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Preparation of Oral Rinse using Ethanolic Extract of Ocimum tenuiflorum and Evaluation of its Antimicrobial Activity and Cytotoxicity

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Background: Antimicrobial resistance (AMR) in oral care is a growing concern, exacerbated by the frequent use of synthetic antiseptics. Ocimum tenuiflorum, commonly known as Tulsi, is known for its antimicrobial properties, offering a potential natural alternative. Aim: This study aimed to formulate an oral rinse using the ethanolic extract of Ocimum tenuiflorum and evaluate its antimicrobial activity and cytotoxicity. Materials and Methods: The ethanolic extract of Ocimum tenuiflorum was prepared by mixing 2 g of dried plant powder with 20 ml of ethanol, followed by shaking for 24 hours. The extract was then used to formulate an oral rinse containing 1000 µg/mL of the extract along with standard rinse components. Antimicrobial activity was assessed using agar well diffusion and time-kill curve assays against four microorganisms: Enterococcus faecalis, Lactobacillus sp., Streptococcus mutans, and Candida albicans. Cytotoxicity was evaluated using the Brine shrimp lethality assay across various concentrations (5-80 µg/mL). Results: The oral rinse showed effective antimicrobial activity, with inhibition zones ranging from 8 to 12 mm at concentrations between 25 and 100 µg/mL, comparable to a commercial mouth rinse. The time-kill curve assay indicated a dose-dependent reduction in microbial load, demonstrating significant bactericidal and fungicidal activity. Cytotoxicity testing revealed no adverse effects on nauplii survival across all tested concentrations. Discussion: The results suggest that the Ocimum tenuiflorum-based oral rinse effectively reduces microbial counts and has no cytotoxic effects, highlighting its potential as a safer, natural alternative to conventional synthetic oral care products.

Its efficacy against both bacterial and fungal pathogens support its application in managing oral hygiene and infections. Conclusion: The ethanolic extract-based oral rinse demonstrated substantial antimicrobial activity and safety, indicating its promise as a natural oral care product.

Keywords: Antimicrobial Resistance, Ocimum tenuiflorum (Tulsi), Oral Rinse Formulation.

1. Introduction

Antimicrobial resistance (AMR) in oral care products is an increasing concern, largely due to the frequent use of antiseptics such as chlorhexidine (CHX) and cetylpyridinium chloride (CPC) [1]. Studies have shown that bacteria in dental plaque can develop decreased sensitivity to CHX, with some strains becoming multidrug-resistant, which complicates the effectiveness of these oral care agents in managing plaque [2]. Moreover, the regular application of antiseptics can alter the microbial composition within oral biofilms, potentially favoring resistant phenotypes that may also exhibit resistance to various antibiotics [3]. Additionally, personal care products, including toothpaste, contribute to the problem of AMR by releasing antimicrobial agents into the environment, impacting microbial communities and encouraging resistance. Therefore, the relationship between oral care products and AMR requires further exploration to reduce the associated risks [4].

Herbal oral rinses have shown efficacy in reducing plaque and gingivitis comparable to traditional mouthwashes, including those containing chlorhexidine [5]. Studies suggest that herbal mouthwashes formulated from plant extracts such as tulsi, clove, and pomegranate possess notable antibacterial properties and effectively manage oral health concerns like plaque buildup and gingivitis, with fewer side effects compared to synthetic mouthwashes [6]. In one comparative study, a commercially available herbal mouthwash demonstrated similar effectiveness to chlorhexidine in reducing salivary Streptococcus mutans and enhancing gingival health over a 14-day period [7,8]. Additionally, another study found that a herbal mouthwash significantly decreased gingival bleeding and plaque in patients with chronic periodontitis, further endorsing its potential as an alternative to chemical mouthwashes [9].

