EVALUATING THE ANTIMICROBIAL EFFICACY OF TITANIUM DISCS COATED WITH NANOSIZED NATURAL ZEOLITE AND PHOTODYNAMIC THERAPY AGAINST Aggregatibacter actinomycetemcomitans - AN IN VITRO STUDY.

By

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This is to certify that the dissertation **" Evaluating the antimicrobial efficacy of titanium discs coated with nanosized natural zeolite and photodynamic therapy against *Aggregatibacter actinomycetemcomitans* - An in vitro study"** *is a bonafide research work done by* **REG NO. IK0221001.**

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LIST OF ABBREVIATIONS

ICG	**Indocyanine green**
PTT	**Photothermal therapy**
aPDT	**Antibacterial Photodynamic therapy**
SLA	**Sand-blasted, large gritted and acid etched**
MB	**Methylene Blue**
BHI agar	**Brain Heart Infusion Agar**
PBS	**Phosphate buffer solution**
D.I W	**Deionized water**
2% NaOCL	**Sodium Hypochlorite**
nm	**Nanometer**
µm	**Micrometer**
W	**Watt**
PI	**Plaque Index**
GI	**Gingival index**
PPD	**Pocket Probing Depth**
RAL	**Residual attachment level**
SRP	**Scaling and Root Planing**
NZ	**Nanosized Zeolite**
CFU	**Colony forming unit**
ANOVA	**Analysis of variance**

ABSTRACT

INTRODUCTION

Peri-implant infections mirror periodontal disease, with *A. actinomycetemcomitans* and *P. gingivalis* as key pathogens. Adjunctive therapies such as systemic and topical antibiotics, antiseptics such as citric acid, hydrogen peroxide, chlorhexidine, ultrasonic devices, and laser treatments have been recommended to improve the non-invasive treatment options of peri-implant mucositis and peri-implantitis. Diode lasers (940 nm) effectively target gram-negative anaerobic bacteria like those causing peri-implantitis. Indocyanine green (ICG) shows promise in Antimicrobial Photodynamic Therapy (aPDT), significantly reducing pathogens. Recently, different kinds of nanoparticles have been used in aPDT to improve photosensitizer solubility, photochemistry, photophysics, and targeting. Zeolite nanoparticles are known for their low cytotoxicity and biocompatibility. Moreover, it has been shown that applying zeolite crystals adjacent to the bone can enhance osteoblast cell attachment and proliferation.

AIM

To assess and compare the effect of a combination of Nanosized natural zeolite and photodynamic therapy using Indocyanine green against *Aggregatibacter actinomycetemcomitans* biofilm on titanium discs.

MATERIALS AND METHODS

This is an in-vitro study wherein 200 pre-sterilized titanium discs of 8mm and thickness of 2mm were inoculated with a strain of *Aggregatibacter actinomycetemcomitans* and kept in an anaerobic chamber for 48 hours. The inoculated discs were randomly allocated into eight groups. Group 1: Positive control (2.5% NaOCl), Group 2: Negative control (Biofilm alone) , Group 3: Indocyanine Green (ICG), Group 4: Nanosized Zeolite (NZ), Group 5: Nanosized Zeolite + Indocyanine Green, Group 6: Nanosized Zeolite + Diode Laser, Group 7: Antibacterial Photodynamic Therapy (aPDT) i.e (ICG+ Diode laser), Group 8: Nanosized Zeolite + aPDT. A 940nm Diode laser was used for aPDT. Colony forming units were assessed for each group. The data was subjected to descriptive statistical analysis and normality was assessed using Kolmogrov Smirnov test. Intergroup comparisons were done by One-way ANOVA. A pairwise comparison of groups was carried out using Tukey's post hoc test. All statistical tests were performed at a significance level of 5% ($p<0.05$).

RESULTS

The results of the present study indicate that there was a significant reduction in *A.actinomycetemcomitans* colony count in NZ, NZ+ ICG, NZ+Diode laser, aPDT and NZ+aPDT group when compared with the negative control group. The maximum reduction in bacterial colony count was noted with NZ+ aPDT while minimum reduction was noted in ICG group. Therefore, it is described that within the test groups the combination of Natural Nanosized Zeolite along with antimicrobial Photodynamic therapy (aPDT) exhibited maximum reduction of *A.actinomycetemcomitans* colonies.

CONCLUSION

In conclusion, our investigation into the antimicrobial efficacy of titanium discs coated with Nanosized Natural Zeolite in combination with Antimicrobial Photodynamic therapy against *Aggregatibacter actinomycetemcomitans* yields promising insights for combating peri-implantitis. The synergistic effect observed between Nanosized Natural Zeolite and aPDT emphasizes the potential of combining antimicrobial therapies to enhance the biocompatibility and longevity of dental implants.

KEYWORDS

Implant, Nanosized natural Zeolite, Indocyanine green, Laser, Peri-implantitis, antimicrobial Photodynamic therapy.

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INTRODUCTION

"All truths are easy to understand once they are discovered; the point is to discover them"

- Galileo Galilei

"Peri-implantitis refers to an inflammatory condition impacting the tissues surrounding an osseo-integrated implant in function, leading to a loss of supporting bone, as defined by the 1st European Workshop on Periodontology."[1,2] The overall incidence of peri-implantitis for specific implant systems has been reported to range from "5% to 8%". [3] Research suggest that anaerobic plaque bacteria are harmful to the health of the tissue surrounding implants and can cause peri-implantitis. [4] The success of a dental implant hinges on various factors including mobility, loss, or peri-implant bone loss surpassing 1.0 mm within the initial year and 0.2 mm in subsequent years. Consequently, the implant's structural integrity may be affected by peri-implantitis. [5,6]

Several factors can elevate the likelihood of developing peri-implantitis. These encompass inadequate oral hygiene, smoking, diabetes, a past occurrence of periodontal disease, and genetic susceptibility.[7,8] Research suggests that the design and surface characteristics of dental implants can influence the probability of peri-implantitis development. Compared to implants with smoother surfaces, those with rough surfaces may encourage bacterial adherence and biofilm formation, hence raising the risk of peri-implantitis. Additionally, the emergence of newer implant materials and surface treatments aim to enhance osseointegration and minimize peri-implant complications. [9,10,11]

Studies have shown a resemblance in the microbiological flora related to peri-implantitis and periodontitis. Gram-negative anaerobes, including "*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacterioides forsythus, Treponema denticola, Prevotella nigrescens, Peptostreptococcus micros*, and *Fusobacterium nucleatum"*, are frequently linked to implant failure. Loss of healthy peri-implant tissue allows bacterial contamination to reach the bone directly, accelerating its breakdown. This healthy tissue acts as a biological barrier against agents contributing to peri-implant disease. [12,13,14,15]

The design of screw-shaped dental implants, with their threads and irregular surfaces, can provide niches where plaque and bacteria can accumulate. These areas may be challenging to access with traditional mechanical debridement tools, such as scalers and curettes, making complete removal of plaque and biofilm difficult. Various surface treatments are applied to titanium implants to enhance osseointegration and implant stability. However, some of these treatments, such as roughened surfaces or coatings, may inadvertently promote bacterial adhesion and biofilm formation. This can further hinder the effectiveness of mechanical debridement in eliminating all adherent microbes. [16,17,18,19]

Ultrasonic and laser treatments, alongside antibiotics and antiseptics, are among the supplementary therapies recommended to enhance non-surgical options for managing peri-implant mucositis and "peri-implantitis".[20] Laser treatment has gained attention as a non-surgical approach for peri-implantitis management. Laser energy can target and ablate bacteria within biofilms while promoting tissue decontamination and bio stimulation. Antimicrobial Photodynamic therapy (aPDT) presents a potential

technique for eliminating antibiotic-resistant bacteria infecting wounds. By using a non-toxic dye (photosensitizer) stimulated at the appropriate wavelength, aPDT generates oxygen radicals that irreversibly damage the target. Compared to antibiotics and antiseptics, aPDT offers advantages such as reduced risk of developing multidrug resistance, ease of administration, fewer adverse effects, and lower cost. [21,22,23,24]

Due to its low toxicity, high near-infrared absorption, quick removal, and other qualities, Indocyanine green (ICG) is an effective photosensitizer. ICG has an absorption peak at approximately 800 nm, which is close to the maximum emission wavelengths of dental diode lasers that are available commercially. ICG experiences physical and chemical alterations in aqueous solutions, including irreversible aggregation and degradation, which is accelerated by heat and light.

These modifications alter the maximum wavelength absorption, colour, and optical absorption while also lowering fluorescence intensity.[25,26,27] The integration of aPDT with nanotechnology has therefore been explored to enhance its effectiveness. Nanomaterials can improve drug solubility, prolong drug circulation in the bloodstream, reduce enzymatic degradation, minimize side effects, and increase bioavailability. [28]

Zeolites, porous aluminium silicate compounds with negative charges on external surfaces, have shown potential in various medical applications. These nanoparticles have been investigated for their remineralizing potential in resin-based dental materials. [28] Studies have also explored zeolite incorporation into dental cements to enhance antimicrobial properties. Their porous structure allows for the encapsulation and controlled release of pharmaceutical compounds, thereby improving the efficacy

and therapeutic outcomes of drug formulations. Zeolites offer the potential for controlled release of drugs due to their ability to adsorb molecules and release them gradually over time. Depending on the physicochemical properties of the drug and the zeolite structure, the release rate can be modulated to achieve sustained drug delivery, reducing the frequency of dosing and minimizing side effects. [29,30]

Encapsulating drugs within zeolite nanoparticles, enhances their bioavailability. It protects the drugs from degradation in the gastrointestinal tract, improve their solubility, and facilitate their absorption into systemic circulation. This can lead to improved therapeutic outcomes and reduced dosing requirements.

Functionalization of zeolite surfaces with targeting ligands or molecules allows for targeted drug delivery to specific tissues or cells. This targeting strategy minimizes off-target effects and enhances the therapeutic efficacy of drugs, particularly in the treatment of localized diseases. Zeolites can be modified to tailor their properties for specific drug delivery applications. Surface modifications, such as coating with polymers or other biomaterials, can further enhance drug loading, release kinetics, and biocompatibility.[31]

There are some studies that have explored the incorporation of zeolite into titanium (Ti) alloys, which are widely used in dental and orthopaedic implants. According to some in vitro studies, Ti alloys can leak some Vanadium (V) and Aluminium (Al) ions despite their biocompatibility and corrosion resistance. This can lead to poor osseointegration and shorten the lifespan of the Ti prosthesis. It has been suggested that a zeolite coating be applied to the Ti alloy to prevent this sort of Al and V ion emission. This prevents the alloy metals from dissolving and lessens the modulus

mismatch with bone challenge, which improves osseointegration. [32,33]

The negatively charged surface of zeolites can interact with the positively charged components of microbial cell membranes, leading to destabilization and rupture of the membrane structure. This disrupts the integrity of microbial cells and ultimately inhibits their viability and growth. Some zeolites have the ability to release antimicrobial ions, such as silver ions, into the surrounding environment. Silver ions are widely recognized for a wide spectrum of antibacterial properties, as they can interfere with essential cellular processes in bacteria, fungi, and other microorganisms. Zeolites loaded with silver ions can gradually release these ions over time, providing sustained antimicrobial effects. They can also modulate the pH of their surrounding environment, creating conditions that are unfavourable for microbial growth. By altering the pH to more acidic or alkaline levels, zeolites can inhibit the growth of certain microorganisms that thrive within specific pH ranges. [34,35]

Zeolites can exhibit synergistic antimicrobial effects when combined with other antimicrobial agents or materials. Incorporating zeolites into dental cements or coatings may enhance their antimicrobial properties, providing additional protection against bacterial colonization and biofilm formation on dental surfaces.

In the present study we have therefore used Zeolite nanoparticles in integration with antimicrobial photodynamic therapy using “Indocyanine green” dye to assess their antimicrobial action against “*Aggregatibacter actinomycetemcomitans”* impregnated on titanium discs.

AIM AND OBJECTIVES

AIM:

To assess and compare the effect of a combination of Nanosized natural zeolite and photodynamic therapy using "Indocyanine green" against "*Aggregatibacter actinomycetemcomitans"* biofilm on titanium discs.

