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Apis mellifera vs Melipona beecheii Cuban polifloral honeys: A comparison based on their physicochemical parameters, chemical composition and biological properties

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ABSTRACT

Several polifloral honeys from the *Apis mellifera* and *Melipona beecheii* bee were analyzed to evaluate their pollen profile, physicochemical parameters, chemical composition and their antioxidant and antimicrobial activity. Flavonoids and other phenolic derivatives, 16 compounds in *Apis mellifera* honeys and 19 compounds in *Melipona beecheii* honeys, were identified using the HPLC-DAD-ESI-MS/MS analysis. The physicochemical parameters were within the ranges reported for these honeys, showing some of the distinctive characteristics of *M. beecheii* honeys compared to *A. mellifera* honeys, such as their high moisture and acidity. *M. beecheii* honey showed the highest values of total antioxidant capacity and total phenolic, flavonoid, carotenoids, ascorbic acid, free amino acid and protein contents compared to *A. mellifera* honey. *M. beecheii* honey also exhibited a higher antimicrobial activity. Our result shows that *M. beecheii* honeys is an important source of bioactive compounds with relevant biological properties compared to *A. mellifera* honey.

1. Introduction

Honey has been used by humans both as food and medical product from ancient times to the modern civilization (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010a), where its use in human nutrition and health is due to its chemical composition. Honey composition is varied and is linked to several factors that directly affect its composition and quality such as the bee species, floral origin, environmental and storage conditions (Gheldof, Wang, & Engeseth, 2002). Honey is a rich source of carbohydrates, making it widely used as a natural sweetener, as well as an important source of other minors constituents, which are more related to its biological properties such as polyphenols, carotenoids, minerals, proteins, free amino acid, enzymes and vitamins (Alvarez-Suarez, Giampieri, & Battino, 2013).

The world honey production and consumption is based on the product obtained from the species *Apis mellifera*, whose producers are principally located in Europe and Asia. However, there are other small productions that are based on products obtained from other species of bees, such as the stingless bee, better

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known and mainly used in Australia, Africa and South America. Stingless bee honey production is limited compared with *A. mellifera* honeys, since it does not reach industrial levels, has lower shelf life, lack of quality standards and little knowledge about the product (Vit, Medina, & Enríquez, 2004). In Cuba, the main production of honey is obtained from *A. mellifera* and, similar to the countries of South America, Africa and Australia, the production of honey from the stingless bees is scarce and without any type of industrialization, with *Melipona beecheii* as the principal stingless bees species distributed throughout the country (Fonte, 2007). The principal studies in Cuban honeys have focused mainly on the physicochemical parameters, chemical composition and biological properties of the main monofloral honeys from *A. mellifera* (Alvarez-Suarez, Giampieri, Damiani, Astolfi, Fattorini, Regoli et al., 2012; Alvarez-Suarez, González-Paramás, Santos-Buelga, & Battino, 2010b; Alvarez-Suarez, Tulipani, Díaz, Estevez, Romandini, Giampieri et al., 2010c), while studies on polyfloral honeys have not gone beyond simple quality controls, such as physicochemical parameters. On the other hand, studies on the physicochemical parameters, chemical composition and biological properties in stingless bee honeys are still very few (Fonte et al., 2013).

Therefore, the aim of the present study was to determine and compare the botanical origin, physicochemical parameters, chemical composition and biological properties in several polyfloral honeys from *A. mellifera* and *M. beecheii* from the central region of Cuba. This work constitutes the first report on the chemical composition and biological properties of stingless bees honey from Cuba.

2. Materials and methods

2.1. Honey samples, melissopalynologycal and physiochemical analysis

A total of 16 polifloral honey samples, 8 for each honey type, of two different bee species (*A. mellifera* and *M. beecheii*) were used in the study. Honey samples were provided by beekeepers and collected between April and August 2013 in the municipalities of Sancti Spíritus, Cabaiguán and Fomento, belonging to the province of Sancti Spíritus in the central region of Cuba. The municipalities were selected on the basis of their melliferous and polliniferous potential, as well as their geographic proximity, according to the floral maps designed by the National Center of Apiculture Research of Cuba, which ensures the similarity between plants serving as sources of pollen and nectar to the bees. On the other hand, the sampling period was selected according to the predominance of several blooms, which guarantees the polyfloral character of honeys. The hives sampled belonged to fixed apiaries, located in the same area, at 100 m distance between both bee species. Samples were stored in sterilized containers and maintained at 6–8 °C in the dark until analysis. The polyfloral character of honeys was confirmed according to the melissopalynologycal methods as previously reported (Louveaux, Maurizio, & Vorwohl, 1978), while physicochemical analysis for quality [ashes (%), electrical conductivity (mS/cm), color (mm Pfund), pH, free acidity (mequiv/kg), humidity (%)], diastases index (U Schade) and HMF test (mg/kg)] were verified by the official methods (AOAC, 1990; IHC, 2002).

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) (Cooper, Molan, & Harding, 2002) was included in the study to evaluate the contribution of the predominant sugars to the assayed activities.

2.2. Determination of total phenolic content (TPC), total flavonoid content (TFC), total carotenoids content (TCC), free amino acids and protein content

For TPC analysis honey samples (1 g) were diluted to 10 mL with distilled water, filtered through Minisart filter of 45 μm (PBI International) and analyzed using a Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). TPC results were expressed as mg of gallic acid equivalents (GAE) per 100 g of honey (mg GAE/100 g of honey). For TFC determination the samples were diluted in methanol (50% w/v), analyzed using the aluminum trichloride method (Chang, Yang, Wen, & Chern, 2002) and results were expressed as mg of (+)-catechin equivalents per 100 g of honey (mg CE/100 g of honey). TCC was determined spectrophotometrically as previously reported (Alvarez-Suarez et al., 2010c). The samples (1 g) were vigorously shaken (500 r m^{-1}) with 10 mL of *n*-hexane–acetone mixture (6:4) for 10 min at room temperature and filtered through Whatman No. 4 filter paper. The absorbance was measured spectrophotometrically at 450 nm and results were expressed as mg of β-carotene equivalents per kilogram of honey (mg βcarotE/kg of honey).

