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Antimicrobial activity of honey produced in the West of the state of Pará, Brazil.

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ABSTRACT

Honey is a natural product that has several benefits for health properties such as anti-inflammatory, antioxidant and antimicrobial. Considering these proprieties, this study aimed to evaluate the antimicrobial activity of honey from *Apis mellifera* bee, produced in eight communities at Santarém, Pará - Brazil, against microorganisms of clinical interest. The antimicrobial activity of honey was tested against the species, such as *Staphylococcus aureus*, *Staphylococcus* spp., *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, using well diffusion method. All honey samples (MIC: 12.5% (v/v)) showed activity against at least one species in the 24 h period, and the different activities may be related to both the floral source of honey as the geographical factors. The M02 sample showed the best results (MIC: 6.25% (v/v)) against the three pathogenic microorganisms tested.

Key words: Anti-bacterial agents, honey, *Apis mellifera*, apitherapy.

INTRODUCTION

Among most of natural products studied, honey has been highlighted since it presents a great therapeutic and food values. It is used for this purpose due to its composition which includes carbohydrates, especially glucose and fructose, phenolic acids, flavonoids and micronutrients such as essentials vitamins and minerals (Adenekan et al., 2010; Borsato et al., 2014). This composition gives it many beneficial health properties, such as anti-inflammatory, antimicrobial, antioxidant, emollient, anti-caries, immune stimulant, healing, anti-cancer and bactericide, in addition to his great energy value (Alvarez-Suarez et al., 2010).

Antimicrobial activities of the honey are related to several factors, including its low water content, high sugar content, low pH, the presence of hydrogen peroxide and several compounds extracted from the plant secondary metabolism, including flavonoids and phenolic derivatives (Kwakman et al., 2010; Halawani and Shohayeb, 2011). This variable composition is directly related to the raw material, that is, the floral origin used for the production of honey, geographical factors, seasonality, bee subspecies and post collection treatment (Kasconiene and Venskutonis, 2010; Mazanares et al., 2011; Escuredo et al., 2013). Many different studies have shown that upland forests have great diversity of species and large percentage of species with only one individual per hectare, and low floristic similarity between nearby portions. The dissimilarity among plants in communities located at Western Amazon is associated mainly with the topography and soil characteristics (Oliveira et al. 2008).

Some botanical species that may exist in geographical region of honey collection is the Braziln nut tree (*Bertholletia excelsa*), from Lecythidaceae family, where bees are the main pollinators. The Jeniparana species (*Gustavia hexapetala*) also might be present in Amazonian region. This specie does not have any kind of structure to difficult the access of bees to nectar, the plants species produce only one type of pollen, so the pollen collected to feed the bee larvae is equal to that fertilizes the flowers next visited plant. The Sapucaia species (*Lecythis pisonis*) also could be present, although, the main pollinators of these species are bees from *Xylocopa frontalis* species (Mori, 2001).

The complex and variable composition of honey confers antimicrobial activity against a broad spectrum of microorganisms, both sensitive and antibiotic-resistant pathogens (Overgaauw and Kirpensteijn, 2006). It has been shown, several benefits provided by honey, either for food and/or therapeutic (Namias, 2003; Vit et al., 2008), but more studies are needed to understand the microbiological action (Souza et al., 2009). In view of this, this study aimed to evaluate the antimicrobial activity of *Apis mellifera* honey produced at Santarém-PA, Brazil, against microorganisms of clinical interest, given the peculiarities of the Amazon region, which has a wide variety of floral species.

EXPERIMENTAL

Sample location

A total of 24 honey samples of *A. mellifera* were collected in triplicate, with diverse floral source on November 2014, through manual extraction without any prior treatment. The samples were obtained from eight apiaries located in: Cipoal (H01) (02º54'13''S and 54º78'37''W), Cedro (H02) (02º64'15''S and 54º77'89''W), Bueira (H03) (02º63'77''S and 54º65'13''W), Boa Fé NA (H04) (02º61'35''S and 54º65'92''W), Boa Fé AP (H05) (02º61'89''S and 54º66'47''W), Tipizal (H06) (02º62'73''S and 54º61'13''W), Jacamim (H07) (02º59'34''S and 54º62'21''W) and Terra Amarela (H08) (02º584'42''S and 54º65'95''W) communities of Santarém, Pará – Brazil. Before analysis, all samples were stored at 4°C.

