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Research Article

Phenotypic and Molecular Characterization of *Candida albicans* and Evaluation of Propolis as an Antifungal Agent

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Authors' Contributions
MJJ and MKA designed the study and planned the experiments. MJJ carried out the laboratory work, including sample collection, phenotypic identification, PCR amplification of virulence genes, antifungal susceptibility testing, and chemical characterization of propolis. MKA supervised the project, provided scientific guidance throughout the research, and helped steer the scientific direction. Both authors analyzed the results and discussing the findings. MJJ wrote the first draft of the manuscript. MKA reviewed and improved the final version.

Keywords
Candida albicans, PCR, 18S rRNA, Virulence factors, Propolis, Antifungal agents

Abstract | This study aimed to determine the phenotypic and molecular characteristics of *Candida albicans* isolates from oral and peri-dental samples and to evaluate the antifungal activity of propolis as a natural alternative agent. Oral and peri-dental samples (146) were collected and cultured on Sabouraud Dextrose Agar. Fungal growth was detected in 53 samples, among which 36 isolates were identified as *C. albicans*. Pure *Candida* isolates were subcultured and their phenotypic identification was done with the help of chromogenic *Candida* agar, microscopic examination, formation of germ tubes, chlamydospore formation and hemolytic activity. The findings indicated the normal morphological and microscopic characteristics of *C. albicans*. Formation of germ tube and chlamydospore further aided species identification, whereas the isolates varied in hemolytic activity indicating differences in traits that are associated with virulence. The VITEK 2 Compact system was found to identify all the tested isolates (10 isolates) as *C. albicans* (100%) with a probable range between 94-99%. Chemical characterization of the ethanolic propolis extract, using GC-MS, detected 15 naturally occurring compounds consisting of sesquiterpenes, fatty acids, phenolic compounds, flavonoids, and triterpenoids. The presence of functional groups containing hydroxyl, carbonyl, aliphatic, and aromatic groups found in the FTIR analysis corroborated the assumption of a complex chemical composition of propolis. PCR amplification of the 18S rRNA gene confirmed the molecular identification that all the tested isolates were *C. albicans*. Moreover, the tested isolates (100%) were positive in the presence of the BCR1, BIP, ALS and HWP1 genes, indicating a strong genetic potential for adhesion, biofilm formation, and hyphal development. Propolis exhibited concentration-dependent antifungal activity against *C. albicans*, with the highest inhibition observed at 2%. However, the relatively elevated MIC (130 ± 30 mg/mL) and MBC (1680 ± 624.8 mg/mL) values indicate that higher concentrations are required for fungicidal activity, suggesting the need for further optimization before clinical application.

Novelty Statement | The study combines phenotypic and molecular characterization to identify oral and peri-dental isolates of *C. albicans*. It also detects key virulence and biofilm-associated genes (BCR1, BIP, ALS, and HWP1) using PCR analysis. In addition, the chemical composition and antifungal activity of locally sourced propolis were evaluated using GC–MS, FTIR, and antifungal susceptibility testing, highlighting its potential as a natural antifungal agent.

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Introduction

Candida albicans is a major opportunistic fungal pathogen and one of the most frequently isolated yeasts in clinical

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samples. It is usually a commensal organism that inhabits the mouth, gastrointestinal tract, and the genitourinary mucosa; but disruption of host defense mechanisms can shift this commensal relationship toward pathogenicity (Sadrossadati *et al*., 2018; Rodriguez-Leguizamon *et al*., 2020). Immunosuppression and diabetes mellitus, as well as long-term antibiotic use, malignancy, hospitalization and invasive medical procedures, are also associated with a high risk of getting infected by *C. albicans* since it allows this pathogen to proliferate and infect host tissues (Díaz-Huerta *et al*., 2025).

The pathogenic success of *C. albicans* is largely attributed to its ability to adapt to diverse host environments. Phenotypic plasticity, and in particular the reversible change between the morphologies of the yeast, pseudohyphal, and hyphae, is one of its most significant biological features. This morphological transition is not merely structural but is closely associated with virulence because the hypha shapes promote penetration of the tissues, tissue injury, and immune resistance (Jayachandran *et al*., 2018). The filamentous growth form also plays a central role in biofilm architecture and stability, contributing significantly to persistent infections.

In addition to morphological plasticity, *C. albicans* expresses multiple virulence factors that promote colonization and disease progression. These consist of adhesins facilitating attachment to epithelial cells and abiotic surfaces, secreted hydrolytic enzymes including proteases and phospholipases that break down host tissues and hemolysins that allow implantation of iron onto the host erythrocytes (Parambath *et al*., 2024; Nailis *et al*., 2010). In addition, biofilm formation represents a key virulence mechanism, contributing to increased resistance to antifungal agents and host immune responses (Lopes and Lionakis, 2022).

Clinical management and epidemiological surveillance of *C. albicans* requires proper identification that is accurate and timely. The traditional phenotypic identification procedures, such as colony morphology, germ tube development, chlamydospore growth, and colorimetric media, are common in the routine laboratories because they are very easy and cheap (Khan *et al*., 2018). Although useful, these methods may occasionally lead to misidentification, particularly among closely related Candida species. Therefore, molecular diagnostic methods have taken on greater significance to ascertain the identity of the species and describe virulence-related genetic determinants (Calgin and Cetinkol, 2018).

Increasing cases of antifungal resistance in *Candida* species especially against the widely used azole drugs have become a problem of global clinical concern. This problem has increased the interest in alternative and complementary antifungal agents with natural origins (Bilal *et al*., 2023). Propolis is a resinous substance produced by honeybees and has received significant interest because of its rich chemical composition, such as flavonoids, phenolic acids, terpenoids, and fatty acids that are known to have antimicrobial properties (Gucwa *et al*., 2018). It has been shown that propolis has antifungal effects in *Candida* species and probably disrupts fungal cell membranes, inhibits the formation of biofilms and the expression of virulence factors, which could explain its effect on fungal cells (Hernandez-Hernandez *et al*., 2025). It is also known that the composition of propolis can depend on the geographical origin, botanical sources and environmental conditions, thus, a study of a locally sourced propolis in Baghdad City can give certain details about its chemical composition and antifungal capacity.

In this respect, the current research sought to examine oral and peri-dental isolates of *Candida albicans* using a combination of phenotypic characterization, molecular identification and assessment of the antifungal action of propolis as a natural alternative agent.

