The inhibitory effects of silver diamine fluoride at different concentrations on matrix metalloproteinases

Short title: Effects of silver diamine fluoride on Matrix Metalloproteinases

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Key words: *matrix metalloproteinases, arrest, caries, dentine, silver diamine fluoride, sodium fluoride, chlorhexidine*

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Abstract

Objective: To study the inhibition effect on matrix metalloproteinases (MMPs) by silver diamine fluoride (SDF) solutions at various commercially available concentrations.

Methods: Three SDF solutions with concentrations at 38%, 30% and 12% were studied. Two sodium fluoride (NaF) solutions at 10% and 3% were prepared, and they had same fluoride concentrations with the 38% SDF and 12% SDF, respectively. Two silver nitrate ($AgNO_3$) solutions at 42% and 13% were also prepared, and they had same silver concentrations with the 38% SDF and 12% SDF, respectively. Assay buffer was used as a reference (control). Ten samples of each experimental solution were used to study their inhibition effect on three MMPs, which were MMP-2 (gelatinase A), MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) using MMP assay kits. The percentage inhibition was calculated from the difference of the mean reading of the tested group and control group divided by control group.

Results: The percentage inhibition of 38%, 30% and 12% SDF on MMP-2 were 79%, 60% and 17%, respectively ($p<0.001$); on MMP-8 were 94%, 85% and 77%, respectively ($p<0.001$); and on MMP-9 were 82%, 65% and 60% respectively ($p<0.001$). The percentage inhibitions on MMP-2, MMP-8 and MMP-9 by 38% SDF were significantly higher than the corresponding percentage inhibition by 10% NaF and 42% $AgNO_3$.

Conclusion: Greater inhibition effect on MMPs was found with higher concentration of SDF solution. The SDF had more inhibition on MMPs than solutions of NaF and $AgNO_3$ containing equivalent concentration of fluoride and silver ions, respectively. *(247words)*

1. Introduction

A variety of chemical agents have been adopted in dentistry to control caries progression without surgical removal of the caries lesion. The first extensively reported agent in arresting caries lesion probably is silver nitrate ($AgNO_3$) solution, which is now considered by many obsolete (1). It was used because some researchers believed that the silver nitrate could mechanically block dentine tubules; and silver could react with the organic material of the dentine to form silver albuminate (2). Chlorhexidine (CHX) inhibits growth and acid production of cariogenic bacteria such as *Streptococci mutans* (3). It may be applied to tooth surfaces for caries control. Sodium fluoride (NaF) in various forms has also been used (4). A case report demonstrated using NaF varnish to arrest caries in a teenager (5). Furthermore, silver diamine fluoride (SDF) was used in clinical trials to arrest dentine caries and the results were promising (6-8).

Apart from clinical studies, laboratory studies were performed to investigate the anti-caries effect of SDF on dentine caries (9). Most laboratory studies focused on changes in mineral content such as the calcium and phosphate level, fluoride content and mircohardness of dental hard tissues (1, 9, 10). In effect, both demineralization of hydroxyapatite and degradation of organic matrix are involved in dentine caries progress. Bacterial enzymes such as collagenases were thought to be responsible for the organic matrix destruction. Recent studies showed that matrix metalloproteinases (MMPs) could play an important part in enzymatic degradation of dentine.

MMPs are metal-dependent endopeptidases commonly known as matrixins. A typical MMP consists of predomain, prodomain, hinge, catalytic domain and hemopexin domain (Figure 1). The small predomain consists of about 80 amino-acids connecting to prodomain. MMPs are usually expressed as inactive zymogens. The prodomain holds a cysteine switch, which is suggested to prevent intracellular degradation of zymogen (11). MMPs can be activated by proteinases, chemical agents and in caries lesion, the low pH of the environment. The activation is likely to be initiated through disturbance of the cysteine-Zn^{2+} interaction of the cysteine switch. The prodomain links up with catalytic domain by a hinge. The catalytic domain contains an active Zn^{2+}-binding site. For MMP-2 (gelatinase A) and MMP-9 (gelatinase B), catalytic domain holds fibronectin domain which has a

strong affinity with gelatin. The catalytic domain connects to hemopexin domain. For MMP-2 and MMP-9, hemopexin is thought to mediate enzyme-tissue inhibitors of metalloproteinases (11).

