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Authentication of strawberry tree (*Arbutus unedo* L.) honeys from southern Europe based on compositional parameters and biological activities

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Author statement:

S.M. Osés: conceptualization, data curation, investigation, methodology, resources, supervision, writing-original draft. **S. Nieto**: formal analysis, investigation, validation. **S. Rodrigo**: formal analysis, investigation, validation. **S. Pérez**: formal analysis, investigation, validation. **S. Rojo**: formal analysis, investigation, validation. **M.T. Sancho**: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing-review & editing. **M.A. Fernández-Muiño**: investigation, methodology, resources, supervision, visualization, writing original draft.

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Highlights

- *A. unedo* honeys from different countries share common features

- Carrez clarification improves arbutin analysis

- Three compounds are proposed as floral markers of *A. unedo* honey

- *A. unedo* honeys show antioxidant and anti-inflammatory properties

- Methanolic extracts have higher AOA, SRS and antimicrobial activities than honey

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Authentication of strawberry tree (*Arbutus unedo* L.) honeys from southern Europe based on compositional parameters and biological activities

Running tittle: Authenticity and biological activities of strawberry tree honeys

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Abstract

Strawberry-tree (*Arbutus unedo* L.) honey is expensive and difficult to characterize by melissopalinology. This study aimed to authenticate strawberry-tree (*A. unedo* L.) honeys from southern Europe, determining arbutin, groups of polyphenols, volatile and semivolatile compounds, as well as biological activities such as trolox equivalent antioxidant capacity, antioxidant activities against both hydroxyl (AOA) and superoxide radicals (SRS), oxygen radical absorbance capacity, anti-inflammatory activity, and antimicrobial activity against 7 microorganisms (*Escherichia coli*, *Streptococcus mutans*, *Staphylococcus aureus*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium* sp., and *Penicillium commune*). Arbutin, analyzed using HPLC-UV, was quantified in 83% of the samples. Polyphenols' contents were high. Norisoprenoids and benzene derivatives were the major compounds determined using gas chromatography-mass spectrometry. Theobromine was detected in 67% of samples. 2,6,6-Trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde, 3,4,5 trimethylphenol and 2-hydroxycyclopent-2-en-1-one were proposed as potential strawberry-tree floral markers. Antioxidant and anti-inflammatory activities were significant. Honeys' extracts showed higher AOA and SRS, and better antimicrobial activities than the honeys. This study highlights the potential of strawberry-tree honeys and/or their phenolic extracts for food, pharmaceutical and cosmetic uses.

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Keywords: Strawberry-tree honey; *Arbutus unedo* L.; arbutin; polyphenols; norisoprenoids; benzene derivatives; antioxidant activities; anti-inflammatory activity; antimicrobial activities.

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1. **Introduction**

Strawberry-tree (*Arbutus unedo* L.) honey is usually harvested in southern Europe and its production is limited. Strawberry-tree honey is appreciated and achieves high prices. Its authentication by melissopalinology is difficult, because the anatomical characteristics of *A. unedo* L. flowers (bell-shaped, down faced with a narrow entrance) complicate both bees access inside the flower and bees contact with stamens where the pollen is produced. Therefore, in strawberry-tree honeys, the percentage of *A. unedo* L. pollen is considerably lower than the usual predominant pollen percentage of other unifloral honeys. Thus, for a proper strawberry-tree honey authentication, complementing melissopalinology with sensory and/or chemical analyses is of importance to avoid fraud.

A. unedo L. honey has distinctive sensory features with original and different nuances, in contrast to other honey types. Its color is amber when liquid, and beige-brown when crystallized. It has an intensive coffee-like smell, and a slight sweet taste with bitter and astringent aftertaste that has been mainly related to its arbutin content (Spano et al., 2006; Tuberoso et al., 2010). Arbutin (4-hydroxyphenyl-β-D-glucopyranoside), is found in several parts of the strawberry-tree bush and is used as a cosmetic ingredient because it prevents the melanin production by inhibiting the enzyme tyrosinase at non-cytotoxic concentrations, thus, whitening the skin (Migas and Krauze-Baranowska, 2015). Arbutin has been described as a component of bitter honeys (Floris et al., 2007). However, literature references about arbutin analysis in honeys are limited and dated (Manunta and Tirillini, 1993). On the other hand, arbutin has been quantified in other products (Jeon et al., 2015).

In traditional folk medicine, both the *A. unedo* shrub, and strawberry tree honey have been used (Miguel et al., 2014). The biological properties of strawberry-tree honeys have been attributed to their high amount of phenolics (Castiglioni et al., 2017; Rosa et al., 2011). Recent studies have shown the potential of strawberry-tree honeys against colon cancer cells and their metastatic activity (Afrin et al., 2017).

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Phenolic extracts/compounds are currently being used as natural additives to improve antioxidant and antimicrobial activities of several commodities. Accordingly, honeys high in pholyphenols could be successfully used in foods, pharmaceuticals or cosmetics. With regard to strawberry-tree honeys, research has been done on melissopalinology, quality control, compositional parameters and homogentisic acid as a characteristic strawberry tree honey phenolic acid (Brčić Karačonji and Jurica, 2017; Deiana et al., 2015; Floris et al., 2007; Miguel et al., 2014; Spano et al., 2006; Tuberoso et al., 2010); some bioactive compounds and antioxidant properties (Aazza et al., 2013; Castiglioni et al., 2017; Petretto et al., 2015; Rosa et al., 2011; Tuberoso et al., 2013; Ulloa et al., 2015); volatile compounds (Bianchi et al., 2005; Dalla Serra et al., 1999; De la Fuente et al., 2007), as well as antimicrobial and anti-inflammatory activities in Portuguese honeys from different botanical origins within the Algarve region (Da Silva et al., 2016). However, the quantities of phenolic-related parameters of strawberry tree honey using simple and rapid methods have not been described, including the amount of *o*-diphenols, whose antioxidant activity was reported in cocoa beans (*Theobroma cacao*) (Afoakwa et al., 2012). There are also no studies on the antioxidant activities of strawberry tree honeys determined with methods based on different reaction mechanisms and against different free radicals. Moreover, anti-inflammatory and antimicrobial activities against a variety of microorganisms have seldom been quantified, and only in a few strawberry-tree honeys of local interest.

Honeys are being mainly characterized on the basis or their botanical and geographical origins (Dżugan et al., 2020; Fechner et al., 2016). To obtain quality standards for protected geographical indications, the current tendency regarding honeys is to study features that help differentiate honeys harvested in different areas. Strawberry-tree honeys studies have been mainly done with honeys from Italy, Portugal, Croatia and Corsica (France). There is only one report about volatile and carbohydrate composition of Spanish strawberry-tree honeys (De la Fuente et al., 2007). To obtain health claims from the European Food Safety Agency (EFSA) of the European Commission, it is necessary to describe potentially healthy attributes that unifloral honeys have in

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common, regardless of geographic origin. Therefore, a study of the concentrations of compositional and bioactive parameters, as well as biological activities in strawberry-tree honeys from different countries might be beneficial.

Strawberry-tree honeys from different geographical origins need to be studied from the point of view of authentication and recognition of possible health claims. Therefore, the main aim of this study was to seek common features of strawberry-tree honeys from different southern European countries, analyzing arbutin, groups of polyphenols, the profiles of volatile and semivolatile compounds, trolox equivalent antioxidant capacity (TEAC), antioxidant activities against both hydroxyl (AOA), and superoxide (SRS) radicals, oxygen radical absorbance capacity (ORAC), as well as anti-inflammatory and antimicrobial (against 7 microorganisms) activities. An additional purpose was to compare the potentially functional activities of strawberry-tree honeys and their phenolic extracts, to determine if these honeys and/or extracts could be used beneficially as an ingredient.