Total free amino acids content was determined spectrophotometrically using the Cd-ninhydrin method (Doi, Shibata, & Matoba, 1981) using L-Leucin as standard and results were expressed as mg of L-leucine equivalents (mg LE/100 g of honey), while the protein content in honey was determined as previously reported (Alvarez-Suarez et al., 2010c). Bovine serum albumin (BSA) was used for the calibration curve and results were expressed as mg of bovine serum albumin equivalents (mg BSAE/100 g of honey).

2.3. HPLC-DAD-ESI-MS/MS analysis of honey flavonols and other phenolic derivatives

Honey samples were fractionated into Sep-Pak C18 Plus Short SPE Cartridge (Waters S.p.A., Milan, Italy) as previously reported (Truchado, Ferreres, Bortolotti, Sabatini, & Tomás-Barberán, 2008) and phenolic eluent was analyzed using an HPLC-DAD-ESI-MS system. An Agilent Poroshell 120 EC-C18 column (2.7 μm, 150 mm × 4.6 mm) thermostated at 35 °C was used. The solvents were: (A) 0.1% formic acid, and (B) acetonitrile. The elution gradient established was isocratic 15% B for 5 min, 15–20% B over 5 min, 20–35% B over 10 min, 35–50% B over 10 min, 50–60% B over 5 min, isocratic 60% B for 5 min and re-equilibration of the column to initial solvent conditions. The flow rate was 0.5 mL/min. Double online detection was carried out in the DAD at 280, 330 and 370 nm as preferred wavelengths and in the MS operated in the negative ion mode. Spectra were recorded between m/z 100 and m/z 1500. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 °C) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupols were set at unit resolution and EMS and EPI analyses were also performed. The EMS parameters were: ion spray voltage 4500 V, DP -50 V, EP -6 V, CE -10 V and cell exit potential (CXP) −3 V, whereas EPI settings were: DP -50 V, EP -6 V, CE -25 V and CES 0 V.

2.4. HPLC analysis of vitamin C

Vitamin C content in honey samples was determined as previously described by our group (Alvarez-Suarez et al., 2010b) using a reversed-phase HPLC system. Honey samples (5 g) were diluted in 10 mL of a dithiothreitol solution (4.2 mM in 0.1 M K_2HPO_4, pH 7.0) mixing thoroughly and filtered through a Minisart filter of 45 μm. The filtrate (1 mL) was mixed with a 4.5% *m*-phosphoric acid solution (1 mL) and 20 μL of this solution was injected onto the HPLC system. The HPLC system (Shimadzu Corporation, Kyoto, Japan)

consisted of a Waters 600 controller, a Waters 996 photodiode array detector (PDA) set at wavelength of 262 and 244 nm, and a column incubator at 30 °C. The stationary phase used was a YMC Pack Pro column (150 × 4.6 mm) and the mobile phase was a solution of 50 mM potassium dihydrogen phosphate (pH 4.5) (solvent A) and methanol (solvent B) starting at 100% A and decreasing to 70% A in 10 min at 0.8 mL/min of flow rate. Ascorbic acid was used as standard and the results were expressed as mg vit. C/100 g of honey.

2.5. RP-HPLC analysis of folic acid

Folic acid content was determined following the previously described methodology for the determination of water-soluble vitamins in honey (Ciulu, Solinas, Floris, Panzanelli, Pilo, Piu, et al., 2011). Honey samples (10 g) were dissolved in 10 mL of ultrapure water and then 12.5 mL of phosphate buffer (1 M, pH 5.5) and 1 mL of NaOH 2M were added. The total volume of each sample was topped up to 25 mL, filtered through a PVDF 0.45 μm filter and injected into an RP-HPLC system. The RP-HPLC system consisted of a Jasco PU-2089 Plus binary pump, a sampling valve, a 20 μL sample loop and a Jasco UV-2070 Plus UV–Vis detector. Separation was carried out on a Mediterranea Sea C18 column 250 mm × 4.6 mm, 5 μm particle size and data were elaborated using ChromNAV Software. Mobile phase consisted in a trifluoroacetic acid aqueous solution (0.025% v/v) (Solvent A) and acetonitrile (100%) (Solvent B) starting at 100% A and decreasing to 75% A in 11 min, 55% at 19 min, and then increasing up to 60% at 20 min and finally 100% at 22 min. The flow rate was 1.0 mL/min, and the detector was set at 210 nm. Vitamin B9 (folic acid) was used as standard and results were expressed as mg folic acid/kg of honey.

2.6. Quantification of the total antioxidant capacity (TAC)

The ferric reducing antioxidant power (FRAP) assays (Benzie & Strain, 1996) and DPPH assay as previously reported by our group (Alvarez-Suarez et al., 2012) were used in parallel to determine TAC of the honey samples. For DPPH assay, Trolox was used for the standard curve preparation and results were expressed as μmoles of Trolox equivalents per 100 g of honey (μmol TE/100 g of honey), while for FRAP assay both Trolox and ferrous sulfate were used as standards curve preparation and results were expressed as μmoles of Trolox equivalents per 100 g of honey (μmol TE/100 g of honey) and μmoles of Fe(II) per 100 g of honey (μmol Fe(II)/100 g of honey).

2.7. Determination of honey minimum active dilution

A collection of 26 clinical isolates was recovered from human specimens in laboratories of Central Italy between April 2013 and September 2015. The isolates belonged to 14 Gram-positive bacterial species (*S. aureus, S. epidermidis, S. pneumoniae, S. pyogenes, S. agalactiae, S. mitis, S. oralis, S. anginosus, S. parasanguinis, S. salivarius, S. gordonii, Enterococcus faecalis, Enterococcus faecium*, and *Listeria monocytogenes*); 9 Gram-negative bacterial species (*Enterobacter cloacae, Citrobacter freundii, Salmonella fyris, Serratia marcescens, Acinetobacter baumanii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli*, and *Proteus mirabilis*); and one fungal species (*Candida albicans*).

