ORIGINAL RESEARCH

# Comparative Assessment of Antimicrobial Activity of Propolis and Chlorhexidine on Salivary Isolates of *Candida albicans* and *Streptococcus mutans* in Children with Severe Early Childhood Caries: An *In Vitro* Study

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## Abstract

**Background:** *Streptococcus mutans* and *Candida albicans* are the chief microbes associated with severe early childhood caries (S-ECC). Diverse antimicrobial agents are widely used to prevent ECC, and a quest for newer natural products has been on the rise in the recent past.

**Aim:** To estimate the antimicrobial activity of propolis with chlorhexidine on salivary specimens from children with S-ECC *in vitro*.

**Materials and methods:** A total of 60 children with S-ECC were designated. Salivary samples of 30 children (group I) were inoculated onto mitis salivarius agar (MSA) to isolate *S. mutans*. Another 30 samples (group II) were inoculated on sabouraud's dextrose agar and subcultured on HiCrome *Candida* differential agar to isolate *C. albicans*. Sensitivity testing for 0.2% chlorhexidine and 10% propolis extract was done using the agar well diffusion technique using Mueller–Hinton agar medium. The antimicrobial effect was evaluated by calculating the diameter of the zone of inhibition surrounding the well.

**Results:** All saliva samples collected from groups I and II showed growth of *S. mutans* and *C. albicans*, respectively. All cultured microbes were sensitive to 0.2% chlorhexidine and 10% propolis extract. The mean inhibition zone for *S. mutans* with chlorhexidine was 14.57 ± 0.63 mm, and with propolis, 11.93 ± 0.52 mm. The mean zone of inhibition for *C. albicans* with chlorhexidine was 12.83 ± 0.59 mm, and with propolis, 9.50 ± 0.73 mm. Chlorhexidine consistently showed statistically significantly larger zones of inhibition and hence appeared to be a more potent antimicrobial agent than propolis extract for both *S. mutans* and *C. albicans*. However, propolis has irrefutable action against both *S. mutans* and *C. albicans*.

**Conclusion:** Propolis may be an acceptable substitute for chlorhexidine for long-term use as it has demonstrated antimicrobial activity and fewer side effects. Hence, this Association of Physicians of India herbal drug can be incorporated into mouthwashes and toothpaste to reduce microbial counts.

**Keywords:** *Candida albicans*, Chlorhexidine, Propolis, Severe early childhood caries, *Streptococcus mutans*.

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## Introduction

Dental caries is a significant menace in third-world countries like India. It is the single most prevalent chronic childhood disease, affecting 60–90% of all children.[1,2] Early childhood caries (ECC) is a persistent, infectious, transmissible, complex disease with a very intricate etiology correlated with early colonization and high quantities of cariogenic bacteria. It encases a direct tribulation of pain, suffering, and poor overall health.[3] Any trace of smooth surface caries in children below the age of three is suggestive of severe ECC (S-ECC).[4]

*Streptococcus mutans* and *S. sobrinus* are the primary etiological agents in the initiation of dental caries, although *C. albicans* and *Lactobacilli* play a part in the development of ECC as these are predominant microorganisms found in dental plaque. It causes demineralization because of its high adherence to the tooth surface and the production of acids following fermentation.[5,6] Caries prevention research has focused on methods for lowering or annihilating cariogenic microbes from the mouth.

Among the various antibacterial agents, chlorhexidine is considered the "gold standard" antimicrobial agent used to prevent S-ECC. It is a wide-spectrum bisbiguanide that has antibacterial activity and is more combative on gram-positive microbes.[5,7] Depending on the dosage, it can be used as a bacteriostatic or bactericidal agent. However, it has some possible side effects, such as brownish discoloration of teeth,

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restorations, and tongue, as stated in the literature.[8] Ingestion of 3–6 mL of chlorhexidine by a child with approximately 10 kg body weight can cause gastric disturbance, nausea, or signs of alcohol intoxication.[9]

