EVALUATION OF ANTI-BACTERIAL EFFICACY OF ZIRCONIUM OXIDE NANOPARTICLES (ZrO_2 NPs) AGAINST *STREPTOCOCCUS MUTANS* AND *ENTEROCOCCUS FAECALIS*– AN IN VITRO STUDY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

MAY 2020

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF ANTI-BACTERIAL EFFICACY OF ZIRCONIUM OXIDE NANOPARTICLES (ZrO_2 NPs) AGAINST *STREPTOCOCCUS MUTANS* AND *ENTEROCOCCUS FAECALIS*– AN IN VITRO STUDY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. R. ANIL KUMAR, M.D.S.,** Professor, Head of the Department, Department of Conservative Dentistry and Endodontics, Ragas Dental College and Hospital, Chennai.

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This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVATIONS

S.NO	ABBREVATIONS	
1.	ZrO_2 NPs	Zirconium Oxide Nanoparticles
2.	S. mutans	Streptococcus mutans
3.	MS	Mutans Streptococci
4.	E. faecalis	Enterococcus faecalis
5.	AEP	Acquired Enamel Pellicle
6.	ATCC	American Type Culture Collection
7.	MHA	Muller Hilton Agar
8.	CAMHB	Cation adjusted Mueller–Hinton broth
9.	BHIB	Brain Heart Infusion Broth
10.	SEM	Scanning Electron Microscopy
11.	GTF	Glucosyltransferase
12.	EDAX	Energy – Dispersive x- ray spectroscopy
13.	AST	Anti-microbial Susceptability Testing
14.	MIC	Minimum Inhibitory Concentration
15.	MBEC	Minimum Biofilm Eradication Concentration
16.	PBS	Phosphate Buffered Saline
17.	RPMI	Rosewell Park Memorial Institute media
18.	EPS	Extracellular Polymeric Substances

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Introduction

INTRODUCTION

Dental caries is the most common and widespread biofilm-dependent oral disease modulated by complex interactions between cariogenic bacteria and host factors. These cariogenic bacteria are present in aggregates and are both acidogenic and aciduric in nature. Their ability to tolerate acidic environment and produce acids like lactic acid results in destruction of tooth structure.[1] *Streptococcus mutans* plays a predominent role in the initiation of caries because of its ability to adhere to the enamel salivary pellicle and to other plaque bacteria causing an acidic environment. This results in the loss of mineralized tooth structures paving an avenue for penetration of bacteria into the pulp space.[2]

Microorganisms breach the enamel, invade dentin and overwhelm the immune response of pulp to establish in the root canal system from a nutritionally rich and diverse environment of the oral cavity. This infected and necrotic pulp offers a selective habitat for bacteria and their by-products along with degradation products of both the microorganisms and the pulpal tissue.[3]

Primary endodontic infections are polymicrobial in nature and are dominated by Gram-negative anaerobic rods. These microrganisms are sensitive to ecological changes caused by chemomechanical preparation and the local intracanal environment. But secondary infections are composed of only few bacterial species which are capable of surviving changing environments and also possess antimicrobial resisitance.[4,5]

One of the predominant species isolated from secondary root canal infections is *Enterococcus faecalis* which was formerly classified as a part of group D Streptococcus system. It is a gram- positive, facultative anaerobe with the prevalence ranging from 24 to 77%. *Enterococcus faecalis* is a normal human commensal that can adapt to nutrient-rich, oxygen-depleted, ecologically complex environments of the oral cavity, gastrointestinal tract, and vagina.[6]

The pathogenic role of *E. faecalis* in chronic endodontic treatment failures can be attributed to its ability to invade and colonize the dentinal tubules under stressful conditions like nutrient deficiency. They grow as chains of cells within the dentinal tubules and remain viable surviving chemo-mechanical instrumentation and intracanal medication, thereby re-infecting the obturated root canals. Resistance to several antimicrobial agents, intracanal disinfection procedures and enduring periods of starvation makes its prevalence in secondary infections nine time more likely than in primary endodontic infections.[6,7] Being a robust microrganism, *E. faecalis* was considered to be a model test organism in our study.

Endodontic treatment is aimed at eliminating or reducing the microbial population and also to prevent introduction of new microorganisms within the root canal system through antiseptic and aseptic means respectively.[3] Mechanical preparation of root canals along with irrigation with antibacterial solutions eliminates the bacteria and its by-products and also necrotic tissues which can serve as a substrate for bacterial regrowth. Inter-appointment

intracanal medicaments can also greatly reduce the number of residual microbes in the canal. Finally, a three- dimensional obturation with a biocompatible material followed by permanent coronal restoration prevents reinfection of the root canal.[8]

Failure ensues when the endodontic treatment falls short of the standard clinical principles resulting in post- endodontic apical periodontitis.[9] Myriad of factors like inadequate mechanical debridement, persistence of bacteria within the canal space, poor obturation quality and coronal leakage contributes to the failure of endodontic treatment.[10] Although procedural errors limits the control and prevention of intracanal endodontic infection they do not jeopardize the outcome of endodontic treatment unless a concomitant infection is present. Thus, the persistence of microbial infection in the root canal system and/or the periradicular area are considered the major causes associated with endodontic failures than non- microbial factors.[9,11]

A major challenge in root canal treatment is the inability of the current cleaning and shaping procedures to eliminate bacterial biofilms surviving within the anatomic complexities and uninstrumented portions of the root canal system.[12] The current level of evidence showed that rate of treatment failure ranged between 18%–26% for the past 4 to 5 decades despite the advancements in treatment strategies.[13] Because of the shortcomings of current antibiofilm strategies in root canal treatment, advanced disinfection strategies have to be developed and tested.

Researchers in the field of endodontics have focused their efforts in developing new nanomaterials against microorganisms that are resistant to conventional treatment protocols. Basic concept of nanotechnology has been utilized for various applications in dental sciences such as tooth sealants and as fillers to improve strength, luster and resist wear. Incorporation of antimicrobial nanoparticles in restorative composite materials improves the health of oral environment apart from preventing dental caries.[14]

Nanoparticles with their enhanced and unique physicochemical properties have led nanodentistry to possess the versatility required to uniquely overcome some of the most challenging impediments in preventing and treating dental diseases. The increased surface to volume ratio and increased number of atoms that are present near the surface compared with micro-/macrostructures are suggested to contribute to the distinctly different properties of nanomaterials.[12,15]

Among nanostructured materials, metal oxide nanoparticles and metal complexes are apparently being studied in the field of medicine because highly ionic metal oxides besides their wide variety of physical and chemical properties also possess antibacterial activity. The metal oxide nanoparticles such as Titanium oxide ($Ti0_2$) , Magnesium oxide (MgO) and Zinc oxide (ZnO) are reported to be superior antimicrobial agents that are safe, durable and heat resistant when compared to conventional organic antibacterial agents.[16]

Zirconium is a sturdy transition metal and the oxides of Zirconia are chemically inert, biocompatible and are extensively used in dentistry for their enhanced mechanical properties. But these zirconium dioxide ($Zr0_2$) commonly known as zirconia have not been studied much for their anti-microbial potency.

Previously the anti-bacterial efficacy of ZrO_2 NPs against few bacterial species namely *Bacillus subtilis*, *Staphylococcus aureus*, *E. coli* and *P. aeruginosa* and fungal strains namely *Candida albicans*, *Aspergillus niger*, *Botrytis cinerea* and Aspergillus species has been evaluated.[17,18,16]

Presently there are no studies that evaluated the antibacterial efficacy of Zirconium Oxide (ZrO_2) Nanoparticles against *Streptococcus mutans*, the primary organism responsible for initiation of caries and *Enterococcus faecalis*, a highly resistant endodontic pathogen.

So, the purpose of this study was to evaluate the Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis*.

Aim and Objectives

AIM AND OBJECTIVES

AIM:

To evaluate the anti-bacterial efficacy of Zirconium Oxide Nanoparticles (ZrO_2 NPs) against *Streptococcus mutans* and *Enterococcus faecalis.*

OBJECTIVES:

1. To determine the morphological and elemental characteristics of ZrO_2 NPs used in our study using Scanning Electron microscopy and Energy-Dispersive X-ray spectroscopy.
2. To determine the Anti-microbial susceptibility of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis* using Agar well diffusion method.
3. To determine the Minimum Inhibitory Concentration (MIC) of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis* using Broth Microdilution method.
4. To determine the ability of *Streptococcus mutans* and *Enterococcus faecalis* to form biofilm by Crystal violet assay.
5. To determine the Minimum Biofilm Eradication Concentration (MBEC) of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis*.

Review of Literature

REVIEW OF LITERATURE

J. K. Clarke (1924)[19] conducted an artificial caries experiment to investigate the presence of a specific group of microrganisms for initiation and progression of caries. The results showed that the most predominant organism isolated from the experiment was *S. mutans* followed by *B. acidophilus*. It was concluded from the study that *S. mutans* was capable of decalcifying the enamel to penetrate into dentin when the conditions in the mouth are to a certain extent approximated and *B. acidophilus* produces only a superficial decalcification of the enamel, which can be accounted for by the acidity of the medium**.**

Hamada et al. (1984)[20] reported that *Streptococcus mutans* possess the abilities to adhere to pellicle coated tooth surfaces to form acids. The two characteristics associated with the cariogenicity of this microorganism are its ability to synthesize insoluble glucan and glucosyltransferase from sucrose which is essential in the adherence process. Therefore, agents that would interfere with the adherence ability of *S. mutans* would be useful for controlling dental caries.

E. K. Siren **et al. (1997)**[21] summarized the relationship between bacteriological findings and clinical treatment procedures by investigating root canal treatment cases that were selected for bacteriological investigation by general dental practitioners in Finland. Two groups of teeth were selected

based on the type of infection present in the root canal system. The 'enteric bacteria' group consisted of 40 sequential cases where *Enterococcus faecalis* and/or other facultative enteric bacteria or *Pseudomonas* sp. were found in the samples in pure culture (35%) or together with other types of bacteria. The group 'non-enteric bacteria' consisted of 40 sequential cases where only non-enteric bacteria were found. Enteric bacteria were more frequently isolated in cases with a high number of appointments before sampling. In addition, the number of retreatment cases was significantly higher in the enteric bacteria group than in non-enteric bacteria. Thus, the results emphasized the importance of controlled asepsis throughout the root canal treatment.

Pinheiro et al. (2003)[9] conducted a study to identify the microbial flora with failed root-canal treatment. Sixty root filled teeth with persisting periapical lesions were selected. During nonsurgical endodontic re-treatment, the root filling material were removed and the canals were sampled. Microbial sampling, isolation and species determination were performed using advanced microbiological techniques for anaerobic species. The association of microbiological findings with clinical features was investigated. The results demonstrated that microrganisms were recovered from 51 teeth. In most cases, one or two strains per canal were found. Of the microbial species isolated, 57.4% were facultative anaerobic species and 83.3% were Gram-positive microorganisms. The most frequently recovered bacterial species from this study was *Enterococcus faecalis*.

Wu et al. (2006)[22] determined the presence and characteristics of the microbiota remaining within prepared root canal space using bacterial sampling. In histologic observations of root apices, bacteria have been found in inaccessible inter-canal isthmuses and accessory canals often in the form of biofilms. There is no in vivo evidence to support the assumption that these bacteria can be entombed effectively in the canal system by the root filling and thus be rendered harmless. As a consequence of this residual root infection, post-treatment apical periodontitis may persist or develop as a defence mechanism to prevent the systemic spread of bacteria and/or their by-products to other sites of the body. Histologic observation of root apices with surrounding bone removed from either patients or human cadavers has demonstrated that post-treatment apical periodontitis is associated with 50–90% of root filled human teeth. Thus, if the objective of root canal treatment is to eliminate apical periodontitis at a histological level, current treatment procedures are inadequate. The continued development of treatments that can effectively eliminate root infection is therefore a priority in clinical endodontic research.

***Gomes et al* (2006)**[23] investigated the presence of *Enterococcus faecalis* in endodontic infections by culture and polymerase chain reaction analysis. Microbial samples were obtained from 50 teeth with untreated necrotic pulps (primary infection) and from 50 teeth with failing endodontic treatment (secondary infection). Culture and PCR detected the test species in 23 of 100

and 79 of 100 of the teeth, respectively. *E. faecalis* was cultured from 2 (4%) of 50 necrotic canals and from 21 (42%) of 50 root-treated canals. PCR detection identified the target species in 41 (82%) and 38 (76%) of 50 primary and secondary infections respectively. It was concluded from the study that *E. faecalis* was detected as frequently in teeth with necrotic pulp as in teeth with failing endodontic treatment when a PCR analysis was used.

Stuart et al. (2006)[24] stated that *Enterococcus faecalis* is a microorganism commonly detected in asymptomatic, persistent endodontic infections. Its prevalence in such infections ranges from 24% to 77%. This finding can be explained by various survival and virulence factors possessed by *E. faecalis*, including its ability to compete with other microorganisms, invade dentinal tubules, and resist nutritional deprivation. Use of good aseptic technique, increased apical preparation sizes, and inclusion of 2% chlorhexidine in combination with sodium hypochlorite are currently the most effective methods to combat *E. faecalis* within the root canal systems of teeth.

Yudovin-Farber et al (2009)[25] tested Quaternary ammonium polyethyleneimine (QA-PEI)-based nanoparticles embedded in restorative composite resin at 1% w/w for antibacterial activity against *Streptococcus mutans* using direct contact test. Activity analysis revealed that the alkyl chain length of the QA-PEI nanoparticles plays a significant role in antibacterial activity of the reagent. The most potent compound was octyl-alkylated QA-PEI embedded in restorative composite resin at 1% w/w that totally inhibited

S. mutans growth in 3-monthaged samples. This data indicated that restorative composite resin with antibacterial properties can be produced by the incorporation of QA-PEI nanoparticles.

Jangra et al. (2012)[16] studied the antimicrobial activities of zirconia (ZrO_2) nanoparticles and zirconium mixed ligand complexes on bacterial strains of *E. coli, S. aureus* and fungal strain of *A. niger*. The antimicrobial studies revealed that zirconia exhibited activity only against *E. coli*, whereas, the Zr(IV) complexes exhibited activity against both gram - ve *E. coli* and gram +ve *S. aureus* as well as fungal strains. The Zr(IV) complexes were found to possess significant antifungal activity against *A. niger*. Based on this study, it could be speculated that the ZrO_2 nanoparticles with the same surface areas but with different shapes i.e., different active facets will show different antimicrobial activity.

Melo M.A.S. et al (2013)[1] discussed the current progress and future applications of functional nanoparticles incorporated in dental restorative materials as useful strategies in dental caries management through his review and also proposed antimicrobial and remineralizing mechanisms. Nanomaterials had a great potential to decrease biofilm accumulation, inhibit the demineralization process, to be used for remineralizing tooth structure, and to combat caries related bacteria. These results are encouraging and open the

doors to future clinical studies that will allow the therapeutic value of nanotechnology-based restorative materials to be established.

N. Chrzanowska et Al (2014)[26] compared the ecotoxicity of commercial nanoparticles of aluminium oxides and zirconium oxides, in relation to two bacterial strains *Pseudomonas putida* and *Aeromonas hydrophila*. These bacteria have an ability to form biofilms, as they are present in planktons and participate in wastewater treatment. The study also included the assessment of aluminium oxide (Al_2O_3) and zirconium oxides (ZrO_2) ecotoxicity in order to compare their harmfulness with the nanoparticular form. It has been found that aluminium and zirconium nano-oxides were more harmful to bacteria compared to aluminium and zirconium oxides. Biofilm-forming bacteria were more resistant than planktonic bacteria to the influence of both types of compounds. Aluminium nano-oxide proved to be more toxic than zirconium nano-oxide in relation to both species of bacteria. Nanoparticles appeared to be less toxic towards bacteria with Extra cellular Polysaccharides. *A.hydrophila* strain showed lower sensitivity than *P. putida* to the studied nanoparticles.

Wu et al. (2014)[27] evaluated the antibacterial efficacy of silver nanoparticles (Ag NPs) as an irrigant and medicament against *Enterococcus faecalis* biofilms formed on root dentin. The results showed that the biofilms treated with 0.02% Ag NP gel as medicament significantly disrupted the structural integrity of the biofilm and resulted in the least number of post-

treatment residual viable *E. faecalis* cells compared with 0.01% Ag NP gel and calcium hydroxide groups. Ag NPs as a medicament and not as an irrigant showed potential to eliminate residual bacterial biofilms during root canal disinfection and the findings from this study suggested that the antibiofilm efficacy of Ag NPs depends on the mode of application.

S. Gowri et al. (2014)[28] demonstrated that tetragonal spherical ZrO_2 NPs can be synthesized by biological material of Aloe vera extract. The antimicrobial and antifungal properties were investigated for the synthesised zirconia nanoparticles and the treated cotton by agar diffusion method against *Staphylococcus aureus* and *Escherichia coli* bacterial pathogens and fungal strains like *Candida albicans* and *Aspergillus niger*, respectively. The results showed that antifungal and antibacterial potential of ZrO_2 nanoparticles and ZrO_2 nanoparticles treated cotton exhibited enhanced activity against the test organisms.

Seneviratne CJ et al. (2014)[29] reported the synthesis of a novel mesoporous silica nanoparticle-encapsulated pure Chlorhexidine (Nano-CHX), and its antimicrobial properties against oral biofilms. The antimicrobial properties of Nano-CHX were evaluated in both planktonic and biofilm modes of representative oral pathogenic bacteria. The Nano-CHX demonstrated potent antibacterial effects on planktonic bacteria and mono-species biofilms at the concentrations of 50–200 mg/mL against *Streptococcus mutans*, *Streptococcus sobrinus*, *Fusobacterium nucleatum*, *Aggregatibacter*

actinomycetemcomitans and *Enterococccus faecalis*. Moreover, Nano-CHX effectively suppressed multi-species biofilms such as *S. mutans, F. nucleatum, A. actinomycetemcomitans* and *Porphyromonas gingivalis* up to 72 hours.

The study demonstrated the potent antibacterial effects of the Nano-CHX on oral biofilms, and it may be developed as a novel and promising anti-biofilm agent for clinical use.

Guerreiro-Tanomaru J M et al (2014)[30] evaluated the antibiofilm activity against *Enterococcus faecalis*, compressive strength and radiopacity of Portland cement (PC) added to zirconium oxide (ZrO_2), as radio opacifier, with or without nanoparticulated zinc oxide (ZnO). The following experimental materials were evaluated: PC, PC + ZrO_2; PC+ZrO_2+ZnO(5%), and PC+ZrO_2 + ZnO (10%). Antibiofilm activity was analysed by using direct contact test (DCT) on *Enterococcus faecalis* biofilm for 5 h and 15 h. The analysis was conducted by using the number of colony-forming units (CFU/mL). The results showed that all materials presented similar antibiofilm activity and the addition of nanoparticulated ZnO decreased the compressive strength of PC. It can also be concluded that the addition of ZrO_2 and ZnO does not interfere with the antibiofilm activity and provides radiopacity to Portland cement. However, the presence of ZnO (5% or 10%) significantly decreased the compressive strength of the materials.

