Portuguese honeys as antimicrobial agents against *Candida* species

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ABSTRACT

Background and aim: Honey has been recognized worldwide for its antioxidant, anti-tumor, anti-in-flammatory and antimicrobial properties. Among them, the antifungal properties associated to honey make it an attractive alternative treatment for *Candida*-associated infections, particularly for topical application to the mucous membranes and skin. In this sense, the main purpose of this work was to evaluate physicochemical properties of five Portuguese honeys and Manuka honey (an Australian honey with well recognized medical proprieties, used as control) and to evaluate the antifungal activity in *Candida* species planktonic and biofilm assays.
Experimental procedure: Pollen analysis, pH determination, color, concentration of protein and methyl-glyoxal, conductivity, total phenolics and flavonoids, hydrogen peroxide concentration, and character-ization by differential scanning calorimetry in honey samples were determined. Additionally, the effect of honeys on planktonic growth of *Candida* was initially evaluated by determination of the minimum inhibitory concentrations. Then, the same effect of those honeys was evaluated in biofilms, by Colony Forming Units enumeration.
Results and conclusion: It has been shown that Portuguese heather (*Erica cinereal*) honey presented the most similar physicochemical properties to manuka honey (specially phenolic and flavonoids contents). The five Portuguese honeys under study, presented in general a potent activity against planktonic multi-resistant yeast pathogens (several clinical isolates and reference strains of *Candida* species) and *S. aureus* and *P. aeruginosa* bacteria cultures. Additionally, it was also concluded that Portuguese heather honey (50% and 75% (w/v)) can also act as a good *Candida* species biofilm reducer, namely for *C. tropicalis*.
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1. Introduction

Since the 19th century, honey has been recognized worldwide for its antioxidant, anti-tumor, anti-inflammatory and antiviral properties.[1] Both *in vitro* and *in vivo* studies have demonstrated that honey is an effective, broad spectrum and active antimicrobial agent against a wide variety of bacteria and fungi.[2] Several studies have investigated the antimicrobial properties of honey against bacteria, few have focused on its antifungal properties.[3]

Honey has been reported to containing about 200 substances, a complex mixture of sugars with small amounts of other constitu-ents, such as minerals, proteins, vitamins, aroma compounds, organic acids, enzymes, phenolic acids, flavonoids, pigments, waxes, pollen grains and other phytochemicals.[4] As a complex natural product, several factors contribute to the antimicrobial activity of honey. Among the factors with strongest antifungal ef-fect is the hydrogen peroxide (H_2O_2), which is produced in diluted honey as a result of glucose oxidation.[5,6] Methylglyoxal (MGO), bee defensin-1 and other compounds derived from bees (e.g. flavonoids and phenolic compounds of floral origin, and lysozyme) have also been associated with the antifungal effect of honey.[7] Also, the acidity (pH 3.2–4.5) of honey due to the presence of organic acids

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List of abbreviations

BSA	Bovine serum albumin
CAN	*Candida non albicans*
CFU	Colony-forming unit
DP	Declustering potential
DSC	Differential scanning calorimetry
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GAE	Gallic acid equivalents
GOx	Glucose oxidase
LOD	Limit of detection
LOQ	Limit of quantification
MGO	Methylglyoxal
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
OD	Optical density
QE	Quercetin equivalents
RT	Room temperature
UHPLC-ToF-MS	Ultra-High Performance Liquid Chromatography combined with Time-of-Flight Mass Spectrometer
UPW	Ultrapure water

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in its composition is known to inhibit the growth of various microorganisms.[8] Additionally, the osmotic effect of honey should be considered as an intrinsic antifungal mechanism. Indeed, because honey contains a high concentration of carbohydrates and a reduced volume of water, bacterial growth can be inhibited by cell dehydration; this action is more difficult in fungi due to a higher resistance to osmotic pressure.[9] The honey's action mode, inhibiting the biofilm formation or promoting the disruption of mature biofilm, includes the destruction of the essential components of the organisms of cells,[10] such as the cell membrane integrity and the reduction of the production of an extracellular polysaccharide matrix. However, it is important to note that the composition, sensory attributes and physical properties of honey are influenced by several factors, mainly botanical and geographic origin, environmental conditions, pollen and nectar of the original plant, moisture and beekeeper contribution.[11]

