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ORIGINAL RESEARCH

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Cytotoxicity and antibacterial effects of sodium phytate- and bromelain-containing experimental toothpastes for pediatric black stain management: an *in vitro* study

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Abstract

Background: Black stain is a common form of extrinsic tooth discoloration in pediatric patients. Recently, interest in home-care strategies, such as toothbrushing with specialized toothpaste formulations, has increased. Natural ingredients, including sodium phytate and bromelain, have been proposed as alternatives to conventional whitening agents for managing black stain; however, their cytotoxic and antibacterial performance remains unclear. Therefore, this study aimed to evaluate the cytotoxicity and antibacterial activity of two experimental toothpaste formulations for black stain management. **Methods**: Three toothpaste groups were tested: Group A (0.85% w/w sodium phytate, fluoride-free), Group B (5% w/w bromelain, fluoride-free), and Group C (commercial children's toothpaste, 500 ppm fluoride), all containing sodium lauryl sulfate (SLS) as a common surfactant. Cytotoxicity was assessed using the XTT (sodium 3′-(1-phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzenesulphonic acid) assay on L929 cells at six serial dilutions (1:1–1:32) with exposure times of 1, 2, and 5 minutes. Cell viability below 70% was considered cytotoxic according to the International Organization for Standardization (ISO) 10993-5. Antibacterial efficacy against *Streptococcus mutans* (*S. Mutans*) was assessed using agar-well diffusion. Penicillin-streptomycin and distilled water served as positive and negative controls, respectively. **Results**: All toothpaste formulations reduced cell viability below 70% at dilutions from 1:1 to 1:8. At the 2-minute exposure and 1:8 dilution, Group A was the most cytotoxic, followed by Group B, whereas Group C exhibited the highest cell viability ($p < 0.001$). All formulations demonstrated significant antibacterial activity against *Streptococcus mutans*. Group A's inhibition zone (19.54 $\pm$ 0.99 mm) was comparable to the positive control ($p > 0.05$) and significantly greater than Groups B and C ($p < 0.05$), which were comparable to each other ($p > 0.05$). **Conclusions**: While the experimental toothpastes demonstrated antibacterial activity, their cytotoxic potential warrants caution, and studies optimizing formulation composition, including SLS-free or fluoride-containing alternatives, are needed to confirm clinical safety and applicability.

Keywords

Bromelain; Sodium phytate; Dentifrices; Sodium lauryl sulfate; Antibacterial efficacy; Iron stain

1. Introduction

Black stain (BS) is a distinct form of extrinsic dental discoloration characterized by dark lines or patches on the enamel surface. It may occur in both adults and children, but is most commonly observed in children [1]. Several risk factors have been linked to BS development in children, including chromogenic bacteria, an iron-rich diet, iron supplements, and iron-containing medications. Despite these associated factors, the exact etiology of BS remains unclear, and its clinical management focuses primarily on removal and prevention [2–4]. Because of their dark appearance, these stains may negatively affect dental aesthetics in children, potentially influencing their social interactions, self-confidence, and overall quality of life [5].

BS deposits contain insoluble ferric compounds and elevated levels of calcium and phosphate, which contribute to strong adhesion to the tooth surface, resistance to removal, and rapid recurrence after professional cleaning [2, 6]. Although

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professional prophylaxis is the standard treatment for BS, its effectiveness is often limited by poor cooperation among pediatric patients. Consequently, interest in home-care strategies for stain removal and prevention has increased [7]. Combining mechanical cleaning through toothbrushing with chemical agents in toothpaste formulations may reduce dental stains and whiten teeth [8].

Modern toothpastes are formulated to prevent caries and periodontal disease. Conventional formulations typically contain abrasives, such as hydrated silica; binders; surfactants or detergents, such as sodium lauryl sulfate (SLS); preservatives; colorants; buffering agents; and active ingredients, such as fluorides. Whitening toothpastes may also contain low concentrations of carbamide peroxide or hydrogen peroxide [9]. However, whitening toothpastes often contain aggressive abrasives and chemicals that may cause irreversible enamel wear, particularly in pediatric enamel, which is thinner and less mineralized [10]. To balance effectiveness and safety, alternative whitening approaches are needed, with increasing interest in naturally derived agents. These agents may reduce stains through diverse biochemical mechanisms, including oxidation by fruit-derived acids, such as malic acid from apples; enzymatic protein degradation by bromelain from pineapple or papain from papaya; and chelation by agents such as phytic acid, which occurs in seeds and grains [11–13]. Compared with conventional abrasive methods, these mechanisms may provide a less aggressive approach to stain control. In this context, bromelain and sodium phytate (the salt form of phytic acid) have emerged as promising agents. Bromelain can degrade the protein matrix of the dental pellicle and loosen stain adhesion, whereas sodium phytate acts as a potent chelating agent by binding calcium and iron ions embedded in the stain [12, 14].

