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Chemical composition and antimicrobial activity of two extract of propolis against isolates of *Staphylococcus* spp. and multiresistant bacterials[1]

Jarbas F. Amarante[2], Márcia F. Ribeiro[3], Mateus M. Costa[2], Fredson G. Menezes[2], Tania M.S. Silva[4], Talita A.B. Amarante[5], Adriana Gradela[2]* and Liliane M.D. Moura[2]

ABSTRACT.- Amarante J.F., Ribeiro M.F., Costa M.M., Menezes F.G., Silva T.M.S., Amarante T.A.B., Gradela A. & Moura L.M.D. 2019. **Chemical composition and antimicrobial activity of two extract of propolis against isolates of *Staphylococcus* spp. and multiresistant bacterials.** *Pesquisa Veterinária Brasileira 39(9):734-743.* Laboratório de Anatomia dos Animais Domésticos e Silvestres, Colegiado de Medicina Veterinária, Universidade Federal do Vale do São Francisco, Rodovia 407 Km 12, Lote 543, Projeto Nilo Coelho C1, Petrolina, PE 56300-000, Brazil. E-mail: agradela@hotmail.com

There is a growing need to discover and develop alternative therapies for the treatment of mastitis caused by *Staphylococcus* spp. and multidrug-resistant bacterial infections. This study examined the chemical composition and antimicrobial potential of two propolis extracts (EPA and EPB) against seventy-seven isolates of *Staphylococcus* spp. obtained from subclinical bovine mastitis; three clinical strains of MRSA and two from clinical strains of *S. aureus* ATCC, identified as *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923. The total phenolic content was determined by the Folin-Ciocalteau method, the total flavonoid content by the Dowd method and the phenolic profile was quantified by HPLC-DAD. The MBC values of the extracts were evaluated by broth microdilution method. The amount of total phenolic and flavonoid compounds was higher in EPA than EPB. Both extracts revealed the presence of caffeic, coumaric, cinnamic, ferulic and 3,4-dihydroxybenzoic acids, with higher concentrations of coumaric and cinnamic acids. *Staphylococcus* spp. isolates were susceptible to EPA (90.9%), EPB (83.1%) and oxacillin (80.5%). The oxacillin susceptible isolates were also susceptible to EPA (70.1%) and EPB (80.6%), whereas those oxacillin-resistant strains were also susceptible to EPA (40.0%) and to EPB (26.7%). MBC ranged from 34.3 to 68.7µm/mL for EPA and from 68.7 to 137.5µg/mL for EPB. Both extracts inhibited significantly (100%) the clinical strains of MRSA, *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 at the concentration of 68.7µg/mL. It is concluded that both extracts of propolis, whose main constituents are coumaric and cinnamic acids, have high antimicrobial activity against the microorganisms studied, and EPA also against oxacillin-resistant strains. These findings reinforce its potential use for the treatment of bovine mastitis.

INDEX TERMS: Chemical composition, antimicrobial activity, extract of propolis, *Staphylococcus* spp., multiresistant bacterials, phenolic compounds, microdilution, oxacillin-resistant *Staphylococcus aureus*, propolis, bacterioses.

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[2] Universidade Federal do Vale do São Francisco (Univasf), Rodovia 407 Km 12, Lote 543, Projeto Nilo Coelho C1, Petrolina, PE 56300-000, Brazil. *Corresponding author: agradela@hotmail.com

[3] Centro de Pesquisa Agropecuária do Trópico Semi-Árido (CPATSA), Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Rodovia BR-428, Km 152, Zona Rural, Petrolina, PE 56302-970.

[4] Departamento de Ciências Moleculares, Universidade Federal Rural de Pernambuco (UFRPE), Rua Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil.

[5] Departamento de Ciências Humanas (DCH), Universidade do Estado da Bahia (UNEB), R. Silveira Martins 2555, Cabula, Salvador, BA 41150-000, Brazil.

