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**Physicochemical parameters, chemical composition, antioxidant capacity,
microbial contamination and antimicrobial activity of *Eucalyptus* honey
from the Andean region of Ecuador**

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Abstract

This study aims to evaluate the pollen profile, physicochemical parameters, chemical composition, and antioxidant capacity, and antimicrobial contamination and antimicrobial activity of *Eucalyptus* honey from the Andean region of Ecuador. Aerobic mesophiles, molds and yeasts, fecal coliforms and sulfite-reducing clostridia contamination were also assessed. All honey samples met the quality standards required by international legislation, except for hydroxymethylfurfural, for which three samples did not satisfy international standards. Total Apparent phenolic and flavonoid content, as well as free amino acid and protein content fell within the ranges previously reported for other types of honey of distinct floral and geographical origins. No microbial contamination was observed, while total antioxidant capacity showed evidence of the *Eucalyptus* honey's ability to reduce Fe^{3+} to Fe^{2+} as well as to verify the superoxide radical scavenging activity and metal ion chelating capacity. *Eucalyptus* honey was able to inhibit the growth of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Escherichia coli*, but not effective against *Candida albicans*.

Keywords

Ecuadorian honey, *Eucalyptus* honey, physicochemical parameters, microbial contamination, antioxidant capacity.

Introduction

Honey is defined as the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants, from secretions of living parts of plants or from excretions of plant-sucking insects on the living parts of plants. These secretions are collected by bees and transformed by combining them with specific substances from their own deposits and leaving them in honeycombs to ripen and mature (EU Council, 2001). The composition and quality of the honey depends on several factors, mainly its floral origin and geographical conditions (Acquarone, Buera, & Elizalde, 2007). Sugars are the main compounds present in honey, with fructose (38%) and glucose (31%) being the two most abundant components (Bogdanov, 2017; Bogdanov, Jurendic, Sieber, & Gallmann, 2008). However, there are several minor compounds that have also been associated with the quality and beneficial effects biological properties of honey, such as polyphenols, enzymes, free amino acids, proteins, minerals and vitamins (Alvarez-Suarez, Giampieri, & Battino, 2013). (Bogdanov, 2017). Current literature has comprehensively shown that honey is an important natural source of bioactive compounds with significant antioxidant properties, which may have applications for human health and nutrition (Alvarez-Suarez et al., 2013; Bogdanov, 2017; Bogdanov et al., 2008).

Throughout the world, *Eucalyptus* honey is a significant commercial monofloral honey and is highly desired by consumers, who are primarily attracted to the aroma and medicinal properties of the plant (Barbosa, Filomeno, & Teixeira, 2016). The aroma compounds of *Eucalyptus* honey (Alissandrakis, Tarantilis, Pappas, Harizanis, & Polissiou, 2011), it's While physicochemical parameters (Moussa, Nouredine, Saad, & Abdelmalek, 2012; Nanda, Kaur, Bera, Singh, & Bakhshi, 2006; Ortega-Bonilla, Chito-Trujillo, & Suárez-Ramos, 2016) and

mineral content (González-Miret, Terrab, Hernanz, Fernández-Recamales, & Heredia, 2005) have been studied previously, of honey from different regions have previously been reported (Alvarez-Suarez, Tulipani, et al., 2010; Lemos, Venturieri, Dantas Filho, & Dantas, 2017; Meda, Lamien, Romito, Millogo, & Nacoulma, 2005) as well as the content correlation between a of a particular variety of bioactive honey components with potential as well as a variety of bioactive components with potential beneficial effects (Ciappini & Stoppani, 2014; Perna, Intaglietta, Simonetti, & Gambacorta, 2013; Yao, Jiang, Singanusong, Datta, & Raymont, 2004) biological properties, such as its antioxidant capacity (Ferreira, Aires, Barreira, & Estevinho, 2009; Gomes, Dias, Moreira, Rodrigues, & Estevinho, 2010; Meda et al., 2005) few researchers have focused on microbial contamination (Gomes et al., 2010; Olaitan, Adeleke, & Ola, 2007). Notwithstanding these studies, several regions with melliferous potential exist for this unifloral honey where its characteristics and properties remain unknown.