In addition to stain-removal efficacy, a clinically viable toothpaste formulation must fulfill its primary therapeutic objectives, including caries prevention and maintenance of oral health through adequate antibacterial activity, particularly against *Streptococcus mutans* (*S. mutans*), the primary cariogenic pathogen associated with dental caries [15, 16]. Furthermore, the ingredients used in these formulations should exhibit an acceptable biocompatibility profile without causing significant adverse biological effects on oral tissues [17, 18]. Therefore, evaluating both the cytotoxicity and antibacterial performance of new experimental formulations is essential, especially for pediatric use, because children are more vulnerable to tissue irritation and accidental product ingestion [19].

The present study, therefore, investigated the cytotoxicity and antibacterial properties of two experimental toothpaste formulations containing bromelain and sodium phytate that were developed for BS management and compared them with those of a conventional children's toothpaste. The aim of this study was to evaluate the antibacterial activity of these formulations against *S. mutans* and their *in vitro* cytotoxicity. The null hypothesis was that the experimental formulations would demonstrate cytotoxicity profiles and antibacterial efficacy comparable to those of the commercial toothpaste.

2. Materials and methods

This *in vitro* study evaluated the cytotoxicity and antibacterial efficacy of two experimental children's toothpastes containing bromelain and sodium phytate. These formulations were originally developed in a previous study to manage iron-induced staining [20] and were used for the present biological evaluation. Ethical approval was not required because commercial cell lines and bacterial strains were used.

2.1 Toothpaste formulations and study groups

Three toothpaste groups were evaluated: two experimental children's toothpastes formulated with 0.85% (w/w) sodium phytate (Group A) and 5% (w/w) bromelain (Group B), and a commercial children's toothpaste used as the control (Group C; Colgate-Palmolive Company). The pH of the experimental formulations was evaluated in triplicate using a calibrated pH meter (pH/Temp MW102 Pro, Milwaukee). The pH meter was calibrated using standard buffer solutions of pH 4 and pH 7 prior to measurements. The toothpastes were diluted with distilled water in a 1:3 weight ratio for pH assessment. The mean pH values were 8.09 $\pm$ 0.05 for the sodium phytate-containing formulation (Group A) and 8.72 $\pm$ 0.03 for the bromelain-containing formulation (Group B), both well below the ISO 11609:2017 upper limit of pH 10.5 for dentifrices [21]. The toothpaste compositions and manufacturer information are summarized in Table 1. The concentrations of 0.85% sodium phytate and 5% bromelain were based on previously developed formulations [20]. The phytate concentration is consistent with the 0.5–1% range reported to be effective in the literature [14, 22]. However, no comparable consensus exists for bromelain in a toothpaste matrix, as previous studies have evaluated it in mouthwash formulations at different concentrations [23].

2.2 Cytotoxicity evaluation

Toothpaste extracts were prepared in accordance with ISO 10993-12 [24]. Briefly, 1 g of each toothpaste was weighed and mixed with 5 mL of Dulbecco's Modified Eagle Medium (DMEM; 233301612, Sigma–Aldrich, St. Louis, MO, USA), yielding a concentration of 0.2 g/mL. The mixtures were homogenized and incubated at 37 °C for 24 h. The extracts were then filtered through a 0.22 μm membrane to maintain sterility. The undiluted extracts (1:1) were serially diluted with DMEM to obtain dilutions of 1:2, 1:4, 1:8, 1:16, and 1:32.

L929 mouse fibroblast cells (American Type Culture Collection (ATCC), USA) were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS; 2251101RP, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (PenStrep; 43030723RAL, Serana, Pessin, BB, Germany) at 37 °C in 5% carbon dioxide (CO_2). After reaching 80% confluence, the cells were seeded into 96-well plates (1×10^4 cells/well) and incubated for 24 h. The cells were then exposed to 100 μL of each prepared toothpaste extract dilution (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) for 1, 2, or 5 min (Fig. 1). Culture medium was used as the control. Cell viability was assessed using the XTT (sodium3′-(1-phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)

TABLE 1. Ingredients of the toothpastes tested in this study.