RESUMO.- [**Composição química e atividade antimicrobiana de dois extratos de própolis contra isolados de *Staphylococcus* spp. e bactérias multirresistentes.**] É cada vez mais oportuna a necessidade de descobrir e desenvolver terapias alternativas para tratamento da mastite causada por *Staphylococcus* spp. e de infecções bacterianas multirresistentes. Este estudo examinou a composição química e o potencial antimicrobiano de dois extratos etanólicos de própolis (EPA e EPB) contra setenta e sete isolados de *Staphylococcus* spp. obtidos a partir de mastite bovina subclínica; três estirpes clínicas de MRSA e duas de linhagens clínicas de *S. aureus* ATCC, identificadas como, *S. aureus* ATCC 6538 e *S. aureus* ATCC 25923, ambas metacilina resistentes. O teor total de fenólicos foi determinado pelo método de Folin-Ciocalteau, o teor de flavonoides totais pelo método Dowd e o perfil fenólico foi quantificado por HPLC-DAD. CBM dos extratos foi avaliada pelo método de microdiluição em caldo. A quantidade total de compostos fenólicos e flavonoides foi maior no EPA do que no EPB. Ambos os extratos revelaram a presença dos ácidos cafeico, cumárico, cinâmico, ferúlico e 3,4-di-hidroxibenzóico, com maiores concentrações de ácidos cumárico e cinâmico. Os isolados de *Staphylococcus* spp. foram sensíveis a EPA (90,9%), EPB (83,1%) e oxacilina (80,5%). Os isolados suscetíveis à oxacilina também foram suscetíveis ao EPA (70,1%) e ao EPB (80,6%), enquanto os do resistente à oxacilina foram suscetíveis ao EPA (40,0%) e ao EPB (26,7%). MBC variou de 34,3 a 68,7µm/mL para EPA e de 68,7 a 137,5µg/mL para EPB. Ambos os extratos inibiram significativamente (100%) as linhagens clínicas de MRSA, *S. aureus* ATCC 6538 e *S. aureus* ATCC 25923 na concentração de 68,7µg/mL. Conclui-se que os extratos etanólicos da própolis, cujos principais constituintes são os ácidos cumário e cinâmico, possuem atividade antimicrobiana contra os micro-organismos estudados, e o EPA também contra as cepas resistentes à oxacilina. Estes achados reforçam seu potencial uso para o tratamento da mastite bovina.

TERMOS DE INDEXAÇÃO: Composição química, atividade antimicrobiana, extrato de própolis, *Staphylococcus* spp., bactérias multirresistentes, compostos fenólicos, microdiluição, *Staphylococcus aureus* oxacilina-resistente, própolis, bacterioses.

INTRODUCTION

In Brazil, milk production is a very important segment, totaling 25.4 billion liters annually. Mastitis is the main disease that affects dairy production, with a prevalence of 48.64% in the subclinical form (Acosta et al. 2016), causing changes in the physical-chemical composition and cellularity of the milk, resulting in high economic damages with the reduction in milk production and significant effects on public health (Langoni 2000, Ribeiro 2008).

Approximately 140 different etiological agents may cause bovine mastitis, mainly in the subclinical form, of which the most prevalent contagious microorganisms have been *Staphylococcus aureus*, *Streptococcus* spp., *Streptococcus agalactiae*, *Staphylococcus* spp. and *Corynebacterium bovis*, in pure culture or in association (Costa 2001, Santos & Fonseca 2007, Ribeiro et al. 2009, Martins et al. 2010, Peixoto et al. 2012, Saeki et al. 2012). *S. aureus* is recognized worldwide as a cause of several purulent diseases in humans and animals (Bean & Griffin 1990) and also an important cause of food poisoning in humans (Omoe et al. 2002). Additionally this, it shows persistence in the mammary tissue, due to the characteristics of its virulence (Dos Santos et al. 2003) and the appearance of resistant strains by the inadequate use of antibiotics in the treatment of diseases (Barberio et al. 2002, Hogeveen et al. 2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are one of the most prevalent in cases of mastitis and have as main characteristic to be multiresistant to the antimicrobials of the beta-lactam group (Freitas et al. 2005).

One of the main tools for the control and treatment of mastitis is antimicrobial therapy (Erskine et al. 2003), but its efficiency is compromised in cases of antimicrobial resistance (Russi et al. 2008, Shi et al. 2010, Spohr et al. 2011). In addition, the use of antimicrobials causes severe economic losses due to the discarding of animals (Silva et al. 2004) and milk (Nero et al. 2007), reduction of milk production and drug costs (Vianni & Lázaro 2003).