2. Material and Methods

2.1. Standards, reagents and apparatus

All the solvents were of analytical grade purity: acetonitrile, ethyl acetate, methanol, sodium carbonate, sodium chloride, sodium hydroxide, potassium hydroxide, potassium persulfate, $Fe(NH_4)_2(SO_4)_2$, EDTA, acetic acid, formic acid, p-dimethylaminobenzaldehyde (DMAB), Carrez I, Carrez II, H_3PO_4, HCl, sodium molybdate, H_2SO_4, H_2O_2 were purchased from VWR International Eurolab, part of Avantor (Llinars del Vallès, Cataluña, Spain). Gallic acid (GA), $NaNO_2$, Na_2SO_4 and catechin (Ct) were purchased from Panreac (Barcelona, Cataluña, Spain). $AlCl_3$ and fluorescein sodium were purchased from Fluka Chemie GmbH, part of Sigma-Aldrich (Buchs, San Galo, Switzerland). Na_2HPO_4, NaH_2PO_4 were purchased from Scharlab (Sentmenat, Cataluña, Spain). Potassium tetraborate, nitro-blue tetrazolium (NBT), sodium benzoate and uric acid (UA) were purchased from Alfa Aesar, part of Thermo Fisher (Kandel, Rheinland-Pfalz, Germany). Nutrient

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broth No. 2 (NB), brain heart infusion (BHI), malt extract broth (MEB), agar technical No. 2 and Ringer solution were obtained from Oxoid, part of Thermo Fisher (Basingstoke, Hampshire, UK). Arbutin, Folin-Ciocalteu reagent, catechol (C), quercetin (Q), dinitrophenylhydrazine (DNP), naringenin (N), methyl undecanoate, methyl heptadecanoate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, T), thiobarbituric acid, xanthine, xanthine oxidase (10110434001), 2,2'-azobis(2-amidopropane) dihydrochloride (ABAP), N-acetyl-D-glucosamine (NAG), hyaluronic acid sodium salt (HA) from *Streptococus equi* (53747), bovine serum albumin (BSA), hyaluronidase from bovine testes type IV-S (1400 U/ml, H3884) were purchased from Sigma-Aldrich, part of Merck (Steinheim, Nordrhein-Westfalen, Germany). Water was deionized using a Milli-Q water purification system (Millipore, part of Merck, Bedford, MA, USA).

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2.2. Honey Samples

Twelve strawberry-tree honey samples harvested in 2015, 2016 and 2017 were collected from professional beekeepers in different areas of Spain (samples 1 to 6), Algarve, Portugal (samples 7 to 9), Sardinia, Italy (samples 10 and 11), and Corsica, France (sample 12). All samples were obtained by centrifugal extraction followed by decantation. After sampling, honeys were stored at 4 ºC (for a maximum of 16 wk) until analysis. Floral origin was determined by melissopalynology, extracting the sediment and counting the pollen grains (Von der Ohe et al., 2004), as well as sensory analyses (Piana et al., 2004), assessing the color, odor, taste, flavor and texture.

2.3. Arbutin analysis

Arbutin was analyzed using the HPLC procedure of Jeon et al. (2015) modified for the purpose of improving reliability. Honey (5.0 g) was dissolved in 20 ml distilled water, transferred with Milli-Q water to a 50 ml volumetric flask and clarified with 0.5 ml Carrez I and 0.5 ml Carrez II reagents (AOAC, 2016). After stirring and filling to the mark, the sample was filtered using Whatman™ 40 (GE Healthcare, Chicago, IL, USA). The solution was then filtered using a 0.45-µm membrane (Whatman™) and 20 µl was injected in the chromatograph (Varian Pro Star, part of

Agilent Technologies, Santa Clara, CA, USA). Chromatography was carried out using a C_{18} column Microsorb-MV 100-5 (4.6 x 150 mm) (Agilent Technologies) at 25 °C. The mobile phase consisted of solvent A: H_2O acidified with H_3PO_4 to pH=3 and solvent B: acetonitrile. Initial solvent composition was 0% B. It increased linearly to 50% B in 10 min. Flow rate was 1 ml/min. Detection was carried out at 280 nm (Varian Pro Star UV-Vis detector). Total analysis time was 10 min. Arbutin was quantified using a calibration curve. The quantification limit was calculated as s_b+10s, (s = standard deviation and s_b= average signal of 10 blank injections). Standard and samples were injected in triplicate. Percentages of arbutin recovery were >90%.

2.4. Phenolics extraction

To remove all sugars and other polar compounds from honey and to obtain the phenolic compounds, an extraction was carried out using the method described by Baltrušaitytė et al. (2007). Honey solutions, previously adjusted to pH 2.0 with 2 M HCl, were filtered using a column (300 x 15 mm) with Amberlite XAD-2 resin (Supelco, Bellefonte, PA, USA), preconditioned with methanol and distilled water. The column was washed with 250 ml of acidified water (pH 2.0) and then with 300 ml of distilled water and then the phenolic compounds were eluted with 250 ml of methanol. Methanolic extracts (ME) were stored at -20 ºC until further use, for a maximum of 3 wk.

2.5. Total phenolics content (TPC)

TPC were measured in honey (5.0 g diluted to 50 ml with distilled water) and phenolic extracts using the Folin-Ciocalteu assay (Osés et al., 2016). Sample (0.5 ml) was mixed with 2.5 ml 0.2 N Folin-Ciocalteu reagent. Sodium carbonate (2 ml, 75 g/l) was added after 5 min and the samples were incubated for 2 h in the dark at room temperature (22-25 ºC). Absorbance was measured at 760 nm against a blank of water (for honeys) or methanol (for phenolic extracts) using a Varian Cary Bio 400 spectrophotometer (Varian). GA (0-300 mg/l) was used as the standard for the calibration curve. The results were expressed as mg GA/100 g honey.

2.6. *o*-Diphenols

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o-Diphenols were only analyzed in ME. Two assays were used. The first was done using the method described by Cert et al. (2007), where 2 ml of methanol extract was mixed with 0.5 ml 5% (w/v) sodium molybdate in methanol and water (1:1) and after stirring the samples absorbance was measured at 370 nm. For each sample, color correction was needed (2 ml of methanol extract with 0.5 ml of methanol:water (1:1)). The second assay was the method used by Afoakwa et al. (2012), using the Arnow reactive (10 g of $NaNO_2$ and 10 g of Na_2MoO_4 in 100 ml of water). Briefly, 1 ml of ME was mixed with 2 ml 0.5 M HCl, 1 ml of Arnow reactive, 10 ml of water and 2 ml 1 N NaOH. Absorbance was measured at 515 nm. For both procedures C (0.001-0.050 µg/ml) was used as the standard for the calibration curve, expressing the results as µg C/100 g honey.

2.7. Total flavonoids content (TFC)

2.7.1. Flavones/Flavonols

Flavones and flavonols were determined in ME, mixing 1 ml of each sample with 1 ml 2% (w/v) $AlCl_3$ in methanol. Absorbance was measured at 415 nm against a blank (1 ml of water with 1 ml of methanol) after 10 min at room temperature. Q (2-80 µg/ml) was used as the standard for the calibration curve and the results were expressed as mg Q/100 g honey. A color correction (1 ml of each sample with 1 ml of methanol) was done (Sancho et al., 2016).

2.7.2. Flavanols

Flavanols were analyzed in ME using the procedure described by Pękal and Pyrzynska (2014). Briefly, 1 ml of sample was mixed with 0.3 ml 5% (w/v) $NaNO_2$. After 5 min, 0.5 ml 2% (w/v) $AlCl_3$ in methanol was added and 6 min later 0.5 ml 1 M NaOH was used to neutralize the solution. Absorbance was measured at 510 nm against a blank (1 ml of methanol) after 10 min at room temperature. Ct (1-80 µg/ml) was the standard use in the calibration curve. Results were expressed as mg Ct/100 g honey.

