

## ANTIMICROBIAL EFFECT OF DIFFERENT ETHANOLIC EXTRACTS OF PROPOLIS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS SPP* ISOLATES

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### ABSTRACT

The emergence of multidrug-resistant bacteria has now become a critical issue in both human and veterinary medicine, and in this way, the antibiotic treatment for the control of these infections has become ineffective. In the current investigation, we studied the antimicrobial effectiveness of five ethanolic extracts of propolis (EEP) from different regions of Colombia against six strains of *Staphylococcus spp.* methicillin-resistant. The analysis of major compounds of propolis showed the presence of diterpenic acids, prenylated benzophenones, and triterpenes in the propolis obtained from Cundinamarca Antioquia, and Huila regions respectively. Minimum inhibitory concentration (MIC) technique was implemented to evaluate the antibacterial activity of the propolis extracts. Was found that all propolis extracts showed inhibitory action against the *Staphylococcus* strains evaluated MIC<sub>90</sub> = 60 – 30 mg/mL, and the average MICs (mg/mL) for each EEP were: EEP1 = 11.88±4.98; EEP2 = 31.25±16.71; EEP3 = 51.25±21.43; EEP4 = 22.81±11.88; EEP5 = 17.50±10.25. EEP displayed varying effectiveness against six *Staphylococcus spp* isolates strains, with minimal bactericidal concentration (MBC) within the range from 1.87 to 30 mg/mL. In conclusion, the extract's antimicrobial effectiveness depends on the origin of propolis and the evaluated *Staphylococcus spp* isolates. Also, these findings suggest that the propolis extracts could be an alternative method to control multidrug-resistant infections caused by *Staphylococcus spp*.

**Keywords:** Antibiotic resistance, Antimicrobial, Methicillin, Propolis, *Staphylococcus spp.*

### INTRODUCTION

The increase in antimicrobial resistance within a broad range of infectious agents has put the world on imminent alert (World Health Organization 2014). Complications of nosocomial and community-acquired infections, mainly in immunocompromised patients and patients with underlying diseases, are due to the emergence of resistant microorganisms to commonly used antibiotics. Perhaps even more important is the emergence of multi-resistant opportunistic infectious agents (Klein and Laxminarayan 2007). Among these opportunistic pathogens are the enterococci, the coagulase-negative staphylococci, methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia* and *Escherichia coli* (Cabrera *et al.* 2011), and these can cause serious and even fatal infections in otherwise healthy hosts.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prominent pathogens causing community and livestock-associated infections (Stefani *et al.* 2012, Xiao *et al.* 2013). They have been the subject of interest in the last two decades due to the increasing resistance to MRSA by most potent glycopeptide antibiotics (Fu and Yao 2013). MRSA has altered penicillin-binding proteins (PBPs) with reduced affinity

to penicillin and other available  $\beta$ -lactam antibiotics (Weese 2010).

Staphylococcal infections are of major importance in both human and veterinary medicine. *Staphylococcus aureus* is a major inhabitant of the human skin. It occasionally lives on domestic animals, although these are usually colonized by other species of staphylococci. Furthermore, it has been a frequent cause of subclinical mastitis in animals (Pantosti and Monaco 2007, Weese 2010).

The development of alternative antimicrobial methods has become one of the top priorities of medicine and biotechnology, to combat these kinds of resistant organisms. In this regard, propolis has proved to be a plausible alternative for this purpose. Propolis is a bee product that contains phenolic substances including cinnamic acid derivatives and some flavonoids (Marcucci *et al.* 2001, Borrelli *et al.* 2002). Flavonoids and cinnamic acid derivatives have been considered the main biologically active components in propolis (Borrelli *et al.* 2002). It has been extensively used in folk medicine and also, because of its antibacterial, antiseptic, anti-inflammatory, and anesthetic activities, in alternative medicine (Krol *et al.* 2013).

Several studies have documented the biocidal functions of propolis, including antibacterial, antifungal, antiprotozoal, antiviral, anti-tumor, immune-modulation

and anti-inflammatory activities (Mello *et al.* 2010, Al-Abbadi *et al.* 2015). It has also been used as an alternative treatment for infections (Sanghani *et al.* 2014). Regarding the bactericidal action of propolis extracts, it has been shown to be effective mainly against yeasts and gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus spp.* (Krol *et al.* 1993). Recent studies demonstrated antimicrobial activity of propolis against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant clinical isolates (Astani *et al.* 2013, Saddiqa and Abouwarda 2016). On the other hand, a minor action against gram-negative bacteria has been demonstrated in previous studies (Silici and Kutluca 2005, Rahman *et al.* 2010). Nonetheless, the chemical composition of propolis will depend on the tree bark and leaf buds taken by the bees (*Apis mellifera*) (Stepanovic *et al.* 2003).

