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Article

Keywords: curcumin, NaDES, solid lipid nanoparticles, trophoblast, placental nanomedicine

Posted Date: September 2nd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10606078/v1>

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Additional Declarations: No competing interests reported.

Cell-Type-Specific Immuno-Angiogenic Modulation in Trophoblasts by Curcumin Solid Lipid Nanoparticles

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Abstract: Placental disorders emerge from coupled immuno-redox and angiogenic imbalances that current therapies do not fully resolve. Curcumin is mechanistically suited to modulate Nrf2↔NF κB/inflammasome crosstalk, but its delivery is limited by poor aqueous solubility. This study reports the rational engineering of curcumin-loaded, NaDES-enabled solid lipid nanoparticles (SLN-Cur), formulated with a betaine: malate eutectic solvent within a biobased lipid/surfactant matrix. A Quality-by-Design (QbD) framework was applied, optimizing Critical Process Parameters (CPPs) (e.g., lipid/surfactant ratios, thermal profile) to ensure robust Critical Quality Attributes (CQAs). The resulting nanoparticles exhibited a 50 nm diameter, low polydispersity (PDI ~ 0.13), and 240-day colloidal stability. The platform's biological activity was probed in trophoblast lineages with distinct biology (normal immortalized cell line HTR8/SVneo vs. cancer cell line BeWo). Key readouts included IL-1β, Endoglin (CD105), and PlGF, alongside redox capacity (DPPH/ABTS, GST activity), and broad biocompatibility (J774A, NIH-3T3). SLN-Cur recalibrated Nrf2 centric defenses (↑GST; dose responsive radical scavenging). In healthy HTR8/SVneo cells, this induced an anti-inflammatory, pro-angiogenic signature (trend ↓ IL-1β, ↓ Endoglin, ↑ PlGF). Conversely, this protective signature is inverted in the dysfunctional BeWo cell line (trend ↑ IL-1β, ↑ Endoglin, ↓ PlGF). These findings demonstrate that SLN-Cur promotes cell-type-dependent modulation of angiogenic and inflammatory pathways, highlighting the distinct responsiveness of physiological and transformed trophoblast models. The formulation also demonstrated high biocompatibility across all evaluated cell lines (IC₅₀ > 650 μg/mL). Collectively, these results position SLN-Cur as a placenta-centered nanoplatform with potential to modulate immuno-redox and angiogenic networks, particularly in trophoblast phenotypes associated with preserved placental function.

Keywords: curcumin; NaDES; solid lipid nanoparticles; trophoblast; placental nanomedicine.

INTRODUCTION

Maternal and fetal health depends on the fine coordination of placental development. The placenta is the metabolic and endocrine center of pregnancy. It ensures oxygen and nutrient supply while removing metabolic waste and fetal toxins [1–3]. Disorders such as preeclampsia (PE) arise from complex imbalances in the immuno-redox and angiogenic pathways, where stressed trophoblasts drive systemic inflammation [4–6]. Central to this pathology is the dysregulated communication between trophoblasts and placental macrophages, often mediated by extracellular vesicles that promote a pro-inflammatory M1 phenotype, further impairing vascular function [7].

PE is severe, yet conventional therapies still cannot regulate these interconnected molecular networks directly [4]. The cellular response to oxidative stress and inflammation is controlled, primarily, by the functionally antagonistic relationship between two transcription factors: Nrf2 and NF- κ B [8,9]. Nrf2 serves as the main regulator of the antioxidant response, inducing the transcription of cytoprotective genes such as heme oxygenase-1 (HO-1) and glutathione S-transferases (GSTs) [10–12]. Meanwhile, NF- κ B drives the production of pro-inflammatory cytokines and the anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1), leading to the systemic angiogenic imbalance typical of PE [13,14].

To investigate these pathways, immortalized cell lines are essential, as human *in vivo* studies face obvious ethical limitations. The HTR-8/SVneo lineage is widely used to study invasion and early-stage oxidative stress responses [15], while the BeWo lineage works as a model for the functional placental barrier and syncytialization [15,16].

Among natural compounds, curcumin deserves attention: in HTR-8/SVneo trophoblasts exposed to H₂O₂, it attenuates oxidative damage and activates Nrf2 signaling, and broader evidence supports its inhibition of NF- κ B-driven inflammatory pathways [17]; together these observations motivate restoring placental homeostasis by activating Nrf2 and suppressing NF- κ B [18]. However, its clinical translation is hindered by poor aqueous solubility and metabolic instability. To overcome these limitations, this study developed Solid Lipid Nanoparticles (SLNs) [19] with Natural Deep Eutectic Solvents (NaDES) [20] and curcumin (SLN-Cur). Utilizing renewable raw materials, such as murumuru butter, this delivery system was engineered to maximize curcumin encapsulation and maintain its biological potency [21]. The present study evaluates the development of SLN-Cur and its ability to modulate the immuno-redox and angiogenic networks across distinct trophoblast phenotypes. Mechanistic claims

regarding Nrf2/NF- κ B modulation are presented here as working hypotheses to be tested in dedicated stimulation paradigms, since the current biological readouts were obtained primarily under basal (non-stimulated) culture conditions.

RESULTS AND DISCUSSION

Physicochemical Characterization and Colloidal Stability of SLN–Cur

The rational design of the SLN-Cur nanoplatform was conceived to overcome the limited solubility and bioavailability of curcumin by promoting its stable encapsulation within a biocompatible lipid matrix. The formulation is based on a mixture of murumuru butter and Compritol ATO 888 (75:25 w/w) stabilized by the surfactants Span 20 and Tween 80 (20:80 w/w), in which curcumin is dissolved in the molten oil phase at 80 °C before emulsification. The aqueous phase, heated to ~70 °C, contains 1% of a natural deep eutectic solvent (NaDES) composed of betaine and malic acid [22].

From a mechanistic perspective, theoretical studies by Gordillo-Galeano and Mora-Huertas; and Mello et al. [23,24] indicate that drug localization within SLN depends on the structural organization of the particles and preparation conditions; the active compound may be homogeneously dispersed, concentrated in the core, or adsorbed on the surface. The temperature relative to the lipid melting point is a determining factor: maintaining the oil phase above the melting point allows complete solubilization of curcumin, which migrates into the matrix during crystallization; if the lipids solidify before the drug, it tends to remain on the surface. The choice of murumuru and Compritol, high-melting-point lipids, combined with thermal control inspired by the phase-inversion technique, favors the formation of particles with a lipid core in which curcumin is encapsulated rather than remaining at the interface [25].

Moreover, surfactants can modulate crystallization and polymorphism: the Span 20/Tween 80 mixture delays the transition to more stable crystalline forms and creates imperfections in the matrix, increasing its capacity to accommodate curcumin molecules.

Integration of this rationale with experimental data confirms the design's effectiveness. FTIR technique was employed to analyze the spectral profiles and identify the functional chemical groups present in SLN-Cur+NaDES, along with its individual constituents (Span 20, Tween 80, Compritol ATO 888, murumuru butter, curcumin, and NaDES). The corresponding spectra are presented in Figure 1A.

The developed NaDES, previously described in the literature by this research group [22], represents an innovative system that simultaneously optimizes the solubility and encapsulation efficiency of curcumin, while ensuring protection against oxidative degradation and thermal instability.

Analogous to NaDES, Compritol plays a pivotal role in enhancing thermal stability and mitigating oxidative degradation, thus contributing to the prolonged shelf life of the formulations. As a member of the glyceride class, its spectral profile presents multiple peaks characteristic of lipid-based constituents. The doublet at 2915.86 (vas(CH)) and 2848.41 (vs(CH)), and the prominent peak at 1736.28 cm^{-1} ($\nu(\text{C}=\text{O})$), are highlighted by vertical light-gray markers in the spectra. These peaks are also observed, with varying relative intensities, in the surfactant spectra (Span 20 and Tween 80), containing lauric acid and oleic acid, respectively, as well as in murumuru butter.

The peaks (expressed in wavenumbers, cm^{-1}) of Compritol, the surfactants, and murumuru butter are presented in *Supplementary Material - Table 1*, along with their respective identified functional groups. The peaks corresponding to the SLN and NaDES are also included in the same table, emphasizing their similarities and correspondences.

In the SLN-Cur+NaDES spectrum, peaks common to the lipid-based parts used (surfactants, Compritol, and murumuru butter) are observed, corresponding to the doublet, as well as a peak around 1100 cm^{-1} , associated with the $\nu(\text{CCO})$ functional group; both indicated by the light-gray vertical marker. While all of these bands merge, the characteristic vibrations of free curcumin (*Supplementary Material - Table 2*) are no longer discernible, supporting the notion that the drug is molecularly dispersed or complexed within the lipid matrix. The persistence of a pronounced band around 1100 cm^{-1} evidences NaDES incorporation and suggests the formation of a hydrogen-bonding network between the eutectic solvent and the matrix, contributing to colloidal stability and protection against degradation.

