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Phenolic Composition, Antioxidant and Antimicrobial Activities of Berberis Honey from Tibet, China

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Abstract

Tibetan *Berberis* honey (BH) represents an underexplored natural raw material with promising development value for functional food applications. The present work characterized the phenolic fingerprint of BH and systematically compared its antioxidant and antimicrobial performance with commercial Manuka honey (MH). After phenolic fraction enrichment using XAD-2 macroporous resin, ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was employed to perform metabolomic characterization. The antimicrobial activity was assessed using the broth microdilution and agar diffusion methods. The phenolic extract obtained from BH possessed markedly elevated total phenolic content (TPC), total flavonoid content (TFC), alongside superior antioxidant capacity in comparison with MH ($P < 0.001$). Untargeted metabolomics identified 1,028 metabolites in BH, with phenolic acids (20.62%) and alkaloids (19.16%) as the predominant classes. Targeted metabolomics quantified 104 phenolic compounds, with syringic acid, kaempferol, and protocatechuic acid as the major components. Furthermore, BH demonstrated stronger antibacterial activity against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, yet exhibited weaker activity against *Escherichia coli* compared with MH. The hydrogen peroxide content of BH was 227.16 ± 8.27 μmol/kg, considerably exceeding the value of MH (109.69 ± 8.26 μmol/kg) ($P < 0.0001$), and this constitutes a key basis for its stronger antibacterial activity. Neither honey exhibited inhibitory activity against *Candida albicans*. Collectively, BH possesses a unique phenolic and alkaloid profile and shows superior selective antibacterial activity against certain pathogens over MH, underscoring its potential as a natural functional food with dual antioxidant and antibacterial properties.

1.Introduction

Honey, a natural sweetener, has been used by humans for thousands of years due to its unique sensory characteristics and medicinal value[1]. Its complex chemical composition, which includes sugars, proteins, organic acids, vitamins, minerals, and phytochemicals such as polyphenols, endows honey with various bioactive properties, including antioxidant, antimicrobial, and anti-inflammatory effects, thereby granting it broad application potential in both traditional medicine and modern clinical practice[2,3]. Botanical origin of nectar resources exerts a remarkable impact on honey's functional characteristics; honeys from different sources exhibit distinct differences in antioxidant capacity, total phenolic content, and phenolic compound composition [4]. The geographical region of honey collection, together with the plant source, jointly determines its phenolic composition and antioxidant activity[5].

While phenolic constituents—predominantly phenolic acids and flavonoids—occur in honey at modest levels, they function as critical indicators of its biological activity, floral source, and sensory attributes[6,7]. Research indicates that phenolic constituents serve as the principal determinants of honey's antioxidant potential, wherein total phenolic levels demonstrate a marked positive association with both DPPH radical scavenging efficacy and FRAP-based iron-reducing power[8,9]. More importantly, phenolic compounds are crucial functional components that endow honey with potent antibacterial properties,

representing the core material basis for honey's antimicrobial efficacy. A study by Cucu *et al*. confirmed that varying contents of resveratrol, gallic acid and ferulic acid inside Fallopia japonica honey correlate positively with the sample's antimicrobial potency[10]. Research by Ramon-Sierra *et al*. further confirmed that phenolic extracts from *Melipona beecheii* honey can bind to bacterial DNA/RNA and interfere with nucleic acid function, revealing the multiple mechanisms underlying the antibacterial activity of phenolic compounds[11].

Berberis, referred to as barberry in folk nomenclature, falls into a sizable genus belonging to Berberidaceae of the order Ranunculales, comprising over 500 species of evergreen or deciduous shrubs widely distributed across temperate to subtropical regions of Asia, Europe, the Americas, and Africa[12]. Plants of the genus *Berberis* have been widely used in traditional medicine, especially for the management of bacterial infectious disorders, alongside alleviating inflammation and metabolic diseases. Modern pharmacological studies have validated that *Berberis* plants are enriched with polyphenols and alkaloids, two major bioactive constituents that endow these plants with remarkable antibacterial capacity, as well as multiple auxiliary pharmacological properties including antioxidant, anti-inflammatory, lipid-lowering and hypoglycemic activities[13–16]. In Tibet and the Himalayan region, the *Berberis* genus exhibits high species diversity, with some species concentrated in specific areas, making them suitable for the large-scale production of *Berberis* honey (BH). Given the unique plant growth environment of the Qinghai-Tibet Plateau, Tibetan *Berberis* honey is expected to possess compositional characteristics and biological activities distinct from those of ordinary honey. Zhang *et al*.[17]conducted the first proteomic and metabolomic characterization of Tibetan *Berberis* honey, identifying 14 phenolic acids and 10 flavonoids. Their analysis demonstrated that total phenolic and flavonoid concentrations in this variety significantly surpass those found in conventional counterparts, such as acacia and eucalyptus honeys. Furthermore, the study established polyphenolics as the principal bioactive constituents driving the honey's potent antioxidant capacity and its observed anti-inflammatory effects in vitro.

Although the medicinal value of *Berberis* species and some of the basic bioactivities of Tibetan *Berberis* honey have been preliminarily confirmed, existing studies remain incomplete regarding the systematic identification of phenolic components in Tibetan *Berberis* honey and the quantification of characteristic phenolic compounds. Furthermore, the antibacterial activity of Tibetan *Berberis* honey—particularly its inhibitory effects against various common pathogenic bacteria—has not yet been systematically investigated. Given the growing threat of antibiotic resistance and the limited availability of ideal natural antibacterial agents, it is particularly critical to explore new honey resources with distinctive and potent antibacterial properties. This study focuses on Tibetan *Berberis* honey, systematically analyzing its phenolic composition, evaluating the antioxidant activity of the raw honey and its phenolic extracts, determining its antibacterial activity, and comparing it with Manuka honey (MH). This work not only fills the research gap in the antibacterial activity of Tibetan *Berberis* honey but also provides a scientific basis for its development as a promising natural antibacterial alternative to conventional antimicrobial agents.

2. Materials and Methods

2.1. Samples and Reagents

2.1.1. Honey Samples

The BH sample was collected from local apiaries in Nyingchi (Linzhi), Tibet, China, during the barberry flowering season in August 2024. The MH sample, graded UMF 10^{+} (MGO 263^{+}), was sourced from Manuka Health in New Zealand. Prior to analytical assessment, the honey specimens were maintained at 4°C under light-excluded conditions.

2.1.2. Main Reagents

Staphylococcus aureus (CMCC(B) 26003), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (CMCC(B) 10104), and *Candida albicans* (ATCC 10231) were obtained from the Guangdong Provincial Culture Collection of Microorganisms. Gallic acid, rutin, DPPH reagent, Folin-Ciocalteu reagent, the ABTS radical scavenging assay kit, and the total antioxidant capacity (T-AOC) assay kit (FRAP method) were purchased from Beijing Solabio Technology Co., Ltd. The hydrogen peroxide quantitative analysis kit (water-compatible) was purchased from Sangon Biotech (Shanghai) Co., Ltd.

2.2. Experimental Methods

2.2.1. Extraction of Phenolic Compounds

Phenolic constituents were isolated from honey matrices following the protocol established by Kassim *et al*.[18]. Briefly, a 50 g aliquot of each honey sample was precisely weighed and dissolved in 500 mL of hydrochloric acid adjusted to pH 2.0. This solution was subsequently loaded onto a glass chromatography column and allowed to percolate at a controlled flow rate. To eliminate sugars and other polar contaminants, the column underwent sequential washing with 500 mL of hydrochloric acid (pH 2.0) followed by 600 mL of deionized water. Thereafter, adsorbed phenolics were recovered by eluting the stationary phase with 500 mL of methanol, and the resulting effluent was collected. The eluate was concentrated via rotary evaporation under reduced pressure at 40°C, after which the residual material was redissolved in 10 mL of deionized water. Liquid-liquid extraction was then performed three times using 10 mL portions of ethyl acetate. The pooled organic layers were evaporated to dryness under a gentle stream of nitrogen to yield the final phenolic fractions, termed *Berberis* honey extract (BHE) and Manuka honey extract (MHE), respectively.

