations [53,54]. Overall, the results suggest that the NEs are not toxic to the larvae at the tested concentrations.

**Conclusion**

The rHLB values for cymene and myrcene were 15 and 16, respectively. These formulations demonstrated good colloidal stability over 60 days with stable values of size, PdI, and zeta potential. In vitro release studies demonstrated that the encapsulation of myrcene or cymene in nanoemulsions led to a sustained release of the compounds, suggesting that they could potentially provide a more efficient method for delivering these compounds compared to free solutions.

Furthermore, the study showed that the nanoemulsification process reduced the cytotoxicity of terpenes, as evidenced by the lower IC\(_{50}\) values of free terpenes compared to the nanoemulsions containing monoterpenes. The in vivo acute toxicity assessment in *G. mellonella* larvae indicated that the nanoemulsions exhibit a good toxicological profile. Finally, bioassays showed that terpene nanoemulsions had equal or greater insecticidal properties than free terpenes and they might facilitate their dispersion in an aqueous environment.

The larvicidal effect of the nanoemulsions, together with their safety and sustained release attributes, holds significant promise for environmentally friendly and effective pest control. Subsequent investigations should further optimize these formulations to unlock their full potential as part of integrated pest management.

**Experimental**

**Preparation of terpene nanoemulsions**

Cymene (Cym-NE) and myrcene (Myr-NE) nanoemulsions were obtained by a low-energy method. Briefly, an oil phase composed of the terpene (cymene or myrcene) (5% w/w) was mixed with the surfactants (Span 80/Tween 20) (5% w/w) using

---

**Table 6:** Inhibitory concentrations 50 (IC\(_{50}\)) of the NEs (Cym-NE and Myr-NE), free terpenes (Cym-free and Myr-free), and surfactants solution (B-Cym and B-Myr) in human keratinocytes.

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50}) (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cym-free</td>
<td>6.43 ± 0.56</td>
</tr>
<tr>
<td>Myr-free</td>
<td>1.86 ± 0.15</td>
</tr>
<tr>
<td>B-Cym</td>
<td>16.98 ± 0.90</td>
</tr>
<tr>
<td>B-Myr</td>
<td>22.37 ± 0.32</td>
</tr>
<tr>
<td>Cym-NE</td>
<td>14.95 ± 0.64</td>
</tr>
<tr>
<td>Myr-NE</td>
<td>2.37 ± 0.33</td>
</tr>
</tbody>
</table>

---

**Figure 3:** Kaplan–Meier survival curve of *G. mellonella* exposed to (A) Cym-Sol, (B) Myr-Sol, (C) Cym-NE, and (D) Myr-NE; DC: death control (100% methanol); SC: solvent control (ethanol 5%); TC: trauma control.
a magnetic stirrer. After homogenization, the aqueous phase of ultrapure water (90% w/w) was added dropwise. The terpenes were obtained commercially from Sigma-Aldrich.

Determination of the rHLB of the terpenes
The rHLB values of cymene and myrcene were determined by mixing different proportions of a lipophilic (sorbitan monooleate, Span 80, HLB 4.3) and a hydrophilic surfactant (polysorbate 20, Tween 20, HLB 16.7). Different formulations were prepared in a HLB range of 10.0–16.7, and the rHLB was the one in which the formulation had the best colloidal stability (Table S1, Supporting Information File 1).

Characterization of the nanoemulsions
Visual appearance
The formulations obtained were maintained at room temperature and evaluated visually 24 h and 7, 14, and 21 days after preparation. Signs of instability such as creaming, sedimentation, and phase separation were recorded, as well as physical aspects such as color, transparency, and fluidity.

Dynamic light scattering analysis
The average hydrodynamic diameter and polydispersity index (PDI) of the NEs were evaluated over a period of 60 days using dynamic light scattering, and the zeta potential was determined via electrophoretic mobility in a Zetasizer 3000 HSA (Malvern Instruments) device, using a 10 mW HeNe laser operated at 633 nm with a detection angle of incidence of 173° at 25 °C. Data analysis was performed in automatic mode. The NEs were diluted in deionized water (1:25) before the analysis.

Nanoparticle tracking analysis
Nanoparticle tracking analysis was performed in a NanoSight NS300 (Malvern Instruments, United Kingdom) apparatus equipped with a sample chamber and a 638 nm laser. The samples were diluted (1:1000 v/v) in ultrapure water. The NEs were injected into the sample chamber with sterile syringes until the liquid extended to the tip of the injector. The measurements were performed in triplicate at room temperature (25 °C) and the data were represented as mean ± standard deviation.

