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Comparative evaluation of the biocompatibility and antibacterial efficacy of various toothpaste formulations

Aslı Aşık[1], Dilşah Çoğulu[2]✉, Sunde Yılmaz Süslüer[3], Ceren Yücel Er[4], Çağla Kayabaşı[5], Besra Özmen Yelken[6], Aslı Topaloğlu[7], Ataç Uzel[8] & Cumhur Gündüz[3]

This study aims to evaluate the biocompatibility of twelve toothpaste formulations using human gingival fibroblast (hGF) cell cultures, and to compare their antibacterial efficacy. In accordance with ethical guidelines, healthy hGF were obtained from individuals. The toothpaste formulations tested included Colgate Total 12 (TP1), Colgate Maximum Cavity Protection (TP2), Curaprox Enzycal (TP3), Elmex Kinder (TP4), Klorhex (TP5), ROCS Baby (TP6), ROCS Kids (TP7), Meridol (TP8), Oral-B Pro Expert Stages (TP9), Sensodyne Pronamel for Kids (TP10), Sensodyne Multi Protection (TP11), and Aquafresh Little Teeth (TP12). The cytotoxic effects of these formulations were evaluated in real-time using the xCELLigence system, which monitored cellular activity at 5-minute intervals over a 72-hour period. The apoptotic effects of the toothpastes at their IC_{50} concentrations on hGF were assessed through Annexin V and Caspase-3 assays. Genotoxicity was investigated using the Alexa Fluor® 488 Mouse anti-H2AX assay. Antibacterial efficacy against *Lactobacillus rhamnosus* and *Streptococcus mutans* was determined using a modified microdilution method. Based on IC_{50} values, TP8 was found to be the most cytotoxic toothpaste, while TP5 exhibited the least cytotoxicity. The cytotoxicity ranking of the formulations is as follows: TP8 (0.062)> TP2 (0.266) > TP3 (0.296) > TP10 (0.359) > TP11 (0.385) > TP6 (0.467) > TP7 (0.525) > TP4 (0.745) > TP12 (0.811) > TP9 (1.016) > TP1 (1.176) > TP5 (2.646) ($p < 0.05$). At the IC_{50} concentrations, the Annexin V assay revealed no significant apoptotic effects on the hGF cells, except for TP2. Similarly, the Caspase-3 assay showed no significant impact on apoptosis, and the H2AX assay did not reveal any genotoxic effects ($p > 0.05$). TP8 and TP4 demonstrated the highest antibacterial efficacy against *L.rhamnosus*,whereas TP1, TP2, TP5, TP7, and TP10 exhibited superior activity against *S.mutans*. The tested toothpaste formulations exhibited marked variability in cytotoxicity and antibacterial performance in human gingival fibroblast cultures. While several formulations demonstrated strong antibacterial effects, these were frequently accompanied by increased cytotoxicity, particularly in products containing aggressive surfactant systems. Among the twelve formulations evaluated, Klorhex (TP5) showed the most favorable biological profile, characterized by the highest IC_{50} value (lowest cytotoxicity), absence of significant apoptotic or genotoxic effects, and effective antibacterial activity against *S. mutans*. Based on the combined assessment of biocompatibility and antimicrobial efficacy, TP5 appears to be the most suitable option for clinical use, particularly for individuals with increased mucosal sensitivity or long-term dentifrice exposure. Clinicians should consider both the biocompatibility and antibacterial efficacy of dentifrices when making personalized recommendations for caries prevention.

Keywords Biocompability, Cytotoxicity, Toothpaste, Antibacterial

[1]Department of Pediatric Dentistry, Faculty of Dentistry, İzmir Tınaztepe University, İzmir, Turkey. [2]Department of Pediatric Dentistry, Faculty of Dentistry, Ege University, İzmir, Turkey. [3]Department of Medical Biology, Faculty of Medicine, Ege University, İzmir, Turkey. [4]Pediatric Dentist, Menemen Oral and Dental Health Center, İzmir, Turkey. [5]Department of Medical Biology, Faculty of Medicine, Balıkesir University, Balıkesir, Turkey. [6]Department of Medical Biology, Faculty of Medicine, İzmir Bakırçay University, İzmir, Turkey. [7]Department of Pediatric Dentistry, Faculty of Dentistry, İstanbul University-Cerrahpaşa, İstanbul, Turkey. [8]Division of Basic and Industrial Microbiology Section, Department of Biology, Faculty of Science, Ege University, İzmir, Turkey. ✉email: dilsah.cogulu@ege.edu.tr

Toothbrushes and toothpastes are among the most commonly used and readily accessible products for maintaining oral hygiene[1]. A toothpaste, or "dentifrice," is defined by the United States Food and Drug Administration (FDA) as "an abrasive-containing dosage form (gel, paste, or powder) for delivering an anticaries drug to the teeth. "An anticaries drug is specifically described as" a drug that aids in the prevention and prophylactic treatment of dental cavities."[2]. The primary purposes of using toothpaste are to aid in the removal of dental plaque and to provide preventive agents that protect against tooth decay. Additionally, certain ingredients in toothpaste can help inhibit tartar formation, prevent tooth discoloration, and provide fresh breath[1]. Toothpastes are composed of two primary categories of ingredients: active and inactive. Active ingredients include anticaries agents, desensitizing agents, and antimicrobial agents, while inactive ingredients consist of abrasives, detergents, humectants, thickening agents, and flavoring agents[3]. Among the active ingredients, fluoride-based remineralization agents and anticaries agents play a crucial role. There is substantial evidence demonstrating that fluoride content in toothpaste effectively prevents dental caries. Various fluoride compounds, such as amine fluoride, stannous fluoride, sodium fluoride, and sodium monofluorophosphate, are commonly incorporated into toothpaste formulations. While the comparative efficacy of these fluoride salts has been widely debated[4], a comprehensive review concluded that their effectiveness is equivalent[5]. In addition to fluoride, other active ingredients with remineralization properties, such as sodium fluoride, amine fluoride, stannous fluoride, triclosan, arginine, and Novamin, are used in toothpaste formulations for caries prevention. In addition to their anticaries benefits, fluoride compounds have been shown to exert dose-dependent cytotoxic effects on oral cells. Studies on human gingival fibroblasts and oral epithelial cells report that sodium fluoride and stannous fluoride may induce oxidative stress, mitochondrial membrane disruption, lipid peroxidation, and apoptosis through caspase-dependent pathways. These effects are particularly evident at higher concentrations or prolonged exposure times, underscoring the importance of evaluating fluoride-containing toothpastes not only for remineralization efficacy but also for their biological safety profile.

Other compounds may be added to fulfill additional purposes, including whitening, erosion prevention, anti-tartar action, anti-malodor, anti-plaque, and anti-gingivitis effects. Triclosan and chlorhexidine are commonly included for their antiplaque and antimicrobial properties[1]. Triclosan exhibits anti-inflammatory effects by suppressing cyclo-oxygenase and lipoxygenase pathways, and it is compatible with other toothpaste ingredients, such as fluoride and surfactants[6]. Chlorhexidine, a powerful antiseptic, is effective in treating periodontal diseases and controlling plaque buildup on teeth. It is included in some toothpaste formulations for its antimicrobial properties[7].

Inactive ingredients in toothpaste include abrasives, detergents, humectants, and thickeners. Abrasive insoluble particles such as silica, aluminum hydroxide, and calcium carbonate are commonly incorporated into toothpaste formulations. These abrasives are uniformly dispersed throughout the toothpaste's soluble matrix and primarily serve to aid in the mechanical removal of plaque and other dental deposits. Detergents, including sodium lauryl sulfate (SLS) and sodium N-lauryl sarcosinate, are added to generate a foaming action, which facilitates the dispersion and solubilization of plaque and other dental accretions during the brushing process. This foaming effect not only enhances the cleaning efficacy but also contributes to the even distribution of active ingredients across the tooth surface[8]. Surfactants constitute one of the most biologically active components of toothpaste formulations and have been increasingly scrutinized for their potential adverse cellular effects. Sodium lauryl sulfate (SLS), a widely used anionic detergent, has been associated with membrane disruption, increased epithelial permeability, and cytotoxic responses in gingival and oral mucosal cells. Previous in vitro studies have demonstrated that SLS can induce oxidative stress, mitochondrial dysfunction, and pro-apoptotic signaling in fibroblasts and keratinocytes, even at low concentrations. Cocamidopropyl betaine (CAPB), an amphoteric surfactant frequently included to enhance foaming properties, has also been reported to contribute to irritation and inflammatory responses in oral epithelial models. Given the widespread use of these agents and their established biological activity, evaluating the cytotoxic and apoptotic effects of toothpaste formulations is clinically relevant, particularly for products intended for daily and long-term use. Humectants, such as glycerol, propylene glycol, and sorbitol, are incorporated into toothpaste formulations to prevent moisture loss and maintain the consistency and stability of the product. These compounds function by retaining water, thereby mitigating the evaporation of moisture during storage and use. Additionally, thickening agents or binders, including mineral or seaweed colloids, natural gums, and synthetic cellulose derivatives, are utilized to provide the necessary viscosity and stabilize the toothpaste formulation. Sugar alcohols, commonly included in toothpaste compositions, serve multiple purposes. They facilitate the incorporation of remineralizing agents and antibacterial ingredients, act as carriers for flavoring agents, and enhance water retention, thus contributing to the overall stability and efficacy of the product[1,3,9].

An ideal toothpaste designed for dental plaque control in oral and dental health must possess several key characteristics. The ingredients should be non-toxic, non-allergenic, and non-irritating to ensure safety during use. It must demonstrate efficacy in controlling dental plaque and reducing the formation of plaque-related periodontal diseases. The antibacterial agents included in the formulation should specifically target pathogenic oral flora while minimizing disruption to beneficial microorganisms. Additionally, the taste and odor of the toothpaste should be acceptable to the user, ensuring a pleasant experience during application[9–11].

Toothpastes contain a variety of chemical compounds, some of which may induce allergic reactions or irritation in susceptible individuals. Conditions such as perioral contact dermatitis, stomatitis, or cheilitis have been reported as adverse effects associated with toothpaste use. These reactions are often attributed to certain ingredients, particularly essential oils like peppermint, cinnamon, and spearmint, which are commonly used for flavoring but may trigger hypersensitivity in some individuals[12–15]. Citric acid (often marketed as potassium or zinc citrate), SLS, propylene glycol, polyethylene glycols (such as PEG-8, PEG-12, and PEG-1450), cocamidopropyl betaine, parabens, and pyrophosphates are additional components commonly found

in toothpaste formulations. These ingredients have been documented in the literature as potential allergens or irritants, capable of inducing allergic reactions or contact dermatitis in susceptible individuals[3].