In a quest for safer and more natural alternatives to chlorhexidine, recent developments have introduced formulations made from natural ingredients like propolis, which have anticariogenic effects.[10,11] Propolis is referred to as "bee glue," a viscid resin produced by honeybees.[12] It is a natural antibiotic material and has drawn interest because of its several pharmacological qualities. Prevention has advanced one step further with the continuously increasing use of propolis. This organic substance has an antibacterial action against a wide range of gram-positive microbes, including *S. mutans*. It blocks the adhesion of cells and the synthesis of water-insoluble glucans by *S. mutans*, which aids in dental caries prevention.[6,13–15]

Thus, this research aimed to assess the antimicrobial efficacy of propolis and chlorhexidine on saliva specimens from S-ECC-affected children in reducing *S. mutans* and *C. albicans* colony counts.

## Materials and Methods

A total of 60 children attending the OPD of the Department of Pediatric and Preventive Dentistry, A. J. Institute of Dental Sciences, Karnataka, India, between 3 and 5 years of age with S-ECC, were selected after securing approval from the Institutional Ethics Committee. The samples were selected by means of nonprobability criterion sampling. Informed consent and assent were obtained from the parent or caretaker of each child, who were also educated about the study. A pretested semistructured proforma was filled out by interviewing the parent or caretaker, including child particulars and oral habits. The children's caries experience [decayed, missing, and filled surfaces (dmfs) index] was documented using a mouth mirror, community periodontal index probe, and visible light. Children with S-ECC, with a decayed, missing, and filled teeth (dmft) index score of <2 and willing to participate in the investigation, were included in the study. Children who were currently using a mouthwash, had any abnormal oral, medical, or allergic conditions, xerostomia, or a history of hypersensitivity to any product used in this study, and children on antibiotic therapy or any medication within 2 weeks before the study started were excluded from the study.

An alcohol-free chlorhexidine gluconate mouthwash containing 0.2% chlorhexidine gluconate (Hexidine, ICPA Health Products Ltd., Ankleshwar) and a 10% propolis mouth rinse were used in the study. About 10 gm of commercially obtainable raw green Ukrainian propolis stick were cut, ground, and mixed in 100 mL of 70% ethanol to obtain an ethanolic extract of propolis (EEP). This mixture was left in a dark area at ambient temperature and shaken frequently for 2 weeks until it was entirely dissolved, then filtered and evaporated to obtain a viscous brown substance. Around 500 mg of this substance were weighed and dissolved in 5 mL of 70% ethanol to obtain a 10% propolis solution. Mitis salivarius agar (MSA) enriched with bacitracin, sabouraud dextrose agar M063, HiCrome Agar, and Mueller–Hinton Agar M173 (HiMedia Laboratories Pvt. Ltd., Mumbai) were the culture media used in the study.

### Salivary Sample Collection

Around 2 mL of unstimulated saliva was collected from each candidate in a sterile container, stored on melting ice, and immediately transferred to the laboratory for microbiological evaluation.

### Sample Culturing

Each saliva specimen was vortexed aggressively for 30 seconds to mix the sample thoroughly before plating. Around 500 µL of the salivary sample were inoculated onto MSA and sabouraud dextrose agar plates using a calibrated loop. Plates of MSA were incubated in a 5% carbon dioxide ($CO_2$) enriched atmosphere at 37°C for 1 day and then left at room temperature for another 24 hours. The colony morphology of *S. mutans* manifested as a convex, opaque, and granular "frosted glass appearance" (Fig. 1). Sabouraud dextrose agar plates were incubated at 37°C for 48 hours. The thriving of *Candida* emerged as creamish or pearly white, even, and pasty colonies (Fig. 2). *Candida* species were subcultured on HiCrome *Candida* differential agar, indicated by the emergence of green colonies.

