Anti-biofilm properties of clover honey against *Candida albicans*

Masfufatun Masfufatun,[1] Budhi Setiawan,[2] Rini Purbowati,[3] Lusiani Tjandra,[2] Noer Kumala Indahsari,[1] Diana Tri Ratnasari,[4] Harya Narottama,[5] Muzaijadah Retno Arimbi[6]

[1]Department of Biochemistry, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya; [2]Department of Pharmacology, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya; [3]Department of Biomolecular, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya; [4]Department of Dermatology, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya; [5]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya; [6]Department of Internal Medicine, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya, Indonesia

Abstract

Candida albicans grows rapidly when the microflora becomes imbalanced due to a variety of factors. Its ability to infect a host is aided by its virulence factors, such as biofilm. This study aimed to evaluate the activity of clover honey in inhibiting and degrading the biofilm formation of *C. albicans in vitro*. This study used a true experimental design with an in vitro post-test-only control group design approach. The microtiter plate assay was used to grow planktonic cells and biofilm. This method was carried out to obtain the Optical Density (OD) value for each test, measured by a Microplate Reader. Cell viability was measured using the MTS Assay kit, the biofilm matrix was measured using the Crystal Violet Assay, and the morphology of *C. albicans* biofilms was observed by scanning electron microscopy (SEM). Probit and One-way ANOVA tests were applied to determine the MIC_{50} of both planktonic and biofilm, as well as statistical analysis. The results showed that clover honey exerted inhibitory activity against *C. albicans* planktonic cells at a MIC_{50} value of 31.60% w/v. At the highest concentration, clover honey exhibited antibiofilm activity by lowering the extracellular matrix and viability of *C. albicans* cells by 64.59% and 72.09%, respectively. Based on SEM observation, clover honey changed the cell morphology of *C. albicans* and reduced the thickness of the biofilm. Overall, our findings concluded that clover honey exhibited antifungal properties against *C. albicans* by inhibiting biofilm formation and degrading mature biofilm.

Correspondence: Budhi Setiawan, Department of Pharmacology, Faculty of Medicine, Universitas Wijaya Kusuma Surabaya, Indonesia.
E-mail: budhisetiawan@uwks.ac.id

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Introduction

Candida albicans is a normal microbiome composition in a healthy human body with a well-functioning immune system.[1] However, a balance disorder caused by a variety of factors allows this fungus to multiply rapidly and lead to infection.[2,3] There are several factors that may contribute to *C. albicans* infection.[4] For instance, *C. albicans*' resistance to the immune and antifungal systems is known to be due to virulence factors, specifically its activity in biofilm formation.[5] A biofilm is a community of microbial cells attached to a surface (or found at the air-liquid interface) that has different properties than other planktonic microbial cells.[6,7] *C. albicans*' biofilm is highly structured, consisting of several types of cells (*e.g.*, round budding yeast-shaped cells, oval pseudohyphal cells, and elongated hyphal cells) enveloped in an extracellular matrix.[3,8]

Biofilm formation by *C. albicans* has significant clinical implications and contributes to higher mortality.[9,10] The major consequences of biofilm formation include the ability of cells in the biofilm to develop resistance to antifungal therapy and the biofilm's protection against the body's defenses, affecting the management of patients with Candida infection.[7] Additionally, the formation of *C. albicans* biofilms leads to an increase in acetaldehyde (ACH) levels (a highly toxic, mutagenic, and carcinogenic product of alcoholic fermentation and microbial metabolism),

exerting a hazardous impact on organs.[11] Fungal infections could become more challenging to treat as antifungal drug resistance increases.[12] Consequently, alternative treatments based on natural substances with antifungal and antibiofilm properties are required.[13] Honey is one such natural product that is commonly used in everyday life and consumed daily.[14] While honey consumption may not significantly alter the metabolic profiles of healthy individuals, it can impact glucose levels and other metabolic parameters in individuals with type 2 diabetes if consumed in large quantities.[15] Honey, a thick liquid produced by honeybees from flower nectar, is believed to be effective in treating various types of diseases, including upper respiratory tract infections,[16] eye disorders,[17] and diabetes mellitus.[18] Additionally, honey promotes the growth of probiotic microbes.[19] Honey contains high sugar content, hydrogen peroxide (H_2O_2), high acidity, and organic compounds (polyphenols, flavonoids, and glycosides), contributing to its antifungal properties.[20] The enzymatically produced hydrogen peroxide (H_2O_2) compounds in honey create a highly acidic pH, which helps eliminate pathogenic microbes.[21]