2.2.2. Determination of Total Polyphenol and Total Flavonoid Content

Total polyphenol content (TPC) was quantified utilizing the Folin–Ciocalteu assay, adhering to established protocols[19]. Briefly, honey samples were dissolved in distilled water to yield a final

concentration of 0.1 g/mL. Subsequently, a 0.5 mL aliquot of this solution was combined with 2.5 mL of 0.2 mol/L Folin-Ciocalteu reagent and permitted to react for 5 minutes. Following the addition of 2 mL sodium carbonate solution (0.1 g/mL), the volume was adjusted to 10 mL with distilled water and homogenized. The resulting mixture was incubated under dark conditions for 60 minutes prior to absorbance measurement at 760 nm. To construct the calibration curve, gallic acid standards ranging from 0.0025 to 0.15625 mg/mL were prepared and analyzed identically. TPC values were derived from this standard curve and reported as milligrams of gallic acid equivalents per gram of honey (mg GAE/g). All analyses were conducted in triplicate.

Total flavonoid content (TFC) was quantified via the sodium nitrite-aluminum nitrate colorimetric assay. The procedure was as follows: 5 g of honey was dissolved in distilled water to a final volume of 25 mL. An aliquot of 1 mL from this solution was combined with 0.3 mL of 15% $NaNO_2$, vortexed vigorously, and incubated for 6 min. Following this, 0.3 mL of 10% $Al(NO_3)_3$ was introduced; the mixture was again agitated and held for an additional 6 min. Next, 4 mL of 4% NaOH was added, and the volume was adjusted to 10 mL using 50% ethanol. After thorough mixing, the reaction proceeded for 15 min prior to absorbance recording at 510 nm. All measurements were performed in triplicate. A calibration curve was constructed using rutin standards ranging from 0.03125 to 1.5 mg/mL, processed identically. Sample TFC values were derived from this standard curve and reported as milligrams of rutin equivalents per gram of honey (mg RE/g).

2.2.3. Qualitative and Quantitative Analysis of Phenolic Compounds

Untargeted metabolomic profiling, 20 mg aliquots of polyphenol extracts derived from Berberis honey (BHE) and Manuka honey (MHE) underwent extraction using 1 mL of a pre-cooled (− 40°C) solvent mixture comprising methanol, acetonitrile, and water (2:2:1, v/v/v), which included an isotope-labeled internal standard. The resulting suspensions were vortexed, homogenized at 35 Hz for 4 min, and subjected to three cycles of sonication in an ice-water bath (5 min per cycle). Following incubation at −40°C for 1 h, samples were centrifuged at 12,000 rpm and 4°C for 15 min, after which the supernatants were collected for analysis. Chromatographic separation and mass spectrometric detection were performed on a Vanquish UHPLC system interfaced with an Orbitrap Exploris 120 high-resolution mass spectrometer (Thermo Fisher Scientific). Polar metabolites were resolved using a Waters ACQUITY BEH Amide column (2.1 × 100 mm, 1.7 μm) employing a mobile phase system consisting of water supplemented with 25 mmol/L ammonium acetate and 25 mmol/L ammonia (Phase A) and acetonitrile (Phase B). Conversely, nonpolar metabolites were separated on a Phenomenex Kinetex C18 column (2.1 × 100 mm, 2.6 μm) utilizing water containing 0.01% acetic acid (Phase A) and a 1:1 (v/v) mixture of isopropanol and acetonitrile (Phase B). The column oven was maintained at 40°C with an injection volume of 2 μL. Electrospray ionization (ESI) was conducted in both positive (3.8 kV) and negative (− 3.4 kV) polarity modes, with sheath and auxiliary gas flows set to 50 and 15 arbitrary units, respectively, and a capillary temperature of 320°C. Data acquisition involved full-scan MS at a resolution of 60,000 and MS/MS fragmentation at 15,000 resolution using stepped normalized collision energies (NCE) of 20, 30,

and 40 eV. Raw data files were converted to mzXML format using ProteoWizard, followed by peak detection, retention time alignment, and area integration via custom R scripts. Metabolite identification was achieved by matching against the BiotreeDB (V3.0) library.

For targeted metabolomics quantification, sample extracts were formulated into 10 mg/mL stock solutions using prechilled (− 20°C) 70% aqueous methanol. After vacuum drying 0.5 mL stock liquid, residues were redispersed in 1 mL identical extraction solvent, followed by 60 Hz homogenization lasting 90 s and 30 min ice incubation prior to centrifugation (4°C, 12,000 rpm, 1 min). A total of 300 µL clarified supernatant was vacuum-evaporated, reconstituted with 100 µL methanol/water (7:3, v/v). After secondary centrifugation, 20 µL of the supernatant was loaded onto the detection system.

Chromatographic separation was completed on Vanquish Core HPLC coupled to Q Exactive HF-X Orbitrap MS (Thermo Fisher). Separation was carried out on Waters CSH C18 column (50 × 2.1 mm, 1.7 µm) at 40°C with 1 µL injection volume. Mobile phase A was 10 mmol/L ammonium acetate aqueous solution and phase B was pure acetonitrile at a flow rate of 0.4 mL/min under the established gradient schedule. The ESI+ source was set at 3200 V spray voltage and 320°C capillary temperature; sheath and auxiliary gas were fixed at 30 Arb and 10 Arb respectively, and auxiliary heater was stabilized at 400°C.External standard calibration served for quantitative measurement; standard curves were prepared using 207 individual flavonoid reference substances covering 0.1–2000 ng/mL, where every calibration curve yielded R^2 > 0.99. LOD (S/N = 3) and LOQ (S/N = 10) were defined based on signal-to-noise values. Final phenolic contents were calculated combining measured readings, sample mass and dilution multiples against calibration equations and presented in µg/100 g honey.

2.2.4. Antioxidant Activity Assay

The antioxidant capacity of both crude honey and its isolated phenolic fraction was evaluated via the DPPH radical scavenging assay, adhering to a slightly modified protocol established in prior literature[20]. Serial dilution of raw honey was prepared with ultrapure water at 10, 25, 40, 55 and 70 mg/mL, whereas phenolic extracts were dissolved in methanol to attain 0.025, 0.05, 0.1, 0.2 and 0.4 mg/mL. Then, 2.0 mL of sample solution was mixed with 2.0 mL of 0.05 mg/mL DPPH methanolic solution. Following 60 min of dark incubation under ambient temperature, absorbance values were recorded at 517 nm. 50% methanol served as blank control for reagents, and another blank group substituting DPPH solvent with pure methanol was arranged to eliminate sample background interference.The DPPH scavenging rate was calculated using Eq. (1):

$$Scavenging\,rate\ (\%)\ =\ [1\ -\ (A_2\ -\ A_3)/A_1]\ \times\ 100$$

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where A_1 is the absorbance of the DPPH solution without the sample, A_2 is the absorbance of the mixture of the sample and DPPH, and A_3 is the absorbance of the sample blank (without DPPH).

The half-maximal inhibitory concentration (IC_{50}) was calculated by three-parameter logistic nonlinear regression using GraphPad Prism 10.1.2 software.

ABTS radical clearance activity and FRAP ferric reduction capability were quantified with ready-to-use test kits sourced from Beijing Solabio Technology Co., Ltd. Raw honey was dissolved in double-distilled water to 0.1 g/mL, while phenolic extracts were diluted with methanol to reach 1 mg/mL concentration.

The ABTS detection followed kit specification protocols; absorbance at 405 nm was read after 6 min of dark ambient-temperature reaction, with distilled water and extraction solvent separately set as blank and control groups to compute radical scavenging percentage.

Consistent with kit guidelines, FRAP reaction lasted 10 min at ambient temperature prior to absorbance reading at 593 nm. A series of $FeSO_4$ standards (0.003125–0.05 μmol/mL) were applied to establish calibration curves for calculating reducing capacity. Final outcomes were presented as μmol Fe^{2+}/g for honey samples and μmol Fe^{2+}/mg for phenolic extracts. Every measurement was conducted in biological triplicate.

2.2.5. Antimicrobial Activity Assay

2.2.5.1. Determination of Minimum Inhibitory Concentration

With slight adjustments referring to the protocol published by Lin et al.[21]broth microdilution assay was adopted to test MIC and MBC values of tested honey samples. Bacterial test strains were cultured in LB broth, whereas fungal strains were incubated in PDA medium until logarithmic growth phase. The microbial suspension concentration was standardized to 2×10^6 CFU/mL prior to experiments. Ten grams of honey sample was accurately weighed and serially two-fold diluted with culture medium to prepare a concentration gradient: 50%, 25%, 12.5%, 6.25%, 3.125%, 1.5625%, and 0.78125% (w/v). In a 96-well plate, 10 μL bacterial suspension was mixed thoroughly with 190 μL serially diluted honey solution; the control group was set by mixing 10 μL inoculum with 190 μL sterile blank medium. After incubation at 37°C for 18 h, the plates were visually inspected under standard lighting. The MIC was defined as the lowest honey concentration with complete clarity and no observable microbial growth in wells.

