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Antimicrobial and wound healing effects associated to cytocompatibility and the relationship with phytochemical profile of selected Portuguese monofloral honeys

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ABSTRACT

Owing to its applications in both food and pharmaceutical industries honey is regarded as a functional product. This study evaluated 32 Portuguese monofloral honeys and their activity was compared with manuka honey 850 + .

The antimicrobial activity was determined against Gram-positive and negative bacteria, and yeast. Antioxidant activity, wound healing ability, and cell viability were studied in the human keratinocyte cell line (HaCaT). Phytochemical profile analysis was performed by UPLC/DAD/ESI-MS^n.

Chestnut, bell heather, eucalyptus, manuka, and strawberry tree honeys demonstrated higher antioxidant activity and were effective against *S. aureus*. Incense and orange honeys showed wound healing rates of 89 % and 86 %, respectively. All the honeys had cell viability above 76 %. Bell heather and strawberry tree honeys showed the highest total phenolic content, while incense and orange honeys, showed the lower ones. Phytochemical profile analysis evidenced two main clusters, one dominated by isoprenoids and norisoprenoids, as well as phenolic acids and derivatives, while the other included flavonoids as main compounds.

Blending different Portuguese monofloral honeys may be an alternative to faster wound healing, by combining their diverse bioactive components. The gathered knowledge can be further explored in formulations that consider the biological activity capacity and composition of each honey.

1. Introduction

Honey is an ancient food product with also long use as a medicine. Several studies recognize honey valuable biological activities (García, 2018; Kuropatnicki et al., 2018). Deeper physicochemical and therapeutic characterization of honey bioactive components have contributed to the understanding of honey's beneficial effect mechanisms (Angioi et al., 2021). A concentrated water solution of the two main sugars, fructose and glucose, along with a variety of minor components like amino acids, carotenoids, enzymes, minerals, organic acids, phenolic compounds like phenolic acids and flavonoids, vitamins, and volatiles make up honey's composition of over 200 constituents (da Silva et al.,

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2016a; Hermanns et al., 2020).

The antimicrobial activity of honey is caused by a combination of different factors, including the low water and high sugar content, along with an acidity level generally between pH 3 and 5, and the occurrence of constituents such as glucose oxidase, hydrogen peroxide, the peptide bee defensin-1 or the dicarbonyl compound methylglyoxal (MGO). MGO was found in *Leptospermum scoparium* derived manuka honey and bee defensin-1, common in several honeys, is also present in the wound dressing Revamil® source (RS) honey (Kwakman et al., 2010; Cooper, 2014; Valachová et al., 2016). *In vitro* studies and clinical trials have shown honey's antioxidant and anti-inflammatory properties which have a beneficial influence on the cardiovascular, nervous, gastrointestinal, and respiratory systems, as well its anticancer potential activity (Schramm et al., 2003; Nolan et al., 2019). Recent meta-analysis reviews have systematically evaluated the potential effects of honey on lipid profiles and cardiometabolic health (Gholami et al. 2022; Ahmed et al. 2023).

Honey also has a long history in wound management owing to its broad spectrum of applications such as antimicrobial, anti-inflammatory and antioxidant activities, deodorizing and debriding actions, osmotic action, and tissue growth stimulation (Molan, 1999; Saikaly and Khachemoune, 2017). Clinical trials involving the use of honey in wound treatment, include a broad variety of wounds, ranging from acute wounds like burns and surgical incision sites, to chronic wounds including leg, pressure and diabetic foot ulcers, as well as malignant wounds (Cooper, 2014). Despite the need for further studies to confirm findings, the systematic review and meta-analysis conducted to evaluate the role of honey in diabetic foot ulcer (DFU) suggests that honey effectively promotes DFU healing (Karadeniz and Serin, 2023). Also drawing attention to study limitations, the updated systematic review and meta-analysis of Tang et al. (2024), points to the efficacy and safety application of honey dressings in managing chronic wounds.

Phenolics are among the most important groups of phytochemicals that contribute to honey antioxidant activity (Cianciosi et al., 2018; Cheung et al., 2019), by reducing the oxidative stress through the neutralization of reactive oxygen species (ROS) and exerting a protective effect on several illnesses like cancer, and cardiovascular, metabolic, or neuro-degenerative disorders (Olas, 2020). Honey phenolic composition and its biological activities are related to honey's botanical origin (monofloral or multifloral) as well to the geographical production region (Estevinho et al., 2008; Feás et al., 2013; Cheung et al., 2019; Hermanns et al., 2020), thus, different properties may be expected according to the production location and floral specificity. Despite different works describing these biological activities (Henriques et al., 2005; Estevinho et al., 2008; Aazza et al., 2013; da Silva et al., 2016b; Gonçalves et al., 2018), the studies focusing on antimicrobial, antioxidant and wound healing activities of Portuguese honeys are scarce.

The present study aimed to assess the *in vitro* biological activities of Portuguese monofloral honeys, as well as to characterize its phytochemical profile of the most active ones. For this purpose, 32 honeys with different botanical origins were initially screened to evaluate their antimicrobial and antioxidant activities. Nine of the thirty-two honeys were chosen for further investigation based on the results of the biological activity assessment. All the activities were evaluated in comparison to manuka honey 850 + (mg/kg MGO). In addition, phytochemical composition of the selected honeys was analysed by ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization tandem mass spectrometry (UPLC/DAD/ESI-MS^n). To the best of our knowledge, this is the first study comparing several Portuguese monofloral honeys from diverse botanical sources to assess their antimicrobial, antioxidant and anti-inflammatory properties, as well as the cytotoxicity and wound healing to ascertain the relation between biological activity and phytochemical profile viewing future combined formulations.

2. Material and Methods

2.1. Materials and Chemicals

Ascorbic acid, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Amberlite® XAD®-2 resin and the phenolic standards caffeic acid, *p*-coumaric acid, 2-*cis*,4-*trans*-abscisic acid, chrysin, naringenin and quercetin were acquired from Sigma Chemical Co (St Louis, MO, USA). 2,7'-Dichlorodihydrofluorescein diacetate (H_2-DCFDA), RPMI 1640 culture medium, penicillin G (sodium salt), streptomycin sulphate, and L-glutamine were purchased from Life Technologies (Warrington, UK). The HaCaT spontaneously immortalized human keratinocyte cell lines were acquired from Cell Line Service (CLS, GmbH, Eppelheim, Germany). Flat-bottom 96-well tissue culture plates were obtained from Greiner (Münster, Austria). New Zealand manuka honey 850 + (mg/kg MGO) from Pure Gold®.

Analytical grade formic acid was obtained from Panreac (Barcelona, Spain), and methanol and acetonitrile, with HPLC purity, were purchased from Lab-Scan (Lisbon, Portugal). TGI Pure Water Systems set (Houston, TX, USA) was used to obtain Mili-Q water. Fischer Chemical Scientific (Loughborough, United Kingdom) was the source of all off the other analytical grade purity reagents and/or solvents.