The minimum active dilution (MAD) of each honey for each clinical isolate was determined by an agar incorporation technique (French, Cooper, & Molan, 2005), with the exception of Mueller-Hinton (MH) agar (Oxoid, Basingstoke, England), supplemented with 5% sheep blood for streptococci, instead of nutrient agar. A dilution series with final honey dilutions in the range 1%–20% (v/v), in 1% increments, was used for susceptibility assays. Duplicate control plates of MH agar with no honey were included to confirm the viability of the cultures. Each culture was inoculated onto the agar plates using an auto-pipettor. The inoculated plates were incubated at 37 °C overnight, and then 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.

2.8. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows version 20.0. One-way ANOVA was used to determine significant differences among honey samples with a Bonferroni correction for multiple sample comparison. In all cases a *p*-value < 0.05 was considered statistically significant. The samples were analyzed in triplicate and results are expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Melissopalynologycal and physiochemical analysis

The study on pollen content in honeys allows to determine their floral origin, classifying them as monoflorales or polyfloral. Table 1 shows the most predominant pollen grain classes identified in samples according to the bee species. Although the presence of pollen grains in the honeys did not reach the recommended percentage to classify these as monoflorales (Louveaux et al., 1978), it was possible to identify a high frequency of pollen of some plant species in the honeys of both bee species, which showed, in some cases, similarity in the type of the melliferous flora visited. According to the pollen grain frequency (Table 1), all the plant species visited by *M. beecheii* were also visited by *A. mellifera*, while the plants *Acacia farnesiana*, *Helianthus annus* and *Ceiba pendrata* were only visited by *A. mellifera*. The species *Mimosa pudica* and *Mimosa pigra* were found to be the most frequent species identified in the honey samples of both bee species, while the species *Helianthus annus* was identified, very frequently, only in *A. mellifera* honeys, results that are in agreement with those previously reported in studies from Cuban honeys from both bee species (Leal-Ramos & León-Sánchez, 2013). According to the authors, these results cannot be attributed to the fact that *M. beecheii* does not use these

Table 1
Frequency of pollen grains classes in polyfloral honeys according to the bee species.

Bee species	Frequency class[a]	Pollen identification (botanical genus)
Apis mellifera	P	*Mimosa pudica*
	P	*Mimosa pigra*
	P	*Manguifera indica*
	P	*Acacia farnesiana*
	P	*Helianthus annus*
	P	*Persea americana*
	S	*Ceiba pendrata*
	S	*Psidium guajava* L
	I	*Bidens pilosa*
Melipona beecheii	P	*Mimosa pudica*
	P	*Mimosa pigra*
	P	*Bidens pilosa*
	P	*Psidium guajava* L
	S	*Manguifera indica*
	I	*Persea americana*

[a] Frequency of pollen grains: P – predominant pollen (Most frequent pollen grains found in polyfloral honey samples); S- secondary pollen (Second most frequent pollen grains in polyfloral honey samples); I – important minor pollen (Low frequency pollen grains in polyfloral honey samples).

species, but they could be due to other reasons, such as the short flight rate of these bees or the difficulty of access to flowers used by *A. mellifera*. On the other hand, other authors have reported that in presence of *A. mellifera*, the stingless bee moves to other floral resources even though the floral resources in the same area do not diminish/are sufficient (Cairns, Villanueva-Gutiérrez, Koptur, & Bray, 2005).

Table 2 shows the results of the physicochemical analysis in honey samples from both bee species. On a color basis, honeys were classified as extra light amber. It is known that the floral origin of honey largely determines its physical and chemical properties, as also the color, so the association of color with the monofloral character of honey has allowed, in many cases, to establish a range of colors for honey with a specific floral origin (Alvarez-Suarez et al., 2010c). In the case of polyfloral honey this relationship becomes more variable, since there is no a predominance of a specific floral type, which makes it impossible to establish a relationship of this kind. In polyfloral Cuban honeys the reports about color do not go beyond quality reports, while monofloral honeys have been studied more, mainly due to their economic importance. In monofloral Cuban honeys, the color values have been reported from 12.2 mm Pfund (Extra White) in *Turbina corymbosa* (L.) honeys to 88.9 mm Pfund (Amber) in *Govania polygama* honeys (Alvarez-Suarez et al., 2010c), while in *M. beecheii* honeys values have been reported that classified this honey as extra light amber (Fonte et al., 2013), which are in agreement with our results.

Since moisture affects honey quality and is closely related to the environmental condition, to manipulation during the harvest period and to storage, the moisture content in both honey types was analyzed, showing significant differences between both honeys ($P < 0.05$) (Table 2). *A. mellifera* showed a moisture content of 16.75%, which is within the international parameters recommended for this honey (<20%) (EU Council, 2002), while the mean of moisture content in *M. beecheii* was significantly higher (28.62%, $P < 0.05$) compared to *A. mellifera* honey values. The quality criteria for *A. mellifera* honeys are well defined in the international legislation (EU Council, 2002), while at present there are no international standards to regulate the quality parameters of stingless bee honeys; there is only one proposal for a standard that could be used as a reference (Vit et al., 2004). Therefore, if the results obtained in *M. beecheii* honeys are compared with the proposed norm, the moisture content for this honey is within the proposed range (<30%), being in the same range of values previously reported for *M. beecheii* honeys from Cuba (Fonte et al., 2013), as well as for other stingless bee honeys from other geographical and floral origins (Chuttong, Chanbang, Sringarm, & Burgett, 2016).

Although it is known that honey is characterized by an acidic character, at present the pH values has not been legislated, hence the importance to compare the results with those previously reported by other authors. The pH values in the analyzed honeys differed significantly ($P < 0.05$) between both species, with the most acidic values present in *M. beecheii* honeys (Table 2). The pH values were within the range previously reported for *A. mellifera* (Alvarez-Suarez et al., 2010c) and *M. beecheii* honeys from Cuba (Fonte et al., 2013), as well as from other regions, both for *A. mellifera* (Acquarone, Buera, & Elizalde, 2007), and for stingless bee honeys (Chuttong et al., 2016; Guerrini, Bruni, Maietti, Poli, Rossi, Paganetto et al., 2009). Together with pH, free acidity is another parameter that plays an important role in honey quality and freshness. Although the acidic character is related to honey antimicrobial properties (Alvarez-Suarez et al., 2010a), high levels of acidity can be an indicator of sugar fermentation processes, thus affecting the organoleptic characteristics and quality of honey. In *A. mellifera* honeys free acidity was within the recommended limit (<50 meq/Kg) (EU Council, 2002), as well as for *M. beecheii* honeys, that were within the range previously reported in stingless bee honeys (Chuttong et al., 2016).

