Optimization of Tea Tree Oil and Eucalyptus Essential Oil Combinations in an Oral Rinse for Managing Oral Ulcers

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Abstract. This investigation sought to determine the optimal mixing ratio of tea tree oil (TTO) together with eucalyptus essential oil (EEO) within a mouthwash formulation. Four experimental preparations underwent evaluation regarding their safety profile, antimicrobial effectiveness, anti-inflammatory properties, and sensory characteristics. Safety testing employed the chick embryo chorioallantoic membrane (CAM) model. Antibacterial activity was assessed against several clinically relevant oral pathogens. Anti-inflammatory potential was quantified through qPCR analysis conducted on human gingival fibroblasts. A future clinical trial framework was also designed. The CAM assay revealed that lower oil concentrations produced minimal irritation, whereas higher concentrations intensified irritant responses. All tested preparations demonstrated potent antibacterial effects, achieving over 99.9% inhibition against E. coli, S. aureus, and S. mutans, together with approximately 93% inhibition against P. gingivalis. qPCR data indicated marked downregulation of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8. A concentration-dependent relationship was observed between essential oil levels and anti-inflammatory potency, although taste acceptability declined at elevated concentrations. In summary, essential oil-based oral rinses exhibit considerable antibacterial together with anti-inflammatory potential suitable for oral ulcer management. The formulation combining 0.2% TTO plus 0.2% EEO delivered the most favorable balance across efficacy, safety, and user acceptability parameters.

1 Introduction

Oral ulcers represent a frequent and uncomfortable pathological condition[1, 2]. Worldwide epidemiological data indicate a substantial disease burden, with periodontal

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disorders—including ulcerative lesions—affecting many millions of individuals. Recurrent aphthous stomatitis (RAS) represents a common subtype for which no definitive cure currently exists. Available therapeutic approaches primarily focus on symptomatic relief, encompassing pain reduction together with shortening of ulcer duration[3, 4].Conventional topical antimicrobial therapy faces growing difficulties due to rising concerns over antimicrobial resistance (AMR), thereby motivating the search for plant-derived alternatives that act through multiple mechanisms[5-7]. Bioactive compounds originating from plants have demonstrated considerable promise for treating oral ulcers. Essential oils, in particular, have attracted research interest within dentistry owing to their diverse biological activities[8-11]. Among these natural products, tea tree oil (TTO) together with eucalyptus essential oil (EEO) stand out because of their well-documented antimicrobial, anti-inflammatory, and wound-healing potential, making them suitable candidates for inclusion in oral ulcer formulations[12].

The primary constituent of TTO is terpinen-4-ol, which disrupts microbial membranes and reduces pro-inflammatory cytokine levels (including TNF-α and IL-1β) through inhibition of signaling pathways such as NF-κB activation. EEO contains high concentrations of 1,8-cineole and exhibits broad-spectrum antimicrobial activity together with significant anti-inflammatory effects. These properties suggest that a mouthwash combining TTO together with EEO could concurrently address both infection and inflammation, which represent key factors in oral ulcer pathology[13-15].

Accordingly, this study developed and evaluated four mouthwash formulations containing different ratios of TTO and EEO. The objective was to systematically characterize their safety profiles, antibacterial efficacy, in vitro anti-inflammatory activity, and predicted clinical acceptability, thereby identifying the most balanced together with effective formulation.

2 Materials and Methods

2.1 Sample preparation

Four mouthwash preparations were manufactured containing varying concentrations of TTO together with EEO (either 0.2% or 0.5% for each oil), as detailed in Table 1. The base composition remained unchanged across all formulations. Briefly, the aqueous phase—comprising purified water, propylene glycol, sorbitol, sodium saccharin, cetylpyridinium chloride (CPC), and potassium sorbate—was heated to 40–45°C. The oil phase (containing TTO, EEO, and polysorbate 20) was premixed and subsequently added gradually to the aqueous phase under high-shear mixing to generate a preliminary emulsion. This mixture underwent high-pressure homogenization (200–500 bar) for 2–3 cycles to produce a stable microemulsion. Following cooling, menthol was incorporated, and the batch volume was adjusted to the final weight using purified water. The finished product was stored within opaque containers.

