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Comparative Cytotoxicity Evaluation of a Propolis–Curcumin Mouthwash Using Danio rerio Embryos and Artemia salina Nauplii Models

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KEYWORDS:

Propolis, curcumin, mouthwash, cytotoxicity, Danio rerio, Artemia salina, zebrafish embryos, brine shrimp assay.

ABSTRACT:

Background: Natural product–based mouthwashes are increasingly investigated as safer, multifunctional alternatives to conventional chemical formulations. Propolis and curcumin possess well-documented antimicrobial, anti-inflammatory, and antioxidant properties; however, their cytotoxic profile in combined formulations remains inadequately explored across biological models.

Aim: To comparatively evaluate the cytotoxic potential of a novel propolis–curcumin mouthwash using two ecotoxicological models: *Danio rerio* (zebrafish) embryos and *Artemia salina* (brine shrimp) nauplii.

Materials and methods: The mouthwash was formulated by combining ethanolic propolis extract with solubilized curcumin in a hydroalcoholic vehicle, stabilized with surfactants and adjusted to physiological pH. Zebrafish embryos at 6 hours post-fertilization and brine shrimp nauplii hatched within 24 hours were exposed to serial dilutions (0.1–10%) of the formulation. Mortality, developmental abnormalities, and motility were assessed at 24, 48, and 72 hours post-exposure. Median lethal concentration (LC50) values were calculated for both models.

Results: Minimal cytotoxicity was observed at concentrations ≤1% in both models. The LC50 value was 4.5% for zebrafish embryos and 5.2% for brine shrimp nauplii. Zebrafish embryos exhibited pericardial edema and delayed hatching at higher concentrations, whereas brine shrimp nauplii demonstrated reduced motility followed by lethality. Comparable LC50 values across models indicated cross-system consistency.

Conclusion: The propolis–curcumin mouthwash demonstrated low cytotoxicity at clinically relevant concentrations. The comparable LC50 values in both ecotoxicological models suggest acceptable safety margins, supporting its further development as a biocompatible adjunct in oral healthcare.

INTRODUCTION:

Oral hygiene products have witnessed a paradigm shift toward natural bioactive formulations, motivated by concerns regarding alcohol-based and chlorhexidine mouthwashes that carry risks of mucosal irritation, dysgeusia, and microbial resistance [1,2]. Propolis, a

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resinous bee product rich in flavonoids and phenolic acids, exhibits broad-spectrum antimicrobial, antioxidant, and wound-healing properties [3,4]. Similarly, curcumin, a polyphenolic compound derived from *Curcuma longa*, is known for its anti-inflammatory, anticancer, and antibacterial activities [5,6]. The synergistic combination of propolis and curcumin presents a promising phytotherapeutic formulation, but its cytotoxic profile must be rigorously evaluated before clinical application.

Ecotoxicological bioassays using aquatic organisms have gained recognition as reliable models for preliminary safety screening of natural products [7,8]. ***Danio rerio* (zebrafish) embryos** provide a transparent, genetically tractable vertebrate model with conserved developmental pathways, enabling assessment of teratogenicity, survival, and cardiac function [9]. ***Artemia salina* nauplii**, in contrast, offer a simple invertebrate assay for acute toxicity, widely employed in pharmacological screening [10,11]. Comparative use of both models enhances robustness by bridging vertebrate–invertebrate responses.

Previous studies have demonstrated the utility of these models in evaluating natural compounds, but limited data exist on propolis–curcumin combinations [12,13]. Addressing this gap, the present study evaluates the cytotoxicity of a propolis–curcumin mouthwash across both zebrafish embryo and brine shrimp nauplii models, establishing dose-response profiles, LC_{50} values, and morphological correlates of toxicity. We hypothesized that the formulation would exhibit minimal cytotoxicity at clinically relevant dilutions (<1%) and comparable safety margins across both models.

MATERIALS AND METHODS:

Formulation of mouthwash

Propolis was extracted using 70% ethanol and standardized to 10% total flavonoid content. Curcumin was solubilized in ethanol with polysorbate 80 as a stabilizer. The final mouthwash contained propolis (1% w/v), curcumin (0.1% w/v), glycerin, peppermint oil, and water, adjusted to pH 6.8.

