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Enhanced Antibiofilm Activity of Endophytic Bacteria Mediated Zirconium Nanoparticles

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Abstract: Unraveling the biofilm formation of oral pathogens by endophytic bacteria-mediated zirconia nanoparticles promotes to exhibits as a dental restorative agent by enhancing antibiofilm activity. To demonstrate the mechanistic studies of biogenic zirconium nanoparticle restricts the growth of oral pathogens. The endophytic bacteria isolated from *Terminalia chebula* have valuable secondary metabolites used as reducing agents for the synthesis of zirconia nanoparticles. Characterization studies were done for the application of dental restorative material. The nanospheres' shape and size were confirmed by SEM/EDAX followed by XRD and FTIR for their chemical groups that contribute as the antagonist for biofilm. Parallelly evaluation of antioxidant, antibacterial, anti-inflammatory, and antibiofilm activity is opposing the disease-causing pathogens and examined for biocompatibility on Human Primary Gingival fibroblast cell lines. Therefore, the endophytic bacteria mediated zirconia nanoparticles were biologically assessed against oral pathogens, promoting results to exhibit dental restorative biomaterial.

Keywords: antibiofilm activity; endophytic bacteria; *Terminalia chebula*; zirconia nanoparticle; human gingival fibroblast cell lines (HGF).

1. Introduction

The scope to formulate matter, devices, and methods by atomic precision is known as nanotechnology [1]. Recently, nanotechnology has handled different physics, chemistry, material science, and biotechnology approaches to develop materials that retain peculiar characteristics as its conviction of structures on the nanometre scale. Nanobiotechnology plays a vital role as bio implant materials and a promising drug delivery system. Nanoparticles have remarkable properties like antibacterial activity, high thermal conductivity, high oxidation resistance, and enhanced corrosive-resistance property [2]. Compared with microparticles, the Nanoparticles are reliable and possess a higher surface area when relating to volume and density. Zirconium oxide is a form of ceramic-based nanoparticles with a wide range of applications, including artificial gemstones, deodorants, oxygen sensors, catalyst supports, furnace bricks, and abrasive applications [3]. In the biological synthesis of nanoparticles, green plants, fruits, and seeds were used to obtain the phytochemical properties to be capped on the nanoparticle. Besides, microbes and microbial byproducts were utilized, and the percentage of the release of a hazardous substance into nature remains null [4]. Thus the green method of synthesis has cost-effective manner and reproducible [5,6]. *Terminalia chebula* is the scientific name of Kadukai, a common name in Tamil that has rich secondary metabolites that were

extensively utilized to treat diabetes milletus, skin disorders, and oral hygiene [7]. *Terminalia chebula* belongs to South Asia. In Ayurveda, Kadukai is proposed for leading a long healthy life and termed as multipurpose herb, which includes the digestive tract's cleansing; elimination of bacteria, worms, and parasites prolonged existence leads to cancer effect in the human body [8,9]. The endophytes present *Terminalia chebula* was less explored, and the fungal species are known for the synthesis of nanoparticles [10,11] and have potent biological activity. Compared to that of extract of seed as reducing agent, the synthesis method was examined to treat the oral pathogens present in the human oral cavity, which causes biofilm-mediated disease [11,12]. Examples of oral pathogens include anaerobic bacteria such as Arachnia, Actinomyces, Bacteroides, etc. [13]. Dental caries and periodontitis are some of the oral diseases caused when there is an imbalance in pH, extracellular polysaccharides, and antibiotic resistance [14,15]. This study focus on the Zirconium nanoparticle synthesized by endophytic bacteria against biofilm formation pathogens and evaluated for its biocompatibility on Human Gingival fibroblasts cell line.

2. Materials and Methods

2.1. Isolation of endophytes.

Terminalia chebula, common name Kadukai seed, was collected from Amarti forest in the Vellore region, Tamil Nadu, India. Surface sterilization was done for the collected samples with a slight modification of the procedure [16]. 4% of sodium hypochlorite solution was used to rinse the seeds, followed by 70% ethanol wash and dried in sterile condition and then transferred into tryptic soy agar medium by incubating for 5 days at 37°C [17]. The colonies were selected by biochemical method and identified by molecular sequencing. The potent strain was submitted in the GenBank database of the National Centre of Biotechnology Information (NCBI) with accession id: SUB5320944 *Bacillus niacini* was used to synthesize zirconium oxide nanoparticles [18].