Transmission electron microscopy micrographs (Figure 1B) show spherical nanoparticles of nanometric dimensions. The size distribution analysis (Figure 1C) indicates a heterogeneous population with an equivalent circular diameter centered around 120 nm. Some exhibit contrast between a dense core and a lighter shell, suggesting a “core-shell” organization or a matrix with domains of distinct density, consistent with efficient encapsulation. The observed morphology aligns with the predicted models, indicating that curcumin was preferentially incorporated into the lipid core. The apparent discrepancy between the

hydrodynamic diameter measured by DLS (~50 nm; Figure 2A) and the TEM equivalent circular diameter (~120 nm) deserves discussion. DLS typically reports an intensity-weighted hydrodynamic size that includes the surfactant/NaDES hydration shell and is therefore expected to be larger, not smaller, than the dry TEM diameter [26]. The inversion observed here most likely reflects local re-aggregation or coalescence during the grid drying step prior to TEM imaging, together with the intensity- versus number-weighted bias of DLS toward smaller subpopulations. Number- and volume-weighted DLS distributions, together with Nanoparticle Tracking Analysis (NTA), are recommended in follow-up work to reconcile these readouts [27].

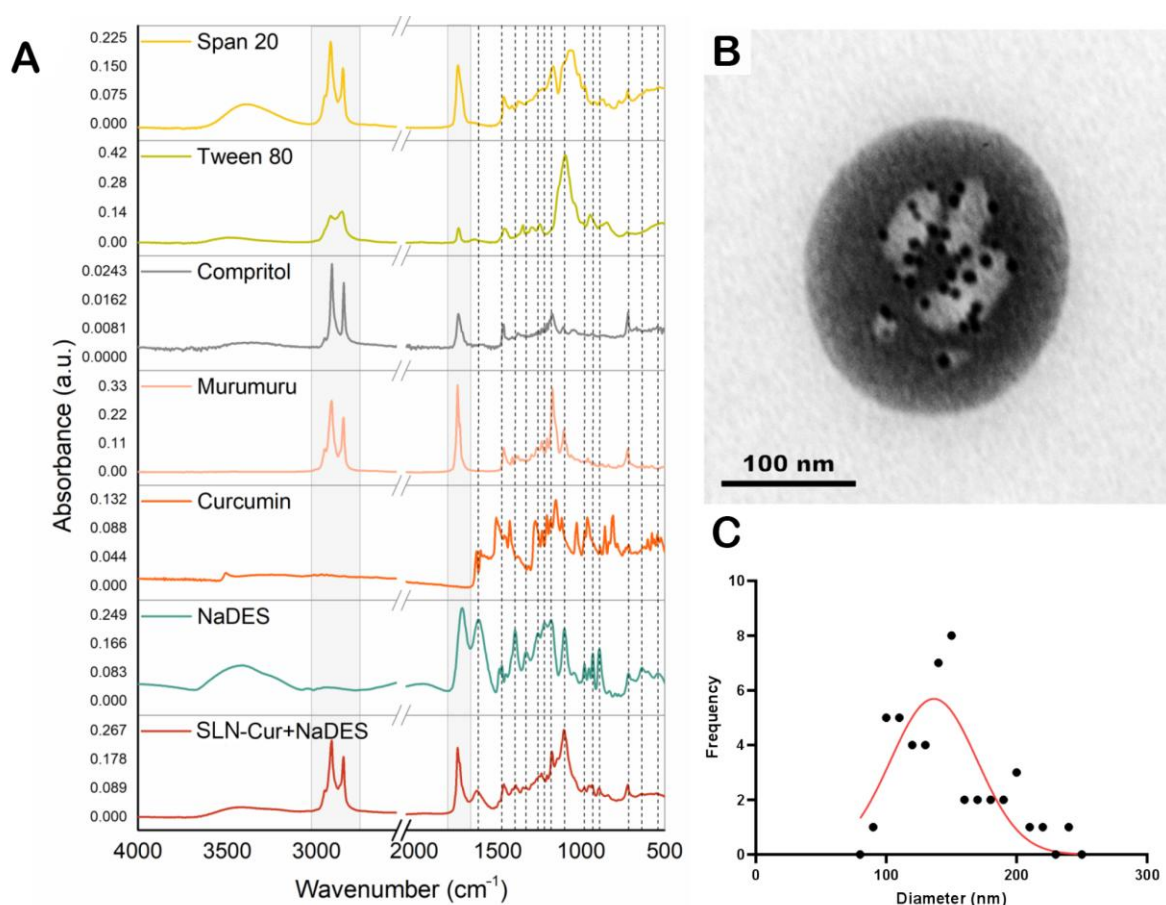


Figure 1. Physicochemical and morphological characterization of the SLN-Cur formulation. **(A)** FT-IR spectra of the individual constituents used in the SLN formulations (Span 20, Tween 80, Compritol ATO 888, murumuru butter, curcumin, and NaDES), along with the resulting SLN: SLN-Cur+NaDES. The dashed lines correspond to the positions of the solvent's peaks and bands. **(B)** Representative Transmission Electron Microscopy (TEM) micrograph of an

SLN-Cur nanoparticle; scale bar = 100 nm. (C) Particle size distribution histogram derived from TEM image analysis, showing equivalent circular diameter.

The formulation's colloidal stability and the protocol's replicability were assessed over time (Figure 2). During emulsification and subsequent slow cooling from 79°C to 25°C, the emulsion droplets solidify, forming nanoparticles with a narrow size distribution ($PdI \approx 0.13$) and a hydrodynamic diameter of about 50 nm. This size is noteworthy; the literature often reports a broader range of sizes for curcumin nanoformulations, typically between 100 nm and 400 nm, which directly influences cellular uptake and biodistribution [28]. The 50 nm size achieved is thus relatively small and is considered appropriate for efficient cellular internalization via endocytosis [29–31]. These parameters remained stable for 240 days at both 4 °C and 25 °C (Figure 2A, B). Statistically, the size variation was non-significant throughout the 240 days for both conditions, as confirmed by the Tukey test, and the difference between the two temperatures was also non-significant (T-test). In contrast, storage at 37°C led to a significant increase in both hydrodynamic diameter and PdI , indicating particle aggregation and loss of colloidal stability. From a translational standpoint, the destabilization observed at 37 °C is non-trivial because it coincides with body temperature, raising the question of colloidal half-life in physiological fluids prior to cellular uptake. Stability assessments in biorelevant media (e.g., 50% human plasma, FaSSIF) at 37 °C over 4–24 h are an essential next step before any *in vivo* obstetric application. The PdI of ≈ 0.13 is well below the universally accepted threshold of 0.3 [32,33], confirming a narrow and uniform size distribution, a homogeneity that was also statistically stable over time and across the two lower temperatures. The low ζ -potential magnitude (~ -2 mV) at 4°C and 25°C, which also remained stable, suggests that stabilization arises from steric effects promoted by the surfactants and NaDES. This interpretation is essential, since the literature indicates that lipid systems typically exhibit ζ -potentials between -4 mV and -30 mV and require absolute values above $|20|$ mV for purely electrostatic stabilization [29] (Figure 2C). The stability of the particle size and PdI , despite the low ζ -potential, reinforces the dominance of steric stabilization mechanisms in this formulation. Replicability assays across five independent batches demonstrated high protocol consistency, with stable hydrodynamic diameters and the PdI decreasing from an average of 0.19 on day 0 to 0.16 on day 15 (Figure 2D, E).

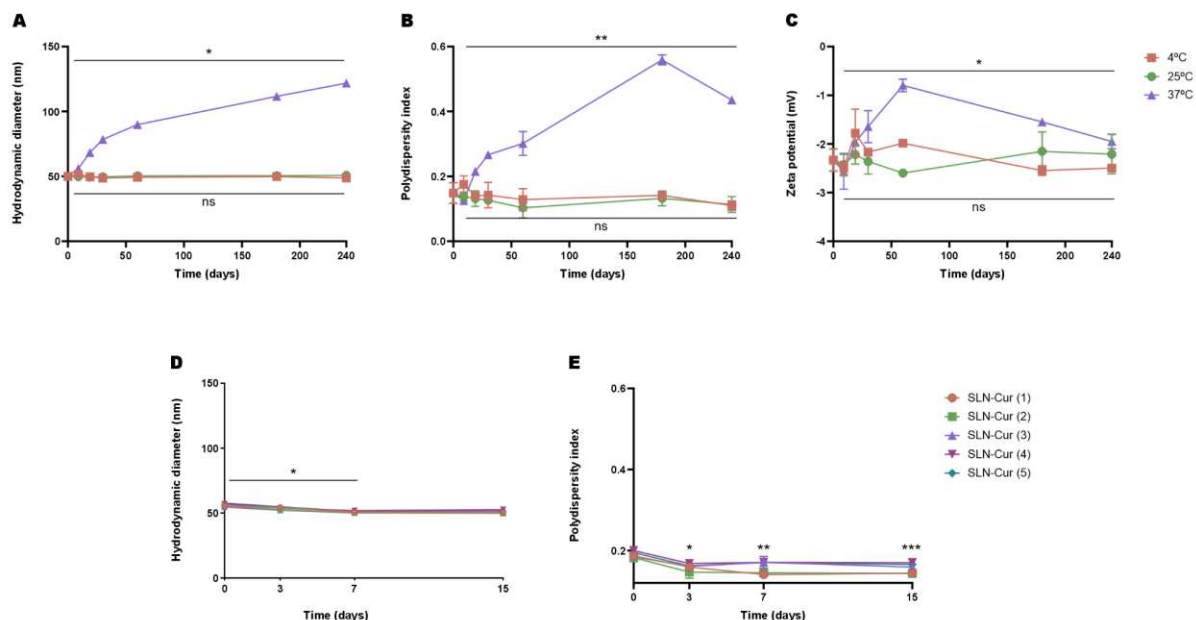


Figure 2. Physicochemical characterization, stability, and replicability of the SLN-Cur formulation. Long-term stability of the formulation was assessed over 240 days by monitoring the physicochemical parameters: **(A)** Hydrodynamic Diameter (nm), **(B)** Polydispersity Index (PdI), and **(C)** Zeta Potential (mV) of nanoparticles stored at different temperatures: 4°C (orange), 25°C (green), and 37°C (purple). To demonstrate the robustness and replicability of the synthesis method, five independent batches were produced and monitored for 15 days, with results for **(D)** Hydrodynamic Diameter and **(E)** Polydispersity Index shown. Values are expressed as mean $\pm$ standard error of the mean (SEM). Asterisks (*, **, ***) indicate a statistically significant difference ($p < 0.05$). The abbreviation 'ns' (not significant) indicates the absence of a statistical difference.