2.2.5.2. Minimum Bactericidal Concentration (MBC) Assay

To determine the MBC, 10 μL aliquots were drawn from wells exhibiting no visible growth during MIC assessment and spread onto LB agar plates. Following incubation at 37°C for 18–24 h, the MBC was identified as the minimum concentration yielding no colony formation.

2.2.5.3. Agar Diffusion Method

The agar well diffusion method was employed. Bacterial suspensions (1×10^7 CFU/mL) were evenly spread onto agar plates using a sterile cotton swab. After wells were created, 100 μL of honey solutions

at varying concentrations (30%, 50%, and 70% w/v) were added to each well. A 5% phenol solution was used as the positive control. Following a 24-hour incubation period at 37°C, the inhibition zone diameter (inclusive of the well size) was quantified and documented in millimeters (mm).

2.2.6. Determination of Hydrogen Peroxide Content

Honey was diluted with purified water to a final concentration of 50% (w/v), and the hydrogen peroxide content was determined using a hydrogen peroxide quantitative analysis kit (water-compatible, C500069, Sangon Biotech (Shanghai) Co., Ltd.). The assay relies on the below reaction mechanism: Fe^{2+} gets oxidized into Fe^{3+} by hydrogen peroxide in acidic surroundings, and the resultant Fe^{3+} further binds with chromogenic reagent to produce colored complexes with maximum absorbance at 595 nm; absorbance intensity presents a positive linear correlation with H_2O_2 content. Serial hydrogen peroxide standards ranging from 1 to 100 μM were employed for calibration curve construction, and all determinations were finished in three parallel replicates.

2.3. Statistical Analysis

All data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism software (version 10.1.2; GraphPad Software, San Diego, CA, USA). For comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was applied, followed by Tukey's post-hoc test for pairwise comparisons. The independent samples t-test was used for comparisons between two groups. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Phenolic Compound Analysis

3.1.1. Total Phenolic and Total Flavonoid Content

The total phenolic content of BH was 0.61 ± 0.01 mg GAE/g honey, significantly lower than that of MH (0.75 ± 0.04 mg GAE/g honey, $P < 0.01$) (Fig. 1a). However, after purification with XAD-2 resin, the total phenolic content of the *Berberis* honey phenolic extract (BHE) was 0.14 ± 0.00 mg GAE/mg extract, significantly exceeding that of the Manuka honey phenolic extract (MHE) (0.10 ± 0.01 mg GAE/mg extract, $P < 0.001$) (Fig. 1b). This result indicates that although the total phenolic content in *Berberis* honey is lower, the phenolic compounds in the extract are present at a higher concentration and purity, suggesting that XAD-2 resin may exhibit superior selective enrichment for *Berberis* honey phenolics. Total flavonoid content (TFC) exhibited similar differences: BH had a total flavonoid content of 0.82 ± 0.00 mg RE/g honey, which was significantly lower than that of MH (0.96 ± 0.03 mg RE/g honey, $P < 0.01$) (Fig. 1c), while that of BHE was 0.40 ± 0.00 mg RE/mg extract, significantly higher than that of MHE (0.14 ± 0.01 mg RE/mg extract, $P < 0.0001$) (Fig. 1d).

3.1.2. Phenolic Composition

Using UPLC-MS/MS-based non-targeted metabolomics analysis, 1,028 and 848 metabolites were identified in BH and MH, respectively (Supplementary Tables S1 and S2). Among these, 596 were common to both samples, 432 were specific to BH, and 252 were specific to MH (Fig. 2a). Among the metabolites in BH, lipids (22.08%) and phenolic acids (20.62%) were the most abundant, followed by alkaloids (19.16%), terpenes (15.47%), and flavonoids (11.67%) (Fig. 2b). Additionally, the profile included amino acids and their derivatives (6.13%) and other components (4.86%). Notably, the proportion of alkaloids was considerably higher in BH than in MH. In the MH samples, phenolic acids (27.36%) and lipids (21.23%) were the most abundant. These were followed by terpenes (16.16%) and flavonoids (15.68%), which were present at comparable levels, while alkaloids (11.91%) represented a notable fraction. Amino acids and their derivatives (5.19%) and other components (2.48%) completed the major categories of the metabolite profile (Fig. 2c).

Targeted metabolomics was subsequently utilized for the quantitative analysis of the phenolic compounds in BH, resulting in the identification of 104 flavonoids and phenolic acids, with concentrations ranging from 0.0006 to 46.98 μg/100 g (Supplementary Table S3). Table 1 lists the top 30 compounds with the highest contents. Among the phenolic acids, syringic acid exhibited the highest concentration (46.98 ± 4.30 μg/100 g), followed by protocatechuic acid (29.46 ± 2.98 μg/100 g) and vanillic acid (28.01 ± 2.59 μg/100 g). Among the flavonoids, kaempferol (30.35 ± 2.27 μg/100 g) and naringenin chalcone (28.75 ± 0.47 μg/100 g) were the most abundant (Table 1).

Table 1
Contents of top 30 phenolic compounds in *Berberis* honey (μg/100 g honey). Data are expressed as mean ± standard deviation (SD) (*n* = 4).

Compound	Concentration (μg/100 g)	Compound	Concentration (μg/100 g)
Syringic acid	46.98 ± 4.30	Quercetin	5.54 ± 0.58
Kaempferol	30.35 ± 2.27	trans-Ferulic acid	5.39 ± 0.69
protocatechuic acid	29.46 ± 2.98	Robinin	4.98 ± 0.41
Naringenin chalcone	28.75 ± 0.47	Chrysin	4.86 ± 0.52
Vanillic acid	28.01 ± 2.59	Rutin	3.88 ± 0.36
Syringaldehyde	23.03 ± 2.33	Salicylic acid	3.63 ± 0.28
4-Hydroxybenzoic acid	22.90 ± 2.16	Sinapic acid	3.49 ± 0.40
Benzoic acid	21.91 ± 2.54	Isorhamnetin	3.22 ± 0.15
Kaempferol-3-neohesperidoside	21.47 ± 2.36	Sakuranetin	2.86 ± 0.23
p-Hydroxycinnamic acid	17.57 ± 1.47	Narcissin	2.54 ± 0.11
Nicotiflorin	12.51 ± 2.13	Cynaroside	1.49 ± 0.11
Pinocembrin	10.68 ± 1.00	Dihydrokaempferol	1.43 ± 0.10
Galangin	7.03 ± 0.66	Astilbin	1.15 ± 0.08
Isorhamnetin-3-O-neohespeidoside	6.96 ± 0.71	Isosakuranetin	1.13 ± 0.06
trans-Cinnamic acid	6.45 ± 0.60	3,7-Di-O-methylquercetin	1.03 ± 0.09

3.2. Antioxidant Activity

The high natural TPC and TFC content of MH contributed to potent activity in the DPPH radical scavenging assay, with an IC_{50} value of 21.96 ± 1.61 mg/mL for DPPH inhibition; whereas the IC_{50} value for BH was 37.74 ± 5.40 mg/mL, significantly higher than that of Manuka honey ($P < 0.05$), indicating that BH exhibits lower DPPH scavenging capacity (Fig. 3a). For the extracts, the IC50 value for BHE was 0.10 ± 0.01 mg/mL, significantly lower than that of MHE (0.56 ± 0.07 mg/mL, $P < 0.01$), thereby confirming the high antioxidant potential of the BHE extract (Fig. 3b).

To further validate their antioxidant capacity, we measured the ABTS radical scavenging activity and iron ion reducing power of BH and MH, as well as BHE and MHE (Fig. 4). At a concentration of 0.1 g/mL, the ABTS radical scavenging activity of MH (32.28% ± 1.96%) was significantly higher than that of BH (8.59%

± 0.68%, $P < 0.0001$) (Fig. 4a). However, after purification and enrichment, at a concentration of 1 mg/mL, the ABTS scavenging rate of BHE (92.90% ± 0.29%) was significantly higher than that of MHE (37.83% ± 1.06%, $P < 0.0001$) (Fig. 4b). Similarly, the ferric reducing power results followed the same trend as the ABTS data. In raw honey, the reducing power of MH (10.25 ± 0.06 μmol Fe^{2+}/g honey) was significantly higher than that of BH (6.27 ± 0.42 μmol Fe^{2+}/g honey, $P < 0.0001$) (Fig. 4c), whereas at the extract level, the reducing power of BHE (3.04 ± 0.10 μmol Fe^{2+}/mg extract) was significantly higher than that of MHE (2.33 ± 0.10 μmol Fe^{2+}/mg extract, $P < 0.001$) (Fig. 4d). These results are consistent with the TPC values, suggesting that phenolics are the primary contributors to antioxidant activity.

