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Antifungal activity against *Candida albicans* biofilm of *Coffea robusta* monofloral honey from Vietnam

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Abstract

Coffee (*Coffea robusta*) flower honey is one of the highest-yielding kinds of honey in Vietnam. Nevertheless, there is little research on the antifungal activity associated with this type of honey. Therefore, this study was conducted to investigate the antifungal activity against *C. albicans* of Vietnamese coffee monofloral honey and its potential effects on biofilm formation. This study was conducted using the broth microdilution method. The results showed that coffee flower honey was resistant to *Candida albicans* strain with minimum inhibitory concentration (MIC) and with minimum fungicidal concentration (MFC) values of 50% and 70% (w/v), respectively. No change in the antifungal activity of honey and the antifungal agent (Amphotericin B) against the *C. albicans* strain occurred after five consecutive exposures. When using SEM, honey at MIC (w/v) strongly affected the two stages of *C. albicans* biofilm formation. The surface structure of the biofilm, deformed, distorted, and atrophied phenotypes impacted by honey were also observed. Expression of transcriptional-level genes involved in biofilm formation, quorum-sensing, and stress survival was analyzed by RT-qPCR in honey-treated and untreated biofilms. The present study showed the antifungal activity of Vietnamese coffee flower honey against *C. albicans.*

Keywords: antifungal activity; biofilm; *Candida albicans*; honey; minimum inhibitory concentration (MIC); transcription level

Abbreviations: CFU: Colony-forming unit; MFC: minimum fungicidal concentration; MIC: minimum inhibitory concentration; RT-qPCR: Real Time qualitative PCR; OD: Optical density; SEM: Scanning Electron Microscopy

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Introduction

Honey is a natural sweetener, not fermented, containing about 200 substances, with the main ingredients including sugar, water, and compounds such as proteins (enzymes), organic acids, vitamins, minerals, antioxidants, antimicrobial agents, and some other substances (Anand *et al*., 2019; da Silva *et al*., 2016; Hermanns *et al*., 2019). Sugar is the main component of honey, accounting for about 95% of the dry weight, which includes fructose (38.5%), glucose (31%), sucrose (1.5%), maltose (7.2%), and some others (4.2%) (da Silva *et al*., 2016; Kamal *et al*., 2019; Santos-Buelga *et al*., 2017). Depending on the plant origin, bee breed, geographical location, weather conditions, temperature, and production method, the composition and proportion of honey ingredients may vary.

The antifungal properties of honey are due to many different components. According to its physical and chemical properties, high viscosity prevents infection, the low water content of honey creates a high osmotic pressure that can affect the fungal cell wall, and its low pH inhibits the proliferation of fungi. Besides, hydrogen peroxide (H_2O_2) is the main agent for antifungal activity in honey, produced by the breakdown of glucose with the enzyme glucose oxidase (Bang *et al*., 2003). Different types of honey have different concentrations of H_2O_2, leading to different antifungal effects. Besides hydrogen peroxide, honey has numerous ingredients that contribute to its antifungal properties, collectively known as non-peroxide antifungal properties. Methylglyoxal, bee defensin-1, and many phenolic compounds derived from bees have also been implicated in the non-peroxide antifungal effects of honey (Chen *et al*., 2012; Montenegro *et al*., 2021). Furthermore, the anti-microorganism activities can also be attributed to the influences on many levels of biofilm development against the initial formation phase, pre-formed stage, or adhesion, including the destruction of essential biofilm components, such as membrane integrity, and impact on extracellular matrix formation (Fernandes *et al*., 2021; Tomičić *et al*., 2022).