OBJECTIVES:

- To assess the effect of "Indocyanine green" (photosensitizer) on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*
- To assess the effect of Nanosized natural Zeolite on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*
- To assess the effect of a combination of Nanosized natural Zeolite and "Indocyanine green" (photosensitizer) on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*
- To assess the effect of combination of Nanosized natural Zeolite and antimicrobial Photodynamic therapy (aPDT) ("Indocyanine green" + Diode laser) on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*
- To compare the effect of Nanosized natural Zeolite, "Indocyanine green" alone and with a combination of Nanosized natural Zeolite and antimicrobial Photodynamic therapy ("Indocyanine green" + Diode laser) on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*

REVIEW OF LITERATURE

Restoring the patient to normal function, speech, health, and appearance is the aim of modern dentistry. The advent of a two-stage threaded titanium root-form implant marked the beginning of the progress of dental implantology in the modern era. The father of contemporary implantology, Dr. P. Branemark, created and evaluated a method utilizing pure titanium screws in his research which he named fixtures. [36] These were the most meticulously maintained and well-researched dental implants when they were initially inserted in 1965. However, in recent years, the most common concern that has come up is the occurrence of peri-implant inflammations, which can cause the implant to fail by influencing the surrounding hard and soft tissues. [37] The microbiota of periodontal disease and peri-implant infections are quite similar, including the species of the red and orange complexes. According to literature the two predominant bacterial species linked to peri-implant deterioration are "*A. actinomycetemcomitans* and *P. gingivalis*." [6]

Lasers have a direct effect on gram negative anaerobic bacteria. Hence using of diode laser (940 nm) can be clinically valuable as a therapeutic modality for peri-implantitis. "Indocyanine green" (ICG) functions as an anionic photosensitizer that is non-toxic. aPDT using ICG as photosensitizer results in substantial reduction of "*P. gingivalis* and *A. actinomycetemcomitans."* [38,39]

One of the recently discovered nanoparticles is zeolite, a porous combination of aluminium silicate. Zeolites have been used in various therapeutic applications, demonstrating their low cytotoxicity and biocompatibility. Applying zeolite crystals next to the bone has also been demonstrated to improve osteoblast cell adhesion and proliferation. [28,29] Of the various available implant surfaces, very few have a significant antimicrobial activity. Thus, it is believed that the use of these nanoparticles with ICG in the aPDT technique will optimize the delivery of the photosensitizer and produce notable results on the surface of SLA implants that are even better than those of traditional aPDT.

R. Haas et al. (1997)[40] conducted an experiment to explore the bactericidal impact of photosensitizing chemicals on micro-organisms around implants. They tested pure suspensions of "*Porphyromonas gingivalis, Prevotella intermedia, or Actinobacillus actinomycetemcomitans"* on various titanium surfaces. After applying toluidine blue solution, they exposed the surfaces to a 905 nm diode soft laser for one minute. While individual treatments didn't affect bacterial growth, combined dye/laser treatment eradicated bacterial cells, as confirmed by electron microscopy. These findings suggest the potential of lethal photosensitization in peri-implantitis therapy.

Parker S. et al. (2013)[41] conducted a comprehensive review on Photodynamic Therapy (PDT) in dentistry, focusing on its historical development, integration into periodontal care, and the clinical use of Indocyanine green. PDT utilizes laser energy to activate a photosensitizing agent, targeting and destroying cellular structures.

It has been widely applied in dental treatments, particularly in addressing endodontic, periodontal, and oral epithelial issues due to its antibacterial properties. Anti-bacterial PDT (aPDT) has been explored as a complement to conventional periodontal therapies, leveraging the broad-spectrum bactericidal nature of a “810 nm diode laser” on Indocyanine green and other photosensitizers. Unlike traditional antibiotics, aPDT operates through oxidative stress-induced cell death, showing minimal impact on dental materials and non-target tissues. The review concluded that PDT represents a promising antibacterial therapy in clinical dentistry.

Topaloglu N et al. (2013)[42] conducted an in-vitro experiment to investigate the bactericidal effects of “Indocyanine green (ICG)” on resistant and wild-type strains of “*Pseudomonas aeruginosa and Staphylococcus aureus”* using an 809-diode laser. Initially, they optimized ICG concentrations and laser doses for “wild-type strains”, then applied the most effective concentrations to resistant strains. Photodynamic treatment (PDT) with ICG successfully killed all bacterial strains, with no effect observed from light or ICG alone. Optimal ICG laser dosages varied by bacteria type; for instance, 84 J/cm2 of light combined with 6μg/mL of ICG eliminated over 95% of wild-type “*S. aureus”* strains. Resistant strains required lower ICG doses for the same effect. Similarly, 125 μg/mL ICG with 252 J/cm2 of light was optimal for 99% elimination of wild-type “*P. aeruginosa”,* with even lower ICG doses effective against resistant strains. The study concluded that combining ICG with an 809 nm laser was an efficient antibacterial technique.

Birang et al. (2017)[43], twenty patients with forty implants affected by primary peri-implantitis underwent periodontal treatment including scaling and root planing (SRP) for the entire oral cavity, and mechanical debridement with titanium curettes for the implant regions. Implants were randomly assigned to receive Laser Therapy (control) or PDT (test). Clinical assessments were conducted at baseline, six weeks, and three months, with microbial samples analyzed via "real-time polymerase chain reaction (PCR)" at baseline and three months post-treatment. Results showed a significant decrease in levels of "*Aggregatibacter actinomycetemcomitans, Tannerella forsythia, and Porphyromonas gingivalis"* in the PDT group, and "*Porphyromonas gingivalis"* in the control group. Both Laser Therapy and PDT demonstrated significant short-term benefits in managing primary peri-implantitis.

Lu Z et al. (2017)[44] proposed that the nano Ag/ZnO hybrid material holds promise as a nanocomposite with potent antibacterial effects and low cytotoxicity, suitable for biomedical use. They employed a lyophilization technique to create a chitosan sponge matrix embedding Ag/ZnO nanocomposites, resulting in a sponge-like dressing loaded with nano Ag/ZnO. The dressing's properties, including porosity, swelling behavior, coagulation ability, and in vitro antibacterial efficacy against drug-sensitive and resistant pathogens, were evaluated. Results show enhanced blood clotting and antimicrobial activity, along with excellent porosity and minimal swelling. In vitro cytocompatibility testing demonstrated low toxicity. Moreover, in vivo studies in mice revealed that the chitosan-Ag/ZnO dressing accelerated wound healing, promoted re-epithelialization, and enhanced collagen deposition. These findings suggest the potential of this innovative dressing for wound care applications.

In their in-vivo study, **Birang E et al. (2019)[45]** randomly assigned contaminated discs to five treatments. Optical density (OD) was measured for aerobic bacteria using a spectrophotometer, while "colony-forming units" were counted for anaerobic bacteria. The results showed that all five approaches - mechanical (plastic curette), chemical (CHX), and laser (810 nm diode and Er: YAG), and aPDT - reduced oral biofilms on rough titanium surfaces. The Er-YAG laser and plastic curette had the highest and lowest effects, respectively.

Partoazar et al. (2019)[46] conducted an in-vitro study to assess the antibiofilm effect and downregulation of the "esp gene" in "*Enterococcus faecalis"* using "nanozinc oxide fabricated on natural zeolite (NanoZnO/Ze)". X-ray diffraction, X-ray fluorescence, and field emission scanning electron microscopy combined with energy dispersive X-ray were utilized to analyze zeolite and "NanoZnO/Ze" materials. Atomic absorption spectroscopy measured zinc release. The study evaluated "*E. faecalis"* biofilm development and esp gene expression in the presence of the nanocomposite. It exhibited dispersed ZnO nanoparticles with an average size of 30 nm on the zeolite surface. Compared to ZnO/Ze, NanoZnO/Ze showed significant and prolonged cationic zinc release. It effectively suppressed "*E. faecalis"* biofilm formation and downregulated the "esp gene expression". These findings suggest that NanoZnO/Ze may enhance defense against "*E. faecalis"* biofilm infections and potentially other pathogen-induced infections.

Sayar F et al. (2019)[47] investigated the impact of aPDT using diode lasers at 635 nm and 808 nm wavelengths, in combination with toluidine blue (TBO) and Indocyanine green (ICG), on "*Aggregatibacter actinomycetemcomitans"* biofilms growth on titanium discs. Eight groups were randomly assigned. Colony forming units (CFUs) on disc surfaces were counted post-intervention. Significant differences in colony counts were observed among the groups. Overall, aPDT proved effective for implant surface cleaning and in vitro biofilm reduction caused by "*A. actinomycetemcomitans"*, with notable reductions observed with "TBO + 635 nm and ICG + 808 nm" diode lasers.

This study conducted by **Pourhajibagher M et al (2021)[48]** aimed to assess the remineralization efficacy on enamel lesions induced by polymicrobial biofilms, as well as the anti-biofilm and anti-metabolic activities of "zeolite-zinc oxide nanoparticles (Zeo/ZnONPs)"-based antimicrobial photodynamic therapy (aPDT) against pre-formed polymicrobial biofilms on the orthodontic brackets. For a period of one and three months, degree of remineralization for the treated enamel slabs were assessed using a microhardness tester and a "DIAGNOdent" pen. Erythrocyte hemolysis and substantial cytotoxicity were not seen in treated cells containing "Zeo/ZnONPs." Additionally, the microhardness value of the exposed enamel surface increased steadily as "Zeo/ZnONP" concentration increased. There were no discernible variations between the aPDT and sodium fluoride varnish control groups, according to statistical analysis. Due to their strong potential to generate ROS, "Zeo/ZnONPs-based aPDT", which has the highest remineralization efficiency of enamel surface, can be utilized as an anti-biofilm therapeutic approach.

El-Telbany M et al. (2022)[49] aimed to isolate multidrug-resistant "*Pseudomonas aeruginosa"* from dental implants and control bacterial growth and biofilm formation using silver nanoparticles (AgNPs). Thirty specimens from individuals with peri-implantitis were collected, and bacterial samples were extracted using sterile paper points. P. aeruginosa isolates with multidrug resistance were identified using the VITEK 2 system and 16S rDNA-based PCR. Susceptibility to sixteen antibiotics was assessed using the VITEK 2 system. The disk-diffusion method evaluated the ability of AgNPs to suppress bacterial growth, while a microtiter plate assay assessed biofilm breakdown. AgNPs demonstrated inhibition of all isolated P. aeruginosa strains and exhibited significant antibiofilm activity against multidrug-resistant P. aeruginosa across all tested concentrations. These findings highlight the potential of AgNPs in suppressing "*P. aeruginosa"* growth and suggest their utility in eliminating "*P. aeruginosa"* biofilms.

Li N et al. (2022)[50] combined hydrogels and metal-organic frameworks (MOFs) to develop an injectable nanocomposite hydrogel. They incorporated zeolitic imidazolate frameworks-8 (DZIF) nanoparticles loaded with dexamethasone into a photo-crosslinking matrix of methacrylic gelatin (GelMA) and methacrylic polyphosphoester (PPEMA). The injectable hydrogel could achieve high local concentrations without inducing antibiotic resistance when injected into deep periodontal pockets. The hydrogel nanocomposite exhibited strong antibacterial activity and downregulated in vitro inflammatory gene expression, providing stable microenvironments conducive to cell viability, proliferation, spreading, and osteogenesis. Micro-computed tomography and histological analyses in an experimental rat model of periodontitis showed that the nanocomposite hydrogel

significantly reduced inflammation of the periodontal mucosa and mitigated inflammation-induced bone loss. These findings suggest that the hydrogel nanocomposite holds promise as a therapeutic option for periodontal disease treatment.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present in vitro study was undertaken to assess and compare the effects of a combination of Nanosized natural zeolite and antimicrobial photodynamic therapy using Indocyanine green against "*Aggregatibacter Actinomycetemcomitans"* biofilm on titanium discs.

This study was conducted in the "Department of Periodontics KAHER's KLE Vishwanath Katti Institute of Dental sciences and KLE's Dr. Prabhakar Kore Basic Science Research Centre (BSRC), KLE Academy of Higher Education and Research, Belagavi, Karnataka."

SOURCE OF DATA:

Titanium Discs: Commercially available, pre-sterilised, Grade 2 machined Titanium discs were procured from Indident^{TM} (Indident Medical Devices, New Delhi).

Natural nanosized zeolite: Commercially available Zeolite Nanoparticles were procured from Nano Research Lab, Jamshedpur, Jharkhand.

Bacterial strain: "*Aggregatibacter actinomycetemcomitans* (AA) – ATCC 33277' was obtained from depository of "KLE's Dr. Prabhakar Kore Basic Science Research Centre (BSRC), KLE Academy of Higher Education and Research, Belagavi, Karnataka."

Indocyanine Green Dye: Commercially available "Indocyanine green" was procured from Aurogreen®, Aurolab, Madurai, Tamil Nadu.

Laser: GaAlAs Diode Laser (BIOLASE, the Diode Laser Therapy System) available in Department of Periodontics, KAHER's KLE Vishwanath Katti Institute of Dental sciences, Belagavi, Karnataka, was used for photodynamic therapy.

