Research Article

Antibacterial and cytotoxic effect of a novel biological Nano-silver fluoride synthesized from moringa oleifera leaf extract

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Abstract: Background: A great dental and biomedical interest had been paid to silver nanoparticles because of their *a*ntimicrobial activity. Objective: To evaluate the antimicrobial and cytotoxic activity of a newly developed Nano-silver fluoride that was synthesized from moringa oleifera leaf extract against *S. mutants*. Material and method: The green synthesis method was used to prepare Nano-silver fluoride from moringa oleifera leaf extract. The minimum inhibitory concentration and the minimum bactericidal concentration were evaluated using brain heart infusion plates, while the cytotoxicity was evaluated by the hemolytic activity. Results: Nano-silver fluoride had a bactericidal and bacteriostatic effect (MIC was 60 ppm and MBC was 120 ppm) the diameter of the inhibition zone increased as the concentration increased. It was toxic at high concentrations and nontoxic at low concentrations. Conclusion: Nano-silver fluoride is a good material to be used in arresting and preventing dental caries and it is safe to be used on humans.

Keywords: Antibacterial activity, cytotoxicity, fluoride, moringa oleifera, silver nanoparticle, *streptococcus mutants*.

Introduction

While one of the most common chronic childhood diseases is dental caries, many decayed teeth remain untreated in underdeveloped countries [(1)]. Despite advances in dental care, surgical removal of the diseased dental tissue accompanied by the placement of appropriate restorative material remains the traditional method of treating dental caries. Recently, traditional methods are replaced by minimally invasive methods [(2,3)].

During the pandemic of COVID-19 and as a protective measure, dental professionals prefer non-aerosol treatment, e.g. the use of topical fluoridated compositions [(4)]. As silver has a bactericidal effect, it had been added as silver diamine fluoride (SDF) that can be used as a remineralizing agent in treating carious lesions. However, its black staining is considered a drawback that limits its use due to esthetic demand. With the development of nanotechnology, Nano- silver fluoride (NSF) is proven to be effective in controlling dental caries [(5)].

Three methods had been found for the synthesis of silver nanoparticles (AgNPs): physical, chemical and biological [4]. Synthesis using the biological method is rapid, low cost, and had less toxic effects than the other traditional methods. This method used different biological materials. Green synthesis is among them that is used in the plants [6]. Green synthesis is defined as the production of nanoparticles using environmentally friendly materials such as bacteria, fungi, and plants [7]. These appealing green strategies are free of the drawbacks that come with the traditional synthetic strategies, they are environmentally friendly [8].

This new material is safe for humans to use and has excellent antimicrobial properties against *Mutans streptococci* and *Lactobacilli*, the main pathogens that cause dental caries. NSF is a yellow solution, this material is both environmentally friendly and inexpensive [9]. The synergistic roles of both silver nanoparticles and fluoride are associated with NSF's efficacy in the prevention of dental caries [10].

The antimicrobial activity of silver nanoparticles had been reported against both Gram-negative and Gram-positive bacteria. Because of their ability to weaken the cell wall, silver nanoparticles accumulate in the bacterial membrane, causing a large increase in its permeability and cell death [10].

Low-concentration NPs were found to be non-toxic, while high-concentration NPs demonstrated more pronounced cytotoxicity [11], also some researchers discovered that the toxicity of NPs was dose-dependent [12,13]. In a previous study, the toxicity of antimicrobial NPs was found to be strongly related to the time rather than the concentration of the antimicrobial NPs [14]. NP toxicity is influenced by a variety of factors, including dosage, shape, particle size, distribution, time of action, interaction with other materials, and so on, according to other studies. Furthermore, because of their small particle size, NPs can easily enter the body and accumulate in organs, resulting in poisoning symptoms. No human cytotoxicity of NPs studies has been performed to date [15,16].

Moringa oleifera Lam (drumstick tree) belongs to the Moringa genus and the Moringaceae family [17]. Its value is related to its tender pods, flowers, and leaves, all of which are safe to consume by humans [18]. The leaves, in particular, are well known for their natural healing properties and they are widely consumed [9]. Extracts prepared from the leaves had been found to have high natural antioxidant properties as well as some antibacterial activity against both gram-positive and gram-negative bacteria [19].