In the presence of zinc (Zn^{2+}) which acts as a co-factor, MMPs mediate the degradation of practically all extracellular matrix molecules, including native and denatured collagen (12). Studies demonstrated that MMPs present in dentine matrix (13, 14) or in saliva (15). They can be activated in an acidic environment or by lactate released by cariogenic bacteria (12). MMP-8 (neutrophil collagenase) is capable of degrading triple-helical fibrillar collagens into distinctive 3/4 and 1/4 fragments. MMP-2 and MMP-9 are gelatinase, which degrades type IV collagen. The activation of MMP-2, MMP-8 and MMP-9 was shown to have a crucial role in collagen breakdown in dentine caries lesions (12). Hence, inhibition of MMP activities may contribute to caries arrest. A laboratory study found CHX could inhibit MMPs (16). However, there is no study found in the literature to investigate the effect of SDF on MMPs. This study aimed to investigate the inhibition effect on MMPs by SDF solutions at various commercially available concentrations.

2. Materials and Methods

2.1 *Preparation of experimental solutions*

Commercially available SDF solutions at 12% (Cariostop, Biodinamica, Brazil), 30% (Cariostop, Biodinamica, Brazil) and 38% (Saforide, Toyo seiyaku kasei, Japan) were selected for the in vitro study. Solutions of $AgNO_3$ at 42% and 13%, and NaF at 10% and 3% were prepared, which contained equivalent concentrations of Silver (Ag^+) and Fluoride (F^-) ions of the 38% and 12% SDF, respectively. Since CHX inhibited MMPs (16), CHX at 0.12% was selected for positive control. Assay buffer was used as reference (negative control) as suggested by the manufacturer. Ten samples of the each experimental solution were used for evaluation in the study. Totally 9 experimental solution were assessed; and they were assigned to be group 1 to 9 as shown in Table 1.

2.2 *Inhibition solution reacts with MMP enzymatic assays*

The inhibition effects of the 9 experimental solutions on MMP-2, MMP-8 and MMP-9 were assessed by using commercially available enzymatic MMP assay kits (Sensolyte, AnaSpec, Fremont, CA, USA). Briefly, each specific MMP substrate was diluted in assay buffer (1:100), and 50 µl diluted substrate was added to each well of a black fluorometric 96-well microtiter plate (Fisher Scientific, Gainesville, FL, USA). Recombinant MMP was diluted in 4-aminophenylmetcuric acetate (APMA) solution. The dilution factor was 1:9 for MMP-2 and MMP-9; and 1:4 for MMP-8. 1µl of the diluted MMP and 10 µl experimental solution was added to well which containing diluted substrate, then mixed gently in dark for 10 sec. Fluorescence intensity of the assay was measured at excitation/emission (340nm/490nm) using (1420 Victor PerkinElmer**,** Boston, MA**,** USA) with an associated computer program (Wallac 1420 manager PerkinElmer**,** Boston, MA**,** USA). The reaction in the essay was terminated by adding cessation solution one hour after the reaction. The data (end-point reading of MMP activity) obtained, initially expressed as relative fluorescence units, were converted to µM (µg/µl) based on standard curves (R^2>0.99) generated with 5-((2-Aminoethyl) amino) naphthalene-1-sulfonic acid (EDANS) fluorescence reference standard. A low value reflected a high inhibition effect on MMPs. The percentage inhibition was calculated from the difference of the value of the tested group and the reference (negative control) group divided by reference group.

2.3 Statistical analysis

All data were assessed for a normal distribution using Shapiro-Wilk test for normality. One-way ANOVA with S-N-K multiple comparison tests were used to detect differences between MMPs reading (µg/µl) of the relevant experimental groups. Analyses were performed with IBM SPSS Statistics - v19.0 (IBM Corporation, Armonk, NY, USA). The levels of statistical significance for all tests were set at 0.05.