Other materials:

- Phosphate buffer solution.
- Phloxin B Stain.
- Microtiter well plates.
- Distilled water.
- Brain heart infusion (BHI) agar.
- De-ionized water

METHOD OF COLLECTION DATA

Inoculation of bacteria on titanium discs

Inoculum preparation was carried out in BHI agar. Standard bacterial colonies of the same morphological type of "*Aggregatibacter actinomycetemcomitans"* were used for the study. This bacterial strain was then transferred onto the titanium discs which were placed in petri plates.

Bacterial strain and colonization

The attachment and maturation of the bacterial colonies on the titanium disc surface eventually led to the formation of a complex organization of cells called the biofilm. The biofilm coated discs were rinsed with 1× PBS (Phosphate buffer solution) to rid them of the planktonic bacteria, leaving the biofilm structure intact. After an incubation period of 48 hours, bacterial formation was assessed by using Phloxin B stain.

Nanosized zeolite suspension procedure

Zeolite nanoparticles were added to distilled water and let sit for a duration of one hour, after which the waste water was filtered out using a cellulose paper filter. This process was repeated three times. The zeolite nanoparticles were then dried at "80°C" and kept away from humidity. The resultant zeolite powder was then suspended in deionized water (DI.W.) for use.

Titanium disc preparation

200 Pre sterilized SLA treated titanium discs of diameter 8 mm and thickness of 2 mm were randomly allocated to eight different group. Each group contained 25 discs.

BHI agar was prepared in petri-plates and the titanium discs were placed within the prepared agar plate. The titanium discs were then inoculated with the "*Aggregatibacter actinomycetemcomitans* (AA)-ATCC 33277" strains. The discs were subsequently placed in an anaerobic chamber for 48 hours. Bacterial biofilm formation was seen on all discs. This biofilm formation on titanium discs was assessed by Phloxin – B stain.

All these inoculated discs in Petri plates were randomly allocated to eight different groups that included:

- Group 1: Positive control (2.5% NaOCl)
- Group 2: Negative control (Biofilm alone)
- Group 3: Indocyanine Green (ICG)
- Group 4: Nanosized Zeolite (NZ)
- Group 5: Nanosized Zeolite + Indocyanine Green
- Group 6: Nanosized Zeolite + Diode Laser
- Group 7: Antimicrobial Photodynamic Therapy (aPDT) i.e (ICG+ Diode laser)
- Group 8: Nanosized Zeolite + aPDT

Laser protocol

Titanium discs were irradiated with "GaAlAs Diode Laser (BIOLASE, the Diode Laser Therapy System) with 400μm fibre optic handpiece at a wavelength of 940 nm, operated at power – 1 W, with a pulse length of 200μm and pulse interval of 200μm in non-contact mode for 30-60s."

Indocyanine green dilution

25mg of ICG was dissolved in 5 mL of sterile water to prepare an initial 5 mg/ mL ICG stock solution. This stock solution was further diluted in saline solution at a ratio of 1:5 to achieve a final ICG concentration of 1mg/mL before implementation.

Photodynamic therapy procedure

Application- Titanium discs were placed within solution for 2 minutes.

Soaking phase- The solution with active ingredient adheres to the bacterial cell membrane and the bacteria then takes up the dye thereby sensitizing them.

Rinsing phase- Excess of the active ingredients were rinsed off. Bacteria stained by the green dye remained on titanium discs.

Activation- The dye-stained bacteria on the discs were activated by laser light energy for 30-60 s

Sample size estimation:

At 95% Confidence interval; 80% Power; 5% Attrition**.** Sample size is calculated by using the formula:

$$\mathbf{n} = \frac{(z_1 - \frac{\alpha}{2} + z_1 - \beta)^2 (p_1 q_1 + p_2 q_2) \times 1.05}{(p_1 - p_2)^2} = \mathbf{25.4} \text{ per group.}$$

Where,

$p_1 = 45.7$; $p_2 = 11.8$; $q_1 = 54.3$; $q_2 = 88.2$; $z_1 - \frac{\alpha}{2} = 1.96$; $z_1 - \beta = 0.85$

With the help of the above formula sample size (n) was calculated as 25.4, the final sample size has been adjusted to **25 discs** per group and **200 discs** for 8 groups.

STATISTICAL ANALYSIS

- The data was entered in Microsoft Excel and analysed statistically using the Statistical package for Social Science (SPSS) software, version 21; SPSS Inc., (Chicago, IL, USA).
- The normality of the data was assessed prior to analysis using the Kolmogorov-Smirnov test.
- Data was found to be normally distributed. Thus, parametric tests were chosen.
- Descriptive analysis was carried out to calculate the mean and standard deviation for all the groups.
- Inter group comparisons was done by One-way ANOVA.
- Pairwise Comparison of all study groups were carried out using Tukey's multiple post hoc test.
- All statistical tests were performed at a significance level of 5% ($p<0.05$). Any value less than or equal to 0.05 was considered as statistically significant.

ARMAMENTARIUM USED IN THE STUDY

Figure 1: Microtiter pipette

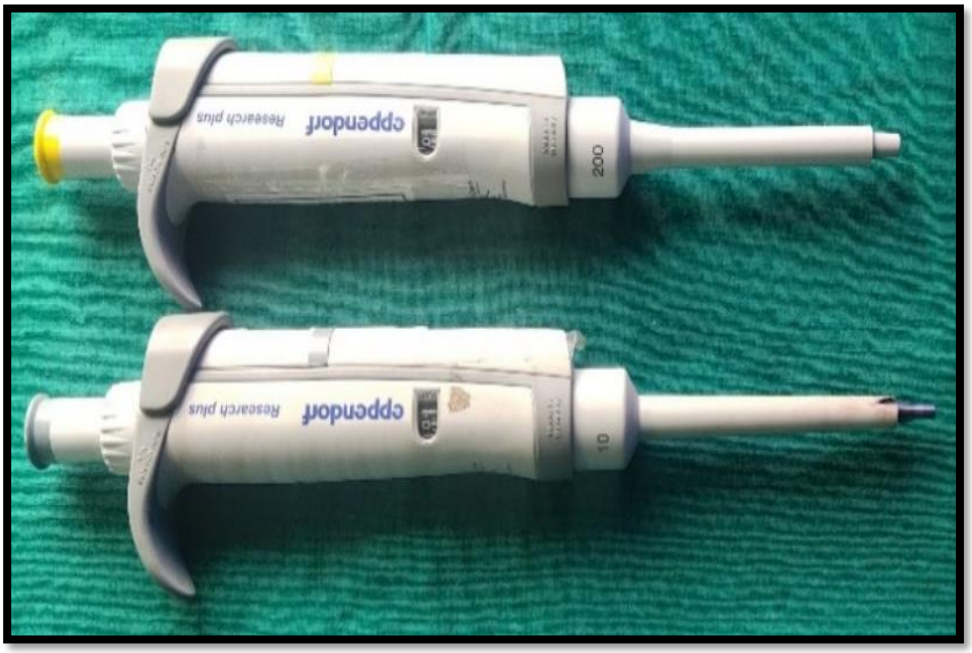


Figure 2: Pre-sterilized titanium discs

Figure 3: Microtiter well plate

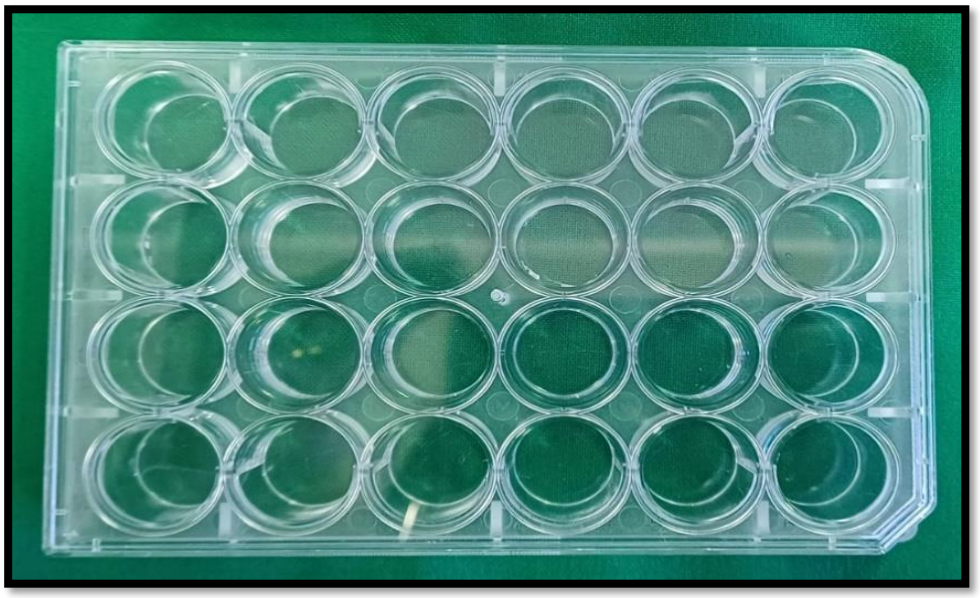


Figure 4: BHI Agar

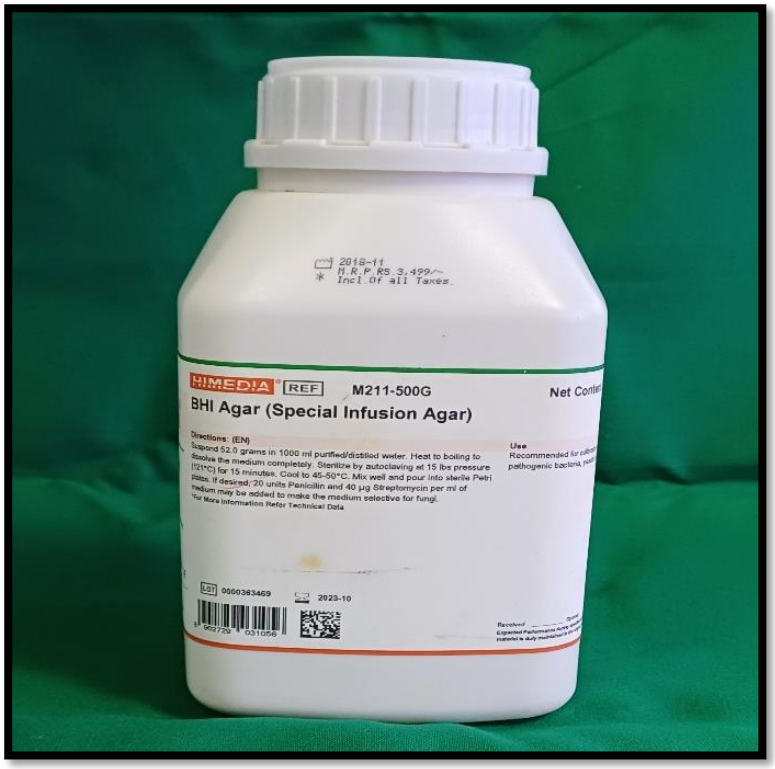


Figure 5: Nanosized Natural Zeolite

Figure 6: Diode laser (Biolase)

