Food Bioscience 62 (2024) 105028

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Evaluating the quality, physicochemical properties, and biological activities of Centauri® honey from Turkey

Márcia Santos Filipe [a,b], Tomasz Kowalczyk [c], Wirginia Kukula-Koch [d], Joanna Wieczfinska [e], Gabrielle Bangay [a,b], Ana María Diaz-Lanza [b], Rossana V.C. Cardoso [f,g], Filipa Mandim [f,g], Soraia I. Falcão [f,g], Miguel Vilas-Boas [f,g], Tomasz Śliwiński [h], Przemysław Sitarek [i], Patrícia Ríjo [a,j,*]

[a] Center for Research in Biosciences & Health Technologies (CBIOS), Universidade Lusófona de, Humanidades e Tecnologias, Campo Grande 376, 1749-024, Lisboa, Portugal
[b] Universidad de Alcalá de Henares. Facultad de Farmacia, Departamento de Ciencias Biomédicas (Área de Farmacología, Nuevos Agentes Antitumorales, Acción Tóxica Sobre Células Leucémicas. Ctra. Madrid-Barcelona Km. 33, 600 28805, Alcalá de Henares, Madrid, Spain
[c] Department of Molecular Biotechnology and Genetics, University of Lodz, Banacha 12/16, 90-237, Lodz, Poland
[d] Department of Pharmacognosy with Medicinal Plants Garden, Medical University of Lublin, 1 Chodzki Str., 20-093, Lublin, Poland
[e] Department of Immunopathology, Medical University of Lodz, Zeligowskiego 7/9, Bldg 2, Rm 177, 90-752, Lodz, Poland
[f] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal
[g] Laboratório Associado para a Sustentabilidade e Tecnologia Em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal
[h] Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236, Lodz, Poland
[i] Department of Medical Biology, Medical University of Lodz, Muszynskiego 1, 90-151, Lodz, Poland
[j] Instituto de Investigação Do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003, Lisboa, Portugal

ARTICLE INFO

Keywords:
Centauri® honey
Honey quality
Promising ingredient
Anti-inflammatory

ABSTRACT

Honey, a natural production of honeybees, underscores Turkey's prominence as one of the world's largest honey producers. The country's diverse flora fosters optimal conditions for beekeeping, resulting in the production of exceptional-quality honey. Centauri® Honey is uniquely sourced from remote alpine regions in Turkey, characterized by bees that inhabit isolated caves with access to medicinal endemic flowers year-round, promoting a unique and pristine production environment. This study evaluates the quality, physicochemical properties, nutritional parameters, and biological activities of five types of Centauri® Honey. The *in vitro* studies highlighted significant nitric oxide inhibition in Centauri® Cave Honey, suggesting it is a promising candidate for further research and potential therapeutic applications, especially in enhancing immune responses by reducing the inflammatory response. This study confirms that Centauri® honey not only adheres to the rigorous standards of the Codex Alimentarius but also offers substantial health benefits, advocating for continued research into its medicinal applications.

1. Introduction

Honey, a natural product created by honeybees through the collection of nectar, plant secretions, or excretions from plant-sucking insects, is a complex substance rich in sugars and diverse components (Rysha et al., 2022; Saxena et al., 2010). Enzymes, amino acids, organic acids, carotenoids, vitamins, minerals and aromatic substances intermingle within this golden liquid (Codex Alimentarius Commission, 2001; Rysha et al., 2022). Blossom honey, also known as nectar honey, is derived from plant nectar. In contrast, honeydew honey primarily originates from the excretions of plant-sucking insects (*Hemiptera*) or from the exudations of living plant parts (Codex Alimentarius Commission, 2001). Notably, honey is abundant in flavonoids and phenolic acids, displaying various biological effects and acting as natural antioxidants (Alqarni et al., 2014; da Silva et al., 2016).

The chemical composition and physical attributes of honey,

* Corresponding author. CBIOS - Board Director for Internationalization & Strategic Networks - Bio.Natural@CBIOS. Escola de Ciências e Tecnologias da Saúde, Centro Universitário de Lisboa, Campo Grande, 376, 1749-024, Lisboa. Portugal.
E-mail address: patricia.rijo@ulusofona.pt (P. Ríjo).

https://doi.org/10.1016/j.fbio.2024.105028
Received 4 June 2024; Received in revised form 26 August 2024; Accepted 31 August 2024
Available online 2 September 2024

including colour, aroma and flavour, as well as the bioactivity, are intricately influenced by features like nectar composition, climate, geographical source, harvesting techniques and storage conditions (Bogdanov et al., 1999; da Silva et al., 2016; Gonçalves et al., 2018). The Codex Alimentarius standard and the EU Honey Directive provide accepted parameters for various characteristics, in order to regulate quality standards of honey (Codex Alimentarius Commission, 2001, The European Union Council Directive 2001/110/EC, 2001).

Throughout history, honey has been employed for its remarkable antimicrobial properties, particularly in the realm of wound healing (Childress & Stechmiller, 2002; Koetzler et al., 2009; Yilmaz & Aygin, 2020). Studies have shown the occurrence of nitric oxide, which accelerates inflammatory and proliferation stages of wound healing by fostering vasodilation, increasing antimicrobial activity and enhancing vascular permeability (Childress & Stechmiller, 2002; Koetzler et al., 2009; Yilmaz & Aygin, 2020). Moreover, honey facilitates re-epithelialization, angiogenesis and stimulation of skin and immune cells, collectively promoting tissue regeneration (Scepankova et al., 2021). Research indicates a correlation between honey's antioxidant and antimicrobial properties, its phenolic content and colour, with darker varieties exhibiting higher concentrations of polyphenols and enhanced activities (Gonçalves et al., 2018).

Turkey, as the second-largest global honey producer, with an annual output of approximately 100,000 tons, boasts a favourable apicultural environment due to the abundance of flowers, which provides an ideal setting for beekeeping endeavours (Food and Agriculture Organization of the United Nations; Mayack & Hakanoğlu, 2022). Centauri® Honey, sourced from remote alpine regions in Turkey's elevated hills, situated 2500 m above the Black Sea, is meticulously harvested by bees inhabiting caves far from human settlements and other bee populations. With unencumbered access to endemic medicinal flowers year-round, Centauri® Honey offers a unique and pristine environment for honey production (Tornuk et al., 2013; Yılmaz & Yavuz, 1999).

This study aims to conduct a comprehensive exploration of the quality, physicochemical properties, nutritional components and biological activities of five types of Centauri® honey. Through a complete examination, we seek to uncover the distinct qualities that make Centauri® Honey a valuable and potentially beneficial natural product.

2. Methods and materials

2.1. Honey samples

In total, five Centauri® honey samples were collected in August 2022 in different regions of Turkey, details of which are shown in Table 1. During production, the nutrition of the bees is manipulated using pre-collected honeys from different regions of Turkey, making it impossible to attribute the final product to a single botanical region.

Storage and Processing: Honey production begins at the entrance of caves within black hives. Once the combs are filled with honey, they are removed from the hives and placed inside the caves at varying depths for an aging process lasting 2–3 weeks. The combs are positioned between or on the rocks during this time. After aging, the combs are hand-squeezed to extract the liquid honey. The extracted honey is then bottled and stored in glass jars within the caves until sold.

2.2. Quality and physicochemical parameters of honey

The colour index, moisture, electrical conductivity, hydroxymethylfurfural (HMF) content, pH, free and total acidity, diastase and proline content were performed using the procedures advised by the International Honey Commission (Bogdanov, 2002). All the determinations were done in triplicate for each sample.

The color index of the honey was analysed using a Honey Color C221 colorimeter (Hanna Instruments, Woonsocket, Rhode Island, USA). Moisture content was determined with a refractometer (Digit-5890, Ref: 8100.5890, Netherlands).

Electrical conductivity was measured using a calibrated Consort C868 conductivity meter (Hanna Instruments, Woonsocket, Rhode Island, USA) by dissolving 5 g of honey in 25 mL of distilled water.

The pH and acidity measurements, a HI902 potentiometer titrator (Hanna Instruments, Woonsocket, Rhode Island, USA) was used. A solution was prepared by dissolving 10 g of honey in 100 mL of deionized water, and 25 mL of this solution was placed in a beaker with a pH electrode to record the initial pH. The solution was then titrated with 0.1 M sodium hydroxide (NaOH) to determine free acidity. To assess lactonic acidity, additional NaOH was added until a final volume of 10 mL was reached, followed by re-titration with 0.025 M sulfuric acid (H_2SO_4) until the equivalence point (pH 7) was achieved. The difference in NaOH consumed in the two titrations was used to calculate lactonic acidity and total acidity, expressed in meq.kg^{-1}.

Diastase activity was measured using the Phadebas method. A solution of 1 g of honey in 100 mL of water was prepared, and 5 mL of this solution was transferred to test tubes and incubated at 40 °C with acetate buffer and Phadebas tablets. After 15 min, the reaction was stopped with 0.5 M NaOH, and absorbance was measured at 620 nm. Diastase number (DN) was calculated using specific equations based on the absorbance value: DN = 28.2 * Abs620 + 2.64 if DN > 8, or DN = 35.2 * Abs620 – 0.46 if DN < 8.

Proline content was evaluated spectrophotometrically by diluting 0.5 g of honey in 10 mL of distilled water. The honey solution, distilled water, and a standard proline solution were reacted with formic acid and ninhydrin, heated in a boiling water bath, and cooled. Absorbance was measured at 510 nm, and proline content was calculated using the equation: Proline = (Abs Sample/Abs standard) x (mass standard/mass Sample) x 80, with results expressed in mg.kg^{-1}.

The 5-HMF content was performed by HPLC according to a previous method (Bogdanov, 2002). The samples were prepared by diluting 5 g of honey samples in a small quantity of distilled water. After transferring the diluted sample to a 25 mL volumetric flask, 500 μL of Carrez I and Carrez II solutions were added. The solutions were filtered through a 0.45 nm membrane directly into a vial. The 5-HMF standard solution was prepared at 0.012 mg/mL in distilled water and five dilution solutions were made, filtered and passed to a vial. The HMF content of the

Table 1
Centauri® honey samples and their respective characteristics.

Characteristics	Honey samples				
	A	B	C	D	E
Altitude (m)	1800	2700	2500	1600	2600
Cave depth (m)	3	1	Cave + Traditional	5	15
Visual colour	Black	Mid color (Dark brown)	Lighter brown	Very Light	Mix Black & Dark Brown
Bee's nutrition	Endemic herbs + Oak honey	Endemic herbs + polyfloral honey	Endemic herbs + polyfloral honey	Endemic herbs + polyfloral honey	Endemic herbs + monofloral honey + polyfloral honey
Location	Western aegean	Eastern black sea	Eastern black sea	Southeastern Anatolia	Western aegean & Eastern black sea
Origin	Beekeeper/Research samples	Beekeeper/Research samples	Beekeeper/Research samples	Beekeeper/Research samples	Commercial

honey samples was calculated by relating the corresponding peak areas with those of the standard solutions, considering the dilution. The results are conveyed in mg/kg.

These methods comprehensively analyze the honey's color, moisture content, electrical conductivity, pH, acidity, diastase activity, and proline content, providing essential quality indicators for various applications.

2.2.1. Protein content

The protein content was given using the Kjeldahl method, which consists of indirect determination based on the quantification of total organic nitrogen (Pascual-Maté et al., 2018).

The assay started with the digestion of 1 g of honey samples by the addition of 15 mL of sulfuric acid and a metallic catalyst, used to accelerate the oxidation process of organic matter, in a digester at 400 °C for 70 min, with an automatic distillation and titration unit (model Pro-Nitro-A, JP Selecta, Abrera, Barcelona) (Fernandes et al., 2023). For the conversion of nitrogen content into total protein, a conversion factor of 6.25 was applied, expressing the results in g/100 g of honey.

2.2.2. Ashes

The ash content was determined indirectly using a formula that relies on previously measured conductivity values. This calculation was performed based on the formula provided by (Sancho et al., 1992) as outlined in the established literature.

$$\text{Ashes}\ (\%) = \frac{\textit{conductivity}\ (\text{mS/cm})}{1000} - 0.14 \div 1.74$$

The equation adjusts the conductivity value to estimate the percentage of ash in the sample, providing an indirect but reliable measure of ash content.