3. Results

The mean end-point reading of the 9 groups were shown in Table 2. A low mean value (MMPs activity) indicated high inhibition of MMPs. There was significant differences in the mean values between groups ($p<0.001$). The mean end-point readings of 38% SDF for MMP-2, MMP-8 and MMP-9 were 2.38, 0.34 and 2.58, respectively. The

mean end-point reading was found significantly lower (hence, higher inhibition effect) in SDF with higher concentration for all 3 MMPs studied. The mean end-point reading of 12% SDF for MMP-2 was 9.17, which was not far from that of the reference (11.08).

Both 38% and 12% SDF had lower mean end-point readings (or greater inhibition) for the 3 MMPs than $AgNO_3$ with the corresponding silver concentrations. With the fluoride concentration at 4.5%, 38% SDF had lower mean end-point readings than the 10% NaF for all 3 MMPs. However, with fluoride concentration at 1.4%, 12% SDF did not showed a significant difference in inhibition of MMP-2 and MMP-9. Compared with the reference, the mean end-point readings of NaF was small for MMP-8 and MMP-9, but not MMP-2. This indicated NaF had notable inhibition effect against MMP-8 and MMP-9 but not MMP-2. Compared with other experimental solutions, the inhibition effect of 0.12% CHX against the MMPs was very low.

The percentage inhibitions of the experimental solutions for MMP-2, MMP-8 and MMP-9 were calculated and they were shown in Figure 1, 2 and 3, respectively. The percentage inhibition of 38% SDF for MMP-2, MMP-8 and MMP-9 were 79%, 94% and 82%, respectively. The percentage inhibitions of 30% SDF for MMP-2, MMP-8 and MMP-9 were not as higher as that of 38% SDF, but were at least 60%. The percentage inhibition of 12% SDF for MMP-2 was only 8%. $AgNO_3$ had low percentage inhibitions on MMPs. The 0.12% CHX, which was intended to be used as a positive control, had very low percentage inhibition values for all 3 MMPs.

4. Discussion

Since information about SDF on MMPs is lacking, this study provides essential information on the inhibition effect of SDF at different concentrations on MMPs. The fluorescent MMPs assay kit used in this study was optimized to detect MMPs activity. Compare with the SDS-gel electrophoresis technique (16), the fluorescent MMPs assay kit was easy to perform. More importantly, the MMPs activity could be quantified to compare the inhibition effect of different experimental solutions. Another advantage of this method

was that the 96-well-plate allowed large numbers of samples to be evaluated at the same time.

Typical MMPs shares common features which include a requirement of Zn^{2+} be bound at their catalytic sites, a family-specific Zn^{2+}-binding motif and a pro-peptide domain to maintain the enzymes as in inactive zymogen (Hannas et al., 2007). They digest extracellular matrix components. In this study, the assay kits for MMP-2 detection used in this study used EDANS and 4-(dimethylaminoazo) benzene-4-carboxylic acid (DABCYL) fluorescence resonance energy transfer peptide as substrate. The MMP-2 activity can be detected

Their activity is often detected in laboratory study by its proteolytic cleavage of EDANS and 4-(dimethylaminoazo) benzene-4-carboxylic acid (DABCYL). The assay kits used in this study detected MMP-2 activity by using EDANS/DABCYL fluorescence resonance energy transfer peptide as substrate with its fluorescence monitored at excitation/emission at 340nm/490nm upon proteolytic cleavage. The assay kits for detection of MMP-8 and MMP-9 share similar principle in the substrate digestion. Based on our pilot study, the kinetic reading (MMPs activity) of the reaction became stable after incubation for 60 minutes. Therefore, the reaction in the essay was terminated by adding cessation solution after one hour incubation to record the end-point readings. However, one of the limitations of this study design was that we did not know to which degree the MMPs activity was inhibited. Despite kinetic reading after 60 minutes was relatively stable, it was an arbitrary time point. Taking end point reading at other time points could give different reading and different percentage inhibition. Another limitation of this study was its *in vitro* nature. Similar to other laboratory study, this *in vitro* experiment was a simplified model to study the reaction between the SDF and other experimental solutions (inhibitor) and MMPs (enzyme). The *in vivo* situation is more complicated, the results therefore need to be interpreted with caution and further studies of SDF on MMPs are necessary to corroborate our findings.