The use of phytotherapics has been shown to be a very useful tool for the prevention and treatment of mastitis, as well as avoiding the elimination of residues in milk (Schiavon et al. 2011). In this sense, propolis, a resinous product produced by honey bees, is being used in the treatment of human and animal diseases (Coelho et al. 2010) and has been shown to be a viable and quite promising alternative in the treatment of infections caused by *S. aureus* (Pinto et al. 2001, Endler et al. 2003, Auricchio et al. 2006, Zeighampour et al. 2014, Shahbaz et al. 2015, Chen et al. 2018) due to its proven antibacterial activity (Barbosa et al. 2009, Araujo & Marcucci 2011). However the results using propolis are still conflicting (Pinto et al. 2001, Loguercio et al. 2006, Peixoto et al. 2012), due to its distinct and complex chemical constitution, necessitating the development of further research (Lustosa et al. 2008, Rufatto et al. 2017). In addition, there are few conclusive results regarding their action against MRSA, that play an important role in nosocomial infections (Taubes 2008) and a serious problem on a global scale (Astani et al. 2013). Brazil is the third largest producer of propolis, contributing 10-15% of the worldwide production (Pereira et al. 2002), for this reason studies about the antibacterial activity of Brazilian propolis would contribute to further increase this participation.

The present study investigated the chemical composition and the antimicrobial activity of two extracts of propolis against isolates of *Staphylococcus* spp. obtained from subclinical bovine mastitis and also clinical strains of MRSA and *Sthaplococcus aureus* ATCC 6538 and ATCC 25923.

MATERIALS AND METHODS

Propolis extracts. The bacterial isolates were tested against two commercial extracts of propolis identified as extract of propolis A (EPA) and extract of propolis B (EPB). The EPA was an 11% green propolis extract (Apis Flora®) originating from the Vassourinha do campo (*Baccharis dracunculifolia*) and obtained from hives of Jundiai, state of São Paulo. It consists of the following ingredients: green propolis, neutral alcohol (ethanol) 95.1% food grade and purified water. The EPB was also in 11% neutral propolis and alcohol solution (Santa Bárbara®). It was obtained from hives in the State of Bahia, containing in its composition 55% plant resins; 30% beeswax; 8 to 10% of essential oils; and 5% pollen. Both extracts had its chemical composition analysed at the Laboratory of Natural Products of the "Universidade Federal Rural de Pernambuco" (UFRPE), in Recife, Pernambuco.

Determination of total phenolic content. Quantification of phenolic compounds in the EPA and EPB was performed by the Folin-Ciocalteau method using gallic acid as the standard (Singleton & Rossi Junior 1965). The standard curve of gallic acid had five points of concentration (4, 8, 16, 24, 36µg/mL), with a wavelength of 760nm, with Y=0.0064x+0.4174, where y is the absorbance and x is the concentration; R^2=0.9577. Quantification of phenolic compounds in extracts of propolis was performed in triplicate, with the quantity of phenols expressed in mg of gallic acid equivalent per gram of propolis extract, given the dry extract content (Roesler et al. 2007).

Determination of total flavonoids. The total flavonoid content in the EPA and EPB was determined by the adapted Dowd method (Meda et al. 2005), with absorbance readings at 300nm, constructing a standard curve of quercetin in five concentrations (1, 5, 10, 20, 40µg/ml), with Y=0.0198x+0.3552, where "y" is the absorbance and "x" is the concentration; R^2=0.9807. The total flavonoid content was expressed as mg of quercetin equivalent per gram of propolis extract, given their dry extract content, as described by Lee et al. (2003).

Determination of dry residue content. An aliquot of 5 mL of EPA and EPB, free of wax, was transferred to a porcelain capsule in dry form (heated in a laboratory oven at 105°C, for 2h, cooled in a desiccator and weighed), and the whole was taken to the oven preheated to 105°C, where it remained for 2h. After cooling in desiccator, the set was weighed. The process of heating, cooling and weighing the assembly was repeated at intervals of 1h until a constant mass was reached (when the difference between two consecutive weighing did not exceed 5mg). This analysis was realized in triplicate and the dry residue content (soluble solids in methanol) calculated by the ratio of the mass of the residue deposited in the crucible to the initial mass of the extracts of propolis crude corresponding to the aliquot of 5mL in percent (Instituto Adolfo Lutz 1976, Brasil 2001, European Pharmacopoeia 2002).