3.3. Antimicrobial Activity

The antimicrobial activities of Tibetan *Berberis* honey (BH) and Manuka honey (MH) were evaluated by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and inhibition zone diameter. As shown in Table 2, both BH and MH exerted strong antibacterial activity against *Staphylococcus aureus*, with identical MIC and MBC values of 6.25%, indicating equivalent inhibitory and bactericidal potency. Against *Escherichia coli*, BH showed an MIC and MBC of 25%, while MH exhibited an MIC of 12.5% and MBC of 25%, suggesting that MH possessed slightly stronger inhibitory activity but similar bactericidal capacity relative to BH.

Notably, BH displayed significantly more potent antibacterial activity than MH against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. For *K. pneumoniae*, the MIC and MBC of BH were both 6.25%, which were markedly lower than those of MH (MIC: 12.5%, MBC: 25%). Similarly, the MIC and MBC of BH against *P. aeruginosa* were both 12.5%, whereas those of MH were both 25%. These results demonstrate that BH inhibits and kills these two Gram-negative pathogens at much lower concentrations than MH. Neither BH nor MH showed antifungal activity against *Candida albicans* at concentrations up to 50%.

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Berberis* honey (BH) and Manuka honey (MH)

Samples	**S. *aureus* (%)**		**E. *coli* (%)**		**K. *pneumoniae* (%)**		**P. *aeruginosa* (%)**		**C. *albicans* (%)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
BH	6.25	6.25	25	25	6.25	6.25	12.5	12.5	> 50	> 50
MH	6.25	6.25	12.5	25	12.5	25	25	25	> 50	> 50

The antimicrobial activities of BH and MH against five pathogenic microorganisms were further verified using the agar well diffusion method at concentrations of 70%, 50%, and 30% (w/v), with a 5% phenol solution as the positive control (Fig. 5). Consistent with the MIC and MBC results, both honeys showed the strongest inhibitory effect against the Gram-positive bacterium *S. aureus*, followed by the Gram-negative bacteria *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. No inhibition zones were observed for either

honey against *Candida albicans*. Moreover, the antibacterial effects of both honeys displayed a clear concentration-dependent manner, with larger inhibition zones at higher concentrations.

For *S. aureus*, the antibacterial activity of BH exhibited significant concentration dependence (Fig. 5a and 5c). The diameters of the antibacterial zones for 70% and 50% BH were significantly larger than those for MH at the same concentrations; at 30% concentration, there was no statistically significant difference between the two. The diameter of the inhibition zone for 70% BH reached (24.33 ± 2.52 mm), which was significantly higher than that of MH (13.00 ± 1.00 mm) and the 5% phenol positive control (16.00 ± 1.00 mm). Overall, BH demonstrated superior antibacterial efficacy against *S. aureus*.

Against *E. coli*, the antibacterial activity of both honeys was relatively weak (Fig. 5b and 5d). The diameter of the antibacterial zone for 70% BH was similar to that of the 5% phenol positive control, while the antibacterial effect of MH at the same concentration was significantly smaller. At 50% concentration, the antibacterial zone of BH was slightly larger than that of MH, though the difference was not statistically significant. At a 30% concentration, BH produced a faint inhibition zone of (7.67 ± 0.58 mm), while MH at the same concentration showed no significant antibacterial activity. With the exception of 70% BH, the antibacterial effects of all other samples were significantly lower than those of the 5% phenol control (13.33 ± 1.53 mm).

Against *K. pneumoniae*, BH exhibited excellent inhibitory activity (Fig. 5e and 5g). At a 70% concentration, the diameter of the BH inhibition zone reached (20.00 ± 2.65 mm), which was significantly higher than that of MH at the same concentration (9.67 ± 0.58 mm), and also significantly superior to the 5% phenol positive control (15.00 ± 2.00 mm). The inhibition zone diameters for 50% and 30% BH were (18.00 ± 2.00 mm) and (15.67 ± 1.15 mm), respectively; in contrast, the inhibition zones for MH at corresponding concentrations were only (8.33 ± 1.15 mm) and (7.33 ± 0.58 mm). BH demonstrated superior antibacterial activity across all tested concentrations.

The overall antibacterial effect of honey against *P. aeruginosa* was relatively weak (Fig. 5f and 5h). BH at 70% and 50% concentrations formed antibacterial zones of (11.00 ± 1.00 mm) and (9.33 ± 1.41 mm), respectively; the 30% concentration showed no antibacterial activity, and MH exhibited no antibacterial effect at any of the tested concentrations. The 5% phenol control exhibited a large antibacterial zone of (25.00 ± 4.24 mm), far exceeding that of the honey samples. However, within the effective concentration range, BH still demonstrated greater antibacterial activity than MH.

Neither BH nor MH produced inhibition zones against *C. albicans* at any concentration, indicating that both honeys lacked antifungal activity at the tested concentrations.

3.4. Hydrogen Peroxide Content

Hydrogen peroxide is one of the key contributors to the antibacterial activity of honey. This study determined the hydrogen peroxide content of the two honeys, as presented in Fig. 6. The hydrogen

peroxide content of BH was 227.16 ± 8.27 μmol/kg, which was significantly higher than that of MH (109.69 ± 8.26 μmol/kg) ($P < 0.0001$).

4. Discussion

4.1. Characteristics and Sources of Phenolic Compounds in *Berberis* Honey

Environmental stress significantly modulates the accumulation of secondary metabolites in nectar-producing plants. Samadi *et al*.[22]reported that environmental stresses such as high altitude, low temperatures, intense UV radiation, and low rainfall can significantly increase the antioxidant activity and the content of secondary metabolites—including total phenolics, total flavonoids, and anthocyanins—in *Berberis* species, thereby enhancing their medicinal and nutritional potential. Wang *et al*.[23]further corroborated that altitude is a key environmental factor regulating the secondary metabolism of nectar-producing plants and further influencing honey composition; conditions such as intense UV radiation and low temperatures in high-altitude regions prompt plants to synthesize and accumulate more antioxidant secondary metabolites, such as flavonoids, and stimulate the enrichment of metabolic precursors such as tryptophan and phenylalanine, thereby increasing the concentration of these bioactive compounds in honey.

The total phenolic content of Tibetan *Berberis* honey determined in this study (0.61 mg GAE/g honey) was higher than that of several previously reported monofloral honeys, such as patchouli honey, hawthorn honey, and pomegranate honey, but lower than that of Manuka honey. The total flavonoid content of Tibetan *Berberis* honey (0.82 mg RE/g honey) was significantly higher than that of all nine Chinese monofloral honeys reported by Zhang *et al*. (0.21–0.61 mg RE/g)[19]and previously reported Tibetan *Berberis* honey (0.14 mg RE/g)[17]. This difference may be attributed to variations in the year of sample collection, geographical altitude, flowering period of the nectar plants, and extraction method. Overall, Tibetan *Berberis* honey is characterized by unique flavonoid enrichment, which may be driven by the natural abundance of flavonoids and alkaloids in its nectar source plants (*Berberis* spp.) and the induction of plant defensive metabolism by high-altitude environmental stress[23,24].

Untargeted metabolomics analysis revealed that the metabolites in Tibetan *Berberis* honey are predominantly lipids and phenolic acids, with the proportion of alkaloids (19.16%) significantly higher than that in Manuka honey (11.91%), consistent with the alkaloid-rich characteristics of *Berberis* spp. Targeted metabolomics enabled the first-time identification of 104 phenolic compounds in Tibetan *Berberis* honey, with relatively high levels of syringic acid, kaempferol, protocatechuic acid, naringenin chalcone, and vanillic acid. These phenolic compounds have been extensively documented to exhibit various biological activities and their high concentration underpins the functional activity of Tibetan *Berberis* honey.

4.2. Relationship between Phenolic Compound Composition and Antioxidant Activity

The total phenolic and total flavonoid contents, as well as the DPPH, ABTS, and FRAP antioxidant activities of raw *Berberis* honey were consistently lower than those of Manuka honey. However, following enrichment with XAD-2 resin, the *Berberis* honey phenolic extract (BHE) exhibited significantly higher total phenolic and total flavonoid contents, as well as superior performance in the three antioxidant assays, compared to the Manuka honey phenolic extract (MHE). This reversal in the total phenolic and total flavonoid contents between the raw honey and the extract may be attributed to the selective enrichment of different phenolic compounds by XAD-2 resin. Previous studies indicate that XAD-2 resin exhibits significant variability in the extraction rates of phenolic and flavonoid compounds, with recovery efficiency being highly dependent on the chemical structure and polarity of the specific compounds[25,26]. As a nonpolar adsorbent, XAD-2 resin co-adsorbs other hydrophobic components while adsorbing phenolics; non-phenolic reducing compounds are eluted along with the phenolics, thereby affecting the purity of the extract[27]. Consequently, despite the low relative abundance of phenolic compounds in raw *Berberis* honey, the resulting extract demonstrates higher enrichment purity and greater antioxidant efficiency per unit mass, indicating a stronger intrinsic antioxidant potential.