Ocimum tenuiflorum, commonly known as Tulsi or Shyama Tulsi, is a widely esteemed medicinal herb recognized for its potent antioxidant, antimicrobial, and allelopathic properties [10]. The antimicrobial activity of Ocimum tenuiflorum extracts is largely due to their rich content of secondary metabolites such as eugenol, caryophyllene, and other aromatic compounds. These bioactive substances exhibit strong antibacterial and antifungal properties, making the extracts effective against a range of pathogens. Research indicates that Ocimum tenuiflorum extracts can inhibit microbial growth in applications such as food packaging, with eucalyptol and caryophyllene being identified as key antimicrobial agents [11,12]. Furthermore, synthesizing silver nanoparticles using Ocimum tenuiflorum enhances its antimicrobial efficacy, showing significant activity against oral pathogens like Streptococcus mutans and Staphylococcus aureus. The extracts also demonstrate a concentration-dependent antimicrobial effect, where higher concentrations result in larger inhibition zones against the microorganisms tested [13]. Collectively, the diverse bioactive compounds present in Ocimum tenuiflorum highlight its potential as a natural antimicrobial agent for both food preservation and medical applications [14].

In this study, an ethanolic extract of Ocimum tenuiflorum was prepared and utilized to formulate an oral rinse. The antimicrobial activity of the oral rinse was evaluated using agar well diffusion and time-kill curve assays, while its cytotoxicity was assessed through the Brine shrimp lethality assay.

2. Materials & Methods

Preparation of ethanolic extract

To prepare the ethanolic extract of Ocimum tenuiflorum, 2 grams of dried plant powder were accurately weighed and added to 20 millilitres of ethanol. The mixture was then transferred to an orbital shaker, where it was continuously agitated for 24 hours to ensure thorough extraction of the bioactive compounds. Following this extraction period, the solution containing the ethanolic extract of Ocimum tenuiflorum was filtered to remove any residual plant material, resulting in a clear extract ready for use in subsequent experiments. The preparation of the ethanolic extract is illustrated in Figure 1.

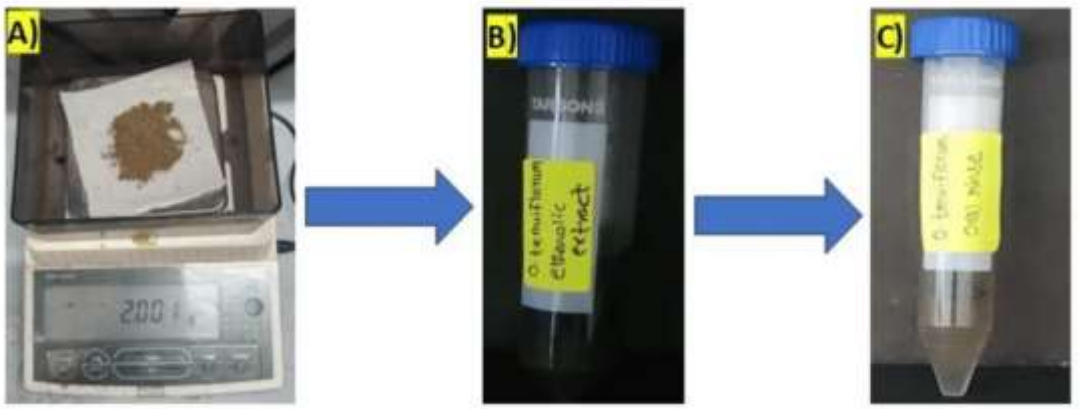

Figure 1: Preparation of Ocimum tenuiflorum ethanolic extract based oral rinse A) Ocimum tenuiflorum powder B) Ethanolic extract C) Ocimum tenuiflorum ethanolic extract based oral rinse

Preparation of oral rinse

To formulate the herbal oral rinse, 1mL of the Ocimum tenuiflorum ethanolic extract was combined with essential oral rinse components. These components included sodium benzoate (used as a preservative), sucrose (as a sweetening agent), sodium lauryl sulphate (a surfactant to enhance cleaning), and distilled water as the solvent base. The ingredients were thoroughly mixed to ensure uniform distribution of the extract and the other components, resulting in a homogenous ethanolic extract-based herbal oral rinse. This formulation was stored in a refrigerator for subsequent antimicrobial and cytotoxicity testing.

