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ORIGINAL RESEARCH ARTICLE

The antimicrobial activity and microbiological safety of stingless bee honeys from Costa Rica

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Summary

Stingless bee honeys from Costa Rica possess etnopharmacological value mainly as a wound dressing. The microbiological study by APHA methods reported that 83 % of the honeys analysed had microbial counts that comply with European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use. All samples reported the absence of *Clostridium botulinum* spores by PCR. Over 90 % of *Tetragonisca angustula* and *Melipona beecheii* honeys inhibited *Pseudomonas aeruginosa* and *Staphylococcus aureus* at minimum inhibitory concentrations lower than Medihoney™. Under the conditions tested, Medihoney™ was not active against *Candida albicans*, whereas 53 % of *T. angustula* honeys rendered inhibition. The melissopalynological analyses reported a homogeneous (monofloral) botanical composition for the Meliponini honeys, which emphasizes the contribution of nectar constituents to the antimicrobial activity and provides foundation to the standardization of a desired inhibitory effect. The traditional use of Costa Rican stingless bee honey as a dressing for burns and wounds reveals the application of a proficient antiseptic agent with low health associated risks.

Actividad antimicrobiana y seguridad microbiológica de mieles de abejas sin aguijón procedentes de Costa Rica.

Resumen

En Costa Rica, las mieles de abejas sin aguijón poseen valor etnofarmacológico principalmente como un tratamiento para heridas. El estudio microbiológico por métodos APHA reportó que 83 % de las mieles analizadas presentaron recuentos microbianos que cumplen con los criterios de calidad microbiológica de sustancias no estériles para uso farmacéutico de la Farmacopea Europea. Todas las muestras reportaron ausencia de esporas de *Clostridium botulinum* por PCR. Más del 90 % de las mieles de *Tetragonisca angustula* y *Melipona beecheii* inhibieron a *Pseudomonas aeruginosa* y a *Staphylococcus aureus* en concentraciones mínimas inhibitorias más bajas que Medihoney™. Bajo las condiciones de ensayo, Medihoney™ no presentó actividad contra *Candida albicans*, mientras que el 53 % de las mieles de *T. angustula* lograron inhibición. Los análisis melisopalinológicos reportaron una composición botánica homogénea (monofloral) para las mieles de meliponinos, lo cual, enfatiza la contribución de los constituyentes del néctar a la actividad antimicrobiana y provee bases para la estandarización de un efecto inhibitorio deseado. El uso tradicional de la miel de abejas sin aguijón de Costa Rica como un tratamiento para quemaduras y heridas devela la aplicación de un eficiente agente antiséptico con reducidos riesgos a la salud.

Keywords: Meliponini, stingless bee, honey, Medihoney™, antimicrobial activity, melissopalynology

Introduction

Honey is a natural product that has more than 4,000 years of pharmaceutical history (Crane, 1999; Cooper, 2005; Jones, 2001; Molan, 2001a). Despite having a long established usage as a wound dressing, its application in medicine was progressively set-aside in mid 1900s due to the advent of antibiotic therapy (Cooper, 2001; 2005). During the last two decades, the antimicrobial resistance phenomenon has boosted research and development of alternative therapies for wound and burn healing (Bowler *et al.*, 2001; Bryskier, 2005a; Howell

- Jones *et al.*, 2005; Salyers and Whitt, 2005). These circumstances led to a resurgence of interest in the application of honey for medicinal purposes (Cooper *et al.*, 2002; Lusby *et al.*, 2002; Molan, 2001a; 2006). The best-known example of the rediscovery of honey as a wound dressing is provided by the studies on the honey produced by the honey bee (*Apis mellifera*) from the nectar foraged from the inflorescences of the manuka tree (*Leptospermum scoparium*) found in Australia and New Zealand (Cooper *et al.*, 2002; Irish *et al.*, 2006; Stephens *et al.*, 2005; Wilkinson and Cavanagh, 2005; Willix *et al.*, 1992).

The concept of "Active Manuka Honey" was developed as a means to standardize its antimicrobial activity. Manuka honey owes its effects to an unidentified constituent that allows honey to exert antimicrobial activity beyond the inhibitory action of osmolarity, pH, or hydrogen peroxide generated by the enzyme glucose oxidase present in *Apis mellifera* honey. A phenol calibration curve was used for the standardization of the UMF ("unique manuka factor") (Allen *et al.*, 1991; Willix *et al.*, 1992). Recently, the identity of the UMF has been attributed to methylglyoxal (Adams *et al.*, 2008; 2009, Atrott and Henle, 2009; Kwakman *et al.*, 2010; Mavric *et al.*, 2008). However, this finding is still a point of discussion (Snow and Manley-Harris, 2004; Stephens *et al.*, 2010; Weston, 2000). Active Manuka Honey has been subjected to clinical trials, where it has demonstrated its efficacy as a wound-dressing agent. In 2008 the Food and Drug Administration (FDA) gave approval for marketing of Medihoney™ (Dermasciences); a manuka honey-based wound dressing (Cooper, 2005; Federal Drug Administration, 2008; Lusby *et al.*, 2002; Simon *et al.*, 2006).