2.2.3. Carbohydrates

The carbohydrate content was calculated using a formula that incorporates the previously determined values for moisture, ash, protein, and lipids. This method is well-established in the literature, as defined by (Shugaba, 2012). The formula used is as follows:

$$\text{Carbohydrates}\ (\%) = 100 - \%\text{moisture} - (\%\text{ash} + \%\text{protein} + \%\text{lipids})$$

This formula provides the percentage of carbohydrates by subtracting the combined percentages of moisture, ash, protein, and lipids from 100%.

2.2.4. Energy

The energy value was calculated using a standard formula from the literature, as described by (Shugaba, 2012). This formula estimates the caloric content of the sample by accounting for the contributions of proteins, carbohydrates, and lipids. The calculation is as follows:

$$\text{Energy}\ (\text{kcal}/100\text{g}) = 4\text{x}\ (\%\text{protein} + \%\text{carbohydrates}) + 9\text{x}\ (\%\text{lipids})$$

By applying this formula, the total energy value per 100 g of the sample can be determined.

2.2.5. HPLC-RI determination of sugars

The sugar content was performed by liquid chromatography coupled to a refraction index detector (HPLC-RI), in accordance with the procedures advocated by the International Honey Commission (Bogdanov, 2002). The samples were prepared by diluting 2.5 g of honey with 20 mL of distilled water. Next, 12.5 mL of methanol was pipetted into a 50 mL volumetric flask, to which the honey solution was then added. Lastly, water was added to make up the final volume. The solutions were filtered through a membrane filter and collected in sample vials. As a mobile phase, a mixture of acetonitrile/water 80:20 (v/v) was used, with a flow rate of 1.3 mL/min. By comparing the retention times of the sample peaks with those of standards, the identification of sugars was obtained. Using standard samples of known composition and concentration, calibration curves were determined as follows: fructose (3.1–60.0 mg/mL, $y = 58.03x - 37.89$, $R^2 = 0.999$), glucose (2.3–45.0 mg/mL, $y = 59.02x - 31.24$, $R^2 = 0.999$), sacarose (0.2–4.5 mg/mL, $y = 59.90x - 12.58$, $R^2 = 0.999$), turanose (0.2–2.8 mg/mL; $y = 24.36x + 0.7668$, $R^2 = 0.999$), maltose (0.2–4.5 mg/mL, $y = 33.98x + 5.568$, $R^2 = 0.998$), trehalose (0.2–4.5 mg/mL, $y = 40.35x + 5.449$, $R^2 = 0.999$), melezitose (0.5–4.5 mg/mL, $y = 39.03x + 6.845$, $R^2 = 0.999$) and raffinose (0.5–4.5 mg/mL, $y = 34.843x + 3.595$, $R^2 = 0.999$). For each of these standards, a calibration line was established by the internal standard method, using a range of concentrations, according to the expected levels for each sugar. The obtained values were calculated from the peak area and are presented in g/100 g of honey.

2.3. HPLC-ESI-QTOF-MS/MS and chemometric approach

2.3.1. Sample preparation and fingerprinting by mass spectrometry

Around 200 mg of each honey sample was transferred to an eppendorf vial and dissolved in ca. 0.2 mL of methanol/water mixture 2:8 (v/v) to achieve the concentration of 200 mg/mL. Next, the eppendorf was vortexed for 5 min and later centrifuged for 10 min at 10,000 rpm and 4 °C. The supernatant was later filtered through a nylon syringe filter, with a pore diameter of 0.1 μm, directly into autosampler vials and subjected to HPLC-MS analysis.

The HPLC-ESI-QTOF-MS/MS chromatograph produced by Agilent Technologies (Santa Clara, California, USA) was employed for the fingerprinting of the extracts and an untargeted analysis. The instrument was composed of a 1200 Series HPLC chromatograph equipped in a degasser, an autosampler, a column thermostat, a DAD detector, a mass spectrometer (G6530B) equipped in a dual AJS ESI ionization source and an isocratic pump for delivering the calibration ion mixture. The separation of the extracts' constituents was achieved at 0.2 mL/min on an RP-18 Zorbax Eclipse Plus column, with dimensions of 150 mm × 2.1 mm and a pore size of 3.5 μm, in the gradient of acetonitrile with 0.1% formic acid in water: 0 min–1%, 10 min–20%, 15 min–40%, 17–18 min – 95%, 19–30 min – 1%. The isocratic pump delivers the reference ion mixture at the flow rate of 0.01 mL/min. The mass spectra were acquired after 10 μL injection of a sample on a freshly calibrated instrument in two ionization modes and in three repetitions for each mode. The following MS settings were applied: gas and shield gas temperatures of 250 and 300 °C, respectively, gas flows of 12 L/min, nebulizer pressure of 35 psig, skimmer voltage of 65 V, capillary voltage of 3000 V, nozzle voltage of 1000 V, *m/z* range of 40–1200 Da and collision energies of 10 and 20 V. Two the most intensive *m/z* signals were fragmented in each scan and were later excluded for the following 0.2 min. The acquisition and analysis of data were recorded by the Mass Hunter Workstation program (version B.10.00, Agilent Technologies), whereas the following sample processing was performed in the Mass Profiler Professional Program (version 15.1., Agilent Technologies). First, the molecular feature extraction was performed from the recorded injections by Profinder program v.10.0.02 (Agilent Technologies) in order to extract the list of *m/z signals* present in the analysed samples, together with their retention times and peak areas in the csv format, later to be compared in the chemometric analysis. The collected data were filtered according to several criterions, which included mass tolerance of 10 ppm, retention time variation of 0.3 min, a peak area of at least 1% of the highest signal and were clustered in five groups following the names of five honey samples – from A to E. Then, the obtained data were analysed by the Mass Profiler Professional Program. The PCA analysis was performed for all analysed samples on the sets of 463 and 169 entities for the positive and the negative ion mode, respectively ($P < 0.05$), using One-way ANOVA with asymptotic p-value computation and Benjamini-Hochberg multiple testing correction – directly by the Mass Profiler Professional Program. Later, the identification of the different signals was achieved at $P < 0.001$ using a list of high-resolution mass

measurements to be identified with the help of the commonly available MS libraries, like Metlin collection of metabolites, lipids, proteins and small molecules, and in relation to the scientific literature.

2.4. In vitro biological activities of honey

2.4.1. Antioxidant activity

2.4.1.1. Thiobarbituric acid reactive substances (TBARS) method. The antioxidant activity of the honey samples was accomplished using the Thiobarbituric Acid Reactive Substances (TBARS) method (Feitor Fernandes, 2010) and DPPH. Initially, the samples were dissolved in water and then consecutively diluted to achieve concentrations ranging from 250 mg/mL to 0.24 mg/mL. The assay focused on assessing lipid peroxidation inhibition within homogenates from porcine (Sus scrofa) brains, by quantifying the reduction of TBARS. The reduction was quantitively measured by the colour intensity produced by the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), with absorbance recorded at 532 nm. The percentage of inhibition was calculated using the formula:

$$\textit{Inhibition Ratio}\ (\%) = \frac{A - B}{A} \times 100\%$$

where 'A' represents the absorbance of the control solution and 'B' the absorbance of the treated sample solution. The results were expressed as EC_{50} values (mg/mL), representing the concentration of the sample that achieves 50% antioxidant activity.

2.4.1.2. Cellular antioxidant activity (CAA). The cellular antioxidant activity (CAA) was subsequently evaluated using a murine macrophage cell line (RAW 264.7). This assay assessed the honey samples' ability to prevent the oxidation of intracellular 7′-dichlorodihydrofluorescein (DCFH) following a previously described methodology. Quercetin served as the positive control, while 7′-dichlorodihydrofluorescein and Dulbecco's modified Eagle's medium (DMEM) was used as negative controls. The eight lipidic extracts were dissolved in a DMSO (50:50, v/v) solution and tested at final concentrations of 8, 2, 0.5, and 0.125 mg/mL. Results were expressed as the percentage inhibition of reactive oxygen species (ROS) formation at the highest concentration tested (2000 μg/mL) (de la Fuente et al., 2022; Silva et al., 2023).

2.4.1.3. DPPH radical scavenging assay. The antioxidant potential was evaluated using the synthetic free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by (Ntungwe et al., 2021). The samples were tested at a concentration of 20% (w/v). Quercetin served as the positive control. Assays were conducted in triplicate, and the free radical scavenging activity was calculated using the following formula:

$$\textit{Scavenging activity}\ (\%) = \frac{\textit{Abs control} - \textit{Abs sample}}{\textit{Abs control}} \times 100$$

2.4.2. NO-production inhibition

The capacity of the studied honey samples to inhibit the production of the inflammatory chemical mediator nitric oxide (NO) was measured thought a murine macrophage cell line (RAW 264.7) stimulated with lipopolysaccharide (LPS). The assay was performed using the Griess Reagent System Kit (Promega, Madison, WI, USA), following the methodology described by (Silva et al., 2020). The samples were dissolved in DMSO:water (50:50, *v/v*) and subsequently diluted to achieve a concentration range of 0.125–8 mg/mL for testing. Dexamethasone at a concentration of 50 μM (Sigma-Aldrich, Saint Louis, MO, USA) served as a positive control, while cells in the presence and absence of LPS were tested as the negative control. Results were conveyed by determining the concentration of the sample responsible for inhibited 50% of NO production (IC_{50}, μg/mL) (Mandim et al., 2022).

2.4.3. Cell cultures

The FHC (normal human colon) cell line was acquired from the American Type Culture Collection (CRL-1831, LGC Standards, Lomianki, Poland) and cultured in DMEM:F12 Medium, containing 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate and 1200 mg/L sodium bicarbonate (American Type Culture Collection, Manassas, Virginia, USA). The A549 (human lung adenocarcinoma) cell line was purchased from Sigma-Aldrich (St. Louis, MO USA) and cultured in Eagle's Minimum Essential Medium with foetal bovine serum in a final concentration of 10% (Sigma-Aldrich, St. Louis, MO USA). The experiments (n = 6) were executed after attaining 80%–90% confluence (passage three to eight) by the cells. The cell viability was assessed using MTT assay.

2.4.3.1. MTT assay. The cells were treated with varying percentages of honey samples (0.5%–10%). After 24, 48 or 72 h of incubation, 50 μL of methyl thiazolyl tetrazolium (MTT, Sigma-Aldrich, St. Louis, MO, USA) was introduced into each well and incubated for 4 h. The contents of the wells were then removed and washed twice with phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific, INC. Waltham, MA, USA). Intracellular formazan crystals were dissolved by adding DMSO. The absorbance was taken at a wavelength of 550 nm.

2.4.3.2. Experimental procedures. Cells were plated out in 100 μL aliquots ($1x10^4$ cells per well) into a 96 well plate and for a 24 h period of incubation at 37 °C and 5% CO_2. Subsequently, cells were treated with 1% of honey (A-E). Moreover, the cells were treated with LPS (10 ng/mL) to stimulate inflammation.

2.4.3.3. RNA isolation and cDNA synthesis. Total RNA was isolated with a Total RNA mini kit (A&A Biotechnology, Gdynia, Poland). Isolated RNA was purified and stored at −80 °C. Briefly, 1 x 10^6 cells were centrifuged and the supernatant removed. 800 μL phenozol was added to the prepared cells and mixed for complete cell lysis. The suspension was incubated for 5 min at 50 °C. To the lysate prepared in this way, 200 μL of chloroform was added, the sample was gently stirred and then left for 3 min at room temperature. It was then centrifuged for 10 min at 12,000 RPM. After centrifugation, the upper fractions were collected into a new Eppendorf tube. 250 μl of isopropanol was added to the supernatant and applied to the mini-column after mixing. Centrifuge for 1 min at 12,000 RPM. The mini-column was transferred to a new tube and 700 μl of A1 wash solution was added. Centrifuge for 1 min at 12,000 RPM. After removal of the filtrate, the rinsing with A1 solution was repeated. After centrifugation for 1 min at 12,000 RPM, the mini-column was removed, the filtrate was removed and 200 μl of wash solution A1 was added. Centrifugation was performed for 2 min at 12,000 RPM. Finally, the mini-column was transferred to a new Eppendorf tube, 100 μl of ultrapure water was added, left for 3 min at room temperature and then centrifuged for 1 min at 12,000 RPM to elute the RNA. Subsequently, reverse transcription using 1 μg of total RNA was carried out with High-Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). The procedures were executed according to the protocol supplied by the manufacturer. Briefly, to the tube was added 10 μL of 2X RT master mix followed by 10 μL of RNA sample. The sample was mixed, briefly swirled and left on ice. The reverse transcription reaction was carried out in a thermocycler using the following parameters: 25 °C/10 min, 37 °C/120 min, 85 °C/5min. The volume of the reaction mixture was 20 μL.

