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RESEARCH ARTICLE

Comparative embryonic toxicology evaluation of *Ocimum tenuiflorum* and *Ocimum gratissimum* herbal formulation based oral rinse and commercial oral rinse

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Abstract

Herbal-based oral care products are gaining attention as safer alternatives to commercial oral rinses. This study evaluates the embryotoxic effects of a herbal-based oral rinse compared to a commercial oral rinse using zebrafish embryos. The objective was to assess differences in hatching and viability rates and the safety profiles of these products. Zebrafish (*Danio rerio*) embryos were exposed to varying concentrations (5, 10, 20, 40 and 80 µg/mL) of both oral rinses and were monitored over 96 hr for hatching rates and viability, evaluated using standard microscopy at 24 hr intervals. The herbal oral rinse showed a gradual decline in hatching and viability rates with increasing concentrations. At 40 µg/mL, the hatching rate was 80 %, which decreased to 60 % at 80 µg/mL. The commercial oral rinse showed a hatching rate dropping to 60 % at 40 µg/mL and 40 % at 80 µg/mL. Viability rates followed a similar trend, with the herbal rinse maintaining 80 % viability at higher concentrations, while the commercial rinse reduced viability to 60 % at the same concentrations. The results indicate that the herbal oral rinse has a lower embryotoxic profile compared to the commercial oral rinse. The higher hatching and viability rates seen with the herbal formulation suggest it may be a safer option for oral applications. The *Ocimum tenuiflorum* and *Ocimum gratissimum* herbal oral rinse exhibited lower toxicity to zebrafish embryos compared to the commercial oral rinse, making it a safer alternative for oral care.

Keywords: antimicrobial activity; genotoxic; hatching rate; stomatitis; viability rate

Introduction

Periodontitis, a prevalent inflammatory condition of the periodontium, remains a significant cause of tooth loss globally. It affects the supporting structures of the teeth, including the gingiva, periodontal ligament and alveolar bone, leading to progressive tissue destruction if left untreated (1). The disease is primarily initiated by the accumulation of bacterial biofilm (plaque) around the teeth and is often exacerbated by a dysregulated host immune response. In addition to mechanical debridement and scaling, adjunctive therapies, including the use of antimicrobial oral rinses (2), are frequently recommended to manage periodontal infections and reduce microbial load in the oral cavity (3).

One of the most widely used adjunctive oral rinses in periodontitis management is chlorhexidine (CHX), a cationic bisbiguanide with broad-spectrum antimicrobial properties. It is effective against both Gram-positive and Gram-negative bacteria and has been regarded as the gold standard in oral rinses for its ability to reduce plaque and gingival inflammation (4). However, while CHX mouthwashes are highly effective in the short-term management of periodontal disease, their long-term use is associated with several adverse effects, which raises concerns about their safety for prolonged use (5, 6).

Among the most prominent issues is the occurrence of dental staining. Prolonged use of chlorhexidine can lead to visible discoloration of the teeth and oral mucosa due to its interaction with dietary chromogens (7). Studies have shown that approximately 56 % of patients using chlorhexidine mouthwash for six months develop significant staining that may require professional cleaning. This cosmetic issue not only impacts patient satisfaction but can also affect compliance, as many individuals are reluctant to continue using products that compromise their dental aesthetics (8).

Another well-documented concern is the increased formation of supragingival calculus. Chlorhexidine alters the oral environment, leading to enhanced mineralization

of plaque into tartar (9). This can complicate periodontal health, requiring more frequent dental visits for professional cleanings. Although chlorhexidine's antibacterial properties help control plaque, the accompanying tartar build up may counteract its long-term benefits, leading to the need for closer monitoring and frequent periodontal maintenance (10).

Taste disturbances are another common side effect of chlorhexidine rinses. Users often report a persistent bitter or metallic taste, which can interfere with their enjoyment of food and beverages (11). This taste alteration can persist even after discontinuing use, further discouraging patients from adhering to recommended oral hygiene regimens. In some cases, oral irritation, such as glossitis, stomatitis and ulcerations, has been reported, which can exacerbate discomfort and contribute to poor patient compliance (12).