The number of methicillin-resistant *Staphylococcus aureus* infections and other multidrug-resistant infections is increasing, and treatment with antibiotics is problematic (Shelburne *et al.* 2004, Rosenberg *et al.* 2012). In the current study, we have investigated for the first time the antimicrobial properties of 5 propolis extracts obtained from different regions of Colombia against isolates of *Staphylococcus spp.* methicillin-resistant.

## MATERIALS AND METHODS

**Propolis:** Five samples of propolis from *Apis mellifera* beehives were collected from different regions (Fusagasugá-Cundinamarca, located at 1.728 Metres Above Sea Level (MASL); Une-Cundinamarca, located at 2.276 MASL; San Luis-Antioquia, located at 1.050 MASL; Betania-Antioquia, located at 1.450 MASL, and Garzón-Huila, located at 828 MASL) from Colombia. We stored under refrigeration (4°C) until they were used in the preparation of extracts.

**Preparation of ethanolic extracts of propolis:** Ethanolic extracts of propolis were obtained using soxhlet extraction according to Cunha *et al.* (2004). Briefly, collected propolis samples were air-dried and powdered in a mortar, and 20g of the powdered sample were processed for 24 h soxhlet extraction at a maximum temperature of 60 °C, using 400 ml of 80% ethanol as solvent. The extracts obtained were left in a freezer overnight to induce the crystallization of dissolved waxes and then filtered through a Whatman no. 4 filter paper at a temperature of approximately 0°C to remove waxes from the extracts. The yield results were calculated based on the initial amount of propolis (w/w). The propolis extracts (EEP) were numbered according to the obtaining region, as follow: EEP 1 from Fusagasugá - Cundinamarca; EEP 2 from Une - Cundinamarca; EEP 3

from San Luis - Antioquia; EEP 4 Betania - Antioquia and EEP 5 from Garzón - Huila.

**Main compounds analysis of propolis extracts:** The analysis of main compounds of the propolis extracts was performed by Gas Chromatography–Mass Spectrometry (GC–MS) in the Laboratory Chemistry of Natural Products at the Institute of Organic Chemistry of Bulgarian Academy of Sciences, according to Popova *et al.* (2010) protocol.

**Bacterial strains:** A total of six methicillin-resistant *Staphylococcus spp* isolates, previously isolated from animal infections were used in this work. All Isolates were identified by conventional methods, including Gram staining, colony morphology, test for catalase, coagulase activity and anaerobic fermentation of mannitol. Staphylococcal isolates were identified by the BBL CRYSTAL™ Identification System (Becton Dickinson Microbiology Systems, Cockeysville, Md.), according to the manufacturer's instructions. Determining the *mecA* gene presence was evaluated by the PCR technique (Vannuffel *et al.* 1995), in the microbiology laboratory of the Veterinary Faculty at Antonio Nariño University – Bogotá Colombia. *Staphylococcus aureus* (ATCC 25923), was used as a control.

All bacterial strains were stored in Trypticase Soy Broth (TSB) medium with 20% of glycerol at –86 °C until further analyses were performed.

**Antimicrobial assay:** Before inoculation, all *Staphylococcus spp* isolates were transferred from the stock cultures to Tryptone Soya Agar (TSA) (Oxoid-CM0131) and incubated overnight at 37 °C. All strains were subsequently subcultured one more time under the same conditions. We used the grown cultures for the preparation of suspensions in sterile phosphate buffered saline (pH 7.2) with densities adjusted to 0.5 McFarland standard.

The MIC of propolis against *Staphylococcus spp* isolates was determined by the tube dilution method according to the procedures recommended by the National Committee for Clinical Laboratory Standards (C.L.S.I. 2013). From a stock solution of 60 mg/mL in 80% ethanol of the propolis, serial dilutions of 1/2 to 1/128 (v/v) were performed in sterile tubes, and they were mixed with equal volume of bacterial suspension.

Positive (broth and inoculum) and negative (simple broth) growth controls were prepared. A control plate with one mL of 80 % ethanol was inoculated with *S. aureus* strains (ATCC 25923) and incubated at 37° C for 24 hours. The MIC was defined as the lowest concentration of EEP that inhibited the growth compared to the control. We read the results on a spectrophotometer (Helios Epsilon, Thermo Spectronic, USA). Where no growth was visible in MIC, the same concentration of propolis tube was tested at TSA plates for further

verification under minimum bactericidal concentration (MBC). Briefly, 20  $\mu$ L of each tube was transferred to TSA plates and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration which could reduce 99.9% of the initial population. We tested all samples in triplicate. Mean values of growth inhibition were calculated.

**Statistical Analysis:** The average values and the standard deviations were calculated from the data obtained from triplicate trials. All data values were normalized to Z points. The mean values were then compared using the one-way ANOVA test. To identify sample means that are significantly different from each other, we used the Student-Newman-Keuls (SNK) test (IBM® SPSS® Statistics 20). A probability level of 5% was considered statistically significant.

