

# Evaluation of Anti-bacterial Effect of Ammonium Hexafluorosilicate on *Streptococcus mutans* and *Lactobacilli*

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## Abstract

**Background:** Although dental caries is a common problem in the world, fluoride application is one of the most effective ways to prevent tooth decay. Ammonium hexafluorosilicate (AHF) has recently been studied as a promising anti-caries and hypo-sensitive agent. **Objectives:** This study was conducted to evaluate the antibacterial activity of AHF against *Streptococcus mutans* (*S. mutans*) and *Lactobacilli* and the cytotoxicity of AHF. **Materials and Methods:** Diffusion well test was used to test the sensitivity of the bacteria. The anti-bacterial effect was evaluated by measuring the inhibition zone of the study (AHF), control +ve (chlorhexidine 0.2%, sodium fluoride 2%) and control -ve (deionized water) groups against the growth of *S. mutans* and *Lactobacilli*. For AHF, different concentrations were used (0.1, 0.32, 0.75, 1.25, 2.5, 5, 10, 15, 20) mg/mL. A serial dilution technique was performed to measure the minimum inhibitory concentration (MIC). The tested solutions were then cultured to the specified minimum bactericidal concentration (MBC). The Mosmann's tetrazolium toxicity assay was used to test the cytotoxic effect of AHF on the normal cell line. Data were analyzed using Shapiro-Wilk, one-way ANOVA, and Tukey's multiple comparison test. **Results:** The AHF had a statistically significant antibacterial effect against *S. mutans* and *Lactobacilli* and increased with increasing concentration. About 10 mg/mL of AHF MIC, 20 mg/mL of MBC, and 10,000 µg/mL of AHF were not toxic. **Conclusion:** AHF exhibited significant antibacterial activity by showing a clear zone against *S. mutans* and *Lactobacilli*. Thus, it can be considered as a promising anticariogenic material in the future.

**Keywords:** Ammonium hexafluorosilicate, cytotoxicity, human *S. mutans*, *Lactobacilli*

## INTRODUCTION

According to the World Health Organization, the most common health problem is untreated tooth (dental) caries in permanent teeth.<sup>[1]</sup> It is a multifactorial chronic infection of dental tissue caused by bacterial plaque.<sup>[2-5]</sup> *Streptococcus mutans* bacteria are the most cariogenic because of its ability to attach primary to the tooth through specific bacterial-surface proteins and thus trigger dental caries.<sup>[6,7]</sup> *Lactobacilli* is considered to be the next most common cariogenic oral bacterium after *S. mutans*. It does not cause caries but plays an important role in progression of caries,<sup>[8,9]</sup> because *Lactobacilli* has a low affinity for the tooth surface and are therefore only present in small numbers in healthy oral cavities. However, with increasing intake of fermentable sugars in the diet, *Lactobacilli* increase in numbers, which contributes to dental caries.<sup>[10]</sup> In individuals with active

dental caries, the diversity and prevalence of *lactobacilli* in dental plaque increase significantly compared to individuals without caries.<sup>[11]</sup>

This is because bacteria are the leading force in demineralization and caries development.<sup>[12]</sup> Therefore, control of dental biofilm has become an important plan for caries control.<sup>[13]</sup>

One of these strategies is the use of fluoride. The anticaries effect of fluoride-containing products can be attributed to the inhibition demineralization processes, improvement

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of remineralization of tooth enamel, and influence on the biological activities of cariogenic microorganisms.<sup>[14]</sup>

In recent decades, ammonium hexafluorosilicate (AHF) has certain advantages over traditional fluoride preparations and has been actively used as a promising anti-caries and hypo-sensitive agent.

As an alternative to the anti-caries agent containing fluoride, AHF solution, has been suggested, the treatment of which does not cause tooth staining upon application. In addition, it has antibacterial effect, its fluoride ions bind to constituents of bacterial cells and influence enzymes, High fluoride concentrations can inhibit biofilm formation.<sup>[15]</sup>

In addition, the AHF solution is acidic, so that after the material is applied to the tooth surface, the acidity of the solution leads to etching of the hard tissues of the tooth and reprecipitation of calcium and phosphate ions as silica-calcium-phosphate crystals.<sup>[16]</sup> In addition, silica-containing compounds induce the formation of apatite from simulated body fluids and artificial saliva.<sup>[17]</sup> In dentistry, preliminary studies show that AHF has the ability to occlude the opened dentinal tubules by eliciting calcium phosphate precipitation.<sup>[18]</sup> To date, no study has investigated the antibacterial effect of AHF alone against *S. mutans* and *Lactobacilli*. This study aimed to test the antibacterial effect of AHF against *S. mutans* and *Lactobacilli*. The null hypothesis to be tested was that the use of AHF has no antibacterial effect against *S. mutans* and *Lactobacilli*.

