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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 spina-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.

## Introduction

Some *Candida* species are found as endosymbionts in most healthy individuals. *C. albicans* is the most common yeast found on the mucosal membranes of humans including in the oral cavity, esophagus, gastrointestinal tract, urinary bladder and genitalia (1). In immunocompromised individuals, *C. albicans* has emerged as a true opportunistic pathogen. This yeast adheres to and colonizes epithelial tissues and causes superficial and life-threatening infections. *C. albicans* has become one of the main causes of morbidity and mortality worldwide among immunocompromised individuals (2). Importantly, *Candida* has been shown to be the third most commonly isolated blood pathogen from patients in U.S. hospitals (3).

According to the National Institutes of Health (USA), more than 60% of all microbial infections are associated with biofilms (4). Biofilms are particularly problematic in the clinical environment and, like bacteria, various fungal species can form biofilms *in vivo* and *in vitro* (5). Among fungi, *C. albicans* is the most common pathogen associated with fungal biofilm infections, especially infections related to implanted medical devices (6). A common issue associated with *C. albicans* biofilms is the increased resistance of these biofilms to antifungal agents such as azole drugs and their derivatives and to host immune defenses. The increased resistance is due to the extracellular matrix secreted by the *Candida* cells, which shields the *Candida* cells from antibodies and prevents drugs from penetrating the biofilm (7,8). The emergence of resistant *C. albicans* has a major

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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 Nahl) 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 (YEPD) medium containing 10 g $l^{-1}$ yeast, 20 g $l^{-1}$ peptone and 20 g $l^{-1}$ 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 $10^3$ 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 $10^8$ 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 buffered 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′-[1-[(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 after treatment with the jujube honey (32). The XTT (Sigma, St. Louis, MO) solution (1 mg $ml^{-1}$ in PBS) 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 (Fluka, UK) for 5 min to prevent further growth. After the fixative was removed, the wells were washed twice with PBS. Then, 1 mL of PBS containing 60 µl of the XTT-menadione solution was added to each well, including the control well without a biofilm. The MTPs were then incubated for 2 h at 37°C in the dark. Following incubation, 75 µl of XTT-menadione solution from each well was transferred to a new microtiter plate, and its absorbance was determined spectrophotometrically at 490 nm (Perkin Elmer, Waltham, MA).

### *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, 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.

### *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

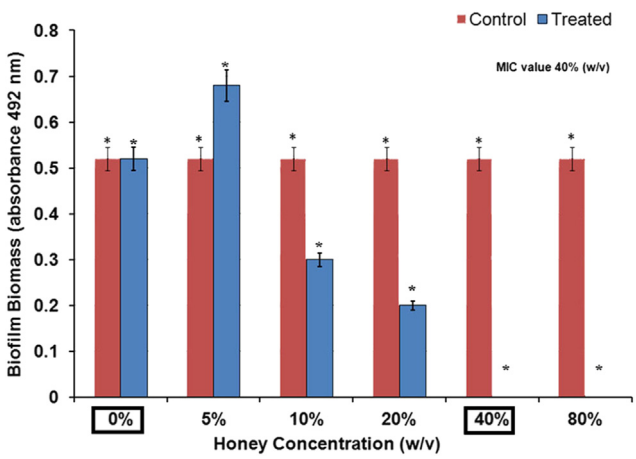


**Figure 2.** The effect of jujube honey on the formation of *C. albicans* biofilms. F = 301, $p < 0.0001$. (A color figure can be found in the online version of this article.)

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

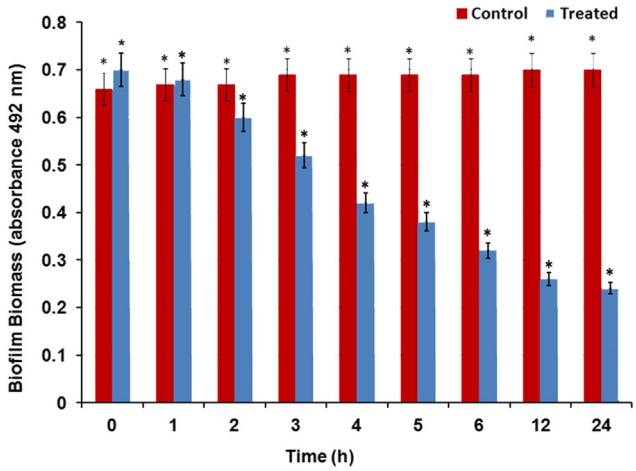


**Figure 1.** Growth analyses of established *C. albicans* biofilms treated with 40% (w/v) jujube honey. F = 612, $p < 0.0001$. (A color figure can be found in the online version of this article.)

