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Effect of Jujube Honey on Candida albicans Growth and Biofilm Formation

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**Background and Aims.** *Candida* species, especially *Candida albicans*, are major fungal pathogens of humans that are capable of causing superficial mucosal infections and systemic infections in humans. The aim of this study was to evaluate the jujube (*Zizyphus spinia-christi*) honey for its *in vitro* inhibitory activity against pre-formed biofilm and its interference with the biofilm formation of *C. albicans*.

**Methods.** The XTT reduction assay, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were employed to determine the inhibitory effect of Jujube honey on *C. albicans* biofilm. Changes in the infrared spectrum after treatment with honey were also determined by Fourier transform infrared (FTIR) spectroscopy.

**Results.** Jujube honey affects biofilms by decreasing the size of mature biofilms and by disruption of their structure. At a concentration of 40% w/v, it interferes with formation of *C. albicans* biofilms and disrupts established biofilms. The SEM and AFM results indicated that this type of honey affected the cellular morphology of *C. albicans* and decreased biofilm thickness.

**Conclusions.** The present findings show that jujube honey has antifungal properties against *C. albicans* and has the ability to inhibit the formation of *C. albicans* biofilms and disrupt established biofilms. © 2013 IMSS. Published by Elsevier Inc.

Key Words: Jujube honey, *Candida albicans*, Biofilm, Scanning electron microscopy, Atomic force microscopy.
Effect of Jujube Honey on Candida albicans

Impact on public health and the economy. Because of the increasing prevalence of drug-resistant C. albicans, there is an urgent need to develop alternative treatments for Candida infections that are safe, effective and inexpensive.

Among all of the strategies that have been exploited to overcome drug resistance, the use of natural substances has shown particular promise, and many natural substances have been found to have antifungal properties (9). Bee products such as honey and propolis are rich sources of essential bioactive compounds. Because of its medicinal qualities, honey has been used for the management of many diseases throughout the ages and has become a traditional remedy for treating microbial infections and wounds (10–14). The Talmud, the Old and New Testaments of the Bible, and the Holy Qur’an (1400 years ago) mentioned honey as a cure for diseases. A large chapter (SORA) presents in the Holey Qur’an named BEE (Al Nahil) and part of it says (And thy Holy LORD taught the bee to build its cells in hills, on trees and in men’s habitations, then to eat of all the produce of the earth and find with skill the spacious paths of its LORD, there issues from within their bodies a drink of varying colors, wherein is healing for men, verily in this is a sign for those who give thought).

The antimicrobial properties of honey depend on its type, flower source, botanical and geographical origins and the harvesting, processing and storage conditions used (12,15,16). Honey is widely used in the Arabian peninsula for nutritional and therapeutic purposes; however, no research has been conducted on the antimicrobial activity of regional honey collected in the Arabian peninsula. The antimicrobial effects of honey on Staphylococcus aureus, Pseudomonas aeruginosa and other bacterial biofilms have been studied (17–20). Honey also reduces the production of an extracellular polysaccharide matrix while promoting the disruption of mature biofilms (21,22). The effect of honey on C. albicans biofilms has not been extensively studied (23–29). To our knowledge, no research has been conducted on the effect of honey on C. albicans biofilms. A better understanding of C. albicans responses to honey may facilitate its use as a biofilm inhibitor. The aim of this study was to use broth dilution assay followed by the determination of the minimum inhibitory concentration (MIC) of jujube honey and use of new techniques like scanning electron microscopy (SEM), atomic force microscopy (AFM) and Fourier transform infrared (FTIR) spectroscopy to investigate the in vitro effects of jujube honey on planktonic states of C. albicans and detachment of biofilm-embedded states.

Materials and Methods

Honey

Natural jujube honey was used throughout this study. This honey was obtained from the beekeepers’ association of Al-Baha, Saudi Arabia in a 1-kg sterile container. The honey was obtained directly from the honeycomb by pressing and was filtered to remove the wax and other impurities. This natural honey was passed through 45-μm-pore-size filters and stored at −4°C until use.

Microorganisms and Culture Conditions

The test organism used in this study, C. albicans ATCC 10231, was provided by the College of Medicine, King Saud University Riyadh, Saudi Arabia. The strain was cultured in yeast peptone dextrose broth (YPD) medium containing 10 g 1−1 yeast, 20 g 1−1 peptone and 20 g 1−1 dextrose. The cultures were incubated for 36 h at 35°C with agitation (120 rev min−1).

