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Evaluation of antimicrobial efficacy of zinc oxide nanoparticles synthesised using oil containing apple seed oil formulation against oral pathogens: An in vitro study

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ABSTRACT

Background: Zinc oxide nanoparticles (ZnO NPs) are known for their antimicrobial properties and are widely used in medical and dental applications. Combining ZnO NPs with natural oils, such as apple seed oil, offers potential benefits against oral pathogens. This study explores the antimicrobial efficacy of ZnO NPs synthesized using apple seed oil.

Aims: To evaluate the antimicrobial efficacy of zinc oxide nanoparticles synthesized using apple seed oil against *Streptococcus mutans*, *Enterococcus faecalis*, and *Staphylococcus aureus*.

Materials and Methods: This in-vitro study was conducted in a controlled laboratory environment to assess the antimicrobial properties of ZnO NPs combined with apple seed oil against selected oral pathogens.

Zinc oxide nanoparticles were synthesized from zinc sulfate heptahydrate and sodium hydroxide, followed by calcination. These nanoparticles were dispersed in apple seed oil. The agar cup diffusion method was used to test antimicrobial activity against Streptococcus mutans, *Enterococcus faecalis*, and *Staphylococcus aureus* by applying different concentrations (25μL,50μL,100μL) of the nanoparticle-oil mixture to bacterial cultures. The diameters of the inhibition zones were measured to determine antimicrobial efficacy. Data were analyzed to compare the effectiveness of varying concentrations of ZnO NPs in apple seed oil against the pathogens.

Results: The study revealed that zinc oxide nanoparticles in apple seed oil in varying concentrations (25μL, 50μL,100μL) did not show significant antimicrobial activity against *Streptococcus mutans*, *Enterococcus faecalis*, or *Staphylococcus aureus*. No discernible zones of inhibition were observed for any tested concentrations, indicating a lack of efficacy.

Conclusions: Zinc oxide nanoparticles synthesized with apple seed oil did not exhibit significant antimicrobial properties against the tested oral pathogens. This combination appears ineffective under the study conditions. Further research is needed to explore alternative formulations and understand interactions between antimicrobial agents and bacterial strains to develop effective treatments for oral infections.

Key Message: The findings of this study indicate the combination of zinc oxide Nano particles and apple seed oil does not show any significant results; it showed neither increase nor decrease in the growth of root canal pathogens. Further in vivo studies are required to gain understanding of its exact efficacy.

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1. Introduction

Zinc oxide non-eugenol (ZONE) cement was created and applied in situations like these, taking the place of eugenol with different materials.[1]

Previously, physicochemical methods were involved in nanoparticle synthesis. The biocompatibility of the Zinc Oxide nanoparticles is due to its molecule estimate and shape, viewpoint proportion and morphology. The time required to synthesize large quantities of nanoparticles using traditional physical and chemical methods is reduced, but toxic capping chemicals are required to maintain stability and are environmentally toxic.[2] The large surface area-to-volume ratio of nanoparticles is a notable differentiator that allows for excellent molecular interaction properties in their small size, distribution, and morphology.[2]

Zinc oxide nanoparticles was tried with a variety of liquids from ozonated chemicals to natural extracts, in this study we have evaluated the antimicrobial efficacy of zinc oxide nanoparticles with apple seed oil.

The antibacterial activity of zinc oxide nanoparticles is primarily derived from the nanoparticles compared to the release of very few free Zn^{2+} ions. The ability of zinc oxide nanoparticles to exhibit antibacterial properties have been demonstrated in many studies.[2]

Triglycerides make up the predominant component of apple seed oil, while tocopherols and phytosterols are the minor bioactive component. Like other vegetable oils, apple seed oil is particularly composed of. Research has shown that gamma tocopherol exhibits comparatively greater anticancer efficacy than alpha tocopherol.[3] The active components of vitamin E are primarily responsible for the biological effects of these substances. It was discovered that apple seeds had a moderate level of antimicrobial activity.[4] Failure of root canal treatment often occurs because of the remnants of infections micro-organisms in .The most prevalent microorganisms even after root canal treatment is completed are *Enterococcus faecalis*, *Streptococcus mutans* and *Staphylococcus aureus*.[5]

