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Apicultural products from the Sibillini Mountains bio-district: Chemical characterization and bioactive potential within a circular economy perspective

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ABSTRACT

Honey composition is influenced by environmental and geographical factors, including altitude. In this study, multifloral honeys collected at different altitudes within the Sibillini Mountains bio-district (Italy), together with associated apicultural by-products (honeycomb and beeswax), were investigated through an integrated chemical and biological approach. Volatile organic compounds were characterized by HS-SPME–GC–MS, while phenolic compounds were quantified by UHPLC–MS/MS. Low-altitude honeys showed higher concentrations of several phenolic acids and volatile compounds compared with high-altitude and commercial samples. Honey polyphenolic extracts reduced intracellular ROS/RNS levels and pro-inflammatory cytokine production in HepG2 and THLE-2 cells, with more pronounced effects observed for low-altitude samples. Honeycomb extracts exhibited higher antioxidant activity than beeswax, suggesting their potential as sources of bioactive compounds. Overall, samples collected at different altitudes displayed distinct chemical and biological profiles. These findings provide insights into honey chemical fingerprinting within a single production area and support the potential valorisation of apicultural by-products in a circular bioeconomy framework.

1. Introduction

Honey is a heterogeneous natural food matrix whose composition reflects botanical origin, geographical and environmental conditions, as well as extraction and storage practices (Becerril-Sánchez et al., 2021; Tafere, 2021). Although its chemical profile is largely dominated by sugars, mainly fructose and glucose (≈60–82%), which together with low water content ensure microbiological stability and long shelf-life (Anjos et al., 2015; Martinello & Mutinelli, 2021), minor constituents play a key role in defining honey quality and functionality. Phenolic compounds and volatile organic compounds (VOCs) represent important quality markers related to antioxidant capacity, sensory properties and geographical identity (Wang et al., 2023). Phenolic acids (e.g., caffeic, ferulic, p-coumaric, ellagic) and flavonoids (e.g., quercetin, galangin, pinocembrin, apigenin, chrysin) are widely recognized as major contributors to honey's antioxidant and anti-inflammatory properties, acting through free-radical scavenging and modulation of oxidative pathways (Becerril-Sánchez et al., 2021). Their concentration and activity can be modulated by environmental factors, including altitude, floral biodiversity and local environmental conditions, due to the combined effects of temperature, and ultraviolet radiation (Grassi et al., 2025; Mohammed et al., 2020; Yayinie et al., 2022). Honeys produced at higher elevations often show distinctive phenolic profiles and altered antioxidant parameters compared to lowland honeys, although outcomes remain highly dependent on botanical source and local microclimatic conditions. Environmental influences also extend to the volatilome. VOCs, including aldehydes, alcohols, ketones, esters, terpenes, norisoprenoids and furanic derivatives, are key determinants of honey aroma and represent sensitive markers for chemical fingerprinting and authenticity assessment (Manyi-Loh et al., 2011; Türk &

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Şen, 2021; Wang et al., 2023). Despite this, studies simultaneously addressing altitude-related variations in both phenolic composition and VOC profiles within the same production area are still limited. Alongside the growing interest in honey chemistry, apicultural by-products such as beeswax residues and discarded combs are emerging as underexplored matrices with significant valorisation potential. Traditionally regarded as low-value waste, these materials exhibit complex chemical profiles, including long-chain hydrocarbons, wax esters, fatty acids, proteins, minerals and phenolic constituents (Peron et al., 2024). Recent studies have reported their antioxidant, antimicrobial and cytotoxic properties, highlighting potential applications in nutraceutical, pharmaceutical, cosmetic and sustainable material sectors (Giampieri et al., 2018; Guan et al., 2012; Hosseini et al., 2023; Mekky et al., 2025; Zhao et al., 2020). Their recovery is fully consistent with circular bioeconomy strategies aimed at reducing waste while generating added value from agro-food by-products (Peron et al., 2024). However, studies simultaneously investigating altitude-related variations in both volatile and phenolic profiles within the same production area, together with the functional characterization of associated apicultural by-products, remain limited. Therefore, the aim of this study was to (i) characterize the volatile and phenolic profiles of honeys collected at different altitudes within the same bio-district, (ii) evaluate their biological activity in hepatic cell models and (iii) assess the antioxidant potential of associated apicultural by-products (comb and beeswax). The observed antioxidant and anti-inflammatory effects are likely associated with the combined contribution of multiple phenolic compounds rather than a single bioactive constituent.

2. Materials and methods

2.1. Samples and reagents

Honey samples, as shown in Fig. 2S, produced at different altitudes, together with beeswax and comb by-products, were supplied by *Azienda Agricola Fratelli Coperchio* (Cessapalombo, Italy). All samples were multifloral honeys. Honeys produced above 1000 m a.s.l. were classified as high-altitude honeys, whereas those harvested below 1000 m a.s.l. were considered low-altitude honeys. A commercially available multifloral honey (product in Central Italy) purchased from the local market was included exclusively as a comparative reference sample and not as a true experimental control. Ultrapure water (18 MΩ cm) was generated using a Milli-Q purification system (Millipore, Bedford, MA, USA). Glassware was pre-treated with methanol and dried before use to avoid contamination. Cyanidin-3-glucoside chloride, delphinidin-3,5-diglucoside chloride, delphinidin-3-galactoside chloride, petunidin-3-glucoside chloride, malvidin-3-galactoside chloride, quercetin-3-glucoside and kaempferol- 3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The remaining 29 analytical standards of the 38 phenolic compounds were supplied by Sigma-Aldrich (Milan, Italy). Individual stock solutions of each analyte, at a concentration of 1000 mg L^{-1}, were prepared by dissolving pure standards in HPLC-grade methanol and storing them in glass stoppered bottles at 4 °C until analysis. Formic acid (99%) was obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric acid (37%) was obtained from Carlo Erba Reagents (Milan, Italy). HPLC-grade methanol was supplied by Sigma-Aldrich (Milano, Italy). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 μm syringeless filter, Phenomenex (Castel Maggiore, BO, Italy).

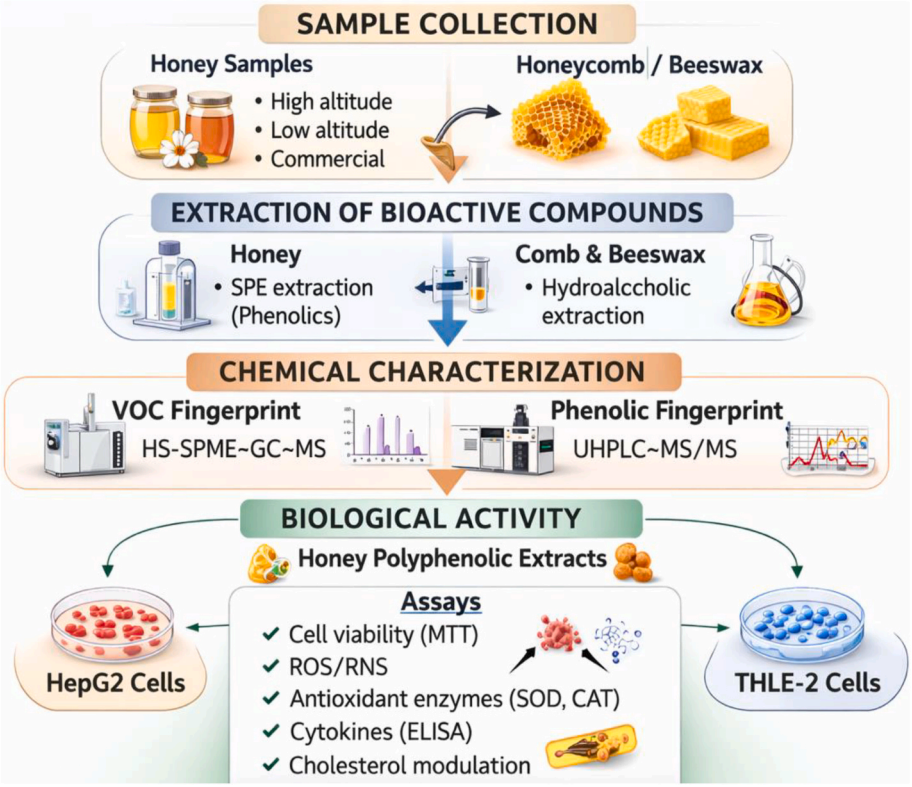

Fig. 1. Schematic overview of the experimental workflow adopted in this study. Multifloral honey samples collected at different altitudes (high altitude and low altitude) together with a commercial honey and apicultural by-products (honeycomb and beeswax) were subjected to extraction of bioactive compounds. Chemical characterization included analysis of volatile organic compounds (VOCs) by HS-SPME–GC–MS and determination of phenolic compounds by UHPLC–MS/MS. The biological effects of honey extracts were evaluated in hepatic cell models (HepG2 and THLE-2) through the assessment of cell viability, intracellular reactive oxygen and nitrogen species (ROS/RNS), cholesterol levels and pro-inflammatory cytokine release.