Caries destruction in dentine is different from enamel because dentine contained about 30% by volume of organic matrix. The destruction of organic matrix was considered

mainly by bacterial collagenases in the past. Kawasaki and Featherstone (17) showed that the bacterial collagenases did not resisted the acidic fall during demineralisation, and hence they might not be the main reason for the matrix destruction. Some researchers now suggested the host MMPs present in dentine matrix (13, 14) or in saliva (15) have may an important role in dentine caries process. In fact, MMPs are widely active on the biological processes and can contribute to both normal and pathological events. MMP-8 (neutrophil collagenases) cleaves interstitial collagen types I, II and III. They are capable of digesting other extra cellular matrix and non- extra cellular matrix molecules.

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) not only degrade the denatured collagen molecules (gelatin), type IV collagen, but also other proteins to a lesser degree (18). Recent studies suggested that the activation of MMP-2, MMP-8 and MMP-9 have a crucial role in the destruction of dentine collagen in caries lesions (12, 15). SDF at 38% is one of the most commonly used agents in clinical trials (8, 19). A concentration at 12% was also used in a clinical study (6) conducted in Nepal. In addition, 30% SDF is available in market and is a common concentration used in South America. These concentrations of SDF were therefore selected for this study. For comparison purpose, freshly prepared $AgNO_3$ and NaF solutions contained equivalent Ag^+ and F^- of 12% and 38% SDF were also selected for this study. To prevent photo-chemical reaction of Ag containing solutions, the solutions were kept in opaque glass bottles and they study was performed in dark room.

CHX was reported to have an inhibition effect on MMP-2, MMP-8 and MMP-9 (16). It was intended to be used as a positive control in this study. However, the results of this study found no strong inhibition effect on all the three MMPs. The percentage inhibitions of CHX on MMP-2, MMP-8 and MMP-9 were 8%, 25% and 5%, and were substantially lower than SDF. The assay kit contained 4-aminophenyl mercuric acetate (APMA), which is a major component of the assay kit to activate the recombinant MMP. APMA is an organomercurial MMP activator which might prevent the CHX inhibition of MMPs (16). Another reason might be that calcium chloride was added to bind CHX in the previous study (16) but not in our study. This addition of calcium chloride helped generating chelating mechanism on MMP and this could contributed to the inhibitory effect of CHX on MMP (20).

Despite clinical studies found SDF is effective in arresting dentine caries, the mechanism is not yet elucidated. SDF has been shown to remineralised caries dentine and increased it microhardness (9) Recent study found SDF has antimicrobial properties to cariogenic bacteria such as *Streptococcus mutans* and *Actinomyces naeslundii* (10, 21). This study showed that 38% SDF had a significant inhibition of MMP-2, MMP-8 and MMP-9, and the inhibition can be important in halting destruction of organic substance in dentine caries lesion. Studies are now in progress to further evaluate these new anti-MMP properties *in situ* and *in vivo.*

Inhibition of MMP activities may attribute to caries arrest. *In vivo* study with rat molars also demonstrated a significant reduction in dentinal lesion progression with MMP inhibitors, along with reduced salivary MMP activity (22). In this study, SDF solutions demonstrated a concentration-dependent inhibition effect on all the 3 MMPs. 38% SDF could inhibit up to 90% in all 3 MMPs which were present in dentine caries. This may be one of the reasons for the success of 38% SDF to arrest caries in clinical trials (8, 19). This study found 12% SDF could hardly inhibit MMP-2 and slightly inhibit MMP-8 and MMP-9. This might give a reason why 12% SDF was not effective to arrest caries in a clinical trial (6).

The percentage inhibitions on MMP-2, MMP-8 and MMP-9 by 38% SDF were significantly higher than the corresponding percentage inhibitions by 10% NaF and 42% $AgNO_3$. Looking into the results of equivalent F^+ and Ag^+ solution, F^+ seems to contribute more to this inhibition effect than Ag^+ does. Theoretically, the reactive series of metals determines the reactivity of metals. Silver is a metal which is less reactive than hydrogen and can hardly replace H in a reaction with water. Thus Ag is averse to bind to specific site of MMPs to inactivate their catalytic functions. However, a dose dependent inhibition of both MMP-2 and MMP-9 with silver particles was demonstrated previously, but the mechanism is not clear (23).

