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Influence of seasonality and production method on the antibacterial activity of propolis

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ABSTRACT. The antibacterial activity of propolis produced throughout the year by different methods of collection ('intelligent' collector of propolis - ICP; plastic screen - PC; conventional scraping - CS) on *Staphylococcos aureus* and *Escherichia coli* is investigated. Fifteen beehives (five per collector) of Africanized *Apis mellifera* were used. Monthly produced propolis, with the same collection technique, was mixed for the preparation of the extract. The ethanol extract of propolis (EEP) was prepared at the ratio of 30% (30 g of propolis, completing the volume for 100 mL with ethanol 70%). Two microorganisms, a positive bacterium Gram *Staphylococcus aureus* and a negative bacterium Gram *Escherichia coli*, through the methodology of diffusion in agar, were used for the biological activity evaluation of EEP. Results show that propolis presented antibacterial activity, affected by seasonality and by collecting method.

Keywords: apiculture, biological properties, quality, bacteria.

Influência da sazonalidade e método de produção na atividade antibacteriana da própolis

RESUMO. O objetivo deste estudo foi investigar a atividade antibacteriana da própolis produzida ao longo do ano por diferentes métodos de coleta (coletor de própolis 'inteligente' – CPI, tela plástica – TP e raspagem convencional – RC) sobre *Staphylococcos aureus* e *Escherichia coli*. Foram utilizadas 15 colmeias de *Apis mellifera* africanizadas. Para o preparo do extrato, foi misturada a própolis produzida mensalmente pela mesma técnica de coleta. O EAP foi preparado na proporção de 30% (30 g de própolis completando o volume para 100 mL com etanol 70%). Para avaliação da atividade biológica do EAP, foram usados dois micro-organismos: uma bactéria gram positivia *Staphylococcus aureus* e uma gram negativa *Escherichia coli*, por meio da metodologia de difusão em ágar. Os resultados mostraram que a própolis apresenta atividade antibacteriana, sendo esta influenciada pela sazonalidade e método de colheita utilizado.

Palavras-chave: apicultura, atividade biológica, qualidade, bactéria.

Introduction

Propolis is a natural resinous mixture produced by *Apis mellifera* L. from leaf, buds and bark, representing a complex set of substances (55% resins and balsams; 30% waxes; 10% volatile oils; about 5% pollen) and mechanical impurities (SALATINO et al., 2011; KUROPATNICKI et al., 2013).

Flavonoids were the principal group of compounds isolated from propolis, but other substances, such as aromatic acids, phenolic compounds, organics acids, minerals, vitamins and amino acids, were also found (SEIDEL et al., 2008; KUROPATNICKI et al., 2013). However, the chemical composition and biological activities of propolis vary and depend on the diversity of plants and geographical locations from which bees collect it (SALATINO et al., 2011).

Propolis production is an inborn trait of honeybees. Several factors, such as seasonality, production method and others, are involved in this process, which must be taken into account when productivity increase is desired (BANKOVA et al., 1998; DAUGSCH et al., 2008; TEIXEIRA et al., 2008).

Propolis has a broad spectrum of biological properties including antifungal, anti-inflammatory, anti-tumoral and antibacterial activities (ARAÚJO et al., 2012; MANNANI et al., 2012; KUROPATNICKI et al., 2013). Several authors have reported that the susceptibility of a range of Gram-positive bacteria to ethanol extracts of propolis may vary according to the site of the propolis collected (GONSALES et al., 2006; MULI et al., 2008).

Current study investigated the microbiological activity of propolis produced by different methods of collection (Intelligent Collector of Propolis, plastic screen and scraping) and in different seasons, on the standard *Staphylococcos aureus* and *Escherichia coli*.

Material and methods

Propolis samples were collected at Nucleus of Education, Sciences and Technology in Rational Beekeeping (NECTAR) in the apiary of Animal Production Department – College of Medicine Veterinary and Animal Sciences – São Paulo State University (UNESP) in Botucatu, São Paulo State, Brazil.

Africanized *A. mellifera* were allocated in fifteen Langstroth beehives (five per collector), distributed randomly and managed only for the production of propolis. The collectors comprised plastic screen (PS), 'intelligent' collector of propolis (ICP) and scraping collector (SC).

The climatic data for spring were: temperature 19.5 ± 2.8°C; humidity 50.7 ± 8.4%; insolation 6.0 ± 3.6 hours; rainfall 3.1 ± 8.3 mm; wind speed 118.9 ± 59.4 km h^{-1}; for summer: temperature 21.3 ± 2.3°C; humidity 55.7 ± 8.5%; insolation 5.8 ± 3.7 hours; rainfall 6.9 ± 13.1 mm; wind speed 91.4 ± 52.5 km h^{-1}; for autumn: temperature 18.9 ± 2.9°C; humidity 56.1 ± 6.2%; insolation 7.5 ± 3.6 hours; rainfall 3.9 ± 8.1 mm; wind speed 73.5 ± 38.1 km h^{-1}; for winter: temperature 17.9 ± 2.9°C; humidity 53.0 ± 8.4%; insolation 6.9 ± 3.3 hours; rainfall 1.3 ± 5.0 mm; wind speed 89.7 ± 47.1 km h^{-1}.