Toothpaste Groups	Manufacturer	Ingredients
Group A: Sodium Phytate-Containing Toothpaste	Experimental	Sodium Phytate, Polyethylene glycol, Sorbitol, Sodium carboxymethyl cellulose, Sodium lauryl sulfate (SLS), Calcium carbonate, Glucite
Group B: Bromelain-Containing Toothpaste	Experimental	Bromelain, Sorbitol, Sodium carboxymethyl cellulose, Sodium lauryl sulfate (SLS), Calcium carbonate, Glucite
Group C: Colgate Kids	Colgate-Palmolive Company, China	Aqua, Hydrated silica, Sodium lauryl sulfate (SLS), Trisodium phosphate, Cellulose gum, Sodium phosphate, Sodium saccharin, Carbomer, Sodium fluoride (500 ppm), Limonene, Polysorbate 80, Sodium hydroxide

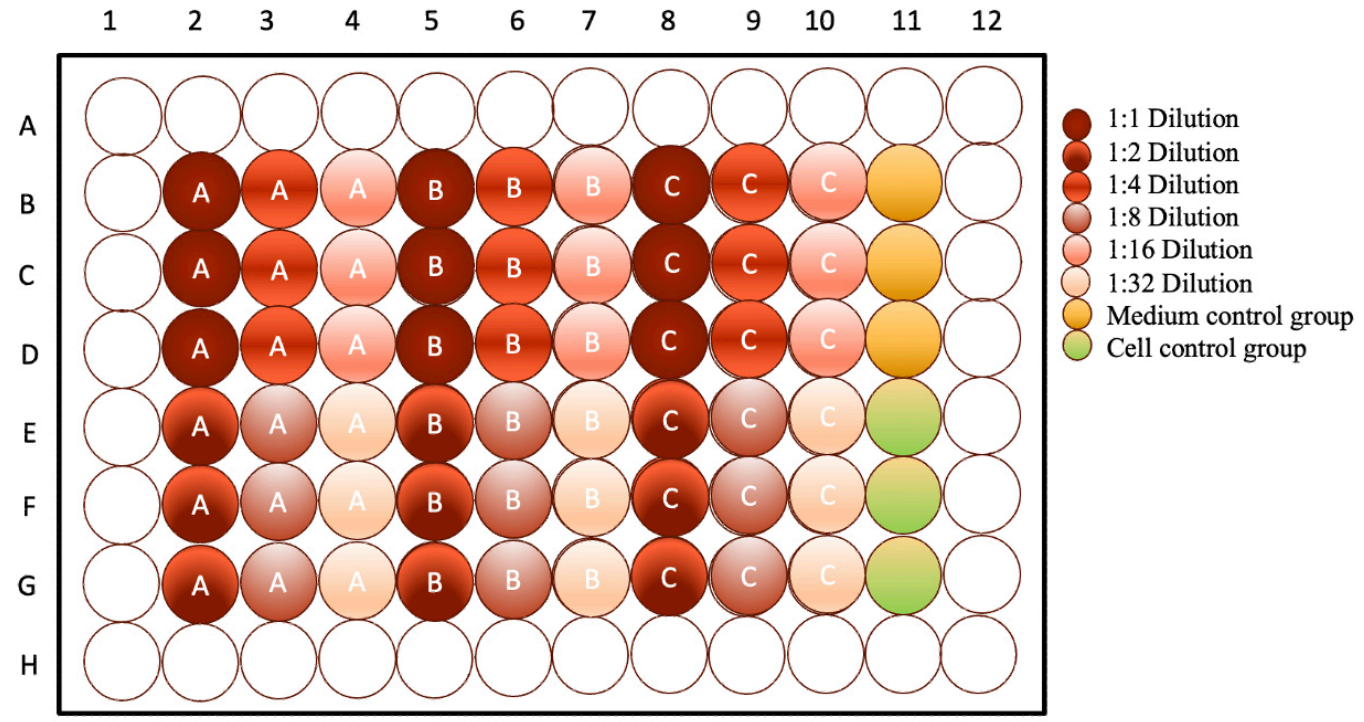


FIGURE 1. Schematic representation of the experimental design for the XTT assay. Group A: sodium phytate-containing toothpaste; Group B: bromelain-containing toothpaste; Group C: commercial toothpaste. Serial dilutions (1:1–1:32), a medium control, and a cell control are shown in the 96-well plate layout.

benzenesulphonic acid) assay. Subsequently, 50 μL of XTT solution and 1 μL of XTT activator were added to each well, and the plates were incubated for 3 h at 37 °C in 5% CO_2. Absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate reader (Varioscan Flash, Thermo Scientific, Waltham, MA, USA). Absorbance readings were normalized to those of untreated control cultures (100%). The experiment was conducted in triplicate and repeated three times. This replication scheme is consistent with standard practice in cell-based cytotoxicity testing and with comparable studies in this field [15, 25–27].

