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MICROBIOLOGY

In vitro comparison between antimicrobial and antibiofilm effects of Green Propolis and *Baccharis dracunculifolia* against *Staphylococcus pseudintermedius* isolate

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Abstract: *Staphylococcus pseudintermedius* is the leading cause of canine pyoderma. Honeybee products are common to treat this and other types of infections. High average annual population loss of bees has been observed. This study evaluated antibacterial and antibiofilm profile of Green Propolis and *Baccharis dracunculifolia* against *S. pseudintermedius* and the chemical similarities among both. Ethanolic extracts were produced and chemically characterized. The isolates were subjected to treatment with the extracts in both planktonic and sessile forms. Green propolis minimum inhibitory concentration (MIC) was 0.156 mg / mL, and minimum bactericidal concentration (MBC) was 0.312mg / mL. *Baccharis dracunculifolia* extract MIC and MBC was 0.312mg / mL and 2.5 mg / mL, respectivelly. Both extracts reduced SD55 formation of biofilm at minimum inhibitory concentration and at 1/8 minimum inhibitory concentration. The results observed in relation to ED99, were similar for both extracts. Besides that, similar chemical indicators between both extracts, including the presence of Artepellin C, suggest that the *Baccharis dracunculifolia* extract could be an alternative to the Green Propolis extract in the treatment of staph infections.

Key words: *Staphylococcus pseudintermedius*, Green Propolis, *Baccharis dracunculifolia*, antimicrobial.

INTRODUCTION

Propolis is a natural product derived from bees, with high nutritional value in addition to its medicinal properties like anti-inflammatory, antimicrobial, and healing of wounds and sunburn. Due to the great exploitation of these insects, a high average annual population loss of bees has been observed and even extinctions in many geographic regions (McMenamin et al. 2018). Green Propolis (GP), also known as Brazilian Green Propolis, comes from foraging by Africanized bees (*Apis mellifera* Lepeletier) in plants of the *Baccharis dracunculifolia* (BD) species belonging to the *Asteraceae* family (Costa et al. 2018). The biological properties of GP are attributed to its chemical constituents originated from plant exudates and substances secreted by the honeybee metabolism (Marcucci et al. 2001). Some authors have stated that the biological activities of Brazilian Green Propolis are mostly due to the high levels of prenylated p-coumaric acids, mainly Artepillin C, which is also present in *B. dracunculifolia* DC (Banskota et al. 2001, Bankova 2005).

Staphylococcus pseudintermedius is part of the normal microbiome of dogs and cats, usually found on the skin and mucous membranes, mainly in the nostrils, mouth, and perineum of asymptomatic animals (Gharsa et al. 2013, Kizerwetter-Świda et al. 2017). Although this, they can also cause opportunistic infections such as pyoderma, otitis, endometritis, urinary tract infections, respiratory tract infections among others (Abraham et al. 2007, Ravens et al. 2014, Ruzauskas et al. 2016). Furthermore, methicillin-resistant *S. pseudintermedius* (MRSP) colonization and infection have been described in many companion animals and humans, corroborating its zoonotic potential (Ruzauskas et al. 2016).

Biofilms, defined as a complex community of microorganisms surrounded by an extracellular matrix of polysaccharides adhered to each other and a surface, are considered a resistance factor (Costerton et al. 1995). Members of the Staphylococcus genus are among biofilm-forming bacteria, which are constantly related to persistent chronic infections (Grant & Hung 2013). The ability to form a biofilm is related to the strength of adhesion to a surface and thus to form a layer, so that the density of this layer is directly related to the resistance of the biofilm produced (Machado et al. 2020). In general, biofilm production in this species can be related to a range of factors and genes. Operon *ica* genes are related to producing a polysaccharide extracellular matrix (PIA) and have been found previously in *S. pseudintermedius* strains (Arciola et al. 2005, Casagrande Proietti et al. 2015). Also, *S. pseudintermedius* can harbor 18 different surface proteins (*Sps*) and it is known that some of them (*SpsD* and *SpsL*) can bind to canine corneocytes and extracellular matrix components like fibrinogen and fibronectin, possibly facilitating initial adhesion (Fitzgerald et al. 2019). The *Agr* System is a quorum-sensing system that regulates the expression of virulence factors in *Staphylococcus* species, including the biofilm. Previous studies have described *Agr* System genes in *S. pseudintermedius* strains (Chitra et al. 2015).