Flavonoids and phenolic acids are the primary contributors to the antioxidant activity of honey. Flavonoids directly scavenge reactive oxygen species (ROS) by donating hydrogen atoms or electrons via their hydroxyl groups; the ortho-dihydroxyl structure can chelate metal ions to inhibit the Fenton reaction, while also regulating the activity of antioxidant enzymes[28]. Phenolic acids primarily neutralize ROS through hydrogen/electron transfer and activate the Nrf2-ARE signaling pathway to upregulate endogenous antioxidant enzymes[29].

The phenolic compounds identified at high concentrations in this study all possess well-defined antioxidant mechanisms. Syringic acid, which was found in the highest concentration, not only scavenges free radicals but also activates the KEAP1/NRF2 pathway and inhibits NF-κB activation, thereby alleviating oxidative stress and inflammation[30]. Furthermore, Ham *et al*. confirmed that this compound exhibits anti-steatotic and anti-inflammatory effects in mice with high-fat diet-induced obesity[31]. Kaempferol, the second most abundant component in *Berberis* honey, exerts its antioxidant effects through multiple pathways: First, it inhibits the expression of the fatty acid transporter CD36 by activating the AMPK signaling pathway, thereby reducing lipid accumulation in hepatocytes, and suppresses ferroptosis to lower ROS levels and improve mitochondrial function[32]; Second, by simultaneously targeting the p53-p21-p16 senescence pathway and the Nrf2/HO-1/NQO1 antioxidant pathway, it enhances the expression of antioxidant enzymes and inhibits the development of atherosclerotic plaques[33]. Due to its ortho-dihydroxyl structure, protocatechuic acid exhibits strong free radical scavenging and metal chelating activity, thereby improving insulin resistance[34]. Vanillic acid also demonstrates multiple protective effects. Naganna *et al*. confirmed that vanillic acid enhances insulin

secretion and protects pancreatic β-cells from H_2O_2-induced oxidative stress damage[35]. Furthermore, Magiera *et al*. revealed that vanillic acid scavenges ROS, inhibits lipid peroxidation and protein carbonylation, and downregulates fMLP-induced oxidative bursts in human neutrophils[36].

In addition, pinocembrin, galangin, and quercetin—all present in quantities exceeding 5 μg/100 g in *Berberis* honey—exhibit significant antioxidant activity. Pinocembrin triggers the Nrf2/HO-1 signaling cascade to elevate the expression of downstream antioxidant proteins including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx); meanwhile, this compound eliminates diverse free radicals like DPPH and ABTS, thereby restraining lipid peroxidation progress[37]. Galangin's antioxidant property mainly originates from the unique substitution arrangement of three hydroxyl groups (3,5,7-trihydroxy) on its B-ring. Such structural feature endows the molecule with remarkable radical elimination and metal chelating effects, meanwhile improving the intrinsic antioxidant defense system via PI3K/Akt signaling stimulation[38]. Quercetin, due to the 3',4'-ortho-dihydroxyl structure on its B ring, scavenges ROS/RNS, chelates Fe^{2+} and Cu^{2+} to inhibit the Fenton reaction and reduce ·OH production, and activates the Nrf2/ARE pathway to upregulate the expression of antioxidant enzymes such as SOD, CAT, GPx, and HO-1[39]. The high concentration of these characteristic phenolic compounds and their synergistic multi-component effects constitute the fundamental material basis for the antioxidant activity of Tibetan *Berberis* honey.

4.3. Antimicrobial Activity of Tibetan *Berberis* Honey and Its Chemical Basis

The antimicrobial activity results demonstrated that Tibetan *Berberis* honey (BH) exhibited strong bacteriostatic and bactericidal effects against *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, but showed no activity against *C. albicans*. In terms of both MIC and MBC values, BH showed significantly stronger bactericidal activity against *K. pneumoniae* and *P. aeruginosa* than Manuka honey (MH). For *S. aureus*, BH and MH showed identical MIC and MBC values, but BH produced significantly larger inhibition zones at concentrations ≥ 50%, indicating stronger antibacterial efficacy at high concentrations. The phenomenon is likely related to the favorable diffusion performance and synergistic action of multiple active components in BH. MH showed a slightly lower MIC against *E. coli* (12.5% vs. 25% for BH), but the MBC values were identical (25%), meaning the two honeys exhibited similar bactericidal capacity against *E. coli*. Both honeys exhibited markedly superior inhibitory activity against Gram-positive bacteria (*S. aureus*) than against Gram-negative bacteria (*E. coli, P. aeruginosa*), which is consistent with the results of most studies[10,40,41]. This difference may be attributed to the more complex outer membrane structure of Gram-negative bacteria, which hinders the penetration of hydrophobic compounds (such as polyphenols) into the cytoplasmic membrane[9,34].

The antibacterial activity of honey results from the synergistic action of multiple factors, primarily including high osmotic pressure, low pH, and compounds such as hydrogen peroxide, methylglyoxal, and phenolic compounds[42]. In the present investigation, *Berberis* honey possessed markedly elevated

hydrogen peroxide levels compared with Manuka honey, which may partially explain its stronger inhibitory effect on specific strains. Notably, the antimicrobial action of hydrogen peroxide in honey does not solely depend on its concentration but is dynamically regulated by the honey's internal enzyme systems and components. Research by Osés *et al*. revealed this complex mechanism: hydrogen peroxide is generated by glucose oxidase (GOx) and subsequently degraded by catalase (CAT), with the balance of their activities determining the net accumulation of hydrogen peroxide. Concurrently, polyphenolic compounds can facilitate its production through redox processes, while non-peroxide components (such as low water activity, acidity, and phenolic compounds) synergistically enhance the effects of hydrogen peroxide[43]. Brudzynski *et al*. further elucidated the underlying chemical mechanism, demonstrating that the Fenton-type coupled reaction system composed of polyphenols, hydrogen peroxide, and transition metal ions (such as Cu^{2+}) is one of the key mechanisms underlying honey's antibacterial activity: polyphenols act as “active intermediates,” undergoing autoxidation in the presence of hydrogen peroxide to generate reactive oxygen species (ROS), which in turn cause irreversible oxidative damage to bacterial DNA[44]. Furthermore, hydrogen peroxide, as a small-molecule oxidizing agent, can penetrate the outer membrane relatively effectively and disrupt cellular structures through oxidative damage[45]. Thus, the antibacterial activity of honey depends on the steady-state equilibrium of hydrogen peroxide and its synergistic interaction with components such as polyphenols and metal ions; the high hydrogen peroxide content in the *Berberis* honey in this study likely enhances the inhibitory effect on specific bacterial strains through the aforementioned multiple mechanisms.

The antibacterial mechanisms of honey polyphenols are multifaceted. Ramon-Sierra *et al*. found that phenolic extracts from *Melipona beecheii* honey can bind to bacterial DNA and RNA, thereby interfering with nucleic acid function[11]; furthermore, membrane disruption and the induction of oxidative stress also contribute to their antibacterial activity[46]. Alarejani *et al.* used molecular docking and molecular dynamics simulations to elucidate the binding mechanism of phenolic compounds with a key bacterial enzyme (Gyrase B), thereby validating the role of polyphenols in inhibiting this enzyme's activity[47]. The antibacterial mechanisms of the abundant phenolic compounds identified in *Berberis* honey in this study have been clearly elucidated in previous research. For example, eugenol exhibits potential against quorum sensing and can inhibit virulence factors of *Serratia marcescens*[48]; protocatechuic acid induces bacterial death by triggering lipid peroxidation and DNA breaks through the induction of oxidative stress (enhancing the production of superoxide anions and hydroxyl radicals)[49]. The antibacterial activity of kaempferol has been validated in multifloral honey extracts[40]; vanillic acid exhibits bacteriostatic activity against various bacteria, with an MIC of 0.467–0.809 mg/mL[50]; the inhibitory effect of eugenol on *Mycobacterium marinum* involves the NRF2/HO-1/NQO-1 and AMPK-α1/AKT/GSK-3β pathways[51]. Chosonone disrupts cell membrane integrity, increases permeability, and induces reactive oxygen species accumulation[37]. Galangin inhibits DNA gyrase, interferes with cell wall synthesis, and depolarizes membrane potential, with particularly significant effects on Gram-positive bacteria[38]. Quercetin inhibits DNA gyrase and topoisomerase IV, disrupts the cell membrane, inhibits biofilm formation, and chelates iron ions[39]. The synergistic effects of these compounds, combined with the high

hydrogen peroxide levels in BH, underlie its superior inhibitory efficacy against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* compared to Manuka honey.