2.3 Antibacterial activity against *Streptococcus mutans*

The antibacterial activity of the toothpaste formulations against *S. mutans* (ATCC 25175) was evaluated using a modified agar-well diffusion method [16]. *S. mutans* was selected because of its well-established role as the primary cariogenic pathogen in dental caries [28]. Its widespread use as a standard test organism in studies of dentifrice antibacterial activity also permits meaningful comparison with previous findings [15, 16, 28]. A bacterial suspension was prepared in Mueller-Hinton broth, incubated at 37 °C for 24 h, and standardized to 0.5 McFarland (1.5×10^8 CFU/mL). Mueller-Hinton agar plates were then inoculated with 100 μL of the bacterial suspension using sterile swabs. Wells measuring 6 mm in diameter and 5 mm in depth were created at equal distances from one another and from the agar edges using a sterile punch. Each well was filled to capacity with the toothpaste formulation or control material using a sterile syringe (approximately 0.14 mL). Penicillin-streptomycin and distilled water served as the positive and negative controls, respectively. After incubation at 37 °C

in 5% CO_2 for 24 h, the inhibition zones were measured in millimeters using a digital caliper. The assay was performed with twelve replicates to ensure reproducibility, informed by the practice in comparable *in vitro* antibacterial studies on toothpaste formulations against *S. mutans* of employing multiple replicates to account for measurement variability [28]. This replicate number was selected because manual caliper-based measurement of inhibition zones is susceptible to plate-to-plate variation in agar thickness, diffusion homogeneity, and well positioning; therefore, additional replicates were required to obtain reliable mean values.

2.4 Statistical analysis

Normality and homogeneity of variance were assessed using the Kolmogorov-Smirnov and Levene tests, respectively. Descriptive statistics are presented as the mean ± standard deviation (SD). A one-sample *t*-test was used to determine whether mean cell viability at each concentration differed significantly from the reference control value of 100%. Differences in cell viability among toothpaste groups and exposure times, and differences in inhibition-zone diameters among groups, were analyzed using one-way analysis of variance (ANOVA). For significant ANOVA results, Tukey's Honestly Significant Difference (HSD) *post hoc* test was used for pairwise comparisons. A Bonferroni correction was applied to control type I error arising from multiple comparisons. Statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA), with statistical significance set at $p < 0.05$.

3. Results

3.1 Evaluation of the cytotoxic effects of the samples

The cytotoxicity of the toothpaste formulations was evaluated using the XTT assay. According to ISO 10993-5 [29], a reduction in cell viability of more than 30% is considered cytotoxic. Cell viability was evaluated at six dilutions and three exposure times and compared with that of the untreated control (100% viability). The results are presented as the mean ± SD in Table 2 and visualized as a heatmap in Fig. 2.

All toothpaste groups showed cell viability below 70% at the higher extract concentrations (dilutions 1:1, 1:2, and 1:4) and differed significantly from the untreated (100%) control ($p < 0.00093$). However, no significant differences among toothpaste groups were detected at these dilutions at any exposure time ($p > 0.0028$). At 1 min, cell viability differed among groups at the 1:8 and 1:16 dilutions (both $p < 0.001$). At 1:8, Groups A and B showed lower viability than Group C, whereas at 1:16, Group A showed lower viability than Groups B and C. Group A was noncytotoxic only at 1:32, whereas Groups B and C were noncytotoxic at both 1:16 and 1:32. A similar pattern was observed at 2 min, the recommended toothbrushing time. At the 1:8 dilution, Group A showed the lowest cell viability, followed by Group B, whereas Group C showed the highest viability ($p < 0.001$). At 5 min, only the 1:16 dilution showed a significant difference among groups ($p < 0.001$): Group A had lower cell viability than Group C, and Group B showed an intermediate profile. At this exposure time, Group A reduced cell viability below 70% at all tested dilutions. No significant differences among exposure times were observed within any dilution or group ($p > 0.0028$).

3.2 Antibacterial activity of the toothpastes

The antibacterial activity of the toothpaste formulations was assessed using the agar-well diffusion method, and the results are summarized in Table 3. All tested toothpastes exhibited significant antibacterial activity against *S. mutans*. The positive control (penicillin-streptomycin) and Group A produced the widest inhibition zones, with no statistically significant difference between them ($p > 0.05$). However, Group A exhibited significantly greater antibacterial activity than Groups B and C ($p < 0.05$), while Groups B and C showed comparable antibacterial activity against *S. mutans* ($p > 0.05$).

