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FULL-LENGTH RESEARCH ARTICLE

Chemical Characterization of Rare Unifloral Honeys of Ailanthus (*Ailanthus altissima*), Fennel (*Foenicum vulgare*), and Raspberry (*Rubus idaeus*) and their Antimicrobial and Antioxidant Activity

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Abstract Characterization of unifloral honey is of great importance for the definition of quality standards and the confirmation of the authenticity of honey. In this study, standard physicochemical analyses, pollen analyses, determination of total phenolic and flavonoid content, analysis of antioxidant capacity and antimicrobial activity, and qualitative and quantitative analyses of phenolic compounds by LC–MS/MS method were performed in three rare uniofloral honeys—ailanthus (*Ailanthus altissima*), fennel (*Foeniculum vulgare*), and raspberry (*Rubus idaeus*) honeys. The results showed that each honey type has specific physicochemical properties and phenolic content, which consequently influence its biological activity. All honey samples showed good characteristics, according to the compositional and quality criteria of the standard codex for honey. Generally, the ailanthus honey samples had a higher total phenolic and flavonoid content, while the fennel samples showed greater variability. The ailanthus honey samples also showed higher DPPH antioxidant activity, and the ABTS and ORAC assays revealed no differences between the honey types analysed, with the exception of the raspberry honey. In ailanthus honey, the flavonoids chrysin, quercetin, and the phenolic acid 3,4-DHBA were the most abundant. Several quercetin derivatives, including quercetin-3-glucuronide, quercitrin, and quercetin methyl ether, were detected in the fennel honey. In addition, raspberry honey exhibited a distinct phenolic profile containing catechin, epicatechin, quercetin rhamnoside, sakuranetin, tectochrysin, quercetin dimethyl ether, rhamnetin, caffeic acid benzy ether, and pinobanksin-3-O-pentanoate. The strongest results for antibacterial activity came from ailanthus honey. The increased antimicrobial activity of ailanthus honey was found especially against S. *aureus* and E. *coli* and moderately against A. *baumannii*. This study is the first step towards a thorough characterization of ailanthus, fennel, and raspberry honeys and may contribute to the recognition of these rare honeys and provide a good basis for their use in the pharmaceutical industry.

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Introduction

Honey contains a high concentration of phytochemicals from plants, which have antimicrobial, antioxidant, and anti-inflammatory properties [1–3]. The chemical composition is primarily determined by its botanical and geographical origins, as well as environmental factors [4]. Honey from a single plant species is typically much more expensive than polyfloral honey due to its biochemical composition [5]. Among the great competition of different honey types, authenticity is a key factor. Therefore, research into honey's composition and bioactive properties is a very important feature for the market and the customer. In our study, we aimed to characterize rare unifloral honey types: ailanthus *(Ailanthus altissima),* fennel *(Foeniculum vulgare)*, and raspberry honey *(Rubus idaeus)* which are not commonly found on the market or easily available.

Ailanthus altissima (Mill.), also known as the tree of heaven, is primarily grown in Eastern Asia. It belongs to the *Simaroubaceae* family and has been used in traditional medicine as an astringent and antiparasitic agent, as a central nervous system depressant, and to treat fever, epilepsy, asthma, infection, and gastric diseases [6–8]. In Korea, *A. altissima* has been used for suppressing inflammation [8]. To date, various bioactive components, such as quassinoids, β-sitosterol, quercetin, luteolin, indole alkaloid, acetilamarolide, merosin, ailanholide, chaparinne, and isoquercitrin [7], have been identified in A. *altissima* plant. It is important to note that this species belongs to the group of invasive plants and that guidelines are currently in force to prevent its spread [9, 10].

Foeniculum vulgare (Mill.) also known as fennel is grown in countries near the Mediterranean Sea. It belongs to the *Umbelliferae* (*Apiaceae*) family [11] and has been used in traditional medicine to treat digestive, endocrine, reproductive, and respiratory system disorders [12, 13]. Phytochemical studies of the fennel plant have revealed the presence of numerous bioactive compounds, including flavonoids, fatty acids, and amino acids [14]. Experiments in vivo and in vitro show that it has antimicrobial, antiviral, anti-inflammatory, antinociceptive, antipyretic, and memory-enhancing properties [15–17].

Rubus idaeus L. also known as red raspberry is a plant that produces red fruits and is primarily grown in Eastern Europe and Northern Asia. It belongs to the *Rosaceae* family, specifically the *Rosoideae* subfamily, which includes a variety of edible fruits [18]. According to the literature, raspberry leaves are high in phenolic compounds. Teleszko and Wojdyło [19] confirmed that the plant's leaves contain more phenolic compounds than its fruits. To date, raspberry leaf extracts have been shown to be cytotoxic to human carcinoma cells, inhibit bacterial growth, lower blood glucose and lipid levels, and have anticoagulant activity [20–23]. Raspberry's biological effects are linked to its high antioxidant activity both in vivo and in vitro, as well as the presence of bioactive compounds like kaempferol, quercetin, and tiliroside [22]. In traditional medicine, raspberry leaf tea and infusion have been used for diarrhoea and colic, in compresses for skin diseases, and furthermore, the raspberry syrup is considered a traditional antipyretic and diaphoretic drug [24].

To date, the detailed characterization and biological activity of various plant parts of *Ailanthus altissima*, *Foeniculum vulgare*, and *Rubus idaeus* have been examined. The findings revealed that these plants have a variety of biological activities as a result of their high concentration of phytochemicals. For this reason, we believe it is critical to analyse honey samples derived from the aforementioned plants. According to our knowledge, characterization and biological potential of *Ailanthus altissima*, *Foeniculum vulgare*, and *Rubus idaeus* honey types were not performed. Therefore, the aim of this study was to analyse pollen content and determine the basic physicochemical parameters of the honey, the total phenolic and flavonoid content and the antioxidant potential using three different methods: DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), ABTS (2,2′-azinobis (3-ethylbenzthiazoline-6-acid)), and ORAC (oxygen radical absorbance capacity) method. Additionally, antimicrobial activity of honey was determined and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) was used for the qualitative and quantitative analysis of phenolic compounds.