Toothpaste not only interacts with the hard dental tissues during mechanical plaque removal but also comes into contact with the gingival and other intraoral mucosal tissues. Literature reviews have highlighted that the primary compounds incorporated into toothpaste formulations can exert cytotoxic effects on cellular structures. Notably, detergents, particularly SLS and cocamidopropyl betaine, have been identified as the most significant contributors to cytotoxicity, with several studies documenting their harmful impact on cells[8,16–18].

Despite their widespread use, there is a notable lack of studies investigating the cytotoxic, apoptotic, and genotoxic effects of toothpaste formulations with varying compositions on human gingival fibroblast (hGF) cells, as well as their comparative antibacterial efficacy. This study aims to address this gap in the literature by conducting a comprehensive evaluation of the biocompatibility and antibacterial efficacy of different toothpaste formulations, an area that remains underexplored in the current body of research. Recent survey-based and epidemiological studies have highlighted growing variability among commercially available toothpaste formulations, including differences in fluoride concentration, surfactant systems, antimicrobial additives, and child-specific formulations. This diversity has raised concerns regarding both the biocompatibility and antibacterial performance of these products, particularly given the increasing reports of surfactant- and fluoride-associated cytotoxicity in oral soft tissues and the inconsistent antimicrobial efficacy observed across formulations[19,20]. These findings underscore the need for systematic comparative evaluations to inform safer and more effective clinical recommendations.

This study aims to compare the cytotoxic, apoptotic, and genotoxic effects of various toothpaste formulations on oral mucosal tissues through in vitro cell culture assays using hGF. Furthermore, the antibacterial efficacy of these toothpastes will be assessed and compared against *S. mutans* and *L. rhamnosus*.

Methods

The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of the Faculty of Medicine, Ege University (protocol number: 14 – 5.1/8).

Isolation and purification of human gingival fibroblast (hGF) cells

The present study employed human gingival fibroblast (hGF) cells derived from two healthy individuals who underwent tooth extractions for orthodontic treatment at the Faculty of Dentistry, Ege University, Department of Periodontology. Gingival tissue samples were aseptically collected under local anesthesia and subsequently transported to the Department of Medical Biology, Faculty of Medicine, Ege University. The tissues were enzymatically digested using Collagenase Type II (Biochrom AG, Berlin, Germany) to generate a cell suspension culture. The culture medium used was Dulbecco's Modified Eagle's Medium (DMEM; Biochrom AG, Berlin, Germany), supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, and 100 U/mL penicillin and 10 µg/mL streptomycin. The cells were incubated at 37 °C in a humidified incubator (Heraeus®, Berlin, Germany) with 95% humidity and 5% CO_2 until they reached the desired confluence. Cell proliferation, passage, and maintenance were monitored using an inverted microscope, with each cell line subcultured into separate flasks. Cells from the third passage were subsequently used for cytotoxicity, apoptosis, and genotoxicity evaluations.

Preparation of toothpaste formulations for experimental investigation

Toothpastes included in this study were selected based on the following criteria: (1) commercial availability in the Turkish and international consumer market, (2) clear labeling of active ingredients, including fluoride content and surfactant systems, (3) relevance to routine pediatric or adult use, and (4) representation of different formulation categories (fluoride-containing adult toothpaste, low-fluoride or fluoride-free pediatric toothpaste, enzyme-based, and chlorhexidine-containing). Toothpastes lacking complete ingredient disclosure, pharmaceutical prescription-only formulations, professional in-office products, and whitening pastes with high abrasivity were excluded from analysis. The toothpaste formulations evaluated in this study were sourced from commercially available products present in the consumer market at the time of acquisition. For each formulation, the ingredient list published by the manufacturer for the corresponding batch was documented, and the brand name, batch (lot) number, and acquisition details were recorded to ensure traceability, acknowledging that commercial formulations may undergo periodic updates. In the present study, commercially available toothpastes were utilized, with the ingredients listed in Table 1.

For clarity, the twelve toothpaste formulations were categorized into ingredient-based groups according to their principal active or inactive components:

- Fluoride-containing adult toothpaste formulations: products with sodium fluoride, stannous fluoride, or monofluorophosphate as active anticaries agents.
- Pediatric formulations: child-targeted products containing reduced fluoride concentrations or no fluoride, typically featuring mild surfactants and low-abrasivity systems.
- Enzyme-based formulations: toothpastes incorporating lactoperoxidase, glucose oxidase, or other enzymatic components in place of conventional fluoride/surfactant systems.
- Chlorhexidine-containing antibacterial formulations: pastes employing chlorhexidine digluconate as the primary antimicrobial agent.

This categorical grouping was used to facilitate comparison of biocompatibility and antibacterial outcomes across formulations with distinct ingredient profiles.

Toothpastes	Group	Manufacturer	Ingredients	Lot Number
TP1: Colgate Total 12	Fluoride-containing Adult toothpaste	Colgate-Palmolive, New York, USA	1450 ppm Sodium Fluoride, Triclosan, Water, Hydrated Silica, Glycerin, Sorbitol, PVM/MA Copolymer, Sodium Lauryl Sulfate, Aroma, Carrageenan, Sodium Hydroxide, Propylene Glycol Cellulose Gum, Sodium Saccharin, Limonene, CI 77, 891.	GT8992
TP2: Colgate Maximum Cavity Protection	Fluoride-containing Adult toothpaste	Colgate-Palmolive, New York, USA	1450 ppm Sodium Monofluorophosphate, Calcium Carbonate, Water, Glycerin, Sodium Lauryl Sulfate, Arginine, Aroma, Sodium Bicarbonate, Cellulose Gum, Tetrasodium Pyrophosphate, Benzyl Alcohol, Sodium Saccharin, Sodium Hydroxide, Cl 77,891.	JB9021
TP3: Curaprox Enzycal	Enzyme based toothpaste	Curaden AG, Kriens, Switzerland	Aqua, Hydrated Silica, Sorbitol, Glycerin, Steareth-20, Titanium Dioxide, Aroma, Disodium Phosphate, Carrageenan, Sodium Chloride, 950 ppm Sodium Fluoride, Citric Acid, Sodium Benzoate, Sodium Saccharin, Amyloglucosidase, Potassium Thiocyanate, Glucose Oxidase, Limonene, Lactoperoxidase	211,121
TP4: Elmex Kinder	Pediatric toothpaste	Gaba International, Therwil, Switzerland	500 ppm Amine Fluoride, Titanyum Dioxite, Su, Sorbitol, Silica Hydrate, Hydroxyethyl-Cellulose, Cocamidopropyl Betaine, Aroma, Limonene, Hydrochloric Acid.	225,111
TP5: Klorhex	Chlorhexidine-containing toothpaste	DentaSave, Drogsan, Istanbul, Turkey	0.2% Chlorhexidine, 1400 ppm Sodium Fluoride, Titanium Dioxide, Water, Hydrated Silica, Glycerin, Sorbitol, Potassium Nitrate, Sodium Cocoamfoacetate, PEG-40, Hydrogenated Castor Oil, Carboxymethyl Cellulose, Sodium Saccharin, Aroma, Phenoxyethanol Ethylhexylglycerin.	07042022
TP6: ROCS Baby	Pediatric toothpaste	R.O.C.S. Trading GmbH, Munich, Germany	Xylitol, Water, Glycerin, Silica, Chamomile Extract, Potassium Alginate, Sodium Benzoate, Xanthan Gum.	201,022
TP7: ROCS Kids	Pediatric toothpaste	R.O.C.S. Trading GmbH, Munich, Germany	500 ppm Amine Fluoride, Xylitol, Water, Silica, Glycerin, Hydroxyethyl Cellulose, Polysorbate-20, Aroma, Titanium Dioxide, Cocamidopropyl Betaine, Sodium Saccharin, Methylparaben, Propylparaben, Potassium Hydroxide, Benzylalcohol.	0760922
TP8: Meridol	Fluoride-containing Adult toothpaste	Gaba International, Therwil, Switzerland	1400 ppm Amine Fluoride/Stannous Fluoride, Water, Sorbitol, Hydrated Silica, Silica Dimethyl Silicate, Hydroxyethylcellulose, Cocamidopropyl Betaine, PEG-40, Hydrogenated Castor Oil, Aroma, Sodium Gluconate, Limonene, PEG-3 Aminopropylamine, Sodium Saccharin, Potassium Hydroxide, Hydrochloric Acid, Cl74160.	251,951
TP9: Oral-B Pro Expert Stages	Pediatric toothpaste	Protecter&Gamble, Ohio, USA	500 ppm Sodium Fluoride, Water, Sorbitol, Hydrated Silica, Sodium Lauryl Sulfate, Cellulose Gum, Aroma, Sodium Saccharin, Carbomer, Trisodium Phosphate, Limonene, Cl 42, 090.	2,365,853,396
TP10: Sensodyne Pronamel for Kids	Pediatric toothpaste	GlaxoSmithKline, London, UK	1450 ppm Sodium Fluoride, Titanium Dioxide, Water, Sorbitol, Hydrated Silica, Glycerin, PEG-6, Cocamidopropyl Betaine, Xanthan Gum, Aroma, Sodium Saccharin, Sucralose, Sodium Hydroxide Limonene.	32,280,057 A
TP11: Sensodyne Multi Protection	Fluoride-containing Adult toothpaste	GlaxoSmithKline, London, UK	1450 ppm Sodium Monofluorophosphate, NOVAMIN, Titanium Dioxide, Glycerin, PEG-8, Hydrated Silica, Aroma, Sodium Lauryl Sulfate, Carbomer, Sodium Saccharin, Eugenol, Limonene.	32,180,079
TP12: Aquafresh Little Teeth	Pediatric toothpaste	GlaxoSmithKline, London, UK	500 ppm Sodium Fluoride, Titanium Dioxide, Water, Sorbitol, Hydrated Silica, Glycerin, Xanthan Gum, Aroma, Cocamidopropyl Betaine, Sodium Methyl Cocoyl Taurate, Carrageenan, Sodium Saccharin, Limonene, Cl 73, 360, Cl74160.	00442881

Table 1. The composition of toothpastes used in the present study.

