Phytofabrication of Zinc Oxide Nanoparticles Synthesis from Acacia Nilotica Bark and its Antibiofilm Activity Against Dental Caries Pathogens

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**Abstract:** Dental caries is a common chronic infectious disease resulting from tooth-adherent cariogenic bacteria, primarily *Streptococcus mutans,* which metabolize sugars to produce acid, demineralising the tooth structure over time. *Acacia nilotica* (L.) trees grow near rivers. Their seeds are traditionally used as an antiseptic or mixed with yogurt to treat dysentery, heal pustules in the skin, and treat sore throat and cough. Biofilm is the self-synthesized, mucus-like extracellular polymeric matrix that acts as a key virulence factor in various pathogenic microorganisms, thereby posing a serious threat to human health. The present study focused on the synthesis of zinc, oxide nanoparticles mediated by *A.nilotica* bark extract, and its anti biofilm activity against dental caries, causing pathogens (*Streptococcus mutans* and *Enterococcus faecalis*). *Acacia nilotica* bark collected from the local market used in the study. Further, a series of morphological, physiological, and conventional biochemical tests were performed to identify the selected microorganisms. In addition to this, the study conducted the following tests: Green synthesis of ZnO NPs from *Acacia nilotica* bark extract, XRD analysis of synthesized ZnO NPs, EDAX images of synthesized ZnoNPs,SEM pictures of synthesized ZnO NPs, Microtiter plates demonstrating the antibiofilm activity of ZnO NPs. Microtiter plate optical density (OD) 600nm reading (mean± standard error) of biofilm formation with ZnO NPs added at concentration of 25 to 100µg/mL against *Streptococcus mutants* and *Enterococcus faecalis.* In this study, Antibiofilm activity of ZnO NPs was evaluated by measuring biofilm growth with crystal violet in the presence of varying concentrations of ZnO NPs. ZnO NPs successfully reduced biofilm formation at concentrations of 100 μg/mL (*p <*0.05). ZnO NPs showed significant dose-dependent antibiofilm activity against S. mutans. The positive control without ZnO NPs exhibited growth at an OD of 1.123. Notable inhibition of colony growth would decrease OD. Bacterial growth was mildly inhibited by the addition of 50 μg/mL ZnO NPs (OD of 0.349) and was decreased further with the addition of increasing ZnO NP concentrations (OD of 0.3 at 100 μg/mL). Compared to previous study,Meroni et al analyzed the antibiofilm abilities of biological-derived AgNPs with a size of 11 nm. The abilities against *S. mutans* were not identified.In this study, we suggest that the *A. nilotica bark* has the potential to be further developed as a novel antibacterial drug.ZnO nanoparticles are commonly used in sunscreens due to their ability to block ultraviolet (UV) radiation. They provide broad-spectrum UV protection and are preferred for their transparency on the skin compared to other sunscreen agents. ZnO nanoparticles exhibit antimicrobial properties and can promote wound healing. They have been incorporated into wound dressings and ointments to prevent infection and accelerate the healing process.

**Keywords:** Anti biofilm activity, *Acacia nilotica,* Zinc oxide nanoparticles, Dental caries, pathogens.

# INTRODUCTION

Dental caries is a common chronic infectious disease resulting from tooth-adherent cariogenic bacteria, primarily *Streptococcus mutans,* which metabolize sugars to produce acid, demineralizing the tooth structure over time[(Wang et al., 2023)](https://paperpile.com/c/WCgzEP/bJm8C).​​ Green synthesis employs a clean, safe, cost effective and environmentally friendly process of constructing nanomaterials[(Srivastava et al., 2020)](https://paperpile.com/c/WCgzEP/MsSym). Microorganisms such as bacteria, yeast, fungi, algal species and certain plants act as substrates for the green synthesis of nanomaterials. The rise of multidrug-resistant bacteria in hospital facilities has left physicians with fewer treatment options, resulting in more expensive therapies[(Aparna et al., 2021; Sarani et al., 2023)](https://paperpile.com/c/WCgzEP/o0Mf+OU84).

