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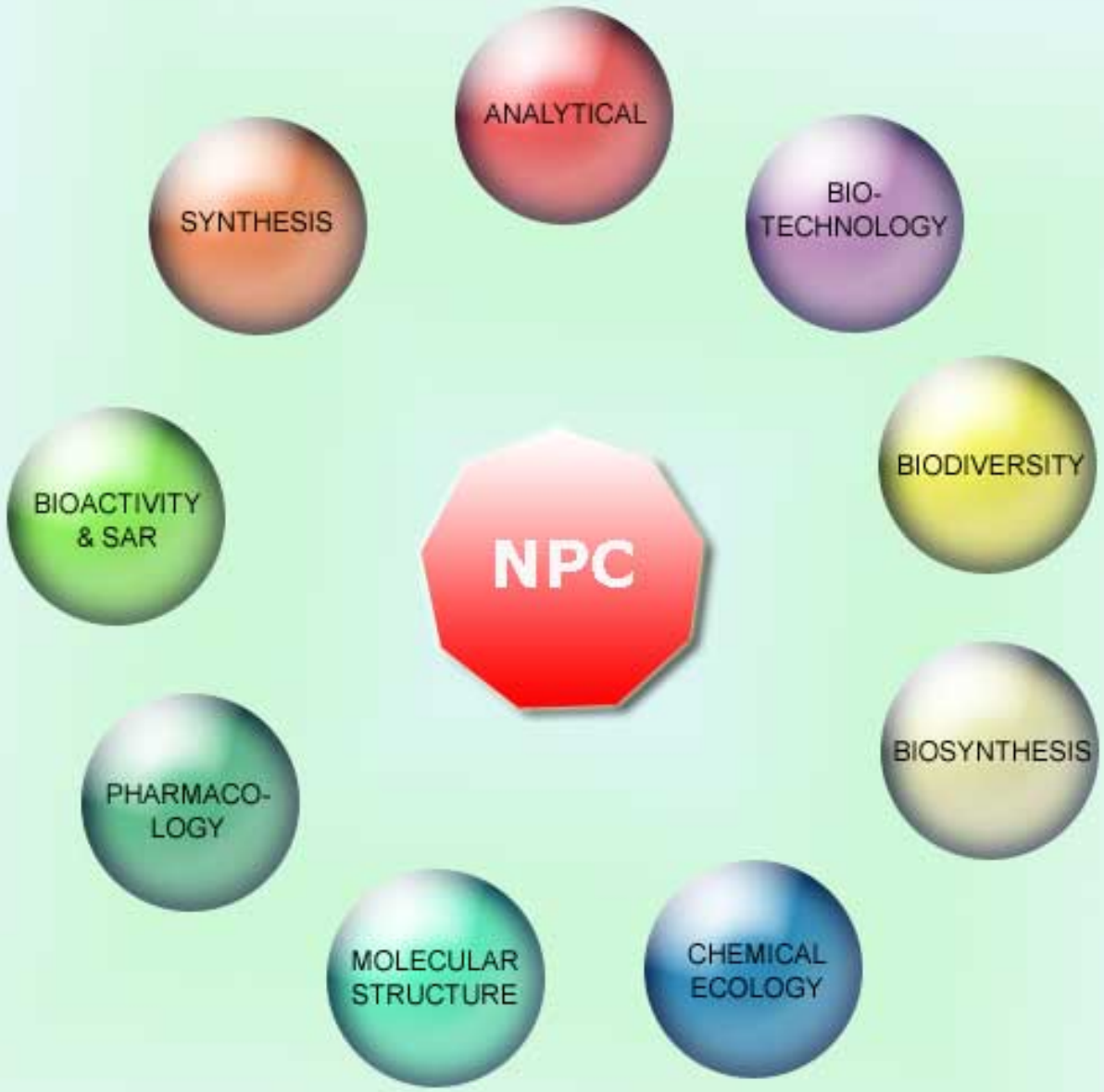