Minimum Inhibitory Concentration (MIC)

MICs of the natural jujube honey against planktonically grown C. albicans ATCC 10231 were determined using a macrobroth dilution assay (30). The honeys were serially diluted (80−5% w/v) in YPD broth. The cultures were incubated for 36 h at 35°C with agitation (120 rev min−1). Following incubation, the broth was used to aseptically inoculate Petri dishes containing Sabouraud dextrose agar (Oxoid) with 103 CFU of Candida. The growth of the colonies was assessed after 48 h, and the MIC was the lowest concentration of honey (w/v) that inhibited the visible growth of C. albicans ATCC 10231.

Establishment of Biofilms

The growth of the biofilms was evaluated using the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-5-tetrazolium-carboxanilide (XTT) reduction assay in 96-well flat-bottomed polystyrene microtiter plates (Jet Biofil, China) using a method based on that described by Lal et al. (31). To determine whether the jujube honey could prevent the formation of Candida biofilms and to determine the lowest concentration of honey capable of preventing biofilm formation, different MIC dilutions of honey in YEPD broth (80% w/v, 40% w/v, 20% w/v, 10% w/v and 5% w/v) were used to study the kinetics of biofilm inhibition. Each MIC dilution was tested in at least seven wells in each microtiter plate. Aliquots of 190 μl of each dilution were dispensed into the wells of the microtiter plate. C. albicans was cultured for 48 h in 10 ml of YEPD broth containing 5 x 108 CFU ml−1. Ten microliters of this 48-h culture was added to each well and incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm to create a homogeneous distribution and adherence to surface of the wells. After 1.5 h, nonadherent cells were removed by gently washing two times with sterilized phosphate buffer saline (PBS) (pH 7.4) without disturbing the adherent cells. After the plates were washed, another aliquot of the same honey dilution in sterile YEPD broth with a final volume of 200 μl was added to each well, and the plates were incubated for 48 h under the
same conditions to allow the colonization and maturation of the biofilms. As a control, 200 μl of autoclaved YEPD broth with Candida (positive control) or without Candida (negative control) was added to each of seven wells of the microtiter plate, which was then incubated at 37°C for 48 h.

To determine whether jujube honey could disrupt established biofilms of C. albicans, biofilms were cultured in 96-well microtiter plates by adding 10 μl of 5 x 10^8 CFU ml^−1 C. albicans in YPD to the microtiter plate. The plate incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm to create a homogeneous distribution and adherence to surface of the wells. After 1.5 h, nonadherent cells were removed by gently washing two times with sterilized phosphate buffered saline (PBS) (pH 7.4). One hundred fifty μl of sterilized YEPD broth was added to the each well and the plate was then reincubated for 24–48 h at 37°C to allow proper adhesion and the establishment of biofilms in the absence of jujube honey. Different concentrations of honey in YEPD broth (80% w/v, 40% w/v, 20% w/v, 10% w/v and 05% w/v) were added to each well in final volumes of 200 μl. The plate was then incubated at 37°C for 48 h. All experiments were performed in triplicate, and quantification was performed using the XTT reduction assay.

**Evaluation of Biofilms Using the XTT Reduction Assay**

A sodium 3′-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) assay was used to quantify the cells in the biofilms. As a control, 200 μl of sterile control was added to each of seven wells of the microtiter plate, which was then incubated at 37°C for 48 h. The XTT-menadione solution (1 mg ml^−1) was prepared, filtered and sterilized using a 0.22-μm pore size filter. Prior to each assay, the XTT solution was thawed and mixed with menadione solution at a ratio of 5:1 (v/v). The biofilms on the microtiter plate wells were washed three times with PBS, and all remaining adherent biofilms were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, at 4°C for 4 h. After washing with distilled water, the biofilms were dried in air. All images were collected in tapping mode using sharpened silicon NSG10S nitride cantilevers with a spring constant of ~10 N m^−1. A constant force of 0.58 N m^−1 was used. The cantilevers had an amplitude range of 5–15 nm, a tip radius of 10 nm and a cone angle of 22°. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz.

**Fourier Transform Infrared Spectroscopy**

Treated and untreated Candida biofilms were analyzed using an IR spectrometer (Thermo Electron Corp., Waltham, MA) using the KBr pellet technique. Biofilm materials were powdered and added to KBr to form a pellet that contained 1% test material. Purified dextran was used as a standard, and the spectrum was taken in the frequency range of 500–1800 cm^−1 at a 4 cm^−1 resolution in absorbance mode. Each final spectrum was the average of 48 scans.

