Original Article

PREPARATION OF IMPLANT-PROSTHETIC DISINFECTANT SOLUTION BY CHITOSAN, GREEN SYNTHESIZED SILVER NANOPARTICLES USING *Cymbopogon citratus*, *Azadirachta indica*, *Melaleuca alternifolia* AND EVALUATION OF ITS ANTIMICROBIAL PROPERTIES-AN INVITRO STUDY

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Abstract

AIM: The aim of the study is to synthesize an implant-prosthetic disinfectant solution by a mixture of green synthesized silver nanoparticles using *Cymbopogan citratus*, *Azadirachta indica, Melaleuca alternifolia* and chitosan and to perform characterization tests and evaluate its antimicrobial activity .

MATERIALS AND METHODS: 1 gram of neem powder was mixed with 100ml of water and made to boil for 10 min and filtered. 2 grams of lemongrass powder was mixed with 100ml of water, boiled for 10 min and filtered. Both the filtered extracts were mixed together and 40ml of the obtained extract was stored separately. 0.018 g of silver nano particle powder was mixed with 60ml of distilled water and chitosan solution with 1 ml of tea tree oil and the 40ml of the stored extract was mixed to finally obtain 100ml of final substrate.The final substrate was allowed to undergo reduction procedure ,centrifuged then dried in hot air over at 80 degree celcius .The obtained powder was scraped and further characterized using UV-Visible spectrophotometer and Scanning Electron Microscope(SEM). The anti microbial activity was also analyzed by incubating the coated prosthetic component with prepared disinfectant solution(test) and ethanol (control) in strains of E.coli,S.Aureus,E.fecalis. and the results were statistically compared using independent-t Test.

RESULTS: The UV-visible-spectroscopy results of the prepared solution showed peak around 400 nm-450 nm confirming the presence of silver nanoparticles. The scanning electron microscope tests revealed that the silver nanoparticles were seen between 37 nm- 43 nm and were biconcave ,triangular rod shaped .The length and the width corresponding to the zone of

inhibition around healing abutment (HA) and the Impression coping (IC) were measured. Independent t-test revealed that the antimicrobial property of the prepared disinfectant solution was superior to that of the standard ethanol solution , signifying the extension of its use in the clinical practice.

CONCLUSION: The present study revealed that the prosthetic disinfectant solution is effective in controlling the risk of peri- implant infection in chair side clinical use. The silver nanoparticles could be synthesized in a simple, eco-friendly method using *Cymbopogon citratus ,Azadirachta indica,* extract. Hence, it can be employed in large scale production for ensuring high patient oriented successful treatments and also can be used to prevent the cross-contamination among dentists and lab professionals.

KEYWORDS: Silver nanoparticles, chitosan disinfectant, chitosan, *Cymbopogon citratus ,Azadirachta indica,* antimicrobial property.

1. NTRODUCTION:

The human oral cavity harbors innumerable species of microorganisms. The complex interaction between the saliva, host tissue products and micro-organisms leads to the formation of acquired pellicles within minutes(1). The cell-to-cell adhesion occurs quickly among the bacterial species following which biofilm formation occurs(2). The biofilm provides the exchange of nutrients and prevents the microorganisms that compete with each other ,thus enabling them to thrive on the surface of implant prosthetic components(3). This becomes a serious concern as it increases the risk of peri-implantitis and also paves way for the pathogenic spread from patients to health care professionals, from patients to the lab technicians and vice versa. Thus the health care professionals should make sure that there is no cross contamination especially during the impression procedure which involves transfer of the 3-dimensional spatial replication of the implant position on the master cast and it is their duty to disinfect and sterilize all the prosthetic components.