Cryogenic transmission electron microscopy
The nanoemulsions were mounted onto a copper grid with lacy carbon film (300 mesh). The acquisition was carried out with a MET Talos Arctica G2 apparatus.

In vitro terpene release profile
The in vitro release assays were conducted assuring sink conditions. Modified Franz cells, equipped with a polyethersulfone membrane (Sigma-Aldrich) and with a diffusion area of 1.77 cm² were used in the assays. A Microette (Hanson Research, USA) was used. The receptor compartment was filled with 7.0 mL of a receptor solution composed of 0.1 M phosphate buffer and ethanol (50:50 v/v), pH 5.5. 1 mL of the formulations was used, as allowed by the Franz cell.

The acceptor solution was constantly agitated at 300 rpm using mini-magnetic agitators. The temperature was maintained at 37 ± 2 °C by utilizing a circulating heating bath in the jacketed cells.

The evaluation of the release of cymene and myrcene from the nanoemulsions was performed at specific time intervals: 30 min and 1, 2, 4, 6, 8, 12, and 24 h. Each measurement was repeated six times to ensure reliability. The released compounds were quantified by high-performance liquid chromatography, following a previously validated method.

In silico molecular and physicochemical properties of the monoterpenes
The ACD/Labs Percepta Platform, particularly the PhysChem Module, was employed to forecast molecular and physicochemical data. The ChemSpider tool facilitated the acquisition of these properties [56,57].

Preliminary larvicidal assay
The protocol involved exposing III–IV-instar larvae to terpenes and terpene-based nanoemulsions; the mortality was recorded after 24 h. The laboratory-susceptible reference strain (Bora) from French Polynesia was utilized. The experimental protocol adhered to WHO guidelines with certain modifications [55]. Each experiment was conducted in triplicate, involving 25 third-instar larvae within each sample. Nanoemulsions diluted in distilled water at concentrations of 5, 25, and 50 mg/L were employed. For the negative control, a surfactant solution was utilized at the highest concentration of the tested samples.

Cytotoxicity in human keratinocytes (HaCAT)
The HaCat cell line (code 341; Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) is a line of non-tumorigenic human epithelial keratinocytes. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine fetal serum and 100 μg/mL of penicillin G/streptomycin. Maintained at 37 °C with 5% CO₂, the cells were grown until they reached a subconfluent density. To detach the cells, a 5 min trypsin treatment with TrypLE™ Express at 37 °C was performed, followed by inactivation using 0.3 mg/mL trypsin inhibitor. The cells were then centrifuged at 500g for 5 min, resuspended in DMEM, and placed overnight in 96-well microplates (200 μL/well, 1 × 10⁶ cells/mL) at 37 °C with 5% CO₂. After incubation, nanoemulsions, free terpenes, and surfactant solutions were administered at concentrations from 0.1 to
250 mg/mL to the cells for 24 h at 37 °C with 5% CO₂. Cell viability was assessed using a colorimetric MTT assay. Cells were exposed to a 10 μL MTT stock solution (5 mg/mL in PBS) and incubated at 37 °C for 2 h. After incubation, the culture medium was replaced with 100 μL of DMSO. The optical density at 570 nm was measured using a microplate reader. Cell viability was determined by comparing the absorbance of each product concentration to untreated cells, with the negative control (DMEM) representing 100% cellular metabolism. The analysis utilized average values.

In vivo toxicity evaluation
The experiment used larvae of the *G. mellonella* species, as described by Allegra et al. and Marena et al. with modifications [54,58]. A minimum of ten larvae per group (*n* = 10) were used, which were fed and raised at 25 °C until they weighed more than 0.2 mg. Larvae between 0.2 and 0.3 mg were used for the experiment, and the samples were administered (10 μL/larva) on the left side of the last proleg using a 10 μL Hamilton syringe. The larvae were then kept in the dark at room temperature and observed after 24, 48, and 72 h to evaluate their behavior, including physical aspects such as color, melanization, or loss of mobility, in response to the treatment. Death was considered when there was no physical reaction after stimulation. The samples were tested at concentrations of 250, 500, and 1000 mg/kg, with controls including trauma control (puncture only, TC), death control (100% methanol, DC), solvent control (5% ethanol, SC), and NE control (Cym-B and Myr-B).

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Data Availability Statement
The data that supports the findings of this study is available from the corresponding author upon reasonable request.

References


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