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Original Article

Evaluation of the Cytotoxicity and Anti-biofilm Activity Of A Novel Dentifrice Containing 4-Hydroxycinnamic Acid: An In-Vitro Study

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ABSTRACT

INTRODUCTION: Evaluating biocompatibility is critical for assessing the safety and therapeutic potential of compounds, often serving as a preliminary step in product development. This study aims to evaluate the biocompatibility and anti-biofilm activity of a novel dentifrice containing 4-hydroxycinnamic acid (4-HCA) against *Streptococcus mutans* (SM) and *Lactobacillus acidophilus* (LA).

MATERIALS AND METHODS: The present in vitro study involved three groups- Group 1- commercially available dentifrice as control, Group 2- 0.2% 4-HCA dentifrice, Group 3- 0.4% 4-HCA dentifrice. The antibiofilm efficacy of the test dentifrices was assessed using colony-forming unit (CFU) counts and the Crystal Violet assay. The cytotoxicity was evaluated using the MTT assay and microscopic examination of human gingival fibroblast (HGF) cells.

RESULTS:

ANTIBIOFILM ACTIVITY: The CFU counts for SM and LA were highest in the control group, followed by the 0.2% 4-HCA group, and lowest in the 0.4% 4-HCA group. Crystal Violet assay results showed significant inhibition of biofilm formation in both 4-HCA groups compared to the control.

BIOCOMPATIBILITY: The MTT assay indicated that Group 3 had the highest cell viability and proliferation rates 24 horse after a 2 minute exposure to the conditioned medium across the different dilutions (1:40, 1:100, and 1:1000), as compared to Groups 2 and 1, indicating better biocompatibility. Morphological analysis of HGF cells showed normal spindle-shaped morphology in cells treated with Groups 2 and 3.

CONCLUSION: The novel dentifrice containing 0.4% 4-HCA demonstrates significant anti-biofilm activity against SM and LA and is biocompatible with HGF cells. This study supports the development of 4-HCA-based dentifrices as safe and effective oral hygiene products.

KEYWORDS: Cytotoxicity, Antibiofilm, Dentifrice, Orthodontic, Hydroxycinnamic Acid

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INTRODUCTION

The field of orthodontics has witnessed significant advancements in recent years, with a growing emphasis on enhancing patient comfort, oral health, and treatment outcomes during orthodontic care.[1] Orthodontic dentifrices are formulated with specific active ingredients aimed at addressing the unique needs of patients undergoing orthodontic care.[2] These include agents such as fluoride for remineralization, triclosan or zinc citrate for antibacterial activity, and non-abrasive components to ensure the integrity of orthodontic appliances. Additionally, some formulations incorporate innovative technologies like bioactive glass, hydroxyapatite, or casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) to promote enamel repair and reduce sensitivity.[3,4] White spot lesions (WSLs) are a significant complication associated with orthodontic treatment, as fixed appliances create more areas for plaque accumulation and hinder effective oral hygiene, resulting in elevated levels of acid-producing bacteria, especially *Streptococcus mutans* (SM).[5,6] It has been reported that metallic brackets, in particular, have the highest critical surface tension, which significantly increases the risk of enamel demineralization.[7] According to a 2023 study by Jha et al., the reported prevalence of WSLs in orthodontic patients after 12 months of treatment is 46.57%. Thus, measures to prevent enamel demineralization during orthodontic treatment must be taken.[8,9]

4-hydroxycinnamic acid (4-HCA), found in various fruits, vegetables, and grains, is a phenolic compound known for its phytochemicals and health benefits.[10] Recent research on hydroxycinnamic acid (HCA) has demonstrated the positive impacts of HCA on conditions such as cardiovascular disease, obesity, diabetes, brain disorders, cancers, and neurodegenerative diseases.[11] Laverty et al. have reported on the activity of 4-HCA against *Staphylococcus aureus* and *Escherichia coli.*[12] Ramasundar et al. recently reported on the anti-quorum sensing and antibiofilm properties of 4-HCA against SM that were isolated from patients undergoing orthodontic treatment.[13] The cytotoxicity of HCA in tumour cell lines was previously assessed by Kozubek et al.,[14] which was followed by a study done by Thorat et al., in which the cytotoxicity of 4-HCA was assessed using the MTT assay and zebrafish test.[10] The study concluded that 4-HCA is found to be non-toxic at a concentration of 40 uL. Ramasundar et al. have suggested that 4-HCA can be incorporated in oral prophylaxis agents.[13]