The intricate ability to reduce the biofilm formation using novel nanoparticles is becoming a spotlight to control oral cavity infections(4). The metal nanoparticles having size less than 100 nm is gaining huge attention as antibacterial, antiviral, antifungal agents because of their nano-size and increase surface to volume ratio enabling them to closely interact with the surface of the micro-organism and the vital organelles present within them(5). In this study, silver nanoparticles were chosen as they interact with the bacterial enzyme's sulfhydryl group and control its activity. Some researchers have proven that they increase the oxidative stress by releasing reactive oxygen species ,thus exerting bactericidal effects(6). *Azadirachta indica*(NEEM) possesses anti-bacterial, anti-cariogenic,anti-inflammatory, cytotoxic, astringent, anti-oxidant,, anti-viral, cytotoxic properties(7).Lemongrass (*Cymbopogon citratus*) growing in tropical and subtropical region is a perennial grass consisting of 55 sub-species, rich in vitamin A,C and E possessing high antimicrobial and anti-infectious properties(8) .Chitosan is being studied extensively in the pharmaceutical and medical fields due to their nontoxicity, ,hemostatic activity, antitumor, antioxidant, antimicrobial, and high biocompatibility properties(9). *Melaleuca alternifolia(*Tea tree oil) primarily made of monoterpenes, alcohols and sesquiterpenes. They disrupt the integrity of the intracellular membrane, affect the homeostasis and inhibit the respiration of the cell bringing about effective antimicrobial property(10). Thus the study aims to synthesize an effective, strong prosthetic disinfectant solution by a mixture of green synthesized silver nanoparticles using *Cymbopogon citratus ,Azadirachta indica, Melaleuca alternifolia* and chitosan and to evaluate the anti microbial activity to ensure maximum success during implant therapy.

2. MATERIALS AND METHODS:

2.1 PREPARATION OF THE PLANT EXTRACT:

1 gram of neem powder was mixed with 100ml of water and made to boil in LABQUEST BOROSIL HME 500ml for 10 min and filtered 2 grams of lemongrass powder was mixed with 100ml of water, boiled in LABQUEST BOROSIL HME 500ml for 10 min and filtered. Both the filtered extracts were mixed together and 40ml of the obtained extract was stored separately. 0.018 g of silver nano particle powder was mixed with 60ml

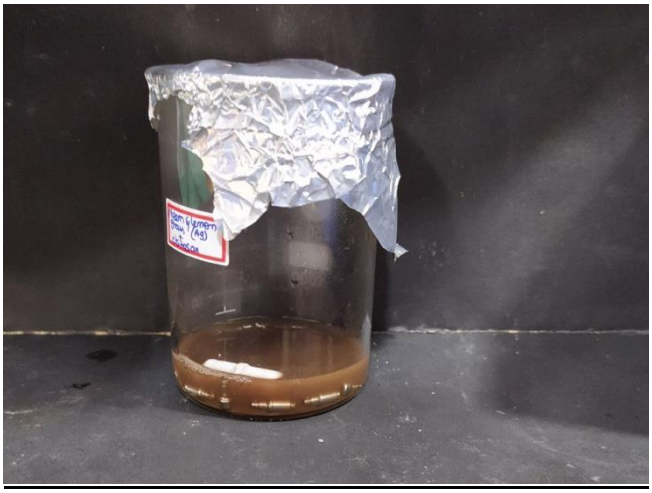
of distilled water and chitosan solution 1 ml of tea tree oil and the 40ml of the stored extract was mixed to finally obtain 100ml of final substrate (Figure 1). The final substrate was kept in the shaker overnight and allowed to undergo a reduction procedure. The reduced solution is equally distributed and centrifuged ,then dried in hot air oven at 80 degree celcius .The obtained powder was scraped and further characterized using SEM(Scanning Electron Microscope) and Ultraviolet-Visible Spectroscopy analysis (Figure 3).

Figure 1: Represents the procedure of synthesis of nanoparticles.The solution before undergoing reduction procedure was yellow in color and the solution after undergoing reduction overnight was brown ,signifying the presence of nanoparticles. The solution was centrifuged to obtain the nanoparticles.

2.2 DISINFECTION OF THE PROSTHETIC COMPONENTS WITH THE PREPARED NANOPARTICLES AND ANTIMICROBIAL ANALYSIS OF THE SOLUTION.

The two commonly used prosthetic components-healing abutments and impression coping ,ten from each category of various companies were chosen. The components were immersed in the prepared solution(test solution) and in the ethanol(control) for about 8 hours(Figure 2) and were immediately incubated by agar well diffusion method in the strains of *E.Faecalis, E.coli ,S.aureus* species. The inoculated plates were incubated overnight at 37 degree celsius and the zone of inhibition around each prosthetic component was measured in terms of length and width (in mm).

Fig 2: Represents the prosthetic components immersed into the prepared prosthetic disinfectant solution.



2.3 STATISTICAL ANALYSIS:

The results were tabulated and statistically analyzed using SPSS SOFTWARE version 2.0. Independent T-tests were performed and results were observed. $p<0.05$ was considered to be statistically significant.