Materials and Methods

Specimen collection

A total of 146 oral and peri-dental samples were collected using sterile cotton swabs from patients attending hospitals in the Governorate of Baghdad. All specimens were aseptically collected and transported to the laboratory where further mycological studies were done.

The samples were collected from patients presenting with clinical signs suggestive of oral candidiasis, including mucosal inflammation, pseudomembranous lesions, or peri-dental irritation. The study population was not randomly screened from the general community. All culture plates were carefully examined to exclude bacterial contamination based on colony morphology and Gram staining.

Primary culturing, isolation and purification

The specimens were inoculated onto Sabouraud Dextrose Agar (SDA) plates and allowed to incubate at 37 °C over a period of 24-48 h. Yeast-like colonies were observed after incubation, having a creamy texture and smooth margins. Pure isolates were obtained by selecting and subculturing individual colonies on new SDA plates and the isolates further used in phenotypic, biochemical and molecular studies.

Phenotypic identification

Pure isolates were grown on Chromogenic *Candida* Agar and developed at 37 °C after 24-48 h. Presumptive identification was done by colony color and morphology. Gram staining was used to examine the morphology and

budding types of the yeast cells under the microscope.

The germ tube test was performed by inoculating yeast cells into human serum and incubating at 37 °C for 2–3 h. Microscopic analysis of germ tube formation was done. The formation of the chlamydospores was tested by cultivating the isolates in Cornmeal Agar and incubation at 25-28 °C in 48-72 h. Hemolytic activity was assessed using blood-supplemented Sabouraud Dextrose Agar plates were incubated at 37 °C (48h) to measure the hemolytic activity.

Identification with the VITEK 2 compact system

The VITEK 2 Compact system was used to perform final identification (bioMerieux, France). Sterile saline solution was used to prepare yeast suspensions and they were adjusted to the recommended McFarland standard. Identification cards of YST were inoculated and left to incubate automatically and the results were analyzed by the VITEK 2 software.

Preparation of propolis extract

Raw propolis was collected locally and extracted using 70% ethanol. The extract was filtered, concentrated and stored under the right conditions until it was utilized during antifungal tests and chemical characterization.

GC–MS analysis

GC–MS analysis was performed using an Agilent Technologies system equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Mass spectra were recorded under electron ionization mode at 70 eV, and the oven temperature was programmed from 60 °C to 280 °C with a 10-min hold.

FTIR analysis

An FTIR spectrophotometer (Shimadzu, Japan) was used to perform the FTIR analysis. FTIR spectra were recorded in the range of 400–4000 cm^{-1} to identify the functional groups present in the propolis extract.

Genomic DNA extraction

Due to cost and resource limitations associated with molecular assays, ten representative isolates were selected for molecular identification and virulence gene detection. These isolates were chosen to reflect phenotypic variation observed among the total recovered isolates, including differences in colony morphology and hemolytic activity.

Standard fungal DNA extraction procedures were used to extract genomic DNA of selected *Candida albicans* isolates. The concentration and purity of DNA were determined spectrophotometrically, and DNA samples were kept at -20 °C until PCR analysis.

PCR amplification and molecular identification

PCR reactions were done in a total of 25 μL mixture consisting of template DNA, forward, and reverse primer, PCR master mix, and nuclease-free water. The amplification was done under the condition of the thermal cycler with the optimum conditions based on gene targets.

18S rRNA gene amplification

PCR of 18S rRNA gene was done with the help of the following primers generated using Primer3 software which were mentioned below:

Forward primer: 5′- GGAAGGGRTGTATTTATTAG -3′

Reverse primer: 5′- GTAAAAGTCCTGGTTCCCC -3′

Expected amplicon size: 550 bp

The thermal cycling conditions were the following: 5 min of denaturation at 95 °C, 30 cycles consisting of the denaturation at 94 °C, annealing at 48 °C and extension at 72 °C, and a final extension at 72 °C.

BCR1 gene amplification

In this work, the Primer3 software was used to generate primers with the BCR1 gene based on the conserved sequences:

Forward primer: 5′-ACAACATGGTCAACATGGG-CA-3′

Reverse primer: 5′-TGTTGCATGTGAACTTGGC-3′

Expected amplicon size: 594 bp

PCR conditions included an initial denaturation phase at 95 °C in 5 min, 30 cycles of denaturation phase with 94 °C at 45 sec, annealing phase with 60 °C at 40 sec and extension phase with 72 °C at 55 sec, and a final extension phase with 72 °C at 10 min.

Biofilm-induced protein gene (BIP) amplification

This study designed the primers to the gene of the biofilm-induced protein gene (BIP):

Forward primer: 5′-ACAAAGACTCCGTGTCGT-CAA-3′.

Reverse primer 5′-TCAAAATCCTTGGTGGCTGGT -3′

Expected amplicon size: 442 bp

Amplification was done with an initial denaturation of 95 degrees Celsius in 5 min, 30 replication of 45 s denaturation, 40 sec annealing and 55 sec extension with the last extension time of 10 min.

ALS gene amplification

In this study, primers that were used against the ALS gene are as follows:

Forward primer: 5′-GCTGCTCCAATTCAACAAG-GA-3′.

Reverse primer: 5′-AGACAGCCCAATTGAGACGA-3′.

Expected amplicon size: 463 bp

The PCR conditions involved initial denaturation at a temperature of 95 °C, 30 cycles of denaturation at 94 °C, 45 sec, 60 °C, 40 s, 72 °C, 55 s, and finally extension in 72 °C, 10 min.

HWP1 gene amplification

The amplification of HWP1 gene was done using PCR with the following primers as described by Romeo and Criseo (2008):

Forward primer: 5′-GCTACCACTTCAGAATCAT-CATC-3′.

Reverse primer: 5′-GCACCTTCAGTCGTAGA-GACG-3′.

Expected amplicon size: 941 bp

The thermal cycling was performed under initial denaturation at 95°C, 30 cycles of denaturation at 94 °C, 45s, annealing 58 °C, 40s, and extension at 72 °C, 55s, and lastly extension, 72 °C, 10 min.

Agarose gel electrophoresis

Electrophoresis was done using 1.5% agarose gel stained in Tris acetate EDTA (TAE) buffer. Electrophoresis was done at a current of 90 to 100 V in 45 to 60 min duration to identify DNA bands in the band gel under ultraviolet light in comparison with a molecular weight marker to identify the size of the DNA band.

Antifungal susceptibility testing

The antifungal activity of propolis was initially evaluated using the agar well diffusion method. Yeast inoculum was standardized to approximately 0.5 McFarland turbidity standard prior to plating. All experiments were conducted in triplicate to ensure reproducibility.