The presence of secondary forest was observed around all apiaries. The point H02 has a high amount of Brazil nut trees and did not have any type of agricultural activity at distance lower than 2000 m. Close to collection points H03, H04, H05, H07 and H08 have livestock areas and close to collection points M01 and M06 have a soybean field, at distances from 500 to 1500 m and 400 m, respectively.

Evaluation of antimicrobial activity

The test organisms used in the antimicrobial assays were *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) (CEFAR®, São Paulo, Brazil) and *Staphylococcus* spp. (bovine mastitis) (UFOPA/LM 003) and *Staphylococcus saprophyticus* (UFOPA/LM 006). They were obtained from Laboratory of Microbiology of the Universidade Federal do Oeste do Pará (UFOPA). All strains were grown in sterilized nutrient medium (NM - 3.0 g bacteriological peptone, 5.0 g of meat extract, 5.0 g NaCl in 1000 mL aqueous solution containing initial pH 7.2-7.4) until the preparation of bacterium suspensions.

In vitro antibacterium activity of honey was evaluated using the agar well-diffusion assays. An aliquot of 100 µL of each bacterium suspension adjusted to 0.5 of the McFarland scale, corresponding to 10^8 CFU mL^{-1} of bacterium, were seeded in sterile petri dishes (15 mm × 90 mm) containing Mueller-Hinton agar (MHA) (Sigma-Aldrich®, St. Louis, USA). After solidification, plates were punched to make the well of 6 mm diameter. 100 µL of each honey sample were poured, in quadruplicate, into the well in assay plates (Thakur, 2009). The negative control was carried out in a Petri dish without addition of honey. Osmotic control was performed replacing honey samples by 65% (w/v) of glucose solution in ultrapure water.

An antibiogram for each microorganism was generated using conventional antibiotics discs (ampicillin, amoxicillin + clavulanate, amikacin, cefepime, ceftazidin, cefalotin, cefuroxime, chloramphenicol, clindamycin, ciprofloxacin, cefoxitin, gentamicin, moxifloxacin, meropenem, nitrofurantoin, norfloxacin, oxacillin, penicillin G, rifampicin, sulfazotrim, tetracycline and vancomycin) (Cefar®, São Paulo, Brazil). The plates were incubated at 35°C for 48 h. The antibacterium activity was detected by the presence of inhibition halos around the wells and expressed in millimeters.

The broth dilution technique was used to ascertain the Minimum inhibitory concentration (MIC) of honey samples (Taveira et al., 2010). A non-diluted honey sample was added in the first tube and a serial dilution of honey in ultrapure water was established to obtain the following honey concentrations: 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78% (v/v).

Subsequently, 100 µL of Mueller Hinton Broth (MHB) (Sigma-Aldrich®, St. Louis, USA), 10 µL of suspension with 10^8 CFU mL^{-1} and 100 µL of each honey solutions were added to each pool for MIC (Khan et al., 2014). 15 µL of resazurin solution (0.01%) was used as chemical colorant. Negative control was performed replacing honey samples by Mueller Hinton Broth (MHB) and positive control was performed replacing honey samples by 100 µL of antibiotic ampicillin (100 µg mL^{-1}). Osmotic control was performed, replacing honey samples by glucose solutions with: 65.00, 32.50, 16.25, 8.12, 4.06, 2.03, 1.02 and 0.51% (w/v) ultrapure water. All plates were incubated at 35°C for 24 h. MICs for bacterium determinate as the lowest concentrations of honey samples are capable of inhibiting bacteria growth. Data were submitted to analysis of variance and averages were compared by Tukey test ($p < 0.05$).

RESULTS

The inhibitory action of honey samples for the species *S. saprophyticus, S. aureus, Staphylococcus* spp. and *S. epidermidis* are shown in Table 1. All samples showed