Bees (Hymenoptera, Apidae) in the Meliponini tribe are commonly known as stingless bees. These social insects are found in the tropical regions of the world. In the neotropics, stingless bees have an important role as native pollinators responsible for the reproduction and conservation of indigenous flora (Cortopassi-Laurino *et al.*, 2006; Michener, 2000; Roubik, 1989; Sommeijer, 1999). The practice of keeping stingless bees (meliponiculture) was developed by the ancient Maya culture. Records of stingless beekeeping and its meaning to Maya society can be found in the Madrid Codex (Vail, 2006). Meliponini bees constituted an important part of their cultural life. The Maya honoured the bee gods and considered the honey sacred. Honey was involved in their religious rituals, used as a sweetener and had medicinal applications (Cortopassi-Laurino *et al.*, 2006; de Jong, 1999; Sommeijer, 1996; Vit *et al.*, 2004).

Meliponiculture nowadays is a practice present in all tropical America. Stingless bee honey is still kept in high regard. The use of these honeys in traditional medicine as a treatment of infected wounds, burns, digestive disorders, respiratory tract infections and eye illnesses like cataracts and conjunctivitis is still common practice (Cortopassi-Laurino *et al.*, 2006; de Jong, 1999; Sommeijer, 1996; 1999; Vit *et al.*, 2004). In Costa Rica, *Melipona beecheii* and *Tetragonisca angustula* are the stingless bee species of most commercial interest due to the unique taste and medicinal value of their honey (Aguilar *et al.*, 2013; Cortopassi-Laurino *et al.*, 2006; Kent, 1984; Sommeijer, 1996; 1999). According to traditions, the application of Meliponini honeys in wound healing is feasible. Nevertheless, there are few studies that report the antimicrobial activity of these products (de Bruijn and Sommeijer, 1997; Dardón and Enríquez, 2008; Dardón *et al.*, 2013; De Mera and Angert, 2004; Miorin *et al.*, 2003; Sgariglia *et al.*, 2010; Temaru *et al.*, 2007; Zamora *et al.*, 2013) and none that evaluate their microbial content and therefore, the health risk associated to the practice of using stingless bee honey as a wound dressing agent.

To our knowledge, this paper is the first report on microbiological safety and antimicrobial activity in comparison to Medihoney™ and the botanical origin of *Tetragonisca angustula*, *Melipona beecheii*, *Melipona costaricensis*, *Scaptotrigona pectoralis*, *Cephalotrigona capitata* and *Tetragona perangulata* honeys from Costa Rica.

Table 1. Water content and density of stingless bee honeys and Medihoney™. Data are expressed as mean values and range per bee species.

Bee species	**Water content (%) + / - 0.1**	**Density (g / cm^3) + / - 0.001**
Apis mellifera (Medihoney™)	18.5	1.416
Overall stingless bee honeys	23.2 (19.8 – 31.8)	1.378 (1.310 – 1.397)
Tetragonisca angustula	22.1 (19.8 – 24.8)	1.381 (1.334 – 1.397)
Melipona beecheii	23.6 (21.6 – 25.4)	1.380 (1.363 – 1.394)
Melipona costaricensis	26.7 (25.2 – 27.8)	1.357 (1.352 – 1.369)
Scaptotrigona pectoralis	25.1 (24.2 – 26.0)	1.373 (1.367 – 1.378)
Cephalotrigona capitata	27.4	1.359
Tetragona perangulata	31.8	1.310

Material and methods

Sample collection

A total of 65 honeys were directly bought from keepers of Meliponini bees. The samples were collected during the harvest season (April, 2008), a period of approximately two to three weeks right after Easter holidays. Every sample consisted of 500 g to 1 kg of honey. The association of each sample to a particular bee species was verified. When necessary, the species origin was confirmed by comparison of bee specimens collected from the hives with Ayala´s taxonomic classification key (Ayala, 1999). The honeys were harvested from hives of the following Meliponini species: *Tetragonisca angustula* (n = 36), *Melipona beecheii* (n = 21), *Melipona costaricensis* (n = 4),

Scaptotrigona pectoralis (n = 2), *Cephalotrigona capitata* (n = 1) and *Tetragona perangulata* (n = 1). The sample collection was in areas where meliponiculture is traditionally practiced (Cortopassi-Laurino *et al.*, 2006; Kent, 1984; Sommeijer, 1996). Commercially available Medihoney™ hydrocolloid wound paste (Dermasciences) was used as a reference for antimicrobial activity.