HMF and diastase represents two important indicators of the freshness of honey. Under normal conditions HMF is absent, while levels may increase during processing or aging, mainly influenced by temperature abuse, pH, storage condition and floral origin (Fallico, Arena, Verzera, & Zappalà, 2006). In both honeys, HMF levels were within the accepted range by *A. mellifera* (<80 mg/kg) (EU Council, 2002), as well as those previously reported in honeys

Table 2
Physicochemical parameters, bioactive compound and total antioxidant capacity in polyfloral honeys from *A. mellifera* and *M. beecheii* bee from Cuba.

Parameters	Values	
	A. mellifera honey	*M. beecheii* honey
Physicochemical parameters		
Color (mm Pfund)	37.35 ± 6.52[a]	41.65 ± 7.68[a]
Moisture (%)	16.74 ± 0.38[a]	28.62 ± 5.61[b]
pH	4.76 ± 0.41[a]	3.2 ± 0.21[b]
Free acidity (meq/Kg of honey)	32.65 ± 4.85[a]	41.52 ± 8.19[b]
HMF (mg/kg of honey)	16.54 ± 3.12[a]	9.23 ± 1.32[b]
Diastase index (°Gothe)	13.75 ± 1.52[a]	1.3 ± 0.12[b]
Electrical conductivity (mS/cm)	0.33 ± 0.02[a]	0.58 ± 0.14[b]
Ashes (%)	0.18 ± 0.04[a]	0.46 ± 0.03[b]
Bioactive compounds		
Total phenolic content (TPC) (mg GAE/100 g of honey)	54.30 ± 7.19[a]	94.39 ± 14.55[b]
Total flavonoid content (TFC) (mg CE/100 g of honey)	2.68 ± 0.38[a]	4.19 ± 0.37[b]
Total carotenoids content (TCC) (mg βcarotE/kg of honey)	4.78 ± 0.34[a]	6.24 ± 0.29[b]
Vitamin C content (vit. C) (mg/100 g of honey)	4.55 ± 0.87[a]	8.84 ± 0.84[b]
Total free amino acids content (mg LE/100 g of honey)	99.15 ± 12.04[a]	119.69 ± 13.95[b]
Total protein content (mg BSAE/g of honey)	1.81 ± 0.22[a]	2.71 ± 0.26[b]
Folic acid content (μg folic acid/100 g of honey)	8.34 ± 0.15[a]	7.37 ± 1.19[a]
Total antioxidant capacity (TAC)		
FRAP (μmol TE/100 g of honey)	159.70 ± 17.28[a]	175.82 ± 10.83[b]
FRAP (μmol Fe(II)/100 g of honey)	21.59 ± 5.57[a]	38.54 ± 11.37[b]
DPPH (μmol TE/100 g of honey)	31.06 ± 2.19[a]	42.23 ± 1.66[b]

Sample was analyzed in triplicate and data are presented as means ± standard deviation. Mean values within a row sharing the same letter are not significantly different for $P < 0.05$.

from stingless bees (Chuttong et al., 2016). Our results are in agreement with those previously reported where HMF content in *A. mellifera* honeys was significantly higher than those reported in stingless bees (Chuttong et al., 2016). Moreover, the diastase index in *A. mellifera* honeys was within the ranges recommended for this honey type (>8 Schade units) (EU Council, 2002). As mentioned above, at present there are no international standards to regulate the quality parameters of stingless bee honeys, so the only reference is the standard proposed by Vit et al. (2004) and the comparison with the results reported by other authors. In this sense, diastase index in *M. beecheii* honeys was significantly lower compared to *A. mellifera* honeys ($P < 0.05$), however the values were within the ranges proposed by Vit et al. (2004) and in agreement with previously reported values (Chuttong et al., 2016).

Another important parameter to consider in honey is the total mineral content, which is generally low and closely related to their floral origin and the characteristic of the soil where the plants are localized (Andrade et al., 1999), which presupposes a wide variability in their ash content. However, current legislation has set the maximum ash value in *A. mellifera* honey at not more than 0.6% (EU Council, 2002), which suggests that honeys within these values have a nectar origin (Andrade et al., 1999). In our study, both honey types were between the suggested limits, with the ash content in *A. mellifera* honeys significantly lower ($P < 0.05$) than those found in *M. beecheii* honeys. Mineral content in honey has also been directly related to the electrical conductivity (Acquarone et al., 2007), which can be used as a criterion to confirm the floral origin of honey. Electrical conductivity in the analyzed honeys showed significant differences ($P < 0.05$) between them, with the highest values in *M. beecheii* honeys. However, both electrical conductivity values were within the established ranges for this parameter (<0.8 mS/cm) (EU Council, 2002), suggesting that both honeys have a nectar origin, which can also be corroborated when analyzing this parameter with total ash content.

3.2. HPLC-DAD-ESI/MS analysis of flavonoids and other phenolic derivatives

Fig. 1 shows the representative HPLC chromatograms of both honeys, while UV spectra, mass characteristics, and tentative identification of the peaks are indicated in Table 3. The compounds were identified on the basis of their UV and mass spectra obtained by HPLC-DAD-ESI/MS in negative ion mode, as well as their chromatographic behavior. Up to 16 compounds were identified in *A. mellifera* honeys, while 19 compounds were identified in *M. beecheii* honeys using the HPLC-DAD-ESI-MS/MS analysis. *M. beecheii* honeys were characterized by the presence of *C*-pentosyl-*C*-hexosyl-apigenin (peak 3), in accordance with previous reports of C-glycosides and *O*-glycosides flavonoids in stingless bee honeys from Venezuela (Truchado, Vit, Ferreres, & Tomas-Barberan, 2011), while the *C*-pentosyl-*C*-hexosyl-apigenin isomer (peaks 5, 6, and 7) was identified in both honey types. Hydroxycinnamic acids were also identified in the samples with caffeic and ferulic acid present only in *A. mellifera* honeys, while coumaric acid was identified in both honey types. These compounds have been previously reported in *A. mellifera* honeys from different geographical and botanical origins (Gheldof et al., 2002; Truchado et al., 2008), as well as in several monofloral Cuban honeys (Alvarez-Suarez et al., 2010b).

