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Original Research Article

Synergistic antibacterial action of nanoparticle-Enhanced royal jelly and pulp capping agents against Streptococcus mutans

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Abstract

Introduction: Nanoparticles can have natural antimicrobial capabilities or be engineered to transport antibacterial chemicals, hence increasing their efficacy. By integrating nanoparticles into these goods, we can leverage the synergistic effects of nanoparticles and bioactive chemicals found in apiarian products, resulting in increased antibacterial activity.

Materials and Methods: Royal jelly was obtained from Queenbees Pvt Ltd, located in Chennai, Tamil Nadu, India. Calcium Hydroxide in the form of Dycal (Dentsply) was used as the base material. The experimental groups were categorized as follows: Group A—Dycal with royal jelly extract, Group B—Dycal with nanoparticle-incorporated royal jelly, and Group C—Dycal with nanoparticle-incorporated calcium hydroxide.

Nanoparticles were synthesized and examined for FTIR, SEM, EDAX, and antibiofilm testing.

Results: Group 1 includes alkanes (702.5), aromatic groups (615.50), and carbonyl groups (ketones and amides) (1636.97). Group 2: The silver nanoparticles included in royal jelly have function and hydroxyl groups. Group 3: The presence of hydroxyl (3300.05) and carbonyl groups (1700). SEM investigation reveals the crystalline structure of nanoparticles spread uniformly in royal jelly. The EDAX analysis reveals that the Royal jelly sample has a considerable amount of silver integrated into the matrix.

Conclusion: Silver Nanoparticle incorporated royal jelly demonstrated maximum biofilm suppression against Streptococcus mutans. The results from FTIR, SEM, and EDAX confirm the successful incorporation of silver nanoparticles into royal jelly, demonstrating their potential for enhanced antibacterial efficacy.

Keywords: Apiarian product, Indigenously developed, Innovative pulp capping agent, Nanoparticle synthesis, Pulp capping agent.

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1. Introduction

The growing worldwide worry over antibiotic resistance has prompted researchers to look at fresh and sustainable alternatives to existing antibacterial drugs. One such area of study is the synergistic antibacterial activity of natural compounds, notably those generated from beekeeping, against notable pathogens such as *Streptococcus mutans.*[1,2] The urgency of addressing bacterial resistance emphasizes the significance of creating creative solutions that not only effectively combat microbial illnesses but also reduce the environmental impact of synthetic antibiotics.[3]

Dental caries is a multifactorial disease initiated by microbial colonization, leading to demineralization and eventual breakdown of the tooth structure. Among the various cariogenic microorganisms, *Streptococcus mutans* (S. mutans) plays a pivotal role in the pathogenesis of dental caries due to its ability to adhere to the enamel pellicle via specific adhesins, facilitating the formation and maturation of cariogenic biofilms. The persistence of *S. mutans* within these biofilms presents a significant challenge in restorative and endodontic treatments, necessitating effective antimicrobial strategies.

Nanoparticles have emerged as promising agents in antimicrobial therapy, offering enhanced efficacy due to their high surface area, unique physicochemical properties, and ability to disrupt bacterial biofilms. In vital pulp therapy, where the goal is to preserve and maintain the integrity of compromised pulp tissue, conventional pulp capping agents such as calcium hydroxide and mineral trioxide aggregate (MTA) have been widely used.[4] Calcium hydroxide (CH), a widely used pulp capping agent, has been regarded as the

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gold standard for decades due to its antimicrobial properties and ability to induce dentin bridge formation. However, recent studies have questioned its long-term prognosis due to inherent limitations. Calcium hydroxide exerts its antibacterial effect through its alkaline pH, which aids in microbial elimination and reduces pulpal irritation. However, its limitations, including dissolution in oral fluids and the presence of tunnel defects in the dentin bridge, necessitate the search for alternative materials, compromising the longevity of the pulp capping treatment.[5] Additionally, calcium hydroxide's high alkalinity can cause tissue necrosis at the site of application, leading to inconsistent regenerative outcomes.[6] Similarly, chlorhexidine (CHX), a widely used antimicrobial agent, has shown therapeutic potential as an oral disinfectant and non-specific matrix metalloproteinase (MMP) inhibitor. Despite their benefits, these materials still have drawbacks, prompting the exploration of novel bioactive alternatives.