2.2. Extraction of bioactive compounds from comb and beeswax

The extraction of phenolic compounds from comb and beeswax was performed following Peron et al., 2024 with minor methodological refinements to increase purification efficiency and minimize lipid interference, as shown in Fig. 1 and S.

2.2.1. Pre-cleaning

Raw beeswax and comb samples were mechanically fragmented into flakes <1 cm to increase surface area. Ten grams of material were suspended in 100 mL of absolute ethanol and magnetically agitated for 10 min to solubilize volatile and low-molecular-weight impurities. The suspension was filtered, and the recovered wax was dried in a ventilated oven at 40–50 °C until the solvent had fully evaporated.

2.2.2. Delipidation

To remove wax esters, free fatty acids and long-chain hydrocarbons known to interfere with spectrophotometric assays, 5 g of pre-purified material were treated with 50 mL of n-hexane (1:10 w/v). The mixture was left under gentle agitation at room temperature for 2–3 h and subsequently filtered. The delipidated fraction was dried in an oven for an additional 2–3 h to evaporate residual organic solvent.

2.2.3. Phenolic extraction

Exactly 1 g of delipidated sample was extracted with 20 mL of ethanol/water (70/30 v/v) in an ultrasonic bath (40 min, room temperature). Sonication was applied to enhance mass transfer of polar compounds from the solid matrix. Samples were centrifuged at 5000 rpm for 10 min, and the supernatant was collected for spectrophotometric analyses. The chosen hydroalcoholic mixture ensures efficient extraction of phenolics while maintaining compatibility with subsequent analytical procedures.

2.3. Spectrophotometric assays

2.3.1. Total phenolic content (TPC)

TPC was determined according to the Folin–Ciocalteu assay (Santanatoglia, Angeloni, et al., 2023), one of the most widely applied methods for total phenolic quantification. Extract aliquots (100 μL) were mixed with 500 μL of diluted Folin–Ciocalteu reagent (1:10 v/v) and allowed to react for 5 min. Then, 400 μL of sodium carbonate solution (7.5% w/v) were added and the mixture incubated at room temperature for 30 min. Absorbance was measured at 765 nm. A gallic acid calibration curve (0–200 mg/L, $R^2 > 0.99$, Table 1S) was used to express results as mg gallic acid equivalents per kg dry weight (mg GAE/kg). Samples were diluted appropriately to obtain absorbance values within the linear range.

2.3.2. Total flavonoid content (TFC)

TFC was determined using the aluminum chloride colorimetric method (Laurita et al. 2021), based on complex formation between Al^{3+} and flavonoid hydroxyl groups. Briefly, 500 μL of extract were mixed with 500 μL of 2% $AlCl_3$ in methanol and incubated for 30 min at room temperature. Absorbance was measured at 415 nm, using rutin as external standard (0–100 mg/L, $R^2 > 0.99$, Table 1S). Results were expressed as mg rutin equivalents per kg dry weight (mg RE/kg).

2.3.3. DPPH radical scavenging activity

Antioxidant activity was assessed following Santanatoglia, Angeloni, et al., 2023. The working DPPH solution (0.1 mM in methanol) was prepared fresh daily. Extract aliquots (100 μL) were added to 3.9 mL of DPPH solution, vortexed and kept in the dark for 30 min. The decrease in absorbance at 517 nm was monitored. A Trolox calibration curve (0–300 mg/L; $R^2 > 0.99$) was used to express antioxidant capacity as mg Trolox equivalents per kg dry weight (mg TE/kg) (Table S1). All measurements were corrected with blank samples containing solvent instead of extract.

2.4. Honey chemical characterization

2.4.1. HS-SPME–GC–MS analysis of VOCs

Volatile organic compounds (VOCs) were extracted using headspace solid-phase microextraction (HS-SPME), following Liang et al., 2020; Abouelenein et al., 2025. Five grams of honey were transferred into 20 mL vials sealed with PTFE/silicone septa. Samples were equilibrated for 40 min at 50 °C to promote volatilization, followed by a 15-min extraction under agitation (5 s on, 2 s off). A DVB/CWR/PDMS fiber - selected for its broad affinity toward polar and apolar volatiles, was preconditioned at 250 °C for 15 min prior to use. After extraction, the fiber was desorbed in the GC injector at 250 °C for 3 min (insertion speed: 100 mm/s; penetration depth: 40 mm). VOCs were analyzed using an Agilent 8890 GC coupled to a 5977 B quadrupole MSD (EI source). The injector operated in splitless mode using a dedicated SPME Ultra Inert liner (0.75 mm i.d.). Helium was used as carrier gas at 1 mL min^{-1}. Separation was performed on an HP-5MS UI column (30 m × 250 μm i.d., 0.25 μm film thickness) The oven temperature program was as follows: initial temperature 35 °C; ramped to 60 °C at 7 °C/min and held for 1 min; then increased from 60 °C to 80 °C at 1.5 °C/min; subsequently increased continuously to 110 °C at 1.5 °C/min without an intermediate holding step; finally ramped to 280 °C at 10 °C/min and held for 1 min. Data were acquired in SCAN mode (m/z 35–450). Compounds were identified by comparison with mass spectra and retention indices from the NIST library, supported by literature RI values. Data processing was carried out using MSD ChemStation (Agilent G1701DA D.01.00).

2.4.2. UHPLC–MS/MS phenolic analysis

2.4.2.1. Extraction by SPE. Phenolics were isolated using solid-phase extraction (SPE) following Kenjerić et al., 2008 with adjustments to improve recovery. Honey samples (2.5 g) were acidified with 2.5 mL of 37% HCl (pH ≈ 2) to ensure matrix liquefaction and stabilization of phenolic acids. SPE was performed using Strata C18-E cartridges (500 mg/5 mL). Cartridges were conditioned with methanol and acidified water before loading 5 mL of sample. Sugars and highly polar components were removed by washing with 4 mL deionized water, while phenolics were eluted with 2 mL of 90% methanol. The eluates were evaporated under nitrogen and reconstituted in 0.5 mL methanol at a fixed volume for chromatographic analysis.

2.4.2.2. Chromatographic analysis. Phenolic compounds were quantified using an Agilent 1290 Infinity UHPLC system coupled to an Agilent triple quadrupole mass spectrometer. Separation was achieved on a Synergi Polar-RP C18 column (250 × 4.6 mm, 4 μm) at 40 °C, following optimized conditions for cereal-bound phenolics. Mobile phases: A: water +0.1% formic acid; B: methanol +0.1% formic acid. Gradient: 0–1 min: 20% B; 1–25 min: linear gradient to 85% B; 25–26 min: 85% B; 26–32 min: return to 20% B; Flow rate: 0.8 mL/min.