In both honey types, three peaks (15, 17 and 20) were assigned to flavonol aglycones (quercetin, isorhamnetin and kaempferol, respectively). These compounds have been previously reported in monofloral Cuban honeys from *A. mellifera* (Alvarez-Suarez et al., 2010b), as well as in honeys from different botanical origins (Gheldof et al., 2002; Truchado et al., 2009; Truchado et al., 2008), whereas in the case of stingless bees honeys reports are scarce (Guerrini et al., 2009). Moreover, two peaks (peak 16 and 19) were assigned to flavonol aglycones (luteolin and apigenin, respectively) in both honey types. Luteolin and apigenin have been previously reported in honeys from *A. mellifera* (Kečkeš et al., 2013), while luteolin has been reported in Ecuadorian stingless bees honeys (Guerrini et al., 2009). The glycosylated forms of quercetin (peak 8), apigenin (peak 10), kaempferol (peak 11) and isorhamnetin (peak 12) were found in both honey types, while the methylated form of luteolin was found in *M. beecheii* honey, and methyl quercetin was identified in both honey types.

3.3. Chemical composition and total antioxidant capacity (TAC)

Chemical composition and TAC of honey samples are shown in Table 2. Similar to physicochemical parameters, the chemical composition of honey is determined by several factors such as bee species, the floral and geographic origin (Alvarez-Suarez et al., 2010a). A significant difference ($P < 0.05$) was found between both honey types in relation to their TPC, TFC, TCC, vit. C content, as well as total protein and free amino acid content, where *M. beecheii* honeys had the highest values. The range of values for the chemical compounds here reported were in agreement with those previously found in Cuban honeys from *A. mellifera* (Alvarez-Suarez et al., 2010b,c, 2012), as well as in honeys from other geographical origins (Bentabol-Manzanares, Hernández-García, Rodríguez-Galdón, Rodríguez-Rodríguez, & Díaz-Romero, 2014; Bueno-Costa et al., 2016; Fyfe, Okoro, Paterson, Coyle, & McDougall, 2017). A similar behavior was also observed in values obtained in *M. beecheii* honeys, which were within the ranges previously reported by other authors in different stingless bee species from different geographical areas (da Silva, da Silva, Camara, Queiroz, Magnani, de Novais et al., 2013; Guerrini et al., 2009).

The presence of vitamins in honey has been studied in the past, mainly by biological assay methods, but at present studies of these compounds in honey are still insufficient (Ciulu et al., 2011). The folic acid content in both honey types was determined by an RP-HPLC method adapted for studies of water-soluble vitamins in honey (Ciulu et al., 2011). No significant differences were found in folic acid content in both honeys, however, the results presented here are encouraging, since they demonstrate that honeys from *A. mellifera* and *M. beecheii* may be a natural source of folic acid (Table 2). To the best of our knowledge, the results here exposed represent the first reports of an extensive study of this compound in honey, so it is difficult to compare our results with others. However, these results were within the concentration range previously reported in *A. mellifera* honeys from different floral origins by the authors who proposed the method used in our study (Ciulu et al., 2011).

TAC was also studied in both honey types (Table 2). The TAC of *M. beecheii* and *A. mellifera* honeys was well evidenced by their ability to reduce Fe^{3+} to Fe^{2+}, as well as their capacity to scavenge $DPPH^{\bullet}$. In both analyzes *M. beecheii* honeys showed a higher TAC than *A. mellifera* honeys ($P < 0.05$) being in agreement with the results previously reported by other authors (Bueno-Costa et al., 2016; da Silva et al., 2013; Alvarez-Suarez et al., 2010c; Bastos, dos Santos, Mendonça, & Torres, 2009).

3.4. A. mellifera and M. beecheii honey antimicrobial activity

Among the beneficial effects of honey on human health, antimicrobial activity, along with its wound healing capacity, is considered one of the most relevant (Alvarez-Suarez et al., 2010a). Several authors have reported on the antimicrobial activity of honey; however, most of these studies used honey produced by