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MBC) of propolis were determined using a broth microdilution method. Serial dilutions of propolis were prepared in appropriate broth medium, and standardized yeast suspensions were added to each dilution. The MIC was defined as the lowest concentration showing no visible growth after incubation at 37°C for 24–48 h. For MBC determination, aliquots from tubes showing no visible growth were subcultured onto Sabouraud Dextrose Agar plates, and the lowest concentration showing no colony growth was recorded as the MBC.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by appropriate post hoc comparison. A P-value less than 0.05 was considered statistically significant.

Results

Specimen collection and primary culturing

A total of 146 oral and peri-dental swabs were collected from patients attending hospitals in the Governorate of Baghdad and cultured on Sabouraud Dextrose Agar under appropriate incubation conditions. Fungal growth was observed in 53 samples. Among these fungal-positive cultures, 36 isolates were identified as *Candida albicans* based on phenotypic and biochemical characteristics. Ten representative *C. albicans* isolates were subsequently selected for molecular characterization. Figure 1 demonstrates the steps of collection of the specimen and initial culturing on Sabouraud Dextrose Agar, and its representative oral samples in the first culturing phase.

Figure 1: Collection and primary culturing of oral specimens on Sabouraud Dextrose Agar.

Candida isolates: Isolation and purification

Following primary culturing on Sabouraud Dextrose Agar, yeast-like colonies with smooth, creamy to whitish surfaces and well-defined margins were observed. Pure isolates were obtained by repeated subculture on fresh Sabouraud Dextrose Agar plates of individual colonies. This step of purification was necessary because of the mixed growth that needed to be removed to be able to assure the accuracy of the further phenotypic, biochemical and molecular identification steps. The recovery of pure *Candida* isolates is a confirmation that the isolation and purification procedure is functional. Figure 2 illustrates representative purified isolates that have grown on Sabouraud Dextrose Agar.

Figure 2: Purified candida isolates grown on sabouraud dextrose agar.

Chromogenic candida agar identification

Pure isolates were cultured on Chromogenic *Candida* Agar to allow preliminary differentiation of *Candida* species based on colony color and morphology. Incubation on such a medium showed specific coloration patterns of colonies which indicated *C. albicans*. Colonies showed the same color as mentioned in the case of *C. albicans*, which confirms that chromogenic media should be used as a quick presumptive identification tool. Figure 3 shows the differentiation of *Candida* species by the color of colonies on Chromogenic *Candida* Agar.

Figure 3: Differentiation of Candida species based on colony color on chromogenic Candida Agar.

Microscopic examination

Microscopic examination of Gram-stained smears revealed oval to spherical yeast cells. The cells were seen as single or in budding form which is a typical microscopic characteristic of the *Candida* species. The culture of the isolates proved to be pure as no bacterial contamination was detected in the inspected smears. These microscopic examinations also supplemented the results of identification using the cultural and chromogenic methods. Figure 4 demonstrates the gram stain appearance of the *Candida* cells under the microscope.

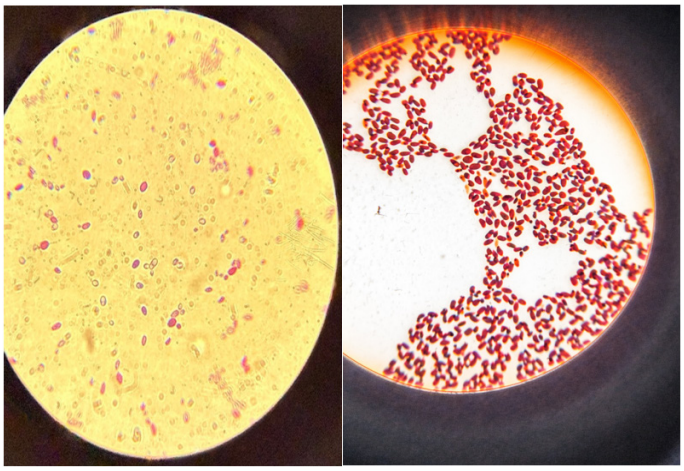

Figure 4: Microscopic appearance of Candida cells after Gram staining (×40).

Germ tube formation test

Germ tube test was done to identify the species of *C. albicans* as opposed to other *Candida* species. Several isolates exhibited germ tube formation following incubation in human serum. Germ tubes were found as filamentous extensions of the yeast cells which were not constrained at the point of origin. The isolation of germ tubes also ruled out other isolates as *C. albicans* since the test is regarded as a major diagnostic parameter of this species. Figure 5 shows representative germ tubes formation observed with a light microscope.

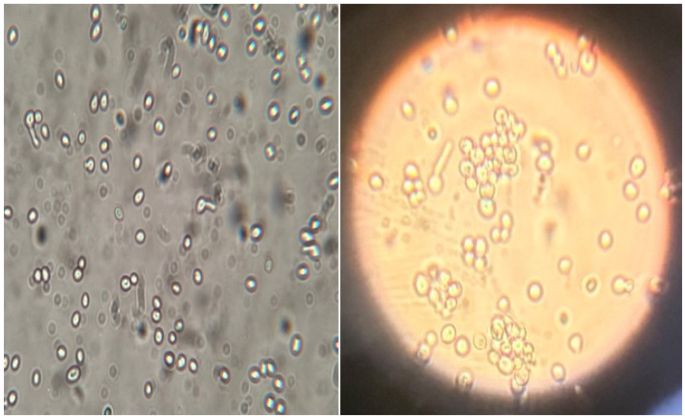

Figure 5: Germ tube formation in *Candida albicans* observed under light microscopy (×40).

Chlamydospore formation

The isolates grew chlamydospores at the ends of pseudohyphae when they were grown on Cornmeal Agar. These thick-walled spherical structures, characteristic of *C. albicans*, were clearly observed microscopically. Chlamydospores production also gave more validity to species identification. Figure 6 will demonstrate the formation of *C. albicans* chlamydospores on Cornmeal Agar.