Materials and Methods

Reagents and Materials

Phenolic standards: 2,5-DHBA, 3,4-DHBA, apigenin, caffeic acid, chrysin, kaempferol, luteolin, myricetin, naringenin, *p*-coumaric acid, *p*HBA, and quercetin were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Methanol (LC–MS purity) and acetonitrile (ACN; LC–MS purity) were purchased from VWR Chemicals BDH® (Radnor, PA, USA). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid (ABTS)) were purchased from Alfa Aesar (Haverhill, MA, USA). The (±)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich, Merck. Aluminium chloride 6-hydrate was purchased from Gram-Mol (Zagreb, Croatia). Milli-Q water was obtained using connected Ultrapure Water Systems (GenPure UV-TOC/UF xCAD plus) and Milli-Q water purification system (< 0.055 µS/cm, Milli-Q Model Pacific TII 12; Thermo Fisher Scientific, Waltham, MA, USA).

Honey Samples

All honey samples were collected directly from producers in different regions of Croatia (Fig. 1). Two ailanthus honey samples (A1 and A2) were collected from Ičići (45°18′53″N 14°17′02″E). Two fennel honey samples (F1 and F2) were collected from Županja (45°04′N 18°42′E). Raspberry honey sample (R) was collected from Vinkovci (45.29°N 18.80°E). A1 honey sample was produced in 2020, while all other samples were produced in 2021.

Physicochemical Parameters

Physicochemical characterization of honey samples was performed by measuring water content, electrical conductivity, pH, free acidity, hydroxymethylfurfural (HMF) content, apparent reducing sugars, and apparent sucrose content according to our previous work [25] in agreement with the International Honey Commission (IHC) [26].

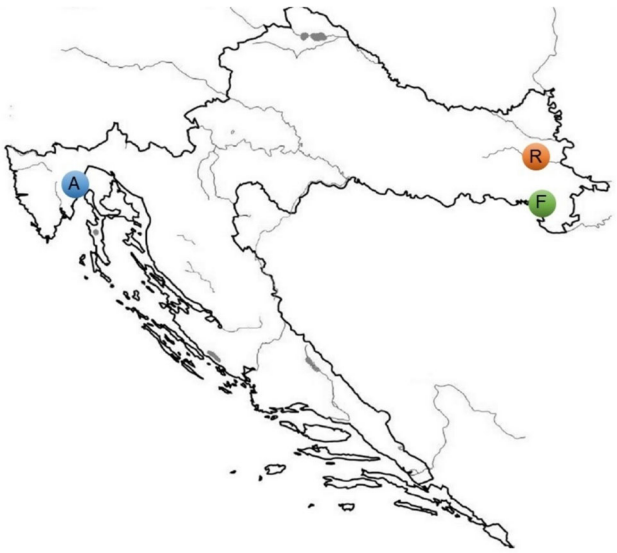

Fig. 1 Locations of ailanthus (A), fennel (F), and raspberry (R) honey producers in Croatia that provided samples for the study

Water Content

Water content was determined by digital refractometer PAL22S (Atago, Tokyo, Japan) according to the method: "Determination of water with digital and Abbe refractometers" [27]. Briefly, honey was dissolved in a heating bath at 50 °C. The refractive index was measured at 20 °C after waiting for 6 min for equilibration.

Electrical Conductivity

The electrical conductivity of honey was determined according to the method: "Determination of electrical conductivity" [27]. A honey sample of 20 g was dissolved in 100 mL of distilled water at 20° C. An electrical conductivity cell was used to conduct the measurements.

pH

pH value was determined according to the method: "Determination of pH and free acidity by titration to pH 8.3" [27]. For pH measurement, pH meter (Mettler-Toledo, Ohio, USA) was used.

Free Acidity

Free acidity was determined according to the official IHC method: "Determination of free acidity" [27]. A honey sample of 10 g was dissolved in 75 mL of water. The solution was then titrated with 0.1 M sodium hydroxide to reach a pH of 8.30.

Hydroxymethylfurfural (HMF) Content

Evaluation of the HMF content was done by use of HPLC/DAD system Agilent 1200 (Agilent Technologies, Santa Clara, USA) by the method for "Determination of hydroxymethylfurfural by HPLC" [27]. The mobile phase consisted of water and methanol (9:1 V/V), with a flow rate of 1.0 mL/min and an injection volume of 20 µL. A honey sample of 10 g was dissolved in a 50 mL beaker. The HMF content of the sample was determined by comparing its corresponding peak areas to those of the standard solutions.

Reducing Sugars and Apparent Sucrose

Apparent reducing sugars and apparent sucrose content of honey are the most determined parameters for honey quality control purposes. Apparent reducing sugars are defined as those sugars that reduce Fehling's reagent. Apparent sucrose content is determined indirectly by calculating the difference in total reducing sugar before and after the inversion of sugars in honey. The determination of

apparent sucrose requires the use of acid hydrolysis to break the glycoside bonds of the disaccharides, thus releasing reducing sugars such as glucose and fructose [27]. The method is based on the difference in concentrations of invert sugars and multiplied by 0.95 to give the apparent sucrose content. Briefly, Fehling I and Fehling II solution were used as A and B solutions. For A solution 69.28 g of copper sulphate pentahydrate was in 1000 mL of distilled water. For B solution, 346 g sodium potassium tartrate and 100 g sodium hydroxide were dissolved in 1000 mL of distilled water. The standard invert sugar solution was prepared by weighing 9.5 g of pure sucrose, adding 5 mL of hydrochloric acid, and diluting the obtained solution with distilled water to 100 mL. After 3 days of incubation at 20 °C to 25 °C, the solution was diluted to 1000 ml. Neutralization was made with 1 M sodium hydroxide solution (40 g/L) and diluted to the required concentration (2 g/L) for standardization. For the indicator solution, methylene blue was used. Sample preparation for the determination of apparent sucrose content was the same as the procedure for reducing sugars. The honey solution was hydrolysed by heating the test sample to 65 °C in a water bath. Afterwards, 10 mL of hydrochloric acid was added. The solution was allowed to cool naturally for 15 min, brought up to 20 °C, and neutralized with sodium hydroxide. The prepared sample was cooled again, and the volume was adjusted to 100 mL (diluted honey solution). Titration procedure was the same as described in method for reducing sugars [33].

Pollen Analysis

The shape and size of the pollen grains, as well as the structure and colour of the pollen outer wall, were used to classify all species [28]. To prepare the sample, 10 g of honey was dissolved in 20 mL of distilled water, heated in a water bath to 45 °C, and centrifuged for 15 min at $\times$1375g. The obtained sediment was used to prepare a sample for microscopy analysis. Two parallel samples of the same honey were prepared [29, 30].