The antifungal properties of honey make it an attractive alternative treatment for *Candida*-associated infections, particularly for topical application to the skin and mucous membranes.[7] Moreover, unlike most antibiotics or antifungals, resistance to honey cannot be induced.[12] The occurrence of fungal infections, such as candidiasis and candidemia, has been increasing significantly in recent decades, contributing to high morbidity and mortality.[13] *Candida* species are part of the normal microbiota of the human's oral cavity, gastrointestinal, urinary and vaginal tracts.[13,14] However, changes in the host's defences can lead to an exaggerated growth and, consequently, colonization by this species, thus inducing superficial and invasive infections.[15] In fact, several factors have been implicated in significantly increasing the incidence of fungal infections, such as parenteral nutrition, immunosuppressive therapy, use of broad-spectrum antibiotics, invasive surgical procedures, and application of internal medical devices (e.g. venous catheters, dental implants, valves cardiac grafts, vascular grafts, ocular lenses and artificial joints).[16] The genus *Candida* includes more than 150 species of yeast, of which at least 15 may be pathogenic to humans, the most well-known *Candida* pathogens are *Candida albicans*, *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*.

Although *C. albicans* is considered the most common pathogen, the incidence of infections due to species of *non-albicans Candida* (NAC) has been increasing, mainly due to the indiscriminate prescription of antifungal agents.[17–19] The increasing incidence of drug's resistant pathogens such as NAC, the limited number of effective therapeutic options and the toxicity of the compounds have drawn attention to the development of alternative treatments such as the use of natural products, as honey.

The main purpose of this work was to evaluate physicochemical properties of different Portuguese honeys related to antifungal activity and evaluate the antifungal effect of these honeys on different *Candida* species and on their biofilms.

2. Material and methods

2.1. Honeys

In this study, we used five Portuguese honeys with different floral origins: chestnut (*Castanea sativa mill*), eucalyptus (*Eucalytus globulus*), orange blossom (*Citrus sinensis*), rosemary (*Lavandura stoechas*) and heather (*Erica cinerea*), supplied by Portuguese beekeepers. The commercially available Manuka (*Leptospermum scoparium*) honey (Medihoney ®, Derma Sciences) was also used as a reference. All honey samples were used as raw and unprocessed and stored in the dark at 4 °C.

2.2. Honey physical chemical characterization

2.2.1. Determination of pH

The pH of honey was determined following to official method described by the International Honey Commission US National Honey Board.[20] Briefly, 5 g of honey, at 20 °C for 24 h, were vortexed with 5 mL of water. The pH of the final solution was measured in a pH meter (Hanna instruments HI 2210).

2.2.2. Total phenolics content

The total phenolic content was determined using the Folin-Ciocalteu method as described by Singleton, Orthofer, and Lamuela-Raventós.[21] Briefly, honey was diluted in water (7.5%, 15%, and 30% (w/v)) and 7.5 mL of Folin-Ciocalteu reagent (10% (w/v) in water) were added to 1 mL of diluted sample. The mixture was homogenized and incubated for 5 min, after which 7.5 mL of sodium carbonate (aqueous solution 60 mg/mL) were added. After incubation in the dark at room temperature (RT) for 2 h, the absorbance of the reaction mixture at 725 nm was determined against the blank (prepared as described previously but without the honey). Gallic acid standard solutions (50–175 μg/mL) were used for the calibration curve. The total phenolics contents were expressed in mg of gallic acid equivalents (GAE) per g of extract (mg GAE/g of extract).

2.2.3. Total flavonoids contents

The total flavonoids content of the samples was determined using the aluminium chloride method described by Yoo et al.[22] Briefly, 1 mL of honey samples (pure and with a dilution of 50% (w/v), in water) were added to 4 mL of ultrapure water (UPW) and 300 μL of sodium nitrite (5% (w/v)). The mixture was homogenized and incubated for 5 min at RT, after which 600 μL of aluminium chloride-water solution (10% (w/v)) were added. Then, 2 mL of 1 M sodium hydroxide and 2.1 mL of water were added to the mixture and vortexed. The sample optical density was measured at 510 nm (OD_{510}) (Synerary HT - BioTek). Quercetin standard solutions (0.1–0.5 mg/mL) were used for the calibration curve. Total flavonoids content was expressed in mg quercetin equivalents (QE) per g of extract (mg QE/g of honey).

2.2.4. Hydrogen peroxide concentration

The hydrogen peroxide (H_2O_2) is released in the reaction of glucose oxidase (GOx), which oxidizes D-glucose, and was quantified by measuring the OD_{510} after using the GOx activity assay kit (Nzytech), according to the manufacturer's instructions. The enzyme GOx (10–40 mU/0.5 mL) was used for the calibration curve.

