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Ecuadorian stingless bee (Meliponinae) honey: A chemical and functional profile of an ancient health product

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ABSTRACT

Stingless bee honey samples from west Amazonian Ecuador were studied for their physiochemical, chemical and functional properties. Reducing sugars (44.9 ± 5.72 g/100 g), water (34.1 ± 4.34 g/100 g), free acidity (31.8 ± 4.05 meq/100 g), diastase activity (1.60 ± 0.20 u.d./g), hydroxymethylfurfural (15.0 ± 1.91 mg/kg), electrical conductivity (0.48 ± 0.06 mS/cm), ash (0.28 ± 0.04 g/100 g), colour (150 mm Pfund) were determined as physicochemical parameters. Melissopalynological analyses were processed evidencing pollen belonging to 14 plant families. Glucose (25.5 ± 3.41 g/100 g), fructose (25.2 ± 3.37 g/100 g) and sucrose (3.72 ± 0.49 g/100 g) contents were determined by HPLC, evidencing equal concentrations between fructose and glucose. Coumarins and flavonoids were determined by densitometric HPTLC: fraxin and bergamotin (0.065 ± 0.009; 0.035 ± 0.005 μg/g) among coumarins; luteolin (0.045 ± 0.006 μg/g), quercitrin (0.020 ± 0.003 μg/g), isoramnetin (0.015 ± 0.002 μg/g) among flavonoids. Among the vitamin E isomers, evaluated by HPLC, the occurrence of the only β-tocopherol (1.12 ± 0.15 μg/g) was noted. All these results were compared with those acquired for two multifloral *Apis mellifera* honeys. DPPH and β-carotene bleaching tests were performed, showing interesting values for Ecuadorian honey samples, higher than those shown by multifloral *A. mellifera* honeys (88.1 ± 11.1 DPPH inhibition%; 70.8 ± 8.90 β-carotene inhibition%). Antibacterial activity, against both Gram positive and Gram negative bacteria, revealed MIC values (10–50 μg/ml) always lower than those of *A. mellifera* honeys. Ecuadorian Meliponinae honey samples also showed anti-mutagenic activity assayed with *Saccharomyces cerevisiae* D7 strain, inhibiting back mutation over the entire range of concentrations.

1. Introduction

In both ancient and modern civilizations, honey represents a natural product of great importance, with many functional applications. In fact, far from being simply used as a sweetener, honey is known both as food with significant nutritional properties and as a natural product with valuable therapeutic applications, topically or orally administered. Honey, probably because it has been so appreciated in all times and cultures, has also been considered as food for Gods and often employed in holy ceremonies as a viaticum towards deity. All these nutritional, therapeutic and social roles have been noted in different cultures even though no distinctions have been observed between *Apis mellifera* and stingless bee honeys, i.e. honeys from *Melipona* spp., *Scaptotrigona* spp. and *Trigona* spp. (Meliponinae). Within the stingless bee genus there are about 500 species, the majority of them present in South America, and some in Australia, Asia and Africa (Vit, Bogdanov, & Kilchenmann, 1994). However, stingless bee honeys are better known and mainly used in South America, Africa and Australia, while honeys derived from *A. mellifera* are mainly produced and distributed in Europe and Asia. The distribution of stingless bee honeys in the world market is limited compared with honeys from *A. mellifera*, as a consequence of their limited industrial production, lower shelf life and lack of an institutional quality standard, due to the scant knowledge about the product (Souza et al., 2006; Vit, Medina, & Enríquez, 2004). In particular, Meliponinae honey from Amazonian Ecuador is still collected by natives without any distinction among different *Melipona* spp., *Trigona* spp. and *Scaptotrigona* spp. beehives. In fact, after harvesting from different Meliponinae hives, honey is preserved as a mixture preparation, and used by Achuar populations mainly for nutritional and health purposes. Native ethnomedicine indicates main use as a sweetener and remedy against colds (throat inflammation, in particular), advising its use

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before blowpipe hunting because it is believed that (halfway between traditional medicine and charming folk belief) "honey limits the power of the poisoned arrows and the pulmonary capacity". Apart from this latter anecdotical aspect, the curative properties reported for stingless bee honeys in Ecuador are similar to those reported for analogous honeys from Venezuela, Guatemala, Mexico and Amazonian Brazil (Vit, 1994; Vit, Rios, Novoa, Reinosa, & Camargo, 1992; Vit et al., 2004).

Notwithstanding the long consumption tradition, the literature concerning composition and nutritional properties of Meliponinae honeys from South America and Africa goes back to 1953 (Nogueira-Neto, 1953). Reports detail typical honey parameters such as shelf life, sugar and water contents, pollen analysis, pH, taste, colour, enzymes, with particular reference to honeys from Brazil, Guatemala and Venezuela (Cortopassi-Laurino & Gelli, 1991; Vit et al., 1994). Recently, increasing interest in stingless bee honeys has led researchers to find food quality standard parameters, showing the high compositional and functional variability mainly due to ethnological and botanical sources, geographical origin and harvesting season (Almeida-Muradian, Hitomi Matsuda, & Markowicz Bastos 2007; Bogdanov, Vit, & Kilchenmann, 1996; Finola, Lasagno, & Marioli, 2007; Souza et al., 2006; Vit, Persano Oddo, Marano, & Salas De Mejìas, 1998; Vit, Soler, & Tomás-Barberán, 1997; Vit et al., 1994; Vit et al., 2004).

