Short Communication

Methanolic Extract of *Clinopodium mexicanum* Enhances Macrophage-Mediated Intracellular Clearance of *Streptococcus mutans* Associated with Increased Nitric Oxide Production

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Abstract | Host-directed antimicrobial strategies may complement conventional approaches against Streptococcus mutans. This study tested whether a methanolic extract of Clinopodium mexicanum enhances macrophage-mediated clearance of Streptococcus mutans in the absence of detectable direct antibacterial activity under the tested conditions. In disk diffusion assays, the extract produced no measurable inhibition halo. In contrast, pretreatment of murine J774A.1 macrophages reduced the number of viable bacteria recovered from inside macrophages after infection. At 4 h post-infection, treatment with the extract reduced intracellular bacterial recovery compared with untreated infected macrophages. At 24 h, intracellular survival was also reduced, with the overall mean intracellular recovery/phagocytic uptake and intracellular survival values reported as 47.68 ± 21.86 and 43.50 ± 21.86, respectively. Extract stimulation increased nitrite accumulation, particularly at 80 µg mL−1, while MTT-based macrophage metabolic activity remained largely preserved under conditions of limited cytotoxicity. LPS was used only as a positive control for nitrite production and not as a direct benchmark of equivalent macrophage activation. These findings indicate that the extract did not show detectable direct antibacterial activity under the tested conditions but was associated with enhanced host-cell-associated antibacterial activity. Because nitric oxide synthesis was not pharmacologically inhibited, increased nitrite accumulation should be interpreted as an activation-associated marker rather than definitive proof of nitric oxide-dependent bacterial killing.

Keywords | *Clinopodium mexicanum*, *Streptococcus mutans*, Macrophages, Nitric oxide, Host-directed antimicrobial strategy, Intracellular bacterial clearance

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INTRODUCTION

Streptococcus mutans is a major cariogenic oral bacterium associated with dental caries and host–microbe interactions in the oral environment (Loesche, 1986; Lemos *et al.*, 2019). Although most antimicrobial strategies against *S. mutans* target bacterial growth or biofilm formation, host-directed approaches may offer

complementary control by enhancing innate immune functions such as macrophage phagocytosis and bacterial clearance. Although this strategy has been studied mainly in tuberculosis and viral infections (Kaufmann *et al*., 2018; Taya *et al*., 2023), evidence also supports its potential against *S. mutans*. For example, Perry *et al*. (2015) showed that streptazolin stimulates macrophage activity and enhances bacterial killing, supporting the evaluation of immunomodulatory compounds as adjunctive therapies against this organism.

Macrophages contribute to bacterial elimination through phagocytosis and intracellular killing, including nitric oxide-dependent mechanisms (Bogdan, 2001; Palmieri *et al*., 2020). *Clinopodium mexicanum* is a Mexican medicinal plant with documented ethnopharmacological relevance and reported flavanone glycosides among its characterized constituents (Estrada-Reyes *et al*., 2010; Alvarado *et al*., 2020). The present study tested the hypothesis that a methanolic extract of *Clinopodium mexicanum* enhances macrophage-mediated intracellular clearance of *Streptococcus mutans* in the absence of detectable direct antibacterial activity under the tested conditions.

MATERIALS AND METHODS

Dried aerial parts of *Clinopodium mexicanum* were subjected to cold methanolic maceration for 24 h to obtain the crude extract. After extraction, the plant material was removed by filtration, and the methanolic phase was concentrated to yield the crude methanolic extract, which was stored at 4°C until use in the biological assays. The selection of C. mexicanum was based on its medicinal relevance and previously reported biological properties (Estrada-Reyes *et al*., 2010; Alvarado *et al*., 2020).

Streptococcus mutans was a laboratory strain provided by the Oral Microbiology Laboratory, Faculty of Dentistry, Universidad Autónoma de Nuevo León. The direct antibacterial activity of the extract against *S. mutans* was evaluated by the disk diffusion assay as an initial screening approach. Briefly, disks containing the extract were placed on agar plates previously inoculated with *S. mutans*, and growth inhibition halos were recorded after incubation. Chlorhexidine (0.2%) was used as a positive control to verify assay performance. In parallel, direct bactericidal activity under infection assay conditions was assessed by incubating the extract with the bacterial inoculum in the absence of macrophages for the corresponding exposure period. A 24 h broth-based liquid growth-curve or time-kill assay was not performed.