2.2. Biogenic zirconium nanoparticles.

Zirconium oxychloride ($ZrOCl_2 \cdot 8H_2O$) was taken as a precursor 2 mM was dissolved in 100 ml of culture containing *Bacillus niacini* and kept under the stirrer for 8 h at 1000 rpm in 60°C this was mentioned as SV2 method. The Zirconium oxide nanoparticle was synthesized by extract of *Terminalia chebula* seeds of about 60 ml of extract was mixed with 40 ml of the same concentration dissolved in MilliQ water and placed in magnetic stirrer with 80°C for at 600 rpm 10 h, which was mentioned as SV1 [19]. The nutrient present in the medium acts as a supplement and enhancement agent for bacteria and sucrose growth and enzymes act as catalysts for the reaction process. This was assessed once every hour by UV readings range 200-800 nm to synthesize zirconium oxide nanoparticles [20,21]. After the reaction progressed, the solutions were centrifuged to get cell-free nanoparticles by washing the pellets with 70% ethanol twice and placed in a hot air oven at 50°C for 24 h. The fine powder was taken for biological evaluation [22].

2.3. Experiment studies.

The biogenic zirconium nanoparticles synthesized via green synthesis was confirmed by characterization method such as UV-visible spectrum, FTIR, XRD, SEM and EDAX After all this characterization, the sample was further analyzed for biocompatibility [23,24].

2.4. Biofilm activity.

The oral pathogenic strains, including *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris*, *Streptococcus mutant*, and *Staphylococcus aureus*, were gifted from Kalvi dental clinic Konavattam Vellore region, Tamil Nadu. All the strains were subcultures in a selective medium. With McFarland standards, the culture stock was prepared with *Luria bertin* broth of about 10 mL, in which 10 µL of culture was added and incubated at 37 °C for 48 h. The matured culture was then diluted to 10-fold in the growth medium to form biofilm in a 24-well plate, 1 mL was seed, and with various concentration of 20, 40, 60 µL of Biogenic zirconium nanoparticle were made up to 1 ml was added and incubated 37 °C for 72 h to form mature biofilms. After incubation, the broth was removed and added with 0.5% phosphate buffer saline to get free from cell debris and rinsed twice, followed by 0.1% crystal violet, this was leftover for 10 min, and then stain was removed by 70% ethanol to takeoff excess dye. Then, 4% glacial acetic acid was added, and OD (optical density) was measured after 15 min at 540 nm using an ELISA reader [25,26].

2.5. MTT assay.

200 µl of human gingival fibroblasts cell suspension was seeded in a 96-well plate at required cell density (20,000 cells per well), without the test agent, and incubated for 24 h. About 40 µl of zirconium nanoparticles were added and allowed for 24 h at 37°C in a 5% CO2 atmosphere to the cell line. After the incubation period, media was removed, and MTT reagent was added and followed by 100 µL of solubilization solution DMSO [27]. Stirring was done in a gyratory shaker which promotes dissolution of MTT formazan crystals in cell culture. An ELISA reader took the absorbance at 630 nm used as reference wavelength [28].

3. Results and Discussion

3.1. UV-Visible spectrometry.

In Figure 1, UV-Visible spectrometry reveals the biogenic zirconium oxide nanoparticle's spectral pattern from *Terminalia chebula* at the peak range between 200-1000 nm. The standard zirconium oxide was used as the reference for the characterization studies that emit at 235 nm. In contrast, biogenic zirconium oxide nanoparticle (SV1) from seed extract of *Terminalia chebula* emit peaks at 260 nm [29], and another synthesized zirconium nanoparticle (SV2) from endophyte *Bacillus niacin* emits at the peak of 290 nm at 5 h [30]. From this synthesis pattern, the synthesis process factors were influenced and enhanced the product formation. The metabolites in endophytic bacteria give an expeditious response when compared with seed extract [21,31].