2.7.3. Flavanones/dihydroflavonols

Flavanones and dihydroflavonols were determined in ME using the procedure described by Popova et al. (2004). Briefly, 1 ml of the working solution and 2 ml of DNP solution (1 g DNP in 2 ml 96%

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sulphuric acid, diluted to 100 ml with methanol) were heated 30 min at 30 °C in a shaking water bath. After cooling to room temperature, the mixture was diluted to 10 ml with 10% (w/v) KOH in methanol. An aliquot (0.5 ml) of the resulting solution was diluted to 25 ml with methanol. Absorbance was measured at 486 nm against a blank, where the sample was substituted with 1 ml of methanol. N (0.10-2.50 µg/ml) was used for the calibration curve and the results were expressed as mg N/100 g honey.

2.8. Volatile and semivolatile compounds

They were analyzed using a modification of the procedure of D'Arcy et al. (1997), in which 10 g honey was weighed in triplicate and extracted with undiluted ethyl acetate, adding two internal standards: 600 µl of methyl undecanoate in ethyl acetate (0.152 mg/ml), before solvent extractions and 600 µl of methyl heptadecanoate in ethyl acetate (0.144 mg/ml). Combined extracts were concentrated using an all-glass rotary evaporator (R-124, Büchi Labortechnik AG, Flawil, Switzerland) at 30 ºC. Extracts were kept in glass vials at -30 ºC for a maximum of 2 wk until the analysis. Sample (1 µl) was injected into the gas chromatography-mass spectrometry (GC-MS) instrument. GC-MS was carried out on a Hewlett Packard (HP) 6890 series GC system (part of Agilent Technologies) coupled to an HP 5973 mass selective detector (Hewlett Packard). Chromatographic separation was achieved using a SGE 50QC2/BPX5 (SGE Analytical Science, part of Trajan, Melbourne, Victoria, Australia) capillary column (5% phenyl polysilphenylene-siloxane; 50 m length, 0.22 mm id, 0.25 µm film thickness). The oven temperature was programmed to start at 50 ºC (4 min initial hold). After that, the temperature was increased at 5 ºC/min to 90 ºC (1 min hold), then at 3 ºC/min to 250 ºC (10 min hold) and finally at 15 ºC/min to 320 ºC (10 min hold). Ultra-pure grade helium (Air Liquide, Burgos, Castilla y León, Spain) at a flow rate of 22.9 ml/min was used as the carrier gas. Temperatures were: 250 ºC for the injector, 280 ºC for the transfer line, 150 ºC for the quadrupole and 230 ºC for the source. The MS detector was operated in the Scan mode between 30 and 300 *m/z*. The identification of volatile compounds was done by comparing the mass spectra for each peak with the data included in the Wiley 275

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library (G1033A, included in the software of the GC equipment). For the semi-quantification the internal standard method was used. Concentration (mg/kg) was based on the ratio of peak areas of each volatile compound to that of the internal standard (methyl undecanoate), multiplied by the final concentration of the internal standard (45.6 mg/l).

2.9. Antioxidant activity

2.9.1. ABTS$^{\bullet+}$ scavenging activity test

TEAC of strawberry-tree honey and their ME was measured using ABTS as the source of free radicals (ABTS$^{\bullet+}$) using the procedure described by Sancho et al. (2016). ABTS$^{\bullet+}$ (1490 µl, obtained mixing 1:1 ABTS with $K_2S_2O_8$, and then, kept in the dark for 16-18 h) was mixed with 10 µl of sample, standard or blank. T was used as the standard for the calibration curve (0.625-3.000 mM). The percentage inhibition after 6 min was calculated using the following equation:

$$\% \text{ inhibition} = [((Ab-As)/Ab) \times 100] \quad (1)$$

where Ab = absorbance of the blank, As = absorbance of the sample or standard.

Results were expressed as µmol T/100 g honey or 100 ml ME.

2.9.2. Radical-scavenging effect on hydroxyl radicals

AOA of honeys (75% w/v, in water) and their ME were assayed using the procedure of Koracevic et al. (2001). Sample (10 µl) was mixed with 490 µl 0.1 M sodium phosphate buffer (pH 7.4), 500 µl 0.01 M sodium benzoate, 200 µl 2 mM $FeSO_4$-EDTA and 200 µl 0.01 M hydrogen peroxide. After 1 h incubation at 37 ºC, the reaction was stopped by adding 1 ml 20% acetic acid, and right after 1 ml 0.8% (w/v) thiobarbituric acid in 50 mM NaOH was added. The solution was boiled 10 min and then cooled in ice. Each sample (A_1) had its own control (A_0), in which 20% acetic acid was added before Fe-EDTA and H_2O_2. For each series of analyses a negative control (K_1 and K_0) was prepared, containing the same reagents as A, but replacing the sample with phosphate buffer. As the standard for the calibration, 1 mM UA in 5 mM NaOH (U_1 and U_0) was used. Antioxidant activity was calculated using the following equation:

$$AOA = \mu mol\ UA/100\ g \text{ of honey or } 100\ ml \text{ of } ME = f \times (C_{UA}) \times 1000 \times (K-A)/(K-U) \quad (2)$$

Where f = the dilution factor (0.15 for honey and 0.1 for ME), C_{UA} = the concentration of UA (1 mM), K = the absorbance of control (K_1-K_0), A = the absorbance of sample (A_1-A_0); U = the absorbance of UA solution (U_1-U_0).

2.9.3. **Superoxide radical-scavenging activity**

SRS was done with the xanthine-xanthine oxidase system using the procedure described by Küçük et al. (2007) slightly modified. For honey samples, concentrations of 75, 50, 25 and 5% were used, while for methanolic extracts 100, 75, 50 and 25% were used. Sample, 250 µl of each concentration, was added to the reaction mixture containing 100 µM xanthine, 600 µM NBT, 0.05 U/ml of xanthine oxidase from cow milk (10110434001, Sigma-Aldrich) and 0.1 M phosphate buffer (pH 7.4) making a final volume of 5.0 ml for honey and 1.0 ml for ME. One unit (U) was 1 µmol of xanthine converted to UA/min at pH 7.5 at 25 ºC. Mixtures were incubated at 25 ºC for 10 min and the absorbance was measured at 560 nm against a blank for each sample, in which the enzyme had not been added. IC_{50} (50% inhibitory concentration in mg/ml) values for the inhibition of the generation of superoxide anions by the samples were calculated by measuring the reduction of NBT to form blue formazan.

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2.9.4. **Oxygen radical absorbance capacity**

The ORAC assays measures antioxidant activity against peroxyl radicals ($ROO^{\bullet}$). ORAC values were determined using the method described by Huang et al. (2002) with modifications, using a fluorometer Varioskan LUX microplate reader (Thermo Fisher). Three µl 4.1 µM fluoresceine disodium were added to 187 µl of sample diluted in buffer (75 mM sodium phosphate, pH 7.4) (sample), or to 187 µl buffer (blank) or to a mixture of 181 µl buffer and 6 µl 0.2 µM T solutions (T) in a 96-well white plate (Greiner Bio-one, San Sebastian de los Reyes, Madrid, Spain) at 37 ºC for 5 min. Then, 10 µl, 0.37 M ABAP was added to the mixture and measured every 5 min for 90 min (excitation wavelength 485 nm and emission wavelength 520 nm). The results were expressed as µmol T/g honey or 100 ml ME using the following equation, with areas obtained using Excel (Microsoft 365. Redmond,WA, USA):

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ORAC = [(area sample – area blank)/((area T- area blank)/µmol T)] (3)