2. Materials and Methods

This in-vitro study was conducted in Department of Paediatric and Preventive Dentistry, NIMS Dental College and Hospital in collaboration with Department of Microbiology and Department of Pharmacy, NIMS university. This study was conducted in a duration of 3 months.(October 2023-December 2023)

2.1. Preparation of zinc oxide nanoparticles

The synthesis of zinc oxide nanoparticles was carried out in a well-equipped pharmacology laboratory. The materials and equipment used included zinc sulfate heptahydrate, distilled water, sodium hydroxide pellets, UV–vis diffuse reflectance spectroscopy, a Büchner funnel, Petri dishes, vacuum flasks, a calcination oven, and gloves.

Zinc Sulfate Heptahydrate Solution (Solution A) was prepared by dissolving 200 grams (1mole) of zinc sulfate heptahydrate in 700 ml of distilled water in a 2-liter, 3-neck round bottom flask. Sodium Hydroxide Solution (Solution B) was then prepared by dissolving 56 grams (2 moles) of sodium hydroxide pellets in 700 ml of distilled water. Solution B was slowly added to Solution A with vigorous stirring, initiating a precipitation reaction that forms a white fine precipitate, presumably zinc hydroxide. By the procedure of Stirring and Precipitation: the mixture was continuously stirred for 12 hours to ensure thorough mixing and reaction. Filter the precipitate using a Büchner funnel under vacuum to separate the solid from the liquid phase leading to filtration phase. To obtain the neutral pH the solution was taken through the process of washing and drying with distilled water. The washed precipitate was transferred to a Petri dish and dried in a furnace at 90°C until all water evaporated. Crush and dry the cake into a fine powder and calcine at 500°C in a furnace for 2 hours to convert zinc hydroxide into zinc oxide nanoparticles. The powder is gradually heated at a rate of 2.5°C per 60 seconds. Allow the powder to cool slowly to room temperature.

The powdered samples were analyzed using X-ray diffraction (XRD) to examine their crystalline structure. UV–vis diffuse reflectance spectroscopy was employed to investigate the optical absorption properties of ZnO, using a Shimadzu UV 3600 UV–vis-NIR spectrometer in diffuse reflectance mode with BaSO4 as a reference material.

2.2. Antimicrobial activity assessment

Zinc oxide nanoparticles with different concentrations of apple seed oil were prepared against microbial samples in the laboratory. The materials and equipment used included a nutrient medium, Petri plates, apple seed oil, bacterial cultures -*Streptococcus mutans*, *Enterococcus faecali*s, *Staphylococcus aureus,* Mueller Hinton broth, and gloves.

The sample included Zinc oxide nanoparticles in apple seed oil (Sample A) with a positive control (PC) – chloramphenicoland a negative control - distilled water.

The synthesized zinc oxide nanoparticles were dispersed in apple seed oil labeled as Sample A. For each test organism, five samples were prepared:

1. *Streptococcus mutans* + zinc oxide nanoparticles in apple seed oil.
2. *Staphylococcus aureus* + zinc oxide nanoparticles in apple seed oil.
3. *Enterococcus faecalis* + zinc oxide nanoparticles in apple seed oil.

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2.3. Procedure

Culture Growth and Standardization: Grow the test organisms in Mueller Hinton broth and adjust their optical density using McFarland standards to ensure consistent starting density for the antimicrobial assay.

Agar Cup Diffusion Method: Load varying volumes (100 μl, 50 μl, and 25 μl) of the sample solutions into wells created in agar plates. Incubate the plates at room temperature for 24 hours to allow diffusion of the samples.

Evaluation of Results: The plates were examined for zones of inhibition, indicating the antimicrobial activity of the samples against the test organisms.

2.4. Sample preparation

The synthesized zinc oxide nanoparticles are dispersed in apple seed oil.