Apiarian products, such as royal jelly, propolis, and honey, have demonstrated promising bioinductive and antimicrobial properties, making them potential candidates for pulp capping applications. Royal jelly, a resinous substance collected by honeybees, has long been recognized for its therapeutic properties, including antimicrobial, anti-inflammatory, and antioxidant activities.[7,8,9] Moreover, advancements in nanotechnology have enabled the enhancement of royal jelly through the development of nanoparticles, unlocking a new dimension of its antibacterial potential.[10,11,12,13] This discovery has sparked curiosity about the potential benefits of royal jelly augmented with nanoparticles against bacterial strains, particularly S. mutans, a major cause of tooth cavities.[14,15]

The antibacterial properties of synthetic pulp capping agents have made them a staple in modern endodontic procedures.[16,17] However, the emergence of antibiotic-resistant strains calls for a re-evaluation of such conventional approaches. This study aims to bridge the gap between traditional and contemporary antibacterial strategies by comparing the efficacy of an indigenously developed nanoparticle-enhanced apiarian product, Royal jelly, with that of widely used pulp capping agents against *S. mutans.*[11,18] It is essential that we take into account the larger context of antimicrobial resistance, the ecological effects of antibacterial agents, and the potential of natural products as sustainable alternatives as we delve into this comparative study. This study has the potential to advance dentistry as well as add to the expanding corpus of knowledge focused on finding environmentally responsible solutions to global health issues.

This study aims to evaluate the potential of nanoparticle-enhanced apiarian products as a superior alternative to calcium hydroxide and other synthetic materials, providing both antimicrobial protection and regenerative benefits. Examining the combined antibacterial activity of apiarian chemicals enhanced by nanoparticles was the primary goal. The main objective of this investigation was to determine whether contemporary pulp capping agents could suppress the biofilm formation of *S.mutans* in the apiarian product Royal jelly, which is made locally using silver nanoparticles. Fourier Transform Infrared Spectroscopy (FTIR) was used to analyze the biochemical composition. Scanning Electron Microscopy (SEM) was employed to assess the shape and distribution of nanoparticles. Energy Dispersive X-ray Analysis (EDAX) was utilized to measure and verify the presence of silver.

2. Materials and Methods

2.1. Preparing nano particle enhanced royal jelly (NPERJ)

Royal jelly was sourced from Queenbees Honey Pvt. Ltd., located at Chennai, Tamil Nadu, India. Royal jelly was cleansed to get rid of impurities prior to experiment. Silver nanoparticles were synthesized and incorporated into royal jelly following a standardized method to ensure uniform dispersion. Silver nanoparticles were synthesized using silver nitrate ($AgNO_3$) as the precursor and a reducing agent, followed by continuous stirring until nanoparticle formation was confirmed. A stabilizing agent such as polyvinylpyrrolidone (PVP) was added to prevent aggregation and ensure stability. The synthesized nanoparticles were then gradually incorporated into royal jelly under constant stirring, followed by ultrasonication to achieve uniform dispersion. The final composite was stored under controlled conditions to maintain its stability and bioactivity. A commercially available pulp capping agent, Dycal (Dentsply), was selected for the study, as it is widely used in dental practice. All agents were prepared in accordance with the manufacturer's guidelines to maintain consistency and uniformity. The bacterial strain of *Streptococcus mutans* (ATCC 25175) was grown in appropriate media under controlled laboratory conditions.