A better understanding of the safety of silver nanoparticles is required to improve their therapeutic application. One of the most important procedures for determining a medication's safety is to assess its haemolytic potential when exposed to blood in vitro [20]. If the red blood cell (RBC) membrane becomes compromised, haemolysis will occur. The resulting release of haemoglobin may cause adverse health events [21]. This work aims to evaluate the antimicrobial activity of the NSF-MOLE, against *S. mutans* which is considered the primary pathogen involved in the development of dental caries, and their cytotoxic effect.

Materials and Methods

Green synthesis of Nano-silver fluoride from moringa oleifera leaf extracts

Preparation of the leaf extract

Fresh M. oleifera plant material was collected from AL-Qasim Green University/College of Agriculture. Leaf material was separated from the stems, washed with distilled water and then air-dried to remove the residual debris [22].

The extract was prepared by using 5 g of a fresh leaf which was transferred to a 250 ml beaker and 100 ml of deionized water was added to the leaf. The mixture was boiled for 15 min. The extract was cooled at room temperature and then filtered using filter paper number 001(THOMAS BAKER, India) to obtain a clear extract that was used for the synthesis of silver nanoparticles [22,23].

Green synthesis of silver nanoparticles (Ag NPs)

Ten ml of the plant extract was added to 100 ml of 1mM $AgNo_3$ (THOMAS BAKER, India). The mixture was stirred and heated using a Hot plate magnetic stirrer (LABINCO, Netherlands) for 20 min at 70 ºC. The color of the aqueous solution was changed from colorless to yellowish brown color which was an indication of the Ag NPs formation [23,24]. At the end of the reaction, sodium fluoride (NAF) (10.104 ppm) was added to improve the stability and the cariostatic efficacy of the solution and the stirring was continued overnight. The final solution Nano- silver fluoride (NSF) was stored in a dark container until further use [25,5].

Characterization of Nano-silver fluoride

The silver nanoparticles were characterized by several techniques to examine their properties and to ensure the green synthesis of the nanomaterial of silver was produced: the X-ray diffraction was used for the characterization of the silver nanoparticle, UV-VIS spectroscopy was performed for the NSF characterization and scanning electron microscope (SEM) was used for the characterization of the morphology and the size of the nanoparticles.

Antimicrobial activity of Nano- silver fluoride

Testing the sensitivity of *streptococcus mutans* to Nano- silver fluoride

To investigate the sensitivity of *streptococcus mutans* to Nano-silver fluoride, the material was prepared in final concentrations of (25,50,75,100,125) ug/ml. chlorhexidine was a positive control and the distilled water was the negative control. Agar well diffusion technique was used in this experiment on streptococcus mutans [26].

Procedure

Twenty-five ml of Mueller Hinton Agar medium was poured into sterile Petri dishes, left to cool, and solidified in sterile conditions at room temperature. Then, incubated at 37◦C for 24 hrs. to get rid of the humidity and ensure the media sterility. The density of activated inoculums was adjusted to that of the standard turbidity (0.5 McFarland standard turbidity to approximate cell density 1.5 x10^8 CFU/ml). Activated *Streptococcus mutans* inoculum was spread using a swab immersed in bacterial broth and left for 10 min at room temperature. Seven wells of equal sizes and depths (6 mm) were prepared in the agar

using Kork borer. Each well was filled with 100µl of different concentrations prepared from NSF and the control agents. After leaving the plates at room temperature for 10 min, the plates were incubated in an incubator for 24 hrs. at 37◦C. The diameters of growth inhibition zones were measured by vernier while the absence of the inhibition zone indicated the resistance of the bacteria to the tested agent.

Determination of Minimum Bactericidal Concentration (MBC)

The method conducted was the preparation of the different concentrations (15,30,60,120,240) ug/ml of NSF incorporated with 10 ml BHI. A to find the minimum bactericidal concentration of the NSF [27].