Initially, 1 mL samples of the toothpastes were mixed with 9 mL of cell culture medium to achieve a 10% dilution. The preparation of 10% (w/v) toothpaste extracts followed established protocols described in previous dentifrice biocompatibility studies, where toothpaste slurries are centrifuged and the clarified supernatant is used for experimental testing[19,20].A vortex mixer was employed to ensure thorough homogenization of the toothpaste with the culture medium. Following preparation, the falcon tubes containing the diluted toothpaste formulations were incubated for 30 min at 37 °C in a 5% CO_2 incubator. Subsequently, the samples were centrifuged at 3500 rpm for 5 min to promote the sedimentation of any particulate matter. The supernatant, containing a well-mixed culture medium, was then transferred to separate falcon tubes and filtered through a 0.22 µm pore size filter, resulting in a sterile DMEM culture medium with a 10% toothpaste concentration. The 10% (w/v) dilution was selected based on its wide use in prior cytotoxicity and biocompatibility studies evaluating dentifrice formulations in vitro[19,20]. This concentration approximates the dilution ratio that occurs when toothpaste is combined with saliva during typical tooth brushing, thereby representing a physiologically relevant exposure scenario. Furthermore, it enables effective comparison of cytotoxicity profiles across formulations and allows for downstream IC_{50} calculations within a dose-response framework. The pH of each 10% (w/v) toothpaste extract was measured using a calibrated digital pH meter to account for potential acidity-related cytotoxic effects. All extracts demonstrated pH values within the physiological range (6.1–7.8), consistent with previously reported dentifrice slurry measurements. These findings indicate that the cytotoxic responses observed in this study were not attributable to extreme pH deviations.

Assessment of the cytotoxicity of toothpaste formulations on human gingival fibroblast (hGF) cell cultures using the xCELLigence Real-Time cell analyzer (RTCA) system

Real-time cell analysis (RTCA-xCELLigence) was utilized for up to 72 h to determine the IC_{50} value, defined as the concentration of toothpaste that inhibits 50% of the biological and metabolic processes in the hGF cell population[21].

The RTCA system comprises four primary components: the RTCA Analyzer, the RTCA SP Station, a computer running the RTCA software, and single-use 96-well E-Plates with gold microelectrodes embedded at the bottom of each well. During the experiment, the electrodes were subjected to an estimated voltage of 20 mV. Initially, 100

µL of Dulbecco's Modified Eagle's Medium (DMEM) was added to each well of the 96-well xCELLigence plate. The plate was then placed in the incubator, and baseline measurements were recorded. Subsequently, 100 µL of a cell-medium mixture containing 3×10^5 hGF cells per mL was added to each well. The cells were allowed to adhere to the well surface for 24 h, during which time measurements were obtained every 15 min[21].

At the end of the incubation period, half of the 200 µL culture medium in the 96-well plates seeded with cells was withdrawn and replaced with toothpaste solutions diluted to concentrations of 1/100, 1/200, 1/400, 1/800, 1/1600, and 1/3200, respectively. Cytotoxicity was evaluated over a 72-hour period using the RTCA-xCELLigence platform. The 72-hour continuous exposure period was selected in accordance with established RTCA-xCELLigence cytotoxicity protocols, which require extended monitoring to capture dynamic alterations in cell adhesion, proliferation, and viability. This exposure duration is intended to provide a sensitive worst-case in vitro scenario for detecting sublethal and cumulative cytotoxic responses, rather than to reproduce the short, intermittent nature of toothbrushing. Continuous exposure enables stable IC_{50} curve generation and allows sublethal cytotoxic, apoptotic, and genotoxic responses to manifest, consistent with prior dentifrice biocompatibility research. The inhibitory concentration values were determined by fitting the cytotoxicity data to a sigmoidal dose-response curve with the equation [Y = bottom + (top - bottom)/(1 + 10^((LogIC$_{50}$ - X) * HillSlope))][21].

Evaluating the apoptotic effects of toothpastes on hGF cell cultures using the Annexin V and Caspase-3 assay

Gingival fibroblast cells were seeded at a density of 0.5×10^6 cells per well in a 6-well culture plate and incubated for 24 h under 95% humidity and 5% CO_2 to facilitate optimal cell attachment. Following this incubation, 1 mL of the existing medium was removed from each well and replaced with 3 mL of fresh medium containing the IC_{50} concentrations of the various toothpaste formulations. The cells were then exposed to the toothpaste-containing medium for an additional 72 h[21].

After the 72-hour exposure period, the culture medium was aspirated, and 500 µL of trypsin-EDTA was added to each well to promote the detachment of the adherent cells. Following a 5-minute incubation with trypsin, 1 mL of medium supplemented with 10% fetal bovine serum was added to neutralize the trypsin activity. The resulting cell suspension from each well (1.5 mL) was then transferred to Eppendorf tubes and centrifuged at 2,500 rpm for 5 min. The supernatant was discarded, and the cell pellets were resuspended in 1 mL of phosphate-buffered saline (PBS). A subsequent 5-minute centrifugation was performed to ensure thorough washing of the cells[21].

To assess annexin V test

In each experimental tube, 100 µL of binding buffer, 1 µL of Annexin V, and 1 µL of propidium iodide were added and thoroughly mixed. The samples were then incubated at room temperature, protected from light, for 5 min. Following the incubation, the extent of apoptosis within the cell populations was assessed at the 72-hour time point using a BD Accuri™ C6 Flow Cytometer (Biosciences, USA). The cell populations were classified into four distinct categories: viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells, based on the respective fluorescence signals corresponding to Annexin V and propidium iodide staining[21].

Caspase-3 assay

Following treatment with phosphate-buffered saline (PBS), the cells were resuspended in BD Cytofix/Cytoperm™ solution at a concentration of 1 million cells per 0.5 mL. The suspension was incubated on ice for 20 min, after which the BD Cytofix/Cytoperm™ solution was carefully removed from the cell pellet. The cells were then washed twice with BD Perm/Wash™ buffer at room temperature to facilitate permeabilization. At the 72-hour time point, apoptosis within the cell populations was assessed using a BD Accuri™ C6 Flow Cytometer (Biosciences, USA). The cell populations were categorized into two distinct groups: apoptotic cells and non-apoptotic cells, based on their fluorescence profiles corresponding to Annexin V and propidium iodide staining[21].

Annexin V detects early apoptotic changes by binding to externalized phosphatidylserine on the outer plasma membrane, indicating the initial stages of programmed cell death. In contrast, Caspase-3 reflects activation of the execution phase of apoptosis, representing irreversible, late-stage apoptotic commitment. Assessing both markers allows differentiation between early apoptotic signaling and downstream apoptotic execution.

Assessment of the genotoxic effects of toothpaste formulations on human gingival fibroblast (hGF) cell cultures using the H2AX assay

The genotoxicity of the IC_{50} concentrations of toothpaste was evaluated using the Alexa Fluor® 488 Mouse anti-H2AX assay. Following PBS washes, the cells were fixed in 3.7% formaldehyde in 100 µL of fresh PBS and incubated for 10 min at room temperature. After removing the fixative, the cells were permeabilized by incubation in 100 µL of 90% methanol at −20 °C for 5 min. The permeabilization buffer was discarded, and the wells were washed with 1000 µL of PBS to remove the supernatant. The cells were then incubated with 100 µL of Triton X-100 for 5 min at room temperature, followed by centrifugation at 2500 rpm for 5 min, with the supernatant being discarded after each step[21].

A diluted pH2AX antibody solution was added to each well and incubated for 30 min. The antibody was prepared by diluting 2.5 µL of pH2AX antibody in 22 µL of PBS, followed by centrifugation to remove any unbound antibody. After incubation, the cells were treated with 100 µL of 0.05% Tween solution, centrifuged once more, and the supernatant was discarded. The cells were then resuspended in 50 µL of PBS. The resulting genotoxic effects within the cells were assessed using a BD Accuri™ C6 Flow Cytometer (Biosciences, USA) at the 72-hour time point[21].

Antibacterial properties of toothpaste formulations

A modified microdilution assay was employed instead of the standardized CLSI/EUCAST MIC/MBC protocol due to the inherent physicochemical characteristics of toothpaste formulations. Toothpaste extracts retain viscosity, non-dissolved particulates, and intrinsic turbidity even after centrifugation and filtration, which makes optical-density–based MIC determination unreliable. Because MIC/MBC assays require optically clear antimicrobial solutions, such matrices would produce false-positive turbidity signals unrelated to bacterial growth. Therefore, serial dilutions of the clarified supernatant were combined with TTC reduction as a metabolic indicator, enabling a specific distinction between bacterial viability and toothpaste-related background coloration. In the present study, a modified microdilution method was utilized to evaluate the antibacterial properties of various toothpaste formulations against *S. mutans* and *L. rhamnosus*. This modified approach is widely recommended for evaluating antibacterial properties of dentifrices with complex multi-component matrices.

S. mutans was selected as a primary colonizer and a well-established initiator of dental caries due to its acidogenic and aciduric properties. While certain strains of *L. rhamnosus* are recognized for their probiotic benefits in gastrointestinal health, lactobacilli species, including the ATCC 7469 strain used in this study, are frequently isolated from advanced carious lesions and are strongly associated with the progression of caries due to their high acid tolerance. Therefore, *L. rhamnosus* ATCC 7469 serves as a relevant model organism for evaluating antimicrobial efficacy against the acidogenic bacteria implicated in caries development. The use of defined, standardized reference strains was essential to ensure the reproducibility and comparability of the antimicrobial assays, which would be challenging to achieve with the complex and variable consortium of normal oral flora.

L. rhamnosus ATCC 7469 and *S. mutans* ATCC 25,175 were cultured in MRS Broth (Merck, Darmstadt, Germany) and Tryptic Soy Broth (Oxoid, Basingstoke, UK), respectively, to activate the organisms. The cultures were incubated in 5 mL of liquid medium at 37 °C for 24–48 h. The microbial inoculum was standardized to a 0.5 McFarland turbidity using a saline solution[21].

The antimicrobial efficacy of the toothpaste formulations was assessed using the modified microdilution method in 96-well U-shaped microplates. All experimental procedures were conducted in triplicate. Positive controls consisted of sterile growth medium and the test microorganism to ensure microbial growth, while negative controls contained only the growth medium to check for any contamination[21].