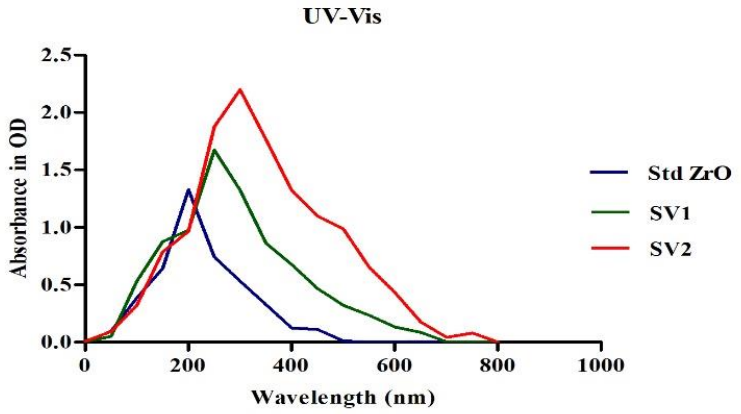


Figure 1. The spectral pattern reveals the speak emitted for SV1, SV2, and Std ZrO.

3.2. XRD.

In Figure 2, the XRD pattern of synthesized biogenic zirconium nanoparticles was compared with JCPDS – 79-1771 reveals that the grain size differs from the synthesis pattern [32]. Crystalite size of the nanoparticle was found to be 15.52 nm for the SV1 method due to the phytochemicals present in the seed extract, and it reduces the charge and stability [33], whereas 60 nm size for SV- 2, bacteria uptakes the nutrients and produces the secondary metabolites which enhance the product formation with aggregation, after the calcination process the synthesized zirconium nanoparticles are reduced in size. This can be achieved by at high temperature in muffle furnace then the particles become fine powder with no aggregation [34].

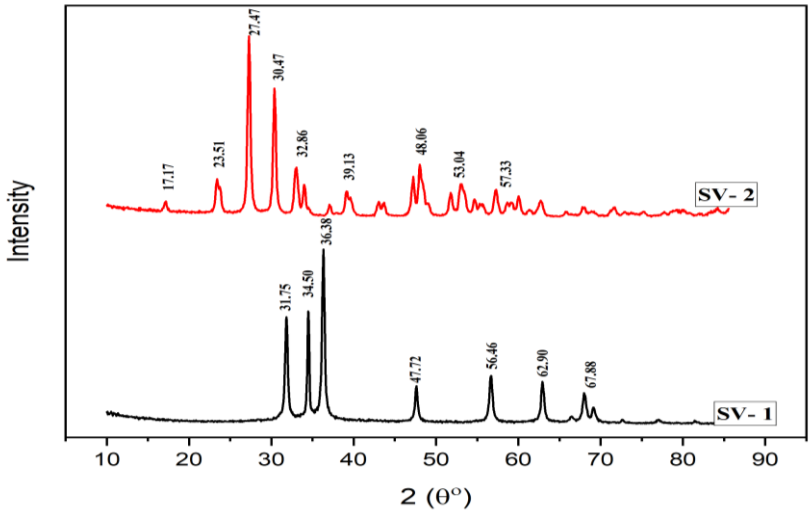


Figure 2. The XRD graph representation for size of biogenic zirconium oxide and nanoparticle.

3.3. FTIR.

In Figure 3, FTIR spectral patterns represent the standard zirconium oxide and SV1 for the green synthesis of zirconium oxide nanoparticles by seed extract. For std, the peak of 673.36 and 746.26 refers to the zirconia metal, and 3438.40 represents oxide. In the SV1 spectral band, the extract acts as a capping agent, and the phytochemicals like alkaloids, flavonoids, phenols, carbohydrates, glycosides, terpenoid, saponin, and acids enhance the formation of the nanoparticles bands at 3327.73 gives the OH stretch [35], 1610.56, 1383.06, 1249.50, 1100.02, 1012.40 corresponds to alcohol/phenol O-H stretch, aromatic C=C bending, aromatic C-H bending and aliphatic iodine compound [29]. For SV2 spectral pattern indicates at 3292.49,

1610.56, 2871.01, and 2713.76 prominent to CH_2. 2434.61 and 2027.01 due to vibration of –C=C– the stretch of alkenes and 1541.17 aromatic C=C bending with aromatic C-H bending 669.13 attributes for aryl disulfides [36].