Procedure

Activation of isolates. Preparation of BHI.A(brain heart infusion agar) which was dispersed into screw capped bottles 10ml in each bottle and autoclaved. Final concentrations of (15,30,60,120,240) ug/ml were prepared from stock and incorporated in the sterile BHI.A to get 10ml of agar and NSF. The experimental bottles were poured into sterile Petri dishes and waited to become hard then inoculated by a streaking loop full of each activated bacteria. All these Petri dishes were incubated for 24 hrs at 37°C including the control plate (positive control) which contained BHI.A with microbial inoculum without the addition of the NSF, and (negative control) plate which contained BHI.A with different concentrations of NFS without bacterial inoculum. The MBC was determined as the lowest concentration of NSF that killed the microorganisms.

Cytotoxicity of Nano-silver fluoride: haemolytic activity in human erythrocytes

According to the method of Pinto et al. in 2012 28., human erythrocytes were obtained from blood discarded from Hilla General Teaching Hospital. Aliquots of human blood (type A, B and O) were mixed with 0.9 % (w/v) NaCl at a ratio of 1:30. The samples were then centrifuged at 2,500 rpm for 5 min to obtain the erythrocytes. This procedure was repeated twice, and the sediment from the last centrifugation was re-suspended in 0.9 % NaCl to a final concentration of 0.5 %. NSF was added to 3 mL of the erythrocyte suspension at various concentrations (400, 240, 120, 60, 20 and 8 ug/mL) for a final volume of 3 ml. The erythrocyte suspension was the negative control (0 % haemolysis), and the erythrocyte suspension plus 50 ul of 1 % Triton X-100 (THOMAS BAKER) was a positive control (100 % haemolysis). The samples were incubated for 1 h at room temperature under slow (100 rpm) and constant agitation (Incubadora Shaker). The samples were then centrifuged at 2,500 rpm for 5 min, and hemolysis was quantified by spectrophotometry at 540 nm. The degree of haemolysis can be measured by spectrophotometry at a wavelength of 540 nm. The tests were performed in triplicate. The results are expressed as the arithmetic mean of three measurements, and the levels of hemolysis were determined as a percentage relative to the positive control (100 % hemolysis)

Statistical analysis

Data was recorded in Excel sheets and statistical analysis was performed using SPSS for Windows release 22 (SPSS Inc., Chicago, IL, USA). ANOVA test was used followed by Games Howell post hoc for the diameter of the inhibition zone among groups. For cytotoxicity, the ANOVA test was used followed by Dunnett T3 post hoc.

Results

Characterization of silver nanoparticles synthesized by green synthesis: The XRD pattern clearly showed the main peaks at 38◦, 44◦, 64◦and 77◦, these angles belong to faced cubic center silver ion. The formation of silver nanoparticles can be visually detected after heating the aqueous solution of silver ions with moringa oleifera leaf extract. The primary solution was colorless and after the reaction of the silver ion with extract the color changed to yellowish-brown this color belonged to surface plasmon resonance (SPR)of silver nanoparticle that can be detected by UV- visible spectroscopy that shows the peak centered around 430 nm and the material was synthesized by the green synthesis method which produced nanoparticles with a small diameter of less than (100 nm) (29). The result showed nanoparticles with spherical or semi-spherical forms and diameters between (19-50) nm. The particle appeared separated with slight agglomeration that occurred because of the presence of fluoride ions.

Antimicrobial activity of Nano-silver fluoride

Testing the sensitivity of *streptococcus mutans* to Nano-silver fluoride

Shapiro-Wilk test showed normal distribution of the diameter of the inhibition zone among groups. The result showed that with the increase in the concentration of NSF-MOLE the diameter of the inhibition zone was increased to reach the maximum diameter (Table 1).

Table 1: Descriptive and statistical test of the diameter of inhibition zone among groups.