2.2. Honey sampling and experimental setup

Of 51 labelled monofloral honeys previously studied by Machado et al. (2021, 2022), 32 honey's were selected according to microbiological quality, Supplementary Material (SM) Table S1. Most honey samples were obtained from beekeepers without processing, Table S1, and others from specialized shops, between 2015 and 2018. The honey samples were stored in a cool, dry place until further assessment, Figure S1.

Antimicrobial activity (AMA) evaluation was performed with honeys from 10 different botanical sources, as detailed in Table S1, using commercial New Zealand manuka 850 + (mg/kg MGO) honey as control.

The 16 honeys showing higher antimicrobial activity were chosen for antioxidant activity (AOA) assessment, Table S1. Based on its antioxidant activity performance, the number of samples was further reduced to 9 to evaluate wound healing ability (WHA) and cell viability (CV) determination, Table S1. To evaluate the potential relation between the biological activities of honey and the relevant compounds found, the phytochemical profile (PP) was also examined. These samples with seven botanical origins, were obtained from different locations in mainland Portugal, and Azores Islands (São Miguel), as detailed in Figure S2. All samples were kept in a cool, dry, and dark room until analysed.

2.3. Antimicrobial activity

2.3.1. Minimum inhibitory concentration (MIC) determination

All the 32 honey samples were evaluated for the lowest concentration of honey that inhibited growth of the tested microorganisms (MIC), after an initial assay of the antibacterial activity by diffusion method, detailed in SM S2.3, S3.1 and Table S3. The MIC values were determined visually against four ATCC strains, specifically, Gram-positive bacteria (*S. aureus* ATCC 43866), Gram-negative bacteria (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027), and yeast (*Candida albicans* ATCC 10231). The assay was run in a 96-well sterile culture plates, containing 100 μL of liquid M-H Broth (Liofilchem). All the samples were diluted in the M-H Broth (50 %, w/v), transferred into the first well, and followed by a serial dilution (50–0.8 %). Following an 18 h incubation at 37 °C, 50 μL of 10^4 CFU/mL microbial suspensions prepared in sterile M-H Broth were added to each well. The microplates were then incubated at 37 °C for 24 h at static condition and microbial growth in each well was compared with that of the positive control. The culture medium without

honey was used a negative control, and gentamicin (GEN) at 1 % as positive control.

2.4. Reactive oxygen species (ROS) production measurement

The fluorimeter technique using 2,7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) (Nunes et al. 2021), was used to determine the capacity of honeys to reduce the intracellular ROS production. Briefly, HaCaT sub-confluent cells seeded in 96-well plates were incubated for 30 min with 20 μM of H_2-DCFDA at 37 °C. The culture medium was then removed, and fresh medium was added to the cells before being exposed to 10 mg/mL of honeys or 1 mg/mL of ascorbic acid (positive control) for 1 h. Cells were exposed to H_2O_2 at 500 μM for 1 h to generate oxidative stress. ROS levels were determined by fluorescence at 485 nm and 520 nm, as excitation and emission wavelengths, respectively, in a FLUOstar Omega microplate reader (BMGLabtech, Ortenberg, Germany). Six replicates were performed and collected data is expressed as the percentage of ROS reduction determined as: 100-(fluorescence of exposed cells/fluorescence of unexposed control from the same experiment) × 100. The concentration effect (logarithmic curve) obtained for each honey sample was used to calculate the half maximal inhibitory concentration (EC_{50}), that is, values that reduced ROS by 50 %.

2.5. Scratch in vitro wound healing migration assay

HaCaT cells were seeded onto 24-well plates and cultured until 100 % confluence. The monolayer was wounded by scraping it with a sterile tip. The wells were washed twice with medium without serum and then with culture medium with 1 % of foetal bovine serum (FBS). Honey samples were tested in two wells at 10 mg/mL, together with a negative control medium with 1 % FBS and a positive control medium with 10 % FBS. An inverted microscope with phase contrast (MEIJI, Japan) and a digital camera attached, was used to monitor the healing process. Microphotographs of the wounds were taken at 0 and 24 h after the wound was made. Area of wounds at 24 h was compared with the wound area at time 0, to evaluate the migration rate of cells. Using NIH ImageJ software, the area was measured as the cleared space between the wound edges, at 40 × magnification. Data were collected from three replicates and expressed as % of closed area, using the formula: [(Wound area t_0 - Wound area t)/Wound area t_0] × 100.

2.6. Cell viability

The cytotoxicity of honeys was evaluated on the HaCaT cells, using general cell viability endpoint of MTT reduction method according to Marto et al. (2016). Sterile flat-bottom 96-well tissue culture plates were used to seed HaCaT cells in RPMI 1640 culture medium, supplemented with 10 % FSB, 100 units of penicillin G (sodium salt) and 100 μg of streptomycin sulphate, and 2 mM L-glutamine. Final cell density was 2×10^5 cells / mL, 100 μL/well, and they were kept at 37 °C and 5 % CO_2. The cells were incubated with 10 mg/mL of honeys for 24 h in the same conditions. The culture medium and sodium dodecyl sulphate (SDS) at 1 mg/mL were, respectively, the negative and positive controls. Following the exposure time, and medium replacement by medium with 0.5 mg/mL MTT, the cultures were incubated for 3 h, under the same conditions. The medium was then removed, and the intracellular formazan crystals were solubilized and extracted with 100 μL dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm in a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany), after 15 min in continuous stirring at room temperature. Data collected from six replicates is expressed as the relative cell viability (%) compared to control cells and was calculated by [(Absorbance) sample/(Absorbance)] control × 100.

2.7. Phytochemical profile

2.7.1. Extraction

Phenolic compounds were extracted following the method of Tomás-Barberán et al. (2001). From each honey sample, about 25 g were mixed with 125 mL of acidified water (pH = 2 with HCl 1 M). The solution was then filtered with cotton to remove any solid particles. To retain phenolic compounds in a selective manner, the filtrate was percolated through a Amberlite® XAD®-2 column. A washing step using acidified water at pH = 2 was used to remove sugars and other polar compounds. The phenolic fraction was then eluted with methanol and the extract was concentrated under reduced pressure at 40 °C using a Büchi R-210 rotatory evaporator (Flawil, Switzerland). The final residue was solubilized in 2.5 mL of distilled water and liquid/liquid extracted with diethyl ether. The extraction procedure was repeated three times, and the ether extracts were combined and concentrated under reduced pressure at 40 °C, as above. The final residue was dissolved in methanol and analysed by ultra-performance liquid chromatography with diode array detection coupled to electrospray ionisation tandem mass spectrometry (UPLC/DAD/ESI-MS^n). The total phenol content per honey sample (mg/100 g honey) was calculated as: [100 ×total extracted phenols (mg)] / honey sample (g).

