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Evaluation of cytotoxicity and embryonic toxicology of fluoride varnish with quercetin - an in vitro study

Avaliação da citotoxicidade e toxicologia embrionária do verniz fluorado com quercetina - um estudo in vitro

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ABSTRACT

Objective: Although fluoride varnish is a standard preventive agent, concerns about its potential cytotoxicity have led to the incorporation of adjunctive bioactive compounds such as quercetin, known for its antioxidant and antimicrobial effects. This study aimed to evaluate the cytotoxic and embryotoxic potential of a newly formulated fluoride varnish containing quercetin, using brine shrimp lethality and zebrafish embryotoxicity assays as preliminary screening methods. **Material and Methods:** An in vitro comparative experimental study was conducted. The test varnish contained sodium fluoride, tricalcium phosphate, xylitol, and quercetin. Five concentrations (5, 10, 20, 40, and 80 μg/L) were tested and compared against a commercial 5% NaF varnish (Profluorid). Brine shrimp (Artemia salina) nauplii were exposed for 24 and 48 hours to assess viability. Zebrafish (Danio rerio) embryos were evaluated over 96 hours for hatching rate, viability, and morphological integrity. Data were analyzed using one-way ANOVA. **Results:** Both brine shrimp and zebrafish embryos demonstrated high survival and hatching rates across all test concentrations. No statistically significant differences were observed between the test and control groups ($p > 0.05$). The highest naupliar viability (99.20 ± 0.84%) and embryo hatching rate (88.00 ± 8.37%) occurred at the lowest concentration. No morphological or teratogenic abnormalities were detected. **Conclusion:** The quercetin-enriched fluoride varnish showed no significant cytotoxic or embryotoxic effects, indicating its biocompatibility and potential as a safer alternative in pediatric caries prevention.

KEYWORDS

Brine shrimp assay; Cytotoxicity; Fluoride varnish; Quercetin; Zebrafish embryo.

RESUMO

Objetivo: Embora o verniz fluoretado seja um agente preventivo padrão, as preocupações com sua potencial citotoxicidade levaram à incorporação de compostos bioativos adjuvantes, como a quercetina, conhecida por seus efeitos antioxidantes e antimicrobianos. Este estudo teve como objetivo avaliar o potencial citotóxico e embriotóxico de um verniz fluoretado recém-formulado contendo quercetina, utilizando ensaios de letalidade em artêmia e embriotoxicidade em peixe-zebra como métodos de triagem preliminar. **Material e Métodos:** Foi realizado um estudo experimental comparativo in vitro. O verniz testado continha fluoreto de sódio, fosfato tricálcico, xilitol e quercetina. Cinco concentrações (5, 10, 20, 40 e 80 μg/L) foram testadas e comparadas com um verniz comercial de 5% de NaF (Profluorid). Náuplios de Artêmia (Artemia salina) foram expostos por 24 e 48 horas para avaliar a viabilidade. Embriões de peixe-zebra (Danio rerio) foram avaliados ao longo de 96 horas quanto à taxa de eclosão, viabilidade e integridade morfológica. Os dados foram analisados usando ANOVA unidirecional. **Resultados:** Tanto os embriões de artêmia quanto os de peixe-zebra demonstraram altas taxas de sobrevivência e eclosão em todas as concentrações testadas. Não foram observadas diferenças estatisticamente significativas entre os grupos de teste e controle ($p > 0,05$). A maior viabilidade naupliar (99,20 ± 0,84%) e a maior taxa de eclosão de embriões (88,00 ± 8,37%) ocorreram na menor concentração. Não foram detectadas anomalias

morfológicas ou teratogênicas. **Conclusão:** O verniz fluoretado enriquecido com quercetina não apresentou efeitos citotóxicos ou embriotóxicos significativos, indicando sua biocompatibilidade e potencial como uma alternativa mais segura na prevenção de cáries pediátricas.

PALAVRAS-CHAVE

Ensaio com artêmia; Citotoxicidade; Verniz fluoretado; Quercetina; Embrião de peixe-zebra.

INTRODUCTION

Dental caries is a pervasive and multifactorial oral health condition that affects individuals across all demographics, geographies, and climates, making it a true "gobendemic"—a term that encapsulates its universal and persistent nature [1]. Despite being largely preventable, dental caries remains a significant global public health challenge, with its prevalence influenced by dietary habits, oral hygiene practices, and socioeconomic factors. Over the years, the paradigm of caries management has evolved substantially, transitioning from a purely restorative approach to a preventive and minimally invasive strategy that incorporates risk-based assessment and intervention [2]. The early identification of caries risk factors, coupled with the implementation of preventive measures, plays a crucial role in mitigating the disease burden.