More serious, though less common, side effects include hypersensitivity and allergic reactions. There have been reports of individuals experiencing severe allergic responses to chlorhexidine, including anaphylaxis. While these reactions are rare, they underscore the need for caution, particularly in individuals with known allergies or sensitivities (13). Moreover, chlorhexidine's broad-spectrum antimicrobial action can disrupt the natural balance of the oral microbiota, leading to dysbiosis. The disruption of beneficial bacteria in the oral cavity may exacerbate oral health issues over time, potentially contributing to the recurrence of periodontal disease (14, 15).

In response to the limitations of commercial chlorhexidine-based rinses, there has been a growing interest in the development of herbal formulation-based alternatives. Herbal oral rinses, which are derived from plant-based ingredients with known therapeutic properties, offer a promising solution by providing antimicrobial, anti-inflammatory and antioxidant benefits without the harsh side effects associated with synthetic chemicals. These formulations are gaining traction due to their perceived safety, efficacy and alignment with natural and holistic health practices (16, 17).

Among the most notable herbal ingredients in oral care are *Ocimum tenuiflorum* (holy basil or tulsi) and *Ocimum gratissimum* (African basil). Both species are rich in bioactive compounds with potent antimicrobial, anti-inflammatory and antioxidant properties (18). *Ocimum tenuiflorum*, traditionally used in Ayurvedic medicine, contains eugenol, ursolic acid and rosmarinic acid, which have demonstrated strong antibacterial activity against key oral pathogens such as *Streptococcus mutans* and *Porphyromonas gingivalis*. These pathogens play a critical role in the etiology of periodontitis and the ability of *O. tenuiflorum* to inhibit their growth positions it as a valuable natural remedy for oral health (19, 20).

Similarly, *Ocimum gratissimum* is known for its high content of thymol and eugenol, which have antimicrobial and antifungal effects. These compounds have been shown to reduce the microbial load in the oral cavity, making *O. gratissimum* a beneficial ingredient in oral care formulations. Together, these two herbs offer a broad spectrum of activity against oral pathogens while providing additional benefits such as reducing inflammation, promoting healing and supporting the natural balance of the oral microbiome (21).

Importantly, herbal formulations are associated with fewer side effects compared to synthetic chemical-based rinses. They are less likely to cause dental staining, taste disturbances, or mucosal irritation, making them more suitable for long-term use. Furthermore, the bioactive compounds in herbal ingredients may selectively target pathogenic bacteria while preserving the beneficial microbiota in the oral cavity, thus maintaining microbial balance and promoting overall oral health (22).

Despite the growing popularity of herbal oral care products, there is limited research on their potential toxicological effects, particularly during sensitive developmental stages. Embryonic toxicology studies, which assess the impact of substances on the early stages of development, provide into the safety of these products (23). Zebrafish (*Danio rerio*) embryos are widely used in toxicology research due to their genetic similarity to humans and the transparency of their embryos, which allows for the observation of developmental processes in real time (24).

This study aims to conduct a comparative embryonic toxicology evaluation of a commercial chlorhexidine-based oral rinse and an herbal formulation-based oral rinse containing *Ocimum tenuiflorum* and *Ocimum gratissimum*. By assessing key toxicological parameters, such as embryonic mortality, hatching rates and developmental abnormalities, this research seeks to provide a comprehensive evaluation of the safety profile of these oral rinses. The findings of this study will offer important insights into the potential risks and benefits of herbal oral care products and contribute to the ongoing development of safer alternatives for managing periodontal disease. Studying embryonic development in this context can provide insights into the potential cytotoxic effects of the oral rinse formulations on living tissues. It is particularly relevant when assessing safety and biocompatibility, as the embryo represents a sensitive model for toxicity studies. For oral rinse, this ensures the formulation does not cause harm to cells or tissues in the oral cavity.

Materials and Methods

Preparation of *Ocimum tenuiflorum* and *Ocimum gratissimum* based herbal formulation

To prepare the herbal formulation, 1 g each of *Ocimum gratissimum* (African basil) and *Ocimum tenuiflorum* (Black tulsi) were combined with 100 mL of distilled water. The solution was heated at 60 °C for 15-20 min using a heating mantle (25). After heating, the mixture was passed through filter paper to separate the extract. The resulting filtrate was reduced to a final volume of 5 mL through controlled heating and then stored for further applications.

