Synergistic Effects of Thyme Essential Oil and Thyme Honey on Biofilm Formation by *Candida albicans*

Running title: Synergistic Effects of Thyme Oil and Honey on Candida Biofilms

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Abstract

Candida albicans is a major pathogenic yeast responsible for numerous systemic infections. Its ability to form biofilms significantly complicates treatment, leading to high rates of treatment failure and mortality. This study investigates the synergistic effects of thyme essential oil and thyme monofloral honey on *Candida albicans* biofilm formation, exploring them as a potential natural therapeutic strategy. The inhibition of biofilm formation was assessed using a crystal violet microtiter plate assay. Various concentrations of thyme monofloral honey (100%, 75%, 50%, and 25% v/v) and thyme essential oil were tested both individually and in combination. A clinical isolate of *Candida albicans* served as the target organism for the study. The effectiveness of the treatments in inhibiting biofilm formation was measured, and fluorescent microscopy was employed to visualize the effects on yeast cell density and morphology. The results indicated a significant synergistic effect of combining thyme essential oil and honey, achieving the highest inhibition rate of 59% at the 75% concentration of honey and thyme essential oil, compared to individual treatment rates of 28% for thyme essential oil and 31% for honey alone. Microscopy imaging revealed a marked reduction in the density of *Candida albicans* cells and changes in cell morphology in treated samples, highlighting the effectiveness of the combined treatment in inhibiting biofilm formation.

The combined action of thyme essential oil's antimicrobial properties and the bioactive compounds found in thyme honey suggests a promising strategy for overcoming *Candida albicans* biofilm-associated infections. These findings support the exploration of natural antimicrobials as alternatives to synthetic antifungal agents, particularly in an era of rising antifungal resistance. Further research, including *in vivo* studies, is necessary to validate the clinical efficacy of these natural products against multidrug-resistant pathogens.

Keywords: *Candida albicans*, Honey, Thyme Essential Oil, Anti-biofilm, Synergistic Effect

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Introduction

Recent estimates indicate that Earth is home to approximately 8.7 million eukaryotic species, with fungi comprising about 7% of this total, which translates to around 611,000 species (Mora et al., 2011). Among these fungi, approximately 600 species are recognized as human pathogens (Brown et al., 2012). This relatively small group includes fungi responsible for mild skin infections, such as Dermatophytes and *Malassezia* species, as well as certain fungi that pose significant risks of life-threatening systemic infections, including notable pathogens like *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Candida albicans* (Köhler et al., 2017).

Candida species are particularly concerning, ranking as the fourth most common cause of hospital-acquired systemic infections in the United States, with an alarming mortality rate that can reach up to 50% (Pfaller and Diekema, 2007). These pathogens are prevalent and can affect skin and mucosal surfaces, and even progress to systemic infections. They are implicated in around 400,000 cases of systemic fungal diseases (Mukaremera et al., 2017). Among the various species, *Candida albicans* is the most common cause of mucosal and systemic infections, accounting for approximately 70% of fungal infections worldwide (Morad et al., 2018). This fungus has been a major contributor to life-threatening invasive infections over the past several decades, and despite available treatments, the mortality rate remains close to 40%, particularly in hospital settings (Enoch et al., 2017).

C. albicans commonly inhabit the human body, especially in the gastrointestinal tract, typically without causing any harm. However, under certain conditions, it can shift to a pathogenic form and lead to infections (Köhler et al., 2017). One of the most significant challenges in treating *C. albicans* infections is its ability to form biofilms (Tsui et al., 2016). Biofilms are complex communities of microorganisms that adhere to surfaces such as medical devices, tissues, or mucosal membranes and are enveloped by a protective matrix of extracellular substances (Blankenship and Mitchell, 2006). This biofilm formation makes *C. albicans* particularly resistant to conventional antifungal treatments, complicating efforts to fully eradicate the infection. Consequently, the presence of *C. albicans* biofilms is often associated with persistent and recurring infections, further complicating treatment strategies (Nobile and Johnson, 2015).