2.7.2. UPLC/DAD/ESI-MS^n

The UPLC/DAD/ESI-MS^n analysis was performed in a Dionex UPLC final 3000 equipment (Thermo Scientific, Waltham, MA, USA) equipped with a photodiode array detector and coupled to a mass detector. The chromatographic system comprised an automatic thermostatic column compartment, an automatic sampler kept at 5 °C, a degasser, a quaternary pump, and a photodiode array detector.

A UV-DSpher PUR C18-E 100 mm × 2.0 mm i.d. column with a particle size of 1.8 μm (VDS Optilab, Germany), kept at 30 °C was used in the chromatographic separation. Formic acid in water and 0.1 % (v/v) (A) and formic acid in acetonitrile 0.1 % (v/v) (B) constituted the mobile phase solvents. A linear gradient program at 0.3 mL/min was used: 0.0–1.0 min 20 % (B), 1.0–11.1 min from 20 % to 95 % (B), 95 % (B) for 2 min, 13.0–13.3 min from 95 % to 20 % (B), and 20 % (B) for 5 min. Injection volume: 3 μL. Spectral data for all peaks were accumulated within 190–600 nm. Each sample was filtered through a 0.2 μm nylon membrane (Whatman).

Mass analysis was performed on a LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA), in negative mode, equipped with an ESI electrospray ionisation source. Spray voltage 5 kV. Capillary voltage −20 V. Capillary tube voltage −65 V. Capillary temperature 325 °C. Gas and auxiliary gas (N_2) flow rate, 50 and 10 (arbitrary units), respectively. Mass spectra were acquired in the mass range of 100–1000 *m/z*. A data dependent scan was performed by deploying collision-induced dissociation (CID) for the fragmentation study. Collision energy in the MS^n experiments 35 (arbitrary units). Data acquisition was carried out with Xcalibur®data system (ThermoFinnigan, San Jose, CA, USA). The samples chemical profile was compared with that of standard individual solutions profile and retention under same operative conditions. The compounds' structure elucidation was achieved by comparing their UV spectra, mass profile and chromatographic behaviour, with that of commercial standards and with the information published in the literature, when these were not commercially available. The quantification was made using the calibration curves for caffeic acid, *p*-coumaric acid, 2-*cis*,4-*trans*-abscisic acid, chrysin, naringenin, and quercetin, as detailed in SM Table S2. Whenever standards were not obtainable, compounds were expressed in equivalents of the most structurally similar phenol as detailed in SM Table S4. The assays linearity, the limit of detection (LOD) and quantification (LOQ) were obtained following in-lab procedures (Falcão et al. 2013), as detailed in SM S2.7.2.

2.8. Statistical analysis

Shapiro–Wilk test was performed for data distribution analysis. One way ANOVA was performed for normal data, followed by multiple comparisons using Tukey's test. Kruskal–Wallis test was used for other non-parametric analysis, followed by multiple comparisons using Dunn's tests. Results are expressed with mean and standard deviation (SD). Differences with $p < 0.05$ or $p < 0.001$ were classified as statistically significant, unless stated otherwise. Statistical analysis was conducted in R (version 4.0.2) and RStudio (version 1.3.1093) using *agricolae* (Mendiburu, 2021), *FSA* (Ogle et al., 2021) and *multcomp* (Hothorn et al., 2021) packages.

The phenolic compounds concentration was used to evaluate the relationship between the various samples by cluster analysis using the Numerical Taxonomy Multivariate Analysis System (NTSYS PC software, version 2.1, Exeter Software, Exeter University, Exeter, UK) (Rohlf, 2000).

The sequential agglomerative hierarchical nested cluster analysis (SAHN) was chosen as agglomerative clustering method. The percent composition data matrix was standardized to eliminate the effects of different scales of identification. For cluster analysis, correlation coefficient was selected as a measure of similarity among all samples, and the unweighted pair group method with arithmetical averages (UPGMA) was used for cluster definition.

Correlation degree used Pestana and Gageiro (2000) scale as very high [0.90, 1.00], high [0.70, 0.90[, moderate [0.40, 0.70[, low [0.20, 0.40[and very low (<0.20).

3. Results and Discussion

3.1. Antimicrobial activity

3.1.1. MIC evaluation

The MIC values of the monofloral honeys ranged from 12.5 % (w/v) to > 50 % (w/v), depending on the microorganism, Table 1. The results showed that only bell heather honey sample H2 had antimicrobial activity against all strains, with the MIC values of 12.5 % (w/v) for *S. aureus*, and 50 % (w/v) for both *E. coli* and *C. albicans*.

In general, darker honeys (carob tree, chestnut, bell heather and strawberry tree honeys) revealed higher antimicrobial activity than the lighter honey samples (incense and orange honeys), according to colour index determined previously by Machado et al. (2022). The commercial manuka honey, showed, for all the microorganisms tested, MIC values of 12.5 % (w/v), like strawberry tree honey sample for *S. aureus*, and MIC ≥ 50 % (w/v) for remaining microorganisms, being less effective than bell heather H2 honey with MIC value of 25 % for *P. aeruginosa*.

The results achieved are similar to those found in a study with Portuguese commercial monofloral honeys obtained from orange, lavender, eucalyptus, and heather, with the latter revealing higher effectiveness against *C. albicans* (MIC 6.3 %), in comparison with honeys from other botanical origins (Gonçalves et al., 2018). However, the MIC values accomplished in the present study were in general higher compared to those obtained by Gonçalves et al. (2018) for *E. coli* (12.5 %), *P. aeruginosa* (12.5 %) and *C. albicans* (6.3–12.5 %). The exception was the behaviour against *S. aureus*, with the MIC values being the double compared to the obtained in the present work (12.5 %) for eucalyptus, bell heather and strawberry tree honeys.

The current study suggests that Gram-positive bacteria *S. aureus* is more susceptible to honey samples, showing a lower MIC, than Gram-negative bacteria, which are in accordance with the results obtained with Northeast Portuguese *Erica* and *Lavandula* honeys (Estevinho et al., 2008). Geographical production region can influence the antimicrobial properties of honey. Nevertheless, studies carried out in other countries, showed similar results to honeys produced in Portugal, namely, the strawberry tree honey from Greece (Graikou et al., 2021) or manuka honeys described by Gośliński et al. (2020). The Sicilian chestnut honeys studied by Ronsisvalle et al. (2019) showed higher MICs values for *E. coli* and *P. aeruginosa*, but the MIC value for *S. aureus* was the same as that found in our results.

The differences observed between Gram-positive and Gram-negative strains, could be related to specific factors, such as cell wall composition: Gram-negative bacteria possess an outer membrane that protects the peptidoglycan layer from lysozyme and other antimicrobial agents. This feature gives Gram-negative bacteria high drug resistance when compared with the outer membrane absent in the Gram-positive bacteria (Johnston et al., 2018).

In addition to the specific characteristics of bacterial cell wall, it is also possible to justify the honey's antimicrobial properties due to the synergistic features of elevated osmolarity and low acidity, as well to other minor constituents, mostly hydrogen peroxide and phenolic compounds (Leyva-Jimenez et al., 2019).