The antifungal properties of honey make it an attractive alternative treatment for *Candida* infections, especially for topical application to the skin and mucous membranes (Chen *et al*., 2012; Fernandes *et al*., 2021). Furthermore, unlike most antibiotics or antifungals, it is less likely to induce honey resistance (Blair *et al*., 2009; Fernandes *et al*., 2021). The occurrence of fungal infections, such as candidiasis and candidemia, has increased dramatically in recent decades, contributing to high morbidity and mortality (Fernandes *et al.*, 2021; Rodrigues *et al*., 2017). There are now more than 200 species of *Candida*, but the most common of these is *Candida albicans*. They are dimorphic species capable of forming biofilms. *Candida albicans* is an opportunistic human fungus that lives harmlessly in the gastrointestinal tract (Gastrointestinal - GI), reproductive tract, oral cavity, and skin of most humans. In people with healthy immune systems, *C. albicans* is harmless, keeping the microbial balance in its habitat. However, changes in the host-microbiome (e.g., use of antibiotics), changes in the host immune response (stress, foreign microbial infection, or use of inhibitory therapy), or there are environmental changes in the body (e.g., changes in pH or nutrient content), which can facilitate *C. albicans* to overgrow and cause infection (Nobile *et al*., 2015). It has been observed that *Candida* spp., primarily *C. albicans*, is one of the four most common causes of blood and cardiovascular infections in US hospitals, accounting for approximately 15% of all infections (Dominic *et al*., 2007; Nobile *et al*., 2015). Mortality is as high as 50% in infected patients. Every year in the United States, an estimated 100,000 deaths and an estimated $6.5 billion in costs are spent on treating this fungal infection, with devastating health and economic consequences (Nobile *et al*., 2015).

Unlike antibacterial drugs, antifungal drugs are somewhat less common (scarce) (Costa-de-Oliveira *et al*., 2020). Many efforts have been made to discover new natural-origin antifungal agents from diverse sources such as plant extracts, essential oils (Tomičić *et al*., 2022), or bacteria (Zabouri *et al*., 2021). Up to date, azoles, polyenes, and echinocandins are the three main classes of antifungal agents considered the last resort in many hospitals for treating invasive candidiasis, leading to the urgent demand for identify novel antifungal and their

activities (Costa-de-Oliveira *et al*., 2020). Moreover, with the increasing resistance and decreasing tolerance of antifungal agents today, susceptibility testing is increasing and plays a role in antifungal drug selection. The main aim of this work was to investigate the antifungal activity against *C. albicans* of Vietnamese coffee monofloral honey, as well as potential effects of the honey against the biofilm formation of the *C. albicans* strain.

Materials and Methods

Vietnamese coffee honey samples

In this study, coffee (*Coffea robusta*) monofloral honey samples were collected directly at wholly coffee-covered breeding sites in Dak Lak province, the main coffee-growing region of Vietnam and the only place where this type of honey can be harvested.

Sampling equipment and containers were clean, dry, and free from effects on the honey's properties such as odor, taste, or physicochemical and microbiological properties. All honey samples were raw and unprocessed and stored in a dry, dark place at room temperature. Honey samples were tested for no antibiotic residues, including Enrofloxacin, Flumequine, Oxytetracycline, Amitraz, Coumaphos, and Chloramphenicol.

Microorganisms and culture conditions

The fungal strain *C. albicans* ATCC 10231 was used and cultured in the Sabouraud Dextrose Broth (SDB) and MH (Mueller-Hinton), which was supplemented with 2% (w/v) agar when required to obtain the respective solid media Sabouraud Dextrose Agar (SDA) and Mueller-Hinton Agar (MHA). The yeast strain used in the study was incubated at 30-37 °C for 18-24 hours.

Antifungal test

The antifungal properties of honey were evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using the broth micro-dilution method. Briefly, 90% sterile honey (w/v) was diluted with sterile SD broth to variable concentrations ranging from 10% to 80% with 1% intervals in a 48-well plate. Then, 10 µL of 1-2 × 10^6 CFU/mL fungal cultured solution was added into the honey dilutions wells, resulting in the final inocula of 1-2 × 10^5 CFU/mL. Negative control wells contained only the cultivation medium. The MIC, expressed in % (w/v), was obtained by visually observing the turbidity gradient after overnight incubation at 30-37 °C for the absence or presence of fungal growth, which was later confirmed by spreading on the SDA plates.