Traditional antifungal therapies frequently struggle to completely eradicate *C. albicans* infections due to the protective characteristics of *Candida* biofilms. The biofilm matrix serves as a barrier, hindering antifungal agents from effectively reaching the underlying fungal cells (Mathé and Van Dijck, 2013). This resistance often results in recurrent infections, necessitating prolonged treatment (Atriwal et al., 2021). Moreover, extended use of antifungal medications can lead to adverse side effects and the emergence of drug-resistant strains of *C. albicans.* It exhibits significant antifungal resistance, particularly to azoles, through mechanisms such as overexpression of membrane transporters. Resistance to other

antifungal classes, including polyenes and echinocandins, has also been observed due to genomic changes and enzyme inactivation, complicating treatment strategies for infections. (Bhattacharya et al., 2020). As a result, there is an urgent need for alternative approaches that can disrupt biofilms and improve the efficacy of antifungal treatments. Recent studies have emphasized a growing interest in natural substances with antifungal properties. More than 300 herbal species are recognized for their pesticidal properties, with numerous specifically demonstrating antifungal efficacies. These natural products have been utilized in clinical practice for centuries, often providing an alternative or complementary approach to conventional antifungal treatments (Liu et al., 2011). The historical use of these herbs, combined with emerging scientific evidence supporting their effectiveness, underscores their potential to enhance treatment strategies against infections caused by *C. albicans* and other fungi (Tseung and Zhao, 2016).

Essential oils (EOs) are secondary metabolites produced by plants, existing in the aromatic and volatile liquids found in various plant parts. Many EOs exhibit antimicrobial properties which are believed to be associated with their phenolic compounds (Nazzaro et al., 2013). Thyme (*Thymus vulgaris*) EO is celebrated for its antimicrobial, antioxidant, and anti-inflammatory properties (Kowalczyk et al., 2020). It contains several bioactive compounds, particularly thymol and carvacrol, which are types of monoterpene phenols. These compounds have demonstrated potential antifungal properties against *C. albicans*. (Alshaikh and Perveen, 2021). Not only do these compounds inhibit the growth of *C. albicans*, but research showed that they also disrupt its biofilm matrix, making thyme effective against biofilm-related infections (Alves et al., 2019). By targeting the fungal cell wall, thymol and carvacrol induce membrane damage, ultimately leading to cell death, which addresses the persistent challenges posed by biofilms in clinical settings (Shariati et al., 2022).

Honey, a natural product produced by *Apis mellifera* bees from the nectar of flowers or from the secretions of plant-sucking insects, is another significant source of bioactive compounds originating from plants (Pattamayutanon et al., 2015). This sweet substance is not only valued for its flavor but also for its rich nutritional profile, comprising numerous vitamins and bioactive compounds. Throughout history, honey has been utilized as a medicinal remedy, prescribed by physicians for a wide array of human health issues (Boukraâ, 2023). One type of honey, known as monofloral honey, is derived when bees predominantly gather nectar from a single type of flower. Certain monofloral honeys have gained recognition as medical-grade honey (MGH). MGH is characterized by its high sugar content, low water activity, acidic pH, and significant bioactive compounds such as hydrogen peroxide, methylglyoxal, and bee-derived peptides (Holubová et al., 2023). These qualities create an unfavorable environment for the growth and survival of various microorganisms, including pathogenic fungi (Mandal and Mandal, 2011). Honey exhibits diverse antimicrobial

properties that can effectively kill or inhibit the growth of a range of microorganisms, including multidrug-resistant pathogens (Mandal and Mandal, 2011). Since the foundational study by Molan in 1992, which highlighted honey's antimicrobial activity (Molan, 1992), extensive research has been conducted to explore the efficacy of honey from differing geographical and botanical origins, its chemical composition, and its therapeutic potential.

Research has indicated that darker honeys, such as thyme honey, are particularly rich in antioxidant compounds, including phenolic compounds and flavonoids, which underpin their powerful antioxidant and antibacterial properties (Alissandrakis et al., 2007, Karabagias et al., 2016). Thyme honey is not only nutritious but also beneficial for various health issues. It contains vitamins B, A, and E, which contribute to the health of the brain and nervous system, alleviate intestinal discomfort, relieve coughs and sore throats, combat joint pain, and reduce menstrual pain. Additionally, thyme honey has been noted for its positive effects on conditions such as epilepsy, convulsions, headaches, and migraines (Alissandrakis et al., 2007). It is also one of the few honey types used in managing diabetes (Lafraxo et al., 2021).

Considering the individual health benefits and bioactive properties of EOs and honey, there has been considerable interest in studying extracts from various plants that are high in phenolic compounds when used alongside honey (Nagy-Radványi et al., 2024). Studies suggest that these two substances may work together synergistically. Additionally, combining EOs, which are typically made up of inedible and poorly soluble components, with honey may enhance the solubility of the EOs and improve their absorption in the digestive system (Assaggaf et al., 2022).