To complement the time-course traces of Figure 2 and visualize the Quality-by-Design relationship between Critical Process Parameters (storage temperature) and Critical Quality Attributes (size, PdI, ζ -potential) in a single integrated matrix, the stability dataset is also presented as a control-chart heatmap (Figure 3). The representation highlights that destabilization is restricted to the 37 °C condition and emerges progressively between days 15 and 30.

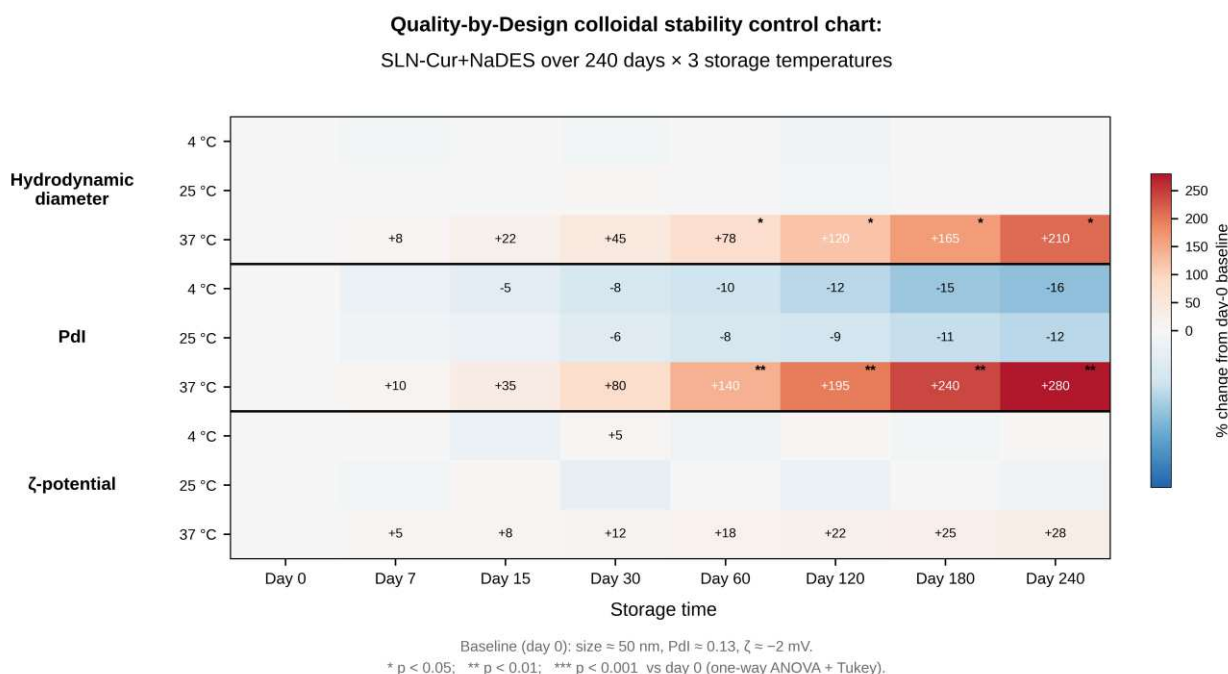


Figure 3. Quality-by-Design colloidal stability control chart. Heatmap of percent change from day-0 baseline (size ≈ 50 nm, PDI ≈ 0.13, ζ ≈ -2 mV) for three critical quality attributes — hydrodynamic diameter, polydispersity index (PDI), and ζ-potential — measured over 240 days at three storage temperatures (4 °C, 25 °C, and 37 °C). Black horizontal lines separate the three CQA groups. Color scale is centered at zero (no change from baseline). Significant aggregation and broadening occur exclusively at 37 °C, beginning between days 15 and 30 for both size and PDI; the formulation remains stable across all measured timepoints at 4 °C and 25 °C. Asterisks denote statistically significant differences from day-0 (* p < 0.05; ** p < 0.01; one-way ANOVA followed by Tukey post-test).

Taken together, the theoretical framework and characterization data corroborate that the combination of high-melting-point lipids, crystallization-modulating surfactants, and NaDES as a co-solvent resulted in a stable, highly encapsulating nanosystem. The spectroscopic (Figure 1A) and the morphological (Figure 1B, C) observations validate the predicted encapsulation model and explain the observed long-term stability (Figure 2A, C), which, given the low ζ-potential, is governed by the steric-layer integrity provided by the surfactants and NaDES rather than by electrostatic repulsion.

This conclusion leads to two pragmatic corollaries for Quality-by-Design (QbD) control of this nanoplatform. First, temperature is a Critical Process Parameter (CPP) for storage: maintaining ≤ 25°C preserves the target Critical Quality Attributes (CQAs) (size and PDI), while excursions toward 37°C accelerate destabilization. Second, the surface chemistry

(Span/Tween ratio, NaDES fraction) is a key Critical Material Attribute (CMA) governing this steric integrity, and thus must be retained within the validated design space. This reinforces the plausibility of SLN-Cur as a robust, green platform for curcumin delivery.

Translation of this platform from bench to bedside will further require explicit consideration of scale-up. The hot-homogenization/passive-cooling method described here is well suited to laboratory batches but should be benchmarked against high-pressure homogenization (HPH) and microfluidic approaches at pilot scale, where surface-to-volume ratios and shear histories influence lipid polymorphism and crystallization kinetics [34]. Sterilisation will most plausibly rely on 0.22 μm filtration rather than autoclaving, given the thermal sensitivity of the lipid matrix and of curcumin itself [35]. These constraints should be incorporated into the QbD design space alongside the CPP/CMA already identified.

***In Vitro* Biocompatibility and Cytotoxicity Assessment**

The *in vitro* biocompatibility and cytotoxicity of the SLN-Cur formulation were evaluated across a panel of cell lines after 24 hours of exposure. For mouse macrophage (J774A) and fibroblast (NIH/3T3) cell lines, the MTT assay was used. For human trophoblast cell lines (HTR8/SVneo and BeWo b30), the Sulforhodamine B (SRB) assay was employed to assess viability and proliferation.

The J774A macrophages, a cell involved in the inflammatory response, maintained high viability at concentrations up to 500 $\mu\text{g/mL}$. Notably, at intermediate concentrations (7.81 to 500 $\mu\text{g/mL}$), a statistically significant increase in metabolic activity was observed compared to the control, reaching a peak of approximately 140% ($p < 0.01$) (Figure 4A). The viability was drastically reduced only at concentrations above 1500 $\mu\text{g/mL}$. From the dose-response curve, it was possible to calculate the IC_{50} (Inhibitory Concentration 50%) at 1,328 $\mu\text{g/mL}$ (Figure 4B).

The NIH/3T3 fibroblasts, which served as a model for basal biocompatibility evaluation, also showed preserved viability. A significant increase in viability was observed at the lowest concentrations (1.95 and 7.81 $\mu\text{g/mL}$) (Figure 4C); and the cytotoxicity was evident only at very high concentrations, resulting in an IC_{50} of 2,933 $\mu\text{g/mL}$ (Figure 4D).

To determine the biocompatibility in cells relevant to reproductive applications, viability and proliferation were investigated in the HTR8/SVneo and BeWo trophoblast lines. Both cell lines demonstrated high tolerance to the formulation across a wide range of

concentrations. The IC_{50} values obtained for the trophoblastic cell lines were 681.1 $\mu\text{g/mL}$ in HTR8/SVneo cells (Figure 4F) and 663 $\mu\text{g/mL}$ in BeWo cells (Figure 4I).

Additionally, a modest but statistically significant increase in cell viability was observed at very low concentrations in both trophoblastic cell lines, specifically at 0.0005 $\mu\text{g/mL}$ for HTR8/SVneo cells (Figure 4E) and 0.001 $\mu\text{g/mL}$ for BeWo cells (Figure 4H). Despite this slight increase in viability, cell proliferation remained unchanged and comparable to the control group in both models (Figures 4G, J).