2.10. Anti-inflammatory activity

Anti-inflammatory activity was assessed using the hyaluronidase inhibition assay using the procedure of Ferreres et al. (2012), based on the mechanism of the Morgan-Elson reaction. Enzyme activity was defined as 1 unit of hyaluronidase that catalyzes the liberation of 1 µmol NAG/min with specified conditions. Stock solution of 5 mg/ml HA from *Streptococus equi* (53747) was prepared in water and stored at 4 ^oC for a maximum of 4 wk. HA stock solution (70 µl) and 100 µl of buffer (0.2 M sodium formate, 0.1 M NaCl and 0.2 mg/ml BSA, pH adjusted to 3.68 with formic acid) were added to 200 µl milliQ water and 50 µl sample. The mixture was heated at 37 ^oC for 10 min before starting the reaction by addition of 50 µl of hyaluronidase from bovine testes type IV-S (1400 U/ml, H3884) prepared in 0.9% NaCl. The mixture was incubated 1 h at 37 ^oC in a water bath. The enzymatic reaction was stopped by adding 100 µl of 0.8 M potassium tetraborate and then, incubated 3 min in a water-bath at boiling. The test tubes were cooled at room temperature and 750 µl of DMAB (2 g of DMAB dissolved in a mixture of 2.5 ml of 10 N HCl and 17.5 ml of glacial acetic acid, further diluted 1:2 with glacial acetic acid immediately before use), was added. The tubes were incubated 20 min at 37 ^oC and the color of the resulting product was measured at 586 nm against a blank sample (where enzyme and sample had been substituted with buffer). NAG standard solutions (in the range between 0 and 2 µmol), were used for the calibration curves. With the NAG formed in each enzymatic reaction and using the linear regression equation, the percentage of enzyme inhibition was calculated using the following equation:

% Inhibition = (A-B/A) x 100 (4)

where A = the µmol of NAG in the positive control (substituting µl of sample with buffer), B = the µmol of NAG.

2.11. Antimicrobial activity

The antimicrobial activity of honeys and phenolic extracts was evaluated using agar well diffusion

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measuring the inhibition halo. Three bacteria and 4 fungi species were used. *E. coli* (CECT 434), *Streptococcus mutans* (CECT 479), *Staphylococcus aureus* NB1 (bacterial collection of the Department of Biotechnology and Food Science, at the Universidad de Burgos (Burgos, Castilla y León, Spain), isolated from food), *Aspergillus flavus* (CECT 2687), *A. niger* NB1, *Fusarium* sp. NB1 (fungi collection of the Department of Biotechnology and Food Science, Universidad de Burgos, isolated from bee pollen) and *Penicillium commune* M35 (fungi collection of the Department of Food Hygiene and Food Technology, Universidad de León (León, Castilla y León, Spain), isolated from food). Stock cultures were maintained on NB (*E. coli* and *St. aureus*), BHI (*Str. mutans*) and MEB for fungi, with glycerol (20%) at -20 ºC. Bacterial inoculi were prepared in NB or BHA for 24 h at 37 ºC. Cell suspensions were diluted in sterile Ringer to 10^8 CFU/ml (determined using plate counts). While fungi were prepared from sporulating 7-day-old cultures grown on MEA at 25 ºC. Dilutions with sterile tween 80 at 0.05% were used to adjust the suspension to 10^5 conidia/ml (determined using microscopic counting on a Neubauer chamber, Brand GMBK, Wertheim, Germany).

Agar plates (NA, BHA) were inoculated with bacterium suspension (8 log cfu/ml) or fungi suspensions (5 log conidia/ml) over the surface of the plate. Two h later, sterile discs (6.0 mm diameter, Oxoid) impregnated with honey or ME were placed on the surface of the agar using a sterile tweezer. Plates were incubated at 37 ºC for 24 h for bacterium and at 25 ºC for 5 days for fungi. Methanol was also tested. Inhibition halos were measured using a Vernier caliper (0.02 mm minimum resolution). The diameter of zones (mm), including the diameter of the discs, was obtained.

2.12. Statistical analysis

All the analyses were done in triplicate. A normality test was done for all the parameters. The parametric values were evaluated using multiple range tests with Tukey HSD test ($p<0.05$), while non-parametric values were analyzed using the Kruskal-Wallis test followed by box-and-whiskers graphic interpretations. Pearson correlations were used to assess the results. Statistical software

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Statgraphics Centurion XVII was used (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results and discussion

3.1. Arbutin

Arbutin was assayed with and without Carrez clarification. Carrez clarification improved chromatographic resolution, providing symmetrical and well-defined peaks. Arbutin was quantified in 10 samples (Table 1). In two samples from Spain (3 and 5 in Table 1), the arbutin content was below the method's limit of quantification. Arbutin is mainly present in the strawberry-tree fruits (Pawlowska et al., 2006) being in low amounts in other parts of the shrub. All samples showed melissopalinological and sensory characteristics of strawberry-tree honeys. One possible explanation for the absence of arbutin in samples 3 and 5 could be that arbutin content can vary in *A. unedo* L. nectar depending on such factors as climatic conditions, among others. As strawberry tree honey is difficult to characterize by melissopalinology, because *A. unedo* pollen is under-represented (Von der Ohe et al., 2004), other possible markers, such as arbutin content should be analyzed more in depth in honeys from different geographic locations and harvesting years. Homogentisic acid, two abscisic acid isomers and unedone should also be considered (Deiana et al., 2015).

3.2. TPC

Honey showed a mean TPC 6 times higher than the TPC of ME (Table 1). Folin-Ciocalteu method also quantifies other reducing compounds (Petretto et al., 2015), so that a prior step for isolation of phenolic compounds would be needed to remove interferences (Pyrzynska and Biesaga, 2009), and properly analyze honey TPC. Other authors observed lower phenolic values for strawberry-tree honeys, ranging from 42 to 118 mg GA/100 g for honey samples (Aazza et al., 2013; Afrin et al., 2017; Castiglioni et al., 2017; Da Silva et al., 2016; Rosa et al., 2011; Tuberoso et al., 2013; Ulloa et al., 2015). Only Petretto et al. (2015) analyzed TPC on honey extracts, obtaining higher values (39 ± 8 mg GA/100 g) than those of this study. Strawberry-tree honeys had high amounts of TPC.

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Thus, phenolic extracts of strawberry-tree honeys could be successfully used as ingredients for food and cosmetic companies, as these compounds have been shown to be useful for food preservation (Martillanes et al., 2017), and are currently used as active constituents of cosmetic creams (Taofiq et al., 2019).

3.3. *o*-Diphenols

Total *o*-diphenols were quantified in strawberry-tree honeys using two different procedures, obtaining similar results (Table 1). Significant correlations were found between both assays (r = 0.7969; $p<0.05$), and between the values of *o*-diphenols by each procedure and TPC (r = 0.6607 and r = 0.6254, respectively; $p<0.05$). Despite the concentration of *o*-diphenols in strawberry-tree honeys being low, these compounds might be responsible for some bioactivity because *o*-diphenols are easily oxidized to quinones, thus showing high antioxidant activity.

3.4. Flavonoids content

Three different groups of flavonoids were measured. Table 1 shows the results for flavones/flavonols, flavanols and flavanones/dihydroflavonols. The values for ME were lower than those observed by Aazza et al. (2013) and Ulloa et al. (2015) in honeys (averages 9.7 and 5.3 mg of Q/100 g honey, respectively). As stated by Sancho et al. (2016), honeys' flavonoids must be measured on alcoholic extracts, to remove interferences. Flavanols were the main group of flavonoids found. Afrin et al. (2017) obtained values ranging between 6.6 and 11 mg Ct/100 g honey in samples from Sardinia, Italy. A significant correlation was observed between flavanols and TPC (r = 0.9113; $p<0.05$). Flavanones/dihydroflavonols were not previously analyzed in strawberry-tree honeys, their contents were lower than the amounts obtained for other flavonoids. The amount of flavonoids depends on various factors, such as botanical and geographical origins, as well as climatic conditions, which can explain the differences that were found among the flavonoids' contents of the strawberry-tree samples.