Volume 4. Issue 9. Pages 1171-1318. 2009
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2009
Vol. 4
No. 9
1221 - 1226

Properties of Honey from Ten Species of Peruvian Stingless Bees

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Received: February 20th, 2009; Accepted: June 30th, 2009

Honey produced by ten stingless bee species (*Melipona crinita*, *M. eburnea*, *M. grandis*, *M. illota*, *Nannotrigona melanocera*, *Partamona epiphytophila*, *Ptilotrigona lurida*, *Scaptotrigona polystica*, *Scaura latitarsis*, and *Tetragonisca angustula*) from Peru has been characterized according to traditional physicochemical standards (color and moisture), biochemical components (flavonoids, polyphenols, nitrites, proteins), and bioactive properties (antibacterial activity, antioxidant capacity). Analytical data are also provided for a sample of *Apis mellifera* and an artificial honey control. For stingless bees, honey color varied between 26 and 150 mm Pfund. *M. illota* produced the lightest honey, while *N. melanocera* and *T. angustula* were the darkest. Moisture varied between 20.8 and 45.8 g water/100 g, confirming higher moisture for stingless bee honey than the *A. mellifera* honey standard of 20 g water/100 g. Flavonoids varied from 2.6 to 31.0 mg quercetin equivalents/100g, nitrites from 0.30 to 2.88 μmoles nitrites/100 g, polyphenols from 99.7 to 464.9 mg gallic acid equivalents/100g, proteins from 0.75 to 2.86 g/100 g, and the antioxidant capacity from 93.8 to 569.6 μmoles Trolox equivalents/100 g. The minimal inhibitory concentration (MIC) was slightly lower against *Staphylococcus aureus* (12.5 -50 g/100 mL) than *Escherichia coli* (50 g/100 mL).

Keywords: antioxidant, antibacterial, color, honey, Meliponini, moisture, Peru.

Stingless bee (Hymenoptera, Apidae, Meliponini) honey has been used in traditional medicine for centuries or more in Peru, collected by cutting down trees with bee colonies, locating the honey pots, and then leaving the nests exposed, instead of collecting them for meliponiculture. Colonies are also common in house walls and around human constructions [1]. Honey pots are usually squeezed with the bare hands to extract honey, which often ends up being mixed with both pollen and brood. In Amazonian Peru, this honey is widely used and often sold at local markets, both as a sweetener, but more often as an ingredient of folk medicine, as in Guatemala, Mexico and Venezuela [2]. Peruvian local names for *Tetragonisca angustula* is "*ramichi*", and for *Melipona grandis* and all other species of *Melipona* "*ronsapilla*".

Honey is a complex matrix with edaphic, botanical and entomological components contributing to the bioactive properties and its value in apitherapy. As a rediscovered ancient remedy, the main attributes of honey dressing were outlined by Molan: 1. It provides a moist healing environment caused by its osmolarity drawing fluid out of tissues. 2. Hydrogen peroxide is released by the action of honey glucose oxidase. 3. Direct antibacterial, anti-inflammatory and antioxidant effect [3].

Considering the vast neotropical biodiversity of 391 stingless bee species [4], only the honey produced by a few has been studied, this work having been recently reviewed [5]. The main differences of stingless bee honey compared with *Apis mellifera* (honeybee) honey are a higher water content and

Table 1: Entomological origin of honey samples.

No.	Stingless bee species	n
1	*Melipona crinita* Moure & Kerr, 1950	2
2	*Melipona eburnea* Friese, 1900	1
3	*Melipona grandis* Guérin, 1844	5
4	*Melipona illota* Cockerell, 1919	1
5	*Nannotrigona melanocera* Schwarz, 1938	1
6	*Partamona epiphytophila* Pedro & Camargo, 2003	1
7	*Ptilotrigona lurida* Smith, 1854	1
8	*Scaptotrigona polystica* Moure, 1950	1
9	*Scaura latitarsis* Friese, 1900	1
10	*Tetragonisca angustula* Latreille, 1811	1
11	*Apis mellifera* Linnaeus, 1758	1

acidity, lower diastase activity, and a different sugar spectrum. They are also known for different sensory characteristics. Stingless bee honey from Peru has not been analyzed previously. In this study, to address the reputed medicinal properties of the honey, we evaluated the color, moisture, flavonoid, nitrite, polyphenol and protein contents, antibacterial activity and antioxidant capacity of honey produced by ten different species of stingless bees collected in localities around Tarapoto, San Martin, from Peru and one sample of *A. mellifera*. Table 1 lists the species studied and the number of honey samples examined for each species.