Results were interpreted based on the presence of a red color following the addition of 10 µL of 2,3,5-Triphenyltetrazolium chloride (TTC) to each well after 24 h of incubation. The appearance of red coloration indicated active metabolism and proliferation of the microorganisms, while the absence of red coloration signified microbial inhibition or death[21]. To minimize interference from toothpaste colorants and opacifiers, only the clarified supernatant obtained after centrifugation was used for antibacterial testing. In addition, for each toothpaste and dilution, non-inoculated blanks containing toothpaste extract, growth medium, and TTC (but no bacteria) were included on the same microplates. These blanks did not develop the characteristic red formazan color, confirming that the TTC signal observed in test wells reflected bacterial metabolic activity rather than intrinsic toothpaste coloration or turbidity. The TTC-based metabolic assay was selected as a high-throughput comparative method because CFU enumeration and qPCR quantification are susceptible to interference from toothpaste excipients that inhibit bacterial recovery, DNA extraction, or amplification. Thus, TTC reduction provided a more robust and reproducible endpoint for screening the antibacterial performance of complex dentifrice matrices.

Statistical analysis

For each toothpaste formulation, cytotoxicity, apoptosis, and genotoxicity experiments were performed using hGF cells obtained from two independent donors, with all measurements conducted in triplicate technical replicates per donor. This combination of biological and technical replication is consistent with recommended standards for RTCA-based IC_{50} estimation and flow-cytometry based apoptosis/genotoxicity assays. A priori power analysis is not commonly applied to nonlinear dose-response modeling; therefore, sample size was determined based on methodological precedents demonstrating that biological duplicates with triplicate technical replicates provide sufficient robustness and reproducibility for in vitro biocompatibility assessments. Statistical analyses were performed using IBM SPSS Statistics software (Version 25.0, Armonk, NY, USA). The cytotoxic properties of the toothpaste formulations were statistically evaluated through sigmoidal dose-response analysis. For the statistical analysis of data obtained from apoptosis and genotoxicity assays, the chi-square test and relative risk analysis were applied. A significance level of 0.05 was adopted to determine statistical significance.

Results

Based on the IC_{50} values obtained through the xCELLigence system, the cytotoxic effects of the toothpastes on human gingival fibroblast (hGF) cell lines were ranked as follows: Meridol (0.062) demonstrated the highest cytotoxicity, followed by Colgate Maximum Cavity Protection (0.266), Curaprox Enzycal (0.296), Sensodyne Pronamel for Kids (0.359), Sensodyne Multi Protection (0.385), ROCS Baby (0.467), ROCS Kids (0.525), Elmex Kinder (0.745), Aquafresh Little Teeth (0.811), Oral-B Pro Expert Stages (1.016), Colgate Total 12 (1.176), and Klorhex (2.646). These values were derived using the "RTCA Software Version 1.2.1" analysis program, which employed a sigmoidal dose-response curve model [Y = bottom + (top - bottom)/(1 + (x/EC50)^HillSlope)]. Statistical analysis revealed significant differences in IC_{50} values among the toothpaste formulations (one-way ANOVA, $p < 0.05$), and post hoc comparisons confirmed that several formulations demonstrated distinct cytotoxicity profiles relative to one another. (Fig. 1)

Bar graph showing normalized IC_{50} cytotoxicity values for the twelve toothpaste formulations. Lower IC_{50} values indicate higher cytotoxic potential under the experimental conditions.

With the exception of Colgate Maximum Cavity Protection toothpaste, the IC_{50} concentrations of the other toothpaste formulations did not induce statistically significant apoptotic effects in the human gingival fibroblast

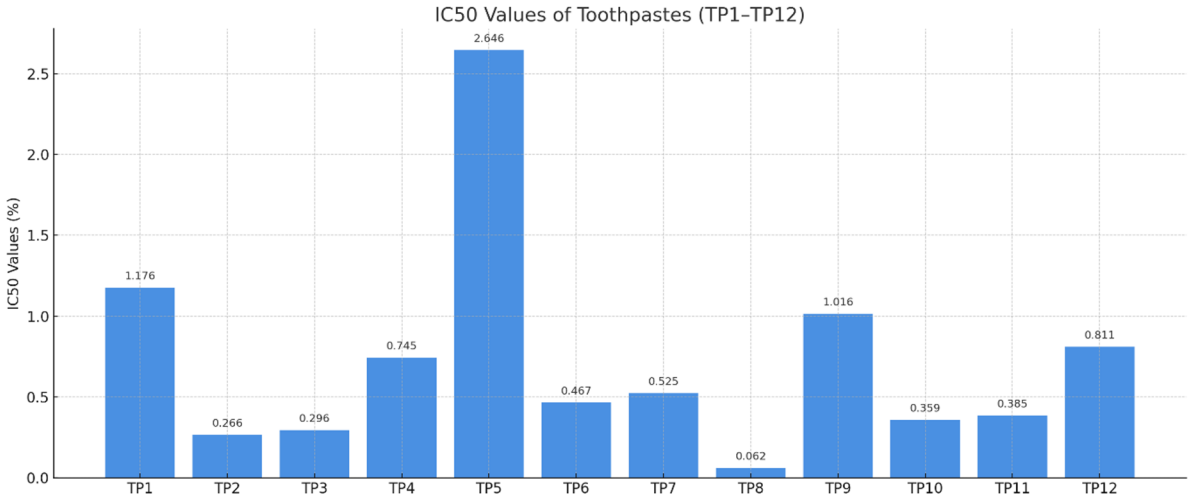

Fig. 1. IC_{50} cytotoxicity values of all toothpaste formulations.

Toothpastes	ALIVE (%)	APOPTOSIS (%)	OR	95% C.I.	*p*
Control	99.50	0.50	1.000	-	-
TP1: Colgate Total 12	98.68	1.32	2.66	0.10–69.80	0.557
TP2: Colgate Maximum Cavity Protection	85.67	14.33	33.29	1.96–566.61.96.61	0.015*
TP3: Curaprox Enzycal	94.88	5.12	10.74	0.58–198.63.58.63	0.111
TP4: Elmex Kinder	98.60	1.41	2.84	0.11–72.38	0.528
TP5: Klorhex	93.76	6.25	13.26	0.73–239.56.73.56	0.080
TP6: ROCS Baby	96.18	3.82	7.90	0.41–152.67.41.67	0.171
TP7: ROCS Kids	94.67	5.33	11.20	0.61–206.19.61.19	0.104
TP8: Meridol	96.40	3.60	7.43	0.38–145.04.38.04	0.186
TP9: Oral-B Pro Expert Stages	99.22	0.79	1.58	0.05–55.21	0.802
TP10: Sensodyne Pronamel for Kids	98.72	1.28	2.58	0.10–68.61.10.61	0.571
TP11: Sensodyne Multi Protection	97.67	2.34	4.76	0.22–102.18.22.18	0.319
TP12: Aquafresh Little Teeth	99.21	0.79	1.59	0.05–55.32	0.800

Table 2. Comparison of apoptotic effects of toothpastes by Annexin V.

(hGF) cell line ($p > 0.05$). Colgate Maximum Cavity Protection, however, significantly triggered apoptosis in the hGF cells, exhibiting a 33.29-fold increase in apoptotic activity (odds ratio [OR] = 33.29, 95% confidence interval [CI] = 1.96–566.61, $p = 0.015$).(Table 2 **and** Fig. 2).

All Annexin V-FITC/PI analyses were performed using the BD Accuri™ C6 flow cytometer, and gating was conducted within the BD Accuri software using its standardized quadrant-based fluorescence discrimination model. Because the instrument was operated in numeric acquisition mode, raw scatter dot plots were not exported. To illustrate the analytical workflow, a standardized schematic representing the gating strategy used to identify viable, early apoptotic, late apoptotic, and necrotic populations is provided as Supplementary Fig. 1.

The study found that the IC_{50} concentrations of the evaluated toothpastes did not induce a significant apoptotic effect on the hGF cell line, as assessed by the Caspase-3 assay ($p > 0.05$).(Table 3 and Fig. 2).

Side-by-side bar graph demonstrating early apoptosis (Annexin V) and late apoptosis (Caspase-3) responses for each toothpaste formulation.

The toothpaste formulations did not demonstrate statistically significant genotoxic effects at the IC_{50} concentrations when assessed on the hGF cell line using the H2AX assay ($p > 0.05$). (Table 4 and Fig. 3)

Bar plot representing γ-H2AX fluorescence intensity as an indicator of DNA double-strand break formation induced by each toothpaste extract.

The antibacterial efficacy of the toothpaste formulations was assessed using the modified microdilution method, with the results summarized in Table 5. The presence of a red hue indicates active microbial metabolism, denoted as (+) in Table 5, while the absence of a red hue signifies microbial inhibition or death, indicated as (-). The findings reveal that Colgate Total 12, Colgate Maximum Cavity Protection, Klorhex, ROCS Kids, Oral-B Pro Expert Stages, and Sensodyne Pronamel for Kids were the most effective toothpastes against *S. mutans*.

The ranking of antibacterial efficacy against *S. mutans*, from highest to lowest, is as follows: Colgate Total 12 = Colgate Maximum Cavity Protection = Klorhex = ROCS Kids = Sensodyne Pronamel for Kids > Elmex Kinder =

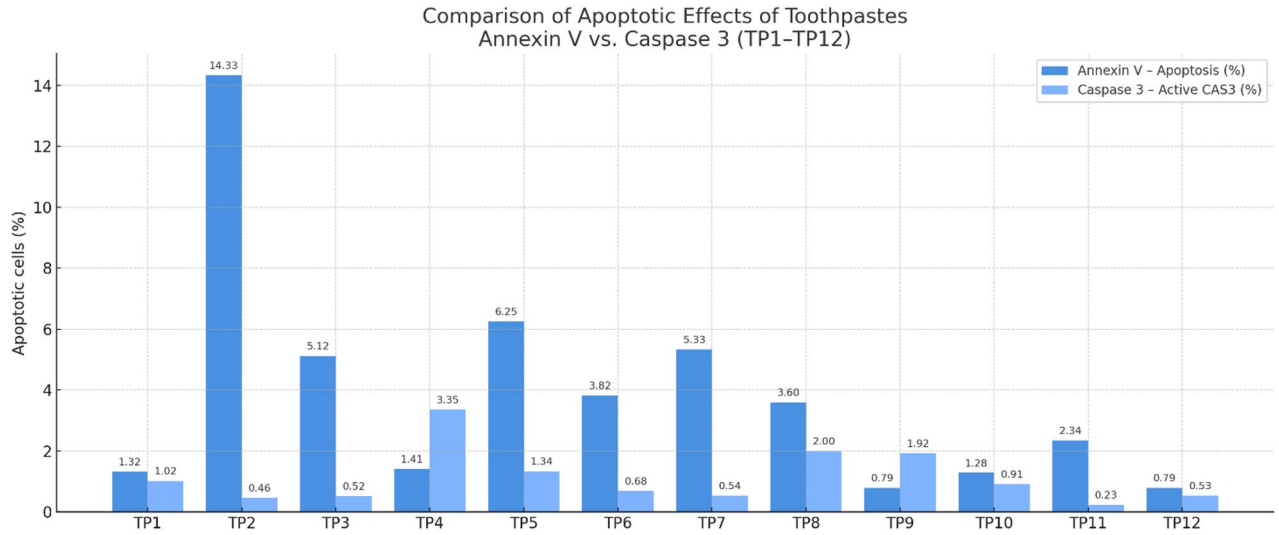

Fig. 2. Comparison of Annexin V and Caspase-3 apoptotic activity.

