BJNS
Brazilian Journal of Natural Sciences

SYNERGISTIC EFFECT OF BACCHARIS DRACUNCULIFOLIA DC AND GREEN PROPOLIS WITH ANTIMICROBIAL DRUGS AGAINST *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Relevance: This study demonstrated the synergism of various natural preparations in combination with antibiotics, which is a matter of high potential therapeutic interest due to the development of bacterial resistance to these antibiotic drugs. This synergism is very relevant because it demonstrates that these extracts and their association with these antibiotics may be viable therapeutic options against the pathogens that are responsible for a high incidence of nosocomial and community infections. Aims: The study investigated the antibacterial activity against Staphylococcus aureus isolates (methicillin sensitive Staphylococcus aureus (MSSA) and methicillin resistant Staphylococcus aureus (MRSA) of green propolis and Baccharis dracunculifolia DC. ethanolic extracts combined with classical antibiotics. Methods and Results: In the test of the antibacterial activity of the combination of classical antibiotics with natural extracts (ten antibiotics plus one association of two antimicrobial disc diffusions), 25 isolates were used. The isolates were inoculated in Müeller Hinton agar culture medium containing the green propolis and Baccharis dracunculifolia DC. Extracts Baccharis dracunculifolia DC ethanolic extract (BDEE) showed higher antibacterial synergistic behavior with the antibiotics tested than green propolis ethanolic extract (PVEE). Conclusions: BDEE was the preparation that showed the best synergistic antimicrobial effect in sub doses with the tested antibiotics. These activities may be related to Artepillin C, the major compound in PVEE, and nerolidol, the major compound in BDEE, in synergism with other minority compounds and related antibiotics.

Keywords: green propolis; Baccharis dracunculifolia; Artepillin C; nerolidol; Staphylococcus aureus; MRSA; MSSA; antibiotics; synergistic.

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DOI: https://doi.org/10.31415/bjns.v5i1.178 - Article received on: July 19, 2023; accepted July 25, 2023; published on August 4, 2023, in the Brazilian Journal of Natural Sciences, ISSN: 2595-0584, Vol. 5, N1, Qualis B4, Online at www.bjns.com.br. All authors contributed equally to the article. The authors declare that there is no conflict of interest.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) bacteria are important etiologic agents of community based and healthcare related infections. They cause infections of the skin, soft tissues, surgical sites and bone tissue, as well as causing pneumonia, bacteremia, endocarditis and joint infections [1,2]. Currently, β-lactam antibiotics are the preferred drugs against *S. aureus* infections. *S. aureus* antibiotic resistant isolates are a major public health concern since these bacteria can easily spread in the environment. β-lactam antibiotics resistance developed through β-lactamases synthesis and penicillin binding protein mutations, encoded by plasmids and chromosomes [3–5]. Antimicrobial resistance has hampered antimicrobial therapy and, more recently, new clones of methicillin resistant bacteria (MRSA) have emerged in different parts of the world, including Brazil [6]. Colonization in humans is very common and present in at least 30–50% of individuals [7]. These colonizing microorganisms act as opportunists, causing endogenous infections and being transmitted to other patients [7]. One strategy to discover new therapeutic actions against multiresistant bacteria is the investigation of natural products [8].

Baccharis spp. is one of the largest genera in the Asteraceae family and includes more than 500 species that are primarily distributed in southern and southeastern Brazil, Uruguay, Paraguay, Argentina and Bolivia [9]. Many species from this genus have been widely used in popular medicine for treatment and prevention of anemia, inflammation, and diabetes, as well as for stomach, liver- and prostate-related diseases [10].

Baccharis dracunculifolia DC. (*B.dracunculifolia*, Asteraceae), a native Brazilian plant popularly known as *"alecrim do campo*", is traditionally used as treatment for inflammation, liver disorders and gastric ulcer. It is the most important plant source for propolis production from the southeast region of Brazil, also known as "green propolis". No signs of acute toxicity were observed following the administration of a single dose of 2000 mg/kg of *B.dracunculifolia* DC. essential oil in rodents [11], indicating its safety.