For the MFC assay, 50 µL suspension of each well of the 48-well plate resulting from the previous assay was placed onto SDA plates. The lowest antifungal concentration yielded no colony growth after incubation at 37 °C for 12-24 h was considered MFC.

Selection for resistance to honey and antifungal

Etest assays

C. albicans was inoculated into test tubes containing sterile SD broth and cultured in shaking condition overnight at 37 °C. Then the fungal strain was measured for the optical density (OD) at 600 nm with the obtained value of about 0.08 to 0.1, corresponding to a fungal density of about 1-2 × 10^6 CFU/mL. 100 µL of cultured strain was spread onto MH (Mueller-Hinton) agar plates using a sterile cotton swab. After the agar surface was completely dry, an Etest amphotericin B strip was applied to the agar surface and incubated at room temperature for 18-24 hours. The amphotericin B MIC was read at the point at which the border of the elliptical inhibition zone intersected the strip.

Antifungal test

Colonies of *C. albicans* strain from the previously spread SDA plate at MIC value were inoculated into test tubes containing 5 mL of sterile SD broth and shaken overnight at 37 °C. Then the antifungal test by broth microdilution method was performed as described above, and the results were reported in the new MICs. This process was repeated to test the potential resistance of *C. albicans* to our honey samples.

Antifungal effect of honey on biofilms

Establishment of biofilms

At the early stage of *C. albicans* biofilm development, the Vietnamese coffee honey was investigated for interfering with biofilm formation onto polystyrene plates (Ansari *et al*., 2013). *C. albicans* was shaken overnight at 37 °C in sterile SD broth. The optical density at 600 nm was measured to get the value of 0.4 to 0.6, corresponding to the density of *C. albicans* about 5-6 × 10^8 CFU/mL. Ten µL of cultured strain were added to 96-well plates having 190 µL of SD medium and shaken at 80 rpm for 2 h at 37 °C. After 2 h, non-adherent fungal cells were removed by gentle washing twice with sterile 0.1 M PBS (pH 7.4). After the washing steps, wells were added with 200 µL of diluted honey at the MIC value, and plates were incubated for 48 h at 37 °C.

For the established biofilm stage, the effect of honey on the mature biofilm was also evaluated (Ansari *et al*., 2013). The assay was performed as described above with slight modifications. After the wells were washed with PBS, 200 µL of sterile SD broth was added to each well, and wells were incubated for 24 h at 37 °C for the adherent cells to proliferate and establish the biofilm. After 24 h, non-adherent fungal cells were removed by gentle washing twice with sterile PBS. After that, wells were added with 200 µL of diluted honey at the MIC value, and plates were incubated for 48 h at 37 °C.

Scanning Electron Microscopy (SEM)

For SEM, microtiter plates with established *C. albicans* biofilms were gently washed three times with PBS (pH 7.4), and then glutaraldehyde of 2.5% (volume of 100 µL) was added to each well. After that, the biofilms were processed and visualized by SEM using FE-SEM S-4800 (Hitachi).

Quantitative reverse transcription PCR (RT-qPCR) analysis of C. albicans biofilm-related genes

RT-qPCR was used to investigate honey-induced changes in the transcript levels of genes involved in biofilm formation. Genes involved in biofilm formation (*EFG1*, *ALS3*, *ZAP1*), quorum-sensing (*DPP3*), and stress-survival (*HSF1*, *HSP90*) pathways were evaluated.

