

Research Article

IN VITRO EVALUATION OF THE COMBINED ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF STANNOUS FLUORIDE AND AMINE FLUORIDE AGAINST STREPTOCOCCUS MUTANS

Author: Annu Sharma ¹, Asif Mallick ², Arpita Soni ³, Amreen Khan ⁴

¹ Assistant Professor, GIP, Geeta University

² Sister Nivedita University, New Town, Kolkata (West Bengal), 700156

³ Aayushman College Ranapur, Jhabua, Ranapur (Madhya Pradesh), 457661

⁴ Associate Professor, BM College of Pharmaceutical Education and Research, Indore (Madhya Pradesh)

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CORRESPONDING AUTHOR

Ms. Annu Sharma

17sharmanu@gmail.com

ABSTRACT

Dental biofilms created by Streptococcus mutans are a key cause of dental caries. This bacteria strongly attaches to teeth, produces extracellular polysaccharides, and forms organized biofilms, which play a major role in its ability to cause disease. Fluoride-based products are commonly used to prevent caries due to their ability to kill bacteria and help restore minerals to teeth. The present study aimed to evaluate the antibacterial and antibiofilm effects of stannous fluoride (SnF₂), amine fluoride, and their combination against Streptococcus mutans ATCC 25175 using agar well diffusion, crystal violet biofilm biomass quantification, and viable cell count assays. Standardized cultures of S. mutans ATCC 25175 were exposed to SnF₂, amine fluoride, and their combination. Antibacterial activity was evaluated using the well diffusion method. Biofilm biomass was quantified using crystal violet staining, while viable bacterial cells were determined by colony-forming unit enumeration. All experiments were performed in triplicate under aseptic laboratory conditions. The combination of SnF₂ and amine fluoride produced the largest inhibition zone, the lowest crystal violet absorbance, and the lowest viable bacterial count compared with the individual fluoride treatments. The combination produced a 75.8% reduction in biofilm biomass and approximately an 83% reduction in viable bacterial count compared with the untreated control. The combination of SnF₂ and amine fluoride demonstrated greater antibacterial and antibiofilm activity against S. mutans than either agent alone under the experimental conditions used. Further studies using multispecies biofilms and clinically relevant models are required to determine the applicability of this combined fluoride approach in oral healthcare.

INTRODUCTION

Dental caries remains one of the most prevalent chronic oral diseases worldwide and is strongly associated with microbial biofilm accumulation on tooth surfaces. *Streptococcus mutans* has been identified as a principal cariogenic microorganism due to its ability to metabolize dietary sugars and produce organic acids that demineralize dental enamel (figure 1). The organism possesses remarkable adherence capabilities mediated through glucosyltransferase enzymes, enabling the formation of complex biofilms that protect bacterial communities from environmental stressors.^[1]



Figure 1: *Streptococcus Mutans*^[2]

Biofilm-associated microorganisms exhibit increased resistance to antimicrobial agents compared with their planktonic counterparts, making effective plaque control a continuing challenge in preventive dentistry^[3]. Fluoride-based compounds have been extensively utilized in oral healthcare because of their ability to inhibit enamel demineralization and enhance remineralization processes^[4]. Among these agents, stannous fluoride (SnF_2) has demonstrated broad-spectrum antimicrobial activity and the capacity to reduce bacterial metabolism and biofilm formation^[5].

Amine fluoride represents another important fluoride-containing compound characterized by its surfactant properties and enhanced retention on oral surfaces^[6]. Previous investigations have shown that amine fluoride can interfere with bacterial adhesion and suppress the growth of cariogenic microorganisms^[7]. The combination of different fluoride compounds has gained attention as a potential strategy for improving antimicrobial efficacy against dental biofilms^[8].

Studies have suggested that synergistic interactions between fluoride formulations may provide superior antibacterial effects while simultaneously strengthening enamel resistance^[9]. Combination therapies have been reported to affect extracellular polysaccharide synthesis and bacterial viability within mature biofilms^[10-13]. Furthermore, fluoride-based antibiofilm approaches remain important components of modern preventive dentistry^[14]. Although the antibacterial and antibiofilm properties of individual fluoride-containing agents have been widely investigated, comparatively limited information is available

regarding the combined activity of stannous fluoride and amine fluoride against *S. mutans* under controlled in vitro conditions. Comparative evaluation of the two individual agents and their combination using antibacterial activity, biofilm biomass, and viable bacterial count as complementary endpoints remains limited. Therefore, further experimental evaluation is warranted to determine whether combining these fluoride agents produces greater antibacterial and antibiofilm effects than their individual use.