Extract was prepared by the mixing of the monthly propolis produced from the same technique of collection. The ethanol extract of propolis (EEP) was prepared in the ratio of 30% (30 g of propolis, completing the 100 mL volume, with ethanol 70%). After a week, extracts were filtered and EEP obtained (ORSI et al., 2000).

To determine dry weight (DW; mg mL^{-1}) in each sample, 2 mL of EEP were weighed and kept at 105°C during two hours for the evaporation of the volatile phase. Contents were weighed again and DW was obtained by the difference between the initial and final weights.

For the evaluation of the biological activity EEP, two standard microorganisms (American Type Culture Collection), namely, Gram positive (*S. aureus* - ATCC 25923) and Gram negative (*E. coli* - ATCC 25922) bacteria were used. The microorganisms had been tested by the methodology of diffusion in agar using paper discs filter. Three antibiotics were used as control: ampicillin, cephalexin and penicillin. A disc containing only alcohol (propolis solvent) was also used as control.

Filter paper discs (8 mm in diameter) saturated with 15 μL of each EEP (one extract to each month) were placed on Mueller Hinton agar plates, which were inoculated with test organisms (10^6 UFC mL^{-1}), following standard protocol described by the National Committee of Clinical Laboratory Standards (NCCLS, 2005).

The antibacterial activity was determined by reading the formation of the inhibitory halo around the discs after 24 hours incubation, at 37°C. Each assay was performed in triplicate.

Results were compared by ANOVA, followed by Tukey's test to verify differences between averages, at statistical significance $p < 0.05$. The results are also evaluated by Correlation of Pearson to verify the existence of relations between the variables analyzed for each extract (ZAR, 1996).

Results

No correlations between climatic variables and propolis production were observed. Seasonality affected the antibacterial activity of EEP against *S. aureus*. It may be observed that in the winter the ICP and SC methods showed an inhibition halo significantly greater than that in the other seasons. Similarly, the collector also influenced the antibacterial activity against *S. aureus*, since ICP and SC showed an inhibition halo significantly greater when compared with that of PC (Table 1).

The season affected the antibacterial activity of EEP on *E. coli*. The propolis produced by ICP in the winter showed an inhibition halo which was significantly greater than that for spring and summer. In the case of SC, the inhibition halo was significantly greater in the winter than in spring, summer and autumn. Collector did not affect the antibacterial activity of EEP on *E. coli* (Table 2).

There was no statistical significance between the dry weight (DW) of EEP produced in the different seasons and that by collectors, for the two bacteria (Tables 1 and 2).

The antibiotics amoxicillin and cephalexin showed antibacterial activity on *S. aureus* and *E. coli*. Penicillin showed antibacterial activity on *S. aureus*. Ethanol 70% failed to influence the two bacteria (Tables 1 and 2).

Table 1. Antibacterial activity of ethanolic extract of propolis (EEP) and dry weight (DW in mg mL^{-1}) produced by 'intelligent' collector of propolis (ICP), scrape (SC) and plastic sreen (PS) in function of the seasonality, Ethanol 70%, Amoxicillin (AM), Cephalexin (CEF) and Penicillin (PEN) against *Staphylococcos aureus*. The results represent the average and the respective standard deviation.

	SPRING		SUMMER		AUTUMN		WINTER	
	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)
ICP	8.7 ± 0.6 aA	3.0 ± 0.2A	8.7 ± 0.6 aA	3.0 ± 0.5A	10.8 ± 2.5 aA	3.5 ± 0.8A	14.3 ± 0.6bB	3.1 ± 1.1A
SC	8.3 ± 0.6aA	3.6 ± 0.7A	8.3 ± 0.6 aA	2.8 ± 0.5A	11.3 ± 3.0 aA	3.0 ± 0.3A	13.8 ± 0.8bB	3.1 ± 0.3A
PC	9.0 ± 0.0aA	2.6 ± 0.2A	8.0 ± 1.7 aA	2.6 ± 0.3A	11.2 ± 2.6 aA	3.0 ± 0.6A	12.0 ± 0.5 aA	1.9 ± 0.6A
Ethanol	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-
AM	14.0 ± 0.0	-	14.2 ± 0.0	-	13.8 ± 0.1	-	14.0 ± 0.0	-
CEF	24.2 ± 0.1	-	24.1 ± 0.0	-	24.0 ± 0.0	-	23.7 ± 0.1	-
PEN	15.0 ± 0.0	-	15.0 ± 0.1	-	14.8 ± 0.0	-	15.2 ± 0.0	-

Different small letters in the same row and different capital letters in the same column indicate statistical difference between the means ($p < 0.05$).