Injection volume: 1 μL. Data acquisition was carried out in Dynamic Multiple Reaction Monitoring (D-MRM), enabling high sensitivity and selectivity. External calibration curves were prepared for each analytical standard (5–2000 μg/L). A total of 38 phenolic compounds were monitored, as shown in Table 2S (Mustafa et al., 2021; Santanatoglia, Cespi, et al., 2023, Santanatoglia, Cespi, et al., 2023). Quantification was based on external calibration, and method performance was assessed through LOD (0.2-5 μg/L), LOQ (0.5-15 μg/L), linearity ($R^2 > 0.99$) and repeatability (RSD%) (intra-day <5%; inter-day <10%).

2.5. Biological assays

2.5.1. Cell cultures

Human hepatocellular carcinoma HepG2 cells (HB 8065™, ATCC) and the THLE-2 cell line derived from normal primary hepatic cells were used in the study. HepG2 cells were grown at 37 °C in a 5% CO_2 atmosphere in MEM growth medium supplemented with 10% fetal bovine serum (FBS), antibiotic and antimycotic, L-glutamine, and 1% sodium pyruvate. Culture media and reagents were purchased from EuroClone S.p.A. (Milan, Italy). The THLE-2 cell line, purchased from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (Brescia, Italy), was cultured in BEBM medium (Lonza, Milan, Italy) supplemented with BEGM (BPE 13 mg/mL, hydrocortisone 0.5 mg/ml, hEGF (human epidermal growth factor) 0.5 μg/mL, transferrin 10 mg/mL, insulin 5 mg/mL, triiodothyronine 6.5 μg/mL, retinoic acid 0.1 μg/mL, plus EGF 5 ng/mL and 10% fetal calf serum (FCS). Flasks and plates used to propagate and grow THLE-2 cells were precoated with a mixture of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin dissolved in BEBM medium.

2.5.2. Cell viability assay (MTT)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to evaluate cell viability upon treatments. This colorimetric assay measures mitochondrial dehydrogenase activity, providing an indirect assessment of viable cell number. Dried extracts were resuspended in DMSO to treat cells. Cells were exposed to increasing concentrations (0–200 μg/mL) of each honey extract. Control cells received the corresponding volume of DMSO. After 24 h of treatment, the cells were washed with phosphate-buffered saline (PBS, pH 7.5), and MTT (final concentration 0.5 mg/mL) was added to culture medium without FBS and incubated for 2 h at 37 °C. The medium was then removed and replaced with 100 μL of DMSO. The amount of MTT-formazan produced was determined spectrophotometrically by measuring the optical density (OD) at 550 nm using a plate reader. The OD550 of the DMSO solution in each well was taken as an index of cell number, and the OD550 of the untreated control was considered 100%.

2.5.3. ROS/RNS measurement

Intracellular ROS/RNS production was assessed using fluorescence-based detection methods (Cellular ROS/RNS Assay Kit, Abcam, Cambridge, UK). Both HepG2 and THLE-2 cells were treated with 10 and 50 μg/mL concentrations of each honey extract dissolved in DMSO for 24 h. Specific fluorescent probes were employed to quantify oxidative and nitrosative stress levels using a SpectraMax Gemini XPS plate reader. Each sample was prepared in triplicate in the assay, and three independent experiments were performed.

2.5.4. Antioxidant enzyme activity

Activities of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were measured in HepG2 cells using colorimetric assay kits obtained from Invitrogen (Thermo Fisher Scientific, Life, Technologies Corporation, MD, USA). Cells were treated with honey extracts dissolved in DMSO (10 and 50 μg/mL) for 24 h. Assays were performed following datasheet indications and plates were read at 450 nm on a microtiter plate reader.

2.5.5. Cytokine release (ELISA)

The effect on inflammation was evaluated in cells treated with honey extracts dissolved in DMSO (10 and 50 μg/mL) by measuring, using ELISA assays, the inflammatory cytokines IL-1β (Abcam, Cambridge, UK), IL-6 (antibodies.com, Stockholm, Sweden), IL-8 (Abcam, Cambridge, UK) and TNF-α (Abcam, Cambridge, UK) and the anti-inflammatory cytokine IL-10 (Abcam, Cambridge, UK) released into the culture medium, following the manufacturer's instructions. Each sample was prepared in triplicate in the assay, and three independent experiments were performed.

2.5.6. Cholesterol assay

Total cellular cholesterol and cytoplasmic cholesterol were measured using the Amplex Red Cholesterol Assay kit following 24 h exposure to 10 and 50 μg/mL honey extract concentrations. After treatment, cells were detached with trypsin, washed with PBS, and centrifuged at 8000×g for 5 min. Briefly, pellets were resuspended in RIPA lysis buffer and kept for 30 min in ice with intermittent vortexing every 5-10 min to ensure uniform cell lysis and detergent action. After lysis, samples were centrifuged at 12,000 x g for 15 min at 4 °C and the supernatant was collected. The working solution was prepared in buffer and contained Amplex® Red reagent (300 μM), horseradish peroxidase (2 U/mL), cholesterol oxidase (2 U/mL), and cholesterol esterase (0.2 U/mL). A cholesterol standard curve was generated by diluting the supplied cholesterol reference standard (5.17 mM) in reaction buffer. Then, 50 μL of cell lysates diluted in reaction buffer and 50 μL of the working solution were added to a 96-well plate and incubated at 37 °C. Fluorescence measurements were recorded after 30 min using a SpectraMax Gemini XPS plate reader (λ_{exc} = 540 nm, λ_{em} = 590 nm). Each sample was prepared in triplicate in the assay, and three independent experiments were performed.

2.6. Statistical analyses

All analyses were performed in biological triplicate (n = 3), and results are expressed as mean ± standard deviation (SD). Statistical differences among samples were assessed by one-way analysis of variance (ANOVA), applied separately for each analytical parameter and extraction condition. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Antioxidant properties of comb and beeswax

The analysis of the comb and beeswax samples revealed their potential as sources of natural antioxidants, in agreement with findings reported in the literature (Giampieri et al., 2018; Zhao et al., 2020). However, differences were observed in the total phenolic and flavonoid contents, as well as in the antioxidant capacity assessed through the DPPH assay, as shown in Table 1. In particular, honeycomb exhibited higher values than wax across all assays performed: the antioxidant activity measured by DPPH was 1305.10 ± 14.61 mg TE/kg for honeycomb and 1176.98 ± 8.35 mg TE/kg for wax; the total flavonoid

Table 1
Antioxidant properties of honeycomb and beeswax extracts determined by spectrophotometric assays. DPPH radical scavenging activity, total phenolic content (TPC), and total flavonoid content (TFC) are expressed as mg Trolox equivalents per kg dry weight (mg TE kg^{-1}), mg gallic acid equivalents per kg dry weight (mg GAE kg^{-1}), and mg rutin equivalents per kg dry weight (mg RE kg^{-1}), respectively. Results are reported as mean ± standard deviation (n = 3). Different superscript letters within the same column indicate statistically significant differences according to one-way ANOVA ($p \leq 0.05$).