## RESULTS AND DISCUSSION

Previous reviews have shown knowledge that over 300 chemical components belonging to the flavonoids, terpenes, and phenolics have been identified in propolis (Bankova *et al.* 2000, Huang *et al.* 2014). In this work, the GC-MS analysis of the propolis extracts showed that major compounds of propolis from Cundinamarca were rich in diterpenic acids; while that of the propolis from Antioquia and Huila regions, were rich in prenylated benzophenones and triterpenes respectively (Table 1). In line with these results, Meneses *et al.* (2009) and Martínez *et al.* (2012) reported the presence of fatty acids and their esters, aromatic acids, sesquiterpenes, diterpenes, triterpenes, and flavonoids among others, on propolis collected from Antioquia. Plants, such as the Pinaceae and Cupressaceae species (*Pinus patula*, *Cupressus lusitanica* Miller) that corresponds to the predominant vegetation in this area were recognized as possible sources of propolis (Meneses *et al.* 2009, Martínez *et al.* 2012). The propolis collected in African and European countries of Mediterranean area also is characterized by its high content of diterpenes (Graikou *et al.* 2016). Likewise, *Clusia* species are important sources of propolis in tropical and subtropical regions, such as Amazon region of Brazil, Cuba, and Venezuela. Generally, this kind of plants contains polyprenylated benzophenones in roots, leaves, and fruits (de Castro Ishida *et al.* 2011).

In this study, all EEP showed an antimicrobial effect against *Staphylococcus spp* isolates,  $MIC_{90} = 60 - 30$  mg/mL, irrespectively of microbial resistance of the isolates. The highest minimum bactericidal concentration we recorded from propolis was at the concentration of 1.87 mg/mL followed by 7.5 mg/mL (Table 2). Similar MBC results from propolis (2.01 to 5.48 mg/mL) were obtained by Miorin *et al.* (2003) and Rahman *et al.*

(2010). Average  $MIC_s$  (mg/mL) for each EEP were: EEP1 = 11.88 $\pm$ 4.98; EEP2 = 31.25 $\pm$ 16.71; EEP3 = 51.25 $\pm$ 21.43; EEP4 = 22.81 $\pm$ 11.88; EEP5 = 17.50 $\pm$ 10.25.

The negative growth was observed in propolis at concentrations of 1.87 to 30 mg/mL, while growth was observed at concentrations of 0.93 mg/mL followed by lower concentrations. These results coincide with those reported by Fernandes *et al.* (1995). In this study, the one-way ANOVA revealed significant differences in microbiology activity between the five propolis extracts ( $P < 0.05$ ), and the SNK test showed the EEP 3 and EEP 1 as different from the others EEPs. EEP3 have shown to be the less efficient inhibitor of growth in 5 of the six isolates of methicillin-resistance *Staphylococcus spp.* isolates. Contrary to observed, EEP1 showed the greatest inhibitory activity against the growth of the six strains (Figure 1). We show that regardless of collection location, 60% (EEP 1, 4 and 5) of the EEP samples exerted antibacterial action on the growth of *Staphylococcus spp* at the dosage of 30 mg/mL (dilution 1/2). It has been observed in previous studies that the antimicrobial activity of natural extracts can be due to the presence of diterpenes (Zamilpa *et al.* 2002). This is consistent with the results obtained for the EEP 1 in this study. Likewise, it has been observed that *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Aeromonas hydrophila*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, show sensitivity to prenylated benzophenones and triterpenes (Mokoka *et al.* 2013, Oya *et al.* 2015). These observations support the results found for EEP 1 and 2 in this study. In this regard, many studies have discovered that the observed effects might be the result of a synergistic action of its complex constituents (Sforcin *et al.* 2005, Bueno-Silva *et al.* 2013). Several works have shown that not even a single component isolated from propolis showed an activity higher than the total extract (Orsi *et al.* 2005, Orsi *et al.* 2012). Nevertheless, EEP 2 (2.276 MASL) and 3 (1050 MASL) displayed lower inhibitory activity against the *Staphylococcus* strain. Conversely, EEP3 and 4 displayed higher antimicrobial activity specifically against *Staphylococcus aureus*, isolates from cows and goat respectively, this may be due to the concentration or another kind of compounds with antagonistic activity (Mani *et al.* 2006). It is also, is important to consider that the chemical composition of propolis varies according to its geographical origin, and to that extent changes its antimicrobial activity, indicating variability in these plant sources.

Ethanol at the concentration used did not interfere with bacterial growth. The same findings were observed in several studies (Sforcin *et al.* 2005, Tukmechi *et al.* 2010, Al-Abbadi *et al.* 2015).