*Acacia nilotica* bark boasts a diverse array of phytochemicals, including alkaloids, flavonoids, and tannins[(Zahid et al., 2023)](https://paperpile.com/c/WCgzEP/CiSr7). These compounds become pivotal in the synthesis of zinc oxide nanoparticles, influencing their properties and potential applications[(Hou et al., 2022)](https://paperpile.com/c/WCgzEP/U3qSz). The use of Acacia nilotica bark aligns with green synthesis principles, emphasizing sustainability in nanoparticle fabrication(Rafi et al., 2024). This eco-friendly approach capitalizes on natural resources, minimizing environmental impact and promoting a greener alternative to conventional synthesis methods[(Zubair et al., 2022)](https://paperpile.com/c/WCgzEP/JKNa6). The biosynthesis of nanoparticles from microbes and plant extracts has attracted researchers' attention because their small size, large surface area, orientation, and physical properties make them suitable to be used in medical sciences[(Karthik et al., 2019; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/WCgzEP/CN5P+Dw9o). Furthermore, they have a low cost and are not harmful to nature.

Antimicrobial resistance (AMR) is a global issue that is thought to be causing an increase in morbidity and mortality (Tuluwengjiang et al., 2024). With the use of nanoparticles (NPs), nanotechnology has developed a cutting-edge answer to the antimicrobial resistance issues of today[(Poornima et al., 2021a; Saravanan et al., 2022)](https://paperpile.com/c/WCgzEP/OUzH+sbsc). Due to their ability to withstand the harsh conditions of many processes, metals and their oxides have garnered a lot of attention in recent decades. Because zinc oxide is harmless, non-toxic, and stable, it is one of the metal oxides that is of particular importance. Because of their uses in biology, such as biological sensing, biological labeling, nanomedicine, gene transport, and drug administration, zinc oxide nanoparticles have gained increased appeal. Zinc oxide nanoparticles are naturally known as a strong resistance of microbes[(Upadhyay et al., 2023)](https://paperpile.com/c/WCgzEP/MHHix). ZnO is a multipurpose material that has been utilized in a multiplicity of applications such as UV absorption, antibacterial treatments, sunscreen lotions, catalysts, photocatalysis, biological applications due to their great biocompatibility, low toxicity, and economic.[(Ganapathy 2022)](https://paperpile.com/c/WCgzEP/7RVM+3fxo) Zinc oxide-eugenol cements are considerably better tolerated by tissue than other dental materials. As they alleviate pain and are bacteriostatic and antiseptic, they are well tolerated by patients.[(Merchant et al., 2022; Setty et al., 2023)](https://paperpile.com/c/WCgzEP/x9As+uakq)

Several studies have employed aqueous extracts of *Acacia nilotica* as a bioreductant to produce nanoparticles from silver-doped TiO2, iron nanoparticles (FeNPs), and gold nanoparticles and to examine their potential as antibacterial and anticancer agents.[(Chokkattu et al., 2022; Marya et al., 2022; Ramamurthy et al., 2022; Rao et al., 2019)](https://paperpile.com/c/WCgzEP/BSw8+9KED+vRpM+sdEq) This survey may pave the way for additional research in this field, and we anticipate that the use of photo-synthesized nanomaterials will broaden the research topics pertaining to the fight against dangerous bacteria and biofilms as well as the range of novel applications for metal oxide nanoparticles[(Barabadi et al., 2023; Jain & Verma, 2022)](https://paperpile.com/c/WCgzEP/kAY9+nO6r). The current study used a green chemical approach to create zinc oxide (ZnO) based nanoparticles (NPs), and the bark extract of *Acacia nilotica* demonstrated potent antibacterial activity against *Streptococcus mutans* and *Enterococcus faecalis*.

# anMATERIALS AND METHODS

## Collection of Acacia nilotica

The bark of *Acacia nilotica* were collected and thoroughly washed under running water, dried at room temperature, and then cut into small pieces to remove adhering dust particles.