Total Phenolic Content

Honey samples preparation for total phenols content determination was performed based on our previous work [25]. Briefly, 1 g of honey sample was dissolved in 10 mL of Milli-Q water and homogenized. The resulting solution was combined with 0.1 mL Folin-Ciocalteu reagent and 0.9 mL Milli-Q water in a volume of 0.1 mL. 0.8 mL of the 7.5% sodium carbonate solution was added after 5 min at room temperature, and after 20 min the absorbance at 760 nm was measured using a monochromator device Infinite M200 PRO (Tecan, Männedorf, Switzerland). The calibration curve was built using solutions of gallic acid in Milli-Q water with concentrations ranging from 0 to 200 mg/L. Absorbance was measured after 20 min of incubation at 760 nm. The results are expressed as gallic acid equivalents (GAE), mg eqGAE/100 g honey.

Total Flavonoid Content

Honey samples preparation for total flavonoid content determination was performed based on our previous work [25]. Briefly, honey samples were dissolved in 80% methanol to a concentration of 0.1 g/mL. Aluminium chloride methanol solution (2 g/100 mL) was mixed with the sample solutions in a 1:1 (V/V) ratio. Following 10 min of incubation, absorbance values were measured at 415 nm. The calibration curve was constructed using quercetin solutions. The results are expressed as quercetin equivalents (QUE), mg eqQUE/100 g honey.

Antioxidant Activity

Antioxidant activity of honey was measured using three different spectrophotometric methods: ABTS, DPPH and ORAC method. ABTS and DPPH methods were performed according to our previous work [25].

ABTS Method

Samples were prepared by dissolving honey in methanol to a concentration of a 50 mg/mL. The calibration curve was constructed using a range of Trolox concentrations from 0 to 0.21 mM. 160 mL of ABTS solution, previously diluted in methanol to an absorbance of 0.7, was mixed with 40 L of Trolox or sample solution. After 7 min of exposure, the absorbance was measured at 734 nm. A series of Trolox solutions were used to construct the calibration curve. The results are expressed as mg eq Trolox/100 g honey.

DPPH Method

Samples were dissolved in methanol to a final concentration of 75 mg/mL. A solution of DPPH radical was prepared by dissolving DPPH in methanol at a concentration of 0.1 mM. 160 mL of the DPPH solution and 40 mL of the honey sample were combined and after 60 min of incubation, the absorbance was measured at 517 nm using a monochromator device. The calibration curve was created using a Trolox solution in methanol at concentrations ranging from 0 to 0.21 mM. The percentage of free radical inhibition was calculated and expressed as mg eqTrolox/100 g honey.

ORAC Method

The antioxidant activity of the honey samples was determined using the oxygen radical absorbance capacity (ORAC) assay according to the study by Elez Garofulić et al. [31]. An automatic plate reader (BMG LABTECH, Offenburg, Germany) with 96-well plates was used for the ORAC procedure, and data were analysed using MARS 2.0 software. The 2,2′-azobis radical (2-amidinopropane) dihydrochloride (AAPH), fluorescein solution, various dilutions of Trolox, and samples were prepared in 75 μM phosphate buffer (pH 7.4). The appropriately diluted samples were placed in a black 96-well plate containing a fluorescein solution (70.3 nM). The plate was incubated at 37 °C for 30 min, and after the first three cycles, AAPH (240 mM) was injected into each well to initiate peroxyl radical generation. On each plate, different dilutions of Trolox were used as reference standard. Fluorescence intensity (excitation at 485 nm and emission at 528 nm) was monitored every 90 s over a total measurement period of 120 min. The measurements were performed in duplicate, and the results were expressed as μM eqTrolox.

Antimicrobial Activity Assay

Antimicrobial activity of honey samples was determined using agar-well diffusion and broth dilution methods. Susceptibility tests were made according to the European Committee for Anti-microbial Susceptibility Testing (EUCAST) recommendations [32]. Honey was dissolved in MHB (Muller Hinton Broth, Biolife, Milan, Italy) to prepare stock solution of 0.8 g/mL.

Bacterial reference strains that were used in the assay were: *Staphylococcus aureus* ATCC 25923, *Acinetobacter baumannii* ATCC BAA–1605 (multidrug resistant), *A. baumannii* ATCC 19606 (drug sensitive), *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 as well as several clinical bacterial isolates such as methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* (multidrug resistant) and extended spectrum beta-lactamase (ESBL)—positive *E. coli*. Antibiotics meropenem (Sigma, St Louis, MO, USA) and vancomycin (Sigma, St Louis, MO, USA) were used as positive controls.

Agar-well Diffusion Assay

For the agar-diffusion test, bacterial suspensions (10^8 CFU/mL) were spread onto the surface of Mueller–Hinton agar (MHA) plates (Difco, MD, USA) using sterile swab. The wells of 6 mm diameter were then cut with sterile borer in the agar and filled with 30 μL of honey (concentration 0,4 g/mL). After 2-h incubation at 4 °C and an overnight incubation at 35 ± 2 °C, the plates were examined, and antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zone around the well. All tests were done in duplicate according to a previously published paper [33].

Broth Dilution Method

Twofold serial dilutions in MHB were prepared from stock solutions of each honey sample to give final concentrations ranging from 0.0125 to 0.4 g/mL. A volume of 100 μL of each diluted sample was mixed with equal volume of bacterial suspension (10^6 CFU/mL). The plates were incubated for 24 h at 35 ± 2 °C and ×200g (Unimax 1010; Heidolph Instruments GmbH&CO., KG, Schwabach, Germany). Positive (broth and bacterial inoculum) and negative (only broth) growth controls were prepared. Minimal inhibitory concentration (MIC) values were the lowest concentration of honey sample that produced no visible bacterial growth compared to the control wells after 24 h of incubation. Minimal bactericidal concentration (MBC) was determined by inoculating the samples used for MIC determinations onto MHA and incubating further for 18–24 h at 35 ± 2 °C. MBC was defined as the lowest concentration of honey sample that killed ≥ 99% of bacteria.

Solid Phase Extraction (SPE)

Phenolic compounds were extracted using solid-phase extraction (SPE) performed according to the modified method of Yung An et al. [34], described in our previous work [25], for which SPE columns CHROMABOND C18 ec, 6 mL, 500 mg, PP with PE filter (Macherey–Nagel, Düren, Germany) were used.