Extracts and essential oil of *B.dracunculifolia* DC. were studied in association with classical antibiotics. In some cases, there was no change in the antibiotic action; however, in many cases, there was a synergistic or an antagonistic interference in the antibiotic action. These results show that the use of plant derived products can interfere in the efficiency of antibiotics [12].

Propolis is a natural resinous substance collected by honeybees (*Apis mellifera*) from plant buds and exudates, and it is used by bees as a barrier to prevent microbial contamination of the hive. In Brazil's southeast, a specific type of propolis is known as "green propolis" because of its color. It has been used in popular medicine to promote health and to prevent various diseases [13,14]. Several biological activities have been attributed to Brazilian green propolis, such as antimicrobial [15], leishmanicide [16], antimutagenic [17] and antioxidant activities [18,19].

The biological properties of propolis result from the components contained in the collected plant exudates [18]. Many authors have argued that green propolis's biological activities are mainly related to its high concentrations of *p*-coumaric acid derivatives, especially Artepillin C (3,5-diprenyl-p-cumaric acid), which is also present in *B.dracunculifolia* DC. [18–21]. Therefore, investigation of the biological properties of various substances responsible for the effects of Brazilian green propolis and *B.dracunculifolia* DC. is important, not only for academic reasons but also for quality issues and characterization of the main constituents present in both [13,14].

In vitro synergism among different propolis types and preparations has been investigated, as associations of propolis with antibiotics are considered to have high potential therapeutic interest. Due to the possible development of bacterial resistance to antibiotic drugs, such synergism is relevant, and it is evident that propolis may be a viable therapeutic option against bacterial diseases [22]. The widespread use of antimicrobial drugs and the ability of certain microbes to acquire accessory genes that can cause diversity in microbes phenotypes and resistance mechanisms has led to an unprecedented crisis in antimicrobial resistance [23]. Ethanol extracts of propolis are of great significance in this context, exhibiting higher antibacterial and antifungal activities against multidrug resistant strains [24]. The combination of propolis and antimicrobial drugs may allow the use of lower doses of these antibiotics by potentializing their effects, presenting high potential especially when used on the skin [25]. The antimicrobial activities of extracts of green propolis and *B.dracunculifolia* DC. Have been demonstrated with MRSA and MSSA clinical isolates, indicating that they could be important tools to treat infections caused by these bacteria [19]. Extracts of propolis, the main constituents of which are coumaric and cinnamic acids, show high antimicrobial activity against *Staphylococcus* spp. Oxacillin resistant strains [26]. The hydroalcoholic extract of *B.dracunculifolia* DC. has the potential to replace green propolis in the treatment of infections caused by *Staphylococcus aureus* (*S.au- reus*), due to the fact that both contain Artepillin C in their compositions [27].

MATERIAL AND METHODS

Baccharis dracunculifolia DC. and Green Propolis Samples

Green propolis resin produced by honeybees (*Apis mellifera*) and *B.dracunculifolia* DC. leaf buds ("*alecrim do campo*") were obtained in February 2014 by Bee Propolis Brasil Ltd., located in Bambuí, Minas Gerais, Brazil. They were stored at room temperature in the dark and protected from humidity.

Preparation of Plant and Green Propolis Extracts

Samples of *B.dracunculifolia* DC. (BD) and green propolis (PV) were dried and extracted with ethanol 95% (Ecibra, Brazil) (BDEE and PVEE, respectively). The extracts concentrations were prepared in accordance with the dry material present in the raw materials. The ethanolic extracts of both were prepared by steeping them for 2 weeks in ethanol and then filtering them through qualitative filter paper (pore size: 8 µm, Whatman®,

 DOI: https://doi.org/10.31415/bjns.v5i1.178

Maidstone, UK). All extracts were stored in the dark in hermetically closed glass bottles under refrigeration at 8 °C [19].

