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Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) Evaluation of Green Synthesised Silver Nanoparticles on *Staphylococcus aureus* and *Streptococcus agalactiae*

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Abstract
In the present study silver nanoparticles were green synthesised using leaf extract of *Moringa oleifera* and fruit extract of *Tamarindus indica*. They were characterised and evaluated for Minimum Inhibitory Concentration and Minimum Bacterial Concentration on *Staphylococcus aureus* and S*treptococcus agalactiae*. 66 (66%) and 27 (27%) of the 100 milk samples obtained from various sources tested positive by the culture method for *Staphylococcus aureus* and *Streptococcus agalactiae*, respectively. Silver nanoparticles prepared with leaf extract of *Moringa oleifera* and fruit extract of *Tamarindus indica* showed a Minimum Inhibitory Concentration (MIC) of 0.312 mg/ml on *Staphylococcus aureus* in both extracts and MIC of silver nanoparticles on *Streptococcus agalactiae* was at 0.312 mg/ml and 0.625 mg/ml concentration when prepared with Tamarind and Moringa extracts respectively. *Staphylococcus aureus* and *Streptococcus agalactiae* did not grow in any concentrations of the Minimum Bacterial Concentration (MBC), proving that it is bactericidal.

Keywords: Green silver nanoparticles, *S. Aureus*, *S. Agalactiae*, MIC, MBC

1. Introduction
Plants can be thought of as Nano factories that produce metallic nanoparticles in a beneficial and safe manner, with the potential for large-scale production. Plants generate more than other biological models, are environmentally friendly and have a higher rate of growth. The presence of different polyphenols and other heterocyclic chemicals affects a plant's ability to reduce (Nair *et al.*, 2010) [1]. The preferred metal is silver nanoparticles because they have the potential to effectively kill microorganisms (Sondi and Salopek, 2004) [2]. The broad spectrum of target areas that the silver nanoparticles affect include both extracellular and intracellular locations. In general, microbes find it more difficult to become resistant to silver than they do to antibiotics (Baker *et al.*, 2005) [3]. The bacterial cell's reactive groups are bound by silver ions, which has an oligo-dynamic impact on germs that causes the cells to precipitate and become inactive. Due to their superior antibacterial characteristics, including their broad range and surface activity, silver nanoparticles stand out when compared to other metallic nanoparticles. In contemporary nanoscience and nanotechnology, plasmon resonance is crucial (Jana and Pal, 2007) [4]. Due to their capacity to deactivate enzymes in microbial cells and changes membrane permeability, which causes lysis and apoptosis, silver cations released during the process of interaction between silver nano-particles and microbial cells have the potential to destroy the microbes (Feng *et al.*, 2000) [5].
The first creation of silver nanoparticles involved the reduction of silver ions present in a silver nitrate solution using an aqueous extract of *Moringa oleifera* leaf, Prasad and Elumalai, (2011) [6]. Jayaprakash *et al.* (2017) [7] reported that *Tamarindus indic*a fruit extract was used to synthesize AgNPs with no need for a capping agent, external surfactant or template. Sudhan *et al.* (2005) [8] investigated the prevalence of subclinical mastitis (SCM) in crossbred cattle from organized dairy farms in India and found that S. *aureus* was the primary pathogen (56.89%), followed by Micrococcus *spp.* (15.51%), Bacillus *spp.* (12.06%), *S. epidermidis* (8.62%), Klebsiella *spp.* (3.44%), *E. coli* (1.72%) and Corynebacterium *spp.* (1.72%).

(Ranjan *et al.*, 2011) [9] found that *S. aureus* was the much common bacterial species in mastitis cases, accounting for 52 (27.37%) isolates, followed by coagulase -ve Staphylococcus *spp.* 24 (12.63%), *E. coli* 17 (8.95%), Pseudomonas *spp.* 15 (7.89%), Streptococcus *spp.* 11 (5.79%), mixed bacterial infection (4.74%), Yeast (3.15%), Klebsiella *spp.* (1.57%) and Bacillus *spp.* (0.52%).

