Original Article

Evaluation of minimum inhibitory concentration and minimum bactericidal concentration of royal jelly against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*

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Abstract

Objective: The objective of this study was to ascertain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of royal jelly (RJ) against three microorganisms frequently linked with endodontic infections: *Staphylococcus aureus*, *Enterococcus faecalis, and Candida albicans.*

Materials and Methods: Freshly harvested RJ was prepared at different concentrations (20%, 10%, 5%, 2.5%, and 1.25%) in distilled water. The microbial cultures of the target organisms were prepared. MIC was determined using a broth dilution technique, monitoring microbial growth. MBC was determined by inoculating agar plates with samples from tubes showing no apparent growth and evaluating the presence of bacterial or fungal growth following the incubation period.

Results: For *S. aureus*, the MIC and MBC were 5 mg/ml of RJ. For *E. faecalis*, the MIC and MBC were 10 mg/ml of RJ. For *C. albicans*, both MIC and MBC were 10 mg/ml of RJ. The findings demonstrated RJ's potential to inhibit and eliminate these pathogenic microorganisms, making it a potential candidate for endodontic infection control.

Conclusion: The antimicrobial properties of RJ against *S. aureus*, *E. faecalis*, and *C. albicans* present a promising avenue for enhancing infection control in endodontics. Additional investigations are needed to refine its use in clinical settings, especially in cases with mixed microbial infections.

Keywords: Antimicrobial efficacy; *Candida albicans*; *Enterococcus faecalis*; intracanal medicament, royal jelly; *Staphylococcus aureus*

INTRODUCTION

Royal jelly (RJ), often referred to as nature's elixir, is a remarkable substance produced by worker honeybees. It serves as the exclusive food source for the queen bee throughout her entire life, enabling her to grow larger, live longer, and maintain her reproductive capacity. This exceptional secretion is revered not only for its crucial role in the hive but also for its potential health benefits for humans. These were reported to have antibacterial, antioxidant, anti-inflammatory, antitumoral, antiviral, immunomodulatory, neuroprotective, and antiaging effects.[1] MRJP's present in RJ provide antimicrobial efficacy against various bacteria.[2] The antimicrobial activity of RJ against *Enterococcus faecalis* can be attributed to the presence of MRJP1,[3] while its effectiveness against

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Staphylococcus aureus is primarily due to the presence of 10-hydroxy-2-decenoic acid (10-HDA).[4,5] In addition, its antifungal activity against *Candida albicans* is associated with the presence of MRJP2.[6]

Endodontic infections are primarily caused by a complex microbial community residing within the root canal system of teeth. Among these microorganisms, *C. albicans*, *E. faecalis*, and *S. aureus* have emerged as key players, contributing to the pathogenesis and persistence of endodontic infections. *C. albicans*, a common fungal pathogen, has been increasingly recognized for its involvement in persistent endodontic infections.[7] This yeast-like fungus can form biofilms within the root canal, protecting it from host defenses and antimicrobial agents.[8] Moreover, *C. albicans* has been associated with treatment-resistant cases of endodontic infections, emphasizing the need for a comprehensive understanding of its role in disease progression.[8] *E. faecalis* is another microorganism frequently implicated in persistent endodontic infections.[9] Its ability to survive in the harsh environment of the root canal, resist antimicrobial treatment, and modulate the host immune response makes it a formidable adversary. The persistence of *E. faecalis* in endodontic lesions has been linked to treatment failures and recurrent infections, highlighting the importance of elucidating its role.[10] *S. aureus*, a notorious pathogen in various infectious diseases, has also been found in endodontic infections.[11] They possess a wide array of virulence factors that may contribute to the persistence and exacerbation of endodontic infections.[12] Calcium hydroxide has been traditionally used as an intracanal medicament in endodontics due to its unique properties, including its ability to disinfect and promote healing within the root canal system. However, when it comes into contact with periapical tissues, there are possibilities for inducing periapical inflammation.[13] The objective of this article is to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of RJ against *E. faecalis*, *C. albicans*, and *S. aureus*.