The problem of multidrug resistance and the lack of expectations for the launch of new antimicrobials by the pharmaceutical industry has led to an increase in studies regarding the combined antimicrobial activity between drugs and natural products, aiming at future alternative therapies (Torres et al. 2018). Although some studies demonstrated that Green Propolis has antimicrobial activity, especially on Gram-positive bacteria (Stepanović et al. 2003, de Lima et al. 2016) there are no reports about its performance against strains of *S. pseudintermedius*. The present study evaluated the components of ethanolic extracts of GP and BD, and the antibacterial and antibiofilm *in vitro* effects of the Green Propolis and *Baccharis dracunculifolia* ethanolic extracts against a *Staphylococcus pseudintermedius* isolated from canine pyoderma. The possible substitution of the propolis extract for the plant extract may be a strategic way to reduce bees' exploitation.

MATERIALS AND METHODS

The present work was approved by the ethics committee on animal use at Comitê de Ética no Uso de Animais - Centro Estadual de Pesquisa em Saúde Animal Geraldo Manhães Carneiro (CEUA-CEPGM), of Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro, Brazil (protocol CEUA-CEPGM 001 / 16). It was registered at Conselho Nacional de Controle de Experimentação Animal (CONCEA), process n° 01200.003805 / 2014-25. The use of the natural material was conducted under authorization of Sistema Nacional de Gestão do Patrimônio

Genético e do Conhecimento Tradicional Associado (SISGEN) (process number A314288).

Samples

S. pseudintermedius SD55 isolate was collected from dogs with pyoderma during a surveillance study. *S. pseudintermedius* ED99 was used as a control (Ben Zakour et al. 2011). Both belongs to the collection of Laboratório de Cocos Gram-Positivos do Instituto Biomédico, Universidade Federal Fluminense.

Antimicrobial susceptibility test

Susceptibility of SD55 was conducted using the disc diffusion method according to Clinical & Laboratory Standards Institute (CLSI) recommendations (CLSI M100-S23 2013). Antimicrobial agents tested were cefoxitin (30μg), ciprofloxacin (5μg), clindamycin (2μg), doxycycline (30μg), enrofloxacin (5μg), erythromycin (15μg), gentamicin (10μg), nitrofurantoin (10μg), penicillin G (10IU), rifampicin (30μg), sulfamethoxazole/trimethoprim (23.75μg/1.25μg), tetracycline (30μg) and tobramycin (10μg). A strain of *Staphylococcus aureus* (ATCC25923) was used as a positive control for the test. The presence of resistance genes (*mecA*) was performed with a PCR as recommended in previous studies (Zhang et al. 2005).

Polymerase Chain Reaction (PCR) for identification of operon *ica, agr* and *Sps* genes

Sample SD55 was characterized regarding virulence genes (*ica* operon, *Sps* and *Agr* System) as recommended in previous studies (Casagrande Proietti et al. 2015, Chitra et al. 2015). All the amplifications were performed in a Veriti thermocycler (Applied Biosystems, California, USA). Visualization of the reaction products was performed using 1% agarose gel electrophoresis in 0.5X TBE buffer (0.05 mM Tris, 1.25 mM EDTA, and 0.05 M boric acid). A 1Kb molecular size marker (Invitrogen) was used. The amplification product was visualized under ultraviolet light after the products were stained with Gel Red (Invitrogen). The strain ED99 was already characterized and for that was used as a positive control for *ica* operon genes, surface proteins genes, and *agr* System genes.