LC–MS/MS Analysis

Chromatographic separation of phenolic compounds was performed on Purospher STAR RP-18 Hibar HR column (50 mm × 2.1 mm, 1.7 μm, Merck, Darmstadt, Germany) on an Agilent 1260 series high performance liquid chromatograph equipped with a degasser, binary pump, autosampler and column oven coupled to Agilent 6460 triple quadrupole mass spectrometer equipped with Jet Stream electrospray source. For quantitative and qualitative analysis of phenolic compounds in honey samples, modified MRM method was established according to our previous work [35]. Briefly, the mobile phase was composed of (A) 0.1% formic acid in Milli-Q water and (B) 0.1% formic acid in acetonitrile. The gradient elution procedure was as follows: 0–0.9 min linear gradient from 1 to 10%B, 0.9–3 min from 10 to 20%B, 3–4.5 min from 20 to 25%B, 4.5–6 min from 25 to 30%B, 6–7.5 30%B, 7.5–9 min from

30 to 90%B, 9–9.30 90%B, 9.30–9.60 from 90 to 10%B and 9.60–12 min from 10 to 1%B and 12–15 1%B. Post time was set to 2 min. The column oven was kept at 30 °C. The injection volume of the sample was 2.5 µL. All samples were injected in triplicate.

The parameters for AJS-ESI-QQQ were specified as follows: The capillary voltage was 3.5 kV in both positive and negative mode, the nozzle voltage was 0.5 kV, ion source temperature was set to 300 °C, gas flow was 5 L/min, nebulizer pressure was 45 psi, drying gas temperature was 250 °C and sheath gas flow was 11 L/min. Nitrogen was used as collision gas. The quantitative method optimization parameters LC–MS/MS are given in Table S1.

Calibration curves were generated for each phenolic standard and the linearity range was determined. In brief, phenolic standards were diluted in methanol at 15 different concentrations, followed by the generation of calibration curves and the determination of the linearity range. Linear regression was used to create calibration curves that were not compelled to pass through zero. To obtain the most trustworthy calibration curves, a 1/x statistical weight was employed. The coefficient of determination (R2) was used to determine linearity. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the guidelines of International Conference on Harmonization (ICH) (ICH Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1) Guideline on Validation of Analytical Procedures: Methodology Developed to Complement the Parent Guideline, 2005). These parameters were determined for optimization of the standard addition method of quantification.

For qualitative analysis of honey extracts, precursor and product ions scan modes were used with the same MS parameters as described above. Fragmentor and collision energies were adjusted for each analysed compound. Phenolic compounds were identified by using the following databases: MassBank [36], *mz*Cloud [37], and ReSpect [38].

Absolute Quantification of Phenolic Compounds by the Standard Addition Method

The standard addition method was performed according to method described in our previous work [25]. Briefly, calibration curves were created in the same matrix as the analysis by adding a series of different concentrations of the target compound to each sample prior to SPE. Linear regression analysis was used to calculate the actual analyte concentration in the sample. *Statistical data processing.*

Statistical data processing was performed in MassHunter Qualitative analysis version B.07.00 (Agilent technologies, Santa Clara, USA) and Microsoft Excel version 16.48 (Microsoft Corporation, Redmond, USA).

Results and Discussion

Standard Chemical Analysis

As a quality control factor for unifloral honey types, pollen analysis is used to determine and control its botanical and geographical origin. Therefore, we first performed pollen analysis of the obtained ailanthus, fennel, and raspberry honey samples, and the results are shown in Fig. 2. Currently, in the Regulation of honey quality, there is no threshold for pollen share in honey to confirm the *Ailanthus altissima*, *Foeniculum vulgare,* and *Rubus idaeus* honey types. The results show that analysed samples have a high proportion of pollen grains representative of their group. Honey samples marked as A1 and A2 had 52% and 46% of pollen grains derived from *Ailanthus altissima* species, respectively. In addition, samples F1 and F2 had 61% and 54% of *Foeniculum vulgare* pollen grains, while the R1 sample had 51% of *Rubus idaeus* L. pollen grains.

When we compare the standard chemical parameters of different types of honey (Table S1), we can see that honey samples A1 and A2 have a slightly lower water content (16.0 and 17.1%, respectively), whereas fennel and raspberry honey have a water content of 19% or higher. Raspberry honey had a lower pH than the other analysed samples (3.81). Ailanthus honey had a lower amount of reducing sugars, 69.63 and 67.29 g/100 g, while fennel and raspberry honey samples had around 74 g of reducing sugars per 100 g of honey. Reducing sugars are responsible for the level of energy provided by honey. In addition, they ensure the longevity of honey by ensuring a high osmotic pressure, reducing water activity, and thus reducing the potential formation and spread of microorganisms. Sucrose content was highest in ailanthus honey type (3.91 and 3.45 g/100 g). Fennel honey had high variability in sucrose content, ranging from 2.84 to 3.81 g/100 g. Overall, analysis of other standard chemical parameters confirmed that all analysed honey samples meet the criteria set by the European Union Directive 2001/110/EC [39].

Spectrophotometric Analysis

Ailanthus honey had a total phenolic content of 53.14 ± 0.58 and 57.75 ± 0.48 mg eqGAE/100 g honey for A1 and A2 samples, respectively (Table 1). Samples of the fennel honey presented higher variability, with values of 42.32 ± 0.75 and 63.36 ± 0.57 mg eqGAE/100 g honey. Raspberry honey had the lowest value of total phenols (39.48 ± 0.59 mg eqGAE/100 g honey), but the