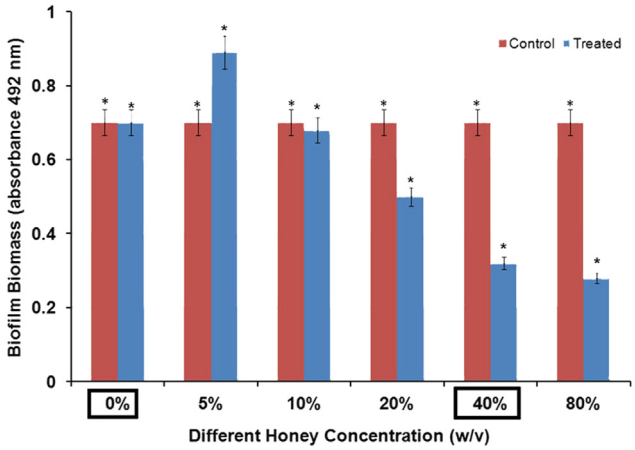


**Figure 3.** The effect of jujube honey on established *C. albicans* biofilms. F = 68.8, $p < 0.0001$. (A color figure can be found in the online version of this article.)

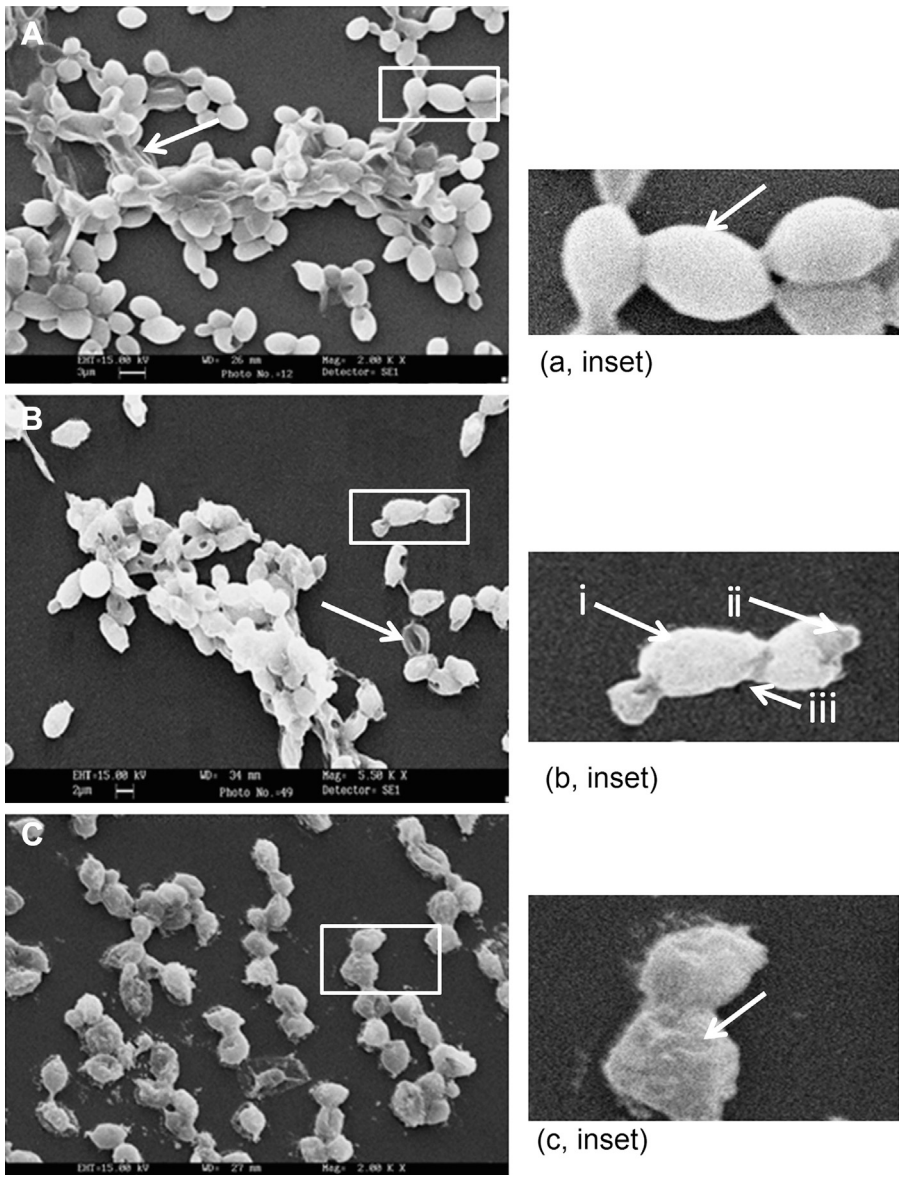


**Figure 4.** Scanning electron microscopy micrographs of the 48 h *C. albicans* biofilms on microtiter plates. (A) Biofilm formed in the absence of honey, showing a dense network of cells and hyphae. White arrow indicated exopolysaccharides material (A, inset). White arrow indicates the smooth cell wall of a normal cell. (B) Inhibition of established biofilm treated with 40% w/v of jujube honey (after 24 h) is illustrated. There is no exopolysaccharide material observed and white arrow indicates the formation of small pores within the cell walls (B, inset). i, white arrow indicates the rough cell wall; ii, vesicle formation due to lytic material; iii, shrinkage in cell membrane due to plasmolysis of cell. (C) Prevention of biofilm formation on microtiter plates after 48 h is illustrated. (C, inset). White arrow shows rough cell wall and shrinkage in cell membrane due to plasmolysis of cell.