Toothpastes	INACTIVE CAS3(%)	ACTIVE CAS3 (%)	OR	95% C.I.	*p*
Control	99.37	0.64	1.000	-	-
TP1: Colgate Total 12	98.99	1.02	1.60	0.07–36.88	0.864
TP2: Colgate Maximum Cavity Protection	99.55	0.46	0.72	0.02–32.03	0.919
TP3: Curaprox Enzycal	99.48	0.52	0.81	0.02–31.85	0.769
TP4: Elmex Kinder	96.65	3.35	5.38	0.37–79.15	0.911
TP5: Klorhex	98.66	1.34	2.11	0.11–41.98	0.220
TP6: ROCS Baby	99.33	0.68	1.06	0.04–32.65	0.443
TP7: ROCS Kids	99.46	0.54	0.84	0.02–31.86	0.972
TP8: Meridol	98.00	2.00	3.17	0.19–53.62	0.625
TP9: Oral-B Pro Expert Stages	98.09	1.92	3.04	0.18–52.16	0.424
TP10: Sensodyne Pronamel for Kids	99.09	0.91	1.43	0.06–35.32	0.673
TP11: Sensodyne Multi Protection	99.77	0.23	0.36	0.00–42.33.00.33	0.927
TP12: Aquafresh Little Teeth	99.47	0.53	0.83	0.02–31.85	0.863

Table 3. Comparison of apoptotic effects of toothpastes by caspase 3.

Toothpastes	H2AX NEG (%)	H2AX POZ (%)	OR	95% C.I.	*p*
Control	98.23	1.77	1.00	-	-
TP1: Colgate Total 12	96.67	3.33	1.91	0.30–12.09.30.09	0.491
TP2: Colgate Maximum Cavity Protection	96.40	3.60	2.07	0.34–12.81	0.433
TP3: Curaprox Enzycal	97.15	2.86	1.63	0.25–10.87	0.612
TP4: Elmex Kinder	96.04	3.96	2.29	0.38–13.77	0.366
TP5: Klorhex	97.91	2.09	1.19	0.16–8.94	0.870
TP6: ROCS Baby	98.16	1.85	1.05	0.13–8.37	0.966
TP7: ROCS Kids	96.77	3.23	1.85	0.29–11.83	0.515
TP8: Meridol	96.97	3.03	1.73	0.27–11.31	0.565
TP9: Oral-B Pro Expert Stages	95.51	4.50	2.62	0.45–15.22	0.285
TP10: Sensodyne Pronamel for Kids	98.22	1.78	1.01	0.12–8.21	0.996
TP11: Sensodyne Multi Protection	98.19	1.81	1.02	0.13–8.28	0.983
TP12: Aquafresh Little Teeth	97.63	2.37	1.35	0.19–9.63	0.767

Table 4. Comparison of genotoxic effects of toothpastes by H2AX.

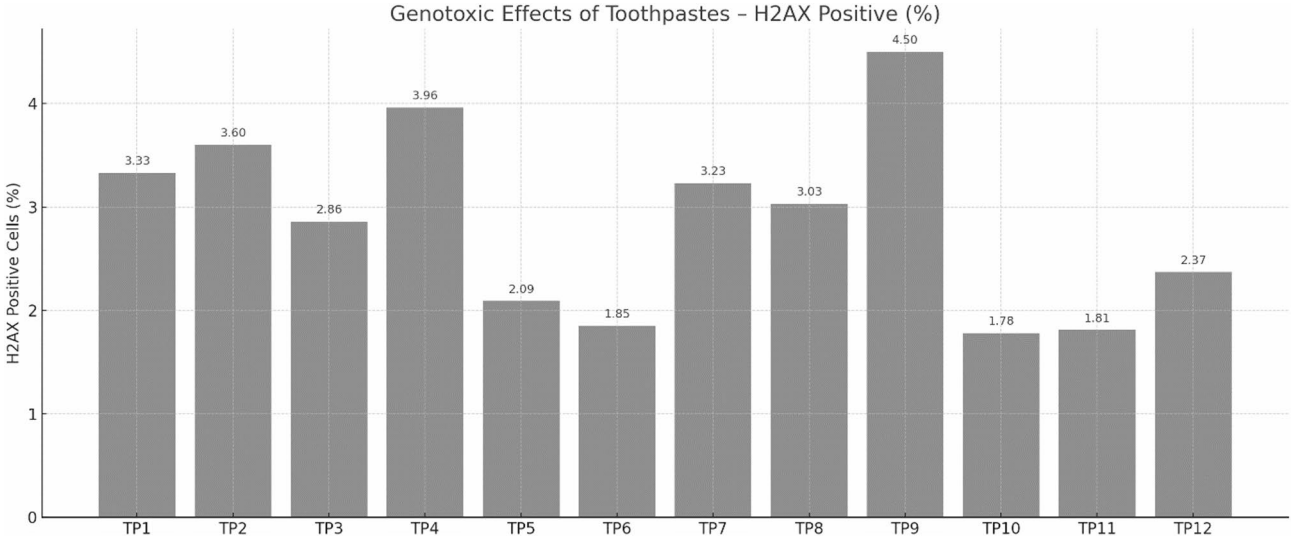

Fig. 3. Genotoxicity assessment using the γ-H2AX assay.

Meridol = Sensodyne Multi Protection > Aquafresh Little Teeth = Oral-B Pro Expert Stages > Curaprox Enzycal = ROCS Baby. (Fig. 4)

For *L. rhamnosus*, Meridol and Elmex Kinder exhibited the highest antibacterial efficacy. The order of antibacterial activity against *L. rhamnosus*, from highest to lowest, is as follows: Meridol = Elmex Kinder > Colgate Total 12 > Colgate Maximum Cavity Protection = Sensodyne Multi Protection > Klorhex > Curaprox Enzycal = Sensodyne Pronamel for Kids = ROCS Baby = Aquafresh Little Teeth > ROCS Kids > Oral-B Pro Expert Stages. (Fig. 4)

Heatmap showing normalized antibacterial activity of each toothpaste formulation against two bacterial species.

To provide a concise overview of the comparative outcomes across all experimental methods, a summary graphic integrating cytotoxic, apoptotic, genotoxic, and antibacterial results for all toothpaste formulations has been included (Fig. 5). This composite figure allows the major findings to be visualized simultaneously and facilitates cross-formulation interpretation.

The graphic synthesizes IC_{50} cytotoxicity values, Annexin V–derived early/late apoptosis percentages, Caspase-3 activation intensity, γ-H2AX genotoxicity signals, and antibacterial activity against *S. mutans* and *L. rhamnosus*. All parameters are normalized to allow direct comparison among toothpastes. Higher cytotoxic and apoptotic responses correspond to increased color intensity, whereas stronger antibacterial activity is indicated by higher suppression values. This integrated visualization enables rapid interpretation of inter-formulation differences across all biological endpoints.

Discussion

The various compounds of toothpaste formulations not only interact with the tooth surface but also make contact with the gingival tissues. Although toothpastes are generally regarded as beneficial for oral cleaning, the inclusion of fluoride and detergents in their formulations may pose a risk of inducing cellular-level damage to the soft tissues[22]. These materials may persist in the oral cavity for several hours following application, and the concentration of active ingredients within mucosal tissues may be higher than in saliva[23]. Considering such knowledge, the objective of this study is to evaluate the cytotoxic, apoptotic, and genotoxic effects of various toothpaste formulations on hGF and to compare their antibacterial efficacy against *S. mutans* and *Lactobacillus*.

Cytotoxicity values indicate the overall reduction in viable cells, whereas apoptosis assays provide mechanistic insight into the pathways associated with programmed cell death. Annexin V identifies early apoptosis through phosphatidylserine externalization, while Caspase-3 activation reflects irreversible execution-phase apoptosis. As cells may also undergo non-apoptotic death mechanisms such as necrosis or pyroptosis, the combined use of viability assays and apoptosis markers offers a more comprehensive evaluation of toothpaste-induced cellular effects.

The results of the present study demonstrated that all tested toothpaste formulations induced varying degrees of cytotoxicity in the hGF cell cultures. Detergents, commonly incorporated into toothpaste formulations for their surfactant, foaming, and antibacterial properties, are known contributors to such effects[24]. As the mechanistic background of surfactant-related cytotoxicity has been added to the Introduction, this section now focuses on integrating the study findings with prior evidence rather than repeating foundational concepts.

The toothpastes utilized in the present study can be primarily classified into four distinct categories based on the nature of their detergent components: anionic, amphoteric, cationic, and non-ionic surfactants. Anionic detergents commonly found in toothpaste formulations include sodium lauryl sulfate (SLS), sodium methyl cocoyl taurate (Addinol), sodium stearate (sodium octadecanoate), sodium lauryl sarcosinate, and sodium C12-14 olefin sulfonate. Among these, SLS is the most prevalent ingredient. SLS is characterized by its stable

		Dilution rates of toothpastes								
Toothpastes	**Microorganisms**	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
TP1: Colgate Total 12	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	-	+
	L. rhamnosus ATCC 7469	-	-	-	-	-	-	+	+	+
TP2: Colgate Maximum Cavity Protection	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	-	+
	L. rhamnosus ATCC 7469	-	-	-	-	-	+	+	+	+
TP3: Curaprox Enzycal	*S. mutans* ATCC 25,175	-	-	+	+	+	+	+	+	+
	L. rhamnosus ATCC 7469	-	-	+	+	+	+	+	+	+
TP4: Elmex Kinder	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	+	+
	L. rhamnosus ATCC 7469	-	-	-	-	-	-	-	+	+
TP5: Klorhex	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	-	+
	L. rhamnosus ATCC 7469	-	-	-	-	+	+	+	+	+
TP6: ROCS Baby	*S. mutans* ATCC 25,175	-	-	+	+	+	+	+	+	+
	L. rhamnosus ATCC 7469	-	-	+	+	+	+	+	+	+
TP7: ROCS Kids	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	-	+
	L. rhamnosus ATCC 7469	-	+	+	+	+	+	+	+	+
TP8: Meridol	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	+	+
	L. rhamnosus ATCC 7469	-	-	-	-	-	-	-	+	+
TP9: Oral-B Pro Expert Stages	*S. mutans* ATCC 25,175	-	-	-	-	-	-	+	+	+
	L. rhamnosus ATCC 7469	+	+	+	+	+	+	+	+	+
TP10: Sensodyne Pronamel for Kids	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	-	+
	L. rhamnosus ATCC 7469	-	-	+	+	+	+	+	+	+
TP11: Sensodyne Multi Protection	*S. mutans* ATCC 25,175	-	-	-	-	-	-	-	+	+
	L. rhamnosus ATCC 7469	-	-	-	-	-	+	+	+	+
TP12: Aquafresh Little Teeth	*S. mutans* ATCC 25,175	-	-	-	-	-	-	+	+	+
	L. rhamnosus ATCC 7469	-	-	+	+	+	+	+	+	+