Groups	N	Mean	±SD	±SE	Minimum	Maximum	F	p value
25	10	.000	.000	.000	.000	.000		
50	10	.000	.000	.000	.000	.000		
75	10	1.000	.115	.037	.800	1.200		
100	10	1.130	.116	.037	.900	1.300	594.017	0.000 **
125	10	1.400	.133	.042	1.200	1.600		
DW	10	.000	.000	.000	.000	.000		
CHX	5	1.4	0	0	1.4	1.400		

*Levene test=8.232, p value=0.000 Sig

Concerning the NSF, results showed a statistically significant difference in the diameter of the inhibition zone for all the concentrations used except for the 75ppm with 100ppm and for 125ppm with chlorhexidine, which showed no significant difference (Fig.1, Table 2).

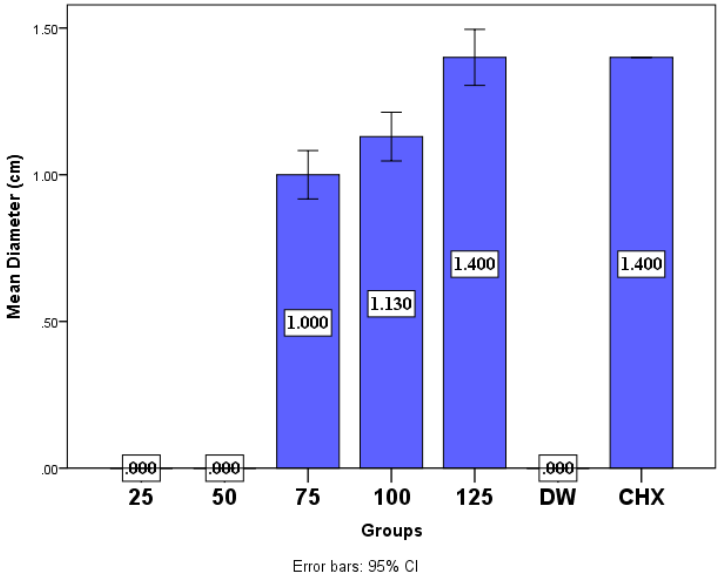

Figure 1: mean diameter of inhibition zone among groups.

B. Determination of Minimum Bactericidal Concentration (MBC)

Results of this study revealed that MBC was 120, this concentration showed no growth after re-culturing on BHI –agar media, this means that (120) concentration had a bactericidal effect and killed the bacteria (Figure 2).

Table 2: Multiple Comparisons of Diameter (cm) among groups using Games-Howell

(I) Groups	(J) Groups	Mean Difference (I-J)	P value
25	50	.000	.
	75	-1.000	**0.000***
	100	-1.130	**0.000***
	125	-1.400	**0.000***
	DW	.000	
	CHX	-1.4	**0.000***
50	75	-1.000	**0.000***
	100	-1.130	**0.000***
	125	-1.400	**0.000***
	DW	.000	
	CHX	-1.4	0.000*
75	100	-.130	0.212^
	125	-.400	**0.000***
	DW	1.000	**0.000***
	CHX	-0.4	**0.000***
100	125	-.270	**0.002***
	DW	1.130	**0.000***
	CHX	-0.27	**0.001***
125	DW	1.400	**0.000***
	CHX	0.000	1.00^
DW	CHX	-1.4	**0.000***

*=significant at p<0.05, ^=not significant at p>0.05.

