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EVALUATING THE ANTIMICROBIAL EFFECTIVENESS OF ETHANOLIC PROPOLIS EXTRACT AGAINST E. FAECALIS, S. AUREUS, AND C. ALBICANS

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ABSTRACT:

Objective: This study aimed to investigate the antimicrobial efficacy of propolis against key endodontic pathogens, such as Enterococcus faecalis, Staphylococcus aureus, and Candida albicans. The primary focus was on determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) of the ethanolic extract of propolis.

Methods: Raw propolis was obtained from beekeepers in Chennai, India, and subjected to maceration. The ethanolic extract was then evaluated for its effectiveness against the specified pathogens through conventional broth dilution techniques. Bacterial and fungal concentrations were adjusted to standardized levels, and MIC and MBC/MFC values were determined through serial dilutions. Statistical analysis, including ANOVA and Tukey's post hoc test, was utilized to evaluate the significance of the results.

Results: Propolis exhibited concentration-dependent antimicrobial effects. For Staphylococcus aureus, the MIC and MBC were determined to be 20 mg/ml. Similarly, Candida albicans showed a MIC and MFC of 20 mg/ml. Enterococcus faecalis displayed susceptibility to the ethanolic extract of propolis, with an MBC of 5 mg/mL.

Conclusion: The concentration-dependent antimicrobial effectiveness of the ethanolic extract of propolis against specific bacteria and fungi provides a promising avenue for enhancing infection control in endodontics. Despite these positive results, further research and clinical trials are necessary to establish optimal application protocols and ensure the safety of propolis as an intracanal medicament.

Keywords: Propolis, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Antimicrobial efficacy.

1. INTRODUCTION

In the ever-evolving realm of dentistry, the quest for effective intra-canal medicaments to combat persistent endodontic pathogens has intensified. Even though these endodontic infections are of polymicrobial origin, several microbes have emerged as key pathogens in persistent endodontic infections. *Enterococcus faecalis* (*E. faecalis*), a gram-positive coccus, typically originates in the human oral cavity, gastrointestinal tract, and vagina. Its ability to adapt effectively to environments with rich nutrients, low oxygen levels, and complex ecological systems allows it to thrive in root canals. [1] *Candida albicans* has a preference for

inhabiting the dentin walls of root canals, where it enters dentinal tubules and forms biofilms. *C. albicans* within biofilms exhibits increased resistance to removal compared to its planktonic form and is frequently encountered in persistent or refractory endodontic infections unresponsive to conventional root canal treatments. [2] *Staphylococcus aureus*, a resilient microorganism, is commonly detected in cases of multiple root canal treatments. It is significantly involved in the onset of initial endodontic infections and persistent infections that occur between treatment sessions, especially when the root canal remains unintentionally exposed during therapy.[3,4]

Propolis is derived from the resinous collection of bees from diverse plant sources. It has long been acknowledged for its varied pharmacological attributes, encompassing antimicrobial, anti-inflammatory, and antioxidant properties. [5] Such inherent qualities position it as a compelling candidate to address the multifaceted challenges posed by endodontic infections.

The goal of this research is to determine the lowest concentration of propolis needed to inhibit bacterial growth (MIC) and to eradicate bacteria (MBC) against key endodontic pathogens like *E. faecalis*, *C. albicans*, and *S. aureus*. The significance of exploring natural alternatives, such as propolis, stems from the growing apprehension regarding antimicrobial resistance and the adverse effects associated with synthetic intra-canal medicaments. [6] Establishing the minimum concentrations at which propolis effectively inhibits and eradicates these microbial threats is crucial to affirming its efficacy as a potential intra-canal medicament. This exploration not only illuminates propolis's antimicrobial prowess but also underscores its potential as a natural alternative to synthetic medicaments commonly utilized in endodontic therapies.

2. MATERIALS AND METHODS

Maceration

In April 2023, raw propolis was obtained from beekeepers in Chennai, India, and subsequently stored at a temperature of -18 degrees Celsius in preparation for the experiment. To extract propolis, 5 grams of finely powdered propolis were mixed with 250 milliliters of 70% ethanol using a maceration technique. The mixture was stirred for 24 hours at room temperature and 250 revolutions per minute (rpm) using an orbital shaker. Afterward, the solution underwent centrifugation at 4000 rpm for 10 minutes, resulting in the separation of the liquid portion from the solid residue. The supernatant was then filtered through the Whatman 5 filter paper. The residue underwent an additional extraction with 250 mL of 70% ethanol.[7]

Bacterial cultivation

The bacterial culture of *S. aureus* (ATCC: 25923) underwent centrifugation at 11,000 rpm for five minutes in the Brain Heart Infusion (BHI) broth after resuscitation. Following supernatant removal, 20 mL of sterile normal saline was added, preserving the resulting bacterial pellet as the stock culture containing viable cells. Following this, the bacterial density was adjusted to an optical density of 0.10 at 625 nm utilizing a spectrophotometer, corresponding to 0.5 McFarland standard.