J774A.1 murine macrophages were maintained in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Antibiotics were withdrawn at least 24 h before infection to avoid interference with bacterial uptake and recovery. The dried extract was dissolved in methanol to prepare stock solutions, and the final methanol vehicle concentration in cell culture was maintained at ≤ 0.1% (v/v). Cells were pre-stimulated for 24 h with C. mexicanum extract at 60, 70, or 80 μg mL^{-1}, washed twice with phosphate-buffered saline, and then transferred to antibiotic-free medium. Wells receiving medium alone were included as negative controls.

Cell viability was evaluated after 24 h exposure to 60, 70, or 80 μg mL^{-1} extract using the MTT assay, which estimates metabolic activity based on tetrazolium salt reduction by viable cells (Mosmann, 1983). Macrophage activation was assessed in parallel by indirect estimation of nitric oxide production through nitrite quantification in culture supernatants after 24 h of stimulation. Nitrite levels were measured with the Griess reaction, as described by Granger *et al*. (1996), and were used as a functional indicator of nitric oxide production in response to treatment (Bogdan, 2001; Palmieri *et al*., 2020).

For intracellular bacterial assays, macrophage monolayers were infected with the *S. mutans* laboratory strain grown in brain heart infusion medium. The infection was performed at an average MOI of 10:1, calculated from the macrophage seeding density and bacterial inoculum; this value represents an average exposure ratio and does not imply that every macrophage contacted exactly 10 bacteria. Thereafter, cultures were washed three times with phosphate-buffered saline, and extracellular bacteria were eliminated by gentamicin protection (100 μg mL^{-1} for 30 min). For determination of intracellular bacterial recovery at 4 h, cells were lysed immediately after this step with 0.1% Triton X-100 for 5 min, lysates were serially diluted, and viable intracellular bacteria were quantified on brain heart infusion agar after 24 h of incubation at 37°C under 5% CO_2. The 4 h endpoint was expressed as the percentage of the initial inoculum recovered intracellularly and was interpreted as intracellular bacterial recovery/phagocytic uptake rather than as direct evidence of intracellular killing. Bacterial replication inside macrophages during the first 4 h was not directly assessed.

For determination of intracellular survival, following the initial gentamicin protection step, cultures were maintained for an additional 24 h in fresh medium containing 10-20 μg mL^{-1} gentamicin and were then lysed, serially diluted, and plated as described above. Intracellular survival was expressed as the percentage of intracellular colony-forming units recovered at 24 h post-infection. A specific gentamicin penetration control and a 0 h post-gentamicin lysis control was not performed. All experiments were performed with technical triplicates for each condition and

in three independent biological replicates. The same dried crude extract batch was used to prepare fresh working dilutions for each independent experiment. Data were analyzed by one-way or two-way analysis of variance, as appropriate, followed by Sidak's, Bonferroni's, or Tukey's multiple-comparison tests, with statistical significance established at $\alpha = 0.05$. All bacterial manipulations were performed under BSL^{-2} conditions using appropriate personal protective equipment.

RESULTS AND DISCUSSION

The extract produced no measurable inhibition halo in the disk diffusion assay, and no direct bactericidal effect was detected under the infection assay conditions. This indicates that the biological effect observed in macrophage infection experiments was unlikely to result from direct growth inhibition of *S. mutans* and instead supports evaluation of the extract as a host-directed intervention. However, because a liquid growth-curve or time-kill assay was not performed, weak or delayed direct antibacterial effects cannot be fully excluded. Future studies should include broth-based growth kinetics and time-kill assays over broader concentration ranges and exposure times.

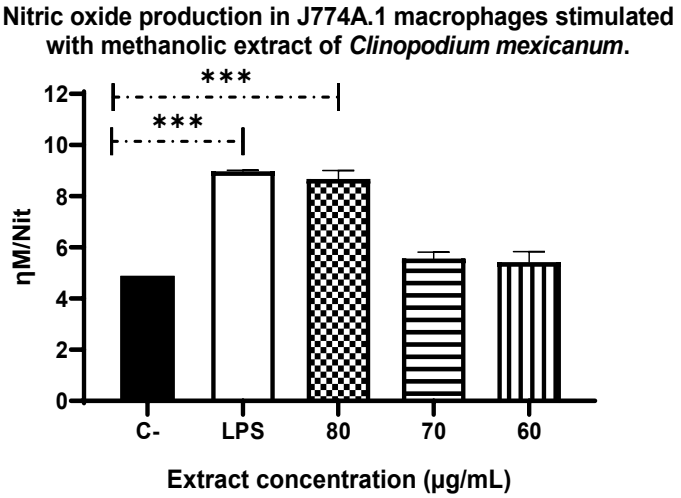

Figure 1: Nitric oxide (NO) production was quantified in culture supernatants of J774A.1 macrophages after 24 h stimulation with *C. mexicanum* extract at 60, 70, and 80 µg/mL. Lipopolysaccharide (LPS, 1 µg/mL) served as positive control and untreated cells (C–) as negative control. Nitrite accumulation was determined using the Griess reaction and expressed as µM nitrite. Data represent mean ± SD of three independent biological experiments performed in technical triplicate. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple-comparison test. ***$p < 0.001$ versus negative control.