C. albicans cells (1×10^8 CFU/mL) were cultured in the absence or presence of honey at MIC values in 24-well plates for 24 h at 37 °C. Next, wells were washed with PBS, and adherent cells were collected for total RNA extraction. The quality and quantity of extracted RNA were determined spectrophotometrically. cDNA was synthesized from 2 µg RNA using the SensiFastTM cDNA synthesis kit (Bioline). The RT-qPCR mixture contained 6µL cDNA, 10µL SYBR Green Master Mix, 1pMol/µL of each primer, and sterile MilliQ water to final volume same 20µL. Primer sequences used to amplify specific genes are presented below (Table 1). RT-qPCR was performed in a 96-well plate on a Real-Time PCR Detection System (95 °C for 60s, followed by 40 cycles of 95 °C for 5s, 60 °C for 30s). Actin (*ACT1*) was used as an internal control.

Table 1. List of gene-specific primers used for Real-time RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)
EFG1	CGCAACATCTCAAGGAA	TGTGGTGGGATAGGTAC	195
ALS3	GGTTATCGTCCATTTGTTGA	TTCTGTATCCAGTCCATCTT	139
ZAP1	ATCTGTCCAGTGTTGTTTGTA	AGGTCTCTTTGAAAGTTGTG	131
DPP3	CTCCTTCGGGTCATTCATCAA	GATTACCACCAAACCAAGGGA	267
HSF1	CCATCAGATGGTATTTCTTCC	GGTGAAACACTTGGAAAGTC	211
HSP90	CTGGTGCTGACGTTTCTA	ACCAGCGTTAGATTCCCA	131
ACT1	TAGGTTTGGAAGCTGCTGG	CCTGGGAACATGGTAGTAC	124

Statistical analysis

The data of gene expression were illustrated as the mean ± standard error of mean (SEM) from three independent replicates and analyzed by One-way ANOVA using GraphPad Prism 7 (GraphPad Prism Software Inc, USA). Differences with the value $P < 0.05$ were considered significant.

Results and Discussion

MIC, MFC

The broth microdilution method was used to evaluate the total antifungal activity of the Vietnamese coffee honey against the *Candida albicans* strain. As a result, the MIC value for *C. albicans* was 50%, while the MFC was 70%, with no fungal colony observed on the SDA plate after 48 h of incubation at 37 °C (Figure 1).

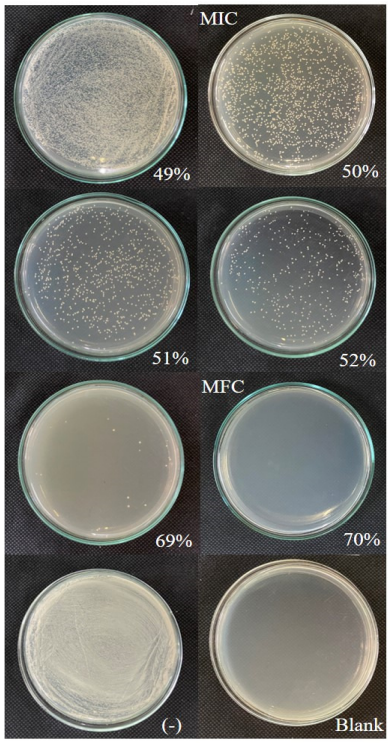

Figure 1. Total antifungal activity of Vietnamese coffee honey at various concentrations against *Candida albicans* ATCC 10231 (% w/v). (-) Negative control