Ecuador is one of the most biodiverse countries in the world, possessing a floral wealth that can be used in the production of honey (Cabrera, 2007). According to the National Beekeeping Health Program run by the Ecuadorian Agency for Agricultural Quality Assurance (AGROCALIDAD), beekeeping activities in Ecuador are primarily concentrated in the central and northern highlands where there is a floral abundance, which allows bees to use different blooms throughout the year and so produce varieties of honey from both specific and non-specific blooms. Despite enjoying an autochthonous flora, the extensive eucalyptus plantations in the country constitute an important melliferous source for honey production; it is therefore not uncommon to find that *Eucalyptus* honey is one of the main monofloral varieties of honey produced in the country (Cabrera, 2007). The main species of *Eucalyptus sp* in Ecuador (*E.*

globulus) was initially introduced in 1865 and was extensively planted on the central plateau between the altitudes of 1.800 and 3.300 m, with the best growth occurring in areas between 2.000 and 2.900 m above sea level. *Eucalyptus* honey is a significant commercial monofloral honey worldwide, and is highly desired by consumers, primarily attracted by its aroma and medicinal properties of the plant (Barbosa, Filomeno, & Teixeira, 2016). The aroma compounds of *Eucalyptus* honey have been studied previously (Alissandrakis et al., 2011), as well as its chemical composition, such as the flavonoids that differentiate it from other varieties (Ciappini & Stoppani, 2014; Ciappini & Vitelleschi, 2013; Martos, Ferreres, & Tomás Barberán, 2000; Moussa et al., 2012; Nanda et al., 2006; Perna et al., 2013; Yao et al., 2004). To date, no studies have been published on the composition and beneficial effects biological properties of different types of Ecuadorian honey; as such, the aim of this study was to confirm the floral origin, as well as determine the physicochemical parameters, antioxidant capacity, microbial contamination and antimicrobial activity of several samples of monofloral *Eucalyptus* honey from Ecuador.

Materials and methods

Honey samples

Samples of honey were collected directly from beekeepers in the province of Pichincha, located in the inter-Andean region of Ecuador. The Pichincha cantons, from where the samples were taken, and their respective districts: Quito (Tumbaco, Chillogallo, Amaguaña and Pintag), Cayambe (Cangahua) and Mejía (Machachi) are shown in **Figure 1**, while the distribution of beekeepers by districts (parishes) sampled is presented in **Table 1**. A total of 10 beekeepers

were chosen, on the basis of number of apiaries kept (≥ 20), which made up approximately 50% of total beehives in the province. For each beekeeper, all apiaries were sampled, by taking three samples at random from each apiary. The samples were kept at room temperature and the different analyses were performed within a time period not exceeding three months from the date of collection. Beekeepers were identified as Beekp1 to Beekp10 and results were expressed as the mean values obtained from the analyses of the three samples from each beekeeper.

A solution of artificial honey (1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose and 33.5 g glucose in 17 mL of de-ionized water) was included to eliminate any interference that might occur from these compounds in the tests performed (Cooper, Molan, & Harding, 2002).

Pollen content and physicochemical analysis

The botanical origin was confirmed by the traditional qualitative microscopic analysis and frequency determination of the classes of pollen grains in the honey samples following a previously reported protocol (Louveaux, Maurizio, & Vorwohl, 1978; Von Der Ohe, Oddo Livia, Piana, Morlot, & Martin, 2004). The pollen grains' morphology was compared to that in a published pollen catalog (Montoya-Pfeiffer, León-Bonilla, & Nates-Parra, 2014).

The honey samples were tested using the usual physicochemical tests, such as ashes (%), color (mm Pfund), Diastase activity (Schade units), free acidity (meq/kg), pH, electrical conductivity (mS/cm), moisture (%), density (g/ml) and the hydroxymethylfurfural (HMF) test (mg/kg) (AOAC, 1990; EU Council, 2002).