4. Discussion

Although home-care approaches, such as toothbrushing with specialized toothpastes, have gained increasing attention for managing BS in children, recent interest has focused on naturally derived stain-removal agents. Phytic acid acts by chelating calcium ions and disrupting chromogen adhesion to the pellicle [30], whereas bromelain removes discoloration through proteolytic degradation of the pellicle protein matrix [31]. Sodium phytate has shown promise for reducing calculus formation and removing stains from permanent teeth [22]. Milleman *et al*. [14] reported that a sodium phytate-containing toothpaste significantly reduced extrinsic stains compared with a conventional toothpaste over 6–12 weeks. Similarly, enzymatic toothpastes containing bromelain are more effective than conventional abrasive formulations for managing extrinsic stains [12, 13]. Furthermore, a recent *in vitro* study reported that toothpastes containing sodium phytate and bromelain effectively reduced BS in primary teeth, with efficacy comparable to that of professional polishing while maintaining favorable surface roughness [20].

Materials intended for oral use require thorough cytotoxicity evaluation. Given the complex composition and frequent daily use of toothpastes, establishing safety and efficacy is essential. Approximately 83% of children aged 3–5 years reportedly swallow toothpaste regularly [32]. Accordingly, the present study evaluated the cytotoxicity and antibacterial efficacy of two experimental children's toothpaste formulations developed for BS management.

Cell-culture models are widely used to evaluate the biocompatibility of oral-care products because they provide a faster, more standardized, and cost-effective alternative to animal testing. Among the available models, the L929 mouse fibroblast cell line is commonly used in cytotoxicity studies of dental materials because of its ease of use and well-characterized response to toxic agents [33]. For cell viability assessment, the XTT tetrazolium-reduction assay offers a sensitive and efficient alternative to the conventional MTT assay by eliminating the formazan-dissolution step and enabling multipoint absorbance measurements [34].

The present findings are consistent with previous reports

TABLE 2. Cell viability (%) of L929 cells exposed to toothpaste extracts (mean ± SD).

Time	Groups	1:1	1:2	1:4	1:8	1:16	1:32
1 min							
	A	32.52 ± 2.47*	34.42 ± 3.22*	37.10 ± 2.90*	38.84 ± 1.08^{A,*}	43.00 ± 4.97^{A}	74.25 ± 6.51
	B	32.45 ± 1.85*	32.78 ± 1.23*	34.74 ± 2.17*	39.05 ± 1.61^{A,*}	76.25 ± 3.32^{B}	93.54 ± 5.09
	C	32.80 ± 2.19*	32.83 ± 2.21*	35.17 ± 1.33*	66.19 ± 2.14^{B}	86.93 ± 10.33^{B}	105.62 ± 8.87
	p-value[1]	0.978	0.645	0.436	<0.001	<0.001	0.004
2 min							
	A	31.57 ± 2.30*	32.84 ± 1.88*	36.74 ± 2.15*	37.70 ± 1.58^{A,*}	49.05 ± 3.27^{A}	73.69 ± 3.33^{A}
	B	31.29 ± 1.54*	33.93 ± 2.85*	36.65 ± 1.29*	50.19 ± 1.21^{B}	74.02 ± 5.79^{B}	87.07 ± 3.48^{A,B}
	C	31.74 ± 0.48*	32.46 ± 0.69*	33.94 ± 0.96*	62.40 ± 1.38^{C}	81.02 ± 3.87^{B}	95.42 ± 4.66^{B}
	p-value[1]	0.944	0.670	0.115	<0.001	<0.001	<0.001
5 min							
	A	32.02 ± 2.53*	34.83 ± 0.71*	35.11 ± 3.10*	36.31 ± 3.25*	42.57 ± 7.20^{A}	67.67 ± 8.30
	B	31.78 ± 2.60*	32.63 ± 2.87*	37.36 ± 1.26*	45.57 ± 5.79	61.87 ± 9.82^{A,B}	76.57 ± 3.18
	C	30.26 ± 0.34*	36.79 ± 3.65*	37.86 ± 3.46*	61.12 ± 6.84	84.56 ± 4.00^{B}	89.06 ± 1.59
	p-value[1]	0.569	0.249	0.479	0.004	<0.001	0.007

*Descriptive statistics are presented as the mean ± standard deviation. [1]One-way ANOVA with Bonferroni correction (significance at $p < 0.0028$). Different uppercase letters within the same exposure time and dilution indicate statistically significant differences ($p < 0.0028$). *Statistically significant difference compared with the control group (100% viability) based on a one-sample t-test (Bonferroni-adjusted $p < 0.00093$).*