2.2.5. MGO determination

MGO determination was performed using an Ultra-High Performance Liquid Chromatography combined with Time-of-Flight Mass Spectrometer (UHPLC-ToF-MS), based on Donarski, Roberts, and Charlton.[23] Honeys samples were diluted to 30% (w/v) in UPW and a set of methylglyoxal calibration standards were dilute, also in UPW to concentrations ranging from 0.125 to 40 μg/mL. For the derivatisation, 120 μL OPD (o-phenylenediamine, 100 mg/mL dissolved in UPW) were added to 800 μL of each honey sample or standard solution, homogenized and heated at 60 °C for 30 min. Samples and standards were cooled down to RT and dissolved in 10 mL using UPW before analysis.

Detection and quantification of MGO was performed with a Nexera X2 Shimadzu UHPLC coupled with a 5600+ ToF-MS detector (SCIEX, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization source working in positive mode (ESI+). In terms of chromatographic conditions, a column Acquity UPLC® HSS T3 (2.1 × 100 mm, 1.8 μm) was used and kept at 40 °C, the autosampler was maintained at 10 °C to refrigerate the samples and a volume of 10 μL of sample extract was injected in the column. The mobile phase consisted of 0.1% (v/v) formic acid [A] and acetonitrile [B] with a flow rate of 0.5 mL/min and with the gradient program described in Table 1.

In terms of mass spectrometry the acquisition was performed in full-scan from 100 to 300 Da using the Analyst® TF (SCIEX, Foster City, CA) software and with the following settings: ion source voltage of 5500 V; source temperature 575 °C; curtain gas (CUR) 25 psi; Gas 1 and Gas 2 of 55 psi; declustering potential (DP) 100 V. The identification and data processing were made through the PeakViewTM and MultiQuantTM (SCIEX, Foster City, CA) softwares.

2.2.6. Protein concentration

The protein content of honey was determined using Bradford method.[24] Briefly, 10 μL of honey sample and 300 μL of Bradford reagent (Sigma) were added to each well of the microplate. For the blank analysis, it was used distilled water. The microplate was stirred and left for stabilization during 10 min at RT. The OD was read at 595 nm.

The calibration curve was constructed with standard solutions of bovine serum albumin (BSA) with concentrations between 250 μg/mL and 2000 μg/mL.

2.2.7. Characterization by differential scanning calorimetry (DSC)

DSC measurements were carried out in liquid nitrogen atmosphere using DSC-822e instrument (Mettler Toledo International Inc., Greifensee, Switzerland) as described by Pinho et al.[25] Samples

Table 1
Elution gradient of the UHPLC-ToF-MS methods used to determine MGO in honey samples.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	97	3
2	97	3
5	0	100
6	97	3
7	97	3

were weighed (2.5 ± 0.2 mg) and sealed in aluminium pans and the calibration was made using indium as standard. Samples were heated from 25 to 350 °C at a scanning rate of 10 °C/min. Data were treated using LAB mettler star SW 8.1 software (Mettler-Toledo International Inc., Switzerland).

2.2.8. Determination of color, conductivity and pollen analysis

The evaluation of color and conductivity, as well as pollen analysis were carried out by APISMAIA company (Porto, Portugal).

2.3. Susceptibility testing

2.3.1. Microorganisms and culture conditions

In this assay several fungal and bacterial strains were used, namely: *C. albicans* SC 5413, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 9001; several clinical isolates from the biofilm/CEB collection; *Pseudomonas aeruginosa* DSM 22644; and *Staphylococcus aureus* ATCC 25923. For culturing, Sabouraud Dextrose Broth (SDB; Liofilchem) and Tryptic Soy Broth (TSB; Liofilchem, Italy), were used for fungi and bacteria, respectively. The broth was supplemented with 2% (w/v) agar (Liofilchem), when required, to obtain the respective solid media Sabouraud Dextrose Agar (SDA) and Tryptic Soy Agar (TSA). All yeast and bacteria strains, used in the study, were incubated at 37 °C for 18–24 h.

2.3.2. Planktonic antimicrobial susceptibilities

Susceptibilities of planktonic-cell cultures were evaluated by determining the minimum inhibitory concentration (MIC), the minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC). The MIC values were determined according to standard European Committee on Antimicrobial Susceptibility Testing (EUCAST, AST for bacteria and AFST for fungi), using the broth microdilution method.[26] Briefly, the initial cell concentration was adjusted for 2×10^5 CFU/mL and dispensed into 96-well plates in a proportion of 1:2 with diluted honey solutions 2-fold the desired concentration. Negative control wells contained only broth medium. The MIC, expressed in % (w/v), was obtained by visual observation of the turbidity gradient after incubation overnight at 37 °C.