5. Conclusion

This work profiled the phenolic components of Tibetan *Berberis* honey (BH) and compared its antimicrobial performance with Manuka honey (MH). BH is rich in polyphenols and alkaloids, providing a favorable material basis for its potent biological activity. BH exhibited prominent selective antibacterial activity: it outperformed MH against *K. pneumoniae* and *P. aeruginosa*. The two honeys had identical MIC and MBC against *S. aureus*, while BH formed larger inhibition zones at high concentrations. For *E. coli*, BH had weaker inhibitory activity yet equivalent bactericidal ability. No antifungal activity was observed for either honey. Its high hydrogen peroxide content and inherent phenolic composition synergistically drive the antibacterial potency of BH. In conclusion, BH is a valuable natural resource with distinctive antimicrobial properties for antibacterial application.

Declarations

Author Contributions: YZ (Yane Zhou): Writing - original draft, Validation, Software, Visualization. WW: Software, Methodology. CY: Writing - review & editing. MZ: Data curation. JL: Writing - review & editing. GH: Writing - review & editing. YZ (Yi Zhang): Writing - review & editing, Investigation, Conceptualization. WL: Writing - review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

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Figures

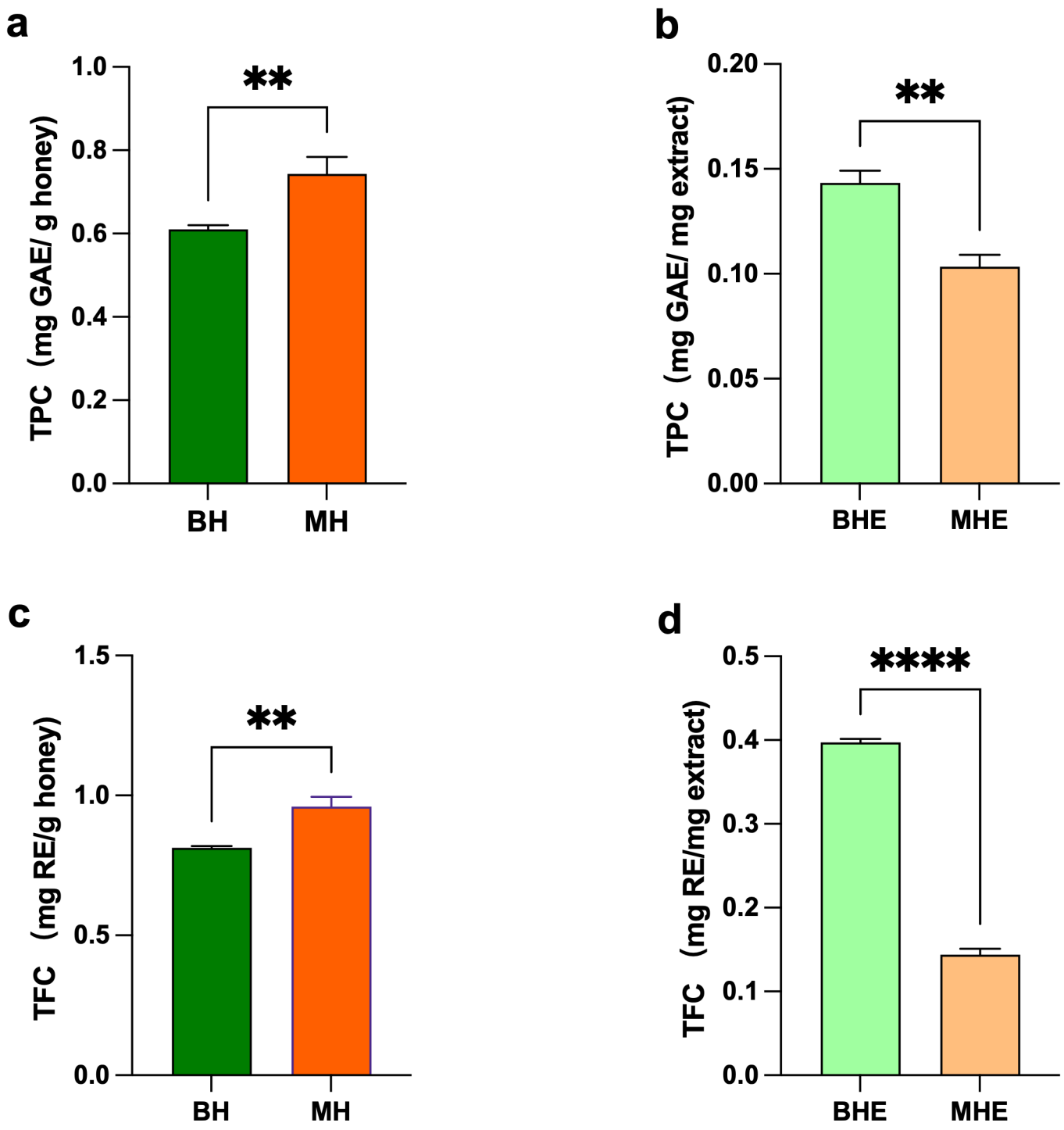


Figure 1

Total phenolic content (TPC) and total flavonoid content (TFC) of *Berberis*honey (BH), Manuka honey (MH), and their polyphenol extracts ((BHE: *Berberis* honey extract; MHE: Manuka honey extract). (a) TPC of BH and MH; (b) TPC of BHE and MHE; (c) TFC of BH and MH; (d) TFC of BHE and MHE. Data are expressed as mean ± standard deviation (SD) (n = 3). Statistical significance was determined by unpaired two-tailed t-test. $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$.

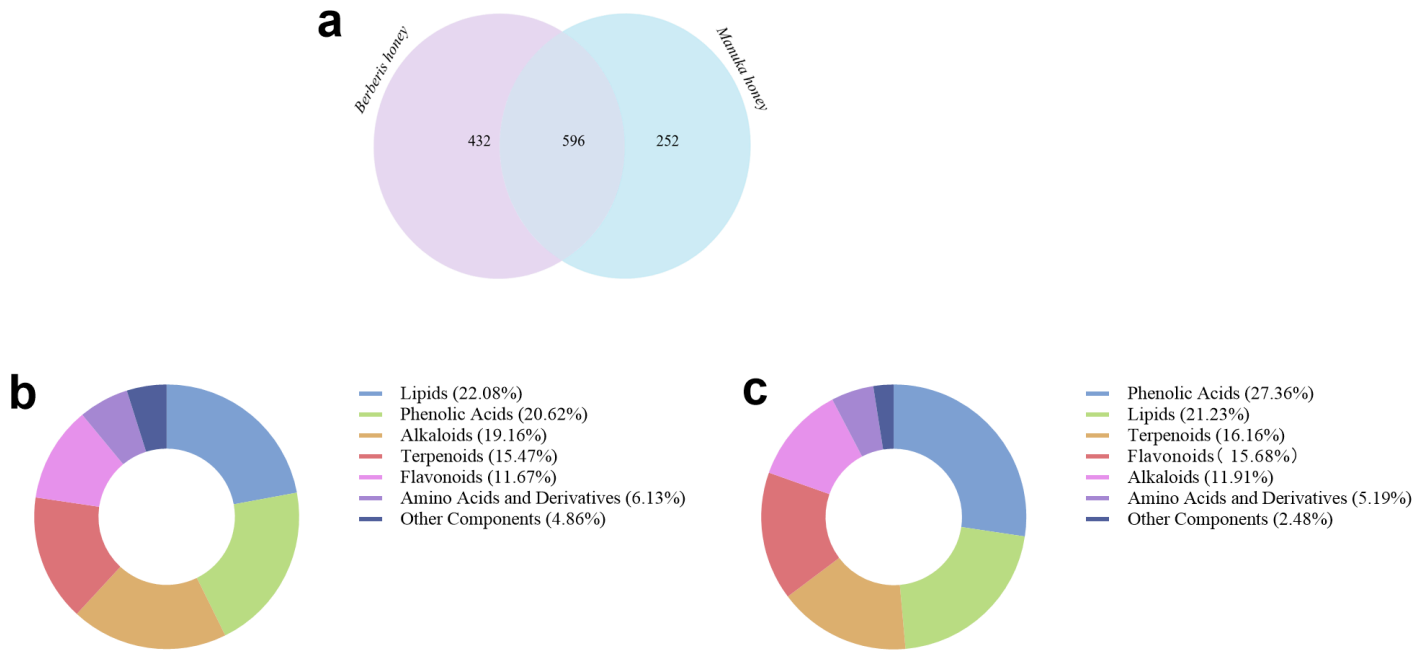


Figure 2

The main components in *Berberis* honey and Manuka honey. (a) Common components between *Berberis*honey and Manuka honey. (b) Main components identified in *Berberis* honey. (c) Main components identified in Manuka honey.