Dianat et al. (2015)[31] compared the antimicrobial activity of Nanoparticle calcium hydroxide (NCH) and Calcium Hydroxide (CH) against *E. faecalis*. Antimicrobial activity of NCH against *E. faecalis* was evaluated by two independent tests: the minimum inhibitory concentration (MIC) of intracanal medicament and agar diffusion test (ADT). The efficiency of the medicament in dentinal tubules was evaluated on 23 human tooth blocks that were inoculated with *E. faecalis*. The tooth blocks were assigned to one control group (saline irrigation) and two experimental groups receiving CH and NCH as intracanal medication. The optical density in each group was assessed with spectrophotometer after collecting samples from dentin depths of 0, 200 and 400μm. The MIC for NCH was 1/4 of the MIC for CH. NCH with distilled water (DW) produced the greatest inhibition zone in agar diffusion test. NCH had greater antimicrobial activity in dentin samples from depths of 200 and 400μm compared to CH. The study revealed that the antimicrobial activity of NCH was superior to CH in culture medium. In dentinal tubules the efficacy of NCH was again better than CH on the 200- and 400-μm samples.

Burns J et at (2015)[32] conducted a Randomised controlled trial, double blind, in a community setting where school children with active caries in primary teeth and no pulpal exposure, fistula or decay in permanent teeth were chosen. Two drops of Nano Silver Fluoride (NSF) or one drop of water were applied to the tooth with a micro brush for two minutes, once in a 12-month period. At one week, five months and 12 months the presence of active caries,

as classified using International Caries Detection and Assessment System (ICDAS II) criteria, was measured. The results concluded that annual application of NSF solution was more effective in hardening and arresting dentine caries in primary teeth than the placebo.

Thakare *et al*. (2016)[33] evaluated the structural and biological properties of nanocrystalline tetragonal zirconia (ZrO_2) synthesized using simple sol–gel method. The sample was characterized by X-ray powder diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), Transmission Electron Microscopy (TEM) and evaluated for cell growth study using 3T3 mouse fibroblast cells and for degradation using Phosphate Buffered Saline (PBS) solution. The synthesized materials were also evaluated for their antibacterial activity against *Escherichia coli* and S*taphylococcus aureus* bacterial strains. The sample of zirconia has excellent tissue biocompatibility, higher cell growth and does not show the toxicity towards normal 3T3 mouse fibroblast cells. The result of qualitative antibacterial tests revealed that the nanocrystalline zirconia had an important inhibitory activity on *E. coli* and *S. aureus.* The sample showed stability at the physiological condition and does not show degradation. Hence, it can be used for various biomedical applications.

Abdullah R M et al. (2016)[34] studied the antibacterial activity of Zirconium Oxide nanoparticles and the combination of Zirconium oxide nanoparticles and Cefotaxime against few isolates. ZrO_2 nanoparticles showed

Zone of inhibition of 37mm for *Staphylococcus epidermidis*, 10mm for *Staphylococcus aureus* and 8mm for *Klebsiella* species. Cefotaxime showed effectivity on the isolates with the zone of inhibition for *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Klebsiella* species of 40, 9, 8mm respectively. Combination of Cefotaxime and Zirconium oxide nanoparticles showed synergistic effect against *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Klebsiella* species with zone of inhibition 43, 11 and 10mm respectively.

Gonzalez-Luna et al. (2016)[35] determined the bactericidal effect of silver nanoparticles as a final irrigation agent in endodontics. This study included 120 single rooted teeth inoculated with *Enterococcus faecalis* (*E. faecalis*) and organized into 4 groups: (A) 30 teeth irrigated with a dispersion of silver nanoparticles (537 μg/mL); (B) 30 teeth irrigated with a sodium hypochlorite solution (2.25%); (C) 30 teeth irrigated with a dispersion of silver nanoparticles (537 μg/mL) + EDTA (17%); and (D) 30 teeth with a saline solution. After the irrigation protocol, the samples were analysed through a spectrophotometer to measure the bactericidal effect and scanning electron microscope and atomic force microscope in order to observe the presence of dental smear layer. The results showed that nanoparticles of 10nm and the sodium hypochlorite at 2.25% were effective for eliminating *E. faecalis*, with no significant difference between them.

Shrestha and Kishen (2016)[12] presented a comprehensive review on the scientific knowledge that is available on the application of antibacterial nanoparticles in endodontics. The application of nanoparticles in the form of solutions for irrigation, medicament, and as an additive within sealers/restorative materials has been evaluated to primarily improve the antibiofilm efficacy in root canal and restorative treatments. In addition, antibiotic or photosensitizer functionalized nanoparticles have been proposed recently to provide more potent antibacterial efficacy. They concluded that the increasing interest in this field warrants sound research based on scientific and clinical collaborations to emphasize the near future potential of nanoparticles in clinical endodontics.

Singla D et al.(2016)[36] detected the presence of *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque by using Polymerase Chain Reaction (PCR) method, quantification of these micro-organisms using Modified Sucrose-Bacitracin (SB-20M) agar medium and to correlate their presence in Caries Active (CA) and Caries Free (CF) pre-school children. Sixty-eight pre-school children, in the age group of 3-5 years were divided equally into 34 CA and 34 CF children. Dental plaque samples were obtained for detection of these microorganisms by PCR method and quantification was done using SB-20M culture medium. The results showed that *S. sobrinus* was significantly higher in CA group as compared to CF group whereas *S. mutans* showed no significant difference. On quantification of these micro-organisms, *S. sobrinus*

was present in significantly higher numbers in CA group as compared to CF group. On correlating the CFU/ml of the micro-organisms with the DMFT index, both the micro-organisms showed a positive correlation.

Fathima J B et al. (2017)[37] determined the antimicrobial activity and anti-tooth decay applications of the synthesized NPs of ZrO_2. The antimicrobial activity of different concentrations of the synthesized ZrO_2 NPs was examined against gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), respectively. The synthesized ZrO_2 NPs displayed a better inhibitory action against *Pseudomonas aeruginosa* (inhibition zone size of 20mm) at the concentration of 100µg/ml compared to other bacteria due to the negatively charged *P.aeruginosa* cell wall readily attracting positively charged ZrO_2 NPs and thereby inhibiting microbial actions. Moreover, the concentration of ZrO_2 NPs was directly proportional to their inhibitory actions against the tested microorganisms. Finally, the preventive role of ZrO_2 NPs in a tooth decay pathway has been elucidated in this study.

Gad et al (2017)[38] investigated the effect of nano-ZrO_2 addition on the translucency and tensile strength of the PMMA denture base material. The tensile strength increased significantly after nano-ZrO_2 addition and the maximum increase seen was in the 7.5% NZ group. The translucency values of the experimental groups were significantly lower than those of the control group. Within the reinforced groups, the 2.5%NZ group had significantly

higher translucency values when compared to the 5%NZ and 7.5%NZ group. Thus, the addition of nano-ZrO_2 increased the tensile strength of the denture base acrylic. The increase was directly proportional to the nano-ZrO_2 concentration. The translucency of the PMMA was reduced as the nano-ZrO_2 increased.

Rajasekar S. et al. (2017)[39] determined the efficacy of ZrO_2 as a promising antibacterial agent against cancer causing *Pseudomonas aeruginosa.* A total of 80 isolates of Pseudomonas spp. were isolated from blood cancer patient from three tertiary hospitals. The biofilm and Metallo beta-lactamase production was tested by combined disc test and tissue culture plate method. ZrO_2 nanoparticle were obtained and tested against 10 Metallo beta-lactamase and biofilm producing carcinogenic isolates. The results showed that ZrO_2 nanoparticles showed appreciable activity at all tested concentrations (0.2, 0.4 and 0.6 mg/ml).

Nina DOSKOCZ et Al (2017)[40] studied the impact of zirconium oxide nanoparticles on the growth of *Pseudomonas Putida* and also compared the harmfulness of different forms of zirconium oxide. Research and literature data showed that presence of the nanoparticles can negatively influence the microorganisms. The EC_{50}-16h determined in this study was 27.2 mg/l, and NOEC was 0.39 mg/l. Nano-ZrO_2 proved to be more toxic than zirconium oxide to *P. putida*. This indicates that the nano form of a given substance displays different properties and may constitute a far greater danger to the

environment than the same substance in its large form. Death of bacteria caused by nanoparticles may affect the normal biological, chemical and nutrient cycle in the ecosystem, and nanoparticles can also have detrimental impact on other organisms.

Soekanto et al. (2017)[41] investigated the potency of propolis fluoride (PPF) and nano silver fluoride (NSF) as fluoride-based varnishes for inhibiting *Streptococcus mutans* and *Enterococcus faecalis* biofilm formation. In this study, both varnishes were compared to silver diamine fluoride (SDF) varnish, the gold standard for anticariogenic agents. The suspensions of *S. mutans* and *E. faecalis* were cultured and then plated into 96 - well plates and combined with SDF (38%), NSF (3.16, 3.66, and 4.16%), or PPF (3, 6, and 10%). *E. faecalis* was incubated in an anaerobic environment for 24 h, and the same protocol was used for *S. mutans*. The amount of biofilm inhibition was evaluated by optical density measurements at 570 nm using a microplate reader. The minimum inhibitory concentration (MIC) of PPF for *S. mutans* was 3%, and minimum bactericidal concentration (MBC) was 10%. The MIC of PPF for *E. faecalis* was at 6%, but no MBC was established. The MIC of NSF for *S. mutans* was 3.16%, and the MBC was 4.16%. The MIC of NSF for *E. faecalis* was 3.16%, while the MBC was 4.16%. Biofilm formation was inhibited dose-dependently by both NSF and PPF. They also showed clear antibacterial effects that are comparable to those obtained with SDF fluoride-based varnish.

Ram kumar P et al (2017)[42] synthesized MgO-ZrO_2 nanoparticles by wet chemical method by mixing the solutions of magnesium sulphate (0.45 M) and zirconium oxychloride (0.25M) in aqueous sodium hydroxide and refluxed at elevated temperature. The prepared mixed oxide nanoparticles were characterized by FT-IR, XRD, SEM, EDAX, DSC and CV studies. Antimicrobial activity of the synthesized mixed oxide was also investigated. The maximum inhibition zone 12 mm was observed in E.coli when compared with other bacterial species. The result showed that all the bacterial species used for the present study were moderately sensitive towards MgO-ZrO_2 when compared with standard tetracycline.

Zhou Z et al (2017)[43] described the pH-positive, doxycycline (DOX)-loaded nanocarriers to combat pathogenic biofilms. The mixed shell-core nanoparticles consisted of quaternary ammonium chitosan (TMC) as section of positive charged, which targeted nanoparticles to negatively charged biofilm surfaces. And liposome was used as DOX loading tool to eradicate multidrug-resistant biofilm. In drug release test, DOX release was pH-dependent with t1/2 = 0.75 h and 2.3 h for release at pH 4.5 and 6.8. Furthermore, TMC-Lip-DOX NPs could adhere biofilm and removal of biofilm from the hydroxyapatite (HA) surface, efficiently. This constituted a highly effective pathway to control oral plaque biofilms and has a good potential use for dental biofilm therapies

Ye and Shi (2018)[44] investigated the toxicological profile of ZrO_2 NPs. In this study, TiO_2 NPs were served as control to evaluate the biocompatibility of ZrO2 NPs. The cytotoxicity of TiO_2 and ZrO_2 NPs in osteoblast-like 3T3-E1 cells were detected and it was also found that reactive oxygen species (ROS) played a crucial role in the TiO_2 and ZrO_2 NP-induced cytotoxicity with concentration-dependent manner. TiO2 and ZrO2 NPs could induce apoptosis and morphology changes after culturing with 3T3-E1 cells at high concentrations. Moreover, TiO_2 and ZrO_2 NPs at high concentrations could inhibit cell osteogenic differentiation, compared to those at low concentrations. The author concluded that TiO_2 and ZrO_2 NPs could induce cytotoxic responses in vitro in a concentration-dependent manner, which may also affect osteogenesis; ZrO_2 NPs showed more potent toxic effects than TiO_2 NPs.

Rostinawati T et al (2018)[45] tested the efficacy of different tooth pastes against caries producing bacteria because the effectiveness of toothpaste in inhibiting the growth of bacteria that causes dental caries will also be influential in the prevention of dental caries. The bacteria that causes dental caries from clinical isolates was identified with experimental observations methods through the phenotype and genotype approach using Polymerase Chain Reaction (PCR) 16s rRNA. The phenotype observation through the observation of the colony and microscopic morphology (Gram stain) revealed that the bacteria had a rod shape and was a Gram-positive bacteria. The results

of DNA sequence of 16S rRNA fragments homology with the DNA sequence of 16S rRNA of the BLAST database showed that the bacteria that cause dental caries had the highest similarity with *Bacillus licheniformis* and the test for the activity of toothpastes showed that the toothpastes were active against this bacteria.

Gad et al (2018)[18] evaluated the effect of zirconia nanoparticles added to cold-cured acrylic resin on *Candida albicans* adhesion. A total of 120 acrylic resin specimens with dimensions measuring 22×10×2.5 mm^3 were prepared and divided into two equal groups. One group (repair) comprised heat-polymerized specimens that were sectioned at the centre and prepared to create a 2 mm repair area that was repaired with cold-cured resin reinforced with 0%wt, 2.5%wt, 5%wt, and 7.5%wt zirconia nanoparticles. The second group contained intact cold-cured acrylic resin specimens reinforced with 0% wt, 2.5% wt, 5% wt, and 7.5% wt zirconia nanoparticles. Specimens were incubated at 37°C in artificial saliva containing *C. albicans*, and the effect of zirconia nanoparticles on *C. albicans* was assessed using two methods: 1) a slide count method and 2) a direct culture test. Variations in the number of living *Candida* were observed in relation to the different concentrations of zirconia nanoparticles. It was found that *C. albicans* adhesion to repaired specimens was significantly decreased by the addition of zirconia nanoparticles in comparison with the control group. Intact cold-cured groups and groups repaired with cold-cured resin reinforced with 7.5%wt zirconia

nanoparticles showed the lowest *Candida* count. It was concluded from the study that addition of zirconia nanoparticles to cold-cured acrylic resin is an effective method for reducing *Candida* adhesion to repaired polymethyl methacrylate (PMMA) denture bases and cold-cured removable prosthesis.

Fan *et al.* (2018)[46] investigated the co-work pattern and optimum ratio between Ag+ and Zn2+ in their synergistic antibacterial activity against *E. faecalis*, the possible mechanisms behind this synergy and the primary application of optimum Ag^{+}–Zn^{2}+ co-work pattern against the *E. faecalis* biofilm on dentin. A serial of Ag^{+}–Zn^{2+} atomic combination ratios were tested on both planktonic and biofilm-resident *E. faecalis* on dentin, their antibacterial efficiency was calculated and optimum ratio determined. And the cytotoxicity of various Ag^{+}–Zn^{2+} atomic ratios was tested on MC3T3-E1 Cells. The role of Zn^{2+}in Ag^{+}–Zn^{2+} co-work was evaluated using a Zn^{2+} Pretreatment study and membrane potential- permeability measurement. The results showed that the synergistically promoted antibacterial effect of Ag^{+}–Zn^{2+} combinations was Zn^{2+} amount-dependent with the 1:9 and 1:12 Ag^{+}–Zn^{2+} atomic ratios showing the most powerful ability against both planktonic and biofilm-resident *E. faecalis.* This co-work could likely be attributed to the depolarization of *E. faecalis* cell membrane by the addition of Zn^{2+}. The cytotoxicity of the Ag^{+}– Zn^{2+} atomic ratios of 1:9 and 1:12 was much lower than 2% chlorhexidine. Thus it was concluded that Ag^{+}–Zn^{2+} atomic ratios of 1:9 and 1:12 demonstrated similar strong ability against *E. faecalis* biofilm on

dentin but much lower cytotoxicity than 2% chlorhexidine. New medications containing optimum Ag^{+}–Zn^{2+} atomic ratios higher than 1:6, such as 1:9 or 1:12, could be developed against *E. faecalis* infection in root canals of teeth or any other parts of human body.

Teixeira J A et al. (2018)[47] evaluated the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), anti-adherence, anti-acid, enamel microhardness, and OCT of an experimental dentifrice containing nano-silver fluoride (NSF) and a sodium fluoride (NaF) toothpaste *in vitro* against *S. mutans*. The microdilution technique was used to determine the MIC and MBC.

Fragments of deciduous enamel were treated with dentifrice slurries, containing bacterial suspension and PBS-treated saliva. The quantification of the microorganisms that adhered to the enamel was determined after 24 hours of incubation, and media pH readings were performed after 2 hours and 24 hours. Deciduous teeth were evaluated for microhardness and OCT during 14 days of pH cycling. Dentifrices containing NSF presented a lower MIC and higher statistically significant results compared to NaF dentifrices with respect to preventing bacterial adhesion and pH decreases. NSF and NaF dentifrices showed the same ability to avoid enamel demineralization corroborated by the OCT images. The NSF formulation had a better antibacterial effect compared to NaF dentifrices and similar action on the demineralization of enamel indicating their potential effectiveness to prevent caries.

Ahmed *et al*. (2019)[48] analyzed the antibacterial efficacy of nanosilver, chitosan, and fluoride as an ingredient in the dentifrices against *S. mutans* strains and comparing them with each other. The antimicrobial activity of the three Toothpastes (Nanosilver (Group 1), chitosan (Group 2) containing toothpastes and fluoride (Group 3) toothpaste) were determined by modified agar well diffusion method. Saline was kept as the control. The results showed that Nanosilver-containing toothpaste had the highest antibacterial efficacy against *S. mutans*, followed by fluoride- and chitosan-containing toothpaste.

Materials and Methods

MATERIALS AND METHODS

The Study was done in the Department of Biotechnology, University of Madras, Guindy Campus, Chennai.

- Zirconium Oxide Nanoparticles (ZrO_2) was obtained from Nano Research Lab **(Figure 1)**
- Standard ATCC strains of *Enterococcus faecalis* (ATCC *29212*) and *Streptococcus mutans* (ATCC 25175) were procured from Sigma Aldrich and sub-cultured onto Brain Heart Infusion Agar (BHIA) plate at 37 ^{0}c for 24 hours.

ARMAMENTARIUM

- Brain Heart Infusion Agar - **Figure 2**
- Petri Dish - **Figure 3**
- Microtitre plates- 96 well - **Figure 4**
- Incubator - **Figure 5**
- Spectrophotometer - **Figure 6**
- Freezer - **Figure 7**
- Micro pipettes (mL)-20, 20-200, 100-1000 microlitre - **Figure 8**
- Laminar air flow chamber - **Figure 9**
- Scanning Electron Microscope - **Figure 10**

METHODOLOGY

A. CHARACTERIZATION OF ZIRCONIUM OXIDE NANOPARTICLES:

i) SCANNING ELECTRON MICROSCOPY

ii) ENERGY – DISPERSIVE X- RAY SPECTROSCOPY

B. ANTI – MICROBIAL SUSCEPTABILITY TESTING OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

C. MINIMUM INHIBITORY CONCENTRATION OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

D. DETERMINATION OF GROWTH CONDITIONS AND BIOFILM QUANTIFICATION OF *E. FEACALIS* AND *S. MUTANS* BY CRYSTAL VIOLET STAINING ASSAY

E. MINIMUM BIOFILM ERADICATION CONCENTRATIONS OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

A. CHARACTERIZATION OF ZIRCONIUM OXIDE NANOPARTICLES:

Morphological analysis of Zirconium Oxide Nanoparticles (ZrO_2 NPs) was done using High Resolution Scanning Electron Microscopy (FEI- Quanta FEG 200F, SAIF, IIT Madras) (**Figure 10**) and elemental analysis was done using Energy Dispersive X-ray spectroscopy (SAIF, IIT Madras).