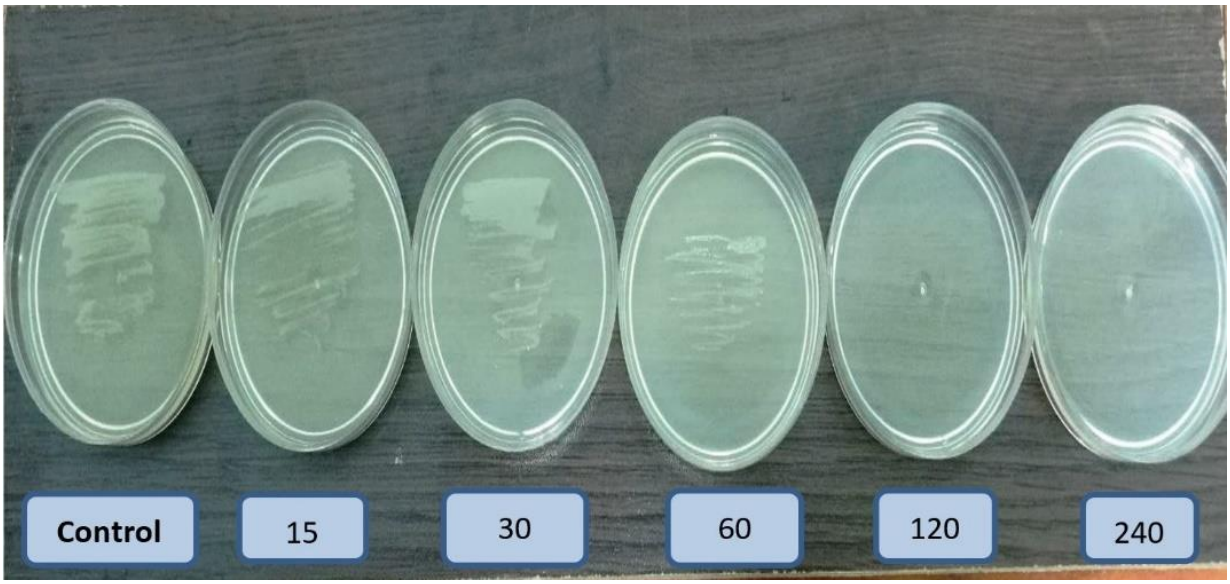

Figure 2: Brain heart infusion shows the minimum inhibitory concentration and minimum bactericidal concentration.

Cytotoxicity of Nano-silver fluoride: haemolytic activity in human erythrocytes

The percentage of vital cells was normally distributed among the ABO groups and concentration using the Shapiro-Wilk test ($p>0.05$).

The vitality of cells among the eight groups of concentrations for each blood group is demonstrated in Table 3. The greatest mean of the vital cells belonged to the first concentration followed by the second one, while it was the lowest in the last concentration with a statistically significant difference. Meanwhile, the highest mean of the vital cells was found in blood group B followed by blood group A, while it was the lowest in blood group O.

Following the multiple pairwise comparisons, it was clear that when comparing the 2nd concentration with the 1st and 3rd and when comparing the 5th with the last one, these results were not significant statistically, while the other comparisons were significant (Table 4). For the experiment, the material was considered to be toxic when it caused hemolysis to 50% of the red blood cell, so NSF-MOLE was considered toxic at the higher concentrations (240, 400) ppm, while it was considered nontoxic at the other concentrations (120, 60, 20), Figure 2.

Table 3: The cell vitality among the concentration groups by ABO blood groups.

ABO Blood Groups	Groups	Minimum	Maximum	Mean	±SD	±SE	F	p-value
A	8	88.570	92.195	90.007	1.926	1.112	36.920	0.000*
	20	85.122	88.310	86.277	1.766	1.020		
	60	69.697	76.360	73.238	3.351	1.935		
	120	28.375	56.620	42.966	14.146	8.167		
	240	33.884	42.340	36.790	4.808	2.776		
	400	.000	29.268	13.219	14.838	8.567		
B	8	93.000	96.140	94.846	1.641	0.947	198.207	0.000*
	20	92.820	95.062	93.914	1.122	0.648		
	60	88.246	89.690	89.118	.768	0.443		
	120	59.649	71.270	64.039	6.310	3.643		
	240	.000	15.789	8.614	7.992	4.614		
	400	.000	13.860	7.383	6.974	4.027		
O	8	90.952	91.111	91.022	.081	0.047	47.876	0.000*
	20	83.333	89.717	86.255	3.226	1.863		
	60	59.126	66.667	64.005	4.231	2.443		
	120	41.645	55.111	48.760	6.765	3.906		
	240	-1.028	28.889	12.461	15.173	8.760		
	400	-11.825	16.000	3.773	14.215	8.207		
	8	88.570	96.140	91.958	2.547	0.849	98.435	0.000*
	20	83.333	95.062	88.815	4.280	1.427		
	60	59.126	89.690	75.454	11.333	3.778		
	120	28.375	71.270	51.922	12.662	4.221		
	240	-1.028	42.340	19.289	15.949	5.316		
	400	-11.825	29.268	8.125	11.609	3.870		

*=Significant at p<0.05, ^=not significant at p>0.05.