The present study provides a comparative in vitro evaluation of stannous fluoride and amine fluoride used individually and in combination against *S. mutans* ATCC 25175. The study evaluates antibacterial activity, biofilm biomass, and viable bacterial counts within the same experimental framework, thereby providing complementary evidence regarding the effect of the fluoride treatments on bacterial growth and biofilm development. The findings provide preliminary experimental information that may support further investigation of combined fluoride formulations for oral biofilm control.

Therefore, the present study aimed to comparatively evaluate the antibacterial and antibiofilm effects of stannous fluoride, amine fluoride, and their combination against *Streptococcus mutans* ATCC 25175 using agar well diffusion, crystal violet biofilm biomass quantification, and viable cell count analysis.

MATERIALS AND METHODS

Pure culture of *Streptococcus mutans* (ATCC 25175) was obtained from a microbiology culture repository. Stannous fluoride (SnF_2) and amine fluoride analytical-grade powders were procured from certified chemical suppliers. Brain Heart Infusion (BHI) broth, BHI agar, crystal violet stain (0.1%), phosphate-buffered saline (PBS), ethanol (95%), and sterile distilled water were used throughout the study.

Preparation of Test Solutions

Stannous fluoride stock solution was prepared by dissolving 1.0 g of SnF_2 in 100 mL of sterile distilled water to obtain a concentration of 10 mg/mL. Amine fluoride stock solution was prepared similarly at a concentration of 10 mg/mL. A combination solution containing equal concentrations of SnF_2 and amine fluoride was prepared immediately before experimentation. The final concentration of each treatment in the respective assay was calculated based on the volume of treatment solution added and should be reported for reproducibility.

Table 1. Experimental Treatment Groups

Group	Treatment	Concentration
G1	Control	0
G2	SnF_2	[actual concentration]
G3	Amine fluoride	[actual concentration]
G4	SnF_2 + amine fluoride	[concentration of each agent]

Preparation of Bacterial Inoculum

A loopful of *S. mutans* culture was inoculated into 50 mL BHI broth and incubated at 37°C for 24 h under microaerophilic conditions. The resulting bacterial suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard, corresponding approximately to 1×10^8 CFU/mL.

Agar Well Diffusion Assay

Antibacterial activity was evaluated using the agar well diffusion method. Sterile BHI agar plates were prepared and inoculated uniformly with 100 μL bacterial suspension. Wells of 6 mm diameter were created using a sterile cork borer. Each well received 100 μL of the designated treatment solution. Plates were

incubated at 37°C for 24 h. Zones of inhibition surrounding each well were measured in millimeters using a digital caliper.

Biofilm Formation Assay

Biofilm formation was evaluated in sterile 96-well microtiter plates. Each well received 180 µL BHI broth supplemented with 1% sucrose and 20 µL bacterial suspension. Subsequently, 20 µL of respective treatment solutions were added to designated wells. Plates were incubated at 37°C for 48 h. Following incubation, planktonic cells were removed and wells were washed three times with PBS. Attached biofilms were fixed using 200 µL methanol for 15 min. Wells were stained with 0.1% crystal violet for 20 min and washed thoroughly. Bound stain was dissolved using 200 µL ethanol and absorbance was recorded at 595 nm.

Viable Cell Count Determination

Biofilms formed on sterile glass slides immersed in bacterial cultures were used for viable count analysis. After treatment exposure for 48 h, biofilms were scraped into 10 mL PBS and serially diluted. Dilutions were spread onto BHI agar plates and incubated at 37°C for 24 h. Colony-forming units (CFU/mL) were determined by standard plate counting procedures.

Table 2. Parameters Evaluated During Study

Parameter	Method
Antibacterial activity	Agar well diffusion
Biofilm biomass	Crystal violet assay
Viable bacterial count	Plate count method
Biofilm reduction percentage	Comparative calculation

Evaluation of Biofilm Reduction

The percentage reduction in biofilm biomass was determined by comparing treated groups with untreated controls. Optical density values obtained from crystal violet staining were used for calculating relative biofilm inhibition.

Experimental Conditions

All experiments were performed in triplicate under aseptic laboratory conditions. Culture media and glassware were sterilized by autoclaving at 121°C for 15 min before use. Fresh bacterial cultures were prepared for each experimental run to maintain consistency and reproducibility.