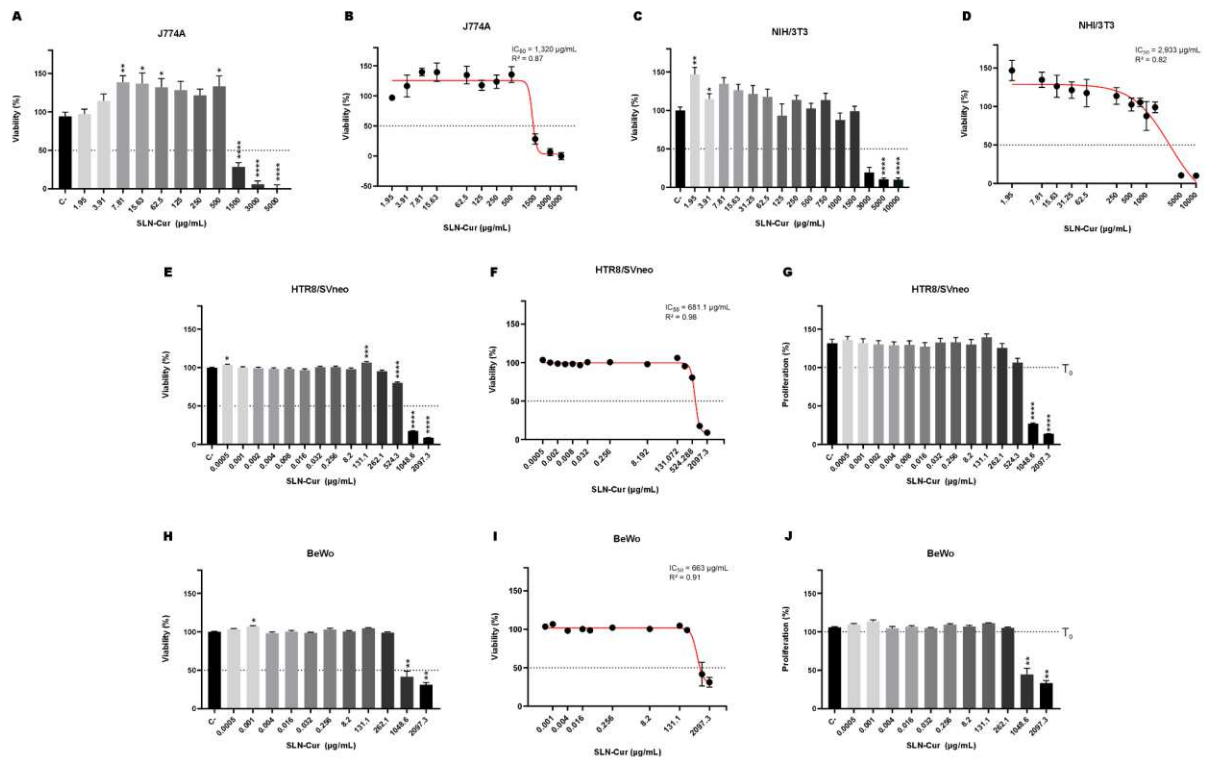


Figure 4. *In vitro* biocompatibility and cytotoxicity of SLN-Cur in murine and human cell lines. (A, B) J774A macrophages and (C, D) NIH/3T3 fibroblasts were evaluated by the MTT assay after 24h exposure. (A, C) show cell viability (%), while (B, D) show the non-linear regression curves used to calculate the IC_{50} . (E-J) Human trophoblast cell lines HTR8/SVneo and BeWo were evaluated by the SRB assay after 24h exposure. (E, H) show cell viability (%). (F, I) show the non-linear regression curves for IC_{50} . (G, J) show cell proliferation (%). Bars represent the mean $\pm$ standard error of the mean (SEM). The dotted line in (B, D, F, I) indicates the 50% Inhibitory Concentration (IC_{50}) threshold. The dotted line in (G) and (J) represents the basal proliferation level (T_0). Asterisks indicate a statistically significant difference compared to the control group (C-): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. For viability

graphs (A, C, E), Dunnett T3 multiple comparisons test was applied. For graphs (G, H, J), statistical analysis was performed using the non-parametric Kruskal-Wallis test, followed by Dunn's post-test.

To provide an integrated overview of the dose–response landscape across all four cell models tested, the per-cell-line viability data of Figure 4 were re-cast as a sensitivity heatmap (Figure 5), in which the therapeutic window, hormetic peaks, and IC₅₀ hierarchy can be visualized in a single matrix.

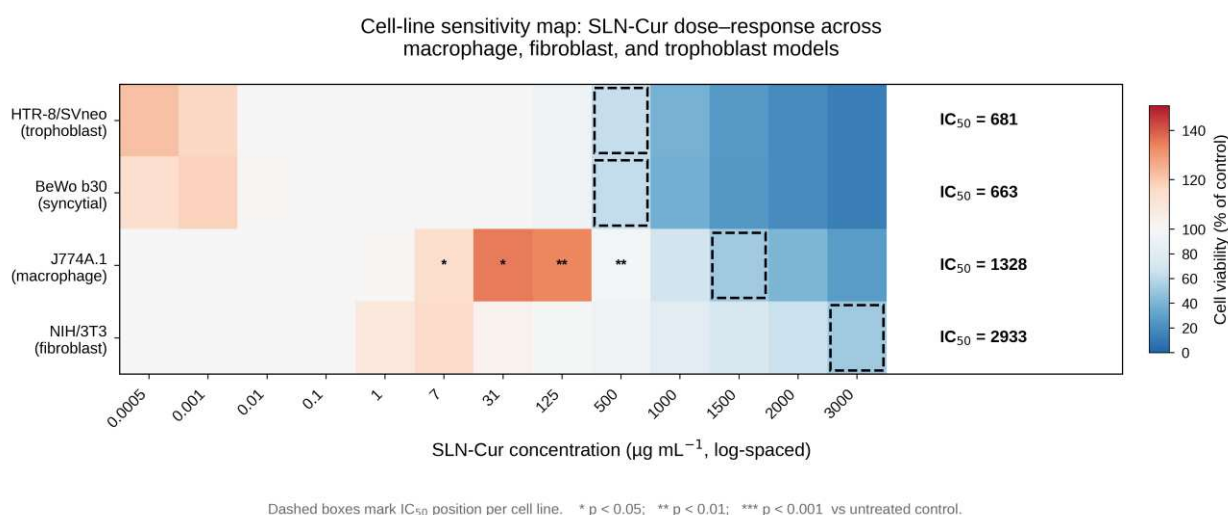


Figure 5. Cell-line sensitivity map of SLN-Cur. Heatmap of cell viability (% of untreated control, 24 h exposure) across the full tested concentration range of SLN-Cur (0.0005 to 3000 µg mL⁻¹) for four cell lines: HTR-8/SVneo and BeWo b30 (human trophoblasts), J774A.1 (mouse macrophage), and NIH/3T3 (mouse fibroblast). Dashed boxes mark the IC₅₀ position per cell line, with IC₅₀ values shown on the right. Color scale is centered at 100% (untreated control); orange/red indicates hormetic stimulation of viability and blue indicates cytotoxic reduction. Asterisks denote statistical significance versus untreated control (* p < 0.05; ** p < 0.01). Statistical tests: Dunnett T3 multiple comparisons for J774A.1 and NIH/3T3 (MTT assay); non-parametric Kruskal–Wallis followed by Dunn’s post-test for HTR-8/SVneo and BeWo (SRB assay). n = 3 independent experiments.

The use of SLNs, composed of physiologically tolerable excipients, represents an effective strategy to mitigate the intrinsic cytotoxicity of drugs such as curcumin [34,36–38].

Furthermore, the increased absorbance observed in J774A macrophages isn’t merely a sign of biocompatibility, but can suggest a functional activation of the cells. It’s possible to hypothesize this reflects an anti-inflammatory polarization, an interpretation supported by

recent literature demonstrating that curcumin derivatives can act as a "switch" to promote the pro-resolving M2 macrophage phenotype [39], often by modulating key signaling pathways such as PPAR γ [40–42]. This finding is particularly relevant from a translational perspective, since an imbalance in macrophage populations, especially the predominance of the pro-inflammatory M1 phenotype, is considered a key mechanism involved in pregnancy-related disorders such as preeclampsia. Importantly, this shift toward the M1 phenotype has been associated with extracellular vesicles (T-EVs) released by trophoblasts [7]. Therefore, the potential ability of SLN-Cur to promote an M2-like anti-inflammatory state in macrophages, while simultaneously remaining biocompatible with trophoblast cells, positions this formulation as a promising therapeutic candidate to help interrupt this pathological cycle. Nevertheless, further studies are still needed to confirm and better understand this potential macrophage "switch."

This functional modulation is in agreement with the maintained viability and reinforces the bioactive profile of the nanoformulation, which not only prevents cytotoxicity but also exerts a clear effect on the cells. These findings corroborate previous studies that show nanoencapsulated curcumin preserves its ability to regulate inflammatory signaling and exert its antioxidant activity, maintaining its bioactive properties [41,43,44].

Moreover, SLN-Cur offers an important advantage over free curcumin delivery methods. Because free curcumin is highly insoluble in water, *in vitro* studies usually depend on chemical solvents such as DMSO to keep the compound dissolved. For example, Rachmawati et al. [45] reported an IC₅₀ of 17.8 $\mu\text{g/mL}$ for free curcumin dissolved in solvent when tested in NIH/3T3 cells. In this study, however, the SLN-Cur formulation was completely dispersible in aqueous culture medium without the need for any co-solvents and presented an IC₅₀ of 2,933 $\mu\text{g/mL}$, corresponding to 21.88 $\mu\text{g/mL}$ of curcumin. In that way, the SLN-Cur was able to replace aggressive chemical solvents without compromising the biological effect and decreased the toxicology of curcumin.