DPPH	*mg TE/Kg*
BEESWAX	1176.98 ± 8.35[a]
COMB	1305.10 ± 14.61[b]
TFC	***mg RE/Kg***
BEESWAX	1134.10 ± 5.76[a]
COMB	1150.11 ± 10.55[b]
TPC	***mg GAE/Kg***
BEESWAX	1145.12 ± 12.20[a]
COMB	1204.82 ± 25.15[b]

content (TFC) reached 1150.11 ± 10.55 mg RE/kg in honeycomb compared to 1134.10 ± 5.76 mg RE/kg in wax; and the total phenolic content (TPC) was 1204.82 ± 25.15 mg GAE/kg in honeycomb and 1145.12 ± 12.20 mg GAE/kg in wax. These results, summarized in Fig. 2, indicate that honeycomb represents a matrix richer in bioactive compounds than wax, likely due to the presence of residual honey, pollen and propolis that enhance its chemical composition embedded within the comb structure, which are rich in phenolic acids, flavonoids and other bioactive compounds. These minor constituents contribute to the chemical profile of honeycomb, enhancing its antioxidant potential relative to beeswax. While phenolic compounds and flavonoids are the main contributors, other constituents such as organic acids, carotenoids and redox-active proteins may also play a role in the observed activity. These findings highlight honeycomb as a promising matrix for nutraceutical, cosmetic, and biodegradable applications, aligning with circular bioeconomy principles. For a more comprehensive understanding of bioactive composition, future studies should employ targeted LC–MS/MS profiling of honeycomb and wax, allowing the identification and quantification of individual phenolic and flavonoid compounds. This approach would complement DPPH, TPC and TFC measurements and provide a deeper insight into the functional potential of these apicultural by-products (Giampieri et al., 2018; Zhao et al., 2020). The valorisation of these materials aligns with circular economy principles, transforming potential waste into functional resources. In nutraceuticals, extracts can be used for natural antioxidant supplements; in cosmetics, for anti-aging and moisturizing formulations; and in environmental applications, beeswax can contribute to biodegradable materials or natural food preservatives. This approach enhances the sustainability and overall value of apicultural products.

3.2. Volatile fingerprint of honeys from different altitudes

To evaluate the influence of altitude on honey aroma, three multifloral honey samples from the same producer with comparable botanical backgrounds were analyzed: high-altitude, low-altitude and commercial honey. It should be noted that, despite efforts to select samples from similar floral sources, some botanical variability cannot be excluded, and the present study represents a pilot fingerprinting comparison rather than a definitive survey. Only volatile compounds common to all samples were considered (Table 2). GC–MS/MS analysis revealed quantitative differences in the concentration of various compounds across heterogeneous chemical classes, including: aldehydes, aromatic alcohols, terpenoids, acids and phenols. Consistent with previous studies, the results indicate that altitude may influence the aromatic profile of honey, altering both the composition and relative concentration of volatile compounds. Recent investigations on honeys of diverse botanical origins, such as Astragalus honey from Turkey and honeys from different regions of Cyprus, have shown that increasing altitude is associated with an overall reduction in aromatic intensity and a simplification of the volatile profile. This phenomenon has been attributed to harsher climatic conditions and lower mean annual temperatures, which reduce the emission of volatile compounds by plants

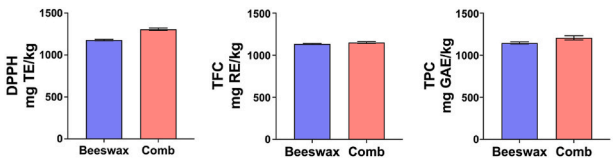

Fig. 2. Antioxidant properties of honeycomb and beeswax extracts evaluated through spectrophotometric assays. Radical scavenging activity was determined using the DPPH assay (mg TE/kg ± SD), while total flavonoid content (TFC) (mg RE/kg ± SD) and total phenolic content (TPC) (mg GAE/kg ± SD) and were quantified using aluminum chloride colorimetric and the Folin–Ciocalteu methods, respectively. Results are expressed as mean ± standard deviation (SD).

and limit the diversity of nectar sources available to bees (Ioannis et al., 2019; Türk & Şen, 2021). The results of the present study are in line with these observations. Low-altitude honeys exhibited higher levels of volatile compounds, particularly aromatic aldehydes (benzaldehyde, benzene acetaldehyde), aromatic alcohols (benzyl alcohol, phenylethyl alcohol) and monoterpenoids (linalool), which are associated with floral, fruity and sweet notes contributing positively to honey's sensory complexity. This suggests that at lower altitudes, where temperatures are higher and flora is more diverse, bees have access to a wider range of nectar sources rich in volatile aromatic precursors. Conversely, high-altitude honey samples displayed a simpler profile, characterized by relatively higher abundance of aliphatic aldehydes (nonanal, decanal) and certain ketones (e.g., 4-oxoisophorone), imparting soapy or slightly green notes. These differences indicate that the montane environment, with lower temperatures and a limited flowering period, results in a less intense but more defined aromatic profile, dominated by the few plant species prevalent at high altitudes. Commercial honey exhibited intermediate or lower levels of most identified compounds, reflecting the blending of different origins and the effects of processing and storage, which tend to reduce the concentration of more labile volatile compounds, as previously reported (Da Silva et al., 2020). The analysis of the aromatic profiles of the honey samples revealed differences between organic honeys (high- and low-altitude) and commercial honey. Organic honeys exhibited higher concentrations of aldehydes and aromatic alcohols, including benzaldehyde, benzeneacetaldehyde, decanal, lilac aldehyde, linalool, benzyl alcohol and phenylethyl alcohol, associated with floral, fruity, sweet and spicy notes (Türk & Şen, 2021). Additional compounds such as linalool oxide, responsible for citrus, herbal and cooked-apple aromas, were more abundant in organic honeys (Fig. 3). These differences suggest that organic honeys may retain a more diverse array of volatile compounds compared to commercial honeys, which generally showed lower levels of fresh volatiles and higher concentrations of degradation markers, including 2,5-furandicarbaldehyde, furyl hydroxymethyl ketone, isophorone and fatty acids (nonanoic and hexadecanoic acids) (Da Silva et al., 2020). HMF (5-hydroxymethylfurfural) was detected in commercial honey, whereas it was virtually absent in organic honeys, consistent with minimal heat treatment and limited prolonged storage (Islam et al., 2014). It should be noted that, although some degradation compounds were detected in organic honeys, their concentrations were relatively low and unlikely to substantially affect the overall volatile composition. This study did not include formal sensory evaluation; however, the chemical data, interpreted in the context of the Leibniz Odorant Database (https://www.leibniz-lsb.de/en/datenbanken/leibniz-lsbtum-odorant-database/odorantdb) provide preliminary insights into the potential aromatic characteristics of the different honey types. Overall, the findings indicate that organic honeys, regardless of altitude, tend to maintain a broader and more diverse profile of positive volatile compounds, whereas commercial honeys are comparatively richer in degradation products and show a reduction in fresh volatiles. These results highlight chemical differences that may underlie the distinctive characteristics of local organic honeys, though further studies including sensory analysis would be needed to confirm their organoleptic attributes.

3.3. Phenolic composition of honey samples

HPLC analysis of honey samples from different altitudes revealed a clear trend in the distribution of phenolic compounds, indicating an altitude related variation. As shown in Tables 3S and 4S, low-altitude honey exhibited higher concentrations of most phenolic compounds compared to high-altitude and commercial honey, the latter representing a reference sample of lower botanical and environmental complexity. Specifically, low-altitude honey contained the highest levels of short-chain phenolic acids, including caffeic acid (20.56 ± 0.62 mg/kg), ferulic acid (9.52 ± 0.24 mg/kg) and *p*-coumaric acid (3.02 ± 0.33 mg/kg), known for their antioxidant activity and their role in protecting

Table 2
Volatile organic compounds detected in high-altitude, low-altitude and commercial honey samples by HS-SPME-GC–MS analysis. Compounds are listed according to retention time (RT), experimental linear retention index (LRI(E)) and literature retention index (LRI). Results are expressed as relative abundance (peak area percentage, %) and reported as mean ± standard deviation (n = 3). Only volatile compounds common to all honey samples are reported to enable direct comparison among honey types. Odor descriptors are assigned based on literature data. Different superscript letters within the same row indicate statistically significant differences according to one-way ANOVA ($p \leq 0.05$).