Table 1:

S.No	Sample	Sample Size
1	*Streptococcus mutans*+ zinc oxide nano particles in apple seed oil	5
2	*Staphylococcus aureus*+ zinc oxide nano particles in apple seed oil	5
3	Entererococcus faecalis+ zinc oxide nano particles in apple seed oil	5

3. Results

In this study all the observation has been mentioned in the tables below:

Table 2 presents the results of the experiment investigating the antimicrobial activity of nanoparticles in apple seed oil, and a positive control against *Streptococcus mutans*. Each row represents a separate trial, with the "Zone of inhibition" column indicating the diameter of the area where bacterial growth was inhibited around the substance being tested. Across all five trials, the nanoparticles in the apple seed oil showed no observable zone of inhibition against *Streptococcus mutans*, suggesting a lack of antimicrobial activity. These results imply that the apple seed oil did not demonstrate significant antimicrobial effects against *Streptococcus mutans* under the tested conditions.

Table 3 presents the findings of an experimental investigation evaluating the antimicrobial properties of nanoparticles in apple seed oil and a positive control against *Enterococcus faecalis*. Each row corresponds to a different trial, with the "Zone of Inhibition" column showing the diameter (in millimeters) of the area where bacterial growth was inhibited by the tested substance.

In all five trials, apple seed oil showed no measurable zone of inhibition against *Enterococcus faecalis*, indicating that this substance did not exhibit significant antimicrobial activity against this bacterium under the conditions tested.

In contrast, the positive control consistently produced substantial zones of inhibition, ranging from 26 mm to 29 mm across the trials. These results suggest that the positive control, which likely contains a known antimicrobial agent, effectively inhibited the growth of *Enterococcus faecalis*.

In all five trials, the nanoparticles in apple seed oil did not show any observable zone of inhibition against *Staphylococcus aureus*. This indicates that these substances did not exhibit significant antimicrobial effects on this bacterium under the tested conditions.

In contrast, the positive control consistently produced zones of inhibition ranging from 15 mm to 19 mm, suggesting that it effectively inhibited the growth of *Staphylococcus aureus*. These findings imply that the nanoparticles in apple seed oil may not have strong antimicrobial properties against *Staphylococcus aureus* at the concentrations or formulations tested.

4. Discussion

Tayel et al. carried out a study to evaluate the antibacterial effectiveness of ZnO nanoparticles compared to conventional ZnO powder against nine different bacterial strains. Nano-sized materials have become significant in the fields of regenerative endodontics and root canal disinfection. Although ZnO is water-insoluble due to its high polarity, this challenge can potentially be overcome by using ZnO nanostructures. The antibacterial effectiveness of ZnO nanoparticles depends on their concentration, with higher concentrations showing the greatest antibacterial activity.[4]

Dentin treated with ZnO nanoparticles exhibited higher levels of protein peptides that link the collagen structure to hydroxyapatite (HAp) crystals. Additionally, these nanoparticles activate Matrix Coagulation enzyme-3, which facilitates the release of small leucine-rich proteins and N-linked glycoproteins that bind to integrins from dentin. Phosphorylated proteins that adhere to the dentin surface enhance mineral production, playing a crucial role in dentin mineralization. Moreover, zinc nanoparticles inhibit collagen degradation mediated by Matrix Metalloproteinase-3 in partially demineralized dentin, thereby supporting dentin remineralization.[6]

These insights contribute to developing new strategies for combating bacterial infections in dental settings and hold promise for future clinical applications. Moreover, adopting green synthesis methodologies offers numerous advantages, including environmental sustainability and compatibility with biomedical applications. By avoiding toxic chemicals in synthesis protocols, green synthesis aligns with eco-friendly and safe principles, making it especially suitable for medical use. Additionally, exploring plant-derived antimicrobial compounds highlights the wealth of bioactive molecules found in nature. Compounds derived from secondary metabolites, such as alkaloids and glycosides, are

Table 2: Zone of inhibition of *streptococcus mutans* with apple seed oil

S.No	Organism	Zone of inhibition(mm)	
		Apple seed oil	Positive Control in apple seed oil
1	*Streptococcus Mutans*	0	20
2	*Streptococcus Mutans*	0	21
3	*Streptococcus Mutans*	0	21
4	*Streptococcus Mutans*	0	19
5	*Streptococcus Mutans*	0	20