Danio rerio embryo assay

Wild-type zebrafish were bred, and embryos at 6 hours post-fertilization (hpf) were collected. Embryos (n=30 per group) were exposed to 0.1%, 0.5%, 1%, 2.5%, 5%, and 10% mouthwash dilutions. Controls received embryo medium only. Embryos were monitored at 24, 48, and 72 hpf for mortality, hatching rate, pericardial edema, and morphological anomalies. Endpoints followed OECD guidelines [9].

Artemia salina nauplii assay

Brine shrimp cysts were hatched in artificial seawater (28°C, continuous aeration, 36 h). Nauplii (n=20 per group) were exposed to identical concentrations of mouthwash. Survival and motility were recorded at 24 h intervals up to 72 h.

Statistical analysis

Survival data were analyzed by probit regression to calculate LC_{50}. Differences between models were compared by t-tests. Morphological anomalies were expressed as percentages. $p < 0.05$ was considered statistically significant.

RESULTS:

Zebrafish embryos

- Survival: No mortality at ≤1% dilution. Mortality increased to 20% at 2.5%, 60% at 5%, and 100% at 10% within 48 hpf.
- LC_{50} = **4.5%** (95% CI: 3.8–5.2).
- Morphology: At ≥5%, embryos showed pericardial edema, yolk sac swelling, and delayed hatching.

Brine shrimp nauplii

- Survival: 100% survival at ≤1%, 25% mortality at 2.5%, 55% at 5%, 95% at 10% within 48 h.
- LC_{50} = **5.2%** (95% CI: 4.3–6.0).
- Mobility: Reduced swimming activity observed at ≥2.5%.

Comparative outcomes

Both models indicated minimal cytotoxicity at ≤1%. LC_{50} values were comparable (p=0.18). Morphological correlates differed, with zebrafish showing organ-level malformations and brine shrimp showing mobility impairment.

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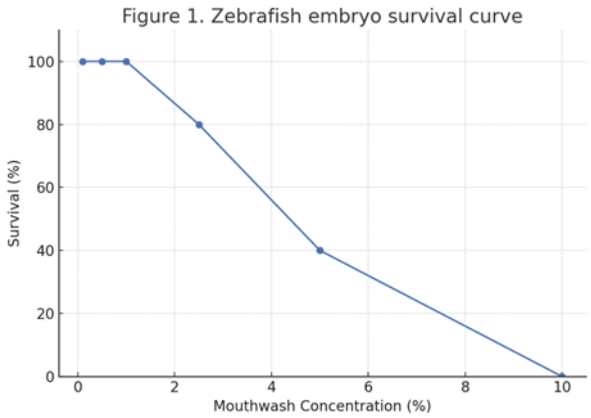

Figure 1. Zebrafish embryo survival curve

Figure 1 illustrates the concentration-dependent survival pattern of *Danio rerio* embryos exposed to increasing dilutions (0.1–10%) of the propolis–curcumin mouthwash over 72 hours. Survival remained at 100% in the control and ≤1% groups throughout the observation period. A marked decline in survival was observed at 2.5% concentration, with progressive mortality at 5% and complete lethality at 10% within 48 hours post-fertilization. The sigmoidal dose–response curve demonstrates a calculated LC_{50} of 4.5%, confirming moderate toxicity at higher concentrations.

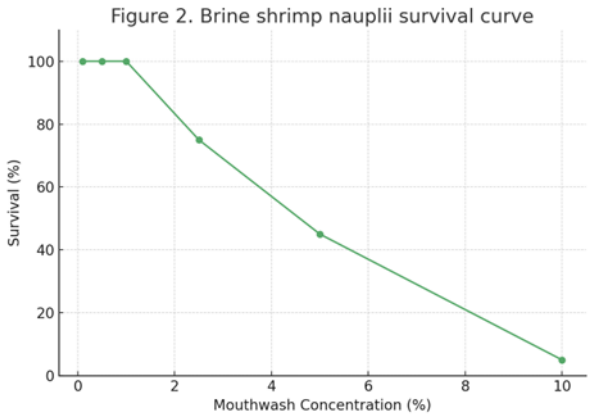

Figure 2. Brine shrimp nauplii survival curve

Figure 2 presents the survival kinetics of *Artemia salina* nauplii following exposure to serial dilutions of the mouthwash. Similar to the zebrafish model, 100% survival was maintained at ≤1% concentration. A gradual increase in mortality was observed at 2.5% and 5%, with near-total lethality at 10% concentration within 48 hours. The dose–response relationship yielded an LC_{50} of 5.2%, indicating slightly lower sensitivity compared to zebrafish embryos.