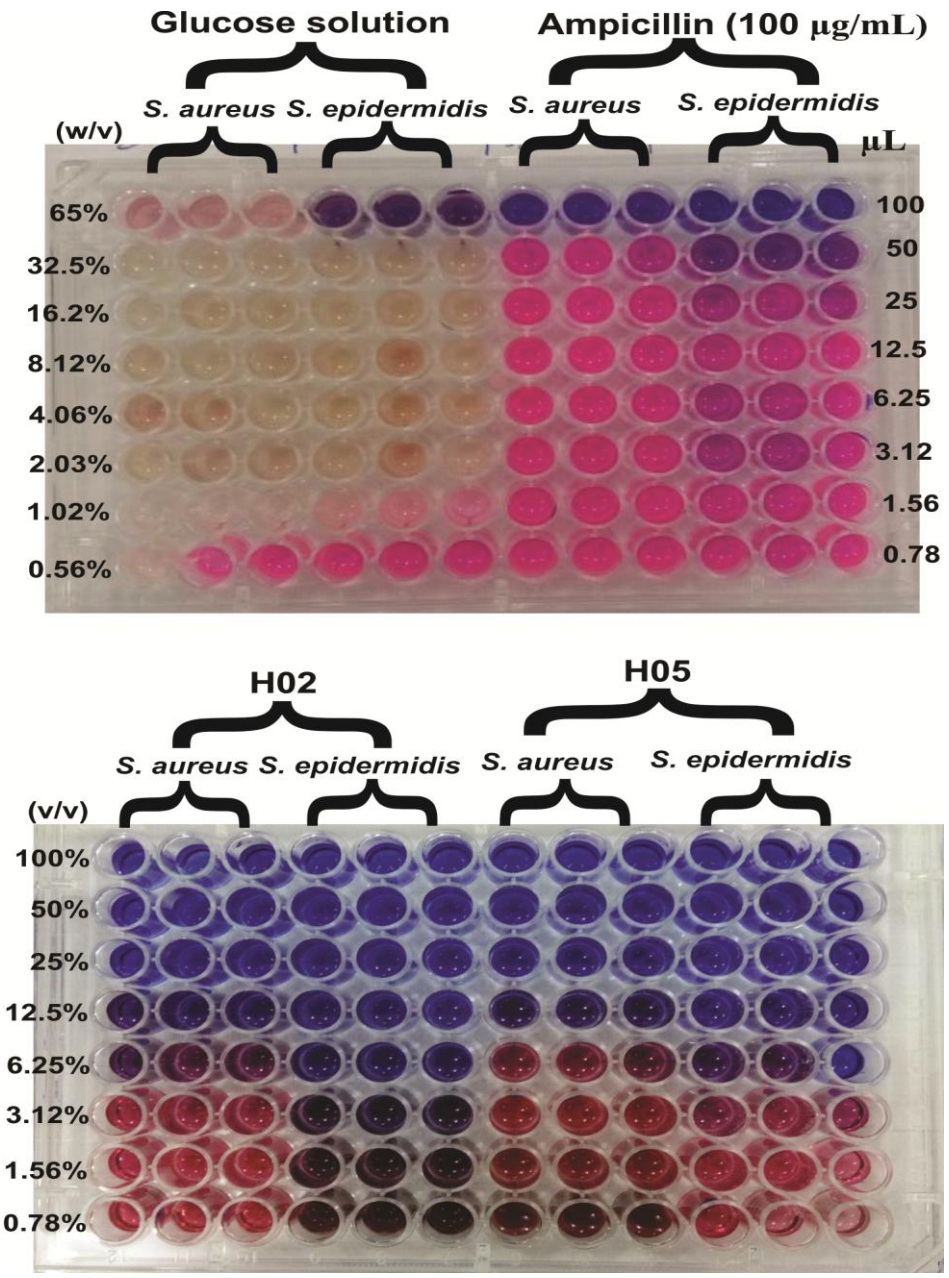

Figure 1: Minimal inhibitory concentration of the glucose, ampicillin and honey solutions against growth of *S. aureus* and *S. epidermidis.* (colour only in online version).

inhibitory activity against at least one tested bacterium. The intensity of antibacterium activity varied, describing the inhibition zones of growth between 7.7 and 17.5 mm, and *Staphylococcus* spp. demonstrated a greater sensitivity. Figure 1 shows the minimal inhibitory concentration of the glucose, ampicillin and honey solutions against growth of *S. aureus* and *S. epidermidis*.

As shown in Table 1, some samples of honey showed no inhibitory activity against the strains of *S. saprophyticus* and *S. aureus* for the 24 h period, and also, some have lost the inhibitory effect after 48 h. To the strains of *Staphylococcus* spp. and *S. epidermidis*, inhibition was obtained for 100% of honey samples tested for both 24 h period and for 48 h. The minimal inhibitory concentration of the honey samples against the four microorganisms tested is shown in Table 2.

The minimal inhibitory concentration obtained, for all honey samples was 12.5% (v/v), but for H02 sample was 6.25% (v/v). In contrast, 65% (w/v) glucose solution only inhibited the growth of *Staphylococcus* spp. and *S.*

Table 1: Inhibition diameter in mm obtained using no diluted honey on staphylococci species.