## MATERIALS AND METHODS

### The sample collection

Collection of unstimulated saliva was performed under standard conditions following instructions cited by Navazesh, 1993.<sup>[19]</sup> Saliva was collected from healthy adult patients attending specialized dental centers in Baghdad city in the morning between 9 and 11 AM. The subject was asked to avoid eating or drinking for 3h. before the procedure of saliva collection, the patient was sitting comfortably and 3–5mL of unstimulated saliva was obtained using a drooling procedure by giving the patient basic steps to spit the saliva by lowering the head and positioning the plane tube near the lower lip to allow the saliva to weep. Place the saliva in a refrigerator box with ice to transport it to the laboratory in less than 1 h to be cultured.

### Identification of bacteria

After isolation of the *S. mutans* and *Lactobacilli* separately, identification for these bacteria was done by colony morphology,<sup>[20]</sup> morphological examination of the microbial cells,<sup>[21]</sup> motility test, catalase production test,<sup>[22]</sup> and carbohydrate fermentation test.<sup>[23]</sup>

### Inhibition zone procedure

Agar well technique was applied to test the sensitivity of *S. mutans* and *Lactobacilli* to different concentrations of

solutions as compared to the control positive which is sodium fluoride 2%, chlorhexidine 0.2% and to the control negative which is de-ionized water. Mueller-Hinton Agar (MHA) media was used and prepared according to the instructions of MHA media was prepared in accordance with Hi-Media's requirements, which involved the suspension of 35 gm of the media in 1000 ml of distilled water, after being dissolved by boiling, it was autoclaved for 15 minutes at 121 °C, at last, it kept firmly closed in a cool dry place until it reaches 45-50 °C and was poured into sterile Petri dishes, then kept at the refrigerator, that involved the suspension of 35 gm of the media in a liter of distilled water, after being totally dissolved with boiling, it was autoclaved for 15min. at 121 C, at last it kept firmly closed in a cool dry place then volume of 25 ml of MHA (pH 7) was poured into sterile Petri dishes, then kept at 37 C for 24 hrs. in an incubator.<sup>[24]</sup> 0.1 mL of activated inoculums was spread into each plate and left at room temperature for 20 min. Then, nine wells of same diameter (6mm) and depth were set in each agar plate using a cork borer; each well was filled with 0.1 mL of the tested agent. For AHF solutions, different concentrations were used (0.1, 0.32, 0.75, 1.25, 2.5, 5, 10, 15, 20 mg/mL).

The plates were then kept at room temperature for 1h, after that incubated aerobically for 24h at 37°C. Zone of inhibitions were measured along the diameter of each well with a ruler, no zone means a complete resistance of bacteria to the tested agents.

### Minimum inhibitory concentration and minimum bactericidal concentration determination of AHF for *S. mutans* and *Lactobacilli*

In this experiment, a serial dilution technique was performed and standardized suspensions of bacterial broth containing 10<sup>8</sup> (McFarland Standard 0.5) were prepared.<sup>[25]</sup> Test tubes were categorized according to the number of different concentrations of AHF solutions, which are 1.25, 2.5, 5, 7.5, 10, and 20 mg/mL, after 1 mL of bacterial suspension was added to each tube and 0.5 mL of the tested agent was then added to the respective tube. The tubes were then incubated anaerobically at 37°C for 48 h. The tubes were examined for turbidity. The tube that revealed turbidity (this result revealed that bacterial growth had occurred) was excluded, while the tube that did not show turbidity to the naked eye (there was no visible growth of bacteria) was identified as the minimum inhibitory concentration (MIC).<sup>[26]</sup>

To measure minimum bactericidal concentration (MBC), 0.5 mL of each concentration was collected separately and spread on Mueller-Hinton agar and then incubated at 37°C for 24h, the MBC (the minimum concentration that can kill the bacteria<sup>[26]</sup>) was the first concentration higher than or equal to the MIC with no growth on the media.<sup>[27]</sup>

### Cytotoxic effect of AHF

In this study, Mosmann's tetrazolium toxicity (MTT) assay was employed to test the cytotoxic effect of AHF on the normal cell line (human dermal fibroblast cell).<sup>[28,29]</sup> MTT assay essentially evaluates cell viability by defining the mitochondrial function of cells.<sup>[30]</sup> This assay was conducted at the Pharmacology Department, College of Medicine, Malaya University, Kuala Lumpur, Malaysia.