**Scanning Electron Microscopy**

For SEM, a microtiter plate with established Candida biofilms was carefully cut into small pieces using a sterile knife and washed with 4% (v/v) formaldehyde and 1% (v/v) PBS at room temperature. These samples were then treated with 1% osmium tetroxide for 1 h and washed in distilled water. The samples were dehydrated in a series of ethanol (30% for 10 min, 50% for 10 min, 70% for 10 min, 95% for 10 min, and absolute alcohol for 20 min). All specimens were air dried to the critical point using a Polaron critical point drier and then sputter coated with gold. After sputter coating, the surfaces of the biofilms were visualized by SEM (Leo 435, Cambridge, UK).

**Atomic Force Microscopy**

Images of biofilms on MTPs were taken with Nanoscope III Multi Mode AFM (NTegra; NT-MDT, Moscow, Russia). Biofilms were established in MTPs. After washing the biofilms with PBS, different concentrations of honey in YEPD broth (80% w/v, 40% w/v, 20% w/v, 10% w/v and 5% w/v) were added to each well in final volumes of 200 μl. One well without any honey was used as a control. After 48 h of incubation, the liquid medium was withdrawn and the wells were washed twice with PBS. The biofilms were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0. Biofilms were analyzed simultaneously at a scan rate of 250 kHz.

**Statistical Analysis**

ANOVA test was used to compare between different means of biofilm biomass (absorbance). Data analysis was carried out using GraphPad software.

**Results**

**Determination of the Minimum Inhibitory Concentration**

Jujube honey inhibited C. albicans ATCC 10231 growth in a concentration-dependent manner. The MIC of jujube honey against biofilm-forming C. albicans ATCC 10231 was 40% (w/v), and the minimal fungicidal concentration (MFC) was 50% (w/v). The MFC is defined as the lowest concentration of honey resulting in the death of 99.9% of the inoculum. In general, the MFC value is greater than the MIC value. The growth curves of yeast exposed to
40% (w/v) jujube honey showed a reduced growth rate and a reduction in the total number of cells (Figure 1) over a 24-h period relative to cell growth without honey. The growth assays conducted with 50% (w/v) jujube honey revealed no *C. albicans* growth.

**Prevention of Biofilm Formation**

In this experiment, to determine whether jujube honey could prevent the formation of *Candida* biofilms and to determine the lowest concentration of honey capable of preventing biofilm formation, different concentrations of honey in YEPD broth (80% w/v, 40% w/v, 20% w/v, 10% w/v and 5% w/v) were used to study the kinetics of biofilm inhibition. The inhibition of biofilm formation was dependent on the concentration of the honey. It was evident that concentrations of honey below 10% w/v did not inhibit the biofilm and even encouraged biofilm development (Figure 2). However, concentrations more than 10% w/v inhibited significantly the biofilm formation.

**Effect of Honey on Established Biofilms**

Similarly, when 24 h established biofilms were treated with different concentrations of jujube honey (80–5% w/v), the *C. albicans* biomass was significantly reduced after 24 h of contact with honey concentrations greater than 10% w/v, but biofilm growth was enhanced at 5% w/v. A higher concentration of jujube honey was required to disrupt established biofilms than to prevent biofilm formation (Figure 3).

**Effect of Contact Time on Established Biofilms Exposed to an Inhibitory Concentration of Honey**

To monitor the effectiveness of jujube honey over time, biofilms that had been established for 24 h were incubated with and without 40% w/v of jujube honey for varying time intervals, after which the biofilm biomass was determined. The biomass of the *Candida* biofilms was determined after exposure to 40% w/v of honey for 1, 2, 3, 4, 5, 6, 12 and 24 h. The results show that after 24 h of exposure to jujube honey, the biofilm biomass detected was significantly reduced compared with the biomass of the untreated established biofilm (Figure 1).

**Scanning Electron Microscopy Analysis of *C. albicans* Biofilms**

To evaluate the prevention and inhibition of *C. albicans* biofilm growth, SEM was performed. SEM images of a control *C. albicans* biofilms and of a biofilm treated with 40% w/v of jujube honey are shown in Figure 4. Untreated sessile cells of biofilm showed a smooth cell wall (Figure 4A, inset) and covered by exopolysaccharide
Visualization of the ultrastructure revealed that reductions in the number of adherent cells and in biofilm development take place when the biofilm is treated with 40% w/v of honey. When a 24-h established biofilm was treated with 40% w/v of honey, growth of the established biofilm was inhibited, and some small pores developed in the cell walls. These pores may be due to bursting of cell membrane of *C. albicans* cells by shrinkage and osmotic effect of honey, which led to cell death and to a reduction in the numbers of established cell (Figure 4B). No exopolysaccharide material is observed and shrinkage of cell membrane due to plasmolysis has been observed (Figure 4B). Biofilm formed in the presence of 40% (w/v) jujube honey, no exopolysaccharide material and cell aggregation are observed. Shrinkage of the cell membrane indicates cell lysis (Figure 4C and 4D).