In this context, examining the interactions between thyme EO and thyme monofloral honey could yield important insights for innovative uses in natural medicine and functional foods. This investigation is part of a larger research project focusedon understanding MGHs and their interactions with herbal EOs. The specific objective of this study is to assess the potential synergistic effects of thyme EO and thyme monofloral honey when used together, particularly by analyzing their antibiofilm activity against clinical strains of *C. albicans*.

Material and methods

Thyme Essential Oil (EO) Preparation

EO from thyme was extracted using steam distillation. Fresh or dried thyme leaves were washed to remove contaminants and placed in a round-bottom flask, which was filled halfway with distilled water. The flask was equipped with a condenser and a receiving flask, and heated to a boiling point of approximately 100 °C to produce steam that passed through the plant material, facilitating the extraction of the essential oil. After 2-4 hours of distillation, the mixture was allowed to cool, and the distillate was collected. The aqueous layer was carefully removed to separate the EO, which was then stored in dark glass bottles, labeled with the extraction date for future use.

Honey Selection

Monofloral honey samples were collected

from 2023 to 2024 directly from beekeepers across various ecological regions of Iran. Each sample, weighing 250-300 g, was sourced from individual colonies and transported to the laboratory at temperatures below 20 °C. To verify authenticity, the samples were tested for physicochemical and phytochemical properties, emphasizing parameters relevant to medical use. Key physicochemical factors, including reducing sugars (before and after hydrolysis), 5-Hydroxymethylfurfural, proline, diastase activity, pH, sucrose, and the fructose/ glucose ratio, were analyzed following International Honey Commission (IHC) guidelines and methodologies from Nayik et al.(Nayik and Nanda, 2016) and Oroian and Ropciuc (Oroian and Ropciuc, 2017). Additionally, melissopalynological analysis was conducted for botanical and geographical identification based on Louveaux et al.'s methods (Louveaux et al., 1978)(data not shown). After a thorough analysis, thyme honey emerged as a candidate for further analysis.

Yeast Cultivation

One clinical isolate of *C. albicans*, previously obtained from a gastric biopsy and stored at -80 °C, was used in this study. Previous research has demonstrated that this isolated yeast exhibits a high capacity for biofilm formation. After thawing, the recruited isolate was inoculated onto Brain Heart Infusion (BHI) agar and incubated at 37°C for 24 hours. The identity of the isolate as *C. albicans* was confirmed by the appearance of green colonies on Chromagar. To evaluate the resistance profile of this isolate, susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, assessing resistance to azoles (e.g., fluconazole), echinocandins (e.g., caspofungin), and polyenes (e.g., amphotericin B) (CLSI, 2020)

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) of thyme essential oil (EO) and thyme honey against *C. albicans* was assessed using the broth microdilution method in 96-well microplates. Fresh yeast cultures were diluted to a 0.5 McFarland standard, and each well received 90 µL of brain-heart infusion (BHI) medium. A 10 µL aliquot of yeast suspension was added to each well, followed by 50 µL of honey at four concentrations (100%, 75%, 50%, and 25% v/v, corresponding to final concentrations of 25%, 18.75%, 12.5%, and 6.25% v/v) and 50 µL of thyme EO. Control wells were included for evaluating the individual effects of thyme EO and honey, as well as negative controls without any additives. Each treatment was replicated three times for statistical reliability, and the plates were incubated for 24 hours at 37 °C in a shaking incubator. The MIC was defined as the lowest concentration of honey that inhibited yeast growth. Following this, 50 µL from wells with no visible yeast growth were inoculated onto BHI agar plates and incubated for another 24 hours at 37 °C. The minimum bactericidal concentration (MBC) was determined as the lowest concentration of honey that showed no bacterial growth on

the agar plates (CLSI, 2020).