MATERIALS AND METHODS

Royal jelly

Freshly harvested RJ was acquired and stored in the freezer at 4°C until the experiment was begun. This stock solution was used to prepare five different concentrations of RJ: 20%, 10%, 5%, 2.5%, and 1.25% in distilled water.

Cultivation of bacteria

The *S. aureus* (ATCC: 25923) bacterial culture, which had been resuscitated, underwent centrifugation at 11,000 rpm for 5 min in brain heart infusion (BHI) broth. Following the removal of the supernatant, 20 ml of sterile normal saline was introduced. The resultant bacterial pellet, containing viable cells, was preserved as the stock culture. Subsequently, the bacterial concentration was adjusted to an optical density of 0.10 at 625 nm using a spectrophotometer, corresponding to 10^8 CFU/ml according to the 0.5 McFarland standard. *E. faecalis* (ATCC: 29212), cultured overnight at 37°C in BHI medium. The *C. albicans* strains (ATCC: 90028) were cultivated on sabouraud dextrose (SD) agar and in SD broth for their growth. Freshly subcultured *C. albicans* that had been incubated for 24 h were employed in all experiments conducted in this study.

Minimum inhibitory concentration determination

The antibacterial efficacy of RJ was assessed using the conventional broth dilution technique, which involved monitoring observable microbial growth in the agar broth. The MIC in BHI broth was determined by subjecting serial two-fold dilutions, spanning concentrations from 20 mg/ml to 1.25 mg/ml, to a bacterial suspension adjusted to a concentration of 10^8 CFU/ml according to McFarland's standard. A control group was established, consisting of inoculation broth incubated solely at 37°C for a duration of 24 h. The MIC endpoint was defined as the RJ concentration at which no visible growth was observed in the test tubes. To validate the MIC value, the optical turbidity of the tubes was measured both before and after the experiment.

Minimum bactericidal concentration determination

Following the determination of the MIC of the RJ, 50 µl aliquots was extracted from all the tubes, in which no observable bacterial growth was detected. These aliquots were then plated onto BHI agar plates and incubated for a period of 24 h at 37°C. The MBC endpoint was established when 99.9% of the bacterial population were eradicated at the lowest concentration of the antimicrobial agent. This determination was made by assessing the presence or absence of bacteria on both the agar plates before and after the incubation period.

Statistical analysis

The MBC result underwent statistical analysis through an analysis of variance (ANOVA), which included descriptive statistics such as mean and standard deviation. Subsequently, Tukey's *post hoc* test was employed to further analyze the MBC of RJ concerning its impact on *C. albicans*, *E. faecalis*, and *S. aureus*. It is important to note that the significance level for all the conducted statistical tests was predetermined at $P < 0.05$.

RESULTS

Staphylococcus aureus

After incubating under aerobic conditions at 37°C for 24 h, turbidity was observed in test tubes containing RJ at concentrations of 1.25 mg/ml and 2.5 mg/ml, indicating

bacterial growth [Table 1a]. However, no turbidity was observed in tubes with concentrations of 5 mg/ml, 10 mg/ml, and 20 mg/ml, indicating inhibition of bacterial growth. Subsequently, suspensions from the tubes containing 5 mg/ml, 10 mg/ml, and 20 mg/ml were inoculated onto BHI agar plates and incubated for 24 h. No bacterial growth was observed in any of these concentrations, confirming their bactericidal effect [Table 1b].

A statistical analysis using ANOVA and Tukey's *post hoc* test was conducted to assess the MBC for different RJ concentrations in relation to *S. aureus*. The results demonstrated significant inhibition of bacterial growth for concentrations of 5 mg/ml, 10 mg/ml, and 20 mg/ml when compared to 1.25 mg/ml and 2.5 mg/ml, respectively. The MIC was determined to be 5 mg/ml.