Microbial content

The total aerobic count, yeast and moulds count and most probable number (MPN) of total coliforms, faecal coliforms (*Escherichia coli* as indicator) followed American Public Health Association (APHA) methods in accordance with Pouch-Downes and Ito (2001). The microbial counts are reported through descriptive statistics.

Determination of Clostridium botulinum by polymerase chain reaction (PCR)

The evaluation of the presence of *C. botulinum* types A, B, E and F was performed following the method developed by Lindstrom *et al.* (2001), as described by Fournier *et al.* (2006).

Water content and total solids percentage

The water content of the reference and samples was performed by refractive index determination in an ABBE-3L refractometer (Milton Roy Company; USA) and expressed as percentage of humidity following the Harmonized Methods of the European Honey Commission as described by Bogdanov *et al.* (2002). Total solids percentage was calculated as the subtraction of the percentage of water content to the total composition of honey (100 %). This value was used in the MIC test interpretation as a correction factor due to the differences in water content that honeys presented.

Density

The density of honey was calculated by means of specific gravity calculations at 25.0 +/- 0.1 °C. This physical parameter was determined with an 11.00 ml picnometer (Cole-Parmer; USA). In brief, the picnometer is filled with distilled water, the temperature of the device brought to 25.0 +/- 0.1 °C in a water bath and its weight determined in an analytical balance (precision +/- 0.0001 g). The device was cleaned and dried, then filled with honey and its weight was measured under the conditions previously described. The density was calculated at 25.0 +/- 0.1 °C according to the following equation:

δ honey = (mass of honey / mass of water) x δ H_20

Where δ honey is the density of honey and δ H_20 is the density of water at 25.0 +/- 0.1 °C (0.997 g / cm^3).

Antimicrobial activity

The evaluation of the antimicrobial activity of the samples and Medihoney™ was developed in a 96-well microtiter plate-based minimum inhibitory concentration (MIC) assay. The tests were performed against American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19116), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 9027), a clinical isolate of *Staphylococcus epidermidis* (UCR 2902) part of the collection of the Faculty of Microbiology of the Universidad de Costa Rica and *Candida albicans* (ATCC 10231).

Every test consisted of eleven serial dilutions (1:1) in Mueller – Hinton Broth (MHB) (Oxoid, UK) of a 500 mg / ml honey test solution in duplicate against the culture tested. Under the template proposed, four tests can be performed per microplate. Three replicates were done for every sample and reference. The final volume contained in every well of the plate was 200 µL. Aseptic technique was maintained during all steps of the assay. The MIC test was carried on a biosafety level II laminar airflow chamber (Labconco; USA).

Sample preparation

An aliquot of 0.900 g to 1.000 g of the sample or Medihoney™ was weighed in an analytical balance (Ohaus; USA) and prepared in a 1.5 ml microcentrifuge tube. A start solution of 1000 mg / ml of honey in MHB was prepared for each test. The latter took in regard the individual density of each sample and reference. Finally, a test solution with a concentration of 500 mg / ml in MHB and a volume of 1 ml was prepared out of every start solution. Both preparations were throughout dissolved in a vortex mixer (Cole-Parmer; USA).

Culture solution

A fresh culture of the microorganism to test was previously prepared in Brain Hearth Infusion Agar (BHIA) (Difco, USA) and subjected to incubation for 24 h at 35°C (48 h at 35°C for *C. albicans*). Colonies were withdrawn of the culture and dissolved in a tube that contained 5 ml of sterile peptone water (peptone, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 7.2 + / - 0.2). The turbidity of this solution was calibrated to a concentration of 1.0×10^8 cfu / ml with a 0.5 Mac Farland nephelometric standard and later an aliquot of 150 µl was dissolved in 14850 µl of sterile MHB. The final concentration in the micro plate was 5.0×10^5 cfu / ml.