The physicochemical data, color and moisture content of the honey produced by each species is shown in Table 2.

Table 2: Physicochemical components of honey.

Bee species[1]	Physicochemical components[2]	
	Color	Moisture
1	88	28.8
2	103	23.8
3	38	27.5
4	26	28.0
5	150	33.4
6	78	45.8
7	120	35.2
8	128	33.0
9	130	20.8
10	150	28.9
11	125	20.0
control	91	19.6

[1] See bee species in Table 1.
[2] Averages of color (mm Pfund) and moisture (g water/100 g honey).
Minimum and maximum values of each variable are highlighted.

Honey color varied between 26 and 150 mm Pfund. The lightest honey was from *M. illota*, and the darkest from *N. melanocera* and *T. angustula*. Besides the entomological origin of the honey in this study, color variations depending on the botanical origin are known for *A. mellifera* honey [6]. Moisture varied between 20.8 and 45.8 g water/100 g for *S. latitarsis* and *P. epiphytophila*, respectively, in agreement with previous studies that report higher moisture contents than those of the *A. mellifera* honey standard of 20 g water/100g [5]. The biochemical components of honey studied in this work were flavonoids, nitrites, polyphenols, and proteins (Table 3).

Table 3: Biochemical components of honey.

Bee species[1]	Biochemical components[2]			
	Flavonoids	Nitrites	Polyphenols	Proteins
1	7.3 ± 0.6	0.37 ± 0.03	155.1 ± 5.1	1.08 ± 0.11
2	8.9 ± 0.7	0.65 ± 0.13	179.6 ± 4.8	1.08 ± 0.04
3	3.1 ± 1.3	0.35 ± 0.12	105.5 ± 18.2	0.54 ± 0.17
4	2.6 ± 0.1	0.30 ± 0.00	99.7 ± 0.8	0.75 ± 0.12
5	31.0 ± 1.2	1.46 ± 0.06	464.9 ± 5.9	2.86 ± 0.01
6	5.9 ± 0.3	1.26 ± 0.08	151.3 ± 17.7	0.94 ± 0.06
7	23.4 ± 1.1	2.88 ± 0.13	240.3 ± 10.4	1.88 ± 0.04
8	17.6 ± 0.7	1.15 ± 0.03	337.0 ± 8.7	1.57 ± 0.04
9	17.7 ± 0.9	1.13 ± 0.15	282.6 ± 4.9	1.77 ± 0.03
10	18.8 ± 0.7	1.07 ± 0.06	260.9 ± 4.6	1.91 ± 0.04
11	5.0 ± 2.9	0.37 ± 0.05	78.3 ± 52.6	1.48 ± 0.03
control	1.6 ± 2.7	0.00 ± 0.00	23.6 ± 4.2	0.27 ± 0.02

[1] See bee species in Table 1.
[2] Averages ± SD of flavonoid (mg quercetin equivalents QE), nitrite (μmoles nitrite), polyphenol (mg gallic acid equivalents GAE), and protein (mg) contents, per 100 g honey.
Minimum and maximum values of each variable are highlighted.

The lowest flavonoid, polyphenol and protein contents were found in *M. illota* honey, and the highest were in *N. melanocera*, ranging from 2.6 to 31.0 mg QE/100g, 99.7 to 464.9 mg GAE/100g, and 0.75 to 2.86 g protein/100g, respectively. Whereas the lowest nitrite content was also found for *M. illota* honey, the highest was for that of *P. lurida*, varying from 0.30 to 2.88 μmoles nitrites/100 g.