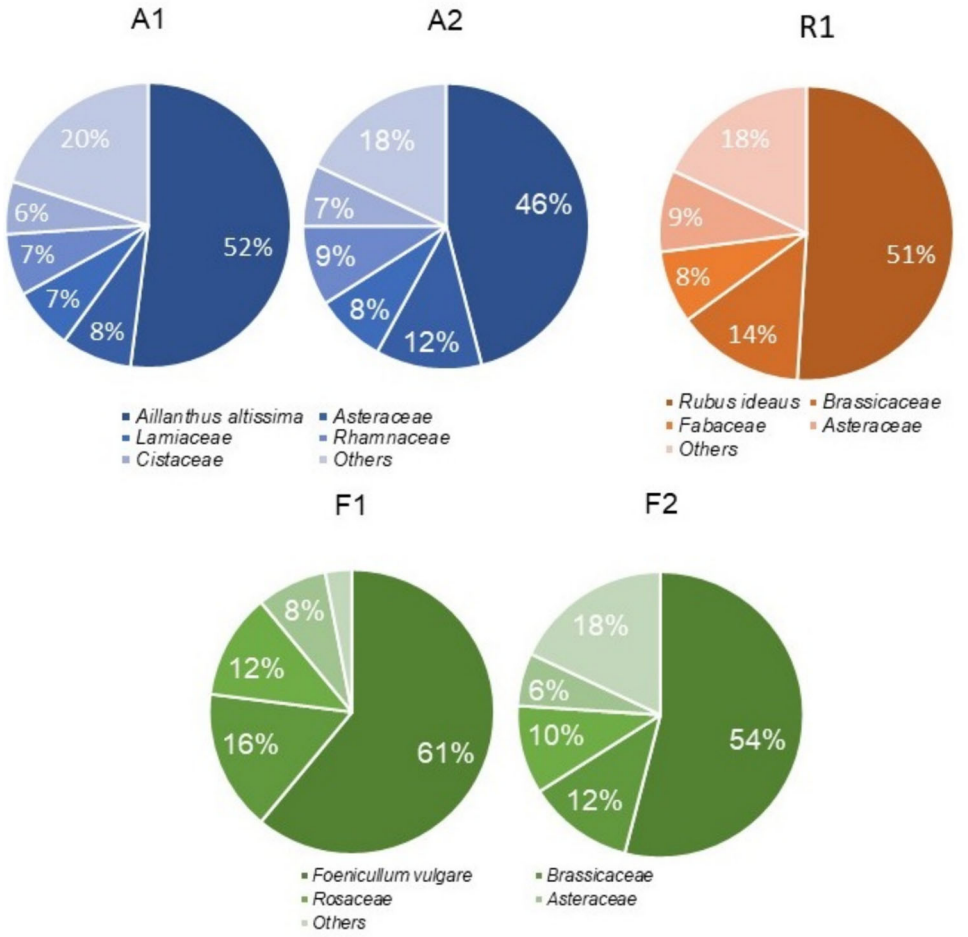

Fig. 2 Results of the pollen analysis for *Ailanthus altissima* (A1 and A2), *Foeniculum vulgare* (F1 and F2), and *Rubus idaeus* (R1) honey samples

highest concentration of total flavonoids that account for around 16.3% of all phenols in the sample. The high concentration of total flavonoids in raspberry honey agrees with the literature, where the high share of flavonoids among phenolic compounds in raspberries was highlighted [40].

The antioxidant activity was analysed with DPPH, ABTS and, ORAC assays (Table 1). ABTS and ORAC assays are hydrogen atom transfer (HAT) based assays, while DPPH is a single electron transfer (SET) based. The strongest antioxidant activity among all honey types, according to DPPH assay, had ailanthus honey. ABTS and ORAC assays did not show differences among analysed honey types, except for raspberry honey, which had lower results (ABTS: 48.99 ± 0.67 mg eqTrolox/100 g honey, ORAC: 172.20 ± 1.15 µM eqTrolox). In comparison with our previous work on Mint honey, all three honey types had lower phenolic content and antioxidant activity [25].

Table 1 Total phenolic content, total flavonoid content and antioxidant activity measured by DPPH, ABTS and ORAC assays of ailanthus (A), fennel (F), and raspberry (R) honey samples honey

Honey sample	Total phenols (mg eqGAE/100 g honey)	Total flavonoids (mg eqQUE/100 g honey)	DPPH (mg eqTrolox/100 g honey)	ABTS (mg eqTrolox/100 g honey)	ORAC (µM eqTrolox)
A1	53.14 ± 0.58	6.73 ± 0.21	33.45 ± 0.71	59.30 ± 0.35	175.16 ± 1.25
A2	57.75 ± 0.48	7.90 ± 0.31	29.64 ± 1.91	59.27 ± 0.39	177.20 ± 1.38
F1	42.32 ± 0.75	4.51 ± 0.16	11.10 ± 0.21	56.16 ± 0.49	178.17 ± 1.42
F2	63.36 ± 0.57	9.57 ± 0.45	19.87 ± 0.89	65.86 ± 0.41	177.83 ± 1.35
R1	39.48 ± 0.59	6.42 ± 0.53	16.37 ± 045	48.99 ± 0.67	172.20 ± 1.15

Results are expressed as mean value ± standard deviation (SD)

Assessment of the Antimicrobial Analysis

In vitro antibacterial activity of ailanthus, fennel, and raspberry honey samples was tested against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) (Fig. 3). Controls in this experiment were two antibiotics: vancomycin and meropenem and their results are given in table S3. Ailanthus honey A2 showed antibacterial activity against *S. aureus* with a zone of inhibition (ZOI) 19.5 ± 0.7 mm for ATCC 25923 strain and 17.5 ± 0.7 mm for methicillin-resistant ATCC 43300 strain (Fig. 2). In addition, the same honey had a moderate antibacterial effect on *A. baumannii*, with inhibitory zones of 9.5 ± 0.7 mm and 14.5 ± 0.7 mm for ATCC BAA-1605 and ATCC 19606 strains. These results are valuable since it was shown that the bacterium *A. baumannii* is one of the leading causes of hospital epidemics in the world in immunocompromised patients due to its ability to quickly develop resistance to various antibiotics [41]. As for other honey types, fennel honey was also potent against *S. aureus*, especially sample F2 with inhibitory zones of 17.5 ± 0.5 mm and 17.5 ± 0.5 depending on the bacteria strain. However, this honey type had a lover antibacterial effect on *A. baumannii* than *Ailanthus altissima* honey. Lastly, raspberry honey showed moderate antibacterial effect on *S. aureus* species. Overall, gram-positive bacteria were found to be more susceptible as compared to Gram-negative bacteria, except *A. baumannii*. The obtained results show the potential of the analysed types of honey as therapeutic agents for wound infection.

The broth microdilution assay for *S. aureus* ATCC 25923 strains gave a MIC value of 0.025 g/mL, while the MBC was 0.05 g/mL for sample 2 of ailanthus honey (Tables 2 and 3). Similar results were observed for the fennel honey, whereas the better effect had sample F2 with MIC of 0.05 g/ml for both *S. aureus* strains. All honey types had weak effects on *E. coli* and *P. aeruginosa* strains. Surprisingly, the A2 sample, which overall showed the highest antibacterial activity, had also a bactericidal effect on the *E. coli* ATCC 25922 strain, with a MIC value of 0.05 g/ml. It would be desirable to obtain more ailanthus honey samples to confirm this valuable result, since it was shown in the literature that honey overall does not influence *E. coli* [42]. Gobin et al. analysed twelve different honey types and the most potent were shown to be the honeydew type of honey and mint honey with MICs ranging from 0.05 to 0.1 g/mL, and MBCs ranging from 0.05 to 0.1 g/mL, respectively. Herein analysed honey types showed that ailanthus and fennel have the same, or even stronger antibacterial activities and therefore can be considered as antimicrobial agents.