Honey Sample	*S. saprophyticus*		*S. aureus*		*Staphylococcus* spp.		*S.epidermidis*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
H01	0±0^{b}	0±0^{c}	0±0^{d}	0±0^{b}	9.7±0.9.0^{d}	8.7±0.9^{d}	11.0±0.0^{a}	10.7±0.5^{a}
H02	8.7±0.9^{a}	8.5±0.5^{a}	7.7±0.9^{c}	0±0^{b}	13.5±0.5^{bc}	13.0±1.1^{b}	11.2±0.5^{a}	10.7±0.9^{a}
H03	0±0^{b}	0±0^{c}	8.7±0.5^{bc}	0±0^{b}	12.5±1.0^{c}	12.5±0.5^{b}	11.5±0.5^{a}	8.0±0.0^{cd}
H04	8.5±0.5^{a}	8.0±0^{ab}	0±0^{d}	0±0^{b}	12.7±0.5^{c}	12.2±0.5^{b}	11.2±0.9^{a}	9.7±0.9^{ab}
H05	0±0^{b}	0±0^{c}	9.7±0.5^{ab}	8.7±0.5^{a}	12.7±0.5^{c}	11.2±0.9^{bc}	11.2±0.5^{a}	9.0±0.8^{bcd}
H06	8.7±0.9^{a}	7.7±0.5^{b}	0±0^{d}	0±0^{b}	13.2±0.9^{bc}	10.2±0.9^{cd}	10.7±0.5^{a}	9.5±0.5^{abc}
H07	8.5±1.2^{a}	0±0^{c}	0±0^{d}	0±0^{b}	14.5±0.5^{b}	12.2±0.5^{b}	10.0±0.8^{a}	7.7±0.9^{d}
H08	0±0^{b}	0±0^{c}	10.2±0.9^{a}	8.5±0.5^{a}	17.5±0.5^{a}	15.7±0.5^{a}	11.2±0.9^{a}	10.5±0.5^{ab}
Glucose[1]	0±0^{b}	0±0^{c}	0±0^{c}	0±0^{c}	0±0^{e}	0±0^{e}	0±0^{e}	0±0^{e}

[1]Glucose solution 65% (w/v) in ultrapure water. Tests were performed in quadruplicate and the results are presented as means ± standard deviation. Means followed the same lower case letter in the columns do not differ by Tukey test at the 0.05 level of probability. H: Honey.

Table 2: Minimal inhibitory concentration (MIC) of the honey samples.

Honey sample	Minimal inhibitory concentration (v/v % honey)[1]			
	S. saprophyticus	*S. aureus*	*Staphylococcus* spp.	*S. epidermidis*
H01	R	R	12.5	12.5
H02	6.25	12.5	6.25	6.25
H03	R	12.5	12.5	12.5
H04	12.5	R	12.5	12.5
H05	R	12.5	12.5	12.5
H06	12.5	R	12.5	12.5
H07	12.5	R	12.5	12.5
H08	R	12.5	12.5	12.5
Glucose[2]	R	R	65	65

[1]Antimicrobial activity by MIC technique, given in % of honey solution (v/v) required to inhibit the microorganism evaluated. [2]Antimicrobial activity by MIC technique, given in % of glucose solution (w/v) required to inhibit the microorganism evaluated. H: Honey. R: Resitant.

epidermidis. The sample honey H02 showed better antimicrobial activity through a strong inhibition effect in all studied strains over the period of 24 and 48 h, except for *S. aureus* for 48 h. Already the H01 sample did not show any activity against the species *S. saprophyticus* and *S. aureus.* An antibiogram was prepared with the microorganism studied using conventional antibiotics, and the sensitivity profiles are shown in Table 3. Table 3 shows that all staphylococci species tested were resistant to the antibiotics such as cefepime, oxacillin, penicillin G and sulfazotrin. The *Staphylococcus* spp. was resistant against most antibiotics.

DISCUSSION

Table 3 shows that *Staphylococcus* spp. isolated from bovine mastitis and *S. epidermidis* were resistant to 66.6 and 81.8% of antibiotics that were submitted, respectively. Interestingly, these species were sensitive to all the honey samples (Tables 1 and 2). Henriques et al. (2010) in his research also found the action of honey on *Staphylococcus* spp. resistant.

It is noteworthy that these bacteria were selected by their frequent association processes of infectious of the varied topography (Al-Waili, 2004; Santos, 2007). In addition, the susceptibility of resistant organisms related to clinical cases is important because it describes the action of a given compound against a specific strain (Cassettari, 2005).