Anti-biofilm Activity Assay

The crystal violet (CV) microtiter plate assay was performed to evaluate the effect of thyme EO and thyme honey on the biofilm formation of *C. albicans*, as described by(Shukla and Rao, 2017). Initially, yeast cultures were incubated on BHI agar plates for 24 hours. Yeast suspensions were then prepared by diluting a 0.5 McFarland standard to obtain a final concentration of 1×10^3 to 3×10^3 colony-forming units per mL, which was utilized for the anti-biofilm assay. Each well of a 96-well plate received 90 µL of liquid BHI medium. Subsequently, 10 µL of the yeast suspension was added to each well, followed by the addition of 50 µL of four concentrations of honey (100%, 75%, 50% and 25% V/V final concentrations 25, 18.75, 12.5, and 6.25% V/V) and 50 µL of thyme EO. Control wells were set up to assess the individual contributions of the components: wells that lacked honey but contained EO and yeast were utilized to evaluate the impact of EO by itself, while wells that lacked EO but included honey and yeast were used to determine the effect of honey independently. Additionally, wells containing neither yeast nor honey nor EO served as negative controls. Each treatment group was replicated three times to ensure statistical validity. The plates were then incubated for 24 hours at 37 °C. After incubation, the culture medium was discarded, and the wells were rinsed with sterile phosphate-buffered saline (PBS). The microplate was inverted and allowed to air dry at room temperature for one hour. After drying, 200 µL of a 2% crystal violet solution was added to each well, and the microplate was incubated for 15 minutes without agitation. Following this, the wells were rinsed with phosphate buffer to eliminate any residual dye. Subsequently, 200 µL of a 30% acetic acid solution was added to extract the bound dye from the wells. Finally, the optical absorbance was measured at 595 nm using a microplate reader. The inhibitory effect on biofilm formation was analyzed using the formula: Inhibitory rate = $(1 - S/C) \times 100\%$, where S represents the average absorbance of the sample group treated with thyme EO and honey, while C reflects the average absorbance of the control group.

Statistical analysis

To assess the effectiveness of each treatment in preventing biofilm formation compared to the control group, a paired samples t-test was performed using Excel version 2021. The optical absorption values obtained from the treatment groups compared with those of the control group following crystal violet staining, with a p-value of ≤ 0.05, was considered statistically significant.

Microscopy imaging

To illustrate the inhibitory effects of honey and thyme EO oil on the biofilm formation of *Candida albicans*, Evans Blue solution (0.01% in PBS) was utilized to stain the treated *Candida albicans* samples that were exposed to a combination of thyme EO and various concentrations of honey. These treated samples were then compared to a control group. Observations were conducted using immersion oil with a fluorescent microscope which was configured with a 1000X objective lens to examine biofilm formation, using a FITC filter set for

effective fluorescence detection.

Results

Resistance profile

The resistance profile of the *C. albicans* isolate was assessed using disk diffusion tests. The results revealed resistance to fluconazole, caspofungin, and amphotericin B, characterized by an average inhibition zone of less than 15 mm, confirming the isolate's multidrug-resistant phenotype.

Susceptibility assay by MIC and MBC

The MIC and MBC tests indicated that the thyme EO and honey were ineffective in inhibiting the growth of *C. albicans*. However, subsequent tests were conducted to evaluate their ability to inhibit biofilm formation.

Anti-biofilm activity assay

The light absorption for biofilm quantification was measured at a wavelength of 595 nm using a microplate reader (Table 1). All p-values obtained from the treatments were below 0.05, demonstrating statistically significant differences when compared to the negative control. Notably, the combination of thyme essential oil and honey, across all concentrations tested, exhibited the lowest p-value. This finding suggests that the synergistic effect of these two components is more effective in inhibiting the biofilm formation of *C. albicans*. Subsequently, the inhibition rate was measured using the formula (Inhibitory rate = (1 − S/C) × 100%), and the mean is presented in Table 1. Notably, the combination treatments exhibited markedly higher inhibition rates than either treatment alone at each concentration level. At the concentration of 75% (final concentration 18.75% V/V), the synergistic treatment achieved an average inhibition rate of 58.40%, while individual treatments with thyme EO and honey yielded average inhibition rates of 27.10% and 22.45%, respectively. Interestingly, the lowest concentration of 25% honey (final

Table 1. Optical Absorption Values and Inhibitory Rates (%) Obtained after Treatment of Clinical *C. albicans* Isolates

samples (Final concentration) % V/V	Optical Absorption Values				
	Repeat 1	Repeat 2	Repeat 3	Mean	Mean of inhibition rate (%)
Honey 100 (25)	0.70	0.71	0.72	0.71	26.10
Honey 75 (18.75)	0.65	0.67	0.68	0.67	30.30
Honey 50 (12.5)	0.75	0.76	0.74	0.75	21.90
Honey 25 (6.25)	0.85	0.84	0.86	0.85	11.5
Thyme EO	0.72	0.69	0.70	0.70	27.10
Honey 100 (25) + Thyme EO	0.50	0.52	0.51	0.51	46.90
Honey 75 (18.75) + Thyme EO	0.40	0.39	0.41	0.40	58.40
Honey 50 (12.5) + Thyme EO	0.55	0.56	0.54	0.55	42.80
Honey 25 (6.25) + Thyme EO	0.65	0.66	0.64	0.65	32.30
Negative Control (no treatment)	0.95	0.93	1.00	0.96	0.00