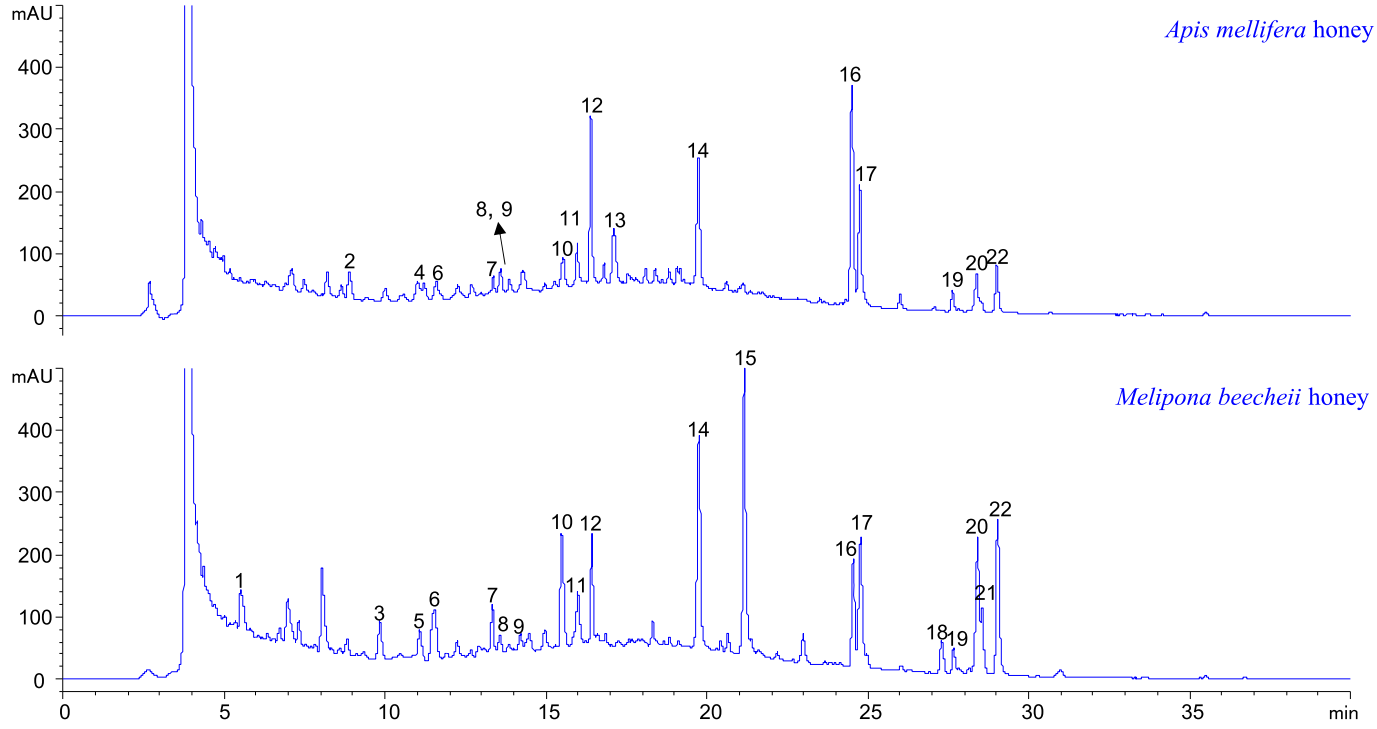

Fig. 1. HPLC-DAD chromatograms of flavonoids and other phenolic derivatives in both honey types (*A. mellifera* and *M. beecheii*) analyzed by HPLC-DAD-ESI/MS.

Table 3
t_R, UV, MS spectra (MS $[M - H]^-$; MS^2 $[M - H]^-$) and tentative identification of flavonoids and other phenolic derivatives in polyfloral honeys from *A. mellifera* and *M. beecheii*.

Peak	Honey type	Tentative identification	R_t (min)	UV–Vis	Ms $[M-H]^-$ m/z	MS^2 $[M-H]^-$ m/z
1	*M. beecheii*	Dihydrocaffeic acid	5.5	320	181	163,135
2	*A. mellifera*	Caffeic acid	8.9	293sh,393	179	135
3	*M. beecheii*	*C*-pentosyl-*C*-hexosyl-apigenin	9.8	–	563	443
4	*A. mellifera*	Ferulic acid	11.0	296,322	193	–
5	*M. beecheii*	*C*-pentosyl-*C*-hexosyl-apigenin isomer	11.0	272,336	563	443
6	*M. beecheii/A. mellifera*	*C*-pentosyl-*C*-hexosyl-apigenin isomer	11.5	270,332	563	–
7	*M. beecheii/A. mellifera*	*C*-pentosyl-*C*-hexosyl-apigenin isomer	13.3	272,336	563	473,443, 383,353
8	*M. beecheii/A. mellifera*	Quercetin deoxyhexosyl hexoside	13.6	255,267sh, 303sh,354	609	301
9	*M. beecheii/A. mellifera*	Coumaric acid	14.2	310	163	119
10	*M. beecheii/A. mellifera*	Apigenin trihexoside	15.5	266,298sh, 334	755	–
11	*M. beecheii/A. mellifera*	Kaempferol deoxyhexosyl hexoside	16.0	263,304sh, 352	593	–
12	*M. beecheii/A. mellifera*	Isorhamnetin deoxyhexosyl hexoside	16.4	268,300sh, 354	623	–
13	*A. mellifera*	Pinobanksin methylether	17.1	287,333sh	285	271,179
14	*M. beecheii/A. mellifera*	Unknown	19.7	260,352, 384sh	241	–
15	*M. beecheii*	Quercetin	21.2	350	301	–
16	*M. beecheii/A. mellifera*	Luteolin	24.5	268,286sh, 348	285	213,133
17	*M. beecheii/A. mellifera*	Isorhamnetin	24.8	255,268sh, 371	315	301
18	*M. beecheii*	Bis-methylated quercetin	27.3	–	329	301
19	*M. beecheii/A. mellifera*	Apigenin	27.6	336	269	183,149, 117
20	*M. beecheii/A. mellifera*	Kaempferol	28.4	265,326sh, 365	285	135,151, 161
21	*M. beecheii*	Methyl luteolin	28.5	280,346	299	285,227, 133
22	*M. beecheii/A. mellifera*	Methyl quercetin	29.0	370	315	301

A. mellifera, while only a few papers reported the activity of stingless bees honey (Mercês, Peralta, Uetanabaro, & Lucchese, 2013; Nishio et al., 2016). Thereby, the antimicrobial activity of Cuban honey from *M. Beecheii* against 26 clinical isolates from human

specimens was investigated.

The agar incorporation technique demonstrated that both honeys tested were able, with different efficiency, to inhibit microbial growth (Table 4), where *M. beecheii* honey exhibited a greater antimicrobial activity (MAD range, 1–10%) compared to *A. mellifera* honey (MAD range, 11–20%). *M. beecheii* honey showed a moderate antibacterial activity against streptococci (MAD range, 4–10%) compared to other bacterial genera both Gram-positive (MAD range, 1–3%), Gram-negative (MAD range, 2–3%), and *C. albicans* (MAD, 3%). Its reduced activity against streptococci was particularly evident in *S. agalactiae* species (MAD, 10%). This honey, however, was able to successfully inhibit the growth of *C. albicans* (MAD, 3%), unlike *A. mellifera* honey which showed a reduced activity (MAD, $\geq$ 20%). Notably, a 2% dilution of *M. beecheii* honey was also able to prevent *P. mirabilis* swarming; conversely, using *A. mellifera* honey, the swarming was inhibited by a 9% dilution.