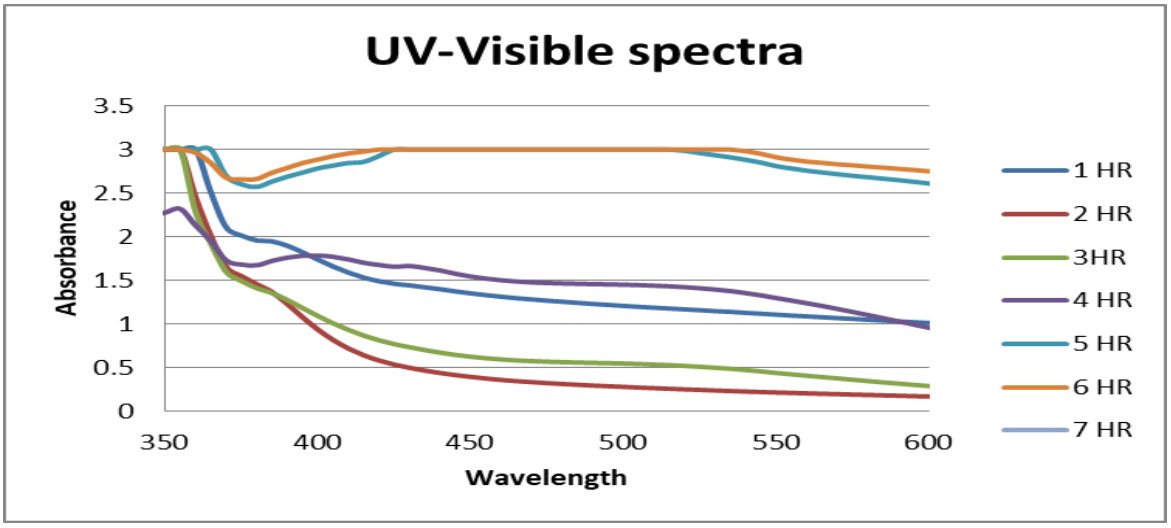
3. RESULTS:

3.1 CHARACTERISATION OF SILVER NANOPARTICLES:

3.1.A: UV-VISIBLE SPRECTROPHOTOMETRY:

The surface plasma resonance phenomenon has occurred leading to the color change of the solution from yellow to brown signifying that the reduction reaction and formation of nanoparticles has taken place . 3 ml of the solution was taken in a cuvette and scanned using double beam UV-Vis-spectrophotometry between the range of 350 nm and 600 nm. It was observed that the peak was around 400 nm-450 nm confirming the presence of silver nanoparticles. The results were studied and recorded graphically.