B. ANTI – MICROBIAL SUSCEPTABILITY TESTING OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

The antimicrobial activity of the Zirconium Oxide nanoparticles were examined against *E. faecalis* (ATCC 29212) and S. *mutans* (ATCC 25175) bacterial strains by Agar well diffusion method. The test cultures were inoculated into nutrient broth and incubated at 37^0C for 24 hours and then swabbed onto Muller Hinton agar plates using sterile swabs **(Figure 13)**. Stock solutions of ZrO_2 NPs with a concentration of 100mg/ml were prepared in deionized water. Since nanoparticles form aggregates, the stock dispersion was sonicated (0.4 kW, 20 kHz) for 30 min to break aggregates and then it was diluted to the exposure concentrations of 100mg/ml, 75mg/ml, 50mg/ml and 25mg/ml using Micro pipettes (**Figure 8**). These dilutions were then added to the agar wells. Antibiotic disc of Gentamycin

was used as positive control. The plates were incubated at 37^0C and observed for zones for inhibition **(Figure 16).**

C. MINIMUM INHIBITORY CONCENTRATION (MIC) OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

MIC is defined as the lowest concentration of the antimicrobial agent that revents visible growth of a microorganism under defined conditions. MIC was determined by M100 broth microdilution method as per the CLSI guidelines 2017.

INOCULUM PREPARATION: ATCC 29212 (*E. faecalis*) and ATCC 25175 (*S. mutans)* were obtained from Sigma-Aldrich and are isolated in pure culture. The strains were sub-cultured onto Brain Heart Infusion agar and incubated at 37°C overnight. The inoculum was prepared by suspension of 2 or more identical colonies in 5mL of sterile saline. The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at 530 nm to yield a stock suspension of 1 x 10^6 to 5 x 10^6 cells/mL. A working suspension is made by 1:100 dilution of the stock suspension

with Cation adjusted Mueller–Hinton broth (CAMHB) medium, which results in 5.0 x 10^2 to 2.5 x 10^3 cells/mL.

PROCEDURE: Dilutions of ZrO_2 NPs was done till well- 10. 100µL of the inoculum suspension in CAMHB were added to the series of wells containing ZrO_2 dilutions. Well 11 was used as a positive media control with only culture media and bacterial strain and Well 12 was used as negative growth control with only media **(Figure 15)**. The MIC values were read at 490nm spectrophotometrically (**Figure 6**) after 24 hours based on the prominent decrease in growth compared to that of the drug free growth control well.

D. DETERMINATION OF GROWTH CONDITIONS AND BIOFILM QUANTIFICATION OF *E. FEACALIS* AND *S. MUTANS* BY CRYSTAL VIOLET STAINING ASSAY

The growth conditions for biofilm formation and Crystal violet staining were adapted according to the modified method described by Melo S. et al. Briefly, all the strains were initially grown on Brain Heart Infusion agar at 37° C for 24 hours and 2-3 identical colony of standard strain was further sub-cultured in RPMI 1640 broth medium without bicarbonate, buffered to pH 7.0 with 3-(*N*-morpholino) propane sulfonic acid (MOPS) overnight, on a rotating shaker at 120 rpm at 37°C. The cell cultures were harvested, washed twice with PBS and adjusted to

~10^8 CFU/mL in RPMI 1640. 100µL of this cell suspension was seeded into the respective wells of the polystyrene, round-bottom, 96-well microtiter plate (**Figure 4**) and the plates were incubated at 37°C for 2hrs at 75rpm for the planktonic cells to adhere onto the inner walls of the microtitre plate wells. Following the attachment phase, non-adherent cells were removed by washing the wells with 150µL of PBS and 100µL of fresh RPMI 1640 medium were added to each well. The plate was incubated at 37°C for 48hrs with shaking at 75 rpm for the growth and maturation of biofilms. The test medium RPMI 1640 without inoculum was added to the final well of each plate as the negative control.

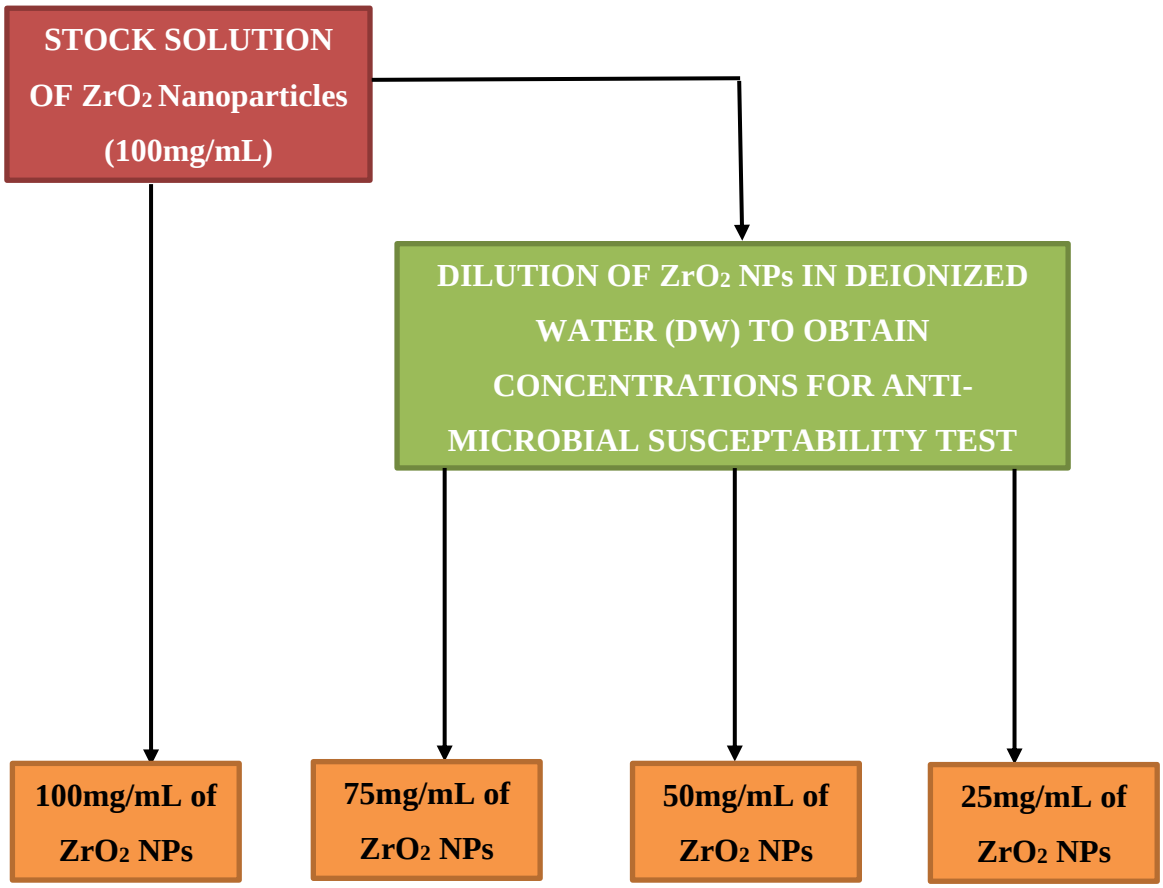
The biofilms formed after incubation were quantified using crystal violet staining method. The biofilms were washed twice with 200µL PBS and the plate was dried for 20 min at 37°C; subsequently the biofilms were stained with 110 µl of 0.4% aqueous CV for 45 mins and then washed thrice with 200µL sterile Milli-Q water and left for destaining with 200µL of 95% ethanol for 45 min. A final volume of 100 µl of the destaining solution from each sample was transferred to a new flat bottom plate and measured with a spectrophotometric plate reader at 595 nm. The absorbance values of the negative control were subtracted from the values of the test wells to minimize background interference. The biofilm production quantities were reported as the arithmetic mean of highest absorbance values of the test wells. The biofilms formed were classified into strong, moderate and weak

based on the spectrophotometric absorbance at 595nm in relative to the corresponding absorbance of the cell-free medium control.

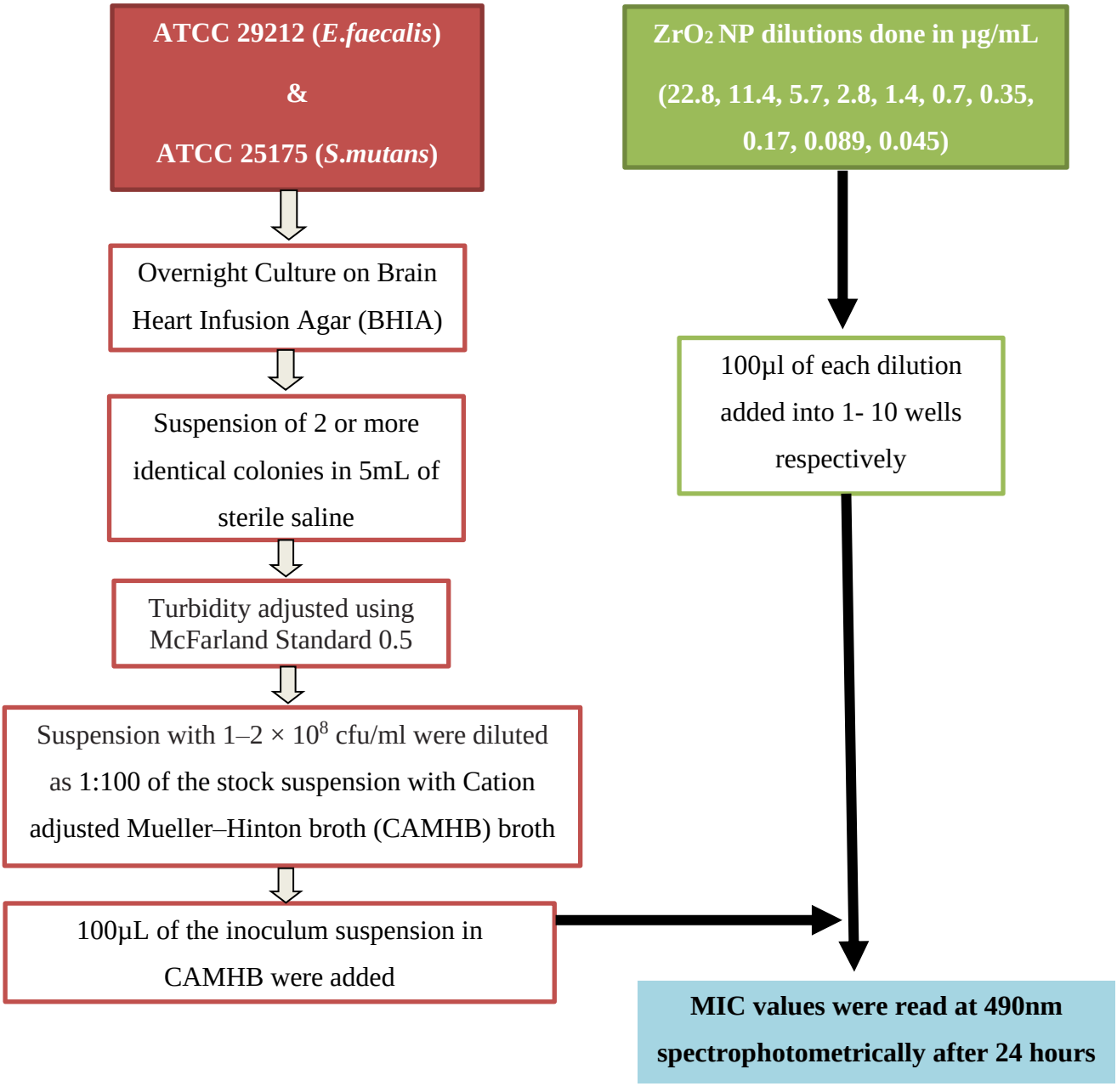
E. MINIMUM BIOFILM ERADICATION CONCENTRATIONS OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

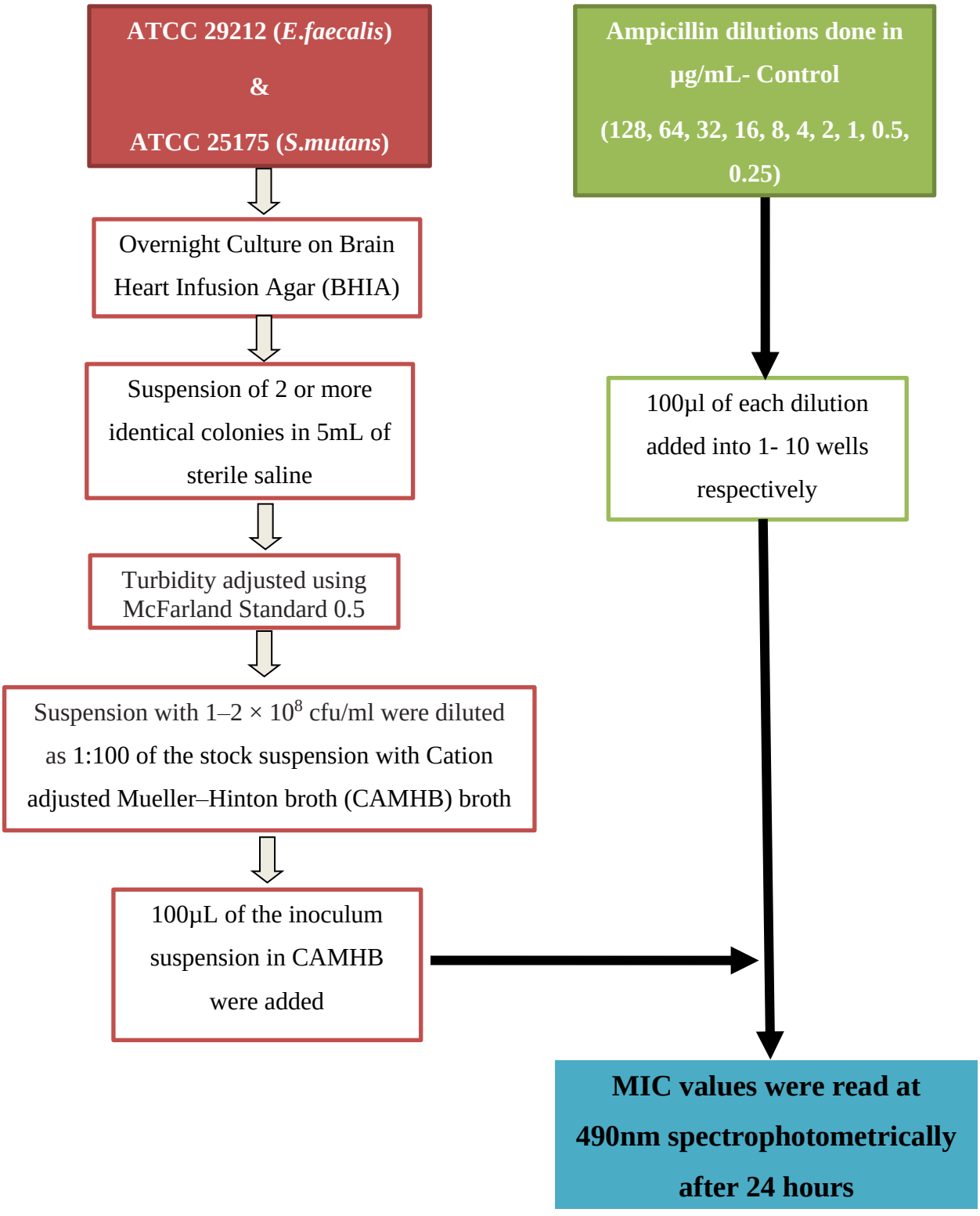
Biofilms were formed in RPMI 1640 medium as described earlier. After 24 hours of biofilm growth, they were washed twice with PBS and the plates were inverted onto absorbent paper to remove residual buffer prior to exposure with ZrO_2 NPs. The final concentrations of ZrO_2 NPs dilutions were added to the respective wells of the microtiter plate which was incubated for 48 hours at 37°C and 75 rpm. Wells containing biofilms, but no drug, served as positive controls for each strain tested. Following drug exposure, biofilms were washed thrice with sterile PBS and metabolic activity was determined using the XTT reduction assay. 100µl of XTT is added to each well and incubated at 37°C in the dark for 1 hour. Before reading, 100 µl of reaction mixture was transferred to a clean flat-bottomed microtiter plate and read in a spectrophotometer at 490 nm. The absorbance values of the negative control wells (containing no cells) were subtracted from the values of the test wells to account for any background absorbance.