Ethanolic extracts

The ethanolic extract of *B. dracunculifolia* was obtained as described by Duarte et al. (2004), with slight modification. The leaves were subjected to desiccation in an oven with forced circulation and temperature between 40°C for three days, followed by processing in a mill to obtain dry and pulverized plant material. This material was subjected to exhaustive maceration with 70% ethanol. After several extractions at 72h intervals, the complete depletion of plant material was obtained. The extractive solution then passed through the distillation of the solvent, obtaining the crude ethanolic extract. The ethanolic extract from Green Propolis was obtained as described by El-Guendouz et al. (2016), with slight modification. One gram of propolis was broken up into small pieces and extracted by maceration using 30 ml of 70% alcohol for one week at 37°C under agitation at 200 rpm. After maceration, the extract was concentrated on a rotary evaporator under reduced pressure. The solution for using the ethanolic extracts was diluted to 10% in dimethylsulfoxide (DMSO - Proquímios, RJ-Brazil), to prepare the stock solution. For dilutions of the stock solution, the Müeller Hinton broth was used to reach the concentrations used in the tests.

Phytochemical screening of ethanolic extract

The chromatographic profile of the ethanolic extracts was analyzed by Thin Layer Chromatography (TLC) on silica gel and obtained using the conditions described in the literature

for flavonoids (Stander et al. 2019). The TLC was visualized using a dark chamber of ultraviolet light at a wavelength of 365 nm. High-performance liquid chromatography (HPLC) was used to determine the extracts' chemical composition using the conditions described in the literature (Sun et al. 2019).

Minimal inhibitory and bactericidal concentrations

According to the Clinical and Laboratory Standard Institute guidelines, the minimum inhibitory concentration (MIC) for GP and BD was determined by following the microdilution method (CLSI M100-S23 2013). In a polystyrene plate with 96 wells), 100 µl of the Agar Müeller Hinton and decreasing serial dilutions from 5 mg / mL to 0.039mg / mL of the extract were added ranging a final volume of 100 µl in each well. 5 µl of the bacterial inoculum was standardized at 0.5 on the McFarland ladder (1.5 x 108 CFU / mL), diluting in saline solution (1:10) was placed in each well. The plate was incubated at 35 ± 2ºC in aerobiosis for 16 to 20 hours. As a control, 2 fold decreasing vancomycin concentrations (8 to 0.06 µg / mL) were performed. After incubation, 15 µl of 0.01% resazurin dye was added in each well, with reading after 1h incubation at 35 ± 2ºC. To determine the minimal bactericidal concentration (MBC), before applying the resazurin dye to the MIC plate, 1µL was removed from the wells. Spots were sown on Müeller Hinton agar plates and incubated at 37 ± 2ºC in aerobiosis for 18 to 24 hours, after which the presence or absence of bacterial growth was observed (Miranda et al. 2015).

Microtiter dish biofilm formation assay

An inoculum from three bacterial colonies in 3 mL of TSB medium with 1% glucose was incubated at 35°C for 24 hours. After that, using 96-well microplates, 100 µL of the bacterial inoculum and 100 µL of the ethanolic extract previously diluted in MH broth were dispensed in each well in the test dilutions (MIC and ½ MIC). As controls, 100 µL TSB medium with 1% glucose and 100 µL of TSB medium with 1% glucose with bacterial inoculum were used. After incubation in a bacteriological oven at 35°C for 20 hours, the cell culture was carefully removed, followed by three washes with sterile distilled water. Then, bacterial growth was fixed in a drying oven for approximately 1 hour. The adhered biomass was stained with 100 µL of 0.1% aqueous gentian violet crystal solution, added to each well, kept at rest for 15 minutes at room temperature. Then the dye was removed, and the plate was washed once with sterile distilled water and dried in a drying oven at 65ºC. After drying, 100 µL of alcohol at 96° GL was added to each well and kept at room temperature, without stirring, for 30 minutes, and then the reading proceeded (Antunes et al. 2010).