materials. 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

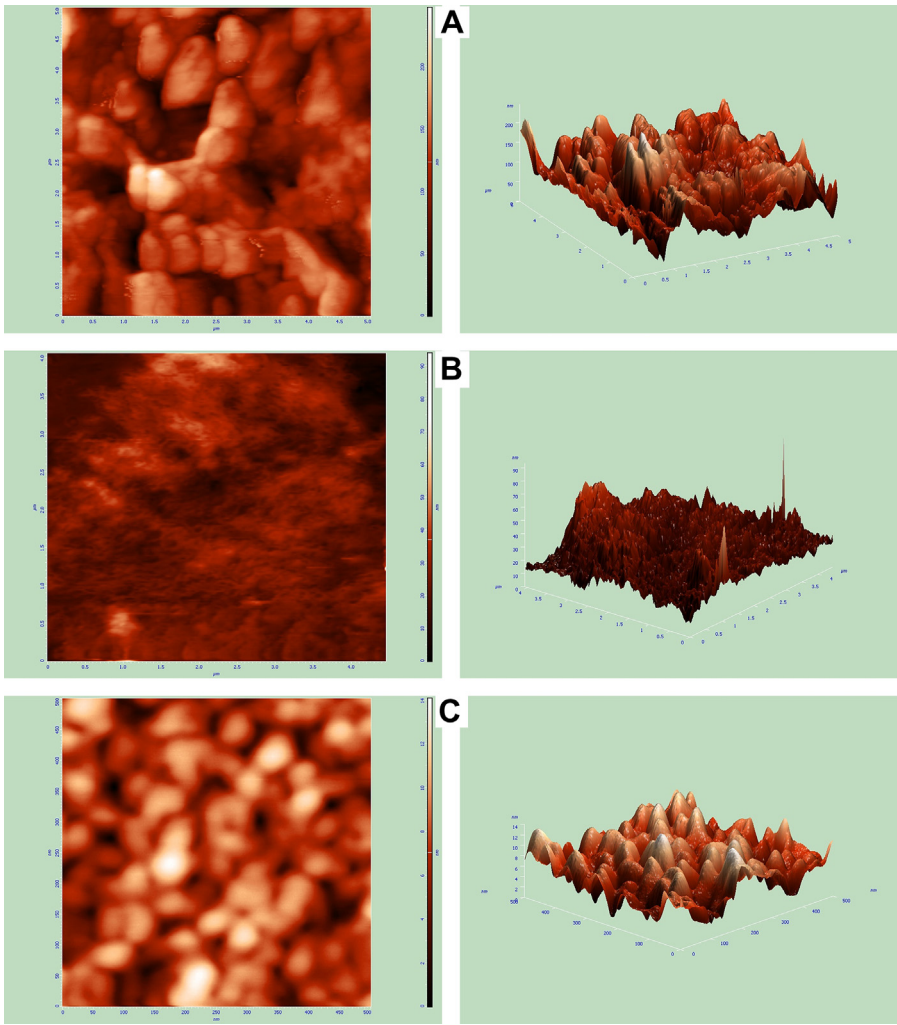


**Figure 5.** Atomic force microscopy micrographs showing the variation in the roughness and height of *C. albicans* biofilms on microtiter plates: (A) untreated biofilm after 48 h (height 200 nm). (B) 40% w/v jujube honey-treated established biofilm (48 h) (height 90 nm). (C) Formation of biofilm after treatment with 40% w/v of jujube honey (48 h) (height 14 nm). (A color figure can be found in the online version of this article.)

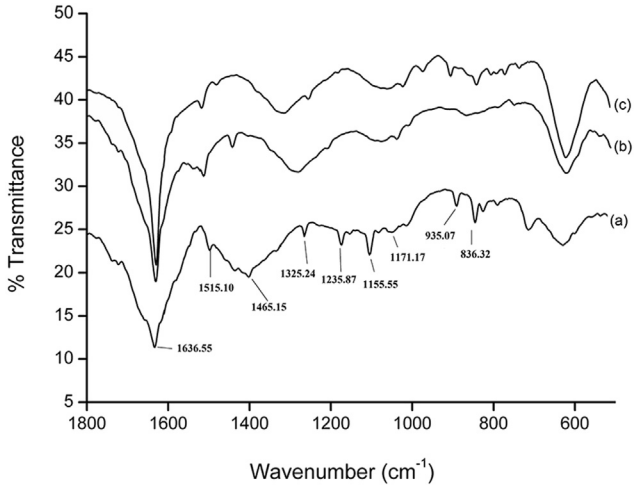


**Figure 6.** FTIR spectra of *C. albicans* biofilms. (a) Untreated biofilm spectra after 48 h. (b) MIC-treated established biofilm spectra after 48 h (c) Spectra of biofilm formed with 40% w/v of jujube honey after 48 h.

comparison of the FTIR spectra of untreated and treated *C. albicans* biofilm showed that there were some differences in the wave number, shape, and the number of absorption peaks within the same range of wave number. The FTIR spectral profile of control (without honey) obtained in 800−1200 $cm^{-1}$ region mainly reflected the absorption of sugars present in the exopolysaccharide matrix secreted by *C. albicans* cells. Absorbance peaks for sugars in the mid-infrared region were present at 836, 935, 1017, 1088, 1155 and 1171 $cm^{-1}$ (Figure 6A). These peaks indicate the presence of β-glucans and mannans moieties with other sugars like arabinose, mannose etc. The FTIR spectra also exhibited specific absorbance bands corresponding to the C = O stretching of carboxylate groups at 1636 $cm^{-1}$. C-C ring stretching at 1465 $cm^{-1}$ and C-H stretching of primary aromatic amines at 1235 $cm^{-1}$ were also observed (Figure 6A). Comparison of the untreated biofilm spectrum with the treated biofilm spectrum showed remarkable differences. Exopolysaccharide sugar specific peaks were not clearly discernible in treated biofilm to that of untreated biofilm, apart from the peaks at 1515 and 1465 $cm^{-1}$ (Figure 6). The major differences of spectra in this region might result from the differences in exopolysaccharide sugar composition. This reflected no production of extracellular polysaccharides in *C. albicans* biofilm in the presence of honey.