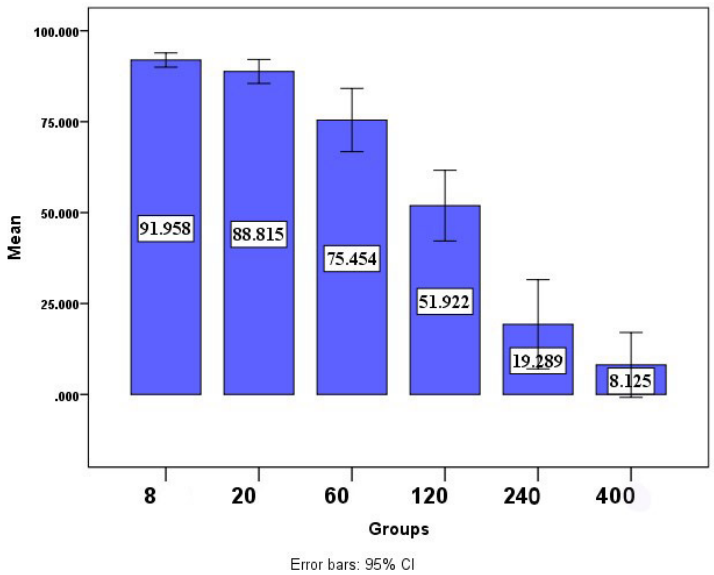

Figure 2: Mean of vital cells among the eight concentrations.

Table 4: Multiple Comparisons of vitality between groups using Dunnett T3

(I) Groups	(J) Groups	Mean Difference (I-J)	P value
8	20	3.143	0.612 ^
	60	16.505	**0.026***
	120	40.037	**0.000***
	240	72.670	**0.000***
	400	83.833	**0.000***
20	60	13.362	0.086 ^
	120	36.894	**0.000***
	240	69.527	**0.000***
	400	80.690	**0.000***
60	120	23.532	**0.011***
	240	56.165	**0.000***
	400	67.329	**0.000***
120	240	32.633	**0.003***
	400	43.797	**0.000***
240	400	11.164	0.740^

^=Not significant at p>0.05, *= significant at p<0.05

Discussion

New comprehensive caries preventive methods should include fluoride and other agents that influence the de- and remineralization balance, as well as antibacterial strategies [9, 30].

In recent years, many researchers had focused on developing modified or unique synthetic procedures for the silver nanoparticles rather than using the traditional methods, which had been linked to toxic environmental impacts [31]. In the present study, NSF-MOLE was synthesized by a green method from leaf extract samples of the medicinal tree species M. oleifera, which was a cost-effective synthesis technique [4,8,22].

To make the new nanoscale biological compound a more effective material affecting the balance between de- and remineralization [32].and to enhance the antimicrobial strategy [33], fluoride was added to reduce biofilm formation and adhesion, as well as reduce acid production to prevent demineralization [34].

This study reported the characterization and testing of the NSF-MOLE, which contained silver nanoparticle synthesized from moringa oleifera leaf extract and fluoride. The color of this material was yellowish-brown. This color belongs to the surface plasmon resonance (SPR) of AgNPs, which is an intrinsic feature of AgNPs (Oda et al., 2019) [29]. Meanwhile, silver nanoparticle produced from cauliflower extract has brownish-red color (Oda et al., 2019) [29], while that produced from Beta vulgaris Extract has a yellow-orange color (Hashim and Oda, 2019) [23], Silver nanoparticle produced from Mace-Arils of *Myristica fragrans* has a pale, brownish solution (Rizwana et al., 2021) [6].