2.4.3.4. Gene expression analysis. The expressions of proteins: IL-1β and IL-6 were evaluated by TaqMan gene expression assays qPCR technique, Hs01555410_m1 (IL-1β) and Hs00174131_m1 (IL-6), purchased from Life Technologies (Carlsbad, CA, USA). Each sample was taken in triplicate and the gene expression was employed with the $2^{-\Delta\Delta C}$t method. The results were normalized utilizing endogenous reference gene -

β-actin (Hs99999903_m1, Life Technologies, Carlsbad, CA, USA).

2.4.3.5. ELISA test. The concentrations of IL-6 and IL-1β were obtained by ELISA using a commercial MyBiosource kit (San Diego, California, USA), according to the manufacturer's protocol.

IL-6: Standard was prepared by adding 50 μl of standard solutions (S1, S2, S3, S4, S5, S6) to the respective standard wells. Test samples were prepared by adding 50 μl of sample to each well and then adding 100 μl of HRP-conjugated reagent to each well except the blank wells. The plate was then sealed and incubated for 60 min at 37 °C. After this time, all wells were washed 4 times, 50 μl of chromogen A solution was added to each well and 50 μl of chromogen B solution was added. The samples were gently mixed and the plate was incubated for 15 min at 37 °C. After incubation, 50 μl of stop solution was added to each well.

The optical density (O.D.) was read at 450 nm using an ELISA reader 5 min after the addition of the stop solution.

IL-1β: Standards or samples were added to the appropriate wells (100 μL per well) and incubated at 37 °C for 90 min. The ELISA plates were washed 2 times and then the prepared biotinylated antibody was added to each well (100 μL per well). The plates were incubated at 37 °C for 60 min. Then the ELISA plates were washed 3 times, the prepared enzyme conjugate was added to each well other than the blank wells (100 μL per well). Plates were incubated at 37 °C for 30 min. The ELISA plates were then washed 5 times and 100 μL of the prepared colour reagent was added to each well (including the blank well). The plates were incubated at 37 °C. The incubation was carried out until a clearly appearing colour gradient in the standard. Then 100 μL of colour reagent C was added to each well (including the blank well). After thorough mixing, the results were read OD 450 nm).

2.5. Statistical analyses

The results were analysed with Statistica software (StatSoft, Tulsa, OK). Levene's test and the Shapiro-Wilk test were used, respectively, to verify the equality of variances and the data distribution. As a multiple comparison procedure, the ANOVA test was used to calculate significant changes, along with the appropriate post hoc tests. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Physicochemical and nutritional parameters of honey

The physicochemical analysis of the five honey samples began with an examination of their colour. As shown in Table 2, the honey samples displayed a diverse range of colours, with honey A being a dark amber, B, C and E exhibiting a delicate light amber hue, and D standing out with its white appearance. The moisture content was also evaluated, revealing results ranging between 14% and 18% (w/w). The electrical conductivity values of the five samples comply with the recommended maximum value of 800.00 mS cm^{-1}, outlined by the Codex Alimentarius (2001).

The pH determination in our analysis (Table 2) revealed that honey samples B and C had slightly lower pH levels than expected (3.049 and 3.100, respectively). In contrast, honey samples A, D, and E exhibited higher pH values (4.348, 3.200, and 3.600, respectively). Free acidity was evaluated, and the results indicated that all the honey samples had values lower than the maximum permitted value of 50.0 meqKg^{-1}. The 5-Hydroxymethylfurfural (5-HMF) content, which indicates honey freshness and potential overheating. The results showed 5-HMF levels ranging between 3.0 and 9.70 mg kg^{-1}. Diastase activity, which is crucial for indicating aging and exposure to elevated temperatures, was measured in the honey samples. All samples, except honey D, met the minimum level of 8 diastase units as set by the Codex Alimentarius and the European Honey Directive. Honey D, however, exhibited a lower diastase index. According to the Codex Alimentarius, the ash content for blossom honey should be below 0.6 g/100 g, while honeydew honey or a mixture of honeydew honey with blossom honey should not exceed 1.2 g/100 g (Codex Alimentarius Commission, 2001). The ash content of honey samples B through E ranged from 0.04 to 0.33 g/100 g, aligning with the standard requirements for blossom honey. Honey sample A exhibited an ash content of 0.87 ± 0.12 g/100 g. This higher ash content aligns with the composition of honey sample A, as the bees were fed with endemic herbs and oak honey. Since oak honey is classified as honeydew honey, which naturally has a higher ash content, this result was expected.

Table 2
Physicochemical and nutritional parameters of honey.

Physicochemical and Nutritional parameters	Honey samples				
	A	B	C	D	E
Colour (mm Pfund)	150 ± 0.0[a] (Dark amber)	63 ± 1.5[b] (Light amber)	57 ± 2.5[c] (Light Amber)	31 ± 1[d] (White)	85 ± 1.7[e] (Light Amber)
Moisture content (%)	14 ± 1[a]	18 ± 1[b]	16 ± 1[a,b]	15 ± 1[a, b]	17 ± 1[a,b]
Electrical conductivity (mS.cm^{-1})	1.65 ± 0.21[a]	0.44 ± 0.02[b]	0.31 ± 0.00[b]	0.22 ± 0.00[b]	0.72 ± 0.01[c]
pH initial	4.348	3.049	3.100	3.200	3.600
Free for pH=7 (meqKg^{-1})	9.6 ± 0.6[a]	6.4 ± 0.1[b]	4.6 ± 0.0[c]	4.1 ± 0.2[c]	7.2 ± 0.0[d]
Free for pH=8.3 (meqKg^{-1})	13.7 ± 0.5[a]	8.8 ± 0.1[b]	6.8 ± 0.2[c]	6.5 ± 0.5[c]	9.9 ± 0.2[d]
Lactonic (meqKg^{-1})	4.1 ± 1.2[a]	4.8 ± 0.2[a]	3.5 ± 0.1[a]	3.5 ± 0.2[a]	4.4 ± 0.2[a]
Total (meqKg^{-1})	13.7	11.2	8.1	7.6	11.6
5-HMF (mg.kg^{-1})	9.70 ± 0.87[a]	3.0 ± 0.16[b]	5.98 ± 0.24[c]	8.67 ± 0.58[a]	6.12 ± 0.21[c]
Diastase index (DN)	8.1 ± 0.4[a]	12.0 ± 1.1[b,d]	12.6 ± 0.7[b]	5.6 ± 0.5[c]	10.7 ± 0.3[d]
Proline (mg.g^{-1})	2.0 ± 0.0[a]	2.3 ± 0.3[a]	1.6 ± 0.4[a,b]	1.2 ± 0.3[b]	2.2 ± 0.1[a]
Ash (g/100g)	0.87 ± 0.12[a]	0.17 ± 0.01[b]	0.10 ± 0.00[b]	0.04 ± 0.00[b]	0.33 ± 0.01[c]
Protein content (g/100g)	0.72 ± 0.03[a]	0.38 ± 0.02[b]	0.28 ± 0.05[b]	0.27 ± 0.01[b]	0.54 ± 0.07[c]
Energy (Kcal)	340.5 ± 0.5[a]	326.9 ± 0.1[b]	335.6 ± 0.0[c]	339.8 ± 0.0[d]	332.3 ± 0.0[e]
Carbohydrates (mg/100g)	84.6 ± 0.1[a]	81.2 ± 0.0[b]	83.3 ± 0.0[c]	84.4 ± 0.0[d]	82.5 ± 0.0[e]

Statistical differences between physicochemical and nutritional parameters of honey were assessed using ANOVA followed by Tukey's post-hoc test. In row, different letters next to values indicate significant differences at $P < 0.05$.

The protein content of the honey samples ranged between 0.27 and 0.72 g/100g. Honey sample A recorded the higher protein content (0.72 g/100g ± 0.03), while honey sample D presented the lowest quantity of protein (0.27 g/100g ± 0.01).

The results of sugar content analysis in honey are shown in Table 3. The levels of fructose, glucose, sucrose, turanose, maltose, trehalose, melizitose and rafinose were measured and the total sugar content, the fructose + glucose (F + G) sum, the fructose/glucose (F/G) ratio, and the glucose/humidity (G/H) ratio were also calculated. The analysis indicated that glucose and fructose are the primary sugar components in the honey examined. Among the samples, honey D exhibited the highest total sugar content at 81.41 g/100 g, whereas honey A contained the lowest, measured at 72.02 g/100g.

According to the Codex Alimentarius Commission, 2001, the sucrose content in honey should not exceed 5 g/100 g (Codex Alimentarius Commission, 2001). In our analysis, honey A was the only sample where sucrose was detected, registering a level of 0.59 ± 0.05 g/100 g, which is well within Codex standards. Following the Codex Alimentarius Commission (2001), the combined sum of fructose and glucose (F + G) in honey should not be less than 60 g/100 g. All the honey samples met this

Table 3
Sugar content of honey samples at the level of g/100g of honey.

Sugars	Honey samples				
	A	B	C	D	E
Fructose	32.64 ± 0.77[a]	40.00 ± 0.77[b,c]	37.76 ± 1.41[b,c]	41.81 ± 2.51[b]	36.95 ± 2.02[a,c]
Glucose	25.38 ± 0.70[a]	31.54 ± 0.72[b]	31.63 ± 1.05[b]	32.25 ± 2.32[b]	28.09 ± 0.40[a]
Sucrose	0.59 ± 0.05	–	–	–	–
Turanose	3.93 ± 0.27[a]	3.23 ± 0.15[a]	3.01 ± 0.11[a]	3.20 ± 0.33[a]	3.3 ± 0.63[a]
Maltose	2.37 ± 0.07[a]	0.82 ± 0.04[b]	1.00 ± 0.13[b,c]	1.37 ± 0.16[c]	1.25 ± 0.28[c]
Trehalose	2.68 ± 0.12[a]	1.74 ± 0.17[b]	1.41 ± 0.06[c]	1.72 ± 0.08[b,c]	2.25 ± 0.13[d]
Melizitose	1.79 ± 0.14[a]	–	0.24 ± 0.14[b]	0.66 ± 0.15[b,c]	1.08 ± 0.28[c]
Rafinose	2.64 ± 0.23[a]	0.57 ± 0.19[b]	–	0.40 ± 0.14[b]	0.21 ± 0.08[b]
Total among of sugars	72.02	77.90	75.05	81.41	73.13
F + G	58.02	71.54	69.39	74.06	65.04
F/G	1.29	1.27	1.19	1.30	1.32
G/H	1.81	1.75	1.98	2.15	1.65

Values were presented as mean ± standard deviation. F + G-frutose glucose sum of both; F/G – frutose, glucose ratio; G/H – glucose, humidity ratio. Statistical differences between sugar content of honey samples were assessed using ANOVA followed by Tukey's post-hoc test. In row, different letters next to values indicate significant differences at $P < 0.05$.

standard, except for honey A, which recorded a value of 58.02 g/100g, falling below the required threshold. The fructose/glucose (F/G) ratio is a commonly used parameter in numerous studies focusing on bee products. In our study, the F/G ratio varied between 1.19 and 1.32. The hight content of fructose, as compared with glucose, in honey results in a F/G ratio over 1, as our research confirmed.

3.2. HPLC-ESI-QTOF-MS/MS and chemometric approach

The samples were assessed in an untargeted metabolomics analysis, to confirm the repeatability and robustness of the injections. The PCA analysis revealed some differences between the five analysed honey samples in both positive and negative ion mode (Fig. 1). The five honey samples were gathered in the centre of the PCA plot, with some outstanding points – especially for the A sample – emphasizing the occurrence of some statistically significant differences between the tested honeys. As presented in the grey brackets of Fig. 1, the three components explained 56.38% and 74.77% of variation for the negative and positive ion modes, respectively. The recorded fingerprints for samples A-E in both ion modes are presented in the Supplementary file (Fig. S1).