HPLC-DAD analysis. The High Performance Liquid Chromatography (HPLC) system consisted of two SCL-10Avp solvent pumps, equipped with a SPDM2O diode array detector (HPLC-DAD; Shimadzu, Corp., Kyoto, Japan). Samples were injected into a Rheodyne 7125i type injector with a 20mL capacity loop. Chromatographic separation was done with a C-18 column (25cm x 4.6mm x 5mm, Shimpack CLC-ODS), pre-column C-18 SULPELCO 4.0mm. Water: formic acid (99: 1, solvent A) and methanol (solvent B) were used as the mobile phase and water was used for the acid derivatives: formic acid (95:5, solvent A) and methanol (solvent B). The chromatographic condition was: 0-15min 20% B, 15-20min 30% B, 20-30min 40% B, 30-40min 40% B, with flow rate of 1.0mL/min. For monitoring, the wavelength of 290nm and temperature of 40°C (Dalmora et al. 1997) were used. The identification of phenolics was based on retention times, UV-spectra and chromatographic comparison (co-injection) with authentic markers.

The caffeic, p-coumaric, ferulic, cinnamic, and 3,4-dihydroxybenzoic acids identified in propolis were quantified using the external standard method based on EPAk area. Analyses were made by drawing a calibration curve. To make the calibration curve of each phenolic compound, appropriate volumes from each stock solution were diluted with methanol to obtain working solutions in the concentration range of 0.5-40mg/mL that were correlated with the measured area. The area of these EPAk was drawn and the corresponding concentration of phenolics was calculated based on the calibration curve. For each sample, the quantitative analyses were performed in triplicate at 290nm.

Reagents and standards. Ferulic, 3-hydroxy-4-methoxycinnamic, caffeic, p-coumaric, cinnamic, sinapic, 4- methoxycinnamic, 3,4- dihydroxybenzoic, 4-hydroxybenzoic and syringic acids were obtained from Sigma-Aldrich (Hamburg, Germany), gallic and vanillic acids were obtained from Fluka Chemie AG (Buchs, Switzerland). All reagents used were analytical grade, as well as formic acid (Vetec, Brazil) and methanol (TEDIA).

Tested samples. The antimicrobial activity of EPA and EPB was analysed at the Animal Microbiology and Immunology Laboratory of the "Universidade Federal do Vale do São Francisco" (Univasf), in Petrolina, Pernambuco State, using seventy-seven isolates of *Staphylococcus* spp. obtained from cases of subclinical bovine mastitis in dairy farms in the Northeast region of Brazil. They were also tested three multiresistant isolates, one being Methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) and two from clinical strains of *S. aureus* ATCC, identified as ATCC 6538 and ATCC 25923, both methicillin-resistant.

Microdilution and minimum bactericidal concentration (MBC). The antimicrobial activity of the EPA and EPB was determined as the minimum bactericidal concentration (MBC) against *Staphylococcus* spp. isolates (n=77) and clinical strains of MRSA (n=3), *S. aureus* ATCC 6538 (n=1) and *S. aureus* ATCC 25923 (n=1). The samples plated on the TSA culture medium (Tryptone Soy Agar), were inoculated in tubes containing 3mL of Mueller Hinton (MH) broth medium, in order to perform microdilution according to Clinical And Laboratory Standards Institute (2006). After 24h, the medium was turbid at 0.5 on the Mac Farland scale (1x108 CFU/ml) and 0.1mL of this suspension was inoculated into tubes containing 9.9mL of MH broth (NCCLS 2002). Subsequently, microdilution was performed by placing 200µL of pure, sterile MH broth in each well of the microplate and then 200µL of each extract in the first well, followed by a 1:2 dilution and discarding the last 200µL, with the concentration varying from 0.26µg/ml to 550µg/ml. Then each well was inoculated with 10µL of the suspension containing the microorganisms. These wells and the positive control wells and negative control were incubated for 24h at 37°C. The contents of each of these wells were inoculated into petri dishes containing MH agar medium and incubated for 24h at 37°C. The lowest concentration of extract in which there was no growth of the microorganisms in the plaques was considered the MBC (NCCLS 2002). All samples were tested in triplicate.