For MFC and MBC, 10 μL of each well of the 96-well plate resulting from the previous assay were placed in SDA or TSA. The lowest antimicrobial concentration that yielded no colony growth after incubation at 37 °C for 12–24 h was considered as MFC or MBC.

2.3.3. Biofilms' antimicrobial susceptibilities

Biofilms were formed according to the modified microtiter plate test described by Stepanovic et al.[27] Briefly, after inoculation at 37 °C for 24 h in SDB, the different cultures were centrifuged twice (3000×g, 4 °C, 10 min) and the pellet resuspended in RPMI 1640 medium (Gibco® by Life Technologies). The concentration was adjusted to 1×10^7 cells/mL using a Neubauer counting chamber (Marienfield Superior, Germany). Then, 200 μL of the cell suspensions were transferred to 96-well flat tissue culture plates. For biofilm formation, microtiter plates were incubated aerobically for 24 h on a horizontal shaker at 120 rpm and 37 °C. Negative controls were performed using wells containing only broth medium.

The effect of each honey on biofilms was evaluated by exposing the 24 h-old biofilms to increasing concentrations of each honey (25%, 50% and 75% (w/v)). Briefly, after biofilm formation, the cell suspension was replaced by the honey solutions prepared. Plates were then incubated aerobically at 37 °C. After 24 h, treated biofilms were removed to assess biofilm-cells cultivability through CFU enumeration. For this, 200 μL of fresh saline solution were added to each well and the biofilms were scraped. The resulting

biofilm-cells suspensions were then serially diluted in saline solution and plated onto SDA plates. After 24 h of aerobic incubation at 37 °C, the cultured cell count was performed. Values of cultivable sessile cells were expressed as Log CFU per area (cm^2).

2.4. Statistical analysis

Data were analyzed using the Prism software package (Graph-Pad Software version 6.01 for Macintosh). One-way ANOVA and t-tests were used to compare MEI means of honey samples; $p < 0.05$ was statistically significant. For all assays, at least three independent experiments were carried out.

3. Results and discussion

3.1. Honey physical and chemical characterization

The physico-chemical properties of the Portuguese honeys (Chestnut, eucalyptus, orange blossom, rosemary and heather) in this study are summarized in Table 2. Manuka honey was also analyzed and used as a control.

According to the pollen analysis, rosemary and eucalyptus honeys have the highest monofloral percentage (63% and 61% (w/v), respectively), with a similar value to manuka honey (>70%). The orange blossom honey has different pollen origins, with only 8% (w/v) of orange blossom. The monofloral status generally refers to the presence of a single type of pollen in amounts greater than 45% (w/v) of the total pollen content in the spectrum.[11] Thus, in this study, all the honeys characterized can be considered monofloral, except for the orange and heather honeys (Table 2). The honey color and conductivity parameters are directly affected by mineral content.[28] A study by Gonzales et al.[29] described that lighter honeys (as rosemary and lavender) had lower amounts of minerals, while darker honeys (as chestnut, avocado and heather) had a higher mineral content. According to this fact, in this study, heather honey has the highest mineral content, since it is one of the darkest honeys (dark amber) which can be confirmed by its high conductivity (622 μS/cm). Orange flower honey has the lowest mineral concentration, with white color and low conductivity (186 μS/cm). In addition, honey samples with electrical values of conductivity lower than 800 μS/cm were considered flower honey or a flower mixture with honeydew, and honey samples with higher values were considered chestnut or honeyed honey.[30] Therefore, all the honeys characterized, except chestnut honey, have conductivity values below the maximum limit indicated by the Portuguese legislation[31] (800 μS/cm), and all of them were considered as honey flower.