The present paper reports, for the first time a study focused on stingless bee (Meliponinae) honey coming from the west Amazonian region of Ecuador, traditionally harvested by natives in the Morona Santiago region with an aim to characterize its functional properties through a physicochemical and chemical investigation, melissopalynological and microbiological analyses, and biological assays aimed to clarify its quality parameters. In particular, chemical characterization was aimed at the detection of bioactive flavonoids, reputed as one among the most responsible for the majority of biological and therapeutic properties of honeys (Vit et al., 1997), coumarins and vitamin E isomers. The research strategy led to a functional profile of Ecuadorian Meliponinae honey by comparison with further stingless bee honeys from other central and south American regions, relating the qualitative and quantitative detection of bioactive compounds to ethnopharmacobotanical uses by checking of in vitro antioxidant properties (DPPH and β-carotene bleaching tests), antibacterial activity, and anti-mutagenic capacity. The anti-mutagenic activity of Ecuadorian Meliponinae honey samples was assessed by using the diploid *Saccharomyces cerevisiae* D7 strain assay (Zimmermann, 1975; Zimmermann, Kern, & Rosemberger, 1975). In particular, the D7 strain assay was performed to evaluate the honey samples' inhibition effect against ethyl methanesulfonate (EMS) induction of mitotic gene conversion and mutational reversion. The understanding of damaging oxidation reactions, or the capacity to prevent them, by the intake of functional foods (e.g. honeys) could help in lowering the risk of severe disease (e.g. heart diseases, different kinds of cancer), which are increasing and are the main cause of death in Europe and North America (Conroy et al., 2003; Jemal et al., 2006).

2. Materials and methods

2.1. Honey samples

Three samples of stingless bee (Meliponinae) honey mixture were purchased from Fundacion Chankuap' (Macas, Morona Santiago region, Ecuador) from three different stocks collected in January, 2006, by Achuar gatherers from wild beehives on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77°.15″ W/2°.35″ S). The samples consisted of mixtures of honey traditionally harvested by natives from different beehives without distinction of the bee species producers. The samples were immediately stocked at 4 °C, in airtight containers, in the dark, to prevent degradations prior to analyses. Successively, the samples were frozen at −20 °C for 1 h to increase the fluency of the honey, and facilitating extractions, chemical analyses and biological tests. Some chemical characterizations and biological properties of the Amazonian samples (MH) were consistently compared to those of two kinds of commercial *A. mellifera* multifloral honeys purchased from the market (CH1 and CH2) and processed in the same way as for stingless bee honey.

2.2. Analytical methods (physicochemical parameters)

Physicochemical parameters of Ecuadorian stingless bee honey samples were determined, following methods recommended by the Codex Alimentarius Commission (Revised Codex Standard for Honey, 2001; http://www.codexalimentarius.net/; http://www.fao.org/docrep/meeting/005/X4616E/x4616e0b.htm) and those reported by Finola et al. (2007). The evaluated parameters were: reducing sugars (g/100 g), water content (g/100 g), free acidity (meq/100 g), diastase activity (u.d./g), hydroxymethylfurfural content (mg/kg), electrical conductivity (mS/cm), ash (g/100 g). Colour (mm) was determined using a Lovibond apparatus.

2.3. Melissopalynological analyses

The melissopalynological qualitative analysis was based on specialized literature (Souza et al., 1993, and references therein) and was performed using a Zeiss Axiophot microscope.

2.4. HPLC and HPTLC analyses

2.4.1. Sample preparations

Aliquots of both stingless bee honey samples (MH) and commercial ones from *A. mellifera* (CH1, CH2) were processed, following the method described by Tomás-Barberán, Ferreres, Blázquez, García-Viguera, and Tomás-Lorente (1993) matched for the qualitative and quantitative analyses of flavonoids, coumarins and vitamin E isomers. In particular, 50 ml of honeys were diluted with acid water (pH 3.0, adjusted with HCl) until completely fluid. The solutions were then filtered, and the filtrate passed through an Amberlite XAD-2 column (Sigma–Aldrich) washed first with 100 ml of acid water and subsequently with 300 ml of distilled water. The fractions were then eluted with 300 ml of methanol and then passed through a Sephadex LH-20 column as purifying procedure. These fractions were dried in a Rotavapor and then stored in the dark at −20 °C prior to being processed for densitometric HPTLC chemical analyses. For HPLC analysis of vitamin E, instead, the methanolic eluate was collected and liquid-liquid-extracted, three times, with 300 ml of *n*-hexane (Sacchetti & Bruni, 2007). The *n*-hexane fraction was dried with a rotavapor and then stored in the dark at −20 °C prior to HPLC analysis. In all procedures, care was taken to preserve the operations from light and oxidation.