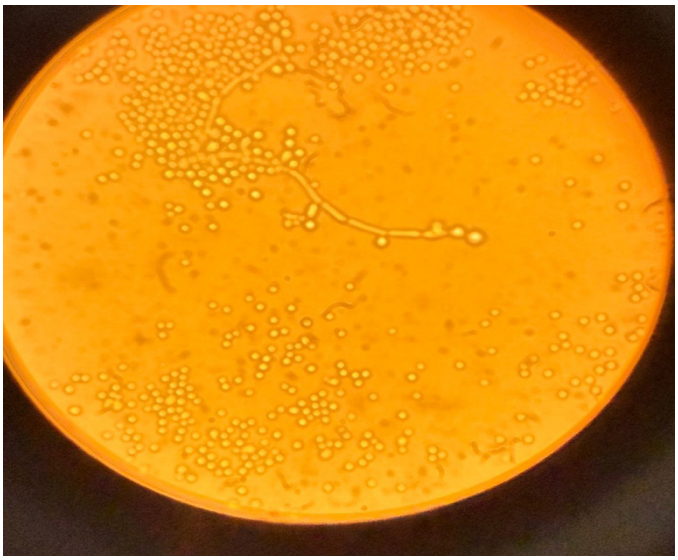

Figure 6: Chlamydospore formation of *Candida albicans* on Cornmeal Agar.

Hemolytic activity

To determine the hemolytic activity of the isolates, blood-supplemented Sabouraud Dextrose Agar was used. Hemolytic activity varied among the isolates, as indicated by the presence of clear or semi-clear zones surrounding fungal colonies. These hemolytic zones depict the capability of the isolates to make hemolysins that are regarded as significant virulence-related factors. These hemolytic patterns observed show inconsistency in the production of hemolysin among isolates of *C. albicans*. The observed representative hemolytic activity on blood-enriched agar is depicted in Figure 7.

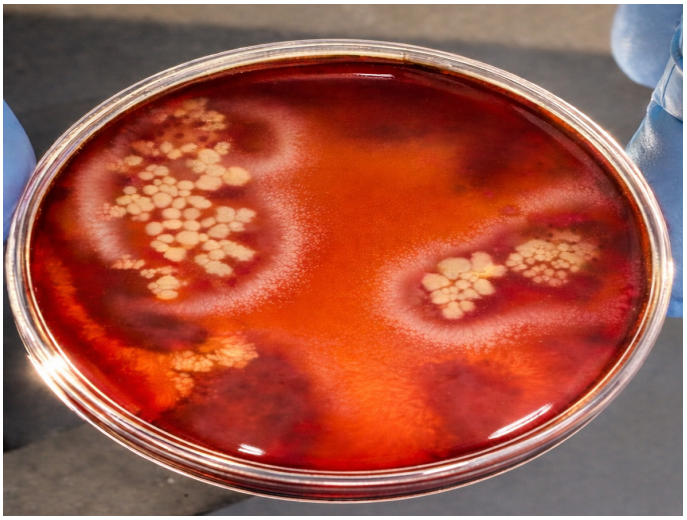

Figure 7: Hemolytic activity of *Candida albicans* isolates on blood-enriched agar.

Identification with VITEK 2 compact system

Ten representative isolates were selected for identification using the VITEK 2 Compact system based on their typical phenotypic characteristics. All ten tested isolates (100%) were identified as *C. albicans* by the VITEK 2 Compact system. The systems yielded a probability of between 94% and 99% showing a high degree of confidence in the identification of the species. There were slight differences in certain biochemical reactions; but these differences did not influence the final result of identification. The VITEK 2 findings were very much consistent with the phenotypic, microscopic and biochemical identification procedures. Table 1 presents the detailed identification results achieved with the help of the VITEK 2 Compact system.

Propolis as a natural antifungal agent

In this research, Propolis was used as an alternative to the natural source to test its antifungal effect against *C. albicans*. Honeybees produce a resinous substance known as propolis which is famous due to its complicated chemical structure and biological functions. In the current research propolis was taken as a possible antifungal agent to examine its inhibitory ability towards *C. albicans* isolates isolated in oral samples.

Table 1: Identification of *Candida albicans* isolates using VITEK 2 compact system.

Isolate No.	Identified organism	Probability (%)	Status
1	*Candida albicans*	99	Final
2	*Candida albicans*	99	Final
3	*Candida albicans*	99	Final
4	*Candida albicans*	99	Final
5	*Candida albicans*	98	Final
6	*Candida albicans*	99	Final
7	*Candida albicans*	94	Final
8	*Candida albicans*	95	Final
9	*Candida albicans*	96	Final
10	*Candida albicans*	94	Final

Propolis extract preparation

The propolis ethanol extract was prepared using the local raw propolis successfully. The extract had appropriate physical properties such as homogeneity and stability and could be used in the further experimental assays. To establish the chemical composition and the functional groups in the prepared extract, the prepared extract was subjected to chemical characterization using the GC-MS and FTIR techniques. The extract was prepared successfully, which attested to its appropriateness in the antifungal testing and the analysis.

Natural compounds in propolis identified by GC–MS

The chemical constituents of ethanolic propolis extract were identified by gas chromatography- mass spectrometry analysis (GC-MS). GC–MS analysis identified a total of 15 naturally occurring compounds in the propolis extract. Compound identification was based on comparison of mass spectra with standard reference libraries. The analysis focused on qualitative identification of major constituents, and relative percentage composition was not quantified in the present study. Table 2 provides these compounds with their retention times, molecular formulas and chemical classes.

The GC–MS profile showed that bioactive compounds of a wide variety were present. Sesquiterpenes carotol, isoaromadendrene and lanceol were observed to have a lower retention time, meaning that they are relatively volatile and resinous plants. Major components of the extract were found to be fatty acids and their esters such as oleic acid, cis-vaccenic acid, hexadecanoic acid ethyl ester and octadecenoic acid methyl ester.

Furthermore, the phenolic compounds and flavonoids in terms of chalcone and flavone derivatives had been detected at an increased retention time. The compounds have been known to help in the biological and antioxidant effect of propolis. In addition, the derivatives of triterpenoid compounds like noroleanone and cyclolanostanes were

Table 2: Natural compounds identified in propolis extract by GC–MS analysis.