The pH range of the honeys studied varied from 3.50 (rosemary honey and manuka honey) to 4.53 (chestnut honey). The pH values of honey should be between 3.2 and 4.5, in order to interfere with the growth of the microorganisms.[32] Therefore, all the honeys tested present potential of antimicrobial activity.
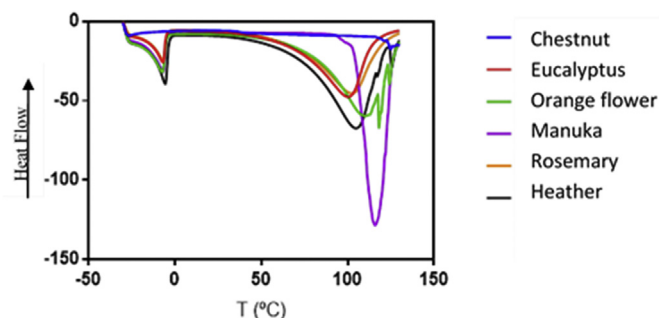

Fig. 1. Differential Scanning Calorimetry (DSC) analysis of the different Portuguese and Manuka honeys.

The protein content of honeys used in this study ranged from 27.5 ± 3.2 mg/100g (orange flower honey) to 374.8 ± 5.1 mg/100g (heather honey). Regarding the total phenolics content, values between 34.8 ± 1.0 and 179.6 ± 14.0 mg of GAE/100 g of honey were obtained for the six honey samples, agreeing with the study carried out by Meda et al.,[33] where they determined the phenolic content of 27 honey samples and observed values between and 32.59–114.75 mg GAE/100 g honey. The quantification of MGO levels in honeys evidenced that there is a large discrepancy between the obtained MGO values, ranging from 6.1 ± 0.4 mg/kg (rosemary honey) to 13.9 ± 1.2 mg/kg (chestnut), as the highest content of 962.8 ± 14.5 mg/kg in manuka honey. The production of H_2O_2 was also evaluated for all honeys as a function of the dilution (50% and 75% (w/v)). It was observed that the production of H_2O_2 decreased with the dilution of honey. Among the honeys studied, chestnut and heather honeys presented the highest H_2O_2 production values (40.0 ± 1.4 μM and 30.6 ± 0.8 μM, respectively), similar to manuka honey (40.6 ± 1.4 μM). Honeys were also analyzed by DSC (Fig. 1).

The analysis by DSC allows the evaluation of the degree of purity of the honeys studied, i.e. pure substances can be characterized by a unique and sharp melting point.[34] This does not happen in orange blossom honey due to its pollinic complexity (Table 2). Besides orange blossom honey, a very intense and wide endothermic peak between 100 °C and 120 °C, corresponding to the melting of sugars (mono-, di-, tri-, and oligosaccharides),[34] is observed.

Observing all the parameters (Table 2), chestnut, eucalyptus and heather honey had higher concentrations of protein and MGO, higher conductivity value, phenol content and total flavonoids, and higher production of H_2O_2 resembling the properties of Manuka honey. On the other hand, orange blossom honey presents the lowest values of all the parameters evaluated in comparison with the remaining honeys. Heather honey was one of the darkest honeys and present the highest values of total phenols (179.6 ± 0.14 mg of GAE/100 g of honey) and flavonoids (61.5 ± 0.01 mg QE/100 g of honey), closest to manuka honey. Due

Table 2
Pollen and physical-chemical characterization of the different honeys.

Honey	Pollen Analysis[a]%	Color[a]	pH[a]	[Protein][a] mg/100g	MGO[a] mg/kg	Conductivity[a] μS/cm	Total phenolic[a]mg of GAE/100 g of honey	Total flavonoid[a]mg QE/100 g of honey	H_2O_2 (μM)	
									50% (W/W) of honey	75% (W/W) of honey
Chestnut	50	Light Amber	4.53	89.2 ± 2.9	13.9 ± 1.2	983	103.9 ± 2	34.4 ± 2	35.7 ± 0.5	40.0 ± 1.4
Eucalyptus	61	Light amber	3.82	78.2 ± 10.6	8.7 ± 0.1	378	111.6 ± 2	49.9 ± 3	20.7 ± 1.2	26.7 ± 0.5
Orange blossom	8	White	3.62	27.5 ± 3.2	6.4 ± 0.5	186	34.8 ± 1	12.3 ± 1	7.1 ± 0.5	13.9 ± 1.7
Rosemary	63	Clear Amber	3.50	60.2 ± 11.3	6.1 ± 0.4	152	55.4 ± 0.3	25.2 ± 4	15.4 ± 0.6	23.7 ± 0.3
Heather	30	Dark Amber	4.32	374.8 ± 5.1	11.4 ± 1.4	622	179.6 ± 14	61.5 ± 1	22.5 ± 0.4	30.6 ± 0.8
Manuka	>70	Clear Amber	3.50	234.6 ± 21.7	962.8 ± 14.5	580	163.1 ± 4	65.4 ± 0.2	27.0 ± 0.5	40.6 ± 1.4

[a] Characteristics evaluated at 100% of honey concentration.

to the potential interest of using these Portuguese and traditional honeys for treatment of microbial infections, their antimicrobial and, particularly, antifungal activity was evaluated.