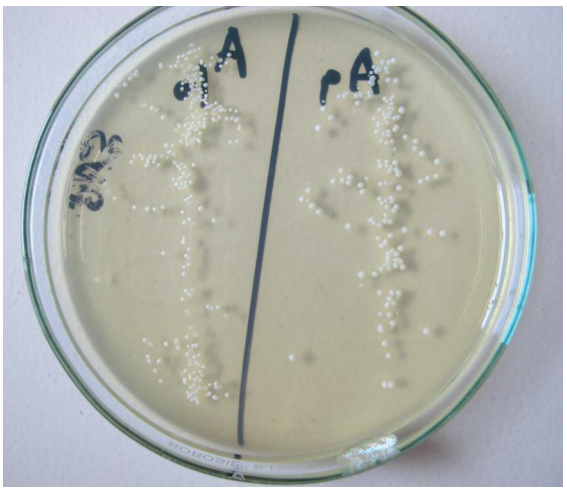

**Fig. 1:** *S. mutans* appeared as a convex, opaque, and granular frosted glass appearance

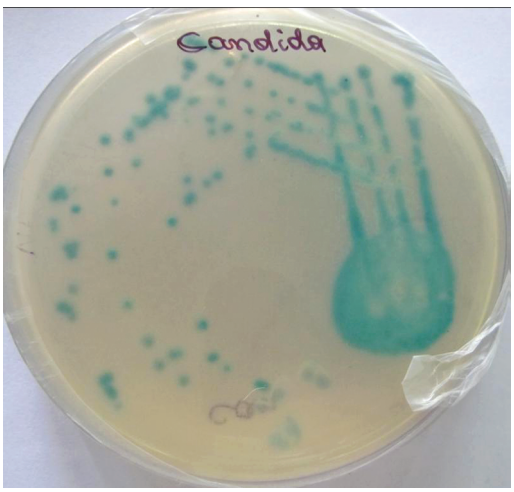


**Fig. 2:** The growth of *Candida* appeared as cream or white, smooth, and pasty colonies

### Sensitivity Testing Using Agar Well-diffusion Technique

Sterile Petri plates with Mueller–Hinton agar medium were prepared. Three wells, each 6 mm in diameter, were cut in each Petri dish using a Durham tube. The bottom of each Petri plate was marked to represent the content of each well. On 30 plates, the bacterial inoculum of *S. mutans* harvested from MSA was evenly spread using a sterile cotton swab. Similarly, *C. albicans* colonies picked from HiCrome *Candida* differential agar were spread on the remaining 30 plates. Around 30 µL of 10% propolis extract, 0.2% chlorhexidine (positive control), and 70% ethanol (negative control) were added to the wells using a calibrated pipette. Plates inoculated with *S. mutans* were incubated in a 5% $CO_2$-enhanced atmosphere at 37°C for 24 hours and left at room temperature for another 24 hours. Plates of *C. albicans* were incubated at 37°C for 48 hours. The antibacterial activity was assessed by estimating the diameter of the inhibition zone that had been established around the well. After the completion of the procedure, all cultures were autoclaved and discarded.

## Results

Data were obtained, sorted, and statistically analyzed using Statistical Package for the Social Sciences (SPSS) version 16.0. The mean and standard deviations were used for age distribution, dmfs scores, and zones of inhibition. The Chi-squared test was employed to ascertain the interrelation in categorical data. The unpaired *t*-test was used to compare the mean values of the zones of inhibition for the 2 mouth rinses. Statistical significance was determined at $p < 0.05$.

In the current study, 60 children participated with a mean age of 49.9 ± 9.9 months. Among them, 61.7% were girls, and 38.3% were boys. The mean age of boys and girls was 50.9 ± 7.6 and 49.2 ± 6.5 months, respectively. There was no significant difference between the age-groups and gender of the children. Upon oral evaluation, the mean dmfs score of the children was 9.40 ± 2.6. There was no significant difference between the mean dmfs scores of girls and boys. The mean dmfs scores in group I were 9.44 ± 3.0 for girls and 8.58 ± 2.4 for boys, and the mean dmfs scores in group II were 9.42 ± 2.6 for girls and 10.18 ± 2.2 for boys, with no significant differences between the two.