3.5. Volatile and semivolatile compounds analysis

Eighty-four out of 108 volatile analytes were identified. To summarize common features about

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volatile and semivolatile compounds in honey, Table 2 reports data for the 33 compounds detected in at least half of the samples including 10 norisoprenoids, 10 benzene derivatives, 7 heat-related compounds, 5 aliphatic compounds and 1 alkaloid (theobromine).

Norisoprenoids were the most important group since 7 of their compounds were found in all the samples and in high quantities, with 2,6,6-trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde and vomifoliol being the major volatile compound. Both compounds were also observed in Sardinian strawberry-tree honeys (Dalla Serra et al., 1999). Vomifoliol was also present in high quantities in heather honeys, minimizing its value as a strawberry tree floral marker, but 2,6,6-trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde could be further investigated as a possible marker for *A. unedo* L. honeys. Other important norisoprenoids were α-isophorone, 4-oxoisophorone (ketoisoforone) detected in all samples and β-isophorone present in 10 honeys. Bianchi et al. (2005) and De la Fuente et al. (2007) proposed these last three compounds as markers for the botanical origin of strawberry-tree honeys. Karabagias et al. (2019) also found α-isophorone, ketoisoforone and 2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one as predominant volatile compounds in Greek strawberry tree honey samples with the latter being quantified in 8 samples and also in Sardinian strawberry tree honeys (Dalla Serra *et al.*, 1999). Finally, 4 norisoprenoids were detected in 12 samples (2,2,6-trimethyl-cyclohexan-1,4-dione, 2,6,6-trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde, 4-hydroxy-2,2,6-trimethyl-5 oxo-3,6-cyclohexadien-1-carboxaldehyde and 2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1,4-dione) that were previously described in Sardinian *A. unedo* honeys (Dalla Serra *et al.*, 1999).

Among benzene derivatives homogentisic acid (2,5-dihydroxyphenylacetic acid) and 3,4,5-trimethylphenol were the most prevalent compounds. Homogentisic acid (2,5-dihydroxyphenylacetic acid) was the most abundant phenolic compound in this type of honey (Rosa et al., 2011; Tuberoso et al., 2010). Homogentisic acid was detected in all the strawberry-tree honeys in similar concentration ranges as those found by Brčić Karačonji and Jurica (2017), who developed a GC-MS method for the analysis of homogentisic acid using ethyl acetate as the solvent,

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improving the recovery with the addition of salt and with a decrease in pH. Two trimethylphenol isomers were found in all samples: 3,4,5 trimethylphenol with a concentration range from 12 to 934 mg/kg and 2,3,5-trimethylphenol with values from 1.3 to 72 mg/kg. Karabagias et al. (2019) also found 3,4,5-trimethylphenol in Greek strawberry-tree honeys. These authors also reported 2,4,6-trimethylphenol. However it was found in 5 samples so it was not included in Table 2.

The aliphatic compound 2-hydroxycyclopent-2-en-1-one was found in all samples. This compound had not been previously described in strawberry-tree honeys.

Among the volatile and semi volatile compounds, it is important to highlight the presence in 8 samples of theobromine. This alkaloid has also not been previously reported in *A. unedo* L. honeys. Theobromine contents were also analyzed in the samples using HPLC-UV, with values ranging from nondetectable to 28 mg/kg honey, with an average concentration of 5.8 mg/kg honey (data not shown).

Considering the presence and abundance of volatile and semivolatile analytes in the samples the components that can be used as markers of botanical origin of strawberry-tree honeys are not only those previously proposed (homogentisic acid, α-isophorone, 4-oxoisophorone (ketoisoforone) and β-isophorone) but also 2,6,6-trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde, 3,4,5 trimethylphenol and 2-hydroxycyclopent-2-en-1-one. Theobromine should be more studied in strawberry-tree honeys.

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3.6. Antioxidant activity

A variety of methods have been proposed to determine the antioxidant capacity of foods. However, there is no universal method that can measure the whole range of antioxidant capacities, because the assays have different reaction mechanisms and test antioxidant activity against different free radicals. To properly assess the antioxidant activity of strawberry-tree honeys, 4 different procedures were used. The antioxidant activity obtained for the strawberry-tree honeys and their ME are shown in Table 3. Strawberry-tree honeys showed better scavenging of $ABTS^{\bullet+}$ activities and $ROO^{\bullet}$ than ME. This was consistent with other non-phenol compounds in honeys also showing

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antioxidant capacity. On the other hand, ME showed better antioxidant activities against $^{\bullet}OH$ and $O_2^{\bullet-}$ radicals. It is likely that glucose oxidase activity in honeys (which produces H_2O_2), minimizes the antioxidant capacity against hydroxyl radicals to some extent, in contrast to what occurs with ME that are free of enzymes. The values of $ABTS^{\bullet+}$ scavenging activity for honey were similar to those obtained by Castiglioni et al. (2017) (750 µmol T/100 g), and higher than the values previously reported by Afrin et al. (2017) (100-390 µmol T/100 g) and Tuberoso et al. (2013) (590 µmol T/100 g), who also described lower TPC. The average ORAC value for honeys was slightly higher than the values reported by Aazza et al. (2013) in other strawberry-tree honeys (40 µmol T/g). Regarding the antioxidant capacity against hydroxyl and superoxide radicals, no data were found in the literature for strawberry-tree honeys, but the results against hydroxyl radicals were similar to those reported for heather, chestnut and honeydew honeys (Osés et al., 2016). Antioxidant values against superoxide radicals were higher than those obtained by Küçük et al. (2007) in chestnut, multifloral and rhododendron honeys. In summary, the analyzed strawberry-tree honeys and their ME showed good antioxidant activities against $ABTS^{\bullet+}$, peroxyl, hydroxyl and superoxide radicals. Significant correlations were observed between TPC and $ABTS^{\bullet+}$, superoxide radicals and ORAC values ($r = 0.6859$, $r = 0.6990$; $r = 0.7068$, respectively; $p<0.05$), as well as between flavanols and $ABTS^{\bullet+}$ ($r = 0.7388$; $p<0.05$), consistent with Ulloa et al. (2015). However, no correlation was obtained between TPC and antioxidant activity against hydroxyl radicals, which shows that other honey constituents, such as vitamins, organic acids, enzymes, peptides, and other minor compounds (Rosa et al., 2011; Ulloa et al., 2015) are likely to contribute to hydroxyl radical scavenging.

3.7. Anti-inflammatory activity

Figure 1 shows the percentage of hyaluronidase inhibition for honeys at 75% and ME (0.1 g/ml). Strawberry-tree honeys showed higher anti-inflammatory activity than their extracts. Similar results for strawberry-tree honeys were obtained by Da Silva et al. (2016), who obtained a hyaluronidase inhibition value of 48 ± 2% at a concentration of 36 g/l. A high correlation was

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observed between some volatile compounds and the ME anti-inflammatory activities. Anti-inflammatory activity was positive correlated with 2-hydroxycyclopent-2-en-1-one (r = 0.8068), β-isophorone (r = 0.8921), ketoisoforone (r = 0.8028), 2,3,5-trimetylphenol (r = 0.7486), 3,4,5-trimetylphenol (r = 0.6658), 5-hydroxy-2,4,4-trimethyl-2,5-cyclohexadien-1-one-3-carboxaldehyde (r = 0.8362) and homogentisic acid (r = 0.8366). As stated in 3.4 GC-MS section, most of these compounds were proposed as possible strawberry-tree honey floral markers.