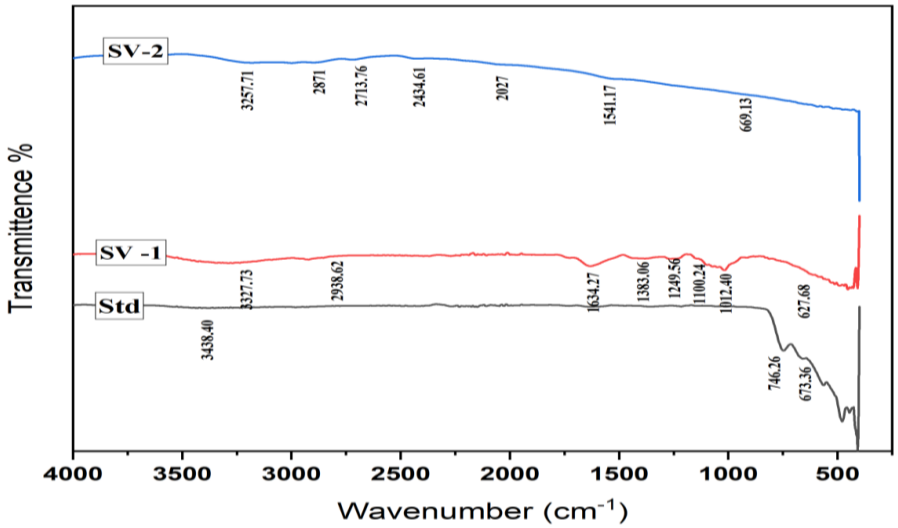
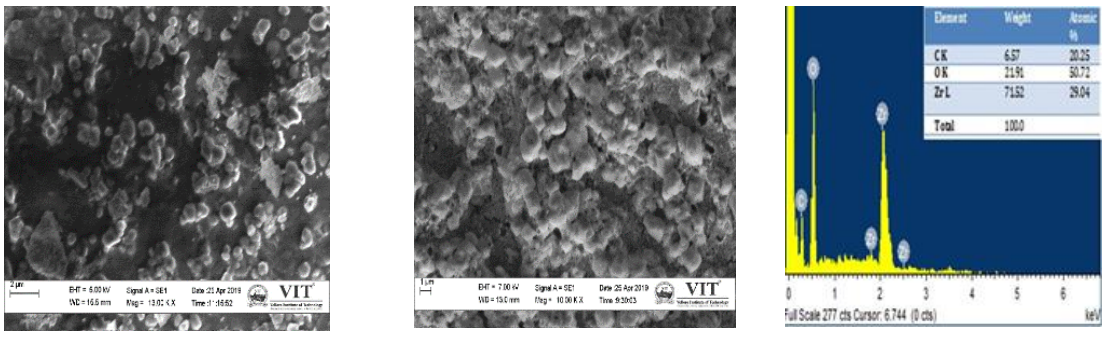


Figure 3. FTIR spectrum for biogenic zirconium nanoparticle.

3.4. SEM/EDAX.

In Figure 4 (a), the synthesized zirconium oxide nanoparticles are eventually distributed in the SEM image with no aggregation. The phytochemicals that are responsible for the reducing agent result in the formation of the oxide nanoparticles, whereas in (b) promptly occurrences with evenly shape was due to the bacterial conjugation of metal-binding sites to the precursor and the production of acids from *Bacillus* sp enhances the synthesis pattern of the nanoparticles. In (c), there was evidence of elements present in the synthesized nanoparticles with zirconia, carbon groups, and oxygen groups were equally distributes [37,38].



(a) **(b)** **(c)**

Figure 4. (**a**) SEM of SV1; (**b**)SEM of SV2; (**c**) EDAX.

3.5. Antibiofilm activity.

In Table 1, the oral pathogens show significant removal of biofilm formation when treated with a respective concentration of 40 µg/mL of zirconium nanoparticles SV2 compared to that of zirconium oxide nanoparticle SV1 for *E.coli* 81% of biofilm removal and 91.5%, similarly prominent results obtain for the virulent strain *S.aureus* 92.5% removal of biofilm treated with SV2 and 80.5% of SV1. The commonly existing pathogenic microbial strain was *S. mutant* has 90.5% removal when treated with SV2 and 83.5 % when subjected to SV1. At

higher concentrations, the viability of the cell may lead to a toxic effect [39]. The exopolysaccharides were responsible for induced biofilm formation with the receptor [40] glucan-binding domains of glucosyltransferase play a vital role in forming biofilms by glucan binding proteins in virulent strains of *S.aures* and *S.mutant* [41]. In some adverse conditions, the exuberance of pathogenic resistance on antibiotics reoccurrence occurs and may lead to rapid cell growth in the oral tissues [42]. This can be addressed using medication with natural products and the combination of the dual role by direct contact killing of pathogens as well as the release of ionic by the capped agents in the nanoparticles with monomers [43].