Identification of Phenolic Compounds and Diterpenes in Baccharis dracunculifolia DC. and Green Propolis Ethanolic Extracts by UHPLC-MS

The BDEE and PVEE solvents were evaporated, and 10 mg of each dry extract was dissolved in 1 mL of ethanol. Aliquots of 4 µL of each sample and standards were analyzed using ultra high performance chromatography with mass spectrometry (UHPLC- MS). The chromatography conditions, solvents and gradient were adapted from [28] and are described in the following. The chromatographic conditions were: Waters UPLC® Acquity chromatograph with a BEH Acquity Waters C18 column (1.7 µm × 2.1 mm × 50.0 mm) and oven temperature of 30 °C. The elution was undertaken by gradient, with a flow of 200 µL/min. The gradient is shown in Table 1.

Table 1. Gradient used in UHPLC analysis of *B.dracunculifolia* DC. and green propolis extracts.

Time (min)	% Solvent A *	% Solvent B **
0.0–8.0	70	30
8.1–10.0	0	100
10.1–12.0	70	30

* Solvent A: purified water (Milli Q) with 0.1% (*v/v*) formic acid PA; ** solvent B: methanol with chromatographic grade Merck®.

A triple quadrupole mass spectrometer (Waters TQD Acquity) with electrospray ionization (ESI) was used to perform negative ion mode scanning under the following conditions: 3000 V capillary, 30 V cone, 3.0 V extractor, source temperature of 150 °C and desolvation temperature of 300 °C [28,29]. The analyzed full-scan mass range was 50 to 1200 Da and peak identification was performed by comparing the retention time, mass spectra and fragmentation patterns with reference to the compounds obtained from various data libraries, networks and standards.

Evaluation of Nerolidol in B.dracunculifolia DC. and Green Propolis Ethanolic Extracts (BDEE and PVEE) by GC-MS

The *B.dracunculifolia* DC. and green propolis ethanolic extracts (BDEE and PVEE) were analyzed using gas chromatography coupled to mass spectrometry (GC/MS). A sample of *B.dracunculifolia* DC. volatile oil was used as a standard for the identification of nerolidol.

The analysis was performed with a gas chromatograph (HP6890) coupled to mass detector (HP5975) equipped with a HP5-MS capillary column (30 m × 0.25 mm × 0.25 µm). The oven temperature was programmed to increase from 60 to 240 °C at 3 °C/min [30]. Carrier gas: He (1 mL/min). The injector temperature was 220 °C and the detector temperature was 280 °C.

The compounds were identified by comparing their mass spectra with data from the GC-EM library of the National Institute of Standards and Technology (NIST); by the retention index (RI), using a homologous series of n-alkanes (C8–C30); and by comparison with the mass spectra of terpene standards in the literature [30].

***Synergistic Antibacterial Activity* / Sensitivity/Resistance Profile of *S.aureus* Isolates**

The *S. aureus* isolates that were used in this study (Table 2) were isolated from blood cultures of hospitalized patients between June 2011 and June 2012 at Hospital Irmandade Santa Casa de Misericordia de São Paulo, Sao Paulo State, Brazil. The 25 isolates (12 MRSAs and 13 MSSAs) were classified according to their sensitivity/resistance to methicillin [31].

Tolerance and resistance to specific antibiotics against *S. aureus*, such as vancomycin, are unstable and induced phenomena; thus, tolerant and resistant strains can return to the regular behavior of the species when stored improperly in laboratories or may even disappear in the absence of the selective pressure of the antimicrobial drug. In order to avoid these situations, all samples were tested immediately after isolation. When MIC determination was not possible within the time limit, the isolates of *S. aureus* were immediately preserved in brain heart infusion (BHI) with 15 to 20% glycerol and stored in a freezer at −80 °C. All the isolates were identified by Gram staining, catalase, coagulase and DNAase tests. Then, they were separated for further tests performed in a specific medium. For susceptibility testing, disc diffusion, the Etest for vancomycin and oxacillin, and vancomycin microdilution were performed. Tests were performed and interpreted according to the European Committee on Antimicrobial Susceptibility Test (EUCAST) recommendations.