A study conducted with Portuguese lavender honey against *Candida albicans*, *Candida krusei* and *Cryptococcus neoformans*, revealed that all the yeast growths were reduced in the presence of honey, not due to the sugar content of honey, but instead related with the presence of minor compounds (Estevinho et al., 2011).

Other mechanisms of action might be involved in honey's antimicrobial effect, for instance, considering the effect of manuka honey on *P. aeruginosa* and *S. aureus*, it was found that inhibitory concentrations caused loss of cell integrity, affecting the normal cell cycle and the cell division process, cell lysis, and death (Henriques et al., 2010, 2011).

Table 1
Minimum inhibitory concentration (MIC) (% w/v) of the studied monofloral honey samples. Comparative EC_{50} values of ROS inhibitory activity of honey samples and ascorbic acid on HaCat cells, and total phenolic content per honey sample.

	MIC values (% w/v)										
	Honey monofloral samples										Controls
	Ct1	Ct4	C2	E3	H2	H4	I3	O5	St2	Mk	GEN*
S. aureus	50	50	25	12.5	12.5	12.5	> 50	50	12.5	12.5	< 3.15
E. coli	50	50	> 50	50	50	> 50	> 50	> 50	50	50	< 3.15
P. aeruginosa	50	50	> 50	50	25	50	50	50	50	50	< 3.15
C. albicans	> 50	> 50	50	> 50	50	50	> 50	> 50	> 50	> 50	na
	Antioxidant (EC_{50}) and Total phenolic content (TPC)										
	Ct1	Ct4	C2	E3	H2	H4	I3	O5	St2	Mk	AA (1 mg/mL)
EC_{50} (mg/mL)	> 10.0	> 10.0	2.6 ± 0.1	6.2 ± 1.3	1.9 ± 0.2	6.0 ± 1.4	> 20.0	> 20.0	1.3 ± 0.2	> 10.0	5.3 ± 0.2
TPC (mg/100 g honey)	26	27	16	16	30	38	11	15	137		

GEN* : gentamicin MIC breakpoint mg/L. na: not applicable. EC_{50}: concentration causing half-maximal neutralization of H_2O_2. AA: ascorbic acid. Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, O: orange, St: strawberry tree, Mk: manuka honeys.

3.2. ROS measurement obtained from the selected honeys

Carob tree, chestnut, eucalyptus, bell heather, and strawberry tree honey samples were able to reduce ROS formation in HaCaT cells by more than 70 %. In contrast, incense, lavender, both orange (O4, O5) and L8 (viper's bugloss) honeys, Table S1, attained a ROS formation reduction of 46, 22, 28, 45 and 36 %, respectively, Fig. 1.

Multifloral honey samples, Table S1, reduced ROS formation between 38 % and 66 % and manuka honey in 68 %. The positive control, ascorbic acid (AA), achieved the highest ROS formation reduction, 93 %. Bell heather (H4) and strawberry tree honeys exhibited values of ROS reduction (%) significantly higher than the obtained with the manuka honey, while lavender, multifloral (M1), both orange (O4, O5) and L8 (viper's bugloss) honeys, Table S1, showed the opposite, Fig. 1.

Different chemical and biochemical studies have been performed *in vitro* to describe the antioxidant properties of honey, including evaluating the capacity for scavenging 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid equivalent antioxidant capacity (AEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, ferric ion reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) or Trolox equivalent antioxidant capacity (TEAC) assay (Escuredo et al., 2013; Dżugan et al., 2018; Gonçalves et al., 2018).

In the present study, the ability of honeys to reduce the intracellular ROS production was evaluated in HaCaT cells. There are, however, only few studies available on the antioxidant properties of honey using this methodology (Karapetsas et al., 2020).

From the 9 selected honeys to continue studies, Ct1, Ct4, C2, E3, H2, H4, I3, O5 and St2, Table S1, bell heather (78 %) and strawberry tree honeys (82 %) showed the best performance in reducing ROS formation, Figure S3.

The free radical-scavenging activity expressed as EC_{50} (mg/mL), showed high variability among the honey samples, Table 1. The lowest values of EC_{50} that corresponds to the highest antioxidant capacity, were obtained with the honeys of strawberry tree (1.3 ± 0.2 mg/mL), bell heather H1 (1.9 ± 0.2 mg/mL), chestnut (2.6 ± 0.1 mg/mL) and eucalyptus (6.2 ± 1.3 mg/mL). For carob tree, incense, and orange honeys, the EC_{50} were above 10 mg/mL, like for manuka honey.

Several works, using different methodologies, including capacity for scavenging ABTS free radical or the ORAC method, reported higher antioxidant activity to Portuguese and Spanish bell heather, chestnut and strawberry tree honeys (Ferreira et al., 2009; Aazza et al., 2013; Escuredo et al., 2013). Likewise, the results were also comparable to those obtained with both Italian and Turkish chestnut honey and also with Turkish orange honey (Sagdic et al., 2013; Gül and Pehlivan, 2018; Ronsisvalle et al., 2019; Aker and Nïsbet, 2020). The results reported in a study with Portuguese chestnut, eucalyptus, and heather honeys, using the DPPH assay, were comparable to those in this work, since chestnut and heather honeys showed the highest *in vitro* antioxidant activity (Karabagias et al., 2018).

Although the observed antioxidant activity was less effective than the positive control of ascorbic acid, the monofloral Portuguese honeys, particularly those from chestnut, eucalyptus, bell heather and strawberry tree, confirmed a significant activity in agreement with the above reported studies, and that activity is related to honey's botanical origin.

3.3. In vitro wound healing

The performance of the selected Portuguese monofloral honeys towards the *in vitro* scratch wound healing assay in HaCaT cells is detailed in Fig. 2 and Figure S4. Eucalyptus and bell heather H4 honey samples showed the lowest closure percentage, 28 and 27 %, respectively, statistically different from the positive and negative controls. Contrarily, incense, and orange honey samples showed the highest percentage of closure, 89 and 86 %, with a better statistical significance performance then the positive control. All the other samples showed a comparable closure area with values around 57 %, Fig. 2, and Figure S4. Manuka honey revealed a wound closure ability of 53 %, statistically lower than the positive control, although like the negative control.