Fluoride has been shown able to alter MMP-2 activity in enamel (24), while the effect of fluoride on MMP-2, MMP-8 and MMP-9 is lacking. This study found F^+ solution

had inhibition effect on MMPs, especially on MMP-8 and MM-9. The percentage inhibitions of 38% SDF for MMP-2, MMP-8 and MMP-9 were significantly higher than the corresponding percentage inhibition on equivalent F^+ solution. One of the explanations was that during caries activity, the release of acids by bacteria rapidly decreased the acidity (pH) of the surrounding. The acidic environment can then activate host-derived pro-MMPs from both dentine and saliva (15). The alkaline environment provided by SDF could be a barrier of MMPs activation.

Conclusion

For the first time, our study reported inhibition effect of SDF at different concentration on MMP activities. The inhibition effect on MMP-2, MMP-8 and MMP-9 was related to the concentration of SDF solution. We found 38% SDF had significantly more MMPs inhibition than 30% and 12%. In addition, it also had significantly more MMPs inhibition than 10% NaF and 42% $AgNO_3$, which had equivalent concentration of fluoride and silver ions, respectively. The significant inhibition effect of SDF on MMPs demonstrated beneficial anti-proteolytic properties which, as well as known antimicrobial properties, can be useful for caries arrestment.

(2680 words)

Clinical Significance

Literature reported SDF is a simple and effective agent that has been used in arresting dentine caries for many years. However, the exact mechanisms of caries arrest is not yet fully understand and need to be elucidated by further studies. Dentine contains hydroxyapatite and organic matrix, which can be digested by matrix metalloproteinases (MMPs). Recent studies found MMPs could play an important part in caries activity. This study demonstrated SDF could inhibit MMP-2, MMP-8 and MMP-9 activities. The inhibition can be important in halting destruction of organic substance in dentine caries lesion.

Acknowledgements

The authors acknowledge Ms. Epasinghe Don Jeevanie for her support in this research. This study was supported by the NSFC/RGC Joint Research Scheme (Grant No. N_HKU 776/10).

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Table 1 Fluoride and silver content and acidity (pH) of the nine experimental solutions

Group	Product	F^-	Ag^+	pH
1	38% SDF	4.5%	25.5%	13
2	30% SDF	3.5%	20.0%	12
3	12% SDF	1.4%	8.0%	12
4	10% NaF	4.5%	-	9
5	3% NaF	1.4%	-	9
6	42% $AgNO_3$	-	25.5%	10
7	13% $AgNO_3$	-	8.0%	10
8	0.12% CHX[#]	-	-	7
9	Assay buffer*	-	-	-

[#] Positive control, * Reference (negative control) solution

Table 2 Mean end-point reading (MMPs activity) of experimental solutions

Group (n=10)	End-point Reading (MMP activity) in µg/µl (mean±SD)		
	MMP-2	MMP-8	MMP-9
1 - 38% SDF	2.38 ± 0.16	0.34 ± 0.08	2.58 ± 0.48
2 - 30% SDF	4.41 ± 0.32	0.77 ± 0.09	4.91 ± 0.52
3 - 12% SDF	9.17 ± 1.51	1.21 ± 0.18	5.60 ± 0.71
4 - 10% NaF	7.89 ± 0.55	1.66 ± 0.43	5.01 ± 0.76
5 - 3% NaF	8.51 ± 1.09	2.30 ± 0.54	5.65 ± 1.39
6 - 42% $AgNO_3$	11.08 ± 1.74	1.49 ± 0.20	6.77 ± 1.27
7 - 13% $AgNO_3$	10.64 ± 1.67	2.87 ± 0.31	12.35 ± 1.89
8 - 0.12% CHX	10.21 ± 1.03	3.95 ± 0.67	13.24 ± 0.52
9 - Assay buffer	11.08 ± 0.68	5.28 ± 0.55	13.97 ± 0.27
Comparing all experimental solutions			
p value	<0.001	<0.001	<0.001
Group 1 to 9	1<2<4,5<8 1<2<3<6,7,9	1<2<3,5,6<4<7<8<9	1<2,4<6<7<9 1<3,5<7,8
Comparing 38%SDF, 30%SDF and 12%SDF			
p value	<0.001	<0.001	<0.001
Group 1,2,3	1<2<3	1<2<3	1<2<3
Comparing 38%SDF (Ag-25.5% & F-4.5%), 10%NaF (F-4.5%), and 42%$AgNO_3$ (Ag-25.5%)			
p value	<0.001	<0.001	<0.001
Group 1,4,6	1<4<6	1<6<4	1<4<6
Comparing 12%SDF (Ag-8% & F-1.4%), 3%NaF (F-1.4%), and 13%$AgNO_3$ (Ag-8%)			
p value	<0.001	<0.001	<0.001
Group 3,5,7	3,5<7	3<5<7	3,5<7