Preparation of herbal formulation based on oral rinse

The mouthwash was prepared using 0.3 g of sucrose as a sweetener and 0.001 g of sodium benzoate as a preservative

and 0.01 g of sodium lauryl sulfate as a foaming agent. These were dissolved in 9 mL of distilled water. Subsequently, 1 mL of *Ocimum tenuiflorum* and *Ocimum gratissimum* herbal formulation were added to the mouth rinse preparation and the mixture was placed in an orbital shaker. Finally, a total volume of 10 mL of *Ocimum tenuiflorum* and *Ocimum gratissimum* herbal formulation based oral rinse was used for further studies. The inclusion of sucrose in the formulation was intended primarily to improve the palatability of the herbal rinse, as *Ocimum tenuiflorum* and *Ocimum gratissimum* extracts, in their raw form, can have a strong, sometimes bitter taste. However, Sucrose can help in the nourishment of the embryo by contributing to unintended effects in the embryonic toxicology evaluation, which is one of the limitations of the study.

Formulation dilution: The extract was then diluted in a suitable solvent, distilled water to match the desired concentration of active ingredients per milliliter of the liquid oral rinse.

While spectrophotometry was initially employed to preliminarily identify the nanoparticles in the oral rinse, GC-MS is the more suitable technique for the detailed analysis of active compounds The active compounds in the oral rinse will be analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) in further studies.

Comparative embryonic toxicology of commercial oral rinse and herbal formulation based on oral rinse

The commercial oral rinse used in our study is Listerine and its composition is Eucalyptol (0.092 %), Menthol (0.042 %), Methyl Salicyclate (0.060 %), Thymol (0.064 %). Wild-type zebrafish were sourced from local vendors in India and housed in separate tanks under controlled environmental conditions, including regulated temperature, light/dark cycles and pH levels. The zebrafish were fed twice daily with either dry blood worms or appropriate food. For embryo collection, one female and three males were placed in a breeding tank and the viable eggs were rinsed with E3 medium. The embryos were then transferred to culture plates of varying well sizes and exposed to different concentrations of the herbal formulation-based oral rinse, as well as a commercial oral rinse for comparison. The experiment was replicated three times, including control groups. Dead embryos were removed from the treated groups at 12-hr intervals and the plates were covered with foil to minimize light exposure while maintaining a temperature of 28 °C.

Zebrafish embryo evaluation

During the post-fertilization observation period, the development of zebrafish embryos was carefully monitored using a stereo microscope. The embryos were exposed to varying concentrations (5, 10, 20, 40, 80 µg/L) of the herbal formulation-based oral rinse and a commercial oral rinse for 24-78 hr. Embryo mortality and hatching rates were assessed every 24 hr. The study aimed to evaluate embryo and hatchling mortality, hatching rates and any developmental abnormalities in both the control and treatment groups. Deformed embryos were imaged using a COSLAB Model: HL-10A light microscope and the percentage of abnormal embryos was recorded daily throughout the experiment.

Statistical analysis

The data collected from the hatching and viability rate experiments were expressed as mean ± standard deviation (SD). Statistical significance between different concentrations and groups was determined using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (version 8.0.1).

Results

Embryonic toxicology Evaluation

In this study, the embryonic toxicology of *Ocimum tenuiflorum* and *Ocimum gratissimum* based herbal formulation oral rinse was compared with a commercial oral rinse using zebrafish embryos. Both hatching rate and viability rate were evaluated at different concentrations (5, 10, 20, 40 and 80 µg/mL) to understand their potential toxicity (Fig. 1).

Hatching rate

The hatching rate was used as a key indicator of embryonic development under the influence of both oral rinses. At lower concentrations (5 µg/mL and 10 µg/mL), the herbal formulation-based oral rinse exhibited a hatching rate of 100 %, which was identical to the control group. This demonstrates that the herbal formulation did not harm embryo hatching at these concentrations (Fig. 2). However,

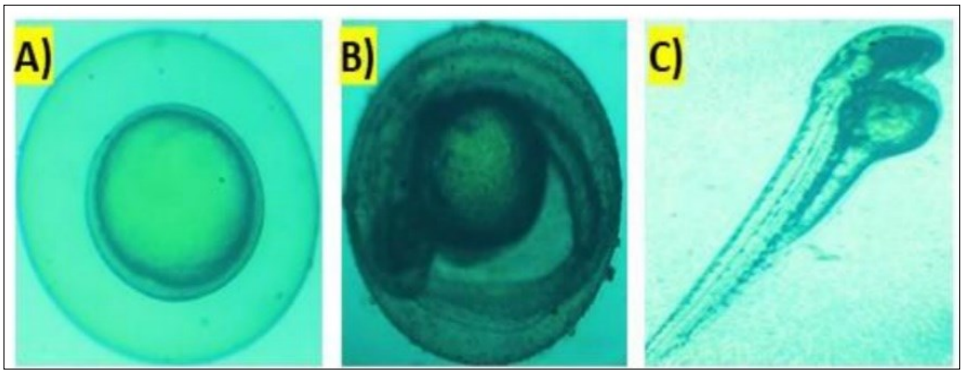

Fig. 1. Zebrafish embryos treated with *Ocimum tenuiflorum* and *Ocimum gratissimum* based herbal oral rinse developed without any malformation or abnormality in day 1, 2, 3.