## 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).

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*.

The mechanism of the antifungal effect of honey is not fully understood; however, several potential pathways have been proposed. One proposed mechanism is that $H_2O_2$, 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 phytocompounds 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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## References

1. Pfaller M, Diekema D. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 2007;20:133−163.
2. Morgan J, Meltzer M, Plikaytis D, et al. Excess mortality, hospital stay, and cost due to candidemia: a case-control study using data from population-based candidemia surveillance. Infect Contro Hospital Epidemiol 2005;26:540−547.
3. Ramage G, Vande Walle K, Wickes B, et al. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother 2001;45:2475−2479.
4. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001;45:999−1007.
5. Cousins B, Allison H, Doherty P, et al. Effects of a nanoparticulate silica substrate on cell attachment of Candida albicans. J Appl Microbiol 2007;102:757−765.
6. Ramage G, Mowat E, Jones B. Our current understanding of fungal biofilms. Crit Rev Microbiol 2009;35:340−355.

7. Silva S, Henriques M, Martins A, et al. Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. Med Mycol 2009;47:681−689.
8. Jain N, Kohli R, Cook E, et al. Biofilm formation by and antifungal susceptibility of Candida isolates from urine. Appl Environ Microbiol 2007;73:1697−1703.
9. Koç A, Silici S, Kasap F, et al. Antifungal activity of the honeybee products against *Candida* spp. and *Trichosporon* spp. J Med Food 2011;14:128−134.
10. Molan P. The antibacterial activity of honey: 2. Variation in the potency of the antibacterial activity. Bee World 1992;73:59−76.
11. Al-Waili N, Salom K, Al-Ghamdi AA. Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. Sci World J 2011;11:766−787.
12. Al-Waili NS, Salom K, Butler G, et al. Honey and microbial infections: a review supporting the use of honey for microbial control. J Med Food 2011;10:1079−1096.
13. Yaghoobi N, Al-Waili N, Ghayour-Mobarhan M, et al. Natural honey and cardiovascular risk factors effects on blood glucose, cholesterol, triacylglycerole, CRP, and body weight compared with sucrose. Sci World J 2008;8:463−469.
14. Al-Waili NS. Investigating the antimicrobial activity of natural honey and its effects on the pathogenic bacterial infections of surgical wounds and conjunctiva. J Med Food 2004;7:210−222.
15. Molan P, Cooper R. Honey and sugar as a dressing for wounds and ulcers. Trop Doct 2000;30:249−250.
16. Sherlock O, Dolan A, Athman R, et al. Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. BMC Complement Altern Med 2010;10:47−51.
17. Alandejani T, Marsan J, Ferris W, et al. Effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Otolaryngol Head Neck Surg 2009;139:107−111.
18. Merckoll P, Jonassen T, Vad M, et al. Bacteria, biofilm and honey: a study of the effects of honey on 'planktonic' and biofilm-embedded wound bacteria. Scand J Infect Dis 2009;41:341−347.
19. Cooper R, Lindsay E, Molan P. Testing the susceptibility to manuka honey of streptococci isolated from wound swabs. J Apiprod Apimed Sci 2011;3:117−122.
20. Nassar H, Li M, Gregory R. Effect of honey on *Streptococcus mutans* growth and biofilm formation. Appl Environ Microbiol 2012;78: 536−540.
21. Okhiria OA. The role of biofilm in wounds. (Thesis; University of Wales; 2010).
22. Irish J, Carter D, Blair S. Honey prevents biofilm formation in microbial pathogens. Malays J Med Sci 2007;14:112.
23. Al-Waili N, Al-Ghamdi A, Ansari MJ, et al. Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in single and polymicrobial cultures. Int J Med Sci 2012;9:793−800.
24. Al-Waili NS. Mixture of honey, beeswax and olive oil inhibits growth of *Staphylococcus aureus* and *Candida albicans*. Arch Med Res 2005;36:10−13.
