Journal of Advanced Veterinary Research

https://advetresearch.com

Comparative Analysis Between the *in vitro* Performances of the Hydroalcoholic Extracts of Green Propolis and *Baccharis dracunculifolia* against *Staphylococcus aureus*

Yasmim de Moraes Assumpção[1], Eunice Ventura Barbosa[2], Renata Freire Alves Pereira[3,4], Leandro Rocha[2], Bruno Penna[1,4*]

[1]*Gram-Positive Cocos Laboratory, Biomedical Institute, Fluminense Federal University, Brazil.*
[2]*Natural Products Technology Laboratory - Fluminense Federal University, Brazil.*
[3]*Laboratory of Molecular Epidemiology and Biotechnology - Rodolfo Albino Laboratory, Fluminense Federal University, Brazil.*
[4]*Post-graduation Program in Applied Microbiology and Parasitology - Fluminense Federal University, Brazil.*

ARTICLE INFO

Original Research

Received:
27 October 2021

Accepted:
02 January 2022

Keywords:

Staphylococcus aureus, Brazilian Green Propolis, Baccharis dracunculifolia, MIC/MBC, Biofilm

ABSTRACT

Staphylococcus aureus is one of the most common opportunistic pathogens in humans and several animals. Some strains can form a biofilm, which is related to chronic diseases. Green Propolis are used frequently in alternative medicine. Several studies have demonstrated the chemical similarities between Green Propolis and its botanical source *Baccharis dracunculifolia*. This study evaluated the antibacterial profile of the hydroalcoholic extracts of Green Propolis and *Baccharis dracunculifolia* against *S. aureus*. The antibiofilm effect of the extracts was also evaluated against a reference strain of *S. aureus*. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) for each isolate were assessed. MIC and MBC values for both extracts were similar against all bacterial strains. The extracts demonstrated good performance against the biofilm of the *S. aureus* ATCC 25923. Green Propolis and *Baccharis dracunculifolia* extract antibiofilm activities were mainly on biofilm's newly formed and consolidated moments.

Introduction

The global epidemic of multidrug-resistant microbes is putting unprecedented pressure on the medical research community. The problem of resistance to multiple drugs depends on factors such as the lack of expectations for the launch of new antimicrobials by the pharmaceutical industry. Besides, microorganisms are becoming increasingly resistant to the arsenal of available antimicrobials. The inappropriate and excessive use of antibiotics to treat microbial infections and the resulting pressure of antibiotic selection only worsened the situation. This fact has led to an increase in studies on the combined activity between antimicrobials and natural products, aiming at a future therapeutic alternative (Mitsugui *et al*., 2008; Torres *et al*., 2018).

Staphylococcus aureus is considered one of the most common opportunistic pathogens in humans and several animals. This bacteria harbor intrinsic virulence determinants and the ability to cause a wide range of infections (Hernández *et al*., 2001; Lowy, 2003). One of the most worries nowadays is microorganisms' ability to form a biofilm (Karaolis *et al*., 2005). Biofilms are virulence factors that hinder and limit bacterial infection treatment, especially that of the *Staphylococcus* genus (Hall-Stoodley *et al*., 2004; Grant and Hung, 2013). The formation of these sessile communities and their inherent resistance to antimicrobial agents are associated with persistent and chronic bacterial infections (Costerton *et al*., 1999). Besides the importance of *S. aureus* in human medicine, it is also the object of study in veterinary medicine due to its zoonotic potential (Hanselman *et al*., 2009). Methicillin-resistant *S. aureus* (MRSA) cases have emerged in production animals (Graveland *et al*., 2011; Loncaric *et al*., 2019).

Green Propolis, also known as Brazilian Green Propolis (GP), comes from foraging by Africanized bees (*Apis mellifera Lepeletier*) in plants of the species *Baccharis dracunculifolia* DC (BD) belonging to the Asteraceae family (Costa *et al*., 2018). Among some known GP properties, one of the most studied is related to antimicrobial activity, being identified greater efficiency on Gram-positive bacteria, especially concerning *S.*

*Corresponding author: Bruno Penna
E-mail address: bpenna@id.uff.br

aureus (Stepanovic *et al*., 2003; Lima *et al*., 2015). Different researchers investigated similarities between the Brazilian Green Propolis and *B. dracunculifolia*, the propolis' botanical origin. Due to its high antioxidant and antimicrobial power, *B. dracunculifolia* has been studied as a possible crucial raw material in the industrial process to manufacture medicines and cosmetics (Casagrande *et al*., 2018). The present study evaluated the components of hydroalcoholic extracts of GP and BD, their antimicrobial, and antibiofilm potential.