The observed differences between the samples prompted further analysis towards the identification of the different features that discriminate the samples. After processing the data, a total of 632 chemical features were chosen for a succeeding statistical analysis. Next, they were transformed logarithmically, transformed by the Mass Profiler Professional Program, normalized and compared, leaving a list of 22 entities out of 463 in the positive ion mode and 24 entities out of 169 in the negative ion mode at $P < 0.001$. The analysis of the responses lead to the creation of tables (Table 4 and Table S3) with the list of differentiating chemical features for both negative and positive ion mode, that were tentatively identified based on their accurate *m/z* measurements (the mass deviation not higher than 10 ppm), MS/MS fragmentation pattern, the nitrogen rule, the retention time and in comparison with the scientific literature (Chemspider, Metlin). The full list of differentiating components is presented in Table S2 in the Supplementary File together with the character of the occurring difference compared in pairs of samples, in relation to sample A, which stands out from the rest of the tested extracts.

The samples investigated in this study were found to contain naringenin, but in varying amounts. Sample D was found to be the richest source of naringin (the peak area of 4.6 million), followed by the samples B (1.5 million), C (670,000) and E (440 000). The compound was not present in the sample A.

Another marker that was found to demonstrate variation among the honey samples was the derivative of glutaric acid, namely the hydroxymethylglutaric acid. The molecule was identified in all analysed honey samples with different peak areas. Interestingly, the simple organic acids were found to be markers for honeydew honey samples in the previously published studies of Tischer Seraglio et al. (Seraglio et al., 2021). The authors had proved that glutaric, malic, gluconic, lactic and acetic acids differentiated the analysed samples regardless from the harvest time.

The presence of picolinic acid in the positive ion mode, which is known to express antioxidant, sleep cycle and pain regulatory action was detected in the honey samples (Liaqat et al., 2022). Its presence may be important for the therapeutic properties of honeys.

Table 4 shows the presence of other discriminating markers, that include other flavonoids, decanoic acid derivatives, glycosylated phenols and sugars, whose content is different among the analysed samples and may be directly related to the conditions where the pollen was collected by the bees. Also, the compound that was tentatively identified as secogalioside with the molecular formula of $C_{17}H_{24}O_{12}$ had its input into the differentiation of the tested samples; honey sample D was found to be a major source of this metabolite, yet in samples B and C no *m/z* signal of 419 was noted. Samples A and D were found to contain the *m/z* of 399 that was recorded in the positive ion spectra that could stand for the pantothenic acid glucoside. The presence of the aglycon was previously reported in the scientific literature by Ciulu and co-investigators (Ciulu et al., 2011).

3.3. In vitro biological activities of honey

3.3.1. Antioxidant and NO-production activities

The TBARS method was applied to detect lipid oxidation. This assay measured malondialdehyde (MDA), a by-product of the endoperoxide of unsaturated fatty acids, created as a consequence of lipid substrate oxidation. The honey samples were examined at different concentrations and the results were conveyed as EC_{50} values, indicating the concentrations in mg/mL at which the samples displayed 50% of their antioxidant activity (Table 5). Honey sample A exhibited higher antioxidant activity with $EC_{50} = 2.0 \pm 0.1$, followed by honey sample E with $EC_{50} = 4.8 \pm 0.2$. It is important to note that while both samples demonstrated antioxidant properties, they did not reach the level of antioxidant activity observed in Trolox. This positive control achieved the lowest EC_{50} value and thus the highest activity. In the CAA assay, the honey samples didn't show antioxidant activity at a concentration of 2 mg/mL. Using the DPPH method, honey sample A exhibited the highest antioxidant activity at 29.07% ± 2.00%, followed by honey sample E at 18.68% ± 2.37% and honey sample B at 14.82% ± 0.72%. This assay did not show significant antioxidant activity in Honey samples C and D.

The NO-production inhibition was measured using RAW 264.7 macrophage cells (Table 5). In comparison to the positive control, dexamethasone, honey sample B exhibited the highest capacity to inhibit the NO production, with $IC_{50} = 17.86 \pm 1.45$, followed by honey sample C which showed an $IC_{50} = 32.74 \pm 2.34$. Considering the results of NO-production inhibition of the honey, the studies continued with different cell lines.

3.3.2. Cell viability

The cytotoxic effect was tested for all honey samples in different concentration ranges (0.5%–10%) and for different times (24, 48 and 72 h) on lung cancer cell line (A549) and normal colon cell line (FHC). For all tested honeys, a cytotoxic effect was demonstrated for the A549 line,

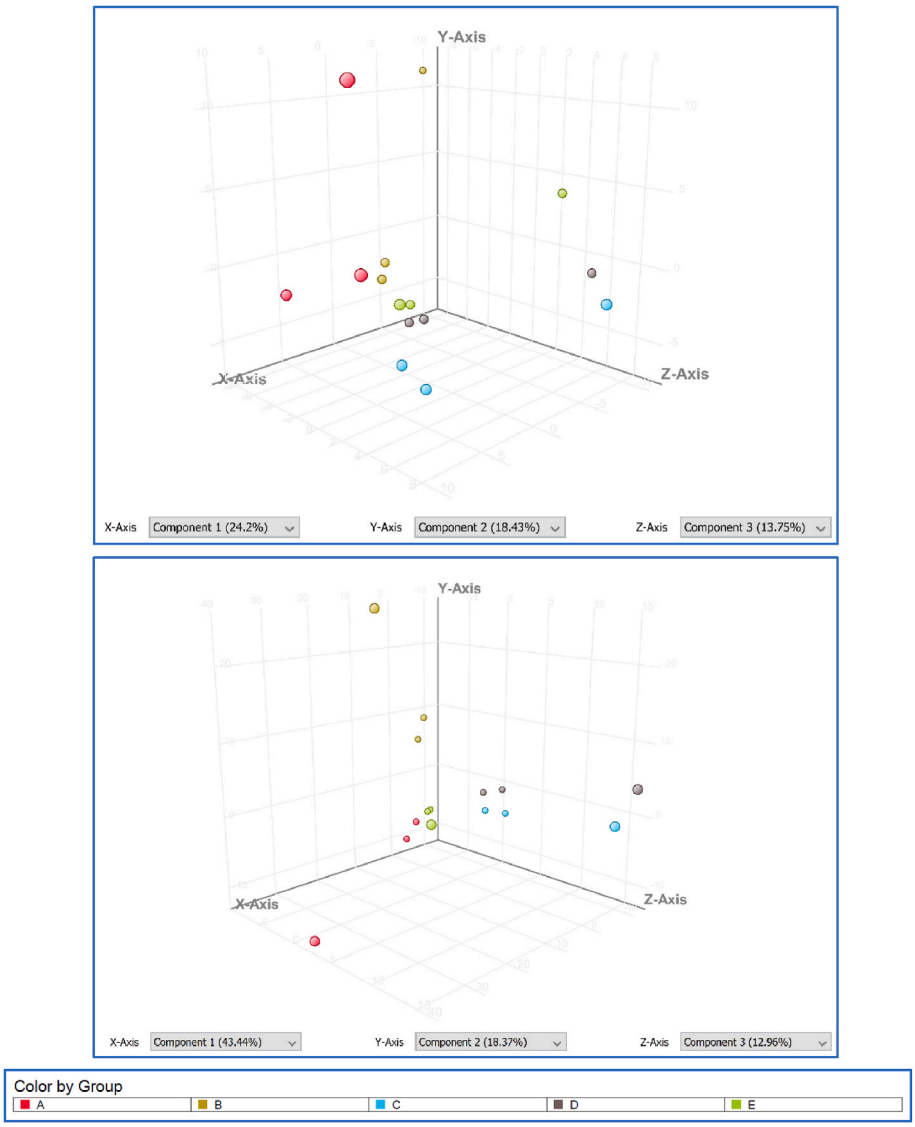

Fig. 1. The results of PCA analysis for the five samples of honey in the negative (left) and positive (right) ion mode ($P < 0.05$) that include (in grey brackets) the scores recorded for the three dimensions.

depending on the concentration and exposure time, however, the weakest effect was observed for honey sample B (Fig. 2). In turn, no cytotoxic effect was demonstrated for the normal FHC cell line for all the tested honeys after 24 h after exposure, with honey samples B and C the least active. This percentage increased over time, with the most sensitive to the effects of the cells after the treatment of honey for 72 h (Fig. 2).

3.3.3. Anti-inflammatory effect of LPS-induced honeys in an in vitro model

The anti-inflammatory effect was assessed in A549 lung cancer cells induced by LPS. After 24 h of LPS induction, there was an increase in the expression of cytokines (Fig. 3A and B), specifically IL-6 and IL-1β, at both the gene and protein levels.

However, honey samples A, D and E (Fig. 4A and B) exerted a strong effect in reducing the levels of these cytokines in LPS-induced A549 cells, indicating their potential anti-inflammatory properties. In the study evaluating the anti-inflammatory activity of honeys induced by LPS in an *in vitro* model, the effects were also investigated in a normal colon cell line (FHC cell line). The honey samples exhibited a decrease in the level of LPS-induced cytokines in these cells, indicating their potential anti-inflammatory properties.

3.3.4. Morphological changes after honey treatment in A549 and FHC cells

Microscopic observations showed morphological changes in A549 cells depending on the concentration of honey samples (Fig. 5A and B). In turn, such changes were not observed for normal FHC cells (normal colon cell line), at the concentration of 1 and 5% (Fig. 5 C). The absence of morphological changes implies that the application of honey at these concentrations did not lead to any discernible modifications in the cellular structure or morphology of the typical FHC cells. This discovery holds significance, affirming that the honey treatment exhibited no detrimental effects on the regular colon cells within the parameters of our study.

4. Discussion

This work provides a comprehensive evaluation of various quality and physicochemical parameters of Centauri® honey, examining five different samples. These assessment factors are crucial for determining commercial appeal, consumer acceptance and regulatory compliance. Parameters analysed included colour, moisture content, electrical conductivity, pH, acidity, 5-HMF content, diastase activity, proline concentration, ash, protein content and sugar analysis.

Table 4
The list of selected differentiating molecular features from the mass chromatograms that were identified as the differentiating ones, with experimental details and tentative identification (Ion – ionization mode, error – error of *m/z* measurement, DBE – double bond equivalent value, MS/MS – registered MS/MS fragments).

Differentiating chemical feature	Ion. ±	Rt [min]	Molecular formula	m/z experimental	m/z theoretical	Error	DBE	MS/MS	Proposed compound
123.0332	[M+H]+	3.1	C6H5NO2	124.039	124.0393	−2,34	5	110, 108	Picolinic acid
161.1051	[M + NH4]+	3.11	C7H12NO3	162.1115	162.1125	6.02	1	149, 120	Hydroxy-isopropylbutanolactone
162.0519	[M-H]-	1.95	C6H10O5	161.0461	161.0455	−3.41	2	147, 113	Hydroxymethylglutaric acid
176.0476	[M+H]+	22.39	C10H8O3	177.0554	177.0564	−4.43	7	149	Herniarin
180.0623	[M-H]-	20.3	C6H12O6	179.0546	179.0561	8.4	1	113, 89, 71	α-D-Glucose
212.0685	[M+H]+	17.66	C10H12O5	213.0761	213.0757	−1.65	5	168, 144	Propyl gallate
272.0681	[M-H]-	20.69	C15H12O5	271.0615	271.0612	−1.11	10	253, 197	Naringenin
286.1405	[M + CH3COO]-	15.8	C12H18O4	285.1348	285.1344	−1.53	4	61, 44	Dimethyldecadien-1,10-dioic acid
378.1877	[M + HCOO]-	15.25	C16H28O7	377.1820	377.1817	−0.78	3	–	Dimethyl-octadiene-diol-glucopyranoside
380.2035	[M + HCOO]-	16.08	C16H30O7	379.1978	379.1974	−1.17	2	334, 316	Rhamnopyranosyl-3-hydroxydecanoic acid
388.1176	[M-H]-	4.95	C20H20O8	387.1075	387.1085	2.68	11	342, 264, 211	Hydroxy-pentamethoxyflavone
388.1231	[M-H]-	3.21	C12H22O11	341.1079	341.1089	3.03	2	–	Isomaltulose
398.1906	[M + NH4]+	1.97	C15H27NO10	399,1982	399.1973	−2.21	2	–	Pantothenic acid glucoside
420.1273	[M-H]-	14.77	C17H24O12	419.1201	420.1278	−0.48	6	211	Secogalioside
440.0989	[M-H]-	3.01	C19H20O12	439.09	439.0882	−4.09	10	–	Dihydroxyphenyl-1-(galloyl-glucopyranoside)
448.1923	[M + HCOO]-	13.1	C19H30O9	447.1833	447.1872	8.67	5	402, 248	Methylepihydroxyjasmonate glucoside

Table 5
Antioxidant activity was tested through TBARS, CAA and DPPH assays. In TBARS method, EC_{50} results are expressed in mg/mL; CAA is expressed in percentage of oxidation at 2 mg/mL; DPPH assay was expressed in percentage. The anti-inflammatory activity was tested using RAW 264.7 macrophages, and the results expressed in IC_{50}, μg/mL. Results are expressed as mean ± standard deviation.