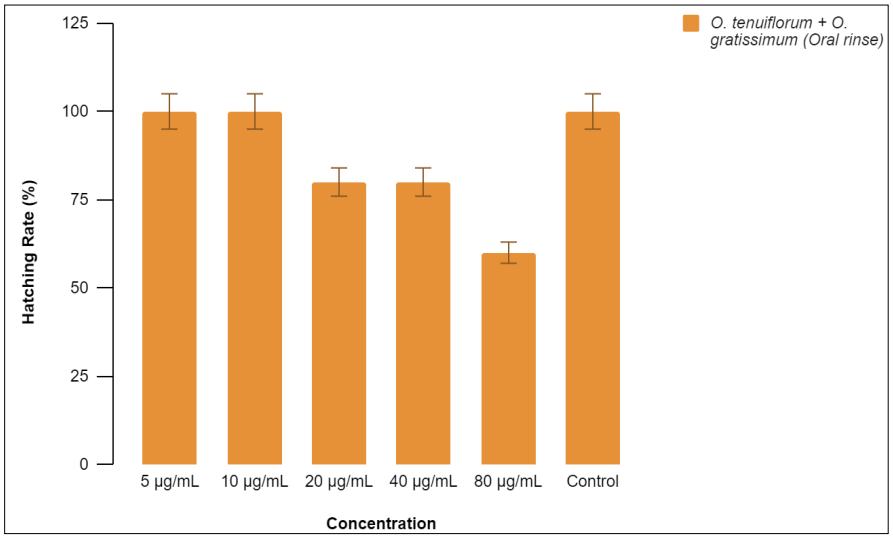

Fig. 2. Hatching rate of zebrafish embryos exposed to *Ocimum tenuiflorum* and *Ocimum gratissimum* oral rinse.

as the concentration increased, a dose-dependent reduction in the hatching rate was observed, with an 80 % hatching rate at 20 and 40 µg/mL and a further decrease to 60 % at 80 µg/mL.

In contrast, the commercial oral rinse exhibited a more pronounced decrease in hatching rate, even at lower concentrations. While the hatching rate at 5 µg/mL remained consistent with the control group at 100 %, it dropped to 80 % at 10 and 20 µg/mL. At 40 µg/mL and 80 µg/mL, the hatching rate declined to 60 % and 40 %, respectively. This indicates that the commercial oral rinse had a greater impact on the embryo development process, reducing the hatching rate more drastically compared to the herbal formulation at similar concentrations.

Viability rate

The viability rate of zebrafish embryos exposed to the herbal formulation-based oral rinse and the commercial oral rinse followed a similar trend to the hatching rate. The herbal formulation did not affect the viability at concentrations of 5 µg/mL, 10 µg/mL, or 20 µg/mL, where the viability remained 100 %. However, a reduction to 80 % viability was noted at 40 µg/mL and 80 µg/mL, the viability remained steady 80 %, indicating a relatively moderate decrease in embryo viability.

In comparison, the commercial oral rinse exhibited a more significant reduction in embryo viability at higher concentrations. The viability remained at 100 % for 5 µg/mL and 10 µg/mL, but it dropped to 80 % at 20 µg/mL. At 40 µg/mL and 80 µg/mL, the viability declined to 60 %, indicating that the commercial oral rinse had a stronger toxic effect on embryo survival as the concentration increased (Fig. 3).

Overall, the herbal formulation-based oral rinse was found to be less toxic to zebrafish embryos in comparison to the commercial oral rinse. The hatching and viability rates were more favorable in the embryos exposed to the herbal formulation, particularly at lower concentrations, where the rates remained comparable to the control. On the other hand, the commercial oral rinse showed a more pronounced negative impact on both hatching and viability rates at relatively lower concentrations (Fig. 4). These findings suggest that the herbal formulation may provide a safer alternative to commercial oral rinses in terms of embryonic toxicity.