Materials and methods

This study was approved by the Ethics Committee on the Use of Animals of the State Animal Health Research Center Geraldo Manhães Carneiro (CEUA-CEPGM), of the Agricultural Research Corporation of the State of Rio de Janeiro (protocol CEUA-CEPGM 001/16) and registered with the National Council for Animal Experimentation Control (CONCEA), process # 01200.003805/2014-25.

Extracts of Brazilian Green Propolis (GP) and Baccharis dracunculifolia D.C. (BD)

Aerial parts of *Baccharis dracunculifolia* were harvested in the mountainous region of Trajano de Moraes, a municipality located in the North of the Rio de Janeiro State, between coordinates 22° 03'48" S - 42° 03'59" W. A plant material voucher was deposited at the herbarium of the State University of Rio de Janeiro, under the number RFFP 19499.

The 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 air circulation at 40 °C for three days, followed by processing in a mill furnishing dry and pulverized plant material. This plant material was extracted by exhaustive maceration with aqueous ethanol 70% (w/w), furnishing the crude hydroalcoholic extract after evaporation of the solvent in a rotary evaporator.

A beekeeper collected green propolis from *Apis mellifera* bees' hive in the same municipality where *B. dracunculifolia* was collected (Trajano de Moraes). The product was taken from beehives located at the following coordinate: 22002'48" S - 42001'55" O. The hydroalcoholic extract of Green Propolis was prepared as described for obtaining the extract of *B. dracunculifolia*.

Bacterial samples

S. aureus ATCC 43300 (MRSA) and *S. aureus* ATCC 25923 (strong biofilm builder) were kindly provided by the Reference Materials Laboratory of the National Institute for Quality Control in Health of the Oswaldo Cruz Foundation -R.J. Two clinical isolates of *S. aureus* BL4 (no subclinical mastitis, tetracycline-resistant) and *S. aureus* LD2AD (MRSA, subclinical mastitis) were kindly provided by the Collection of Pathogenic Microorganisms for Production Animals (CMPAP) of PESAGRO-RIO. Bacteria were cultivated in Agar Mueller Hinton medium and incubated aerobically at 37ºC for 24 h. After growth, cultures were stored at 4 °C.

Minimum Inhibitory Concentration (MIC)

CLSI (2018) determinations were undertaken with some modifications to access the MIC. In the first row of a 96 wells polystyrene plate containing 190 µL of Mueller Hinton broth, 10 µL of the extract diluted to 10% in DMSO were added. 100 µL of the medium was added to the wells of the second row onwards. From the first well of the column to the last, double serial dilutions were performed. After that, 5 µL of the inoculum 0.5 McFarland were added in each well. The final concentration of the extract in the same column varied from 5 mg/ml to 0.039 mg /ml. In the last column, Vancomycin from 8 to 0.06 µg/mL was used as a positive control. After 24 hours of incubation at 37 °C, 15 µL of Resazurin (7-hydroxy-3H-phenoxazine-3-one-10-oxide) were added into each well, and after 1 to 4 h of incubation at 35.0±2.0 ºC in aerobiosis, they were analyzed. The experiments were carried out in three replicates of triplicates, and MIC was considered the lowest concentration of the substance that prevented visible bacterial growth (pink indicates bacterial growth and the blue, absence of growth).

Minimum Bactericidal Concentration (MBC)

For MBC, 1 µL of each serial dilution obtained at the MIC wells, was removed after the incubation period, and before adding Resazurin solution. Spots were sown on Mueller Hinton agar plates and incubated at 37.0±2.0 ºC in aerobiosis for 18 to 24 h, followed by observation of the presence or absence of bacterial growth (Miranda *et al*., 2015).

Evaluation of the Antibiofilm Activity of Hydroethanolic Extracts

Microtiter dish biofilm formation assay

An inoculum from three bacterial colonies in 3 mL of TSB medium with 1% glucose was incubated in an oven at 35 °C for 24 h. After that, using 96-well microplates, 100 µL of the bacterial inoculum, and 100 µL of the hydroethanolic extract previously diluted in Mueller Hinton broth corresponding to specific concentrations (MIC, 1/2MIC, 1/4MIC, and 1/8MIC) were applied in 96-well plates. After incubation in a bacteriological oven at 35 °C for 20 h, 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 h. The adhered biomass was stained with 100 µL of 0.1% aqueous gentian violet crystal solution, added to each well, and kept for 15 min 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 65 ºC. After drying, 100 µL of alcohol at 96 ° GL was added to each well and kept at room temperature, without stirring for 30 min, and then the Optical Density (OD) reading was performed (Antunes *et al*., 2010).