2.4.2. HPLC analysis of sugars

For separation and chromatographic analysis of Ecuadorian honey (MH) sugars, techniques reported in the literature were followed (Bogdanov et al., 1996, and references therein). The HPLC instrument consisted of a modular HPLC Jasco apparatus (Tokyo, Japan) composed of a pump (Model PU-980), a ternary gradient unit (Model LG-1580-02), a 3 line degasser (Model DG-980-50), and a RI detector (Model RI-1530), linked to an injection valve with a 20μl sampler loop. The injection volume was 40 μl. The column was a 25 × 0.46 cm, 5 μm Spherisorb-Amino type, eluted with 80% acetonitrile at a flow rate of

1.5 ml/min. The temperatures of the column and detector were constantly at 40 °C. All solvents used were chromatographic grade from Sigma–Aldrich. Chromatograms were recorded and compound peaks were identified by comparing their spectra with those obtained with pure standards of glucose, fructose and sucrose (Sigma–Aldrich). Peak area was determined by integration, using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France). The qualitative and quantitative analysis of each extract was performed 3 times.

2.4.3. HPLC analysis of vitamin E isomers

Normal Phase-HPLC analyses of the *n*-hexane fractions (Section 2.4.1) of Ecuadorian honeys (MH) and commercial *A. mellifera* ones (CH1, CH2) were performed using a modular Jasco HPLC unit (Tokyo, Japan) which consisted of a PU-980 pump, a LG-1580-02 ternary gradient unit, a DG-980-503-line degasser, and a UV/VIS 975 detector set at an excitation wavelength of 295 nm, linked to an injection valve with a 20 μl sampler loop. A Lichrosorb silica gel Si 60 (5 μm and 25 × 0.46 cm; Teknokroma, Barcelona, Spain) column was used and the mobile phase was 0.05% isopropanol/hexane at a flow rate of 1.0 ml/min. The injection volume was 40 μl. All solvents used were of chromatographic grade (Sigma–Aldrich). Chromatograms were recorded and α-, β-, γ-, and δ-isomer peaks from all honey samples (MH, CH1, CH2) were identified by comparing their spectra with those of pure tocopherol standards (Matreya Inc., Pleasant Gap, PA 16823 USA); tocotrienol standards were kindly supplied by Prof. Stefano Manfredini (Department Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ferrara, Italy). The peak areas were determined by integration using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France). For each extract, qualitative and quantitative analysis was performed in triplicate.

2.4.4. HPTLC densitometric analyses of coumarins and flavonoids

The NP-HPTLC (normal phase-high performance thin layer chromatography) densitometric analysis was performed using NP-HPTLC plates (Merck, 5641, silica gel 60, 10 × 20 cm, without fluorescence indicator). The methanolic fractions (Section 2.4.1) of the samples (MH, CH1, CH2) were applied to the plates, together to the commercial standards of flavonoids and coumarins as 10 mm wide bands, with a Linomat IV (Camag, delivery speed 4 μl/s). The plate development was performed by employing a modified chromatographic chamber (Camag), achieving a separation comparable to that obtained with AMD (automated multiple development) (Fang, Wan, Jiang, & Cao, 2005, and references therein). The plate development occurred in five steps using two different solvent solutions.

Solvent solution 1; ethyl acetate:formic acid:acetic acid:water (100:11:11:27).
Solvent solution 2; toluene:ethyl acetate:acetic acid (50: 45: 5).

The detection was performed with a Camag Reprostar II apparatus. The plates were scanned with a Camag Scanner II, equipped with Cats software 3 (Camag). Fluorescence was induced at 365 nm with a mercury vapour lamp. Plates were examined for both fluorescence emission, (measured through a cut-off filter at 400 nm, sensitivity 220, span 7, offset 5%), and for absorbance (λ = 240 nm; cut-off filter at 400 nm, sensitivity 230, span 7, offset 10%). Coumarins gave an inherent bluish fluorescence while flavonoids exhibited different nuances in colour from yellow-orange to yellow–green after being sprayed with a water solution of 4% aluminium sulphate. Flavonoids and coumarins were identified by chromatographic comparison with authentic commercial standards. For coumarins, the following standards were checked: bergamotin, bergapten, bergaptol, fraxidin, fraxin, imperatorin, isopimpinellin, rhamnetin, saponarin, xanthotoxol (Extrasynthese), 6,7-dimethoxycoumarin (Sigma–Aldrich), 4-methyl-umbelliferone, 5,7dimetossicoumarin, 6-methylcoumarin, 7-methylcoumarin, angelicin, daphnetin, dihydrocoumarin, herniarin, esculetin, fraxetin, isoscopoletin, santonin, scoparone, scopoletin, and xanthotoxin (Roth). For flavonoids the standards employed were: apigenin, apigenin-7-glucoside, hesperidin, isoquercitrin, isorhamnetin, kaempferol, kaempferol-3-glucoside, quercetin dihydrate, quercitrin (Extrasynthese), luteolin, luteolin-7-glucoside, neohesperidin, naringenin (Roth). Qualitative determination was achieved by comparing the bands obtained from honey samples with those of pure standards, while quantitative data were obtained by integration of the peak areas using dedicated Cats 3 software (Camag). For each sample, qualitative and quantitative analysis was performed in triplicate.