Samples	Antioxidant activity			Anti-inflammatory activity
	TBARS (EC_{50}, mg/mL)	CAA (% inhibition of oxidation at 2 mg/mL)	DPPH (%)	NO-production Inhibition (IC_{50}, μg/mL)
A	2.0 ± 0.1[a]	>2000	29.07 ± 2.00[a]	62.75 ± 6.03[a]
B	>166.7	>2000	14.82 ± 0.72[b]	17.86 ± 1.45[b]
C	>166.7	>2000	8.46 ± 2.37[c]	32.74 ± 2.34[c]
D	>166.7	>2000	7.82 ± 2.23[c]	63.76 ± 3.30[a]
E	4.8 ± 0.2[b]	>2000	18.68 ± 2.37[b]	>400
Positive control	0.0058 ± 0.0006	95.3 ± 4.6	96.04 ± 0.57[d]	6.3 ± 0.4

Positive controls: TBARS- Trolex; CAA and DPPH- Quercetin. Anti-inflammatory- Dexamethasone. DPPH - 2,2-diphenyl-1-picrylhydrazyl. Statistical differences honey samples were assessed using ANOVA followed by Tukey's post-hoc test. In collum, different letters next to values indicate significant differences at $P < 0.05$.

The colour of honey is a crucial factor for its commercial appeal, playing a significant role in determining quality, consumer acceptance and preferences (da Silva et al., 2016). According to the Codex Alimentarius (2001) honey's colour should range from nearly colourless to dark brown. The colour of the honey samples ranged from nearly colourless to dark brown, aligning with the Codex Alimentarius standards (Codex Alimentarius Commission, 2001).

Another essential parameter in evaluating honey quality is its moisture content, which affects stability and resistance to spoilage. The results (14%–18.1%, w/w) fall below the 20% (w/w) limit set by European Union's Council Directive 2001/110/EC, ensuring the quality and safety of honey (Gonçalves et al., 2018; The European Union Council Directive 2001/110/EC, 2001). Similar to (Gürbüz et al., 2020; Kahraman et al., 2010; Yılmaz & Yavuz, 1999). The difference in moisture content of honey is influenced by various factors including the level of maturity achieved, harvesting season, and ecological conditions (Sajid et al., 2020).

Electrical conductivity, linked to ash content and acidity, serves as a key indicator in honey quality control. The electrical conductivity values of the five samples comply with the recommended maximum value of 800.00 mS cm^{-1}, outlined by the Codex Alimentarius (2001).

The pH determination, while lacking a specific limit set by regulatory committees, commonly falls within the range of 3.2–4.5. In our analysis, Table 2, all honey samples exhibited acidity. Samples B and C show slightly lower pH levels than expected, while honey samples A, D and E fall within the anticipated range, emphasizing their adherence to quality standards (da Silva et al., 2016; Karabagias et al., 2014).

Free acidity is an important parameter related to honey's deterioration, regulated by the Codex Alimentarius Commission (2001) , wi th a maximum permitted value of 50.0 meqKg^{-1}. The honey samples in this study exhibited typical levels of free acidity, range of 7.6–13.7 meq/kg. Kahraman et al. consucted a study on Turkish honey, reporting higher acidity values ranging from 6.94 to 29.6 meq/kg (Kahraman et al., 2010). Similarly, Gürbüz et al. tested various types of Turkish honeys from different regions, finding mean acidity levels ranging from 12.12 to 19.25 meq/kg (Gürbüz et al., 2020). This parameter is influenced by factors such as organic acids, geographical origin and harvest season (Codex Alimentarius Commission, 2001; da Silva et al., 2016).

The 5-Hydroxymethylfurfural (5-HMF) content, a marker of honey freshness and potential overheating, adheres to the standards set by the Codex Alimentarius Commission's (2001). According to these guidelines, 5-HMF content in honey should not exceed 40 mg/kg. In our study, the honey samples ranged between 3.0 and 9.70 mg/kg, indicative of fresh honey (Codex Alimentarius Commission, 2001; Tornuk et al., 2013). In contract, studies by Sajid et al., Boussaid et al., and Parviz et al. reported higher values ranging from 24.45 to 40.68 mg/kg, 27.43 ± 1.50 mg/kg and 37.31 ± 17.13 mg/kg, respectively (Boussaid et al., 2018; Parviz et al., 2015; Sajid et al., 2020).

Diastase activity, crucial for indicating aging and exposure to elevated temperatures, meets the minimum level of 8 diastase units set by Codex Alimentarius and The European Honey Directive for all

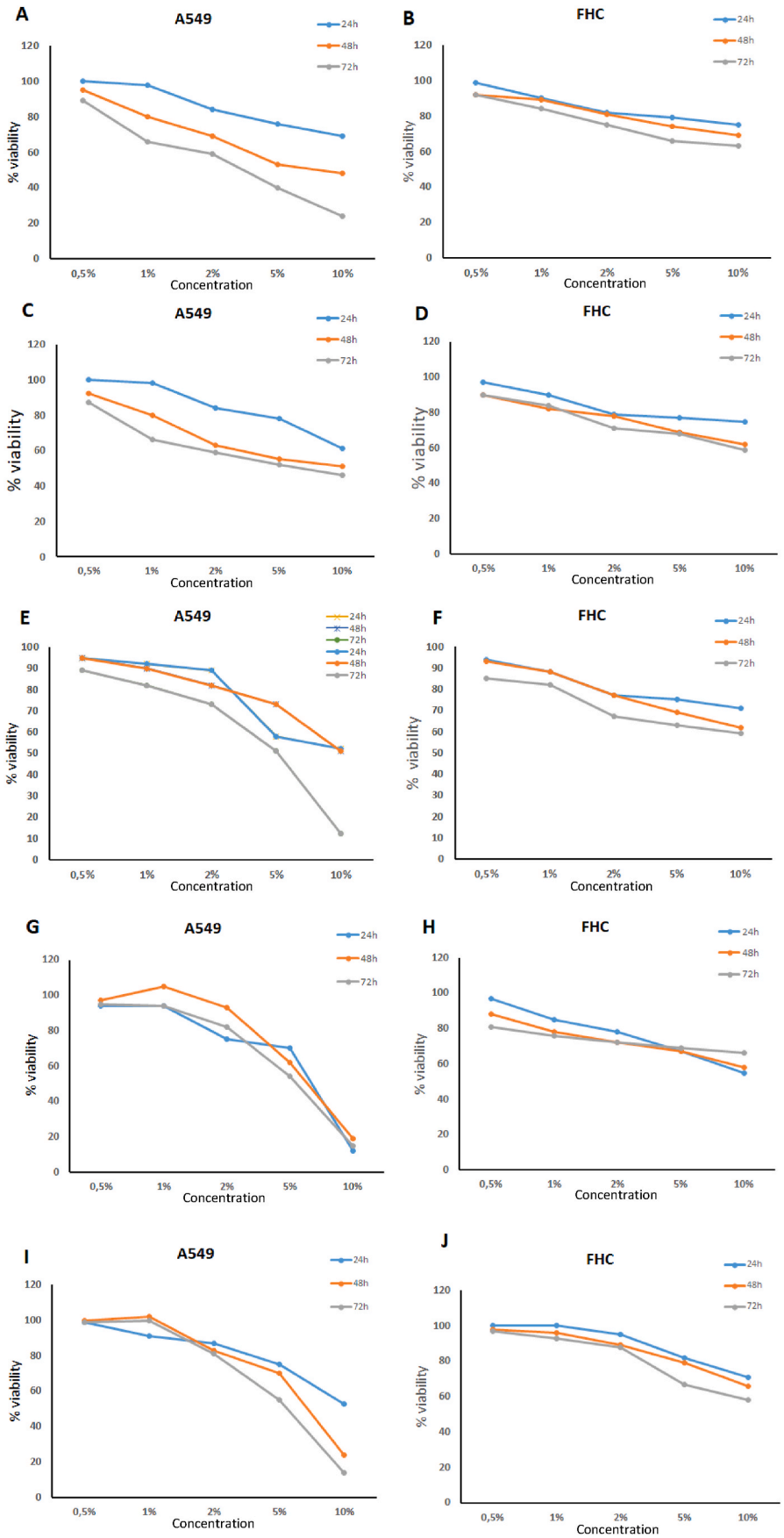

Fig. 2. Cell viability determined by MTT assay after stimulation for 24h, 48h and 72h: with honey A in A549 (A), FHC cells (B); with honey B in A549 (C) and FHC cells (D); with honey C in A549 (E) and FHC cells (F); after stimulation with honey D in A549 (G) and FHC cells (H); with honey E in A549 (I) and FHC cells (J).

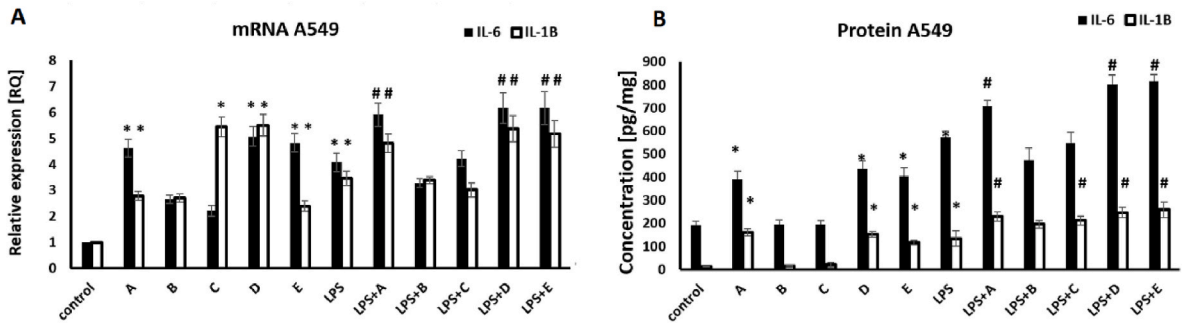

Fig. 3. The effect of the tested honeys on the expression of pro-inflammatory cytokines in A549 cells. Expression at the mRNA (A) and protein (B) levels. Data presented as mean; *P < 0.05 comparing to control, #P < 0.05 comparing to LPS.

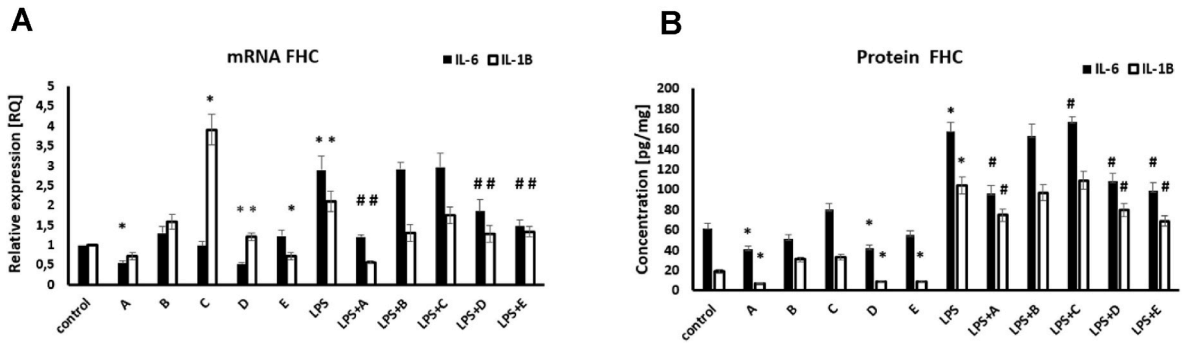

Fig. 4. Evaluation of the effect of selected honeys on mRNA (A) and protein expression of IL-1β and IL-6 in FHC cells. Data presented as mean; *P < 0.05 comparing to control, #P < 0.05 comparing to LPS.

samples except honey D, which exhibits a lower diastase index, possibly associated with extended storage or heat exposure (Bogdanov, 2016; Codex Alimentarius Commission, 2001; Fechner et al., 2016).