For *Enterococcus faecalis*

Following a 24-h incubation period under aerobic conditions at 37°C, it was observed that turbidity developed in test tubes containing RJ at concentrations of 1.25, 2.5, and 5 mg/ml, indicating the proliferation of *E. faecalis*. In contrast, no turbidity was observed in tubes with concentrations of 10 mg/ml and 20 mg/ml, signifying the inhibition of *E. faecalis* growth [Table 2a]. Subsequently, suspensions from the 10 mg/ml and 20 mg/ml tubes were introduced onto BHI agar plates and incubated for 24 h. Remarkably, there was no evidence of *E. faecalis* growth in any of these RJ concentrations, thus confirming their bactericidal activity against *E. faecalis* [Table 2b]. Statistical analysis, employing ANOVA and Tukey's *post hoc* test, was conducted to ascertain the MBC for varying RJ concentrations in relation to *E. faecalis*. The results consistently indicated an MBC of 10 mg/ml for *E. faecalis*.

Minimum fungicidal concentration against *Candida albicans*

Following a 24-h incubation period under aerobic conditions at 37°C, turbidity was observed in the test tubes containing RJ at a concentration of 5 mg/ml, indicating the presence of *C. albicans* growth at this concentration. In contrast, no turbidity was observed in tubes with concentrations of 10 mg/ml and 20 mg/ml, suggesting the inhibition of *C. albicans* growth [Table 3a]. To confirm these findings, suspensions from the tubes containing RJ at 10 mg/ml were plated onto Sabouraud's agar medium for *C. albicans* and incubated for 24 h. Consistent with the earlier observations, no discernible growth of *C. albicans* was detected in the 10 mg/ml concentration, thus confirming its fungicidal activity against *C. albicans* [Table 3b].

This outcome aligns with the determination of both the MIC and minimum fungicidal concentration (MFC), which were found to be 10 mg/ml for *C. albicans*.

Table 1a: Minimum inhibitory concentration and turbidity for different concentrations of royal jelly after 24 h (*Staphylococcus aureus*)

Dilution of RJ	1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL
Set 1	+	+	−	−	−
Set 2	+	+	−	−	−
Set 3	+	+	−	−	−
Set 4	+	+	−	−	−
Set 5	+	+	−	−	−

+: Indicating growth, −: Indicating absence of growth, RJ: Royal jelly

Table 1b: Minimum bactericidal concentration of royal jelly after 24 h

Dilution of RJ	5 mg/mL	10 mg/mL	20 mg/mL
Set 1	−	−	−
Set 2	−	−	−
Set 3	−	−	−
Set 4	−	−	−
Set 5	−	−	−

−: Indicating absence of growth, RJ: Royal jelly

Table 2a: Minimum inhibitory concentration and turbidity for different concentrations of royal jelly after 24 h (*Enterococcus faecalis*)

Dilution of RJ	1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL
Set 1	+	+	+	−	−
Set 2	+	+	+	−	−
Set 3	+	+	+	−	−
Set 4	+	+	+	−	−
Set 5	+	+	+	−	−

+: Turbidity indicating growth, −: No turbidity indicating absence of growth, RJ: Royal jelly

Table 2b: Minimum bactericidal concentration of royal jelly after 24 h

Dilution of RJ	10 mg/mL	20 mg/mL
Set 1	−	−
Set 2	−	−
Set 3	−	−
Set 4	−	−
Set 5	−	−

−: Indicating absence of growth, RJ: Royal jelly

DISCUSSION

This study was conducted with the primary objective of ascertaining the MIC and MBC of RJ in relation to *S. aureus, E. faecalis,* and *C. albicans*. The evaluation of the antibacterial properties of substances often involves standard methods such as agar diffusion assays and MIC tests. It is worth noting that direct contact tests offer advantages over the agar diffusion method, primarily because they are not influenced by the diffusion characteristics of the tested material within the medium.[14] In MIC tests, serial dilutions of a substance are employed to pinpoint the lowest concentration at which it retains its antibacterial efficacy.