2.5. Antioxidant activity

2.5.1. DPPH test

Free radical-scavenging activity of methanolic fractions (Section 2.4.1) of the honey samples (MH, CH1, CH2) was performed according to the procedure of Wang et al. (1998). The honey methanolic fractions were dried with a Rotavapor, weighed and then added to a 10^{-4} M methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH**.**, Sigma–Aldrich) to obtain assay sample solutions at concentrations of 0.3, 0.4, 0.5, 0.6, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 mg/ml. The assay solutions were then placed in an orbital shaker (Universal Table Shaker 709; 200 rpm) at room temperature. After 30 min of incubation, the absorbance of each sample was measured at 517 nm with a spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK). Butylated hydroxyanisole (BHA; Sigma–Aldrich), at the same concentrations as employed for honey samples, was used as positive control. Antioxidant activity was expressed as DPPH**.** inhibition percentage ($Ip^{DPPH}\%$) as calculated by:

$$Ip^{DPPH}\% = 100(1 - AbsDPPH_1/AbsDPPH_2)$$

where, $DPPH_1$:DPPH solution with methanolic honey fraction; $DPPH_2$ = DPPH solution with methanol only (negative control). For each sample, assays were performed in triplicate.

2.5.2. β-Carotene bleaching test

Antioxidant activity of methanolic fractions (Section 2.4.1) of honey samples (MH, CH1, CH2) was determined through the β-carotene bleaching test (Taga, Miller, & Pratt, 1984). An aliquot of 10 mg of β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in 10 ml of $CHCl_3$ with 20 μl of linoleic acid (Sigma–Aldrich) and 200 μl of Tween 40 as emulsifier. $CHCl_3$ was removed using a rotary evaporator (Büchi 461 Switzerland) at 40 °C, and distilled water (50 ml), previously O_2-saturated for 30 min, was added to obtain an emulsion. The methanolic fraction of honeys (0.2 ml), at a concentration of 100 mg/ml, was then added to the emulsion and the assay samples incubated at 55 °C. Absorbance was monitored with a spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) at time 0', and after 120' at 470 nm. To set up the spectrophotometer, 20 μl of linoleic acid, 200 μl of Tween 40 and 50 ml of distilled water solution, without honey methanolic fractions, were employed. As positive control, a methanolic solution of 2 mg/ml of butylated hydroxyanisole (BHA; Sigma–Aldrich) was used. Negative controls were set up with appropriate aliquots of methanol only. The antioxidant activity (AA) expressed as inhibition percentage of β-carotene oxidation, was calculated as:

$$AA = 100(DRc - DRs)/DRc$$

where, DRc [=ln(a/b)/120] is control percentage degradation; DRs [=ln(a/b)/120] is sample percentage degradation; a = absorbance at time 0'; b = absorbance after 120 min of incubation.

For each sample, assays were performed in triplicate.

2.6. Microbiological analyses

2.6.1. Antibacterial activity

The biological activity of the methanolic fractions (Section 2.4.1) of honey samples (MH, CH1, CH2) against Gram positive and Gram negative bacteria, purchased from the American Type Culture Collection, was determined by employing the standard disk diffusion technique (Okeke, Iroegbu, Eze, Okoli, & Esimone, 2001). The activity was assessed against *Enterococcus foecalis* ATCC 29212, *Staphylococcus aureus* subsp. *aureus* ATCC 29213, *Escherichia coli* ATCC 4350 and *Pseudomonas aeruginosa* ATCC 17934 strains. Mother cultures of each microorganism were set up 24 h before the assays, in order to reach the stationary phase of growth. The tests were assessed by inoculating Petri dishes from the mother cultures with proper sterile media with an aim to obtain a microorganism concentration of 10^6 CFU (colony forming units)/ml. Sterile paper disks (6 mm diameter, Difco), previously deposited with methanol solutions (10 μl) of each honey sample at 1, 5, 10, 50, 100, 500, 1000 μg/ml, were placed in the centre of the inoculated petri dishes. The plates were then incubated at 37 °C for 24 h and the growth inhibition zone diameter (IZD) was measured to the nearest mm. The lowest concentration of each assay sample deposited on the sterile paper disk showing a clear zone of inhibition was taken as the MIC (minimum inhibitory concentration) (Okeke et al., 2001). Negative controls were set up with 10 μl of methanol, while positive ones prepared with ampicillin (25 μg/plate), which induced a complete (100%) growth inhibition of bacteria.

2.6.2. Yeast strain assay (Saccharomyces cerevisiae D7 assay): antimutagenic activity of honey extracts

A toxicity and mutagenic pre-test was performed on the honey samples, using yeast cells (*S. cerevisiae* D7 strain); complete liquid (YEP), solid (YEPD) and minimal media were prepared according to Zimmermann et al. (1975). The inhibitory effect of honey extracts on mutagenic activity and gene conversion, instead, was evaluated against direct acting mutagen ethyl methanesulfonate (EMS) (2 μg/plate) by using the D7 diploid strain of *S. cerevisiae* ATCC 201137 assay (Zimmermann et al., 1975). Cells from a culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28 °C until they reached the stationary growth phase. The yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain a final mixture of 2×10^8 cell/ml. The mixture (4 ml) contained 1 ml of cell suspension, potassium phosphate buffer and honey methanolic extracts in order to reach final concentrations of 0.15, 0.30 and 0.60 mg/ml. The mixture was incubated under shaking for 2 h at 37 °C. Then the cells were plated in complete and selective media to ascertain survival, *trp* convertants and *ilv* revertants.