MIC test preparation

Once the dilutions of the samples or Medihoney™ were prepared, 100µl of the culture solution were dispensed in all the wells of the microplate with exception of the negative control. The negative control of the test consisted of four wells containing 200 µl of MHB only (0% growth). Another four wells containing 100 µl of MHB and

100 µl of the culture solution performed as the positive control (100% growth). After the plates were prepared, the lid and bottom borders of the plates were sealed with parafilm (Pechiney; USA) to prevent evaporation during the incubation period (24 h at 35°C for bacteria, 48 h at 35°C for *C. albicans*) in a gravimetric airflow incubator (Digisystem Laboratory Instruments Inc.; Taiwan).

MIC test interpretation

After incubation, the test plates were opened in the laminar airflow chamber and visually inspected over a dark background. Microbial growth inhibition was evidenced as absence of turbidity as established by the negative control. The MIC was determined as the lowest concentration that inhibited the growth of the culture in test. As a confirmatory phase, a loopful of the MIC value well of every sample was seeded in a BHIA plate. Absence of growth after incubation for 24 h (48 h for *C. albicans*) at 35°C was considered confirmation of results. The minimum inhibitory concentration results presented herein are expressed in milligrams of total solids of honey per millilitre of solution. The MIC of every sample was multiplied by its percentage of total solids. The values obtained were subjected to this correction in order to achieve a quantitative result that allows a direct comparison between the antimicrobial activity of stingless bee honeys and Medihoney™ despite the differences of water content and density.

Botanical origin

The botanical origin of the stingless bee honey samples was determined through a melissopalynological analysis. Pollen grains were concentrated from samples, mounted on microscope slides and quantified (between 200 to 300 grains). Relative amounts were expressed in percentages, and classified according to frequency in the following classes: predominant pollen (>45 %), secondary pollen (16 % – 45 %), minor secondary pollen (3 – 15%) and minor pollen (< 3 %) as described by Hodges (1984). The pollen grains were taxonomically identified to species level by means of capturing digital images from the slides and comparisons to pollen grain identification keys (Bush and Weng, 2007; Martínez *et al.*, 1993; Palacios *et al.*, 1991).

Statistical analyses

Normal distribution evaluation, statistical inference and all descriptive statistics were executed with the InfoStat software (InfoStat Group, Universidad Nacional de Córdoba; Argentina).

Results

All Meliponini samples presented a low total aerobic count (< 1.0 x 10^1 cfu / g; EST of honey), absence of total coliforms, faecal coliforms and *E. coli* (< 3 MPN / g of honey) in 25 grams. Concerning the total yeast and moulds counts, 83 % of samples reported a count lower than 1.0 x 10^1 cfu / g of honey. The remaining 17% reported count values in the range of 9.0 x 10^2 to 9.0 x 10^6 cfu / g. The *C. botulinum* types A, B, E and F used as references produced by PCR the expected amplification products. On the contrary, none of the samples gave positive results for any serotype under examination.

The density, water content and MIC results of the *T. angustula* and *M. beecheii* samples were analysed through a normality test. A modified Shapiro – Wilks test was performed for each parameter. The *M. costaricensis*, *S. pectoralis*, *C. capitata* and *T. perangulata* honeys were scarce in number, therefore not considered for this test. The density of *M. beecheii* honeys ($p = 0.8684$) and the humidity of *T.*

Table 2. Percentage of *T. angustula*, *M. beecheii* and overall stingless bee honeys that obtained a MIC under the conditions tested and percentage of samples that achieved a MIC lower than Medihoney™ (< MDHY).

Microorganisms tested	Stingless bee species				Overall stingless bee honeys (n = 65)	
	Tetragonisca angustula (n = 36)		*Melipona beecheii* (n = 21)			
	Total (%)	< MDHY (%)	Total (%)	< MDHY (%)	Total (%)	< MDHY (%)
S. aureus	94	92	95	90	95	91
S. epidermidis	94	92	100	95	97	91
L. monocytogenes	78	78	100	100	88	88
E. coli	83	47	100	57	91	52
S. enteritidis	50	50	100	100	71	71
P. aeruginosa	97	97	100	100	98	98
C. albicans	53	53	5	5	33	33

Table 3. Minimum inhibitory concentrations of honeys against the microorganisms tested. Data are expressed as median values and range per bee species (mg / ml) and for stingless bees as a group (overall). S B = stingless bee species, *T. a = Tetragonisca angustula, M. b = Melipona beecheii, M. c = Melipona costaricensis, S. p = Scaptotrigona pectoralis, C. c = Cephalotrigona capitata, T. p = Tetragona perangulata,* MDHY = Medihoney™: reference, >250 mg/ml = no inhibition under the conditions tested, * = only one sample caused inhibition.