Table 3a: Minimum inhibitory concentration and turbidity for different concentrations of royal jelly after 24 h (*Candida albicans*)

Dilution of RJ	1.25 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL
Set 1	+	+	+	−	−
Set 2	+	+	+	−	−
Set 3	+	+	+	−	−
Set 4	+	+	+	−	−
Set 5	+	+	+	−	−

+: Turbidity indicating growth, −: No turbidity indicating absence of growth, RJ: Royal jelly

Table 3b: Minimum fungicidal concentration of royal jelly after 24 h

Dilution of RJ	10 mg/mL	20 mg/mL
Set 1	−	−
Set 2	−	−
Set 3	−	−
Set 4	−	−
Set 5	−	−

−: No turbidity indicating absence of growth, RJ: Royal jelly

Table 4: Minimum inhibitory concentration and minimum bactericidal concentration/minimum fungicidal concentration of royal jelly against respective micro-organisms

RJ	MIC (mg/mL)	MBC/MFC (mg/mL)
Enterococcus faecalis	10	10
Staphylococcus aureus	5	5
Candida albicans	10	10

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration, RJ: Royal jelly

One of the key challenges in endodontics is effectively managing and eliminating bacterial infections within the root canal system. Bacteria, particularly species such as *S. aureus*[12] and *E. faecalis*,[15] are commonly associated with persistent endodontic infections, which can lead to treatment failure and discomfort for patients. The results of this study demonstrate the potential antimicrobial efficacy of RJ against *S. aureus*, *E. faecalis*, and *C. albicans*. The MIC and MBC of 5 mg/ml for *S. aureus*, 10 mg/ml for *E. faecalis*, and 10 mg/ml for *C. albicans* [Table 4] highlight RJ's ability to inhibit and effectively eliminate these pathogenic bacteria. In an antibacterial study by Eshraghi and Seifollahi concluded the MIC of ether soluble fraction of RJ to be 30 mg/ml against various bacteria including *E. faecalis* and *S. aureus*.[16] This antibacterial effect is majorly because of bactericidal action by 10-HDA present in ether soluble fraction of RJ.[16] This antimicrobial activity could have promising applications in endodontics, where controlling and eradicating bacterial infections is paramount.[17]

In endodontic procedures, infected pulp tissue is removed from the root canal, and the canal is disinfected to prevent further infection and promote healing. Traditional disinfection methods often involve the use of chemical agents such as calcium hydroxide and chlorhexidine.[18] While effective, these chemicals may have limitations, including cytotoxicity and potential damage to periapical tissues.[18] The use of natural products such as RJ, which exhibit antimicrobial properties, could offer an alternative or complementary approach to disinfection in endodontics without any side effects. In addition, the anti-inflammatory properties of RJ may aid in lessening periapical inflammation, promoting tissue repair and regeneration. Further, the antioxidant properties of RJ might contribute to overall periapical health by neutralizing oxidative stress.

Moreover, the potential bactericidal effect of RJ against *E. faecalis* is particularly noteworthy. *E. faecalis* is notorious for its ability to survive and persist in the root canal system, even after conventional treatments.[19] If RJ can effectively eliminate *E. faecalis* at a concentration of 10 mg/ml, it may hold promise as an adjunctive treatment in cases of persistent endodontic infections where *E. faecalis* is implicated. In the field of endodontics, where infections can involve various microorganisms,[20,21] having an antimicrobial agent effective against both bacteria and fungi such as *C. albicans* is highly advantageous.

However, it is important to acknowledge that the transition from laboratory studies to clinical applications in endodontics requires further research and clinical trials. Factors such as the safety, biocompatibility, and practicality of RJ as an intracanal medicament need to be thoroughly investigated. In addition, the optimal concentration and mode of application must be determined to ensure effective and safe use in clinical settings.

CONCLUSION

As this field of research evolves, it opens doors to further investigations. These may include exploring RJ's interactions with other microorganisms commonly found in endodontic infections, assessing its long-term effects, and refining protocols for its use in endodontic procedures.

In conclusion, the antimicrobial properties of RJ, as demonstrated against *S. aureus*, *E. faecalis*, and *C. albicans*, present a promising avenue for enhancing infection control in endodontics. While these findings are encouraging, additional research and clinical trials are necessary to determine the optimal application and safety of RJ as an intracanal medicament.

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Conflicts of interest
There are no conflicts of interest.

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