Green Synthesis and Antimicrobial Activity of Silver Nanoparticles Synthesised Using *Terminalia chebula*

Running Title: Antimicrobial Activity of Silver Nanoparticles Synthesized using *Terminalia* chebula against *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis*.

Abstract

Background: When left unsupervised, dental pathogens cause chronic diseases like gingivitis, periodontitis and dental caries that lead to the irrevocable loss of tooth structure. However, we can curb the excessive proliferation of pathogenic oral flora such as *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis* with sound preventive measures such as using promising herbs with well documented health benefits like *Terminalia chebula*.

Aim: The aim of this study was to evaluate the potential antimicrobial activity of silver nanoparticles synthesized using *Terminalia chebula* against *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis* in different concentrations.

Materials and Methodology: *Terminalia chebula* was used to prepare the plant extract, followed by the synthesis of silver nanoparticles. The Agar-Well Diffusion Method was used to determine the antimicrobial efficacy of Ag-NPs in different concentrations, namely 25, 50 & 100 μL. Amoxicillin was used as a positive control. The zone of inhibition was recorded for each plate and the results were analysed statistically with one way anova followed by post hoc analysis using IBM SPSS software (Version 20.0)

Results: A colour change was observed after the synthesis of Ag-NPs. The prepared particles were then characterised by a peak seen at 440 nm in UV-vis-spectroscopy. The zone of inhibition

increased in size with an increase in Ag-NP concentration. For S. aureus, the zone of inhibition was significantly higher at all concentrations of Ag-NPs than Amoxicillin (p<0.05), whereas for E.faecalis, it was significantly Lower than amoxicillin (p<0.05). For S. mutans at 25 microliter concentration the zone of inhibition was significantly lower than amoxicillin (p<0.05) whereas at higher concentrations there was no significant difference with amoxicillin (p>0.05)

Conclusion: Ag-NPs synthesized using *Terminalia chebula* is an effective antimicrobial agent against dental pathogens, especially *Staphylococcus aureus* and *Streptococcus mutans*.

Keywords: Silver Nanoparticles; Terminalia chebula; Streptococcus mutans; Staphylococcus aureus and Enterococcus faecalis.

Introduction

Nanotechnology is currently an enthusiastically growing field in the epoch of modern science. The size-dependent characteristics of nanoparticles give it its multidimensional nature that has been efficiently employed in the past in fields such as agriculture (1), physics, optics, medicine and electronics as catalysts, imaging probes, delivery systems, energy sources, scavengers, biological labels, diagnostic biosensors and as automotive parts (2) among several others. It has also been acknowledged that nanomaterials divulge new mechanical and chemical properties to everyday products, thus exponentially multiplying their functionality and longevity (3,4).

It is a well-documented fact that silver has a relatively low toxicity and a high biocompatibility when compared to most metals, thus greatly increasing its potential to be used in the pharmaceutical and healthcare industry (5). One amongst its many beneficial properties is its antimicrobial efficacy. Interestingly, it was found that bacteria killed by silver showed significant antibacterial potential against a viable population of the same bacterium. This is because the killed bacterium acts as a reservoir for silver and promotes further bactericidal activity - earning for itself a rather amusing term - the "Zombie's Effect". Thus, it exhibits a desirably long lasting

and persistent bactericidal effect (6). This subsequently prevents bacterial re-colonisation and proliferation. With the intention of harnessing the health benefits of silver, its nanoparticles were synthesized using metallic silver nitrate. Herbal formulations are being used in many systemic and oral diseases due to its therapeutic benefits with less side effects.(7–9)This was mediated by an herbal formulation of *T. chebula*. Although silver nanoparticles can be conventionally synthesised by availing physical and chemical methods, biosynthesis proves to be a better choice as it does not involve the use of hazardous chemicals (10) and it is economical, simple, high yielding, rapid and non-toxic. Biologically synthesised silver nanoparticles also exhibit exalted levels of stability and solubility (11).

Terminalia chebula is a native, cheap and commonly found plant with established health benefits. It has an extensive history of importance in Ayurveda (12) due to its use in kidney and liver dysfunctions, antitussive, cardiotonic, homeostatic, diuretic and laxative functions (13)(14). Thus, by using silver nanoparticles, we have aimed to exploit the merits of *Terminalia chebula*.