S.B.	Microorganisms tested						
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Listeria monocytgenes***	***Escherichia coli***	***Salmonella enteritidis***	***Pseudomonas aeruginosa***	***Candida albicans***
T. a	49.2 (23.5 – 195.5)	96.6 (23.5 – 200.5)	97.3 (47.0 – 200.5)	99.0 (47.0 – 199.3)	196.0 (47.4 – 200.5)	97.8 (47.3 – 200.5)	195.0 (189.5 – 200.5)
M. b	24.5 (23.4 – 192.0)	24.2 (11.8 – 191.5)	93.8 (23.4 – 98.0)	97.5 (93.3 – 196.0)	188.5 (46.9 – 196.0)	48.6 (46.8 – 194.5)	188.5*
M. c	103.8 (45.1 – 187.0)	126.5 (45.1 – 187.0)	126.5 (45.1 – 187.0)	160.3 (92.3 – 187.0)	123.2 (90.3 – 187.0)	137.8 (90.3 – 187.0)	>250 mg/ml
S. p	23.4 (23.1 – 23.7)	17.5 (11.8 – 23.1)	23.4 (23.1 – 23.7)	70.6 (46.3 – 94.8)	46.9 (46.3 – 47.4)	46.9 (46.3 – 47.4)	185*
C.c	45.4*	45.4*	45.4*	45.4*	45.4*	45.4*	>250 mg/ml
T. p	21.3*	10.6*	10.6*	42.6*	21.3*	42.6*	170.5*
Overall	48.4 (21.3 – 195.5)	48.6 (10.6 – 200.5)	94.4 (10.6 – 200.5)	98.3 (42.6 – 199.3)	188.5 (21.3 – 200.5)	95.8 (42.6 – 200.5)	194.5 (170.5 – 200.5)
MDHY	101.9	101.9	203.8	101.9	203.8	203.8	>250 mg/ml

angustula samples (p = 0.1669) were normally distributed. The remaining parameters, including the MIC results did not follow a normal distribution (p < 0.0001). These conditions exclude the possibility of an analysis of variance (ANOVA) and consequently all the mentioned results were evaluated through descriptive statistics. The water content and density results are described in Table 1. The mean water content of all Meliponini honeys (overall) was 25 % higher than the value obtained for Medihoney™. Likewise, the mean value reported per bee species was higher. A correlation analysis was performed between the density and the water content parameters of *T. angustula* and *M. beecheii* honeys. The statistical inference returned a Pearson coefficient of – 0.62 for *T. angustula* and – 0.84 for *M. beecheii* honeys respectively. Both are negative correlations.

All the Meliponini honeys under scope presented inhibitory activity at least against one of the microorganisms tested and 100% accomplished an MIC lower than the reference against one of the type culture strains assayed as a minimum. The results comprised in Table 2 and Table 3 disclose the broad-spectrum antimicrobial activity of the Meliponini honey under trial against culture strains of medical importance.

In general, a honey is considered produced mainly from one floral resource (monofloral) if the pollen of the plant is predominant (Hodges, 1984; Sawyer, 1988). All the Meliponini honeys analysed possess a predominant nectar source (pollen frequency > 45 %). The predominant botanical source for the *T. perangulata* sample corresponded to *Brosimum alicastrum* (pollen frequency 63 %). The *M. costaricensis*, *S. pectoralis* and *C. capitata* honeys shared the same major nectar resource: *Miconia argentea* (pollen frequency range 67 % to 79 %). Ten floral sources constituted the main nectar origin of the *T. angustula* and *M. beecheii* samples (pollen frequency range 45 % to 94 %) (see Fig. 1). The major botanical resource of 52 % of the *T. angustula* honeys is concentrated in *Spondias purpurea* and *Gliricidia sepium* flora. *M. beecheii* samples presented as predominant nectar source *Tabebuia ochracea* and *Andira inermis* species in 52 % of the analysed cases.

Discussion

The harvest of stingless bee honey is still rudimentary (Cortopassi-Laurino *et al.*, 2006; Sommeijer, 1999). Although all the samples were collected in meliponaries that use wooden boxes for housing the bee colonies, the lack of innovative tools for honey extraction means a slow and manual process outdoors or inside the keepers house. With the aid of suction devices, honey is deposited in pitchers or jars during harvest and later dispensed in bottles or small dropper containers for commerce. The absence of adequate extraction facilities and tools could lead to spoilage of honey due to pathogen vectors like flies or excessive manipulation. Therefore, the microbiological quality and safety of the product was analysed.