Cytotoxic effect using brine shrimp lethality assay:

The cytotoxicity of the herbal oral rinse, formulated with Ocimum tenuiflorum ethanolic extract, was tested through the Brine Shrimp Lethality Assay. Artemia salina nauplii, which

were hatched from cysts in synthetic seawater, were used as the test organisms. Varying concentrations of the oral rinse (5, 10, 20, 40, and 80 µg/mL) were created by diluting the stock solution in seawater.

A control group containing only seawater, without any herbal oral rinse, served as the reference for baseline comparison. For each concentration, 10 nauplii were placed in separate vials containing 5 mL of the respective test solution. These vials were maintained under constant light exposure and observed at intervals of 24 and 48 hours. The survival of the nauplii was recorded at each time point, and the mortality rate was determined by comparing the number of dead nauplii in each test vial to the control group.

Antimicrobial activity- agar well diffusion technique

The antimicrobial efficacy of the Ocimum tenuiflorum ethanolic extract-based oral rinse was assessed using the agar well diffusion method. The herbal formulation was tested at three concentrations: 25 µg/mL, 50 µg/mL, and 100 µg/mL, with a commercially available mouth rinse serving as a standard for comparison. The microbial strains evaluated included Enterococcus faecalis, Lactobacillus species, Streptococcus mutans, and Candida albicans.

For the assay, standardized bacterial inoculate were prepared at approximately 106 CFU/mL. Mueller-Hinton agar was used for bacterial strains, and Rose Bengal agar was utilized for C. albicans. The inocula were uniformly spread across the agar plates using sterile cotton swabs. Wells, 9 mm in diameter, were created in the agar with a sterile cork-borer, and the different concentrations of the herbal rinse were added to the respective wells. The commercial mouth rinse was similarly applied as a control. The plates were incubated at 37°C for 24 hours.

Post-incubation, the antimicrobial activity was determined by measuring the zones of inhibition around the wells, with the diameter recorded in millimetres using a digital calliper. Each experiment was performed in triplicate to ensure reliability and reproducibility. Statistical analysis was conducted, and the results were presented as mean ± standard deviation, with statistical significance set at a p-value < 0.05.

Time kill curve assay

The time-kill curve assay was performed to assess the antimicrobial activity of the Ocimum tenuiflorum ethanolic extract-based oral rinse against prominent oral pathogens, including Enterococcus faecalis, Lactobacillus species, Streptococcus mutans, and Candida albicans. The bacterial inocula were standardized to approximately 106 CFU/mL and exposed to the herbal oral rinse at concentrations of 25 µg/mL, 50 µg/mL, and 100 µg/mL. A commercial oral rinse was used as a reference, while an untreated inoculum served as the control. Samples were taken at intervals of 0, 1, 2, 3, 4, and 5 hours, followed by serial dilution, and the optical density was measured at 600 nm using an ELISA plate reader to monitor the reduction in microbial load over time.

3. Results

Cytotoxic effect

The cytotoxicity assessment of the herbal oral rinse was conducted across various

concentrations (5, 10, 20, 40, and 80 µg/mL) by measuring the percentage of live nauplii after 24 hours (Day 1) and 48 hours (Day 2) and the results are presented in Figure 2.