Statistical Analysis

All experiments were performed in triplicate and the results were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using [software name and version]. Differences among treatment groups were analyzed using [actual statistical test]. A p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study evaluated the antibacterial and antibiofilm efficacy of stannous fluoride (SnF₂), amine fluoride, and their combination against *Streptococcus mutans*. The findings demonstrated that both fluoride agents exhibited inhibitory activity against the tested microorganism; however, the combined treatment produced the most pronounced effects on bacterial growth inhibition, biofilm suppression, and reduction of viable bacterial cells.

Antibacterial Activity by Agar Well Diffusion Assay

The agar well diffusion assay demonstrated antibacterial activity for both fluoride treatments, while the combination produced the largest inhibition zone of 21.4 ± 0.8 mm. SnF₂ produced a greater inhibition zone than amine fluoride under the present experimental conditions. These findings are consistent with recent investigations showing that fluoride-containing oral-care formulations can inhibit *S. mutans* growth and biofilm development. However, direct comparison of inhibition-zone values between different studies should be made cautiously because differences in fluoride concentration, inoculum density, agar composition, diffusion characteristics, and incubation conditions can influence the measured inhibition zone.

Table 3. Zone of Inhibition Against *S. mutans*

Group	Treatment	Zone of Inhibition (mm)
G1	Control	0.0 ± 0.0
G2	SnF ₂	15.8 ± 0.7
G3	Amine Fluoride	13.9 ± 0.6
G4	SnF ₂ + Amine Fluoride	21.4 ± 0.8

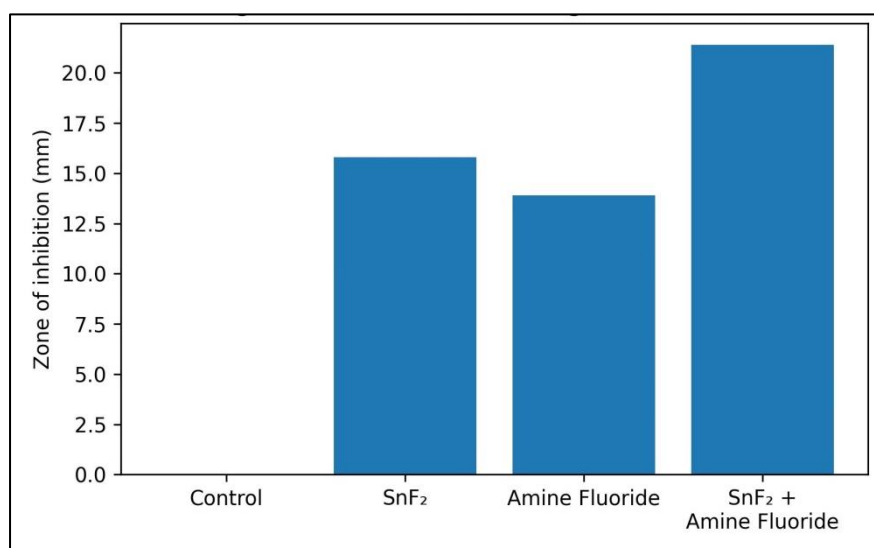


Figure 2: Zone of Inhibition Against *S. mutans*

The larger inhibition zone observed in the combination group suggested that simultaneous exposure to both fluoride compounds enhanced bacterial susceptibility. Stannous fluoride alone exhibited greater antibacterial activity than amine fluoride, likely due to the antimicrobial action of stannous ions. Nevertheless, the combined formulation demonstrated a broader inhibitory effect than either agent used independently.

Biofilm Biomass Quantification

Crystal violet staining demonstrated reduced biofilm biomass following treatment with both fluoride agents. The combination treatment produced the lowest absorbance at 595 nm, indicating the greatest

reduction in total attached biomass among the tested groups. The calculated biofilm reduction for the combination treatment was approximately 75.8% relative to the untreated control. These results support the antibiofilm activity of the tested fluoride treatments under the experimental conditions. Recent studies investigating fluoride-containing oral-care preparations have likewise reported reductions in *S. mutans* biofilm formation, although the magnitude of inhibition varies according to formulation and experimental model.