It can be hypothesized that the absence of *C. botulinum* spores reflects the scarcity of these sporulated anaerobe in the neotropical environment where the indigenous bees collected the nectar to produce honey. The biodiversity present in Mesoamerica and in particular in Costa Rica, might create a highly competitive microbial niche that in addition to the presence of antimicrobial agents in

honey, do not offer suitable conditions for *C. botulinum* survival.

At present there are no microbiological quality standards specified for stingless bee honeys. Nevertheless, as stressed by the Codex Alimentarius Commission, the absence of food borne pathogens is mandatory (Codex Alimentarius Commission, 1997; 2001). In addition, for wound healing applications the product must be free of viable *C. botulinum* spores (Lusby *et al.*, 2002; Molan and Allen, 1996; Molan, 2001b; Wahdan, 1998). Our results show that 83 % of the honeys analysed had microbial counts that comply with the European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use (European Directorate for the Quality of Medicines and HealthCare, 2011). The absence of *C. botulinum* spores suggests that the prevalence of this microorganism in Meliponini honeys proceeding from Costa Rica is low. Both conditions indicate that the health risk associated to the practice of using Costa Rican stingless bee honey as a wound-dressing agent is low.

Several authors have reported the high water content of stingless bee honeys compared to that of *A. mellifera* (de Bruijn and Sommeijer, 1997; Souza *et al.*, 2006; Vit *et al.*, 1994). In addition, the percentage of humidity of Meliponini honey can vary according to the species and the geographical area where it is produced (Bijlsma et al., 2006; Vit *et al.*, 2004).

The statistical inference performed between the density and the water content parameters returned negative correlations, thus the density value decreases along with an increase in the percentage of humidity. The latter justifies the determination of density and water content parameters of every honey sample and their suggested role in achieving quantitative and comparable minimum inhibitory concentrations.

Burn and wound patients are high-risk groups for infection. Strains of *S. aureus*, *P. aeruginosa* and *C. albicans* are the most common agents that severely undermine the healing process (Howell-Jones *et al.*, 2005; Méan *et al.*, 2008). In addition, infected burns and wounds can play a role as reservoir and source of dissemination of antibiotic resistant microorganisms (Ha *et al.*, 2011; Howell-Jones *et al.*, 2005; Wilkinson and Cavanagh, 2005).

The broad-spectrum antimicrobial activity (Cooper *et al.*, 1999; 2002; Irish *et al.*, 2006), clinical efficacy (Simon *et al.*, 2006) and subsequent Federal Drug Administration (FDA) approval of the use of Medihoney™ as a dressing (Federal Drug Administration, 2008; Lusby *et al.*, 2002) set a milestone in burn and wound healing therapy. To our knowledge, this is the first report of the antimicrobial activity of stingless bee honeys directly compared to Medihoney™ by means of a minimum inhibitory concentration assay.

The commerce of stingless bee honey in Costa Rica has a blooming market. This trade is mainly sustained by *T. angustula* and *M. beecheii* honeys owe to their use as a traditional medicine