No.	RT (min)	Compound	Molecular formula	Chemical Class
1	13.416	Carotol	$C_{15}H_{26}O$	Sesquiterpene alcohol
2	14.250	2-Naphthalenemethanol (octahydro derivative)	$C_{15}H_{26}O$	Sesquiterpenoid
3	16.787	Isoaromadendrene	$C_{15}H_{24}$	Sesquiterpene hydrocarbon
4	17.863	Cyclotetradecatetraene derivative	$C_{15}H_{24}$	Sesquiterpene hydrocarbon
5	18.053	Lanceol (cis)	$C_{15}H_{26}O$	Sesquiterpene alcohol
6	18.777	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	Fatty acid ester
7	19.742	Oleic acid	$C_{18}H_{34}O_2$	Unsaturated fatty acid
8	21.066	Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Fatty acid ester
9	21.157	cis-Vaccenic acid	$C_{18}H_{34}O_2$	Unsaturated fatty acid
10	21.353	Ethyl 14-methyl-hexadecanoate	$C_{19}H_{38}O_2$	Branched fatty acid ester
11	24.307	Chalcone derivative	$C_{15}H_{12}O_3$	Phenolic compound (flavonoid precursor)
12	26.864	Flavone derivative	$C_{15}H_{10}O_2$	Flavonoid
13	28.123	Tetradecanoic acid, methyl ester	$C_{15}H_{30}O_2$	Fatty acid ester
14	29.342	28-Norolean-17-en-3-one	$C_{29}H_{46}O$	Triterpenoid ketone
15	29.786	Cyclolanostane derivative	$C_{30}H_{50}O$	Triterpenoid

also detected, which confirms the complicated resinous structure of the propolis sample under consideration in the current study. In general, the GC-MS findings prove that the propolis extract is abundant in naturally occurring compounds that can have biological activity.

Propolis extract characterization fourier transform infrared (FTIR)

FTIR spectroscopy was used to identify the functional groups present in the ethanolic propolis extract. The FTIR spectrum showed the presence of a number of typical absorption bands which corresponded to various functional groups of different bioactive compounds.

The presence of hydroxyl (–OH) groups, characteristic of phenolics and flavonoids, is indicated by the presence of a broad absorption band at around 3363 cm^{-1}. The presence of C-H stretching vibrations of aliphatic hydrocarbons identified as absorption bands at 2974 cm^{-1} and 2889 cm^{-1} indicates the presence of fatty acids and terpenoid structures.

The absorption band at 1649 cm^{-1} is C=O stretching vibrations which can be due to the presence of carbonyl groups that are usually available in flavonoid, chalcones and other phenolics. Other bands within the range of 1446–1384 cm^{-1} are related to C H bending vibration and this further confirms the existence of the aliphatic chains. Intense absorption bands in the range of 1327–1047 cm^{-1} are C-O stretch vibrations of alcohols, ester, and phenols. Figure 8 displays the FTIR spectrum of the ethanolic propolis extract that contained these functional groups.

Candida albicans *molecular identification by 18S rRNA gene*

The molecular identification of *C. albicans* isolates was done by PCR amplification of 18S rRNA gene. The gel electrophoresis of agarose showed clear and specific amplification of the products at about 550 bp in all the samples (n = 10). The size of the amplicon was matched to the size of the 18S rRNA gene which was expected.

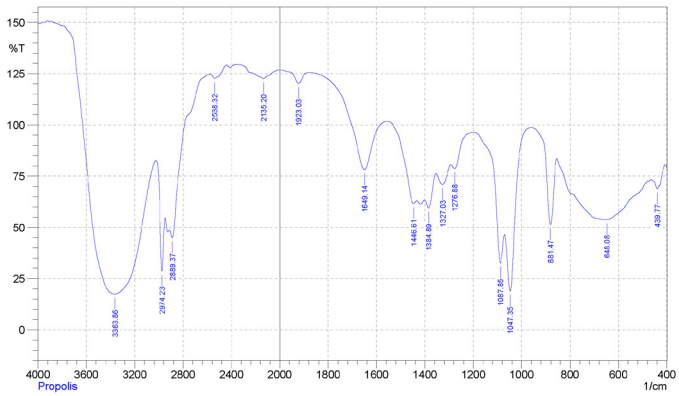

Figure 8: FTIR spectrum of ethanolic propolis extract showing major functional groups.

The consistent detection of the expected amplification product in all analyzed isolates confirmed the reliability of the PCR assay. These findings validate the fact that all the tested isolates were identified as *C. albicans* molecularly. Figure 9 shows the PCR amplification results of the 18S rRNA gene.

Identification of the biofilm and cell wall regulator 1 (BCR1) gene

PCR was used to identify Biofilm and Cell Wall Regulator 1 (BCR1) gene in *C. albicans* isolates. Agarose gel electrophoresis revealed that all investigated isolates had a clear and specific amplified product at the size of about 594 bp (n = 10). This amplicon is a proof of successful amplification of the BCR1 gene.

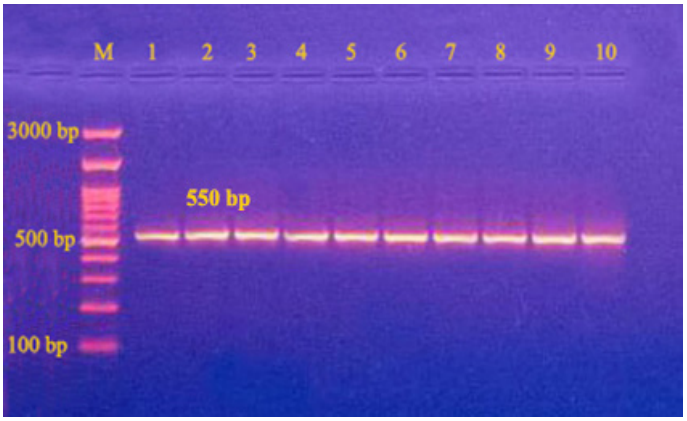

Figure 9: PCR amplification of the *18S rRNA* gene (550 bp) in *Candida albicans* isolates. A specific amplification product of approximately 550 bp was detected in all examined samples (n = 10), confirming the molecular identification of *Candida albicans* isolates.

The BCR1 gene was detected in all examined isolates (100%) and this means that the BCR1 gene is widely spread among the *C. albicans* isolates tested. Figure 10 shows the PCR amplification of the BCR1 gene results.

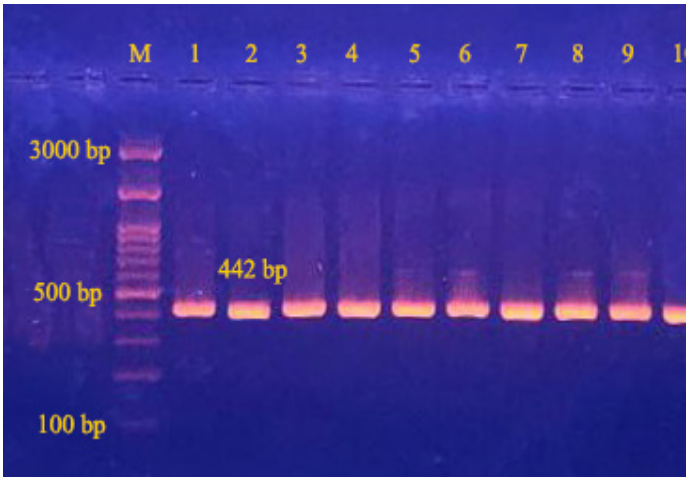

Figure 10: Detection of the Biofilm and Cell Wall Regulator 1 (BCR1) gene (594 bp) in *Candida albicans* isolates by PCR analysis. A specific PCR amplification product of approximately 594 bp was observed in all examined samples (n = 10), indicating that all *Candida albicans* isolates are positive for the BCR1 gene.