Table1. Composition of Mouthwash Formulations

Ingredient	Function	Percentage (w/w %)
Water (Purified)	Solvent / Vehicle	q.s. to 100%
Propylene Glycol	Solvent, Humectant	20.0%

Ingredient	Function	Percentage (w/w %)
Sorbitol (70% Solution)	Humectant, Sweetener	0.02%
Sodium Saccharin	Sweetener	0.2%
Menthol	Cooling Agent, Flavoring	0.2%
Cetylpyridinium Chloride (CPC)	Antimicrobial Agent	0.2%
TTO	Active (Antimicrobial/Anti-inflammatory)	0.2 - 0.5%
EEO	Active (Antimicrobial/Anti-inflammatory)	0.2 - 0.5%
Polysorbate 20 (Tween 20)	Surfactant, Emulsifier	0.8%
Potassium Sorbate	Preservative	0.2%

2.2 Safety assessment (CAM assay)

The Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay was employed to evaluate the irritation potential of 2% dilutions prepared from each mouthwash formulation. Fertilized chicken eggs underwent incubation for 7–10 days. After careful exposure of the CAM, 0.3 mL of each test sample was applied. Reactions including hemorrhage, coagulation, and lysis were monitored over a 5-minute observation period. The Irritation Score (IS) was computed based on the time required for onset of each effect. Scores were categorized as follows: $IS < 1$ (non-irritant); $1 \leqslant IS < 5$ (slight irritant); $5 \leqslant IS < 9$ (moderate irritant); $IS \geqslant 9$ (corrosive). A 0.9% NaCl solution together with 0.1 mol/L NaOH served as negative and positive controls, respectively.

2.3 Antibacterial activity test

Antibacterial testing was conducted using the quantitative suspension method (plate count technique). Test microorganisms comprised Escherichia coli 8099, Staphylococcus aureus ATCC 6538, Streptococcus mutans ATCC 25175, and Porphyromonas gingivalis ATCC BAA-308. Bacterial suspensions (approximately $1–5 \times 10^8$ CFU/mL) were combined with equal volumes of the test mouthwash (0.2% TTO + 0.2% EEO formulation) and incubated for specified contact periods (48 hours for aerobic species, 72 hours for P. gingivalis under anaerobic conditions). Following neutralization, serial dilutions were plated, incubated, and colonies enumerated. The antibacterial rate (R%) was calculated using the formula: $R\ (\%) = [(A - B) / A] \times 100\%$, where A represents the mean colony count in control groups and B represents the mean colony count in test groups. All tests were performed in triplicate[16].

2.4 2.4 Anti-inflammatory activity assay (qPCR)

Human gingival fibroblasts (HGFs) received pre-treatment with non-cytotoxic dilutions (1:100) of the four mouthwash formulations or vehicle control for 1 hour. This was followed by stimulation using P. gingivalis LPS (1 μg/mL) for 6–8 hours[17]. Total RNA was extracted, reverse-transcribed into cDNA, and analyzed by quantitative real-time PCR (qPCR) employing primer sets targeting TNF-α, IL-1β, IL-6, IL-8, together with the reference gene GAPDH[18]. Relative gene expression changes were calculated using the $2^{\Delta\Delta Cq}$ method. Statistical significance was determined by one-way ANOVA combined with Tukey's post-hoc test ($p < 0.05$)[19]:

TNF-α:
Forward: 5'-CCTCTCTCTAATCAGCCCTCTG-3'
Reverse: 5'-GAGGACCTGGGAGTAGATGAG-3'

IL-1β:
Forward: 5'-ATGATGGCTTATTACAGTGGCAA-3'
Reverse: 5'-GTCGGAGATTCGTAGCTGGA-3'

IL-6:
Forward: 5'-ACTCACCTCTTCAGAACGAATTG-3'
Reverse: 5'-CCATCTTTGGAAGGTTCAGGTTG-3'

IL-8:
Forward: 5'-ACTGAGAGTGATTGAGAGTGGAC-3'
Reverse: 5'-AACCCTCTGCACCCAGTTTTC-3'

Reference Gene (GAPDH):
Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'
Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'

2.5 Clinical trial design

A future randomized, double-blind, placebo-controlled clinical trial was designed to evaluate efficacy together with safety. Individuals diagnosed with recurrent aphthous stomatitis (RAS) or mild-to-moderate gingivitis would be assigned to use either the test mouthwash or a placebo rinse twice daily over a 2–4 week period. Primary evaluation criteria would include ulcer healing or gingivitis improvement, safety assessment (irritation, allergic reactions), and sensory attributes (taste, cooling sensation, perceived cleanliness) rated on a 0–4 scale[20, 21].