Oxacillin susceptibility test. The sensitivity test was performed in all samples, in triplicate, by the modified Kirby-Bauer disk diffusion method (Clinical And Laboratory Standards Institute 2006), with microbial turbidity on the 0.5 scale of Mac Farland in MH broth. Samples were transferred with sterile swab to MH agar plates, in which the oxacillin-containing discs (1µg) were applied. The plates are incubated in an oven for 24h at 37°C. The breakpoint was determined according to (Clinical And Laboratory Standards Institute 2006).

Statistical analysis. The hypotheses are related to the antibacterial activity potential *in vitro* of two commercial extracts of propolis against bovine mastitis caused by *Staphylococcus* spp., in addition to relating them to resistance to oxacillin. The analyzes were performed observing the significance levels of the samples at 1% and 5%. The results were analyzed using analysis of variance

(ANOVA) and the probability p=0.05 was considered the critical value for all tests. The Tukey post-hoc test was used to separate statistically significant means. The SAS software Proc Gun model software was used for statistical analysis.

RESULTS

Analysis of the propolis extracts

The content of total phenolic and of total flavonoid varied between the samples. The EPA had a total phenolic content of 126.22mg (12.62%) and of total flavonoids of 51.06mg (5.10%) and the EPB of 73.12mg (7.31%) and 17.45mg (1.74%), respectively. The dry residue (soluble solids in methanol) content was 11.52% to the EPA and 10.37% to the EPB.

The components of each extract were identified by comparison with retention times of known chemical standards commonly found in propolis. The HPLC-DAD chromatograms of EPA and EPB indicated a similar profile of phenolic compounds in both extracts, with the presence, mainly, of cinnamic, ferulic, caffeic, coumaric, and 3,4-dihydroxybenzoic acids in all samples, which were detected according to their retention time and the UV spectral characteristics in comparison to those of standards.

The HLPC-DAD analysis revealed that the EPA presented a concentration of 226.55µg of coumaric acid, 222.55µg of cinnamic acid, 106.87µg of caffeic acid, 7.04µg of ferulic acid and 2.2µg of 3,4-hydroxybenzoic in 5mg of dry extract of propolis A, and for the EPB the concentrations were, respectively, 130.03µg, 130.03µg, 54.86µg, 3.99µg and 1.05µg in 5mg of dry extract of propolis B.

Antimicrobial activity of the propolis extracts

MBC against *Staphylococcus* spp. isolates ranged from 8.6µg/mL to 275µg/mL for the EPA, with a higher (P<0.01) number of sensitive isolates at the concentration of 68.7µg/mL (22/70, 31.4%), followed by the concentration of 34.3µg/mL (19/70, 27.1%). MBC ranged from 2.1µg/mL to 275µg/mL for the EPB, with a higher (P<0.01) number of sensitive isolates at the concentration of 137.5µg/mL (19/70, 56.2%), followed by concentration of 68.7µg/mL (19/64, 29.7%). These results indicated that EPA was more effective (P<0.01) in equal (22/70, 31.4%) or lower (33/70, 47.1%) concentration than 68.7µg/mL, while the EPB was more effective (P<0.01) in a higher concentration (38/64, 59.4%), presenting similar results at equal concentration (19/64, 29.7%) and lower result at concentrations below 68.7µg/mL (7/64, 10.9%) (Table 1). MBC against clinical strains of MRSA, ATCC 6538 and ATCC 25923 was also 68.7µg/ml for both EEP-A and EEP-B.

The analysis of the antimicrobial activity of the extract of propolis showed that 90.9% (70/77) of the 77 isolates of *Staphylococcus* spp. were susceptible to EPA, 83.1% (64/77) to EPB, 80.5% (62/77) susceptible to oxacillin and 19.5% (15/77) resistant to oxacillin. Among *Staphylococcus* spp. isolates susceptible to oxacillin 70.1% (44/62) were also susceptible to EPA and 80.6% (50/62) to EPB, whereas among those resistant to oxacillin 40.0% (6/15) was susceptible to EPA and 26.7% (4/15) to EPB (Fig.1).

Clinical strains of MRSA and *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 exhibited 100% susceptibility to both extracts.

DISCUSSION

This study brings important contributions to the scientific community due to the number of clinical strains of *Staphylococcus* spp. evaluated, as well as by the potential use of the propolis in the treatment of clinical cases of bovine mastitis and other infections caused by MRSA.