Biofilm-induced protein (BIP) gene

To identify the biofilm induced protein gene in *C. albicans* isolates, PCR amplification was done. The findings showed that it was possible to observe the specific amplification product at about 442 bp in all the samples analyzed (n = 10), which is the size of the target gene.

The uniform occurrence of this gene in all the isolates suggests that it is commonly found and helps argue its role in biofilm formation in *C. albicans*. All examined isolates (100%) were positive for the BIP gene. Figure 11 presents the results of the amplification.

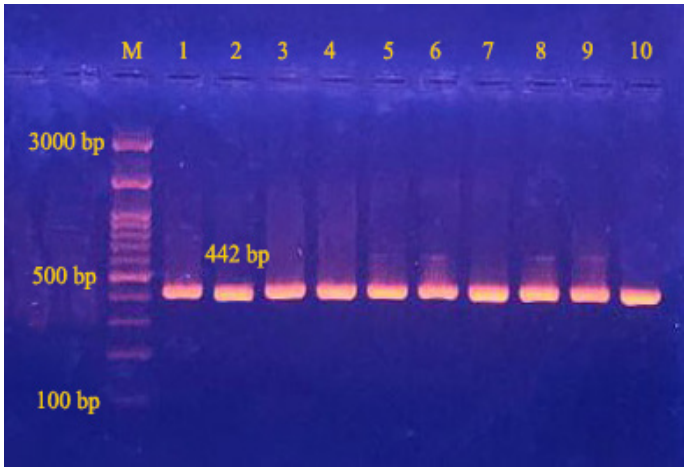

Figure 11: Detection of the biofilm-induced protein gene (442 bp) in *Candida albicans* isolates by PCR analysis. A specific PCR amplification product of approximately 442 bp was observed in all examined samples (n = 10), confirming that all Candida albicans isolates are positive for the biofilm-induced protein gene.

Identification of the protein necessary to communicate cohesion, adhesion and biofilm formation (ALS) gene

The *C. albicans* isolates were analyzed through PCR of the gene encoding the protein needed to that of cohesion, adhesion and formation of biofilms (ALS). The electrophoresis of the agarose gel revealed a specific product of amplification at about 463 bp in all the samples under analysis (n = 10).

The uniform presence of the ALS gene in all isolates underscores its essential role in adhesion and biofilm formation. PCR analysis confirmed that all the examined isolates (100 %) had the ALS gene. Figure 12 shows the amplification results.

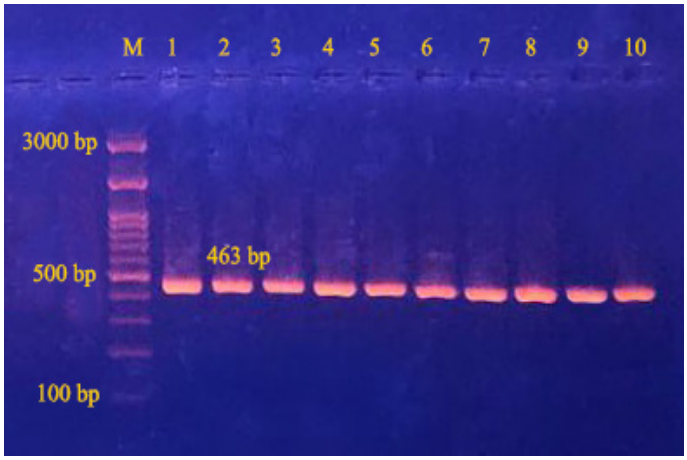

Figure 12: Detection of the protein required for cohesion, adhesion, and biofilm formation gene (463 bp) in *Candida albicans* isolates by PCR analysis. A specific PCR amplification product of approximately 463 bp was observed in all examined samples (n = 10), indicating that all *Candida albicans* isolates are positive for this biofilm-associated gene.

Identification of hyphal wall protein 1 (HWP1) gene

The PCR amplification of the gene of Hyphal Wall Protein 1 (HWP1) was done to further identify isolates of *C. albicans*. Agarose gel electrophoresis revealed a distinct and specific amplification product and specific amplification product in all the samples examined (n = 10) with approximate length of 941 bp.

The HWP1 gene is conserved in the *C. albicans* isolates as the presence of the gene is consistent across all of the isolates. This germ tube making gene is linked with hypha formation. The HWP1 gene was positive in all of the isolates studied (100%). The amplification findings of the PCR are represented in Figure 13.

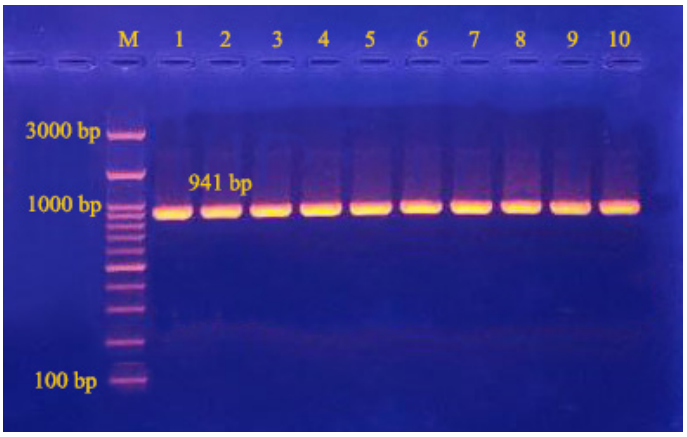

Figure 13: Detection of the Hyphal Wall Protein 1 (HWP1) gene (941 bp) in *Candida albicans* isolates by PCR analysis. A specific PCR amplification product of approximately 941 bp was observed in all examined samples (n = 10), confirming that all *Candida albicans* isolates are positive for the HWP1 gene.

Table 3: Antifungal activity of different concentrations of propolis against *Candida albicans*.