Stimulation with the extract increased nitric oxide production in J774A.1 macrophages, with the strongest response observed at 80 µg mL^{-1} (Figure 1). Importantly, exposure to 60–80 µg mL^{-1} did not significantly reduce macrophage viability, and cell survival remained above 80% relative to untreated controls (Figure 2). The coexistence of largely preserved metabolic activity and enhanced nitrite accumulation suggests that the extract induced macrophage activation under conditions of limited cytotoxicity rather than causing broad nonspecific metabolic damage. However, because viability at the highest concentration decreased by approximately 15–20%, we cannot fully exclude that reduced macrophage numbers contributed partially to lower intracellular CFU recovery.

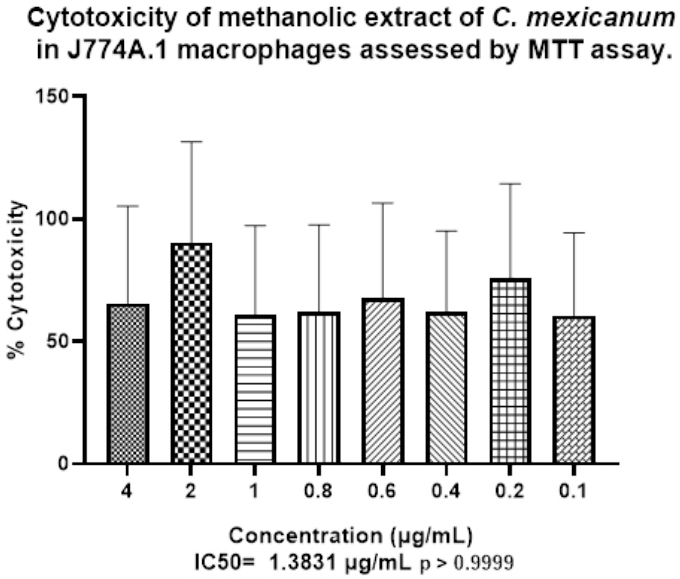

Figure 2: Cell viability was analyzed after 24 h exposure to *C. mexicanum* extract at 60, 70, and 80 µg/mL using the MTT assay. Data represent mean ± SD of three independent biological experiments performed in technical triplicate. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's multiple-comparison test. No statistically significant differences were observed compared with untreated control cells ($p > 0.9999$). Because MTT reflects metabolic activity rather than direct cell number, these data were interpreted as evidence of largely preserved metabolic activity under conditions of limited cytotoxicity, rather than as definitive evidence of complete cell safety.

A limitation of the present study is that macrophage viability was evaluated by MTT assay, which reflects metabolic activity rather than direct cell counts. Although viability remained above 80% and no statistically significant reduction was detected, a moderate decrease in viable or metabolically active macrophages could influence intracellular CFU recovery. Therefore, future studies should normalize bacterial recovery to the number of viable macrophages at the time of lysis and confirm viability using complementary approaches, such as trypan blue exclusion, direct cell counting, or live/dead staining. Higher nitrite accumulation was associated with fewer viable intracellular bacteria recovered after extract treatment against *S.*

mutans (Figure 3). The 4 h endpoint was interpreted as intracellular bacterial recovery/phagocytic uptake rather than direct evidence of intracellular killing, whereas the 24 h endpoint was used to evaluate intracellular survival after gentamicin protection. Earlier uptake time points, such as 30 min post-infection, were not included; therefore, future studies should incorporate early uptake assays to distinguish bacterial internalization from subsequent intracellular killing. In untreated infected macrophages, viable intracellular *S. mutans* was still recovered at 24 h post-infection, indicating that the strain used in this study remained detectable inside J774A.1 macrophages under our assay conditions. Therefore, the reduction in intracellular recovery observed after extract treatment is consistent with enhanced macrophage-associated bacterial clearance. However, because a detailed intracellular persistence kinetic was not performed, future studies should report absolute CFU counts over multiple time points to determine whether intracellular bacterial numbers remain stable or decline spontaneously in untreated macrophages. The reduction in intracellular bacterial recovery was observed consistently across the three independent biological replicates, each performed in technical triplicate, indicating that the effect was not driven by a single outlier experiment. Data are presented as mean ± SD to show the variability among replicates.