RT (min)	LRI(E)	LRI	Compound	Aromatic Notes	High	Low	Commercial
Aldehydes							
11.353	960	962(3)	Benzaldehyde	bitter almond-like, marzipan-like	6.71 ± 0.81^{a}	27.09 ± 0.34^{c}	15.26 ± 0.31^{b}
15.966	1043	1045(4)	Benzeneacetaldehyde	bitter almond-like, marzipan-like	0.39 ± 0.01^{a}	5.99 ± 0.79^{c}	1.31 ± 0.42^{b}
18.223	1077	1076(5)	2,5-Furandicarbaldehyde	caramel-like, sweet, almond-like	0.35 ± 0.01^{a}	n.d	1.39 ± 0.16^{b}
27.949	1205	1206(2)	Decanal	soapy, citrus-like	1.42 ± 0.04^{b}	0.84 ± 0.36^{a}	0.56 ± 0.16^{a}
32.73	1266	1270(3)	Cinnamaldehyde	cinnamon-like	0.39 ± 0.01^{a}	0.51 ± 0.35^{a}	0.47 ± 0.11^{a}
23.537	1151	1145	Lilac aldehyde	floral, sweet	1.84 ± 0.13^{b}	0.65 ± 0.24^{a}	0.98 ± 0.02^{a}
20.192	1104	1104(2)	Nonanal	citrus-like, soapy	15.30 ± 0.42^{b}	2.47 ± 0.44^{a}	n.d
36.368	1310	1316(4)	2-Methoxy-4-vinylphenol	smoky, clove-like	0.17 ± 0.01^{a}	0.27 ± 0.08^{a}	0.09 ± 0.04^{a}
Alcohols							
5.617	795	788(7)	2,3-Butanediol	butter-like, sweet	1.6 ± 0.09^{a}	1.94 ± 0.19^{a}	1.04 ± 0.25^{a}
15.387	1034	1036(4)	Benzyl alcohol	bitter almond-like, fruity	6.81 ± 0.19^{ab}	7.64 ± 0.96^{b}	5.50 ± 0.01^{a}
20.609	1110	1116(5)	Phenylethyl alcohol	floral, rose-like, sweet	6.77 ± 0.18^{b}	10.44 ± 0.40^{c}	5.88 ± 0.27^{a}
19.881	1099	1099(2)	Linalool	citrus-like, floral	0.64 ± 0.02^{a}	2.66 ± 0.90^{b}	0.38 ± 0.16^{a}
26.092	1183	1183(2)	p-Cymen-8-ol	petrol-like, musty	0.76 ± 0.02^{a}	n.d	0.51 ± 0.14^{a}
Ketons							
18.454	1080	1087(4)	Furyl hydroxymethyl ketone	caramel-like, burnt sugar, nutty	0.43 ± 0.07^{a}	n.d	0.75 ± 0.51^{a}
21.113	1118	1123(5)	Isophorone	woody, floral, slightly minty	0.99 ± 0.23^{b}	0.18 ± 0.05^{a}	0.50 ± 0.22^{ab}
22.907	1143	1145(5)	4-Oxoisophorone	floral, violet-like, woody	3.34 ± 0.67^{c}	1.87 ± 0.58^{b}	0.44 ± 0.41^{a}
44.732	1460	1453(2)	Geranyl acetone	floral, fruity, rosy, green, citrus-like	0.1 ± 0.05^{a}	0.26 ± 0.36^{a}	n.d
Acids							
33.577	1276	1273(6)	Nonanoic acid	moldy, pungent	0.83 ± 0.3^{b}	n.d	0.24 ± 0.21^{a}
52.237	1959	1968(7)	Hexadecanoic acid	moldy, pungent, green	0.22 ± 0.01^{a}	n.d	0.12 ± 0.31^{a}
Lactones							
15.81	1041	1049	4-Methyl-4-vinylbutyrolactone	sweet, creamy, caramel-like	0.37 ± 0.05^{a}	0.53 ± 0.24^{a}	0.61 ± 0.35^{a}
16.701	1055	1056(4)	Caprolactone	creamy, buttery, coconut-like	0.23 ± 0.01^{b}	n.d	0.05 ± 0.03^{a}
Phenols							
46.125	1510	1514(5)	2,4-di-tert-butylphenol	phenolic-like, leather-like	4.48 ± 0.41^{c}	3.43 ± 0.47^{b}	2.34 ± 0.37^{a}
54.427	2187	2173(1)	3,4′-Isopropylidenediphenol	phenolic, plastic-like	5.22 ± 0.28^{b}	1.68 ± 0.37^{a}	1.64 ± 0.14^{a}
Terpenes/oxygenated terpenes							
17.903	1072	1074(4)	Linalool oxide	floral, lavender-like, citrus, sweet	1.67 ± 0.29^{a}	2.66 ± 0.90^{b}	0.51 ± 0.14^{a}
41.934	1387	1386(4)	Damascenone	cooked apple-like	n.d	0.25 ± 0.09^{b}	0.09 ± 0.04^{a}
Alkenes/hydrocarbons							
42.58	1399	1392(2)	Tetradecene	waxy, oily, fatty, green	1.56 ± 0.22^{b}	0.95 ± 0.86^{a}	0.69 ± 0.23^{a}
Altri							
22.554	1138	1144(5)	Benzyl nitrile	almond-like, bitter	0.53 ± 0.14^{a}	0.87 ± 0.03^{b}	0.08 ± 0.07^{a}
29.346	1224	1232(7)	HMF	caramel, burnt sugar, toasty	n.d	n.d	0.43 ± 0.07^{a}

nectar from oxidative degradation. Benzoic acid derivatives, such as 4-hydroxybenzoic acid (2.89 ± 0.20 mg/kg) and 3-hydroxybenzoic acid (2.47 ± 0.50 mg/kg), were also elevated, reflecting a general enrichment in phenolic metabolites. In contrast, high-altitude and commercial honeys displayed progressively lower concentrations, approximately two-to three-fold lower than low-altitude honey for the major phenolic acids. This trend aligns with literature reports on the impact of geographical and environmental factors on honey phenolic composition (Grassi et al., 2025; Mohammed et al., 2020). The findings of the present study are consistent with these observations. Low-altitude honeys were enriched in phenolic acids (caffeic, ferulic, *p*-coumaric) and flavonoids, such as kaempferol-3-glucoside, quercetin, and isorhamnetin, whereas high-altitude and commercial honeys exhibited lower concentrations of these compounds, as illustrated in Fig. 4. This decrease likely reflects not only harsher climatic conditions and reduced light intensity at higher altitudes, but also lower floral diversity in montane environments, which limits the availability of phenolic precursors in nectar. Overall, the results indicate that altitude is a key factor modulating honey composition, affecting both the quantity and type of bioactive metabolites. The observed progressive reduction in phenolic and volatile content with increasing altitude supports the hypothesis that the pedoclimatic and floristic conditions of lowland areas favor the biosynthesis and accumulation of antioxidant compounds, potentially associated with higher bioactive content.

3.4. Biological activity of honey polyphenolic extracts

3.4.1. Cell viability

The effects of honey polyphenolic extracts on the viability of HepG2 hepatocellular carcinoma cells were evaluated using the MTT assay after 24 h exposure to increasing extract concentrations (0–200 μg mL^{-1}). As shown in Fig. 5, all tested honey extracts produced a concentration-dependent reduction in HepG2 cell viability. At lower concentrations, cell viability remained relatively high, whereas progressive reductions were observed at higher concentrations approaching 200 μg mL^{-1}. Among the tested samples, the low-altitude honey extract exhibited the most evident effect on HepG2 cell viability, suggesting a higher biological activity compared with the high-altitude and commercial honey extracts. These findings are consistent with previous studies reporting antiproliferative and cytotoxic effects of honey and honey-derived polyphenols in cancer cell models, including HepG2 cells (Aryappalli et al., 2017; Nguyen et al., 2021; Sakač et al., 2022). Such effects have been associated with the presence of phenolic compounds capable of modulating cellular signaling pathways related to oxidative stress and apoptosis (Cianciosi et al., 2018). Polyphenols are known to interfere with proliferation pathways such as PI3K/Akt and ERK signaling while enhancing antioxidant defense mechanisms within cells (Mirza-Aghazadeh-Attari et al., 2020). Moreover, the antiproliferative activity of honey extracts has been correlated with their total phenolic content and antioxidant capacity, supporting the hypothesis that compositional differences among honey types may influence their