2.6 Statistical analysis

Data are presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 8.0. For comparisons of anti-inflammatory gene expression, one-way ANOVA followed by Tukey's test was applied. Significance levels are indicated as $*p < 0.05$, $**p < 0.01$, $*p < 0.001$, with n.s. denoting $p \geqslant 0.05$.

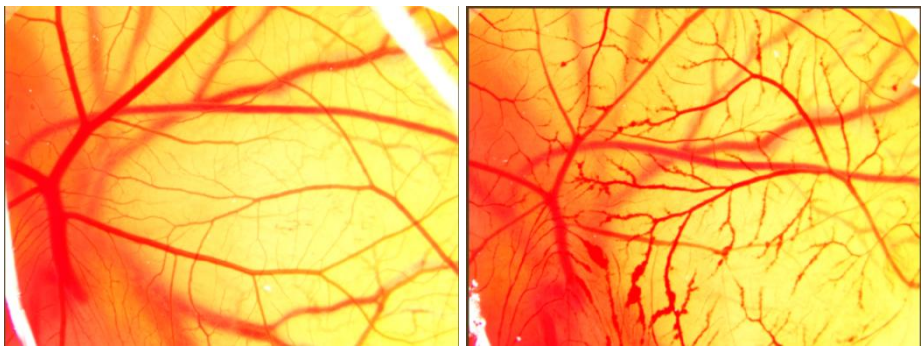
3 Results

3.1 Irritation Potential (CAM Assay)

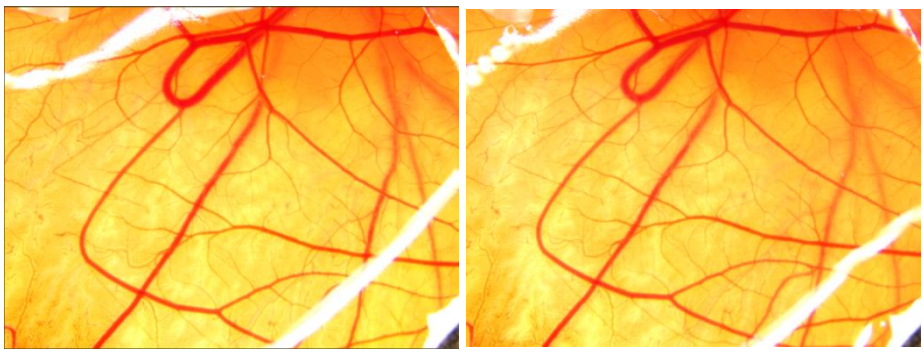
Table 2 together with Figure 1 present the Irritation Scores (IS) obtained for the 2% diluted formulations. The preparation containing 0.2% TTO plus 0.2% EEO was classified as non-irritant, with an IS value of 0.73. Formulations containing one oil at the 0.5% concentration level (0.2% TTO + 0.5% EEO, IS = 1.70; 0.5% TTO + 0.2% EEO, IS = 3.22) were categorized as slight irritants. The formulation containing 0.5% TTO together with 0.5% EEO produced a moderate irritant classification (IS = 5.27). Control samples performed as anticipated. These findings indicate that irritancy increases proportionally with essential oil concentration, and that TTO contributes more strongly to irritation compared with EEO at equivalent concentrations.