concentration 6.25% V/V) demonstrated an enhanced inhibition rate of 32.30% when combined with thyme EO, compared to an average of 12% for the honey treatment alone, further emphasizing the synergistic interaction between these treatments. The control group, which received no treatment, exhibited the highest optical absorption value of 0.96, confirming the absence of any inhibitory effect in untreated samples (Table 1, Figure 1).

Microscopy imaging

The fluorescent imaging analysis demonstrated that treatment with a combination of honey 75% V/V (final concentration 18.75% V/V) and thyme EO led to a notable decrease in the populations of *C. albicans* yeast cells. In contrast to the control group, the treated samples exhibited significantly fewer fluorescently labeled yeast cells. The cells appeared more scattered, exhibiting reduced numbers and altered cell shapes, which suggests effective inhibition of yeast biofilm formation (Figure 2).

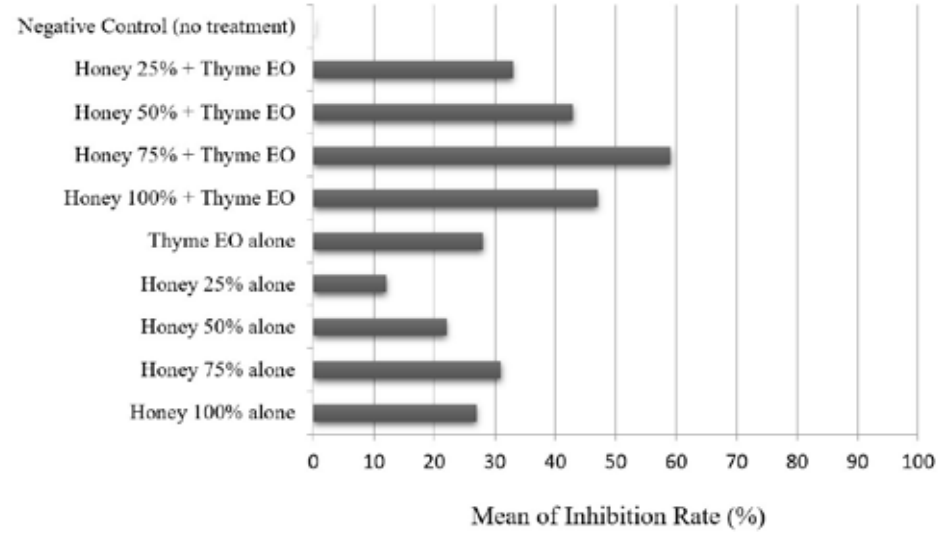

Figure 1. Comparison of inhibition rates (%) for various combinations of Thyme EO and honey against clinical isolates of *C. albicans*

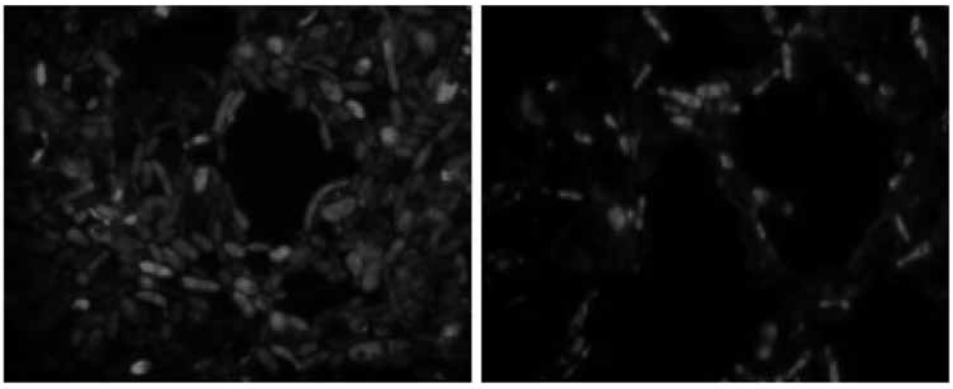

Figure 2. A: dense populations of *C. albicans* oval cells within biofilm; B: significant reduction in *C. albicans* cell density following treatment with a combination of thyme EO and honey 75% V/V, final concentration 18.75% V/V, (Magnification 1000X)

Discussion

Biofilms are critical to the pathogenesis of *Candida albicans* infections, as they significantly enhance resistance to antifungal agents, immune responses, and mechanical removal efforts (Pierce et al., 2017). The biofilm matrix offers a protective environment for *Candida* cells, complicating eradication efforts with conventional therapies. Thus, disrupting this matrix is essential for rendering *Candida* cells susceptible to antifungal treatment and ensuring successful therapeutic outcomes (Sardi et al., 2013).