Determination of ~~apparent~~ total phenolic ~~content~~ (TPC) and total flavonoid content (TFC)

Apparent total phenolic content (ATPC) was determined using the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1998). Aliquots (0.5 mL) of honey solution (1g/10 mL) were mixed with 2.5 mL of Folin–Ciocalteu reagent (0.2 N) for 5 min and then 2 mL of sodium carbonate solution (0.7 M) was added. The solution was allowed to stand in the dark at room temperature for 2 hours and the absorbance was measured spectrophotometrically at 760 nm against the **artificial honey analogue-sugar** blank. Gallic acid was used as a standard to produce the calibration curve (0.5-3 mM, $R^2=0.9954$) and TPC was expressed as mg of gallic acid equivalents (GAE) per kg of honey (mg GAE/kg of honey).

Apparent total flavonoid content (ATFC) was determined by previously established methods (Chang, Yang, Wen, & Chern, 2002). Honey samples were diluted in methanol (50% w/v) and 0.25 mL of each was mixed with distilled water (1.25 mL) and a 5% NaNO_2 solution (75 μL). The solution was incubated for 6 min, then 150 μL of a 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution was added and incubated for a further 5 min before 0.5 mL of 1 M NaOH was added. Distilled water was added to give a final volume of 2.5mL and the absorbance was determined spectrophotometrically at 510 nm **against the artificial honey blank**. (+)-Catechin was used as a standard to produce the calibration curve (0.037-0.25 mM, $R^2= 0.9916$) and the TFC was expressed as (+)-catechin equivalents (CE) per kg of honey (mg of CE/kg of honey).

Determination of protein and free amino acids content

The protein content was determined as previously reported (Azeredo, Azeredo, De Souza, & Dutra, 2003). Honey samples were diluted in distilled water (50% w/v) and 0.1 mL of this solution was added to 5 mL of Coomassie Brilliant Blue reagent. This solution was allowed to stand for 2 minutes at room temperature, after which its absorbance was measured spectrophotometrically at 595 nm. Bovine serum albumin was used for the calibration curve (10–100 µg/0.1 mL, $R^2 = 0.9980$) in 0.15 M of sodium chloride and the protein content was expressed as mg of bovine serum albumin (BSA) per 100 grams of honey (BSA/100 g of honey).

Free amino acid content was determined using the Cd-ninhydrin method (Doi, Shibata, & Matoba, 1981). Honey samples (1.25 g) were diluted in 25 mL of distilled water and 1 mL of this solution was added to 2 mL of the Cd-ninhydrin reagent. The reaction mixture was incubated in a water bath at 84°C for 5 minutes and then cooled in ice, after which the absorbance was determined spectrophotometrically at 507 nm. L-Leucine was used for the calibration curve (2.4–42 mg/L, $R^2 = 0.9935$) and the free amino acid content was expressed as mg of L-leucine equivalents (LE) per 100 grams of honey (mg LE/100 g of honey).

Determination of total carotenoid content

Total carotenoid content (TCC) was determined following a previously published method (Ferreira et al., 2009). Briefly, 1 gram of honey was shaken ($500 \text{ r} \cdot \text{m}^{-1}$) in 10 mL of extraction solution (n-hexane-acetone, 6:4) at room temperature for 10 minutes and then filtered (Whatman No. 4 filter paper). The absorbance was determined spectrophotometrically at 450

nm. β -carotene was used for the calibration curve (0.25–10 $\mu\text{g/mL}$, $R^2= 0.9982$) and results were expressed as mg of β -carotene equivalent (βcarotE) per kg of honey (mg $\beta\text{carotE/kg}$ of honey).