Figure 1 Diagrammatic representation of a typical matrix metalloproteinases (MMP)

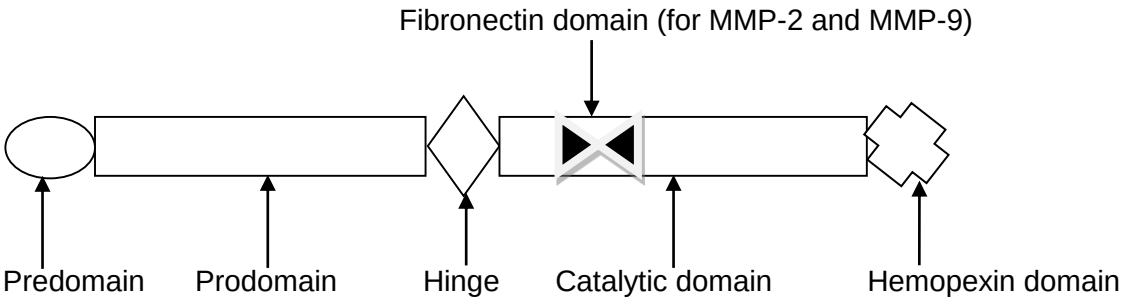

Figure 2 Percentage inhibition of MMP-2 of the experimental solutions