Table 4. Biofilm Biomass Determined by Crystal Violet Assay

Group	Treatment	Absorbance at 595 nm
G1	Control	1.28 ± 0.05
G2	SnF ₂	0.63 ± 0.03
G3	Amine Fluoride	0.72 ± 0.04
G4	SnF ₂ + Amine Fluoride	0.31 ± 0.02

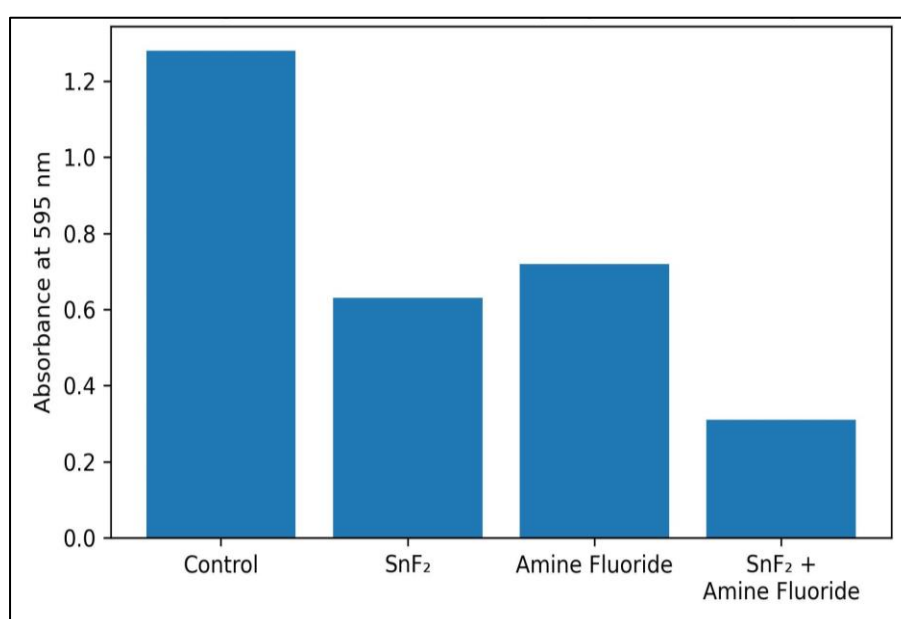


Figure 2: Biofilm Biomass Determined by Crystal Violet Assay

The optical density values indicated that biofilm biomass was substantially reduced in fluoride-treated groups. The combined fluoride treatment reduced biofilm formation by approximately 75.8% compared with the untreated control. These findings suggested that the combined formulation effectively interfered with bacterial attachment and extracellular polysaccharide production.

Percentage Reduction in Biofilm Formation

Biofilm inhibition percentages were calculated relative to the untreated control. The results demonstrated a progressive increase in inhibition from individual fluoride treatments to the combined formulation.

Table 5. Percentage Biofilm Inhibition

Group	Treatment	Biofilm Inhibition (%)
G2	SnF ₂	50.8
G3	Amine Fluoride	43.8

G4	SnF ₂ + Amine Fluoride	75.8
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The results confirmed that both fluoride compounds possessed antibiofilm properties. However, the combination treatment exhibited markedly higher inhibition than either agent alone. This enhanced activity may be attributed to complementary mechanisms of action. While stannous fluoride disrupted bacterial metabolism and enzyme activity, amine fluoride improved surface interaction and retention, facilitating prolonged antimicrobial contact.

Viable Cell Count Analysis

Viable cell enumeration further demonstrated the inhibitory effect of the fluoride treatments. The combination treatment produced the lowest viable bacterial count, corresponding to an approximately 83% reduction compared with the untreated control. This finding complements the crystal violet assay because crystal violet primarily estimates total attached biomass, whereas viable-cell enumeration provides an estimate of recoverable living bacteria. The agreement between these two independent endpoints strengthens the evidence that the combination treatment reduced both biofilm biomass and viable bacterial populations under the conditions tested.

Table 6. Viable Cell Count in Biofilms

Group	Treatment	Viable Count (CFU/mL × 10 ⁶)
G1	Control	9.4 ± 0.5
G2	SnF ₂	4.2 ± 0.3
G3	Amine Fluoride	5.0 ± 0.2
G4	SnF ₂ + Amine Fluoride	1.6 ± 0.1