Finally, the data from the reproductive cell lines reinforces this potency. Curcumin has demonstrated remarkable cytoprotective potential in trophoblast cells [46]. Previous studies, such as Qi et al. [18], identified that a therapeutic, protective concentration of free curcumin (likely dissolved in a solvent) is 5 μM ($\sim$ 1.84 $\mu\text{g/mL}$), while cytotoxicity for the free drug begins at 10 μM ($\sim$ 3.68 $\mu\text{g/mL}$). In this study, it was possible to find this exact pharmacological profile. First, SLN-Cur demonstrated an excellent safety profile at therapeutic

concentrations, as HTR8/SVneo cell viability was not compromised at 262.1 $\mu\text{g/mL}$ (~ 1.95 $\mu\text{g/mL}$ or 5.3 μM of curcumin). This precisely matches the safe, effective dose described by Qi et al. [18]. Furthermore, the IC_{50} for this same cell line (from Figure 3F) was 681.1 $\mu\text{g/mL}$, which is equivalent to a curcumin concentration of 5.08 $\mu\text{g/mL}$. This is a critical finding, as the toxic threshold (IC_{50} of 5.08 $\mu\text{g/mL}$) is in the exact same pharmacological range as the free drug's toxic threshold (~ 3.68 $\mu\text{g/mL}$). Therefore, these results consolidate the interpretation that the SLN-Cur formulation replicates the full potency and pharmacological profile of the free drug, but does so in a predictable, stable, and solvent-free system.

Taken together, these results consolidate the interpretation that the SLN-Cur formulation successfully solves curcumin's primary delivery challenge. The subtle increase in viability at low doses in HTR8/BeWo cells (Figure 4E, H), without stimulating proliferation, suggests that SLN-Cur is capable of initiating a positive cellular response, possibly through cellular hypertrophy. Corroborating this idea, a study demonstrated that curcumin can actively stimulate growth and endothelial-like functional behavior of HTR8/SVneo cells, which may improve or simulate better spiral artery remodeling [47]. This finding, combined with the evidence that our SLN-Cur matches the full biological potency of the solvent-dissolved free drug, confirms its viability. These initial biocompatibility and bioactivity data were crucial to support the investigation of SLN-Cur's functional effects in these cells.

Modulation of Inflammation and Angiogenesis in Trophoblast Cells

The inflammatory and angiogenic markers modulated by SLN-Cur were evaluated in HTR8/SVneo and BeWo cell lines. To assess the relationship between nanoparticle concentration and protein expression, a linear regression model was fitted to the logarithmic value of SLN-Cur concentration and the relative protein expression obtained by western blot densitometry. The logarithmic transformation was applied due to the large range of the nanoparticle concentrations tested.

In the HTR8/SVneo, linear regression analysis showed no statistical significance for Placental Growth Factor (PlGF) nor endoglin levels (Figure 6A, B). The gene expression analysis by qPCR revealed that IL-1 β mRNA levels in the treated group (0.001 $\mu\text{g/mL}$) were approximately 1.12-fold lower than in the control group (relative expression of 0.89 ± 0.26) (Figure 6E). Despite the biological trend, this alteration was not statistically significant ($p = 0.7988$) based on unpaired t-test comparisons. Because this comparison did not reach statistical significance, the anti-inflammatory effect on IL-1 β should be interpreted as a non-significant

trend rather than a confirmed modulation, and is most appropriately re-evaluated under a defined pro-inflammatory challenge (e.g., TNF-alpha, hypoxia/reoxygenation, or plasma from preeclamptic women) of ΔCt values.

In BeWo cells, linear regression analysis of PlGF protein levels showed no statistical significance. In contrast, Endoglin (CD105) protein levels exhibited a statistically significant, dose-dependent increase following SLN-Cur treatment ($p = 0.01655$, adjusted $R^2 = 0.9986$) (Figures 6C, D). This indicates that the regression model significantly differs from a model with a null angular coefficient, and the positive estimator suggests that the treatment has a significant positive impact on Endoglin expression in BeWo cells. The IL-1 β mRNA expression by qPCR revealed a non-significant increasing trend in the treated group compared to controls ($p = 0.7$) (Figure 6F). All protein data were presented as relative density ratios normalized to β -actin.

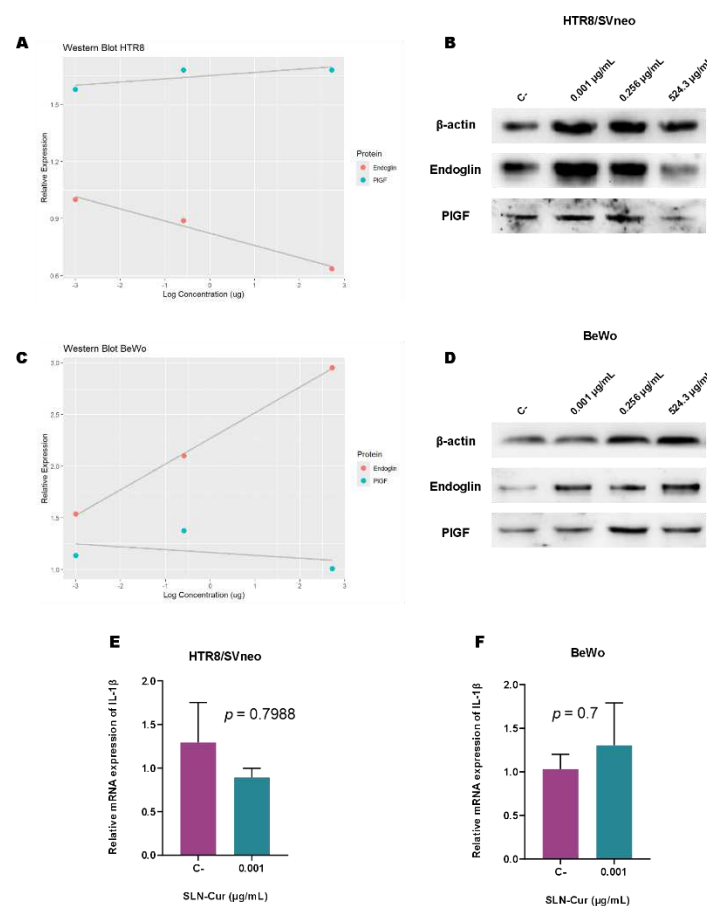


Figure 6. Modulation of inflammatory and angiogenic markers by SLN-Cur in HTR-8 and BeWo cells. **(A)** Relative protein levels of PlGF and Endoglin in HTR-8 cells. **(B)** Representative Western Blot bands for PlGF, Endoglin, and β -actin in HTR-8 cells. **(C)** Relative protein levels of PlGF and Endoglin in BeWo cells. **(D)** Representative Western Blot

bands for PlGF, Endoglin, and β -actin in BeWo cells. **(E)** Relative mRNA expression of IL-1 β in HTR-8 cells, analyzed by qPCR. **(F)** Relative mRNA expression of IL-1 β in BeWo cells, analyzed by qPCR. Cells were treated with the indicated concentrations of SLN-Cur and Control (C-). Values represent the mean $\pm$ standard error of three independent experiments (n=3). Mean values of the relative expression for each treatment group (expressed in log value) with the fitted linear model as the gray line for A and C; unpaired t-test for E and F. For panels B and D, each blot strip is a single, contiguous crop from one membrane and one exposure; no lanes were spliced from different gels or exposures. Full-length, uncropped blots for β -actin, Endoglin/CD105 and PlGF (HTR-8/SVneo and BeWo) are provided in *Supplementary Figures S1–S6*.

A consolidated view of the angiogenic and inflammatory marker response across both trophoblast lines is provided in Figure 7, which expresses the data of Figure 6 as $\log_2$ (fold-change vs Control) heatmaps. This representation makes immediately apparent the cell-line-specific divergence: BeWo cells display a robust, dose-dependent Endoglin up-regulation, whereas HTR-8/SVneo cells remain largely unresponsive across the markers tested.

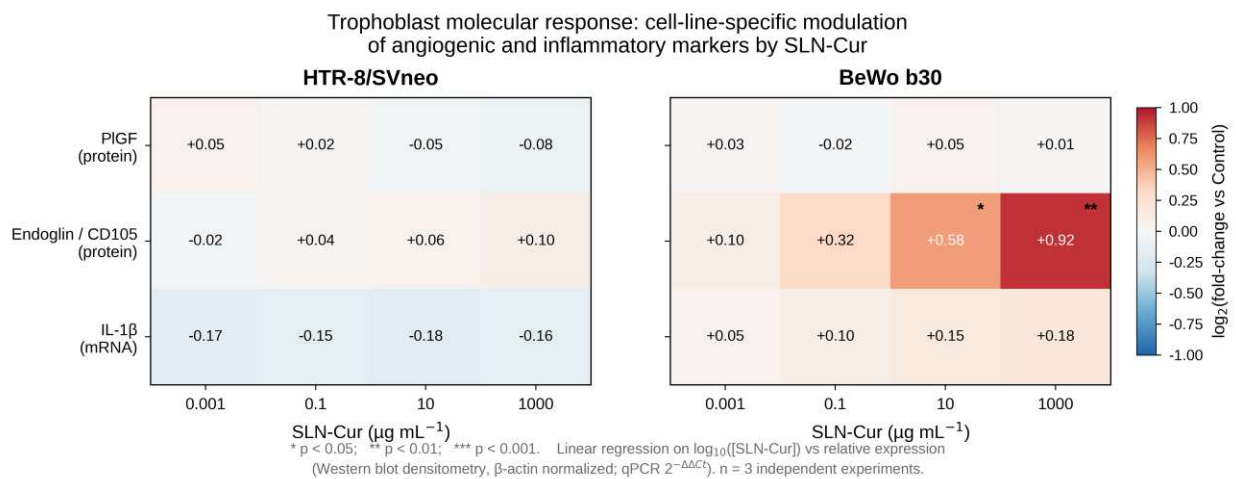


Figure 7. Cell-line-specific modulation of angiogenic and inflammatory markers by SLN-Cur. Heatmaps of $\log_2$ (fold-change vs untreated Control) for three molecular markers measured in HTR-8/SVneo (left panel) and BeWo b30 (right panel) cells exposed to increasing concentrations of SLN-Cur (0.001, 0.1, 10, and 1000 $\mu\text{g mL}^{-1}$): PlGF (protein, Western blot, β -actin normalised), Endoglin/CD105 (protein, Western blot, β -actin normalised), and IL-1 β (mRNA, qPCR, $2^{-\Delta\Delta C_t}$). Endoglin in BeWo cells exhibits a statistically significant dose–response (linear regression on $\log_{10}[\text{SLN-Cur}]$: $p = 0.01655$; adjusted $R^2 = 0.9986$). All other comparisons did not reach statistical significance. Asterisks indicate dose-points consistent with the significant regression slope (* $p < 0.05$; ** $p < 0.01$). The contrast between the two

trophoblast models — silent response in HTR-8/SVneo versus dose-dependent Endoglin up-regulation in BeWo — supports the interpretation discussed in the main text. $n = 3$ independent experiments.