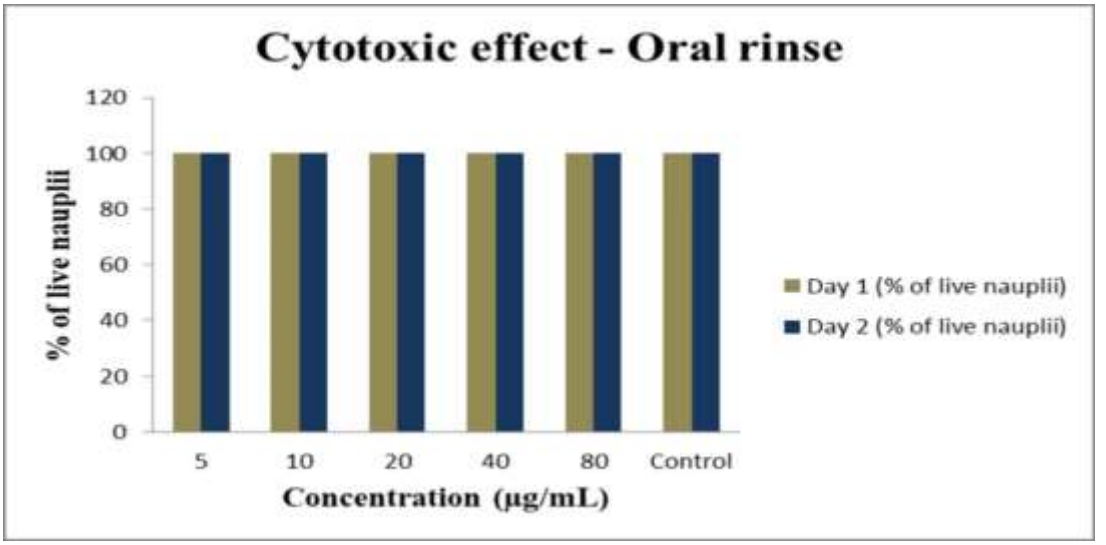

Figure 2: Brine shrimp lethality assay of Ocimum tenuiflorum ethanolic extract mediated oral rinse

The cytotoxicity of the herbal oral rinse was evaluated at different concentrations (5, 10, 20, 40, and 80 µg/mL) by determining the percentage of surviving nauplii at 24 hours (Day 1) and 48 hours (Day 2), as shown in Figure 3. At the lowest concentration of 5 µg/mL, the survival rate of the nauplii was 100% on both Day 1 and Day 2, indicating no observable cytotoxicity at this dose.For the 10 µg/mL concentration, the survival rate of nauplii was also 100% on both Day 1 and Day 2, suggesting the oral rinse did not affect nauplii viability. At 20 µg/mL, the percentage of live nauplii was maintained at 100% across both days, further supporting the non-cytotoxic nature of the oral rinse at this concentration. The 40 µg/mL concentration similarly showed no reduction in the percentage of live nauplii, with 100% survival recorded on both Day 1 and Day 2.

At the highest tested concentration of 80 µg/mL, the nauplii survival rate remained at 100% on both Day 1 and Day 2, indicating that even at elevated concentrations, the oral rinse did not exhibit any cytotoxicity. The control group, which did not contain the oral rinse, also showed 100% live nauplii on both days, serving as a baseline to confirm the assay reliability. These results indicate that the herbal oral rinse, across all tested concentrations, does not exert a cytotoxic effect on nauplii, demonstrating its safety and biocompatibility for potential therapeutic use.

Antimicrobial activity

The antimicrobial activity of the herbal oral rinse was evaluated against four microorganisms: Enterococcus faecalis, Lactobacillus sp., Streptococcus mutans, and Candida albicans using Agar well diffusion technique, shown in Figure 3.

Figure 3: Agar well diffusion technique for Ocimum tenuiflorum ethanolic extract mediated oral rinse against A)Lactobacillus B)C. albicans C) E. faecalis D) S. mutans

The results, presented as zones of inhibition (in mm), demonstrate the efficacy of the oral rinse at concentrations of 25, 50, and 100 µg/mL, compared to a commercial mouth rinse used as a control, as shown in Figure 4.