Discussion

This study provides a comprehensive comparative evaluation of the embryonic toxicology of *Ocimum tenuiflorum* and *Ocimum gratissimum* based herbal formulation oral rinse versus a commercial oral rinse. Zebrafish embryos were used

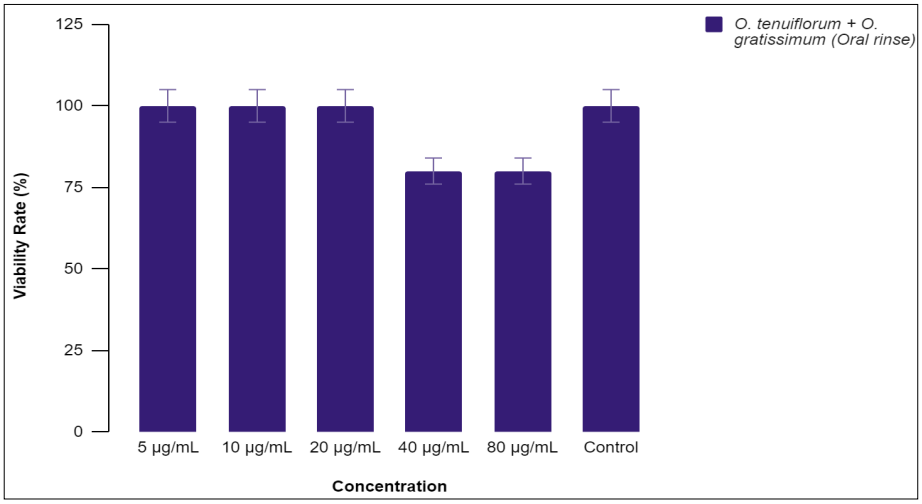

Fig. 3. Viability rate of zebrafish embryos exposed to *Ocimum tenuiflorum* and *Ocimum gratissimum* oral rinse.

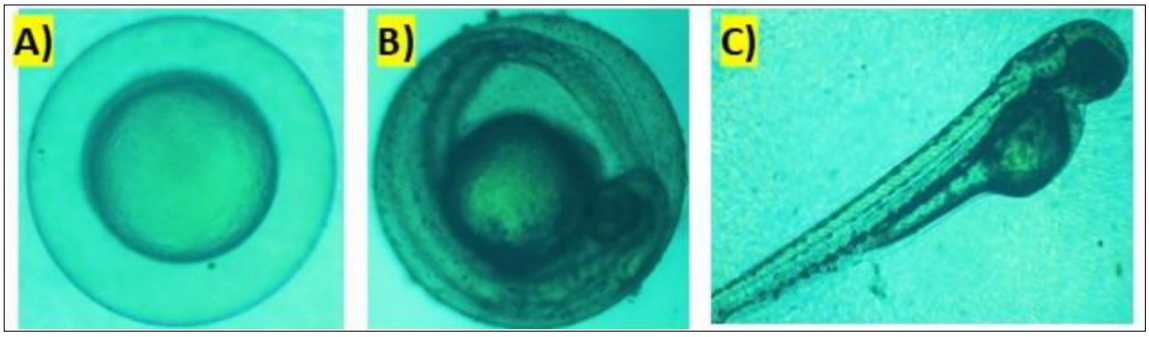

Fig. 4. Zebrafish embryos exposed to commercial oral rinse developed without any malformation or abnormality in day 1, 2, 3.

as a model system to assess the effects on hatching and viability rates at various concentrations. The results demonstrate the reduced embryotoxicity of the herbal formulation compared to the commercial oral rinse, particularly at higher concentrations.

Hatching rates at higher concentrations

At higher concentrations, significant differences were observed in the hatching rates of zebrafish embryos exposed to the two oral rinses. The herbal formulation exhibited a gradual decline in hatching rates as the concentration increased. At 40 µg/mL, the hatching rate was 80 %, which further decreased to 60 % at 80 µg/mL. In contrast, the commercial oral rinse showed a sharper decrease in hatching rates, with the hatching rate dropping to 60 % at 40 µg/mL and further falling to 40 % at 80 µg/mL (Fig. 5). These findings indicate that, while both oral rinses negatively impact hatching rates at higher concentrations, the herbal formulation is less detrimental than the commercial oral rinse. This suggests that the herbal rinse may be a safer alternative, especially at higher concentrations, where the commercial oral rinse exhibits significantly greater toxicity. These observations are consistent with previous studies that have shown the lower toxicity of natural products compared to synthetic formulations (26).