The effectiveness of honey as an antimicrobial agent has been widely characterized.[22–25] For instance, clover and Manuka honey exhibited favorable activity against planktonic cells and biofilms of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella spp.,* and *Proteus mirabilis*. All the tested bacterial strains were sensitive to clover honey.[26] Despite numerous studies on honey antibiofilm effects, the impact of honey on *C. albicans* biofilms has not been extensively studied.[27–31] This knowledge gap may result in increased morbidity, limited treatment options, recurrent infections, and therapeutic failures. Therefore, this study aimed to investigate the inhibitory activity of clover honey on the biofilm formation and degradation of *C. albicans* isolated from Candidiasis Vulvovaginitis (CVV) patients.

Materials and Methods

Research design

This study employed an experimental research approach, specifically a true experimental design, utilizing an *in vitro* post-test-only control group design. The methods involved microdilution broth to determine the Minimum Inhibition Concentration (MIC) and a microtiter plate biofilm assay to test the anti-biofilm activity of clover honey. This procedure aimed to obtain the Optical Density (OD) value for each test, measured through a Microplate Reader. The study comprised 6 treatment groups and 1 control group.

Honey

The clover honey was purchased from the distributor of HDI Group of Companies, PT. Harmoni Dinamik Indonesia. This honey was harvested from cold, dry, and fertile areas in the Arizona Desert Mountains, United States.

Isolation of *C. albicans* and its culture conditions.

To grow the *C. albicans* culture, a single colony of *C. albicans* was transferred into Sabouraud Dextrose Broth (SDB) liquid medium. Then, the culture was shaken for 18-24 h with a rotary shaker at 150-160 rpm. After 24 h of incubation, the cell cultures were centrifuged for 15 min at 10,000 rpm, 4°C. The collected cells were washed twice with 0.1 M sterile phosphate-buffered saline (PBS) to remove the remaining media. The precipitate of *C. albicans* was suspended in PBS until it reached a turbidity of 0.5 (OD ~0.5) at λ 600 nm UV-Vis spectrophotometer. This suspension is defined for biofilm formation.[32]

Minimum Inhibitory Concentration (MIC)

The MIC of clover honey on the growth of planktonic *C. albicans* was determined using the macro broth dilution test using 96 wells of a polystyrene microtiter plate. Briefly, Clover honey was serially diluted (50-1.56% w/v) in Sabaroud Dextrose Broth (SDB) medium. 190 µL of different concentrations of the honey were put into 96 well microplates and then added 10 µL of *C. albicans* inoculum on each of the samples. Plates were incubated for 24 h at 35°C on a rotary shaker at 120 rpm. After incubation, the growth of planktonic *C. albicans* was analyzed using an MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. MIC was defined as the lowest concentration of honey (w/v) that causes inhibition of *C. albicans* growth.

Biofilm growth and development inhibition essay

The test was carried out using a 96-well polystyrene microtiter plate. A volume of 100 µL of *C. albicans* culture containing $5x10^8$ CFU/mL was added to each well and incubated for 1.5 hours at 37°C. After incubation, non-adherent cells were removed by gently washing with sterilized saline phosphate buffer (PBS; pH 7.4) twice without disturbing the attached cells. Briefly, 100 mL of clover honey samples in RPMI 1640 medium (100-3.125%) was added to each well, and then the plates were incubated for another 48 hours. Cell viability was analyzed using the MTS Assay, while biofilm biomass was determined using the Crystal Violet Assay. This method was modified from Ansari *et al.*'s study in 2013.[33]