Although there are no scientific data on the antifungal ability of Vietnamese honey, including Vietnamese coffee honey, the anti-candidal effect of our honey sample was potent compared to published data on types of honey worldwide. In the publication of Fernades *et al.* (2020) the authors used the diffusion method in a modified liquid medium to investigate the antifungal ability of Portuguese honey and Manuka honey for *C. albicans* strains. Most honey samples also had MIC values of 50% (w/v) and MFC values of >50%, which were compatible with the honey in this present study. In addition, Anand *et al.* (2019) studied a range of honey with similar results. MICs of some important commercial honey were reported as Tea tree (45%), Jelly bush (<50%), Super Manuka (60-70%), and Jarrah (75-80%). Compared to them, the highest antifungal activity against *C. albicans* was recorded in Agastache (Australia) honey, with a MIC value of 40%. The *in vitro* antifungal activity of Turkish unprocessed honey samples also revealed their MICs in the range of 40% to 80% (w/v)(Koc *et al*., 2009). Accordingly, the antifungal activity of different types of honey varies among the botanical source and geographical origin. The composition of a particular honey sample dramatically depends on the composition of the nectar and has a positive correlation to the inhibition capacity (Fernandes *et al*., 2021; Montenegro *et al*., 2021; Vica *et al*., 2022). The element with the most potent antifungal effect found in honey is hydrogen peroxide (H_2O_2), which is produced by the oxidation of glucose (Fernandes *et al*., 2021). In addition, the phenolic profile of honey contributed to its non-peroxide antibacterial activity, given its potential to become a therapeutic alternative against resistant pathogenic microorganisms (Montenegro *et al*., 2021).

Honey resistance ability

Theoretically, repeated use of honey could promote honey's resistance to fungi. Thus, the sensitivity of *C. albicans* to repeated honey was performed in parallel with Etest Amphotericin B.

Etest Amphotericin B, after five exposures to the antifungal agent, obtained a consistent MIC value of approximately 0.5 mcg/ml (Table 2). Similarly, after five consecutive sensitivity tests of *C. albicans* to Vietnamese coffee honey, the MIC and MFC values were maintained at 50% and 70%, respectively (Figure 2). The results indicate no resistance of *C. albicans* to either the repeatedly exposed drug or honey.

Table 2. The susceptibility for long-term exposure of *Candida albicans* ATCC 10231 to amphotericin B antibiotic by MIC breakpoints

Amphotericin B	Mean MIC [µg/mL] ± SD*
Control	0.50 ± 0.00
(I)	0.50 ± 0.00
(II)	0.58 ± 0.14
(III)	0.54 ± 0.19
(IV)	0.50 ± 0.00
(V)	0.58 ± 0.14

*SD, Standard Deviation for three replicates

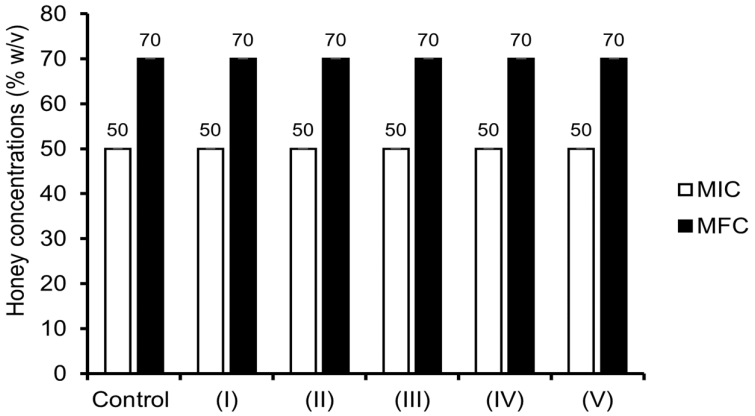

Figure 2. The susceptibility for long-term exposure of *Candida albicans* ATCC 10231 to Vietnamese coffee honey by MIC and MFC values (% w/v)

However, the secondary resistance of amphotericin B to *C. albicans* has been reported during extended treatment (Perea *et al*., 2002). The rising number of drug-resistant microorganisms has recently emerged as a major threat to human health. Moreover, when treating *Candida* infection with antifungal agents, it is common to use a combination of multiple antifungal drugs to affect many pathways easily and maximize the treatment effectiveness. As a drawback, it can easily lead to numerous undesirable side effects and a risk of multidrug resistance. With regard to honey, this is a product of natural origin, with many ingredients and bioactive compounds existing and possessing antifungal properties from its intrinsic nature. The high sugar content and osmotic pressure of honey can also fight fungus in contrast to amphotericin B, which acts by only one pathway (membrane action). As far as we know, there are no scientific publications on the sensitivity of fungi when consecutively exposed to honey. However, for bacteria, Blair *et al*. (2009) showed that even though the two strains of *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) rapidly produced resistant phenotypes after repeatedly exposed to antibiotics such as tetracycline, oxacillin, and ciprofloxacin, they were not resistant to honey after repeatedly exposure at concentrations below lethal levels.

