Original Research article

Terminalia chebula mediated silver nanoparticles and its antibacterial activity against oral pathogens.

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

Abstract

Background: Dental infections cause chronic disorders such as gingivitis, periodontitis, and dental caries when left untreated, resulting in irreversible tooth loss. However, solid preventive methods, such as employing promising herbs with well-documented health advantages like *Terminalia chebula*, can help to reduce the excessive expansion of dangerous oral flora like *Streptococcus mutans, Staphylococcus aureus, and Enterococcus faecalis.*

Aim: The goal of this study is to see how effective silver nanoparticles made from *Terminalia chebula* are against oral pathogens including *Streptococcus mutans, Staphylococcus aureus, and Enterococcus faecalis* at various doses.

Materials and Methodology: The plant extract was made from Terminalia chebula, followed by the synthesis of silver nanoparticles. The antibacterial efficacy of Ag-NPs in varied concentrations, namely 25, 50, and 100 L, was determined using the agar-well diffusion method, with amoxicillin acting as a positive control. For each plate, the zone of inhibition was recorded, and the findings were statistically analysed using one way anova and post hoc analysis using IBM SPSS software (Version 20.0)

Results: Following the synthesis of Ag-NPs, a colour shift was noticed. UV-vis spectroscopy was used to characterise the synthesized particles, which revealed a peak at 440nm. With a rise

in Ag-NP concentration, the antibacterial activity indicated an increase in zone of inhibition. The zone of inhibition for *S. aureus* was substantially higher than Amoxicillin at all doses of Ag-NPs ($p< 0.05$), whereas it was significantly lower for *E. faecalis* ($p<0.05$). The zone of inhibition for *S. mutans* was substantially lower than amoxicillin at 25µL concentration ($p< 0.05$), but there was no significant difference at higher concentrations ($p>0.05$).

Conclusion: Antibacterial activity of Ag-NPs derived from *Terminalia chebula* against dental pathogens, particularly *Staphylococcus aureus and Streptococcus mutans, Enterococcus faecalis*.

Keywords: Silver Nanoparticles, *Terminalia chebula*, Green synthesis.

1. Introduction

Nanotechnology is currently an enthusiastically growing field in the epoch of modern science. The size-dependent characteristics of nanoparticles give it its multi-dimensional 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 dextran-based 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 antibacterial activity in different concentrations in comparison with the standard antibiotic amoxicillin.

2.Materials and Methodology

2.1Preparation 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°C . The resulting solution was filtered using a Whatman No. 1 filter paper. Finally, the filtered plant extract was collected and stored at 4°C for further use.

2.2Synthesis of Silver Nanoparticles

To make a metallic solution, 1 mM silver nitrate was dissolved in 90 ml double distilled water. To this, 10 mL of *Terminalia chebula* fruit extract was added, resulting in a 100 mL formulation. Its colour change was very appealing, and photographs were taken to capture it methodically. For the creation of its nanoparticles, the formulation was held in a magnetic stirrer at 700 rpm for 48 hours.

2.3Characterisation 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.

2.4 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 using a hot air oven. Finally, the silver nanoparticle powder obtained was collected and stored in an air-tight Eppendorf tube.

2.5 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*. The fresh bacterial suspensions were dispersed on the surface of Muller-Hinton Agar containing plates. Ag-NP concentrations of (25, 50 & 100µL) 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 zone of inhibition were recorded for each well.

2.6 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%.

3. Results and Discussion

3.1 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.