**Table 1. Main chemical constituents identified in Colombian propolis by GC-MS (percent of Total Ion Current, TMS derivatives).**

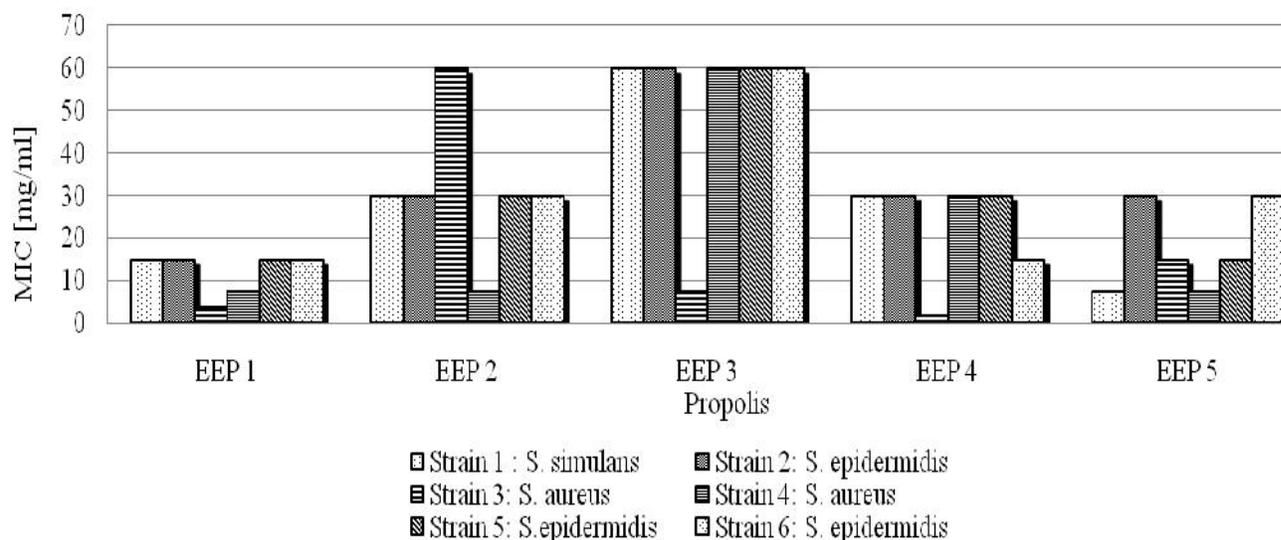
T(min)	COMPOUND	EEP-1	EEP-2	EEP-3	EEP-4	EEP-5
12.1 – 39.6	Sugars	1.0	1.6	-	13.4	5.6
28.9 – 32.5	Fatty acids	-	29.5	7.1	3.9	10.6
38.7 – 41.5	Arachidic acid	-	9.1	0.5	-	9.1
32.3 – 40.2	Diterpenes	10.7	14.9	-	9.7	0.7
44.5 – 44.9	Flavonoids	-	2.2	-	-	-
46.5 – 54.1	Benzophenones	10.0	-	14.0	39.7	-
49.8 – 51.0	Triterpenes	-	-	5.0	2.7	57.2

**Table 2. Minimum Bactericidal Concentration (MBC) of EEP against *Staphylococcus spp.***

ISOLATED STRAIN	EEP(mg/mL)					
	EEP1	EEP2	EEP3	EEP4	EEP5	
1	15	30	60	30	7.5	
2	15	30	60	30	30	
3	3.75	60	7.5	1.87	15	
4	7.5	7.5	60	30	7.5	
5	15	30	60	30	15	
6	15	30	60	15	30	

Strains: (1) *S. simulans*; (2) *S. epidermidis*; (3) *S. aureus*; (4) *S. aureus*; (5) *S. epidermidis*; (6) *S. epidermidis*.

### Minimum inhibitory concentration of EEP



**Figure 1. Survival of *Staphylococcus spp.* to five ethanolic extracts of propolis (EEP) from different regions of Colombia. (MIC: Minimum Inhibitory Concentration. Average MIC: EEP1 = 11.88±4.98; EEP2 = 31.25±16.71; EEP3 = 51.25±21.43; EEP4 = 22.81±11.88; EEP5 = 17.50±10.25)**

**Conclusion:** In conclusion, all EEP tested in this work have shown antibacterial activity against the *Staphylococcus spp.* isolates. However, antimicrobial effectiveness of these depended on its concentration and the collecting areas. In this study, propolis obtained from the regions of Fusagasugá - Cundinamarca, Une - Cundinamarca, Betania - Antioquia and Garzón - Huila

showed the greatest inhibitory activity against the growth of the six strains. These findings suggest, that these propolis extracts could be an alternative method to control multidrug-resistant infections caused by *Staphylococcus spp.*

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