Inoculum

S. aureus isolates were tested with penicillin, oxacillin, clindamycin, gentamicin, trimethoprim plus sulfamethoxazole and vancomycin discs using Müeller Hinton agar plates inoculated with a suspension (equivalent to 0.5 McFarland standard) of *S. aureus* isolates. The plates were incubated at 37 °C for 24 h and inhibition zones were measured.

Evaluation of Antibacterial Activity of Ethanol Extracts in Association with Classical Antibiotics

This study evaluated the synergism of ethanolic extracts (BDEE and PVEE) in com- bination with ten classical antibiotics (oxacillin, erythromycin, clindamycin, chloramphenicol, tetracycline, gentamicin, ciprofloxacin, vancomycin, penicillin and cefoxitin) plus one association of antimicrobials (sulfamethoxazole + trimethoprim − cotrimoxazole) in *S. aureus* isolates [22,32].

The inoculum was prepared with fresh cultures of *S. aureus* with turbidity compared to the 0.5 scale of the McFarland standard (10^8 CFU/mL) in BHI broth.

 DOI: https://doi.org/10.31415/bjns.v5i1.178

Table 2. *S. aureus* isolates used in tests of synergistic antibacterial activity of ethanolic extracts with antimicrobial drugs.

	S.aureus isolates	Type		*S.aureus* isolates	Type
1	ATCC 43300	MRSA	**14**	11202024	MRSA
2	ATCC 29213	MSSA	**15**	11161831	MSSA
3	11188308	MRSA	**16**	11140905	MSSA
4	11149669	MRSA	**17**	11175830	MSSA
5	11145131	MSSA	**18**	11175921	MSSA
6	11172486	MRSA	**19**	11171695	MSSA
7	111722853	MRSA	**20**	11147733	MSSA
8	11176032	MRSA	**21**	11140902	MSSA
9	11173791	MRSA	**22**	811641	MSSA
10	11183712	MRSA	**23**	11180960	MSSA
11	11190401	MRSA	**24**	11187226	MSSA
12	11189367	MRSA	**25**	11198474	MSSA
13	11196262	MRSA			

MRSA: methicillin resistant *S.aureus*, **MSSA**: methicillin sensitive *S.aureus.*

Acrylic petri dishes (90 × 15 mm) containing 19.5 mL of Müeller Hinton agar medium plus 0.5 mL of these extracts were prepared by adopting one quarter MIC90 concentration, while the BDEE and PVEE final concentrations in the dishes were 128.35 and 61.59 µg/mL, respectively. As control, dishes containing only Müeller Hinton agar medium (negative control) and dishes containing 19.5 mL of this medium plus 0.5 mL ethanol were used to observe the interference of ethanol in the results.

To observe the growth of the 25 isolates, they were suspended in saline solution (0.9% NaCl, 100 µL in each well) and then inoculated into 25 wells containing Müeller Hinton broth in triplicates. Using a Steers replicator, the isolates were incorporated into growth dishes containing only Müeller Hinton culture medium in agar (negative control) and this same medium with the addition of ethanol, BDEE and PVEE [33].

The synergism test was performed in triplicate with the disc-diffusion method in agar by seeding the isolates in dishes (containing only Müeller Hinton culture medium in agar (negative control) and the same medium with the addition of ethanol, BDEE and PVEE) in three homogenous directions, and then the antibiotics discs were applied.

The dishes were incubated at 37 °C for 24 h. Growth was observed, and the inhibition halos were annotated and statistically calculated using the Friedman method.

RESULTS

Identification of Phenolic Compounds and Diterpenes in B.dracunculifolia DC. and Green Propolis Ethanolic Extracts by UHPLC-MS

Figure 1 shows the chromatograms obtained by UHPLC-MS with the phytochemical profiles of the *B.dracunculifolia* DC. and green propolis ethanolic extracts.

Several phenolic compounds and diterpenes were identified by UHPLC-MS, as de- scribed in Table 3.

Figure 1.