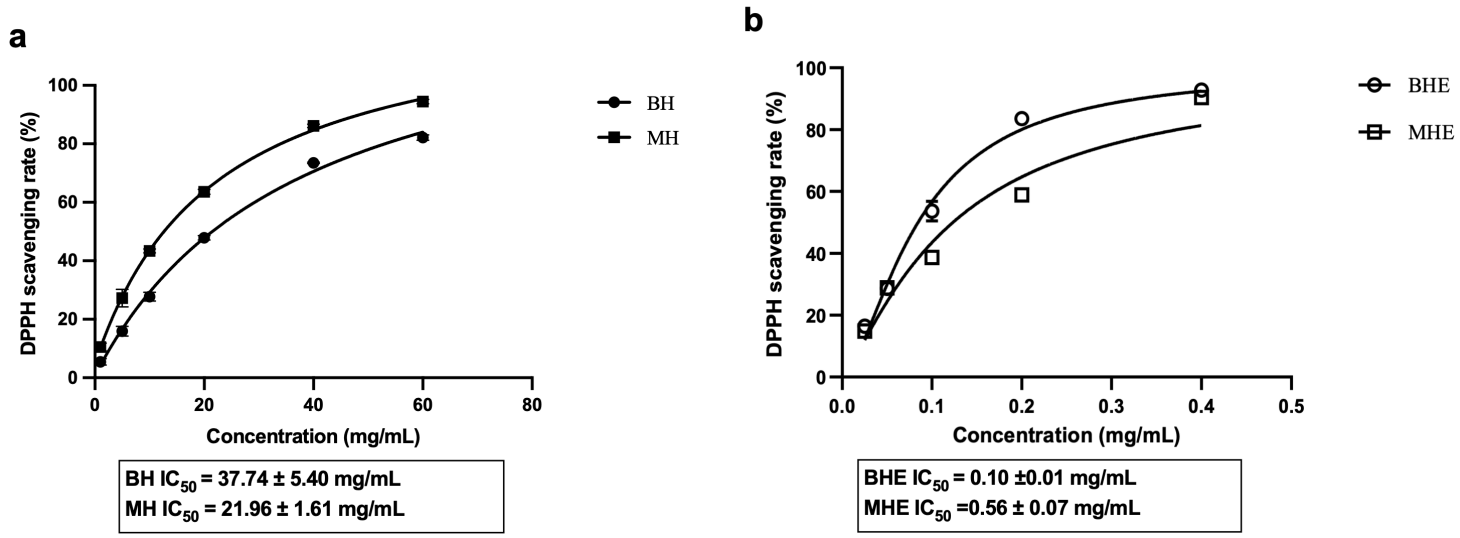


Figure 3

DPPH radical scavenging activity of BH, MH, BHE and MHE. (a) BH vs MH; (b) BHE vs MHE. The IC_{50} of BH was 37.74 ± 5.40 mg/mL, significantly higher than that of MH (21.96 ± 1.61 mg/mL, $P < 0.05$). The IC_{50} of BHE was 0.10 ± 0.01 mg/mL, significantly lower than that of MHE (0.56 ± 0.07 mg/mL, $P < 0.01$).

Data are mean ± SD (n = 3). Curves were fitted by three-parameter logistic regression. Statistical significance was determined by unpaired two-tailed t-test. $P < 0.05$, $P < 0.01$.

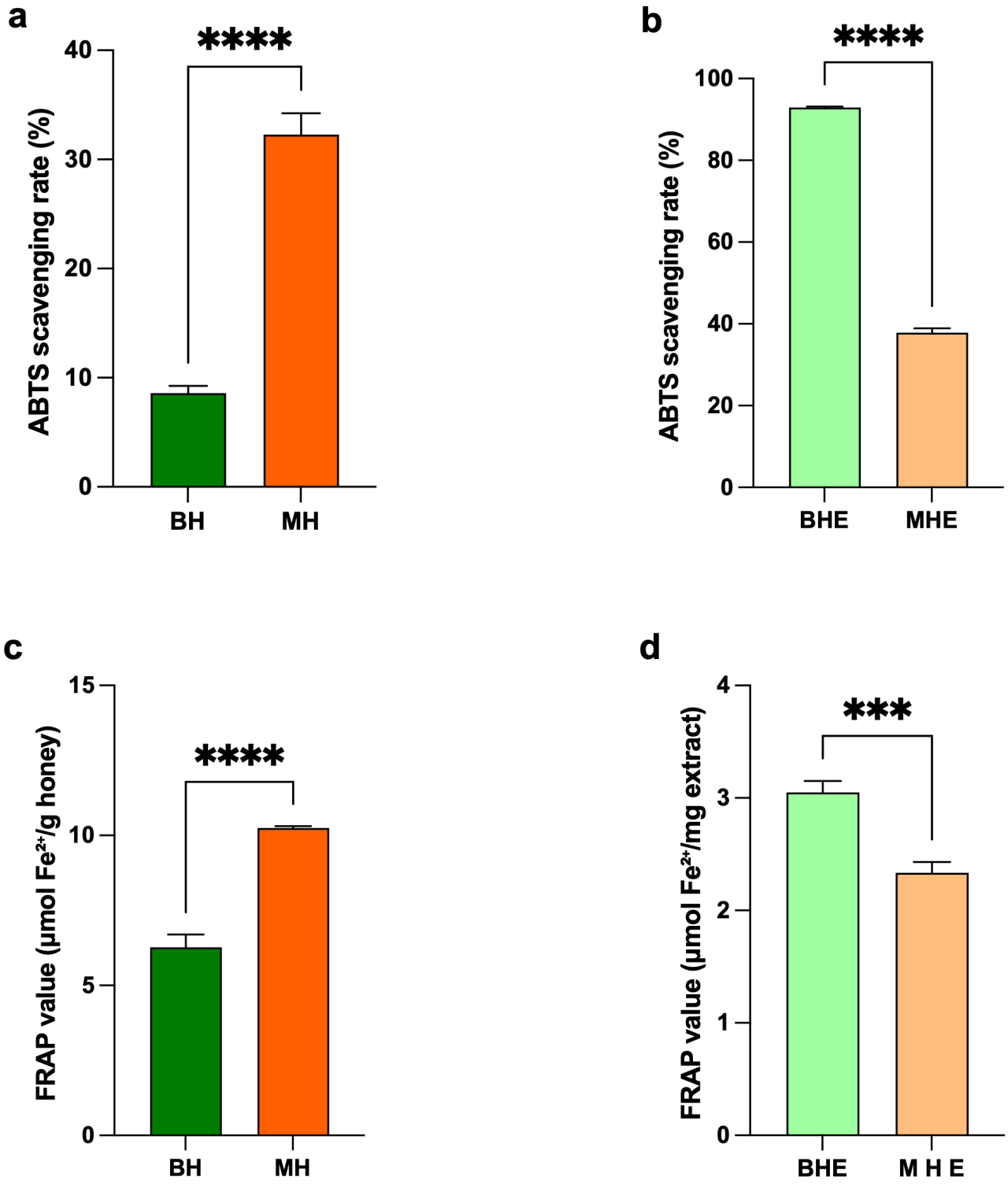


Figure 4

ABTS radical scavenging capacity and FRAP iron reducing antioxidant activity of BH, MH, BHE and MHE. (a) ABTS scavenging rate of BH vs MH; (b) ABTS scavenging rate of BHE vs MHE; (c) FRAP reducing

capacity of BH vs MH; (d) FRAP reducing capacity of BHE vs MHE. Data are mean ± SD (n = 3). Statistical significance was determined by unpaired two-tailed t-test. $P < 0.001$, $P < 0.0001$.