3.8. Antimicrobial activity

Honey at 75% did not show antimicrobial activity against bacteria and fungi. On the other hand, all ME showed antimicrobial activities (Table 4). The results were different from those of Da Silva et al. (2016), who found high inhibitory activities against *St. aureus* MRSA strains and *Ps. aeruginosa* of strawberry-tree honeys at 25 and 50% (w/v). Fungi, and specifically *P. commune* and *Fusarium* sp. were the most sensitive microorganisms with ME, showing the highest halo (10 and 11 mm, respectively), while the antibacterial activity was low. These results could be due to non-phenolic honey compounds that might inhibit the antimicrobial activity of honey, or it could be due to a "dilution factor" in honeys. Methanol did not have any antimicrobial activity. Correlations were observed between antimicrobial activity against *E. coli* and TPC (r = 0.5730; $p<0.05$) and between *A. flavus* and dihydroflavonols (r = 0.5808; $p<0.05$) and *o*-diphenols (r = 0.6713; $p<0.05$), suggesting that each microorganism could be sensitive to different compounds.

4. Conclusions

Arbutin analysis using HPLC should be carried out after Carrez clarification before chromatography. Arbutin was quantified in 83% of samples, so it might be a marker for strawberry-tree honeys.

GC-MS analysis of volatile and semivolatile compounds showed that 2,6,6-trimetyl-4-oxo-2-cyclohexen-1-carboxaldehyde, 3,4,5 trimethylphenol and 2-hydroxycyclopent-2-en-1-one can be proposed as new floral markers of strawberry-tree honeys. Theobromine was found in 67% of

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samples. This alkaloid should be studied as a possible additional marker of strawberry-tree honeys. Strawberry-tree honeys from different southern European countries shared high polyphenols' contents, also showing potentially antioxidant and anti-inflammatory activities, with averages of 764 µmol T/100 g for TEAC, 55 µmol T/g for ORAC, 0.06 mmol UA/100 g for AOA, 725 mg/ml for SRS and 58% hyaluronidase inhibition for anti-inflammatory activity. On the other hand, unlike honeys, ME showed higher antioxidant activities for AOA and SRS, as well as higher antimicrobial activities, being more sensitive against fungi. In honeys, TEAC, ORAC and anti-inflammatory activities were about twice as high as in ME, which highlights the importance of non-phenolic honey compounds.

Strawberry-tree honeys could be considered for possible future health claims. Apart from their use in foods, *A. unedo* honeys could also be appealing for cosmetics and pharmaceuticals because of their biological properties and arbutin content.

Conflicts of interest: The authors declare that they have no conflicts of interest with respect to the work described in this manuscript.

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Figure caption

Figure 1. Anti-inflammatory activity of strawberry-tree honeys at 75% (w/v) and their phenolic extract (0.1 g/ml). a-e: different letters show significant differences ($p<0.05$) for each type of sample.

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Figure 1.

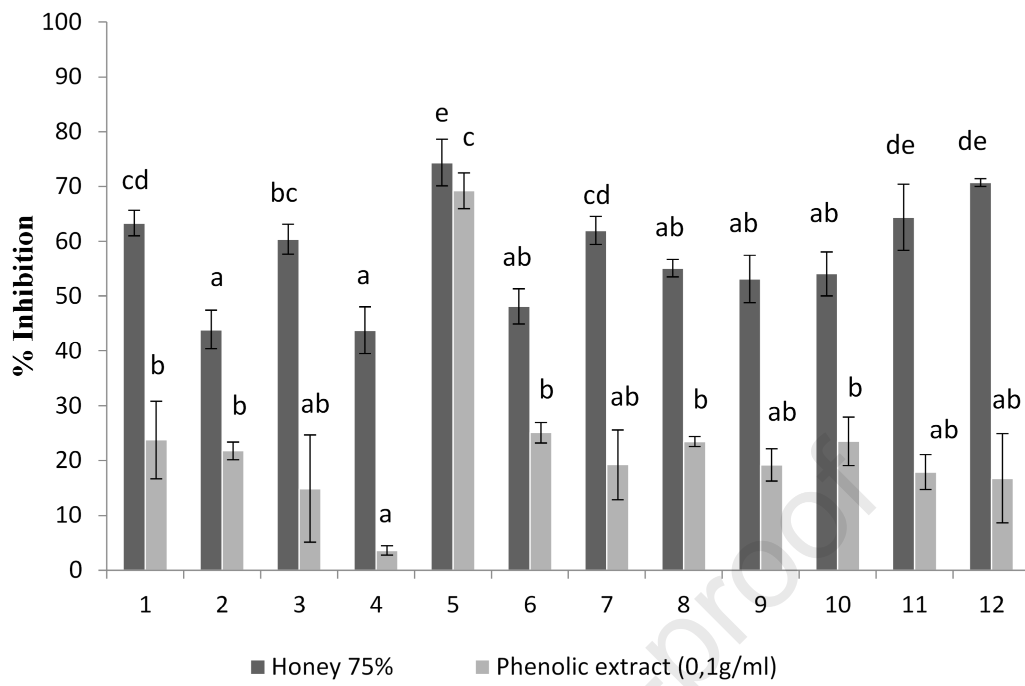

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Table 1. Arbutin, total phenols, flavonoids and o-diphenols of the strawberry-tree honey samples.

Sample	Arbutin (mg/kg)	Total phenols in honey (mg GA/100 g) (x 10^2)	Total phenols in methanolic extract (mg GA/100 g)	Total flavones /flavonols (mg Q/100 g)	Total flavanols (mg Ct/100 g)	Total flavanones /dihydroflavonols (mg N/100 g)	Total o-diphenols (procedure 1) (µg C/100 g)	Total o-diphenols (procedure 2) (µg C/100 g)
1	16 ± 3[b]	0.92 ± 0.01[a]	25 ± 1[d]	1.8 ± 0.2[abc]	2.2 ± 0.1[a]	0.70 ± 0.01[ab]	3.9 ± 0.4[a]	3.0 ± 0.5[a]
2	55 ± 1[f]	1.33 ± 0.01[c]	26 ± 1[d]	2.9 ± 0.3[d]	4.9 ± 0.7[bc]	0.98 ± 0.01[cd]	5.2 ± 0.4[bcd]	5.3 ± 0.4[cd]
3	<QL	1.48 ± 0.01[d]	21.4 ± 0.2[b]	2.33 ± 0.02[cd]	5.4 ± 0.3[bcd]	1.1 ± 0.2[de]	6.1 ± 0.2[cde]	5.5 ± 0.1[d]
4	36 ± 6[d]	2.01 ± 0.02[f]	23.2 ± 0.3[c]	2.2 ± 0.4[bcd]	6.8 ± 0.2[d]	1.83 ± 0.04[f]	8.6 ± 1.3[f]	6.3 ± 0.1[e]
5	<QL	1.44 ± 0.02[d]	20.2 ± 0.4[ab]	2.0 ± 0.6[abcd]	4.7 ± 0.1[b]	2.19 ± 0.04[g]	7.38 ± 0.04e[f]	5.2 ± 0.1[cd]
6	59 ± 2[g]	1.21 ± 0.03[b]	19.4 ± 0.1[a]	1.2 ± 0.1[a]	4.6 ± 0.1[b]	2.15 ± 0.01[g]	5.5 ± 0.4[bcd]	3.85 ± 0.03[b]
7	17 ± 5[b]	1.7 ± 0.1[e]	32.6 ± 0.2[g]	1.5 ± 0.4[ab]	6.2 ± 0.2[cd]	0.64 ± 0.03[a]	5.7 ± 1.3[cd]	4.9 ± 0.1[c]
8	57 ± 1[fg]	1.67 ± 0.03[e]	36 ± 1[h]	1.2 ± 0.1[ab]	5.79 ± 0.04[cd]	0.98 ± 0.03[cd]	5.6 ± 0.3[cd]	4.8 ± 0.4[c]
9	14 ± 2[b]	1.47 ± 0.04[d]	29.5 ± 0.3[f]	1.0 ± 0.1[a]	4.9 ± 0.7[bc]	0.83 ± 0.01[bc]	4.3 ± 0.2[ab]	3.0 ± 0.1[a]
10	28 ± 1[c]	1.48 ± 0.01[d]	27.4 ± 0.4[e]	2.5 ± 0.7[cd]	5.77 ± 0.02[cd]	0.61 ± 0.03[a]	4.9 ± 0.4[abc]	3.3 ± 0.1[a]
11	2.0 ± 1.2[a]	1.51 ± 0.01[d]	23 ± 1[c]	1.93 ± 0.01[abc]	5.2 ± 0.3[bc]	1.02 ± 0.01[d]	6.2 ± 0.5[de]	5.48 ± 0.04[d]
12	52 ± 1[e]	1.50 ± 0.01[d]	19.6 ± 0.1[a]	1.7 ± 0.3[abc]	4.9 ± 0.3[bc]	1.25 ± 0.02[e]	4.9 ± 0.3[abc]	4.1 ± 0.2[b]
Mean ± SD	**34 ± 22**	**1.5 ± 0.3**	**25 ± 5**	**1.9 ± 0.6**	**5.1 ± 1.1**	**1.2 ± 0.6**	**5.7 ± 1.3**	**4.6 ± 1.1**

a-h: different letters means significant differences ($p<0.05$) for each parameter. QL: quantification limit.