Collectively, it can be concluded that *C. albicans* ATCC 10231 was susceptible to Vietnamese coffee honey, which may represent an effective method for treating *C. albicans* infections.

Scanning Electron Microscope (SEM)

The ability of honey to affect the biofilm structure of *C. albicans* fungus was observed using a scanning electron microscope (SEM). SEM is a type of electron microscope that provides detailed, high-resolution images of fungal phenotypes, as well as biofilm surface structures when impacted by honey at magnification levels of difference. The results are shown in Figure 3.

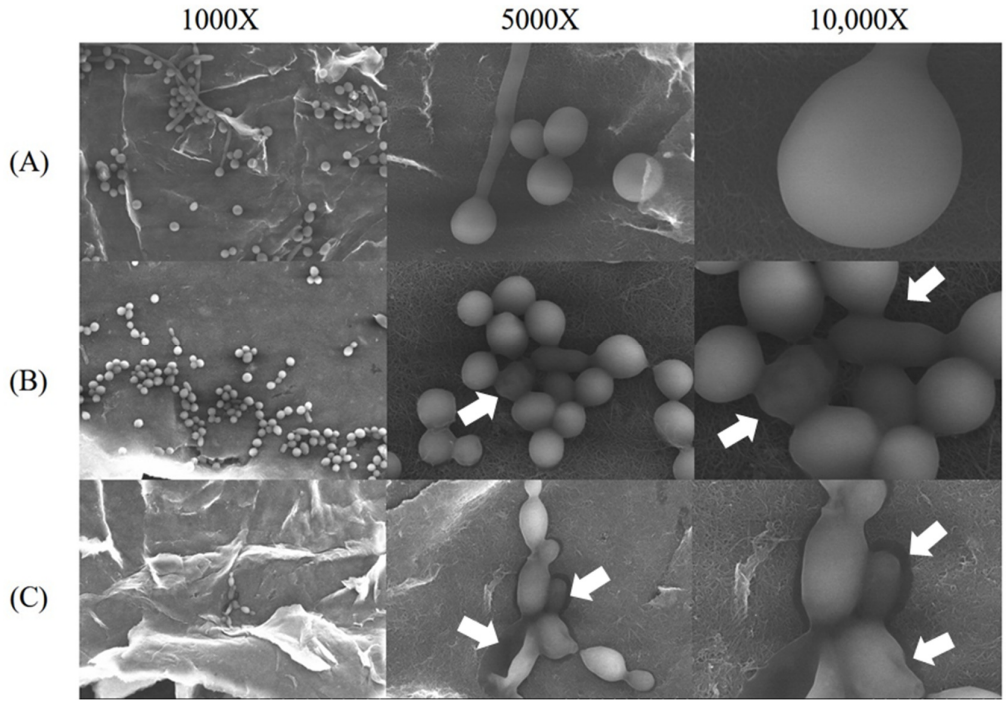

Figure 3. Representative SEM images of Vietnamese coffee honey's effect on the cell wall structure and biofilm formation against *Candida albicans* ATCC 10231 at different magnifications indicated
Biofilms were formed after 24 hours of incubation in a 96-well plate prior to honey exposure. The images represent *C. albicans* cells in (A) control (without honey); (B) early stage of biofilm formation treated with honey (MIC = 50% w/v); and (C) established biofilm treated with honey (MIC = 50% w/v)