The use of honey in wound healing has been described since antiquity, and several recent studies were performed to attest wound closure effects of honey, mainly with manuka honey (Hixon et al., 2019; Rossi and Marrazzo, 2021). The antibacterial, anti-inflammatory, and antioxidant properties of honey contribute to wound healing, particularly in ulcers and burns (Hadagali and Chua, 2014). Besides, additional mechanisms may be involved in which phenolic compounds are not the main actors. For example, in HaCaT cells, Ranzato et al. (2012) concluded that keratinocyte re-epithelialization during wound repair using honey samples was done by activating regulatory pathways of cell proliferation and locomotion. Ranzato et al. (2012) also reported an

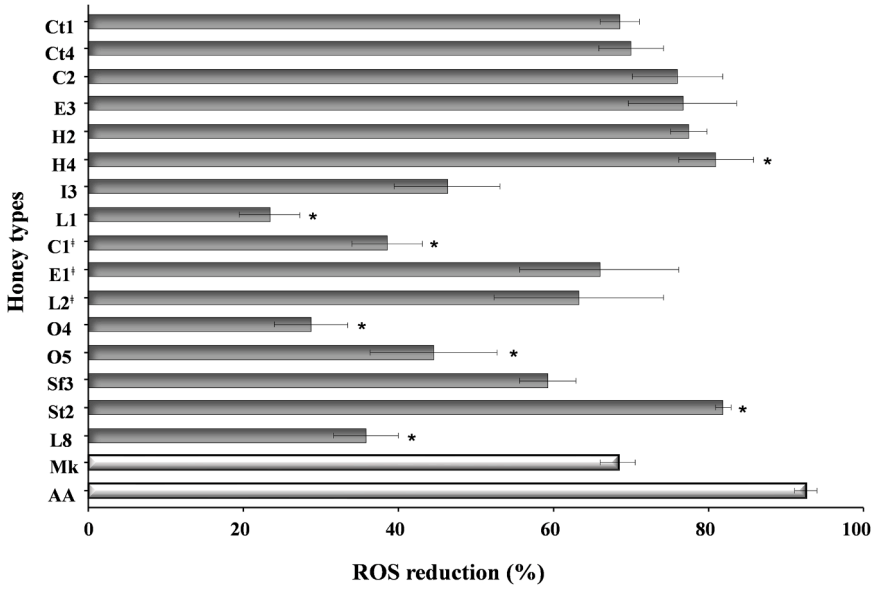

Fig. 1. Reactive oxygen species (ROS) formation reduction (%) after HaCaT cells exposure to H_2O_2. HaCaT cells treated with honey samples (10 mg/mL) or ascorbic acid (AA) solution (1 mg/mL) as positive control. Results are mean ± SD, n = 6. Statistical significance: * $p < 0.05$ in comparison to Mk honey. ‡ Multifloral honeys. Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, L: Lavender, O: orange, S: Sunflower, St: strawberry tree, Mk: manuka honeys.

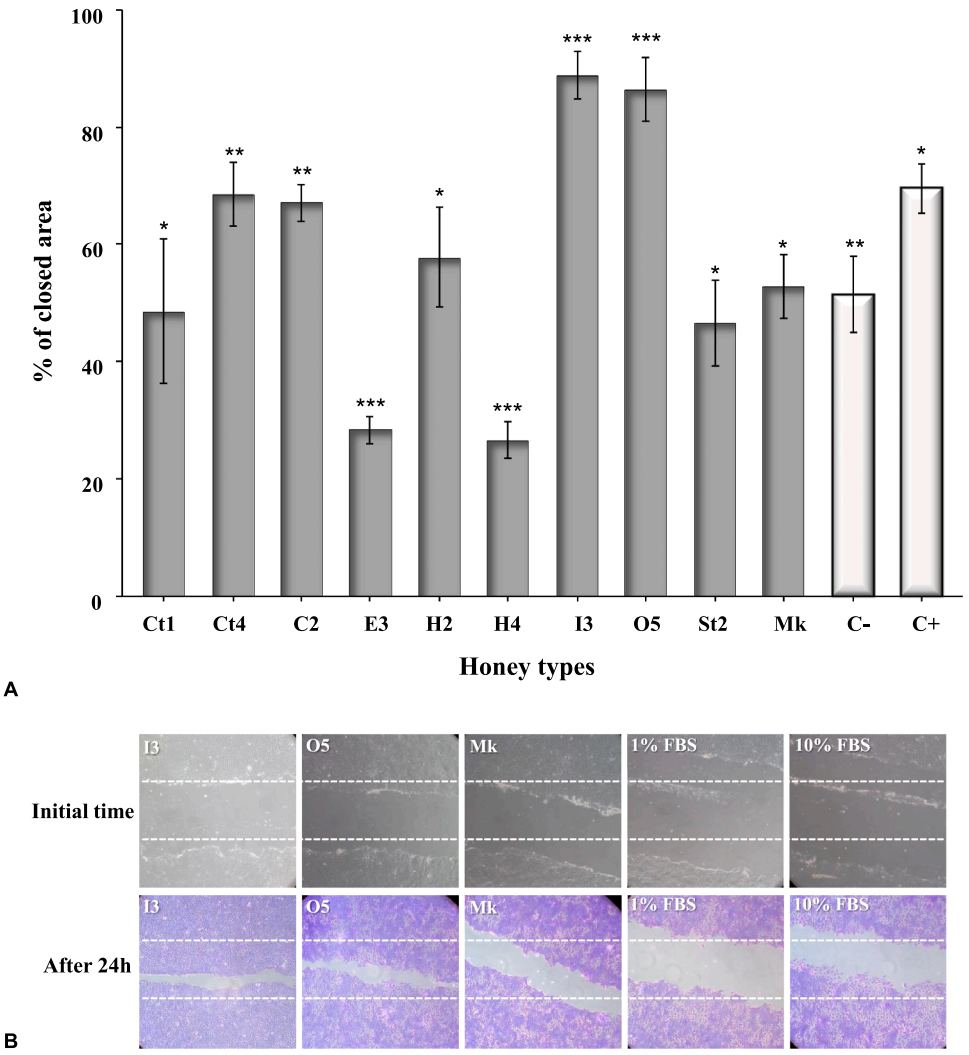

Fig. 2. A - Graphical representation of the percentage of wound closure in all the experimental samples in a scratch assay using HaCaT cells. Results are mean ± SD, n = 3. Statistical significance: * $p < 0.001$ in comparison to positive control (Medium 10 % FBS), * * $p < 0.001$ in comparison to negative control (Medium 1 % FBS), * ** $p < 0.001$ in comparison to positive and negative controls. C-: negative control. C+ : positive control. Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, O: orange, St: strawberry tree, Mk: manuka honeys. B - Representative time-lapse images of HaCaT keratinocyte scratch assays immediately after the scratches were made and after 24 h in the presence of 10 mg/mL of different honey samples or control medium: 1 % FBS: negative control, 10 % FBS: positive control. The cells were allowed to migrate for 24 h, fixed and photographed. Samples after 24 h were stained with crystal violet dye. The outlines of the original wounds are marked with dashed lines. Original magnification 40 × . FBS: foetal bovine serum. I: incense, O: orange, Mk: manuka honeys.

increase of wound closure induced by acacia, buckwheat and manuka honeys, where the cellular mechanisms of induction depended on honey botanical origin. Another study with Calabrian honeys showed that orange honey increased the wound healing rate compared to chestnut and acacia honeys (Governa et al., 2019). The therapeutic use of honey in skin diseases is well documented, being appropriate as a dressing for burns and wounds, decreasing inflammation and pain, stimulating fibroblast migration and keratinocyte closure (Martinotti and Ranzato, 2018). These properties give honey a potential role in tissue engineering, being suitable to be incorporated in biomaterials for tissue regeneration (Hixon et al., 2019; Rossi and Marrazzo, 2021).