Streptococcus mutans is a gram-positive bacterium which is commonly found in the oral cavity and it plays an essential role in the pathogenesis of dental decay (15). It is one of the most vital initial colonizers of tooth surfaces that seeks to change its immediate local environment in terms of pH, coaggregation and substrate availability among several other factors – thereby allowing for more fastidious organisms to colonize and aggregate even further (16)(17)(18), finally resulting in the formation of dental plaque and calculus. This is because it is one of the few bacteria furnished with uncommon specialised receptors that enhance adhesion to tooth surfaces. It employs the use of the enzyme glucansucrase which converts sucrose into a sticky dextranbased polysaccharide that helps other organisms to cohere. As it metabolises dietary sucrose to lactic acid, an acidic environment with a low pH is created in the mouth which leads to the demineralisation of enamel – causing an increase in the vulnerability of teeth to dental decay (19). Other bacteria such as Staphylococcus aureus, Streptococcus mitis, Streptococcus sanguinis and Enterococcus faecalis have also been implicated in the development of dental caries, endodontic infections, peri-implantitis and gingival diseases (20)(21).

It was estimated that approximately 2.3 billion adults had dental caries in their permanent teeth and that more than 530 million children had decayed primary teeth in 2015 and its incidence only

continues to increase despite having worldwide access to dental care (22). Also, with our current scientific footing we cannot regenerate natural tooth structure once it has been lost (23). Thus, it is important to devise effective and inexpensive measures against dental distress causing pathogens such as *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis* – subsequently giving this study its aim.

Our team has extensive knowledge and research experience that has translated into high quality publications (24–36),(37–41)(42)(43). Here, we have attempted to synthesize silver nanoparticles using an herbal formulation prepared from the fruits of *Terminalia chebula* and studied its antimicrobial activity in different concentrations in comparison with the standard antibiotic amoxicillin.

Materials and Methodology

Preparation of the Plant Extract

Fruits of *Terminalia chebula* were freshly collected from Chennai in the month of January, 2021 and were thoroughly washed 3-4 times in double distilled water. They were then shade-dried for 7-14 days and were powdered using a mortar and pestle and stored in an airtight utensil. Later, 1 g of the powder was dissolved in distilled water and was boiled for 5-10 minutes at (60-70)°. The resulting solution was filtered using a Whatman No. 1 filter paper. Finally, the filtered plant extract was collected and stored at 4°C until further use.

Synthesis of Silver Nanoparticles

1 millimolar of silver nitrate was dissolved in 90 ml of double distilled water to make a metallic solution. To this, the plant extract of *Terminalia chebula* was added and was made into a formulation of 100 ml. Its colour change was visually appreciated and photographs were taken for its methodical documentation. The formulation was later kept in a magnetic stirrer for the synthesis of its nanoparticles.

Characterisation of the Synthesised Silver Nanoparticles

The synthesis of silver nanoparticles was initially characterised by using UV-vis-spectroscopy, where 3 ml of the solution was taken in a cuvette and scanned in a UV-vis-spectrometer within a wavelength field limit of 250 nm to 750 nm. The results were recorded for its graphical analysis.

Preparation of the Silver Nanoparticle Powder

The silver nanoparticle formulation was centrifuged in a Lark refrigerated centrifuge. The centrifugation was done at 8000 rpm for a time duration of 10 minutes and the resultant pellet was collected and then washed twice with distilled water. It was later purified and dried at 60°C. Finally, the silver nanoparticle powder that was obtained was collected and stored in an air-tight Eppendorf tube.

Study of Anti-bacterial Activity of Ag-NPs Against Streptococcus mutans, Staphylococcus aureus and Enterococcus faecalis

The Agar-Well Diffusion Method was used to assess the antimicrobial efficacy of Ag-NPs. Different dilutions of Ag-NPs were tested against 3 organisms - *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis*. Their fresh bacterial suspensions were dispersed on the surface of Muller-Hinton Agar containing plates. Ag-NP concentrations of (25, 50 & 100) μL were incorporated into the wells and the plates were then incubated at 37°C for 24 hours. Antibiotics were used as a positive control and the zones of inhibition were recorded for each well.

Statistical Analysis

The data was recorded in a Microsoft Excel 2016 (Microsoft Office 10) spreadsheet and was later exported to the Statistical Package for the Social Sciences for Windows (Version 20.0, SPSS, Inc., Chicago, USA) where it was subjected to statistical analysis. A one-way ANOVA test was used with the level of significance set at p<0.05 followed by a Post Hoc (Tukey HSD) test with the confidence interval set at 95%.