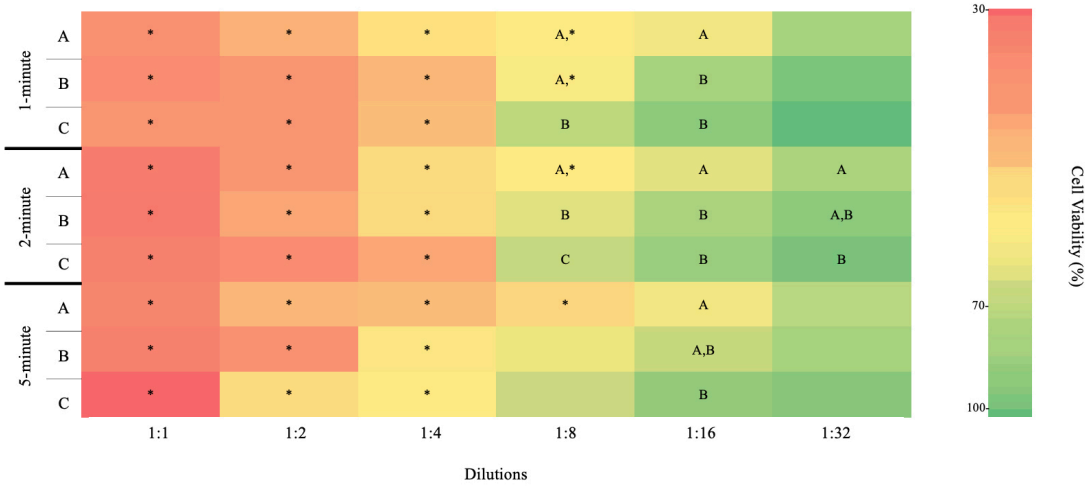


FIGURE 2. Cell viability (%) heatmap for toothpastes A, B, and C across exposure times and dilutions. Different uppercase letters within the same exposure time and dilution indicate statistically significant differences ($p < 0.0028$). *Statistically significant difference compared with the control group (100% viability) based on a one-sample *t*-test (Bonferroni-adjusted $p < 0.00093$).

TABLE 3. Comparison of the antibacterial activity of the toothpastes against *S. mutans* (inhibition-zone diameter, mm; mean ± SD).

Groups	Inhibition zones (mm)
A	19.54 ± 0.99^{A}
B	17.42 ± 1.00^{B}
C	17.71 ± 0.84^{B}
Negative control (Distilled water)	0.00 ± 0.00^{C}
Positive control (Penicillin-streptomycin)	20.50 ± 0.50^{A}
p-value[1]	<0.001

Descriptive statistics are presented as the mean ± standard deviation. [1]One-way ANOVA (significance at $p < 0.05$). No statistically significant difference was detected between groups denoted by the same uppercase letter ($p > 0.05$).

[15, 18, 35] showing a strong association between toothpaste concentration and cytotoxic potential and further highlight the influence of formulation composition on cytotoxicity. Sodium lauryl sulfate (SLS) is widely used in toothpastes because of its foaming and plaque-emulsifying properties. However, its antimicrobial action may involve cytotoxic mechanisms, such as membrane disruption. Such disruption in fibroblasts and epithelial cells may contribute to oral mucosal irritation and desquamation *in vivo*. Numerous studies have identified SLS as one of the most cytotoxic toothpaste components [17, 35, 36]. Previous studies have also shown that SLS-containing pediatric toothpastes are more cytotoxic to L929 fibroblasts than SLS-free formulations [18, 37]. Moore *et al*. [38] investigated toothpastes containing different detergents in immortalized normal oral keratinocyte (TERT-1) cells after a 2-min exposure and found that higher detergent concentrations significantly reduced cell viability. Similarly, Birant *et al*. [39] examined the effects of different children's toothpastes on dental stem cell viability and differentiation, concluding that SLS-containing toothpastes significantly reduced cell viability compared with other formulations. Beyond direct cellular damage, Karaman *et al*. [40] reported that SLS, either alone or incorporated into toothpaste, significantly influenced the expression of genes related to odontogenesis in zebrafish embryos. Together, these findings emphasize the need for more biocompatible detergent alternatives in experimental pediatric formulations to preserve cellular health without compromising efficacy.

Although several studies have reported time- and concentration-dependent toxicity, differences among experimental models suggest that cytotoxic responses may vary according to the cell line and formulation tested. In the present study, no statistically significant differences in cytotoxicity were observed among exposure times ($p > 0.0028$). By contrast, Ghapanchi *et al*. [15] evaluated the *in vitro* cytotoxicity of 16 commercial toothpastes in HeLa and oral epithelial cells after 1, 2, and 5 min of exposure. They found that SLS-containing toothpastes were cytotoxic and that cytotoxicity increased over time. These discrepancies may reflect differences in the cell models and the complex compositions of the toothpaste formulations evaluated.