Table 5. Comparison of antibacterial effects of toothpastes by modified microdilution method.

molecular structure and low surface tension, which enhances the paste's ability to spread uniformly across the tooth surface. Additionally, SLS exhibits antimicrobial properties, inhibiting the growth of various bacterial species. This antibacterial action is attributed to SLS's ability to adsorb onto the bacterial cell wall, penetrate its porous structure, and interact with cell membrane components, including lipids and proteins. By compromising the integrity of the bacterial cell membrane, SLS increases cell permeability, leading to the leakage of intracellular components and, ultimately, cell lysis[25,26]. In the present study, two toothpastes; Colgate Total 12 and Colgate Maximum Cavity Protection demonstrated the most potent antibacterial effects against *S. mutans*, both of which contained SLS. However, despite their antibacterial efficacy, certain adverse cellular effects associated with SLS were also observed. Specifically, four toothpastes; Colgate Total, Colgate Maximum Cavity Protection, Sensodyne Multi Protection, and Oral-B Pro Expert Stages exhibited varying degrees of cytotoxicity, and one toothpaste, Colgate Maximum Cavity Protection, induced significant apoptotic effects in the Annexin V assay.

Existing literature suggests that SLS may contribute to the reduction of keratinization in the human oral epithelium. Research indicates that SLS can penetrate deeper into the mucosal layers, compromising the mucin layer, denaturing proteins in epithelial cells, and altering the solubility of structural lipids within the cell membrane. These disruptions hinder the proper functioning of living tissue and may lead to increased

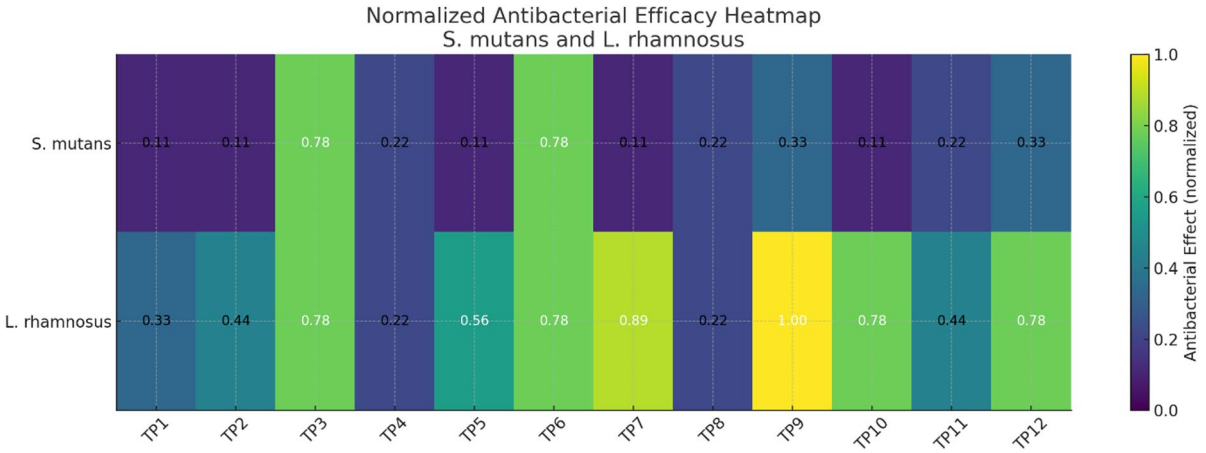

Fig. 4. Antibacterial efficacy heatmap against *S. mutans* and *L. rhamnosus*.

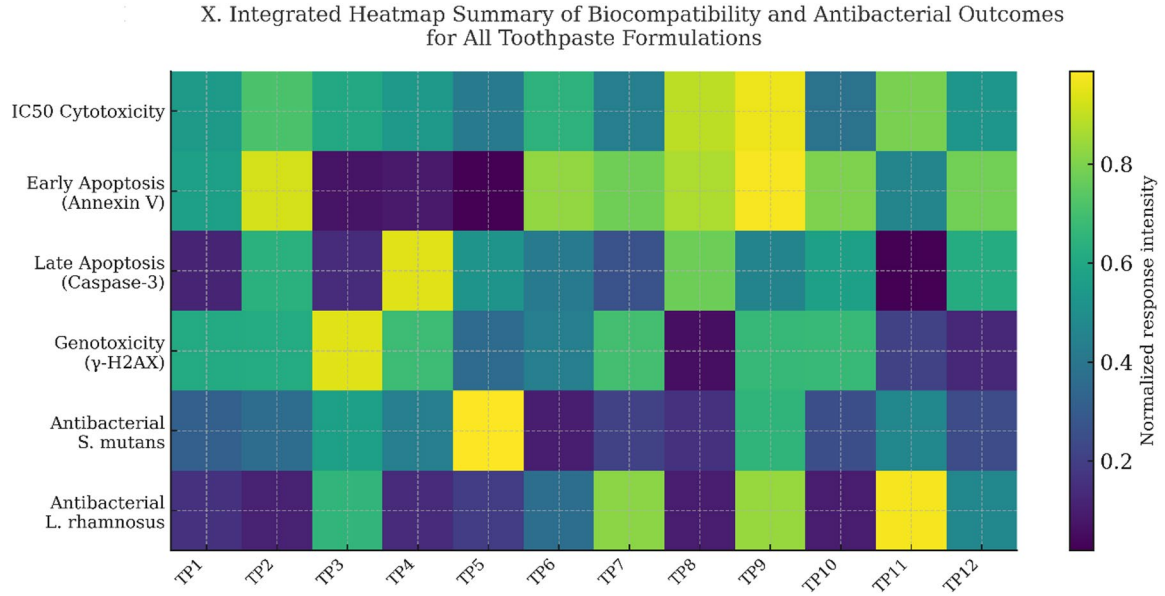

Fig. 5. Integrated summary of biocompatibility and antibacterial outcomes across all toothpaste formulations.

permeability of the cell membrane, further exacerbating cellular damage. These findings highlight the dual nature of SLS in toothpaste formulations, as it offers antibacterial benefits while potentially inducing harmful effects at the cellular level[16].

Cocamidopropyl betaine (CABP), an amphoteric surfactant, is frequently employed in toothpaste formulations due to its relatively mild irritancy profile and enhanced biocompatibility compared to other surfactants, such as SLS. In the present study, the toothpastes containing CABP included Meridol, Sensodyne Pronamel for Kids, ROCS Kids, Elmex Kinder, and Aquafresh Little Teeth. CABP is noted for its reduced potential to induce mucosal irritation and its lower foaming capacity relative to SLS. Furthermore, it has been documented to demonstrate improved biocompatibility, making it a preferable alternative in formulations aimed at minimizing adverse effects on oral tissues. Its use in toothpaste is beneficial for individuals with heightened sensitivity to harsher detergents, contributing to a more favorable safety profile for daily oral care. Contrary to earlier studies[19,27], the outcomes of the present study demonstrated that the Meridol toothpaste containing CABP had the strongest cytotoxic effect and exhibited cytotoxicity levels comparable to SLS-containing formulations.

Steareth-20, Steareth-30, and Polyxamer 470 are examples of non-ionic surfactants commonly found in toothpaste formulations. In the present study, only Curaprox Enzycal toothpaste, which contains Steareth-20, was tested. While this toothpaste showed cytotoxic effects, the primary cause of its toxicity was the acidic enzymatic compounds in its ingredients, rather than the surfactant itself. Non-ionic surfactants like Steareth-20 are generally less irritating than anionic detergents. However, the presence of additional active ingredients, such as enzymes, can increase the overall cytotoxicity of the product. These enzymatic components are more likely to interact with and damage cellular structures, potentially causing harm to oral mucosal tissues.

This study did not include isolated positive control surfactants such as pure sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), or other detergent standards. As a consequence, the specific contribution of individual surfactants to the overall cytotoxic and apoptotic responses cannot be directly inferred. Our conclusions are therefore restricted to the composite effects of the complete commercial formulations evaluated. Future studies integrating purified SLS, CAPB, and additional surfactant controls would allow more detailed mechanistic dissection of ingredient-specific cytotoxicity and help distinguish the relative contributions of detergents from other toothpaste components.

It is important to note that the IC_{50} values obtained in this study represent the cytotoxic potential of the complete toothpaste extracts rather than isolated surfactant molecules. Because manufacturers do not routinely disclose the exact quantitative concentrations of detergents such as sodium lauryl sulfate or cocamidopropyl betaine on product labels, it is not possible to derive precise IC_{50} thresholds for the individual surfactants from these data. Nevertheless, the overall pattern observed was that formulations containing classical anionic surfactants (e.g., sodium lauryl sulfate) tended to exhibit lower IC_{50} values, consistent with higher in vitro cytotoxicity, whereas low-foaming or enzyme-based formulations without SLS generally demonstrated higher IC_{50} values and therefore comparatively better biocompatibility under the conditions tested. These in vitro IC_{50} ranges should be interpreted as conservative indicators of relative cytotoxic risk, acknowledging that actual intraoral exposure involves considerable dilution by saliva and brief contact times during routine toothbrushing.

It should be emphasized that the biological outcomes observed in this study reflect the integrated effects of the complete toothpaste formulations. Although detergents such as sodium lauryl sulfate or cocamidopropyl betaine are known contributors to cytotoxicity and may partly explain the lower IC_{50} values observed in certain formulations, these effects cannot be ascribed solely to surfactants. Commercial dentifrices contain numerous functional ingredients including fluoride salts, abrasives, humectants, preservatives, and pH modifiers that may act additively or synergistically. Therefore, the observed cytotoxic, apoptotic, and genotoxic responses should be interpreted as the combined result of multi-component formulations rather than the isolated action of a single ingredient class.