## Leaf extract preparation

In a 500-mL Erlenmeyer flask, 10 g of chopped leaves was combined with 100 mL of double distilled water to produce an aqueous extract of A. nilotica. The mixture was allowed to stand at 60 °C for 20 min before being filtered through a Whatman no. 1 filter paper. The filtered bark extract was used to synthesize Zinc oxide nanoparticles. Until it was required again, extra leaf solution was stored at − 20 °C.

## Biosynthesis of silver nanoparticles

Ten milliliters of the leaf extract was mixed with 90 mL of a 1.0mM in distilled water solution. Within 24 h, a color change from yellow to brown was observed. This indicated the formation of colloidal ZnO NPs. After 15 min of centrifugation at 10,000 rpm, ZnO NPss were obtained. Before the pellet was characterized, it was spun in a centrifuge three times, freeze- dried, and ground into a powder. Only then was it put back into deionized water.

## Green Synthesis of ZnO NPs

The supplier of the zinc nitrate hexahydrate (Zn (NO3)2. 6H2O) was Sigma-Aldrich Chemicals in India. Fresh leaves were washed three times in the presence of distilled water to remove dust, chopped, and added to water (1:10) at 60ºC while being continuously stirred for 30 min. After filtering, the mixture was cooled and kept at 40ºC for additional use[(Nasim et al., 2022)](https://paperpile.com/c/WCgzEP/mAbjz). 24h spent shaking the leaf extract with 0.2M zinc nitrate (1: 9). The colour change of the liquid from brown into a semi-solid creamy colour indicated the formation of ZnO NPs. The phytochemicals found in biomaterials (such plant extract) can function as reducing agents, transforming the metal precursors into metal nanoparticles (NPs). Materials having phytochemicals may act as both reducing and stabilizing agents because they include antioxidants and toxic-free substances.

## Anti biofilm susceptibility screening by 96-microtiter well plate method

A 96-microtitre well plate was used to conduct a quantitative investigation on biofilm development. Freshly grown bacteria were added to Brain Heart Infusion (BHI) broth and the mixture was then incubated for 72 h at 37 ºC. The cell suspensions were diluted at a ratio of 1:100 in the freshly made BHI broth medium after 24 h. Bacterial cells that were not exposed to ZnO NPs were regarded as the positive control. ZnO NPs were also added to the treated bacterial cultures at a concentration ranging from 25, 50, 75 and 100 µg/mL. The sterile BHI broth medium remained empty. Then, 200 µL culture suspensions with and without ZnO NPs treatment were added to the sterilised 96-well microplates, which were then incubated for a further 24 h at 37 ºC without shaking. Three replicates of each bacterial suspension were stored. By rotating the plates over, all of the treated and untreated cells in the microtiter wells were discarded. Free-floating cells and undesirable material were then removed by washing the plates three times in phosphate buffered saline (PBS, pH 7.2).

## Minimal inhibitory concentrations

MICs of AgNPs against different bacterial strains were determined using an amended broth macro-dilution method. For the estimation of MIC, the stock solutions of AgNPs (0.5, 1, 2.5, 5, 7.5, 10, 12.5, 15, 25, 50, 100, and 200 μg/mL) were prepared. The sterile Mueller–Hinton broth was transferred into a sugar test tube (13×100 mm) containing 2.0 mL of bacterial inoculum (culture density of 5×105 CFU/mL) in two sets for each bacterial strain[(Kamath et al., 2022)](https://paperpile.com/c/WCgzEP/iDMqN). Subsequently, each test tube was mixed with 2.0-mL individual concentrations of AgNPs, limiting the final tube volume to 4.0 mL, resulting in a 1:2 dilution followed by incubation for 24 h at 37 °C. The optical density (O.D.) of microbial growth was measured at 600 nm. The lowest dose of AgNPs exhibiting no growth after incubation was considered the MIC endpoint. The turbidity of bacterial growth and MBC values were determined by swabbing the bacterial culture on MHA plates followed by incubation at 37 °C for 24 h. The MBC is considered the concentration of extract at which bacteria are completely killed.