Current dentifrices for orthodontic patients face several limitations in effectively addressing their unique oral care needs. Many formulations lack targeted antimicrobial agents specifically designed to combat the heightened risk of white spot lesions common among orthodontic patients.[15,16] Recent studies have explored natural agents such as nutmeg-based gels,[17] herbal mouthwash formulations,[18] and modified soft liners with enhanced antibacterial properties,[19] highlighting the potential of plant-derived compounds in improving oral health outcomes. Previous studies have only evaluated the cytotoxicity of 4-HCA, but the present study is the first of its kind, wherein 4-HCA has been incorporated into a dentifrice and subsequently tested for the cytotoxicity of the newly formulated dentifrice. The present study aimed at evaluating the biocompatibility and anti-biofilm activity of a novel dentifrice containing two different concentrations of 4-HCA against a commercially available dentifrice using MTT assay and microscopic examination of human gingival fibroblast cells (HGFs).

MATERIALS AND METHODS

This in-vitro study was conducted in a university setting at Chennai, in the month of June 2024.

Dentifrice Formulation

A newly formulated dentifrice was developed at the Mahavir Health in Morbi, Gujarat, India. 4-HCA was purchased from Sigma-Aldrich (St. Louis, MO) in powder form to synthesize the novel dentifrice. The ingredients used for synthesis of the novel dentifrice were calcium carbonate- 8- 20% w/w, silica- 8-20% w/w, sorbitol- 20-30% w/w, Demineralized (DM) water, sodium carboxymethyl cellulose (CMC)- 1-2% w/w, sodium lauryl sulfate- 0.5-2% w/w, sodium benzoate- <1% w/w, and 4-Hydroxycinnamic acid- 0.2-0.4% w/w.

The test compounds were grouped into three categories such as Group 1 - Commercially available dentifrice (control), Group 2 - Novel dentifrice containing 0.2% 4-HCA, and Group 3 - Novel dentifrice containing 0.4% 4-HCA.

Microbiological processing of the plaque sample

Dental plaque sample collection was done for the purpose of isolation of the following organisms - *SM, Lactobacillus acidophilus (LA), Candida albicans (CA),* and *Enterococcus faecalis (EF)*. Dental plaque samples were collected from 15 patients undergoing fixed orthodontic treatment with 0.022-inch MBT (McLaughlin, Bennett, Trevisi) metal brackets. After informing the patients about the purpose of the plaque sample collection, written consent was obtained for the same. A sterile cotton swab was swiped across the buccal surface of banded upper molars and stored in sterile plastic tubes. The collected samples were inoculated in sterile Brain Heart Infusion (BHI) broth for *SM* and *EF*, De Man-Rogosa-Sharpe (MRS) broth was used for *LA*, and Sabouraud Dextrose (SD) broth was used for *CA.* The turbidity of the cultures was adjusted to match the 0.5 McFarland standard and incubated for 24 hours at 37°C.

Antibiofilm activity

Colony forming unit

6 extracted teeth were immersed in sterile brain heart infusion (BHI) and De Man-Rogosa-Sharpe (MRS) broth with fresh suspensions of the test organisms *SM* and *LA* that were isolated from orthodontic patients. The samples were incubated at 37º C for 72 hours for the formation of biofilms. After incubation, the samples were treated with 50 mg/ml of test samples for 6 hours and then gently agitated with sterile distilled water, and swabs were collected, and the lawn culture was made on the sterile Mutans Sanguis agar (MSA) and MRS agar. The plates were incubated at 37ºC for 24 hrs - 48 hrs. After incubation, the colonies were counted and recorded as colony-forming units per ml (cfu/ml).