3.2. Antimicrobial role of honeys on planktonic populations

The susceptibility of *Candida* species to the different honeys was determined by MIC and MFC evaluation. In addition, due to the fully described action of honeys against bacteria, two refence strains, *S. aureus* and *P. aeruginosa*, were also used (Table 3).

It was verified that all honeys at 50% (w/v) induced inhibition of a range of pathogenic *Candida* species in planktonic state (Table 3). However, no significant difference was observed among the candidacidal activities of all honeys tested. Khosravi[35] concluded the similar findings for testing 28 locally produced honeys from plants of two floral sources against some pathogenic *Candida* species. Comparing the MIC and MFC of the different fungi species, *C. tropicalis* was the most susceptible to honeys, especially heather honey. On the other hand, the other *Candida* species were more tolerant, which is in accordance with the literature,[3,36,37] that describes *C. glabrata* the most resistant to currently antifungal agents used, and *C. parapsilosis* with an antifungal susceptibility similar to *C. albicans*. Moreover, our results are in accordance with other studies[10,35] that have reported that honey has antifungal activity against *Candida* species such as *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *Candida kefyr*, and *Candida dubliniensis*.

As expected, *S. aureus* and *P. aeruginosa* proved to be more susceptible to the six honeys assayed, compared to fungi species. Indeed, Wahdan[38] observed that fungi are generally much more tolerant than bacteria, due to the high osmotic effect obtained with the honey. In addition, it was, also, found that there were differences on the MICs and MBCs of the six honeys. The same was observed by Allen et al.[39] in which the antibacterial activity of honey from 26 different floral sources was tested in *S. aureus*, and it was shown that differences among floral sources were considerable.

Although there is some antimicrobial potential of honeys against planktonic cultures, the importance of MIC and MBC/MFC values for any potential therapeutic agent whether antibacterial or antifungal is highly questionable, since most bacteria and fungi demonstrate the ability to form biofilms, and the biofilm eradication concentration is generally 10 to 1000 times higher than MIC for the same strain.[40] Therefore, since we obtained positive results in the susceptibility assays with planktonic *Candida*, heather and manuka honey were selected for the treatment of biofilms. The manuka honey was used, due to its well-known antimicrobial properties and the heather honey was also selected due to the promising antimicrobial characteristics shown in the planktonic susceptibility assays and the physical and chemical properties similar to manuka honey.

3.3. Effect of honey in Candida biofilms

The effect of honey (heather and manuka) on 24 h and 48 h biofilms (mature biofilms) of *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis* was evaluated (Fig. 2).

Concerning 24 h biofilms assays, for *C. albicans*, a significant reduction of the biofilm occurred as compared with control, after treatment with manuka honey at 50% (w/v) ($p < 0.1$) and 75% (w/v) ($p < 0.01$), and with heather honey at 75% (w/v) ($p < 0.01$). For *C. tropicalis* biofilms, significant reductions were obtained with both honeys at concentrations of 50% (w/v) and 75% (w/v) ($p < 0.0001$). Regarding *C. glabrata*, a significant reduction was observed only when using 75% (w/v) of manuka honey ($p < 0.0001$). Finally, *C. parapsilosis* species showed that, similarly to *C. glabrata* biofilms, manuka honey only presents a significant reduction of 1.5 log CFU/cm^2 at 75% (w/v) ($p < 0.001$). On the other hand, heather honey showed significant reductions at 50% (w/v) concentration ($p < 0.001$) (Fig. 2D).

The effect of honey on 48 h biofilms was similar for both honeys and a significant reduction was obtained only with honey at 75% (w/v) in all strains studied (Fig. 2), except for *C. glabrata* species against which only manuka honey was effective ($p < 0.1$) (Fig. 2C).

Both manuka and Portuguese heather honeys have a higher antifungal effect in 24 h biofilms than 48 h biofilms (Fig. 2). The antifungal effect of both honeys was also observed to be dose-dependent, i.e., in general 75% (w/v) is the most effective concentration of honey in the reduction of biofilms. In fact, it was recognized that high concentrations of sugar, cause hypertonic conditions leading to lysis of the walls of microbial cells, becoming toxic to microorganisms and honey has about 70% (w/v) of sugar in its composition.[38,41,42]

Table 3
The minimum inhibitory concentrations (MICs) and minimum fungicidal/bactericidal concentrations (MFC/MBC) of different Portuguese honeys and manuka honey against *Candida* species (% w/v).