Increased nitric oxide production was accompanied by improved macrophage antibacterial performance against *S. mutans* (Figure 3). At 4 h post-infection, pretreatment with 80 µg mL^{-1} extract reduced intracellular bacterial recovery to 37.3% of control levels, and at 24 h intracellular survival remained further reduced, indicating a sustained effect on bacterial clearance. Because *S. mutans* can persist within phagocytic cells depending on the activation state of the host cell, this reduction in intracellular recovery is biologically consistent with enhanced macrophage effector function (Chen *et al*., 2008). The reduction in intracellular bacterial recovery was observed consistently across the three independent biological replicates, each performed in technical triplicate, indicating that the effect was not driven by a single outlier experiment. Data are presented as mean ± SD to show the variability among replicates.

Taken together, the absence of direct antibacterial activity, the preservation of macrophage viability, and the increase in nitric oxide production support a host-mediated mechanism of action. Although the present design does not establish nitric oxide as the only effector responsible for intracellular killing, the concordance between nitrite induction and reduced intracellular recovery supports its participation in the observed phenotype. In this context, *C. mexicanum* appears better positioned as a source of immunomodulatory molecules than as a direct antimicrobial extract, which is conceptually aligned with current host-directed therapeutic strategies for difficult-to-control bacterial infections (Kaufmann *et al*., 2018; Taya *et al*., 2023).

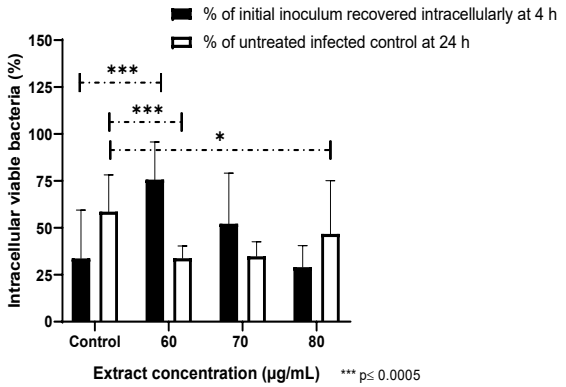

Figure 3: J774A.1 macrophages were pre-treated for 24 h with methanolic extract of *C. mexicanum* at 60, 70, and 80 µg/mL and then infected with *S. mutans* at an average MOI of 10:1. The 4 h endpoint was expressed as the percentage of the initial inoculum recovered intracellularly and was interpreted as intracellular bacterial recovery/phagocytic uptake. Intracellular survival was quantified at 24 h post-infection after gentamicin protection. Data are expressed as mean ± SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparison test. For intracellular recovery/phagocytic uptake, significant differences were observed for control vs. 60 µg/mL ($p < 0.0001$) and 60 vs. 80 µg/mL ($p < 0.0001$). For intracellular survival, significant differences were observed for control vs. 60 µg/mL ($p = 0.0356$) and control vs. 70 µg/mL ($p = 0.0472$). Other comparisons were not statistically significant. Estimated CFU values were calculated from the percentage of initial inoculum recovered, assuming an initial inoculum of 1×10^5 CFU/well based on an MOI of 10:1 and 1×10^4 macrophages/well. These values represent estimated CFU derived from the initial inoculum and not raw plate-count CFU data.

CONCLUSION

Methanolic extract of *Clinopodium mexicanum* enhanced macrophage-mediated intracellular clearance of *Streptococcus mutans* in vitro and was associated with increased nitric oxide production in the absence of direct antibacterial activity. These data support further fractionation and compound identification to define the active immunomodulatory constituent(s).

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NOVELTY STATEMENT

This study provides preliminary evidence that a methanolic extract of Clinopodium mexicanum enhances macrophage-associated intracellular clearance of Streptococcus mutans in a murine macrophage model. The findings suggest that this plant extract may act mainly through host-cell-associated immunomodulatory mechanisms rather than direct antibacterial activity under the tested conditions. This work supports further investigation of C. mexicanum as a potential source of immunomodulatory compounds for host-directed antimicrobial strategies.

AUTHOR'S CONTRIBUTION

Ameyalli Jocelyn Martínez Delgado contributed to the experimental work, data acquisition, analysis, and initial manuscript drafting. Uziel Castillo-Velázquez contributed to conceptualization, study design, supervision, data interpretation, manuscript writing, critical revision, and correspondence. María Argelia Akemi Nakagoshi Cepeda contributed to methodology, experimental support, data analysis, and manuscript revision. Sonia Martha López Villarreal contributed to methodology, validation, data interpretation, and manuscript revision. Osvelia Esmeralda Rodríguez Luis contributed to conceptualization, supervision, project coordination, data interpretation, manuscript writing, critical revision, and correspondence. All authors reviewed and approved the final version of the manuscript

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Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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