Among the most extensively studied and clinically validated preventive agents, fluoride has remained the cornerstone of caries prevention for decades. Fluoride varnish, in particular, has been widely adopted in professional dental care due to its superior substantivity, ease of application, and sustained release of fluoride ions [3]. The mechanism of action of fluoride involves enhancing enamel remineralization, inhibiting demineralization, and exerting antibacterial effects by disrupting bacterial metabolism and reducing acid production [4]. Numerous studies have demonstrated that the professional application of fluoride varnish, even in communities with fluoridated drinking water, significantly reduces caries incidence, particularly among high-risk pediatric populations [5].

Despite the well-established benefits of fluoride varnish, concerns regarding potential adverse effects at the cellular level have necessitated the exploration of adjunctive bioactive compounds that can enhance its therapeutic efficacy while mitigating cytotoxicity [6]. One such promising agent is quercetin, a naturally occurring flavonoid with potent antioxidant, anti-inflammatory, and antimicrobial properties. Quercetin exerts anti-inflammatory effects by modulating the expression of cytokines, pro-inflammatory mediators, and key regulatory enzymes involved in inflammatory pathways. Enhancing the bioavailability of flavonoids is critical to maximizing their therapeutic potential and expanding their application in pharmaceutical formulations [7-9].

Quercetin exhibits a remarkable ability to inhibit matrix metalloproteinases (MMPs), specifically MMP-2, MMP-8, and MMP-9, which are activated in the acidic environment of demineralized dentin and contribute to collagen matrix degradation [10,11]. By incorporating quercetin into fluoride varnish formulations, it is hypothesized that its MMP-inhibitory properties may confer additional protective effects on the dentin organic matrix, thereby enhancing the longevity and effectiveness of fluoride-based therapies [12].

In addition to quercetin, tricalcium calcium phosphate (TCP) has gained recognition for its role in enamel remineralization. Quercetin when combined with tricalcium phosphate have demonstrated the ability to act as a scaffold in bone regeneration [13,14]. However, before such formulations can be considered for clinical application, a comprehensive evaluation of their cytotoxic and embryotoxic potential is imperative to ensure their biosafety.

Given the increasing emphasis on biocompatibility in dental materials, in vitro toxicity assays serve as a critical initial screening tool. The brine shrimp lethality assay, widely regarded as a simple yet reliable method for preliminary cytotoxicity assessment, provides valuable insights into the toxicological profile of bioactive compounds. Additionally, the zebrafish embryotoxicity model has emerged as a robust and ethically viable alternative for evaluating developmental toxicity, offering high genetic homology to humans and real-time visualization of embryogenesis.

The present study aimed to evaluate the cytotoxicity and embryotoxicity of fluoride varnish containing quercetin using the brine shrimp lethality assay and zebrafish embryonic toxicology evaluation. This investigation seeks to elucidate the biocompatibility profile of this novel formulation, contributing to the ongoing efforts in optimizing fluoride-based caries preventive strategies while ensuring safety for clinical applications.

MATERIAL AND METHODS

Study design and ethical considerations

This laboratory-based in vitro comparative experimental study was executed at the Gold Research Laboratory, Department of Pharmacology. Ethical clearance was obtained from the Institutional Scientific Review Board prior to study initiation (Approval No.: SRB/SDC/PEDO-2304/24/110). The investigation commenced in April 2024 and was completed over a duration of four weeks. The timeframe encompassed the procurement of chemicals and zebrafish embryos, formulation of the quercetin-integrated fluoride varnish, and execution of subsequent cytotoxicity and embryotoxicity assays.

EXPERIMENTAL GROUPS AND FORMULATION

Test group: fluoride varnish incorporating quercetin

A novel bioactive varnish was formulated by integrating quercetin into a fluoride-based matrix. Sodium fluoride (Cat.no.-57010) was procured from RANKEM, Avantor Performance Materials India (Maharashtra), while tricalcium phosphate (Cat.no.-34878), quercetin powder (Cat.no.-71923), xylitol (Cat.no.-64949), and ethanol (Cat.no.-77141) were sourced from Sisco Research Laboratories Pvt. Ltd. (Maharashtra, India).