Crystal Violet assay

Samples from all three test dentifrices were diluted to concentrations ranging from 10^{-1} to 10^{-6}. BHI broth for SM & MRS broth for LA were dispensed in the wells of a 96-well microtiter plate along with overnight cultures of SM and LA and samples of the various prepared dilutions of groups 1, 2, and 3. The optical density was adjusted to 0.4 at 600 nm using spectrophotometry. The plates were incubated without agitation at 37 °C for 24 hours and then cleaned three times with deionized water. Crystal violet was dissolved in 95% ethanol, and the absorbance was measured at 595 nm as described earlier.

Growth Inhibition (%) = (Sample OD value)/(Control OD value) × 100.

Biocompatibility

Cell Culture

Human Gingival Fibroblast (HGF) cultures were performed with DMEM (Invitrogen Corporation, CA, USA) supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO2. The culture medium was changed every three days and subcultured at 80% confluence. In passage 2, cells were subjected to seed in culture dishes in all *in vitro* experiments for this study.

Dentifrice extract preparation:

Dentifrice samples were eluted as per the ISO format. Eluates of these materials were prepared following ISO 10993-5 recommendations; 0.2 g of each sample was mixed with 1 mL of DMEM culture medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA), centrifuged at 4200 rpm, and the supernatant was collected and filtered. This conditioned medium was sterilized by exposure to ultraviolet light for two hours and prepared with different ratios of dilution, respectively 1:10, 1:20, 1:40, 1:100, and 1:1000 for the subsequent experiments.

MTT Assay

To assess the biocompatibility of dentifrice samples with different ratios, respectively, 1:10, 1:20, 1:40, 1:100, and 1:1000 dilutions treated on human gingival fibroblast cells along with the control group was determined over 24 hours by MTT assay as we previously described. Briefly after elution, different ratios of dentifrice were incubated on HGF cells and seeded on 96-well culture plates for 24 hr., respectively. To determine percent viability, the post-incubated cells were replaced with 10 µl of stock MTT dye (10 mg/ml) added in each well, and the plate was incubated again at 37 °C for 4 h. The medium was replaced with 100 µl DMSO in each well to dissolve the formazan crystals, and absorbance was recorded at 570 nm with Synergy hybrid Multi-Mode Reader (BioTek, Winooski, VT, US). The percent cell viability was calculated using the following equation:

Cell viability (%) = O.D. of cells treated with CLC NPs/OD of control cells X 100

Morphometric Analysis by Phase Contrast Microscopy

The cytocompatibility of dentifrice extract treatment on HGF cells was evaluated by analysis of cell morphology capturing high-resolution images captured under a phase contrast microscope at a magnification of 100 µm (10X) to check whether any morphological changes caused by the dentifrice extract on cell structure.

RESULTS

Antibiofilm activity

Colony-forming units

The highest number of CFU of SM and LA were shown by Group 1, followed by Group 2, and then Group 3. (Table 1) (Figure 1).

Organisms	Group 1	Group 2	Group 3
SM	**9.93×10^2**	**1.07×10^2**	**0.74×10^2**
LA	**2.315×10^3**	**5.81×10^2**	**4.36×10^2**

Table 1. Colony-forming units of SM and LA for Groups 1, 2, and 3.

SM- *Streptococcus mutans*; LA- *Lactobacillus acidophilus*.