Results and Discussion

Visual Observation

A colour change was visually observable after the synthesis of nanoparticles. The extract had turned a dark brown from its initial white colour. This colour change from white to dark brown caused by the excitation of surface plasmon resonance primarily confirms the formation of Ag-NPs.

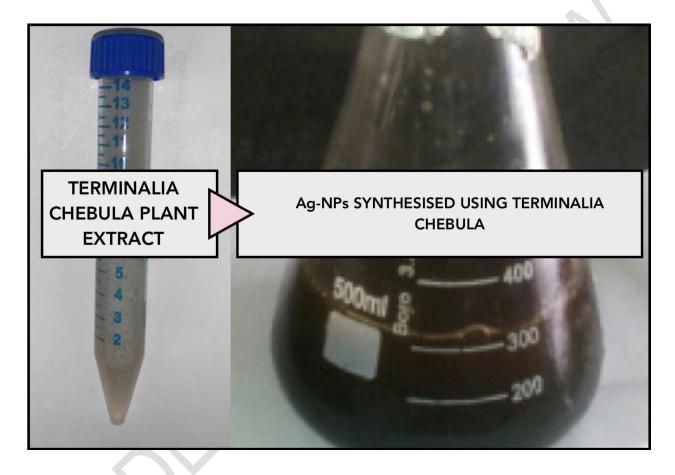


Figure 1 - Showcasing a visually observable colour change from white to dark brown after the synthesis of nanoparticles from a metallic formulation of silver nitrate using *Terminalia chebula*.

UV-vis-Spectroscopy

The bioreduction of pure silver nitrate to silver nanoparticles was characterised by employing UV-vis-spectroscopy. This was carried out to assess the successful formation and stability of the Ag-NPs. It was done using a UV-vis-spectrometer within a wavelength field limit of (250 - 750) nm. Its peak (Surface Plasmon Resonance) was noted at 440 nm. This is in line with the

comparable findings of 437 nm, 400 nm - 440 nm and 450 nm in similar studies conducted by Vidhya *et al.* in 2021 (44), Chandra Sekhar *et al.* in 2016 (45) and Prathibha *et al.* in 2015 (46) respectively.

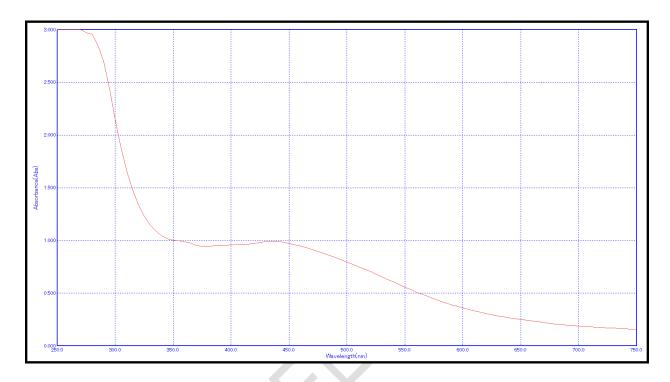


Figure 2 - Showing the characterisation of Ag-NPs post bioreduction using UV-vis-spectroscopy, showing a peak at 440 nm with wavelength given in nanometer units in the 'X' axis and absorbance in the 'Y' axis.

Antimicrobial Activity

The antimicrobial activity for three different concentrations of our silver nanoparticle solution were measured and recorded in 3 plates containing unique microbes - *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis* - simultaneously being scaled against the antibiotic drug control in use. All measurements were done in triplicate.