### Statistical analysis

Data description, analysis, and presentation were performed using Statistical Package for Social Science (SPSS version 21) (Chicago, IL). Minimum, maximum, mean, standard deviation (SD) and standard error (SE), simple chart bar, Levene test, Shapiro-Wilk, one-way ANOVA with Dunnett T3 post hoc test, and Tukey's multiple comparisons test were used. Statistical significance was defined as a *P* value of <0.05.

### Ethical approval

This research was approved by the Research Ethics Committee of University of Baghdad, College of Dentistry, according to a decision report (ref. number: 562, Date: April 17, 2022).

## RESULTS

### Identification of bacteria

*S. mutans* colonies were rough, with granular surfaces, and adherent to the mitis salivarius bacitracin agar plates. *S. mutans* cells were Gram-positive, purple-stained, and organized in a diplococcal or chain pattern [Figure 1A,B].

In the catalase production test, they gave negative results because no bubbles formed in carbohydrate fermentation test. *S. mutans* is capable of fermenting mannitol, raffinose, and melibiose, so the color of the reaction changed from red to yellow, indicating a positive reaction.

For *Lactobacilli*, colonies were spindle star, ovoid, round, or heart-shaped, and white or slightly yellow in color, which appeared on the selective Rogosa agar plate [Figure 2A,B].

*Lactobacilli* cells were Gram's positive, nonmotile, and nonspore-forming rods [Figure 2C]. The catalase test was negative in all *Lactobacilli* colonies (there were no bubbles).

### Antimicrobial effect of the AHF against *S. mutans*

Using Shapiro-Wilk test, all results (diameter of inhibition zone) for *S. mutans* were normally distributed among groups at *P* > 0.05 (ranged 0.068–0.310).

ANOVA test showed a statistically significant difference (*P* < 0.05) as shown in Table 1 and Figure 3. The diameter of inhibition zone of *S. mutans* increased with increasing concentration of AHF from 2.5 mg/mL (10, 15, and 20 mg/mL), showing a higher inhibition zone than CHX. NaF and DW showed no inhibition zone (mean 6 mm represents no inhibition as it means the diameter of the well).

### Groups

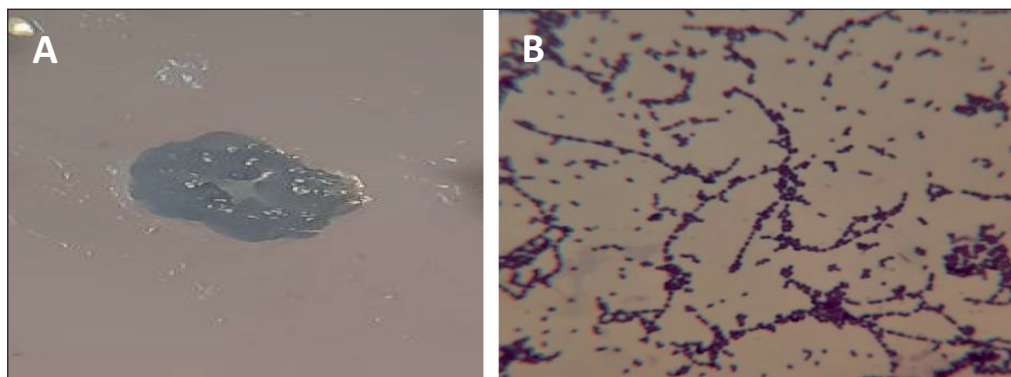
Error bar: 95% CI.

Dunnett's T3 test was used for multiple comparisons of the inhibition zones between groups. There was a statistically significant difference between the inhibition zones, except for 2.5 mg/mL of AHF, which showed no significant difference with 0.1, 0.3, 0.75, and 1.25 mg/mL of AHF, NaF, and DW. In addition to that, there were no significant differences between all the groups that showed no inhibition zones, 0.1, 0.3, 0.75, and 1.25 mg/ml of AHF, NaF, and DW [Table 2].