## Antibacterial activity

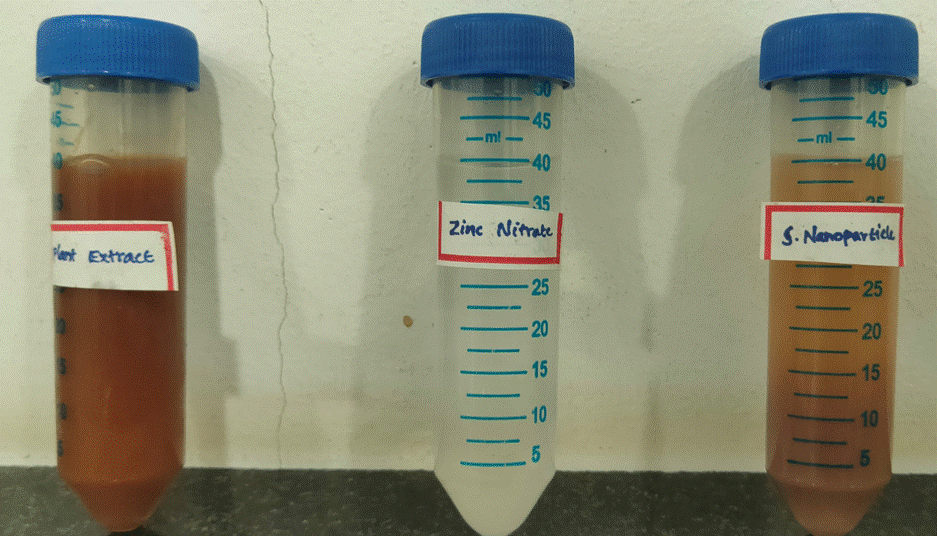
The *E. alba*-mediated silver nanoparticles were tested for antibacterial activity by employing the agar well diffusion method. The clinical pathogenic strains of *S. mutans* and *C. albican*s were acquired from the Department of Cariology. The pathogenic cultures were properly sub-cultured and maintained in our laboratory. In the antibacterial assays, AgNPs (50 and 100 μg/mL) were poured into the wells of Mueller–Hinton agar (MHA) plates, respectively, after which they were incubated for 24 h at 37 °C and 25 °C, respectively. The antibiotic chloramphenicol was used as a positive control. The growth inhibition zones were measured by the zone inhibition scale (Hi- Media, India).

## Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used to do statistical analyses after the information that was assessed was graph in a Microsoft Excel spreadsheet (SPSS Statistics; version 23, IBM, SPSS Inc, Chicago, United States of America). Analysis of the group samples' means and standard deviations was done using descriptive statistical methods. Three sets of samples' means were compared using a one-way analysis of variance (ANOVA) test for numerical data. To identify which of the three groups is in charge of the significant difference, the post hoc Tukey's test was used.

# RESULTS

Physiochemical changes take place in the aqueous solution when zinc acetate dihydrate is added to *A. nilotica* extracts. The mixture's hue shift is one of the clearest signs that the green synthesis of ZnO NPs was effective. Within 50 minutes of the synthesis process, this alteration is noticeable. This was regarded as the first stage in the NP synthesis process. The color shifts from yellow to light brown in the current study. The chemical components flavonoids and phenolics found in peel extract are thought to be the ones that convert zinc ions into zinc oxide nanoparticles. The color of the solution stopped changing after three hours, meaning that ZnO salt has fully bio-reduced into NPs (Fig.1).

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**Fig. 1:** Green synthesis of ZnO NPs from *Acacia nilotica* bark extract.