Table 2. Antibacterial activity of ethanol extract of propolis (EEP) and dry weight (DW in mg mL^{-1}) produced by 'intelligent' collector of propolis (ICP), scrape (SC) and plastic screen (PS) as a function of seasonality, Ethanol 70%, Amoxicillin (AM), Cephalexin (CEF) and Penicillin (PEN) against *Escherichia coli*. Results represent average and the respective standard deviation.

	SPRING		SUMMER		FALL		WINTER	
	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)	Inhibition Halo (mm)	DW (mg)
ICP	6.0 ± 0.0 abA	3.0 ± 0.2A	4.7 ± 0.6aA	3.0 ± 0.5A	6.5 ± 0.9bA	3.5 ± 0.8A	8.2 ± 0.8bcA	3.1 ± 1.1A
SC	5.3 ± 1.1aA	3.6 ± 0.7A	4.3 ± 0.6aA	2.8 ± 0.5A	6.3 ± 0.3abA	3.0 ± 0.3A	7.8 ± 1.0bA	3.1 ± 0.3A
PC	5.3 ± 0.6aA	2.6 ± 0.2A	5.7 ± 1.7aA	2.6 ± 0.3A	7.0 ± 1.8aA	3.0 ± 0.6A	8.0 ± 0.9aA	1.9 ± 0.6A
Ethanol	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-
AM	20 ± 0.0	-	20.0 ± 0.0	-	20.2 ± 0.1	-	19.8 ± 0.0	-
CEF	15.0 ± 0.0	-	15.0 ± 0.1	-	14.8 ± 0.0	-	15.2 ± 0.0	-
PEN	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-

Different small letters in the same row and different capital letters in the same column indicate statistical difference between the means ($p < 0.05$).

Discussion

Several researchers have studied the antibacterial properties of propolis and evidenced its efficient activities against Gram positive and its limited activity against Gram negative bacteria (TOSI et al., 2007; PROBST et al., 2011). Current assay showed that EEP had a greater activity against Gram positive bacteria and less activity against Gram negative bacteria. Above differences might be due to the bacterial wall cell constitution. In fact, gram positive bacteria have a less complex wall cell and lower lipid contents (LOGUERCIO et al., 2005) and thus might have a higher susceptibility to the propolis's chemical constituents.

Other evidences also suggest that propolis composition may be altered as a function of bioactive complex compounds in vegetal sources. Analyzing the possible vegetal sources of the propolis produced in Botucatu, São Paulo State, Brazil, Bankova et al. (1999) observed that the propolis was usually derived from *Baccharis dracunculifolia*, popularly known as 'vassourinha', *Araucaria angustifolia* (Paraná pine tree) and *Eucalyptus citriodora*, even though other secondary vegetal resinous sources may occur around the apiary.

Trusheva et al. (2011) reported that propolis produced in different Brazilian states showed activities against *S. aureus* and verified a positive co-relationship between the antibacterial activity and total flavonoid contents. Other researchers underscored a relationship between the flavonoid concentration and the propolis's antibacterial activity (BARBARIC et al., 2011; ISLA et al., 2012). However, Souza et al. (2010) researched flavonoid contents in the propolis produced in the same region as current research and failed to observe any difference due to seasonality and production method. They suggested that other substances could have caused the antibacterial activity observed, which may vary according to seasons.

In a study on Brazilian propolis, seasonality affected the antibacterial activity of the propolis produced in the northeastern and southeastern regions of Brazil (CASTRO et al., 2007). The authors suggested that propolis composition may alter as a function of bioactive complex compounds in vegetal sources and may vary over the year (EREMIA; DABIJA, 2007).

Activity mechanism of propolis compounds is complex and may be attributed to the synergy among some of its components. Takaisi-Kikuni and Schilder (1994) verified that the ethanol extract propolis interfered on the growth of *Streptococcos agalactie* by inhibiting protein synthesis. Koo et al. (2002) suggested that propolis and its components may interfere with the enzymatic activity of some bacteria such as *Streptococcos mutans* and *Streptococcos sangui*. A significant synergy may be verified between clinical antibiotics and propolis from two geographical sources against *Salmonella typhi* (ORSI et al., 2006, 2012a and b).

S. aureus and *E. coli* were sensitive to the antibiotics amoxicillin, cephalexin and penicillin. Since the alcohol used as solvent for EEP preparation did not present antibacterial activity, the activity against *S. aureus* was due to compounds present in the propolis, as has been reported by other researchers (FERNANDES JR. et al., 2006; AYALA et al., 2008; MAIA-ARAÚJO et al., 2011).

Conclusion

Propolis showed antibacterial activity against *S. aureus* and *E. coli*, influenced by seasonality and the collector method employed.

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