Biofilm degradation essay of *C. albicans*

C. albicans biofilm was cultivated on a 0.2 µm cellulose nitrate filter membrane. In brief, the cellulose nitrate filter membrane was sterilized by UV radiation for 15 minutes on both sides, serving as an in vitro model of the semipermeable biological membrane. Subsequently, the sterilized cellulose nitrate filter membrane was positioned on top of the spider medium, a nutrient-limited medium, using sterile tweezers. Next, 10 µL of *C. albicans* suspension (turbidity 0.5 at λ 600 nm) was applied to the surface of the cellulose nitrate filter membrane and incubated for 1 hour at 37°C. The petri dish was then inverted and further incubated for another 24 hours. After incubation, the membrane containing the biofilm was transferred to a new spider medium using sterile tweezers and incubated for an additional 24 hours. After 48 hours of incubation, the *C. albicans* biofilm was exposed to a 20 µL honey solution. Conversely, in the control group, sterile distilled water was applied. Following this, the *C. albicans* biofilm was incubated for 24 hours at 37°C. The cell morphology of the *C. albicans* biofilm, subsequent to degradation with clover honey, was analyzed using a Scanning Electron Microscope (SEM).[32]

Cell viability analysis

MTS reduction analysis was employed to assess cell viability by measuring metabolic activity. Prior to analysis, the CellTiter 96® AQueous One Solution reagent (Promega) was prepared. This procedure took approximately 90 minutes at room temperature or 10 minutes in a water bath at 37°C. While awaiting the liquid reagent, the microplate containing the *C. albicans* biofilm was initially washed with distilled water three times. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Reagent was dispensed into each well of the washed 96-well assay plate. The plates were then incubated for 1-4 hours at 37°C. A microplate reader ELISA was utilized to measure absorbance at λ 490 nm.

Biomass analysis of *C. albicans* biofilms

After treatment and incubation, the microplates were washed with running water three times. Subsequently, 200 µL of 0.01% Crystal Violet solution was added to each well and incubated at room temperature for 15 minutes. The microplate was then re-washed in the same manner with running water three times. Two hundred µL of ethanol (96%) solution was added to each well and re-incubated for 15 minutes at room temperature. Optical Density (OD) was recorded using an iMark Bio-Rad microplate reader at λ 595 nm.

SEM analysis

The biofilms were dried at 37°C overnight and dehydrated with a gradient of ethanol (50% for 10 min, 70% for 10 min, 96% for 20 min). The dried biofilm was then coated with palladium and imaged using SEM.[32]

Data analysis

One-way ANOVA and LSD post hoc tests were employed to analyze the mean comparison of optical density results at various concentrations of clover honey in both planktonic cells and biofilm. The Kolmogorov-Smirnov and Levene tests were utilized to assess normality and homogeneity of the data, respectively. A significance threshold of $p<0.05$ was applied to determine the significant difference between the various samples. Probit analysis was conducted to determine the MIC_{50} of planktonic cells and biofilm of *C. albicans*. All statistical analyses were performed using the SPSS software program.[34]

Results

Minimum Inhibitory Concentration (MIC50) of Clover Honey

It was found that clover honey, at varied doses, prevented the formation of *C. albicans* planktonic cells. According to Probit analysis, the clover honey MIC_{50} for *C. albicans* planktonic cells was 31.60% (w/v). The inhibitory effect of higher concentrations of clover honey increased, preventing *C. albicans* planktonic cells from growing by 74.88% at a concentration of 50% (Figure 1).

Extracellular matrix of *C. albicans* biofilm

The determination of the effect of clover honey on *C. albicans* biofilm, especially its impact on the extracellular matrix, was performed using a crystal violet solution. The procedure was intended to measure the biofilm mass, and the solution colors the microbial cells as well as the extracellular matrix.[35] As a result, the measured absorbance value was proportional to the number of cells and extracellular matrix in the *C. albicans* biofilm. Figure 2 displays that 100% Clover honey was able to inhibit the growth of *C. albicans* biofilm by reducing its extracellular matrix by 64.59%. In addition to that, Probit analysis revealed that the MIC_{50} of Clover honey for *C. albicans* biofilm was 58.25%.