To our knowledge, the cytotoxic potential of phytic acid and bromelain in toothpaste formulations has not been extensively investigated. However, the available literature generally describes these agents as biocompatible [30, 31]. Nassar *et al*. [41] reported that low concentrations of phytic acid (0.01%–0.1%) were not cytotoxic to pulp cells after 6 h, whereas prolonged exposure significantly reduced cell viability after 24 h. The authors attributed this delayed toxicity to possible iron chelation in the FBS-enriched culture medium. In the present study, exposure times were limited to 1, 2, and 5 min; therefore, this mechanism may not have substantially influenced the findings. In addition, studies evaluating bromelain-containing whitening formulations have reported no significant cytotoxicity in L929 or primary human fibroblast cells while demonstrating effective stain removal [42, 43].

In the present study, all toothpaste formulations showed greater cytotoxicity at higher extract concentrations ($p < 0.00093$), consistent with previous reports of SLS-containing formulations [15, 17, 18, 27, 37]. At the highest extract concentrations (dilutions 1:1–1:4), no significant differences in viability were detected among the three formulations, which may reflect a nonspecific ceiling effect dominated by osmotic or physical stress. By contrast, the differences observed among groups at intermediate dilutions (1:8 and 1:16) suggest that formulation-specific chemical effects became more apparent once this ceiling effect was reduced. Because SLS was present as a common surfactant in all groups, baseline cytotoxicity related to cell-membrane disruption was expected. Nevertheless, significant differences in cell viability were observed among groups, suggesting distinct biological effects of the active ingredients and overall formulations. Notably, Group A (0.85% w/w sodium phytate) generally showed lower cell viability than Groups B (5% w/w bromelain) and C (Table 2). At the recommended toothbrushing time of 2 min and a 1:8 dilution, Group A showed the lowest cell viability, followed by Group B, whereas Group C showed the highest viability ($p < 0.001$). The lower viability observed for Group A may be attributable to the strong calcium-chelating capacity of sodium phytate, which may alter the cellular ionic microenvironment, or to differences in the overall formulation, including possible differences in SLS concentration. Despite its 5% (w/w) bromelain content, Group B showed an intermediate cytotoxicity profile that varied across dilutions and exposure times (Table 2). At 1:16, it resembled Group C at 1 and 2 min, but showed an intermediate profile at 5 min. At 1:8, Group B was comparable to Group A and lower than Group C at 1 min, and significantly lower than Group C at 2 min (50.19% *vs.* 62.40%, $p < 0.001$), with no differences at 5 min. This variability may partly reflect the enzymatic and structurally labile nature of bromelain, whose proteolytic activity may be influenced by interactions with other excipients present in the toothpaste matrix, as well as by concentration-dependent effects across dilutions. Overall, Group C showed higher cell viability under most conditions despite lacking the natural agents present in Groups A and B, suggesting that toothpaste cytotoxicity depends on complex interactions among ingredients—including flavoring agents, binders, and pH adjusters—rather than on a single active ingredient.

Antimicrobial activity is an essential property of modern dentifrices because mechanical plaque removal alone is often insufficient for effective caries prevention [44]. Although numerous studies have investigated the antibacterial effects of synthetic toothpaste ingredients, evidence regarding plant-derived alternatives remains limited [15, 44].

The *in vitro* evaluation of antibacterial efficacy is highly dependent on the method used. Although disk-diffusion methods are widely used, they may yield inconsistent results for viscous or poorly water-soluble materials, such as toothpastes, because active components may not diffuse efficiently through the disk matrix [45]. Therefore, the agar-well diffusion method was used in the present study because it permits more homogeneous diffusion of toothpaste suspensions and may improve reproducibility for complex dental formulations [46].

The present findings are consistent with previous studies suggesting that the antibacterial properties of toothpastes may depend on synergistic interactions among formulation com-

ponents [44, 47]. Several studies have also associated the antibacterial properties of toothpastes with surfactants such as SLS [28, 47, 48]. As with the cytotoxicity findings, the presence of SLS in all groups may have provided a baseline antimicrobial effect; nevertheless, significant differences in antibacterial efficacy indicated that the active ingredients and overall formulations also played substantial roles. Notably, the commercial control toothpaste (Group C) contained fluoride as its primary active ingredient, which can exert antimicrobial and metabolic-inhibitory effects against *S. mutans*. Although the experimental formulations (Groups A and B) were fluoride-free, their antibacterial activity was either greater than that of the fluoride-containing commercial toothpaste (Group A, $p < 0.05$) or statistically comparable to it (Group B, $p > 0.05$). These findings suggest that alternative active ingredients, such as sodium phytate and bromelain, may contribute to the antimicrobial activity of dentifrice formulations.