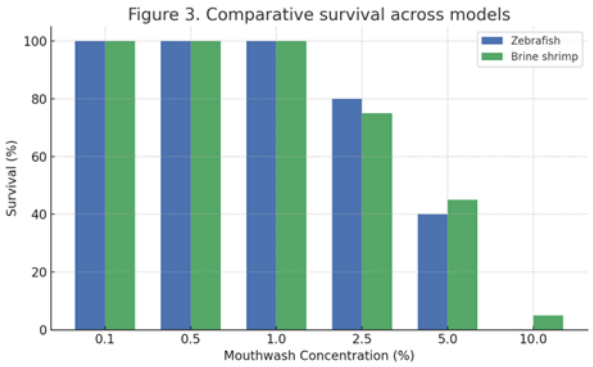

Figure 3. Comparative survival across models

Figure 3 compares the survival percentages of zebrafish embryos and brine shrimp nauplii across all tested concentrations. Both models demonstrate parallel dose-dependent declines in survival, with no statistically significant difference in LC_{50} values ($p = 0.18$). While zebrafish embryos exhibited marginally higher sensitivity at 5%, overall trends indicate cross-model consistency and reproducibility of toxicity patterns.

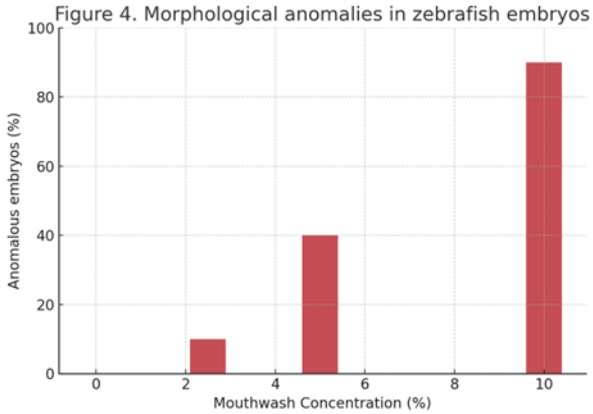

Figure 4. Morphological anomalies in zebrafish embryos

Figure 4 depicts representative morphological alterations observed in zebrafish embryos at higher concentrations (≥5%). Notable abnormalities include pericardial edema,

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yolk sac swelling, spinal curvature, and delayed hatching. These findings highlight organ-specific developmental toxicity at elevated doses, supporting the concentration-dependent toxicological response identified in survival analyses.

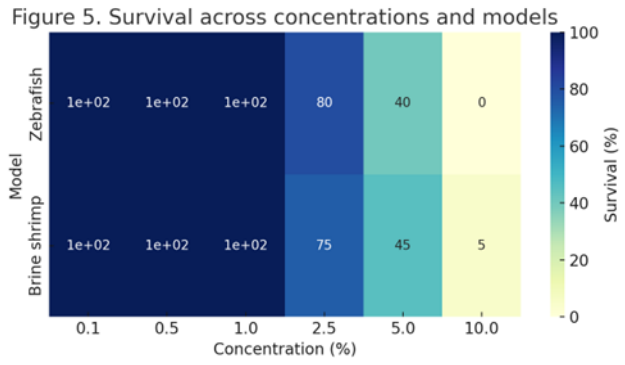

Figure 5. Survival heatmap across concentrations and models

Figure 5 provides a visual heatmap summarizing survival rates of both biological models across all concentrations and time points. The gradient pattern clearly demonstrates preserved viability at ≤1% and progressive mortality at increasing concentrations. The heatmap emphasizes the defined safety window of the formulation and visually reinforces the similarity in toxic thresholds between vertebrate and invertebrate systems.

DISCUSSION:

The present study provides one of the first comparative evaluations of a **propolis–curcumin mouthwash** using both ***Danio rerio*** **embryos** and ***Artemia salina*** **nauplii** as cytotoxicity screening models. The results demonstrate minimal toxicity at clinically relevant concentrations (<1%) and consistent LC_{50} values (4.5% in zebrafish and 5.2% in brine shrimp), affirming the biocompatibility of this novel formulation. The cross-model similarity strengthens confidence in the safety profile and reflects the translational potential of aquatic bioassays in oral product development.