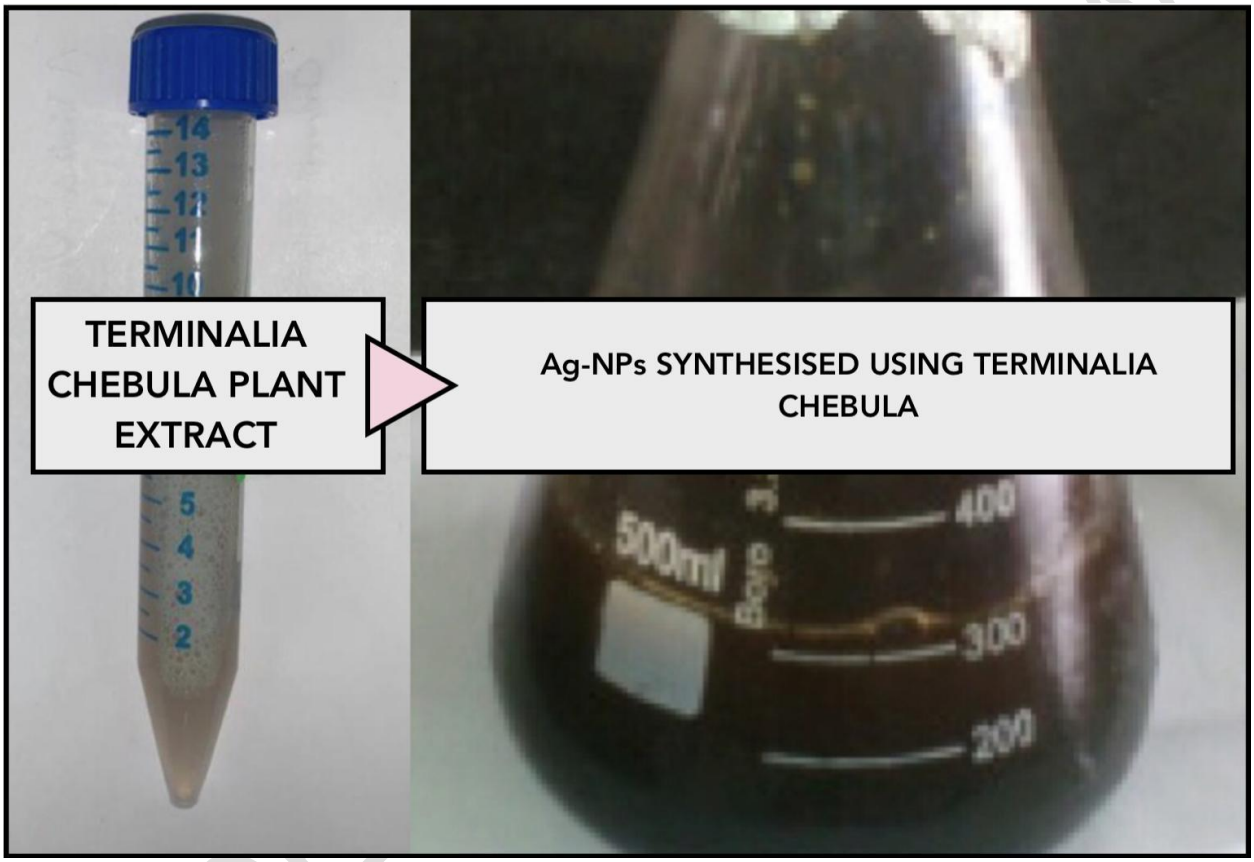

Figure 1 – Colour change image of *T. chebula* mediated silver nanoparticles.

3.2 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 – 750nm). The Surface Plasmon Resonance peak 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.