The varnish stock solution (10 mL) was synthesized by accurately weighing 300 μg of sodium fluoride, 300 μg of tricalcium phosphate, and 100 μg of quercetin powder. These constituents were mechanically stirred in 10 mL of absolute ethanol containing 100 μg of xylitol until a homogenous mixture was achieved. The resulting suspension was subjected to centrifugation at 8000 rpm for 15 minutes at ambient temperature to enhance dispersion and stability of the formulation.

Subsequently, serial dilution was done with ethanol to obtain five experimental concentrations:

- Group 1: 5 μg/L
- Group 2: 10 μg/L
- Group 3: 20 μg/L
- Group 4: 40 μg/L
- Group 5: 80 μg/L

Control group

A commercially available fluoride varnish 5% NaF, Profluorid® (VOCO, Cuxhaven, Germany) was utilized as the control standard (Group 6).

Brine shrimp lethality assay

The protocol used for Brine Shrimp Lethality Assay followed a similar protocol used in the previous studies [15,16].

The preliminary cytotoxic potential of the experimental formulation was assessed utilizing the brine shrimp (Artemia salina) lethality bioassay. Artificial seawater was prepared by dissolving 2 g of iodine-free salt in 200 mL of distilled water. Nauplii were hatched and collected 24 hours post-incubation under continuous aeration.

Ten viable nauplii were randomly introduced into each well of 6-well ELISA plates containing 10–12 mL of the prepared saline solution. Each test group (Group 1 to Group 5) received its respective concentration of the test varnish, and the plates were maintained undisturbed for 24 hours at room temperature. Mortality was recorded by direct visualization under a magnifying lens, and the percentage lethality was calculated using the formula:

$$\%\ Mortality = \left[\frac{Number\ of\ dead\ nauplii}{(Dead + Live\ nauplii)} \right] \times 100 \quad (1)$$

The same protocol was repeated at the 48-hour timepoint. All tests were performed in triplicate to ensure reproducibility, and concentration-dependent cytotoxic effects were analyzed and depicted graphically.

Zebrafish embryotoxicity assay

The protocol used for Zebrafish Embryotoxicity Assay followed a similar protocol used in the previous studies [17].

Fish maintenance and breeding conditions

Wild-type *Danio rerio* specimens were procured from licensed aquaculture vendors in India. The fish were acclimatized and housed in dedicated aquaria maintained under optimal environmental parameters: temperature (28 ± 2°C), pH (6.8–8.5), and a photoperiod of 14 hours light and 10 hours dark. The fish were fed twice daily with commercial dry flakes and freeze-dried bloodworms.

Controlled breeding was initiated by transferring three males and one female into breeding tanks equipped with mesh separators to prevent predation of fertilized eggs. Embryos were collected the following morning and rinsed thrice with E3 medium devoid of methylene blue.

Exposure protocol

Fertilized eggs were sorted under a stereomicroscope to ensure viability and transferred into multiwell culture plates (6-, 12-, or 24-well plates), with 20 embryos per 2 mL of E3 medium in each well. Experimental treatments included five graded concentrations (5, 10, 20, 40, and 80 μg/mL) of freshly prepared quercetin-fluoride varnish. The varnish suspensions were ultrasonicated for 15 minutes to ensure nanoparticle dispersion before being introduced into the wells. Each experimental group and the control group were conducted in triplicate.

Embryos were incubated for 96 hours post-fertilization (hpf) at 28°C in darkened conditions to prevent photodegradation. Non-viable embryos were systematically removed at 12-hour intervals to maintain optimal culture conditions.

Embryo evaluation and endpoint criteria

Embryonic development was scrutinized at 24-hour intervals using a COSLAB light microscope model: HL-10A (Ambala Cantt, India). Morphological integrity, viability, and developmental progression were documented and photographed. Observational endpoints included embryo mortality rate, hatching efficiency, incidence of developmental deformities and larval viability and cumulative mortality.

Malformed embryos and larvae were identified, cataloged, and quantified to assess potential teratogenic effects. Data were statistically analyzed to determine dose-dependent embryotoxic responses to the test formulations.

STATISTICAL ANALYSIS

The collected experimental data underwent rigorous statistical scrutiny to ascertain the significance and distributional properties of the observed outcomes. Preliminary assessment of data normality was conducted utilizing the Shapiro–Wilk test. For intergroup comparisons involving multiple treatment concentrations, one-way analysis of variance (ANOVA) was employed to detect statistically meaningful differences among the means of the independent groups. All statistical computations were executed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). A p-value less than 0.05 was interpreted as indicative of statistical significance.