Table 2. Irritation Scores (IS) from the CAM Assay

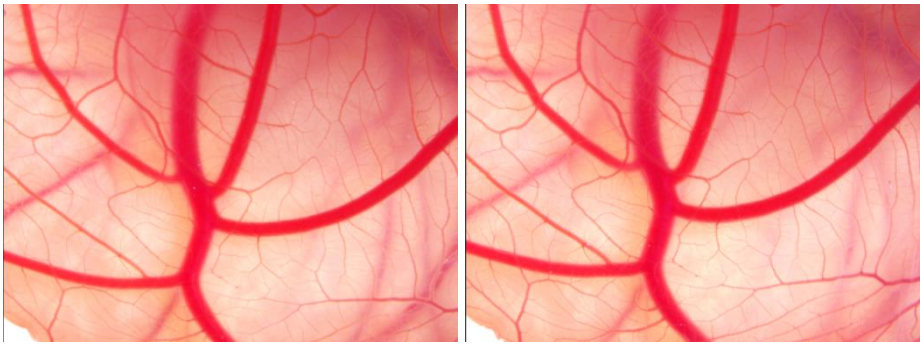
Product (2% diluted)	IS/ES scores	Irritation judgement
0.2% TTO, 0.2% EEO	IS=0.73	Non irritant
0.2% TTO, 0.5% EEO	IS=1.70	Slightly irritant
0.5% TTO, 0.2% EEO	IS=3.22	Slightly irritant
0.5% TTO, 0.5% EEO	IS=5.27	Moderately irritant
Negative control	ES=0.00	Non irritant
Positive control	ES=18.12	Strong irritant/ corrosive