Several honey components were hypothesized as contributors to the stimulation of keratinocytes' proliferation and consequent wound re-epithelialization, such as nitric oxide content, high osmotic pressure, and hydrogen peroxide. The latter also stimulates angiogenesis during the healing process, providing suitable tissue oxygen and nutrients. The presence of trace elements, in particular Zn, Cu and Mn, could stimulate keratinocyte proliferation during re-epithelialization (Tenaud et al., 2000). Bucekova et al. (2017) identified defensin-1 from royal jelly, but which also occurs in honey, as the component that improves wound closure and wound healing *in vivo* and *in vitro*.

To the best of our knowledge this is the first report that compares wound healing in HaCaT cells in such a diversity of Portuguese monofloral honey types. Although no compounds responsible for this activity have been identified yet, this study reveals that the *in vitro* wound healing activity is dependent on the honeys type and, therefore, they could be used in medical devices for this purpose.

3.4. HaCaT cell viability

All honey samples tested showed cell viability higher than 76 % at 10 mg/mL concentration, Fig. 3, confirming their cytocompatibility.

Afrin et al. (2017) assessed the cytotoxic effects of Sardinian strawberry tree and manuka honeys on human dermal fibroblast, showing that both honeys do not exhibit toxic effects in the concentrations from 3 to 40 mg/mL. In the present study, cytotoxicity of honey samples was not detected after 24 h, which may indicate that the tested honeys can be used at non-cytotoxic doses, at the assessed concentrations, 10 mg/mL, although higher concentrations should also be tested.

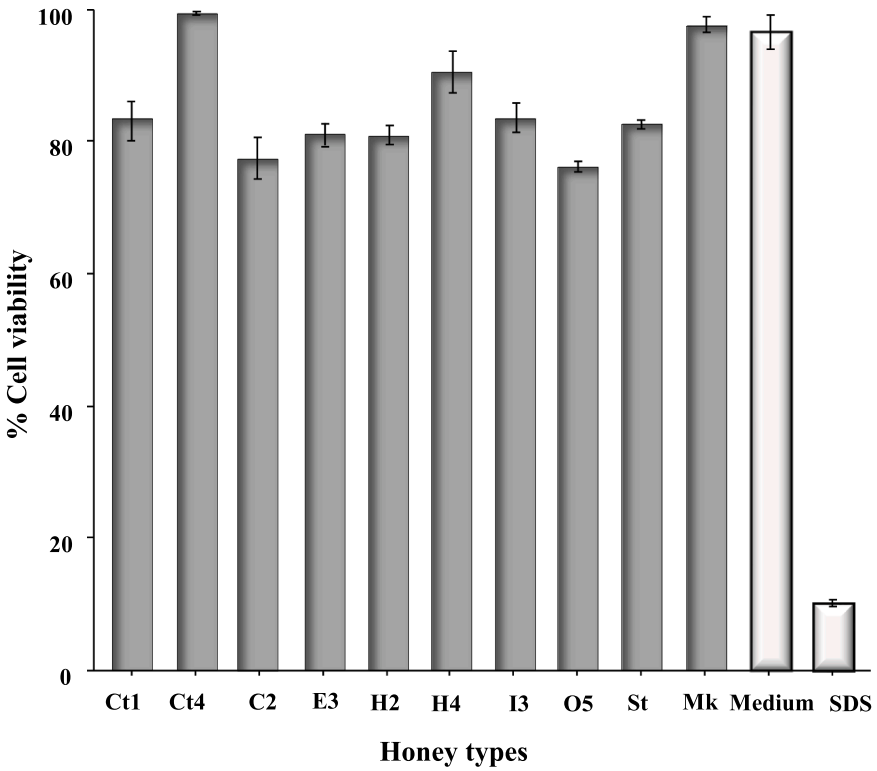

Fig. 3. Viability of HaCaT cells after 24 h of incubation with the honey samples at 10 mg/mL. SDS: sodium dodecyl sulphate at 1 mg/mL. Results are mean ± SD, n = 6. Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, O: orange, St: strawberry tree, Mk: manuka honeys.

3.5. Phytochemical profile analysis

Aiming to identify the putative compounds responsible for the biological activities previously evaluated, the phenolic content and the respective UPLC/DAD/ESI–MS^n profile analysis were performed on the nine selected honeys.

3.5.1. Total phenolic content

Despite the Folin-Ciocalteu (FC) assay being the most commonly used procedure to determine the total phenolic content of food extracts, including honey, with the results expressed in gallic acid equivalents (Afrin et al., 2017; El-Haskoury et al., 2018; Mohammedi, 2021), in the present study the phenol content was expressed as the total fraction obtained after phenolic extraction, as described in the materials and methods section. Several works report that FC assay does not measure the polyphenol content of a sample but rather determines its reducing capacity. Additional compounds should be considered when total phenol content is measured following this procedure, such as proteins, reducing sugars, or vitamins, since they are also able to reduce the FC reagent (Everette et al., 2010). To avoid this interference, other methods have been proposed, including a preliminary partial purification of the phenolic extracts using solid phase extraction columns, before the FC assay is applied (Sánchez-Rangel et al., 2013).

In view of the above, the total phenolic quantification in the present work was not performed using the FC method and the results comparison was done with studies where the quantification was not achieved by FC assay directly on honey, to disregard the possible overestimation of its amount in honeys with the same botanical origin.

Strawberry tree, bell heather and carob tree honeys, presented the highest phenolic content, Table 1. Strawberry tree honey showed values of 137 mg/100 g honey, while both incense and orange honeys the lowest, 11 mg/100 g honey and 15 mg/100 g honey, respectively. Carob tree and bell heather honeys produced in different geographical locations demonstrated similar results, 26–27 mg/100 g honey and 30–38 mg/100 g honey, respectively. Eucalyptus honey showed a phenolic content of 16 mg/100 g honey. Comparatively, similar total phenolic content was found in Spanish eucalyptus honey, 18 mg/100 mg honey (Cheung et al., 2019), although lower values were detected in Portuguese eucalyptus honey obtained from Caramulo region, ranging between 4 and 6 mg/100 g (Silva et al., 2020). Orange honey total phenolic content, 15 mg/100 g honey, was higher than that obtained with the same type of honey from Spain, 8 mg/100 g honey (Cheung et al., 2019).

Previous studies with honeys having the same botanical origin as those analysed in this work, in which the whole phenolic content was determined with the FC assay on the phenolic fraction, reported similar proportional ratios to those found in this study. Carob tree honey from Morocco, chestnut honeys from Italy and Turkey, bell heather honeys from Ireland, Poland and Portugal, and strawberry tree honeys from Croatia and Italy showed higher total phenolic content. On the other hand, eucalyptus and orange honeys from Algeria and orange honeys from Italy showed lower total phenolic content (Ferreira et al., 2009; Pichichero et al., 2009; Afrin et al., 2017; El-Haskoury et al., 2018; Mohammedi, 2021).