Microtiter dish on preformed biofilm assay

100 µL of the bacterial inoculum was dispensed in each well (96 wells plate) and directed to incubation in a bacteriological oven at 35°C for 6 hours for young biofilm or 24h for mature biofilm. After that time, the inoculum was carefully removed, the wells washed twice with sterile distilled water, and maintained in drying processes for 15 minutes at room temperature. After drying, 100 µL of the ethanolic extract, previously diluted in MH broth, was added in the test dilutions (MIC and ½ MIC). Following incubation at 35°C for 24 hours. The material was removed, washing the wells three times with sterile distilled water. After drying and fixing in an oven at 65ºC for one hour, it was stained with a 0.1% aqueous violet crystal solution for 15 minutes at room temperature. A further wash with sterile distilled water and drying in an oven at 65ºC was carried out. Then alcohol at 96° GL was added, waiting at rest for 30 minutes at room temperature and then reading (Pierce et al. 2008).

Statistical analysis

Analysis of variance (ANOVA-one way) was used to evaluate the treatment data with the extracts, followed by Tukey's post-hoc test. The software used was the R commander licensed by the GPL (Louis, Missouri, USA), and p values <0.05 were considered with statistically significant differences. The graphs were created using the Microsoft Excel® program.

RESULTS

SD55 demonstrated resistance to only two of the tested antimicrobials (penicillin and tetracycline) and was considered a Methicillin Susceptible *S. pseudintermedius* (MSSP) strain due to the absence of the *mec*A gene. SD55 presented all 4 genes of *ica* operon (ADBC) and all four genes of *Agr* System (ABDC), and it was also positive for seven surface protein genes (*Sps*) tested (*SpsK, SpsL, SpsA, SpsN, SpsP, SpsQ, SpsR*). ED99, the strong biofilm builder used as a control although it showed a higher resistance profile, and the 4 genes of *ica* operon (ADBC) and of *Agr* System (ABDC) it also revealed the absence of the *mec*A gene. Regarding surface proteins, according to Ben Zakour et al. (2011), the isolate has 18 genes including those found in this study for SD55 (Table I).

According to the extracts, they were analyzed by a HPLC validated method. Brazilian Green Propolis and *B. dracunculifolia* ethanolic extracts presented a considerable similarity in their chromatograms (Figure 1). The comparison between the retention times and ultraviolet spectra of the extracts compounds with the authentic standards allowed identifying the derivatives of phenolic acids and flavonoids (Figure 2).

About the antimicrobial results for GP ethanolic extract displayed MIC value of 0,156 mg/mL for both ED99 and SD55, and MBC of 1,25mg/mL for ED99 and 0,312 mg/ mL for SD55. Concerning BD ethanolic extract, MIC for ED99 was 0,625mg/ mL and for SD55 was 0,312mg/ mL. MBC displayed the same value for both (2,5 mg/ mL) (Table II).

Regarding the extracts' action, on SD55 biofilm the Green Propolis extract showed better results, still the *Baccharis dracunculifolia* extract was better at MIC and 1/8 MIC at in formation biofilm and at 1/8 MIC in mature biofilm (Figure 3). About the ED99 a greater influence of the GP extract was also observed, although BD was better at 1/4 and 1/8 MIC at in formation biofilm. Like what happened with the SD55 isolate the influence of BD in the mature ED99 biofilm was greater at 1 / 8 MIC (Figure 4).

Table I. Characterization of *S. pseudintermedius* strains SD55 regarding its resistance profile and the presence of resistance and virulence genes.