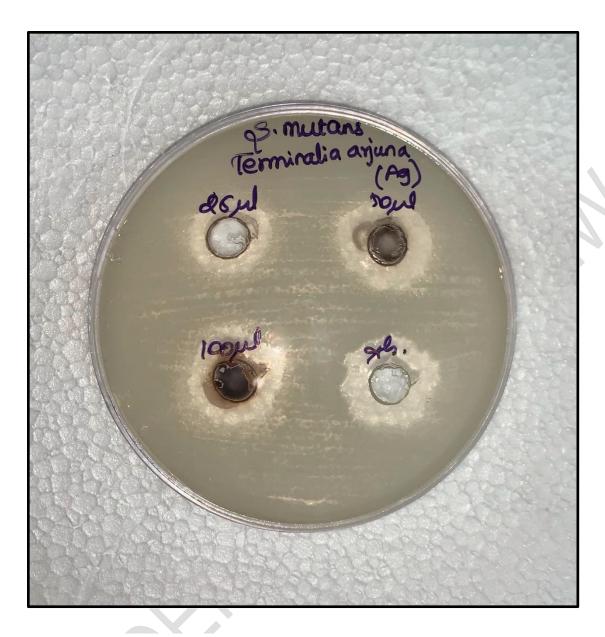


Figure 3 - Showcasing the effect of various concentrations of Ag-NPs on *Streptococcus mutans* with their zones of inhibition:

- 1. Effect of 25 μL of Ag-NPs on *Streptococcus mutans* with its moderately sized zone of inhibition
- 2. Effect of 50 μ L of Ag-NPs on *Streptococcus mutans* with its large sized zone of inhibition
- 3. Effect of 100 µL of Ag-NPs on Streptococcus mutans with its large zone of inhibition
- 4. Effect of Amoxicillin on Streptococcus mutans with its apparent zone of inhibition



Figure 4 - Showcasing the effect of various concentrations of Ag-NPs on *Staphylococcus aureus* with their zones of inhibition:

- 1. Effect of 25 μ L of Ag-NPs on *Staphylococcus aureus* with its moderately sized zone of inhibition
- 2. Effect of 50 µL of Ag-NPs on Staphylococcus aureus with its apparent zone of inhibition
- 3. Effect of 100 μ L of Ag-NPs on *Staphylococcus aureus* with its large sized zone of inhibition

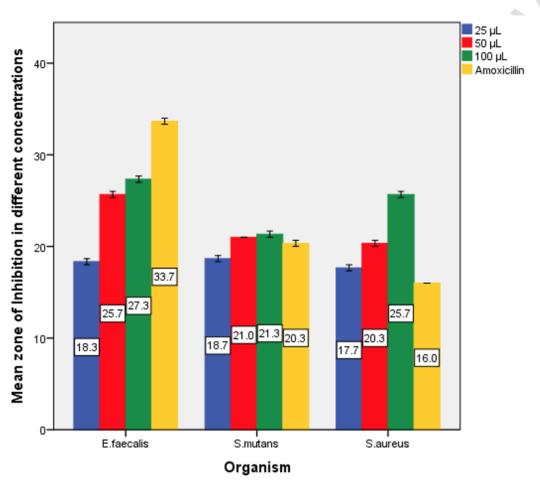
4. Effect of Amoxicillin on Staphylococcus aureus with its apparent zone of inhibition



Figure 5 - Showcasing the effect of various concentrations of Ag-NPs along with the positive control (Amoxicillin)on *Enterococcus faecalis* with their zones of inhibition:

- 1. Effect of 25 μ L of Ag-NPs on *Enterococcus faecalis* with its moderately sized zone of inhibition
- 2. Effect of 50 μ L of Ag-NPs on *Enterococcus faecalis* with its large sized zone of inhibition

- 3. Effect of 100 µL of Ag-NPs on *Enterococcus faecalis* with its large sized zone of inhibition
- 4. Effect of Amoxicillin on Enterococcus faecalis with its highly apparent zone of inhibition



Error Bars: +/- 1 SE

Figure 6 - Bar graph with error bars showing the zones of inhibition for all three dilutions of Ag-NPs and the standard antibiotic (Amoxicillin) on the three different organisms. The three organisms - *Enterococcus faecalis*, *Streptococcus mutans* and *Staphylococcus aureus* are grouped in the 'X' axis and the measurements of the zones of inhibition in millimetre units are denoted on the 'Y' axis. The blue bars depict the zones of inhibition for 25 microliter units of the

Ag-NPs, red depicts 50 microliters, green depicts 100 microliters and yellow depicts the positive control Amoxicillin. For S. aureus, the zone of inhibition was significantly higher than Amoxicillin at all the tested concentrations of Ag-NPs (p<0.05), whereas for E.faecalis, it was significantly lower than amoxicillin (p<0.05). For S. mutans at 25 microliter concentration the zone of inhibition was significantly lower than amoxicillin (p<0.05) whereas at higher concentrations there was no significant difference with amoxicillin (p>0.05)