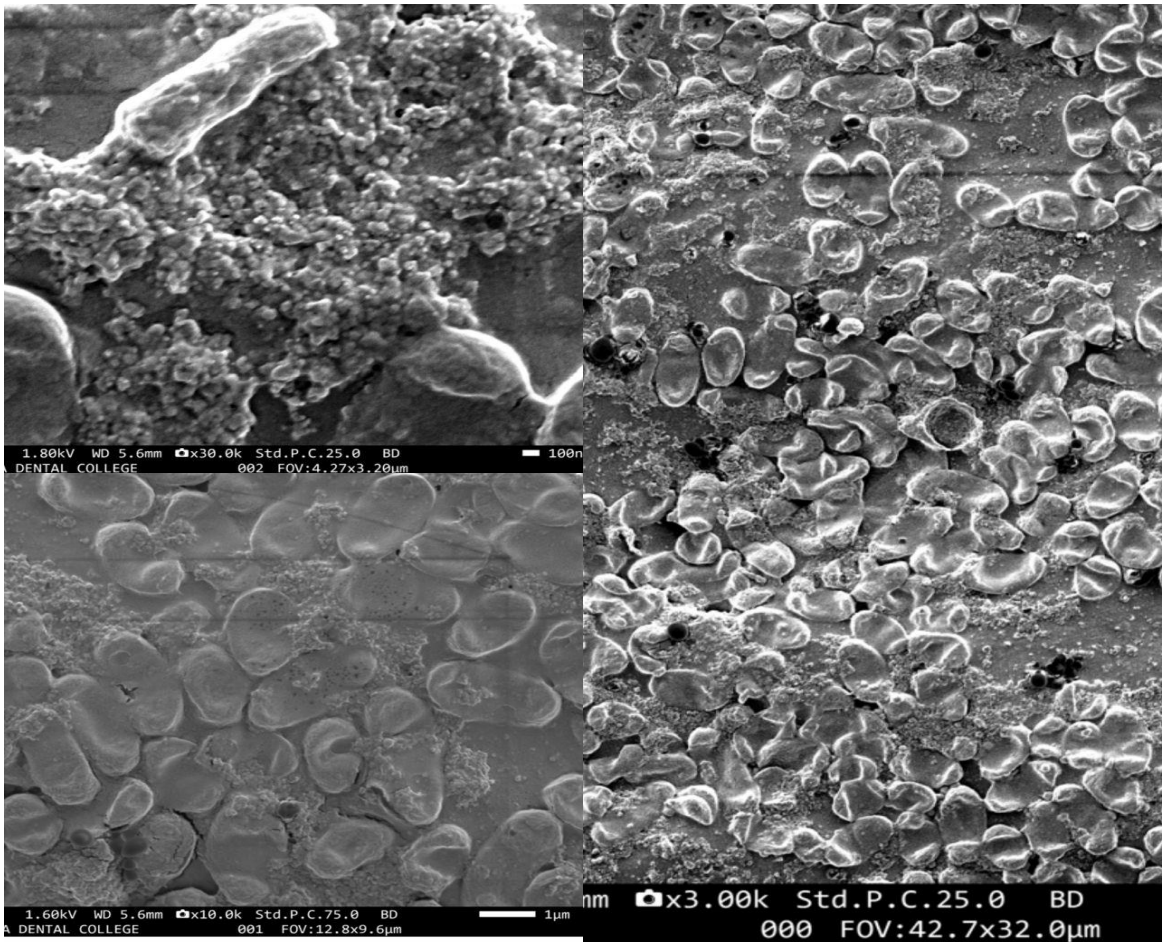
Figure 3:Represents the UV- Visible spectra results . The peak was present between 400 nm - 450 nm confirming the presence of silver nanoparticles.



3.1.B SEM ANALYSIS:

Scanning Electron microscope(SEM) image shows the morphological appearance of silver nanoparticles synthesized using *Cymbopogon citratus, Azadirachta indica,Melaleuca alternifolia*. The image under various magnifications depicts the biconcave appearance of the silver nanoparticles along with rod and spherical appearances in the range of 1nm-10nm, confirming its presence (Figure4). The silver nanoparticles also appeared to be clustered in groups when observed under 30k magnification.

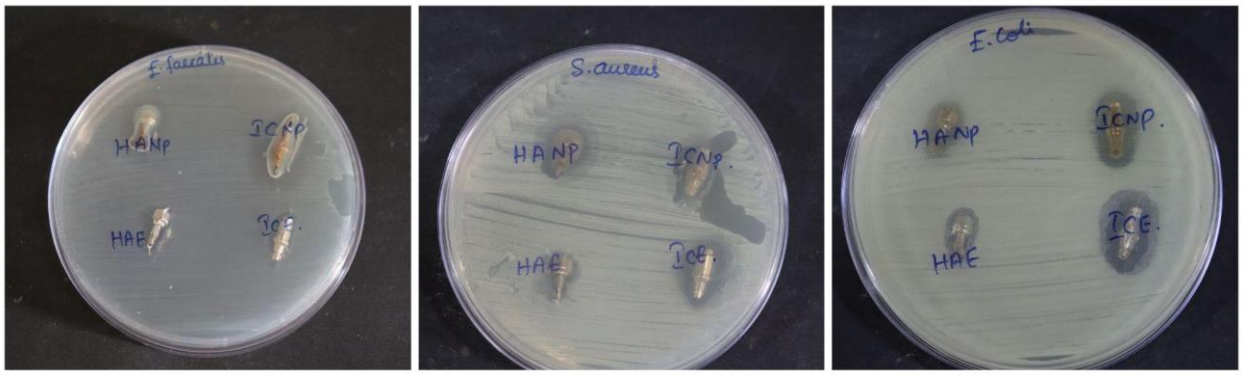
Fig 4: SEM analysis of silver nanoparticles at various magnifications. The biconcave morphological appearance confirms the presence of silver nanoparticles at 3 K magnification.