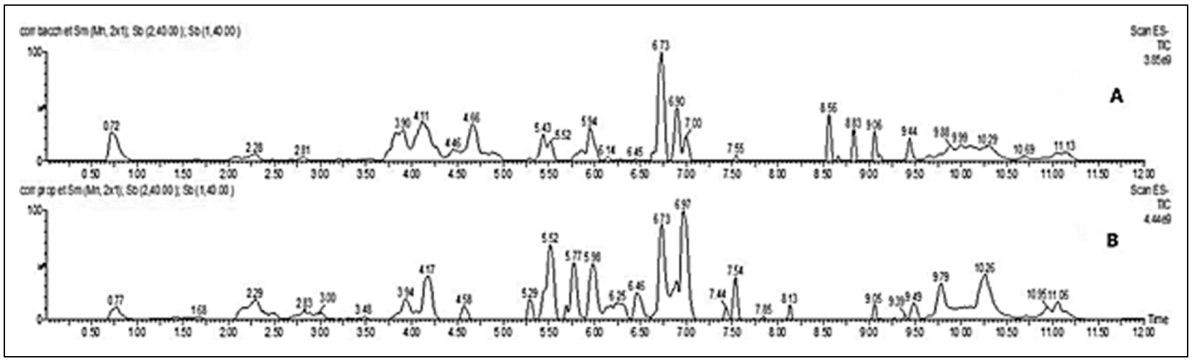

Figure 1. Phytochemical profiles using UHPLC-MS: (**A**) *B.dracunculifolia* DC. ethanolic extract (BDEE); (**B**) green propolis ethanolic extract (PVEE).

 DOI: https://doi.org/10.31415/bjns.v5i1.178

Table 3. Phenolic compounds and diterpenes identified in the *B.dracunculifolia* DC. and green propolis ethanolic extracts.

Compounds	***B.dracunculifolia* DC. Ethanolic Extract**	**Green Propolis Ethanolic Extract**
	(BDEE)	**(PVEE)**
Caffeic acid	+	+
p-coumaric acid	+	+
Dicafeoylquinic acid	+	+
Propol	+	+
3-Prenyl-p-coumaric acid	+	+
3,4-Dihydroxy-5-prenylcinnamic acid	+	+
Kaempferol	+	+
Pinocembrin	nq	+
Kaempferide	+	+
Nerolidol	+	+
Betuletol	+	+
3,5-diprenyl-p-coumaric acid (Artepillin C)	+	+
Diterpenes	nq	+

nq: below the limit of quantification. +: present.

Evaluation of Nerolidol in B.dracunculifolia DC. and Green Propolis Ethanolic Extracts (BDEE and PVEE) with GC-MS

Figure 2 shows the chromatograms obtained with GC-MS, which confirmed the presence of nerolidol in the *B.dracunculifolia* DC. and green propolis ethanolic extracts.

Figure 2.