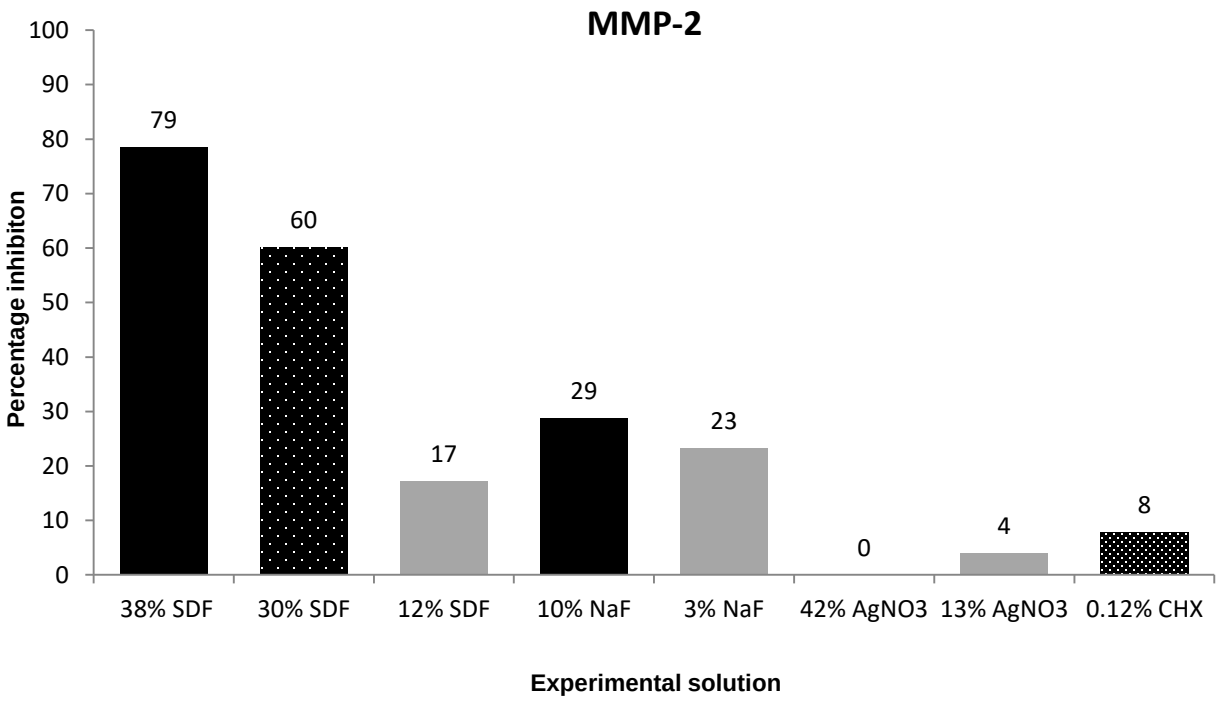

Figure 3 Percentage inhibition of MMP-8 of the experimental solutions

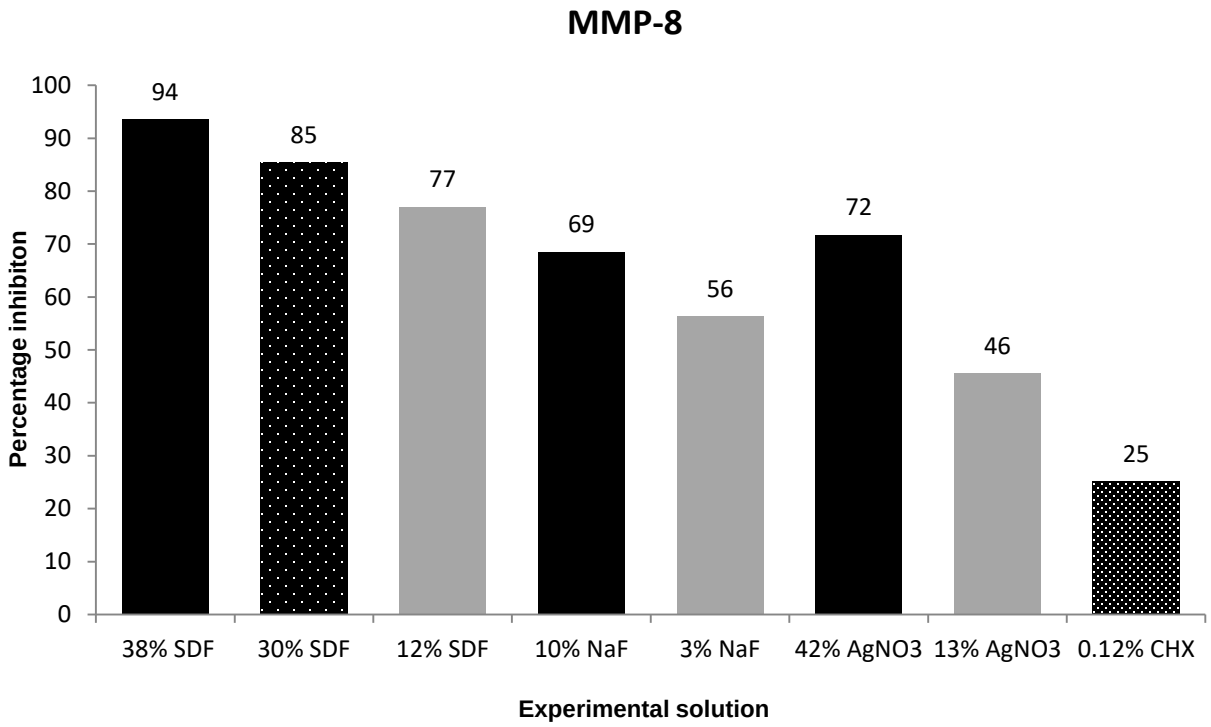

Figure 4 Percentage inhibition of MMP-9 of the experimental solutions