Proline concentration serves as a valuable indicator for honey maturation, with levels below 180 mg kg^{-1} raising concerns about purity. In our study, all honey samples meet the minimum threshold, indicating their authenticity and quality (Bentabol Manzanares et al., 2014; Bogdanov et al., 1999; da Silva et al., 2016; Hermosín et al., 2003).

In turn, the ash content is a quality parameter that evaluates the mineral content in honey. It depends on factors such as geographical origin, nutritional properties and the methods employed in harvesting and beekeeping. Consequently, the ash content is influenced by the soil composition where flowers have flourished and the type of nectar gathered by bees (Rajindran et al., 2022; Rysha et al., 2022). According to the Codex Alimentarius, the ash content for blossom honey should be below 0.6 g/100 g, whereas for honeydew honey or a mixture of honeydew honey with blossom honey shouldn't exceed 1.2 g/100 g (Codex Alimentarius Commission, 2001). In our study, ash content varied with samples B to E displaying typical levels for blossom honey, and sample A indicating a possible mix of honeydew and blossom honey due to its higher ash content.

The sugar content of the honey samples was analysed according to the Codex Alimentarius Commission (2001). Fructose and glucose are the primary sugars found in honey. The ratio and composition of these sugars in honey are influenced not only by the local plant flora but also by geographical conditions and climate factors (Gürbüz et al., 2020). The Commission stipulates that the sucrose content in honey should not exceed 5 g/100 g. In our analysis, only honey sample A showed detectable sucrose, with a level of 0.59 ± 0.05 g/100 g, well within the Codex standards. Exceeding this limit would suggest honey adulteration (Demir Kanbur et al., 2021). The combined fructose + glucose content in the honey samples ranged from 58.02% to 74.54%. Honey sample A exhibited a value slight below of 60 g/100 g threshold set by the EU directive, whereas the other samples met this standard. Statistically significant differences were observed in the sugar content among the honey samples. Honey A, sourced from Western Turkey, exhibited the lowest levels of fructose and glucose, and was unique in containing sucrose. In contrast, honey D, originating from Eastern Turkey, showed the highest levels of fructose and glucose. Gürbüz et al. studied 68 floral honey samples from Southeastern Anatolia of Turkey and the results showed F + G ranging from 62.55% to 77.25% (Gürbüz et al., 2020).

The *in vitro* biological activities of honey, which include antioxidant, NO-production inhibition, and cell viability, were studied.

The TBARS method was employed to detect lipid oxidation by measuring malondialdehyde (MDA), a by-product formed during the oxidation of unsaturated fatty acids. Honey samples were tested at various concentrations, with results reported as EC_{50} values. Honey sample A exhibited the highest antioxidant activity with an EC_{50} of 2.0 ± 0.1 mg/mL, followed by honey sample E with an EC_{50} of 4.8 ± 0.2 mg/mL. Despite their significant antioxidant properties, neither sample matched the activity of the positive control, Trolox, which had the lowest EC_{50} value, indicating the highest antioxidant activity. In the CAA assay, honey samples did not exhibit antioxidant activity at a concentration of 2 mg/mL.

Using the DPPH method, the antioxidant activity of the honey samples ranged from 8% to 29%. Honey sample A showed the highest antioxidant activity at 29.07 ± 2.00 % (honeydew honey), followed by honey sample E at 18.68 ± 2.37% (polyfloral honey), and honey sample B at 14.82 ± 0.72% (polyfloral honey). Honey samples C and D did not demonstrate significant antioxidant activity in this assay. Studies have shown a strong correlation between antioxidant capacity and the content of flavonoids and polyphenols, which depend on the botanical origin of the honey (Chirsanova et al., 2021; Pătruică et al., 2022; Pauliuc et al., 2020). Chirsanova et al. investigated sunflower, rapeseed, polyflora, and honeydew honeys, reporting DPPH values ranging from

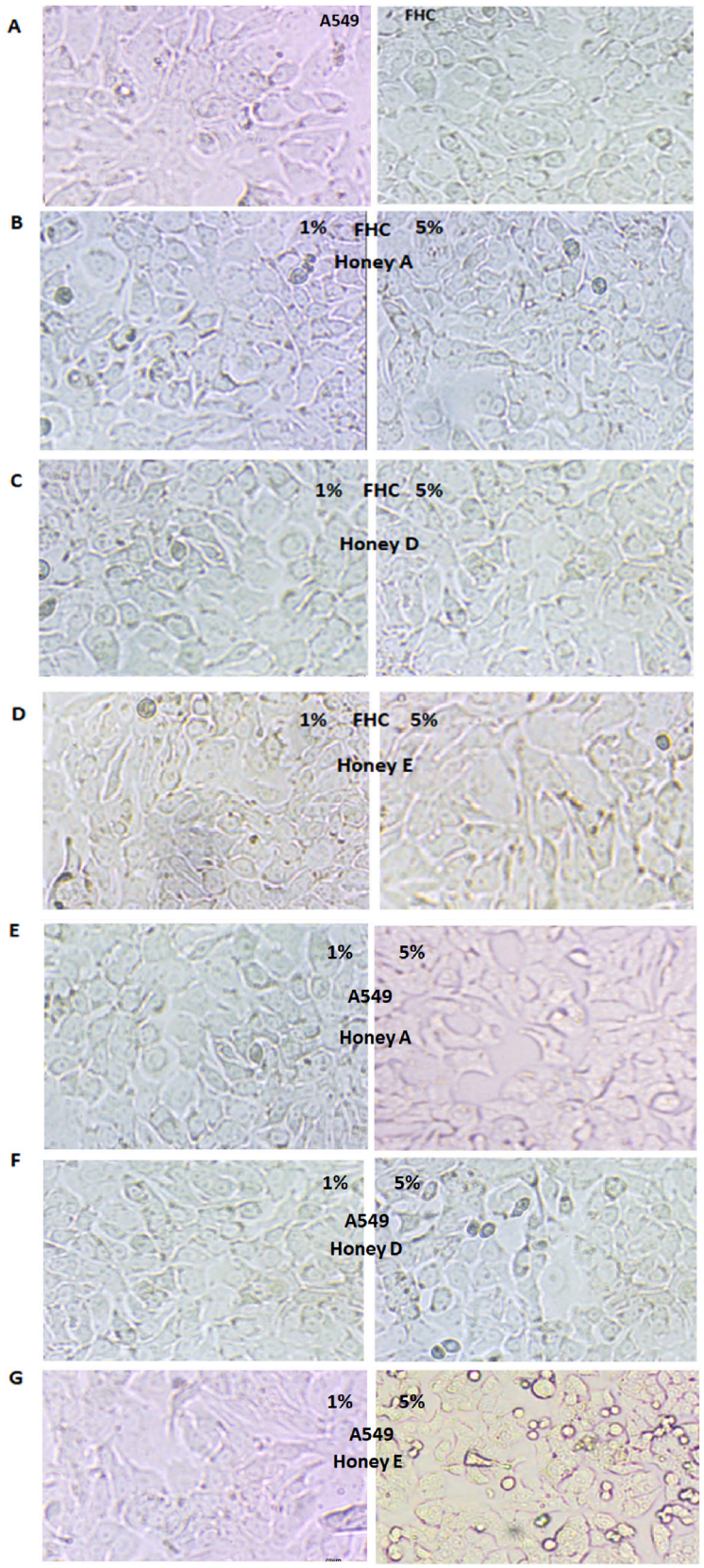

Fig. 5. Morphology of the cells before and after honey stimulation: control picture of A549 and FHC cells (A); FHC cells after stimulation with honey sample A (B); FHC with honey sample B (C); FHC and honey sample E (D). A549 cells after stimulation with honey sample A (E); A549 cells with honey sample D (F); and A549 cells with honey sample E (G). Magnification x40.

16.03% for rapeseed honey to 72.03% for honeydew honey (Chirsanova et al., 2021). Their analysis of the antioxidant activity of rapeseed, sunflower, and polyfloral honeys yielded DPPH values from 55.4% for rapeseed honey to 70.7% for polyfloral honey. DPPH values for honeydew honey vary by botanical origin and geographical area. Flores et al. reported DPPH values of 52.9%–95.6% for Spanish honeydew (Flores et al., 2015). Karabagias et al. found antioxidant activity percentages ranging from 56.8% to 72.4% in Greek honey (Karabagias et al., 2014). Vasic et al. reported Croatian honey with antioxidant activity between 12.2% and 48.89% (Vasić et al., 2019). Mracevic et al. showed Serbian honey with antioxidant activity ranging from 75.89% to 79.1% (Mračević et al., 2020). Pătruică et al. tested Romanian honey, finding DPPH values between 41.88% for acacia honey and 79.20% for honeydew honey (Pătruică et al., 2022).

NO-production inhibition was measured using RAW 264.7 macrophage cells. Compared to the positive control, dexamethasone, honey sample B exhibited the highest NO-production inhibition, with an IC_{50} of 17.86 ± 1.45 μg/mL, followed by honey sample C with an IC_{50} of 32.74 ± 2.34 μg/mL. Several honeys have been reported as anti-inflammatory agents in cell models. Gasparrini et al. investigated the protective effects of Manuka honey against *E. coli* LPS-induced oxidative stress and inflammation in murine RAW 264.7 macrophages, concluding that Manuka honey serves as a natural agent for preventing oxidative stress and inflammation-related diseases. Their study showed that pre-treatment with Manuka honey significantly inhibited LPS-induced ROS and nitrite accumulation, thus protecting cellular biomolecules. Pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, as well as other inflammatory mediators (iNOS), were upregulated following LPS treatment, but Manuka honey effectively suppressed these inflammatory markers (Gasparrini et al., 2018). Similarly, Silva et al. investigated the anti-inflammatory activity of bracatinga (*Mimosa scabrella* Bentham) honeydew honeys at various concentrations. Their study found that treatment with samples Bhh 2, 3, 4, and 8 suppressed IL-6 secretion, with reductions ranging from 19% for Bhh 2 (at 30 μg/mL) to 35% for Bhh 4 (at 30 μg/mL). In contrast, the other honey samples did not inhibit IL-6 production by macrophages (Silva et al., 2022).

The final step in our research was to determine, for the first time, the LC-MS phytochemical panel and biological activity of the Centauri® honeys tested in an *in vitro* model. The health-promoting properties of honeys have been known for a very long time in traditional medicine. Numerous studies confirm that honey can have anticancer, antioxidant, anti-inflammatory and wound healing effects (El-Senduny et al., 2021; Ranneh et al., 2021). This may be due to the fact that they contain various secondary metabolites, which include flavonoids, phenolic acids, as well as macro- and microelements, including sugars, free amino acids, proteins, enzymes, essential minerals and vitamins. Our results displayed that all of the Centauri® honeys tested showed a cytotoxic effect against the A549 lung cancer line in the tested concentration range. Our results are in agreement with Bazaid et al. who showed a cytotoxic effect against MCF-7 breast and A549 lung cancer cells with IC_{50} values of 9.05 and 9.37 mg/mL for Manuka honey, respectively (Bazaid et al., 2022). Similarly, Amran et al. showed that Tualang honey also demonstrated a cytotoxic effect and induced apoptosis in human lung adenocarcinoma cells (H23 and A549) (Amran et al., 2020). In another study by Siti Norfitrah et al., they revealed that another type of honey, Trigona itama honey, also exhibited cytotoxic effects on A549 lung cancer lines (Salim et al., 2019). We hypothesize that active compounds contained in the analysed honeys belonging to different classes such as flavonoids, terpenoids, fatty acids or sugars may be responsible for this effect.