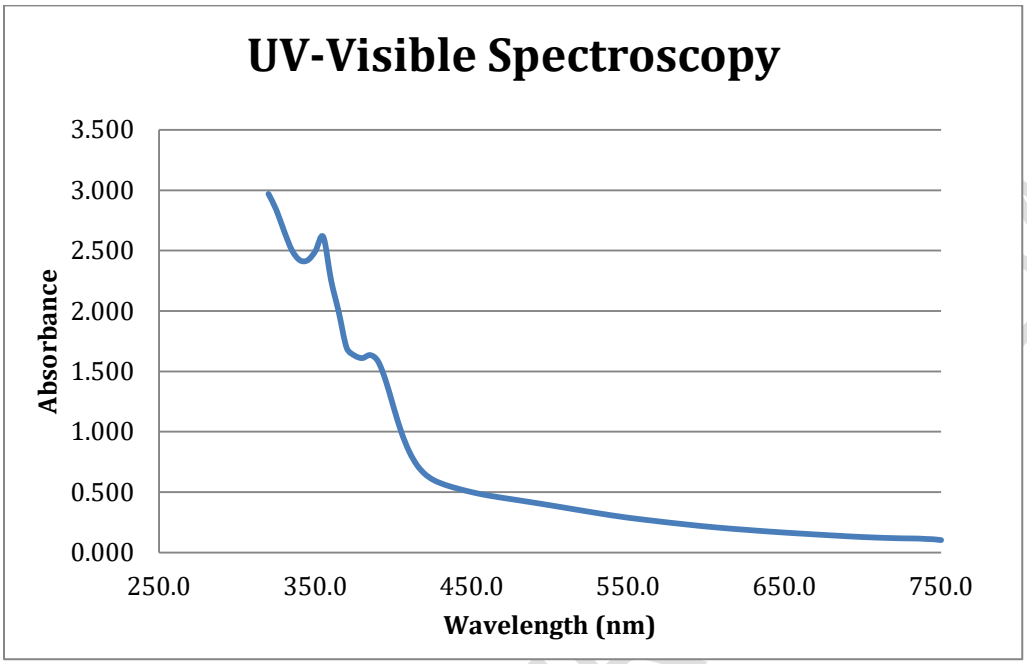

Figure 2 - UV visible spectra of biosynthesized silver nanoparticles

3.3 Antibacterial 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.

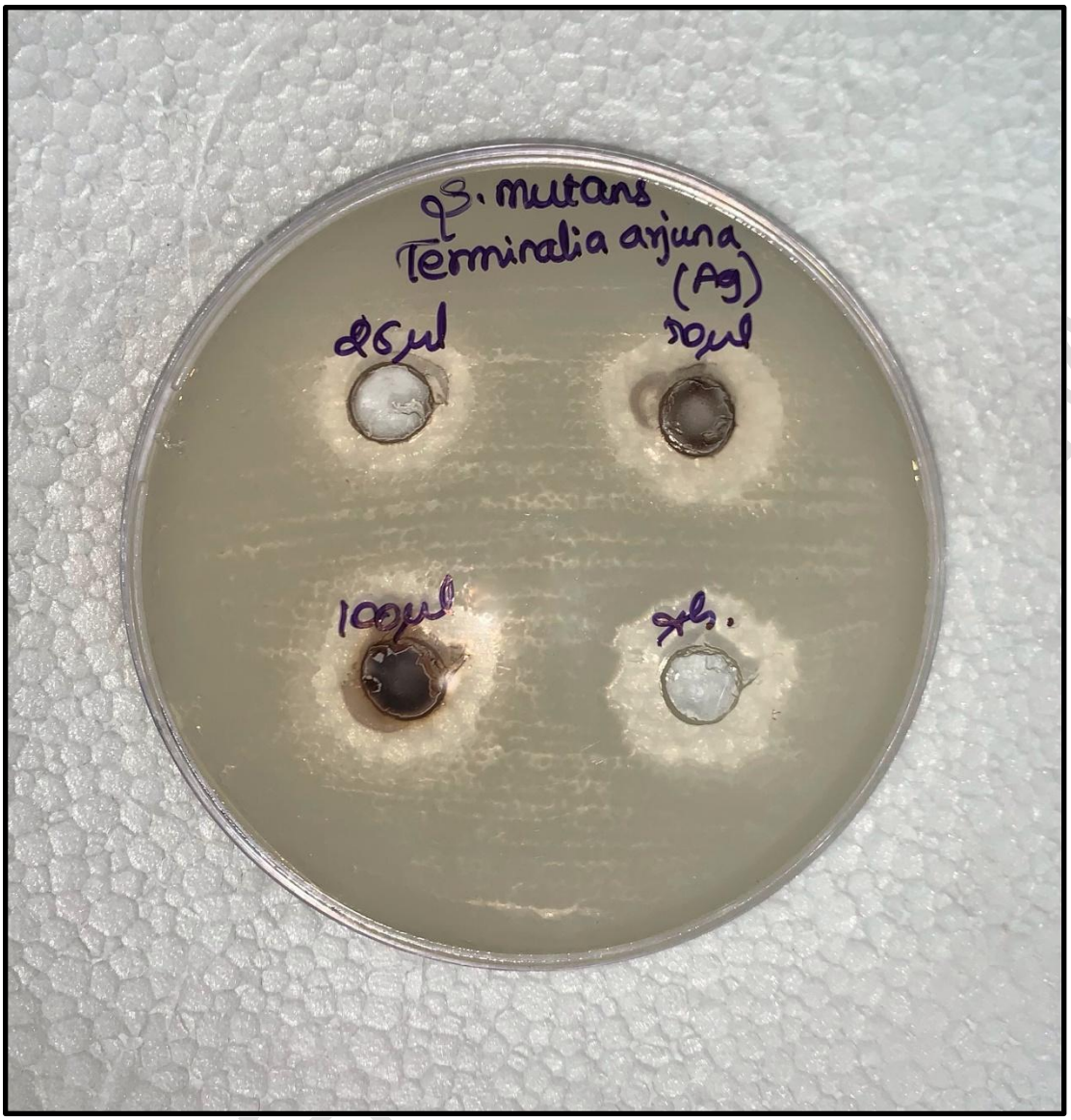

Figure 3 - Showcasing the effect of various concentrations of Ag-NPs on *Streptococcus mutans* with their zones of inhibition:

1. Effect of 25 µL of Ag-NPs on *Streptococcus mutans* with its moderately sized zone of inhibition
2. Effect of 50 µL 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

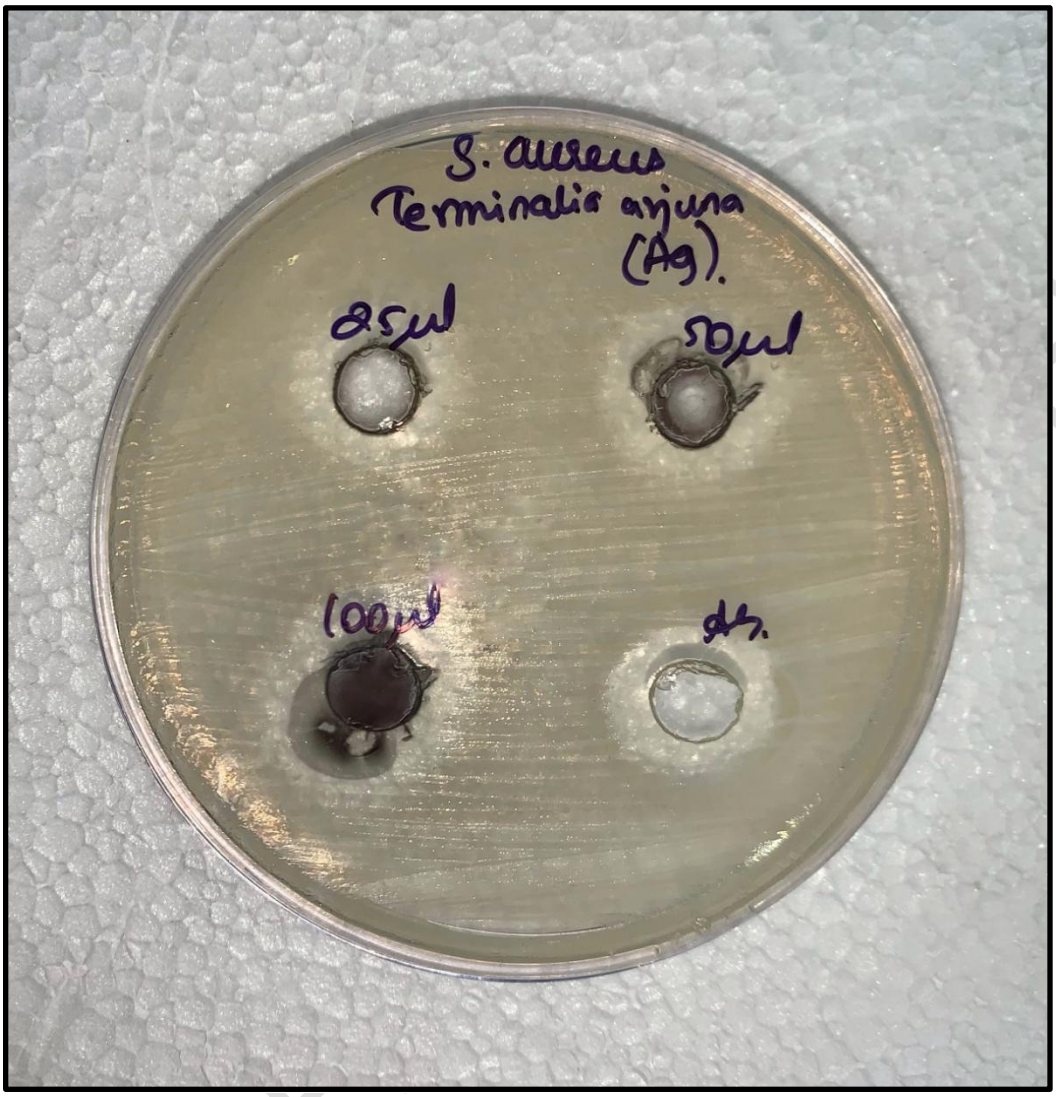

Figure 4 - Showcasing the effect of various concentrations of Ag-NPs on *Staphylococcus aureus* with their zones of inhibition:

1. Effect of 25 µL 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 µL 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