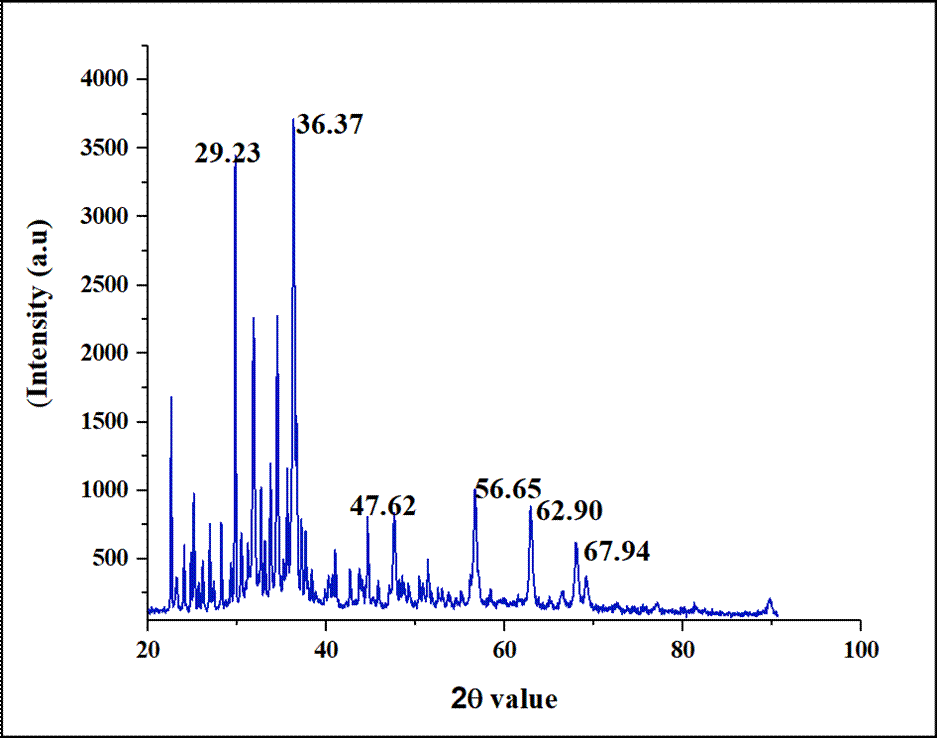
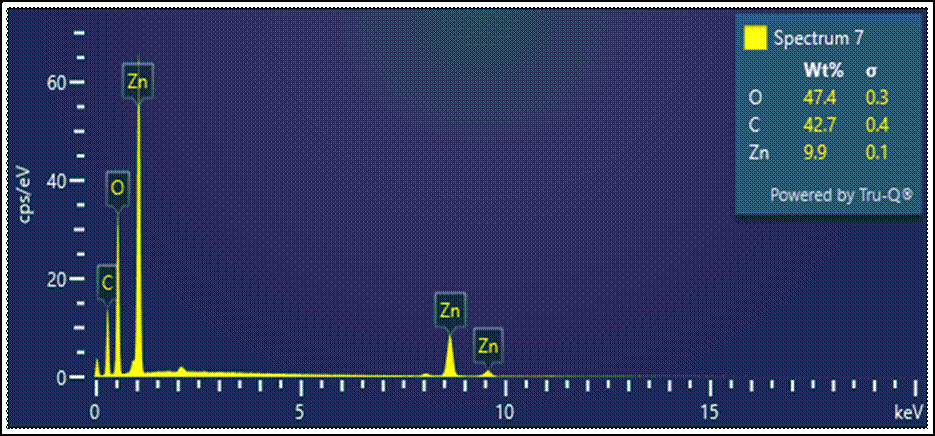