Material and Methods

Isolation and identification of gram-positive food borne pathogens

Staphylococcus aureus

The procedure used to isolate *S. aureus* was described by Safdar *et al.* (2003) [10]. For enrichment, to BHI broth 1 ml of material was added and incubated for 24 hours at 37 °C. On Mannitol salt agar (Hi-Media, Mumbai) plates, enriched samples were streaked before being incubated for 24 hours at 37 °C. Plates having colonies of golden yellow hue on MSA were chosen, and Gram staining was evaluated. Gram-positive cocci-containing organisms were subsequently cultivated in duplicate on nutrient agar (NA) slants and kept at 4 °C for additional biochemical assays and characterisation. The following biochemical identification tests were run on the potential colonies (Table 01). The Clinical Veterinary Microbiology textbook's recommended methods were followed for all biochemical assays by Markey *et al.* (2013) [11].

Table 1: Biochemical identification tests

S. No	Tests	Response of *S. Aureus*
01.	Indole	-ve
02.	Methyl Red	+ve
03.	Voges Proskauer	+ve
04.	Citrate (Simmons)	+ve
05.	Catalase	+ve
06.	Coagulase	+ve

Streptococcus agalactiae

Streptococci were isolated by enriching the sample in streptococcus selection broth for 6 hours, followed by streaking on blood agar (BA) plates and incubation in 5% CO2. The colonies were then re-streaked onto blood agar plates and incubated at 37 °C for 48 hours in 5% CO_2 to obtain pure cultures, after observing the hemolysis pattern and colony morphology. The pure cultures were then streaked onto BHI agar for further identification.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Evaluation

Determination of MIC

The standard broth dilution method (CLSI M07-A8) was used to test the antimicrobial activity of silver nanoparticles made from leaf extract of *Moringa oleifera* and fruit extract of *Tamarindus indica* by observing the visible growth of bacteria in the agar broth.

- 7 Eppendorf tubes were taken and filled with 1 ml of BHI broth.
- Serial two-fold dilutions of silver nanoparticles (prepared with leaf extract of *Moringa oleifera* and fruit extract of *Tamarindus indica*) in concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 mg/ml were added serially to 7 eppendorf tubes.
- Bacterial culture (*Staphylococcus aureus* and *Streptococcus agalactiae*) with a concentration of (0.5 McFarland's standard) 108 CFU/ml was inoculated into all the Eppendorf tubes containing silver nanoparticles and BHI broth.
- A control tube containing only inoculated broth was maintained.
- All tubes were incubated at 37 °C for 24 hrs.
- The tubes were then observed for turbidity (visible growth) both before and after incubation.
- The MIC endpoint is the silver nanoparticle concentration at which there is no discernible growth in the tubes.

Determination of MBC

After the measurement of the silver nanoparticles MIC, 50 µl aliquots from all the tubes that showed no discernible bacterial growth were plated on BHI agar plates and cultured for 24 hours at 37 °C. The pre- and post-incubated agar plates were checked for the presence of any bacteria. The MBC endpoint is reached when the lowest concentration of the antimicrobial agent eliminates 99.9% of the bacterial population.

Results and Discussion

Around the world, food-borne illnesses have become a significant public health concern. Every year, food-borne illnesses afflict roughly 30 percent of the population in industrialised nations. (WHO, 2014) [12]. It is often agreed that eating foods infected with bacteria, viruses and other pathogens that spread through food is the main factor contributing to human food-borne illness. The ideal medium for harmful microorganisms and food deterioration is milk. Streptococcus and Staphylococcus species are the most frequent infections identified in milk. Due to the promiscuous use of antibiotics in both clinical settings and as feed additives, numerous drug resistant serotypes have appeared. The resulting infections and food pollutants are now very challenging to treat. Thus, a novel, all-natural anti-microbial agent is required due to the multi-drug resistance of food-borne pathogens, which is a developing problem. The metallic nanoparticles do have anti-microbial activity, which is a well-known fact. Even though the mechanism and mode of action are still unclear, silver nanoparticle is a powerful anti-microbial agent that is effective against bacteria, viruses and fungi (Malarkodi *et al*., 2013) [13].