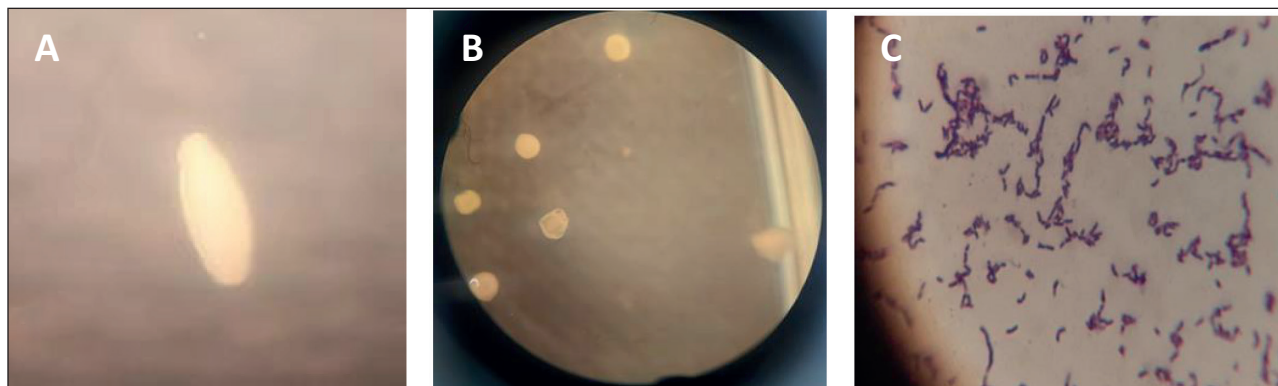
### Antimicrobial effect of the AHF against *Lactobacilli*

Shapiro-Wilk test was used and the results showed that all groups' findings (diameter of the inhibition zone) were normally distributed at *P* > 0.05, which ranged from 0.074 to 0.321.

ANOVA test showed a statistically significant difference (*P* < 0.05) [Table 3; Figure 4]. The diameter of the inhibition zone increases with the increase in concentration of AHF starting from 2.5 mg/mL (10, 15, and 20 mg/mL). AHF showed a higher inhibition zone than the CHX 0.2%. NaF 2% and DW showed no inhibition zone (mean 6 mm represents no inhibition as it means the diameter of the well).



**Figure 1:** (A) Colonies of *S. mutans* on MSB agar under a dissecting microscope (magnification 15×); (B) *S. mutans* cells under light microscope with 100× magnification

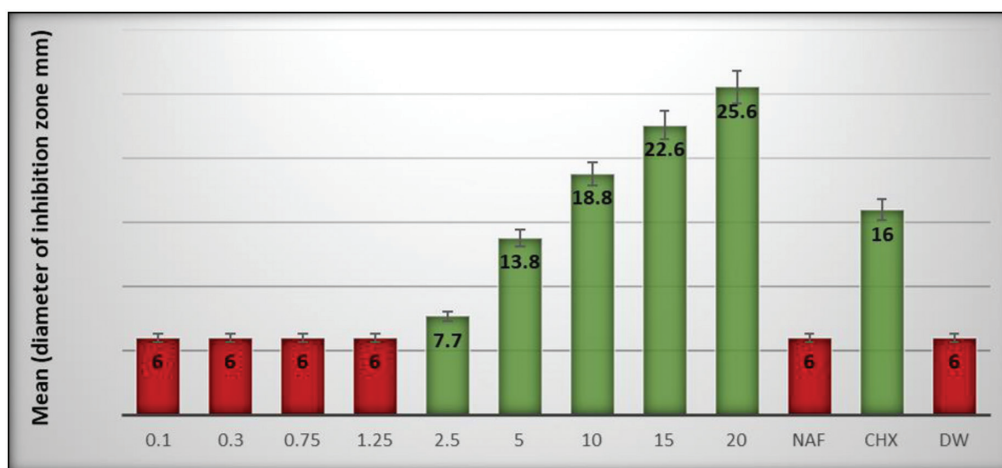


**Figure 2:** (A,B) Colonies of *Lactobacilli* on selective Rogosa agar under dissecting microscope (magnification 15X); (C) *Lactobacilli* cells under light microscope with 100× magnification