The antimicrobial activity of five monofloral Cuban honeys from *A. mellifera* against two Gram-positive and two Gram-negative bacteria was previously reported by our group (Alvarez-Suarez et al., 2010c), while the antimicrobial activity of *M. beecheii* honey from Cuba has not yet been reported. The results showed that *M. beecheii* honey is more active than the five monofloral Cuban honeys previously tested against *S. aureus*, *E. coli* and *P. aeruginosa*. Only Linen vine honey showed an antimicrobial activity comparable to *M. beecheii* honey on *S. aureus* (Alvarez-Suarez et al., 2010c). The antimicrobial capacity of *M. beecheii* honey was generally higher than *A. mellifera* honey like those previously reported by other authors for the same bacteria types (Cooper et al., 2002). Our results clearly suggest that *M. beecheii* honey shows a more efficient antimicrobial capacity compared to *A. mellifera*, results that are in agreement with those previously reported in *M. beecheii* honeys from Mexico (Chan-Rodríguez et al., 2012). Although the antimicrobial activity of honey is related to its osmotic properties and its content of hydrogen peroxide (Alvarez-Suarez et al., 2010a), the results here showed it may be also related to the highest content in polyphenols (Daglia, 2012) and the lower pH shown in *M. beecheii* honey compared with *A. mellifera*. Finally, as far as we know, this is the first time that a Cuban honey proves to be active against yeasts and *P. mirabilis* swarming.

Table 4
MADs of *A. mellifera* and *M. beecheii* polyfloral honeys against 26 microorganisms.

Strain	*A. mellifera* honey	*M. beecheii* honey
S. aureus 13	15%	2%
S. epidermidis 35	14%	1%
S. pneumoniae 9	11%	4%
S. pyogenes 12	14%	7%
S. pyogenes C-105	14%	7%
S. pyogenes m46	14%	7%
S. agalactiae 1357	19%	10%
S. mitis 22	14%	7%
S. oralis 1235	11%	6%
S. anginosus 2513	14%	8%
S. parasanguinis 2761	15%	8%
S. salivarius 14	14%	7%
S. gordonii 143	11%	6%
E. faecalis 212	>20%	3%
E. faecium 17	19%	2%
L. monocytogenes 49	17%	3%
E. cloacae 19902	15%	2%
C. freundii 55	14%	2%
S. fyris 3813	20%	3%
S. marcescens 28315	16%	2%
A. baumanii 8	19%	3%
K. pneumoniae 15	17%	2%
P. aeruginosa 24	12%	3%
E. coli 23	13%	3%
P. mirabilis 112	13%	2%
	9%[a]	2%[a]
C. albicans 18	>20%	3%

[a] The value indicates the honey dilution able to prevent *P. mirabilis* swarming.

4. Conclusions

To the best of our knowledge, this is the first report on chemical composition and biological properties of the polyfloral honey from two bee species from Cuba. From the study of the pollen content it was evident that both types of bees visit certain species of plants, while other types of flowers attract a specific bee species. Both honey types proved to be important sources of bioactive compounds, where *M. beecheii* honeys showed the highest values in the concentrations of these compounds. *M. beecheii* honey also demonstrated a greater ability to inhibit bacterial and yeast growth compared to *A. mellifera* honey. Our data are particular interesting in defining the effect of bee species in the composition and biological properties of honey. The results here reported allows also to propose *M. beecheii* honey as an alternative in the topical treatment of bacterial infections, especially due to yeasts like *C. albicans*, to avoid the indiscriminate use of antibiotics and the phenomena of antimicrobial resistance currently reported.

Conflict of interest

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

References

Acquarone, C., Buera, P., & Elizalde, B. (2007). Pattern of pH and electrical conductivity upon honey dilution as a complementary tool for discriminating geographical origin of honeys. *Food Chemistry, 101*(2), 695–703.

Alvarez-Suarez, J. M., Giampieri, F., & Battino, M. (2013). Honey as a source of dietary antioxidants: Structures, bioavailability and evidence of protective effects against human chronic diseases. *Current Medicinal Chemistry, 20*(5), 621–638.

Alvarez-Suarez, J. M., Giampieri, F., Damiani, E., Astolfi, P., Fattorini, D., Regoli, F., et al. (2012). Radical-scavenging activity, protective effect against lipid peroxidation and mineral contents of monofloral cuban honeys. *Plant Foods for Human Nutrition, 67*(1), 31–38.

Alvarez-Suarez, J. M., González- Paramás, A. M., Santos-Buelga, C., & Battino, M. (2010b). Antioxidant characterization of native monofloral cuban honeys. *Journal of Agricultural and Food Chemistry, 58*(17), 9817–9824.

Alvarez-Suarez, J. M., Tulipani, S., Díaz, D., Estevez, Y., Romandini, S., Giampieri, F., et al. (2010c). Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food and Chemical Toxicology, 48*(8–9), 2490–2499.

Alvarez-Suarez, J. M., Tulipani, S., Romandini, S., Bertoli, E., & Battino, M. (2010a). Contribution of honey in nutrition and human health: A review. *Mediterranean Journal of Nutrition and Metabolism, 3*(1), 15–23.

Andrade, P. B., Amaral, M. T., Isabel, P., Carvalho, J. C. M. F., Seabra, R. M., & Proença da Cunha, A. (1999). Physicochemical attributes and pollen spectrum of Portuguese heather honeys. *Food Chemistry, 66*(4), 503–510.

AOAC. (1990). In K. Helrich (Ed.), *Official methods of analysis of the association of official analytical chemists* (15th ed.). Arlington: VA..

Bastos, D. H. M., dos Santos, M. C. M., Mendonça, S., & Torres, E. A. F. S. (2009). Antioxidant capacity and phenolic content of stingless bee honey from amazon in comparison to apis bee honey. *Acta Horticulturare, 841*, 483–486.

Bentabol-Manzanares, A., Hernández-García, Z., Rodríguez-Galdón, B., Rodríguez-Rodríguez, E., & Díaz-Romero, C. (2014). Physicochemical characteristics of minor monofloral honeys from Tenerife, Spain. *LWT - Food Science and Technology, 55*(2), 572–578.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant Power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76.