More than 300 constituents have been identified in different propolis samples (Marcucci 1995, Bankova et al. 2000, De Castro 2001, Park et al. 2002, Pietta et al. 2002, Alencar et al. 2007), which proportions depend upon of the place and time of collection (Park et al. 2002). Although the chemical composition and biological properties of propolis are variable, it generally has in its composition resin and vegetable balsam (50%), wax (30%), essential and aromatic oils (10%), pollen, as well as several other substances (5%) (Burdock 1998, Park et al. 2002, Pietta et al. 2002). The most important active compounds are flavonoids, terpenoids and phenylpropanoids (García-Lafuente et al. 2009), aromatic acids and phenolic compounds (Garza-González et al. 2010). The total phenolics and flavonoids contents may vary due to different factors, such as flora ecology (Park et al. 2002);

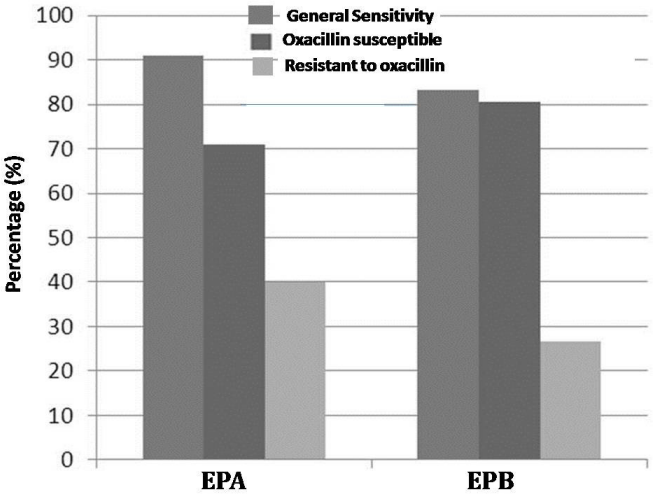

Fig.1. Antimicrobial activity of extract of propolis A (EPA) and B (EPB) on isolates of *Staphylococcus* spp. susceptible to several antibiotics and susceptible or resistant to oxacillin.

Table 1. Minimum bactericidal concentration (MBC) of the extracts of propolis against isolates of *Staphylococcus* spp.

	Total no./ sensitive	Minimum bactericidal concentration (µg/mL)											
		550	275	137.5	68.7	34.3	17.1	8.6	4.3	2.1	1.1	0.53	0.26
EPA	77/70	0^{d}	2^{d}	13^{c}	22^{a}	19^{b}	12^{c}	2^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
EPB	77/64	0^{e}	2^{d}	36^{a}	19^{b}	4^{c}	1^{e}	0^{e}	1^{e}	1^{e}	0^{e}	0^{e}	0^{e}

$^{a, b, c, d, e}$ Values are different (P<0.01).

resin collection period (Dos Santos et al. 2003); genetics of the queen bee (Park et al. 1998); local flora and collection region (Bankova 2005), among others. In spite of this, the values found in this paper were in accordance with the minimum limits set by the "Ministério de Agricultura, Pecuária e Abastecimento" (MAPA) of 5% for total phenolic and 0.5% for total flavonoids (Brasil 2001).

The obtained results confirmed that, the EPA and EPB contain considerable amounts of phenolic and flavonoids compounds, which were higher in EPA than EPB. In both extracts the total phenol content was higher than the total flavonoids content, a common finding for propolis from Brazil (Alencar et al. 2007, Moreira et al. 2008, Kalogeropoulos et al. 2009, Nunes et al. 2012, Coelho 2013), Greece, Cyprus (Kalogeropoulos et al. 2009), Czech Republic, Ireland and Germany (Al-Ani et al. 2018). The total phenol and flavonoids contents observed for EPA were similar to those of Kumazawa et al. (2004), El Sohaimy & Masry (2014) and Silva et al. (2015), lower than Alencar et al. (2007), Cabral et al. (2012), Peixoto et al. (2012) and El Sohaimy & Masry (2014), and higher than Tiveron et al. (2016). Cunha et al. (2004) observed a variation of 6.41 to 15.24% in the total phenol content, while Gonsales et al. (2006) verified variations between 0.05 and 0.63% in the contents of flavonoids and Sousa et al. (2007) verified a total flavonoid content of 0.06 to 0.38% for samples from São Paulo (Franca region) and from 0.12 to 2.11% for those from Minas Gerais (Passo region). This high variability in the total phenol and flavonoid content occurs due to the different sources of vegetable exudate, as well as by the location of the apiary (Park et al. 2002, Gonsales et al. 2006, Sousa et al. 2007). The soluble solids content in the both extract of propolis tested were higher than those observed by Sousa et al. (2007) and met the current legislation standards (Brasil 2001).