The image of the biofilm, formed in the absence of honey in the control sample, showed a dense network of yeast cells, filamentous hyphal formation, and cells with the typical phenotype of the unaffected biofilm. The treatment had a spherical/oval shape, and the mycelial phenotype was detected as smooth, regular, and unchanging (Figure 3A). When the biofilm was formed for 24 h, then treated with honey at a concentration of MIC = 50% (w/v), the fungal cells were distributed in clusters and clustered together. The growth of biofilm was inhibited, and the morphology of the fungal cell wall changed. These abnormal phenotypes were distorted, wrinkled, or disrupted, possibly due to the destruction of the cell membranes of *C. albicans* by honey osmotic contraction, leading to death and a decrease in cell number (Figure 3B). The effect of honey concentration of MIC on the early stage of biofilm formation caused yeast cells to be isolated, discrete, less clustered, and less aggregated (Figure 3C). The constriction of the cell and the roughness of the cell wall suggest that the osmotic pressure exerted by the honey induces a phenotypic change in the fungus. Honey at a concentration of 50% (w/v) was not enough to exert a strong effect on biofilms, so honey at a higher concentration was suggested [70-80% (w/v)] to be able to prevent biofilm formation. Observations from SEM demonstrated the interference of Vietnamese coffee honey on the integrity of cell membranes. In the work of Ansari *et al*. (2013), the authors tested the effect of jujube honey [40% (w/v)] on the biofilm of *C. albicans* observed by SEM. Jujube honey causes cell membrane shrinkage, depolarization, and damage to cell walls. This result may indicate that honey may have different mechanisms of action that interfere with the metabolic and structural pathways of the cell wall in *C. albicans*. Besides, honey can interfere with any stage of biofilm formation (initial and established stages) of microorganisms. Therefore, Vietnamese coffee honey could inhibit the biofilm formation of *C. albicans*.

qPCR analyses

The results in Figure 4 showed honey-induced changes at the transcriptional level of six genes involved in biofilm formation, as assessed by RT-qPCR. The expression level of each gene was analyzed when the biofilm was treated with honey at a concentration of MIC value of 50% (w/v) for 24 h and compared with the untreated biofilm (Figure 4).

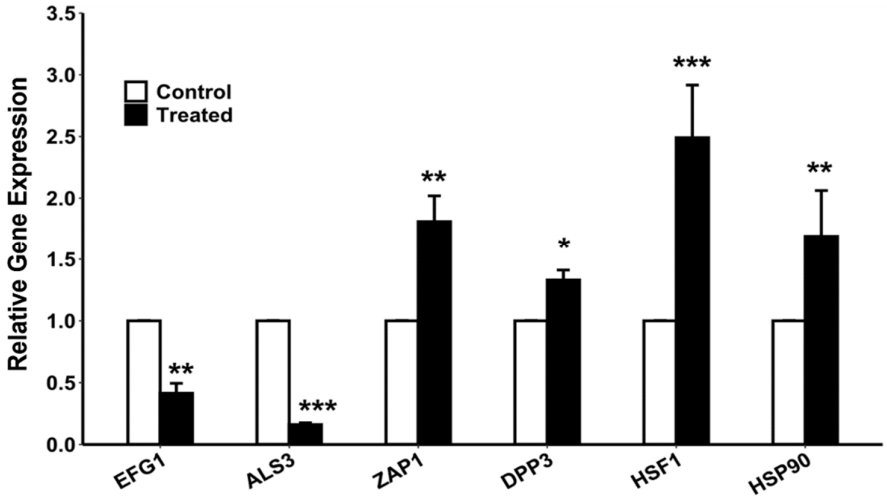

Figure 4. Alterations in gene expression profiles associated with exposure of *Candida albicans* ATCC 10231 to the tested honey as determined by qPCR. The gene expression level (closed column) was presented as a fold change relative to the control group (untreated biofilms with honey, open column). ACT1 expression was used for normalization (Housekeeping gene)

The experiment was performed in triplicate, and bars represent means ± Standard error of the mean (SEM) from three independent experiments. * P≤0.05, ** P<0.01, *** P<0.001.