~~Determination of total antioxidant capacity (TAC)~~ *Ferric reducing antioxidant power (FRAP) assay, metal ions chelating capacity and the superoxide radical ($\text{O}_2^{\cdot-}$) scavenging activity*

~~TAC was determined using the FRAP assay was conducted as previously reported (Benzie & Strain, 1996), the metal ions chelating capacity (Wang, Lien, & Yu, 2004) and the detection of $\text{O}_2^{\cdot-}$ scavenging activity (Oyanagui, 1984).~~

The FRAP assay was conducted as previously reported (Benzie & Strain, 1996). Honey samples were diluted in distilled water (1:10 w/v), then aliquots of 200 μL were added to 1.8 mL of FRAP reagent, allowed to stand for 5 minutes at room temperature and the absorbance was determined spectrophotometrically at 593 nm. Trolox was used as a standard (0.03–2.5 mM, $R^2=0.9902$) and TAC was expressed as μmoles of Trolox equivalents (TE) per 100 g of honey ($\mu\text{mol TE/100 g}$ of honey).

The metal ions chelating capacity was determined as previously reported (Wang et al., 2004). ~~For the chelating metal ions capacity,~~ Honey samples were incubated with 0.05 mL of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (2 mM). The reaction was initiated by adding 5mM of ferrozine (0.2 mL) and after 10 min of incubation at room temperature; the absorbance was determined spectrophotometrically at 562 nm. Chelating ability results were expressed as percentages and were calculated using the following equation:

$$\text{ChA} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

in which A_0 was the absorbance of the control and A_1 the absorbance of each honey sample.

The $\text{O}_2^{\cdot -}$ scavenging activity of honey was investigated using a modified nitrite method based on the ability of the $\text{O}_2^{\cdot -}$ generated by hypoxanthine/xanthine oxidase (HX/XO) to oxidize hydroxylamine to nitrite ion at pH 8.2 (Oyanagui, 1984). The nitrite ion generated was measured spectrophotometrically at 550 nm and results were expressed as the percentages of inhibition (PI) of nitrite ion generation in the presence of honey, calculated by the change in the slope of increasing spectrophotometric absorption (A_m) of the samples compared to the blank (A_b , with distilled H_2O) using the following equation:

$$\text{PI} = [(A_b - A_m)/A_b] \times 100 \quad (2)$$

The results were expressed as the concentration of honey solution required to inhibit the rate of nitrite ion generation by 50% (IC_{50}) (mg/ml).

Microbial contamination

Microbial contamination was determined following a previously reported method (Gomes et al., 2010). Honey samples (10 g) were diluted in 90 mL of peptone water. A general trypticase soy agar media (TSA) was incubated at 37°C for 48 hours and used for the primary screening of bacterial contamination in honey. The presence of aerobic mesophilic bacteria was determined using a standard plate count agar (PCA) incubated at 37°C for 48 hours, while yeasts and molds were studied by using sabouraud dextrose agar (SDA), supplemented with chloramphenicol and potato dextrose agar (PDA) media, both incubated at 25°C for 96 hours.

For detecting and enumerating *Enterobacteriaceae*, the violet red bile glucose (VRBG) agar was used as previously described (ISO 21528-2:2006, 2004). For determining sulphite-reducing clostridia, SPS (sulfite-polymixin-sulfadiazine) agar media were used (ISO 15213:2003, 2003). Aliquots of the initial honey suspension (10, 5, 1 and 0.1 mL) were thermally treated at 80°C for 5 minutes in a test tube, covered with the SPS agar media and then incubated at 37°C for 5 days.

Antimicrobial activity

The antimicrobial activity of the honey samples was studied using four ATTC bacterial strains (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) and one fungal strain (*Candida albicans* ATCC 90028).

The minimum active dilution (MAD) of the honey samples was determined by an agar incorporation technique (French, Cooper, & Molan, 2005) in nutrient agar for bacterial strains and Yeast Mold (YM) agar for fungal strains. A dilution series using final honey samples or artificial honey dilutions in the range of 1–20% (v/v) in 1% increments was used for susceptibility assays. The inoculated plates were incubated at 37°C overnight and the complete growth inhibition was recorded at each inoculation position. The MAD was taken to be the lowest honey dilution at which microbial growth was completely inhibited.