The trend of IL-1 β reduction suggests an anti-inflammatory effect, while the regulation of Endoglin protein levels indicates a beneficial effect in promoting angiogenesis [48–50]. This set of responses is compatible with the hypothesis of Nrf2 pathway activation by curcumin, a mechanism known to orchestrate antioxidant and anti-inflammatory responses [51]. The Nrf2 is the main regulator of cellular defense against oxidative stress, kept inactive in the cytoplasm by its inhibitory chaperone, Keap1. Compounds like curcumin are potent activators of this pathway, interacting with Keap1 to destabilize the complex and allow Nrf2 to translocate to the nucleus. Once in the nucleus, Nrf2 activates the transcription of an arsenal of cytoprotective and antioxidant genes [52,53]. Consequently, there is a decrease in IL-1 β gene expression, since the Nrf2 pathway antagonizes the activity of NF- κ B, the main transcription factor for IL-1 β [9,13].

Curcumin's benefits extend beyond protection. It actively promotes angiogenesis, a mechanism with therapeutic potential in pregnancy complications like IUGR and endometriosis [47,54,55]. The same study showed that curcumin promotes immune tolerance at the maternal-fetal interface by increasing the HLA-G molecule. Thus, the profile observed in HTR8/SVneo cells is not one of passive defense, but of an active promotion of healthy placental function, orchestrated by Nrf2 [47].

In contrast, the BeWo cells exhibited a significant increase in endoglin expression (Figures 6C, D) and a non-significant upward trend in IL-1 β ($p = 0.7$). The literature indicates that trophoblast-derived cell lines display distinct expression profiles, molecular markers, and functional behaviors. In particular, HTR8/SVneo cells are commonly used as a physiologically relevant, endothelial-like model, whereas BeWo cells can reproduce features associated with a preeclampsia-like phenotype under specific stress conditions, such as hypoxia [47,56,57].

The dose-dependent increase in total Endoglin (CD105) observed in BeWo cells warrants careful interpretation. In preeclampsia pathophysiology, soluble endoglin (sEng) — generated by MMP-14-mediated shedding from syncytiotrophoblast — is a well-established anti-angiogenic factor that amplifies the sFlt-1/PlGF imbalance and contributes to maternal endothelial dysfunction [58,59]. Membrane-bound CD105 in extravillous trophoblast and endothelial cells can act as a context-dependent pro-angiogenic TGF- β co-receptor, but in

syncytial-like BeWo models elevated CD105 may equally prime cells for increased sEng release. Because our Western blot quantifies total CD105 without discriminating membrane-anchored from cleaved soluble forms, two non-exclusive scenarios remain open: (i) SLN-Cur up-regulates full-length CD105 as part of a TGF- β -tolerogenic, pro-implantation programme consistent with the cytoprotective effects of curcumin reported in trophoblast biology; or (ii) it primes cells for increased sEng shedding, which would be undesirable. Disambiguation requires conditioned-media ELISA for sEng, MMP-14 activity assays, and parallel readouts of TGF- β 1/3 signaling (SMAD2/3 phosphorylation) — experiments we plan to perform under stimulated, PE-mimicking conditions.

This methodological choice has a direct corollary: the magnitude of the Nrf2/NF- κ B and angiogenic effects of SLN-Cur is likely underestimated in the present setting, since PE pathophysiology is fundamentally driven by oxidative and pro-inflammatory cues [60,61]. Ongoing follow-up experiments include TNF- α challenge (10–50 ng/mL), hypoxia/reoxygenation cycles (1% $\rightarrow$ 21% O₂), and exposure to plasma from preeclamptic women, which will allow proper testing of the Nrf2/NF- κ B modulation hypothesis advanced here. Within this context, SLN-Cur did not significantly modulate the measured inflammatory marker in BeWo cells ($p = 0.7$), while the more consistent response observed in HTR8/SVneo cells supports their suitability as a responsive model for assessing the modulatory effects of SLN-Cur on angiogenic balance.

Activation of Nrf2 and reduction of Reactive Oxygen Species (ROS) are proposed here as the central upstream events, on the basis of the *in vivo* GST induction in zebrafish larvae and the chemical DPPH/ABTS scavenging data; direct demonstration in trophoblasts will require nuclear Nrf2 translocation assays, Keap1 quantification, HO-1/NQO1 induction by Western blot or qPCR, and parallel p-p65/I κ B α measurements [18,62]. Placental hypoxia drives PE, causing mitochondrial ROS overproduction and dysfunction [63,64]. This oxidative stress activates pro-inflammatory pathways [65]. The literature shows ROS reduction inhibits NF- κ B and other redox-sensitive pro-inflammatory pathways, such as p38 MAPK and ERK1/2 [66,67]. The antioxidant effect of nanocurcumin promotes a complete cytokine environment remodeling. For example, curcumin-loaded lipid nanocarriers demonstrated a potent anti-inflammatory profile, characterized by TNF- α and IL-6 suppression, with a significant IL-10 upregulation [44].

This complex interplay explains how a single primary antioxidant event can trigger a complete remodeling of the cytokine environment. It has been shown to improve placental function and redox balance *in vivo* in animal models of IUGR [68–72]. Studies mimicking the maternal circulatory environment of PE have demonstrated that curcumin can directly correct the systemic angiogenic imbalance by decreasing sFlt-1 secretion and increasing PlGF secretion [68]. This provides a strong rationale that our SLN-Cur, as a potent Nrf2-activating antioxidant, could have a dual benefit: protecting the healthy placenta while mitigating systemic vascular dysfunction in the mother.

Antioxidant Activity and Induction of Detoxification Enzymes

The basis for the potent modulatory effect of SLN-Cur lies in its robust antioxidant capacity, which operates through two distinct and synergistic mechanisms: a direct chemical action, evaluated in cell-free assays (Figures 8A, B), and an indirect biological action, demonstrated in an *in vivo* system zebrafish larvae (Figure 8C).

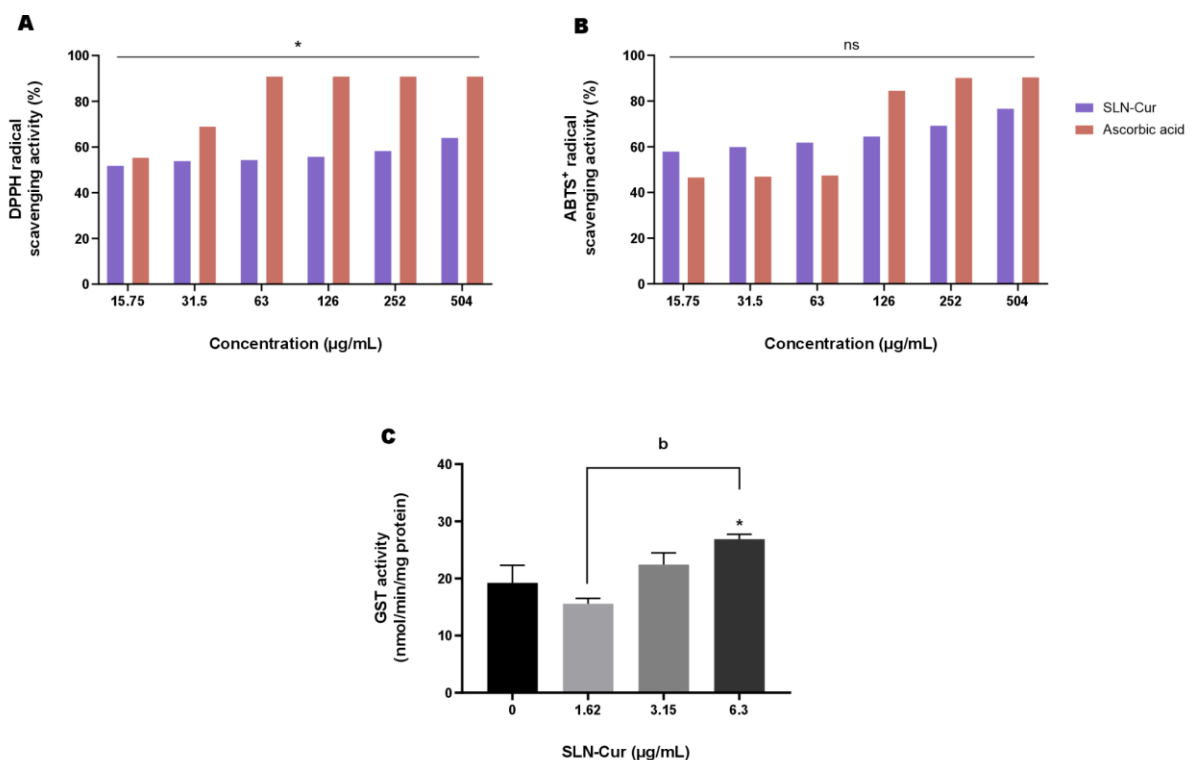


Figure 8. Antioxidant properties of the SLN-Cur formulation. The direct, chemical radical-scavenging capacity of SLN-Cur was evaluated at various concentrations using the (A) DPPH and (B) ABTS assays. The indirect, biological antioxidant response was assessed by measuring the (C) *in vivo* activity of glutathione S-transferase (GST) in zebrafish larvae following

exposure to SLN-Cur. Data are presented as the mean $\pm$ SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by a post-hoc test. * $p < 0.05$ compared to the control group (0 $\mu\text{g/mL}$). ns: not significant. The letter “b” indicates a significant difference between the bracketed groups $p < 0.01$.