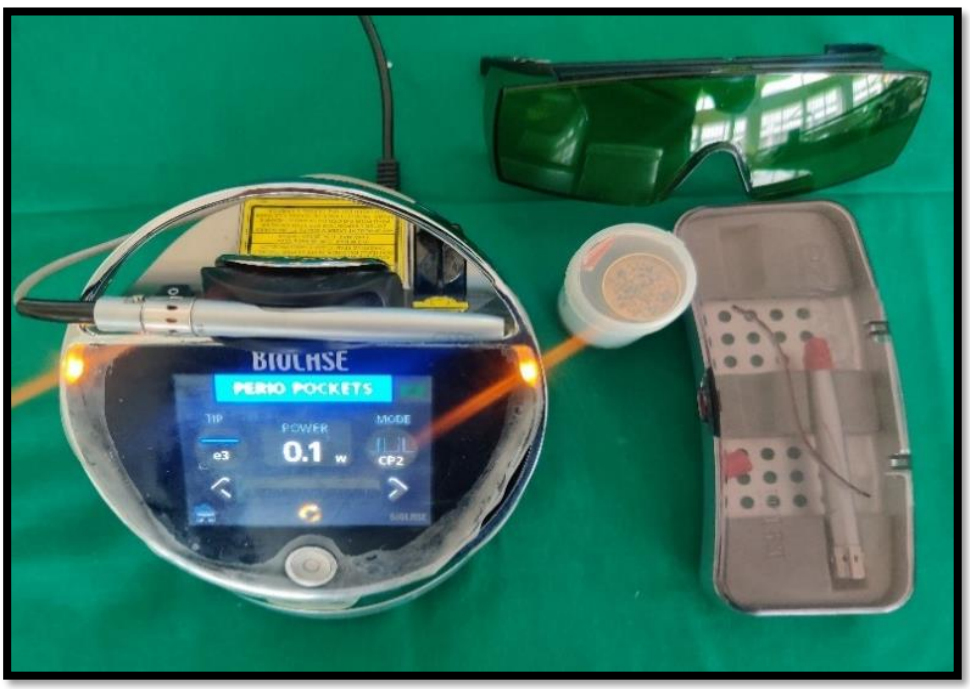


Figure 7: Photosensitizer dye - Indocyanine Green

Figure 8: Titanium discs inoculated with *A. Actinomycetemcomitans* strain

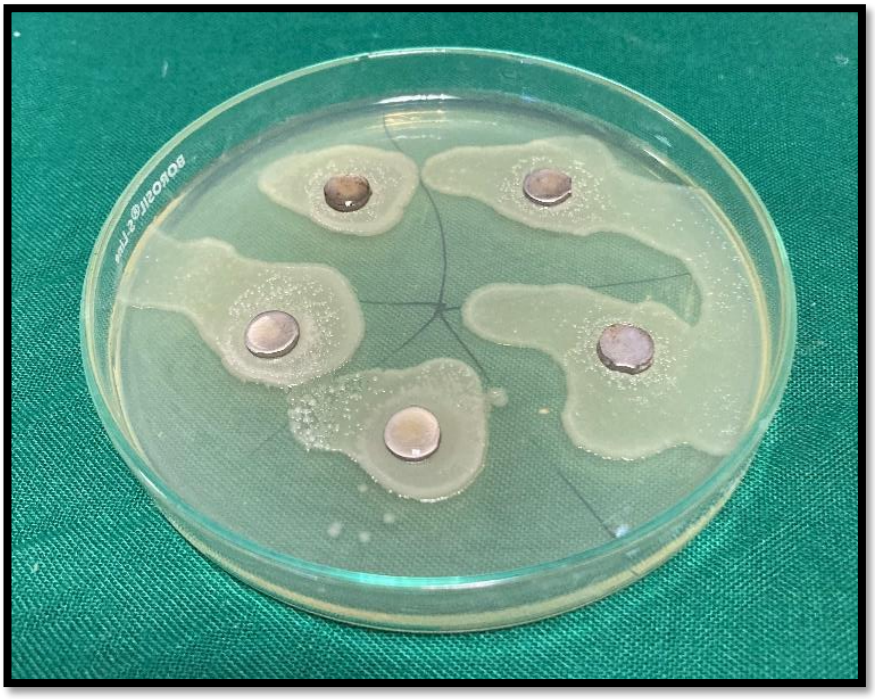


Figure 9: Inoculated discs treated with Nanosized zeolite and Indocyanine green

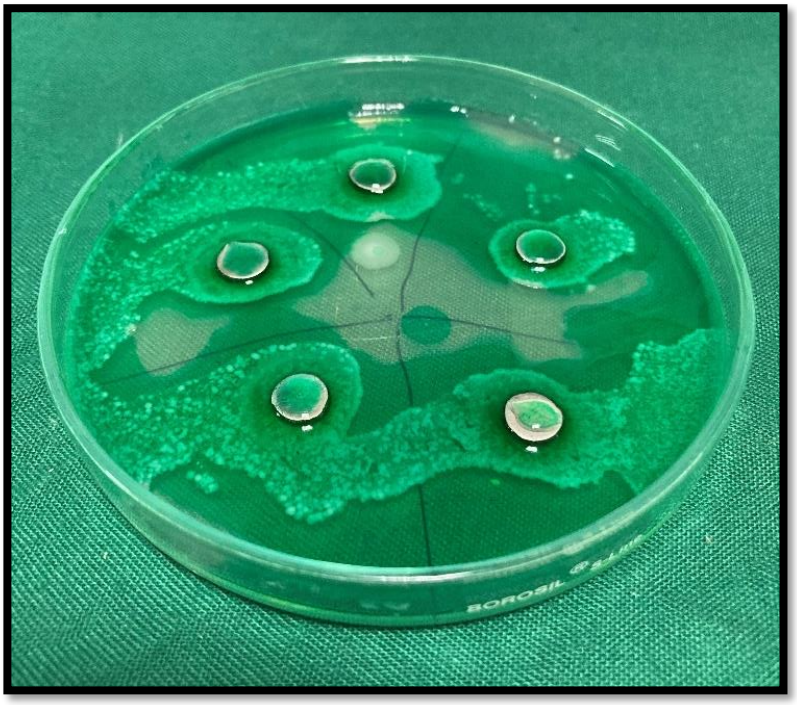


Figure 10: Inoculated discs treated with Nanosized zeolite and Diode Laser

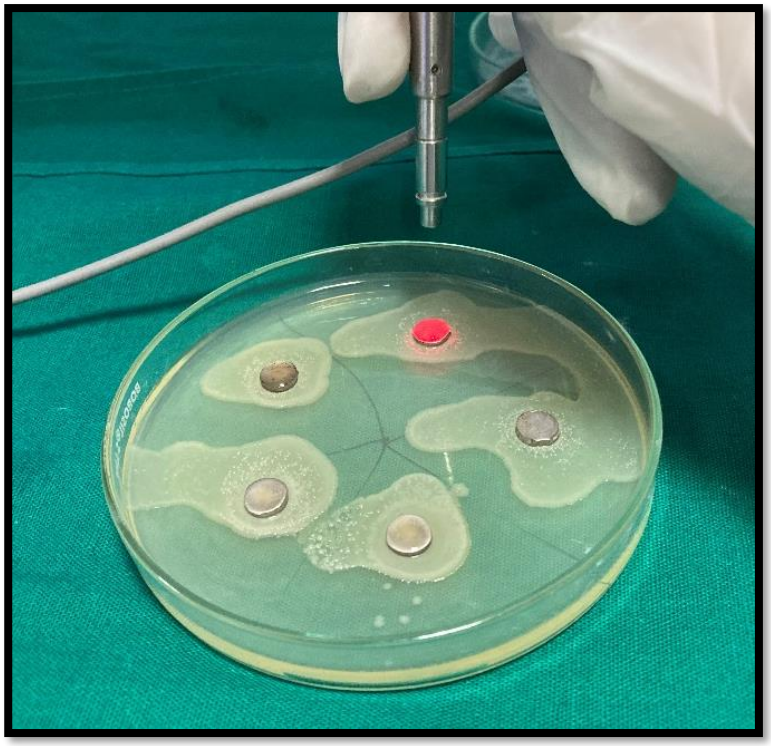


Figure 11: Inoculated titanium disc treated with Antimicrobial photodynamic therapy

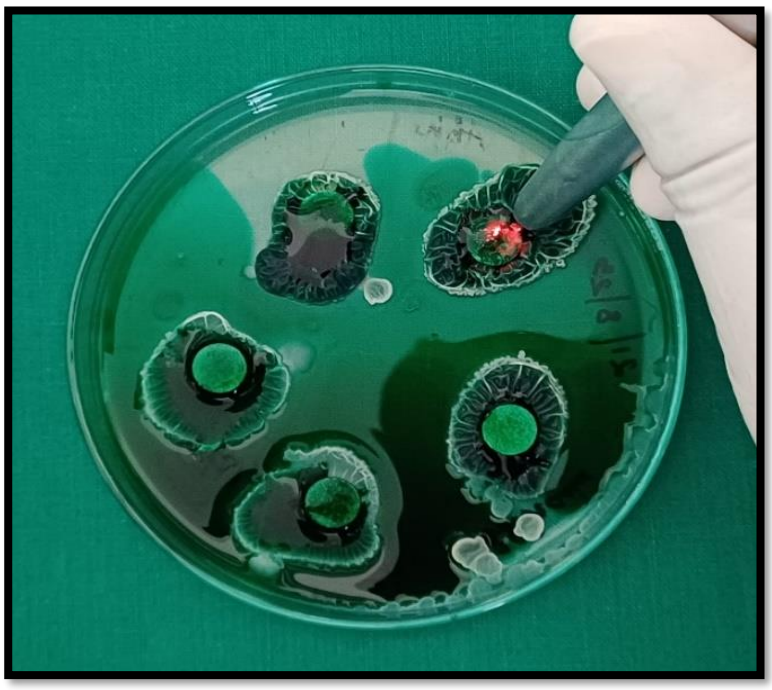


Figure 12: Inoculated discs treated with Nanosized zeolite and with Antimicrobial photodynamic therapy