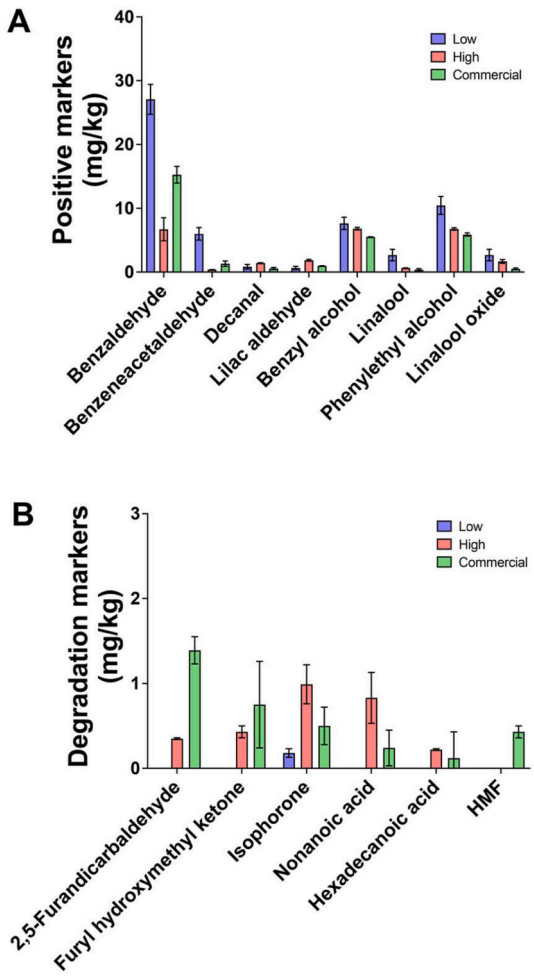

Fig. 3. Volatile organic compounds identified in the analyzed honey samples by HS-SPME–GC–MS. (**A**) Positive aroma markers associated with honey quality and characteristic floral notes. (**B**) Volatile degradation markers potentially related to thermal processing or storage conditions. Relative abundance values are reported for high-altitude, low-altitude, and commercial honey samples.

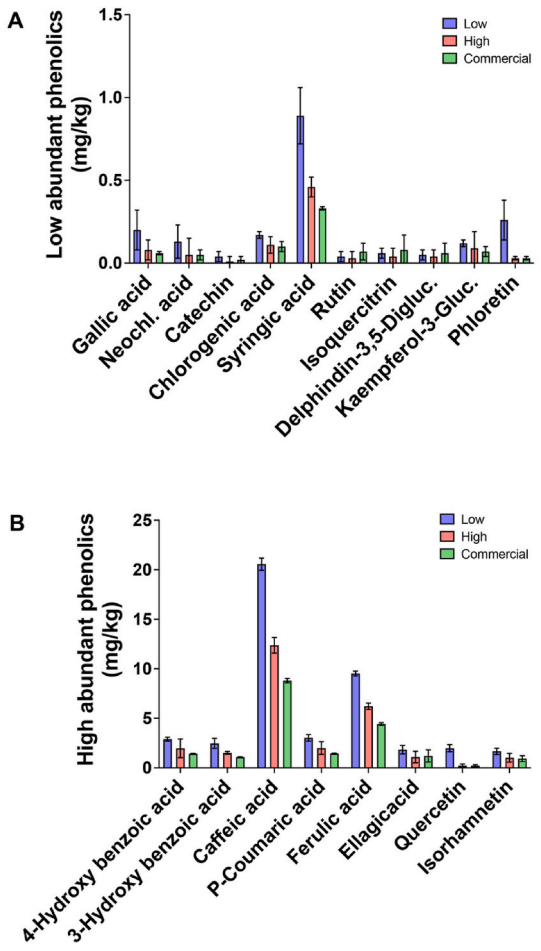

Fig. 4. Phenolic composition of the analyzed honey samples determined by UHPLC–MS/MS. (**A**) Phenolic compounds detected at lower concentrations. (**B**) Major phenolic compounds present at higher concentrations. Values are expressed as mg kg^{-1} ± standard deviation (SD) for high-altitude, low-altitude, and commercial honey samples.

biological effects. To evaluate the potential selectivity of the honey extracts, we exposed THLE-2 normal human liver epithelial cells to the same treatments under identical experimental conditions. As illustrated in Fig. 5, THLE-2 cells displayed higher resistance to the treatments compared with HepG2 cells. Only the highest extract concentrations (150 and 200 μg mL^{-1}) resulted in significant reductions in cell viability, while lower concentrations maintained viability levels close to those observed in untreated control cells. The observed difference in sensitivity between normal and cancer cells may reflect distinct metabolic and oxidative stress responses between transformed and non-transformed hepatocytes. Polyphenols can exert both antioxidant and pro-oxidant effects depending on the cellular context, a phenomenon frequently reported in cancer models where cancer cells exhibit greater susceptibility to redox modulation (Mileo & Miccadei, 2016). These findings therefore suggest a potential selective biological activity of honey polyphenolic extracts toward cancer cells, which has also been reported for several honey types and their isolated phenolic components.

3.4.2. *Oxidative stress modulation*

Oxidative stress arises when intracellular ROS, radical species produced during normal cellular metabolism, overwhelm antioxidant defence systems, leading to damage in lipids, proteins, and DNA finally contributing to aging and disorders (Cecarini et al., 2007). Previous studies demonstrated the ability of honey to scavenge ROS/RNS in different cell types highlighting its antioxidant properties (Beretta et al., 2007). In the present study, intracellular levels of radical species were evaluated in both HepG2 and THLE-2 cells after 24 h exposure to honey extracts at concentrations of 10 and 50 μg mL^{-1}. As shown in Fig. 6, treatment with the extracts resulted in a reduction of intracellular ROS/RNS levels in both cell lines, with a more pronounced effect observed at the higher concentration (50 μg mL^{-1}). Notably, HepG2 cells exhibited a stronger response to the treatments compared with THLE-2 cells, indicating greater sensitivity to the antioxidant activity of the extracts. Among the tested samples, the low-altitude honey extract induced the most evident decrease in radical species, whereas the high-altitude and commercial honey extracts showed comparatively smaller effects. These observations are consistent with the well-established antioxidant properties of honey polyphenols, which can scavenge free radicals and modulate cellular oxidative stress pathways (Cianciosi et al., 2018). Honey polyphenols have been shown to reduce ROS production and influence inflammatory mediators such as cyclooxygenase-2 and nitric oxide synthase, thereby limiting oxidative and inflammatory cellular responses (Ahmed et al., 2022; Wilczyńska & Żak, 2024). The ability of honey extracts to reduce intracellular ROS and RNS may therefore contribute to the biological effects observed in the viability assays. To better characterize the antioxidant ability of the three honey extracts in HepG2 cells, we measured the activity of two key antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD)