Concentration (mg/mL)	Mean ± SE
0.5%	8.85±1.49bc
1%	12.5±0.81ab
2%	15.73±1.15a
DMSO	0±0d
Antifungal (Nystatin)	7.42±1.98c
Amphotericin B	12.66±2ab
Fluconazole	9.38±2.49b
Ketoconazole	14.09±1.54a
Calculated P value	<0.0001
LSD(P<0.05)	4.10

Different letters between any two means denote to the significant difference.

Antifungal effect of propolis against Candida albicans

The propolis extract was assessed on the antifungal activity against *C. albicans* using varying concentrations of the extract. The findings indicated that there was a definite concentration-related antifungal activity as shown in Table 3. The highest antifungal activity was observed at 2%, with a mean inhibition zone of 15.73 ± 1.15 mm with a statistically significant difference found to those at lower concentrations and some standard antifungal agents (one-way ANOVA, $P < 0.0001$).

The moderate activity was exhibited at 1% and 0.5% and the means of inhibition at 12.5 ± 0.81 and 8.85 ±1.49 respectively. The negative control (DMSO) did not exhibit any activity with antifungal effect, which proves that the observed inhibitory effects could be attributed to propolis. Figure 14 has shown representative inhibition zones of *C. albicans* treated with propolis. The standard antifungal agents used for comparison included nystatin, amphotericin B, fluconazole, and ketoconazole at concentrations routinely applied in susceptibility testing. Their inhibition zone values are presented in Table 3 for comparative purposes.

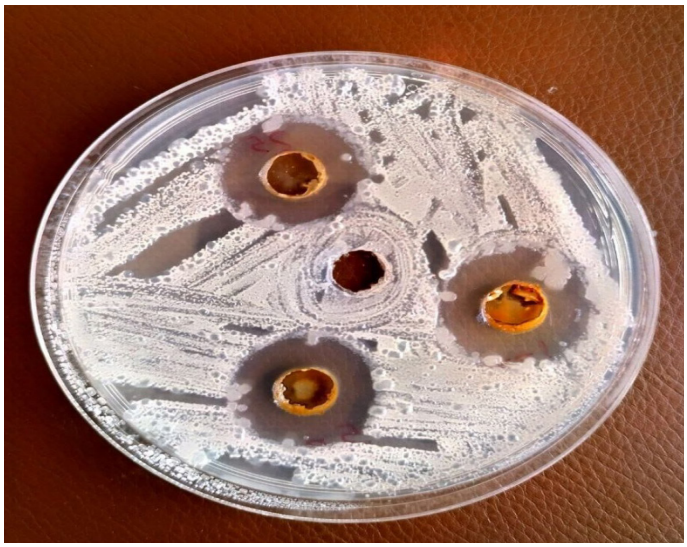

Figure 14: Inhibition zones of *Candida albicans* isolates treated with propolis on Sabouraud Dextrose Agar (SDA).

Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MBC)

Five isolates of *C. albicans* were tested using the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MBC) values of propolis. Table 4 presents the results in a summary. The value of MIC was determined as 130 ± 30 mg/mL and that of MBC was greater at 1680 ± 624.8 mg/mL.

Table 4: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MBC) values of propolis against *Candida albicans*.

Test	Mean ± SE
MIC value	130±30
MBC value	1680±624.8

These results indicate that propolis exhibited inhibitory activity at lower concentrations, whereas higher concentrations were required to achieve fungicidal effects.

Discussion

The present study integrates phenotypic characterization, molecular analysis, and antifungal evaluation of oral and peri-dental *Candida albicans* isolates, highlighting both their virulence potential and the antifungal activity of propolis. The isolation of *C. albicans* from a considerable proportion of fungal-positive samples underscores its role as a major etiological agent in oral candidiasis (De Barros *et al*., 2021). The fact that the isolates continue to grow consistently on Sabouraud Dextrose Agar is another evidence that this medium is suitable in the primary recovery of *Candida* species.

Phenotypic characterization revealed typical morphological features of *C. albicans*, including yeast-like colonies and oval-shaped budding cells. These findings explain why traditional phenotypic methods remain relevant and can be seen as effective initial diagnostic techniques. It was found that the application of chromogenic *Candida* agar allowed quick presumptive identification on the basis of characteristic colony coloration, and the results were similar to those obtained with *C. albicans*, which confirms the diagnostic usefulness of chromogenic media in everyday mycological practice (Aboulghazi *et al*., 2022). The microscopic and chromogenic results with the cultural results show high levels of phenotypic uniformity of the isolates.

A significant proportion of isolates were positive by the germ tube test, which validates the usefulness of this particular technique as a rapid and inexpensive way of identifying *C. albicans*. The formation of germ tubes is interconnected with the transition of yeast to hypha, which is one of the virulence mechanisms and increases tissue invasion and damage to host cells. Therefore, positive germ tube formation not only supported species identification but also reflected the invasive potential of the isolates (Martins *et al*., 2020). In the same manner, the production of chlamydospores in cornmeal agar also supported the identification of *C. albicans* and indicated a survival strategy in an adverse environment (Stevens *et al*., 2018).

The isolates were found to be hemolytic, which is another virulence-related characteristic. The production of hemolysins enables the absorption of iron through the host erythrocytes and this is necessary in the metabolism and the development of the fungi in the host tissues. The variability in hemolytic activity of the isolates was observed, which implies heterogeneity of the virulence expression, which can affect the severity of the infection and its clinical outcome (Lockhart, 2014). This variability reflects the complex pathogenic nature of *C. albicans* and its ability to modulate virulence factors in response to environmental conditions.

PCR amplification of the *18S rRNA* gene was used to provide definitive evidence that all analyzed isolates were *C. albicans*, showing complete agreement with phenotypic identification. Although the *18S rRNA* gene is highly conserved among fungal species and may not provide the same level of discrimination as the internal transcribed spacer (ITS) region, it was selected in this study as a confirmatory molecular marker to support phenotypic identification. However, sequencing analysis was not performed, which represents a limitation of the molecular approach applied. The uniform presence of the amplification product in all samples also indicates good genomic DNA quality and robust PCR conditions.

Molecular detection of virulence- and biofilm-associated genes in all the tested isolates, such as BCR1, the biofilm-induced protein gene, ALS and HWP1 is a critical discovery of this study. BCR1 is an important transcriptional controller of biofilm formation, and the universal availability indicates that there is a great genetic predisposition to biofilm formation among the isolates. Biofilms play a central role in persistent infections as they increase resistance to antimycosis therapy and shield fungi cells against host immunity (Bassetti *et al*., 2013). This observation is also supported by the fact that ALS genes are detected, and they are the ones that facilitate attachment to host tissues and abiotic surfaces which are the first step of colonization and biofilm formation.