Cell viability of *C. albicans* biofilm

The tetrazolium salt (MTS) was used to determine the effect of clover honey treatment on the viability of *C. albicans* biofilm cells. This test is often used as a method to estimate cell viability in biofilms and to determine the impact of biofilm treatment. This method was used to estimate the number of surviving microbes in biofilms through their metabolic activities. MTS works by reducing MTS into an orange formazan compound with mitochondrial dehydrogenase enzyme activity. The measured absorbance value corresponds to the number of living cells in the polymicrobial biofilm. The mitochondrial dehydrogenase enzyme is an indicator of living cells because it is not present at the time of cell death. Figure 3 depicts that clover honey can reduce the growth of *C. albicans* biofilm cell viability by 72% at the highest concentration of 100%.

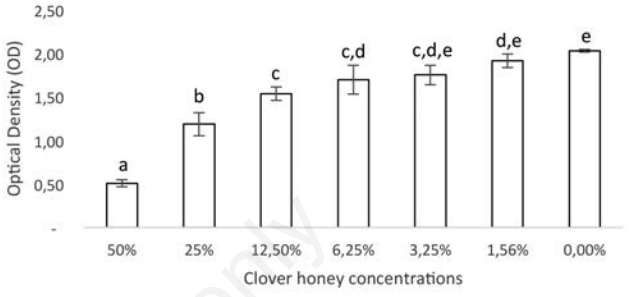

Figure 1. The viability of *C. albicans* planktonic cells by Clover Honey at various concentrations. Note: Letters a-e indicate statically significant different ($p<0.05$).

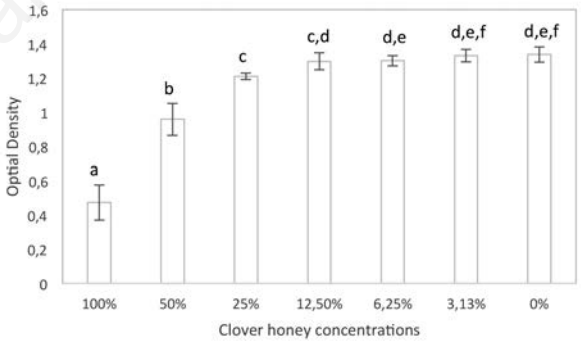

Figure 2. Absorbance values of bio-film growth of *C. albicans* biofilm. Note: Letters a-f indicate statically significant different ($p<0.05$).

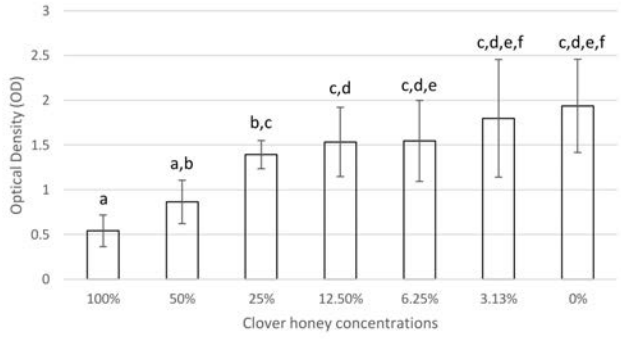

Figure 3. The absorbance value of viability cell on the growth of C. albicans biofilm. Note: Letters a-f indicate statically significant different ($p<0.05$).

Biofilm degradation test of *C. albicans*

The effect of clover honey on *C. albicans* biofilm morphology was determined using SEM. The overall representation of the biofilm-treated control of *C. albicans* and the biofilm-treated with 80% w/v clover honey is shown in Figure 4. Compared to the biofilm-treated control that had an intact membrane morphology with a smooth and regular surface covered by exopolysaccharide material, showing a dense network of cells and hyphae (Figure 4A), the biofilm of *C. albicans* treated with clover honey exhibited noticeable shrinkage, or obvious indentations and breakage on their cell membrane due to the osmotic effect of honey. This result reveals that clover honey disrupts the cell membrane integrity of *C. albicans*, leading to the death of the cells. Exopolysaccharide material was not visible, but small pores were formed on the cell walls, and rough cell walls (Figure 4B). The SEM analysis revealed the absence of exopolysaccharide material as well as plasmolysis-induced cell membrane shrinkage. Cell lysis was indicated by the shrinkage of the cell membrane.