Statistical analyses

The samples were analyzed in triplicate and results were expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics for Windows version 20.0. ~~One-way ANOVA was used to determine significant differences between samples using a Bonferroni correction for multiple sample comparison.~~ A multivariate analysis (MANOVA) was done for the physico-chemical properties and the chemical composition. The bonferroni correction was used in both cases for post-hoc analysis adjusting for multiples groups' comparison. The correlations between the variables were calculated using Pearson's coefficient. In all cases a *P*-value < 0.05 was considered as statistically significant.

Results and discussion

Pollinic analysis and physicochemical analyses

Pollen analysis was used to confirm the floral origin of the different honey samples. **Table 2** shows the most predominant pollen grain classes identified in the samples from each beekeeper. All samples showed a high content of pollen grains specific for *Eucalyptus ssp.* (between 70-90 %), which is in accordance with previous reports for this monofloral honey (Ciappini & Vitelleschi, 2013; Gomez, Hernandez, Gomez, & Marin, 1993; Rodríguez Flores, Escuredo Pérez, & Seijo Coello, 2014; Terrab, Díez, & Heredia, 2003). Moreover, secondary pollen grain classes were also identified in the honey samples showing, in some cases, common plant species between the honeys from different beekeepers from the same region. The species *Mimosa c.f. albida* was the secondary vegetal species identified in honey from the district of Tumbaco, Quito canton, while in the district of Pitag, the secondary vegetal specie was

Brassica napus. It was also found that a group of plant species, including *Persea Americana*, had a low prevalence, as did pollen grains from the genus *Poulsenia*, *Trifolium*, and *Weinmannia*. These species represented only between 5-10% of the total pollen grains identified in the honey samples.

Table 3 shows the physicochemical analysis of the different samples of *Eucalyptus* honey. The moisture content (%) ranged from 11.74 to 19.42 %, all of which were within internationally recommended parameters ($\leq 20\%$) for quality of commercial honey (EU Council, 2002). Since the moisture content in honey is closely linked to environmental and geographical conditions, apiary management and honey storage (Acquarone et al., 2007), the results suggest appropriate handling and storage of the honey. High moisture content may cause fermentation during storage and affect the organoleptic characteristics and biological properties of honey, thus affecting its overall quality (Chirife, Zamora, & Motto, 2006).

The acceptable range of pH values for honey has yet to be legislated, so it is important to compare the present results with those published previously. All samples were found to be acidic, exhibiting pH values ranging between pH 3.1 and pH 4.2 (**Table 3**), which corresponds to those previously reported in *Eucalyptus* honey from other geographical origins (Gomez et al., 1993; Moussa et al., 2012; Nanda et al., 2006; Ortega-Bonilla et al., 2016; Rodríguez Flores et al., 2014). The acidity of honey affects the growth and proliferation of several microorganisms, which can affect organoleptic characteristics, such as texture, flavor, odor, stability and shelf-life (Terrab, Díez, & Heredia, 2002). In most of the samples tested, the values for free acidity were within the established limits (50 meq/Kg) (Codex Alimentarius, 2001; EU Council, 2002) and similar to other *Eucalyptus* honey from other geographical origins (Moussa et al., 2012; Nanda et al., 2006; Ortega-Bonilla et al., 2016). Only one sample

yielded values higher than those established (**Table 3**). Moreover, it is important to note that, in general, honey is naturally acidic regardless of its geographical origin (Acquarone et al., 2007). However, high acidity values are indicative of fermentative processes of different sugars that convert to organic acids and can be used as an indicator of the freshness of honey (Subramanian, Umesh Hebbar, & Rastogi, 2007). All the samples showed electrical conductivity values within the established range, i.e. between 0.24 and 0.78 mS/cm, and not exceeding 0.8 mS/cm. Furthermore, the total ash content, which did not exceed 0.6 %, suggests that all the honey samples were from floral nectar (Codex Alimentarius, 2001; EU Council, 2002).

Results of the color analysis on the samples of honey are also presented in **Table 3**. All samples were classified as having an extra light amber shade. The color of honey can be influenced by several factors, such as mineral content, TPC and TCC; light-colored honeys have been associated with low concentrations of these compounds, while dark-colored honey usually presents a higher content (Al et al., 2009; ~~Alvarez-Suarez, Tulipani, Díaz, et al., 2010;~~ Gomes et al., 2010).