MINIMUM INHIBITORY CONCENTRATION OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

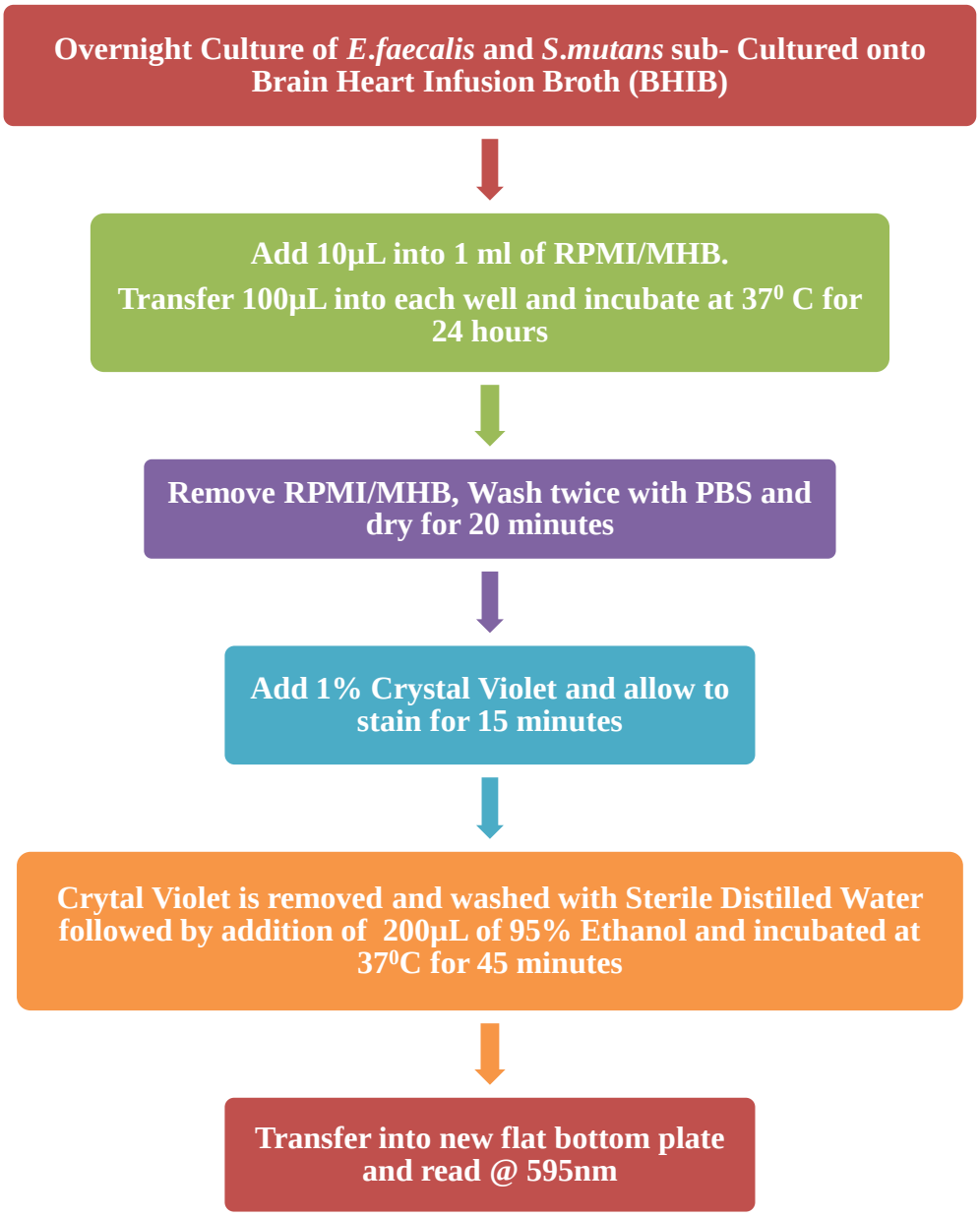


DETERMINATION OF GROWTH CONDITIONS AND BIOFILM QUANTIFICATION OF *E. FEACALIS* AND *S. MUTANS* BY CRYSTAL VIOLET STAINING ASSAY

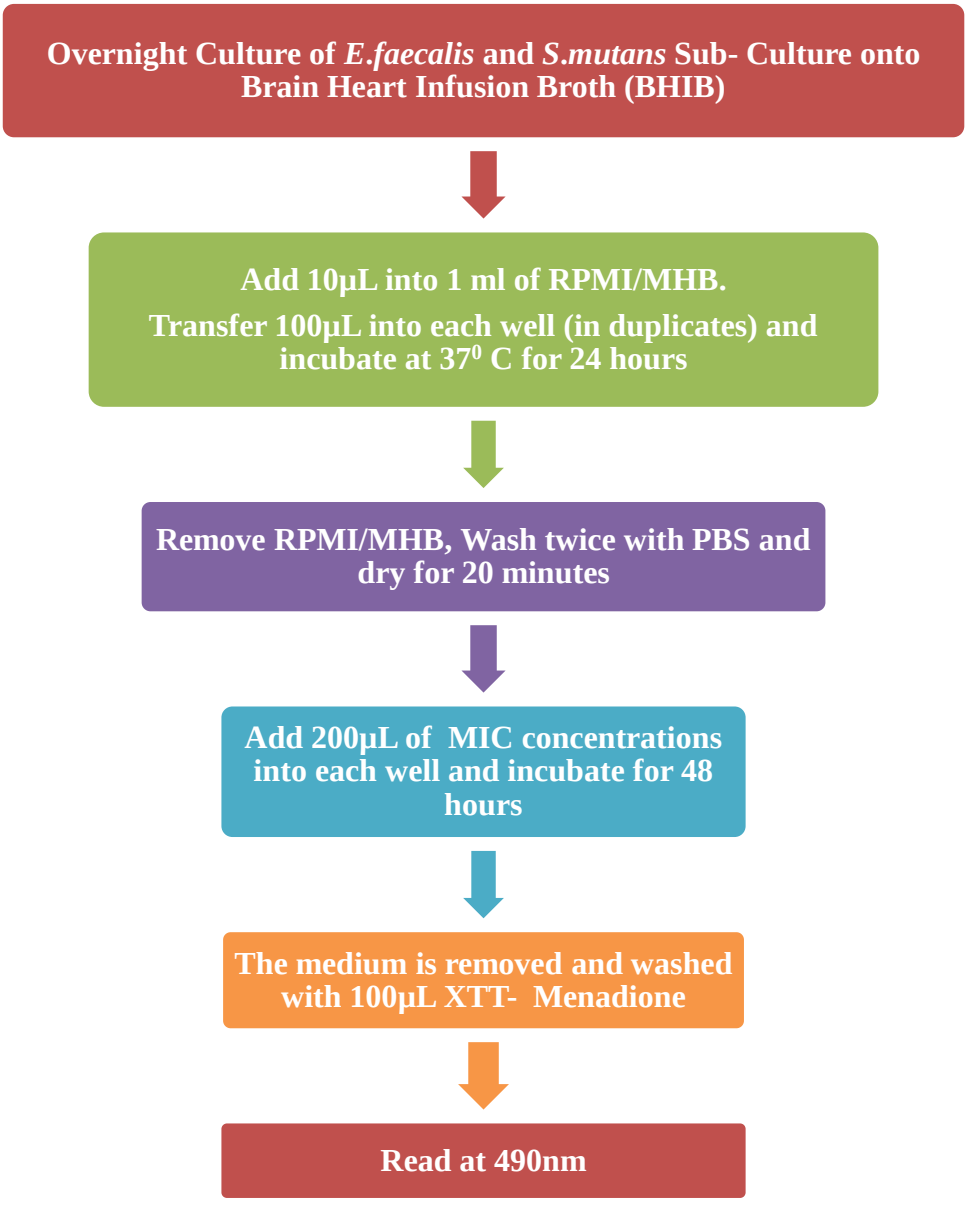



ATCC 29212 (*E.faecalis*)
&
ATCC 25175 (*S.mutans*)
Overnight Culture on Brain Heart Infusion Agar (BHIA)
Suspension of 2 or more identical colonies in 5mL of sterile saline
Turbidity adjusted using McFarland Standard 0.5
Suspension with 1–2 × 10^8 cfu/ml were diluted as 1:100 of the stock suspension with Cation adjusted Mueller–Hinton broth (CAMHB) broth
100µL of the inoculum suspension in CAMHB were added
Ampicillin dilutions done in µg/mL- Control
(128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25)
100µl of each dilution added into 1- 10 wells respectively
MIC values were read at 490nm spectrophotometrically after 24 hours

DETERMINATION OF GROWTH CONDITIONS AND BIOFILM QUANTIFICATION OF E.FEACALIS AND S.MUTANS BY CRYSTAL VIOLET STAINING ASSAY



MINIMUM BIOFILM ERADICATION CONCENTRATIONS OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST E.FAECALIS AND S.MUTANS



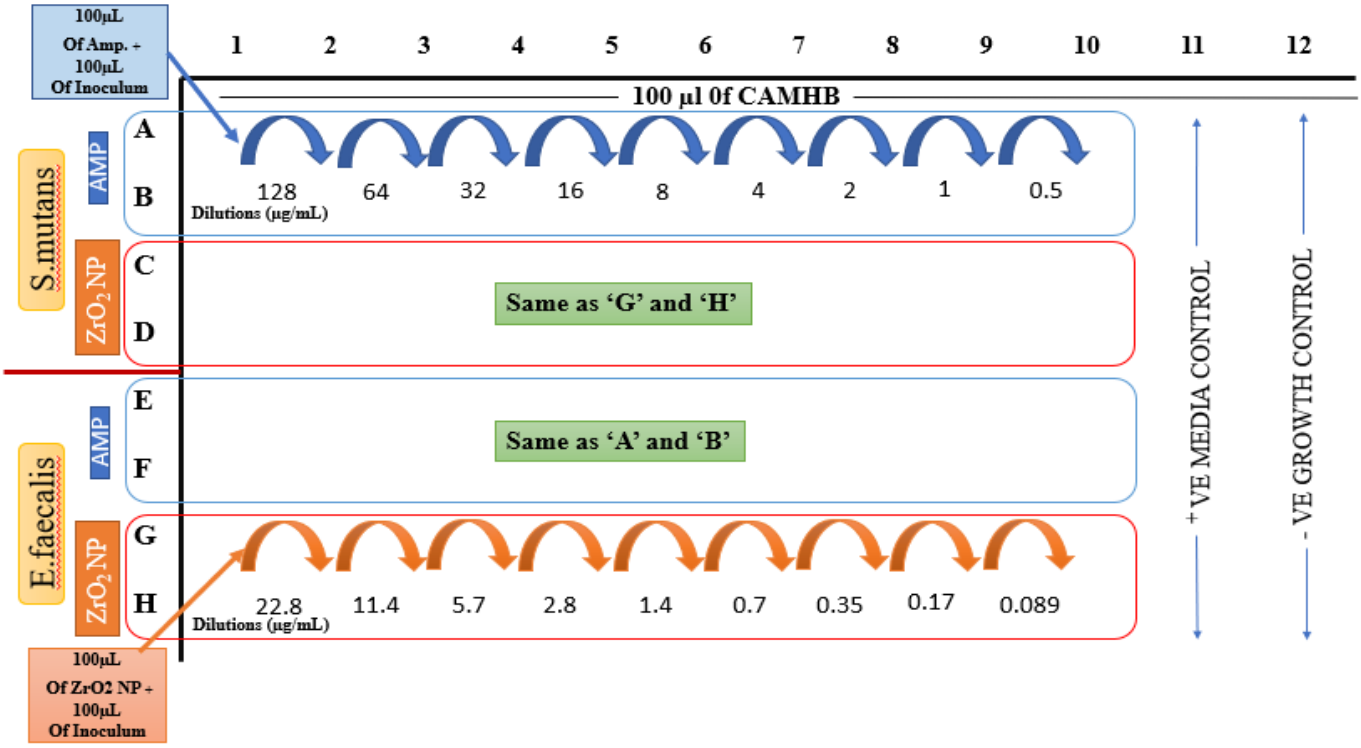


FIGURE 15: SCHEMATIC REPRESENTATION OF DILUTIONS IN MICRO TITRE PLATES

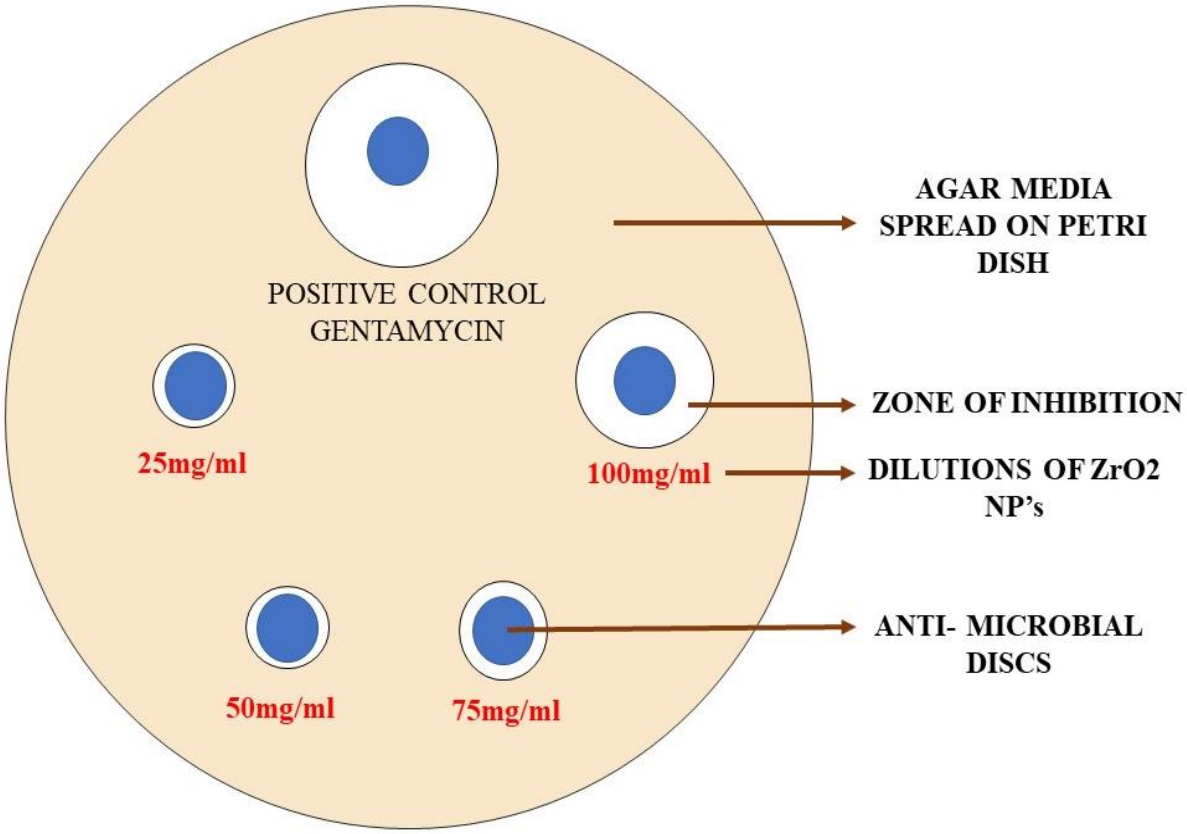


FIGURE 16: SCHEMATIC REPRESENTATION OF AGAR DIFFUSION TEST FOR ANTI MICROBIAL SUSCEPTABILITY

Figures

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FIGURE 1: ZIRCONIUM OXIDE NANOPARTICLES