Table 1. Antibiofilm activity of synthesized SV1 and SV2 ZrO nanoparticles.

Oral Pathogenic Strain isolated from human	Biofilm removal % for SV-1	Biofilm removal % for SV-2
Escherichia coli	81	91.5
Klebsiella aerogenes	62.5	71
Proteus vulgaris	73.5	83.25
Staphylococcus aureus	80.5	92.5
Streptococcus mutant	83.5	90.5

3.6. MTT assay.

In Figure 6, MTT assay was done on human gingival fibroblasts (HGF) cell line, the biomaterial synthesized was found to be less toxicity and the viability was higher [44] the cell count was accepted to be in 94% for SV1 [45]. In Figure 6 (b) and 98% for SV2 in figure 6. (d). The morphological changes were not noticed and remain the same when compared with control cells [46]. In the graph, SV2 treatment reveals that at the higher concentration of 60 µg/ml was treated to HGF. The viability percentage was slightly down to 94.34% when correlated with the lower concentration of 40 µg/ml by 98.18% [23]. The biomaterials are generally less toxic when used at the standard permissible limitation followed. With these standards, the biomaterials expose the valuable biological effects that promote biocompatibility and can be implemented in the drug delivery systems [47].

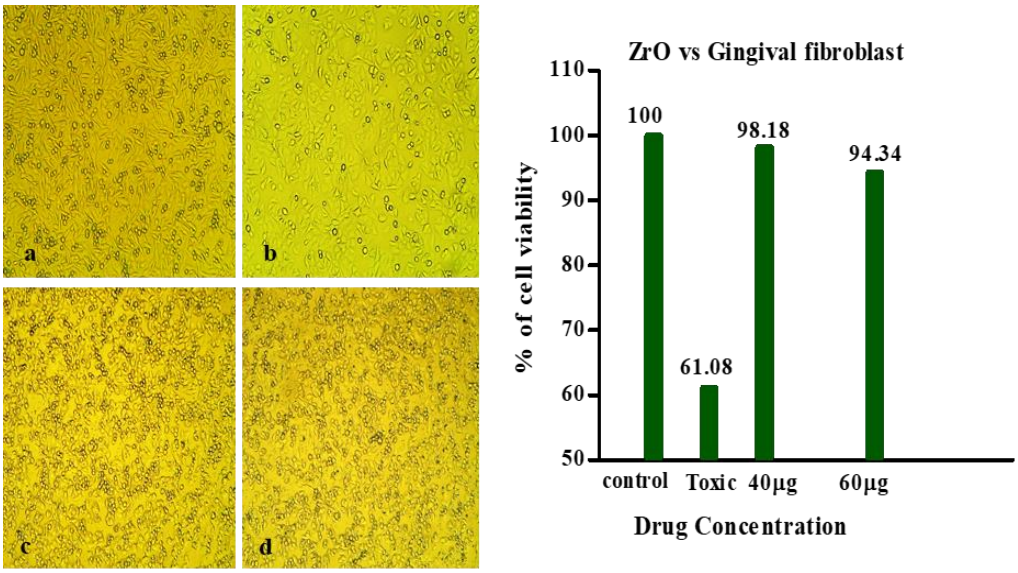


Figure 6. (**a,b**) Gingival fibroblast cells treated with SV1; (**c,d**) Gingival fibroblast cells treated with SV2. The graph represents the % cell viability in the human gingival fibroblast cell line.

4. Conclusions

Nowadays, generally, the dental crowns and restorative materials are made up of zirconia and titanium metals which are not favorable to microbial adhesion. The drawback of these materials was expensive. To overcome this financial crunch on the biomaterials, these kinds of biosynthetic zirconia materials can fetch up in combining both antibiofilm and dental restorative materials. Further *in vivo* studies have to be carried followed by the release mechanism of biogenic zirconium nanoparticles.

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Conflicts of Interest

The authors declare no conflict of interest.

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