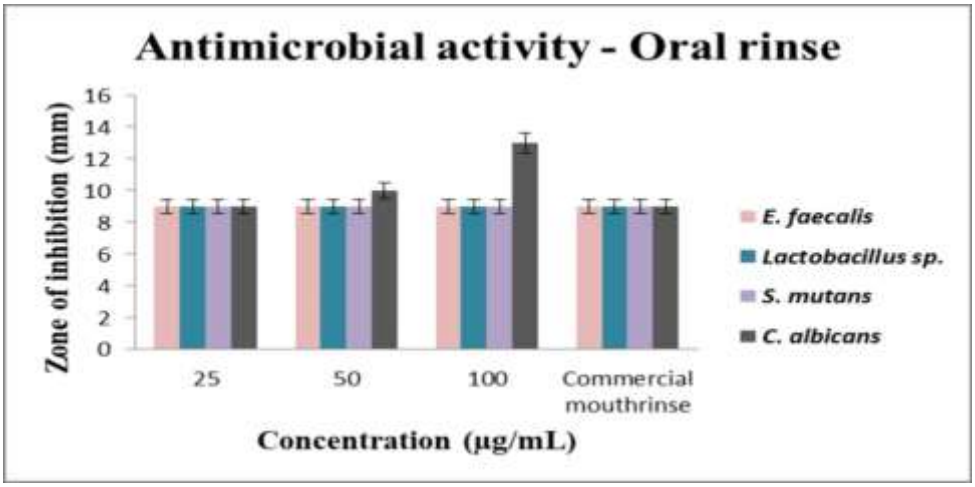

Figure 4: Antimicrobial activity of Ocimum tenuiflorum ethanolic extract mediated oral rinse against different oral pathogens.

The antimicrobial activity of the herbal oral rinse was evaluated against four microorganisms: Enterococcus faecalis, Lactobacillus sp., Streptococcus mutans, and Candida albicans. The results, presented as zones of inhibition (in mm), demonstrate the efficacy of the oral rinse at concentrations of 25, 50, and 100 µg/mL, compared to a commercial mouth rinse used as a control. At 25 µg/mL, the oral rinse exhibited moderate antimicrobial activity, with inhibition zones ranging from approximately 8 to 9 mm for all tested microorganisms. C. albicans showed slightly higher sensitivity compared to the bacterial strains, indicating a broader spectrum of activity at this concentration. Increasing the concentration to 50 µg/mL enhanced the antimicrobial activity, with zones of inhibition ranging from 9 to 10 mm across all microorganisms. Notably, C. albicans continued to exhibit the highest sensitivity, followed closely by S. mutans and Lactobacillus sp., while E. faecalis displayed slightly lower inhibition.

At the highest concentration of 100 µg/mL, the oral rinse demonstrated significantly increased

antimicrobial activity, with inhibition zones expanding to 10-12 mm. C. albicans showed the most substantial inhibition, with a zone size comparable to that of the commercial mouth rinse. Lactobacillus sp. and S. mutans also showed strong inhibition, while E. faecalis again had slightly smaller zones of inhibition.The commercial mouth rinse, used as a standard for comparison, exhibited consistent antimicrobial activity across all microorganisms, with inhibition zones approximately 10-12 mm. The results indicate that the herbal oral rinse, particularly at higher concentrations, has a comparable antimicrobial effect to the commercial product, suggesting its potential as an effective alternative for oral hygiene purposes.

Time kill curve assay

The time-kill curve assay was conducted to evaluate the bactericidal and fungicidal activity of the oral rinse formulated with the ethanolic extract of Ocimum tenuiflorum against four oral pathogens: Enterococcus faecalis, Lactobacillus sp., Streptococcus mutans, and Candida albicans. The assay measured the microbial count (log CFU/mL) over a 4-hour period at different concentrations of the oral rinse (25, 50, and 100 µg/mL) and compared these with a standard commercial mouth rinse and a control. The graph depicting the time kill curve of the oral rinse is depicted in Figure 5.