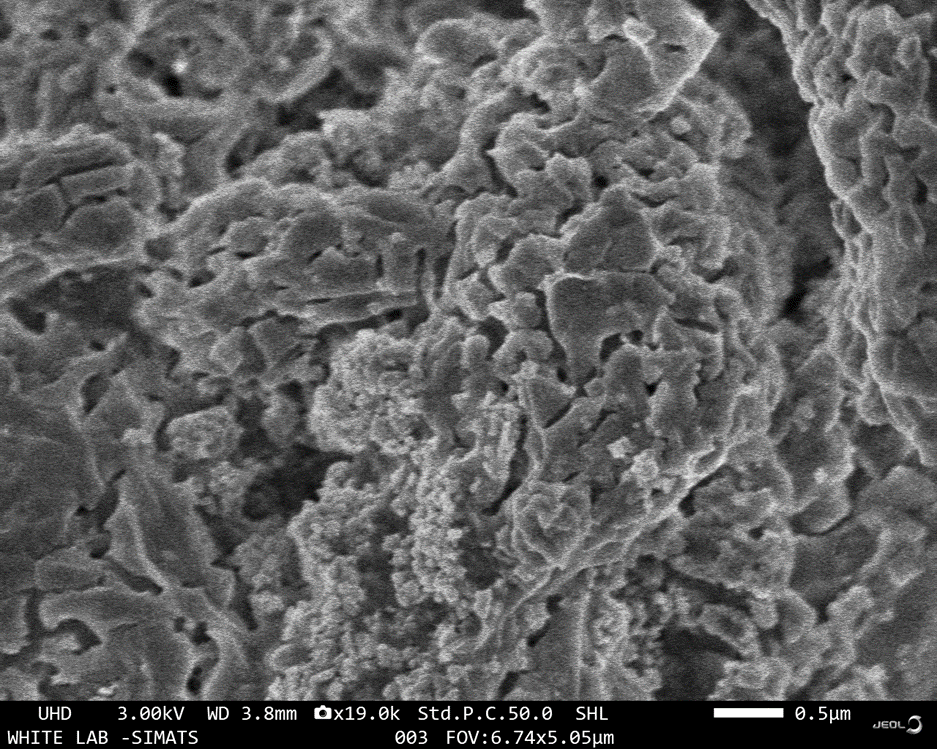
Fig. 2: XRD analysis of synthesized ZnO NPs

The ZnoNPs were subjected to an XRD analysis in order to determine their size, phase identification, and crystalline nature. The results of the XRD pattern demonstrated an unambiguous confirmation of the cubic crystalline lattice of ZnoNPs in the 2θ values of 20–80°. The XRD spectrum exhibited three distinct diffractive peaks corresponding to the lattice planes of the Bragg’s reflection of ZnoNPs 29.23°,36.37°,47.62°,56.65°,62.90°,67.94° (fig. 2).



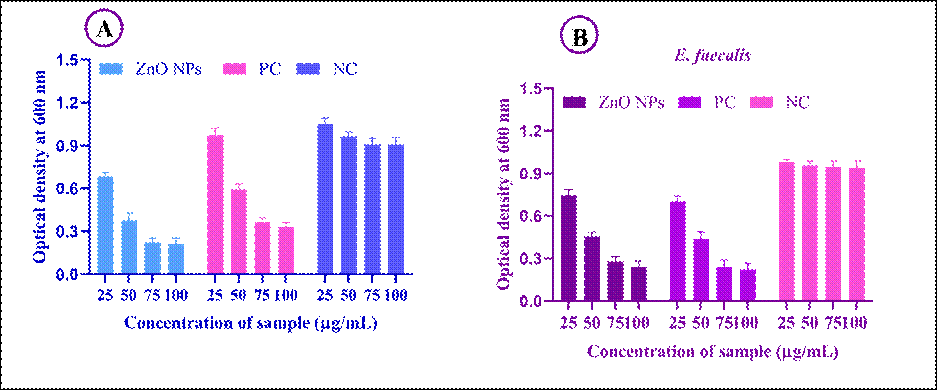
**Fig. 3:** EDAX images of synthesized ZnO NPs

Energy-dispersive X-ray analysis (EDAX) is a technique used for the measurement of nanoparticles by SEM. In this technique, the nanoparticles are analyzed by activation using an EDS X-ray spectrophotometer, which is generally present in modern SEM. The individual separated nanoparticles are deposited on a suitable substrate that does not interfere in the characterization of nanoparticles. This method has found some limitations with regard to accurate dimensional and elemental characterisation (fig. 3).