3.2 ANTI BACTERIAL ANALYSIS:

The antibacterial activity was examined against three bacterial strains - E.coli, E.fecalis, S.aureus by agar well diffusion method .The length and the width corresponding to the zone of inhibition around healing abutment (HA) and the Impression coping (IC) were measured at the maximum diameter and the statistical analysis was done(Fig 5).

Fig.5:Represents the comparison of ZONE OF INHIBITION around the prosthetic components- healing cap and impression coping between the nano-particle coated group and the control group(ethanol) after incubation periods of about 24 hours. The selected bacterial strains were *E.coli,S.Aureus,E.fecalis*.



Independent t-Test was used to analyze the statistical significance for the measured zone of inhibition values between control (ethanol) and the Nanoparticle coated group . The statistical analysis results have been shown in Table 1 and 2.

The maximum zone of inhibition in the healing abutment group was obtained by the antibacterial activity of the Nps against S.Aureus which was 24±0.89mm. In the impression coping group also the maximum zone of inhibition was obtained by Nps disinfectant solution against S.Aureus which was 28±0.89mm The results proved that the difference is statistically significant as the p value obtained is <0.05.

TABLE1: Represents the statistical observation of independent t-test performed for the resultant values of zone of inhibition around the healing cap for three different strains of bacteria comparing the ethanol solution and the prepared nanoparticle disinfectant solution.

HEALING CAP				
		MEAN	SD	SIGNIFICANCE
E.FAECALIS	LENGTH(E)	15.00	0.894	0.000
	WIDTH(E)	9.00	0.894	0.000
	LENGTH(NP)	21.50	1.049	0.000
	WIDTH(NP)	15.17	1.169	0.000
	LENGTH(E)	14.67	0.816	0.000
E.COLI	WIDTH(E)	08.83	0.983	0.000
	LENGTH(NP)	26.23	0.816	0.000
	WIDTH(NP)	15.67	1.033	0.000
	LENGTH(E)	15.67	1.033	0.000
S.AUREUS	WIDTH(E)	09.00	0.894	0.000
	LENGTH(NP)	24.00	0.894	0.000
	WIDTH(NP)	15.17	0.753	0.000

TABLE 2: Represents the statistical observation of independent t-test performed for the resultant values of zone of inhibition around the impression coping for three different strains of bacteria comparing the ethanol solution and the prepared nanoparticle disinfectant solution.

IMPRESSION COPING				
		MEAN	SD	SIGNIFICANCE
	LENGTH(E)	21.67	1.211	0.000
E.FAECALIS	WIDTH(E)	11.00	0.894	0.000
	LENGTH(NP)	27.33	0.816	0.000
	WIDTH(NP)	19.83	0.753	0.000
	LENGTH(E)	21.83	0.753	0.000
E.COLI	WIDTH(E)	11.00	0.894	0.000
	LENGTH(NP)	30.83	1.472	0.000
	WIDTH(NP)	20.50	0.548	0.000
	LENGTH(E)	22.33	1.211	0.000
S.AUREUS	WIDTH(E)	11.00	0.894	0.000
	LENGTH(NP)	28.00	0.894	0.000
	WIDTH(NP)	18.33	3.141	0.000

DISCUSSION:

In this golden era of modern dentistry, implant therapy has become a great alternative to removable dentures(11). Despite having a huge success and survival rate, peri-implant infections pose a serious threat due to the destruction of the supporting peri-implant hard and soft tissue due to the biofilm formed on the implant surface and the surrounding periodontium(12). Researchers have been keen in bringing about the effectiveness of the novel nanoparticles into the field of medicine, of which silver nanoparticles carry a huge literature background(13). The industry of biomedical science has contributed and has started extending its interest into the field of implantology, although a huge range of uses are being proven in other fields of medicine(14).The SEM analysis done in this study revealed the presence of the biconcave , rod, triangle shaped nanoparticles at various magnifications and UV-VIs showed peak absorption at 400 nm-450 nm . This is in accordance with study done by Rajeshkumar et al ,Agarwal et al(8,15). The antibacterial analysis performed in this study revealed that the antibacterial property is better than the efficiency of ethanol solution. This is in accordance with a study conducted by Feng et al where he exhibited the effect of silver nanoparticles on E.coli and S.aureus species(16). Studies conducted by Pal et al and Salopek-sondi et al revealed that silver nanoparticles of size 12-15nm targeted the E.coli species and it was concentrated over the surface of the cell membrane and has also penetrated into the cell's interior architecture.(17,18)The silver nanoparticle's invasion into the organelles leads to decreased ATP(AdenosineTriphosphate) synthesis, depletion of DNA replication products leading to damage of the intracellular organelle ultimately resulting in death of the micro-organism. In few situations, silver ions have also been found to cause arrest of the respiration at cellular level leading to "live, but non-reproducible" state of the organism in which bacteria do not thrive after reaching a certain threshold point. Infection control by the use of nanoparticle solution(19)