The main signaling pathways involved in the induction of filamentous hyphal formation and biofilm formation are Ras1-cAMP-Efg1(Biswas *et al*., 2007; Ramage *et al*., 2002). Interestingly, honey significantly reduced the expression level of *EFG1*, a key component of the Ras1-cAMP-Efg1 pathway. Ras1 GTPase stimulates the enzyme Cyr1 adenylate cyclase to synthesize cAMP, promoting activation of the transcription factor Efg1(Morici *et al*., 2016). As expected, the *ALS3* gene was also downregulated. This result was consistent with previous studies, in which authors showed that the active effect of antibiotics was induced through inhibition of the Ras1-cAMP-Efg1 pathway (Sun *et al*., 2015; Zhao *et al*., 2013). In line with filamentous hyphal inhibition, the *DPP3* gene increased expression. The *DPP3* gene is involved in the conversion of farnesyl pyrophosphate to farnesol synthesis. This is an important quorum-sensing molecule (Hornby *et al*., 2001). Farnesol is an extracellular compound secreted by *C. albicans* that inhibits the conversion of filamentous hyphal formation. It also plays a major role in biofilm formation(Kebaara *et al*., 2008).

Although the components of the extracellular matrix of *C. albicans* biofilms have not been completely characterized, the presence of carbohydrates, proteins, and nucleic acids has been described (Morici *et al*., 2016). The major extracellular carbohydrate was identified as β-1,3-glucan. However, little is known about biofilm substrate production, which relies on complex regulatory mechanisms involving several Zap1 (Zinc-responsive activator protein) transcription factors, which produce an extracellular matrix to promote cell dispersal. Zap1 directly activates the expression of several genes that promote matrix production (Morici *et al*., 2016). In this study, data indicated that coffee honey increased the transcriptional level of *ZAP1*, i.e., the

negative transcriptional regulator Zap1, which activated several genes that reduce extracellular matrix production.

Hsf1 is an essential determinant for viability and virulence in *C. albicans* and is a key regulator of the heat stress response. It is established to activate heat shock proteins (HSPs) through standard heat shock elements (HSEs)(Leach *et al*., 2016). Hsf1 mainly coordinates stress response signaling and affects Hsp90 function, making cells more responsive to antifungal treatment (Nair *et al*., 2018). These two genes increased expression when the cells were treated with the coffee honey. It was explainable that when *C. albicans* encountered coffee honey, the honey could cause stress to the fungus, causing the fungus to increase the expression of *HSF1* and *HSP90* to adapt, react, and counteract the negative effects of honey. In addition to the prevention of heat stress, *HSF1* and *HSP90* are now implicated in additional roles in viability, possibly due to their role in the activation of core gene expressions, drug response, toxicity, and filamentous hyphal formation. Thereby, it is shown that the role of these two genes is not only in responding to heat stress but also in response to other processes mediating biofilm formation.

Conclusions

Our data showed that Vietnamese coffee monofloral honey could effectively inhibit *C. albicans*. The effectiveness of honey was observed in the initial stage of biofilm formation through the exterior phenotype of the fungal cells, which was in line with the expression of genes involved in biofilm formation upon honey exposure. It suggested that honey effectively affected the early stage of biofilm formation in *C. albicans* by influencing the transition from yeast to filamentous. However, our honey at the MIC value did not have much effect on the established biofilm. Additionally, the honey could act on different sites of the cell and minimize drug resistance via up or down regulations of related genes and transcription factors. Collectively, the results indicated that the antifungal effect of Vietnamese coffee honey was potential and ancient yet powerful for application in combined treatment with antifungal drugs.

Authors' Contributions

Conceptualization: QDD, TTTN, HTV; Data curation: QDD, TTTN; Formal analysis: QDD, TTTN; Methodology: QDD, TTTN; Project administration: HTV; Validation: TTTN, HTV; Visualization: QDD, CDB; Writing - original draft: QDD; Writing - review and editing: TTTN, CDB, HTV.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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