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**Fig. 4:** SEM pictures of synthesized ZnO NPs

SEM micrographs showed that as the amount of MEA increases, the grain sizes steadily decrease. The findings of the UV-visible diffuse reflectance spectroscopy show that the ZnO NPs have a large band gap energy and wider absorption bands (fig. 4).



**Fig 5:** Microtiter plates demonstrating the antibiofilm activity of ZnO NPs. Microtiter plate optical density (OD) 600nm reading (mean± standard error) of biofilm formation with ZnO NPs added at concentration of 25 to 100µg/mL. A) *Streptococcus mutans* and B). *Enterococcus faecalis* (fig. 5).

# DISCUSSION

The Acacia nilotica bark aqueous extract (AN-ZnO NPs) was applied in order to generate zinc oxide nanoparticles using the phytonanotechnology approach. Pathogenic microorganisms that are developing resistance to chemically manufactured antibiotics are the real threat to humanity's survival[(Raheel et al., 2013; Sreevarun et al., 2023; Wadhwani et al., 2022)](https://paperpile.com/c/WCgzEP/XShC+At7W+t1Hp). However, a great deal of systemic toxicity[(Tulsani et al., 2021; UmaMaheswari et al., 2022)](https://paperpile.com/c/WCgzEP/VWQsF+sCdr6)is caused by medicinal medicines that are chemically manufactured. Antibiofilm activity of ZnO NPs was evaluated by measuring biofilm growth with crystal violet in the presence of varying concentrations of ZnO NPs[(Adel et al., 2023; Meky et al., 2023)](https://paperpile.com/c/WCgzEP/AOMm+uFdv). ZnO NPs successfully reduced biofilm formation at concentrations of 100 μg/mL (p = 0.005). ZnO NPs showed significant dose-dependent antibiofilm activity against S. mutans (Fig.). The positive control without ZnO NPs exhibited growth at an OD of 1.123. Notable inhibition of colony growth would decrease OD. Bacterial growth was mildly inhibited by the addition of 50 μg/mL ZnO NPs (OD of 0.349) and was decreased further with the addition of increasing ZnO NP concentrations (OD of 0.3 at 100 μg/mL). [(Chokkattu et al., 2023; Muthuswamy Pandian et al., 2022)](https://paperpile.com/c/WCgzEP/xz9i+CPL8)Antibiofilm activity of ZnO NPs was evaluated by measuring biofilm growth with crystal violet in the presence of varying concentrations of ZnO NPs[(Subramanian & Harikrishnan, 2023; Ushanthika et al., 2021)](https://paperpile.com/c/WCgzEP/kL7z+cqus). ZnO NPs successfully reduced biofilm formation at concentrations of 100 μg/mL (p = 0.005). ZnO NPs showed significant dose-dependent antibiofilm activity against *S. mutans*.[(Aparna et al., 2021; Goyal et al., 2023)](https://paperpile.com/c/WCgzEP/Ddm84+OU84) The positive control without ZnO NPs exhibited growth at an OD of 1.123. Notable inhibition of colony growth would decrease OD. Bacterial growth was mildly inhibited by the addition of 50 μg/mL ZnO NPs (OD of 0.349) and was decreased further with the addition of increasing ZnO NP concentrations (OD of 0.3 at 100 μg/mL)[(Nasim et al., 2020)](https://paperpile.com/c/WCgzEP/osziB)[(Nasim et al., 2020; Ushanthika et al., 2021)](https://paperpile.com/c/WCgzEP/kL7z+osziB)