2.7. Statistical analysis

Relative standard deviations and statistical significance (Student's *t* test) were obtained, where appropriate, for all data collected. One-way ANOVA and LSD post hoc Tukey's honest significant difference test were used for comparing the bioactive effects of different honey samples. All computations were done using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

3. Results and discussion

Stingless bee honeys attract attention of researchers for their importance as foodstuffs and traditional remedies in folk medicines of countries in which *Melipona* spp., *Scaptotrigona* spp. and Trigona spp. (Meliponinae) are endemic (Vit et al., 1994). Curiously, notwithstanding the ethnopharmacobotanical tradition linked to these honeys, they lack clear parameters for quality control by institutional food control authorities comparable to that of *A. mellifera* honeys. A systematic chemical and functional characterization, aimed at typification of these natural products from different Amazonian areas is also lacking. Relatively recent are the most advanced researches about physicochemical parameters, chemical characterization and functional properties with the aim to better define their nutritional effects. However, the stingless bee honeys studied with this functional and applicative target were mainly from Guatemala, Mexico, Venezuela and Brazil (Souza et al., 2006; Vit et al., 2004 and references therein). Therefore, starting from a physicochemical point of view, the Ecuadorian honey samples examined (MH) showed a noticeable fluid consistency and a high water content, in analogy with other stingless bee honeys (Table 1). In fact, stingless bee honeys, can be distinguished from *A. mellifera* honeys by always higher average water content. Such evidence can be directly related to the fact that stingless bee species plunder both flowers and mature fruits, the latter being richer in water (Vit, 1994). However, our samples evidenced a uniform water content but nevertheless higher (13.3% on average) than that of honeys from other South American regions (Vit et al., 2004) (Table 1). This could depend on many factors, e.g. the harvesting season, the degree of maturity reached in the hive, climatic factors (Finola et al., 2007), the different plundered flora, or dilution for sophistication. Excluding the latter cause, this result represents a negative quality attribute (Vit et al., 2004) as the maximum endurable amount of water is considered to be 30 g/100 g of honey. Above such a limit there is a high risk of induction of fermentative processes and of subsequent alteration of organoleptic factors, physicochemical, chemical and functional characteristics of the honeys. However, from the perspective of traditional medicinal use, it must be stressed that fermented honeys – or honeys particularly rich in water – are considered by natives to be particularly indicated to treat respiratory disorders. This ethnomedical aspect is consistent with other studies performed on stingless bee honeys from other South American regions (Vit et al., 2004). However, despite the high water content compared to *A. mellifera* samples, stingless bee honeys are fairly resistant to spoilage by unwanted fermentation, because of the presence of polyphenolic compounds derived from the plundered flora and because of cerumen enzymatic processing by bees in the hive (Vit et al., 1994). This property is underlined in our samples by the hydroxymethylfurfural (HMF) values (15.0 ± 1.91 mg/kg; Table 1), considered as indicative of honey freshness being marginally lower than those considered as maximum (40.0 mg/kg) (Vit et al., 2004). Likewise, free acidity values (Table 1) may be considered as a freshness indicator (Finola et al., 2007). Also other physicochemical parameters

Table 1
Physicochemical analyses of Ecuadorian stingless bee (Meliponinae) honey samples.

Colour	150.00	mm Pfund
Water	34.1 ± 4.34	g/100 g
Reducing sugars	44.9 ± 5.72	g/100 g
Free acidity	31.8 ± 4.05	meq/100 g
Diastasic activity	1.60 ± 0.20	u.d./g
Hydroxymethylfurfural (HMF)	15.0 ± 1.91	mg/kg
Electrical conductivity	0.48 ± 0.06	mS/cm
ash	0.28 ± 0.04	g/100 g

(Table 1) fall within the values reported for quality standards of stingless bee honeys (Vit et al., 2004), reflecting a good profile of the Ecuadorian Meliponinae honey as a foodstuff. This is also underlined by the microbiological analyses performed on all Ecuadorian samples which evidenced negative results (under the limit reported by MercoSur and European Community) for the detection of coliform bacteria, yeasts, moulds, Clostridium spores, *Paenibacillus larvae* spores (American foulbrood) and *Bacillus cereus* spores (data not shown).

Interesting parameters to consider for characterizing honeys, as a foodstuff in particular, are colour and organoleptic properties. While smell and taste mainly depends on the contents of volatile compounds with low molecular weight, the colour of honey depends on other chemical components (e.g. mineral content) or physicochemical ones. Our samples, amber-brown coloured (15.00 mm Pfund) with an aromatic smell and a fruity sweet taste, gave higher ash values (Table 1) than those reported for light-coloured honeys, confirming literature suggestions (Finola et al., 2007).

Melissopalynological analyses performed on Ecuadorian stingless bee honey samples (Table 2) evidenced different types of pollen belonging to 14 botanical families (data consistent with the phyto-geographical distribution of the plant species in the sampling and plundered area).

Carbohydrate analyses (Table 3) showed glucose, fructose and sucrose concentrations generally consistent in with average values reported in the literature, but slightly lower than those reported by Almeida-Muradian et al. (2007). Interesting considerations emerged when glucose and fructose concentrations were compared and found to be very similar, suggesting a possible distinction criterion between our Ecuadorian Meliponinae samples and those from other central and South American countries, in which fructose was always detected in a higher concentration than glucose (Almeida-Muradian et al., 2007; Souza et al., 2006). These data also lead us to consider the F/G results, which suggest that Ecuadorian Meliponinae honey samples were not adulterated by addition of any kinds of syrup (e.g. sugar cane syrup), since the F/G ratio is considered as an important criterion to detect this kind of sophistication, especially with values lower than 1.0 (Almeida-Muradian et al., 2007).