The HLPC-DAD analysis revealed mainly the presence of simple phenolic acids as caffeic, coumaric and cinnamic acids and methylated phenolic acids such as ferulic acid, and also 3,4-dihydroxybenzoic acid. These results agree with Al Naggar et al. (2016) that also found coumaric acid, ferulic acid and caffeic acid in Canadian propolis. Previous studies also have had describe presence of caffeic acid (Marcucci et al. 2001, Bankova et al. 2002, Kartal et al. 2002, 2003, Salomão et al. 2004, Ahn et al. 2007, Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Niculae et al. 2015, Oldoni et al. 2015, Tiveron et al. 2016, Al-Ani et al. 2018); coumaric acid (Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, Niculae et al. 2015, Oldoni et al. 2015, Tiveron et al. 2016, Afrouzan et al. 2018, Al-Ani et al. 2018); cinnamic acid (Salomão et al. 2004, Yang et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Tiveron et al. 2016, Al-Ani et al. 2018); and ferulic acid (Bankova et al. 2002, Kartal et al. 2002, Salomão et al. 2004, Ahn et al. 2007, Alencar et al. 2007, Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Niculae et al. 2015, Oldoni et al. 2015, Bakdash et al. 2018).

Coumaric and cinnamic acids were the main components in EPA and EPB, contrasting with the results by previous reports that found ferulic acid (Alencar et al. 2007, Barbarić et al. 2011, El Sohaimy & Masry 2014, Niculae et al. 2015) caffeic acid (Kartal et al. 2002, Melliou & Chinou 2004, El Sohaimy & Masry 2014); p-coumaric acid (Jorge et al. 2008, Salomão et al. 2008, Afrouzan et al. 2018) and cinnamic acid (Katircioglu & Mercan 2006, Mohammadzadeh et al. 2007, Silva Filho et al. 2009) as the main component. Therefore, this was the first study to relate the joint action of cinnamic and coumaric acids to the antimicrobial activity of propolis, confirming that the propolis composition varies according to botanical and geographical origin (Bankova et al. 2002, Salomão et al. 2004, Bankova 2005, Popova et al. 2005, Sahinler & Kaftanoglu 2005, Barbarić et al. 2011, Huang et al. 2014, Shahbaz et al. 2015, Afrouzan et al. 2018, Bakdash et al. 2018).

The results showed that both EPA and EPB showed antimicrobial activity against isolates of *Staphylococcus* spp., in agreement with previous studies indicated that the antibacterial activity of propolis is more pronounced against Gram-positive bacteria (Langoni et al. 1996, Sforcin et al. 2000, Pinto et al. 2001, Fernandes Júnior et al. 2003, Auricchio et al. 2006, Trusheva et al. 2010, Aguiar et al. 2014). The antibacterial activity of the EPA, with 90.9% susceptibility, was similar to the Loguercio et al. (2006) and Coelho et al. (2010) which showed susceptibility of 94.4% in coagulase-positive *Staphylococcus*. When comparing the two extracts, it was verified that isolates of *Staphylococcus* spp. susceptible to common antibiotics, as well as those resistant to oxacillin, showed higher susceptibility to EPA, while isolates susceptible to oxacillin showed greater susceptibility to EPB. It is believed that the higher levels of phenolic compounds in EPA have been responsible for the greater susceptibility of isolates resistant to oxacillin, since phenolic compounds to bind to bacterial cell walls and prevent cell division and gr owth, as also observed in previous reports (Stapleton et al. 2004, El Sohaimy 2014, Al-Ani et al. 2018).