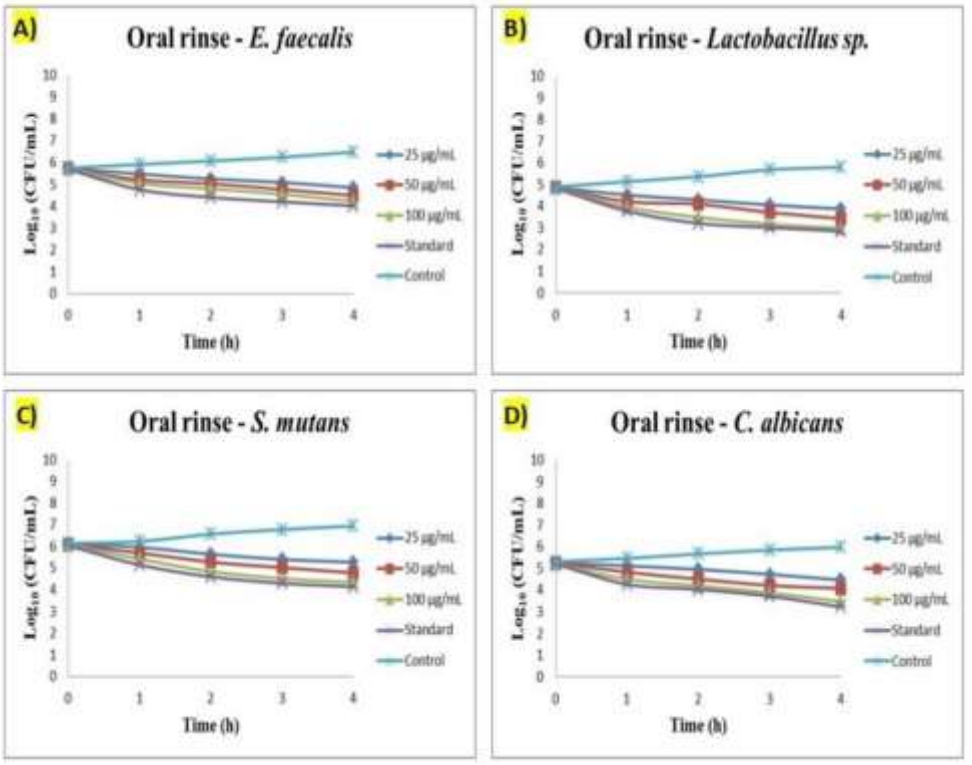

Figure 5: Time kill curve assay of Ocimum tenuiflorum ethanolic extract mediated oral rinse against different oral pathogens A) E. foecalis B) Lactobacillus sps C) S. mutans D) C. albicans

Enterococcus faecalis

The results for E. faecalis showed a time-dependent reduction in bacterial count at all concentrations of the oral rinse. At 25 μg/mL, there was a slight decrease in CFU/mL over time, with a reduction from 5.8 log CFU/mL at 0 hour to approximately 5.2 log CFU/mL at 4 hours. At 50 and 100 μg/mL, a more pronounced reduction was observed, with the bacterial count dropping to 4.8 and 4.5 log CFU/mL, respectively, at 4 hours. The standard mouth rinse exhibited a similar reduction pattern, while the control showed no significant reduction in bacterial count.

Lactobacillus sp.

For Lactobacillus sp., a similar trend was observed. The microbial count at 25 μg/mL decreased from 5.0 log CFU/mL to about 4.5 log CFU/mL after 4 hours. At 50 and 100 μg/mL, the counts dropped further to 4.3 and 4.1 log CFU/mL, respectively. The standard commercial oral rinse again showed comparable effectiveness, while the control group maintained a stable count with no significant reduction.

Streptococcus mutans

The oral rinse demonstrated significant antimicrobial activity against S. mutans as well. At 25 μg/mL, the bacterial count decreased from 6.0 log CFU/mL at the start to 5.4 log CFU/mL after 4 hours. Higher concentrations of 50 and 100 μg/mL resulted in further reductions, with final counts of 5.0 and 4.7 log CFU/mL, respectively. The standard mouth rinse showed a similar efficacy, whereas the control group remained largely unchanged.

Candida albicans

The antifungal activity of the oral rinse was particularly noteworthy against C. albicans. At 25 μg/mL, the fungal count dropped from 5.0 log CFU/mL at 0 hours to 4.5 log CFU/mL after 4 hours. At 50 and 100 μg/mL, the oral rinse exhibited enhanced activity, reducing the fungal count to 4.2 and 4.0 log CFU/mL, respectively, by the end of the assay. The standard mouth rinse showed similar reductions, while the control group displayed minimal change.

Overall, the oral rinse containing the ethanolic extract of Ocimum tenuiflorum demonstrated effective time-dependent antimicrobial activity against all tested microorganisms. The higher concentrations (50 and 100 μg/mL) were particularly effective, with results comparable to the standard commercial mouth rinse, suggesting its potential as an alternative in oral care for reducing microbial loads.