Microtiter dish on preformed biofilm assay

For the evaluation of extracts regarding the breakdown of bacterial cells, 100 µL of the bacterial inoculum was dispensed in each well (96 wells plate) and directed to incubation at 35 °C for 6 h for newly formed biofilm or 24 h for consolidated biofilm. After the time for the formation of the biofilm, the inoculum was carefully removed. The wells were washed twice with sterile distilled water and maintained in drying processes for 15 min at room temperature. After drying, 100 µL of the hydroethanolic extract, previously diluted in Mueller Hinton broth, were added into the test dilutions (MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC), following the previously evaluation protocol (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.

Results

Regarding the antimicrobial results, all the strains displayed similar MIC values ranging between 1.25 and 2.5 mg/mL. The MBC values were similar for all isolates for both extracts (Table 1).

Regarding the antibiofilm effects against *S. aureus* ATCC 25923, GP hydroethanolic extract showed significant density reduction until concentration of 1/8MIC. Although it has shown activity against the consolidated biofilm, the best results were shown for newly formed biofilm at all concentrations (Fig. 1). BD hydroalcoholic extract inhibited the biofilm newly formed at 1/8 of MIC, as well (Fig. 2). Both BD and GP did not demonstrated capacity to act in biofilms that were in formation.

Table 1. MIC and MBC results of Brazilian Green Propolis (GP) and *Baccharis dracunculifolia* (BD) hydroalcoholic extracts against *Staphylococcus aureus* isolates.

Isolates	GP		BD		Vancomycin (PC)
	MIC	MBC	MIC	MBC	MIC
S. aureus ATCC 25923	2.5	5	2.5	5	0.001
S. aureus ATCC 43300	1.25	>5.0	1..25	>5.0	0.001
S. aureus BL4	1.25	5	2.5	5	0.001
S. aureus LD2AD	2.5	5	2.5	5	0.001

*PC Positive Control. Values expressed in mg/mL

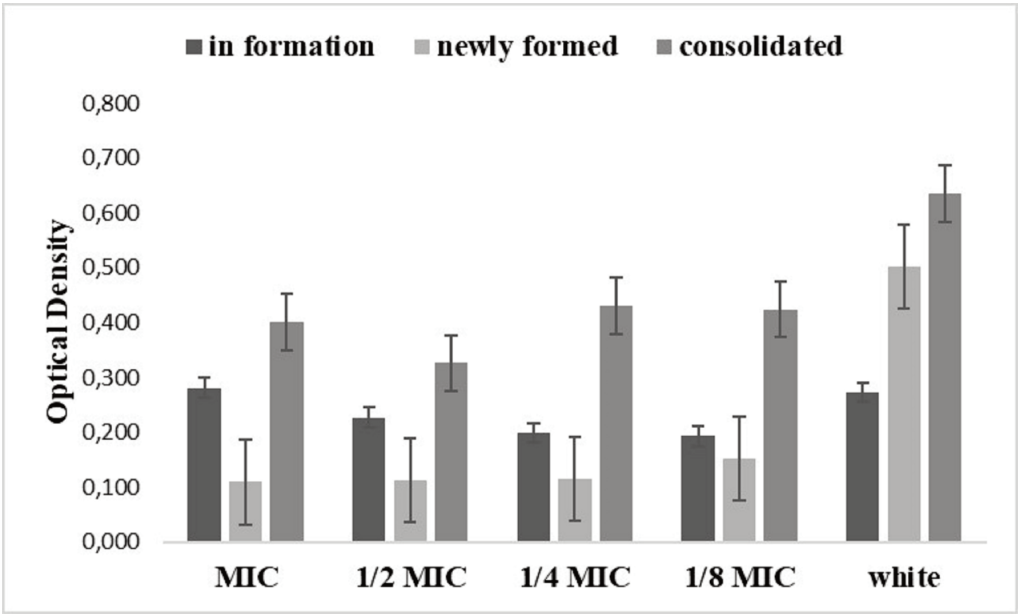

Fig. 1. Optical Density of the Influence of Brazilian Green Propolis hydroalcoholic extract on *Staphylococcus aureus* ATCC 25923 biofilm in formation, newly formed and consolidated ($p < 0.05$).