Cvick et al. conducted a study using the MTT assay to evaluate the cytotoxic effects of toothpaste formulations containing different detergents (SLS, amine fluoride, CABP, and Steareth-20) on various cell lines. The results indicated that toothpastes with SLS and amine fluoride significantly decreased cell viability, while those containing CABP and Steareth-20 exhibited higher LC50 values, indicating lower cytotoxicity. These findings highlight the variable cytotoxicity associated with different detergent types in toothpaste formulations[19]. Moore et al. examined the cytotoxic effects of toothpastes containing Adinol, SLS, Tego Betaine, and Pluronic on immortalized normal oral keratinocyte (TERT-1) cells. The study found that, except for Pluronic, higher concentrations of all the detergents caused a significant decrease in cell viability. These results emphasize the potential cytotoxic effects of common detergent ingredients in toothpaste, suggesting that their concentration may directly impact cellular health, especially in oral epithelial cells[8]. The study by Birant et al. explored the effects of six different kids toothpaste formulations on the viability, osteogenic, and chondrogenic properties of human dental periodontal ligament stem cells and gingival stem cells. The results showed that toothpaste formulations containing SLS had a significantly greater negative impact on cell viability compared to other formulations. This highlights the potential cytotoxicity of SLS, particularly in oral stem cell populations, suggesting that its presence in children's toothpaste could pose a risk to cellular health in the oral cavity[18]. Furthermore, common toothpaste chemicals and found that SLS resulted in the least favorable outcomes[11]. Klorhex, in contrast, demonstrated the highest biocompatibility scores, likely due to its formulation, which excludes SLS and CABP. These detergents are typically linked to cytotoxic effects, and their absence in Klorhex may contribute to its superior biocompatibility. As such, Klorhex appears to be a suitable option for individuals seeking oral care products with minimal risk of adverse effects on oral tissues.

The study examined the compositional characteristics of twelve distinct toothpaste formulations and found that, while eleven of these twelve products contained fluoride, the ROCS Baby toothpaste did not. The majority of the toothpastes featured sodium fluoride, although variations in fluoride types and concentrations were observed across the formulations. Colgate Maximum Cavity Protection incorporates additional ingredients such as arginine, calcium carbonate, and sodium bicarbonate. It is important to note that calcium carbonate has the potential to bind sodium fluoride, thereby reducing its efficacy as an anti-caries agent. In contrast, sodium monofluorophosphate remains unaffected by calcium carbonate binding, indicating that the formulation of Colgate Maximum Cavity Protection may have been developed with this interaction in mind, thereby ensuring the optimal efficacy of the fluoride component in combating dental caries[3].

In a study conducted by Pecci-Llorer et al., the cytotoxic effects of five commercially available children's toothpastes; Oral B Kids 3+, Fluor Kin Calcium, PHB Junior, Colgate 3+, and Vitis Kids on human gingival fibroblasts were assessed using the MTT assay. In addition, the apoptotic effects of these toothpastes were evaluated using the Annexin V assay. The results highlight the significant variability in cytotoxicity among children's toothpaste formulations and emphasize the need for careful consideration of their biocompatibility in oral health applications[20].

The study conducted by Ghapanchi et al. examined the cytotoxic effects of 16 different toothpaste formulations on primary epithelial cells of the oral cavity and HeLa cells, utilizing the MTT assay. In addition, the antibacterial efficacy of these toothpastes against *S. mutans* was assessed. The results revealed significant variability in the cytotoxic effects of the toothpastes on cultured cells, with a notable increase in toxicity levels corresponding to prolonged exposure. The results demonstrated that all toothpastes tested exhibited substantial antibacterial activity against *S. mutans*[28], whereas present investigation revealed significant variations in the antibacterial efficacy of the tested toothpastes on the same bacterial species.

In a study by de Mello Rode et al., the cytotoxic effects of five commercially available whitening toothpastes were evaluated using hGF cell cultures. The MTT assay was employed to assess cell viability, and genotoxicity

was evaluated using the micronucleus test. The researchers concluded that the cytotoxicity observed in these formulations was likely due not only to the detergent compounds but also to the fluoride content. Fluoride has been implicated in inducing oxidative stress, disrupting intracellular homeostasis, and promoting lipid peroxidation mechanisms that can lead to alterations in gene expression and, ultimately, apoptosis. These findings highlight the dual impact of detergent agents and fluoride in contributing to the cytotoxic effects of toothpaste formulations. Interestingly, despite the observed cytotoxicity, the study found that none of the toothpastes exhibited statistically significant genotoxic properties. This aligns with the findings of the present study, where no genotoxic effects were observed in the evaluated toothpastes. This suggests that, while some toothpastes may cause cellular damage at the cytotoxic level, they may not necessarily induce genetic mutations or damage at the molecular level, further suggesting the importance of assessing both cytotoxicity and genotoxicity in the evaluation of oral care products[29].

A critical comparison with previous work reveals both concordances and divergences that help contextualize the present findings. Studies reporting pronounced cytotoxic effects of SLS-containing formulations[19,20] are broadly consistent with the lower IC_{50} values observed in SLS-based products in the current study. However, other reports describing minimal cytotoxicity at similar extract concentrations appear to differ from our observations; such discrepancies may arise from variations in exposure time, the use of primary versus immortalized cells, or differences in formulation additives that modulate detergent activity. Likewise, the relatively lower apoptotic and genotoxic signatures noted in enzyme-based or low-foam formulations in our dataset challenge earlier reports suggesting negligible differences across formulation categories, indicating that product-specific matrix effects may play a larger role than previously assumed. By integrating these contrasting findings, our results highlight the need to interpret toothpaste biocompatibility within the broader chemical and formulation context rather than attributing effects solely to individual ingredients.

Beyond detergent-associated cytotoxicity, fluoride-mediated toxicity must also be considered. Several investigations demonstrate that sodium fluoride and stannous fluoride can impair fibroblast viability, trigger oxidative stress responses, and alter intracellular redox homeostasis. Fluoride ions have been shown to modulate apoptotic protein expression, including Caspase-3 activation, and to reduce mitochondrial membrane potential in vitro. These findings support the interpretation that part of the cytotoxicity observed in fluoride-containing formulations may be attributable not only to surfactants but also to fluoride-induced metabolic stress, particularly under conditions of prolonged mucosal exposure. Although fluoride remains the cornerstone of caries prevention, its potential for inducing soft-tissue cytotoxicity should not be overlooked. Therefore, interpretation of fluoride-containing formulations should balance therapeutic benefit with potential biological cost.

The addition of enzymes in toothpaste formulations has been shown to enhance immune system responses and support overall oral health. Curaprox Enzycal, one of the most widely recognized enzyme-containing toothpastes, was the focus of a study by Paque et al., which compared the effects of enzyme-containing toothpastes to enzyme-free alternatives on dental plaque formation, both in vitro and in vivo. The agar diffusion test results revealed that Colgate Total 12, a non-enzyme toothpaste, exhibited the strongest antibacterial activity against *S. mutans*. In contrast, Curaprox Enzycal, despite containing enzymes, demonstrated the least antibacterial effect. These findings highlight the varying levels of antibacterial efficacy between enzyme-based and non-enzyme-based toothpastes. While enzymatic components may play a role in modulating the oral microbiome, their influence on plaque formation and oral health can differ significantly from that of traditional antibacterial agents. The results of this study align with the data observed in the present investigation, further confirming the differences in antibacterial properties between enzyme-containing and non-enzyme-containing formulations[29]. These inter-formulation differences underscore the need for reliable antimicrobial testing methods, particularly for dentifrice matrices. In present study, the potential interference of toothpaste pigments and opacifiers with TTC reduction was controlled by using clarified supernatants and toothpaste-only TTC blanks, which did not show red formazan formation, thereby supporting the specificity of the colorimetric readout for bacterial metabolic activity. Standard MIC/MBC protocols are not optimized for dentifrice matrices, which contain non-soluble abrasives and surfactants that interfere with turbidity-based detection; thus, a modified microdilution TTC system provided a more accurate and reproducible endpoint for comparative antibacterial assessment. A limitation of the present study is the absence of quantitative bacterial load measurements such as CFU enumeration or qPCR, which could enhance comparability across studies. However, the complex excipient composition of toothpaste formulations may inhibit culture-based recovery or PCR amplification, reducing data reliability. Future studies will incorporate complementary quantitative approaches to validate and extend the metabolic TTC-based findings reported here.

This investigation was limited by its in vitro design, which does not fully represent the oral environment. The 72-hour exposure period does not mimic the short, intermittent nature of real toothbrushing, prolonged exposure is a standard in vitro toxicological approach designed to reveal cumulative or delayed cellular responses. However, this model reflects a 'worst-case scenario,' enabling the detection of subtle cytotoxic or apoptotic effects that may not be observable under brief exposure but remain relevant for assessing biocompatibility, particularly in susceptible or inflamed oral tissues. Another limitation of the present study is the use of gingival fibroblasts derived from only two biological donors. Although this approach aligns with standard practices in in vitro cytocompatibility research where primary cell availability, expansion capacity, and phenotypic stability restrict donor numbers it inherently underrepresents inter-individual biological variability. As such, the generalizability of the findings should be interpreted with caution, and future studies incorporating a broader donor pool would help strengthen population-level extrapolation. Only a restricted number of toothpaste formulations were tested, and the results may not be applicable to all products. Furthermore, the assays focused on short-term cytotoxicity and antibacterial effects, while long-term clinical performance requires further study.

In addition to these methodological limitations, biological factors unique to the oral cavity further constrain the extrapolation of in vitro findings to clinical conditions. Factors such as blood circulation, saliva composition, the mucosal barrier, creatine levels, and the presence of normal microbial flora play critical roles in modulating the protective capacity of the oral cavity against harmful substances. Consequently, the findings of the present study should be interpreted with these variables in consideration.

The findings of this study carry several clinical implications. First, the marked variability in IC_{50}, apoptotic, and genotoxic profiles among commercially available toothpastes suggests that formulation-specific differences particularly in surfactant systems may meaningfully influence soft-tissue compatibility. Products containing anionic detergents such as SLS demonstrated lower biocompatibility in vitro, indicating that their use may warrant caution in patients with mucosal sensitivity, recurrent aphthous stomatitis, or compromised epithelial barriers. Conversely, enzyme-based or low-foam formulations showed comparatively higher biocompatibility, supporting their suitability for pediatric users and individuals prone to irritation. Second, the antibacterial data reinforce the need to balance antimicrobial efficacy with cellular tolerance, as formulations with strong antibacterial effects did not always exhibit favorable cytotoxic profiles. Third, given that toothpaste is used daily and lifelong, even modest differences in biocompatibility may acquire clinical relevance over time. These results therefore underscore the importance of personalized dentifrice selection and highlight the need for manufacturers to optimize surfactant content, buffering capacity, and overall formulation design to enhance both safety and therapeutic performance.