The current results were higher than those found by Bueno-Costa et al. (2011) in honey samples from Rio Grande do Sul, Brazil (MIC: 10 mg mL^{-1}). However, in this study were used some pathogenic bacteria obtained from clinical cases.

Bacterium pathogens, especially those causing hopitalar infections, have developed resistance to one or all synthetic antibiotics introduced in clinical pratice (Levy

Table 3: Sensitivity profile of the strains analyzed against conventional antibiotics.

Antibiotic	Amount per disc (µg)	Tested bacteria			
		S. saprophyticus	*S. epidermidis*	*S. aureus*	*Staphylococcus* spp.
Ampicillin	10	-	-	-	R
Amoxicillin +Clavulanate	30	-	-	-	R
Amikacin	30	-	-	-	S
Cefepime	30	R	R	R	R
Ceftazidina	30	-	-	-	R
Cefalotin	30	-	-	-	R
Cefuroxime	30	-	-	-	R
Chloramphenicol	30	R	I	S	R
Clindamycin	2	R	R	I	R
Ciprofloxacin	5	I	S	S	S
Cefoxitin	30	-	-	-	R
Gentamicin	10	R	S	S	S
Moxifloxacin	5	-	-	-	S
Meropenem	10	-	-	-	S
Nitrofurantoin	300	-	-	-	R
Norfloxacin	10	-	-	-	S
Oxacillin	1	R	R	R	R
Penicillin G	10	R	R	R	R
Rifampicin	5	S	S	I	S
Sulfazotrim	25	R	R	R	R
Tetracycline	30	R	S	S	R
Vancomycin	30	R	R	S	-

R: Resistant, S: Sensitive, I: Intermediate, - Not tested. According to: Clinical and Laboratory Standards Instituter (CLSI 2013).

and Marshall, 2004; Paterson, 2006; Payne et al., 2007). Among these bacterium pathogens, *S. aureus* is one of the most commonly acquired and it is particularly problematic in skin and wound infections, once emerged methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant strains (Quentin et al., 2004; Cui et al., 2006; Goldstein, 2007). Blair et al. (2009) evaluated the antibiotic efect of a medical-grade honey (Medihoney®) against eight species of pathogens with high levels of antibiotic resistance, including *S. aureus*, and found the minimal inhibitory concentration (MIC), ranging from 4.0 to 14.8% (w/v) of honey solution, which is a concentration that can be mantained in the wound environment. It is importante to note that some microoganisms were resistant to some synthetic antibiotics and sensivity to some studied honey samples. However, it is necessary to take in consideration that the antibiotic are synthetic substances, produced and designed with specific actions, while honey has variable composition and needs better analysis of their benefits, making it difficult to define the acceptable level of inhibition for natural products (Tajik et al., 2009).

The different distribution of phenolic compounds (e.g. caffeic acid, ferrulic acid, benzoic acid, gallic acid, quercetin, chrysin, muricetin, pinocembrim and kaempferol) (data obtained by High performance liquid chromatography with diode array detection (HPLC-DAD) (Bandeira et al. 2018)) could be related to the different response of honey against the staphylococci species tested.

This distribution of phenolic compounds could infer that the honey samples presented different floral origins (Halawani and Shohayeb, 2011). In addition, next to the H01 point have a soybean field at distance lower than 400 m and the H02 point was located in a secondary forest, with high amount of Brazil nut trees, at distance higher than 2000 m of any agricultural activity. Although the proximity of the H04 and H05 and, H07 and H08, it is important to note that Amazon soils have very variable composition, which influences the physiology of each plant and can change the honey components (Akujobi, 2010).

Detailed laboratorial tests may confirm the beneficial activities of honey and thus favour its indicated use. In some countries, this process of pre-defined therapeutic indications is already used, as in New Zealand and Australia that sell honey for use in various industries (e.g. pharmaceutical and chemical industries), with prior knowledge of their properties (Weston, 2000; Blair et al., 2009; Khan et al., 2014). And in other countries, such as

Germany, honey is used in therapeutic practice in the public health system (Escuredo et al., 2013). However, despite the frequent use of this product in the Amazon, there are few studies proving their pharmacological and biological properties.

Conclusions

The present study showed that *A. mellifera* honey produced in Santarém are promising in the control of microorganisms related to clinical cases with potential therapeutic action. The antimicrobial activity varied for each honey sample, and some samples showed no inhibitory activity against strains of *S. saprophyticus* and *S. aureus*. This variation may be related to the amount and distribution of phenolic compounds in honey.

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