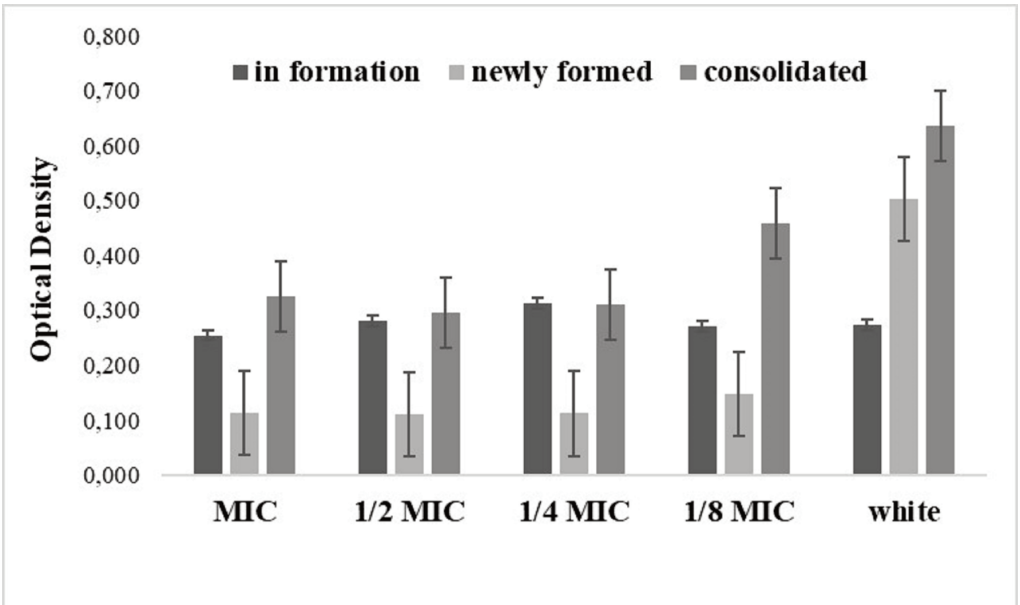

Fig. 2. Optical Density of the Influence of *Baccharis dracunculifolia* hydroalcoholic extract on *Staphylococcus aureus* ATCC 25923 on biofilm in formation, newly formed and consolidated ($p < 0.05$)

Discussion

The Food and Agriculture Organization of the United Nations (FAO) urges countries and people to protect bees and other pollinators because of the risk of a drastic decline in food diversity (www.fao.org/news/story/es/item/1132362/icode/). Bees are seriously threatened by the combined effects of climate change, intensive agriculture, pesticides, loss of biodiversity, and pollution. To replace propolis products derived from bees, plant extracts used as its botanical source would be an alternative raw material for these products, minimizing the losses suffered by these pollinating insects.

Both GP and BD hydroalcoholic 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 (Maróstica *et al*., 2008; Sun *et al*., 2019; Beserra *et al*., 2020). According to the results, it is suggestive that artepillin C might contribute to most biological activities displayed by GP and BD.

Regarding MIC observed values, these results reinforce the hypothesis that the similarity between both extracts' composition guarantees that they have similar inhibitory and bactericidal activities against *S. aureus*. Casagrande *et al*. (2018) reported BD's antimicrobial activity against the reference strain of *S. aureus* ATCC 25923. They found a higher value for MIC (12.75 mg / mL) than ours (2.5 mg/mL), using the same inoculum size. This difference might be explained, in part, by the different locations and season of the plant material collection (Valencia *et al*., 2012), being ours from Rio de Janeiro State and theirs from Paraná State. The chemical profile may also be influenced by plant genotype and differences in the active constituents (Sun *et al*. 2019).

The clinical strains from animal samples were obtained from milk. The results, ranging from 1.25 for the sample of no subclinical mastitis to 2.5 mg/mL, for the subclinical mastitis, are higher than those reported by Diaz *et al*. (2010). They found 0.8 mg/mL for MIC of BD extract against a clinical strain of *S. aureus* isolated from bovine mastitis. The obtained findings suggest great potential of *B. dracunculifolia* for antimicrobial activity (Duarte *et al*., 2004; Diaz *et al*., 2010; Pereira *et al*., 2016; Casagrande *et al*., 2018). The GP and BD had similar values for both MIC and MBC, which confirms the same chemical profile concept due to the sharing of origin.