**Table 1: Descriptive and statistical test of the diameter of inhibition zone against *S. mutans* among groups (10 isolates)**

Groups	Mean	±SD	F	P value
0.1mg/mL AHF	6	0.00	664.504	0.00
0.3mg/mL AHF	6	0.00		
0.75mg/mL AHF	6	0.00		
1.25mg/mL AHF	6	0.00		
2.50mg/mL AHF	7.7	1.703		
5mg/mL AHF	13.8	1.229		
10mg/mL AHF	18.8	1.398		
15mg/mL AHF	22.6	1.075		
20mg/mL AHF	25.6	0.966		
NaF 2%	6	0.00		
CHX 1.2%	16	1.155		
DW	6	0.00		

Levene test = 4.075, P value < 0.05 significant



**Figure 3:** Inhibition zones for *S. mutans* among groups (in mm)

**Groups**

Error bar: 95% CI.

Dunnett’s T3 test was used for multiple comparisons of the inhibition zones between groups. There was a

statistically significant difference between the inhibition zones of all the groups, except for the groups that showed no inhibition zones 0.1, 0.3, 0.75, and 1.25mg/mL of AHF, NaF 2%, and DW, and between 10mg/mL of AHF with CHX [Table 4].

**Table 2: Multiple pairwise comparison of *S. mutans* among groups using Dunnett's T3**

(I) Groups	(J) Groups							
	2.5 mg/mL AHF	5 mg/mL AHF	10 mg/mL AHF	15 mg/mL AHF	20 mg/mL AHF	NAF 2%	CHX 0.2%	DW
0.1 mg/mL AHF	MD	-1.7	-7.8	-12.8	-16.6	-19.6	-10	
	<i>P</i> value	0.294	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	
0.3 mg/mL AHF	MD	-1.7	-7.8	-12.8	-16.6	-19.6	-10	
	<i>P</i> value	0.294	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	
0.75 mg/mL AHF	MD	-1.7	-7.8	-12.8	-16.6	-19.6	-10	
	<i>P</i> value	0.294	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	
1.25 mg/mL AHF	MD	-1.7	-7.8	-12.8	-16.6	-19.6	-10	
	<i>P</i> value	0.294	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	
2.50 mg/mL AHF	MD		-6.1	-11.1	-14.9	-17.9	1.7	-8.3
	<i>P</i> value		<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	0.294	<b>0.00</b>
5 mg/mL AHF	MD			-5	-8.8	-11.8	7.8	-2.2
	<i>P</i> value			<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.034</b>
10 mg/mL AHF	MD				-3.8	-6.8	12.8	2.8
	<i>P</i> value				<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.007</b>
15 mg/mL AHF	MD					-3	16.6	6.6
	<i>P</i> value					<b>0.00</b>	<b>0.00</b>	<b>0.00</b>
20 mg/mL AHF	MD						19.6	9.6
	<i>P</i> value						<b>0.00</b>	<b>0.00</b>
NAF 2%	MD							-10
	<i>P</i> value							<b>0.00</b>
CHX 0.2%	MD							
	<i>P</i> value							<b>1</b>
								<b>0.00</b>

Bold values indicate  $p < 0.05$  significant

**Table 3: Descriptive and statistical test of the diameter of inhibition zone against *Lactobacilli* among groups (10 isolates)**

Groups	Mean	±SD	<i>F</i>	<i>P</i> value
0.1 mg/mL AHF	6	0.00	605.723	<b>0.00</b>
0.3 mg/mL AHF	6	0.00		
0.75 mg/mL AHF	6	0.00		
1.25 mg/mL AHF	6	0.00		
2.5 mg/mL AHF	8.2	0.919		
5 mg/mL AHF	12.4	0.516		
10 mg/mL AHF	16.8	1.549		
15 mg/mL AHF	20	0.816		
20 mg/mL AHF	23.4	1.647		
NAF 2%	6	0.00		
CHX 0.2%	15	1.054		
DW	6	0.00		

Levene test = 12.387,  $P$  value < 0.05 significant

### MIC and MBC of AHF against *S. mutans* and *Lactobacilli*

*S. mutans*  
The AHF tubes containing concentrations of 1.25, 2.5, 5, and 7.5 mg/mL had turbidity; therefore, these tubes were excluded. The MIC tube, on the other hand, with a concentration of 10 mg/mL had no visible turbidity.