Viability rates and toxicological mechanisms

The viability rate of zebrafish embryos exposed to both oral rinses followed a similar trend to the hatching rates. The herbal formulation-based oral rinse maintained higher viability rates at all concentrations compared to the commercial oral rinse. The herbal formulation exhibited no significant effect on viability at concentrations up to 20 µg/mL, maintaining a 100 % viability rate. At higher concentrations of 40 µg/mL and 80 µg/mL, the viability rate dropped to 80 %. In comparison, the commercial oral rinse showed a significant reduction in viability, with rates dropping to 80 % at 20 µg/mL and 60 % at both 40 µg/mL and 80 µg/mL (Fig. 6).

Several potential mechanisms may explain the reduced viability observed in embryos exposed to the commercial oral rinse. First, oxidative stress may play a key role, as commercial oral rinses can induce the production of reactive oxygen species (ROS), which damage cellular components and impair embryonic development (27). Additionally, neurotoxic effects from ingredients like sodium benzoate could disrupt neural signalling pathways, leading to developmental defects and decreased viability rates (28). Furthermore, cardiotoxicity, such as pericardial edema, could compromise the circulatory system of developing embryos, reducing nutrient and oxygen delivery. Genotoxic effects from compounds like triclosan and fluoride, commonly found in commercial oral rinses, may also contribute to developmental abnormalities and reduced viability by damaging DNA. Lastly, metabolic disruption caused by synthetic chemicals may impair growth and development, leading to delayed hatching and reduced body size (29).

Protective compounds in herbal formulation

The reduced toxicity observed in the herbal formulation can be attributed to specific compounds found in *Ocimum tenuiflorum* and *Ocimum gratissimum*, which exhibit beneficial pharmacological properties. Eugenol, a major active constituent in both plants, is known for its anti-inflammatory and antioxidant effects, which may help mitigate oxidative stress in embryos and reduce toxicity (30). Rosmarinic acid, a phenolic compound, has strong antioxidant properties, which may scavenge free radicals and protect embryonic cells from oxidative damage. Flavonoids

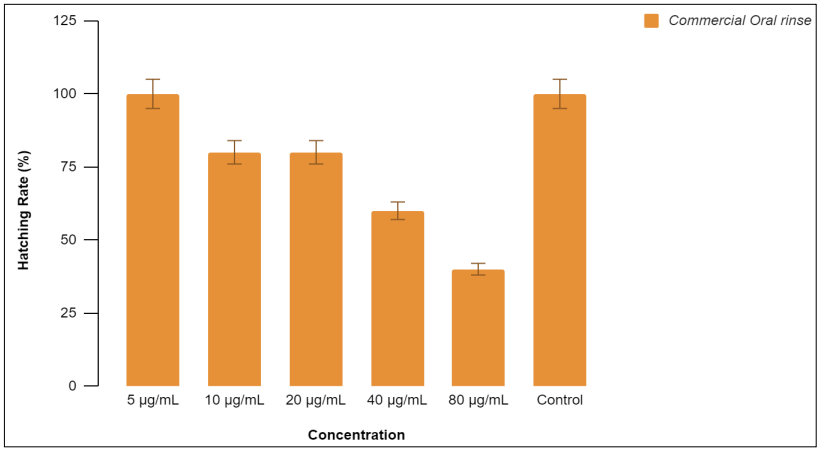

Fig. 5. Hatching rate of zebrafish embryos exposed to commercial oral rinse.