3.5.2. Compounds identification and quantification

UPLC/DAD/ESI-MS^n analysis of the nine selected honeys enabled the identification of 30 compounds of that 16 were flavonoids, 10 phenolic acids, 2 isoprenoids, 1 norisoprenoid, namely 2-hydroxyisophorone, and a camphor derivative, camphoric acid, Table 2, Table S4 and Figure S5.

The identified compounds varied between the different samples, in which flavonoid aglycones were the most abundant flavonoids, particularly pinobanksin (0.3–24 mg/100 g honey), quercetin (0.1–16 mg/100 g honey), and pinobanksin-5-methyl-ether (0.4–8 mg/100 g honey), Table 2. This is consistent with other reports, specifically that flavonoid aglycones are the main flower-derived honey flavonoids resulting from nectar glycosides hydrolysis by bee saliva enzymes (Estevinho et al., 2008), with more than 90 % of honey flavonoids

Table 2
Quantification of the compounds (mg/100 g of extract) identified by UPLC/DAD/ESI-MS^n present in the honey samples.

Components (mg/100 g extract)	Ct1	Ct4	C2	E3	H2	H4	I3	O5	St2
Homogentisic acid									0.3
Protocatechuic acid		2.4		0.1	1.7	5.2			
p-Hydroxybenzoic acid	4.5	1.7		0.5	2.1	4.7		0.6	
Caffeic acid	0.1	0.2	0.2	3.7	0.4	0.4	8.4	0.7	0.3
Quercetin−3-*O*-rutinoside							4.5		
p-Coumaric acid	1.5	1.2	1.5	1.3	11.3	16.2	0.1	1.1	0.1
Cinnamic acid derivative			0.1						
2-Hydroxyisophorone									0.5
Ellagic acid			5.4	1.2	7.1				
Quercetin rhamnoside					6.6	6.8			
Salicylic acid				0.1	0.6	0.1			
Myricetin				0.5					
trans,*trans*-Abscisic acid			2.3		7.8	24.6			7.0
Camphoric acid	0.6	0.7	1.2	1.1	0.8	0.9	3.3	1.2	0.6
cis,*trans*-Abscisic acid	1.8	1.5	3.2	1.1	13.8	24.0		2.8	6.7
Quercetin	0.1	0.2			9.4	16.2	9.8	4.4	
Isorhamnetin				0.9			0.7		
Luteolin				1.4					
Pinobanksin−5-methyl-ether	5.1	7.8	5.6					0.4	
Pinobanksin	0.4	0.5	0.7	23.9	1.6	0.3	5.4	11.7	0.5
Naringenin-trimethyl-ether	0.1	0.2	1.2	0.2	5.4				
Kaempferol-methyl-ether				1.6				0.3	
Quercetin-dimethyl-ether				0.7		0.3	3.8	0.2	
Caffeic acid isoprenyl ester								0.2	
Chrysin	0.3	0.2	0.2	0.9	0.3	0.1	0.5	1.5	0.1
Pinocembrin	t	t			0.1	0.1	0.4	0.1	t
Caffeic acid phenylethyl ester							0.2		
Pinobanksin−3-*O*-acetate	t	t						0.2	
Galangin	t	t	0.2	0.3	0.2	0.2		0.2	
6-Methoxy-chrysin				0.2				0.1	
Grouped components									
Phenolic acids and phenolic acid derivatives	6.1	5.9	7.2	6.9	23.2	26.6	8.7	2.6	0.7
Flavonoids	6.2	8.9	7.9	30.6	17	17.2	20.6	19.1	0.6
Flavonol glucosides					6.6	6.8	4.5		
Isoprenoids and norisoprenoids	2.4	2.2	6.7	2.2	22.4	49.5	3.3	3.9	14.8

t: traces (< 0.05 mg/100 g of extract). Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, O: orange, St: strawberry tree honeys.

derived mainly from propolis, such as pinobanskin, pinocembrin and chrysin (Tomás-Barberán et al., 1993). Two flavonol glycosides were also identified, specifically quercetin-3-*O*-rutinoside (5 mg/100 g honey) in incense honey and quercetin rhamnoside detected in both bell heather honeys (7 mg/100 g honey). In lower amounts, the flavone chrysin (0.1–2 mg/100 g honey) was present in all honey samples.

The flavonol myricetin, and the flavone luteolin were only found in eucalyptus honey, similar to that reported by Martos et al. (2000) for eucalyptus honey obtained in Spain, Italy and Portugal. A methylated kaempferol derivative was also identified in both eucalyptus (2 mg/100 g honey) and orange honeys (0.3 mg/100 g honey).

Concerning phenolic acids composition, caffeic acid (0–8 mg/100 g honey) and *p*-coumaric acid (0–16 mg/100 g honey), were both detected in all the analysed honeys, with the latter being found in higher amounts in bell heather honey, as reported by Andrade et al. (1997) for Portuguese *Erica* spp. honeys. Ellagic acid was found in chestnut honey (5 mg/100 g honey), eucalyptus honey (1 mg/100 g honey), and bell heather honey from Boticas (7 mg/100 g honey), although in larger amounts in the last one, as mentioned by Ferreres et al. (1996a) for Portuguese heather honey. Isoprenoids, such as abscisic acid isomers, *trans*, *trans*- and *cis*, *trans*-abscisic acid, were identified in all honey samples, except in incense honey, with the highest values occurring in bell heather honeys (8–25 mg/100 g honey). It would be interest to explore whether these differences could indicate the mentioned compounds as bell heather floral origin putative markers (Ferreres et al., 1996b), due to its higher contents when compared to the other honey types (nd-7 mg/100 g honey). The absence of these isoprenoids in incense honey could, on the other hand, represent a marker for this honey.

Homogentisic acid (0.3 mg/100 g honey) and 2-hydroxyisophorone (0.5 mg/100 g honey) were only detected in strawberry tree honey consistent with previous studies with Sardinian honeys (Cabras et al., 1999; Montoro et al., 2021).

Higher concentrations of caffeic acid, quercetin-3-*O*-rutinoside, quercetin and quercetin-dimethyl-ether were identified in the incense honey produced at São Miguel, when compared to the other analysed honeys. A study on the phenolic content of Portuguese incense honey from Terceira (Azores archipelago), only includes its determination by the FC method, not comprising the respective compounds identification (Santos et al., 2023). Therefore, in the present study, the phytochemical profile description of Portuguese incense honey is reported for the first time.

Altogether the phenolic compounds identified in the analysed honeys does not provide evidence of a pattern between the total phenolic content per honey sample shown in Table I, and the quantification of the phenolic compounds in each honey sample, evidenced in Table 2. According to Popova et al. (2004), a mixture of flavanones and dihydroflavonols (pinobanksin) had much lower amounts of total phenols if caffeic acid was used in the calibration.