HMF could be found in low quantities or was completely absent in fresh honey but increased during processing and/or aging, influenced by several factors, such as temperature, pH, storage conditions and floral source (Fallico, Arena, Verzera, & Zappalà, 2006). Honey samples from three beekeepers (Beekp 6, 7 and 8) had HMF values outside the allowable range for honey with declared origin from tropical climates (lower than 80 mg/kg), in accordance with international standards for quality (EU Council, 2002), suggesting that these samples might have been exposed to temperature abuse during processing and/or were subject to poor storage practices (EU Council, 2001). All other honey samples were within the

international limits (≥ 80 mg/kg) (EU Council, 2001) and are in agreement with the values previously reported in *Eucalyptus* honeys from other geographical regions (Moussa et al., 2012; Nanda et al., 2006; Terrab et al., 2003). The content of diastase depends on the honey's freshness as well as its geographic and floral origin. The diastase index can be used as a marker of aging and temperature abuse in honey, but should be interpreted with care, as the diastase content of fresh unheated honey can vary greatly depending on its origin and processing conditions (Fallico et al., 2006). According to the results presented here, all samples were within the established limits, in line with international regulations for quality, which state diastase should not be less than 8 Schade units (EU Council, 2002) and with the diastase values previously reported for this type of honey in other geographical regions (Nanda et al., 2006; Ortega-Bonilla et al., 2016; Terrab et al., 2003).

Phytochemical and nutrient content

Polyphenols are a group of phytochemicals that have been to be closely related to the appearance and functional properties of honey (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010; Bogdanov, 2017; Bogdanov et al., 2008) and have been also proposed as potential floral markers for eucalyptus honey (Martos et al., 2000; Perna et al., 2013; Yao et al., 2004). The phytochemical content of the different honey samples was determined and is shown in **Table 4**, while the correlation matrix for quantitative determination is shown in **Table 5**. The A_{TPC} and A_{TFC} content demonstrate significant differences among the various samples; however, the average values were within those previously reported in monofloral *Eucalyptus* honey from other geographical zones (Ciappini & Stoppani, 2014; Rodríguez Flores et al.,

2014; Serrano, Villarejo, Espejo, & Jodral, 2004). In relation to ATFC results, several authors have reported that sugars in honey can interfere with the ATFC assay (Denni & Mammen, 2012; Sancho et al., 2016), so this interference must be corrected in this assay. According to Sancho et al., 2016, total flavonoid content in neutral media must be determined on extracts instead of on honeys. ~~so the results exposed here cannot be considered completely accurate in confirming the actual flavonoid content in the samples analyzed; nevertheless we think that they can serve to give an approximation of the actual content.~~ Our assay can overestimate the actual flavonoid content. Therefore, our results should not be considered completely accurate in confirming the actual flavonoid content in the samples analyzed; nevertheless we think that they can serve to give an approximation of the actual content.

TCC was also analyzed and the results are shown in **Table 4**. The levels of TCC in monofloral *Eucalyptus* honey from Ecuador range between 0.28-0.43 mg β CarotE/100 g of honey, which corresponds to those previously reported by other authors in honey from different floral and geographical origins (Alvarez-Suarez, Tulipani, Díaz, et al., 2010; Ferreira et al., 2009; Gomes et al., 2010). A significant correlation was found between ~~color vs TCC ($P < 0.01$)~~, ~~color vs Prot ($P < 0.05$)~~, ATFC vs ~~FRAP~~ ATPC ($P < 0.01$), ATFC vs FRAP ($P < 0.01$) and ATFC vs TCC ($P < 0.01$), as well as a correlation between ATPC vs FRAP, ATPC vs TCC and ATPC vs Prot ($P < 0.01$). TFC showed a correlation vs MiChec and $O_2^{\bullet-}$ RSA ($P < 0.05$), suggesting that these compounds could be partly responsible for the antioxidant properties of the samples. TCC was also correlated with Prot and MiChec ($P < 0.05$), while MiChec showed a negative correlation vs $O_2^{\bullet-}$ RSA (**Table 5**).