EPA demonstrated potent antibacterial activity against *Staphylococcus* spp. isolates in lower concentrations than EPB. These results agree with Kareem et al. (2015) who also observed this activity in Iranian propolis against *Staphylococcus* spp. MBC for EPA (34.3-68.6µg/mL) was similar to Cabral et al. (2009) and Rhajaoui et al. (2001); lower than Hayacibara et al. (2005), Alencar et al. (2007) and Tiveron et al. (2016) and higher than Santos et al. (2002) and Zeighampour et al. (2014). According to Cos et al. (2006), the ideal anti-infective concentration would be generally below 100µg/ml for extracts, confirming the excellent efficiency of the EPA in relation to EPB and to others studies.

Staphylococcus spp. have a high ability to develop mechanisms of antimicrobial resistance, which makes them a serious problem of global public health (Ratti & Sousa 2009, Garza-González et al. 2010, Reddy et al. 2017). This is the case with methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which are resistant to all beta lactam antimicrobials (Adelman 2005), and often presents the multiple resistance phenomenon (Ratti & Sousa 2009). EPA and EPB inhibited significantly (100%) the clinical strains of MRSA and of *S. aureus* ATCC 6538 and ATCC 25923. Our results were similar to others reports in relation to the MRSA (Vera et al. 2011, Astani et al. 2013, Aguiar et al. 2014) and *S. aureus* ATCC 6538 (Hazem et al. 2017). In opposition, AL-Ani et al. (2018) observed a moderate anti-MRSA efficacy and Katircioglu & Mercan (2006) a low efficacy against *S. aureus* ATCC 25923, reinforcing the importance of the evaluation of multiresistant strains in the tests of antimicrobial activity of natural products (Cos et al. 2006). Low MBC, like observed in this study, might be advantageous in therapeutic level for appropriate treatment

of bacterial infections with regard to toxicity and stability of formulations (Astani et al. 2013).

The individual phenolic compounds present in the EPA and EPB were not identified and quantified, because submitting the entire extracts to antimicrobial activity studies seems to be more beneficial than to submit isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extract (Borchers et al. 2004), corresponding to a synergistic effect.

The way propolis exerts its antimicrobial action is complex and occur, among other things, through inhibition of the bacteria growth by inhibiting of its enzymatic activity diminishing their effects on biological systems (Zeighampour et al. 2014); inhibition of cell division; collapse of the bacterial cytoplasm, cell membranes, and cell walls; bacteriolysis; and protein synthesis inhibition (Takaisi-Kikuni & Schilcher 1994 apud Fernandes Junior et al. 2005). *In vitro* studies have attributed the antimicrobial activity of propolis to the presence of phenolic compounds, flavonoids, phenolic acids and their esters (Pinto et al. 2001, Freitas et al. 2005, Katircioglu & Mercan 2006, Mohammadzadeh et al. 2007, Barbarić et al. 2011) or to the synergistic action of flavonoids and other active principles (Santos et al. 2002, Da Silva Filho et al. 2004, Barros et al. 2007, Sousa et al. 2007). Flavonoids (Uzel et al. 2005, Alencar et al. 2007, Cushnie & Lamb 2011) and phenylethyl ester of caffeic acid apEPAr to act by inhibiting bacterial RNA polymerase (Uzel et al. 2005), while phenolic compounds, such as caffeic acid, benzoic acid and cinnamic acid, cause functional and structural damages on the membrane or cell wall of microorganisms (Scazzocchio et al. 2005) or inhibition of bacterial replication (Rastogi et al. 2008) and galagin and caffeic acid cause enzymatic inhibition in bacteria (Koo et al. 2002). The results suggest that the antibacterial activity of EPA and EPB occurred mainly due to the content of phenolic compounds as previously described (Bankova et al. 1995, Marcucci et al. 2001, Popova et al. 2005, Estevinho et al. 2008, Ristivojević et al. 2016, Al-Ani et al. 2018), in particular by the content of cumaric and cinnamic acids (Popova et al. 2005, Sforcin 2007). However, it can not be ruled out that different substances may be involved in the antimicrobial activity, since Cabral et al. (2012) observed that G6 propolis, which had the lowest phenolic compound and flavonoid contents, showed the best antibacterial effect.

CONCLUSION

We could confirm that both extracts of propolis, whose main constituents are cumin and cinnamic acids, have high antimicrobial activity against the microorganisms studied, and EPA had also a high antimicrobial activity against oxacillin-resistant strains. These findings reinforce its potential use for the treatment of bovine mastitis.

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