Analyses of coumarins and flavonoids were performed by densitometric HPTLC on MH samples and compared with data acquired from two different commercial *A. mellifera* multi-floral honeys (CH1, CH2) (Table 4). Coumarins and flavonoids are compounds typically characterizing phytocomplexes or plant-derived products, i.e. honeys, both from a chemical and functional point of view. While flavonoids represent one of the most investigated chemical classes in honeys, both from stingless bees and *A. mellifera* (Sato & Miyata, 2000; Vit et al., 1997), coumarins have never been investigated in stingless bee honey, although their botanical origin and biological properties might suggest their presence in honeys and their relationship with honey functional properties. All the honey samples (MH, CH1, CH2) were processed for extraction as reported by Vit et al. (1997, and references therein) and HPTLC-densitometric analysis was chosen as an analytical method, being particularly suitable for checking a large number of compounds at the same time (Fang et al., 2005, and references therein). The chemical profile for coumarin content differed among the three tested honeys (MH, CH1, CH2). Fraxin and bergamotin were identified only in MH honey samples; in particular, fraxin concentration was 46.2% higher if compared to bergamotin. Scopoletin was only detected in commercial *A. mellifera* honey CH2, while no coumarins were found in CH1. The flavonoid analyses also involved compounds previously checked in stingless bee honeys from other South American countries (Vit et al., 1997); luteolin, quercitrin and isorhamnetin were the flavonoids detected in MH Ecuadorian honeys (MH), with luteolin as the most abundant compound, it was 66.7% higher in concentration than isorhamnetin, and 55.6% higher than quercitrin. Similarities were found to commercial *A. mellifera* honey CH1 in which luteolin and quercitrin were detected, although at concentrations 73.3% and 91.1%, respectively, lower than that of MH. The flavonoids, luteolin-7-glucoside and naringenin, were identified only in commercial honey CH2. Our results partly agree with other papers showing greater qualitative and quantitative variability in flavonoid contents of stingless bee honeys from different geographical areas (Vit et al., 1997). Therefore, in our opinion the differences could be attributed (i) to the different geographical origins of honeys, (ii) to the problematic determination of bee specie and (iii) to the different botanical sources (flowers and mature fruits) plundered by bees. The ethnomedical use of the MH honeys examined, i.e. to treat respiratory diseases, instead corresponds with the flavonoids detected, which are known for their activity against mucosa inflammation (Iwahashi et al., 2004; Chaabi et al., 2007).

The analysis of vitamin E isomers by HPLC, is the first report of this kind for stingless bee honeys. It revealed, in our Ecuadorian samples, only the presence of β-tocopherol. *A. mellifera* commercial

Table 2
Melissopalynological analyses of Ecuadorian stingless bee (Meliponinae) honey samples.

Botanical families	Genera
Aquifoliaceae	*Ilex* spp.
Burseraceae	*Protium* spp.
Combretaceae	
Compositae	
Cruciferae	
Fabaceae	*Acacia* spp.
Malvaceae	
Moraceae	
Myrtaceae	
Polygonaceae	
Proteaceae	
Rhamnaceae	
Scrophulariaceae	
Vitaceae	*Parthenocissus* spp.

Table 3
Sugars detected by HPLC in Ecuadorian stingless bee honeys. Data are expressed as g/100 g honey samples ± standard deviation.

Sugars	g/100 g honey
Sucrose	3.72 ± 0.49
Glucose	25.5 ± 3.41
Fructose	25.2 ± 3.37

Table 4
NP-HPTLC-densitometric analyses of coumarins and flavonoids in methanolic fractions of Ecuadorian Meliponinae (MH) and of commercial *Apis mellifera* honey samples (CH1, CH2). Values are expressed as μg/g honey ± standard deviation. Results are derived by triplicate determinations.

	MH	CH1	CH2
Coumarins			
Fraxin	0.065 ± 0.009	–	–
Scopoletin	–	–	0.064 ± 0.008
Bergamotin	0.035 ± 0.005	–	–
Flavonoids			
Luteolin-7-glucoside	–	0.029 ± 0.004	0.008 ± 0.001
Luteolin	0.045 ± 0.006	0.012 ± 0.002	–
Quercitrin	0.020 ± 0.003	0.004 ± 0.001	–
Naringenin	–	–	0.035 ± 0.005
Isorhamnetin	0.015 ± 0.002	–	–

showed instead the presence of all the four tocopherol isomers (Table 5). It is noteworthy that, in MH, only the β-isomer is present, whose total abundance is 21.1% lower than the total tocopherol content detected in CH2, but 53.6% higher than the total vitamin E isomers found in CH1. In both *A. mellifera* commercial honeys, β-tocopherol was always the least abundant isomer. In all the honey samples, tocotrienol isomers were not detected.