For *E. faecalis* (ATCC: 29212), cultured overnight at 37℃ in Brain Heart Infusion (BHI) medium, and *C. albicans* strains (ATCC: 90028), cultivation occurred on Sabouraud Dextrose (SD) Agar and in SD Broth for growth. Freshly subcultured Candida albicans (C. albicans) incubated for 24 hours were utilized in all experiments conducted in this study.

Determination of Minimum inhibitory concentration

The effectiveness of Propolis against bacteria was assessed using the traditional broth dilution method, which involves monitoring visible bacterial growth in agar broth. The MIC in Brain

Heart Infusion (BHI) broth was determined by subjecting serial two-fold dilutions (ranging from 20 mg/ml to 1.25 mg/ml) to bacterial suspensions of E. faecalis [8] and S. aureus [9] adjusted to a concentration of 10^8 colony-forming units per milliliter (CFU/ml) according to McFarland's standard.. For the determination of MIC against *C. albicans*, SD broth is used.[10] A control group was established, consisting of inoculation broth incubated solely at 37°C for 24 hours. The MIC endpoint was defined as the propolis concentration where no visible growth was observed in the test tubes. To validate the MIC value, the optical turbidity of the tubes was assessed before and after the experiment.

Determination of MBC & minimum fungifidal concentration (MFC)

After determining the MIC of the ethanolic extract of propolis, 50 microliter samples were taken from all tubes without observable bacterial growth. These samples were then applied to BHI agar plates for *E. faecalis* [8] and *S. aureus* [9] and incubated at 37°C for 24 hours. For determination of MFC against C. albicans, SD agar plates are used. [10] The MBC endpoint was identified as the concentration of the antimicrobial agent where 99.9% of the bacterial population was eradicated. This determination involved examining the presence or absence of bacteria on both agar plates before and after the incubation period.

Statistical analysis

The statistical analysis of the MIC and MBC results involved applying an ANOVA, which incorporated descriptive statistics like mean and standard deviation. Subsequently, Tukey's post hoc analysis was utilized to further investigate the MIC and MBC of Propolis and its effects on *E. faecalis, S. aureus, and C. albicans*. Importantly, a predetermined significance level of $p < 0.05$ was established for all statistical evaluations.

3. RESULTS

S. aureus

After 24 hours, turbidity was evident in test tubes with propolis concentrations of 10 mg/mL and lower. Conversely, no turbidity was noted in tubes containing 20 mg/ml, indicating a hindrance to bacterial growth. Following this step, the solutions from the tubes with a concentration of 20 mg/ml were applied onto Brain Heart Infusion (BHI) agar plates. These plates were then placed in an incubator for a period of 24 hours. Remarkably, upon examination after the incubation period, no signs of bacterial growth were observed. This absence of growth signifies a potent bactericidal effect induced by the Propolis at this concentration.

To delve into these findings, a statistical analysis was conducted using ANOVA and Tukey's post hoc test to assess the minimum bactericidal concentration (MBC) in relation to different propolis concentrations concerning Staphylococcus aureus. The results underscored a significant inhibition of bacterial growth at the 20 mg/mL concentration. The minimum inhibitory concentration (MIC) was consequently determined to be 20 mg/ml.

C. albicans

After incubation in aerobic conditions at 37°C for 24 hours, propolis concentrations of 10 mg/ml and below in test tubes exhibited turbidity, while tubes with 20 mg/ml showed no turbidity, indicating an inhibition of fungal growth. Subsequently, when suspensions from the 20 mg/ml concentration tubes were introduced to SD agar plates and incubated for 24 hours, there was no observed fungal growth, demonstrating a fungicidal effect.

To delve deeper into these observations, a statistical analysis using ANOVA and Tukey's post hoc test was performed to assess the MFC in relation to various propolis concentrations against *Candida albicans*. The findings revealed a significant inhibition of fungal growth at the 20

mg/mL concentration. As a result, the MIC was determined to be 20 mg/mL for *Candida albicans*.

E. faecalis

After a 24-hour incubation under aerobic conditions at 37°C, it was observed that turbidity developed in test tubes containing propolis at concentrations of 1.25 and 2.5 mg/ml, indicating the proliferation of *Enterococcus faecalis (E. faecalis*). Conversely, no turbidity was observed in tubes with concentrations of 5 mg/ml, 10 mg/ml, and 20 mg/ml, signifying the inhibition of *E. faecalis* growth. Subsequently, suspensions from the 5 mg/ml, 10 mg/ml, and 20 mg/ml tubes were introduced onto Brain Heart Infusion (BHI) agar plates and incubated for 24 hours. Remarkably, there was no evidence of *E. faecalis* growth in any of these concentrations, confirming their bactericidal activity against *E. faecalis*.