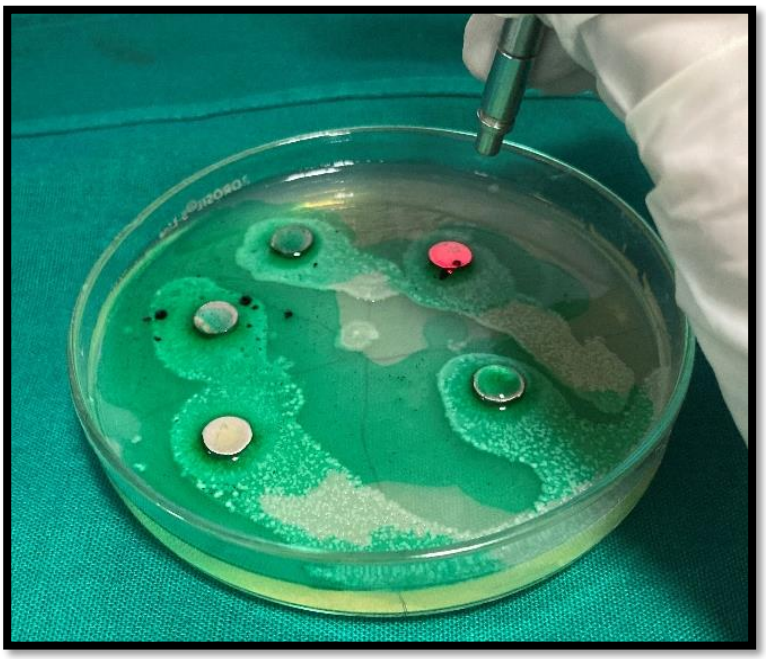


PLATE COUNT METHOD FOR COLONY FORMING UNITS

Figure 13: Group 1-Negative control

Figure 14: Group 2- Positive control

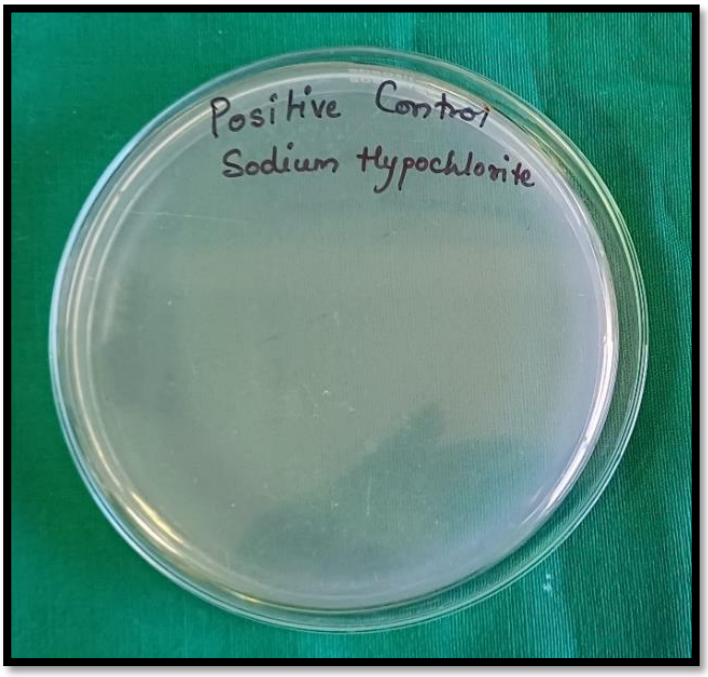


Figure 15: Group 3- Indocyanine green

Figure 16: Group 4- Nanosized Natural Zeolite

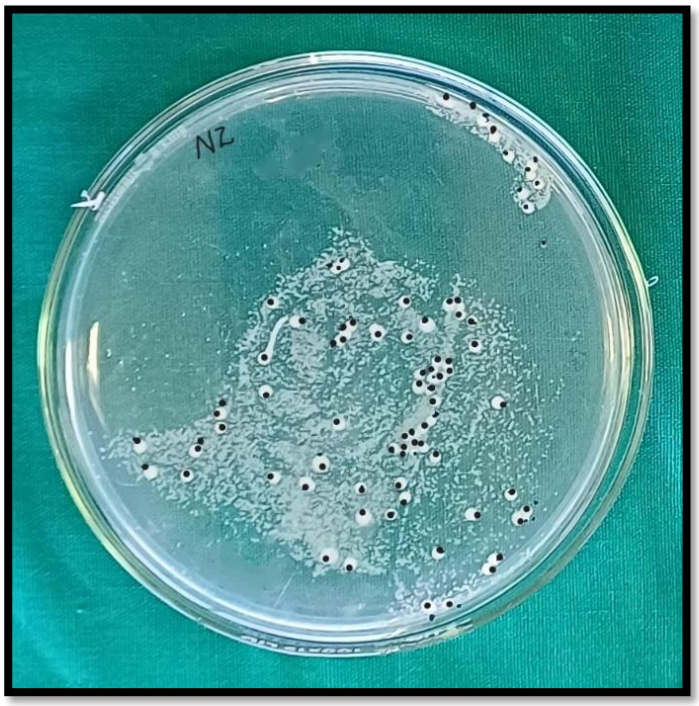


Figure 17: Group 5- Indocyanine green + Nanosized zeolite

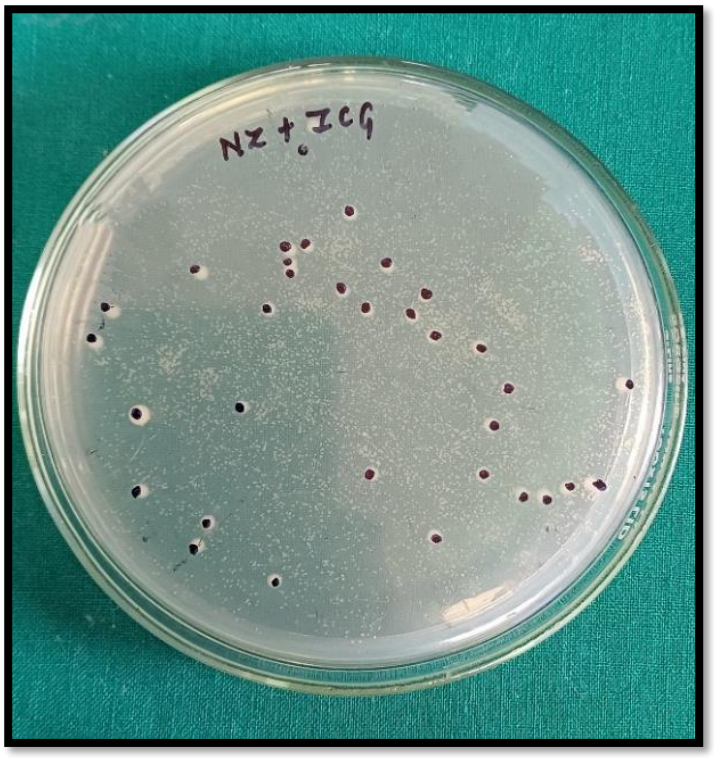
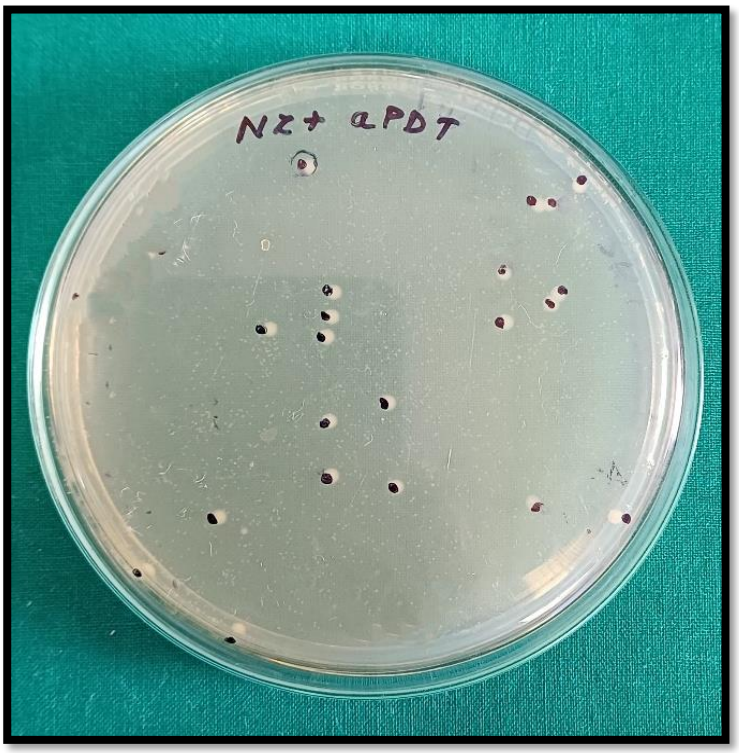


Figure 18: Group 6- Nanosized zeolite + Diode laser

Figure 19: Group 7- Antimicrobial Photodynamic Therapy

Figure 20: Group 8- Nanosized Zeolite + Antimicrobial Photodynamic Therapy



RESULT AND OBSERVATIONS

Table 1: Colony Forming Unit counts/ml in all the study groups.

COLONY FORMING UNIT COUNTS – CFU/ml (x10^4)								
	Study groups							
S.no	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	0	145	120	60	35	86	28	13
2	0	159	122	48	36	74	26	12
3	0	161	121	56	40	80	25	18
4	0	160	118	62	28	86	26	15
5	0	148	120	52	35	74	30	10
6	0	145	116	48	30	68	28	14
7	0	156	123	52	32	85	26	16
8	0	148	118	60	30	80	25	13
9	0	174	124	48	35	74	25	15
10	0	168	121	56	38	80	26	18
11	0	178	120	62	32	86	28	16
12	0	155	115	52	36	74	30	15
13	0	145	122	48	40	68	28	12
14	0	143	118	52	32	70	26	11
15	0	165	120	62	40	78	25	16
16	0	145	117	48	35	74	26	10
17	0	164	122	56	38	80	28	15
18	0	173	119	52	32	76	25	13
19	0	156	122	60	35	84	25	14

20	0	166	120	48	28	68	26	17
21	0	172	121	52	32	78	30	10
22	0	147	116	48	30	70	25	12
23	0	158	120	62	36	70	28	11
24	0	145	123	52	38	74	27	15
25	0	148	122	48	32	80	26	11

*(**Group 1**: 2.5% NaOCl, **Group 2**: Biofilm alone, **Group 3**: ICG, **Group 4**: NZ, **Group 5**: NZ + ICG, **Group 6**: NZ + Diode Laser, **Group 7**: aPDT, **Group 8**: NZ+ aPDT)

Observations:

Table no 1 shows the count of colony forming unit/ml in all the groups. It was observed that most impregnated group was the negative control group, in which no treatment protocol was applied and the least impregnated group that showed absolute antibacterial efficacy was the positive control group, wherein 2.5% NaOCL was used.

*Of the 8 study groups only 7 were included in the statistical analysis as group 1 was positive control and has shown absolute antibacterial efficacy i.e no colony forming units were seen.

Table 2: Normality of CFU/ml counts ($x10^4$) in study groups by Kolmogorov Smirnov test.

Groups	Z-value	p-value
Biofilm alone	0.9790	0.2940
Indocyanine Green (ICG)	0.9000	0.3930
Nanosized Zeolite (NZ)	1.1430	0.1470
NZ+ICG	0.8390	0.4820
NZ +Diode laser	0.7710	0.5920
ICG+ Diode laser (aPDT)	1.3330	0.0570
NZ + aPDT	0.7200	0.6770

Observations:

Table 2 depicts the normality assessment performed among the groups to establish the normal distribution of data. Normality was assessed using Kolmogorov Smirnov test.

It was noted that, the CFU/ml counts ($x10^4$) in the study groups follow normal distribution. Therefore, parametric one-way ANOVA and Tukeys multiple posthoc procedures were applied for further analysis.

Table 3: Descriptive analysis of CFU/ml counts (x10^4) of the study groups.

Groups	Mean	Std.Dev.	Std.Err.	95% CI for mean	
				Lower	Upper
Biofilm alone	156.96	10.84	2.17	152.48	161.44
Indocyanine Green (ICG)	120.00	2.38	0.48	119.02	120.98
Nanosized Zeolite (NZ)	53.76	5.36	1.07	51.55	55.97
NZ+ICG	34.20	3.63	0.73	32.70	35.70
NZ +Diode laser	76.68	5.94	1.19	74.23	79.13
ICG+ Diode laser (aPDT)	26.72	1.67	0.33	26.03	27.41
NZ + aPDT	13.68	2.46	0.49	12.66	14.70

Observations:

The descriptive analysis of all groups was done to estimate the mean and standard deviation at a confidence interval of 95%, which is displayed in **Table 3**. All test groups showed a significant difference compared to the control group. The intergroup comparison of all test groups revealed that the mean and standard deviation in NZ+ aPDT group was 13.68 ± 2.46 ×10^4; suggesting that this group was superior in reducing microbial load.

The mean and standard deviation of ICG group was 120 ± 2.38×10^4, NZ group was 53.76 ± 5.36 ×10^4, NZ+ICG group was 34.20 ± 3.63×10^4, NZ+ Diode laser group was 76.68 ± 5.94 ×10^4, and ICG + aPDT group was 26.72 ± 1.67 ×10^4; suggesting that ICG + aPDT protocol was better at reducing the microbial load compared to

NZ+ICG group followed by NZ group, NZ+ Diode laser group and ICG group respectively. Among test groups; the combination protocol of NZ+ aPDT showed highly statistically significant reduction in microbial load compared to the other test groups, which suggests that it had the highest antibacterial efficacy amongst the test groups.

Table 4: Comparison of the CFU/ml counts ($x10^4$) within the study groups by one way ANOVA.

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between groups	6	417199.9890	69533.3314	2326.3446	0.0001*
Within groups	168	5021.4400	29.8895		
Total	174	422221.4290			

*p<0.05

Observations:

Table 4 provides detailed description of the Parametric Analysis which was done using One way ANOVA to evaluate the difference between and within the groups. Between the groups the mean sum of squares was 69533.3314 and within groups the mean sum of squares was 29.8895. The intergroup comparison revealed a highly statistically significant difference between the study groups with a p-value of 0.0001.

Table 5: Pair wise comparison of study groups with CFU/ml counts (x10^4) by Tukeys multiple posthoc procedures.

Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	p-value	95% CI for mean Diff.	
					Lower Bound	Upper Bound
Biofilm alone vs	Indocyanine Green (ICG)	36.96	1.5463	0.0001*	32.35	41.57
Biofilm alone vs	Nanosized Zeolite (NZ)	103.20	1.5463	0.0001*	98.59	107.81
Biofilm alone vs	NZ+ICG	122.76	1.5463	0.0001*	118.15	127.37
Biofilm alone vs	NZ +Diode laser	80.28	1.5463	0.0001*	75.67	84.89
Biofilm alone vs	ICG+ Diode laser (aPDT)	130.24	1.5463	0.0001*	125.63	134.85
Biofilm alone vs	NZ + aPDT	143.28	1.5463	0.0001*	138.67	147.89
Indocyanine Green (ICG) vs	Nanosized Zeolite (NZ)	66.24	1.5463	0.0001*	61.63	70.85
Indocyanine Green (ICG) vs	NZ+ICG	85.80	1.5463	0.0001*	81.19	90.41
Indocyanine Green (ICG) vs	NZ +Diode laser	43.32	1.5463	0.0001*	38.71	47.93
Indocyanine Green (ICG) vs	ICG+ Diode laser (aPDT)	93.28	1.5463	0.0001*	88.67	97.89
Indocyanine	NZ + aPDT	106.32	1.5463	0.0001*	101.71	110.93

Green (ICG) vs						
Nanosized Zeolite (NZ) vs	NZ+ICG	19.56	1.5463	0.0001*	14.95	24.17
Nanosized Zeolite (NZ) vs	NZ +Diode laser	-22.92	1.5463	0.0001*	-27.53	-18.31
Nanosized Zeolite (NZ) vs	ICG+ Diode laser (aPDT)	27.04	1.5463	0.0001*	22.43	31.65
Nanosized Zeolite (NZ) vs	NZ + aPDT	40.08	1.5463	0.0001*	35.47	44.69
NZ+ICG vs	NZ +Diode laser	-42.48	1.5463	0.0001*	-47.09	-37.87
NZ+ICG vs	ICG+ Diode laser (aPDT)	7.48	1.5463	0.0001*	2.87	12.09
NZ+ICG vs	NZ + aPDT	20.52	1.5463	0.0001*	15.91	25.13
NZ +Diode laser vs	ICG+ Diode laser (aPDT)	49.96	1.5463	0.0001*	45.35	54.57
NZ +Diode laser vs	NZ + aPDT	63.00	1.5463	0.0001*	58.39	67.61
ICG+ Diode laser (aPDT) vs	NZ + aPDT	13.04	1.5463	0.0001*	8.43	17.65