The presence of HWP1 in all isolates is equivalent with the consistent phenotype of germ tube formation as HWP1 strongly relates with hyphae growth and adhesion. The combination of phenotypic and molecular evidence supports the pathogenic importance of the isolates and provides evidence of the coordination of the expression of morphological and genetic virulence factors in *C. albicans* (Ünal *et al*., 2025). Collectively, the uniform presence of virulence- and biofilm-associated genes suggests a strong genetic potential for adhesion, biofilm formation, and hyphal development among the isolates. However, it should be noted that the detection of virulence-associated genes reflects genetic potential rather than active gene expression. The present study did not include quantitative gene expression analysis, and therefore functional activity cannot be definitively inferred from gene presence alone.

In addition, no direct correlation analysis was performed between virulence gene detection and phenotypic characteristics such as hemolytic activity variation. Future studies integrating molecular findings with quantitative phenotypic assays, including biofilm

formation measurements, would provide deeper insight into genotype–phenotype relationships.

The antifungal evaluation demonstrated a clear concentration-dependent inhibitory effect of propolis against *C. albicans* isolates. An increased concentration of propolis led to a much higher antifungal activity, and it means that its effectiveness is highly dependent on the dose. This trend indicates that antifungal activity of propolis is as a result of combination or synergy of various bioactive compounds present in it (Cisterna *et al*., 2010). The negative control test showed no antifungal activity which validates that the inhibition was only due to propolis.

Although propolis demonstrated measurable antifungal activity, the relatively high MIC and especially the MBC values observed in this study indicate that substantially higher concentrations are required compared to conventional antifungal agents. This may limit its direct clinical applicability in its crude extract form. The observed disparity between the MIC and MBC values indicates that propolis primarily exhibits a fungistatic effect at lower concentrations, while substantially higher concentrations are required to achieve fungicidal activity. This concentration-dependent behavior suggests that propolis may be more effective as an adjunct or supportive antifungal agent rather than a standalone fungicidal treatment.

The antifungal activity of propolis can be attributed to its complex chemical composition, which includes flavonoids, phenolic compounds, fatty acids, and terpenoids. It should be noted that the GC–MS analysis in this study provided qualitative identification of compounds without quantitative determination of their relative abundance. Therefore, the contribution of individual compounds to the observed antifungal activity cannot be precisely determined. The antifungal activity of propolis can be attributed to its complex chemical composition, which includes flavonoids, phenolic compounds, fatty acids, and terpenoids that contribute to its antifungal activity through multiple mechanisms (de Almeida *et al*., 2025). Flavonoids and chalcone derivatives are known to disrupt fungal cell membrane integrity, increasing membrane permeability and leading to leakage of intracellular components. Phenolic compounds may inhibit key enzymatic processes and denature cellular proteins, thereby interfering with essential fungal metabolic pathways (Al-Azzawi *et al*., 2024). The FTIR findings further support these mechanisms, as the presence of hydroxyl (–OH) and carbonyl (C=O) functional groups is characteristic of phenolic and flavonoid structures known to interact with fungal cell membranes and intracellular proteins. These functional groups may contribute to membrane destabilization, increased permeability, and interference with enzymatic activity in fungal cells.

The fatty acids and their esters in the propolis extract could also contribute to the antifungal effect in a major way. These compounds have the ability to incorporate into fungal membranes, disrupt lipid organization, and an ability to cause changes in membrane fluidity, which eventually leads to a decrease in fungal viability. Particularly unsaturated fatty acids are proven to improve membrane disruption and antifungal effects (Zulhendri *et al*., 2021). Terpenoid compounds can also play a supplementary role in causing mitochondrial dysfunction and inhibiting the energy production process in fungal cells, thus increasing the antifungal effect of propolis (Murtaza *et al*., 2014).

The propolis activity has an important feature of an antibiofilm potential. Infections that are attached to biofilms are also infamously resistant to antifungal agents, which makes it very difficult to cure them. Some of them have shown that initial adhesion, mature biofilms, and the expression of biofilm-related genes in *C. albicans* can be prevented by propolis (Argüelles *et al*., 2024). This observation is particularly relevant given the universal presence of biofilm-associated genes among the analyzed isolates, implying that propolis can be useful in opposing one of the most devastating virulence factors of *C. albicans*.

Moreover, the concentration-related character of the propolis activity described in this paper is consistent with the previous reports suggesting that low concentrations of propolis have fungistatic but not fungicidal activity. This dose-response association is typical of most natural antimicrobial agents and indicates that propolis can possibly be most effective as an addition agent to standard antifungal therapy instead of a single agent (Ding *et al*., 2021). Propolis also has another benefit, and it is a multitarget mode of action that can potentially decrease the chances of resistance emergence in comparison to single-target antifungal agents (Sasidharan *et al*., 2023).

In summary, the integrated phenotypic, molecular, and antifungal findings of this study highlight the virulence potential of oral *C. albicans* isolates and support the antifungal activity of propolis. The anti-fungal effects, including propolis as an ability to inhibit fungi, disrupt virulence, and likely disrupt biofilm, have shown the potential of the propolis as a natural antifungal agent and prompts further research on its therapeutic use.

A limitation of this study is that molecular and virulence gene analyses were performed on a limited number of isolates. Although these isolates were selected to represent phenotypic diversity, the relatively small molecular sample size may limit the generalizability of the findings. Future studies involving larger numbers of isolates are recommended to confirm these observations.

Conclusion

The present study demonstrated that oral and peri-dental *Candida albicans* isolates exhibit important virulence-related phenotypic traits and harbor biofilm-associated genes, although molecular characterization was performed on a limited number of representative isolates. Propolis showed concentration-dependent antifungal activity against *C. albicans*, primarily exerting inhibitory effects at lower concentrations, while higher concentrations were required for fungicidal activity. The relatively elevated MIC and MBC values suggest that further formulation optimization and in vivo investigations are necessary before considering clinical applications. Overall, the findings highlight the potential of propolis as a complementary antifungal agent and underscore the need for expanded molecular and functional studies to better define its therapeutic relevance.

Declarations

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IRB approval

The study protocol was reviewed and approved by the Institutional Review Board of the Department of Biology, College of Education, University of Al-Qadisiyah, Iraq.

Ethical statement

Oral and peri-dental samples were collected from patients under appropriate ethical considerations. All samples were handled anonymously, and no identifiable personal information was recorded. The study was conducted in accordance with institutional ethical guidelines.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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