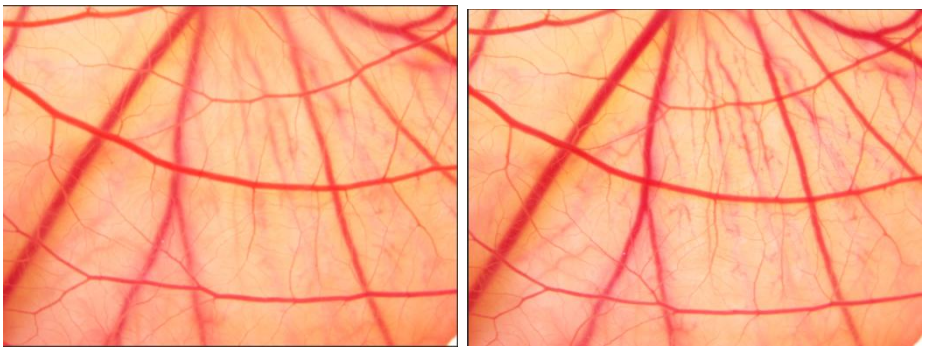
Positive control: 0s 300s



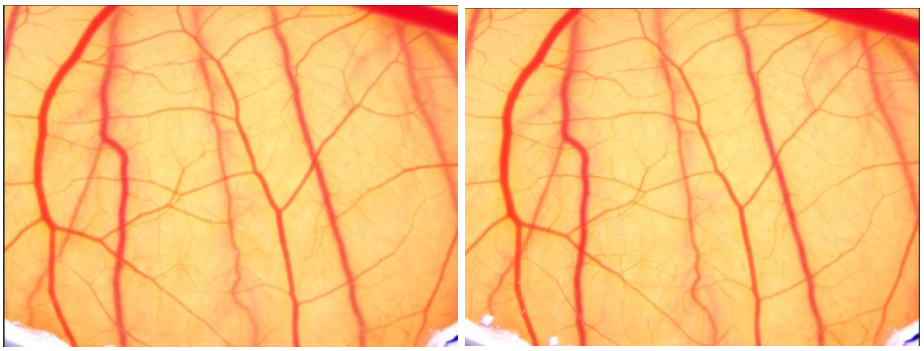
Negative control: 0s 180s



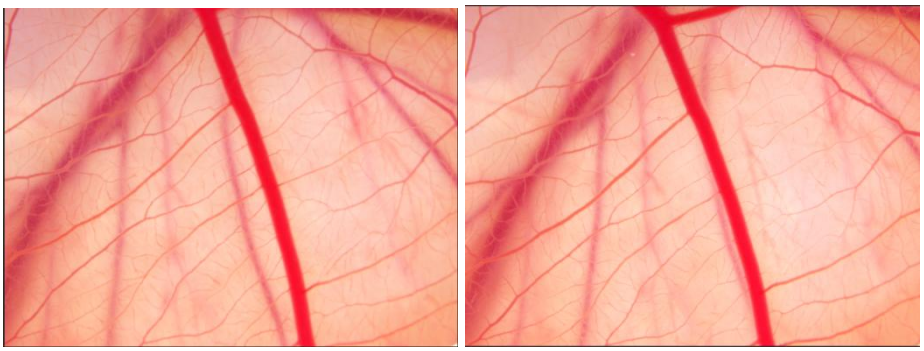
0.2% TTO, 0.2%EEO: 0s 300s



0.2% TTO, 0.5%EEO: 0s 300s



0.5% TTO, 0.2%EEO: 0s 300s



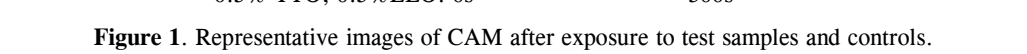


Figure 1. Representative images of CAM after exposure to test samples and controls.

3.2 Antibacterial activity

The 0.2% TTO + 0.2% EEO formulation demonstrated potent antibacterial activity, as summarized in Table 3. Greater than 99.97% inhibition (approaching complete sterilization) was achieved against E. coli, S. aureus, and S. mutans. Against the periodontal pathogen P. gingivalis, a substantial bacteriostatic effect of approximately 93% inhibition was observed.

Table 3. Antibacterial Rate of the 0.2% TTO + 0.2% EEO Formulation

Test Strain	Number of Trials	Mean Colony Count of Test Group (CFU/ tablet)	Mean Colony Count of Control Group (CFU/ tablet)	Antibacterial Rate/%
Escherichia coli 8099 (incubation time 48h)	1	＜5	7.25×10^4	＞99.99
	2	＜5	4.75×10^4	＞99.98
	3	＜5	1.38×10^4	＞99.96
	Average		4.46×10^4	＞99.98
Staphylococcus aureus ATCC 6538 (incubation time 48h)	1	＜5	3.60×10^4	＞99.98
	2	＜5	1.80×10^4	＞99.97
	3	＜5	1.23×10^4	＞99.95
	Average		2.21×10^4	＞99.97
Streptococcus mutans ATCC 25175 (incubation time 48h)	1	＜5	2.55×10^4	＞99.98
	2	＜5	2.95×10^4	＞99.98
	3	＜5	1.17×10^4	＞99.95
	Average		2.22×10^4	＞99.97
Porphyromonas gingivalis ATCC BAA-308 (incubation time 72h)		＜5	8.20×10^4	＞89.02
		＜5	2.00×10^4	＞93.75
		＜5	1.09×10^4	＞96.23
	Average		3.76×10^4	＞93.00

3.3 Anti-inflammatory Effects (qPCR)

All tested formulations significantly suppressed LPS-induced mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) in HGFs, as illustrated in Figure 2. A clear concentration-dependent relationship was evident, with the inhibitory effect strengthening as total essential oil concentration increased. The formulation containing 0.5% TTO plus 0.5% EEO exhibited the most pronounced suppression across all four cytokines measured.

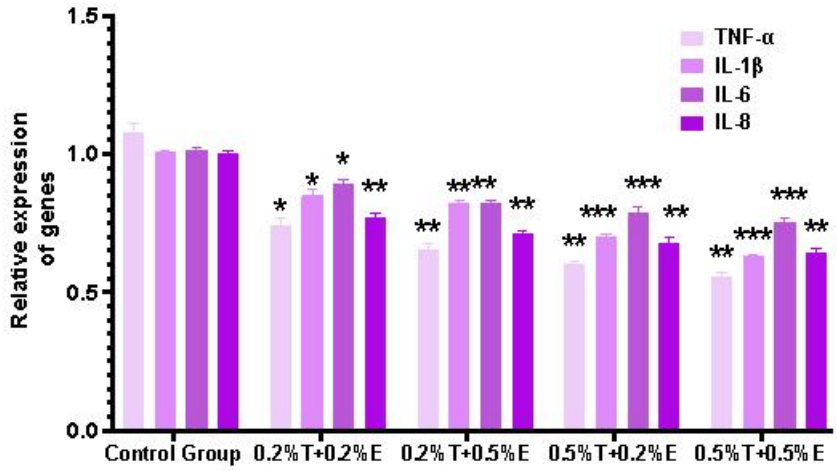


Figure 2. Relative mRNA expression levels of pro-inflammatory cytokines in HGFs after treatment with different mouthwash formulations and LPS stimulation. Data normalized to vehicle control (unstimulated cells). *p < 0.05, **p < 0.01, ***p < 0.001 vs. LPS-stimulated control group.