Doganli (2016) analyzed a propolis extract against *S. aureus* ATCC 33862 at a 20 mg/mL concentration and managed to reduce 60.4% of the biofilm formed, and after chemical analysis, it was verified the presence of flavonoids and phenolic acids in its composition. These results agree with Veiga *et al*. (2017) reports, who 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*.

Green Propolis and *Baccharis dracunculifolia* extracts displayed similar bactericidal and bacteriostatic activities against *Staphylococcus aureus* strains. The chemical HPLC chromatographic profile of both hydroalcoholic extracts showed compounds with antimicrobial activity, such as artepillin C, baccharin, and drupanin. The antibiofilm activity for the extracts of Green Propolis and *B. dracunculifolia* was evidenced in the newly formed and consolidated moments against the clinical and reference strains. These results may suggest using the molecules from the extracts as a strategy to destroy or prevent the formation of bacterial biofilms. Also, *Baccharis dracunculifolia* could be an additional option for Green Propolis to supply market demand.

Conclusion

Results indicate that *Baccharis dracunculifolia* hydroalcoholic extract has the potential to replace Green Propolis for the treatment of infections caused by *Staphylococcus aureus*, including the fact that both contain Artepelin C in their composition. Further studies are needed to confirm this evidence.

Acknowledgement

The authors are grateful for the financial support of the CNPq, FAPERJ, CAPES, and São Paulo Research Foundation (FAPESP), grant # 2017/04138-8. The authors thank the beekeeper Dirceu Thomaz Azevedo for providing the Green Propolis samples.

Conflict of interest

Authors declared that they have no conflict of interest.

References

Antunes, A.L.S., Trentin, D.S., Bonfanti, J.W., Pinto, C.C.F., Perez, L.R.R., Macedo, A.J., Barth, A.L., 2010. Application of a feasible method for determination of biofilm antimicrobial susceptibility in staphylococci. Apmis. 118, 873–877.

Beserra, F.P., Gushiken, L.F.S., Hussni, M.F., Ribeiro, V.P., Bonamin, F., Jackson, C.J., Pellizzon, C.H., Bastos, J.K., 2020. Artepillin C as an outstanding phenolic compound of Brazilian green propolis for disease treatment: A review on pharmacological aspects. Phyther Res. 35, 2274-2286.

Casagrande, M., Zanela, J., Wagner, A., Busso, C., Wouk, J., Iurckevicz, G., Montanher, P.F., Yamashita, F., Malfatti, C.R.M., 2018. Influence of time, temperature and solvent on the extraction of bioactive compounds of *Baccharis dracunculifolia*: In vitro antioxidant activity, antimicrobial potential, and phenolic compound quantification. Ind Crops Prod. 125, 207–219.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing., 2018. 28th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

Costa, P., Almeida, M.O., Lemos, M., Arruda, C., Casoti, R., Somensi, L.B., Boeing, T., Mariott, M., da Silva, R. de C.M.V. de A.F., Stein, B.D.P., Souza, P., dos Santos, A.C., Bastos, J.K., da Silva, L.M., Andrade, S.F., 2018. Artepillin C, drupanin, aromadendrin-4'-O-methyl-ether, and kaempferide from Brazilian green propolis promote gastroprotective action by diversified mode of action. J. Ethnopharmacol. 226, 82–89.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: A common cause of persistent infections. Science 284, 1318–1322.

Diaz, M.A.N., Rossi, C.C., Mendonça, V.R., Silva, D.M., de Ribon, A.O.B., Aguilar, A.P., Muñoz, G.D., 2010. Screening de plantas medicinais com atividade antimicrobiana contra cepas de *Staphylococcus aureus* isoladas de mastite bovina. Brazilian J. Pharmacogn. 20, 724–728.

Doganli, G.A., 2016. Phenolic content and antibiofilm activity of propolis against clinical MSSA strains. Rec. Nat. Prod. 10, 617–627.

Duarte, M.C.T., Figueira, G.M., Pereira, B., Magalhães, P.M., Delarmelina, C., 2004. Atividade antimicrobiana de extratos hidroalcólicos de espécies da coleção de plantas medicinais CPQBA/UNICAMP. Rev. Bras. Farmacogn 14, 6–8.

Grant, S.S., Hung, D.T., 2013. Persistent bacterial infections, antibiotic tolerance, and oxidative stress response. Virulence 4,273–283.

Graveland, H., Wagenaar, J.A., Bergs, K., Heesterbeek, H., Heederik, D., 2011. Persistence of livestock associated MRSA CC398 in humans is dependent on intensity of animal contact. PLoS One 6, 1–7.