The geographical, seasonal, and botanical origin of honey, as well as harvesting, processing, and storage

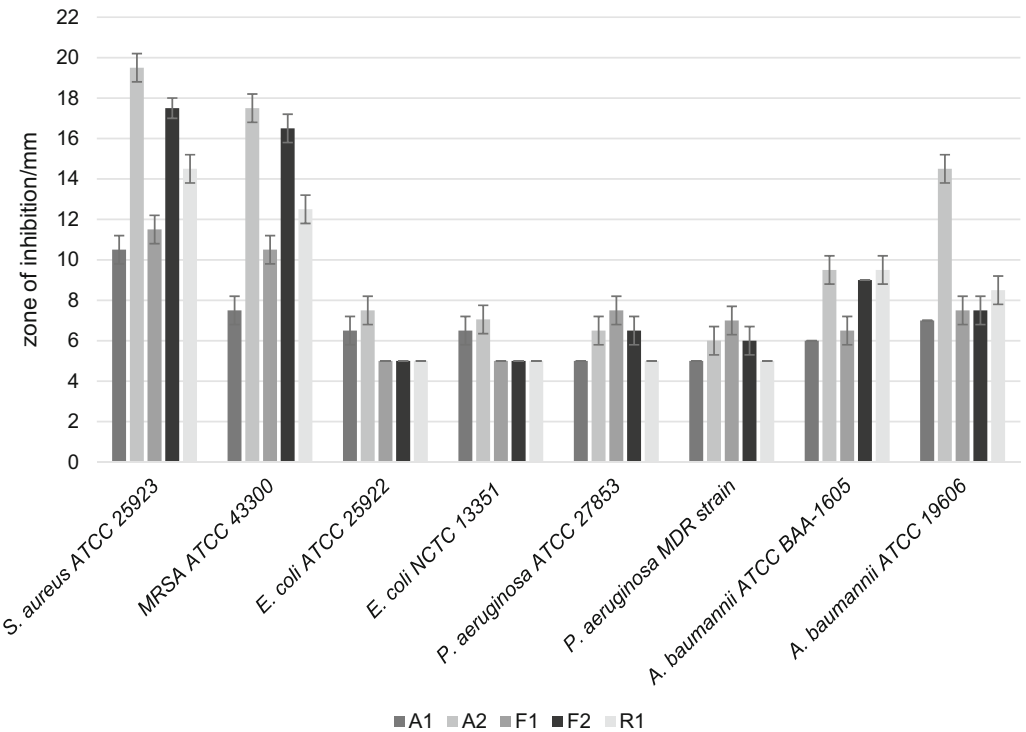

Fig. 3 Inhibition zones of ailanthus (A), fennel (F), and raspberry (R) honey samples against different bacteria

Table 2 The minimum inhibitory concentrations (MICs)[1] of antibiotic controls on different bacteria

Bacteria	Honey samples					Antibiotic control	
	A1	A2	F1	F2	R1	Vancomycin	Meropenem
S. aureus ATCC 25923	0.2	0.025	0.05	0.05	0.1	10^{-6}	ND
MRSA ATCC 43300	0.2	0.025	0.1	0.05	0.1	$5{\cdot}10^{-7}$	ND
E. coli ATCC 25922	0.2	0.05	0.1	0.1	0.1	ND	$6{\cdot}10^{-9}$
E. coli NCTC 13351	0.2	0.1	0.1	0.1	0.1	ND	$1.2{\cdot}10^{-8}$
P. aeruginosa ATCC 27853	0.2	0.1	0.2	0.2	0.2	ND	$6.4{\cdot}10^{-8}$
P. aeruginosa MDR strain	0.2	0.1	0.2	0.2	0.2	ND	$3.2{\cdot}10^{-5}$
A. baumannii ATCC BAA-1605	0.2	0.1	0.1	0.1	0.1	ND	$> 3.2{\cdot}10^{-5}$

Results are expressed in g/mL

[1]MIC—Concentration (g/ml) required for 99% bacteriostatic effect

ND—Not done

Table 3 The minimum bactericidal concentrations (MBCs)[1] of antibiotic controls on different bacteria

Bacteria	Honey samples					Antibiotic control	
	A1	A2	F1	F2	R1	Vancomycin	Meropenem
S. aureus ATCC 25923	0.2	0.05	0.1	0.05	0.2	10^{-6}	ND
MRSA ATCC 43300	0.2	0.05	0.2	0.1	0.2	10^{-6}	ND
E. coli ATCC 25922	0.2	0.05	0.2	0.1	0.1	ND	$6{\cdot}10^{-9}$
E. coli NCTC 13351	0.2	0.1	0.2	0.1	0.2	ND	$1.2{\cdot}10^{-8}$
P. aeruginosa ATCC 27853	0.2	0.1	0.2	0.2	0.2	ND	$6.4{\cdot}10^{-8}$
P. aeruginosa MDR strain	0.2	0.2	0.2	0.2	0.2	ND	$3.2{\cdot}10^{-5}$
A. baumannii ATCC BAA-1605	0.4	0.1	0.2	0.1	0.2	ND	$> 3.2{\cdot}10^{-5}$

Results are expressed in g/mL

[1]MBC—Concentration (g/ml) required for 99% bacterial killing effect

ND—Not done

conditions, can all have an impact on its antimicrobial activity. Honey's antibacterial action is due to the combined action of several factors, including low pH, high osmolarity, hydrogen peroxide formation, and the presence of various phenolic compounds. However, it is thought that the formation of hydrogen peroxide is one of the key mechanisms underlying honey's antimicrobial properties. Research has shown that this process is related to MIC and MBC [43].

Analysis of the Bioactive Compounds

The chemical profile of honey can vary even if there is the same floral source. Namely, although honey is characterized as unifloral and has a high proportion of pollen grains of the same species, the rest of the composition also affects its biological action. To get a deeper insight into the content of the bioactive constituents of ailanthus, fennel, and raspberry honey types, we performed a detailed quantitative and qualitative LC–MS/MS analysis. To overcome the matrix effect and compensate for low recovery in quantitative LC–MS/MS analysis, we employed the standard addition method.