FIGURE 2: BRAIN HEART INFUSION AGAR

FIGURE 3: PETRI DISH

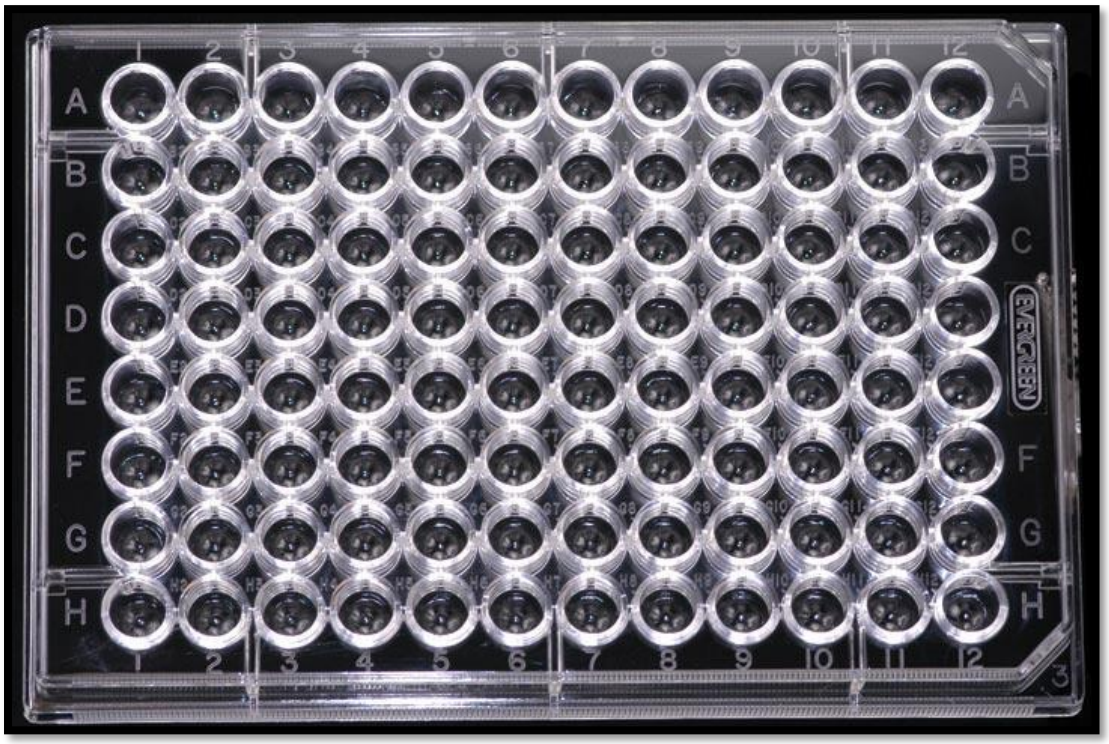


FIGURE 4: MICROTITRE PLATES- 96 WELL

FIGURE 5: INCUBATOR

FIGURE 6: SPECTROPHOTOMETER

FIGURE 7: FREEZER

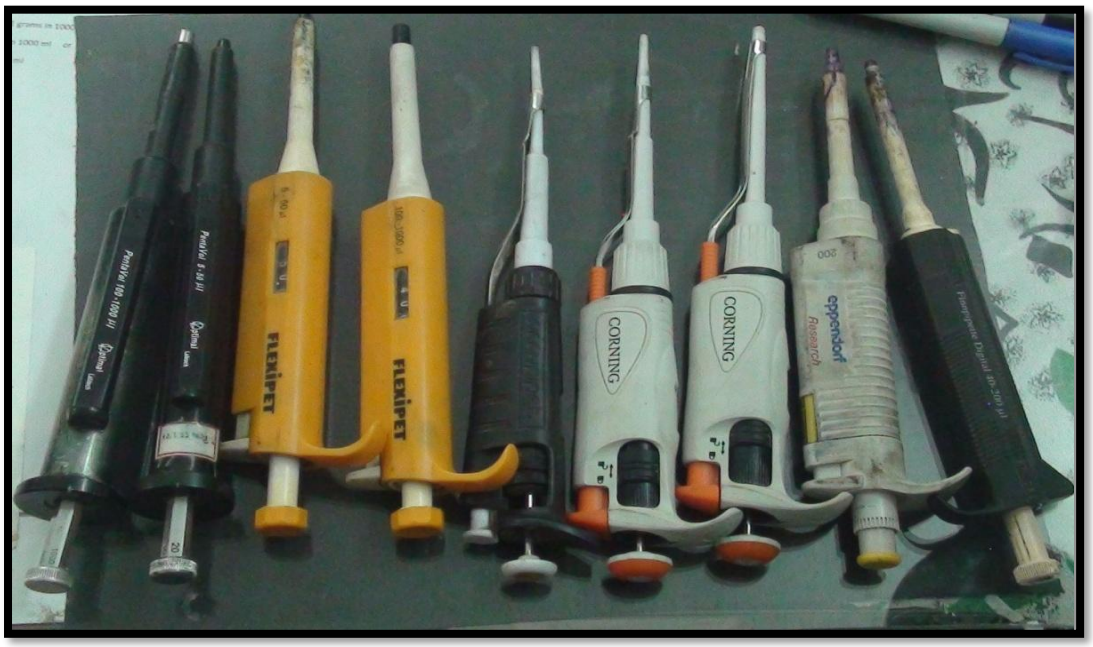


FIGURE 8: MICRO PIPETTES (mL)- 20, 20-200, 100-1000 MICROLITRE

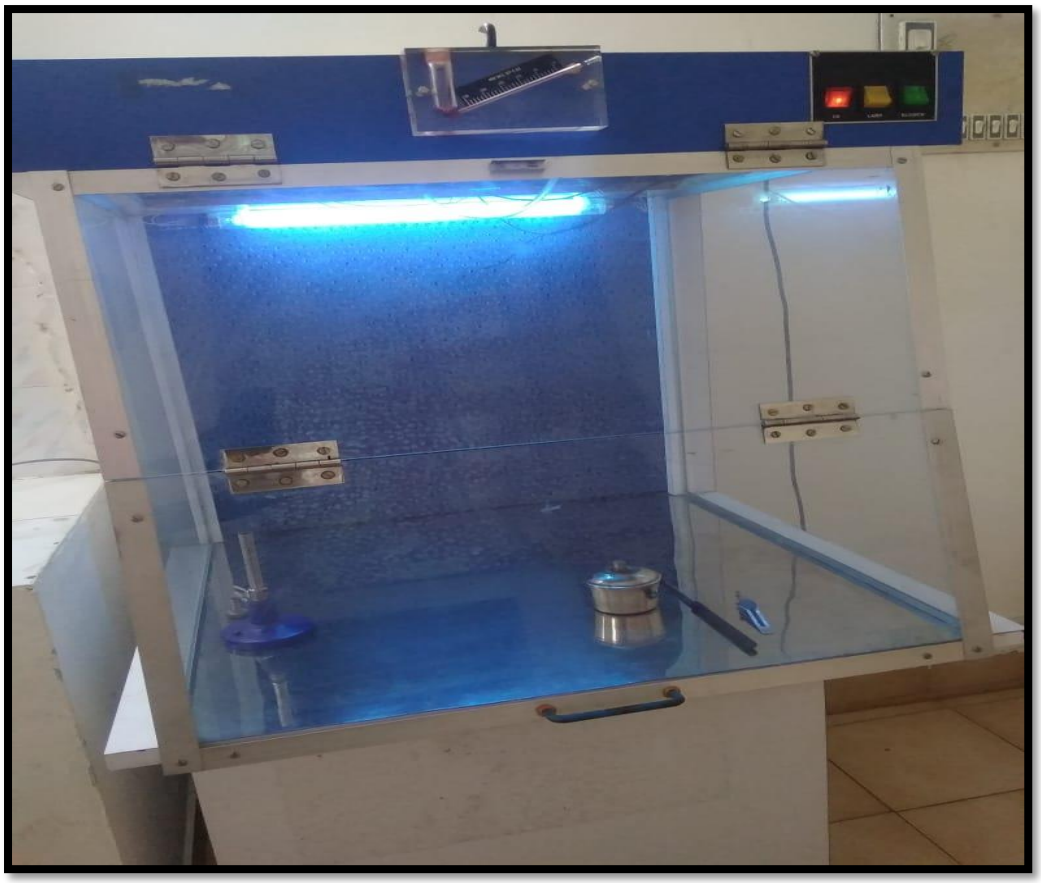


FIGURE 9: LAMINAR AIR FLOW CHAMBER

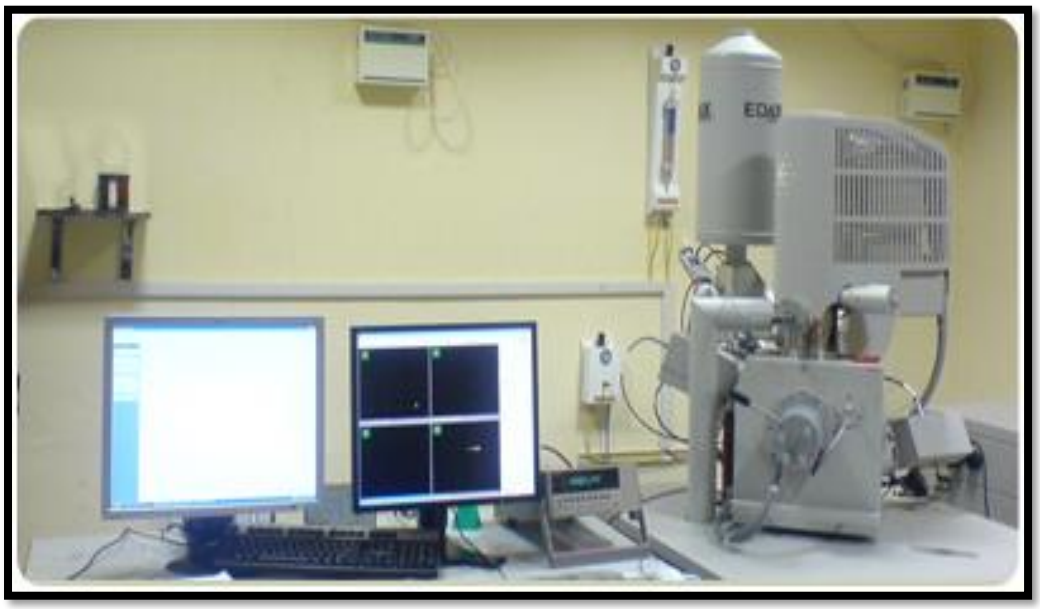


FIGURE 10: SCANNING ELECTRON MICROSCOPE

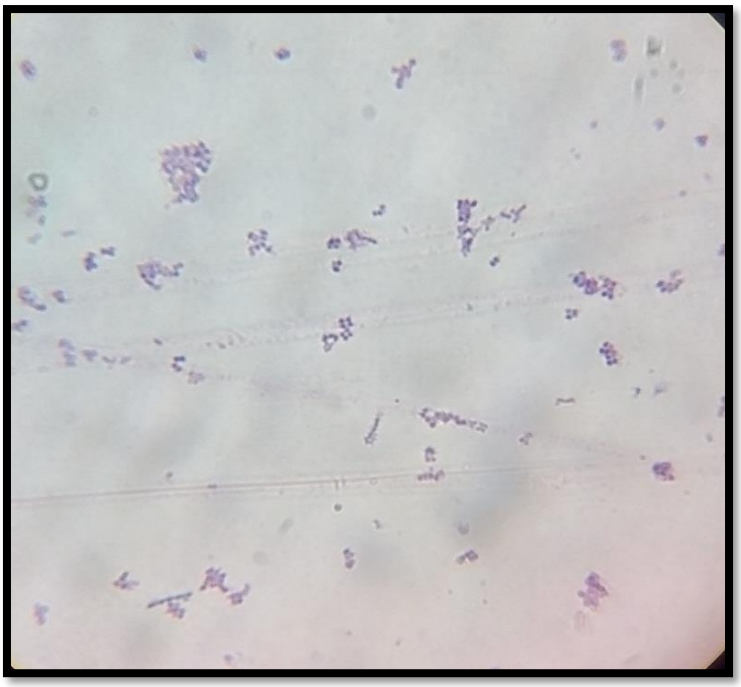


FIGURE 11: GRAM STAINING FOR S. MUTANS

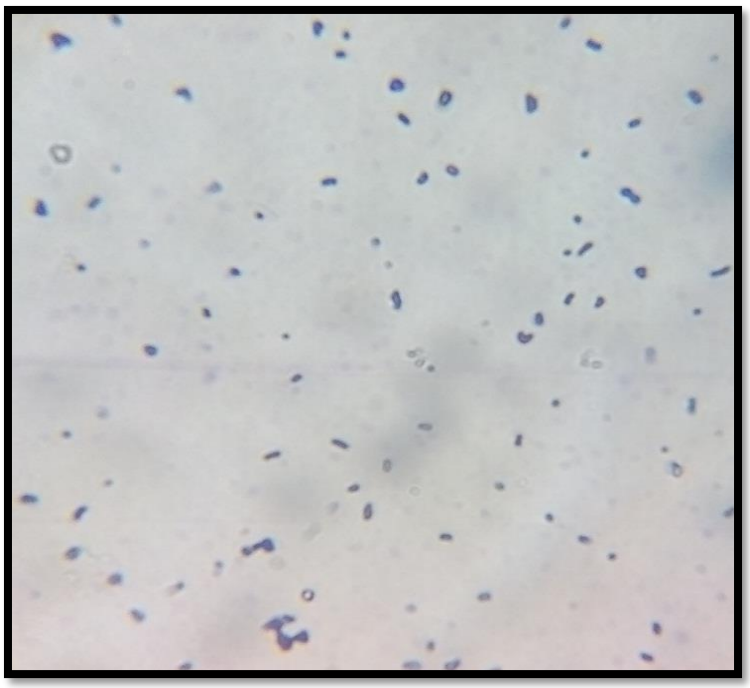


FIGURE 12: GRAM STAINING FOR E. FAECALIS

FIGURE 13: COLONIES OF E. FAECALIS ON NUTRIENT AGAR

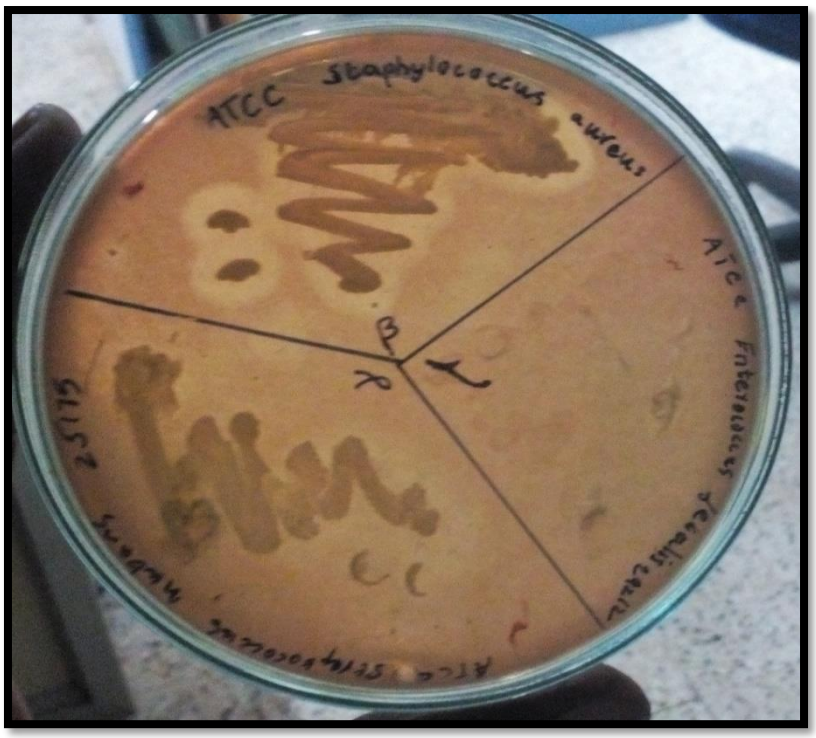


FIGURE 14: GROWTH OF S. MUTANS ON BLOOD AGAR

Results

RESULTS

A. CHARACTERIZATION OF ZIRCONIUM OXIDE NANOPARTICLES:

The powdered ZrO_2 NPs used in this study were morphologically and chemically analyzed using Scanning Electron Microscopy (SEM) and Energy –Dispersive X- ray Spectroscopy (EDAX) respectively.

i. SCANNING ELECTRON MICROSCOPY:

The micrographs of ZrO_2 NPs nanostructures under various magnifications (30,000X, 60,000X, 80,000X and 1,20,000X) are shown in **Figure 17.**

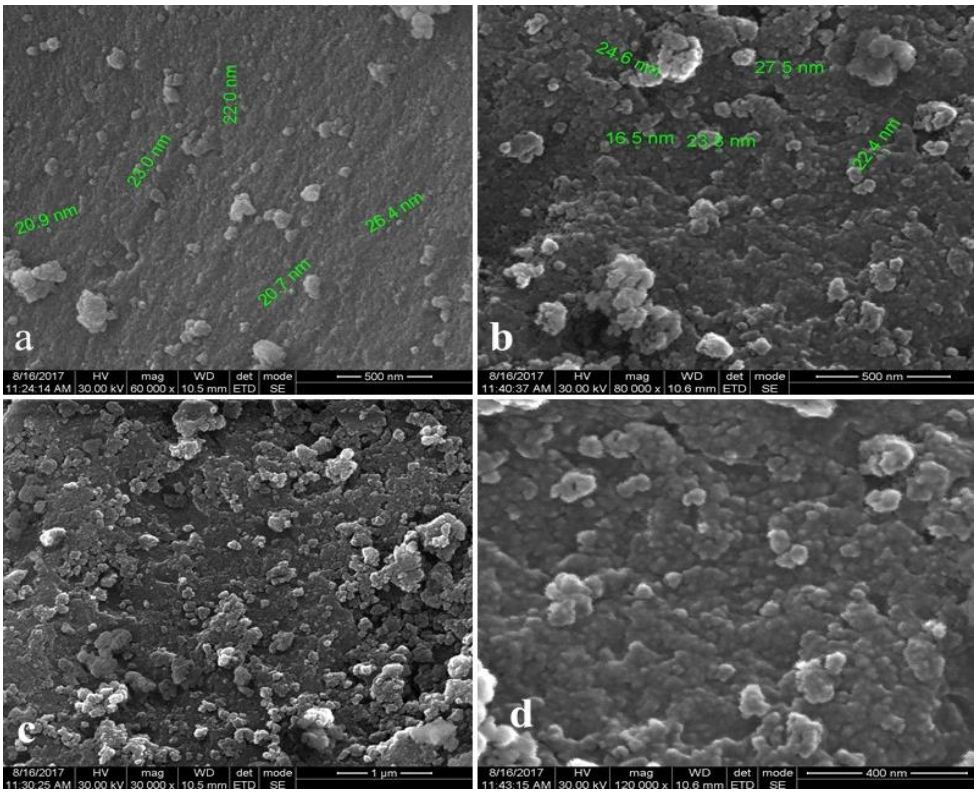
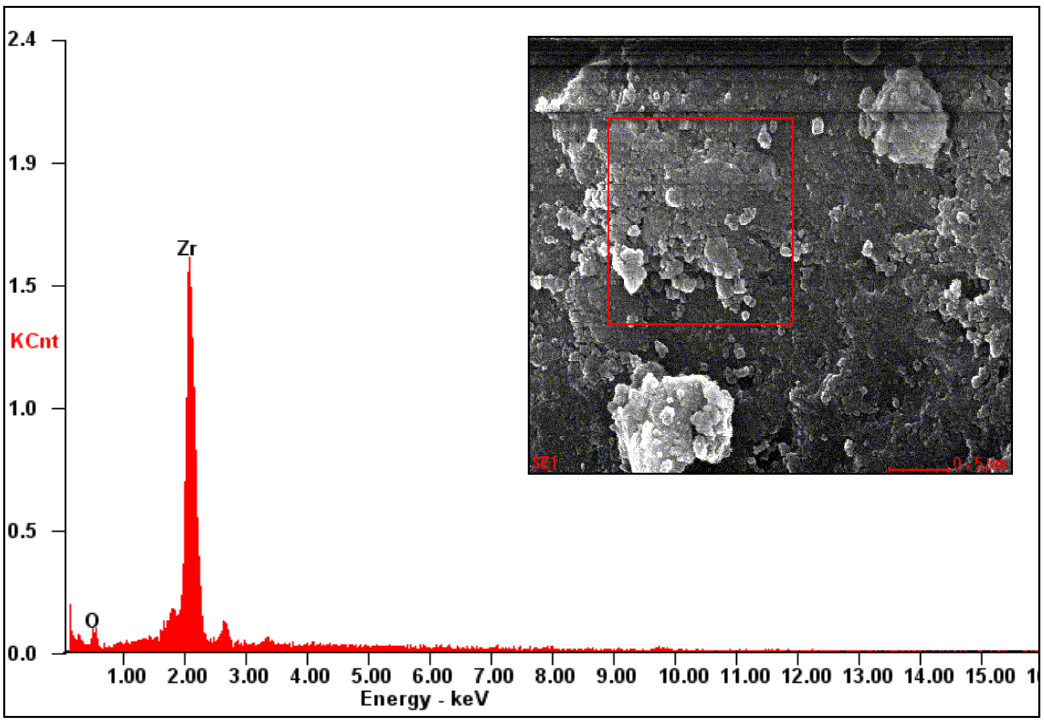


FIGURE 17: SEM MICROGRAPHS OF ZrO_2 NPs

SEM analyzes revealed that the ZrO_2 NPs had a cluster appearance with a spherical structure as shown in **Figure 17c & Figure 17d** and the average particle size of these nanoparticles ranged from 16.5nm to 27.5nm which is depicted in **Figure 17a & Figure 17b.**

ii. ENERGY – DISPERSIVE X- RAY SPECTROSCOPY:

The elemental analysis of ZrO_2 NPs was done using EDAX and the peak values represented that the predominant element in the sample was Zirconium which is evident in Graph 1



GRAPH 1: ELEMENTAL ANALYSIS OF POWDERED ZrO_2 NPs USING EDAX

B. ANTI – MICROBIAL SUSCEPTABILITY TESTING OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

Agar diffusion wells with four dilutions of ZrO_2 NPs (100mg/ml, 75mg/ml, 50mg/ml, 25mg/ml) and a positive control of 10mg/ml Gentamycin against *S. mutans* is shown in **Figure 18**. ZrO_2 NPs exhibited anti- microbial property at 100mg/ml with 12mm clear zones around the wells and these zones were similar in diameter for concentrations less than 100mg/ml.

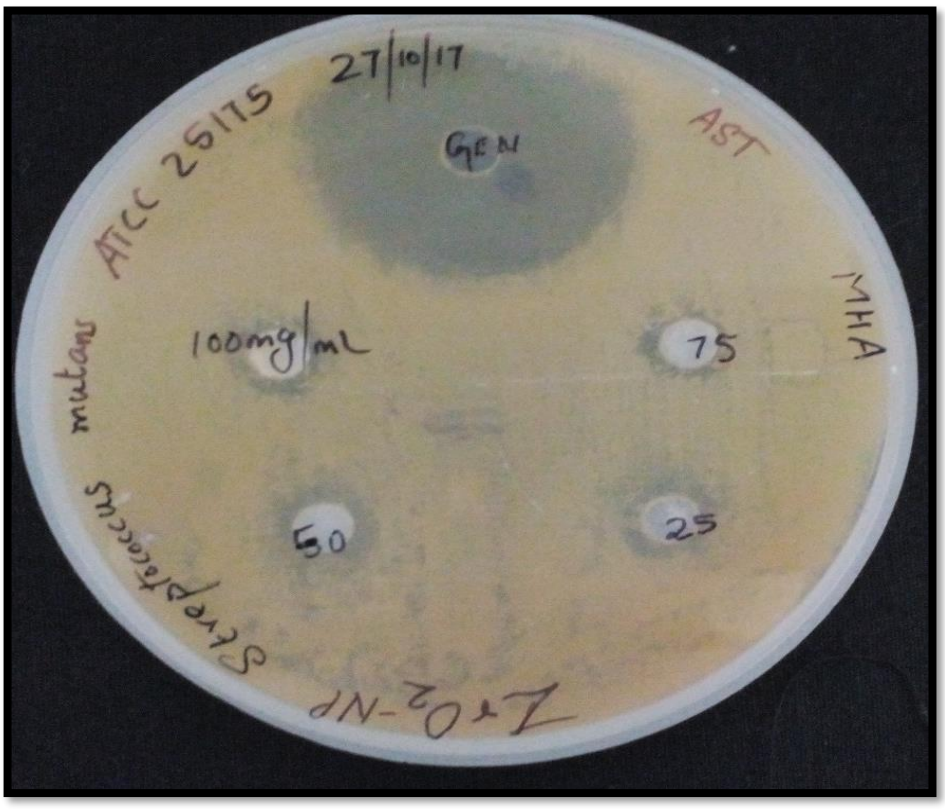


FIGURE 18: ZONE OF INHIBITION OF ZrO_2 NPs AGAINST S. MUTANS

Similarly, **Figure 19** shows that *E. faecalis* is sensitive to ZrO_2 NPs at a concentration of 100mg/ml which is evident from the clear zone of 15mm around the well.