RESULTS

Brine shrimp lethality assay

At 24 hours post-exposure, naupliar viability remained substantially high across all tested concentrations, exhibiting minimal cytotoxic insult. The mean viability at the lowest concentration (5 μg/L) reached 99.20 ± 0.84%, whereas a marginal decrease was observed at the highest concentration (80 μg/L), with viability recorded at 98.20 ± 1.30%. The control group yielded a survival rate of 98.60 ± 1.14%, statistically comparable to all experimental groups. The overall variation in naupliar survival across groups failed to reach statistical significance, as evidenced by the one-way ANOVA output ($F = 0.53$, $p = 0.74$), thereby affirming the non-cytotoxic nature of both the test and commercial varnishes under acute exposure conditions (Table I).

Following 48-Hour exposure, the highest viability was observed at 40 μg/L (98.60 ± 1.14%), while the lowest was recorded at 80 μg/L (97.60 ± 1.95%). The control group presented a comparable survival rate of 98.20 ± 1.92%, reflecting no overt toxic effect from either formulation. Statistical analysis indicated no significant intergroup deviation ($F = 0.36$, $p = 0.86$), thereby underscoring the cytological

safety of fluoride varnish with quercetin, even under extended exposure (Table II). The findings demonstrated consistently high survival percentages across all groups, albeit with a slight reduction at higher concentrations.

Zebrafish embryotoxicity assay

The highest hatching percentage was observed at 5 μg/L and 10 μg/L (88.00 ± 8.37%), while a moderate decline was noted at 40 μg/L and 80 μg/L (82.00 ± 14.83%). The control group exhibited a hatching success of 86.00 ± 8.94%, suggestive of physiological baseline rates. The statistical output ($F = 0.30$, $p = 0.90$) failed to reveal significant differences among the groups, indicating the absence of any embryotoxic liability attributable to the experimental or commercial varnish formulations at the tested concentrations (Table III).

Larval viability following embryonic hatching remained optimal at lower concentrations (5 μg/L and 10 μg/L: 88.00 ± 8.37%), with a minor reduction noted at higher doses (40 μg/L and 80 μg/L: 82.00 ± 14.83%). These outcomes were statistically analogous to the control (86.00 ± 8.94%). One-way ANOVA results reaffirmed the absence of significant differences among treatment groups ($F = 0.30$, $p = 0.90$), indicating that neither acute nor delayed larval mortality was provoked by exposure to fluoride varnish with quercetin (Table IV).

Figure 1 depicts microscopic observation of zebrafish embryos showing no impairment in embryonic development treated with test and control groups. Across all bioassays—naupliar viability at 24 and 48 hours, zebrafish hatching success, and post-hatching larval survival—the test formulation elicited no statistically significant adverse biological effects. The uniformity and resilience of biological response across graded concentrations suggest a robust safety margin, endorsing the material's potential translational applicability in pediatric dental therapeutics.

Table I - Nauplii viability on 24hr exposure of various concentrations of fluoride varnish with quercetin and commercial dental varnish

Concentration (µg/L)	Mean ± Standard Deviation (%)	One-way ANOVA (F)	p-value
5	99.20 ± 0.84	0.53	0.74
10	98.60 ± 0.89		
20	98.60 ± 0.89		
40	98.80 ± 0.84		
80	98.20 ± 1.30		
Control	98.60 ± 1.14		

Table II - Nauplii viability on 48hr exposure of various concentrations of fluoride varnish with quercetin and commercial dental varnish

Concentration (µg/L)	Mean ± Standard Deviation (%)	One-way ANOVA (F)	p-value
5	98.80 ± 1.64	0.36	0.86
10	98.00 ± 1.58		
20	97.80 ± 1.92		
40	98.60 ± 1.14		
80	97.60 ± 1.95		
Control	98.20 ± 1.92		

Table III - Hatching rate on 24hr exposure of various concentrations of fluoride varnish with quercetin and commercial dental varnish

Concentration (µg/L)	Mean ± Standard Deviation (%)	One-way ANOVA (F)	p-value
5	88.00 ± 8.37	0.30	0.90
10	88.00 ± 8.37		
20	86.00 ± 8.94		
40	82.00 ± 14.83		
80	82.00 ± 14.83		
Control	86.00 ± 8.94		

Table IV - Larval Viability Post-Hatching on 24hr exposure of various concentrations of fluoride varnish with quercetin and commercial dental varnish