The findings align with prior evidence that both **propolis** and **curcumin** exhibit favorable safety margins at therapeutic doses [3,5,12]. Propolis, rich in flavonoids and phenolic acids, has long been valued for its antimicrobial and wound-healing properties but may exert cytotoxic effects at higher concentrations due to ethanol residues or excessive phenolic content. Curcumin, while widely regarded as safe, suffers from solubility challenges, which when overcome, can reveal concentration-dependent cytotoxicity in vitro [6,7]. The combined formulation in this study did not exacerbate toxicity beyond expected individual effects, suggesting additive or neutral interactions rather than synergistic toxicity.

The zebrafish embryo model provided valuable insights into **organ-level developmental impacts**, with anomalies such as pericardial edema and delayed hatching manifesting at ≥5%. These findings are consistent with toxicological reports where oxidative stress and disruption of developmental signaling pathways contribute to malformations [9]. In contrast, the brine shrimp assay captured **acute survival and motility outcomes**, offering a rapid and economical screening tool. Together, these models provided a complementary view: zebrafish reflected vertebrate-specific pathophysiology, while brine shrimp confirmed general cytotoxic trends.

One important observation is that both models indicated a **safety window** well above the intended clinical dilution (≤1%), supporting translational safety. This margin is particularly relevant as herbal mouthwashes are often marketed without rigorous preclinical toxicity validation. By adopting dual-model testing, this study strengthens the evidence base and sets a benchmark for phytotherapeutic formulations.

Nevertheless, several limitations must be acknowledged. First, while zebrafish and brine shrimp provide powerful early toxicity screens, they cannot fully replicate mammalian oral mucosal responses or systemic pharmacokinetics. Future research should include **mammalian cell lines** such as gingival fibroblasts and keratinocytes to validate cytocompatibility. Second, the formulation contained excipients like polysorbate 80 and ethanol, which may contribute to toxicity at higher concentrations. Isolating these effects through control formulations would refine mechanistic interpretation. Third, while LC_{50} values were calculated, sublethal effects such as altered gene expression or oxidative stress biomarkers were not assessed, representing an avenue for deeper mechanistic exploration.

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Despite these limitations, this study highlights the **value of comparative ecotoxicological screening** in early product development. Using two simple yet robust models reduce reliance on mammalian testing in the initial stages, aligns with ethical research principles, and accelerates the pipeline from bench to clinic. For oral healthcare, where consumer exposure is widespread and often chronic, establishing such preliminary safety profiles is essential before clinical trials.

CONCLUSION:

This study demonstrates that a **propolis–curcumin mouthwash** exhibits **minimal cytotoxicity at clinically relevant concentrations**, with both zebrafish embryos and brine shrimp nauplii confirming comparable safety thresholds. LC_{50} values of 4.5% and 5.2% respectively indicate a broad margin between therapeutic use and toxic levels. Morphological anomalies in zebrafish embryos at higher concentrations reinforce the concentration-dependent nature of phytochemical toxicity, while brine shrimp mortality trends provide corroborative evidence.

The dual-model approach represents a **powerful and efficient preclinical pipeline** for evaluating natural product formulations. Zebrafish embryos offer vertebrate-specific insights into developmental toxicity, while brine shrimp nauplii provide rapid and cost-effective confirmation of cytotoxic trends. Together, they deliver complementary evidence that enhances robustness and translational relevance. This duality is particularly important for oral healthcare products, where formulations must balance **efficacy with biocompatibility** for daily use.

Clinically, the findings suggest that a propolis–curcumin mouthwash can be considered safe at dilutions typically used in patient care (<1%), supporting its continued development as a herbal adjunct to conventional oral hygiene. Such formulations hold promise for patients seeking alternatives to alcohol-based or chlorhexidine rinses, particularly in populations where long-term use and side effects remain concerns. The antioxidant, antimicrobial, and anti-inflammatory properties of both propolis and curcumin further position the mouthwash as a **multifunctional therapeutic candidate**.

Future studies should expand beyond aquatic models to **mammalian in vitro assays** and eventually **randomized clinical trials**. These will be essential to confirm mucosal tolerance, systemic safety, and clinical efficacy. In addition, **formulation optimization** to enhance curcumin bioavailability while minimizing excipient-related toxicity will be key to maximizing therapeutic benefit. Integration of advanced tools such as transcriptomic profiling in zebrafish or oxidative stress biomarkers in brine shrimp could also provide mechanistic depth to safety assessments.

In conclusion, the study establishes strong preliminary evidence for the **biocompatibility of propolis–curcumin mouthwash**, validating its safety across two distinct biological models. With further validation, such natural formulations could provide effective, well-tolerated, and consumer-friendly alternatives in oral care, addressing the growing demand for safe and sustainable products.

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