25. Ahmed M, Noureddine D, Hammoudi S, et al. Additive potential of ginger starch on antifungal potency of honey against *Candida albicans*. Asia Pacific J Trop Biomed 2012;2:253−255.
26. Koc A, Silici S, Kasap F, et al. Antifungal activity of Turkish honey against *Candida* spp. and *Trichosporon* spp: an in vitro evaluation. Med Mycol 2008;2:1−6.
27. Feas X, Estevinho M. A survey of the in vitro antifungal activity of heather (Erica sp.) organic honey. J Med Food 2011;14:1−5.
28. Candiracci M, Citterio B, Piatti E. Antifungal activity of the honey flavonoid extract against *Candida albicans*. Food Chem 2012;131: 493−499.
29. Irish J, Carter D, Shokohi T, et al. Honey has an antifungal effect against Candida species. Med Mycol 2006;44:289−291.
30. Agarwal V, Lal P, Pruthi V. Effect of plant oils on *Candida albicans*. J Microbiol Immunol Infect 2010;43:447−451.
31. Lal P, Sharma D, Pruthi P, et al. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. J Applied Microbiol 2010;109: 128−136.
32. Roehm N, Rogers G, Hatfield S, et al. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 1991;142:257−265.
33. Chen L, Mehta A, Berenbaum M, et al. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. J Agric Food Chem 2000;48:4997−5000.
34. Mundo M, Padilla-Zakour O. Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. Int J Food Microbiol 2004;9:1−8.
35. Biswal B, Zakaria A, Ahmad N. Topical application of honey in the management of radiation mucositis: a preliminary study. Support Care Cancer 2003;11:242−248.
36. English H, Pack A, Molan P. The effects of manuka honey on plaque and gingivitis: a pilot study. J Int Acad Periodontol 2004;6:63−67.
37. Motallebnejad M, Akram S, Moghadamnia A, et al. The effect of topical application of pure honey on radiation-induced mucositis: a randomized clinical trial. J Contemp Dent Pract 2008;9:40−47.
38. Lerrer B, Zinger-Yosovitch K, Avrahami B, et al. Honey and royal jelly, like human milk, abrogate lectin-dependent infection-preceding Pseudomonas aeruginosa adhesion. ISME J 2007;1:149−155.
39. Maddocks S, Lopez M, Rowlands R, et al. Manuka honey inhibits the development of Streptococcus pyogenes biofilms and causes reduced expression of two fibronectin binding proteins. Microbiology 2012; 158:781−790.
40. Ramage G, Saville S, Thomas D, et al. *Candida* biofilms: an update. Eukaryot Cell 2005;4:633−638.
41. Agarwal V, Lal P, Pruthi V. Prevention of *Candida albicans* biofilm by plant oils. Mycopathologia; 2007;. http://dx.doi.org/10.1007/s11046-007-9077-9.
42. Runyoro D, Matee M, Ngassapa O, et al. Screening of Tanzanian medicinal plants for anti-Candida activity. BMC Complement Altern Med 2006;6:11−21.
43. Scheinin A, Makinen K, Ylitalo K. Turku sugar studies. V. Final report on the effect of sucrose, fructose and xylitol diets on the caries incidence in man. Acta Odontol Scand 1976;34:179−216.
44. Molan P. Re-introducing honey in the management of wounds and ulcers—theory and practice. Ostomy Wound Manage 2002;48: 28−40.
45. Cushnie T, Lamb A. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343−356.
46. Kwakman P, te-Velde A, de Boer L, et al. How honey kills bacteria. FASEB J 2010;24:2576−2582.
47. Arnold J, Bailey G. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. Poult Sci 2000;79:1839−1845.
48. Khan MSA, Ahmad I. Antibiofilm activity of certain phytocompounds and their synergy with fluconazole against *Candida albicans* biofilms. J Antimicrob Chemother 2012;67:618−621.
49. Orsini F, Ami D, Villa AM, et al. FT-IR microspectroscopy for microbiological studies. J Microbiol Methods 2000;42:17−27.
50. Galichet A, Sockalingum GD, Belarbi A, et al. FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype. FEMS Microbiol Lett 2001;197:179−186.
51. Nett JE, Sanchez H, Cain MT, et al. Andes genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. J Infect Dis 2010;202:171−175.