3.5.3. *Cluster analysis*

Cluster analysis evidenced two poorly correlated (Scorr<0.04) clusters, with the first including carob tree, chestnut, bell heather, and strawberry tree honeys and the second cluster comprising eucalyptus, incense and orange honeys, Fig. 4, and Table 3.

Cluster I contained both carob tree and chestnut honeys moderately correlated (Scorr < 0.55) and both bell heather and strawberry honeys also moderately correlated (Scorr < 0.70). Cluster I honeys were dominated by isoprenoids and norisoprenoids (2–50 %), specifically *trans*, *trans*- (2–25 mg/100 g honey) and *cis*,*trans*-abscisic acids (2–24 mg/100 g honey), followed by phenolic acids and phenolic acid derivatives (1–27 %), namely *p*-coumaric acid (0.1–16 mg/100 g honey), ellagic acid (5–7 mg/100 g honey), protocatechuic acid

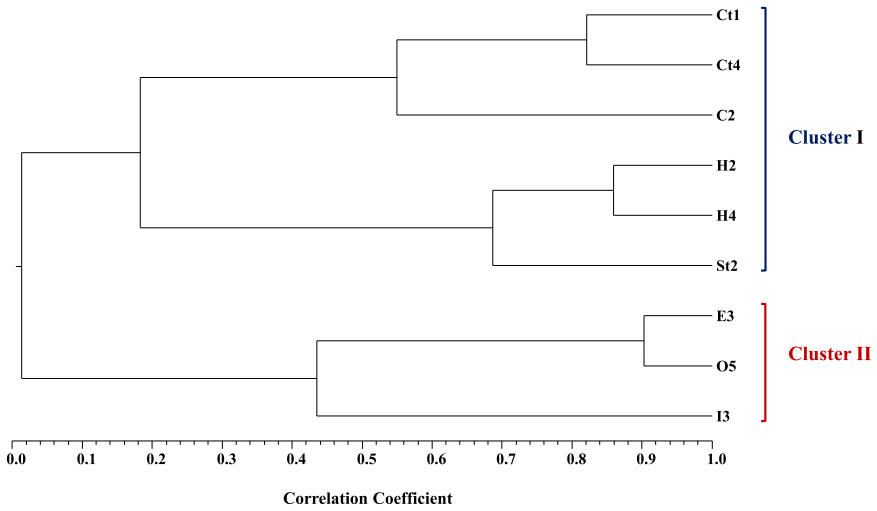

Fig. 4. Dendrogram obtained by cluster analysis of the compounds (mg/100 g) identified by UPLC/DAD/ESI-MS^n, based on correlation, and using the unweighted pair group method with arithmetic average (UPGMA). Ct: carob tree, C: chestnut, E: eucalyptus, H: bell heather, I: incense, O: orange, St: strawberry tree honeys.

Table 3
Minimum and maximum percentage range of the compounds identified by UPLC/DAD/ESI-MS^n from the different honey samples grouped according to cluster analysis. For samples grouped in each of the clusters, see Fig. 4.

Compounds (%)	Cluster I		Cluster II	
	Min.	Max.	Min.	Max.
Homogentisic acid		0.3		
Protocatechuic acid		5.2		0.1
p-Hydroxybenzoic acid		4.7		0.6
Caffeic acid	0.1	0.6	0.7	8.4
Quercetin−3-*O*-rutinoside				4.5
p-Coumaric acid	0.1	16.2	0.1	1.3
Cinnamic acid derivative		0.1		
2-Hydroxyisophorone		0.5		
Ellagic acid		7.1		1.2
Quercetin rhamnoside		6.8		
Salicylic acid		0.6		0.1
Myricetin				0.5
trans,*trans*-Abscisic acid		24.6		
Camphoric acid	0.6	1.2	1.1	3.3
cis,*trans*-Abscisic acid	1.5	24.0		2.7
Quercetin		16.2		9.8
Isorhamnetin				0.9
Luteolin				1.4
Pinobanksin−5-methyl-ether		7.8		0.4
Pinobanksin	0.3	1.6	5.4	23.9
Naringenin-trimethyl-ether		5.4		0.2
Kaempferol-methyl-ether				1.6
Quercetin-dimethyl-ether		0.3	0.2	3.8
Caffeic acid isoprenyl ester				0.2
Chrysin	0.1	0.3	0.5	1.5
Pinocembrin		0.1		0.4
Caffeic acid phenylethyl ester				0.2
Pinobanksin−3-*O*-acetate		t		0.2
Galangin		0.2		0.3
6-Methoxy-chrysin				0.2
Grouped components				
Phenolic acids and phenolic acid derivatives	0.7	26.6	2.6	8.7
Flavonoids	0.6	17.2	19.1	30.6
Flavonol glucosides		6.8		4.5
Isoprenoids and norisoprenoids	2.2	49.5	2.2	3.9

Min.: minimum. Max.: maximum. t: traces (< 0.05 mg/100 g of extract).

(2–5 mg/100 g honey) and *p*-hydroxybenzoic acid (2–5 mg/100 g honey).

Cluster II comprised eucalyptus and orange honeys very highly correlated (Scorr = 0.90) and incense honey moderately correlated to both (Scorr<0.45). These honeys were characterised by the dominance of flavonoids (9–31 %), evidencing pinobanksin (5–24 mg/100 g honey) and quercetin (nd-10 mg/100 g honey) as the main components.

4. Conclusions

The assessed biological activities, the total phenolic content, and phytochemical profile, varied between the analysed honey types, and with different botanical origins. The results highlighted the highest phenolic content in carob tree, and bell heather honeys, and particularly in strawberry tree honey. The results also evidenced that honeys with higher phenolic compounds content, namely bell heather and strawberry tree honeys, showed higher antimicrobial and antioxidant activity, while the honeys with lower phenolic compounds amount, the incense and orange honeys, revealed higher wound healing ability. Therefore, the bioactivity seems not just depend on the phenolic content, since orange and incense honeys, despite being poorer in phenols compared to bell heather and strawberry tree honeys, showed the best ability in wound closure.

This study revealed that the *in vitro* wound healing activity is dependent on the honey type and, therefore, could be useful in medical devices for this purpose. Moreover, blends of diverse honeys may be an alternative to faster wounds healing, combining honey samples with good antimicrobial and antioxidant/anti-inflammatory activities (chestnut, eucalyptus, bell heather, and strawberry tree honeys) with those with good tissue regeneration ability (incense and orange honeys). All honey samples tested showed cell viability higher than 76 % at 10 mg/mL concentration, confirming their cytocompatibility.

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CRediT authorship contribution statement

Figueiredo Ana: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Marto Joana:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Machado Alexandra:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Miguel Maria:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Vilas-Boas Miguel:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Falcão Soraia:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Ribeiro Helena:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Gonçalves Lídia:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Tomás Andreia:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Duarte Aida:** Writing – original draft, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.107659.

Data availability

Data will be made available on request.

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