Table 1 - Table depicts the descriptive statistics of the zone of inhibition of the various concentrations of the biosynthesised silver nanoparticle's antimicrobial activity along with the standard antibiotic on the three microbial species

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Absorbance of S. mutans	25 µl	3	18.67	.577	.333	17.23	20.10	18	19
	50 µI	3	21.00	.000	.000	21.00	21.00	21	21
	100 µl	3	21.33	.577	.333	19.90	22.77	21	22
	AB	3	20.33	.577	.333	18.90	21.77	20	21
	Total	12	20.33	1.155	.333	19.60	21.07	18	22
Absorbance of S. aureus	25 µl	3	17.67	.577	.333	16.23	19.10	17	18
	50 µI	3	20.33	.577	.333	18.90	21.77	20	21
	100 µl	3	25.67	.577	.333	24.23	27.10	25	26
	AB	3	16.00	.000	.000	16.00	16.00	16	16
	Total	12	19.92	3.848	1.111	17.47	22.36	16	26
Absorbance of E. faecalis	25 µl	3	18.33	.577	.333	16.90	19.77	18	19
	50 µl	3	25.67	.577	.333	24.23	27.10	25	26
	100 µl	3	27.33	.577	.333	25.90	28.77	27	28
	AB	3	33.67	.577	.333	32.23	35.10	33	34
	Total	12	26.25	5.723	1.652	22.61	29.89	18	34

Table 2 - Table depicts the one way ANOVA analysis performed for the various concentrations of the silver nanoparticle's antimicrobial activity on the three microbial species as observed in this study, where we have found statistically significant differences between the groups (p<0.05).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Absorbance of S. mutans	Between Groups	12.667	3	4.222	16.889	.001
	Within Groups	2.000	8	.250		
	Total	14.667	11			
Absorbance of S. aureus	Between Groups	160.917	3	53.639	214.556	.000
	Within Groups	2.000	8	.250		
	Total	162.917	11			
Absorbance of E. faecalis	Between Groups	357.583	3	119.194	357.583	.000
	Within Groups	2.667	8	.333		
	Total	360.250	11			

Table 3 - Tabulation showing pairwise comparison between groups using Post Hoc (Tukey HSD) test results, with the confidence interval set to 95%.

Dependent Variable (Tukey HSD)	(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
•		O _N				Lower Bound	Upper Bound
Absorbance of S. mutans	25 μΙ	50 μΙ	-2.333*	.408	.002	-3.64	-1.03
		100 μ1	-2.667*	.408	.001	-3.97	-1.36
		Amoxicillin	-1.667 [*]	.408	.015	-2.97	36
	50 μl	100 μ1	333	.408	.845	-1.64	.97
		Amoxicillin	.667	.408	.414	64	1.97

	100 μl	Amoxicillin	1.000	.408	.144	31	2.31
Absorbance of S. aureus	25 μΙ	50 μl	-2.667 [*]	.408	.001	-3.97	-1.36
•		100 μ1	-8.000*	.408	.000	-9.31	-6.69
		Amoxicillin	1.667*	.408	.015	.36	2.97
	50 µl	100 μ1	-5.333 [*]	.408	.000	-6.64	-4.03
		Amoxicillin	4.333*	.408	.000	3.03	5.64
	100 μl	Amoxicillin	9.667*	.408	.000	8.36	10.97
Absorbance of E. faecalis	25 μl	50 μl	-7.333*	.471	.000	-8.84	-5.82
		100 μ1	-9.000 [*]	.471	.000	-10.51	-7.49
	10	Amoxicillin	-15.333 [*]	.471	.000	-16.84	-13.82
	50 μ1	100 μ1	-1.667 [*]	.471	.031	-3.18	16
		Amoxicillin	-8.000*	.471	.000	-9.51	-6.49
	100 µl	Amoxicillin	-6.333*	.471	.000	-7.84	-4.82

As deduced from Figures 3 and 6, 25 μ L of the Ag-NP solution had the least efficacy against *Streptococcus mutans* with only a moderately sized zone of inhibition measuring up to 18.7 mm.

50 μ L of the Ag-NP solution had a considerable efficacy against *Streptococcus mutans* with a large zone of inhibition of 21.0 mm. 100 μ L of the Ag-NP solution had the best effect on *Streptococcus mutans* out of all the test samples, with a decently apparent zone of inhibition of 21.3 mm. The positive control used was Amoxicillin which had the second best action against *Streptococcus mutans* with its slightly smaller zone of inhibition of 20.3 mm.