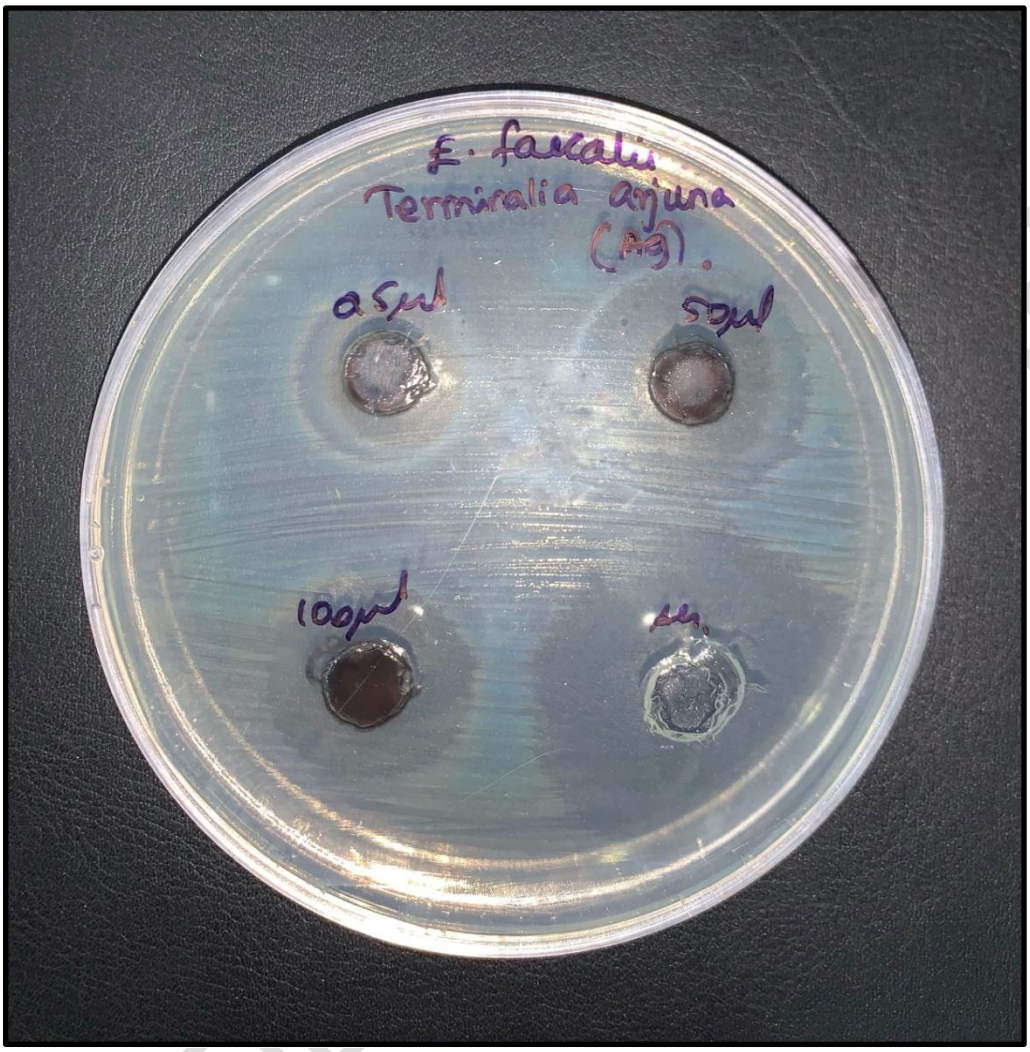

Figure 5 - Showcasing the effect of various concentrations of Ag-NPs along with the positive control (Amoxicillin)on *Enterococcus faecalis* with their zones of inhibition:

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

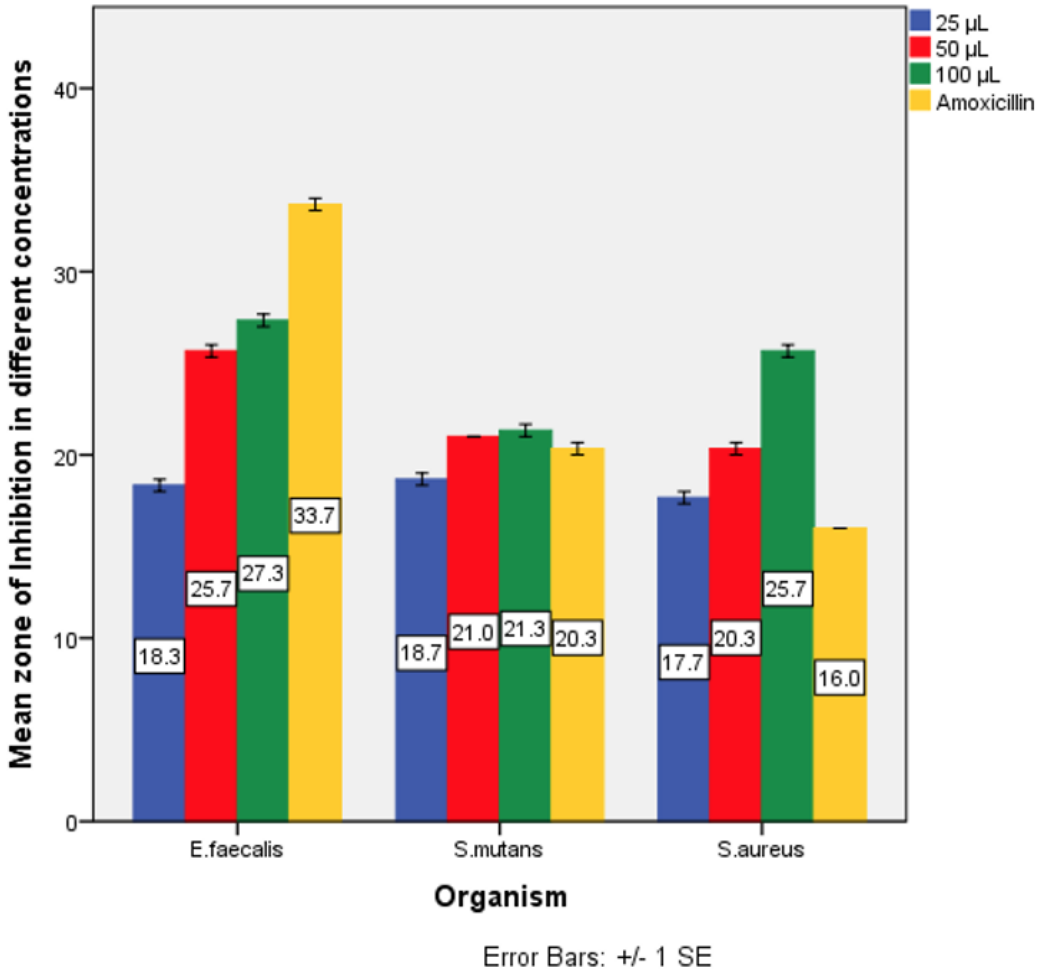

Figure 6 – Antibacterial activity of *T. chebula* mediated silver nanoparticles

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 µL units of the Ag-NPs , red depicts 50µL , green depicts 100 µL 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 µL 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 – Antibacterial activity of *T. chebula* mediated silver nanoparticles- Descriptive statistics

Table 1 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

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Absorbance of S. mutans	25 µl	3	18.67	.577	.333	17.23	20.10	18	19
	50 µl	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 µl	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 – One way ANOVA

Table 2 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 – Post Hoc (Tukey HSD) test results

Tabulation 3 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**	
						Lower Bound	**Upper Bound**
Absorbance of S. mutans	**25 µl**	50 µl	-2.333*	.408	.002	-3.64	-1.03
		100 µl	-2.667*	.408	.001	-3.97	-1.36

		Amoxicillin	-1.667*	.408	.015	-2.97	-.36
	50 µl	100 µl	-.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 µl**	50 µl	-2.667*	.408	.001	-3.97	-1.36
		100 µl	-8.000*	.408	.000	-9.31	-6.69
		Amoxicillin	1.667*	.408	.015	.36	2.97
	50 µl	100 µl	-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 µl	-9.000*	.471	.000	-10.51	-7.49
		Amoxicillin	-15.333*	.471	.000	-16.84	-13.82
	50 µl	100 µl	-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 µL 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 µL of the Ag-NP solution had a considerable efficacy against *Streptococcus mutans* with a large zone of inhibition of 21 mm. 100 µL 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 µL of the Ag-NP solution had the second lowest antimicrobial potential with a moderately sized zone of inhibition measuring 17.7 mm. 50 µL of the Ag-NP solution had a good efficacy with a visibly apparent zone of 20.3 mm. 100 µL 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 Multi Drug Resistant (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.

4. Conclusion

Recent research has found that biogenic synthesis of silver nanoparticles using fruit extract has various advantages, and that these materials have considerable potential for a variety of health-related applications. The nanoparticles have cappings made of plant extract that provide stability. This capping may have biological activity depending on the fruit extract employed, working in tandem with the nanoparticle core's action. These nanoparticles have a lot of potential in terms of controlling pathogenic oral germs. As a result, silver nanoparticles may be utilised to replace traditional antibacterial therapy.

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