The situation is similar for the anti-inflammatory effect of Centauri® honey in an LPS-induced *in vitro* model. The results we presented showed that the tested honeys exhibited a bidirectional mechanism of action, i.e., in LPS-induced A549 lung cancer cells we observed an increase in the inflammatory cytokines IL-6 and IL-1β, which can be regarded as an induction of apoptosis, while for normal FHC intestinal epithelial cells induced with LPS a decrease in these cytokines was observed, which may confirm the anti-inflammatory effect, both at gene and protein level. Similarly to the cytotoxic effect, they are similar for all Centauri® honey samples tested, but a stronger trend can be observed for honey A and D in the tested combinations for the normal FHC cell line and the cancer A549 cell line. We suggest that this effect may be related to the different content of active compounds found in the tested honeys. LC-MS analysis showed that honey sample A has less sugars, i.e. hexose and maltulose derivatives, compared to other honey samples B-E, which may better demonstrate the effects of its other ingredients. In turn, honey sample D, compared to A, contained more naringenin, secogalioside, dimethyl-octadien-diol-glcopyranoside, dimethyldecadien-1,10-dioic acid and dihydroxyphenyl-1-(galloyl-glucopyranoside). Naringenin, a flavanone, demonstrates an extensive range of biological activities, such as anti-inflammatory, antioxidant and skin-healing effects. Naringenin was discovered to be present in the study's samples; nevertheless, it was also discovered that this flavonoid could be used to differentiate the tested samples. The presence of naringenin was previously confirmed by Kurtagic et al. who underlined that this flavonoid may be an indicator of honey originality (Kurtagić et al., 2016). In the samples from Bosnia and Herzegovina, naringin was found to be a leading flavonoid with measured concentration of 41.4 μg/100 g honey.

The hydroxymethylglutaric acid derivative of glutaric acid was another marker intended to distinguish between the honey samples. Each of the samples that were analysed contained this metabolite, albeit with distinct peak areas. Interestingly, the simple organic acids were found to be markers for honeydew honey samples in the previously published studies of Tischer Seraglio et al. (Seraglio et al., 2021). The authors had proved that glutaric, malic, gluconic, lactic and acetic acids differentiated the analysed samples regardless of the harvest time.

Badrulhisham et al. showed that at a concentration of 1% Kelulut honey significantly inhibited the production of NO in LPS-induced RAW 264.7 cells compared to control cells (Badrulhisham et al., 2020). In turn, Nooh et al. demonstrated an anti-inflammatory effect induced by sodium dextran sulfate in honey-treated patients by significantly reducing the levels of oxidative, inflammatory and apoptotic markers (interleukin-1β and −6, superoxide dismutase, reduced glutathione, tumor necrosis factor-α, NO synthase, caspase-3, CD34, Ki67, S100, c-kit and neuron-specific enolase) (Nooh & Nour-Eldien, 2016). In other studies, Gasparrini et al. showed that manuka honey inhibited LPS-induced ROS and nitrite accumulation, by reducing TNF-α, IL-1β, IL-6 and iNOS (Gasparrini et al., 2018). Additionally, Hussein et al. in an *in vivo* model, showed that Gelam honey had anti-inflammatory effects by reducing the size of rat paw swelling and inhibiting the production of pro-inflammatory mediators NO, PGE2, TNF-α and IL-6 in the plasma, as well as inhibiting the expression of iNOS, COX-2, TNF-α and IL -6 in paw tissue (Hussein et al., 2012). Our results confirm the anti-inflammatory effect of Centauri® honey, which may be a good prognosis for further biological tests.

In a recent review by Ayoub et al. focused on the exploitation of polyphenolic compounds in honey and their potential in preventing chronic diseases. The authors highlighted honey's health-promoting benefits across various systems: it supports respiratory health against asthmatic and bacterial infections, protects the duodenal system, and safeguards the blood-vascular system by preventing the oxidation of low-density lipoproteins. Additionally, honey was noted for its positive effects on brain health (Ayoub et al., 2023).

5. Conclusions

This study assesses the quality, physicochemical properties, and biological activities of five types of Centauri® honey from Turkey. Known for its rich biodiversity and unique geography, Turkey's robust apiculture yields honey with a complex matrix of sugars, enzymes, amino acids and other bioactive compounds. The antioxidant capacities were determined, revealing that both honey samples A and E exhibited

antioxidant activities, with sample A performing better than E, while antimicrobial and antifungal assays indicated limited activity. Additionally, cytotoxic effects against the A549 lung cancer line were significant across all samples, corroborating honey's potential anticancer benefits. Anti-inflammatory effects were also noted, particularly with honey samples A and D in an LPS-induced model. Phytochemical analysis via LC-MS highlighted bioactive compounds such as naringenin and hydroxymethylglutaric acid, suggesting diverse therapeutic potentials. Overall, Centauri® honey meets Codex Alimentarius standards and shows promising health benefits, meriting further research for clinical applications.

CRediT authorship contribution statement

Márcia Santos Filipe: Writing – original draft, Investigation. **Tomasz Kowalczyk:** Visualization, Methodology. **Wirginia Kukula-Koch:** Writing – review & editing, Investigation. **Joanna Wieczfinska:** Writing – review & editing, Visualization, Investigation. **Gabrielle Bangay:** Writing – review & editing. **Ana María Diaz-Lanza:** Visualization. **Rossana V.C. Cardoso:** Visualization, Investigation. **Filipa Mandim:** Writing – review & editing, Visualization, Investigation. **Soraia I. Falcão:** Writing – review & editing, Validation, Methodology. **Miguel Vilas-Boas:** Visualization, Validation. **Tomasz Śliwiński:** Writing – review & editing, Validation, Methodology. **Przemysław Sitarek:** Writing – review & editing, Validation, Supervision, Methodology. **Patrícia Ríjo:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, 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.

Data availability

Data will be made available on request.

Acknowledgements

The authors are grateful to the Fundação para a Ciência e Tecnologia (FCT, Portugal) for their financial support through the projects with DOI 10.54499/UIDP/04567/2020 and DOI 10.54499/UIDB/04567/2020 (https://doi.org/10.54499/UIDP/04567/2020), awarded to CBIOS; This work was supported by national funds through FCT/MCTES (PIDDAC): CIMO, UIDB/00690/2020 (DOI: 10.54499/UIDB/00690/2020) and UIDP/00690/2020 (DOI: 10.54499/UIDP/00690/2020); and SusTEC, LA/P/0007/2020 (DOI: 10.54499/LA/P/0007/2020), and the contracts of Soraia I. Falcão through the institutional scientific employment program-contract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.105028.

References

Alqarni, A. S., Owayss, A. A., Mahmoud, A. A., & Hannan, M. A. (2014). Mineral content and physical properties of local and imported honeys in Saudi Arabia. *Journal of Saudi Chemical Society, 18*(5), 618–625. https://doi.org/10.1016/j.jscs.2012.11.009

Amran, N., Wan-Ibrahim, W. I., Rashid, N. N., Ali, J. M., & Abdul-Rahman, P. S. (2020). Tualang honey inhibits cell proliferation and promotes apoptosis of human lung adenocarcinoma cells via apoptosis signaling pathway. *Eur. J. Integr. Med., 37*, Article 101149. https://doi.org/10.1016/j.eujim.2020.101149

Ayoub, W. S., Ritu, Zahoor, I., Dar, A. H., Farooq, S., Ahmad Mir, T., Ahmad Ganaie, T., Srivastava, S., Pandey, V. K., & Altaf, A. (2023). Exploiting the polyphenolic potential of honey in the prevention of chronic diseases. *Food Chem. Adv, 3*. https://doi.org/10.1016/j.focha.2023.100373

Badrulhisham, N. S. R., Ab Hamid, S. N. P., Ismail, M. A. H., Yong, Y. K., Muhamad Zakuan, N., Harith, H. H., Saidi, H. I., & Nurdin, A. (2020). Harvested locations influence the total phenolic content, antioxidant levels, cytotoxic, and anti-inflammatory activities of stingless bee honey. *Journal of Asia-Pacific Entomology, 23*(4), 950–956. https://doi.org/10.1016/j.aspen.2020.07.015

Bazaid, A. S., Alamri, A., Almashjary, M. N., Qanash, H., Almishaal, A. A., Amin, J., Binsaleh, N. K., Kraiem, J., Aldarhami, A., & Alafnan, A. (2022). Antioxidant, anticancer, Antibacterial, antibiofilm properties and gas chromatography and mass spectrometry analysis of manuka honey: A nature's bioactive honey. *Applied Sciences, 12*(19), 9928. https://doi.org/10.3390/app12199928

Bentabol Manzanares, A., Hernández García, Z., Rodríguez Galdón, B., Rodríguez Rodríguez, E., & Díaz Romero, C. (2014). Physicochemical characteristics of minor monofloral honeys from Tenerife, Spain. *LWT - Food Science and Technology, 55*(2), 572–578. https://doi.org/10.1016/j.lwt.2013.09.024

Bogdanov, S. (2002). Harmonised methods of the international honey commission. *International Honey Commission (IHC)*, 1–62.

Bogdanov, S. (2016). Honey composition. In *The honey book*.

Bogdanov, S., Lüllmann, C., Martin, P., von der Ohe, W., Russmann, H., Vorwohl, G., Oddo, L. P., Sabatini, A.-G., Marcazzan, G. L., Piro, R., Flamini, C., Morlot, M., Lhéritier, J., Borneck, R., Marioleas, P., Tsigouri, A., Kerkvliet, J., Ortiz, A., Ivanov, T., & Vit, P. (1999). Honey quality and international regulatory standards: Review by the international honey commission. *Bee World, 80*(2), 61–69. https://doi.org/10.1080/0005772X.1999.11099428

Boussaid, A., Chouaibi, M., Rezig, L., Hellal, R., Donsì, F., Ferrari, G., & Hamdi, S. (2018). Physicochemical and bioactive properties of six honey samples from various floral origins from Tunisia. *Arabian J. Chem., 11*(2), 265–274. https://doi.org/10.1016/j.arabjc.2014.08.011

Childress, B. B., & Stechmiller, J. K. (2002). Role of nitric oxide in wound healing. *Biological Research For Nursing, 4*(1), 5–15. https://doi.org/10.1177/1099800402004001002

Chirsanova, A., Capcanari, T., Boistean, A., & Siminiuc, R. (2021). Physico-chemical profile of four types of honey from the south of the Republic of Moldova. *Food and Nutrition Sciences, 12*(9), 874–888. https://doi.org/10.4236/fns.2021.129065

Ciulu, M., Solinas, S., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Spano, N., & Sanna, G. (2011). RP-HPLC determination of water-soluble vitamins in honey. *Talanta, 83*(3), 924–929. https://doi.org/10.1016/j.talanta.2010.10.059

Codex Alimentarius Commission. (2001). Codex Alimentarius commission. Rev. 1(1987). *Rev, 2*, 2001). Revised Codex Standard for Honey. Codex Stan 12-1981.

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry, 196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.05

de la Fuente, B., Pinela, J., Mandim, F., Heleno, S. A., Ferreira, I. C. F. R., Barba, F. J., Berrada, H., Caleja, C., & Barros, L. (2022). Nutritional and bioactive oils from salmon (*Salmo salar*) side streams obtained by Soxhlet and optimized microwave-assisted extraction. *Food Chemistry, 386*, Article 132778. https://doi.org/10.1016/j.foodchem.2022.132778

Demir Kanbur, E., Yuksek, T., Atamov, V., & Ozcelik, A. E. (2021). A comparison of the physicochemical properties of chestnut and highland honey: The case of Senoz Valley in the Rize province of Turkey. *Food Chemistry, 345*, Article 128864. https://doi.org/10.1016/j.foodchem.2020.128864

El-Senduny, F. F., Hegazi, N. M., Abd Elghani, G. E., & Farag, M. A. (2021). Manuka honey, a unique mono-floral honey. A comprehensive review of its bioactives, metabolism, action mechanisms, and therapeutic merits. *Food Bioscience, 42*, Article 101038. https://doi.org/10.1016/j.fbio.2021.101038

Fechner, D. C., Moresi, A. L., Ruiz Díaz, J. D., Pellerano, R. G., & Vazquez, F. A. (2016). Multivariate classification of honeys from Corrientes (Argentina) according to geographical origin based on physicochemical properties. *Food Bioscience, 15*, 49–54. https://doi.org/10.1016/j.fbio.2016.05.002

Feitor Fernandes, A. S. (2010). *Propriedades nutricionais, nutracêuticas e antioxidantes de espécies silvestres condimentares utilizadas na gastronomia tradicional do nordeste transmontano*. Instituto Politécnico de Bragança.