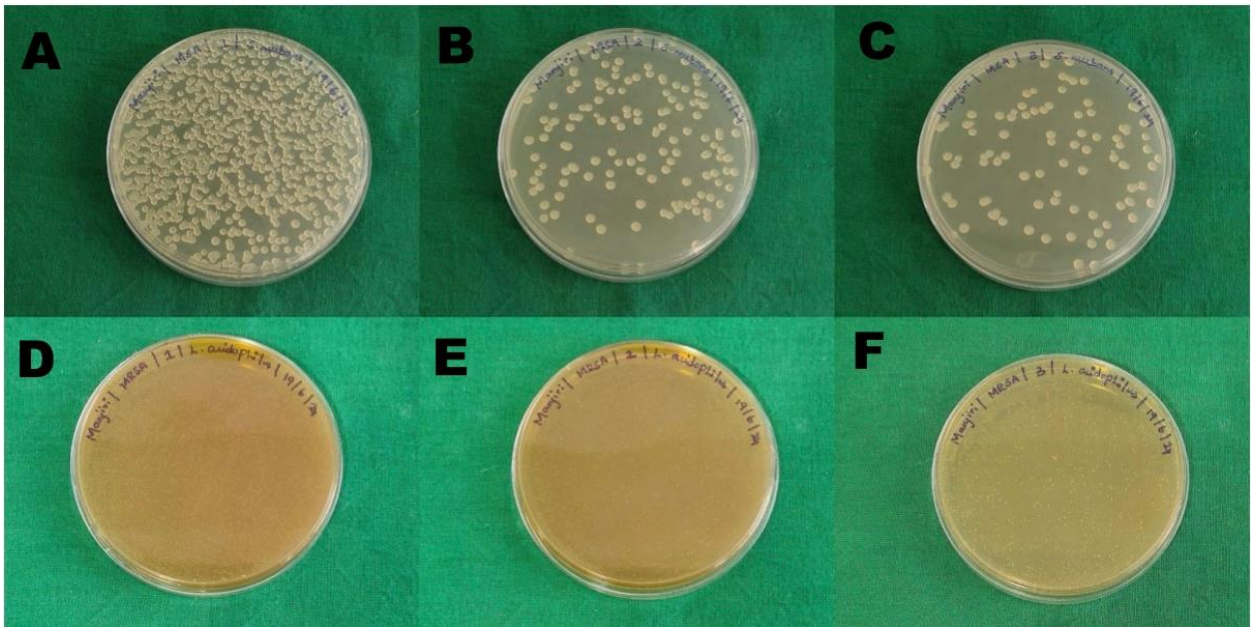

Figure 1. Plates containing MSA and MSRA agar to check for CFU

A- CFU of Streptococcus mutans for Group 1; B- CFU of Streptococcus mutans for Group 2; C- CFU of Streptococcus mutans for Group 3; D- CFU of Lactobacillus acidophilus for Group 1; E- CFU of Lactobacillus acidophilus for Group 2; F- CFU of Lactobacillus acidophilus for Group 3

Crystal Violet Assay

At a concentration of 40L, % growth inhibition for *S. mutans* for Group 1 is 76%, Group 2 is 81%, and Group 3 is 93%. For *L. acidophilus*, Group 1 is 85%, Group 2 is 91%, Group 3 is 100% (Table 2) (Figure 2).

Concentrations	SM			LA		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
10^{-1}	100%	99%	100%	100%	100%	100%
10^{-2}	100%	79%	83%	83%	96%	91%
10^{-3}	0%	9%	10%	59%	61%	73%
10^{-4}	0%	0%	0%	0%	0%	19%
10^{-5}	0%	0%	0%	0%	0%	0%
10^{-6}	0%	0%	0%	0%	0%	0%

Table 2. % growth inhibition of SM and LA for Groups 1, 2, and 3.