Discussion

Many studies have shown that the ability of microbes to form biofilms has implications for microbial resistance to antimicrobial agents. To enhance the permeability of antibiotic agents for killing microbes, a treatment technology is needed by utilizing natural substances that have the potential to inhibit or degrade the formation of biofilms. Clover honey is one of the natural ingredients that can currently be used to damage or inhibit the formation of biofilms. Clover honey inhibited the growth of *C. albicans* planktonic cells at different concentrations. The greater the concentration of clover honey, the greater the inhibitory activity. The MIC_{50} value of clover honey in inhibiting the growth of *C. albicans* planktonic cells was 31.60%, which is lower than the MIC of clover honey against Proteus Mirabilis (40%) from a previous study.[36] The inhibition activity can be attributed to its phenol and flavonoid content. Ciappin study (2014), reported phenol and flavonoid levels of 100.4 mg GAE/100 g and levels of 3.9 mg

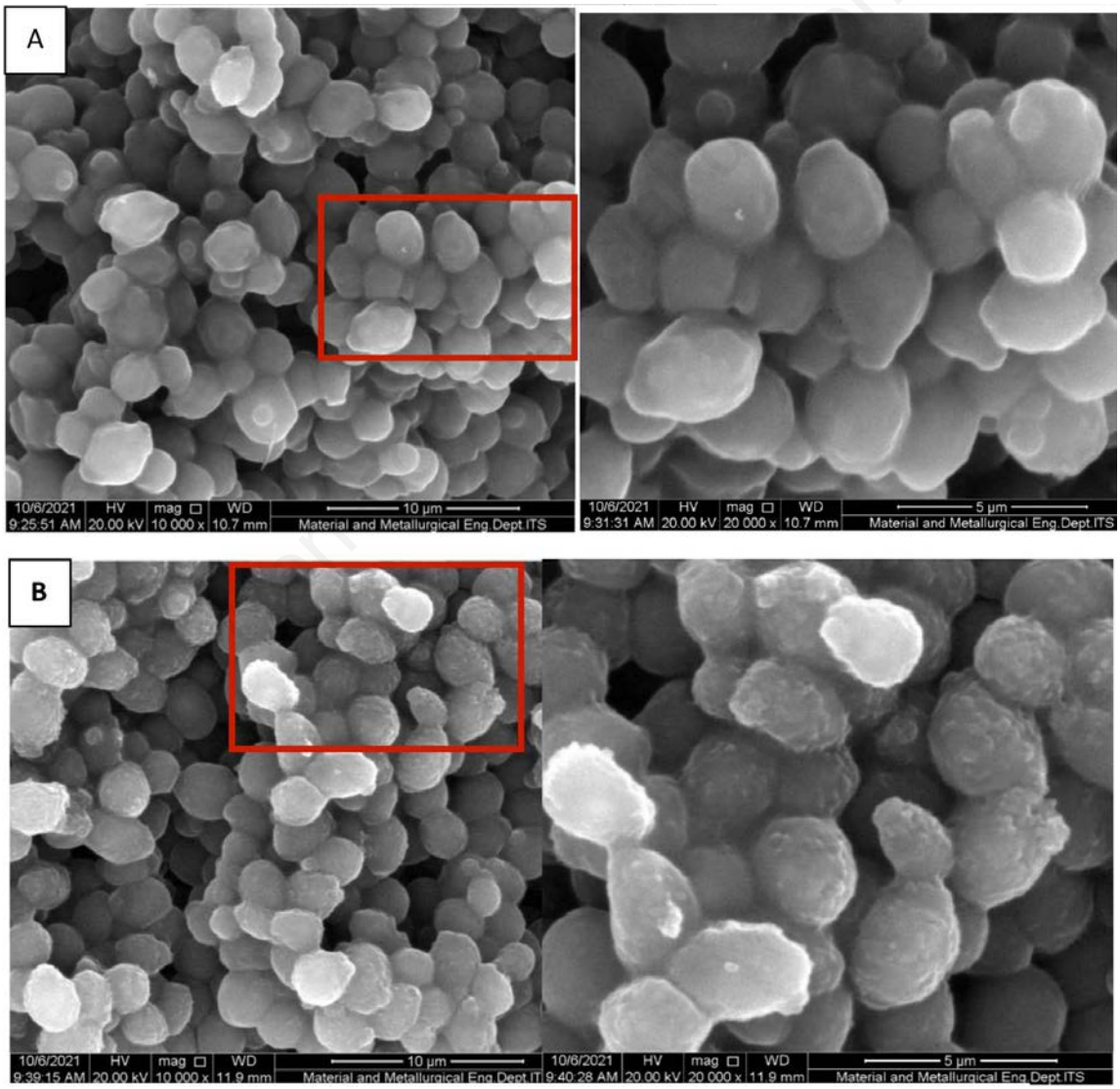

Figure 4. Scanning electron microscope profile of *C. albicans* biofilm on nitrocellulose membrane. (**A**) Biofilm formed in the absence of honey. (**B**) Biofilm was treated with 100% w/v clover honey.

QE/100 g in clover honey. Flavonoid compounds exhibit antifungal effects by degrading cell structures during the replication process, resulting in a lower rate of proliferation.[37] Flavonoid compounds can inhibit the activity of the efflux pump, causing the accumulation of compounds that function as therapy.[38]

Meanwhile, the presence of phenol, which is also observable in clover honey, can inhibit the activity of the Matrix metalloproteinase (MMP) enzyme, facilitating the attachment of the fungus's hyphae to the surface of its host. Phenolic compounds cause the formation of reactive oxygen species (ROS) in fungal cells, resulting in the fragmentation of fungal genetic material. This mechanism is also capable of overcoming multiple drug resistance (MDR) in *C. albicans*.[39,40] Instead of exerting antifungal activity, phenol can inhibit the enzymes related to fungal metabolic functions, isocitrate lyase, and hyphae formation via the DPP3 (dipeptidyl peptidase 3) gene and prevent fungal dimorphism, or changes in the structural shape of fungi that have the potential to reduce the effectiveness of therapy.[41]