Compared to Czech honeys of *A. mellifera*, both the flavonoid content (1.9 to 15.7 mg Q/100 g) and the polyphenol content (47.4 to 265.5 mg GA/100 g) were lower than those of stingless bee honey from Peru. A value of 2.5 mg flavonoid/100 g honey from Australia, Italy, Portugal, and Spain is reported by the Nutrient Data Laboratory of the US Department of Agriculture, consisting of 0.05 mg apigenin, 0.63 mg luteolin, 0.17 mg isorhamnetin, 0.11 mg kaempferol, 1.03 mg myricetin, and 0.51 mg quercetin [8].

Although flavonoids in honey for nutrition and health are considered as the main polyphenols of *Apis mellifera* honey [9], flavonoids only account for 1.57 to 9.73% of the total polyphenols in the Peruvian Meliponini honeys. Therefore, the bioactive properties of honey need to be explained in combination with other compositional parameters. Non conventional approaches could expand routine understanding limited to honey regulations.

The lowest protein content was found in the *M. grandis* honey, and the highest in that of *N. melanocera*, ranging from 54 to 286 mg/100 g.

These values are similar to the Venezuelan stingless bee honeys, ranging from 10.6 to 162.6 mg N/100 g [10], after transforming the nitrogen content into protein content according to Kjedahl, and considering a protein factor of 6.25.

To our best knowledge this is the first work where the nitrite content of stingless bee honey has been reported. The nitrite content was examined because this is a metabolite of nitric oxide. Nitric oxide and/or nitrite might be responsible, in part, for the biological and therapeutic effects of honey [11]. In this research, we could establish that all the stingless bee honey samples had nitrite, along with antioxidant compounds such as polyphenols and flavonoids. Nitrite reduces lipid peroxidation in simulated gastric fluid [12]. Interestingly, Al-Waili [13] demonstrated that, in healthy individuals, honey solutions increased total urinary nitrite content whilst artificial honey decreased it.

Table 4: Antibacterial and antioxidant activity of honey.

Bee species[1]	Bacterial strains		Antioxidant capacity
	E. coli	*S. aureus*	
	MIC[2] (g/100 ml)		(μmoles TE[3]/ 100 g)
1	50	37.5	237.4 ± 13.1
2	50	50	206.0 ± 9.9
3	45	40	1 07.0 ± 17.3
4	50	50	93.8 ± 10.1
5	50	12.5	569.6 ± 7.3
6	-	-	115.7 ± 3.5
7	50	12.5	205.7 ± 11.3
8	50	12.5	330.2 ± 14.8
9	50	25	255.8 ± 5.0
10	50	25	327.7 ± 2.9
11	50	50	81.0 ± 50.1
control	50	50	17.4 ± 9.2

[1] See bee species in Table 1.
[2]Averages of MIC (Minimal inhibitory concentration).
[3]Averages ± SD of TEAC (Trolox equivalent antioxidant capacity).

Minimal Inhibitory Concentration (MIC) was measured to test the antibacterial activity of honey against *Escherichia coli* and *Staphylococcus aureus*. The antioxidant properties were measured as Trolox equivalent antioxidant capacity (TEAC). Bioactive properties of stingless bee honeys from Peru are given in Table 4, compared with a negative control (artificial honey in the Experimental section).

The minimal inhibitory concentration was lower against *S. aureus* (12.5 -50% w/v) than *E. coli* (50% w/v), similar to a study with *A. mellifera* honey from Argentina [14]. Compared with the MICs of stingless bee honey from Guatemala, 2.5- 10% v/v against *S. aureus* and 5% v/v against *E. coli* [15], the Peruvian stingless bee honeys were less active.

The lowest TEAC was found for *M. illota* honey, and the highest for *N. melanocera*, ranging from 93.8 to 569.6 μmoles Trolox equivalents/100 g, respectively. The classes of TEAC suggested for Czech *A. mellifera* honey [7] require further grades on the scale to position *Nannotrigona melanocera* honey from Peru more accurately than very high (>300), as it was higher than 500 μmoles TE/100 g.