Table 3: Zone of inhibition of *Enterococcus faecalis* with Apple seed oil

S.No	Organism	Zone of inhibition (mm)	
		Apple seed oil	Positive Control in apple seed oil
1	*Enterococcus faecalis*	0	28
2	*Enterococcus faecalis*	0	26
3	*Enterococcus faecalis*	0	29
4	*Enterococcus faecalis*	0	28
5	*Enterococcus faecalis*	0	26

Table 4: Zone of inhibition of *Staphylococcus aureus* with a Apple seed oil

S.No.	Organism	Zone of inhibition(mm)	
		Apple seed oil	**Positive control in apple seed oil**
1	*Staphylococcus Aureus*	0	15
2	*Staphylococcus Aureus*	0	18
3	*Staphylococcus Aureus*	0	19
4	*Staphylococcus Aureus*	0	15
5	*Staphylococcus Aureus*	0	16

commonly found in herbs and exhibit strong antimicrobial properties.[7]

Traditionally, zinc oxide has been combined with eugenol for root canal obturation and sealing, but due to the cytotoxicity of eugenol, researchers are seeking alternative mediums that offer similar functionality without the toxic effects. Zinc oxide nanoparticles have been tested with various oils derived from spices and herbs (natural sources) to assess their antimicrobial activity and explore potential substitutes for eugenol.

Apple seed oil is a natural oil that has long been recognized for its health benefits. This experiment aimed to determine if, when combined with zinc oxide nanoparticles, it exhibits any antimicrobial effectiveness against oral pathogens.

Apple seed oil is notable for its high content of oleic and linoleic acids, which are fatty acids with powerful antioxidant properties. These antioxidants play a crucial role in protecting the body against oxidative stress and cellular damage, thereby reducing the risk of chronic illnesses and promoting overall health.

4.1. Key components and antioxidant properties

Oleic Acid: This monounsaturated fatty acid is known for its ability to enhance cardiovascular health and reduce inflammation. It also has potent antioxidant properties that help neutralize free radicals. Research published highlights oleic acid's role in protecting cells from oxidative damage and improving metabolic health.[8]

Linoleic Acid: As a polyunsaturated fatty acid, linoleic acid is essential for maintaining the integrity of cell membranes and skin barrier function. It also has significant antioxidant capabilities. A study found that linoleic acid helps to reduce oxidative stress and inflammation, which are linked to various chronic diseases.[9]

4.2. Health benefits of antioxidants

Integrating apple seed oil into the diet or using it in nutritional supplements can provide an effective way to benefit from its antioxidant properties. The regular consumption of these fatty acids can support various aspects of health, including:

1. Cardiovascular Health: Oleic acid has been shown to improve heart health by lowering bad cholesterol levels and increasing good cholesterol levels.
2. Skin Health: Linoleic acid is crucial for maintaining healthy skin by supporting barrier function and hydration.
3. Inflammation Reduction: Both oleic and linoleic acids have anti-inflammatory effects that can help manage

conditions like arthritis and other inflammatory diseases.[10]

Some studies have indicated that apple seed oil possesses a certain degree of efficacy against the tested microorganisms. These findings underscore the potential of apple seed oil as a natural antimicrobial agent, particularly against bacterial pathogens.[11]

Hegde and Pallavi conducted a study to determine whether microorganisms were present in the pulp area of human deciduous teeth. Of the samples examined, Staph. aureus was found in 5%, E. faecalis in 35%, anaerobes in 20%, and C. albicans in 15%.[12]

Researchers have explored how S. mutans adapts to withstand various harmful substances while thriving within the oral biofilm. As a result, S. mutans has developed mechanisms to establish and maintain dominance in the oral cavity over time. While antimicrobial treatments have been utilized for centuries to prevent and manage this bacterial infection, their effectiveness is often hindered by the bacteria's ability to form robust and resilient biofilms. Given the increasing prevalence of multidrug resistance (MDR) in microbial diseases and the slow development of new anti-infective medications, there is a growing need to investigate alternative therapeutics.[13]

In this particular investigation, we examined the antimicrobial properties of zinc oxide nanoparticles (ZnO NPs) combined with apple seed oil against *Streptococcus mutans*, a common bacterium associated with dental decay. The experiment involved testing different concentrations of the nanoparticle-oil combination: 100μL, 50μL 25μL.