2.2. Silver nanoparticle (AgNP) synthesis

100 mL of 1 x 10^3 M silver nitrate ($AgNO_3$) and 10 Ml of deionized milliQ water with 0.01 g of gallic acid were combined in a 250 Ml Erlenmeyer flask. The pH of the solution was brought to 11 by dropwise addition of 1N NaOH to the reaction mixture.[19]

2.3. Procedure for preparing the inoculum

In a 250-mL Erlenmeyer flask, 100 mL of Brain Heart Infusion (BHI) broth was inoculated with 4 - 5 distinct colonies of *Streptococcus mutans* from the mother culture. Preparation of Test Samples: Test samples for pulp capping agent and NPERJ at different concentrations were made. The test samples were exposed to a standardized inoculum of *S. mutans* for the specified durations. The crystal violet-based assay and other standard protocols were employed to assess the viability of *S. mutans*. Microscopic studies were

employed to examine the morphological and structural alterations in the cells.

2.4. Antibiofilm assay

Microtitre plate based approach was used to perform the antibiofilm assay. Initially, the microtitre wells were inoculated with sterile Mueller Hinton Broth. The samples (R, R-NPs and R-C-NPs) were prepared using broth constituting the stock solution. From the stock solution, 100 ul of the sample was added initially to the first well and mixed thoroughly. From the first well, 100µ of the sample was added to the next well constituting doubling dilution in the range of 0.1 ml to 0.003 ml concentration. After the sample dilution, 50µ of the log phase *S. mutans* culture was added to all the wells. The plate was agitated gently for thorough mixing of the sample with the culture and incubated at 37ºC for 24 – 48 hours. After the incubation period, the contents of the wells were emptied by gentle aspiration. Then the wells were washed with PBS to remove the debris and sample traces. This was followed by the addition of 150µ of 0.2% crystal violet and incubated for 15 to 20 minutes. Then the contents in the well were emptied, washed with PBS to remove the unbound and excess dye. Then the wells were pipetted with 150µ of 30% glacial acetic acid for the dissolution of crystal violet dye. The plate was gently rocked and the absorbance measured at 570 nm in an ELISA plate reader.

Before adding the samples (EO and EO-NRJs) to the MHI broth in the wells, they were diluted to the necessary concentrations (0.1 ml to 0.003 ml). Following that, the samples were treated for 48 hours with a 50µ broth culture. The broth from the wells was aspirated and cleaned with a PBS solution after incubation. Following that, each well received 150 L of crystal violet (0.2%), which was left to stand for 15 to 20 minutes. After that, the excess and unbound color are eliminated by removing it and washing it with PBS. After that, each well's color was removed using 150µ of 30% glacial acetic acid. Readings at 570 nm were taken using an ELISA Plate Reader, and the absorbance value was noted.

2.5. Antibacterial assays

Disk diffusion and minimum inhibitory concentration (MIC) tests were carried out for NPERJ and pulp capping agents. Inhibition zones were assessed, and minimum inhibitory concentrations (MIC) were determined using the microdilution method in a 96-well microtiter plate. Serial two-fold dilutions of the test materials (NPERJ and pulp capping agents) were prepared in a suitable broth medium. A standardized bacterial inoculum i.e. broth culture of *Streptococcus mutans* was added to each well. After incubation at 37°C for 24 hours, the MIC was recorded as the lowest concentration that showed no visible bacterial growth.

For the disk diffusion assay, sterile discs impregnated with the test materials were placed on Mueller-Hinton agar plates inoculated with the bacterial strain. After 24 hours of incubation at 37°C, the diameter of the inhibition zones around each disc was measured using a digital caliper to evaluate antibacterial efficacy.

2.6. FTIR, SEM, and EDAX Analyses

To characterize the functional groups associated with nanoparticles incorporated royal jelly, Fourier Transform Infrared Spectroscopy (FTIR) was conducted in ATR mode. Scanning Electron Microscopy (SEM) was utilized to visualise the morphological and structural characteristics of the synthesized nanoparticles and their uniform dispersion within royal jelly. Energy Dispersive X-ray Analysis (EDAX) was utilized to determine the elemental composition of the sample.