Hall-Stoodley, L., Costerton, J.W., Stoodley. P., 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nat Rev Microbiol. 2, 95–108.

Hanselman, B.A., Kruth, S.A., Rousseau, J., Weese, J.S., 2009. Coagulase positive staphylococcal colonization of humans and their

household pets. Can. Vet. J. 50, 954–958.

Hernández, J.L., Calvo, J., Sota, R., Agüero, J., García-Palomo, J.D., Fariñas, M.C., 2001. Clinical and microbiological characteristics of 28 patients with *Staphylococcus schleiferi* infection. Eur J Clin Microbiol Infect Dis. 20, 153–158.

Karaolis, D.K.R., Rashid, M.H., Chythanya, R., Luo, W., Hyodo, M., Hayakawa, Y., 2005. c-di-GMP (3J-5J-Cyclic Diguanylic Acid) Inhibits. Society. 49, 1029–1038.

Lima, M.F.P., Borges, M.A., Parente, R.S., Júnior, R.C.V, de Oliveira, M.E., 2015. *Staphylococcus aureus* and nosocomial infections - literature review. 21, 32–39.

Loncaric, I., Lepuschitz, S., Ruppitsch, W., Tristan, A., Andreadis. T., Bouchlis, N., Marbach, H., Schauer, B., Szostak, M.P., Feßler, A.T., Künzel, F., Licka, T., Springer, B., Allerberger, F., Monecke, S., Ehricht, F., Schwarz, S., Spergser, J., 2019. Increased genetic diversity of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from companion animals. Vet. Microbiol. 235, 118–126.

Lowy, F.D., 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. J. Clin. Invest. 111, 1265–1273.

Maróstica, M.R., Daugsch, A., Moraes, C.S., Queiroga, C.L., Pastore, G.M., Park, Y.K., 2008. Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin *Baccharis dracunculifolia*. Cienc e Tecnol Aliment. 2, 178–181.

Miranda, J.A., Rocha, J.A., Araújo, K.M., Quelemes, P.V., Mayo, S.J., Andrade, I.M., 2015. Atividade antibacteriana de extratos de folhas de *Montrichardia linifera* (Arruda) Schott (Araceae). Rev Bras Plantas Med. 17, 1142–1149.

Mitsugui, C.S., Tognim, M.C.B., Carrara-Marrone, F.E., Garcia, L.B., 2008. Efeito antimicrobiano *in vitro* da associação de polimixina b e ceftazidima em amostras clínicas de *Pseudomonas aeruginosa*. 7, 76–81.

Pereira, C.A., Costa, A.C.B.P., Liporoni, P.C.S., Rego, M.A., Jorge, A.O.C,. 2016. Antibacterial activity of *Baccharis dracunculifolia* in planktonic cultures and biofilms of Streptococcus mutans. J Infect Public Health 9, 324–330.

Pierce, C.G., Uppuluri, P., Tristan, A.R., Wormley, F.L., Mowat, E., Ramage, G., Lopez-Ribot, J.L., 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 3, 1494–1500.

Stepanovic, S., Daki, I., Milena, Š., 2003. In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. Microbiol. Res. 158, 353–357.

Sun, S., Liu, M., He, J., Li, K., Zhang, X., Yin, G., 2019. Identification and determination of seven phenolic acids in Brazilian green propolis by UPLC-ESI-QTOF-MS and HPLC. Molecules. 24, 1–13.

Torres, A.R., Sandjo, L.P., Friedemann, M.T., Tomazzoli, M.M., Maraschino, M., Mello, C.F., 2018. Chemical characterization, antioxidant and antimicrobial activity of propolis obtained from *Melipona quadrifasciata quadrifasciata* and *Tetragonisca angustula* stingless bees. 51, 1–10.

Valencia, D., Alday, E., Robles-Zepeda, R., Garibay-Escobar, A., Galvez-Ruiz, J.C., Salas-Reyes, M., Jiménez-Estrada, M., Velazquez-Contreras, E., Hernandez, J., Velazquez, C., 2012. Seasonal effect on chemical composition and biological activities of Sonoran propolis. 131, 645–651.

Veiga, R.S., De Mendonça, S., Mendes, P.B., Paulino, N., Mimica, M.J., Lagareiro Netto A.A., Lira, I.S., López, B.G-C., Negrão, V., Marcucci, M.C., 2017. Artepillin C and phenolic compounds responsible for antimicrobial and antioxidant activity of green propolis and *Baccharis dracunculifolia* DC. J Appl Microbiol. 122, 911–920.