In the first group (30 samples), salivary samples were tested for the growth of *S. mutans*, and in the second group (30 samples), for the growth of *C. albicans*. All samples collected from group I revealed the growth of *S. mutans*. Likewise, there was a 100% growth of *C. albicans* in group II, substantiating that *S. mutans* and *C. albicans* are invariably linked with S-ECC. *S. mutans* and *C. albicans*, which were isolated from groups I and II, respectively, were subjected to culture sensitivity testing for 0.2% chlorhexidine and 10% propolis extract. The zones of inhibition obtained for the two microorganisms for the tested antimicrobial agents were as follows.

*S. mutans* were sensitive to the test solutions in each of the 30 isolates. The mean zone of inhibition for *S. mutans* with chlorhexidine was 14.57 ± 0.63 mm, and with propolis was 11.93 ± 0.52 mm. Applying the independent sample *t*-test, it was found that the zone of inhibition for chlorhexidine was significantly higher ($p < 0.001$) in contrast to propolis extract (Table 1). Nevertheless, it is notable that propolis also showed antimicrobial activity against all isolated *S. mutans* samples consistently. *C. albicans* isolates from group II were subjected to culture sensitivity testing for 0.2% chlorhexidine and 10% propolis extract. *C. albicans* were sensitive to both test solutions in all isolates. The mean zone of inhibition for *C. albicans* with chlorhexidine was 12.83 ± 0.59 mm, and with propolis was 9.50 ± 0.73 mm. Applying the independent sample *t*-test, it was found that the zone of inhibition for chlorhexidine was significantly higher ($p$-value < 0.001) compared to propolis extract (Table 2). However, propolis also showed consistent antimicrobial activity in all isolated *C. albicans* samples.

## Discussion

Although a wealth of research supports the use of chlorhexidine in several oral diseases, it also possesses certain drawbacks. A vital detail to consider is the instigation of bacterial resistance, which is a severely detrimental outcome. After 15 days of use of 0.2% chlorhexidine mouthwash, Solís et al. reported tooth discoloration. Other self-reported adverse drug reactions (ADRs) associated with chlorhexidine use included taste alteration, xerostomia, numbness or pain in the tongue and mouth, hypogeusia, and discoloration of the tongue.[16] Over long-term use, calculus and extrinsic tooth staining were also reported, though less frequently than parotid gland swelling, paresthesia, glossodynia, and desquamation of oral mucosa. Surprisingly, there is also limited insight into the threats of chlorhexidine in the dental community, even though chlorhexidine has been popularly used in dental practice as the "gold standard" antiseptic for over 40 years. These pitfalls have necessitated the search for substitutes.[6,17]

Propolis is a plant-based sticky bee product that ancient civilizations have traditionally used. It has various applications and is considered a folk medicine due to its numerous antioxidants, as well as its antibacterial, antifungal, anti-inflammatory, antiviral, immunomodulatory, antiulcer, and wound-healing effects.[14,15] Propolis is an excellent option for creating groundbreaking, effective, and cost-effective remedies due to its broad-spectrum antimicrobial potential. Based on the chemical composition and physical characteristics related to their geographic origin and plant sources, different forms of propolis (such as Poplar, Brazilian, and Mediterranean) may be categorized into distinct subcategories. The main elements that distinguish the various forms of propolis are flavonoids, phenols, diterpenes, and aliphatic compounds. Researchers are continuously exploring the properties of propolis due to its complex composition and extensive range of activities, unveiling its biological potential.[17] Three mechanisms by which

**Table 1:** Zones of inhibition produced on *S. mutans* ($n = 30$)

| | *S. mutans* | | |
|---|---|---|---|
| *Test solution* | *Mean zone of inhibit (mm)* | *Standard deviation* | *Standard error* |
| 0.2% chlorhexidine | 14.57 | 0.63 | 0.11 |
| 10% propolis | 11.93 | 0.52 | 0.10 |
| Test statistic | $t_{58} = 17.71$, $p$-value < 0.001 | | |