The results revealed that none of these concentrations exhibited any significant antimicrobial activity against *Streptococcus mutans*. In this specific context, the combination of zinc oxide nanoparticles and apple seed oil did not effectively combat the growth of *Streptococcus mutans*.(Figure 1)

Nuramirah Azizan et al whose study suggested that the oils extracted from O. stamineus and F. deltoidea exhibited moderate to potent antibacterial effects against seven strains of pathogenic oral bacteria.[14]

Studies have demonstrated that oral E. faecalis possesses various virulence attributes, such as gelatinase activity, haemolysin expression, reaction to pheromones, bacteriocin synthesis, biofilm development, adhesion factor expression, aggregation substances, and antibiotic resistance.[15]

In this experimental setup the area around wells containing different concentrations (100 μL., 50 μL 25 μL.) of zinc oxide nanoparticles mixed with apple seed oil showed no signs of inhibition against Enterococcus faecalis, indicating a lack of antimicrobial activity. This suggests that the combination of zinc oxide nanoparticles and apple seed oil may not antimicrobial activity against Enterococcus faecalis under the conditions. The result of the experiment indicates that at all three concentrations tested (100 μL., 50 μL 25 μL.), there was no observable antimicrobial activity against Enterococcus faecalis.(Figure 2)

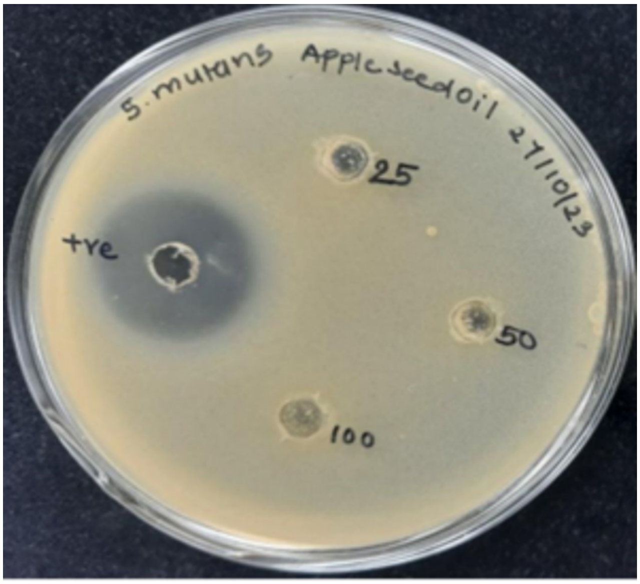

Figure 1: *Streptococcus mutans* against zinc oxide nano particles with apple seed oil

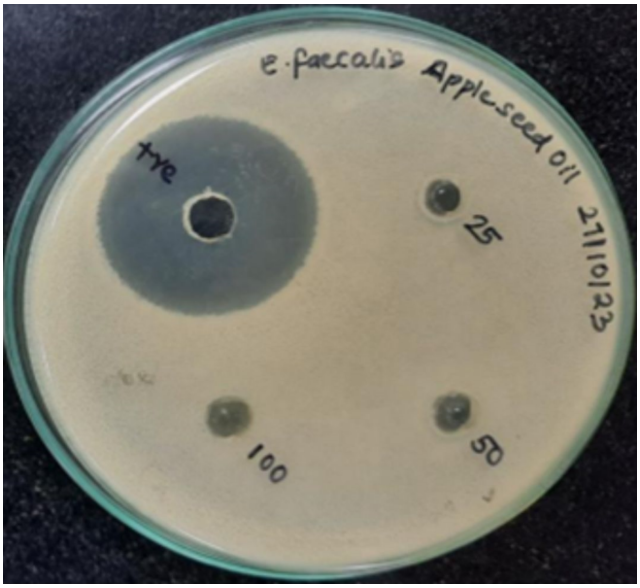

Figure 2: Enterococcus faecalis against zinc oxide nano particles with apple seed oil

Samiei and colleagues conducted research on various formulations involving zinc oxide nanoparticles (ZnO NPs) and their study revealed that when the root canal exterior was treated with these cationic antibacterial nanoparticles, such as ZnO-NP, CS/ZnO-NP, or CS-layer-ZnO-NP, it resulted in a decrease in the attachment of E. faecalis bacteria to the dentin treated with nanoparticles.