Curcumin is a well-known fat-soluble antioxidant with strong radical scavenging ability [73,74]. Among the spectrophotometric methods available, DPPH and ABTS are widely used for assessing the antioxidant potential of bioactive compounds, due to their simplicity, sensitivity, and reproducibility [73].

In the present work, both DPPH and ABTS assays demonstrated the intrinsic ability of the nanoformulation to neutralize free radicals in a dose-dependent manner. The ABTS and DPPH radical scavenging capacity of SLN-Cur gradually increased with increasing concentrations. At 504 $\mu\text{g/mL}$, ABTS scavenging activity reached 76.60% (Figure 8B), while DPPH reached 63.96% (Figure 8A). The concentration-dependent increase suggests that the antioxidant potential of SLN-Cur is related to the availability of curcumin on the nanoparticle surface, facilitating its interaction with free radicals. The significant scavenging activity observed at lower concentrations further indicates that encapsulation preserved the antioxidant properties of curcumin.

When comparing SLN-Cur with ascorbic acid (AA), a significant difference was observed in the DPPH assay across all tested concentrations, indicating distinct antioxidant kinetics. Although DPPH radical scavenging was lower for SLN-Cur, this may reflect a slower but sustained reaction pattern, as described for polyphenols with prolonged kinetic phases in DPPH and ABTS systems [75,76]. Conversely, in the ABTS assay, no significant difference was observed between SLN-Cur and AA, reinforcing the efficiency and stability of the nanoencapsulated curcumin in radical scavenging reactions.

In the *in vivo* zebrafish model, SLN-Cur treatment significantly increased the activity of Glutathione S-Transferase (GST), a key Phase II detoxification enzyme. The increase in GST activity provides functional evidence that curcumin acted as a signaling molecule, activating a genetic defense program governed by the master transcription factor Nrf2 [18]. Activation of GST thus represents a shift from direct chemical protection to adaptive cellular resilience.

The observed biological response of SLN-Cur contrasts with the oxidative stress mechanisms reported for several environmental nanoparticles, which typically cause toxicity

by overwhelming antioxidant defenses and suppressing enzyme activity [77–79]. The finding that SLN-Cur treatment instead resulted in a significant *increase* in GST activity (Figure 8C) suggests a protective, adaptive mechanism rather than a toxic depletion pattern. This effect is consistent with prior studies showing that curcumin supplementation enhances the activities of GST, CAT, and SOD while downregulating stress-related markers such as iNOS, HSP70, and NF- κ B [80]. Such coordinated enzyme induction reflects the broad activation of the Nrf2 pathway, which governs antioxidant, cytoprotective, and anti-inflammatory responses.

The Nrf2 not only upregulates enzymatic antioxidants like GST but also induces the synthesis of its substrate, reduced glutathione (GSH), by increasing Glutamate-Cysteine Ligase (GCL) expression, the rate-limiting enzyme in GSH production [81]. Through this dual mechanism, curcumin provides both the enzymatic machinery (GST) and the metabolic substrate (GSH) necessary for detoxification. This adaptive response has been experimentally confirmed in zebrafish, where curcumin treatment restored the expression and activity of GST in previously oxidatively damaged livers [82]. This axis also has significant anti-inflammatory implications, since Nrf2 activation antagonizes NF- κ B signaling, preventing the expression of pro-inflammatory genes and promoting redox homeostasis.

In summary, SLN-Cur combats oxidative stress through a dual and synergistic mechanism: first, by directly neutralizing reactive radicals in a chemical context (DPPH/ABTS assays), and second, by biologically strengthening the antioxidant defense system through the Nrf2/GSH/GST axis. These findings align with previous reports demonstrating that curcumin, whether free or nanoencapsulated, acts both as a radical scavenger and as an inducer of cellular defense responses, confirming its role as a multifunctional antioxidant agent [44,75,76,80,83,84].

Study Limitations

Several limitations should be acknowledged. First, the angiogenic readouts did not include soluble factors (sFlt-1, soluble endoglin) by ELISA in trophoblast-conditioned media, and free-form PlGF/VEGF were not directly quantified; future work will close this gap. Second, biological assays were conducted under basal conditions and therefore do not interrogate the very redox-inflammatory imbalance that defines preeclampsia. Third, the Nrf2/NF- κ B axis is currently inferred from *in vivo* zebrafish GST induction and indirect chemical antioxidant assays, rather than from direct readouts in trophoblasts (nuclear Nrf2, Keap1, HO-1, NQO1, p-p65, I κ B α). Fourth, encapsulation efficiency and drug loading by

HPLC, although consistent with internal validation, are not reported in the present manuscript and should be added in a dedicated quality table. Fifth, no *in vivo* preeclampsia model (e.g., reduced uterine perfusion pressure [85], L-NAME, or sFlt-1 overexpression) was performed; this is a necessary next translational step. Sixth, transplacental passage of SLN-Cur was not evaluated; in an obstetric indication, the desirable profile is maternal exposure with minimal fetal transfer, which must be probed in *ex vivo* human placental perfusion or established preclinical models before any clinical consideration.

Future Perspectives

Building on these results, four lines of investigation are envisaged: (i) mechanistic validation of the Nrf2/NF- κ B axis in HTR-8/SVneo and BeWo cells under TNF- α and hypoxia/reoxygenation, including nuclear Nrf2 translocation, HO-1 and NQO1 induction, p-p65 phosphorylation, and rescue experiments with the Nrf2 inhibitor ML385 or NFE2L2 siRNA; (ii) parallel ELISA quantification of sFlt-1, free PlGF and soluble endoglin in conditioned media, with MMP-14 activity assays to disambiguate the membrane-bound versus shed CD105 pools observed here; (iii) *in vivo* evaluation in established preeclampsia models such as RUPP in rats [85] and adenoviral sFlt-1 overexpression in pregnant mice, with maternal blood pressure, proteinuria, fetal weight and placental histology as primary outcomes; and (iv) safety profiling of transplacental passage and fetal exposure, ideally using *ex vivo* human placental perfusion. Beyond efficacy, the platform's sustainable footprint — combining Amazonian murumuru butter with a betaine/malic acid NaDES — aligns with several of the 12 principles of green chemistry [86] and constitutes an additional translational and bioeconomic differentiator worth quantifying explicitly in future manufacturing assessments [87].

CONCLUSION

This study shows that solid lipid nanoparticles built from murumuru butter, Compritol ATO 888, a Span 20/Tween 80 blend, and a betaine/malic acid NaDES offer a green, biocompatible way to deliver curcumin. The particles hit nanometric sizes that favor cellular uptake, stay colloidally stable for months under ambient conditions, and retain curcumin's antioxidant and anti-inflammatory activity without cytotoxic cosolvents. The context-dependent modulation of angiogenic and inflammatory markers in HTR-8/SVneo cells, together with hormetic metabolic activation and Nrf2-linked GST induction *in vivo*, makes this NaDES-enabled SLN system a strong candidate for targeting the redox-inflammatory networks that drive placental dysfunction. These findings lay groundwork for moving SLN-Cur into

preclinical preeclampsia models, where its proposed capacity to rebalance Nrf2-NF- κ B signaling and angiogenic homeostasis can be formally tested and, if confirmed, would offer a mechanistically grounded therapeutic angle for a disease that still lacks treatments that modify its course.

METHODS AND MATERIALS

Preparation of Curcumin-Loaded Solid Lipid Nanoparticles (SLN-Cur)

Solid lipid nanoparticles containing curcumin (SLN-Cur) were prepared using murumuru butter and Compritol ATO 888 (75:25, w/w) as the lipid matrix and Span 20/Tween 80 (20:80, w/w) as surfactants. Curcumin was used as the lipophilic active compound, and purified water was used as the aqueous phase. A Natural Deep Eutectic Solvent (NaDES) composed of betaine and malic acid (NaDES-A-Bet:Ma) was incorporated during formulation optimization [22].

The NLS-Cur was produced by hot homogenization followed by passive cooling. The oily phase was melted at 80°C under magnetic stirring (300 rpm) for 20 min. The aqueous phase, heated to 70°C, was slowly added to form the emulsion, which was maintained under heating and stirring before cooling to room temperature. The optimized formulation included 1% (w/v) NaDES-A-Bet:Ma added to the aqueous phase. Reproducibility was evaluated in five independent syntheses (n = 5).

Physicochemical Characterization and Stability

Particle size, polydispersity index (Pdl), and zeta potential were measured by Dynamic Light Scattering (DLS) using a ZetaSizer Nano ZS (Malvern Instruments, UK). Samples were diluted 1:10 in distilled water and analyzed in triplicate at 25°C.