A statistical analysis was conducted, employing ANOVA and Tukey's post hoc test, to ascertain the minimum bactericidal concentration (MBC) for varying propolis concentrations in relation to *Enterococcus faecalis*. The results consistently indicated an MBC of 5 mg/ml for *E. faecalis*.

4. DISCUSSION

The main goal of this study was to ascertain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) / minimum fungicidal concentration of propolis against *Staphylococcus aureus, Enterococcus faecalis,* and *C. albicans*. Standard methods such as agar diffusion assays and MIC tests are commonly employed to evaluate antibacterial properties. It's important to note that direct contact tests offer advantages over the agar diffusion method as they are not influenced by the diffusion characteristics of the tested material in the medium. [11] In MIC tests, serial dilutions are used to identify the lowest concentration at which a substance maintains its antibacterial efficacy.

Effectively managing and eliminating bacterial infections within the root canal system poses a significant challenge in the field of endodontics.

Numerous laboratory studies have investigated the resistance of *E. faecalis* to endodontic treatment, revealing a notable resistance to antimicrobial agents. Furthermore, *E. faecalis* has demonstrated its ability to survive in challenging environments with poor nutrient supply and elevated alkaline pH levels reaching up to 11.5. [12] The ability of *E. faecalis* to form biofilm on the walls of root canals and persist as a mono-infection in treated canals independent of other bacteria adds to its formidable resistance to antimicrobial agents, presenting a substantial challenge in the context of root canal treatments. [13] Fungi are commonly implicated and have been identified in around 3–18% of infected root canals, with Candida species predominantly prevailing. [14] A systematic review and meta-analysis revealed *Candida albicans* as the most frequently isolated fungus from infected root canals. [15] *Candida albicans* has an affinity for binding to both living (tooth dentin) and non-living surfaces (dental prostheses). The circular cells of *C. albicans* adhere to dentin surfaces within a timeframe of 60–90 minutes. [16] Following attachment, these cells proliferate, forming a foundational biofilm layer that matures within 24 hours. Mature biofilms encompass numerous layers of polymorphic cells, comprising hyphal, pseudohyphal, and yeast forms embedded within extracellular matrices, forming robust and physiochemically resilient structures. Subsequently, yeast cells from mature biofilm in spherical form disperse to initiate infections at distant sites. *C. albicans* within biofilms exhibit a resistance 10–100 times greater to host immune responses and antifungal treatment. This heightened resistance is attributed to slowed cell growth and metabolism, safeguarded by extracellular polymeric substances (EPS) and other defensive factors. [17]

Several studies have identified multiple benefits of choosing calcium hydroxide as a medicament, highlighting its notable features, such as high alkalinity, capacity for tissue dissolution, effectiveness in neutralizing endotoxins, and antibacterial properties. Calcium hydroxide (Ca(OH)2) is a widely employed intra-canal medicament. [18] While Ca(OH)2 exhibits some antimicrobial action, its effectiveness against both *E. faecalis* and *C. albicans* is limited. [19] Addressing multi-drug-resistant pathogenic bacteria has been designated a priority by the World Health Organization (WHO). Consequently, there is an increasing demand for innovative, non-conventional antimicrobial treatments. [20]

The experimental results highlight the concentration-dependent antimicrobial effectiveness of the ethanolic extract of Propolis against specific bacteria. The ethanolic extract of Propolis at 20 mg/mL demonstrated potent inhibitory and bactericidal effects on *Staphylococcus aureus*, with MIC and MBC of 20 mg/ml. The ethanolic extract of propolis displayed significant antimicrobial properties against *Enterococcus faecalis*, with concentrations of 5 mg/ml, 10 mg/ml, and 20 mg/ml showing consistent bactericidal activity and a Minimum Bactericidal Concentration (MBC) of 5 mg/ml. Within the limitations of our study, the MIC and MFC of the ethanolic extract of propolis against *C. albicans* is 20mg/ml.

5. CONCLUSION

As the realm of research progresses, it opens avenues for further exploration. These may involve investigating how Propolis interacts with other microorganisms commonly found in endodontic infections, assessing its prolonged effects, and refining protocols for its utilization in endodontic procedures.

In conclusion, the demonstrated antimicrobial effectiveness of Propolis against *Staphylococcus aureus (S. aureus), Enterococcus faecalis (E. faecalis),* and *Candida albicans (C. albicans)* offers a promising prospect for enhancing infection control in endodontics. Despite these encouraging findings, additional research and clinical trials are essential to determine Propolis's optimal application and safety as an intra-canal medicament.

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Conflict of Interest
There is no conflict of interest.

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Table 1

Royal Jelly	MIC	MBC/MFC
E. Faecalis	5mg/ml	5mg/ml

S. aureus	20mg/ml	20mg/ml
C. Albicans	20mg/ml	20mg/ml

MIC and MBC/MFC of Propolis against respective micro-organisms.