*$p<0.05$

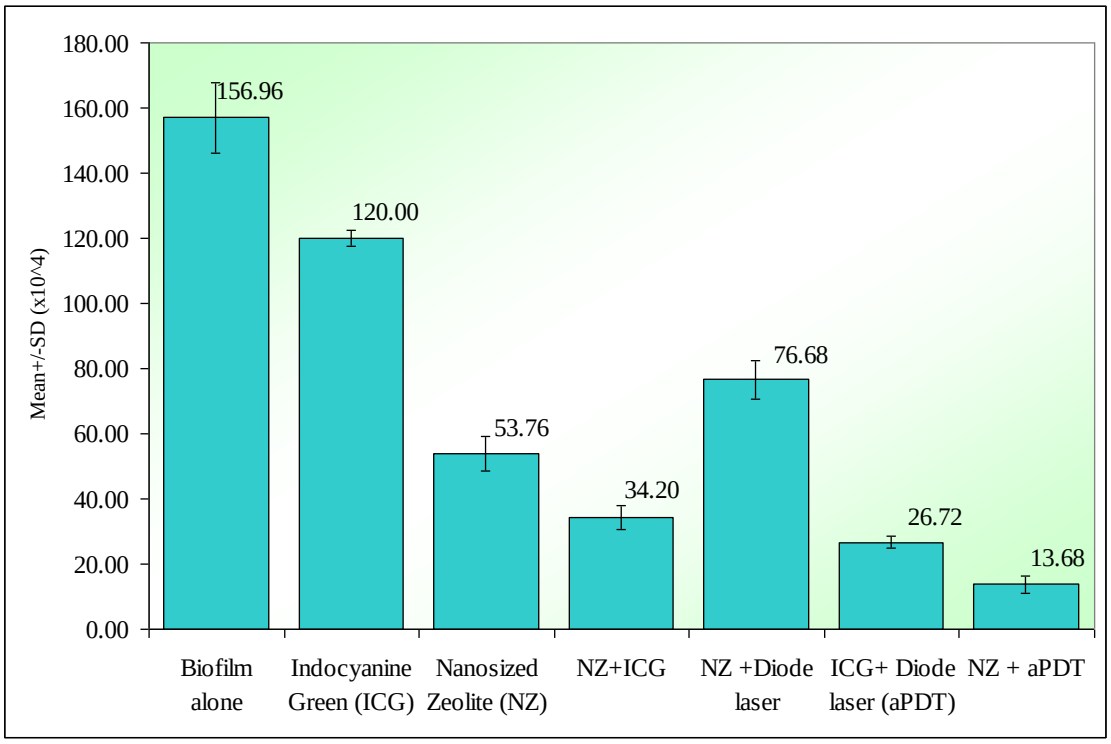
Observations:

Table 5 shows the Tukey's multiple posthoc analysis which was carried out for pairwise comparison of the study groups. The inter group comparisons between Control and other test groups showed highly significant mean difference, i.e, ICG - 36.96; NZ-103.20; ICG + NZ - 122.76; NZ + Diode Laser - 80.28; aPDT - 130.24 and NZ + aPDT- 143.28 respectively. In comparison with ICG and the other test groups, the mean difference values were; NZ- 66.24; ICG + NZ - 85.80; NZ + Diode Laser - 43.32; aPDT - 93.28 and NZ + aPDT- 106.32 respectively and the difference was statistically significant. (*p value - 0.0001)

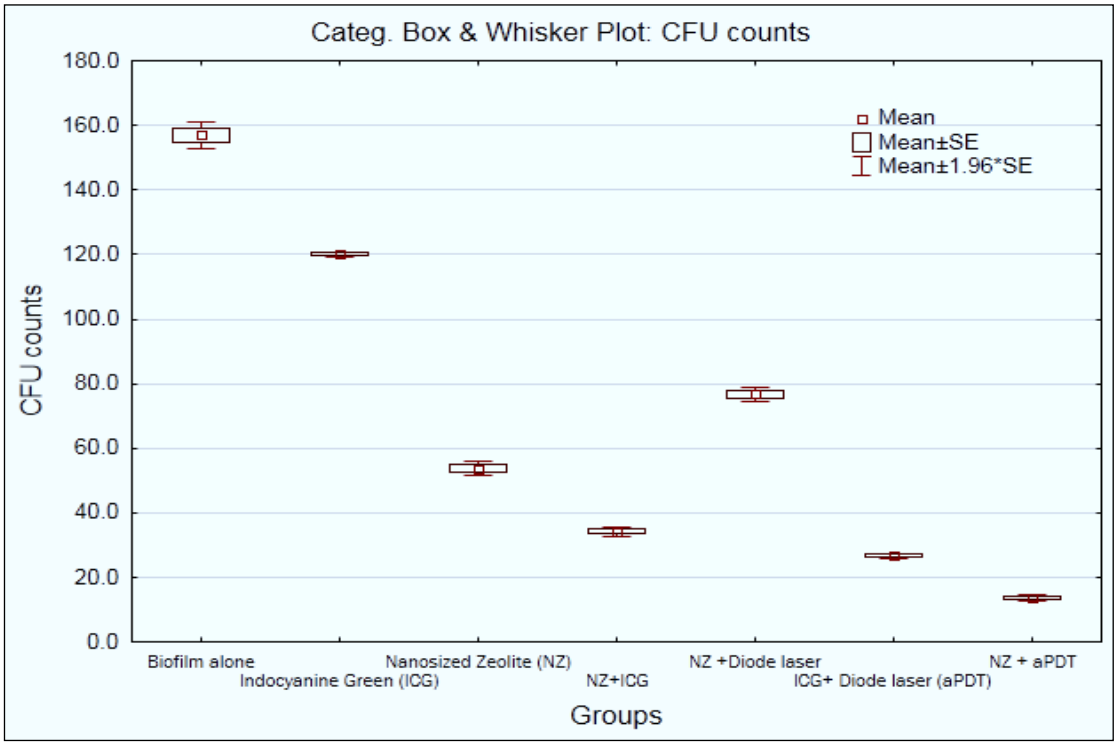
There was a statistically significant difference in the mean difference values between NZ and other test groups and the values were; NZ+ICG - 19.56 ; NZ + Diode laser- -22.92; aPDT - 27.04 and NZ + aPDT - 40.08. On comparing NZ + ICG with the other test groups the mean difference values were as follows; NZ +Diode laser - -42.48; aPDT - 7.48; NZ + aPDT - 20.52, and they were statistically significant. The mean difference values in comparison with NZ + Diode laser and other test groups showed statistically significant differences and the values were: aPDT - 49.96 and NZ + aPDT - 63.00 respectively. Lastly, the intergroup comparison between aPDT and NZ + aPDT the mean difference value was 13.04, which was highly statistically significant. (*p value - 0.0001).

Furthermore, the intergroup posthoc analysis shows that among the test groups NZ + aPDT was superior in reducing bacterial load followed by aPDT, NZ +ICG, NZ, NZ + Diode laser and ICG respectively. Therefore, the combination of Nanosized Natural Zeolite and Antibacterial Photodynamic therapy had the highest antibacterial efficacy when compared to other treatment groups. (*p value - 0.0001).

Graph 1: Comparison of study groups with CFU/ml counts (x10^4)



Graph 2: Whisker plot of comparison of study groups with CFU/ml counts (x10^4)



DISCUSSION

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more so that we may fear less."

- Marie Curie.

True to the quote above, ample literature and evidence is available validating the use of "antimicrobial photodynamic therapy (aPDT)" against the various gram negative periodontal pathogens like "*A.actinomycetemcomitans, P.gingivalis, P.intermedia*."[51,52,53] In particular the use of indocyanine green as the photosensitizer for aPDT owing to its superior phototoxicity, biocompatibility and penetration into tissues over the other photosensitizer molecules like Methylene blue and Toluidine blue is also well documented. Although Indocyanine green is a very powerful photosensitizer molecule, there have been several reports of its instability in aqueous solutions like water, saline, plasma, and blood. Moreover, it experiences irreversible physical and chemical changes that can alter its color and maximum wavelength absorption as well as reduce its optical absorption and fluorescence intensity. [54,55,56]

Therefore, to overcome these limitations, recently, in the field of aPDT a wide variety of nanoparticles have been used to enhance "photosensitizer solubility, photochemistry, photophysics, and targeting". Zeolite which is a porous compound of aluminium silicate with a negatively charged external surface, is one such nanoparticle that has come up in recent research. Depending on the chemical and physical makeup, the primary characteristics of zeolites are their ability to trap water and the localization of water inside their networks. Zeolite crystals also have been demonstrated to improve osteoblast cell adhesion and proliferation.[28,29]

These properties of the zeolite nanoparticles help overcome the limitations of Indocyanine green and also enhance its antibacterial action.

The current study was therefore undertaken to assess and compare the effect of a combination of "Nanosized natural zeolite" and photodynamic therapy using Indocyanine green against "*Aggregatibacter Actinomycetemcomitans"* biofilm on titanium discs. This study involved various test groups using "Nanosized natural zeolite", Indocyanine green and a Diode laser individually as well as in combination with each other to examine which among these combinations would exhibit the highest antibacterial efficacy.

The results indicate that the combination of Nanosized natural zeolite along with antibacterial photodynamic therapy resulted in most significant reduction of bacterial load i.e 13.68 x 10^4 CFU/ml. Other test groups that showed significant decrease in bacterial load were antimicrobial photodynamic therapy, the combination of nanosized natural zeolite and indocyanine green followed by nanosized natural zeolite with colony counts of 26.72 x 10^4 CFU/ml, 34.20 x 10^4 CFU/ml and 53.76 x 10^4 CFU/ml respectively. **(Table 3)**

A considerable amount of evidence demonstrates the superiority of aPDT over the use lasers alone. The individual antibacterial efficacy of diode laser and photosensitizers are much less when compared to their combined use. ICG as a photosensitizer dye has recently been demonstrated to be effective against "*A. actinomycetemcomitans* and *P. gingivalis"* in in-vitro experiments when triggered by an 810 nm diode laser. ICG can cause potent photosensitized cellular damage by means of photon-induced electron transfer.

Therefore, the use of ICG in aPDT against periodontitis and peri-implantitis have been on the rise and these findings have been validated by the studies conducted by Chambrone L et al, [57] Sayar F et al.[47]

In another research work conducted by Shingnapurkar et al, the impact of adjunctive photodynamic treatment (PDT) for chronic periodontitis utilizing an 810 nm diode laser and ICG as a photosensitizer was evaluated. Three months following therapy, statistically significant difference was noted between the test group and the control group in terms of PPD and RAL. They concluded that adjunctive PDT can enhance the clinical results of standard scaling and root planning in individuals with persistent periodontitis.[58]

In this study, we have therefore used ICG as the photosensitizer for aPDT, and we also tested each of them individually as well as in combination with each other. It was noted that the combined use of diode laser and ICG in the form of photodynamic therapy has a superior antimicrobial efficacy (p value 0.0001) (Table 4) and these observations were in tandem with the results of the aforementioned studies.

Gram-positive species ("*S. mutans, S. sanguis, A. viscosus*, and *S. aureus"*) have three to twenty times more peptidoglycan in their cell walls than gram-negative bacteria ("*P. gingivalis, P. intermedia*, and *A. actinomycetemcomitans"*). Given that peptidoglycans are negatively charged, part of the silver ions in the broth is likely bound by them. Gram-positive bacteria may therefore permit fewer silver ions to pass through the plasma membrane than gram-negative bacteria.[59,60] Of all metal ions, metallic silver has the strongest antibacterial action. Zeolite is a hydrated sodium aluminosilicate substance that is porous and crystalline and has a great affinity for silver.

Silver ions are electrostatically bound to zeolite, which causes a slow, steady, and durable release of silver ions from the zeolite, which may be the cause of the antibacterial activity. [61]

Ghatole et al, [62] used a direct contact test to assess the antibacterial properties of calcium hydroxide, calcium hydroxide combined with silver-zeolite, and calcium hydroxide mixed with 2% chlorhexidine against "*E. faecalis"*. According to the results, the antibacterial activity of calcium hydroxide and silver-zeolite was the highest. Similarly, Kawahara et al, [63] carried out a study aimed to assess the antibacterial activity of silver-zeolite against oral bacteria in anaerobic environments. The bacteria that were evaluated under anaerobic conditions had their growth impeded by silver-zeolite, according to the results. These findings showed that silver zeolite might be a helpful carrier for enhancing the antibacterial activity of dental materials, even when they are utilized in anaerobic environments like the periodontal pocket. These studies substantiate the use of Zeolite against the periodontal pathogens owing to its antimicrobial activity.