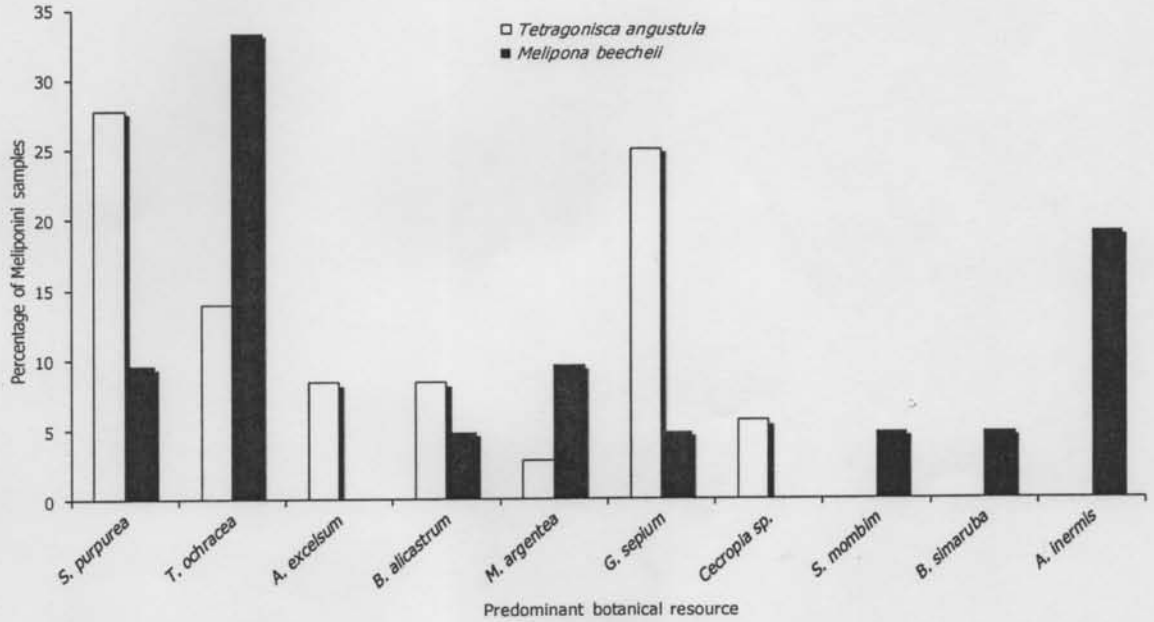

Fig. 1. Predominant nectar resources of *M. beecheii* and *T. angustula* samples. *S. purpurea = Spondias purpurea, T. ochracea = Tabebuia ochracea, A. excelsum = Anacardium excelsum, B. alicastrum = Brosimum alicastrum, M. argentea = Miconia argentea, G. sepium = Gliricidia sepium, S. mombim = Spondias mombim, B. simaruba = Bursera simaruba, A. inermis = Andria inermis.*

(Cortopassi-Laurino *et al.*, 2006; Sommeijer, 1996). A high percentage of the samples produced by these two bee species inhibited *S. aureus* and *P. aeruginosa*, the two major causes of morbidity and mortality due to burn and wound infection (Howell-Jones *et al.*, 2005) at MICs lower than Medihoney™. Under the conditions tested, the reference was not active against *C. albicans*, the dominant cause of opportunistic mycoses (Pfaller and Diekema, 2007; Warnock, 2006), whereas 19 *T. angustula* honeys (n = 36) rendered inhibition. *Candida* infections in burn and wound patients represent a major risk for invasive candidiasis (Ha *et al.*, 2011; Lim *et al.*, 2012; Pfaller and Diekema, 2007). Therefore, in accordance with results, *T. angustula* honey proceeding from Costa Rica represent a suitable subject for further research oriented towards the development of a burn and wound dressing comprising selected broad-spectrum antibacterial and anti-*Candida* activity honey. The microbiological safety and inhibitory activity of the stingless bee honey samples reported here, justify their traditional use as a dressing for infected burns and wounds. Furthermore, the determination of the antimicrobial activity is the cornerstone of upcoming research and development (Bryskier, 2005b).

There are only three previous reports of the inhibitory activity of Meliponini honey from Costa Rica. de Bruijn and Sommeijer (1997) analysed through an agar diffusion assay the antimicrobial activity of stingless bee honeys against strains of *S. aureus*, *Bacillus cereus*, *E. coli* and *P. aeruginosa*. The honeys under trial included an undisclosed number of *M. beecheii* honeys from Costa Rica. The authors solely reported that the growth of most bacteria was inhibited. De Mera and Angert (2004) by a method similar to the described by de Bruijn and Sommeijer (1997) reported inhibition of *P. aeruginosa*, *B. cereus*, *Candida albicans* and *Saccharomyces cerevisiae* by *A. mellifera* and *T. angustula* honeys from Costa Rica. None of the samples under study inhibited *S. aureus*. Contrary to this report, Estrada *et al.* (2005) determined that 80 % of the *A. mellifera* samples studied caused inhibition of *S. aureus*. In addition, 94 % of the *T. angustula* samples subject of the present trial had antimicrobial activity against the mentioned gram-positive cocci. The inhibitory effects of honey from the two mentioned bee species against *S. aureus* was also reported for samples proceeding from Brazil (Miorin *et al.*, 2003). Up to present, the most recent studies of antimicrobial activity of honey produced by Mesoamerican stingless bees are the manuscripts published by Dardón et al. (2013) and Zamora *et al.* (2013). Dardón *et al.* (2013) reported the inhibitory activity of honey produced by eight bee species from Guatemala against eight pathogen microorganisms. Zamora *et al.* (2013) compared the antimicrobial activity of *Apis mellifera* honey and stingless bee honey from Costa Rica. The authors reported a higher biological activity in the honey produced by Meliponini bees.