Figures 4 and 6 demonstrate the efficacy of Ag-NPs on *Staphylococcus aureus*, where 25 μ L of the Ag-NP solution had the second lowest antimicrobial potential with a moderately sized zone of inhibition measuring 17.7 mm. 50 μ L of the Ag-NP solution had a good efficacy with a visibly apparent zone of 20.3 mm. 100 μ L of the Ag-NP solution had the best effect, with 25.7 mm as the zone of inhibition. Amoxicillin had the smallest zone of inhibition among all the test samples measuring only 16.0 mm, suggesting that Ag-NPs is an excellent solution for controlling *Staphylococcus aureus*.

The antimicrobial activity of Ag-NPs synthesized using *Terminalia chebula* against *Enterococcus faecalis* was concluded in Figures 5 and 6 - 25 μL of the sample yielded a zone of inhibition of 18.3 mm, 50 μL stood at 25.7 mm with a large zone of inhibition and 100 μL had a similar but slightly larger zone of inhibition of 27.3 mm. Amoxicillin had the highest antimicrobial activity against *Enterococcus faecalis*, with a zone of inhibition measuring up to 33.7 mm. Out of all the three organisms, it would appear that our Ag-NPs are the least effective against the *Enterococcus* species, birthing the need for better solutions.

Tables 1 and 2 depict the one way ANOVA analysis performed for the various concentrations of the silver nanoparticle's antimicrobial activity on the three species as observed in this study, where we have obtained statistically significant results. (p<0.05) Table 3 shows the values obtained following a Post Hoc test (Tukey HSD), where we have obtained statistically significant differences between the antimicrobial activities of the plant extract and the control on the various organisms.

In a study conducted by Kesarla *et al.* in 2012 where they synthesized similar silver nanoparticles using *Terminalia chebula* in order to study its various properties, they concluded that the nanoparticles showed a good antimicrobial activity towards the gram positive bacteria, *Staphylococcus aureus* and that it was an industrially smart idea to exploit said efficacy (47).

This is in accordance with our findings as depicted i. Another such study carried out by Nandagopal *et al.* in 2014 said that silver nanoparticles synthesised using the benefits of *Terminalia chebula* had excellent antimicrobial and anticancer potentials at all concentrations, especially against MDR bacteria such as *Staphylococcus aureus* (48). Ankegowda *et al.* in 2020 put forward that Ag-NPs mediated by *T. chebula* had a decent but not extremely sensitive antimicrobial potential against *Streptococcus mutans* (49), which is also at par with our findings. Carounanidy *et al.* in 2007 attempted to synthesise a herbal mouthwash using *T. chebula* reduced Ag-NPs. They concluded that using this formulation helped to increase the pH of the oral environment and notably reduced the microbial count of *Streptococcus mutans*, thus playing a crucial role in the destruction of cariogenic bacteria (50).

Recently, there has been an increase in the resistance of *Staphylococcus aureus* and *Streptococcus mutans* towards normal antimicrobial therapy, leading to the failure of treatment modalities and the recurrence of infections (51). Hence, the need to design unconventional antimicrobial remedies is vital. Due to the intricate mode of action of silver nanoparticles, it is strenuous for pathogens to build effective resistance against it (52). It is rapid, high providing and effective against these bacteria which have been known to cause even cardiovascular disorders when left unsupervised in the past. Also, silver nanoparticles when compared to most antimicrobial agents, are less noxious because they inhibit the growth of bacteria at a much lower concentration (53). Considering the large number of advantages, it is admissible to suggest that silver nanoparticles are environmentally benign and economically attractive choices for antimicrobial agents used against *Staphylococcus aureus* and *Streptococcus mutans*. But since our study is an in-vitro investigation in essence and as it is tested against only a few chosen bacteria of the oral cavity, further research is needed to validate and increase the generalisability of our findings.

Conclusion

Through the outcome of our study we have speculated that silver nanoparticles synthesized using *Terminalia chebula* possess an evident antimicrobial potential against *Staphylococcus aureus*

and *Streptococcus mutans*. Thus, silver nanoparticles could possibly be used as a replacement for traditional antibacterial therapy.

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