Nanoparticles have come up recently as an efficient method for drug delivery and the use of nanosized natural zeolite against pathogens has been documented to be effective. In a study conducted by Partoazar et al,[46] he evaluated the impact of nanozinc oxide zeolite (NanoZnO/Ze) produced on natural zeolite (ZnO/Ze) on the antibiofilm effect and "esp gene" downregulation of "*Enterococcus faecalis"*. The results showed that when compared to ZnO/Ze, the cationic zinc leakage from "NanoZnO/Ze" showed a significant and prolonged release. "*Enterococcus faecalis"* biofilm formation was effectively suppressed by NanoZnO/Ze, which also had an impact on the downregulation of esp genes.

Their findings indicate that NanoZnO/Zeolite can strengthen the defences against E. faecalis biofilm infections and perhaps other pathogen-induced biofilm infections.

Apart from the antimicrobial efficacy, studies have also shown that zeolite nanoparticles can be used to enhance the effect of aPDT using ICG. ICG is a water soluble, anionic Tri carbocyanine that belongs to the large family of cyanine dyes. The ICG molecule has an amphiphilic molecular structure with hydrophilic and lipophilic characteristics. Due to its high hydrophilicity its physical and chemical properties are altered in the presence of water-based solutions like saline and blood. These alterations affect its instability, phototoxicity and also its ability to reduce microbial load. [54,55]

Thus, in this study Nanosized Natural Zeolite was used along with ICG in aPDT and the results showed that antibacterial efficacy of the combination was much superior when compared to aPDT using ICG alone. **(Table 4 & 5)**

These results were similar to that of a study conducted by Nagahara et al, [64] where they investigated indocyanine green (ICG), which exhibits strong absorption between 800 and 805 nm in wavelength. They used ICG-loaded nanospheres with low-level diode laser irradiation at an 805 nm wavelength to study the bactericidal effect of PDT on "*Porphyromonas gingivalis*." They noticed that the surface of "*P. gingivalis"* could hold onto ICG-Nano/c. "*P. gingivalis"* was significantly reduced by laser irradiation with ICG-Nano/c (i.e., nearly 2-log10 bacterial death).

Zeolites also have been found to have osteoinductive qualities, and their unique topology makes them an ideal material for the attachment and development of bone cells.

Enhancing osteoblast differentiation and proliferation is thought to be a crucial characteristic of zeolite that needs to be considered while investigating bone regeneration in various implant-related therapeutic techniques. Zinc oxide coatings on titanium implants have been shown to improve osseointegration and reduce modulus mismatch with bone tissue. Zeolite surfaces exhibit complete fibroblast attachment because of their biocompatibility, suggesting that zeolite coatings could be used on dental implants to extend their lifespan and improve post-operative patient recovery.[65,66]

Peri-implantitis is characterized by an increasingly complicated biofilm forming on the implant and many different periodontal pathogenic bacteria can readily colonize the surface of a failed implant. It has been demonstrated that a significant amount of "*P. gingivalis* and *A. actinomycetemcomitans"* can colonize abrasive titanium surfaces, with the help of plasma proteins. Several in vitro and in vivo investigations have suggested that peri-implantitis may be treated by removing these periodontal bacteria from the implant surface using an aPDT.[67,68] This study concentrated on the bactericidal effectiveness of nanosized zeolite-based aPDT on "*A. actinomycetemcomitans"* biofilm on titanium discs as there are very few studies that have utilized this treatment protocol for peri-implantitis. Future research is necessary to substantiate the observations of this study and to further employ it clinically against peri-implantitis and to aid with better osseointegration.

LIMITATIONS

Although this in vitro study provides valuable insights into the antimicrobial efficacy of Nanosized Natural Zeolite and Antimicrobial photodynamic therapy, it may not be entirely possible to replicate these results in the complex in vivo environment. This study focuses solely on "*Aggregatibacter actinomycetemcomitans"*, which is only one of the many pathogenic species that can cause periodontal and per-implant infections. Therefore, the effectiveness of the treatments against other relevant bacterial species also needs to be assessed.

A limited timeframe for evaluating antimicrobial efficacy was employed here, thus, longitudinal studies are necessary to assess the sustainability of the antimicrobial effects of the used agents over time. The current investigation may not have explored all possible parameters for "Nanosized Natural Zeolite" coatings and Antimicrobial photodynamic therapy. Variations in coating thickness, light intensity, photosensitizer concentration, and other parameters can also influence treatment efficacy. Addressing these limitations in future studies can reinforce the validity and applicability of the observations seen in our study.

SUMMARY AND CONCLUSION

The present in vitro investigation was undertaken to assess and compare the effect of a combination of "Nanosized natural zeolite" and photodynamic therapy using Indocyanine green against "*Aggregatibacter actinomycetemcomitans"* biofilm on titanium discs.

200 pre-sterilized titanium discs of 8mm and thickness of 2mm were inoculated with a strain of "*A. actinomycetemcomitans"* and kept in an anaerobic chamber for 48 hours. The inoculated discs were randomly allocated into eight groups. Group 1: Positive control (2.5% NaOCl), Group 2: Negative control (Biofilm alone), Group 3: "Indocyanine Green" (ICG), Group 4: Nanosized Zeolite (NZ), Group 5: Nanosized Zeolite + Indocyanine Green, Group 6: Nanosized Zeolite + Diode Laser, Group 7: Antibacterial Photodynamic Therapy (aPDT) i.e (ICG+ Diode laser), Group 8: Nanosized Zeolite + aPDT. A 940nm Diode laser was used for aPDT.

Colony forming units were assessed for each group. The data was subjected to descriptive statistical analysis and normality was assessed using Kolmogrov Smirnov test. Intergroup comparisons were done by "One-way ANOVA". A pairwise comparison of groups was carried out using Tukey's post hoc test. All statistical tests were performed at a significance level of 5% ($p<0.05$).

In light of the observations made in the study it can be concluded that:

- When used alone Indocyanine green had a very minimal antimicrobial effect on the biofilm coated titanium discs.

- The individual antibacterial effect of Nanosized natural Zeolite on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans"* was noted to be substantial and statistically significant.

- The combination of Nanosized natural Zeolite and Indocyanine green (photosensitizer) showed better reduction in microbial load as compared to their individual use against a biofilm of "*Aggregatibacter actinomycetemcomitans".*

- The most significant antimicrobial efficacy was noted with the combination of Nanosized natural Zeolite and antimicrobial Photodynamic therapy (aPDT) (Indocyanine green + Diode laser) on titanium discs coated with a biofilm of "*Aggregatibacter actinomycetemcomitans."*

In conclusion, our investigation into the antimicrobial efficacy of titanium discs coated with Nanosized Natural Zeolite in combination with Antimicrobial Photodynamic therapy against "*Aggregatibacter actinomycetemcomitans"* yielded promising insights for combating peri-implantitis. The synergistic effect observed between "Nanosized Natural Zeolite" and aPDT emphasizes the potential of combining antimicrobial therapies to enhance the biocompatibility and longevity of dental implants. Our findings suggest that the "Nanosized Natural Zeolite" coating acts as a potent antimicrobial agent, facilitating prolonged antimicrobial activity. Moreover, aPDT offers a non-invasive and targeted approach to bacterial eradication, harnessing the oxidative power of light-activated photosensitizers to disrupt bacterial

membranes and cellular structures. Future studies in longitudinal study models, including animal studies and clinical trials need to be carried out to validate the observations seen in our study.

Within the limitations, our study highlights the potential of combined antimicrobial strategies involving Nanosized Natural Zeolite and Antimicrobial Photodynamic therapy in mitigating peri-implant infections. Advancing our understanding of the complex interplay between biomaterials, microbial pathogens, and antimicrobial therapies, we can pave the way for innovative approaches to improve the success rates and longevity of dental implants, ultimately benefiting patient outcomes and quality of life.

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ANNEXURES

ANNEXURE 1: ETHICAL CLEARANCE

Research and Ethics Committee
KLE VK INSTITUTE OF DENTAL SCIENCES
A Constituent Unit of KLE Academy of Higher Education & Research
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Sl. No.: 1568

CERTIFICATE

EC/NEW/INST/2021/2435
Research & Ethics Committee

This is to Certify that the synopsis titled

Evaluating the antimicrobial efficacy of titanium disks coated with Nanosized Natural Zeolite and Photodynamic therapy against Aggregatibacter actinomycetemcomitans

Submitted by

REG NO. IK0221001

P. G. Student / Staff, Guided by

from Department of

Periodontics

has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study

Date : 27/2/24

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belgaum

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belagavi

ANNEXURE 2: COLONY FORMING UNIT – RESULT

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA.
(Formerly known as KLE University) (Deemed to be University u/s 3 of the UGC Act, 1956)
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Report **Date: 22-02-2024**

Title of Research: "Evaluating the antimicrobial efficacy of titanium disks coated with Nanosized Natural Zeolite and Photodynamic Therapy against *Aggregatibacter actinomycetemcomitans* - An *in-vitro* study"

Student Name: **REG NO. IK0221001**

Guide:

Co-Guide:

Objective Parameters:

1. To assess the effect of Indocyanine green (photosensitizer) on titanium disks coated with a biofilm of *Aggregatibacter Actinomycetemcomitans.*
2. To assess the effect of Nanosized natural Zeolite on titanium disks coated with a biofilm of *Aggregatibacter Actinomycetemcomitans.*
3. To assess the effect of a combination of Nanosized natural Zeolite and Indocyanine green (photosensitizer) on titanium disks coated with a biofilm of *Aggregatibacter Actinomycetemcomitans.*
4. To assess the effect of combination of Nanosized natural Zeolite and antimicrobial Photodynamic therapy (aPDT) (Indocyanine green + Diode laser) on titanium disks coated with a biofilm of *Aggregatibacter Actinomycetemcomitans.*
5. To compare the effect of Nanosized natural Zeolite, Indocyanine green alone and with a combination of Nanosized natural Zeolite and antimicrobial Photodynamic therapy (Indocyanine green + Diode laser) on titanium disks coated with a biofilm of *Aggregatibacter Actinomycetemcomitans.*

Experimental methodology

Pre sterilized SLA treated Titanium disks of diameter 8 mm and thickness of 2 mm were used. *Aggregatibacter Actinomycetemcomitans* were coated on titanium disks. Biofilm formation on titanium disks were assessed after 24 hours. Incubated Titanium disks were placed individually in microtiter well plates. The disks were then subjected to the various test groups.

Group 1: Positive control (2.5% NaOCl)

Group 2: Negative control (Biofilm alone)

Group 3: Indocyanine Green (ICG)

Group 4: Nanosized Zeolite (NZ)

Group 5: Nanosized Zeolite + Indocyanine Green

Group 6: Nanosized Zeolite + Diode Laser

Group 7: Antibacterial Photodynamic Therapy (aPDT) i.e (ICG+ Diode laser)

Group 8: Nanosized Zeolite + aPDT

After 48 hours the colony forming units were assessed by plate count method.

RESULT:

Colony forming units were assessed and counted after test procedures.