Fungal species		Chestnut MIC	MFC	Eucalyptus MIC	MFC	Orange flower MIC	MFC	Rosemary MIC	MFC	Heather MIC	MFC	Manuka MIC	MFC
C. albicans	SC 5314	50	>50	50	>50	50	>50	50	>50	50	>50	50	50
	201	50	>50	50	>50	50	>50	50	>50	50	>50	50	50
	575,541	50	>50	50	>50	50	>50	**38**	>50	50	>50	>50	>50
	945,541	50	>50	50	>50	50	>50	**38**	>50	>50	>50	50	>50
C. tropicalis	ATCC 750	**25**	50	**38**	>50	**38**	>50	**38**	>50	**38**	50	**38**	50
	571,475	>50	50	**38**	>50	**38**	50	**38**	50	**38**	50	**38**	50
	579,025	**38**	50	50	50	**38**	50	50	50	**38**	50	**38**	50
	ATCC 750	**25**	50	**38**	>50	**38**	>50	**38**	>50	50	>50	**38**	50
C. glabrata	ATCC 2001	50	>50	>50	>50	>50	>50	50	>50	50	>50	50	>50
	354,784	50	>50	>50	>50	50	>50	50	>50	>50	>50	>50	>50
	513,100	50	>50	>50	>50	50	>50	50	>50	50	>50	>50	>50
	553,877	50	>50	50	>50	50	>50	50	>50	>50	>50	>50	>50
C. parapsilosis	ATCC 20019	>50	>50	50	>50	50	>50	50	>50	>50	>50	50	50
	491,861	>50	>50	**38**	>50	**38**	>50	50	>50	>50	>50	50	>50
	555,343	>50	>50	>50	>50	50	>50	>50	>50	>50	>50	50	>50
	592,401	>50	>50	**38**	>50	**38**	>50	50	>50	>50	>50	50	>50
Bacterial species		**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus	ATCC25923	**12,5**	**25**	**25**	>50	**12.5**	**25**	**12.5**	**25**	**12.5**	**25**	**12,5**	**25**
P. aeruginosa	DSM 22644	**25**	**38**	**25**	**38**	**25**	**38**	**25**	**25**	**12.5**	**25**	**12.5**	**25**

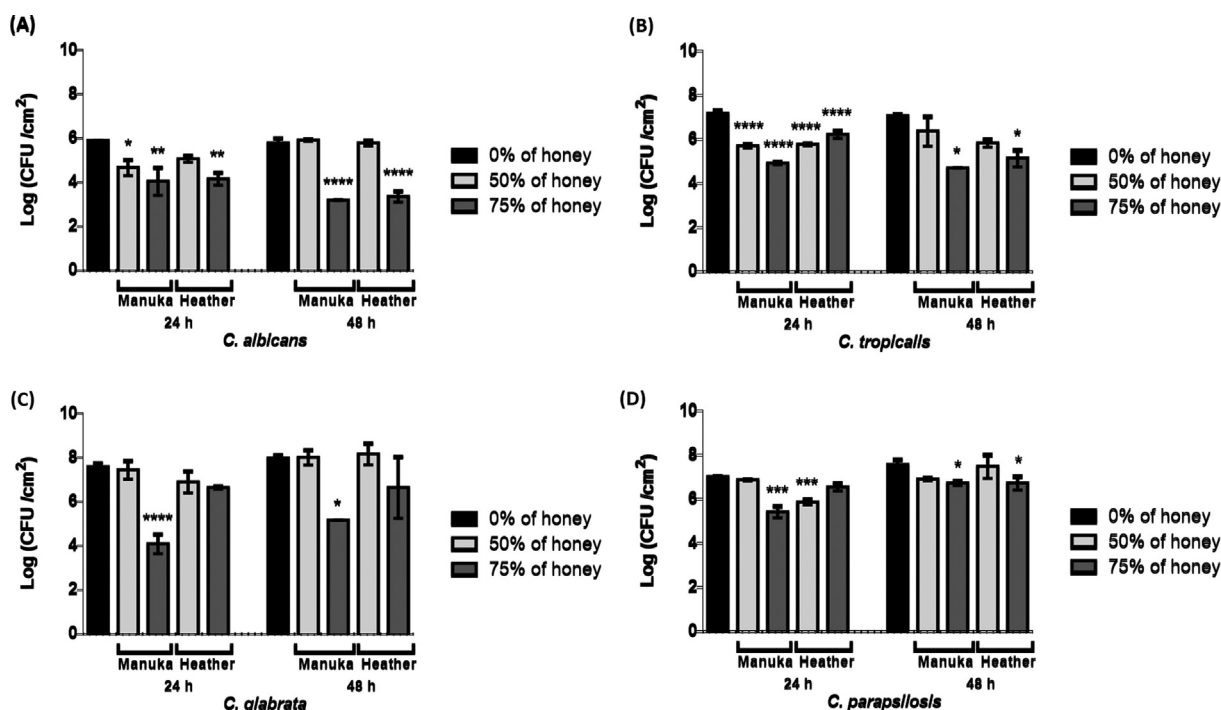MIC and MFC/MBC values below 50% w/v are highlighted in bold.