Bueno-Costa, F. M., Zambiazi, R. C., Bohmer, B. W., Chaves, F. C., Silva, W. P. d., Zanusso, J. T., et al. (2016). Antibacterial and antioxidant activity of honeys from the state of Rio Grande do Sul, Brazil. *LWT - Food Science and Technology, 65*, 333–340.

Cairns, C. E., Villanueva-Gutiérrez, R., Koptur, S., & Bray, D. B. (2005). Bee populations, forest disturbance, and africanization in Mexico. *Biotropica, 37*(4), 686–692.

Chan-Rodríguez, D., Ramón-Sierra, J., Lope-Ayora, J., Sauri-Duch, E., Cuevas-Glory, L., & Ortiz-Vázquez, E. (2012). Antibacterial properties of honey produced by *Melipona beecheii* and *Apis Mellifera* against foodborn microorganisms. *Food*

Science and Biotechnology, 21(3), 905–909.
Chang, C.-C., Yang, M.-H., Wen, H.-M., & Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis, 10*(3), 178–182.
Chuttong, B., Chanbang, Y., Sringarm, K., & Burgett, M. (2016). Physicochemical profiles of stingless bee (Apidae: Meliponini) honey from South east Asia (Thailand). *Food Chemistry, 192*, 149–155.
Ciulu, M., Solinas, S., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., et al. (2011). RP-HPLC determination of water-soluble vitamins in honey. *Talanta, 83*(3), 924–929.
Cooper, R. A., Molan, P. C., & Harding, K. G. (2002). The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. *Journal of Applied Microbiology, 93*(5), 857–863.
Council, E. U. (2002). Council directive 2001/11 O/EC of 20 December 2001 relating to honey. *Official Journal of European Communities, L10*, 47–52.
Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology, 23*(2), 174–181.
Doi, E., Shibata, D., & Matoba, T. (1981). Modified colorimetric ninhydrin methods for peptidase assay. *Analytical Biochemistry, 118*(1), 173–184.
Fallico, B., Arena, E., Verzera, A., & Zappalà, M. (2006). The European Food Legislation and its impact on honey sector. *Accreditation and Quality Assurance, 11*(1), 49–54.
Fonte, L. (2007). *The "bees of the earth" in areas of the western provinces of Cuba: Hives, their honey and the "meliponiculture"*. Cuba: Universidad Agraria de La Habana.
Fonte, L., Díaz, M., Machado, R., Blanco, D., Demedio, J., & García, A. (2013). Physical-chemical and organoleptic characterization of *Melipona beecheii* honey obtained from different agroforestry systems. *Pastos Y Forrajes, 36*, 345–349.
French, V. M., Cooper, R. A., & Molan, P. C. (2005). The antibacterial activity of honey against coagulase-negative staphylococci. *Journal of Antimicrobial Chemotherapy, 56*(1), 228–231.
Fyfe, L., Okoro, P., Paterson, E., Coyle, S., & McDougall, G. J. (2017). Compositional analysis of Scottish honeys with antimicrobial activity against antibiotic-resistant bacteria reveals novel antimicrobial components. *LWT - Food Science and Technology, 79*, 52–59.
Gheldof, N., Wang, X.-H., & Engeseth, N. J. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. *Journal of Agricultural and Food Chemistry, 50*(21), 5870–5877.
Guerrini, A., Bruni, R., Maietti, S., Poli, F., Rossi, D., Paganetto, G., et al. (2009). Ecuadorian stingless bee (meliponinae) honey: A chemical and functional profile of an ancient health product. *Food Chemistry, 114*(4), 1413–1420.
IHC. (2002). *Harmonised methods of the international honey commission. FAM, Liebefeld, CH-3003 bern*. Switzerland: Swiss Bee Research Centre.
Kečkeš, S., Gašić, U., Veličković, T.Ć., Milojković-Opsenica, D., Natić, M., & Tešić, Ž. (2013). The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry. *Food Chemistry, 138*(1), 32–40.
Leal-Ramos, A., & León-Sánchez, L. A. (2013). Antagonism of *Apis mellifera* and *Melipona beecheii* for the sources of feeding. *Revista Cubana de Ciencias Forestales, 1*(2), 102–109.
Louveaux, J., Maurizio, A., & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World, 59*(4), 139–157.
Mercês, M. D., Peralta, E. D., Uetanabaro, A. P. T., & Lucchese, A. M. (2013). Atividade antimicrobiana de méis de cinco espécies de abelhas brasileiras sem ferrão. *Ciência Rural, 43*, 672–675.
Nishio, E. K., Ribeiro, J. M., Oliveira, A. G., Andrade, C. G. T. J., Proni, E. A., Kobayashi, R. K. T., et al. (2016). Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and *S. postica Latreille*, 1807. *Scientific Reports, 6*.
da Silva, I. A. A., da Silva, T. M. S., Camara, C. A., Queiroz, N., Magnani, M., de Novais, J. S., et al. (2013). Phenolic profile, antioxidant activity and palynological analysis of stingless bee honey from Amazonas, Northern Brazil. *Food Chemistry, 141*(4), 3552–3558.
Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.
Truchado, P., Ferreres, F., Bortolotti, L., Sabatini, A. G., & Tomás-Barberán, F. A. (2008). Nectar favonol rhamnosides are floral markers of Acacia (*Robinia pseudacacia*) honey. *Journal of Agricultural and Food Chemistry, 56*(19), 8815–8824.
Truchado, P., Martos, I., Bortolotti, L., Sabatini, A. G., Ferreres, F., & Tomas-Barberan, F. A. (2009). Use of quinoline alkaloids as markers of the floral origin of Chestnut honey. *Journal of Agricultural and Food Chemistry, 57*(13), 5680–5686.
Truchado, P., Vit, P., Ferreres, F., & Tomas-Barberan, F. (2011). Liquid chromatography–tandem mass spectrometry analysis allows the simultaneous characterization of C-glycosyl and *O*-glycosyl flavonoids in stingless bee honeys. *Journal of Chromatography A, 1218*(42), 7601–7607.
Vit, P., Medina, M., & Enríquez, M. E. (2004). Quality standards for medicinal uses of Meliponinae honey in Guatemala, Mexico and Venezuela. *Bee World, 85*(1), 2–5.