Strain	Resistance Profile	*mecA*	*ica* operon genes	*Agr* System genes	Surface proteins genes (*Sps*)
SD55	pen, amo, amp, pip, tet	-	*icaA, icaD, icaC and icaB*	*agrA, agrB, agrD, agrC*	*SpsK, SpsL, SpsA, SpsN, SpsP, SpsQ, SpsR*
ED99	StrepB, ery, ami, rib, str, kan, liv, par, neo, pen, amo, amp, pip, tet, dox, cli, lin	-	*icaA, icaD, icaC and icaB*	*agrA, agrB, agrD, agrC*	All Sps genes including *SpsK, SpsL, SpsA, SpsN, SpsP, SpsQ, SpsR* (Ben Zakour et al. 2011)

pen = penicillin; tet = tetracycline; StrepB = streptogramyn B; ery = erythromycin; ami = amikacin; rib = ribostamycin; str = streptomycin; kan = kanamycin; liv = lividomycin; par = paromomycin; neo = neomycin; amo = amoxicillin; amp = ampicillin; pip = piperacillin; dox = doxycycline; cli = clindamycin; lin = lincomycin.

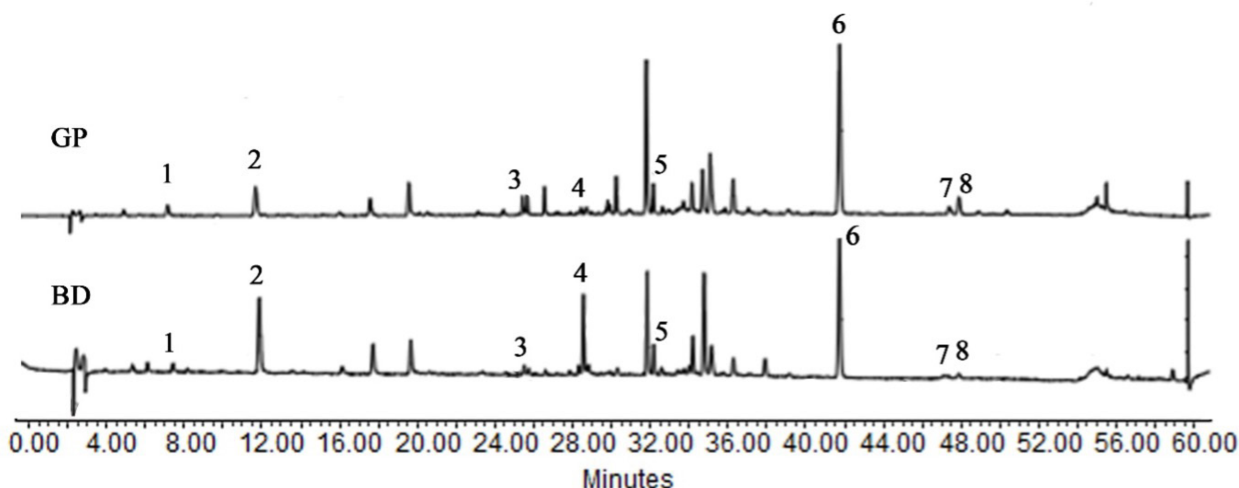

Figure 1. RP-HPLC-DAD chromatograms of Green Propolis (GP) and *B. dracunculifolia* (BD) hydroalcoholic extracts. 1: caffeic acid (RT: 7.596); 2: coumaric acid (RT: 11.947); 3: cinnamic acid (RT: 25.202); 4: dihydrokaempferide (RT: 28.297); 5: drupanin (RT: 32.018); 6: artepillin C (RT: 41.555); 7: baccharin (RT: 46.904); 8: 2,2-dimethyl-6-carboxyethyl-2H-1-benzopyran (RT: 47.612).