Quantitative analysis of phenolic compounds revealed that the most abundant flavonoids in ailanthus honey are chrysin and quercetin with concentrations of 0.0976 ± 0.0140 and 0.1364 ± 0.0083 mg/100 g honey for chrysin and 0.1213 ± 0.0176 and 0.0795 ± 0.0022 mg/100 g honey for quercetin (Table 4). Among the analysed phenolic acids, 3,4-DHBA was the most abundant, with concentrations up to 1.4585 ± 0.0265 mg/100 g honey. Among all flavonoids, apigenin, chrysin and quercetin had concentrations higher than 0.1 mg/100 g honey. Quercetin, one of the strongest antioxidants and molecule with ability to prevent the oxidation of low-density lipoproteins (LDL) by scavenging free radicals and chelating transition metal ions, had the highest mass fraction in fennel honey samples with

0.4811 ± 0.0663 and 0.4219 ± 0.1449 mg/100 g honey. Among the analysed phenolic acids, *p*-coumaric acid was the most abundant (up to 0.2320 ± 0.0166 mg/100 g honey). Raspberry honey had the highest mass fraction of chrysin among all analysed phenolics with a concentration of 0.7143 ± 0.1642 mg/100 g honey. In addition, from the group of flavonoids, both quercetin and apigenin concentrations were high: 0.2733 and 0.3243 mg/100 g honey, respectively. Interestingly, raspberry honey had a high concentration of chlorogenic acid (0.3369 ± 0.0061 mg/100 g honey), in comparison with other analysed honey samples.

Finally, to identify the presence of other bioactive constituents in ailanthus, fennel, and raspberry honey types, we performed MS screening with the same LC–MS/MS instrument and parameters. Table 5 lists only identified compounds. In total, 18 compounds were identified in ailanthus, 29 in fennel and 38 in raspberry honey samples. Malic acid, caftaric acid, 3-hydroxytyrosol, taxifolin, ferulic acid, dimethyl caffeic acid, morin, kaempferide, kaempferol methoxy methyl ether, and *p*-coumaric cinnamyl ester were identified in all three honey types. In addition to the compounds found in all tested honey samples, only a few additional phenolic acids and flavonoid esters were discovered in ailanthus honey, which are listed in Table 5. In fennel honey, the presence of several acids has been confirmed: caffeic, rosmarinic, syringic, vanillic, ellagic, azelaic, abscisic, and sebacic acid. This type of honey is also high in flavonoid aglycones like pinobanksin, pinocembrin, acacetin, and galangin. The results of the quantitative analysis revealed that fennel honey is high in quercetin, and we confirmed the presence of other quercetin derivatives such as quercetin-3-glucuronide, quercitrin, and quercetin methyl ether.

Raspberry honey's unique phenolic composition has been confirmed. This honey contained phenolics not found in other samples, including catechin, epicatechin, quercetin rhamnoside, sakuranetin, tectochrysin, quercetin dimethyl ether, rhamnetin, caffeic acid benzyl ether, and pinobanksin-3-O-pentanoate. LC–MS/MS analysis revealed the entire spectrum of quercetin derivatives as well as numerous flavonoid aglycones. Our findings are consistent with polyphenol profiling of *Rubus ideaus* fruits, which also revealed a significant number of quercetin glycosylated derivatives [44].

Conclusions

In this paper, we determined the biological potential of three rare types of honey that have never been studied. These preliminary findings indicated that these three honey types have strong antimicrobial and antioxidant properties. However, it was not possible to link them to specific phenolic compounds. In the future, it will be necessary to use a high-resolution mass spectrometer to conduct a detailed characterization of biologically active components, create a profile of volatile components, and analyse a larger number of representatives of each type of honey, so that the presence of certain components can be linked to changes in biological activity. Unfortunately, because this manuscript investigates extremely uncommon types of

Table 4 Mass fractions of specific phenolic acids and flavonoids in ailanthus (A), fennel (F), and raspberry (R) honey samples obtained by use of LC–MS/MS method

Phenolic compound	Honey sample				
	A1	A2	F1	F2	R1
2,5-DHBA	< LOQ	< LOQ	0 ± 0	0 ± 0	0.004 ± 0.000
3,4-DHBA	0.3911 ± 0.0743	1.4585 ± 0.0265	0.0859 ± 0.0056	0.0792 ± 0.0031	0 ± 0
Apigenin	0.0266 ± 0.0021	0.0277 ± 0.0009	0.0885 ± 0.0099	0.1047 ± 0.0031	0.2733 ± 0.0025
Chlorogenic acid	0 ± 0	0 ± 0	0.0180 ± 0.0004	0.0179 ± 0.0003	0.3369 ± 0.0061
Chrysin	0.0976 ± 0.0140	0.1364 ± 0.0083	0.3079 ± 0.0516	0.1690 ± 0.0331	0.7143 ± 0.1642
Diosmetin	0 ± 0	0 ± 0	0.0229 ± 0.0057	0.0222 ± 0.0002	0.0502 ± 0.0024
Kaempferol	0.0633 ± 0.0064	0.0574 ± 0.0017	0.0690 ± 0.0030	0.1251 ± 0.0042	0.0593 ± 0.0020
Luteolin	0.0167 ± 0.0010	0.0117 ± 0.0002	0.0077 ± 0.0001	0.0125 ± 0.0018	0.0071 ± 0.0001
Myricetin	0.0048 ± 0.0005	0.0087 ± 0.0001	0.0029 ± 0.0001	0.0071 ± 0.0001	0.0025 ± 0.0001
Naringenin	0.0152 ± 0.0001	0.0249 ± 0.0002	0.0257 ± 0.0008	0.0269 ± 0.0013	0.0886 ± 0.0027
p-coumaric acid	0.1405 ± 0.0066	0.1839 ± 0.0078	0.2320 ± 0.0166	0.1704 ± 0.0037	0.0904 ± 0.0035
Quercetin	0.1213 ± 0.0176	0.0795 ± 0.0022	0.4811 ± 0.0663	0.4219 ± 0.1449	0.3243 ± 0.0253
Rutin	0 ± 0	0 ± 0	0.0143 ± 0.0003	0.0105 ± 0.0002	0.0044 ± 0.0001

Results are expressed as a mean value of mass concentration mg/100 g honey ± standard deviation (SD)

Table 5 Identified compounds in ailanthus (A), fennel (F), and raspberry (R) honey samples obtained by use of LC–MS/MS method