SM- *Streptococcus mutans*; LA- *Lactobacillus acidophilus*

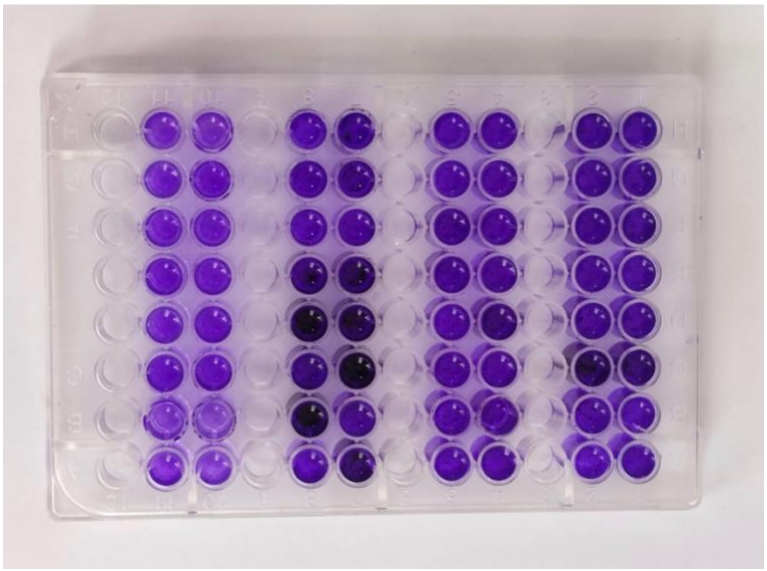

Figure 2. Microtiter well plate.

Biocompatibility by MTT assay

Group 3 generally shows the highest percentage of cell viability across the different dilutions (1:40, 1:100, and 1:1000) compared to Groups 2 and 1, indicating better biocompatibility. (Figure 3)

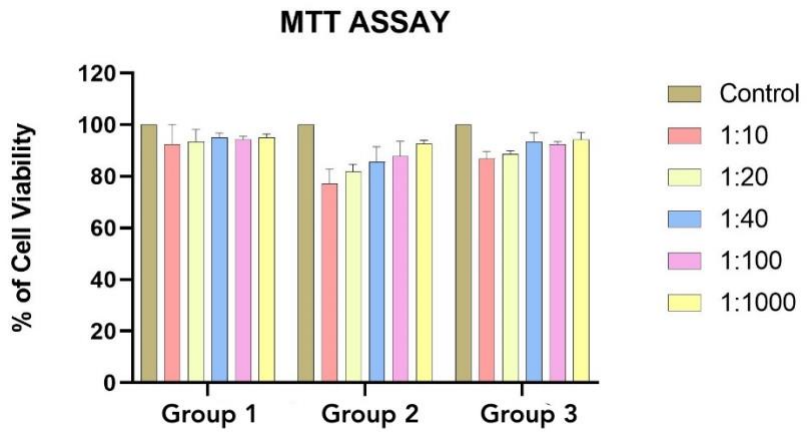

Figure 3. Cell viability of HGF cells was estimated 24 h after a 2 min exposure to the conditioned medium of Groups 1, 2, and 3 and measured by MTT assay.

Cell Morphology

Microscopic examination revealed that HGFs maintained their normal spindle-shaped morphology when treated with Groups 2 and 3. (Figure 4)

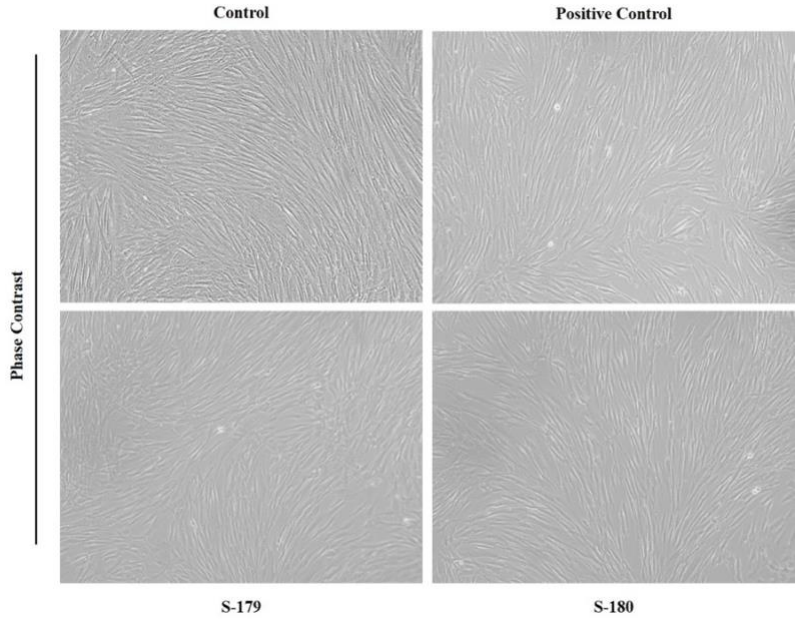

Figure 4. Morphological evaluation of HGF cells on Groups 1, 2, and 3 incubated for 24 hrs, and all the images were captured at a magnification of 100 µ (10X). S-179- Group 2; S-180- Group 3.