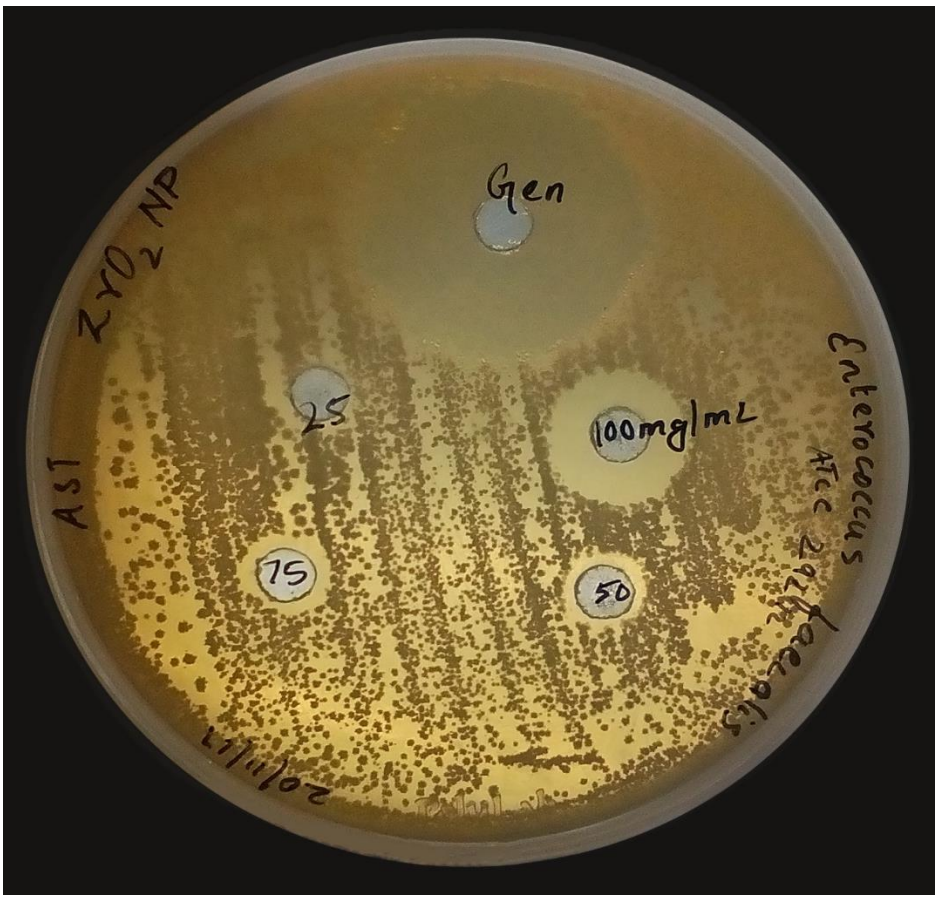


FIGURE 19: ZONE OF INHIBITION OF ZrO_2 NPs AGAINST

E. FAECALIS

Gentamycin was used as a positive control against both *S. mutans* and *E. faecalis* which showed zones of inhibition of 33mm and 31mm respectively.

The diameter of Zones of inhibition for various concentrations of ZrO_2 NPs against *S. mutans* and *E. faecalis* are listed in **Table 1**

TABLE 1: ANTI- MICROBIAL SUSCEPTABILITY TESTING

(AGAR WELL DIFFUSION METHOD)

S.NO	ATCC	DRUG TESTED (mg/mL)		ZONE OF INHIBITION (DIAMETER IN mm)
1.	S. *mutans* (25175)	GENTAMICIN		33 (S)
		ZrO_2 NPs	100	12
			75	11
			50	11
			25	10
2.	E. *faecalis* (29212)	GENTAMICIN		31(S)
		ZrO_2 NPs	100	15
			75	11
			50	11
			25	10

C. MINIMUM INHIBITORY CONCENTRATION OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

Minimum Inhibitory Concentration was done using Broth Microdilution method and the serial dilutions of ZrO_2 NPs against *S. mutans* and *E. faecalis* is shown in **Figure 20.** The microtiter plate is placed in a spectrophotometer to read the optical density values of each well at 490nm which are presented in **Table 2.**

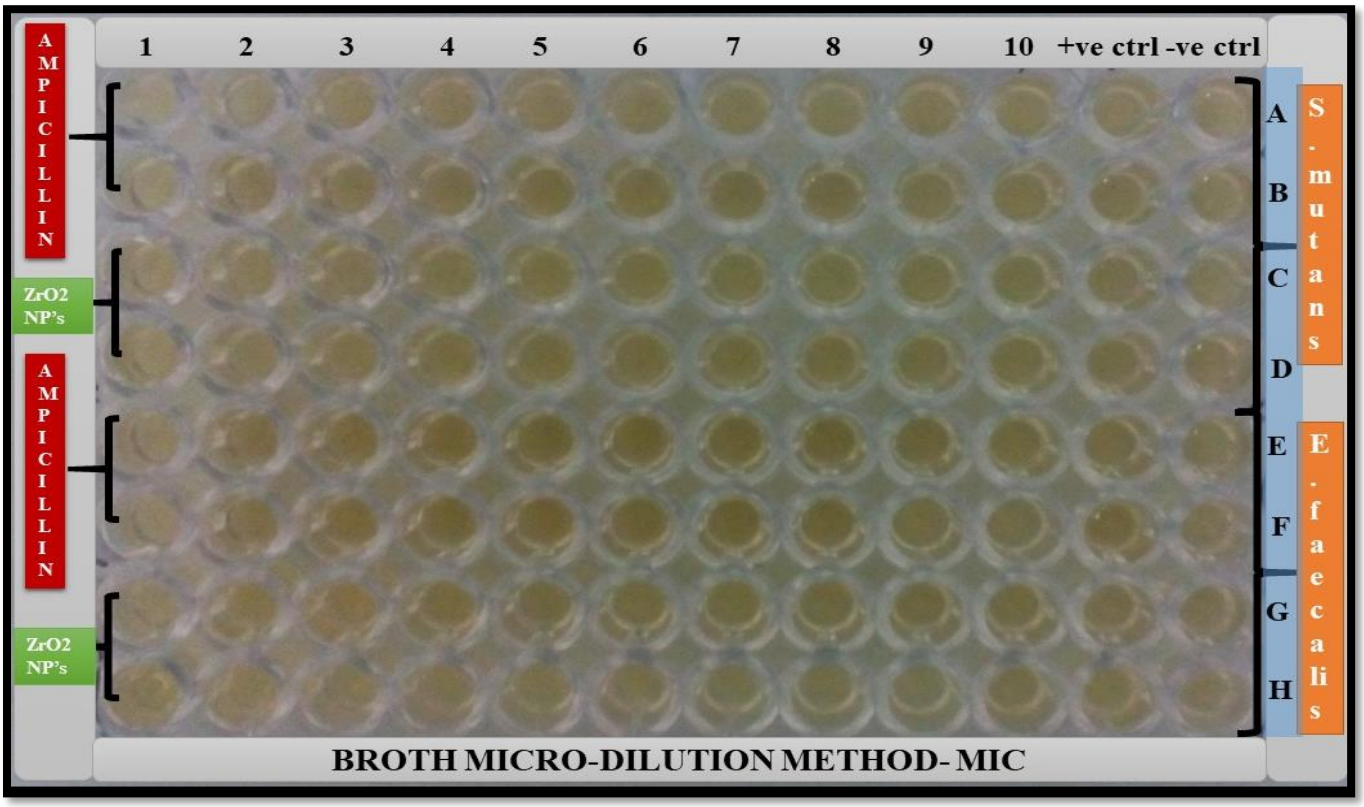


FIGURE 20: SERIAL DILUTIONS OF ZrO_2 NPs AGAINST S. MUTANS AND E. FACECALIS

TABLE 2: SPECTROMETRIC VALUES FOR MIC READ AT 490nm

			SPECTROMETRIC READINGS (OPTICAL DENSITY)											
			1	2	3	4	5	6	7	8	9	10	11	12
S. *mutans*	**AMPICILLIN**	A	0.227	0.312	0.286	0.288	0.3	0.291	0.286	0.274	0.286	1.643	1.624	0.392
		B	0.237	0.249	0.228	0.23	0.306	0.385	0.294	0.301	0.982	1.779	1.009	0.466
	ZrO_2 NPs	C	0.436	0.864	0.888	0.863	0.972	0.934	1.115	1.004	1.062	1.053	1.307	0.374
		D	0.463	0.369	1.026	1.123	1.144	1.354	1.087	1.062	0.995	0.936	1.099	0.323
E. *faecalis*	**AMPICILLIN**	E	0.305	0.294	0.3	0.32	0.316	0.311	0.308	0.295	0.259	1.027	1.557	0.824
		F	0.42	0.334	0.337	0.346	0.332	0.311	0.306	0.294	1.473	1.62	1.205	0.311
	ZrO_2 NPs	G	0.568	0.943	1.591	1.097	0.836	1.233	1.196	1.108	1.053	1.198	0.991	0.286
		H	0.653	1.037	1.444	1.254	1.062	0.986	1.238	1.353	1.199	1.27	0.901	0.281

MIC was calculated using the formula,

$$\mathbf{MIC} = \frac{\mathbf{OD}_{(untreated)} - \mathbf{OD}_{(test)}}{\mathbf{OD}_{(untreated)} - \mathbf{OD}_{(blank)}} \times \mathbf{100}$$

where, **OD**- Optical Density; **$OD_{(untreated)}$**- Optical density in Positive Media Control wells with only media and the bacterial strain; **$OD_{(test)}$**- Optical density in wells treated with ZrO_2 NPs against *E. faecalis* and *S. mutans*; **$OD_{(blank)}$**- Optical density in Negative Growth Control wells with only culture media

The optical density values were substituted in the formula and the percentage thus obtained was taken close to 50% and 90% to calculate MIC_{50} and MIC_{90}.

EQUATION 1: CALCULATIONS FOR MIC 50 AND MIC 90

BACTERIAL STRAIN	**MIC_{50}**	**MIC_{90}**
S. *mutans*	$\frac{1.307 - 0.864}{1.307 - 0.374}\ X\ 100 = \mathbf{46}\%$	$\frac{1.307 - 0.436}{1.307 - 0.374}\ X\ 100 = \mathbf{93}\%$
E. *faecalis*	$\frac{0.991 - 0.568}{0.991 - 0.286}\ X\ 100 = \mathbf{60}\%$	$\frac{0.991 - < 0.568}{0.991 - 0.286}\ X\ 100 = < \mathbf{60}\%$

The concentration at which ZrO_2 NPs eradicated 50% and 90% growth of *S. mutans* and *E. faecalis* is listed in **Table 3**

TABLE 3: MINIMUM INHIBITORY CONCENTRATION

(BROTH MICRODILUTION METHOD)

S.NO	ATCC	MINIMUM INHIBITORY CONCENTRATION			
		AMPICILLIN		ZrO_2NPs	
		MIC $_{50}$ (µg/mL)	MIC 90 (µg/mL)	MIC $_{5O}$ (µg/mL)	MIC $_{90}$ (µg/mL)
1.	*S. mutans* (25175)	0.5	1	11.4	22.8
2.	*E. faecalis* (29212)	0.5	1	22.8	> 22.8

D. DETERMINATION OF GROWTH CONDITIONS AND BIOFILM QUANTIFICATION OF *E. FEACALIS* AND *S. MUTANS* BY CRYSTAL VIOLET STAINING ASSAY

The ability of *S. mutans* and *E. faecalis* to form biofilm was determined using Crystal Violet Staining Assay.

Figure 21: shows crystal violet staining of *S. mutans* and *E. faecalis* indicating the formation of biofilm

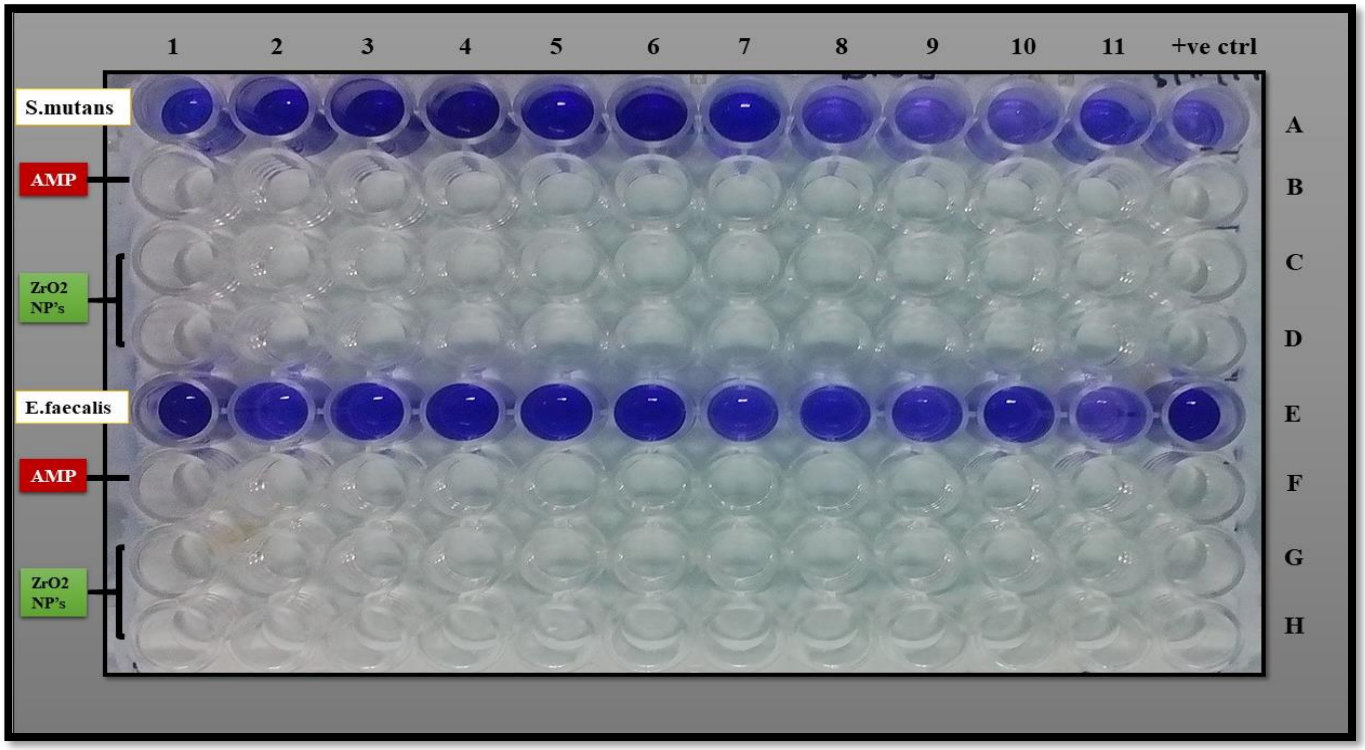


FIGURE 21: CRYSTAL VIOLET ASSAY FOR S. MUTANS AND E. FACECALIS

The spectrophotometric values of optic density of the biofilms formed by *S. mutans* and *E. faecalis* are given in **Table 4**

TABLE 4: SPECTROMETRIC VALUES FOR CRYSTAL VIOLET ASSAY READ AT 595nm

		SPECTROMETRIC READINGS (OPTICAL DENSITY)											
		1	2	3	4	5	6	7	8	9	10	11	12
S. *mutans*	A	2.19	***	***	***	***	***	***	1.17	0.792	0.725	1.433	0.835
AMPICILLIN	B	0.061	0.119	0.043	0.048	0.046	0.041	0.044	0.042	0.046	0.044	0.05	0.055
ZrO_2 NPs	C	0.052	0.057	0.043	0.053	0.05	0.063	0.046	0.047	0.402	0.043	0.04	0.049
	D	0.051	0.049	0.041	0.056	0.064	0.052	0.046	0.041	0.401	0.045	0.043	0.047
E. faeccalis	E	***	1.931	3.086	***	***	***	1.522	2.046	1.342	2.085	0.816	***
AMPICILLIN	F	0.054	0.09	0.043	0.046	0.043	0.042	0.049	0.049	0.042	0.042	0.048	0.044
ZrO_2 NPs	G	0.045	0.062	0.042	0.051	0.04	0.041	0.04	0.04	0.039	0.045	0.042	0.04
	H	0.051	0.082	0.043	0.041	0.04	0.054	0.039	0.04	0.04	0.039	0.042	0.044

*** - Dense Growth where OD were not read at 590nm

The density of biofilm formed by *S. mutans* and *E. faecalis* was calculated by taking the arithmetic mean of three highest OD_{595nm} values and comparing it with the inoculum OD_{595nm}.

EQUATION 2: EVALUATES THE DENSITY OF BIOFILM FORMATION

BACTERIAL STRAIN	**AVERAGE OF OD_{595nm}**	**EVALUATION WITH INOCULUM OD_{595nm}**
S. *mutans*	$\frac{2.046 + 2.085 + 1.931}{3} = \mathbf{2.021}$	> 0.8
E.*faecalis*	$\frac{2.190 + 1.170 + 1.433}{3} = \mathbf{1.590}$	> 0.8

Based on the spectrophotometric absorbance at 595nm in relative to the corresponding absorbance of the cell-free medium control, biofilm formation was classified into four types:

<0.2: Non- Adherent (-) ;

0.2 ≤ 0.4: Weakly Adherent (+)

0.4 ≤ 0.8: Moderately Adherent (+ +)

>0.8: Strongly Adherent (+ + +)

(NOTE: Inoculum OD_{595nm} ˜ 0.2)

The arithmetic mean of optical density values for both *S. mutans* and *E. faecalis* is presented in **Table 5.** The mean for *S. mutans* was 2.021 with a Standard deviation of 0.08 and the mean for *E. faecalis* was 1.590 with a standard deviation of 0.53

TABLE 5: BIOFILM QUANTIFICATION USING CRYSTAL VIOLET METHOD

S.NO	STRAIN	OD_{595nm}	INTERPRETATION
1.	*S. mutans* Average of OD_{595nm}	2.021	Strong (+++)
2.	*E. faecalis* Average of OD_{595nm}	1.590	Strong (+++)

E. MINIMUM BIOFILM ERADICATION CONCENTRATIONS OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

MBECs for ZrO_2 NPs is defined as the lowest drug concentration which inhibits the metabolic activity of *S. mutans* and *E. faecalis* by 50% ($MBEC_{50}$) and 80% ($MBEC_{80}$), relative to the drug-free growth control well. Two replicate tests were conducted with each of the isolates (*S. mutans* and E. *faecalis*) as shown in **Figure 22.**