Culture-based *Staphylococcus aureus* Isolation and Identification

Using Mannitol salt agar media and BHI broth for enrichment, *Staphylococcus aureus* was isolated. The colonies were golden yellow in color and small in size (Figure 01). The supposed colonies of S.*aureus* colonies were subjected to gram staining (Figure 02) and Biochemical tests (Figure 03), including the Catalase Test (Figure 04), for confirmation.

Fig 1: Staphylococcus aureus on MSA plate

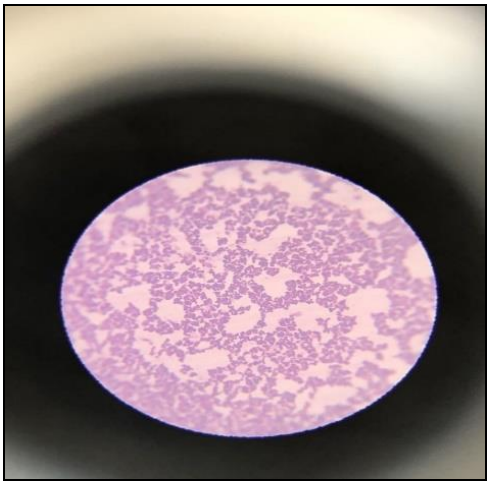

Fig 2: Gram staining (Bunch of grapes appearance)

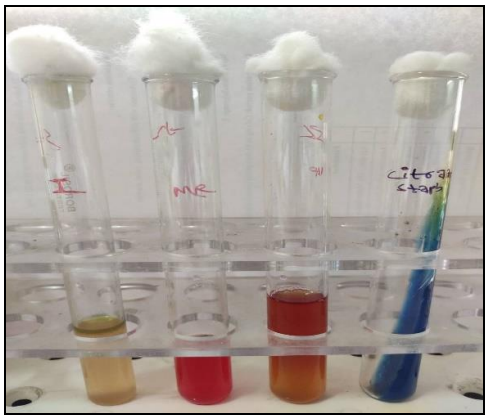

Fig 3: IMVi C test for *S. aureus*

Fig 4: Catalase test

Culture-based *Streptococcus agalactiae* Isolation and Identification:

Streptococcus agalactiae was isolated using BHI broth for enrichment and Blood agar media. The colonies showed a narrow zone of beta hemolysis and they were glistening grey-white in color (Figure 05). The supposed colonies of S.*agalactiae* colonies were subjected to gram staining (Figure 06) and the CAMP test for confirmation.

Fig 5: Beta-hemolysis on Blood agar

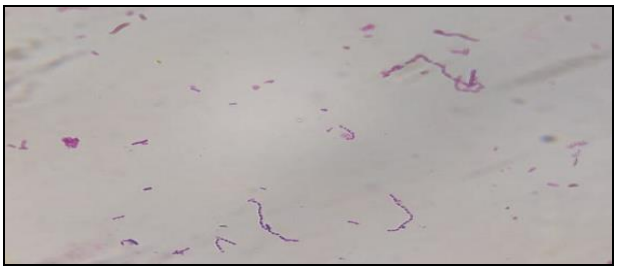

Fig 6: Gram staining (Chain like appearance)

Incidence of *Staphylococcus aureus* and *Streptococcus agalactiae* in Milk Samples

66 (66%) and 27 (27%) of the 100 milk samples obtained from various sources tested positive for *Staphylococcus aureus* and *Streptococcus agalactiae*, respectively, using the culture method. Hegde *et al.* (2013) [14] reported an incidence of 38.6% of *Staphylococcus aureus* in the milk samples collected from different types of dairy farms by cultural method, which was less than the incidence (66%) observed in the present study, In contrast, Preethirani *et al.* (2015) [15] from Bangalore reported a much lower incidence of *Staphylococcus aureus* (7.3%) compared to the current study. Hegde *et al.* (2013) [14] reported an incidence of 34.5% in the milk samples procured from various types of dairy farms by cultural method, which is higher than the 27% incidence observed in the current study. The incidence of *Streptococcus agalactiae* in the present study (27%) was less than the incidence (35.08%) reported by Elango *et al.* (2010) [16] from Namakkal by cultural method. Compared to milk samples from both organised and unorganised dairy farms, the incidence was lower in samples of milk from individual farmers.