3.4 Predicted Clinical Acceptability

A predictive subjective evaluation of key clinical parameters is summarized in Table 4. A clear trade-off was projected: higher essential oil concentrations predicted better ulcer improvement but also increased irritation together with reduced taste acceptance. The 0.2% TTO + 0.2% EEO formulation was predicted to offer the most favorable balance, providing marked improvement with acceptable irritation and the highest taste compliance among the active formulations.

Table 4. Predictive Subjective Evaluation Scores (Scale 0-4)

Clinical Parameter	**0.2%T+ 0.2%E**	**0.2%T+ 0.5%E**	**0.5%T+ 0.2%E**	**0.5%T+0 .5%E**	**Score(0-4)**
Irritation	1.0	2.0	2.0	3.0	0 = None; 1 = Very slight; 2 = Mild; 3 = Moderate; 4 = Severe / Intolerable
Ulcer Improvement	3.0	3.3	3.2	3.5	0 = No effect / Worsening; 1 = Slight relief; 2 = Some improvement; 3 = Marked improvement; 4 = Significant and rapid healing

Clinical Parameter	**0.2%T+ 0.2%E**	**0.2%T+ 0.5%E**	**0.5%T+ 0.2%E**	**0.5%T+0 .5%E**	**Score(0-4)**
Taste Acceptance	208	2.5	2.3	2.0	0 = Extreme dislike; 1 = Dislike; 2 = Neutral / Acceptable; 3 = Like; 4 = Like extremely
Cooling Sensation	3.5	3.5	3.5	3.5	0 = None; 1 = Soft / Gentle; 2 = Moderate; 3 = Strong; 4 = Pungent / Stinging
(Perceived Cleanliness	3.8	3.9	3.9	4.0	0 = None; 1 = Slight; 2 = Clean; 3 = Very clean; 4 = Extremely clean

4 Discussion and conclusion

This study provides a comprehensive in vitro evaluation of TTO and EEO-based mouthwashes for oral ulcer management. The CAM assay established a clear safety-concentration relationship, identifying 0.5% for both oils as the upper tolerable limit due to moderate irritancy. The 0.2% TTO + 0.2% EEO formulation was non-irritant, supporting its safety profile for mucosal application.

The selected formulation exhibited excellent broad-spectrum antibacterial activity. The near-complete eradication of S. mutans and S. aureus, together with the strong suppression of P. gingivalis (approximately 93%), is clinically promising. This efficacy likely results from the synergistic action of CPC (membrane disruption) together with the essential oils (membrane penetration and fluidity alteration), effectively targeting both Gram-positive and Gram-negative oral pathogens.

Furthermore, qPCR results confirmed potent dose-dependent anti-inflammatory activity, with significant suppression of key cytokines (TNF-α, IL-1β, IL-6, IL-8). This aligns with literature suggesting that terpinen-4-ol (TTO) and 1,8-cineole (EEO) can inhibit NF-κB and other pro-inflammatory pathways. While our study measured the downstream gene expression outcome, the observed suppression is consistent with these previously reported mechanisms.

The predictive clinical assessment highlighted a critical balance. Higher oil concentrations enhanced efficacy but at the cost of increased irritancy and reduced palatability, which could hinder long-term patient compliance. Therefore, the 0.2% TTO + 0.2% EEO formulation emerges as the optimal candidate, offering a strong combination of antimicrobial and anti-inflammatory effects, favorable safety, and the best predicted user acceptability.

In conclusion, essential oil-based mouthwashes, particularly the 0.2% TTO + 0.2% EEO formulation, show significant potential as multi-targeted agents for managing oral ulcers. The in vitro findings of safety, potent antibacterial action, and anti-inflammatory activity support its therapeutic promise. These results warrant validation through the proposed randomized controlled clinical trial to confirm efficacy and tolerability in patients.

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