Using SPSS 30.0 software, data from viability tests and antimicrobial assays were statistically examined. One-way ANOVA was utilized to compare the means of the various groups, and Tukey post-hoc tests were then performed. A significant threshold of p 0.05 was selected. Evaluation of the Synergistic Impact: To investigate potential synergistic effects of pulp capping agents and NPERJ, combination tests were conducted.

2.7. Ethical considerations

All necessary institutional review board clearances were obtained, and the study protocol complied with ethical guidelines.

2.8. Data display

The report includes tables summarizing the main findings along with a graphic representation of the data. Against *Streptococcus mutans*, this comprehensive technique offers a thorough investigation of NPERJ and pulp capping agents' antibacterial action, offering insights into possible synergistic effects.

3. Results

Group 1 includes alkanes (702.5), aromatic groups (615.50), and carbonyl groups (ketones and amides) (1636.97). (**Figure 1**, **Figure 2**) Group 2: The silver nanoparticles included in royal jelly have function and hydroxyl groups. (**Figure 3**) Group 3: The presence of hydroxyl (3300.05) and carbonyl groups (1700). (**Figure 4**) SEM investigation reveals the crystalline structure of nanoparticles spread uniformly in royal jelly. (**Figure 5**) The EDAX analysis reveals that the Royal jelly sample has a considerable amount of silver integrated into the matrix. (**Figure 6**)

Using the agar diffusion method, the antibacterial efficacy of modern pulp capping agents and royal jelly augmented with nanoparticles against Streptococcus mutans was assessed. When Royal jelly augmented with nanoparticles was contrasted with conventional pulp capping agents, the inhibitory zone diameter grew considerably. This

result suggests that the Royal jelly formulation made locally has a more potent antibacterial effect.

ANOVA and post-hoc testing using Tukey's Honest Significant Difference (HSD) test were employed in the statistical analysis to verify the differences between the treatment groups. The improved antibacterial activity of Royal jelly boosted with nanoparticles is statistically significant, as confirmed by the reported p-values.

The most effective biofilm suppression was demonstrated by a nanoparticle-enhanced combination against *S mutans*. The existence of hydroxyl ions and amides was revealed by FTIR analysis. SEM analysis revealed equally distributed SNPs in royal jelly. The presence of silver nanoparticles in the matrix was confirmed by EDAX.

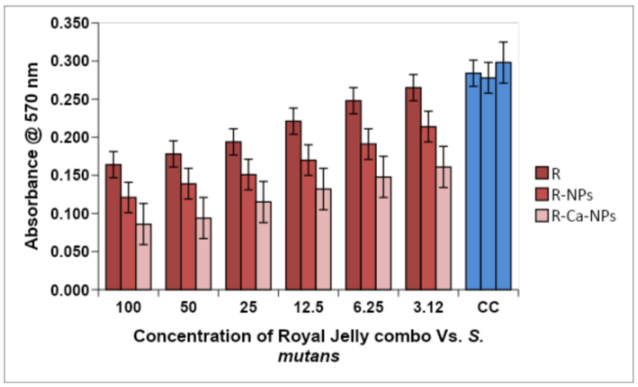

Figure 1: Antimicrobial efficacy against various concentrations.

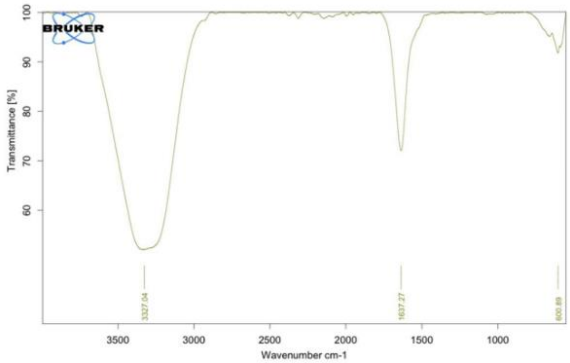

Figure 2: FTIR analysis: Presence of alkynes, alcohol, ethers, and aminoamides.