Tentative identification	t_R	Precursor (*m/z*)	Fragments (*m/z*)	Molecular formula	Honey type		
					A	F	R
Malic acid	0.8	133.0 (−)	114.9, 70.9	$C_4H_6O_5$	+	+	+
Gallic acid	2.1	168.8 (−)	125.0, 78.9	$C_7H_6O_5$	+	−	+
Salvianolic acid G	4.5	417.0 (−)	219.0, 237.0	$C_{20}H_{18}O_{10}$	+	−	−
Caftaric acid	4.9	311.0 (−)	178.9, 148.9, 134.9	$C_{13}H_{12}O_9$	+	+	+
3-hydroxytyrosol	5.1	152.9 (−)	95.0	$C_8H_{10}O_3$	+	+	+
*p*HBA	5.2	136.8 (−)	92.7, 64.8	$C_7H_6O_3$	+	−	+
Catechin	5.5	289.0 (−)	244.8, 204.9, 122.7, 108.8	$C_{15}H_{14}O_6$	−	−	+
Caffeic acid	5.8	178.8 (−)	135.0, 116.9, 88.9	$C_9H_8O_4$	−	+	+
Epicatechin	6.0	289.1 (−)	244.9, 108.9	$C_{15}H_{14}O_6$	−	−	+
Rosmarinic acid	6.0	359.0 (−)	197.0, 179.0, 161.0, 135.0, 121.0	$C_{18}H_{16}O_8$	+	+	−
Syringic acid	6.7	197.0 (−)	181.8, 166.9, 122.6	$C_9H_{10}O_5$	−	+	+
Vanillic acid	6.7	166.9 (−)	151.8, 122.9, 107.9	$C_8H_8O_4$	−	+	+
Ellagic acid	7.0	301.0 (−)	283.7, 244.6, 228.4	$C_{14}H_6O_8$	−	+	+
Taxifolin	7.1	303.0 (−)	185.0, 273.0, 227.0, 151.0	$C_{15}H_{12}O_7$	+	+	+
Quercetin 3-O-glucuronide	7.1	477.0 (−)	301.0, 271.0, 151.0	$C_{21}H_{18}O_{13}$	−	+	+
Ferulic acid	7.2	192.9 (−)	177.9, 149.0, 134.0	$C_{10}H_{10}O_4$	+	+	+
Quercetin rhamnoside	7.8	447.0 (−)	301.0, 271.0, 151.0	$C_{21}H_{20}O_{11}$	−	−	+
Apigenin-7-O-glucoside	7.9	430.9 (−)	310.9, 268.1	$C_{21}H_{20}O_{10}$	+	−	+
Azelaic acid	8.4	287.0 (−)	125.0	$C_9H_{16}O_4$	+	+	−
Quercitrin	8.5	447.0 (−)	301.0, 300.0, 271.0, 151.0	$C_{21}H_{20}O_{11}$	−	+	+
Dimethyl caffeic acid	8.6	209.0 (+)	190.7, 162.9, 133.0, 118.9	$C_9H_8O_4$	+	+	+
Abscisic acid	8.9	263.0 (−)	219.1, 203.8, 152.9	$C_{15}H_{20}O_4$	+	+	−
Sebacic acid	9.2	200.9 (−)	182.8, 138.8, 110.9	$C_{10}H_{18}O_4$	−	+	+
Pimaric acid	9.4	301.0 (−)	257.0, 121.0	$C_{20}H_{30}O_2$	−	−	+
Morin	9.5	301.0 (−)	271.0, 151.0	$C_{15}H_{10}O_7$	+	+	+
Pinobanksin methyl ether	9.6	285.0 (−)	252.9, 240.9, 239.0, 226.9, 223.9	$C_{16}H_{14}O_5$	−	+	+
Sakuranetin	9.6	284.9 (−)	164.9, 136.0, 118.9, 108.0, 92.8	$C_{16}H_{14}O_5$	−	−	+
Quercetin methyl ether	10.0	315.0 (−)	300.0, 270.7, 255.0	$C_{16}H_{21}O_7$	+	+	−
Tectochrysin	10.5	267.0 (−)	252.0, 224.0	$C_{16}H_{12}O_4$	−	−	+
Pinobanksin	10.8	271.0 (−)	252.9, 225.0, 196.6, 160.7	$C_{15}H_{12}O_5$	−	+	+
Kaempferide	11.0	299.0 (−)	284.0, 255.0, 227.0	$C_{16}H_{12}O_6$	+	+	+
Quercetin dimethyl ether	11.0	329.0 (−)	313.7, 298.7, 270.8	$C_{17}H_{14}O_7$	−	−	+
Kaempferol methoxy methyl ether	11.0	329.0 (−)	314.0, 298.8, 284.7	$C_{17}H_{14}O_7$	+	+	+
Rhamnetin	11.2	314.9 (−)	165.0, 121.0	$C_{16}H_{12}O_7$	−	−	+
Kaempferitrin	11.2	577.0 (−)	431.0, 285.0, 283.0, 255.0	$C_{27}H_{30}O_{14}$	−	+	+
Caffeic acid benzyl ether	11.4	269.0 (−)	178.0, 161.0, 134.0	$C_{16}H_{14}O_4$	−	−	+
Caffeic acid phenylethyl ether (CAPE)	11.5	283.0 (−)	179.0, 135.0^-	$C_{17}H_{16}O_4$	−	+	+
Pinocembrin	11.5	254.8 (−)	212.9, 150.8	$C_{15}H_{12}O_4$	−	+	+
Acacetin	11.6	283.0 (−)	268.0	$C_{16}H_{12}O_5$	−	+	+
Galangin	11.6	268.9 (−)	252.0, 168.9, 142.9	$C_{15}H_{10}O_5$	−	+	+
Pinobanksin-3-O-butyrate	12.0	341.0 (−)	303.2, 271.0, 252.9, 196.7, 146.5	$C_{19}H_{18}O_6$	−	+	+
p-coumaric acid cinnamyl ester	12.0	281.0 (+)	241.0, 192.6, 162.1, 147.0, 118.6	$C_{18}H_{16}O_3$	+	+	+
Pinobanksin-3-O pentanoate	12.5	355.0 (−)	271.2, 253.0	$C_{20}H_{18}O_6$	−	−	+

Table lists tentative identification of compound, retention time (t_R), precursor ions (*m/z*), fragments (*m/z*), molecular formula, and presence in each analysed honey type

honey, it was impossible to obtain further samples. Results revealed that there are significant variances even within the same type of honey (particularly in terms of antibacterial activity), and future analyses and studies should aim to gather as many monovarietal types of ailanthus, raspberry, and fennel honey as possible. In addition to the foregoing, it is desirable to identify compositional trends in order to confirm biomarkers for each type of honey examined.

Author Contributions Lara Saftić Martinović designed the study. Tomislav Pavlešić procured honey samples. Lara Saftić Martinović and Nada Birkić wrote the manuscript and performed analyses of antioxidant activity, total phenolic content, total flavonoid content, and quantitative LC–MS/MS analysis. Lara Saftić Martinović performed qualitative LC–MS/MS analysis and prepared tables. Dijana Mišetić Ostojić and Tomislav Pavlešić performed methods for determination of standard physicochemical parameters. Ana Planinić and Ivana Gobin did antimicrobial analyses. Sandra Pedisić obtained ORAC procedure. Lara Saftić Martinović and Nada Birkić shaped the paper concept and performed the final revision.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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