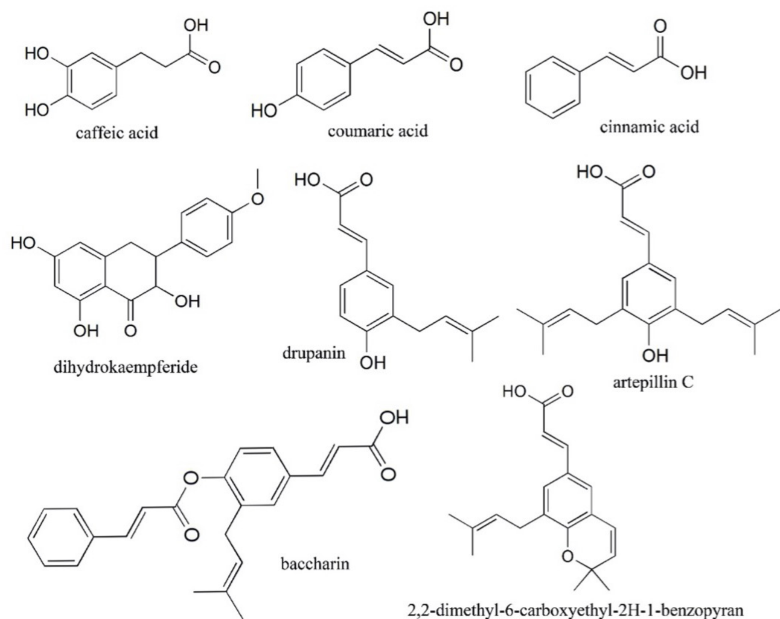

Figure 2. Identified compounds in Green Propolis and *B. dracunculifolia* hydroalcoholic extracts.

Table II. MIC and MBC results of Brazilian Green Propolis (GP) and *Baccharis dracunculifolia* (BD) ethanolic extracts against *Staphylococcus pseudintermedius* isolates.

	GP		BD		Vancomycin (PC)
Isolates	MIC	MBC	MIC	MBC	MIC
S. pseudintermedius ED99	0,0156	1,250	0,625	2,5	0,001
S. pseudintermedius SD55	0,0156	0,312	0,312	2,5	0,001

PC – Positive control; Values expressed in mg/mL; MIC – minimun inhibitory concentration; MBC – minimal bactericidal concentration.

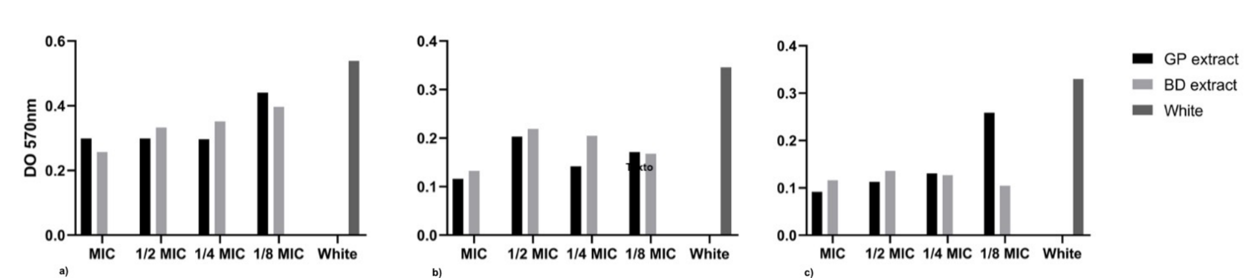

Figure 3. **Optical Density of the Influence of Brazilian Green Propolis (GP) and *Baccharis dracunculifolia* (BD) ethanolic extracts on *Staphylococcus pseudintermedius* SD55 biofilm. a) in formation biofilm; b) young biofilm; c) mature biofilm ($p < 0.05$) (MIC-minimun inhibitory concentration).**