**Table 2:** Zones of inhibition for *C. albicans* ($n_{2=30}$)

| | *C. albicans* | | |
|---|---|---|---|
| *Test solution* | *Mean zone of inhibit (mm)* | *Standard deviation* | *Standard error* |
| 0.2% chlorhexidine | 12.83 | 0.59 | 0.12 |
| 10% propolis | 9.50 | 0.73 | 0.13 |
| Test statistic | $t_{58} = 19.41$, $p$-value < 0.001 | | |

propolis disrupts bacterial membranes are binding to the bacterial cell wall, resulting in cell lysis and bacterial termination; interaction between the hydrophobic parts of the membrane and the polar head group of propolis; and inhibition of protein synthesis.[18] Additionally, propolis extracts exhibit minimal cytotoxicity to human cells while preventing yeast cells from forming biofilms.[19] Red and green Brazilian propolis are popular in contrast to brown and yellow propolis. Based on its chemical and physical locations, Brazilian propolis is divided into 12 types.[3] Raw propolis can't be utilized directly in treatment or analysis. Firstly, it should be extracted to release the most potent and active ingredients.[17] Likewise, the solvent chosen for extraction has a major role in enhancing its pharmacological potential.[19]

Extracts contain approximately 70% concentration of propolis. In an observation by Devequi-Nunes et al., the concentrations of phenolic compounds in ethanolic extracts of red, brown, and green propolis were twice as high as those in extracts obtained by supercritical extraction.[17,20] In addition, ethanolic extracts of red, green, and brown propolis had higher quantities of flavonoids compared to those obtained by supercritical extraction, although brown propolis had lower levels. Propolis ethanol extracts exhibit greater levels of antibacterial activity than ester, chloroform, and water fractions, making them more effective. All the above research justifies the use of ethanolic extract of Brazilian propolis in the current study. However, more research is needed to substantiate the action of propolis on anaerobic organisms.[16] However, this groundwork indicates a strong action of propolis against *C. albicans*. Alkhaled et al. found that 0.12% chlorhexidine was effective >5% propolis in reducing the levels of *S. mutans*.[21] Similarly, Marya et al. found that the antimicrobial efficacy of 0.2% chlorhexidine against *S. mutans* was higher compared to that of propolis, which is in accordance with the current study. However, our research shows that this bee product has a potent anti-*C. albicans* effect. According to Marya et al., 0.2% chlorhexidine had a more potent antibacterial activity against *S. mutans* than propolis, which is consistent with the findings of the present investigation. However, the antimicrobial efficacy of propolis against *C. albicans* was higher than that of chlorhexidine in the same study and did not agree with the current test results.[10] The variation in cariostatic activity may be attributed to the different flavonoid compositions obtained from various sources of propolis. Furthermore, despite the fact that various studies have assessed the antibacterial properties of propolis, it is difficult to compare the results due to the differing methodologies employed.[5,22] Although these findings are *in vitro*, substantial evidence of the antimicrobial activity of propolis related to S-ECC supports the relevance of our results. This research explores the potential of propolis as a safer alternative to chlorhexidine.

## Conclusion

This preliminary *in vitro* study compared the efficacy of propolis against *S. mutans* and *C. albicans*. However, this finding raises interest from a clinical perspective. Further research is obligatory to confirm these results with other oral microorganisms and to overcome the study's limitation of its lack of *in vivo* studies, which aim to evaluate propolis at various concentrations and its effects on patients over time. Nevertheless, the calibration and standardization of propolis extracts and their application in dentistry remain a question. Although several studies have assessed the antimicrobial properties of propolis, it is a challenge to compare the results of these studies owing to the different methods used. More importantly, propolis has shown promising results in controlling caries. Hence, it can serve in the field of dentistry in manifolds.

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