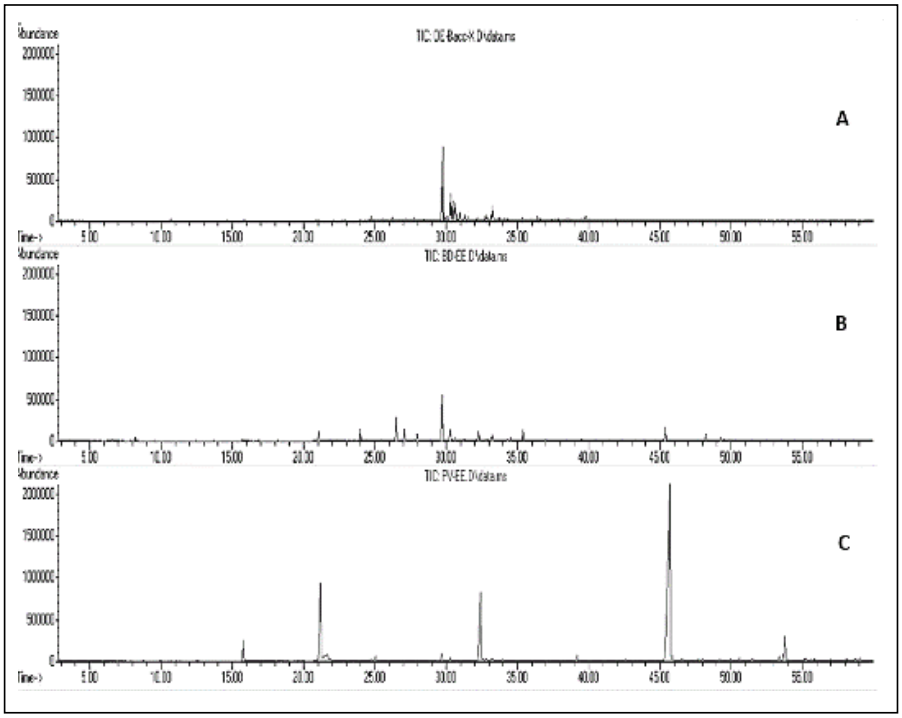

Figure 2. Phytochemical profiles using GC-MS: (**A**) *B.dracunculifolia* DC. volatile oil; (**B**) *B.dracunculifolia* DC. ethanolic extract (BDEE); (**C**) green propolis ethanolic extract (PVEE).

 DOI: https://doi.org/10.31415/bjns.v5i1.178

The *B.dracunculifolia* DC. essential oil fragmentation was similar to nerolidol fragmentation [30].

Table 4 shows the evaluation of the nerolidol in the volatile fraction of *B.dracunculifolia* DC. and in PVEE.

Table 4. Percentage of nerolidol (%, relative area) in the volatile fraction of *B.dracunculifolia* DC. and in the green propolis ethanolic extract determined using GC-MS.

Percentage of nerolidol in volatile fraction	BDEE	PVEE
(%, rel. area)	25.095%	0.680%

BDEE: *B.dracunculifolia DC Ethanolic Extract;* PVEE: *Green Propolis Ethanolic Extract.*

Evaluation of Antibacterial Activity of Ethanolic Extracts in Association with Classical Antibiotics

S. aureus isolates tested in dishes containing only ethanol in the culture medium showed no interference with growth inhibition in the presence of antibiotics diffusion discs; thus, the data obtained from these samples were not used in the statistical calculations of this test.

The data presented in **Figures 3 and 4** show there was a varied synergism in the antibacterial activity of the tested antibiotics in the presence of BDEE and PVEE in methicillin sensitive (MSSA) and resistant (MRSA) *S. aureus* isolates. The 25 tested isolates demonstrated statistical significance ($p < 0.05$).

Figure 3.

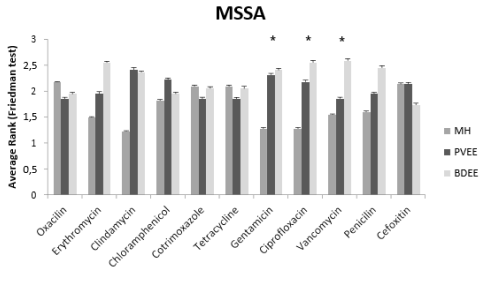

Figure 3. Average ranks of methicillin-sensitive *S. aureus* growth inhibitory zones (MSSA) in dishes with antibiotic discs in the absence (MH) and presence of ethanolic extracts of green propolis (PVEE) and *B.dracunculifolia* DC. (BDEE). * $p < 0.05$.

Figure 4.

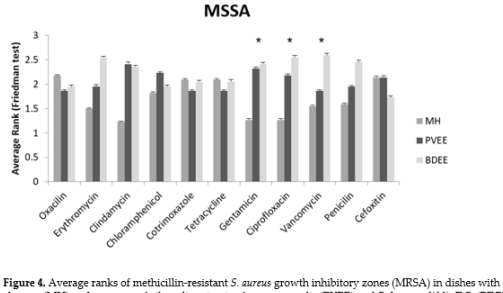

Figure 4. Average ranks of methicillin-resistant *S. aureus* growth inhibitory zones (MRSA) in dishes with antibiotic discs in the absence (MH) and presence of ethanolic extracts of green propolis (PVEE) and *B.dracunculifolia* DC. (BDEE). * $p < 0.05$.