Although honey is not recognized as a powerful antioxidant food like pomegranate or green tea, in this study positive Pearson correlations (P<0.01) were found between flavonoids-TEAC (0.879), polyphenols-TEAC (0.942), proteins-TEAC (0.911), color-TEAC (0.771), and nitrites-TEAC (0.422). These correlations point out the components to explain the mechanisms of the antioxidant action of stingless bee honey, similar to the positive correlation observed between total phenolics and antioxidant activity of Yemeni and imported *A. mellifera* honey [16]. Besides the phenolic and flavonoid profiles of honey, protein content has also been associated with its antioxidant activity [17]. In our honey samples, the water content of honey was not correlated with any other parameter. However, water content (14.2-18.4%) and color (6.18-15.00 mm Pfund) were positively correlated to the water-soluble antioxidant capacity of *A. mellifera* honey from 14 different floral sources [18].

A novel functional profile of commercial stingless bee honey marketed by Achuar natives in the Ecuadorian Amazon compared with *A. mellifera* honey recently addressed a number of bioactivities (i.e. antibacterial against Gram positive and Gram negative bacteria, antimutagenic by yeast strain assays, antioxidant by DPPH and β-carotene bleaching tests) besides routine physicochemical and melissopalynological studies. Special chemical analysis of vitamin E isomers detected the presence of β-tocopherol. The coumarins fraxin and bergamotin were found, as well as the flavonoids luteolin, quercitrin and isorhamnetin [19]. Commercial stingless bee honeys could be admixtures of several species or even unripe *A. mellifera* honey. However, with current efforts to commercialize stingless bee honey in Australia, Brazil, Guatemala, Mexico, Venezuela, Peru and Costa Rica, it is expected that the honey will become more readily available to consumers and for research. In some regions stingless bee honey is sold with the local name of the bee, which may correspond to a single biological species, whereas in other areas, such a name may encompass multiple distinct species or

even several genera. Nevertheless, the identification of the bee species is a valuable addition to any pharmaceutical screening of native honeys, both for basic information about the behavior of the bees and for comparative purposes. In our study, as well as in the results reported by Guerrini *et al*. [19], stingless bees produced honey with higher antioxidant activity than that of *A. mellifera*.

Compositional variations of honey from different species are potential markers for entomological origin, and deserve further multidisciplinary studies for medical applications. Peruvian stingless bee honeys contain variable concentrations of nitrite, and, therefore, this metabolite could be used as an authentication index of honey source. Most notably, the emerging biological functions attributed to the nitrite-nitrate-nitric oxide pathway [20] deserve our attention to understand roles of less investigated honey components.

Antioxidant systems in saliva seem to be of importance locally in the oral cavity as well as in the acidic stomach, where oxidative and nitrosative stress is considerable [21,22] Pietraforte *et al*. [23] reported that urate in saliva is critically involved in the defense against potentially toxic nitrogen species generated from the reaction of salivary nitrite (NO_2^-) with acidic gastric juice. Stomach cancer has been linked to the formation of nitrosamines derived from dietary nitrate and nitrite; ingested nitrate is rapidly absorbed in the upper gastrointestinal tract [24]. For largely undetermined reasons, about 25% of all circulating nitrate is actively taken up by the salivary glands and secreted in saliva [25]. This massive uptake results in salivary nitrate levels that are at least 10-fold higher than plasma levels. In the mouth, commensal bacteria reduce part (about 20%) of the nitrate to nitrite by the action of nitrate reductase enzymes [25]. The vast majority of dietary nitrate comes from vegetables, which are particularly rich in this anion. There has been great concern about the harmful effects of dietary nitrate because the nitrite that is formed will react with acidic gastric juice to form nitrosylating agents (N_2O_3), which in turn promote the generation of potentially carcinogenic nitrosamines [26]. Over the past decade, however, researchers have started to explore possible beneficial effects of nitrate and nitrite locally in the stomach [26,27] and, more recently, also systemically [28,29,30]. Interestingly, it has now been shown that salivary-derived nitrite stimulates gastric mucosal blood flow and mucus generation, which may help to protect the mucosa against luminal aggressors [31]. In addition, acidified nitrite has potent antimicrobial effects, which help in the defense against swallowed pathogens [26, 32]. Many of these effects are clearly related to the formation of NO from acidified nitrite. Antioxidants such as urate, ascorbic acid and polyphenols help to direct the reaction of nitrite in acid toward NO. The absence of these antioxidants, however, allows the formation of potentially harmful reactive nitrogen intermediates with nitrating/ nitrosylating properties. This would promote tissue damage and possibly mutagenesis. Since honey contains nitrite and antioxidants, it is attractive and tempting to think that honey might potentiate the formation of NO and have many positive effects in the stomach and other organs.