The antioxidant activity tested by DPPH**.** and the β-carotene bleaching test revealed a higher radical-scavenger capacity of the Ecuadorian MH honey compared to both *A. mellifera* samples, CH1 and CH2 (Fig. 1). In particular, MH honey evidenced an antioxidant capacity 55% and 32% higher ($P < 0.05$) than CH1 and CH2, respectively, and an activity similar to the positive control butylated hydroxyanisole (BHA) at the highest concentration tested (100 mg/ml). Unlike commercial *A. mellifera* honeys, for which the bioactivity revealed a dose-dependent trend, Ecuadorian stingless bee samples showed a higher radical-scavenger increasing at 100 mg/ml compared to 50 mg/ml. The results of the β-carotene bleaching test (Fig. 2) confirmed the DPPH**.** data, showing the most interesting and significant capacities at 100 mg/ml. In fact, MH samples were always more active than CH1 and CH2 (71% and 47%, respectively; $P < 0.05$), showing absorbance values near to those given by BHA. Even if other undetected chemicals (e.g. ascorbic acid, phenolic compounds, carotenoids, nitrogen compounds) might be co-responsible for the radical scavenger-activity of honey (Pérez, Rodríguez-Malaver, & Vit, 2006), it is undeniable that the important antioxidant capacity might reflect the synergistic action of flavonoids, detected with a similar chemical profile in other stingless bee honeys (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Vit et al., 1997), and of β-tocopherol. It must be also stressed, however that, from a general point of view, colour of honeys is related to antioxidant capacity: in particular, a marked amber-colour reflects a higher antioxidant activity (Frankel, Robinson, & Berenbaum, 1998), and this could be related to the interesting results obtained with MH compared to CH1, CH2 samples.

The antibacterial activity evidenced by MH samples was always better than that by CH ones against both gram positive and gram negative bacteria assayed (Table 6). Particularly interesting is the lowest MIC value detected for the gram positive *S. aureus* subsp. *aureus*, one of the most representative bacteria involved in respiratory diseases and related symptoms. This evidence could be directly related to the main ethnomedical use of Ecuadorian stingless bee honey, i.e. to treat sore throat. Interesting also is the MIC evidence. The gram negative *P. aeruginosa* and *E. coli*, are generally more resistant than are gram positive bacteria to treatments.

All the MH samples were then tested and compared to CH honeys for mutagenic and anti-mutagenic effects (Table 7) in order to determine protective activity of honeys using *Saccharomyces cerevisiae* D7 (Zimmermann, 1975; Zimmermann et al., 1975). This is

Table 5
Vitamin E isomers content checked by NP-HPLC, expressed as μg/g honey ± standard deviation; – = not detected. MH: Ecuadorian stingless bee honey samples; CH1, and CH2: commercial *Apis mellifera* honey samples. Results are derived by triplicate determinations.

	MH	CH1	CH2
α- Tocopherol	–	0.090 ± 0.020	0.040 ± 0.005
β- Tocopherol	1.12 ± 0.150	0.090 ± 0.020	0.020 ± 0.003
γ- Tocopherol	–	0.140 ± 0.018	0.660 ± 0.086
δ- Tocopherol	–	0.201 ± 0.030	0.700 ± 0.092
Tocotrienols	–	–	–
Total isomers	1.12 ± 0.150	0.520 ± 0.070	1.42 ± 0.180

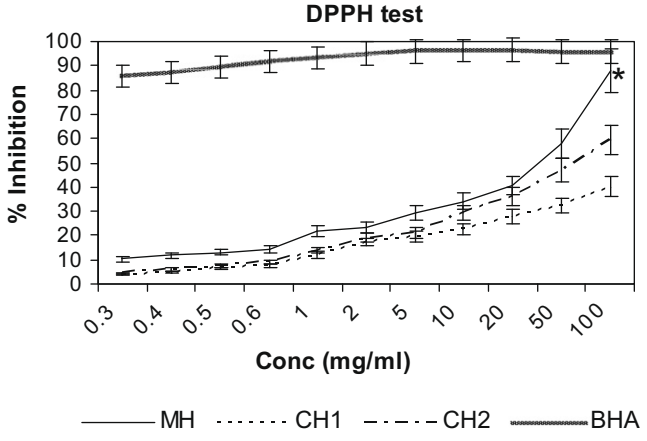

Fig. 1. Antioxidant activity of methanolic fractions of MH, CH1 and CH2 honey samples expressed as inhibition percentage of the DPPH radical at different concentrations. BHA: butylated hydroxyanisole (positive control). Results are derived by triplicate determinations; means ± standard deviation. (*) Significant statistical differences according to Student's *t* test ($P < 0.05$).

Table 6
Antibacterial activity of the methanolic fractions of MH, CH1 and CH2 honey samples, expressed as minimum inhibitory concentration (MIC; μg/ml), considered as the lowest concentration of each sample showing a clear zone of inhibition. Ampicillin (25 μg/ml) induced a complete (100%) growth inhibition. Results are derived by triplicate determinations.

	MIC (μg/ml)		
	MH	CH1	CH2
Staphylococcus aureus subsp. *aureus* ATCC 29213	10	100	50
Enterococcus foecalis ATCC 29212	50	100	100
Pseudomonas aeruginosa ATCC 17934	50	500	100
Escherichia coli ATCC 4350	100	500	100

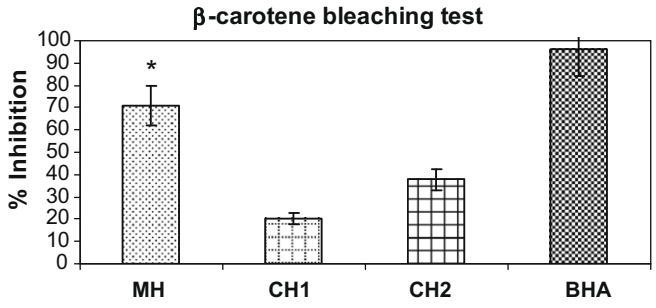

Fig. 2. Antioxidant activity of methanolic fractions of MH, CH1 and CH2 honey samples expressed as inhibition percentage of the β-carotene oxidation. BHA: butylated hydroxyanisole. Results are derived by triplicate determinations; means ± standard deviation. (*) Significant statistical differences according to Student's *t* test ($P < 0.05$).