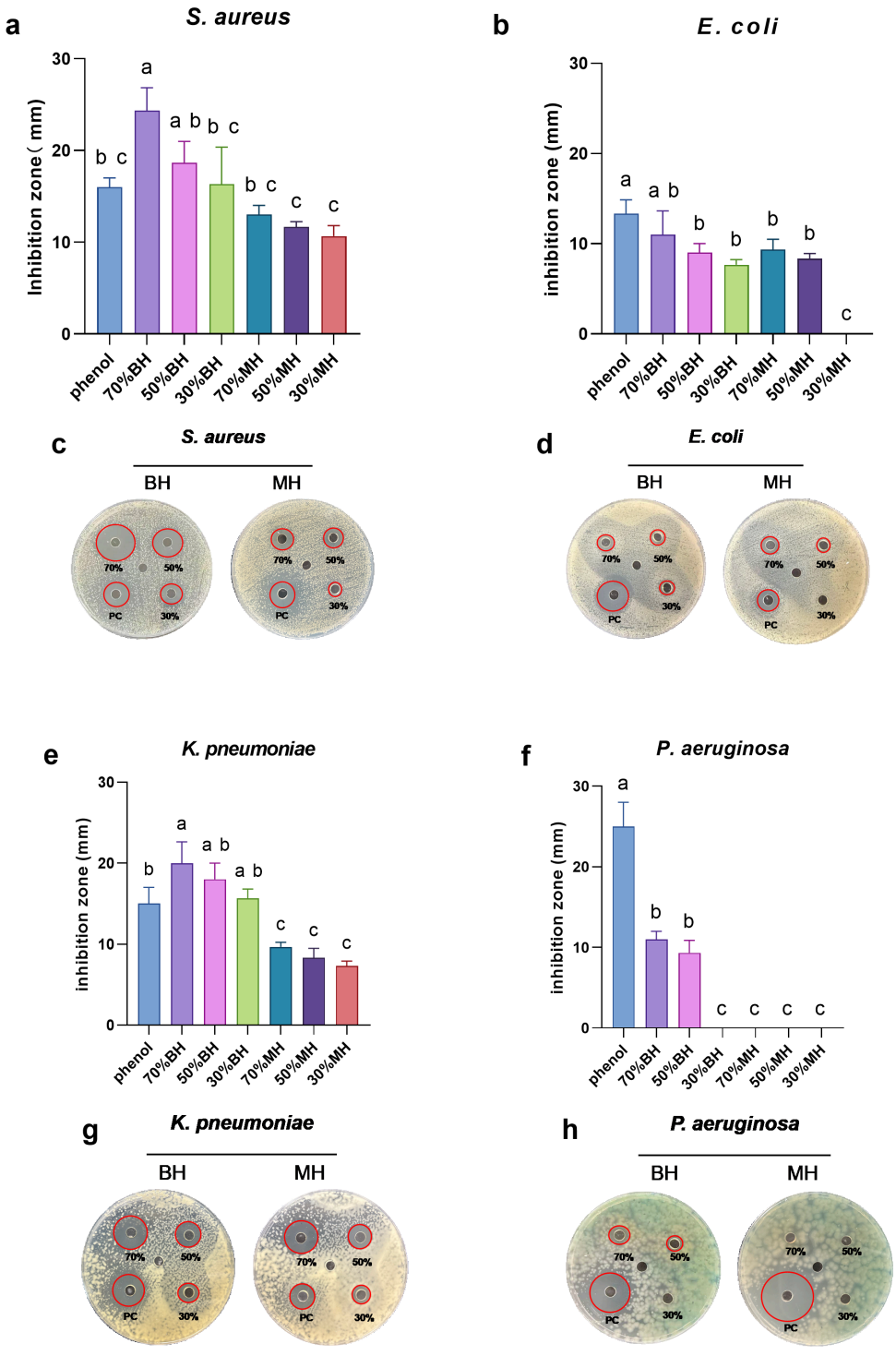


Figure 5

Antibacterial activity of *Berberis* honey (BH) and Manuka honey (MH) against four pathogenic microorganisms: *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. (a, b, e, f) Inhibition zone diameters

at 70%, 50%, and 30% (w/v) against four tested microorganisms. Data are mean ± SD (*n* = 3). 0 indicates no inhibition. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Different letters above the bars indicate significant differences ($P < 0.05$). (c, d, g, h) Representative agar well diffusion images. Wells: 70% BH, 50% BH, 30% BH, 70% MH, 50% MH, 30% MH, and 5% phenol (positive control, PC). Clear zones surrounding the wells indicate bacterial growth inhibition.

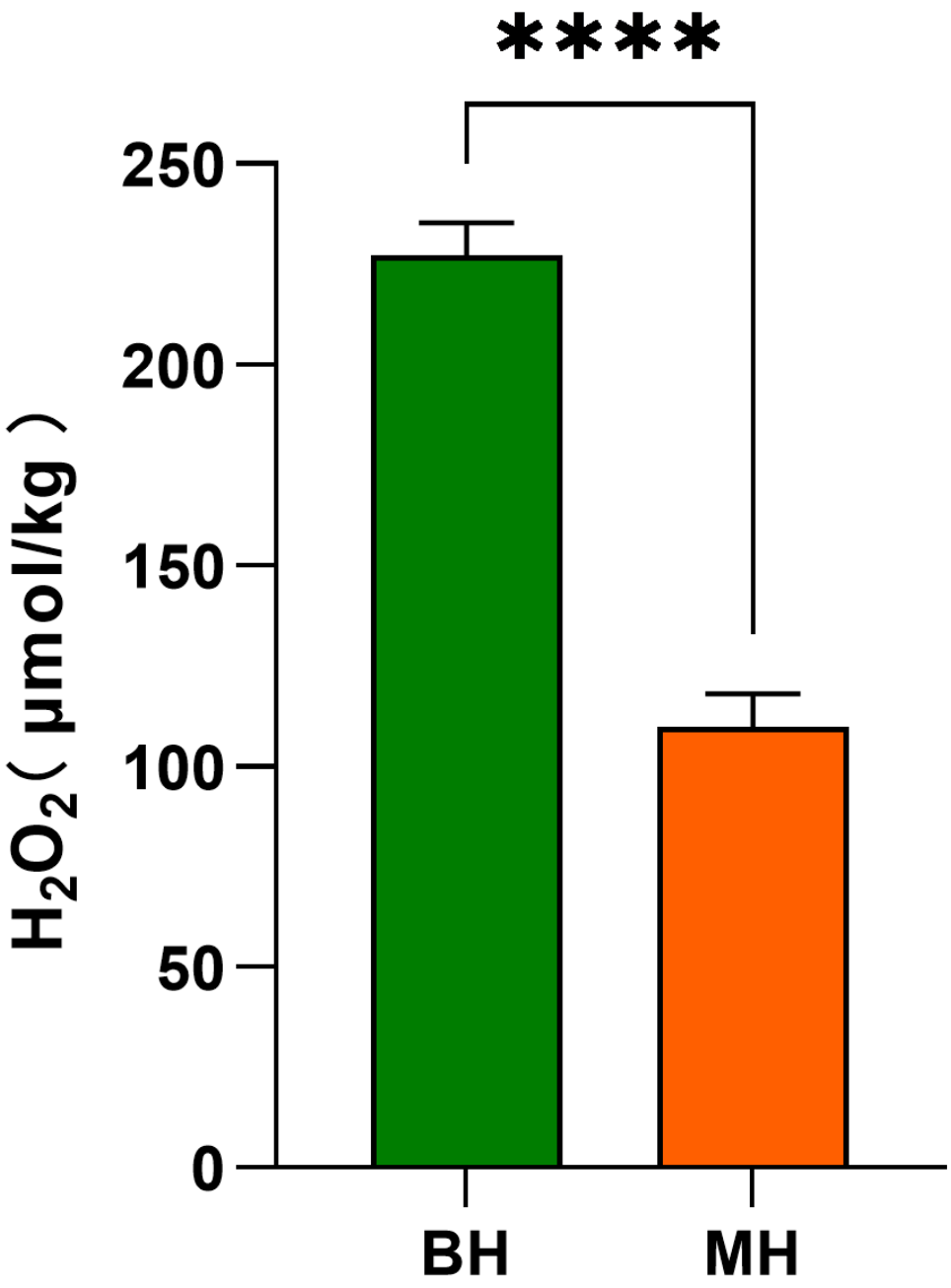


Figure 6

Hydrogen peroxide (H_2O_2) content in *Berberis* honey (BH) and Manuka honey (MH). Data are expressed as mean ± standard deviation (SD) (n = 3). Statistical significance was determined by unpaired two-tailed t-test. $P < 0.0001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS3.xlsx
- SupplementaryTableS2.xlsx
- SupplementaryTableS1.xlsx