R	R-NPs	R-C-NPs
3327.04 cm-^{1} – Broad peak represents O-H group	3277.40 cm-^{1} – Broad peak represents O-H group	3200 – 3500 cm-^{1} – Broad peak represent O-H group
1637.27 cm-^{1} – A strong peak in between 1560 and 1640 cm-1	1636.97 cm-^{1} – A strong peak and a slight shift indicates the involvement of	1500 – 1600 cm-^{1} – A strong peak in this region
indicates the presence of primary amines N-H attributing to the protein	primary amines N-H attributing to the protein capping of the NPs.	indicates the presence of N-H bending vibrations attributing for amide II.
600.89 cm-^{1} – indicates the presence of C-H bending vibrations attributing for the structure of royal jelly.	1060.68 cm-^{1} – indicates the presence of –OH groups that could have been due to the presence of phenolic compounds or sugar broken down from the royal jelly.	
	615 cm-^{1} – strong peak representing the C-H stretching vibrations accounting for royal jelly.	500 – 600 cm-^{1} – medium peak signifies the presence of alkanes in the formulation

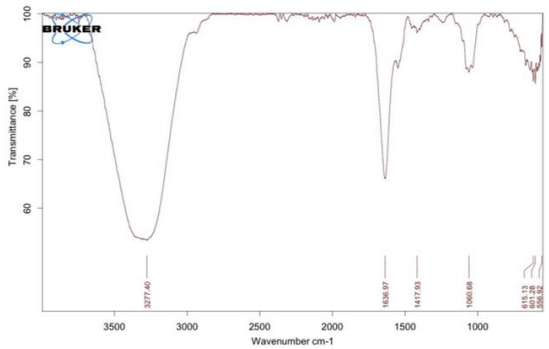

Figure 3: Presence of functional groups and hydroxyl groups.

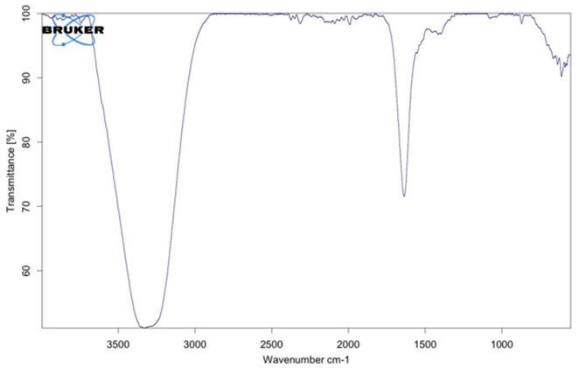

Figure 4: Presence of hydroxyl groups.

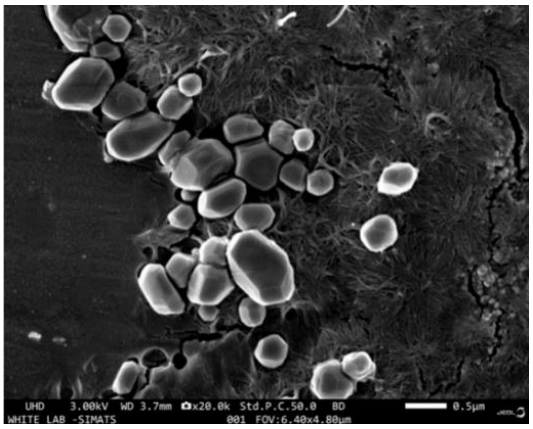

Figure 5: SEM Analysis: Crystalline structures of NPs dispersed in royal jelly.

FESEM micrograph showed the presence of silver nanoparticles with spherical to oval structure exhibiting hexagonal lattice. It was also evident that these silver nanoparticles were dispersed in the protein matrix accounting for effective encapsulation. The size of the nanoparticles were found to be less than 0.1 micron as demonstrated from the micrograph.