Table 7
Effect of survival and antigenotoxicity (mutagenesis and gene conversion) in *Saccharomyces cerevisiae* D7 assay from treatment with Ecuadorian stingless bee honeys (MH) and commercial ones (CH1, CH2) as methanolic extracts. Genotoxic agent: ethyl methanesulfonate (EMS) at 0.5% concentration.

	Honey concentrations (mg/l)	EMS (%)	Survivals (%)	Gene conversion colonies/10^5	Mutants colonies/10^6
	0	0	100.00	0.80 ± 0.10	0.16 ± 0.02
	0	0.50	88.24 ± 11.40	52.90 ± 6.80	426.6 ± 55.10
MH	0.15	0.50	84.96 ± 13.10	25.41 ± 3.20*	71.25 ± 9.20*
	0.30	0.50	83.69 ± 10.80	26.93 ± 3.40*	68.76 ± 8.80*
	0.60	0.50	80.25 ± 10.30	16.32 ± 2.10*	71.42 ± 9.20*
	0	0	100.00	0.73 ± 0.10	0.34 ± 0.04
	0	0.50	85.80 ± 11.10	57.60 ± 7.40	380.60 ± 49.20
CH1	0.15	0.50	81.25 ± 10.50	34.67 ± 4.40*	298.67 ± 38.60
	0.30	0.50	83.00 ± 10.70	36.11 ± 4.60*	333.33 ± 43.10
	0.60	0.50	85.25 ± 10.90	41.00 ± 5.30*	407.20 ± 60.80
	0	0	100.00	0.80 ± 0.20	0.50 ± 0.05
	0	0.50	87.00 ± 11.20	51.70 ± 4.20	374.70 ± 48.47
CH2	0.15	0.50	109.32 ± 14.10	32.40 ± 4.10*	405.20 ± 52.40
	0.30	0.50	111.83 ± 14.40	35.60 ± 4.60*	423.00 ± 54.70
	0.60	0.50	117.56 ± 15.20	44.50 ± 5.70	490.00 ± 63.30

* Significant statistical differences according to Student's *t* test ($P < 0.05$).

the first report of stingless bee honey protection against DNA damage and could represent interesting evidence in relation to the antioxidant capacity determined. The anti-genotoxic effects on *S. cerevisiae* bioassay of methanolic extracts of MH honey and of the two commercial honey samples up to 0.60 mg/ml in a 0.5% fixed ethyl methanesulfonate (EMS) concentration are shown in Table 7. Significant differences between MH and commercial honey samples have been found by the ANOVA test and Tukey's honest significant difference test ($P < 0.05$). Although all samples lower the frequencies of gene conversion according to the Student's *t* test (except commercial CH2 honey samples at 0.60 mg/ml), only MH honeys significantly inhibit back mutation over the entire range of concentrations. Since EMS is an alkylating agent and a direct-acting mutagen, the inhibition effect of stingless bee honey extracts on mutagenic activity and conversion events of EMS on D7 *S. cerevisiae* strain can hardly be explained by the antioxidant activity of the honey flavonoids (alkylating agents are not generators of free radicals). The trapping of the ethyl radical by reaction with reactive honey chemicals might be a possible effective mechanism of inhibition. In particular, direct mispairing, due to the addition of an ethyl group of EMS to the O6 position of guanine and the O4 position of thymine in the DNA, could be scavenged by reaction with the glucoside bond of the fraxin which is present in MH honey. Furthermore, enhanced steric hindrance of the possible chemical complex could reduce access of the genotoxic agent to the yeast cell. Indeed, lower yeast cell permeability (Staleva, Waltscheva, Golovinsky, & Venkov, 1996) could discriminate free EMS from complexed. Presence of fraxin as a complexing agent in MH honey samples, which is not present in commercial honeys (Table 4), would be reflected in the Ecuadorian stingless bee methanolic fractions' chemical reactivity against EMS, and would explain observed differences in anti-mutagenic effect.

4. Conclusion

From a physicochemical and chemical point of view, this first report on Ecuadorian stingless bee (Meliponinae) honey has revealed many similarities to other previously evaluated central and South American honeys. However, some aspects, for example the equal concentrations of glucose and fructose, or the high abundance of β-tocopherol and the coumarin profile, can be considered as possible starting points to typify this product and discriminate it from other Meliponinae honeys. The coumarin and tocopherol analyses go towards this target, evidencing also how these, alongside flavonoids, could be related to the bioactive properties of honey. The relevant results, shown by in vitro antioxidant and antibacterial assays for Ecuadorian honey, reflect ethnomedical uses and indicate how traditional knowledge can be related to the same modern functional properties as are already supporting the health and dietary claims ascribed to the more diffuse *A. mellifera* honeys. On the other hand, anti-mutagenic properties could evidence new functional perspectives of this natural product underlining new profiles both in terms of safety and efficacy.

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