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

Minimum Inhibitory Concentration (MIC): The results of the determination of Minimum Inhibitory Concentration (MIC), for two pathogenic isolates (*Staphylococcus aureus* and *Streptococcus agalactiae*) using Silver Nanoparticles (AgNPs) prepared from *Moringa oleifera* and *Tamarindus indica* were presented in Tables 02 and 03, respectively.

Table 2: *Staphylococcus aureus* and *Streptococcus agalactiae's* Minimum Inhibitory Concentration (MIC) for AgNPs made from *Moringa oleifera* leaf extract.

Pathogen	No of isolates	Concentration of AgNPs (mg/ml)						
		10	5	2.5	1.25	0.625	0.312	0.156
Staphylococcus aureus	42	_	_	_	_	_	_	+
	24	_	_	_	_	_	+	+
Streptococcus agalactiae	20	_	_	_	_	_	+	+
	7	_	_	_	_	+	+	+

Table 3: *Staphylococcus aureus* and *Streptococcus agalactiae's* Minimum Inhibitory Concentration (MIC) for AgNPs made from *Tamarindus indica* fruit extract.

Pathogen	No of isolates	Concentration of AgNPs (mg/ml)						
		10	5	2.5	1.25	0.625	0.312	0.156
Staphylococcus aureus	66	_	_	_	_	_	_	+
Streptococcus agalactiae	21	_	_	_	_	_	_	+
	6	_	_	_	_	_	+	+

Determination of MIC on *Staphylococcus aureus* Isolates: Out of 66 *Staphylococcus aureus* isolates, turbidity was observed in 42 isolates at 0.156 mg/ml concentration after incubation at 37 °C under aerobic conditions for 24 hrs, and the remaining 24 isolates had shown turbidity at concentrations of 0.312 mg/ml and 0.156 mg/ml containing silver nanoparticles prepared with *Moringa oleifera*, indicating the growth of bacteria. Other amounts showed no turbidity (Figure 07). All 66 *Staphylococcus aureus* isolates were tested in tubes containing silver nanoparticles produced with *Tamarindus indica* at a concentration of 0.156 mg/ml; other concentrations did not show any growth (Figure 08).

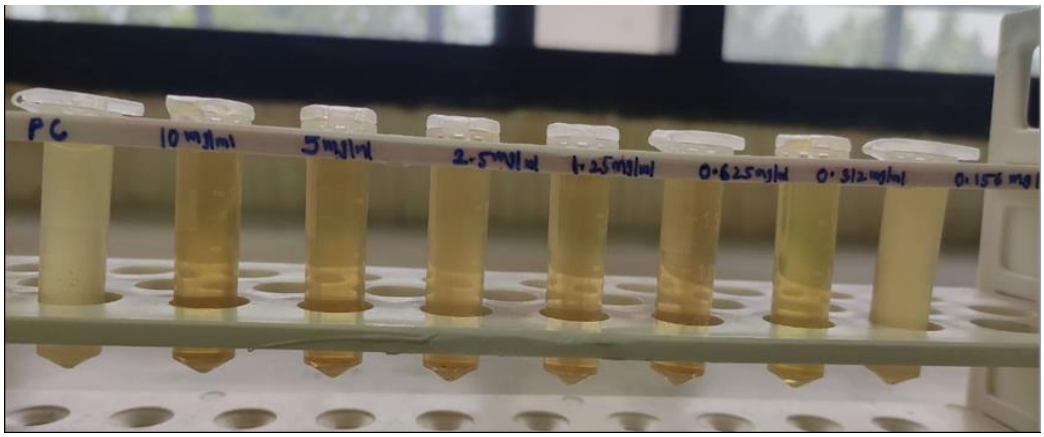

Fig 7: MIC for AgNPs prepared from *Moringa oleifera* leaf extract on *Staphylococcus aureus* (showing turbidity at 0.156mg/ml concentration).