**Atomic Force Microscopy (AFM) Analysis of *C. albicans* Biofilms**

The inhibition of *C. albicans* biofilms was also analyzed using AFM. AFM images of untreated *C. albicans* biofilms
on microtiter plates revealed that the *Candida* cells were embedded within a sticky layer of exopolysaccharides distributed around the cell surface, whereas this layer was absent in treated *Candida* biofilms. The 3D images of *C. albicans* biofilms revealed that this layer surrounded the cells residing in the biofilm (Figure 5). The 3D images provide significantly better image resolution than SEM, providing both the height and roughness of the biofilm on the microtiter plate. The roughness analysis of *Candida* biofilms treated with 40% w/v of honey compared with untreated biofilms was also conducted. The root mean square (rms) values of the untreated and treated biofilms were 216.29 nm and 431.28 nm, respectively. A significant variation in the height of the biofilms was observed. The heights of the untreated and treated biofilms were 200 nm and 90 nm, respectively (Figure 5A and 5B). A significant reduction in the height observed in the biofilm formed in the presence of 40% w/v of jujube honey (Figure 5C).

The thickness of the honey-treated biofilm was reduced to approximately half of that of the control. The three-dimensional structure of the *Candida* biofilms also exhibited significant differences in the Z axis value, with values of 200 nm/div, 90 nm/div and 14 nm/div for the untreated and treated established biofilms and biofilm formed in the presence of 40% w/v of jujube honey, respectively (Figure 5).

**Fourier Transform Infrared Spectroscopy**

To visualize the main spectral differences between untreated and treated *C. albicans* biofilm, averages of spectra from all three experiments were calculated and offset-corrected (Figure 6). Distinctive absorption maxima in the mid-infrared region of 800–1200 cm⁻¹ were found to be useful to study the differences in the absorbance between untreated and treated *C. albicans* biofilms. Results from the

![Figure 5](image-url)
Several studies conducted on the antimicrobial properties of honey have confirmed that honey is effective at treating some oral infections such as ulcers, mucositis, and periodontal diseases (12,35–37). Several reports demonstrating the effectiveness of honey in the treatment of various bacterial biofilms have been published (17–19,38,39). However, little information is available on the effect of honey on C. albicans biofilms. The primary aim of this study was to determine whether honey can prevent the establishment of C. albicans biofilms and/or disrupt established C. albicans biofilms.

Among several known human pathogens, Candida sp. are known to be a part of the endosymbiotic community in humans. However, in immunocompromised patients, C. albicans can cause severe nosocomial infections (40). In most of these infections, C. albicans forms a biofilm and becomes resistant to azole drugs, which are commonly used as antifungal agents to treat Candida infections (41). Currently, some Candida strains show resistance to these drugs, which have a limited ability to penetrate the matrix of C. albicans biofilms. The increased resistance of Candida against azole drugs and the few drugs available for Candida treatment has led to search for new therapeutic alternatives (42). One of these alternatives is honey, which has a wide range of antifungal properties.

We selected jujube honey for this study because it is commonly used as a folk medicine to treat several infections and diseases in the Arabian peninsula. Some honeys from different plant sources and geographical origins were found to be effective against planktonic C. albicans cultures; the most effective was jujube honey, with a 40% MIC (w/v) and a 50% MFC (w/v). Jujube honey was thus selected for further testing against C. albicans biofilms. The MIC of jujube honey effectively prevented the formation of C. albicans biofilms and inhibited established C. albicans biofilms. We further tested different MIC dilutions of jujube honey in YEPD broth (80% w/v, 40% w/v, 20% w/v, 10% w/v and 05% w/v). It was found that 20% w/v and 40% w/v of jujube honey significantly prevented biofilm formation, and 80% w/v completely prevented biofilm formation. In contrast, 5% w/v of honey slightly increased biofilm formation. This result indicates that the active antimicrobial ingredients in jujube honey were diluted to a degree that rendered them ineffective. A similar effect has been reported previously (20,43). When evaluating the time- and concentration-dependent effects of honey at different concentrations on 24-h established biofilms, we found that 5% w/v of honey had no inhibitory effect on biofilms and concentrations of 10% w/v and higher significantly reduced the established biofilm after 12 h of treatment at room temperature. These results are supported by the study of Cooper et al. (19) in which manuka honey at concentrations below 10% (w/v) promoted the growth of established biofilms of Staphylococcus aureus.