The antimicrobial activity of silver nanoparticles was tested and compared to chlorhexidine, which could reduce *streptococcus mutans* in saliva [35].

The nanoparticle produced from green synthesis was spherical and the particle size was in the range of 19 to 50 nm proving that the antibacterial activity of silver nanoparticles against *S. mutans* increases as the particle size decreases. This result met the findings of Espinosa-Cristobal et al. in 2009 [36], Morones et al. in 2005 [37], and Baker et al. in 2005 [38].

Chlorhexidine is a chemical substance, which is considered a bactericidal, antiseptic, and antifungal substance that is active against both gram-positive and gram-negative bacteria. It also possesses bacteriostatic properties, preventing bacterial proliferation by disrupting cell membranes rather than inactivating ATPase, as previously thought [39].

Due to the differences in the characteristics of the various bacterial strains and the reduction in the size of silver Nanoparticles [40], it was difficult to compare the MIC values with those of other researchers. Systematic reviews, on the other hand, concluded that chlorhexidine had limited scientific evidence to be used as a dental caries preventative agent [39]. Silver-containing compounds, such as SDF, had been used in dentistry with excellent clinical efficacy [41]. Thus, silver nanoparticle-containing compounds have a great deal of potential for preventing dental caries.

The hemolytic test is an effective in vitro system that may be used to study the toxic and protective effects of a wide range of substances or conditions linked with oxidative stress [9].

Cytotoxicity of NSF-MOLE was tested and found that there is a significant concentration-related cytotoxicity of NSF-MOLE (the number of vital cell decrease as the concentration increase) [42]. NSF-MOLE was nontoxic at low concentrations (120, 60,20, 8) ppm this finding was consistent with that of Targino et al. in 2014 who tested the cytotoxicity of Nano-silver fluoride on human erythrocyte and found that Nano-silver fluoride was safe in all concentrations tested regardless of blood group type [9]. While the high concentration (200, 400) ppm caused hemolysis to more than 50% of the cells, thus considered toxic. Hernández-Sierra et al. in 2011 found that AgNPs <20 nm increased cytotoxicity in human periodontal fibroblasts in a concentration- and time-dependent manner [42].

Conclusion

The current study showed that NSF-MOLE had a good bacteriostatic and bactericidal effect on *streptococcus mutans* similar to chlorohexidine with no cytotoxic effect on living human erythrocytes at the low concentration, thus it is considered a good noninvasive technique for the treatment of dental caries in children.

Conflict of interest: None.

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العنوان: الفعالية المضادة للبكتريا والتأثير السام لمادة فلوريد الفضة النانوي المصنع من مستخلص أوراق شجرة المورينغا

الباحثون: دعاء جواد كاظم , اسيل حيدر محمد جواد

المستخلص:

الخلفية: اهتمام كبير في طب الأسنان والطب الحيوي اولي للجسيمات النانوية الفضية بسبب نشاطها المضاد للميكروبات. الهدف: تقييم النشاط المضاد للميكروبات والسموم للخلايا لفلوريد الفضة النانو المطوَّر حديثًا والذي تم تصنيعه من مستخلص أوراق المورينجا أوليفيرا ضد المكورات العقدية. الطريقة: تم استخدام طريقة التخليق الأخضر لتحضير فلوريد الفضة النانوية من مستخلص أوراق المورينجا أوليفيرا. تم تقييم الحد الأدنى للتركيز المثبط والحد الأدنى من تركيز مبيد الجراثيم باستخدام الاجار ، بينما تم تقييم السمية الخلوية (MBC120 PPM,MIC 60 PPM) من خلال النشاط الانحلالي. النتيجة: كان لفلوريد الفضة النانوي تأثير مبيد للبكتريا ومقاوم للبكتريا ، ويزداد قطر منطقة التثبيط مع زيادة التركيز. فلوريد الفضة النانوي كان سام عند التركيز العالي وغير سام عند التركيز المنخفض. الخلاصة: فلوريد الفضة النانوي مادة جيدة لاستخدامها في إيقاف ومنع تسوس الأسنان وهي آمنة للاستخدام على الإنسان.