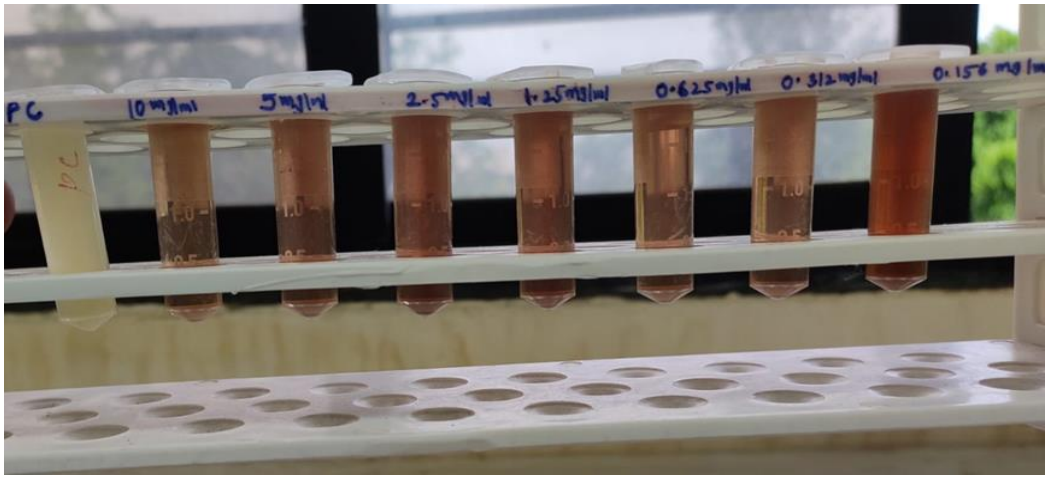

Fig 8: MIC for AgNPs prepared from *Tamarindus indica* fruit extract on *Staphylococcus aureus* (showing turbidity at 0.156mg/ml concentration).

Parvekar *et al.* (2020) [17] reported that test tubes with silver nanoparticles of 0.156 and 0.312 mg/ml concentrations showed turbidity, with the MIC for silver nanoparticles being 0.625mg/ml, which is similar to the antibacterial activity of silver nanoparticles prepared using *Moringa oleifera* and *Tamarindus indica* in the present study. (Moodley *et al.*, 2018) [18] identified AgNPs from *Moringa oleifera* leaf preparations at 0.025mg/ml concentration restricted the growth of *S. aureus*, *P. Aeruginosa*, and *K. pneumoniae* strains, which is lower than the values observed in the present study.

Determination of MIC on *Streptococcus agalactiae* Isolates Twenty of the 27 isolates of *Streptococcus agalactiae* showed turbidity in the tubes, which were incubated anaerobically for 24hrs at 37 °C and the remaining seven isolates showed turbidity at 0.156, 0.312, and 0.625 mg/ml concentrations with silver nanoparticles prepared with *Moringa oleifera*, indicating bacterial growth. Other amounts showed no turbidity (Figure 09). Out of 27 *Streptococcus agalactiae* isolates, turbidity was observed in the tubes for 21 isolates at a concentration of 0.156 mg/ml, and for remaining 6 isolates at concentrations of 0.312 mg/ml and 0.156 mg/ml that contained silver nanoparticles prepared with *Tamarindus indica*, indicating bacterial growth (Figure 10).