Experimental

Honey samples: Honey was collected by syringe extraction from 15 colonies of stingless bees located in San Martin, Peru. One sample of *Apis mellifera* honey was collected from a feral colony and centrifuged.

Artificial honey control: A control syrup was made from 40 g fructose, 30 g glucose, 8 g maltose and 2 g sucrose, made up to 100 mL with distilled water at 121°C for 15 minutes [33].

Antibacterial activity: According to the Clinical and Laboratory Standards Institute [34], the minimal inhibitory concentrations (MIC) were measured against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 chosen as Gram positive and Gram negative strains, respectively.

Antioxidant capacity: Trolox equivalent antioxidant capacity (TEAC) was measured by decoloration of the $ABTS^{+\bullet}$ radical cation. A calibration curve with 0.000-0.625-1.250-2.500 mM Trolox was used to measure the percentage of decoloration, in order to estimate μmoles Trolox equivalents TE/100 g [35].

Color: Color was measured in liquid honey with the C221 Hanna honey color analyzer (mm Pfund), with the following scale provided by the USDA [36]: 1. Water white (≤ 8). 2. Extra white (>8 a ≤17). 3. White (>17 a ≤ 34). 4. Extra light amber (>34 a ≥ 50). 5. Light amber (>50 a ≥ 85). 6. Amber (>85 a ≥ 114). 7. Dark amber (> 114), up to 150 mm Pfund.

Flavonoids: To 0.1 mL of the honey sample solution (10% w/v), 0.5 mL of 20 mg/mL $AlCl_3$ ethanol

solution was added. After 20 min at 37°C, the absorbance was measured at 420 nm. Total flavonoids were calculated, as mg quercetin equivalents QE/100 g honey, from a calibration curve [37].

Moisture: Moisture was estimated by refractive index [38].

Nitrates: Nitrites were estimated by the Griess reaction; absorbance was measured at 540 nm to report μmoles nitrite/100g honey [39].

Polyphenols: Total polyphenol contents in each sample were determined using the Folin-Ciocalteu colorimetric method [40]. The sample solution (0.1 mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, USA) and 0.4 mL of 7.5% Na_2CO_3, and the absorbance was measured at 765 nm after 10 min at 37°C. Total polyphenol contents were expressed as mg gallic acid equivalents GAE/100 g honey.

Proteins: Proteins were measured by the Lowry method [41] using the Folin-Ciocalteu reagent (Sigma, St. Louis, MO).

Supplementary data - Detailed data and images are available at www.saber.ula.ve/stinglessbeehoney

Acknowledgments - Stingless bees were kindly identified by Prof. JMF Camargo, Departamento de Biologia, Faculdade de Filosofia, Ciência e Letras, Universidade de São Paulo, Ribeirão Preto, Brasil. Authors would like to thank CDCHT-ULA for support in the research of stingless bee honey, grants CVI-ADG-M-09-05 and CVI-ADG-FA-04-97.

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Review/Account

Natural Product Communications
2009
Volume 4, Number 9

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