Concentration (µg/L)	Mean ± Standard Deviation (%)	One-way ANOVA (F)	p-value
5	88.00 ± 8.37	0.30	0.90
10	88.00 ± 8.37		
20	86.00 ± 8.94		
40	82.00 ± 14.83		
80	82.00 ± 14.83		
Control	86.00 ± 8.94		

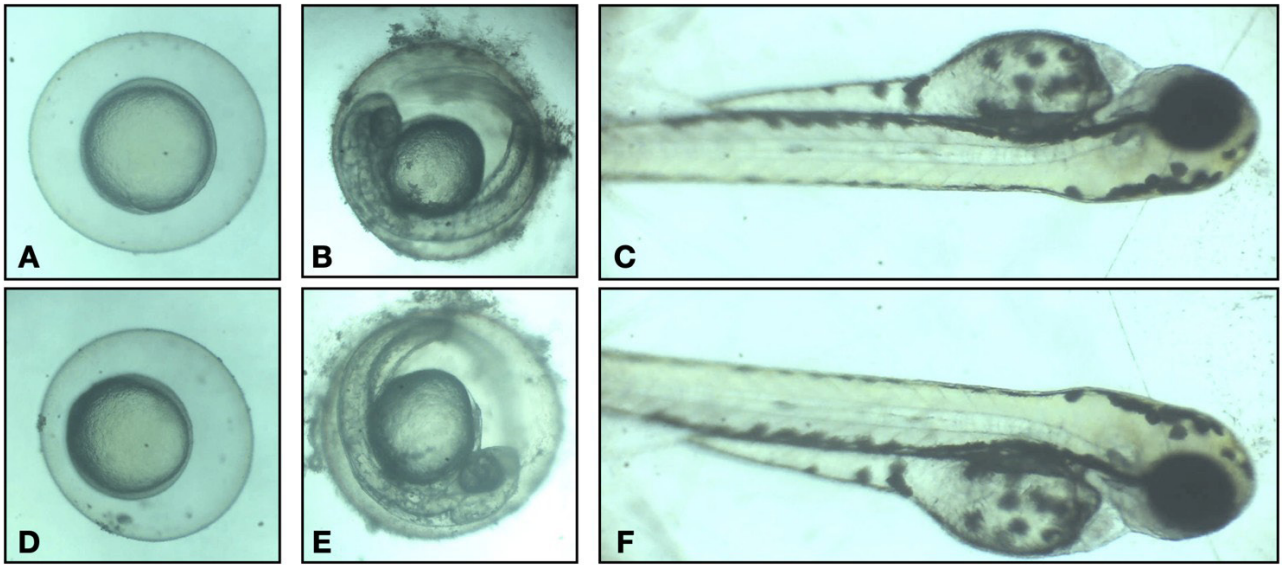

Figure 1 - Microscopic observation showing no impairment in embryonic development of zebrafish treated with test (A-Day 1; B-Day 2; C-Day 3) and control (D-Day 1; E-Day 2; F-Day 3) groups.

DISCUSSION

The present in vitro investigation comprehensively evaluated the cytotoxic and embryotoxic potential of a fluoride varnish incorporated with quercetin using *Artemia salina* lethality bioassay and zebrafish (*Danio rerio*) embryotoxicity assay. The findings revealed consistently high survival and hatching rates across all tested concentrations, with no statistically significant differences when compared to controls. These outcomes suggest that the quercetin-based fluoride varnish exhibited no discernible cytotoxic or embryotoxic liabilities, even at higher dosages.

The rationale for employing *A. salina* and zebrafish embryos stems from their well-established utility in preliminary toxicity screening, providing a rapid, cost-effective, and ethically favorable alternative to mammalian models. Brine shrimp bioassays offer high sensitivity to toxic insults at the cellular level, whereas zebrafish embryotoxicity testing facilitates real-time visualization of developmental anomalies owing to their optical transparency and genomic homology with humans [17].

In the brine shrimp lethality assay, the absence of significant naupliar mortality at both 24 and 48 hours post-exposure indicates minimal acute toxicity of the test varnish. These findings aligned with the cytocompatibility profiles reported in similar formulations containing nanoparticle-incorporated herbal extracts. For instance, Aardra (2023) [18] demonstrated that Camellia sinensis-mediated copper oxide nanoparticles did not induce cellular deformities, nor did they compromise larval viability below certain concentrations [18]. The current study mirrored this trend, exhibiting high naupliar survivability and no apparent morphological aberrations.