DISCUSSION

The present in vitro study evaluates the biocompatibility and anti-biofilm activity of a novel dentifrice containing 4-HCA. Results showed that the novel dentifrice significantly reduced SM biofilm formation, with the highest reduction observed in the 0.4% 4-HCA group. The study further assessed cytotoxicity, demonstrating high biocompatibility with HGF, ensuring safety alongside antimicrobial efficacy, even at higher concentrations of 4-HCA. Ramasundar et al.[13] specifically reported on the inhibitory effects of 4-HCA on quorum sensing and biofilm formation in SM. The current research advances the application of 4-HCA by incorporating it into a dentifrice formulation and testing its biocompatibility with HGFs, which had not been previously addressed.

The study by Yoshikawa et al.,[20] on the antibacterial activity and biofilm dispersion of an isopropyl methyl phenol (IPMP)-containing dentifrice aimed to evaluate its efficacy against oral biofilm formation and bacterial viability. IPMP, an antimicrobial agent, was incorporated into a dentifrice formulation and tested for its ability to inhibit bacterial growth. The IPMP-containing dentifrice effectively disrupted biofilm structure and significantly reduced the viability of key oral pathogens, including *SM* and *LA.* In the present study, the novel 4-HCA dentifrice demonstrated superior antibiofilm activity, with 0.4% 4-HCA showing the least CFUs of *SM* and *LA.* Similarly, the IPMP-containing dentifrice showed significant antibacterial efficacy by reducing biofilm mass and bacterial viability. While the IPMP dentifrice targets bacterial cell membrane integrity, the 4-HCA dentifrice offers additional benefits, such as cytocompatibility with human gingival fibroblasts, confirmed by high cell viability percentages and maintenance of normal cell morphology in microscopic analysis. These results suggest that the 4-HCA dentifrice might provide comparable, if not enhanced, antibiofilm effects alongside improved biocompatibility, making it a promising candidate for use in orthodontic oral care.

Cytotoxicity is a critical factor to consider, as these products come into direct contact with oral tissues. Studies have demonstrated that certain ingredients commonly found in toothpastes can exhibit varying degrees of cytotoxic effects on human gingival fibroblasts (HGFs).[21] In vitro assays, such as the MTT fibroblast assay, have demonstrated that 4-HCA exhibits minimal cytotoxicity towards HGFs, indicating its potential safety for inclusion in dentifrice formulations.[10] These findings suggest that incorporating 4-HCA into toothpaste could provide therapeutic benefits without adversely affecting gingival cell viability. Another study by Kasi et al.[22] assessed the cytotoxicity of various toothpaste ingredients, including xylitol, propylene glycol (PEG), sodium metaphosphate (SMP), lemon, peppermint, fluoride, cinnamon, triclosan, and sodium dodecyl sulfate (SDS), using reconstructed human gingiva (RHG) models. The study found that while xylitol, PEG, and SMP did not affect cell viability or tissue histology, lemon, peppermint, cinnamon, and SDS at concentrations present in toothpastes exceeded the EC50 value, indicating potential harm to the oral mucosa. These studies highlight the importance of evaluating the cytotoxic effects of toothpaste ingredients to ensure their safety and compatibility with oral tissues. The present study not only builds upon earlier research but also fills critical gaps by assessing both the safety and efficacy of a novel 4-HCA dentifrice in an orthodontic context. The findings suggest that this formulation has the potential to address the dual challenges of microbial control and biocompatibility, offering a significant advancement in oral care for orthodontic patients.

CONCLUSION

The MTT assay results play an integral role in validating the biocompatibility of a dentifrice containing 4-HCA. And it shows greater biocompatibility and preserves high cell viability; it supports its development as a safe and effective oral hygiene product, aligning both antibacterial effectiveness and tissue compatibility.

CONFLICT OF INTEREST

None.

SOURCES OF FUNDING

None

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