This finding suggests the potential of these nanoparticle formulations in preventing bacterial attachment to dentin surfaces, which is crucial for enhancing the effectiveness of root canal disinfection and improving the outcomes of endodontic treatments.[16] The virulence and pathogenicity of these skin and mucous membrane inhabitants are attributed to their ability to produce a variety of exotoxins and extracellular enzymes. These factors enable the microorganism to colonize the host, induce the lysis of phagocytic cells, and hinder the phagocytosis process. Moreover, most strains of S. aureus quickly develop resistance to antimicrobials like penicillin by producing penicillinase (β-lactamase), an enzyme that hydrolyzes the β-lactam ring of penicillin, rendering the antibiotic ineffective.[17]

In this particular experimental setup various concentrations (100 μL., 50 μL 25 μL.), the mixture of zinc oxide nanoparticles and apple seed oil did not demonstrate any ability to inhibit the growth of *Staphylococcus aureus*. This suggests that the combination of zinc oxide nanoparticles and apple seed oil may not be effective against *Staphylococcus aureus* in the tested conditions.(Figure 3)

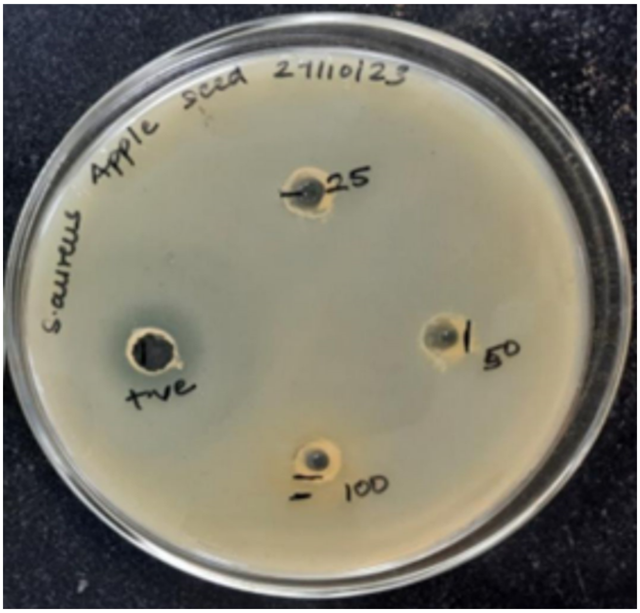

Figure 3: *Staphylococcus aureus* against zinc oxide nano particles with apple seed oil

5. Conclusion

The experiment involved testing different concentrations of zinc oxide nanoparticles mixed with apple seed oil against the aforementioned bacterial strains. Contrary to expectations, none of the tested concentrations exhibited significant antimicrobial activity against *Streptococcus mutans*, *Enterococcus faecalis*, or *Staphylococcus aureus*. This suggests that the combination of zinc oxide nanoparticles with these natural oils may not be effective against these specific pathogens under the conditions tested. However, the current study did not observe similar antimicrobial effects with the combination of zinc oxide nanoparticles and natural oils.

Overall, the research underscores the complexity of antimicrobial interactions and the need for further investigation into alternative therapies for combating oral pathogens. Despite the promising properties of zinc oxide nanoparticles and natural oils, their combined efficacy against specific bacterial strains may vary, highlighting the importance of tailored approaches in dental care and infection management.

6. Ethical Approval

The institutional ethics committee of the university had approved the application to conduct the study under the following proposal number IEC/P-28/2022.

7. Conflict of interest

None.

8. Source of funding

None.

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None.

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