Research has evaluated the antimicrobial properties of thyme EO against several bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Mycobacterium smegmatis*, as well as fungal species like *C. albicans* (Nazzaro et al., 2013). The results indicate that thyme EO exhibits significant bactericidal and antifungal activity against these microorganisms. Thymol, the main bioactive element found in thyme, demonstrates its antimicrobial properties by engaging with membrane proteins via hydrophobic interactions and hydrogen bonding, which subsequently modifies the permeability of cellular membranes. (Kowalczyk et al., 2020). Furthermore, thymol and carvacrol interfere with the biofilm matrix and inhibit the production of extracellular substances necessary for biofilm formation (Swetha et al., 2020).

Thyme honey demonstrate bacteriostatic and microbicidal effects that are affected by its distinctive characteristics, the concentration of active compounds, and the type of bacteria present. Phenolic compounds, including flavonoids and phenolic acids, which confer antioxidant and antibacterial effects, can disrupt microbial membranes, inhibit essential enzymes, interfere with DNA, and ultimately lead to microbial death (Özkök et al., 2016).

The results of this study demonstrated a significant reduction in *C. albicans* biofilm formation when treated with a combination of honey and thyme EO. This combined treatment showed markedly higher inhibition rates compared to treatments with thyme EO or honey alone across all concentration levels tested. In this regard, Thyme honey combined with thyme EO shows promise in disrupting *C. albicans* biofilms through various mechanisms. While thyme EO is rich in potent monoterpenes, thyme honey contains a diverse array of phenolic compounds, and their interaction could yield a broader spectrum of biological activity, addressing complex health issues more efficiently than either component alone (Assaggaf et al., 2022). In this study, the combination of 75% honey and thyme EO showed a greater inhibitory effect compared to a 100% honey mixture with EO. This phenomenon may be attributed to the dilution of honey, which could activate certain bioactive compounds. When honey is diluted, the changes in osmotic pressure and the solubility of its components might enhance the availability and reactivity of specific phytochemicals and enzymes that exhibit antimicrobial and anti-biofilm properties (Mandal and Mandal, 2011).

In an era of increasing interest in natural

remedies as alternatives to synthetic pharmaceuticals, understanding the synergistic effects of thyme EO and honey may facilitate the development of novel natural therapeutics that harness their combined benefits. Additionally, the implications for functional foods are considerable, as the synergistic properties of these products could enhance their applications in promoting health and wellness through innovative nutritional strategies. Given the substantial challenges posed by biofilms in clinical settings, investigating the combined effects of thyme EO and honey could provide valuable insights into new strategies for managing infections stemming from multidrug-resistant pathogens, ultimately contributing to improved healthcare outcomes. Despite these encouraging findings, it is imperative to highlight the necessity for comprehensive studies to validate the clinical efficacy of these natural products. Future research should encompass in vivo experiments and clinical trials to fully unravel the therapeutic potential of honey and its derivatives in combination with other natural agents against *Candida* infections. This is especially important in light of the increasing antifungal resistance, necessitating the development of innovative strategies to combat multidrug-resistant pathogens effectively.

Conclusion

The study demonstrates that the combination of honey and thyme EO significantly reduces biofilm formation by *C. albicans*, highlighting the potential of these natural substances as effective antifungal agents. The synergistic effects of thyme EO and honey contribute to a promising therapeutic strategy to combat the challenges posed by biofilms in clinical settings. As the search for alternative treatments intensifies, the integration of natural antimicrobials into therapeutic protocols provides a multifaceted approach to enhance treatment efficacy against biofilm-associated infections. Increasing the number of strains examined would enhance the applicability of the results to other *Candida* strains and various clinical conditions. Continued exploration of these natural remedies is deemed essential for the development of innovative treatment options aimed at managing infections related to *C. albicans* and other multidrug-resistant pathogens, especially through studies involving a broader spectrum of clinical isolates.

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