COLONY FORMING UNIT – CFU / ml								
S.no	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**
1	0	145×10^4	120×10^4	60×10^4	35×10^4	86×10^4	28×10^4	20×10^4
2	0	159×10^4	122×10^4	48×10^4	36×10^4	74×10^4	26×10^4	20×10^4
3	0	161×10^4	121×10^4	56×10^4	40×10^4	80×10^4	25×10^4	18×10^4
4	0	160×10^4	118×10^4	62×10^4	28×10^4	86×10^4	26×10^4	15×10^4
5	0	148×10^4	120×10^4	52×10^4	35×10^4	74×10^4	30×10^4	20×10^4
6	0	145×10^4	116×10^4	48×10^4	30×10^4	68×10^4	28×10^4	14×10^4
7	0	156×10^4	123×10^4	52×10^4	32×10^4	85×10^4	26×10^4	16×10^4
8	0	148×10^4	118×10^4	60×10^4	30×10^4	80×10^4	25×10^4	20×10^4
9	0	174×10^4	124×10^4	48×10^4	35×10^4	74×10^4	25×10^4	15×10^4

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GSTIN/UIN: 29AABTK0881E1ZN

10	0	168 × 10^4	121 × 10^4	56 × 10^4	38 × 10^4	80 × 10^4	26 × 10^4	18 × 10^4
11	0	178 × 10^4	120 × 10^4	62 × 10^4	32 × 10^4	86 × 10^4	28 × 10^4	16 × 10^4
12	0	155 × 10^4	115 × 10^4	52 × 10^4	36 × 10^4	74 × 10^4	30 × 10^4	15 × 10^4
13	0	145 × 10^4	122 × 10^4	48 × 10^4	40 × 10^4	68 × 10^4	28 × 10^4	20 × 10^4
14	0	143 × 10^4	118 × 10^4	52 × 10^4	32 × 10^4	70 × 10^4	26 × 10^4	20 × 10^4
15	0	165 × 10^4	120 × 10^4	62 × 10^4	40 × 10^4	78 × 10^4	25 × 10^4	16 × 10^4
16	0	145 × 10^4	117 × 10^4	48 × 10^4	35 × 10^4	74 × 10^4	26× 10^4	20 × 10^4
17	0	164 × 10^4	122 × 10^4	56 × 10^4	38 × 10^4	80 × 10^4	28 × 10^4	16 × 10^4
18	0	173 × 10^4	119× 10^4	52 × 10^4	32 × 10^4	76 × 10^4	25 × 10^4	16 × 10^4
19	0	156 × 10^4	122 × 10^4	60 × 10^4	35 × 10^4	84 × 10^4	25 × 10^4	20 × 10^4
20	0	166 × 10^4	120 × 10^4	48 × 10^4	28 × 10^4	68 × 10^4	26 × 10^4	17 × 10^4
21	0	172 × 10^4	121 × 10^4	52 × 10^4	32 × 10^4	78 × 10^4	30 × 10^4	20 × 10^4
22	0	147 × 10^4	116× 10^4	48 × 10^4	30 × 10^4	70 × 10^4	25 × 10^4	20 × 10^4
23	0	158 × 10^4	120 × 10^4	62 × 10^4	36 × 10^4	70 × 10^4	28 × 10^4	16 × 10^4
24	0	145 × 10^4	123 × 10^4	52 × 10^4	38 × 10^4	74 × 10^4	27 × 10^4	15 × 10^4
25	0	148 × 10^4	122 × 10^4	48 × 10^4	32 × 10^4	80 × 10^4	26 × 10^4	20 × 10^4

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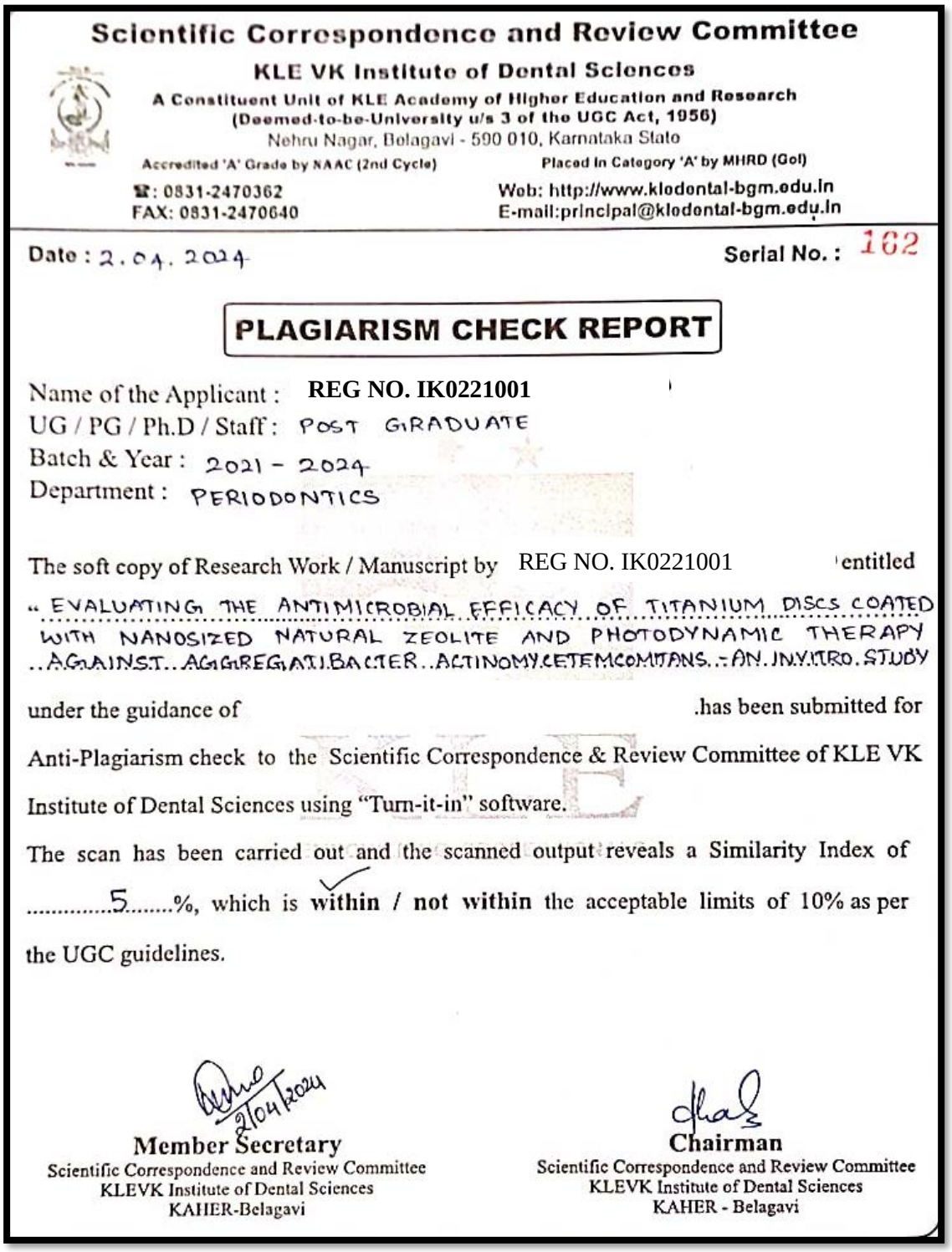
KLE Academy of Higher Education and Research, Belagavi.

ANNEXURE 3: PLAGIARISM CERTIFICATE



Scientific Correspondence and Review Committee

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Date : 2.04.2024 Serial No. : 162

PLAGIARISM CHECK REPORT

Name of the Applicant : **REG NO. IK0221001**
UG / PG / Ph.D / Staff : POST GRADUATE
Batch & Year : 2021 - 2024
Department : PERIODONTICS

The soft copy of Research Work / Manuscript by REG NO. IK0221001 entitled

" EVALUATING THE ANTIMICROBIAL EFFICACY OF TITANIUM DISCS COATED WITH NANOSIZED NATURAL ZEOLITE AND PHOTODYNAMIC THERAPY AGAINST AGGREGATIBACTER ACTINOMYCETEMCOMITANS - AN INVITRO STUDY

under the guidance of .has been submitted for

Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

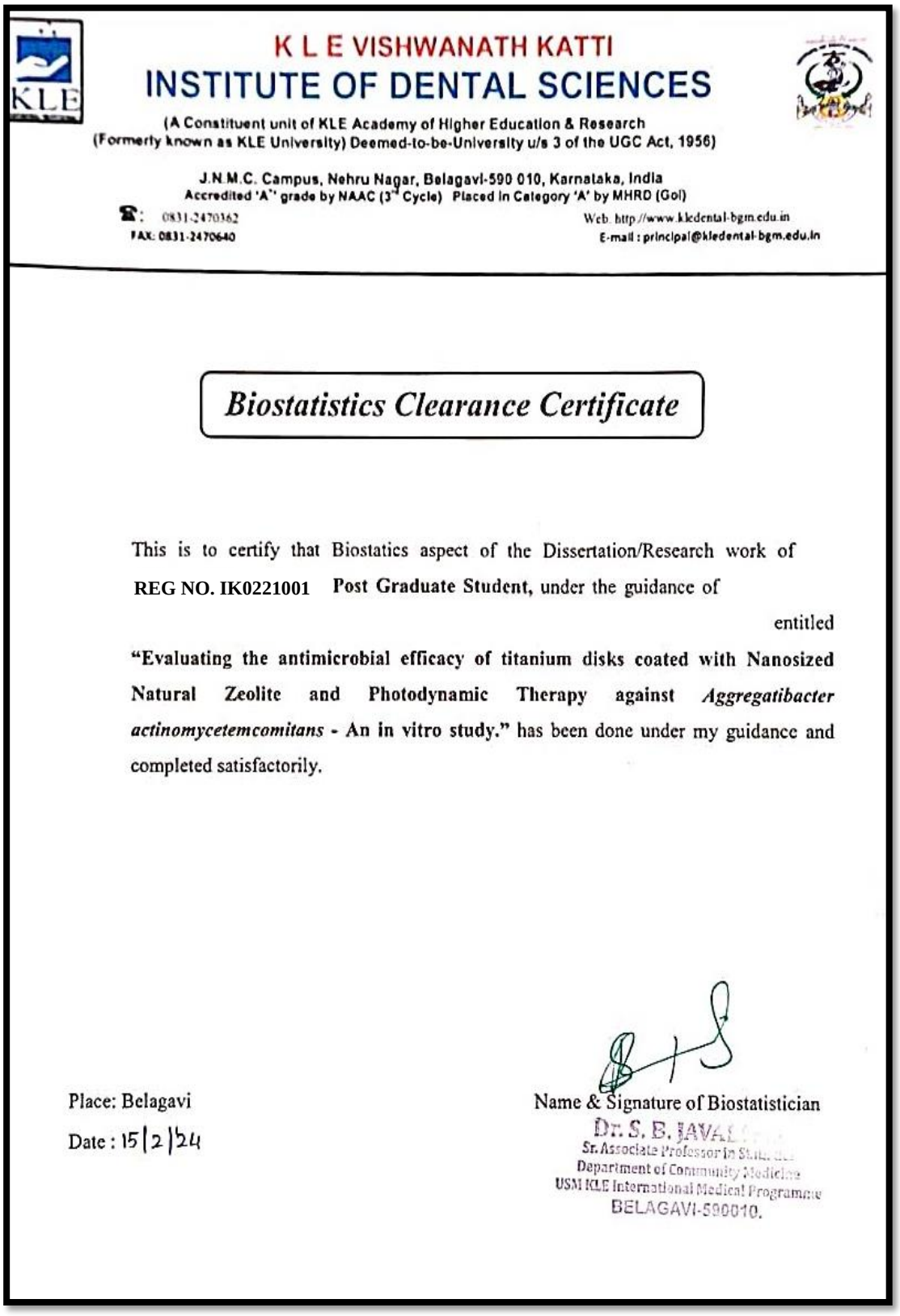
The scan has been carried out and the scanned output reveals a Similarity Index of .....5.....%, which is **within / not within** the acceptable limits of 10% as per the UGC guidelines.

2/04/2024

Member Secretary
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER-Belagavi

Chairman
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER - Belagavi

ANNEXURE 4: BIOSTATISTICIAN CERTIFICATE



K L E VISHWANATH KATTI
INSTITUTE OF DENTAL SCIENCES

(A Constituent unit of KLE Academy of Higher Education & Research
(Formerly known as KLE University) Deemed-to-be-University u/s 3 of the UGC Act, 1956)

J.N.M.C. Campus, Nehru Nagar, Belagavi-590 010, Karnataka, India
Accredited 'A++' grade by NAAC (3rd Cycle) Placed in Category 'A' by MHRD (GoI)

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Biostatistics Clearance Certificate

This is to certify that Biostatics aspect of the Dissertation/Research work of **REG NO. IK0221001** **Post Graduate Student,** under the guidance of

entitled

"Evaluating the antimicrobial efficacy of titanium disks coated with Nanosized Natural Zeolite and Photodynamic Therapy against *Aggregatibacter actinomycetemcomitans* - An in vitro study." has been done under my guidance and completed satisfactorily.

Place: Belagavi
Date : 15|2|24

Name & Signature of Biostatistician
Dr. S. B. JAVALI
Sr. Associate Professor in Statistics
Department of Community Medicine
USM KLE International Medical Programme
BELAGAVI-590010.

ANNEXURE 5: WAIVER FORM

Waiver form

Department of Periodontics
KAHER'S V.K Institute of Dental Sciences, Nehru Nagar, Belagavi.

EVALUATING THE ANTIMICROBIAL EFFICACY OF TITANIUM DISKS COATED WITH NANOSIZED NATURAL ZEOLITE AND PHOTODYNAMIC THERAPY AGAINST *Aggregatibacter Actinomycetemcomitans* - AN IN VITRO STUDY

Waiver of informed consent form

Standard bacterial strains from BSRC will be used in this study. It is not feasible to obtain informed consent from the donors of these strains. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/publication arising from the study.

REG NO. IK0221001

DEPARTMENT OF PERIODONTICS
KAHER'S KLE VK INSTITUTE OF
DENTAL SCIENCES,
BELAGAVI - 590010