4. Discussion

The present study focused on the preparation of an oral rinse using the ethanolic extract of Ocimum tenuiflorum and the subsequent evaluation of its antimicrobial activity and cytotoxicity. The findings suggest that the formulated oral rinse exhibits significant antimicrobial properties against a variety of oral pathogens, including Enterococcus faecalis, Lactobacillus sp., Streptococcus mutans, and Candida albicans, without displaying cytotoxic effects, thereby highlighting its potential as a safe and effective alternative to conventional oral hygiene products. The antimicrobial efficacy of the oral rinse was evaluated using the

zone of inhibition technique and the time-kill curve assay. The zone of inhibition results demonstrated that the oral rinse, even at lower concentrations, was effective in inhibiting the growth of the tested microorganisms [15]. Notably, Candida albicans exhibited the highest sensitivity, which is particularly relevant given the increasing incidence of oral candidiasis in immunocompromised patients and the challenges posed by antifungal resistance [16].

The time-kill curve assay offered additional understanding of the oral rinse's bactericidal and fungicidal properties over time. The findings showed a noticeable dose-dependent decrease in microbial load, with the highest concentration (100 μg/mL) demonstrating the greatest efficacy [17]. The rapid decrease in the log CFU/mL of the pathogens suggests that the bioactive compounds in Ocimum tenuiflorum disrupt microbial cell membranes, leading to cell death [18]. This result aligns with previous studies highlighting the antimicrobial strength of Ocimum tenuiflorum, which is attributed to its abundant phytochemicals such as eugenol, ursolic acid, and rosmarinic acid compounds well-recognized for their broad-spectrumantimicrobial properties. Key compounds like eugenol, apigenin, and isothymusin have demonstrated notable antibacterial properties, with eugenol being particularly effective in disrupting bacterial biofilms by inhibiting quorum sensing [19]. The extraction method is also pivotal; for example, ethanol extracts have shown varying levels of antimicrobial efficacy depending on the concentration and preparation techniques employed [20]. Furthermore, the geographical conditions in which the plant is cultivated can influence the concentration and effectiveness of its secondary metabolites [21-23].

The comparison with a standard commercial mouth rinse revealed that the Ocimum tenuiflorum based oral rinse has comparable, if not superior, antimicrobial efficacy. This suggests that the herbal formulation could be a viable alternative, particularly for individuals seeking natural or plant-based oral care solutions [24,25]. In addition to its antimicrobial efficacy, the safety of the oral rinse was evaluated through cytotoxicity testing using a nauplii survival assay. The results indicated no cytotoxic effects across all tested concentrations, with 100% survival of nauplii observed at both 24 and 48 hours. This is a critical finding as it highlights the biocompatibility of the oral rinse, making it suitable for regular use without adverse effects on human tissues [26]. The absence of cytotoxicity can be attributed to the natural origin of the active compounds in Ocimum tenuiflorum, which are known for their therapeutic benefits and minimal toxicity. This is in contrast to some synthetic antimicrobial agents used in commercial mouth rinses, which can cause mucosal irritation or other side effects with prolonged use [27].

5. Implications and future scope

The findings from this study have important implications for the development of new oral care products. The Ocimum tenuiflorum based oral rinse offers a promising alternative to synthetic formulations, especially in the context of increasing consumer preference for natural and sustainable products. The broad-spectrum antimicrobial activity, coupled with the absence of cytotoxicity, suggests that this formulation could be particularly beneficial for managing oral infections, including those caused by antibiotic-resistant pathogens. However, while the in vitro results are promising, further research is needed to evaluate the clinical efficacy of the oral rinse. In vivo studies involving human participants would be essential to confirm its

effectiveness in reducing oral microbial load and preventing dental caries, gingivitis, and other oral diseases. Additionally, the stability and shelf-life of the formulation should be assessed to ensure its practical applicability in commercial products. Moreover, exploring the synergistic effects of Ocimum tenuiflorum with other herbal extracts could enhance the antimicrobial potency and broaden the spectrum of activity. The potential for such combinations to reduce the risk of developing microbial resistance should also be investigated.

6. Conclusions

The oral rinse containing Ocimum tenuiflorum ethanolic extract exhibited strong antimicrobial activity against major oral pathogens while showing minimal cytotoxicity, highlighting its safety. These results suggest that Ocimum tenuiflorum could be a promising ingredient for natural oral care products. Future studies should concentrate on conducting clinical trials and developing stable formulations to enable the incorporation of this herbal rinse into conventional dental care routines.

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