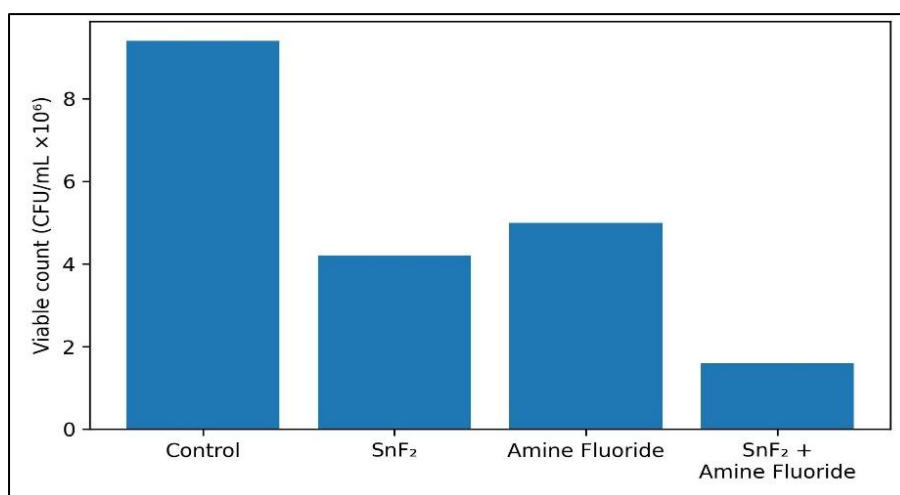


Figure 4: Viable Cell Count in Biofilms

The untreated control exhibited the highest bacterial population, reflecting robust biofilm development. Treatment with SnF₂ reduced bacterial viability by more than 50%, while amine fluoride also demonstrated substantial antibacterial activity. The combined fluoride treatment produced the greatest reduction in viable cells, lowering bacterial counts by approximately 83% relative to the control.

Morphological Observation of Biofilm Development

Visual examination of stained microtiter plate wells supported quantitative findings. Control wells displayed dense violet staining indicative of extensive biofilm formation. Wells treated with SnF₂ and amine fluoride exhibited noticeably lighter staining, indicating reduced biofilm biomass. The combination treatment resulted in minimal stain retention, suggesting marked suppression of bacterial adherence and matrix production.

Table 7. Visual Biofilm Assessment

Group	Observation
G1	Dense and thick biofilm layer
G2	Moderate biofilm formation
G3	Moderate to low biofilm formation
G4	Minimal biofilm formation

The reduction in visible biofilm accumulation indicated that fluoride exposure interfered with biofilm maturation processes. The combined treatment appeared to impair initial bacterial adhesion and subsequent extracellular matrix development.

Overall Comparative Evaluation

A comprehensive comparison of all evaluated parameters demonstrated the superiority of the combined fluoride formulation.

Table 8. Comparative Performance of Treatments

Parameter	SnF ₂	Amine Fluoride	Combination
Inhibition zone (mm)	15.8 ± 0.7	13.9 ± 0.6	21.4 ± 0.8
Biofilm biomass (OD595)	0.63 ± 0.03	0.72 ± 0.04	0.31 ± 0.02
Biofilm inhibition (%)	50.8	43.8	75.8
Viable count (×10⁶ CFU/mL)	4.2 ± 0.3	5.0 ± 0.2	1.6 ± 0.1

The synergistic interaction between SnF₂ and amine fluoride was evident throughout the study. Stannous fluoride contributed potent antimicrobial action through disruption of bacterial metabolic processes and membrane integrity. Amine fluoride enhanced adherence of fluoride ions to oral surfaces and prolonged antimicrobial exposure. The combination of these complementary mechanisms resulted in greater suppression of *S. mutans* growth and biofilm formation than either agent alone.

The findings suggested that dual-fluoride formulations could provide improved protection against cariogenic biofilms. Reduced biofilm biomass and lower viable bacterial counts indicated effective interference with critical stages of dental plaque development. Since *S. mutans* is a primary contributor to enamel demineralization and caries progression, inhibition of its biofilm-forming ability may have significant preventive implications.

CONCLUSION

The present in vitro study demonstrated that stannous fluoride and amine fluoride individually exhibited antibacterial and antibiofilm activity against *Streptococcus mutans* ATCC 25175. The combination treatment produced the largest inhibition zone, the greatest reduction in crystal violet-measured biofilm

biomass, and the lowest viable bacterial count among the tested groups. These findings indicate enhanced combined antibacterial and antibiofilm activity of SnF₂ and amine fluoride under the experimental conditions used. However, because formal synergy analysis was not performed, the observed effect should not be interpreted as definitive synergism. Further studies using multispecies biofilms, validated combination-analysis methods, enamel-based models, and in vivo or clinical approaches are required to determine the practical relevance of the combined fluoride treatment.

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