The mechanism of action of *Melaleuca alternifolia(* Tea tree oil*)* is by disrupting the functional and structural integration of the cell membrane and when used in less concentration, it is proven to be bacteriostatic and bactericidal by mainly disrupting the glycolysis pathway , inducing lysis and viability of the cell(20) . The prime constituent of the *Melaleuca alternifolia oil is* Terpinen-4-ol and it is mainly responsible for the antibacterial action on both aerobic and anaerobic species(21). Terpinolene, α- and γ-terpinene, 1–8 cineole are the other

constituents of the *Melaleuca alternifolia* oil that primely exhibits the antioxidant, anti-inflammatory, antimicrobial properties(22).The local drug delivery enhances the targeted action of the tea tree oil over the desired site of action by increasing the monocyte production resulting in increased superoxide production in cases of moderate periodontal disease(23). Since the peri-implantitis microbiota is similar to that of periodontitis, it can be effectively used in reducing the risk of peri-implantitis.

Dating back to centuries, neem has been used as powerful and active Ayurvedic medicine in Indian subterrain. Neem contains high antioxidant properties and it can be attributed to azadirachtin ,quercetin, nimbin, salannin(24). Neem scavenges the free radical exhibiting high antioxidant property. Studies have proven that it effectively regulates the cell signaling pathways by controlling various genes such as p53,Pten(tumor suppressor genes),angiogenesis and apoptosis(25). Neem also regulates the lipoxygenase(lox) and cyclooxygenase(cox) pathways thus exerting anti-inflammatory property(26)

Lemongrass has been proven to inhibit the growth of advanced periodontal pathogens such as P.gingivalis ,Actinomyces species that are even resistant to tetracycline hydrochloride(27). In a study conducted by Kukkamalla et al, anti-biofilm and antibacterial properties of the lemongrass oil was proven, as it inhibited the overall plaque accumulation. It has also been proven to reduce the adherence of the bacteria and can efficiently disrupt the biofilm formation(28). In a study conducted by Dany et al, 0.25 %lemongrass oil mouthwash increased the levels of glutathione in saliva which usually plays a pivotal role in oxidative stress defense reaction. Lemongrass also contains Citrol which increases formation of vitamin A ,C that are powerful antioxidants capable of reducing the host tissue breakdown during the infectious phase(29,30).

Chitosan binds to the bacterial cell wall's negatively charged membrane, alters its permeability, gets attached to the DNA resulting in DNA replication inhibition leading to cell death(31). Chitosan is also a powerful chelating chemical agent that results in toxin production and reduced growth of microbes by binding to the trace metal elements(31,32). The effective antibacterial property can be attributed to the polycationic surface of the chitosan. This interacts with the anionic structure of micro-organism and the antimicrobial property is directly proportional to he positive charge density(33).In a study by Cai et al, a nanofibrous regenerative membrane was synthesized by chitosan and it was proved to be efficient against E.coli ,with increased effects seen with increasing proportion of chitosan.(34) It is also efficient against fungus as it inhibits the germination of spores(35)

This prosthetic disinfectant formulation possesses the above mentioned innumerable effects and was statistically proven to be more efficient than the ethanol solution. Thus it can be extended to clinical use to decrease the risk of peri-implantitis and improve the efficiency of implant therapy.Further research on the clinical studies would validate the results.

CONCLUSION:

The present study revealed that the prosthetic disinfectant solution is effective in controlling the risk of peri-implant infection and inflammation in chair side clinical use. The silver nanoparticles can be synthesized in a simple, eco-friendly method using *Cymbopogan citratus ,Azadirachta indica* extract. These nanoparticles have the potential to be used as an effective antibacterial agent. Hence, it can be employed in large scale production for ensuring high patient oriented successful treatments and also can be used to prevent the cross-contamination.

CONSENT: NOT APPLICABLE

ETHICAL APPROVAL: It is not applicable.

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CONFLICTS OF INTEREST: NIL

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