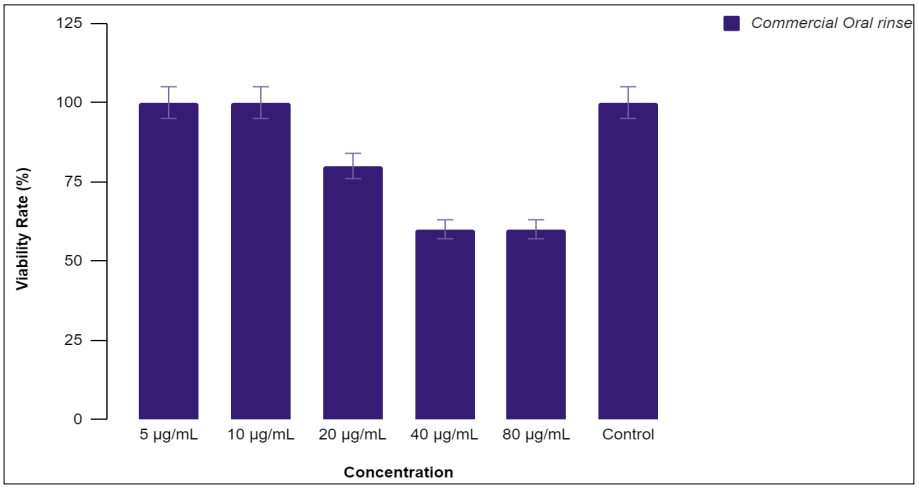

Fig. 6. Viability rate of zebrafish embryos exposed to commercial oral rinse.

like apigenin and orientin, also present in these herbs, enhance cellular defense mechanisms, further contributing to the higher viability rates observed in embryos exposed to the herbal formulation. Additionally, volatile oils and caryophyllene, known for their anti-inflammatory properties, may reduce cellular damage, thereby promoting normal developmental processes in embryos.

Implications for oral health products

The findings from this study have several important implications for the development and safety of oral health products. Herbal formulations like the one based on *Ocimum tenuiflorum* and *Ocimum gratissimum* demonstrate lower toxicity compared to commercial oral rinses, particularly at higher concentrations. This suggests that herbal products may offer a safer alternative for consumers concerned about the potential harmful effects of synthetic ingredients. Furthermore, the antimicrobial properties of compounds like eugenol and flavonoids in the herbal formulation provide additional benefits, such as preventing dental caries and promoting oral hygiene, without the adverse side effects associated with synthetic antimicrobials. This study provides a comprehensive comparative evaluation of the embryonic toxicology of *Ocimum tenuiflorum* and *Ocimum gratissimum*-based herbal formulation oral rinse versus a commercial oral rinse. Zebrafish embryos were used as a model system to assess the effects on both hatching and viability rates at various concentrations. The results demonstrate the reduced embryotoxicity of the herbal formulation compared to the commercial oral rinse, particularly at higher concentrations. High-Performance Liquid Chromatography (HPLC) studies can also be done for phytochemical studies to know the exact chemical constituents of the herbal extract and to reduce the adverse effects of higher concentrations of the herbal extract on embryo hatching. HPLC studies could potentially identify compounds that might be present in high concentrations and assess if those compounds could be causing adverse effects. This would offer a deeper understanding of the formulation's impact on the embryonic development of zebrafish and help identify safer concentration levels to minimize any toxicity risks for further investigations (31).

The growing consumer preference for natural and herbal products in oral care highlights the potential for herbal formulations to meet market demands while maintaining safety and efficacy (32). The relatively low toxicity of herbal formulations, combined with their beneficial properties, makes them ideal candidates for developing innovative oral care products, such as poly-herbal toothpastes or mouthwashes that are both effective and safe for long-term use (33).

Oral rinses are typically used in a diluted form by consumers, as most commercial formulations recommend dilution with water before use. Many mouthwashes are formulated with a concentration that, when diluted with water, results in a safe and effective concentration for oral use. In the context of our study, we chose to test the herbal mouthwash formulations at dilutions that more closely resemble real-world use, where the mouthwash would likely be used in diluted form, as per typical consumer instructions.

Conclusion

In conclusion, the *Ocimum tenuiflorum* and *Ocimum gratissimum* herbal formulation-based oral rinse exhibited lower toxicity in zebrafish embryos compared to the commercial oral rinse, as indicated by higher hatching and viability rates. The findings suggest that herbal formulations could serve as safer alternatives to commercial oral rinses, particularly for consumers seeking natural oral hygiene solutions with fewer adverse effects. The presence of protective compounds like eugenol and rosmarinic acid further enhances the potential of herbal formulations to promote oral health while minimizing toxicity. Further research is recommended to explore the long-term effects of herbal formulations in clinical settings and their potential applications in oral care products.

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Authors' contributions

AS contributed to the conceptualization, methodology, writing of the original draft, editing and project administration; RMV contributed to the data curation, formal analysis, visualization and writing - review and editing; AKS contributed to investigation, software, validation and review; and RS contributed to the resources, supervision, project administration and editing. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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