In addition to phytochemical content, the presence of ~~certain nutrients (~~ protein and free amino acid ~~)~~ content was also analyzed ~~to determine a possible contribution to the nutritional quality and biological properties of honey~~ (Table 4). The protein and free amino acid content of honey has been associated with two main factors: the presence of certain enzymes introduced by bees from their salivary glands and pharynx, as well as floral sources, nectar and pollen (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). The levels of total proteins and free amino acids in the samples of honey were within the range previously reported by other authors in honey from different floral and geographical origins (Alvarez-Suarez, Tulipani, Díaz, et al., 2010; Azeredo et al., 2003; Iglesias, De Lorenzo, Polo, Martín-Álvarez, & Pueyo, 2004; Pérez, Iglesias, Pueyo, González, & De Lorenzo, 2007). It was, however, not possible to draw a comparison with other types of *Eucalyptus* honey because no reports have been published of such analyses.

TAC FRAP assay, metal ions chelating capacity and superoxide radical ($O_2^{\cdot-}$) scavenging activity

Antioxidant activity in honey was analyzed using three different methods and the results are shown in Table 4. A solution of artificial honey was included in the study to evaluate the contribution of the predominant sugars to the assayed activities. ~~TAC analysis~~ (Cooper et al., 2002). Artificial honey showed significantly lower antioxidant activity ~~in the TAC values~~ (FRAP values, metal ions chelating capacity and $O_2^{\cdot-}$ scavenging activity) than all other analyzed honey samples ($P < 0.05$) (data not shown), which led to the inference that the main sugars in honey did not contribute significantly to their antioxidant capacity. The antioxidant

capacity analysis The TAC analysis demonstrated the capacity of *Eucalyptus* honey to reduce Fe^{3+} to Fe^{2+} (Ciappini & Stoppani, 2014), as well as its $\text{O}_2^{\bullet-}$ scavenging activity and metal ions chelating capacity. The antioxidant capacity of honey has been closely associated with its floral origin, due to differences in the content of bioactive compounds such as polyphenols, carotenoids and enzyme activities (Alvarez-Suarez, Tulipani, Romandini, et al., 2010; Bogdanov, 2017; Bogdanov et al., 2008), which directly influences its biological properties, such as the prevention of diseases related to oxidative stress (Bogdanov et al., 2008). The FRAP values in monofloral *Eucalyptus* honeys from Ecuador were within the ranges previously reported in different monofloral honeys, indicating a similarity in its properties compared with other types of monofloral honey (Alvarez-Suarez, González-Paramás, Santos-Buelga, & Battino, 2010; Estevinho, Pereira, Moreira, Dias, & Pereira, 2008; Gomes et al., 2010; Meda et al., 2005). Moreover, *Eucalyptus* honey was able to react with $\text{O}_2^{\bullet-}$, demonstrating its ability to block the radical, as well as chelate metal ions, thereby exhibiting significant antioxidant mechanisms. The analysis of artificial honey showed that honey sugars have low $\text{O}_2^{\bullet-}$ scavenging activity and chelating metal ions compared to the honey samples ($P < 0.05$), demonstrating their low contribution to the antioxidant capacity of honey. The results were in agreement with those previously reported in monofloral honey from Cuba where the $\text{O}_2^{\bullet-}$ scavenging activity of five monofloral honey samples varied between 3.8 - 5.2 mg/mL (Alvarez-Suarez, Giampieri, Damiani, et al., 2012). On the other hand, the ability of *Eucalyptus* honey to block other radicals, such as $\text{OH}^{\bullet}$ (Ciappini & Stoppani, 2014), has been demonstrated, thus showing its broad antioxidant potential. The radical scavenging activity of honey against $\text{O}_2^{\bullet-}$ could be attributed, in part, to the presence of several bioactive compounds

performing the scavenging activity of free radicals, such as polyphenols and carotenoids (Del Rio et al., 2013).