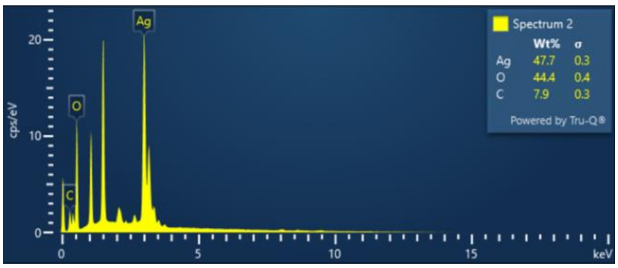

Figure 6: EDAX Analysis: Presence of silver incorporated into royal jelly in the matrix.

EDAX spectrum revealed the presence of signals from Ag, O and C with corresponding weight percentages of 47.7%, 44.4% and 7.9% respectively. Strong signal from Ag revealed the nanoform of silver followed by oxygen which could be due to the release of phenolic compounds rich in oxygen molecules. The presence of carbon peak might be due to the presence of carbon coated grids used for sample loading.

Table 1: Biofilm formation in concentration

% Biofilm formation			
Concentration	**RJ (Royal jelly)**	**RJ-NRJ**	**RJ-Ca-NRJ**
100	57.75	43.53	28.86
50	62.68	50.00	31.54
25	68.31	54.32	38.59
12.5	77.82	61.15	44.30
6.25	87.32	68.71	49.66
3.12	93.31	76.98	54.03
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RJ	Royal jelly
NRJ	Nano particle enhanced royal jelly
Ca	Calcium hydroxide

Table 2: Statistical analysis results

Comparison	**p-value**
Nanoparticle-Enhanced Royal Jelly vs. Contemporary Pulp Capping Agents	<0.05
Combined Treatment vs. Individual Treatments	<0.05

4. Discussion

Nanotechnology has emerged as a revolutionary field in medicine and dentistry, offering innovative solutions to combat microbial infections. Nanoparticles, due to their small size and large surface area-to-volume ratio, exhibit unique physicochemical properties that enhance their interaction with bacterial cells, leading to superior antimicrobial efficacy. Silver nanoparticles (AgNPs), in particular, have demonstrated potent antibacterial activity against a wide spectrum of pathogens, including *Streptococcus mutans*, the primary etiological agent of dental caries. The ability of AgNPs to disrupt bacterial cell membranes, generate reactive oxygen species (ROS), and inhibit metabolic pathways makes them an attractive component for antimicrobial applications in dentistry.[2]

Apiarian products such as honey, propolis, and royal jelly have long been recognized for their bioactive properties, including antibacterial, anti-inflammatory, and wound-healing effects. Royal jelly, a secretion produced by worker bees, contains a rich composition of proteins, vitamins, flavonoids, and antimicrobial peptides, making it a promising candidate for therapeutic applications. However, its limited bioavailability and rapid degradation in biological environments have hindered its widespread use. By integrating nanoparticles into royal jelly, we can enhance its stability, controlled release, and antibacterial efficacy, thereby expanding its potential as a novel biomaterial in endodontics and restorative dentistry. Due to the limitations of Calcium hydroxide, there is a pressing need for alternative pulp capping agents that offer superior antimicrobial properties, enhanced biocompatibility, and improved durability.[5]

The integration of AgNPs into royal jelly (NPERJ) addresses many of the shortcomings associated with conventional pulp capping agents. Our study demonstrated that NPERJ exhibited significantly greater antibacterial efficacy against *S. mutans* than traditional materials. This is likely due to the increased surface area of nanoparticles, which facilitates sustained release and enhances interaction with bacterial biofilms.

The findings of this study show that nanoparticle-enhanced royal jelly has a significant improvement in antibacterial activity against Streptococcus mutans. The bigger inhibitory zones seen in the nanoparticle-enhanced Royal jelly group indicate a stronger antibacterial action. These encouraging findings point to the possibility of nanoparticle-enhanced Royal jelly as a viable antibacterial agent, emphasizing its superiority over current pulp capping treatments and the appearance of synergistic effects when combined.