Fig. 2. Biofilms of 24 h and 48 h of (A) *C. albicans*, (B) *C. tropicalis*, (C) *C. glabrata* and (D) *C. parapsilosis* treated with 50% (w/v) and 75% (w/v) of manuka and heather honeys. *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001 indicates a statistically different reduction in comparison with the respective control.

The mechanism of the antifungal effect of honey is not yet fully understood, in this sense several potential hypotheses have been proposed.[43] These include, for example, the presence and concentration of MGO, an effective antimicrobial agent against planktonic cells and biofilm.[44] In this study, the quantification of MGO levels in honeys evidenced that there is a large discrepancy between the obtained MGO values (11.4 ± 1.4 mg/kg and 962.8 ± 14.5 mg/kg in heather and manuka honey, respectively (Table 2)). Despite this great discrepancy, similar results were obtained both, in planktonic cell susceptibility tests (Table 3) and 24 h or 48 h biofilms treated with manuka and heather honeys (Fig. 2). Another proposed mechanism is the presence and concentration of H_2O_2, produced in honey by the enzyme glucose oxidase.[45] The production of H_2O_2 is one of the honey components that most influence its antimicrobial activity. The different concentrations of this compound in different honeys could result in its varied antimicrobial effects.[46–48] However, in the antifungal activity this honey does not stand out, which means that H_2O_2 alone is no answer to explain its antimicrobial activity. Non-peroxide factors that contribute to the antimicrobial and antioxidant activity of honey are lysozyme, phenolic acids and flavonoids.[49] Several studies have shown that phenolic compounds induce growth inhibition in a wide range of Gram-positive and Gram-negative bacteria.[50,51] In fact, the antimicrobial action of phenolics is related to their ability to denature proteins and are generally classified as surface active agents[51] and the heather honey has the highest values of total concentration of phenols (179.6 ± 14.0 mg of GAE/100 g of honey) and flavonoids (61.5 ± 1.0 mg QE/100 g of honey).

However, it is complicated to quantify the contribution of the different factors to the antimicrobial activity of honey, since these factors may have redundant activity, be mutually dependent or have additive or synergistic activity depending on the microbial species.[52] Several studies have demonstrated that different honey samples differ from the degree of antimicrobial activity. However, the sensitivity of the species to each other can be validly determined in a single study with the same conditions and the same honey tested.[53]

4. Conclusion

Infections caused by *Candida albicans* and NAC species have increased in recent years. In order to overcome such problems, there is much interest in the potential use of natural compounds as alternative antifungal agents. In this sense honey have emerged as an alternative therapy option for some infections. Honey has been reported for years due to its associated antibacterial activity, but less is known about its antifungal activity. After the characterization of several Portuguese honeys (Chestnut, eucalyptus, orange blossom, rosemary and heather), the present study showed that PH honey was the honey with high phenolic and flavonoids contents, which is an important characteristics associated to antimicrobial, specially to antifungal activity. Regarding the susceptibility tests, the six honeys under study, presented, in general, a potent activity against multi-resistant human pathogens, such as the *Candida* species (MIC between 25 and 50% (w/v)) and bacteria (MIC between 12.5 and 38% (w/v)) such as *S. aureus* and *P. aeruginosa*. In addition, this study revealed that the Portuguese honey (heather honey) can act against *Candida* biofilms, especially on *C. tropicalis*. A future hypothesis of this work is to add honey to existing antifungal formulations in order to increase their effect.

Declaration of competing interest

The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2020.02.007.

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