For stability analysis, formulations were stored at 4°C, 25°C, and 38°C for up to 240 days. Morphology was evaluated by Transmission Electron Microscopy (TEM) using a JEOL® 1011 microscope (Tokyo, Japan) microscope operating at 80 kV. Observations were carried out in bright-field mode at magnifications exceeding 10,000x [24].

Molecular structure and interactions were investigated by vibrational spectroscopy. FTIR-ATR spectra of undiluted liquid samples (2 μ L) were recorded on a Vertex 70 spectrometer (Bruker, USA) equipped with a diamond crystal accessory, in absorbance mode

(4,000 to 500 cm^{-1} , 128 scans, 4 cm^{-1} resolution). Complementary Raman measurements were performed in confocal mode on a LabRam HR Evolution spectrometer (Horiba, France) equipped with a CCD detector and an 1800 lines/mm grating, using a 532nm laser (1mW, 50x objective). The final spectra were compiled from 20 acquisitions of 2s each.

Cell Viability Assay

The viability of J774A.1 and NIH/3T3 cells was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [88], which measures mitochondrial activity. Cells were seeded in 96-well plates at a density of 10,000 cells/well and allowed to adhere for 24 hours before treatment. The cells were then exposed to various concentrations of SLN-Cur for 24 hours at 37°C and 5% CO_2 .

After the treatment period, MTT solution was added to each well, and the plates were incubated for 4 hours. Finally, the MTT solution was removed, and the formazan crystals were dissolved by adding 100 μL of dimethyl sulfoxide (DMSO) to each well. The absorbance was read at 595 nm using a spectrophotometer. Each concentration was tested in six replicate wells, and experiments were performed in triplicate.

Viability of BeWo and HTR-8/SVneo cells was assessed using the Sulforhodamine B (SRB). Cells were plated in 96-well microplates at densities of 10,000 cells/well and 12,000 cells/well, respectively, in 200 μL of medium. After a 24-hour incubation period to allow for adhesion, the medium was replaced with fresh medium containing the specified treatments for an additional 24 hours.

Following treatment, cells were fixed with 10% (w/v) trichloroacetic acid (TCA) for 30 minutes at 4°C. The plates were then washed, dried, and stained with a 0.4% (w/v) SRB solution for 30 minutes. Excess stain was removed with 1% acetic acid, and the protein-bound stain was solubilized with a 10 mM Tris-base solution. Absorbance was measured at 488 nm excitation and 585 nm emission using a Synergy HTX Multi-Mode Reader.

Protein and Gene Expression Analyses

For protein expression analysis, HTR-8/SVneo (100,000 cells/mL) and BeWo (150,000 cells/mL) cells were seeded in 6-well plates (2 mL/well), allowed to adhere for 24 h, and treated for 24h. Total proteins were extracted using RIPA buffer supplemented with protease

inhibitors, 1mM sodium orthovanadate, and sodium fluoride, followed by three freeze-thaw cycles and centrifugation (13,000x g, 15 min, 4 °C). After quantification by Bradford assay [89], 50µg of protein was resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk (1 h, room temperature) and incubated overnight with primary antibodies against Endoglin (1:1000), PlGF (1:1000), and β -actin (1:1000), followed by peroxidase-conjugated secondary antibodies (1:3000, 1 h). Bands were developed using ECL substrate, visualized on a ChemiDoc system, and quantified by densitometry. Target protein levels were normalized to β -actin, and all experiments were performed in triplicate across three independent assays.

For interleukin-1 β (IL-1 β) gene expression analysis, cells were seeded and treated under the same conditions described for Western blotting. Total RNA was extracted using TRIzol, quantified via NanoDrop for concentration and purity, and 1µg of RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit. Quantitative Real-Time PCR (qPCR) was performed on a StepOne Real-Time PCR system utilizing SsoAdvanced™ Universal SYBR® Green master mix and specific primers for IL-1 β and a reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [90].

Antioxidant Activity

The antioxidant capacity of SLN-Cur (15.63–500 µg/mL) was evaluated via DPPH [91–93] and ABTS+ radical scavenging assays [94,95] in 96-well plates in triplicate, using a Varioskan™ LUX spectrophotometer (Thermo Scientific, Singapore) with sample blanks for background correction. For the DPPH assay, 150 µL of sample was mixed with 150 µL of 0.1 mM DPPH ethanolic solution, incubated for 30 min in the dark, and measured at 517 nm. For the ABTS+ radical scavenging assay, the radical was generated by reacting ABTS (3.84 mg/mL) with potassium persulfate (37.84 mg/mL, 17.6 L/mL of ABTS) for 12–16 h in the dark, and diluted in ethanol to an absorbance of 0.8–1.0 at 734 nm. Samples (150 µL) were mixed with 150 µL of the ABTS+ radical working solution, incubated for 7 min protected from light, and measured at 734 nm.

Zebrafish Care and Larvae Biochemical Analysis

Zebrafish (*Danio rerio*) embryos were obtained from the University of Brasília facility, where adults were maintained in an automated system (ZebTec, Tecniplast, Italy) under a 12-

h light:12-h dark photoperiod at $27 \pm 1^\circ\text{C}$, pH 7.2–7.6, 6.7° dH hardness, and 728 μS conductivity, fed commercial and live food. Larvae were exposed to blank nanoformulation concentrations (0, 1.56, 3.13, and 6.25 $\mu\text{g/mL}$) for 72 h post-fertilization (hpf), followed by thermal shock euthanasia ($0\text{--}4^\circ\text{C}$). Pools of 30 larvae per group were homogenized on ice in 1.5 mL phosphate buffer (0.1 M, pH 7.2) via ultrasonication (30 s) and centrifuged ($2,000\times g$, 10 min, 4°C). Supernatants were used for Bradford protein quantification (γ -globulin standard) [89] and glutathione S-transferase (GST) activity assays [96], performed in quadruplicate in 96-well plates using a spectrophotometer (Thermo, USA). GST activity was determined by mixing 100 μL of sample with 200 μL of reaction solution (10 mM glutathione and 10 mM CDNB in 0.1 M phosphate buffer, pH 6.5), monitoring the absorbance increase at 340 nm at 40-s intervals for 5 min.

Statistical Analysis

Data were analyzed using GraphPad Prism 8.0 and expressed as mean $\pm$ SEM from at least three independent experiments. Statistical analyses included Shapiro–Wilk and Brown–Forsythe tests, followed by Student’s t-test, One-way or Two-way ANOVA with appropriate post-hoc tests, or Kruskal–Wallis test when applicable. Statistical significance was considered at $p < 0.05$.

Linear regression [97] was fitted and analyzed using the R language, RStudio and the ggplot2 package. Adjusted R^2 method, given as $1 - \frac{SSE/(N-P)}{SST/N-1}$, was used as the determination coefficient [98,99], with SSE being the error sum of squares, SST being the total sum of squares, N being the observations and P being the parameters of the regression.

FUNDING

This research was funded by FAP-DF/Brazil (00193-00000165/2023-20; 0193.00002231/2022-15; 00193.00001066/2021-1; 00193-00000759/2021-79), the National Council for Scientific and Technological Development—CNPq (403033/2023-3; 140035/2022-2), the Financier of Studies and Projects (01.08.0457.00), and the Higher Education Personnel Improvement Coordination—CAPES (0001; 88881.981074/2024-01).

INSTITUTIONAL REVIEW BOARD STATEMENT

This study adheres to Brazil's legal framework for genetic resources and associated traditional knowledge and follows the principles of the Nagoya Protocol on Access and Benefit-Sharing (ABS). It is registered with the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under authorization codes A002D4B and A235193. The lipids used in the formulations were sourced from Amazon Oil, a supplier committed to sustainable and fair-trade practices. At the current laboratory scale (milligrams to grams), benefit-sharing is achieved through regulated access and ethical sourcing. The zebrafish studies were previously approved by the ethical committee of University of Brasilia. Looking ahead, we plan to broaden this framework by formalizing benefit-sharing agreements with local cooperatives and integrating traceability and sustainability certifications into our production pipeline.

INFORMED CONSENT STATEMENT

NOT APPLICABLE.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ACKNOWLEDGMENTS

I would like to thank the laboratory technicians at the University of Siena, Laura and Roberta, for their attention, care, and support throughout the development of this work. I also extend my gratitude to the entire laboratory team for their warm welcome and collaboration during my sandwich PhD period. I am sincerely grateful to Prof. Cesar Koppe Grisolia, who, even after his retirement, continues to play an important role in my academic and scientific training.

CONFLICTS OF INTEREST

Not applicable.

AUTHOR CONTRIBUTIONS

T. B. P.: conceptualization, project administration, investigation, methodology, data curation, writing and editing.

T. E. S. A.: investigation, methodology

L. M. T.: investigation, methodology

A. C. G. S.: investigation, methodology
 D. G. S.: investigation, methodology
 M. R.: Writing: review and editing
 S. P.: investigation, methodology
 L.F.R.: methodology.
 A. P. S.: investigation, methodology.
 C. D. G.: investigation, methodology
 S. N. B.: funding acquisition, writing: review and editing
 C. K. G.: supervision, resources.
 F. I.: project administration, supervision, resources.
 L. E.: supervision, project administration.
 F. P.: project administration, supervision, funding acquisition, review and editing.
 V. C. M.: conceptualization, project administration, supervision, funding acquisition, writing - review and editing.

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