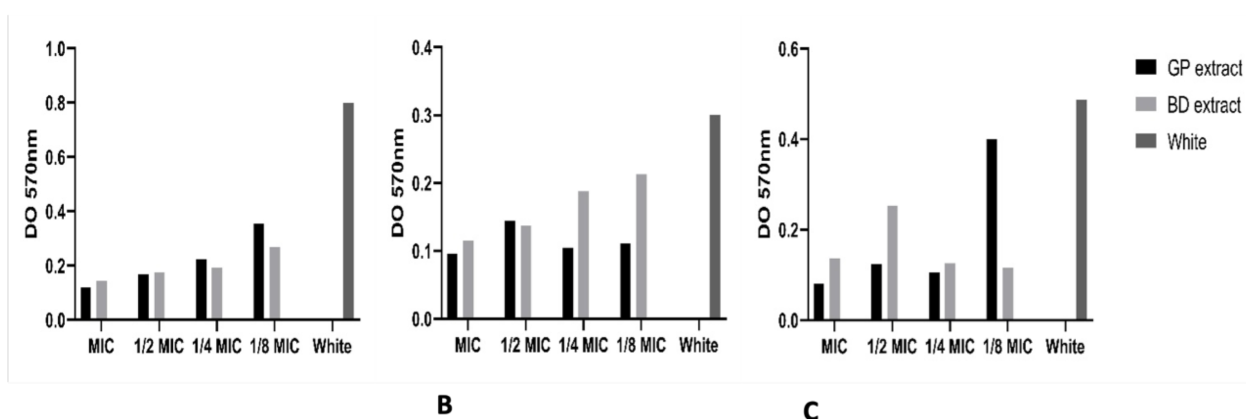

Figure 4. **Optical Density of the Influence of Brazilian Green Propolis (GP) and *Baccharis dracunculifolia* (BD) ethanolic extracts on *Staphylococcus pseudintermedius* ED99 biofilm. a) in formation biofilm, b) young biofilm, c) mature biofilm ($p < 0.05$) (MIC-minimun inhibitory concentration).**

DISCUSSION

Bees are seriously threatened by the combined effects of climate change, intensive agriculture, pesticides, loss of biodiversity, and pollution. Besides that, honeybee products are widely used as arthropod repellents, antimicrobials, and antifungals. Plant extracts used as its botanical source would be an alternative raw material, minimizing the losses suffered by these pollinating insects.

Baccharis dracunculifolia DC and Green Propolis present comparable biological activities. Both GP and BD ethanolic extracts chemical profiles indicate the presence of Artepillin C as a chemical marker, used in the classification and identification of the botanical origin of Green Propolis (Beserra et al. 2020), and Artepillin C has previously been identified as one of the bioactive substances present in propolis of *A. mellifera* with antimicrobial activity (Castro et al. 2009).

Regarding MIC and MBC, the results reinforce the hypothesis that the similarity between both extracts' composition guarantees that they have similar inhibitory and bactericidal activities against *S. pseudintermedius*. These results agree with Veiga et al. (2017) that found better antimicrobial activities in Green Propolis than in other extracts. They suggested that this activity might be related to Artepillin C, which is also present in the extract of *B. dracunculifolia*, but at a lower concentration. Also, Artepillin C might be acting in combination with the other compounds in Green Propolis and *B. dracunculifolia* (Veiga et al. 2017).

The strength to form biofilm is related to the bacteria ability to stick to a surface and form a layer, so the density of this layer is directly related to the strength of biofilm formation (Machado et al. 2020). Biofilms are considered a form of bacterial resistance, also because they hinder the access of antimicrobials to bacterial cells protected by the exopolysaccharide framework. Even with this arsenal to produce biofilm, both extracts analyzed had an interesting and similar activity both in the phase of aggregation of cells to the substrate (biofilm in formation), in the phase of cell-cell adhesion (young biofilm) and the final phase corresponding to cellular displacement (mature biofilm). These results suggest that *Baccharis dracunculifolia* could be an additional option for Green Propolis to supply market demand. Knowing that propolis is widely used, we expect that deeper studies, including cytotoxicity of the different components of BD extract, can direct the possible substitution of the propolis extract by the plant and minimize the exploitation of bees. Also, both extracts presented encouraging results against bacteria and their biofilm and could be considered an interesting alternative treatment to avoid antimicrobial usage.

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