Fernandes, Â., Polyzos, N., Mandim, F., Pereira, C., Petrović, J., Soković, M., & Petropoulos, S. A. (2023). Combined effect of biostimulants and mineral fertilizers on crop performance and fruit quality of watermelon plants. *Horticulturae, 9*(7), 838. https://doi.org/10.3390/horticulturae9070838

Flores, M., Escuredo, O., & Carmen Seijo, M. (2015). Assessment of physicochemical and antioxidant characteristics of Quercus pyrenaica honeydew honeys. *Food Chemistry, 166*, 101–106. https://doi.org/10.1016/j.foodchem.2014.06.005

Food and Agriculture Organization of the United Nations, FAOSTAT, Crops and Livestock Products. Retrieved July 15, 2024, from https://www.fao.org/faostat/en/#data/QCL/visualize.

Gasparrini, M., Afrin, S., Forbes-Hernández, T. Y., Cianciosi, D., Reboredo-Rodriguez, P., Amici, A., Battino, M., & Giampieri, F. (2018). Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation. *Food Chem. Toxicol., 120*, 578–587. https://doi.org/10.1016/j.fct.2018.08.001

Gonçalves, J., Ribeiro, I., Marçalo, J., Rijo, P., Faustino, C., & Pinheiro, L. (2018). Physicochemical, antioxidant and antimicrobial properties of selected Portuguese commercial monofloral honeys. *Journal of Food and Nutrition Research, 6*(10), 645–654. https://doi.org/10.12691/jfnr-6-10-5

Gürbüz, S., Çakıcı, N., Mehmetoğlu, S., Atmaca, H., Demir, T., Arıgül Apan, M., Atmaca, Ö. F., & Güney, F. (2020). Physicochemical quality characteristics of southeastern Anatolia honey, Turkey. *Int. J. Anal. Chem.*, 1–6. https://doi.org/10.1155/2020/8810029

Hermosín, I., Chicón, R. M., & Dolores Cabezudo, M. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry, 83*(2), 263–268. https://doi.org/10.1016/S0308-8146(03)00089-X

Hussein, S. Z., Mohd Yusoff, K., Makpol, S., & Mohd Yusof, Y. A. (2012). Gelam honey inhibits the production of proinflammatory. *Mediators NO, PGE2, TNF- α, and IL-6 in Carrageenan-Induced Acute Paw Edema in Rats. Evid.-Based Compl. Alt.*, 1–13. https://doi.org/10.1155/2012/109636

Kahraman, T., Buyukunal, S. K., Vural, A., & Altunatmaz, S. S. (2010). Physico-chemical properties in honey from different regions of Turkey. *Food Chemistry, 123*(1), 41–44. https://doi.org/10.1016/j.foodchem.2010.03.123

Karabagias, I. K., Badeka, A., Kontakos, S., Karabournioti, S., & Kontominas, M. G. (2014). Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical parameters and chemometrics. *Food Chemistry, 146*, 548–557. https://doi.org/10.1016/j.foodchem.2013.09.105

Koetzler, R., Zaheer, R. S., Newton, R., & Proud, D. (2009). Nitric oxide inhibits IFN regulatory factor 1 and nuclear factor-κB pathways in rhinovirus-infected epithelial cells. *J. Allergy Clin. Immun., 124*(3), 551–557. https://doi.org/10.1016/j.jaci.2009.04.041

Kurtagić, H., Memić, M., & Barudanović, S. (2016). Determination of type of honey produced in the different climatic regions of Bosnia and Herzegovina. *International journal of Environmental Science and Technology*, 2721–2730. https://doi.org/10.1007/s13762-016-1101-5

Liaqat, H., Parveen, A., & Kim, S. Y. (2022). Neuroprotective natural products' regulatory effects on depression via gut–brain Axis targeting tryptophan. *Nutrients, 14*(16), 3270. https://doi.org/10.3390/nu14163270

Mandim, F., Petropoulos, S. A., Pinela, J., Dias, M. I., Kostic, M., Soković, M., Ferreira, I. C. F. R., Santos-Buelga, C., & Barros, L. (2022). Phenolic composition and antioxidant, anti-inflammatory, cytotoxic, and antimicrobial activities of cardoon blades at different growth stages. *Biology, 11*(5), 699. https://doi.org/10.3390/biology11050699

Mayack, C., & Hakanoğlu, H. (2022). Honey bee pathogen prevalence and interactions within the marmara region of Turkey. *Veterinary Sciences, 9*(10), 573. https://doi.org/10.3390/vetsci9100573

Mračević, S., Krstić, M., Lolić, A., & Ražić, S. (2020). Comparative study of the chemical composition and biological potential of honey from different regions of Serbia. *Microchemical Journal, 152*, Article 104420. https://doi.org/10.1016/j.microc.2019.104420

Nooh, H. Z., & Nour-Eldien, N. M. (2016). The dual anti-inflammatory and antioxidant activities of natural honey promote cell proliferation and neural regeneration in a rat model of colitis. *Acta Histochemica, 118*(6), 588–595. https://doi.org/10.1016/j.acthis.2016.06.006

Ntungwe, E., Domínguez-Martín, E. M., Teodósio, C., Teixidó-Trujillo, S., Capote, N., Saraiva, L., Díaz-Lanza, A. M., Duarte, N., & Rijo, P. (2021). Preliminary biological activity screening of plectranthus spp. extracts for the search of anticancer lead molecules. *Pharmaceuticals, 14*, 402. https://doi.org/10.3390/ph14050402

Parviz, M., Karimi, F., Rezaei, M., Javanmard, M. R., Javadzadeh, M., & Allahdadi, G. (2015). Assessment of the physicochemical quality of Iranian honey. *Qual. Assur. Saf. Crop, 7*(5), 629–634. https://doi.org/10.3920/QAS2014.0467

Pascual-Maté, A., Osés, S. M., Fernández-Muiño, M. A., & Sancho, M. T. (2018). Métodos analíticos en mieles. *J. Apic. Res., 57*(1), 38–74. https://doi.org/10.1080/00218839.2017.1411178

Pătruică, S., Alexa, E., Obiștioiu, D., Cocan, I., Radulov, I., Berbecea, A., Lazăr, R. N., Simiz, E., Vicar, N. M., Hulea, A., & Moraru, D. (2022). Chemical composition, antioxidant and antimicrobial activity of some types of honey from banat region, Romania. *Molecules, 27*(13), 4179. https://doi.org/10.3390/molecules27134179

Pauliuc, D., Dranca, F., & Oroian, M. (2020). Antioxidant activity, total phenolic content, individual phenolics and physicochemical parameters suitability for Romanian honey authentication. *Foods, 9*(3), 306. https://doi.org/10.3390/foods9030306

Rajindran, N., Wahab, R. A., Huda, N., Julmohammad, N., Shariff, A. H. M., Ismail, N. I., & Huyop, F. (2022). Physicochemical properties of a new green honey from banggi Island. *Sabah. Molecules, 27*(13), 4164. https://doi.org/10.3390/molecules27134164

Ranneh, Y., Akim, A. M., Hamid, H. A., Khazaai, H., Fadel, A., Zakaria, Z. A., Albujja, M., & Bakar, M. F. A. (2021). Honey and its nutritional and anti-inflammatory value. *BMC Complementary Med. Ther, 21*(1), 30. https://doi.org/10.1186/s12906-020-03170-5

Rysha, A., Kastrati, G., Biber, L., Sadiku, V., Rysha, A., Zogaj, F., & Kabashi-Kastrati, E. (2022). Evaluating the physicochemical properties of some kosovo's and imported honey samples. *Applied Sciences, 12*(2), 629. https://doi.org/10.3390/app12020629

Sajid, M., Yamin, M., Asad, F., Yaqub, S., Ahmad, S., Mubarik, M. A. M. S., Ahmad, B., Ahmad, W., & Qamer, S. (2020). Comparative study of physio-chemical analysis of fresh and branded honeys from Pakistan. *Saudi Journal of Biological Sciences, 27*(1), 173–176. https://doi.org/10.1016/j.sjbs.2019.06.014

Salim, S. N. M., Ramakreshnan, L., Fong, C. S., Wahab, R. A., & Rasad, M. S. B. A. (2019). In-vitro cytotoxicity of Trigona itama honey against human lung adenocarcinoma epithelial cell line (A549). *Eur. J. Integr. Med., 30*, Article 100955. https://doi.org/10.1016/j.eujim.2019.100955

Sancho, M. T., Muniategui, S., Sánchez, P., Huidobro, J. F., & Simal-Lozano, J. (1992). Evaluating soluble and insoluble ash, alkalinity of soluble and insoluble ash and total alkalinity of ash in honey using electrical conductivity measurements at 20 °C. *Apidologie, 23*(4), 291–297. https://doi.org/10.1051/apido:19920403

Saxena, S., Gautam, S., & Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry, 118*(2), 391–397. https://doi.org/10.1016/j.foodchem.2009.05.001

Scepankova, H., Combarros-Fuertes, P., Fresno, J. M., Tornadijo, M. E., Dias, M. S., Pinto, C. A., Saraiva, J. A., & Estevinho, L. M. (2021). Role of honey in advanced wound care. *Molecules, 26*(16). https://doi.org/10.3390/molecules26164784

Seraglio, S. K. T., Bergamo, G., Brugnerotto, P., Gonzaga, L. V., Fett, R., & Costa, A. C. O. (2021). Aliphatic organic acids as promising authenticity markers of bracatinga honeydew honey. *Food Chemistry, 343*, Article 128449. https://doi.org/10.1016/j.foodchem.2020.128449

Shugaba, A. (2012). Analysis of biochemical composition of honey samples from north-east Nigeria. *Biochemistry & Analytical Biochemistry, 2*(3). https://doi.org/10.4172/2161-1009.1000139

Silva, B., Caon, T., Mohr, E. T. B., Biluca, F. C., Gonzaga, L. V., Fett, R., Dalmarco, E. M., & Costa, A. C. O. (2022). Phenolic profile and *in vitro* anti-inflammatory activity of *Mimosa scabrella* Bentham honeydew honey in RAW 264.7 murine macrophages. *Journal of Food Biochemistry, 46*(2). https://doi.org/10.1111/jfbc.14076

Silva, G. C. C., Machado, M. de A., Sakumoto, K., Inumaro, R. S., Gonçalves, J. E., Mandim, F., Vaz, J., do Valle, J. S., Faria, M. G. I., Ruiz, S. P., Piau Junior, R., Gonçalves, D. D., & Gazim, Z. C. (2023). Cellular antioxidant, anti-inflammatory, and antiproliferative activities from the flowers, leaves and fruits of *Gallesia integrifolia* spreng harms. *Molecules, 28*, 5406. https://doi.org/10.3390/molecules28145406

Silva, A. R., Pinela, J., Dias, M. I., Calhelha, R. C., Alves, M. J., Mocan, A., García, P. A., Barros, L., & Ferreira, I. C. F. R. (2020). Exploring the phytochemical profile of *Cytinus hypocistis* (L.) L. as a source of health-promoting biomolecules behind its *in vitro* bioactive and enzyme inhibitory properties. *Food Chem. Toxicol., 136*, Article 111071. https://doi.org/10.1016/j.fct.2019.111071

The European Union Council Directive 2001/110/EC. (2001). *J. Eur. Communities* (pp. 47–52).

Tornuk, F., Karaman, S., Ozturk, I., Toker, O. S., Tastemur, B., Sagdic, O., Dogan, M., & Kayacier, A. (2013). Quality characterization of artisanal and retail Turkish blossom honeys: Determination of physicochemical, microbiological, bioactive properties and aroma profile. *Industrial Crops & Products, 46*, 124–131. https://doi.org/10.1016/j.indcrop.2012.12.042

Vasić, V., Gašić, U., Stanković, D., Lušić, D., Vukić-Lušić, D., Milojković-Opsenica, D., Tešić, Ž., & Trifković, J. (2019). Towards better quality criteria of European honeydew honey: Phenolic profile and antioxidant capacity. *Food Chemistry, 274*, 629–641. https://doi.org/10.1016/j.foodchem.2018.09.045

Yilmaz, A. C., & Aygin, D. (2020). Honey dressing in wound treatment: A systematic review. *Complement. Ther. Med., 51*, Article 102388. https://doi.org/10.1016/j.ctim.2020.102388

Yılmaz, H., & Yavuz, Ö. (1999). Content of some trace metals in honey from south-eastern Anatolia. *Food Chemistry, 65*(4), 475–476. https://doi.org/10.1016/S0308-8146(98)00205-2