MBC of AHF was at the concentration of 20 mg/mL, this concentration showed no growth when culturing on Mueller-Hinton agar.

### *Lactobacilli*

Results showed that there was turbidity in the tubes that contained AHF with concentrations of 1.25, 2.5, and 5 mg/mL, these tubes were excluded, whereas the tube that contained AHF with a concentration of 7.5 mg/mL showed no turbidity on the naked eye as a result, this tube was the MIC.

MBC of AHF was at the concentration of 10 mg/mL, this concentration showed no growth after re-culturing on Mueller-Hinton agar.

### Cytotoxicity study (MTT assay)

The results showed that the viability of the HdFn cells decreased as the concentration of AHF increase starting from (95.25%) at 1250 µg/mL of AHF to (69.41%) at 20000 µg/mL of AHF. The viability of cells at 10000 µg/mL of AHF was 74.76%, and according to the INTERNATIONAL STANDARD ISO 10993-5 third edition 2009-06-01, which states "If the cell viability was reduced to under 70%, it could be regarded as having cytotoxic potential."<sup>[31]</sup> Therefore, this concentration and all the concentrations that showed cell viability more than 70% are considered as non-toxic.

Table 5 shows the concentrations of tested AHF in µg/mL and the means of cell viability percentage for each concentration.

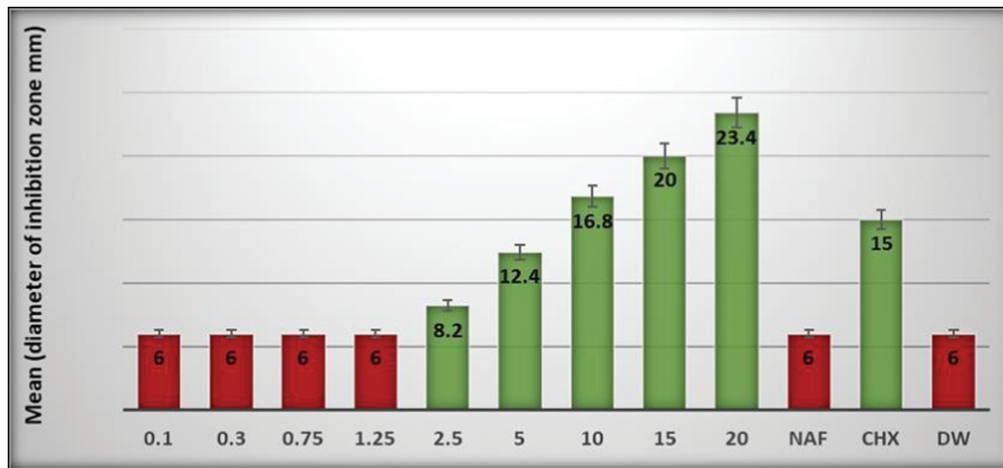


Figure 4: Inhibition zones for *Lactobacilli* among groups (in mm)

Table 4: Multiple pairwise comparison of *Lactobacilli* among groups using Dunnett's T3

(I) Groups	(J) Groups							
	2.5 mg/mL AHF	5 mg/mL AHF	10 mg/mL AHF	15 mg/mL AHF	20 mg/mL AHF	NAF 2%	CHX 0.2%	DW
0.1 mg/mL AHF MD	-2.2	-6.4	-10.8	-14	-17.4		-9	
0.1 mg/mL AHF P value	<b>0.001</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>		<b>0.00</b>	
0.3 mg/mL AHF MD	-2.2	-6.4	-10.8	-14	-17.4		-9	
0.3 mg/mL AHF P value	<b>0.001</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>		<b>0.00</b>	
0.75 mg/mL AHF MD	-2.2	-6.4	-10.8	-14	-17.4		-9	
0.75 mg/mL AHF P value	<b>0.001</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>		<b>0.00</b>	
1.25 mg/mL AHF MD	-2.2	-6.4	-10.8	-14	-17.4		-9	
1.25 mg/mL AHF P value	<b>0.001</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>		<b>0.00</b>	
2.5 mg/mL AHF MD		-4.2	-8.6	-11.8	-15.2	2.2	-6.8	2.2
2.5 mg/mL AHF P value		<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.001</b>	<b>0.00</b>	<b>0.001</b>
5 mg/mL AHF MD			-4.4	-7.6	-11	6.4	-2.6	6.4
5 mg/mL AHF P value			<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>
10 mg/mL AHF MD				-3.2	-6.6	10.8	1.8	10.8
10 mg/mL AHF P value				<b>0.003</b>	<b>0.00</b>	<b>0.00</b>	0.281	<b>0.00</b>
15 mg/mL AHF MD					-3.4	14	5	14
15 mg/mL AHF P value					<b>0.003</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>
20 mg/mL AHF MD						17.4	8.4	17.4
20 mg/mL AHF P value						<b>0.00</b>	<b>0.00</b>	<b>0.00</b>
NAF 2% MD							-9	
NAF 2% P value							<b>0.00</b>	
CHX 0.2% MD								9
CHX 0.2% P value								<b>0.00</b>