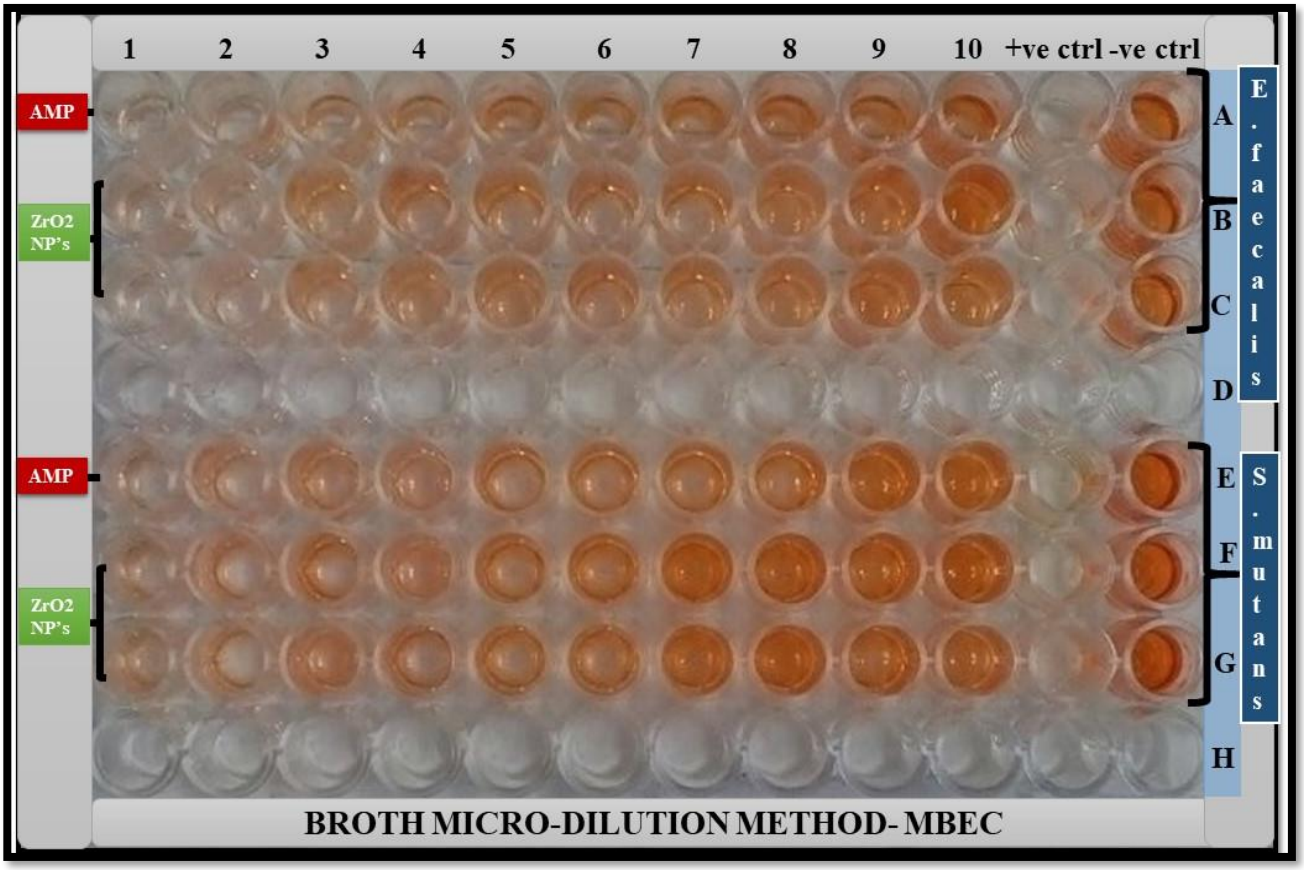


FIGURE 22: SERIAL DILUTIONS OF ZrO_2 NPs AGAINST S. *S.MUTANS* AND *E. FACECALIS* BIOFILM

The visual turbidity of the wells for Minimum Biofilm Eradication Concentration is spectrometrically read at 490nm as shown in **Table 6.**

MBEC was then calculated using the formula,

$$\textbf{MBEC} = \frac{\textbf{OD}_{\textbf{(untreated)}} - \textbf{OD (test)}}{\textbf{OD}_{\textbf{(untreated)}} - \textbf{OD}_{\textbf{(blank)}}} \times \textbf{100}$$

where, **OD**- Optical Density; **OD$_{(untreated)}$**- Optical density in Positive Media Control wells with only media and the bacterial strain; **OD$_{(test)}$**- Optical density in wells treated with ZrO_2 NPs against *E. faecalis* and S. *mutans*; **OD$_{(blank)}$**- Optical density in Negative Growth Control wells with only culture media.

The optical density values were substituted in the formula and the percentage thus obtained was taken close to 50% and 80% to calculate MIC_{50} and MIC_{80}.

EQUATION 3: CALCULATION OF MBEC 50 AND MBEC 80

BACTERIAL STRAIN	**MBEC$_{50}$**	**MBEC$_{80}$**
S. *mutans*	$\frac{0.471 - 0.258}{0.471 - 0.056}\ X\ 100 = \mathbf{51.3}\%$	$\frac{0.471 - < 0.162\ )}{0.471 - 0.056}\ X\ 100 => \mathbf{74.4}\%$
E. *fecalis*	$\frac{0.331 - 0.146}{0.331 - 0.068}\ X\ 100 = \mathbf{55.1}\%$	$\frac{0.331 - 0.135}{0.331 - 0.068}\ X\ 100 = \mathbf{75}\%$

TABLE 6: SPECTROMETRIC VALUES FOR MBEC READ AT 490nm

			SPECTROMETRIC READINGS (OPTICAL DENSITY)											
			1	2	3	4	5	6	7	8	9	10	11	12
E. *faecalis*	**AMPICILLIN**	A	0.161	0.273	0.2	0.241	0.216	0.267	0.288	0.189	0.442	0.498	0.147	0.425
		B	0.093	0.149	0.179	0.231	0.267	0.209	0.235	0.202	0.182	0.259	0.068	0.331
	ZrO_2 NPs	C	0.135	0.121	0.146	0.131	0.182	0.218	0.229	0.188	0.101	0.152	0.064	0.222
		D	-	-	-	-	-	-	-	-	-	-	-	-
S. *mutans*	**AMPICILLIN**	E	0.249	0.491	0.258	0.172	0.501	0.476	0.508	0.49	0.259	0.253	0.047	0.449
		F	0.174	0.836	0.603	0.267	0.427	0.434	0.111	0.552	0.332	0.367	0.041	0.359
	ZrO_2 NPs	G	0.162	0.632	0.258	0.12	0.337	0.416	0.206	0.364	0.384	0.21	0.058	0.471
		H	-	-	-	-	-	-	-	-	-	-	-	-

The concentration at which ZrO_2 NPs eradicated 50% and 80% of *S. mutans* and *E. faecalis* biofilm is listed in **Table 7**

TABLE 7: MINIMUM BIOFILM ERADICATION CONCENTRATION (MBEC)

S.NO	ATCC	MBEC (µg/mL)			
		AMPICILLIN		ZrO_2- NPs	
		MBEC $_{50}$	MBEC $_{80}$	MBEC $_{50}$	MBEC $_{80}$
1.	***S. mutans* (25175)**	16	128	5.7	>22.8
2.	***E. faecalis* (29212)**	8	128	5.7	>22.8

INTERPRETATION OF RESULTS:

1) ZrO_2 NPs used in our study had a purity of 99.9% (certified by Nano Labs) with an average particle size of spherical structures ranging from 16.5nm to 27.5nm.

2) Anti- microbial susceptibility testing showed that both *S. mutans* and *E. faecalis* are sensitive to ZrO_2 NPs indicated by clear zone of inhibition in the agar disc. But this diameter of the microbial inhibition zone may also depend on the solubility and infusibility of ZrO_2 NPs, thereby impeding its full potential to exhibit antimicrobial activity.

3) Although Agar diffusion method can identify the anti-microbial activity, it cannot quantify the amount of the antimicrobial agent (ZrO_2 NPs) that diffused into the agar medium to determine the minimum inhibitory concentration (MIC). Hence, MIC was used as a key indicator to test the potency of anti-microbial agent by making serial dilutions of a ZrO_2 NPs using Broth Microdilution method, which showed MIC of ZrO_2 NPs against both *S. mutans* and *E. faecalis* was observed to be 22.8 µg/ml where 90% of bacterial eradication was observed. Since the objective of the study was to quantify the minimum concentration of ZrO_2 NPs potency against *S. mutans* and *E. faecalis*, concentrations greater than 22.8 µg/ml can produce 99% inhibition of bacterial growth.

4) Crystal Violet Assay revealed both *S. mutans* and *E. faecalis* produced strongly adherent biofilms as their optical density values were greater than 0.8.
5) The results from Minimum Biofilm Eradication Concentration by Broth Microdilution method showed that ZrO_2 NPs eradicated 50% of these biofilms at 5.7µg/mL and concentrations greater than 22.8µg/mL can eradicate 80% of the biofilms formed by both *S. mutans* and *E. faecalis*.

Discussion

DISCUSSION

Dental plaque is a structurally and functionally organized biofilm which consists of microrganisms embedded in a self-organized matrix of extracellular polysaccharides (EPS). The surface of tooth being unique non-shedding hard surface, selectively absorbs various acidic glycoproteins like mucins from the saliva to form an amorphous membranous layer called acquired enamel pellicle (AEP). The AEP increases the net negative charge of the tooth surface to provide an innate defense mechanism by causing initial repulsion of negatively charged bacteria to the tooth surface. This defense mechanism breaks down when plaque formation occurs followed by initial colonization of the AEP by Mutans Streptococci (MS).[49]

The oral micro flora is a complex ecosystem which contains a wide variety of microbial species. The structural, metabolic and chemical interactions between microorganisms in the biofilm plays an important role in maintaining community homeostasis and provides a balanced equilibrium with host defenses for maintaining the integrity of oral tissues. Disease occurs when this balance is disturbed to the benefit of the biofilm.[50]

Dental caries is one of the most common biofilm-mediated, chronic infectious disease affecting 80–90% of the world population.[51] It results in localised destruction of dental hard tissues by complex biological interactions of acidogenic bacteria, fermentable carbohydrates and host factors such as the teeth and saliva.[52]

Anatomic differences in the oral cavity influence the ecology of commensal bacterial and the predominant species accounting for 79% on the tooth surface includes S*treptococcus mitis* and *S. oralis.*[53] These bacterial species are the primary colonizers of the teeth which forms a loose, irreversible bond to the acquired pellicle. Initially, they compete for essential nutrients and creates an unfavorable condition for pathogenic organisms to displace them from the plaque community. As the formation of plaque continues, the proliferation and metabolism of pioneer bacterial species modifies the local environmental conditions to favor the secondary colonizers like *S. mutans* and lactobacilli to adhere to the early colonizers through a process called coaggregation or co-adhesion. Thus a limited number of pioneer microbial species are replaced to a complex flora in the mature plaque.[54,55]

S. mutans behaves as an opportunistic bacteria by proliferating under favourable conditions and initiate the formation of dental caries.[55] The MS represents a group of seven closely related species that are found in plaque. They are gram-positive facultative cocci (**Figure 11**), usually arranged in chains and are capable of fermenting mannitol and sorbitol, producing extracellular glucans from sucrose. J. Kilian Clarke in 1924 isolated such organisms from human carious lesions and called them *S. mutans* because on Gram stain they were more oval than round and their high caries- inducing potential characterized them to be a mutant form of streptococcus species.[49]

S. mutans are highly acidogenic producing short-chain acids like lactic acid which dissolves the dental hard tissue. The acid production of the bacterium serves as a virulence factor because the acidic products formed during the metabolism of dietary carbohydrates are essential for the development of caries. Synthesis of insoluble extracellular polysaccharides from sucrose through glucosyltransferases (GTFs) is also considered an another important virulence factor because it not only facilitates the adhesion and accumulation of the organism on the tooth surface but also provides protection against host immune defences and increased resistance against antibiotics and gene expression.[47] This combination of virulence properties allow *S. mutans* to colonize the surface of tooth and modify the non-pathogenic communities to highly cariogenic dental biofilm that ultimately leads to caries formation.[56] Besides acidogenic potential, *S. mutans* are capable of surviving in acidic environments by functioning at pH 6 and can carry out the glycolysis process at pH 4 or below.

Various treatment strategies have been developed to interfere with colonization of *S. mutans* to have a positive impact on the incidence of dental caries. Contemporary approaches relies primarily on early prevention of dental caries and minimally invasive approaches.[48]

Bacterial invasion from carious lesion initially causes pulpal inflammation and further progression of the disease process leads to ingress of microorganisms into root canal system. This results in Endodontic infections which can be

classified into primary and secondary infections, where primary endodontic infections frequently occurs secondary to dental caries providing a selective habitat for the establishment of a mixed microbiota with predominance of anaerobic bacteria within the root canal system.[57] The microbial etiology of primary endodontic infection depends on the communication channel between the endodontic environment and the microbial source. Root canal treatment is aimed at eliminating the bacteria and their by-products along with necrotic tissues of the canal space which might serve as a substrate for bacterial regrowth. Insufficient cleaning and disinfection of root canals can lead to bacterial persistence resulting in secondary endodontic infection.

Microbes in secondary infection resists intracanal disinfection procedures and adapt to the changing environment to cause persistent intra-radicular infection. *Enterococcus faecalis* is the most consistently reported organism in secondary endodontic infection which is a gram -positive, facultative anaerobic bacteria measuring about 0.5–1μm in diameter (**Figure 12**). They are spherical or ovoid in shape occurring in pairs or short chains forming creamy white colonies (**Figure 13**).[58]

E. faecalis possess a number of virulence factors that permit adherence to host cells and extracellular matrix, colonizing the root canal system. Principal cause for *E. faecalis* to be associated with endodontic failure is its ability to invade dentinal tubules and strongly get adhered to collagen, which is abundantly present in root dentin and cementum through collagen binding

protein called Ace. A study conducted by Nair et al using confocal laser scanning microscope showed that the depth of viable *E. faecalis* ranged from 100 to 400μm into dentinal tubules, resisting eradication and leading to secondary infections.[4] *E. faecalis* also exists in nutrient deficit ecological conditions of the root-filled teeth by forming biofilms which are more resistant to antibacterial agents, phagocytosis, and antibodies.[59]

As current instrumentation systems fail to reach anatomical complexities and to predictably touch all canal walls, proper cleaning and disinfection of isthmuses, recesses, and ramifications usually depends on the chemical effects of irrigants and interappointment medicaments. But conventional irrigants and intra canal medicaments have minimal penetrability into the dentinal tubules, leaving the bacteria within the tubules undisturbed leading to persistence of infection within the canal space. Therefore, complete disinfection can be achieved by developing newer materials and techniques that not only improves infection control in the main canal lumen, but also in the entire root canal system.[8]

S. mutans and *E. faecalis* which were considered the predominant species in the development of dental caries and secondary endodontic infections were selected as the test organisms. Their ability to form biofilms were also evaluated in our study using crystal violet assay as both *S. mutans* and *E. faecalis* possess biofilm mode of bacterial growth as an adaptive process to survive. These sessile biofilm bacteria also show higher antimicrobial

resistance compared to their free-floating ‘‘planktonic’’ counterparts and this resistance has been attributed to the protective barrier provided by the extracellular polymeric matrix (EPM). The results from our study proved that both *S. mutans* and *E. faecalis* form strongly adherent biofilms emphasizing the need for development of newer preventive and therapeutic agents that would disrupt the EPM and allows better penetration of antibacterial agents into the biofilm structure, resulting in significant anti-bacterial action.[15,60]

Nanomaterials have gained attention in recent years for its inherent antimicrobial properties and specific affinity to tooth surfaces.[61] Nanoparticles with a diameter of about 10^{-9} meter has an extremely tiny size which gives them unique physical, chemical, mechanical, magnetic and electric characteristics to enter the cells freely and interfere in their natural activities. They exhibit higher antibacterial activity as a result of their polycationic/polyanionic nature with higher surface area and charge density, resulting in greater degree of interaction with the bacterial cell.[60] The antimicrobial nanomaterials have distinct advantages over conventional antibiotics in terms of overcoming resistance and efficient drug delivery.

Historically, nanoparticles have been categorized into metallic and non-metallic nanoparticles of which metallic nanoparticles are most commonly used in insecticides and bactericides.[62] Having lowest levels of toxicity in the ecosystem, metal nanoparticles are being studied extensively in the field of modern pharmacological sciences.

One of the metal oxides with excellent electrical, mechanical, optical and thermal properties used in wide areas of scientific and technical fields is Zirconium oxide (Zirconia). It is used as an abrasive and is incorporated in many cutting tools and also to make components of engines and furnaces. In the field of medicine, ZrO2 is emerging as a catalyst and is also used for dental fillings, for making dental crowns and for therapeutic purposes. They are currently being employed as an alternative to titanium-based materials as the latter leads to severe localized reactions and also pain. Additionally, ZrO_2 was reported to have anti-microbial activity against a specific group of microorganism which would be beneficial against antibiotic resistant strains posing a major health concern.[63]

Since nanostructured materials exhibited unique physiochemical properties that are unseen in conventional bulk materials, nano- structured ZrO_2 particles were synthesized for enhanced properties. Nanoparticles can be artificially synthesized (engineered), formed naturally or they can originate from an anthropogenic source as accidental by-products. ZrO_2 was found naturally in its impure form and is usually extracted chemically from two minerals: zircon and babbelyite whereas nanostructured ZrO_2 can be synthesized by various methods like co-precipitation, Glycothermal Processing, Solid-State Reaction, Pechini Method, microwave-assisted sol-gel synthesis, bio-phase protocol, hydrothermal method and sol-gel processing.[33]

Many nanomaterials, such as silver, copper oxide, zinc oxide, titanium oxide, and graphene can be used to control bacterial biofilm formation. Quaternary ammonium polyethylenimine, chitosan, and silica nanoparticles have also been suggested effective in controlling biofilms.

Being an inorganic nanomaterial, evaluating the antibacterial efficacy of ZrO_2 NPs would be beneficial because of improved safety and stability when compared with organic antimicrobial agents.[28] Since various studies have proven the versatility of nanotechnology in preventing dental caries, our study was carried out to determine the anti- microbial and anti- biofilm properties of Zirconium oxide nanoparticles (ZrO_2 NPs) against *S. mutans* and *E. faecalis.*[15]

Our study used Zirconium oxide nanoparticle, one of the metal oxides with increased mechanical and electrical properties used in wide areas of scientific and technical fields. Since ZrO_2 were studied predominantly in its microcrystalline form, the nano-crystalline form of ZrO_2 used in our study was characterized for their physical, chemical and optical properties. Combination of Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray Spectroscopy (EDX) is the most reliable characterization techniques capable of analyzing the morphology, size and chemical composition at the surface and in the bulk of single nanoparticle. The results showing spherical and irregularly spherical ZrO_2 NPs with an average size of 16.5nm to 27.5nm has an influence on anti-microbial activity as the size of metallic nanoparticles ensures a significantly large surface area of the particles to come in contact

with the bacteria when compared to their micro structured versions. Such a large contact surface enhances the extent of antimicrobial activity of ZrO_2 NPs.[15,64,60]

Thus, the characterized ZrO_2 NPs was evaluated for their anti- bacterial activity against *E.faecalis* and *S. mutans*.

i. **ANTI- MICROBIAL SUSCEPTABILITY TESTING:**

Agar Diffusion assay is the most important technique for assessing microbial susceptibility to antibiotics and it has a number of variations of which Well- and disc-diffusion methods are the qualitative indicators standardized by the Clinical and Laboratory Standards Institute (CLSI) for antibiotic testing (The Clinical and Laboratory Standards Institute M100-S22, Volume 32 No 3. January 2012).