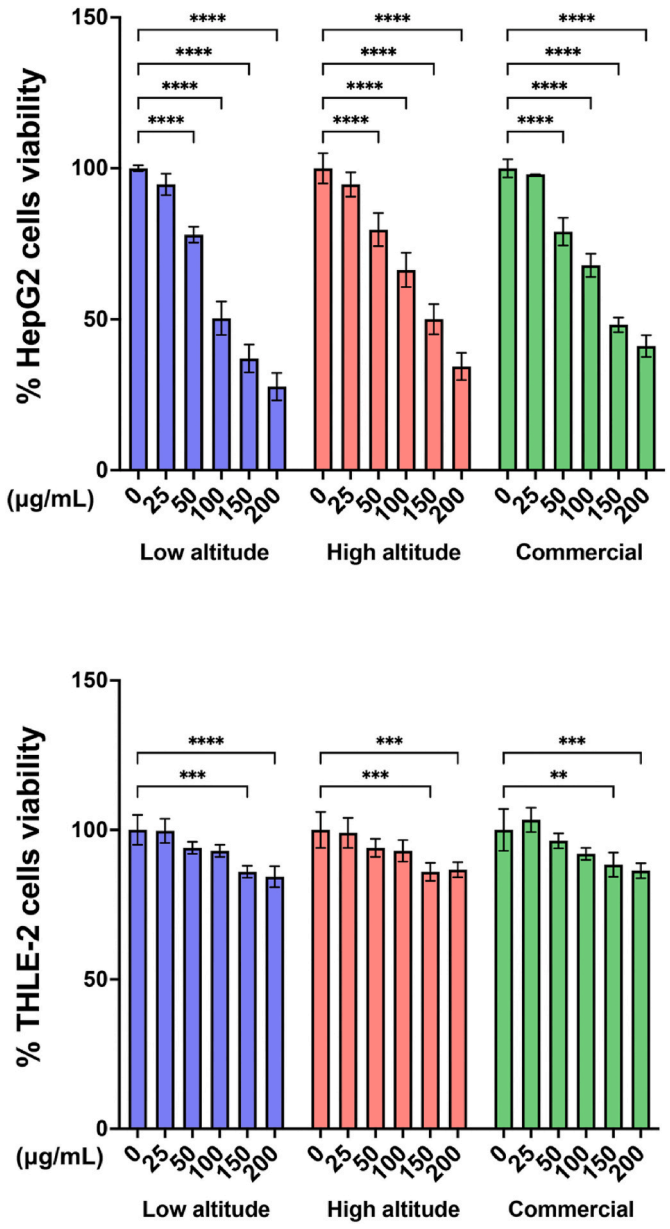

Fig. 5. Effects of honey polyphenolic extracts on cell viability after 24 h exposure. (Left panel) HepG2 hepatocellular carcinoma cells. (Right panel) THLE-2 normal human hepatocytes. Cell viability was determined using the MTT assay following treatment with increasing concentrations of honey extracts (0–200 μg mL^{-1}). Data are expressed as percentage of viable cells relative to untreated control cells and asterisks indicate significant differences compared to controls (****$p < 0.0001$).

(Fig. 6). The two enzymes play critical roles in neutralizing ROS: SOD converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen, preventing oxidative damage to proteins and DNA, while CAT subsequently decomposes H_2O_2 into water and oxygen, avoiding further ROS accumulation and lipid peroxidation (Cecarini et al., 2007). Interestingly, extracts were able to upregulate the activity of both enzymes, mainly at the highest concentration tested (50 μg mL^{-1}). Again, the low-altitude honey extract induced the clearest effect, likely due to its superior phenolic content.

3.4.3. Anti-inflammatory activity

Oxidation and inflammation are two intimately correlated processes often sharing metabolic pathways. The anti-inflammatory potential of honey polyphenolic extracts was assessed by measuring the production of the pro-inflammatory cytokines TNF-α, IL-6, IL-8 and IL-1β in HepG2 cells after 24 h treatment. As shown in Fig. 7, treatment with the three honey extracts resulted in a reduction in cytokine production compared with untreated cells. Among the tested samples, the low-altitude honey extract induced the strongest decrease in TNF-α, IL-6 and IL-1β levels, indicating a more pronounced anti-inflammatory effect. The high-altitude and commercial honey extracts also reduced cytokine levels, although for TNF-α the effect was mainly observed at the highest tested concentrations. Additionally, we evaluated medium concentration of IL-10, an anti-inflammatory cytokine, whose levels significantly increased in cells exposed to highest extract concentrations. Again, low altitude honey extract induced the most pronounced effect. These results are consistent with previous in vitro and in vivo studies demonstrating that honey polyphenols can modulate inflammatory pathways and reduce pro-inflammatory cytokine production (Candiracci et al., 2012; El-Seedi et al., 2022; Ren et al., 2023).

3.4.4. Cholesterol modulation

Cellular lipid metabolism was investigated by measuring total cellular cholesterol and cytoplasmic cholesterol in both HepG2 and THLE-2 cells after 24 h treatment with honey extracts at concentrations of 10 and 50 μg mL^{-1}. As reported in Fig. 8, HepG2 cells showed modulation of cholesterol levels relative to control cells, suggesting that honey polyphenolic extracts may influence lipid metabolism pathways in cancer cells. In contrast, THLE-2 cells showed minimal variations in total and cytoplasmic cholesterol, indicating that the treatments exert a selective action on cholesterol levels in hepatocytes. Again, the low-altitude honey extract exhibited the most evident effect on cellular cholesterol levels, consistent with its stronger biological activity observed in previous assays. These findings align with previous reports demonstrating that certain honey varieties can modulate cholesterol homeostasis and lipid metabolism, potentially through regulation of key metabolic pathways associated with cholesterol synthesis and transport (Nguyen et al., 2021).

Collectively, the biological data provide preliminary evidence that the honey polyphenolic extracts modulate hepatic cell responses in a concentration- and cell type-dependent manner, rather than acting as uniformly protective or cytotoxic agents. The low altitude extract consistently showed the most pronounced activity, in line with its higher phenolic content, suggesting that the greater abundance of phenolic acids and flavonoids may contribute to the observed bioactivity, although the present data do not allow attribution to individual compounds or to a single molecular mechanism. Under the experimental conditions used here, the extracts reduced ROS/RNS accumulation, influenced cell viability, attenuated inflammatory signalling, and modulated cellular cholesterol, supporting a redox-sensitive, immunomodulatory, and lipid-related mode of action. In HepG2 cells, the decrease in TNF-α, IL-6, and IL-1β, together with the increase in IL-10 at the highest doses, is consistent with a shift toward a less inflammatory phenotype, although this should be regarded as a partial and context-dependent response rather than a definitive anti-inflammatory effect. The same extracts also affected cholesterol homeostasis, with the low-altitude sample showing the clearest response, which is consistent with earlier evidence that honey-derived matrices may influence lipid-related pathways in hepatic models (Becerril-Sánchez et al. 2021; Cianciosi et al., 2018; Navaei-Alipour et al. 2021). Because oxidative stress and inflammation are closely interconnected, the cytokine modulation may partly reflect the antioxidant effects observed in the same setting, although a direct action on inflammatory pathways cannot be excluded. As these assays were conducted in vitro, the findings should be interpreted as mechanistic evidence of biological potential rather than proof of in vivo efficacy.