Table 5: Means of cell viability (%) of AHF at different concentrations

AHF concentration	Mean (viability, %)	SD
20,000 µg/mL	69.41	3.07
10,000 µg/mL	74.46	4.40
5000 µg/mL	86.34	2.31
2500 µg/mL	93.44	1.72
1250 µg/mL	95.25	1.03

## DISCUSSION

Dental caries is a multimicrobial disease caused by interactions between the food (diet) and microbiota matrix occurring on a solid surface over a period of time.<sup>[4]</sup> Sugars promote pathogen emergence, the assembly of the matrix, and the acidification of the biofilm microenvironment, and promoting ecological changes leads to acid damage of the mineralized tooth tissue.<sup>[32]</sup>

*S. mutans* is the most cariogenic microorganism in dental biofilm because of its acidogenic and aciduric characteristics,<sup>[33,34]</sup> in addition to its ability to utilize dietary carbohydrates and formation of extracellular polysaccharides.<sup>[35]</sup> Moreover, individuals with active dental caries have significantly increased levels of *Lactobacilli* in dental plaque as compared to caries-free individuals.<sup>[36]</sup>

*Lactobacilli* are known for their ability to quickly ferment sugars producing acidic end-products, at least half of which are lactic acid,<sup>[37]</sup> and their ability to tolerate low pH environments.<sup>[37]</sup> Therefore, acid production is an important virulence factor of *S. mutans* and *Lactobacilli*. Biofilms that deserve attention in dental caries prevention studies.<sup>[38]</sup>

The present results revealed significant differences between the negative control (DW and NaF) and positive control (CHX) and different concentrations of AHF. DW and NaF had no antibacterial effect, while CHX and 2.5, 5, 10, 15, and 20 mg/mL of AHF had antibacterial effects. The antibacterial effect of AHF against *S. mutans* and *Lactobacilli* increased with increasing the concentration and the 10, 15, and 20 mg/mL of AHF showed a higher effect than CHX.

The antibacterial effect of the AHF may be attributed to ions of fluoride, which can bind to constituents of bacterial cells and affect their enzymes, such as enolase and proton-extruding adenosine triphosphatase.<sup>[39]</sup> Therefore, fluoride can inhibit bacterial enzymatic processes thereby inhibiting bacterial growth responsible for tooth decay.<sup>[40]</sup>

Although AHF can potentially be used in dental practice due to its benefits,<sup>[18]</sup> the cytotoxicity of AHF on human cells is essential to perform *in vitro* tests to recognize the potentially harmful impact of this material on oral tissues before its clinical use. The study examined *in vitro* cytotoxicity of AHF on human fibroblast cells using MTT assay. The MTT is a colorimetric assay for assessing cell metabolic activity. This assay evaluates essentially cell viability by the definition of the mitochondrial function of cells by quantifying the activity of mitochondrial enzymes like succinate dehydrogenase.<sup>[29]</sup>

The results of the MTT assay showed that AHF influences cell viability in a dose-dependent manner. As the concentration of AHF increased, the viability of human fibroblast cells decreased, and the cytotoxicity increased. 20,000 µg/mL of AHF showed a higher effect on the viability of the cells. The other concentrations have no cytotoxic potential as their cell viability is higher than 70%.<sup>[31]</sup>

## CONCLUSION

The present study found that AHF has antibacterial effects against *S. mutans* and *Lactobacilli* and MIC of AHF was not toxic.

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Nil.

## Conflicts of interest

The authors reported that they have no conflicts of interest.

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