Sodium phytate and bromelain, the active ingredients in the experimental toothpastes, have previously exhibited antibacterial and antibiofilm activity against oral microorganisms, including *S. mutans*. Nassar *et al*. [49] evaluated phytic acid against 11 bacterial strains, including *S. mutans*, and reported bactericidal activity against all tested microorganisms. Goudarzi *et al*. [50] evaluated pineapple extracts against oral pathogenic bacteria *in vitro* using an agar-diffusion method and found antibacterial activity against *S. mutans* and *Streptococcus sanguinis* (*S. sanguinis*). Rahmi *et al*. [23] evaluated mouthwashes containing different concentrations of bromelain and found that the formulation containing 35% enzyme inhibited *S. mutans* growth. Similarly, all toothpaste formulations in the present study showed significant antibacterial activity against *S. mutans*. Group A (0.85% w/w sodium phytate) exhibited the greatest antibacterial activity ($p < 0.05$), consistent with its lower cell viability, whereas Groups B and C showed statistically comparable antibacterial effects ($p > 0.05$). These findings indicate that toothpaste antibacterial efficacy reflects the collective chemical profile of the formulation rather than the isolated effect of sodium phytate or bromelain.

The present study has several limitations. First, SLS was incorporated into the experimental formulations as part of the standardization process, and its presence in all groups may have obscured the individual biological effects of sodium phytate and bromelain. Furthermore, SLS concentrations could not be directly compared between the experimental and commercial formulations because the precise composition of the commercial toothpaste is not publicly available. Future studies employing alternative detergent systems or SLS-free formulations may help isolate the true biological behavior of sodium phytate and bromelain independent of the shared SLS background. Second, the experimental formulations were fluoride-free; therefore, their clinical applicability should be interpreted cautiously, and they should not be considered substitutes for fluoride-based caries prevention unless combined with an appropriate fluoride source for routine pediatric use. Third, sodium phytate may have interacted with calcium ions (Ca^{2+}) from the calcium carbonate in the paste, though its low aqueous solubility may have limited this interaction. However, free-ion and phytate concentrations were not directly characterized. The extent and impact of this interaction remain unclear and should be evaluated in future studies. Fourth, antibacterial activity against *Actinomyces spp.*, which are more directly implicated in BS pathogenesis [1], was not evaluated and should be investigated in future work. Finally, the pH values of the experimental toothpastes (8.09 for Group A and 8.72 for Group B) were below the ISO 11609:2017 upper limit of pH 10.5 for dentifrices [21], but their associations with the biological outcomes were not statistically assessed.

5. Conclusions

Within the limitations of this *in vitro* study, the experimental toothpastes demonstrated antibacterial activity against *S. mutans* and concentration-dependent cytotoxicity in L929 cells. Group A showed the greatest antibacterial activity, but also the greatest cytotoxicity. The null hypothesis was therefore partially rejected: the antibacterial activity of the experimental formulations was comparable to or superior to that of the commercial control, whereas their cytotoxicity profiles differed significantly among the groups. These findings are preliminary because *in vitro* conditions cannot fully reproduce the complex biological environment of the oral cavity, including salivary flow, tissue barriers, and immune responses. Further studies should confirm the biocompatibility and clinical applicability of these formulations and optimize their composition to achieve effective stain management while maintaining essential anticaries properties.

ABBREVIATIONS

SLS, sodium lauryl sulfate; XTT, sodium 3′-(1-phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzenesulphonic acid; BS, Black stain; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PenStrep, penicillin-streptomycin; ANOVA, analysis of variance; TERT-1, human oral keratinocyte cell line; ISO, International Organization for Standardization; ATCC, American Type Culture Collection; CO_2, carbon dioxide; HSD, Tukey's Honestly Significant Difference; SD, standard deviation; SPSS, Statistical Package for the Social Sciences; *S. mutans*, *Streptococcus mutans*; *S. sanguinis*, *Streptococcus sanguinis*; Ca^{2+}, calcium ions.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

AS—contributed to the conceptualization of the study, investigation, formal analysis, visualization, resources, and the drafting of the original manuscript. AS, DA, SIT and ESSA—contributed to the study methodology. AS and ESSA—participated in the formal analysis. AS and DA—contributed to the review and editing of the manuscript. DA

and SIT—supervised the study. All authors read and approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

As the work did not involve primary human subjects, patient data, or animal material, it is not classified as human subjects research and therefore did not require institutional ethics committee approval.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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