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Table 2. Volatile and semi-volatile compounds (mg/kg honey and relative occurrence %) identified in strawberry-tree (*Arbutus unedo* L.) honeys.

RT (min)	Compounds	Nº samples	Range (mg/kg)	Mean±SD (mg/kg)	Range (%)	Mean±SD (%)
8.18	2,3-butanediol (levo)	11	n.d.-23	5.0 ± 6.7	n.d.-1.4	0.25 ± 0.46
8.32	2,3-butanediol (meso)	11	n.d.-35	5.5 ± 9.6	n.d.-0.91	0.17 ± 0.31
9.06	2-furancarboxaldehyde (furfural)	12	0.32-3.60	1.2 ± 1.0	0.01-0.11	0.03 ± 0.04
9.96	5-metyl-2(5H)-furanone	10	n.d.-15	2.7 ± 4.5	n.d.-0.19	0.04 ± 0.06
11.96	2-hydroxycyclopent-2-en-1-one	12	5.7-42	12 ± 10	0.85-1.2	0.33 ± 0.33
12.13	1,2-propanediol (propylene glycol)	9	n.d.-86	18 ± 24	n.d.-0.71	0.25 ± 0.24
14.14	phenol	10	n.d.-7.1	1.4 ± 2.1	1.00-0.39	0.05 ± 0.11
14.64	3,3-dimetyl-oxetane	12	8.3-93	31 ± 26	0.19-1.2	0.63 ± 0.33
15.25	3,4-heptadiene	10	n.d.-310	70 ± 90	n.d.-2.4	0.94 ± 0.69
15.37	3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone)	10	n.d.-310	47 ± 85	n.d.-2.4	0.71 ± 0.65
15.79	phenylacetaldehyde	6	n.d.-13	1.3 ± 3.8	n.d.-0.10	0.02 ± 0.03
18.86	3,5,5-trimethyl-2-cyclohexen-1-one (α-isophorone)	12	7.8-207	79 ± 62	0.46-3.3	1.4 ± 0.8
19.60	2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one	8	n.d.-38	8.2 ± 11.4	n.d.-0.50	0.19 ± 0.19
19.76	2,6,6-trimethyl-2-cyclohexen-1,4-dione (ketoisoforone)	12	5.4-145	42 ± 41	0.38-1.2	0.70 ± 0.25
20.03	2-butanoyl-5-methylfuran	12	1.0-21	7.7 ± 6.2	0.06-0.49	0.17 ± 0.12
20.46	2,3-dihydro-3,5-dihydroxy-6-metyl-4H-pyran-4-one	9	n.d.-53	11 ± 15	n.d.-0.90	0.29 ± 0.31
21.02	2,2,6-trimethyl-cyclohexan-1,4 dione	12	5.1-100	29 ± 27	0.24-1.2	0.53 ± 0.27
23.45	2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1,4 dione	12	1.9-80	20 ± 22	0.11-0.71	0.35 ± 0.23
23.58	dihydro-4,4-dimethyl-2H-pyran-2,6-(3H)-dione	7	n.d.-73	15 ± 22	n.d.-0.62	0.24 ± 0.25
24.02	4-hydroxy-cinnamic acid	11	n.d.-9.8	3.1 ± 3.3	n.d.-0.15	0.07 ± 0.06
24.73	5-(hydroxymethyl)-2-furaldehyde (hydroxymethylfurfural, HMF)	6	n.d.-139	16 ± 39	n.d.-2.5	0.41 ± 0.74
25.58	2,3,5-trimethylphenol	12	1.3-72	21 ± 22	0.14-0.89	0.35 ± 0.22
26.66	1-methoxy-4-propyl-benzene	8	n.d.-8.6	2.9 ± 3.0	n.d.-0.20	0.05 ± 0.06
27.60	2,6,6-trimethyl-4-oxo-2-cyclohexen-1-carboxaldehyde	12	25-1150	340 ± 320	3.6-37	8.7 ± 9.0
27.81	3,4,5 trimethylphenol	12	12-934	320 ± 310	1.7-11	5.0 ± 3.3
27.98	2,6,6-trimethyl-4-oxo-1-cyclohexen-1-carboxaldehyde	8	n.d.-97	24 ± 29	n.d.-0.88	0.35 ± 0.35
30.18	4-hydroxy-2,2,6-trimethyl-5 oxo-3,6-cyclohexadien-1-carboxaldehyde	12	2.4-217	55 ± 61	0.14-2.0	0.83 ± 0.55
33.16	2,5-dihydroxybenzaldehyde	6	n.d.-3.9	0.32 ± 1.12	n.d.-0.08	0.01 ± 0.02

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RT (min)	Compounds	Nº samples	Range (mg/kg)	Mean±SD (mg/kg)	Range (%)	Mean±SD (%)
40.96	2,5-dihydroxyphenyl acetic acid (homogentisic acid)	12	22-374	170 ± 110	0.89-35	5.5 ± 9.4
41.24	1-methoxy-4-methyl-benzene	10	n.d.-93	36 ± 30	n.d.-1.5	0.61 ± 0.48
47.00	4-hydroxy-4-3-hydroxybut-1-enyl]-3,5,5-trimethylcyclohex-2-en-1-one (vomifoliol)	12	62-572	280 ± 130	2.1-17	7.3 ± 3.5
51.01	3,7-dimethyl-xanthine (theobromine)	8	n.d.-44	5.7 ± 12.4	n.d.-0.33	0.07 ± 0.10
53.24	3-[(4-hydroxy-3,5-dimethyl)-benzoyl] propionic acid	7	n.d.-114	32 ± 37	n.d.-1.6	0.65 ± 0.69

RT: Retention time; Min.: Minimum; Max.: Maximum; SD: Standard deviation. mg/kg: mg methyl-undecanoate/kg honey). n.d. Non detected

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Table 3. Antioxidant activity of strawberry-tree honeys and their phenolic extract against different radicals (ABTS•+, •OH, O_2^- and ROO•).