This conclusion is consistent with prior research highlighting Royal jelly's antibacterial capabilities and the ability of nanoparticles to boost its bioavailability and potency.[20,21,22,23] The enhanced antibacterial activity could be attributed to the increased surface area and sustained release of active compounds facilitated by the nanoparticle formulation.[18] Sharma et al.2009 reported effective antibacterial activity of silver nanoparticles against various bacterial strains.[24] Khalil et al.2006 highlighted the potential of propolis against bacteria and fungi.[25]

4.1. Comparison with Contemporary Pulp Capping Agents

The comparison of nanoparticle-enhanced Royal jelly and modern pulp capping agents highlights the value of natural materials in dental applications. The decreased MIC of nanoparticle-enhanced Royal jelly suggests that it can suppress bacterial growth at lower concentrations than traditional treatments.[23] This discovery is consistent with the increased interest in natural compounds as alternatives to manufactured antibiotics, particularly in the setting of antibiotic resistance.[26] The results challenge the conventional paradigm and advocate for further exploration of Royal jelly-based formulations in endodontic applications.

4.2. Synergistic effects and combined treatment

Katheeja et al.2022 used SEM and EDAX to investigate the shape and crystal structure of nanoparticles. Additionally, FTIR analysis was employed to identify the functional groups present in the nanoparticle-enhanced royal jelly and pulp capping agents, providing crucial information on the chemical composition and potential interactions. A notable component of our study is the discovery of synergistic effects in the combination treatment with nanoparticle-enhanced royal jelly and modern pulp capping agents. These medicines' combined action resulted in a considerable rise in antibacterial activity. Other natural product combinations have shown synergistic interactions, highlighting the potential for multiple approaches to fighting bacterial infections.[27,28,29,30] This finding opens avenues for future research exploring the synergistic potential of natural products and conventional agents for enhanced therapeutic outcomes.[31] Our research also demonstrated that a significant amount of nanoparticles are consistently incorporated in Apiarian products, with a large number of hydroxyl and amide groups involved.

4.3. Clinical implications and longevity

The study's favorable findings hold potential for clinical applications in dentistry. Royal jelly, a natural substance with antibacterial activity, offers an environmentally friendly alternative to synthetic agents. Royal Jelly's eco-friendliness accords with the global movement for sustainable healthcare methods. Furthermore, the lower MIC of nanoparticle-enhanced Royal jelly suggests that the amount of antimicrobial agents necessary for medicinal purposes may be lowered, reducing the environmental impact associated with their production and disposal.

4.4. Limitations and future directions

Despite the hopeful results, it is critical to recognize the study's limitations. The experiments' in vitro nature may not adequately depict the intricacies of the oral environment. In vivo investigations should be conducted in the future to evaluate the efficacy of nanoparticle-enhanced propolis in a more clinically relevant situation. Before NPERJ can be translated into clinical practice, several critical steps must be undertaken. Animal studies are essential to evaluate its biocompatibility, pulp healing potential, and long-term effects. Further mechanistic research is needed to understand its antibacterial action and synergistic interactions with conventional agents. Optimization of its formulation, such as developing nanoparticle-infused hydrogels or scaffolds, can enhance stability and controlled release. Finally, well-structured clinical trials must be conducted to assess its real-world efficacy and safety in dental applications.

5. Conclusion

Finally, this work shows that nanoparticle-enhanced royal jelly has the potential to be a powerful antibacterial agent against Streptococcus mutans. The improved efficacy when compared to current pulp capping agents, as well as the discovery of synergistic effects in coupled treatments, highlight the importance of natural ingredients in endodontic applications. The findings urge a paradigm shift toward long-term and synergistic ways to fight bacterial infections, thereby tackling the important issue of antibiotic resistance. More research is needed to investigate the underlying mechanisms and enhance the formulation for therapeutic applications.

6. Source of Funding

Self-funded.

7. Conflicts of Interest

The authors declare no conflicts of interest.

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