~~Previous reports confirm that polyphenols contribute significantly to radical scavenging activity and biological properties of honey (Alvarez Suarez, Giampieri, González Paramás, et al., 2012; Gheldof, Wang, & Engeseth, 2002).~~

Microbial contamination

Microbial contamination of the honey samples is presented in **Table 6**. Honey is subject to microbial contamination from various sources, such as nectar, pollen, microbiological flora from the honeybees' digestive tract, environmental factors (dust, air, soil etc), and handling and processing (Gomes et al., 2010). The most commonly occurring microbial contamination involves molds and yeasts, as well the spores of *Bacillus spp.* and *Clostridium spp.* (Snowdon & Cliver, 1996). For this reason, their presence was taken into account when determining the microbiological quality of the honey. In all analyzed honey samples, the level of aerobic mesophiles (molds and yeast) was low. In samples where growth had occurred, the number of cfu/g of honey was significantly lower (< 10 CFU/g of honey). Moreover, for fecal coliforms, sulphite-reducing clostridia and *Salmonella sp.*, all samples tested negative. The results were consistent with those previously published for commercial honey from Portugal, where low or negative growths of these types of microorganisms were reported (Gomes et al., 2010).

Antimicrobial activity

The antimicrobial activity of honey has been considered to be one of its most relevant biological properties (Alvarez-Suarez, Tulipani, Romandini, et al., 2010; Bogdanov et al., 2008). Here, the antimicrobial capacity of *Eucalyptus* honey was determined against four bacterial strains (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Escherichia coli*) and one fungal strain (*Candida albicans*) using the agar incorporation technique.

The results demonstrated that *Eucalyptus* honey was able to inhibit microbial growth (Table 7), with MAD values ranging between 1 - 20%. *S. aureus* proven to be the most sensitive to *Eucalyptus* honey, with a MAD mean value of 6% v/v, followed by *S. pyogenes* (MAD: 9% v/v), *E. coli* (MAD: 11% v/v) and lastly *P. aeruginosa*, with the highest MAD value (14% v/v). Moreover, *Eucalyptus* honey was unable to affect the growth of *C. Albicans*, yielding a high MAD (> 20%), compared to those found in the three bacteria strains, signifying this honey's low effectiveness against this particular pathogen. The results were in accordance with those previously reported by others authors using similar bacteria strains and methods for the determination of the antibacterial activity of honey, who demonstrated that *S. aureus* was the most sensitive bacteria to different types of monofloral honey (Agbaje, Ogunsanya, & Aiwerioba, 2006; Alvarez-Suarez, Tulipani, et al., 2010; Cooper et al., 2002; Mundo, Padilla-Zakour, & Worobo, 2004; Osés et al., 2016). Pathogen susceptibility to honey has also been demonstrated in other studies using honey from different geological regions and of dissimilar floral origins (Basualdo, Sgroy, Finola, & Marioli, 2007; C. I. da Silva, Aazza, Faleiro, Miguel, & Neto, 2016; Estevinho et al., 2008; Mundo et al., 2004; Taormina, Niemira, & Beuchat, 2001). These yielded results similar to those obtained in this study, despite being carried out

under distinct experimental conditions. The antimicrobial activity of honey has previously been related to its osmotic properties, hydrogen peroxide content and low pH, as well as bee defensin-1 peptide (Alvarez-Suarez et al., 2013) and polyphenol content (Daglia, 2012).

Conclusions

The results here exposed constitute the first report on investigation of Ecuadorian *Eucalyptus* honey's floral origin, phytochemical composition, antioxidant capacity, quality and safety parameters, as well as its antimicrobial capacity. These results demonstrate that *Eucalyptus* honey from Ecuador meets the quality parameters established by international regulations and contains **important** bioactive compounds (phenolic, flavonoid and carotenoid), which fall within the range previously reported for different types of honey, and specifically for *Eucalyptus* honey. The results also demonstrate that *Eucalyptus* honey from Ecuador possesses significant biological properties, such as antioxidant and antimicrobial capacity and may therefore be used as a natural source of such compounds displaying these properties.

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Conflict of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript

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