Building upon our formulation-specific findings, a broader perspective on dentifrice biocompatibility can be summarized as follows for clinical consideration: For clinicians who may not routinely follow the rapidly evolving literature on dentifrice formulation and biocompatibility, assessing the safety and antibacterial efficacy of toothpastes can be challenging. Current evidence indicates that caution should be exercised with certain ingredient classes and formulation strategies, particularly when recommending products for pediatric patients, individuals with mucosal sensitivity, or long-term daily use.

Anionic surfactants, most notably sodium lauryl sulfate (SLS), represent one of the most consistently reported contributors to soft-tissue cytotoxicity. Although SLS enhances foaming and antimicrobial dispersion, numerous in vitro studies have demonstrated its ability to disrupt cell membranes, increase epithelial permeability, and reduce fibroblast viability in a dose-dependent manner. These effects may be clinically relevant in patients with compromised mucosal barriers, recurrent aphthous stomatitis, or inflammatory oral conditions.

Amphoteric surfactants, such as cocamidopropyl betaine (CAPB), are often considered milder alternatives to SLS; however, accumulating evidence suggests that CAPB-containing formulations may still exhibit notable cytotoxic effects depending on concentration and interaction with other formulation components. Therefore, surfactant class alone should not be assumed to predict biocompatibility.

Fluoride compounds, while indispensable for caries prevention, have also been shown to induce oxidative stress, mitochondrial dysfunction, and reduced cell viability in oral fibroblasts under prolonged or concentrated exposure conditions. Although these findings do not contraindicate fluoride use, they highlight the importance of cautious selection in patients with inflamed or ulcerated oral tissues, where soft-tissue exposure may be prolonged.

Toothpastes incorporating potent antimicrobial agents, such as chlorhexidine or triclosan, may offer strong antibacterial efficacy but can also contribute to increased cytotoxic or irritative potential, particularly when combined with aggressive surfactant systems. In such cases, antimicrobial benefit should be weighed against possible adverse effects on oral soft tissues.

Finally, flavoring agents, preservatives, and excipients, including essential oils, parabens, propylene glycol, and polyethylene glycols, have been associated with allergic contact reactions and mucosal irritation in susceptible individuals. These components warrant particular attention when treating patients with a history of oral hypersensitivity.

Collectively, these findings underscore that toothpaste selection should prioritize the overall formulation profile, favoring products that demonstrate low cytotoxicity, absence of significant apoptotic or genotoxic effects, and adequate antibacterial activity, rather than focusing solely on individual active ingredients.

Fluoride remains the cornerstone of modern caries prevention, and concentrations of 1000, 1450 and 5000 ppm are widely endorsed by dental associations worldwide for different risk groups. However, from a biocompatibility perspective, current evidence indicates that fluoride-related effects on oral soft tissues are not governed by concentration alone, but rather by a combination of dose, exposure duration, formulation composition, and mucosal condition.

In vitro studies have demonstrated that commonly used fluoride salts, including sodium fluoride and stannous fluoride, can induce oxidative stress, mitochondrial dysfunction, and reduced fibroblast viability when exposure is prolonged or concentrations are elevated. Nevertheless, under conditions that approximate routine toothbrushing characterized by short contact times and significant dilution by saliva toothpastes containing approximately 1000–1450 ppm fluoride are generally considered biologically well tolerated, particularly when formulated with mild or low-irritancy surfactant systems.

In contrast, high-fluoride dentifrices (≈ 5000 ppm), although highly effective for caries control in high-risk individuals, may increase the likelihood of soft-tissue irritation or cytotoxic stress if used indiscriminately or for extended periods. Accordingly, such formulations should be restricted to professionally indicated use and applied under clinical supervision, especially in patients with compromised oral mucosa.

Importantly, these findings underscore that no single fluoride concentration can be universally defined as completely non-harmful to oral tissues. Instead, fluoride safety should be evaluated within the broader context of overall formulation design, frequency of use, patient-specific risk factors, and mucosal health status. A balanced approach that maximizes anticaries efficacy while minimizing soft-tissue exposure remains essential for optimal clinical outcomes.

Although IC_{50} values derived from real-time cell analysis provide a sensitive and quantitative indicator of overall cytotoxicity, they do not exclusively reflect apoptotic cell death. Cytotoxicity represents a composite biological outcome encompassing apoptosis, necrosis, membrane disruption, metabolic inhibition, and loss of cell adhesion. In contrast, Annexin V and Caspase-3 assays selectively identify programmed apoptotic processes and therefore interrogate a more specific mechanistic pathway.

In the present study, TP8 (Meridol) exhibited the lowest IC_{50} value, indicating the highest overall cytotoxic potential. This finding suggests that TP8 induces rapid and pronounced cellular stress, likely mediated by the combined action of amine fluoride, stannous fluoride, and amphoteric surfactants, resulting in loss of cell viability through mechanisms that may not predominantly involve classical apoptosis. Such mechanisms may include membrane destabilization, oxidative stress, or mitochondrial dysfunction leading to non-apoptotic cell death.

In contrast, TP2 (Colgate Maximum Cavity Protection) demonstrated the strongest apoptotic response in the Annexin V assay despite exhibiting a higher IC_{50} value. This pattern indicates a preferential activation of apoptosis-related signaling pathways rather than immediate non-specific cytotoxicity. The presence of sodium lauryl sulfate, arginine, and monofluorophosphate fluoride may facilitate phosphatidylserine externalization and early apoptotic signaling without inducing rapid metabolic collapse.

These findings underscore that the most cytotoxic formulation is not necessarily the most apoptotic, and that discrepancies between IC_{50}-derived cytotoxicity and apoptosis-specific assays reflect fundamental differences in the underlying mechanisms of cell injury. Accordingly, comprehensive biocompatibility assessment of dentifrice formulations requires integration of viability, apoptosis, and genotoxicity endpoints rather than reliance on a single metric.

To facilitate clinically meaningful interpretation, the tested dentifrices were categorized into four formulation groups: fluoride-containing adult toothpastes, pediatric formulations, enzyme-based toothpastes, and chlorhexidine-containing formulations. Because this investigation was conducted under standardized in vitro conditions, the terms safe and unsafe should be interpreted as relative biocompatibility profiles rather than absolute clinical judgments. Within the present dataset, the chlorhexidine-containing category (TP5) demonstrated the most favorable biological profile, characterized by the highest IC_{50} value (lowest cytotoxicity), absence of statistically significant apoptosis or genotoxicity signals, and strong antibacterial performance against *S. Mutans*. In contrast, the fluoride-containing adult category included the formulation with the highest cytotoxic potential (TP8) and also contained the only toothpaste (TP2) that triggered a statistically significant apoptotic response at IC_{50} exposure, suggesting that this category may pose greater soft-tissue compatibility concerns under prolonged exposure conditions.

Pediatric and enzyme-based formulations exhibited non-uniform responses, indicating that these categories cannot be presumed intrinsically biocompatible; rather, outcomes appear to be driven by formulation-specific determinants, particularly surfactant systems (e.g., SLS and CAPB), which have been repeatedly implicated as key contributors to cytotoxicity in oral cell models. Accordingly, evidence-based dentifrice selection should prioritize formulations that combine low cytotoxicity with sufficient antibacterial activity against cariogenic bacteria, especially for children and individuals with mucosal sensitivity or compromised epithelial barriers.

The observation that fluoride-containing formulations may induce metabolic stress in gingival fibroblasts while simultaneously providing substantial protection against dental caries represents an apparent but biologically explicable paradox. Fluoride's anticariogenic effects are predominantly mediated through topical interactions with dental hard tissues and the biofilm, including promotion of enamel remineralization, inhibition of demineralization, and reduction of bacterial acidogenicity. These mechanisms occur primarily at the tooth surface and do not require sustained penetration into oral soft tissues.

In contrast, the metabolic stress responses observed in fibroblast cultures reflect conditions of direct, continuous cellular exposure at defined concentrations, as employed in standardized in vitro toxicological models. Under such conditions, fluoride ions have been shown to induce oxidative stress, mitochondrial dysfunction, and alterations in cellular energy metabolism, particularly when exposure is prolonged or concentrations are elevated. These effects are indicative of cellular stress responses rather than definitive predictors of clinical tissue damage.

Importantly, routine toothbrushing involves short contact times and substantial dilution by saliva, resulting in fluoride concentrations at the soft-tissue interface that are considerably lower and transient compared with in vitro exposure models. Consequently, the metabolic stress detected in fibroblast assays should be interpreted as a conservative indicator of relative biological reactivity, rather than as evidence against the clinical safety or efficacy of fluoride-containing dentifrices.

From a clinical perspective, fluoride should therefore be viewed as a therapeutic agent with a wide but finite safety margin, whose benefits in caries prevention clearly outweigh potential soft-tissue risks when used appropriately. Optimization of fluoride delivery through controlled concentration, formulation design, and patient-specific risk assessment remains essential to maximizing anticaries efficacy while minimizing unnecessary cellular stress.

Conclusion

The differences observed in the cytotoxic responses among the tested toothpaste formulations appear to be associated with variations in detergent ingredients and fluoride compounds. These factors not only affected biocompatibility but also influenced antibacterial activity in vitro. Formulations containing SLS and CABP showed a tendency toward reduced cell viability, whereas other formulations demonstrated comparatively lower cytotoxic effects. While these findings provide useful insights into the biological impact of commonly used toothpaste components, the results are limited to an in vitro model and should not be directly generalized to clinical practice without further confirmation through in vivo and clinical studies.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

S.Y.S., C.Y.E., A.U. and C.G. collected data and performed the measurements.A.A, D.Ç., S.Y.S, C.Y.E, C.K., B.Ö.Y., A.T., A.U. and C.G. contributed to the design and implementation of the research, to the analysis of the results of the manuscript. A.A. and D.Ç wrote the manuscript draft and prepared tables and figures. All author reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics Approval and consent to participate
The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Ege University, Faculty of Medicine (protocol number:14 – 5.1/8). The two participants from whom the human gingival fibroblast samples were collected provided written informed consent to take part in the present study.

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Correspondence and requests for materials should be addressed to D.Ç.

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