As we compared to another study, MRSA and ESBL are examples of bacteria that form biofilms that have a protective layer to survive antibiotic treatment. Larger concentrations of *C. sinensis* extracts (14%–68%) in PS11 are associated with superior biofilm removal by ESBL-producing *P. aeruginosa*. Abraham et al.'s findings[(Xia et al., 2023)](https://paperpile.com/c/WCgzEP/PGsXL) show that methanolic caper extraction significantly reduced the production of extracellular polymeric substances (EPS) and the generation of biofilms in *Proteus mirabilis, P. aeruginosa, Serratia marcescens, and E. coli.*[*(Aditya et al., 2021)*](https://paperpile.com/c/WCgzEP/9qaPJ)Similarly, MRSA biofilm reduction is better at higher concentrations (12%–59%) in SA2. Compared to another study, the role of antibiofilms[(S et al., 2023; Solanki et al., 2023)](https://paperpile.com/c/WCgzEP/cXAt+0l8P) are consistent with earlier research on different terrestrial plant species from around the globe. For instance, an Indian study found that the root extract of Vetiveria zizanioides inhibited the production of MRSA biofilms[(Seth et al., 2022)](https://paperpile.com/c/WCgzEP/J3S5U). Likewise, a different Brazilian study discovered that the dichloromethane extract from *Piper regnellit* inhibits the production of biofilms[(Laghari et al., 2023; Poornima et al., 2021b)](https://paperpile.com/c/WCgzEP/gXxc+ZX0q). Biofilm infections are important from a clinical standpoint, even though bacteria are resistant to antibiotics[(*Anti-Inflammatory Potential of a Mouthwash Formulated Using Clove and Ginger Mediated by Zinc Oxide Nanoparticles: An In Vitro Study*, n.d., “Effect of Cervical Lesion Centered Access Cavity Restored with Short Glass Fibre Reinforced Resin Composites on Fracture Resistance in Human Mandibular Premolars- an in Vitro Study,” 2021)](https://paperpile.com/c/WCgzEP/FrDm+2IHR). It might take high antibacterial concentrations to get rid of biofilm producers. This may not always be possible in vivo due to toxicity and accompanying side effects, although low-concentration combination therapy can be useful in the treatment of staphylococcal biofilm-related infections[(Du et al., 2023)](https://paperpile.com/c/WCgzEP/RR9zo) such as those caused by MRSA. Early screening and identification of biofilm producers, together with subsequent testing for antimicrobial sensitivity, are critical steps in the selection of an appropriate antimicrobial agent[(Phillips, 1991)](https://paperpile.com/c/WCgzEP/6fMIt).

As compared to other articles, the bark extract of *A. nilotica* was reported to have greater antibiofilm action, according to the comparative analysis[(Neelakantan et al., 2013)](https://paperpile.com/c/WCgzEP/r1r2c). For this reason, it is advised to utilize *A. nilotica* as a model to find better medications or as a way to track the formation of microbial biofilms. However, bacteria exhibit resistance to antimicrobial agents[(Ragavendran et al., 2022)](https://paperpile.com/c/WCgzEP/Mmtsv). Clinically, biofilm infections are important. In order to eradicate biofilm producers, high antibiotic concentrations can be needed[(Rasha et al., 2021)](https://paperpile.com/c/WCgzEP/LuUQQ). Low-concentration combination therapy can potentially be useful in eliminating staphylococcal biofilm-related infections[(Neelakantan et al., 2015)](https://paperpile.com/c/WCgzEP/fiytd), including those caused by MRSA. However, this may not always be possible *in vivo* due to toxicity and accompanying adverse effects. Finding and evaluating biofilm producers early on is crucial for choosing an appropriate antimicrobial agent[(De Backer, 2019)](https://paperpile.com/c/WCgzEP/DVNeK).

# CONCLUSION

In conclusion, aqueous extract from the bark of *Acacia nilotica* was used as a reducing agent during the manufacturing of ZnONPs. ZnONPs had demonstrated the strongest antibacterial activity against clinical pathogens in studies. This is inexpensive and employs environmentally beneficial practices. ZnONPs have been shown to exist based on results from several analytical characterization techniques, including green synthesis of ZnONPs, SEM, EDAX, and anti-biofilm activity. According to our research, A. nilotica bark may one day be converted into a cutting-edge antimicrobial medication.

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