The agar diffusion method is commonly used for determination of minimum inhibitory concentration (MIC) in solid media that involves the application of different concentrations of antibiotic solutions (ZrO_2 NPs) punched into agar plates seeded with the test bacterial strain (*S. mutans* and *E. faecalis*). The diffusion of antibiotic from these wells into the agarose medium leads to inhibition of bacterial growth in the vicinity of the source leading to the formation of clear ’zones’ which has no bacterial growth.[65]

ZrO_2 NPs possessed anti-microbial activity against *S. mutans* and *E. faecalis* at a concentration of 100mg/mL in the agar diffusion wells which can be

attributed to the electromagnetic attraction between positively charged zirconium ions from ZrO_2 NPs and negatively charged bacterial cell wall, leading to oxidation and death of microorganisms.[37] ZrO_2 NPs being chemical oxide and insoluble in water also aids in minimizing the bacterial adhesion to the tooth surface by binding to the surface of bacteria and inhibiting the synthesis of acids, thereby preventing dental caries.[66,37,28]

The antimicrobial activity of ZrO_2 nanoparticles can also be due to the following assumptions: The active oxygen species generated from the ZrO_2 NPs inhibit the growth of *S. mutans* and *E. faecalis* cells by accumulation or deposition on the surface of these cells. It is also suggested that ZrO_2 nanoparticles are able to slow down the growth due to disorganization of cell membranes, which increases membrane permeability leading to accumulation of nanoparticles in the bacterial membrane and cytoplasmic regions of the cells.[33]

ii. MINIMUM INHIBITORY CONCENTRATION (MIC):

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of antimicrobial agent that visually inhibits 99% growth of microorganisms under defined growth conditions The antimicrobial efficacy of Zirconium oxide nano particles (ZrO_2 NPs) were examined using the standard broth microdilution method (CLSI M07-A3).[67,68] The MIC was determined in BHI broth using serial two-fold dilutions of ZrO_2-NPs in concentrations ranging from 0.045 µg/ml to 22.8 µg/ml with adjusted bacterial concentration of 0.10

at 625 nm (1×10^6 CFU/ ml, 0.5 McFarland's standard). The positive control used in this study contained Cation adjusted Mueller–Hinton broth (CAMHB) medium with tested bacterial concentrations (*S. mutans* and *E. faecalis*) and negative control contained only inoculated broth and the time and temperature of incubation being 24 h and 37ºC respectively. A minimum concentration of 22.8µg/ml of ZrO_2 NPs eradicated 90% growth of *S. mutans* and *E. faecalis* indicating that the antibacterial efficacy of ZrO_2 NPs is efficient even at lowest concentrations eliminating its chance of causing toxicity at higher concentrations.

iii. MINIMUM BIOFILM ERADICATION CONCENTRATIONS OF ZIRCONIUM OXIDE NANOPARTICLES AGAINST *E. FAECALIS* AND *S. MUTANS*

MBEC was evaluated for ZrO_2 NPs against *S. mutans* and *E. faecalis* because biofilms formed by these organisms are more resistant to antimicrobial agents than their planktonic forms. The disruption of 50% and 80% biofilm by ZrO_2 NPs was evident from broth microdilution method done to determine minimum biofilm eradication concentration. The $MBEC_{80}$ of ZrO_2 NPs against *S. mutans* and *E. faecalis* was observed at 22.8 µg/ml. Although ZrO_2 NPs had antibacterial activity at a lower concentration against *S. mutans* and *E. faecalis* in their planktonic form, higher concentrations were needed to eradicate the biofilm formed by them. This can be attributed to the negatively charged biofilm EPM which would provide resistance to the penetration of ZrO_2 NPs.

It acts as a chemical barrier by adsorbing the harmful reactive oxygen species from ZrO_2 NPs from reaching the cell surface, thereby decreasing the effect of ROS. The disruption of biofilm structure formed by both *S. mutans* and *E. faecalis* after treatment with the ZrO_2 NPs was evident from our study.

Fathima et al studied the anti-bacterial efficacy of ZrO_2 NPs against *Bacillus subtilis*, *Staphylococcus aureus*, *E. coli* and *P. aeruginosa* and the results concluded that ZrO_2NPs had potential inhibitory action against *P. aeruginosa* and *S. aureus* and a good inhibition against *E. coli* while there was no action against *B. subtilis.*[17]

Jangra et al also evaluated the antibacterial and antifungal property of two bacterial strains, *Staphylococcus aureus* and *Escherichia coli* and three fungal strains namely *Aspergillus niger*, *Botrytis cinerea* and Aspergillus species isolated from soil respectively. He found that nano-ZrO_2 worked preliminarily on bacterial strains of *Escherichia coli* and *Staphylococcus aureus* and on the fungal strain of *A. niger* and mainly showed activity against *E. coli*. The study also concluded that antimicrobial activity is due to crystal plane-dependent interaction of ZrO_2 nanoparticles with the micro-organisms emphasizing smaller sized nanoparticles plays a crucial role in contributing to the surface energy aspect as more surfaces are available for the surface related activities.[16]

Gad et al studied the antifungal effect of ZrO_2 nanoparticles against *Candida albicans* as a possible approach for prevention of denture stomatitis when ZrO_2 nanoparticles are incorporated as reinforcements. He suggested that addition of zirconia nanoparticles to cold-cured acrylic resin is an effective method for reducing the adhesion of *Candida albicans* to repaired polymethyl methacrylate (PMMA) denture bases and cold-cured removable prosthesis.[37]

As there are only limited studies on the antimicrobial activity of ZrO_2 NPs, our study has proved the Anti-bacterial and anti-biofilm property of ZrO_2 NPs against *S. mutans* and *E. faecalis*. The activities of oral microflora being responsible for oral diseases must be kept to a level consistent with oral health by antimicrobial agent inclusion in dentifrices. As ZrO_2 NPs are capable of disrupting the biofilm formed by *S. mutans*, it can be effectively used in formulations for topical antimicrobial oral use, like dentifrices. As the size of ZrO_2 NPs used in our study is smaller than the size of bacterial cell or eukaryotic cell, even with shorter duration of exposure, it can penetrate the biofilm matrix and deliver the active agents quickly to disrupt the cell walls of *S. mutans*, inhibiting their enzymatic activity and bacterial aggregation, thereby preventing dental caries.[60]

In endodontic infections, bacterial biofilm within the depths of dentinal tubules would remain inaccessible for conventional irrigants, medicaments and sealers contributing to the persistence of infection within the root canal

system. Therefore, nanoparticles with unique properties such as enhanced surface area, chemical and biological activity can be used as an adjunct to provide complete disinfection of the root canal system. ZrO_2 NPs having an anti-bacterial and anti- biofilm activity against *E. faecalis* can be incorporated into irrigant solutions or intracanal medicaments in an attempt to reach the un-instrumented areas at an effective concentration and volume. Because of higher diffusibility, ZrO_2 NPs can also diffuse into the biofilm structure and anatomical complexities within the root canals where the bacteria are harbored. But this requires further genotoxic studies on ZrO_2 NPs.

Anti-microbial susceptability testing was used as a screening test for ZrO_2 NPs against *S. mutans* and *E. faecalis* and the further quantitative analysis were done to evaluate the minimum concentration to bring about the anti-bacterial activity. Since the interactions of ZrO_2 nanoparticles with biosystems are just beginning to be understood, the mechanism of bactericidal actions of ZrO_2 NPs has to be studied in future. As all the tests were conducted in vitro, it cannot be assumed that the results of antimicrobial efficacy could be proportional or transferable to the oral cavity and translated into clinical effectiveness. Nevertheless, the in vitro method is a well-established technique that is used commonly in screening the antimicrobial efficacy of chemicals before in vivo testing.

As this is the first study to evaluate the anti-bacterial efficacy of ZrO_2 NPs against *S. mutans* and *E. faecalis* carried out in vitro, this will serve as one of the most effective methods in controlling dental infections caused by *S. mutans* and *E. faecalis*. Further studies are recommended, to address the efficacy of ZrO_2 NPs against cariogenic and endodontic pathogens, to elucidate the interactions of ZrO_2 NPs in the dental structures, its cytotoxicity, mutagenicity and other potential long-term effects of ZrO_2 NPs.

Summary

SUMMARY

The purpose of the present study was (i) to determine the Anti-microbial susceptibility of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis* using Agar well diffusion method (ii) to determine the Minimum Inhibitory Concentration (MIC) of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis* using Broth Microdilution method (iii) to determine the Minimum Biofilm Eradication Concentration (MBEC) of ZrO_2 NPs against *Streptococcus mutans* and *Enterococcus faecalis*

Standard ATCC strains of *Streptococcus mutans* (ATCC 25175) and *Enterococcus faecalis* (ATCC *29212*) were procured from Sigma Aldrich. Zirconium Oxide nanoparticles used in our study was obtained from Nano Research with an average particle size of 15-20nm. The antimicrobial susceptibility testing of ZrO_2 NPs against *S. mutans* and *E. faecalis* was done using Agar well diffusion method. Four dilutions of ZrO_2 NPs (100mg/ml, 75mg/ml, 50mg/ml and 25mg/ml) were tested and the zones of inhibition in the agar plates were measured. Since agar Diffusion test cannot quantify the anti- microbial agent needed to inhibit bacterial growth, Broth microdilution method was used where serial dilutions of ZrO_2 NPs were seeded in microtiter plates against *S. mutans* and *E. faecalis*. The MIC values were read at 490nm spectrophotometrically after 24 hours based on the prominent decrease in growth compared to that of the drug free growth control well. These values are then substituted in the formula to obtain MIC_{50} and MIC_{90}.

The ability of *S. mutans* and *E. faecalis* to form biofilm was evaluated using crystal violet assay and the biofilm formed were classified based on the optical density values using a spectrophotometer at 595nm. The concentration of ZrO_2 NPs to eradicate the biofilm formed by *S. mutans* and *E. faecalis* was evaluated as Minimum Biofilm Eradication concentration using broth microdiltion method. The optical values were spectrometrically read at 490nm which was then substituted in the formula to calculate $MBEC_{50}$ and $MBEC_{80}$.

Conclusion

CONCLUSION

Within the limitations of this invitro study it can be concluded that:

1) Both Streptococcus *mutans* and Enterococcus *faecalis* were sensitive to Zirconium Oxide Nanoparticles (ZrO_2 NPs) at a concentration of 100mg/mL in the agar well diffusion method of Anti-microbial susceptibility testing.
2) The Minimum Inhibitory Concentration of Zirconium Oxide Nanoparticles (ZrO_2 NPs) to inhibit 50% growth of S. mutans and E. faecalis was found to be 11.4µg/mL and 22.8µg/mL respectively and concentrations higher than 22.8µg/mL of ZrO_2 NPs are required to inhibit 90% growth of these two strains.
3) The ability of S. mutans and E. faecalis to form biofilm was confirmed using crystal violet assay which showed strongly adherent biofilms in the microtitre plates evaluated using spectrophotometry.
4) ZrO_2 NPs have the potential to disrupt the biofilms formed by S. mutans and E. faecalis at a concentration greater than 22.8µg/mL.

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Annexures

ANNEXURE –I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Reconized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA
Tele : (044) 24530002, 24530003 - 06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOM SO EVER IT MAY CONCERN

Date: 24th January, 2020

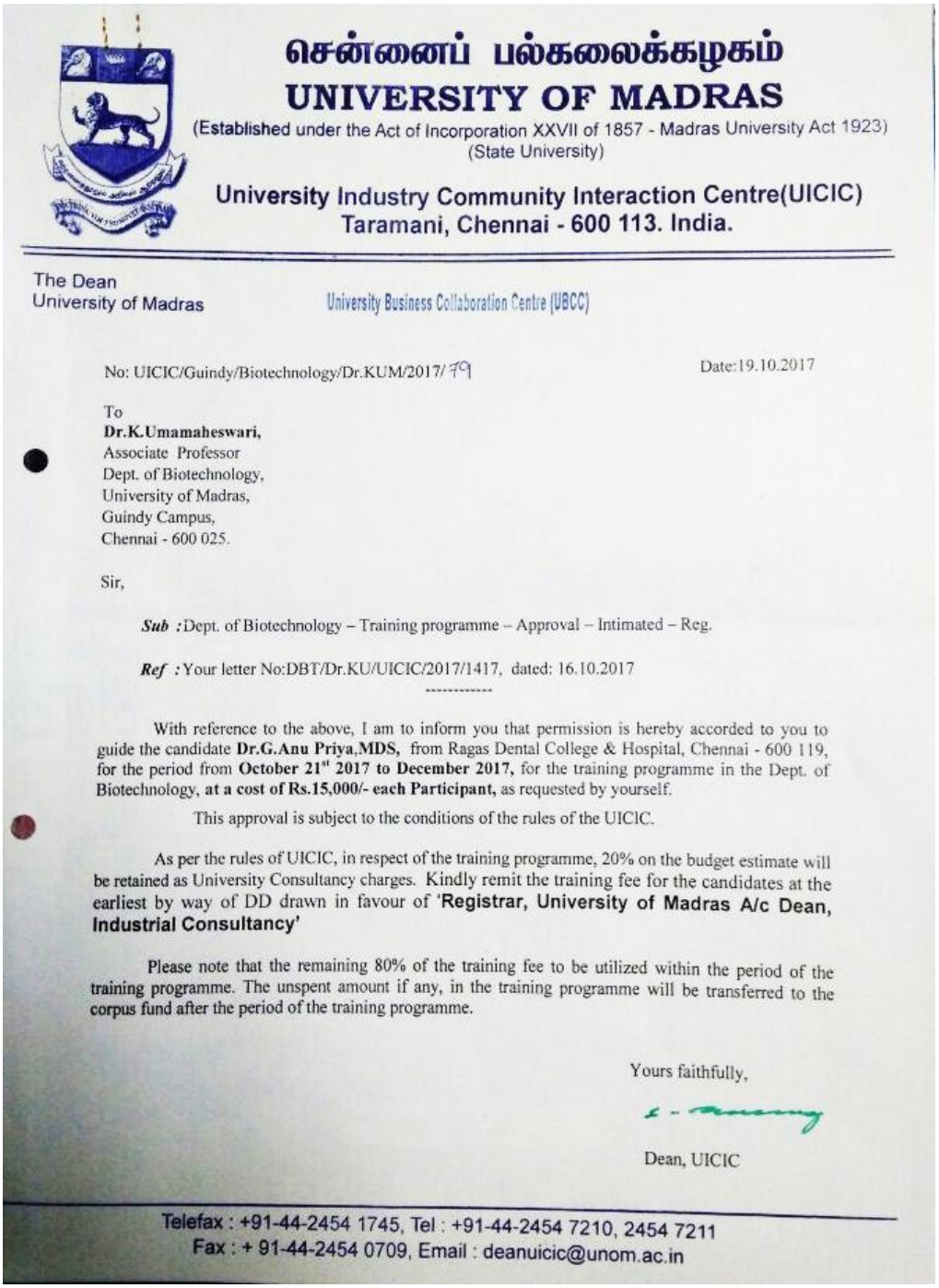
Place: Chennai

From
The Institutional Review Board,
Ragas Dental College and Hospital,
Uthandi,
Chennai- 119

The project titled **"EVALUATION OF ANTI-BACTERIAL EFFICACY OF ZIRCONIUM OXIDE NANOPARTICLES (ZRO_2 NP's) AGAINST STREPTOCOCCUS MUTANS AND ENTEROCOCCUS FAECALIS – AN INVITRO STUDY"** submitted by **Dr. Anu Priya. G** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.

DR. N.S. AZHAGARASAN, MDS
Member secretary,
The Institutional Review Board
Ragas Dental College and Hospital
Uthandi,
Chennai-119

ANNEXURE II



சென்னைப் பல்கலைக்கழகம்
UNIVERSITY OF MADRAS
(Established under the Act of Incorporation XXVII of 1857 - Madras University Act 1923)
(State University)

University Industry Community Interaction Centre(UICIC)
Taramani, Chennai - 600 113. India.

The Dean
University of Madras

University Business Collaboration Centre (UBCC)

No: UICIC/Guindy/Biotechnology/Dr.KUM/2017/79

Date:19.10.2017

To
Dr.K.Umamaheswari,
Associate Professor
Dept. of Biotechnology,
University of Madras,
Guindy Campus,
Chennai - 600 025.

Sir,

Sub **:** Dept. of Biotechnology – Training programme – Approval – Intimated – Reg.

Ref **:** Your letter No:DBT/Dr.KU/UICIC/2017/1417, dated: 16.10.2017

With reference to the above, I am to inform you that permission is hereby accorded to you to guide the candidate **Dr.G.Anu Priya,MDS,** from Ragas Dental College & Hospital, Chennai - 600 119, for the period from **October 21st 2017 to December 2017,** for the training programme in the Dept. of Biotechnology, **at a cost of Rs.15,000/- each Participant,** as requested by yourself.

This approval is subject to the conditions of the rules of the UICIC.

As per the rules of UICIC, in respect of the training programme, 20% on the budget estimate will be retained as University Consultancy charges. Kindly remit the training fee for the candidates at the earliest by way of DD drawn in favour of **'Registrar, University of Madras A/c Dean, Industrial Consultancy'**

Please note that the remaining 80% of the training fee to be utilized within the period of the training programme. The unspent amount if any, in the training programme will be transferred to the corpus fund after the period of the training programme.

Yours faithfully,

Dean, UICIC

Telefax : +91-44-2454 1745, Tel : +91-44-2454 7210, 2454 7211
Fax : + 91-44-2454 0709, Email : deanuicic@unom.ac.in

ANNEXURE III

Nano Labs

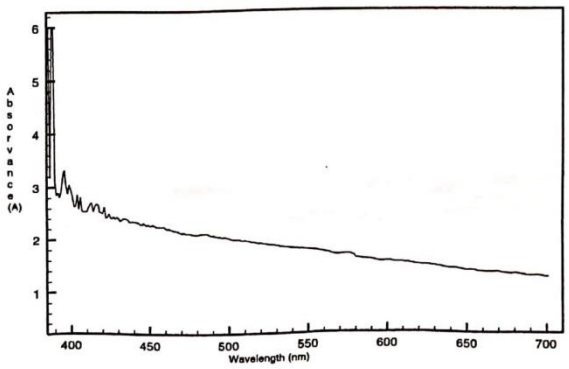
Certificate of analysis

Nanoparticles Name: Zirconium Oxide Nanoparticles/Nanopowder (ZrO2, 15-20nm, purity 99.9%)
Synonyms: Zirconia
Molecular formula: ZrO2
CAS no: # 1314-23-4
Appearance: white
APS: 15-20nm
Purity: 99.9%
SSA: 40-45 m2/g
True Density: 5.89 g/cm3
Bulk Density: 1.3 g/cm3
Morphology: Spherical
Melting point: 2715°C

ZrO2 Nanoparticles Certificate of Analysis--%

ZrO2	Al	Fe	Pb	-
>99.9%	<0.06%	<0.02%	<0.02%	-

UV Spectrophotometer analysis



ANNEXURE – IV

NRL Nano Research Lab

Safety clearance Certificate

Reference: A/16914

This is to certify that the content in this pack are suitable for courier/cargo/freight. The contents are intended purely for research and development purpose.

The materials are non-toxic, non-pathogenic and do not promote fire catching at its normal conditions.

For more information please refer materials safety data sheet and technical data sheet of the respective products available in the packs.

For emergency call us +8757368901 or email us at nanolabs@hotmail.com.

This is being sending for Research and development purpose only not for commercial usage at the following address:

M/s. Anu priya
SARADHALAYAM NO :45, 7 TH STREET,
KAMARAJ NAGAR, AVADI,
CHENNAI - 600071
PH NO : 9940563532,9444642122.

Thanks and regards

Nano Labs

NANO RESEARCH LAB H 21, GOPALPUR

ANNEXURE –V

URKUND

Urkund Analysis Result

Analysed Document:	Anu Thesis- Final.pdf (D63051137)
Submitted:	1/27/2020 3:04:00 PM
Submitted By:	dr.anupriya1993@gmail.com
Significance:	3 %