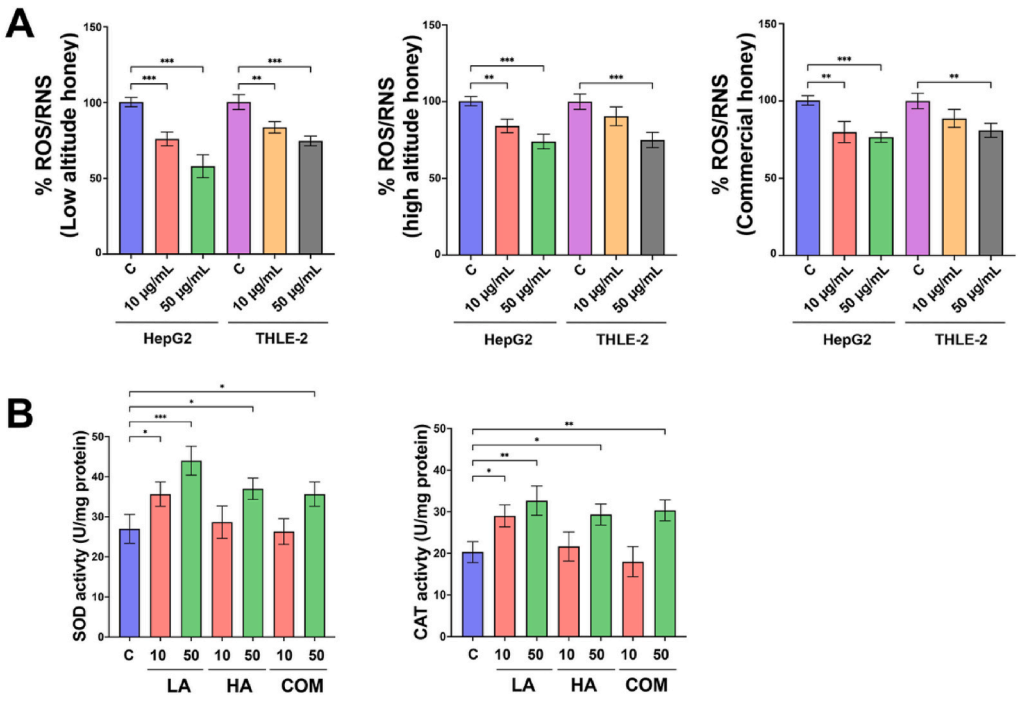

Fig. 6. Antioxidant effects of honey polyphenolic extracts (10 and 50 µg mL^{-1}) in hepatic cell models after 24 h exposure. (**A**) Intracellular ROS and RNS production in HepG2 and THLE-2 cells measured with fluorimetric assays. (**B**) SOD and CAT activities in HepG2 cells measured with colorimetric assays. LA, low altitude, HA, high altitude, COM, commercial. Data are expressed as mean ± standard deviation (SD) relative to control cells (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

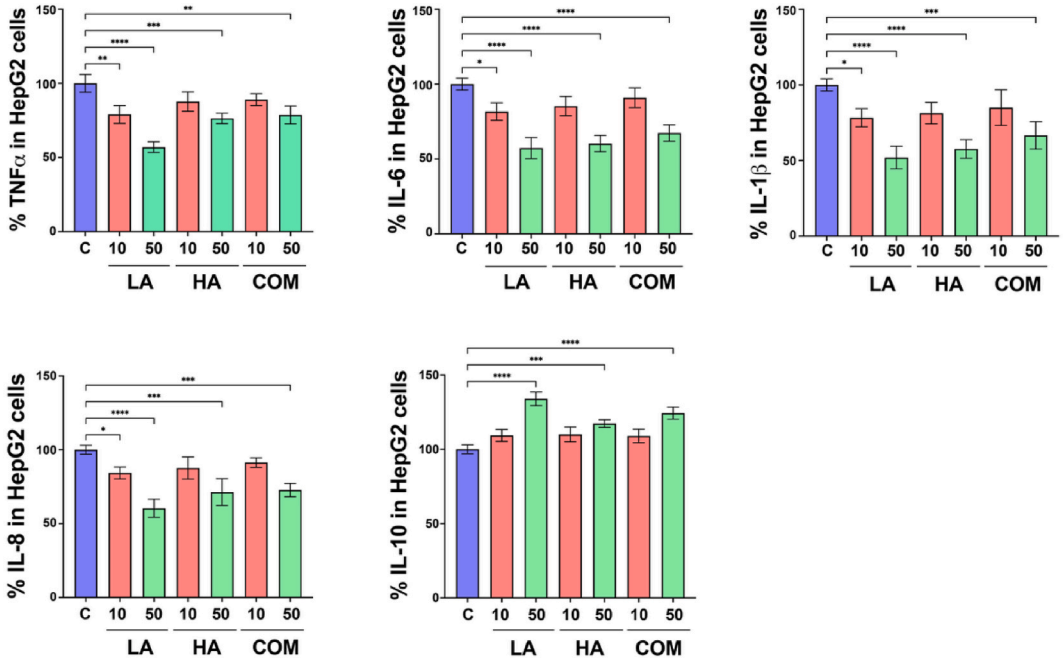

Fig. 7. Anti-inflammatory effects of honey polyphenolic extracts (10 and 50 µg mL^{-1}) in hepatic cell models after 24 h exposure. Production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and anti-inflammatory cytokine IL-10 in HepG2 cells determined by ELISA. Data are expressed as mean ± standard deviation (SD) relative to control cells (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001). LA, low altitude, HA, high altitude, COM, commercial.

4. Conclusions

Although this study provides novel insights into the chemical and biological characterization of multifloral honeys and apicultural by-products from the Sibillini Mountains bio-district, some limitations should be acknowledged. The investigation was conducted on a limited number of representative honey samples collected within a single production area and season. Therefore, the observed differences cannot be exclusively attributed to altitude, as floral biodiversity, environmental conditions and local ecological factors may also contribute to the distinct chemical and biological profiles. Future studies including broader geographical sampling, seasonal variability and detailed

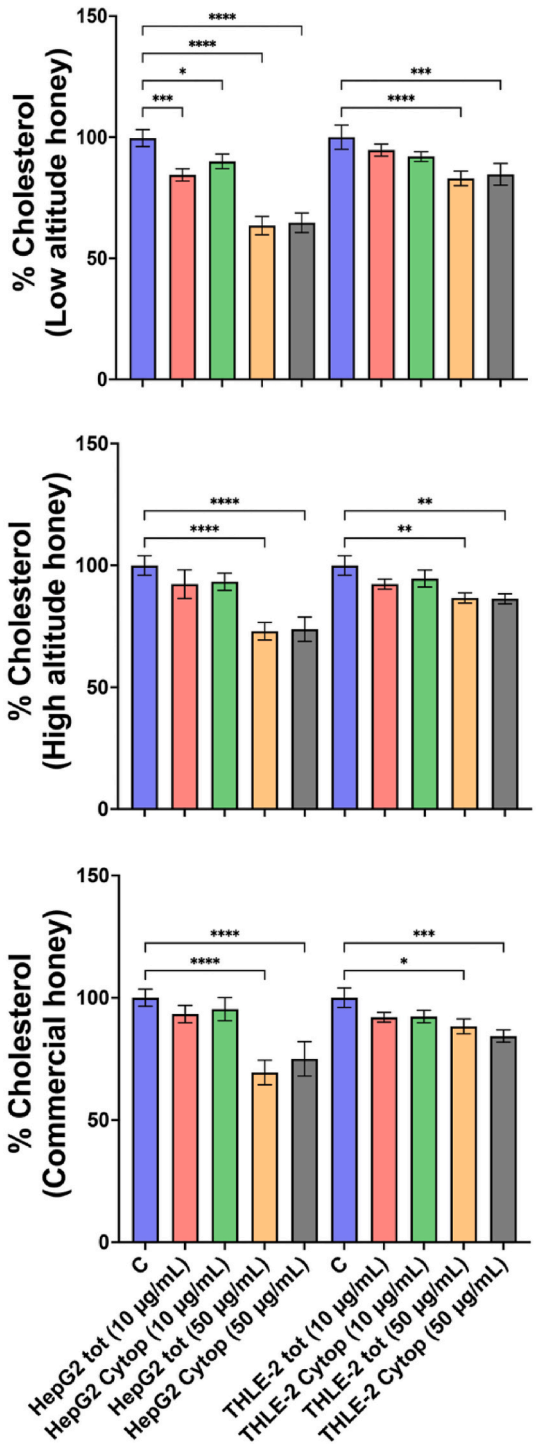

Fig. 8. Total and cytoplasmic cholesterol levels measured using the Amplex Red assay in HepG2 and THLE-2 cells treated with the three honey extracts (10 and 50 μg mL^{-1}). Data are expressed as mean ± standard deviation (SD) relative to control cells (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001).

melissopalynological characterization would further strengthen the interpretation of altitude-related effects and improve the understanding of the relationship between botanical origin, environmental conditions and honey bioactivity.

CRediT authorship contribution statement

Agnese Santanatoglia: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Valentina Cecarini:** Writing – original draft, Methodology, Investigation. **Massimiliano Cuccioloni:** Methodology, Investigation, Formal analysis. **Camilla Antolini:** Methodology, Formal analysis, Conceptualization. **Giovanni Caprioli:** Writing – review & editing, Visualization, Validation, Supervision. **Gianni Sagratini:** Supervision, Resources, Funding acquisition.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2026.109263.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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