Temaru *et al.* (2007), Dardón and Enríquez (2008), Sgariglia *et al.* (2010) and recently Zamora *et al.* (2013) reported inhibition by *T. angustula* and *M. beecheii* honeys against the same type culture strains of *S. aureus*, *E. coli* and *P. aeruginosa* subject of the present study. Unfortunately, with the exception of Miorin *et al.* (2003) and Sgariglia *et al.* (2010), all the studies mentioned performed antimicrobial activity determinations by means of agar diffusion. These methods are low in sensitivity, do not determine a MIC and since non-polar constituents may not diffuse through agar, do not represent the total antimicrobial activity of honey (Boorn *et al.*, 2010). Moreover, none took in regard the differences of water content and density between samples or applied the same methodology. Additionally, they did not use a proper reference (medical grade honey) or determined up to species level the botanical origin of the honey studied. Under these conditions, a proper results comparison between studies is not possible. We suggest the minimum inhibitory concentration assay described herein as a standard to achieve comparable results between Meliponini and *Apis mellifera* honeys. A quantitative measure of the antimicrobial activity is fundamental in setting quality parameters for future research and wound dressing developments.

The proposed method rules out the osmotic effect of honey. As a matter of example, the first concentration in the serial dilution of the MIC test is of 250 mg / ml of honey (volume 200 µl). In the case of the Medihoney™ used as a reference (density 1.416 g / cm^3) this would represent a solution of 18 % (v/v). As reported by Cooper *et al.* (1999) a 22 % (v/v) sugar solution is the minimum concentration capable to inhibit microbial growth due to an osmotic shock. Consequently, the antimicrobial activity reported for the reference and the stingless bee honey samples under study cannot be explained by the extent of the microbial inhibition attributable to sugar content. Similar findings were reported for *Apis mellifera* honey by Molan (1992) and Snow *et al.* (2004).

Several authors have hypothesized over the nature of the antimicrobial activity caused by stingless bee honeys. Vit *et al.* (1994) stated that the stability to fermentation despite high water content might be related to the inhibitory activity. Moreover, the mentioned effect was suggested to find a partial explanation through diffusion of secondary plant metabolites from the cerumen walls of the storage pots to the honey, symbiotic bacteria, enzymes and other non-identified constituents that bees may add during the process of transforming nectar into honey (de Bruijn and Sommeijer, 1997, Dardón and Enríquez, 2008, Nogueira-Neto, 1997, Temaru *et al.*, 2007).

Although the cited explanations may play a role in the nature of the antimicrobial activity of Meliponini honeys, all the mentioned articles on the subject share the same weak point: absence of a proper identification of the botanical origin of honey to species level; which at the same time disregards the contribution of nectar sources to the effect caused by stingless bee honeys. Only the melitopalynological examination can reveal the true nature of the bee forage in an area and its contribution to the honey (Sawyer, 1988).

The botanical resources influence the antimicrobial activity of

honey (Henriques *et al,* 2005; Vit and Tomás-Barberán, 1998; Wilkins *et al.*, 1993). Nectar is the prime material of honey production and its composition depends on the plant species. Among nectar constituents, secondary plant metabolites like flavonoids and terpenoids play important roles as signalling molecules for pollinators, control of microbial contaminants due to their antimicrobial activity and are part of the metabolic offering for flower visitors (Nicholson and Thornburg, 2007). The identification of the nectar resources foraged by stingless bees to produce honey is a key point towards antimicrobial activity standardization. The latter since a determined botanical origin may define the presence and concentration of specific plant derived antimicrobials.

The melissopalynology results reflect the optimum forage behaviour pattern of stingless bees. A reduced number of botanical species constitute the bulk of the diet resources (Beismeijer and Slaa, 2004; van Nieuwstad *et al.*, 1997). This behaviour is strengthened by the massive blooming syndrome that numerous flora exhibit in the neotropics. An abundant supply of resources that are eagerly collected by bees and generate homogenous and convergent dieting strategies that focuses on more profitable sources in close proximity (Eltz, 2001; Ramalho *et al.,* 1990; 2004; Roubik *et al.*, 1986). A defined and monofloral botanical composition, as the one reported herein, emphasizes the contribution of nectar constituents to the antimicrobial activity.

The stingless bee honeys studied are predominantly of high microbiological quality, report absence of *C. botulinum* spores and exhibit antimicrobial efficacy comparable to Medihoney™. Hence, the traditional use of Costa Rican Meliponini honey as a dressing for burns and wounds reveals the application of a proficient antiseptic agent with low health associated risks.

Finally, the monofloral botanical composition reported herein provides a foundation for further research on the antimicrobial activity against antimicrobial-resistant clinical isolates, the standardization of a desired inhibitory effect, and sets the cornerstone for upcoming studies over other beneficial bioactivities that stingless bee honeys could render to wound care.

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