Furthermore, in this study, clover honey also inhibited the growth of *C. albicans* biofilm. The MIC_{50} value of clover honey in inhibiting the formation of a 50% biofilm matrix was 58.25%. This value was higher compared to inhibiting the biofilm matrix of *Pseudomonas aeruginosa*.[42] This activity can be observed in a decrease in the extracellular matrix analysis and cell viability analysis of *C. albicans* biofilm. The potential ability to degrade *C. albicans* biofilm was consistent with morphological SEM analysis results. The antibiofilm activity of clover honey could be attributed to the content of Methylglyoxal, dihydroxyacetone, and H_2O_2. Studies have shown that the presence of these compounds exhibited inhibitory activity of clover honey against *P. aeruginosa* biofilms.[42] According to cell morphology analysis using SEM, clover honey also demonstrated degradation activity against *C. albicans* biofilm. The honey degraded the biofilm by destroying the cell wall structure and its constituent exopolysaccharides, increasing the permeability of the cell wall surface of *C. albicans* (Figure 4). This resulted in the loss of the structure of the exopolysaccharide layer that covers the *C. albicans* biofilm. This layer is accountable for maintaining the biofilm's surface smooth and flat, preventing the penetration of antifungal drugs into the biofilm. The result of the morphology of *C. albicans* biofilm structure was comparable to that reported by Ansari (2013) using jujube honey.[33]

The in vitro method has been chosen due to its ability to control various conditions and focus on specific factors influencing biofilm formation, as well as being more cost-effective. More in-depth studies are needed to determine the efficacy of clover honey as an antifungal agent in humans, as the findings might not be directly applicable to the human body as a whole. Without baseline data, it might have been more difficult to compare the before-and-after treatment in this study, and it could have been more challenging to ascertain if the observed changes were the result of the treatment or other factors.

Conclusions

Clover honey exhibits remarkable properties as it not only inhibits the formation of C. albicans biofilms but also effectively disrupts pre-existing biofilms. The notable antifungal activity observed in clover honey underscores its potential as a promising alternative source of compounds for combating infections associated with Candida biofilms. This finding opens avenues for exploring clover honey as a potential component in the formulation of medical-grade honey aimed at addressing fungal infections. However, to solidify these findings and explore the full extent of clover honey's efficacy, further research and clinical studies are warranted. This ongoing investigation will contribute valuable insights into the potential applications and mechanisms underlying the antifungal properties of clover honey in medical contexts.

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