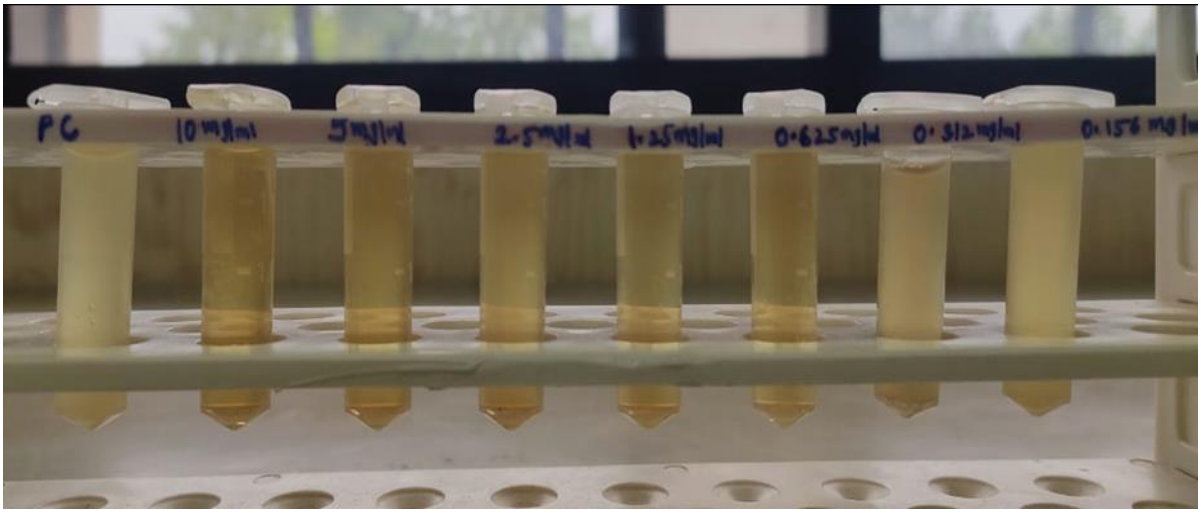

Fig 9: MIC for AgNPs prepared from leaf extract of *Moringa oleifera* on *Streptococcus agalactiae* (showing turbidity at 0.312 and 0.156 mg/ml concentration).

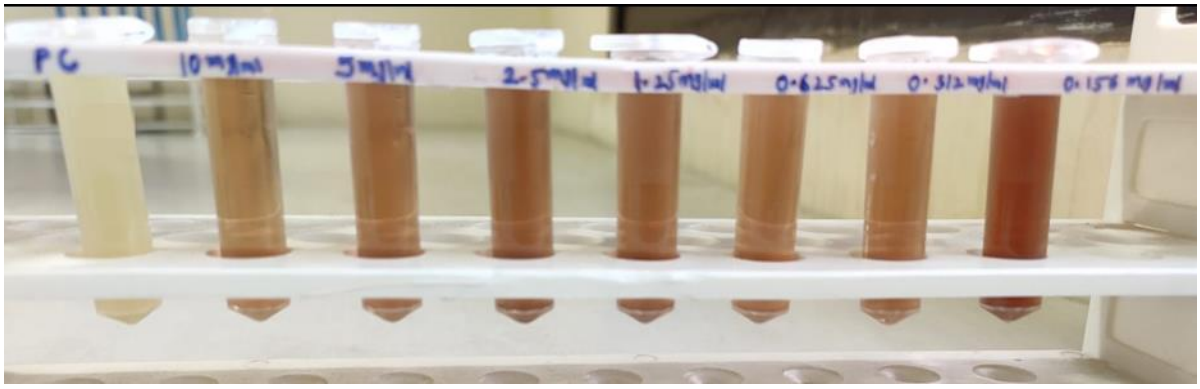

Fig 10: MIC for AgNPs prepared from fruit extract of *Tamarindus indica* on *Streptococcus agalactiae* (showing turbidity at 0.156 mg/ml concentration).

Espinosa-Cristóbal *et al.* (2009) [19] reported Minimum Inhibitory Concentration of silver nanoparticles against *Streptococcus spp.* similar to the present study.

Minimum Bacterial Concentration

Minimum Bacterial Concentration for *Staphylococcus aureus* and *Streptococcus agalactiae*:

The suspension from the tubes that showed no turbidity was plated on BHI agar and incubated for 24 hours. No bacterial growth was observed at any of the concentrations, confirming the bactericidal properties of the substance. Parvekar *et al.* (2020) [17] and Moodley *et al.* (2018) [18] also reported no growth of bacteria on BHI agar plates inoculated with the contents from tubes that showed no turbidity with AgNPs.

Conclusion

According to the findings of this investigation, *M. oleifera* and *T. Indica*-prepared AgNPs were nearly equally effective against *S. aureus* and *S. agalactiae*.

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