Sample	TEAC ($ABTS^{•+}$)		AOA (•OH)		SRS (O_2^-)		ORAC (ROO•)	
	Honey (µmol T/100 g) (x 10^2)	**Phenolic extract (µmol T/100 ml)**	**Honey (µmol UA /100 g)**	**Phenolic extract (µmol UA /100 ml)**	**Honey (mg/ml) (x 10^2)**	**Phenolic extract (mg/ml) (x 10)**	**Honey (µmol T/g)**	**Phenolic extract (µmol T/100 ml) (x 10^2)**
1	2.8 ± 0.1^{a}	21.0 ± 0.2^{a}	78 ± 6^{g}	91 ± 6^{cd}	2.6 ± 0.4^{a}	12 ± 1de	41 ± 1^{b}	3.3 ± 0.2^{de}
2	7.4 ± 0.4^{bc}	28 ± 2^{bc}	60 ± 2de	84 ± 1^{bcd}	6.9 ± 0.3^{bc}	8.4 ± 0.2^{bc}	62 ± 3de	2.3 ± 0.5^{bc}
3	7.7 ±0.3^{bc}	33 ± 9^{bcd}	50 ± 4^{bc}	101 ± 5de	7.14 ± 0.02^{bc}	7.0 ± 0.2^{ab}	31.1 ± 0.4^{a}	0.7 ± 0.1^{a}
4	8.2 ± 1.7^{bc}	26 ± 2^{ab}	51 ± 1^{bc}	77 ± 5^{bc}	1.7 ± 0.1^{a}	9.6 ± 0.1^{cd}	35 ± 4^{ab}	0.9 ± 0.3^{a}
5	7.9 ±0.4^{bc}	25 ± 2^{ab}	56 ± 1^{cd}	57 ± 13^{a}	6.4 ± 0.5^{b}	6.7 ± 0.4^{ab}	41 ± 1^{b}	3.4 ± 0.2^{ef}
6	5.8 ± 0.6^{b}	22 ± 1^{ab}	67 ± 2^{ef}	77 ± 1^{bc}	8.29 ± 0.01^{bc}	6.8 ± 0.2^{ab}	44 ± 1^{b}	0.6 ± 0.3^{a}
7	8.7 ± 1.0^{c}	55 ± 2^{f}	63 ± 1de	87 ± 2^{bcd}	2.4 ± 0.2^{a}	12 ± 2^{e}	69 ± 3^{e}	2.53 ± 0.04^{bc}
8	8.1 ± 0.7^{bc}	48 ± 5^{ef}	74 ± 2^{fg}	95 ± 4^{cd}	7.4 ± 0.6^{bc}	12 ± 2de	113 ± 2^{f}	4.0 ± 0.2^{f}
9	9.0 ± 0.6^{c}	31 ± 4^{bc}	49 ± 5^{b}	75 ± 2^{b}	18 ± 4^{d}	10 ± 1^{cde}	58 ± 2^{cd}	4.8 ± 0.2^{g}
10	9.3 ± 1.3^{c}	40 ± 3^{cde}	40 ± 1^{a}	82 ± 4^{bcd}	10 ± 1^{c}	9.8 ± 0.2^{cd}	28 ± 3^{a}	3.0 ± 0.2^{cd}
11	7.9 ± 0.6b^{c}	44 ± 3^{def}	63 ± 1de	100 ± 5de	6.9 ± 0.2^{bc}	6.2 ± 0.1^{a}	63 ± 4de	0.8 ± 0.1^{a}
12	9.0 ± 0.1^{c}	34 ± 8^{bcd}	40 ± 1^{a}	111 ± 5^{e}	9.4 ± 0.4^{bc}	9.9 ± 0.4^{cd}	51 ± 3^{c}	2.2 ± 0.2^{b}
Mean ± SD	**7.6 ± 1.9**	**34 ± 11**	**58 ± 12**	**86 ± 15**	**7.3 ± 4.3**	**9.2 ± 2.2**	**53 ± 24**	**2.4 ± 1.4**
	Honey (µmol T/g)	**Phenolic extract (µmol T/10ml)**	**Honey (µmol UA /g)**	**Phenolic extract (µmol UA /10 ml)**			**Honey (µmol T/g)**	**Phenolic extract (µmol T/10ml)**
Mean ± SD	**7.6 ± 1.9**	**3.4 ± 1.1**	**0.6 ± 0.1**	**8.6 ± 1.5**			**53 ± 24**	**24 ± 14**

a-g: different letters means significant differences ($p<0.05$) for each parameter.

Table 4. Antimicrobial activity by agar well diffusion (mm) of phenolic extract from strawberry tree honeys from different Mediterranean countries (n = 3). Diameter of the disk 6 mm.

Sample	***St. aureus***	***E. coli***	***Str. mutans***	***A. niger***	***A. flavus***	***P. commune***	***Fusarium* sp.**
1	8.1 ± 0.8^{ABb}	9.3 ± 1.5^{ABb}	7.0 ± 1.7^{Aa}	11 ± 1^{Bb}	8.4 ± 0.2^{ABab}	10 ± 1^{ABb}	10 ± 1^{ABab}
2	8.6 ± 1.6^{ABb}	8.2 ± 0.2^{Aab}	7.3 ± 0.0^{Aa}	9.4 ± 0.7^{ABab}	7.5 ± 0.1^{Aa}	13 ± 1^{Cc}	11.0 ± 0.1^{BCab}
3	7.2 ± 0.8^{Aab}	8.1 ± 0.6^{ABab}	7.3 ± 0.8^{Aa}	9.7 ± 0.5^{BCb}	8.8 ± 0.7^{ABCbc}	10.1 ± 0.0^{BCb}	10.7 ± 0.0^{Cab}
4	7.2 ± 0.2^{Aab}	8.0 ± 0.9^{Aab}	6.5 ± 0.0^{Aa}	8.6 ± 0.5^{ABab}	11.0 ± 0.3^{Ce}	9.7 ± 0.4^{BCb}	10.6 ± 0.5^{Cab}
5	6.1 ± 0.1^{Aa}	6.5 ± 0.4^{ABa}	6.8 ± 0.6^{ABa}	8.8 ± 1.8^{BCab}	9.5 ± 0.4^{Cd}	7.3 ± 0.9^{ABCa}	9.3 ± 0.8^{Ca}
6	6.0 ± 0.0^{Aa}	6.0 ± 0.0^{Aa}	7.1 ± 1.6^{Aa}	8.3 ± 0.4^{ABab}	9.3 ± 0.3^{BCcd}	11 ± 1^{Cbc}	11 ± 1^{Cab}
7	7.2 ± 0.2^{ABab}	8.3 ± 0.8^{ABab}	6.4 ± 0.1^{Aa}	9.2 ± 0.8^{BCb}	8.1 ± 0.1^{ABab}	10.8 ± 0.4^{Cbc}	11 ± 2^{Cab}
8	7.8 ± 0.2^{ABb}	9.5 ± 0.7^{ABb}	7.0 ± 1.2^{Aa}	9.8 ± 1.4^{ABb}	9.0 ± 0.3^{ABbc}	10.6 ± 0.1^{BCbc}	12 ± 2^{Cb}
9	7.5 ± 0.5^{ABab}	9.3 ± 1.4^{Bb}	6.4 ± 0.1^{Aa}	9.5 ± 1.5^{Bb}	8.7 ± 0.7^{ABab}	10 ± 1^{Bb}	10 ± 1^{Bab}
10	6.0 ± 0.0^{Aa}	6.0 ± 0.1^{Aa}	7.1 ± 0.5^{ABa}	11 ± 1^{Cb}	9.4 ± 0.0^{BCcd}	11 ± 1^{Cbc}	10.3 ± 0.4^{Cab}
11	6.8 ± 0.7^{Aab}	6.0 ± 0.0^{Aa}	6.0 ± 0.0^{Aa}	9.1 ± 0.4^{Bb}	9.0 ± 0.1^{Bbc}	10.9 ± 0.3^{Cbc}	9.6 ± 0.2^{Bab}
12	7.8 ± 0.4^{ABb}	6.8 ± 0.7^{ABa}	6.8 ± 1.1^{ABa}	6.0 ± 0.0^{Aa}	8.2 ± 0.2^{BCab}	10.4 ± 0.00^{CDbc}	11 ± 1^{Cab}
Mean ± SD	**7.1 ± 0.9**	**7.6 ± 1.5**	**6.8 ± 0.9**	**8.6 ± 3.0**	**8.9 ± 0.9**	**10 ± 1**	**11 ± 1**

a-e: different letters show significant differences ($p<0.05$) in the same column

A-D: different letters show significant differences ($p<0.05$) in the same row

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Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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