In the zebrafish embryotoxicity assay, hatching success and larval viability remained within physiological norms across all tested concentrations. This paralleled the observations made by Ramakrishnan et al. (2023) [19],

wherein a herbal dental varnish containing TiO_2 nanoparticles and plant extracts (ginger and clove) induced no teratogenic effects such as edema, axial malformations, or delayed hatching, provided the concentration did not exceed embryonically safe thresholds [19]. The subtle reduction in hatching percentages at elevated concentrations in our study (80 μg/L) did not attain statistical significance, further affirming the innocuity of the experimental varnish. The findings of Chokkattu et al. (2022) [20] also corroborated these outcomes, emphasizing that short-term exposure to titanium dioxide nanoparticles—common in dental formulations—did not adversely impact zebrafish embryogenesis, largely due to the protective role of the chorion and the lack of direct cellular interaction during early developmental phases [20].

Notably, the present study's novelty lies in the inclusion of quercetin, a potent flavonoid renowned for its antioxidative, anti-inflammatory, and cytoprotective properties. This marks a pivotal deviation from prior studies which predominantly explored metallic nanoparticles or polyherbal combinations. The incorporation of quercetin may have contributed to the stabilization of cellular and embryonic structures, potentially mitigating oxidative stress-induced damage—a hypothesis supported by previous in vitro analyses demonstrating quercetin's free radical scavenging activity.

In a previous study, Quercetin has shown promising effects in reducing erosive dentin wear by stabilizing the collagen matrix and inhibiting matrix metalloproteinases activity. Its ability to preserve dentin structure under erosive conditions supports its potential as an adjunctive agent to enhance the remineralizing properties of therapeutic formulations in preventive dental care [21]. A study presents novel evidence indicating that quercetin can penetrate dentin up to a depth of approximately 25–30 μm, where it contributes to erosion and abrasion resistance by inhibiting dentin-derived matrix metalloproteinase activity and promoting collagen crosslinking within the demineralized organic matrix [22,23].

Contrasting our results with studies employing high concentrations of metallic nanoparticles reveals crucial insights. While TiO_2 and CuO NPs have demonstrated biocompatibility at low dosages, excessive exposure has been associated with developmental retardation or reduced hatching viability, as noted in the higher-dose arms [24]. In contrast, the current formulation maintained bio-physiological parameters across all concentration ranges, thereby indicating a superior safety margin likely conferred by the non-metallic antioxidant scaffold of quercetin.

The clinical implications of this study are particularly relevant to pediatric dentistry, where safety and biocompatibility of preventive agents are of paramount importance. The findings substantiate the potential translational application of quercetin-enriched fluoride varnish in young populations, without the risk of systemic toxicity or interference with developmental biology. Furthermore, the non-cytotoxic nature of the formulation ensures its compatibility with oral epithelial tissues and supports long-term usage without adverse effects.

Nonetheless, this study is not devoid of limitations. The sole reliance on invertebrate and zebrafish embryo models may constrain direct extrapolation to human physiology. Additionally, the absence of molecular assays to quantify apoptotic markers or oxidative stress indicators limits mechanistic interpretation [25]. Variability in nanoparticle behavior under different environmental stimuli—such as pH or UV exposure—was not explored and could influence toxicity profiles.

Future research should encompass expanded in vivo models and molecular-level investigations to validate the biosafety of quercetin varnishes in mammalian systems. Longitudinal studies assessing chronic exposure, immunogenic potential, and histopathological effects in rodent oral mucosa could provide a more holistic risk assessment. Further, advanced characterization of quercetin's interaction with fluoride ions and potential synergistic benefits in remineralization should be investigated.

CONCLUSION

Within the limitations of this in vitro study, the fluoride varnish incorporated with quercetin demonstrated excellent biocompatibility, exhibiting no significant cytotoxic or embryotoxic effects across all tested concentrations. The inclusion of quercetin, a bioactive flavonoid with known antioxidant and cytoprotective properties, likely contributed to the favorable biological response.

Author's Contributions

BN: Writing – Original Draft Preparation. VR: Writing – Review & Editing.

Conflict of Interest

No conflicts of interest declared concerning the publication of this article.

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Regulatory Statement

This study was conducted in accordance with ethical guidelines governing the use of animal materials in research. Zebrafish (*Danio rerio*) embryos up to 120 hours post-fertilization were used, which do not require IAEC approval under CPCSEA regulations. The Institutional Animal Ethics Committee was informed of the study and the developmental stage of the embryos used.

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