Discussion

Honey is widely used in a variety of household recipes. Honey is an excellent natural food product rich in minerals, antioxidants and simple sugars. Honey can prevent deteriorative oxidation reactions in foods such as the browning of fruits and vegetables and lipid oxidation in meat. Honey inhibits growth of foodborne pathogens and microorganisms that cause food spoilage (33,34).
The mechanism of the antifungal effect of honey is not fully understood; however, several potential pathways have been proposed. One proposed mechanism is that H$_2$O$_2$, a potent antimicrobial agent, is produced in honey by glucose oxidase enzyme (12,44). Flavonoids, a group of plant pigments that are found in honey, are also considered a potential source of the antimicrobial properties of honey (45). Methylglyoxal, a compound present in manuka honey, may be responsible for the antimicrobial activity of this honey (46). The high sugar content has also been thought to be involved (20), challenging this theory. To propose a mechanism that explains how honey might affect C. albicans biofilms at the cellular level, we performed SEM, AFM and FTIR analyses of treated and untreated C. albicans biofilms. The results indicate that jujube honey has not only prevented C. albicans biofilm formation and disrupted established biofilms but also caused changes to the cell wall and exopolysaccharides.

In our study, SEM observations demonstrated the interference of jujube honey with cell membrane integrity, which was obvious with shrinkage of the cell surface in biofilm cells. A similar mode of action was also observed against planktonic cells of C. albicans. Other authors have also shown that some phytochemicals affect cell membrane integrity of yeast cells (47).

These results also indicate that jujube honey interferes with the metabolism of the C. albicans biofilm. Honey may interfere in any step of biofilm formation and thereby inhibit C. albicans biofilm formation.

In the past decade, AFM has been used to study microbial biofilms without the need for time-consuming sample preparation steps (48). AFM-based methodology can potentially reveal the effects of subtle changes in cell surface composition and of interactions with biomaterials. AFM also provides surface information regarding the exopolysaccharides that cover the Candida cells in biofilms. AFM studies have indicated that the C. albicans biofilm thickness decreases by more than half after treatment with honey. At the same time, the roughness of the C. albicans biofilm also increases significantly. This increase in roughness may be due to the removal of the exopolysaccharides layer that covers the C. albicans biofilm. This layer maintains the smooth texture of the biofilm and inhibits the penetration of antifungal drugs into the biofilm. These results are also supported by the data of Lal et al. (31), which show that C. albicans biofilms secrete a thick layer of exopolysaccharides in which cells remain embedded and protected from their outer surroundings.

FTIR spectroscopy allows analysis of molecular composition through the interaction between the infrared radiation and the sample (49). FTIR spectroscopy has been proven very simple to use and very sensitive to small changes in the composition of cells (50). Here FTIR spectroscopy analysis was performed for comparative biochemical composition of exopolysaccharides matrix of treated and nontreated C. albicans biofilm. The FTIR spectra in the region of 800–1200 cm$^{-1}$ primarily reflected the different sugars present in the C. albicans biofilms. The spectral differences between the untreated and treated C. albicans biofilms in this region indicated that honey affected the formation and secretion of exopolysaccharide matrix by altering the sugars (major constituents of C. albicans biofilm exopolysaccharides) composition and deposition. Thus, there is direct evidence that honey affects the exopolysaccharide composition of C. albicans biofilms.

Mature C. albicans biofilms are very difficult to eradicate and are recalcitrant to antifungals. The extracellular glucan present in extracellular matrix is required for C. albicans biofilm resistance and it acts by sequestering antifungals, rendering cells resistant to their action (51). Many antimicrobials have been isolated from naturally occurring substances over the years. Our findings indicate that jujube honey inhibits the initial phase of biofilm formation and has fungistatic, fungicidal and antibiofilm potential. This potential is superior to that of most of the commonly used antifungals. Because biofilms are multifactorial phenomena, multiple mechanisms that target different steps in biofilm development are probably involved in the effects of honey on biofilms. This intriguing observation may have important clinical implications that could lead to a new approach for the management of C. albicans biofilm-related infections.

In conclusion, the findings indicate that jujube honey can inhibit C. albicans biofilms. The significant antifungal activity of jujube honey suggests that this could serve as a source of compounds which have a therapeutic potential for the treatment of Candida-related infections. Further evaluation in vivo is required to determine whether these findings can be exploited in treating biofilm-associated candidiasis.

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