DISCUSSION

Previous studies showed via HPLC analysis of *B.dracunculifolia* DC. and green propolis that the prenylated derivatives of p-coumaric acid, 3-prenyl-p-coumaric acid and 3,5- diprenyl-p-coumaric acid (Artepillin C - **Figure 5**) are the major compounds.

Figure 5.

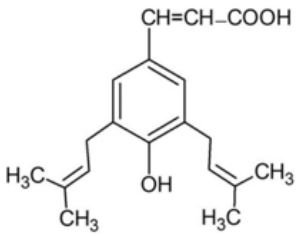

Figure 5. Chemical structure of Artepillin C (3,5-diprenyl-*p*-coumaric acid).

It has been reported that prenylated derivatives of *p*-coumaric acid have significant antifungal and antibacterial activities [29,34]. The major compound quantified by RP-HPLC in the *B.dracunculifolia* DC. and propolis ethanolic extracts from the southeastern region of Brazil was Artepillin C [35]. According to [19], high concentrations of the phenolic compound Artepillin C in PVEE (2549 µg/g) were demonstrated by HPLC analysis. In this study, the presence of other phenolic compounds, such as dicaffeoylquinic acid, 3-prenyl-p-coumaric acid, 3,4-dihydroxy-5-prenyl cinnamic acid, kaempferol, kaempferide and betuletol, were also identified and confirmed, as well as diterpenes, probably from various plant species of the *Anacardiaceae* family (Figure 1, Table 3). The presence of caffeic acid, *p*-coumaric acid, Propol and pinocembrin (Figure 1, Table 3) was found and confirmed. According to [19], a high concentration of Artepillin C (656 µg/g respectively) was also found in BDEE. In this study, the presence of other phenolic compounds, such as p-coumaric acid, dicaffeoylquinic acid, 3-prenyl-p-coumaric acid, 3,4-dihydroxy-5-prenyl cinnamic acid, kampferide and betulol, was observed in BDEE (Figure 1, Table 3). Caffeic acid and Propol were also found in BDEE (Figure 1, Table 3). According to [35], the concentrations of nerolidol (Figure 6) quantified by GC-MS in essential oil were much higher in samples of *B.dracunculifolia* DC. (14.82%) than in samples of propolis (6.64%) from the Brazilian

 DOI: https://doi.org/10.31415/bjns.v5i1.178

southeast. In this study, there was an even higher proportion of nerolidol quantified by GC-MS in the tested *B.dracunculifolia* DC. (BDEE) sample in comparison to the tested volatile fraction of the green propolis sample (PVEE) (Figure 6, Table 4). Among the identified and quantified volatile compounds of the *B.dracunculifolia* DC. (BDEE) and green propolis ethanolic extract (PVEE) used in the antibacterial activity evaluation in association with classical antibiotics (Figure 6, Table 4), CG- MS showed that nerolidol was the major compound found in BDEE (25.95%). In PVEE, the nerolidol concentration was 0.68%. **Figure 6.**

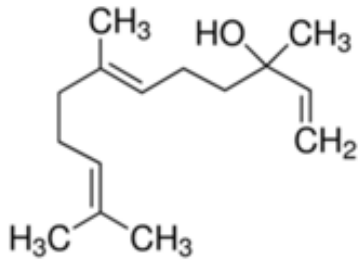

Figure 6. Chemical structure of nerolidol.

In addition to *S. aureus*'s potential for virulence and pathogenicity, this bacteria's ability to develop several antimicrobial resistance mechanisms is remarkable, as evidenced by methicillin resistance. The major mechanisms of resistance are: (1) enzymatic degradation or modification of the antibiotic; (2) alteration of the antibiotic target; and (3) reduction of the intracellular concentration of the antibiotic through changes in cell mem- brane permeability or overexpression of efflux pumps [36].

According to [37], antimicrobial associations can be evaluated for their ability to sup- press the emergence of mutant resistant microorganisms and to produce synergistic effects in vivo. Extension of the shelf life of currently used antimicrobials may be possible when used in combination with natural products. These associations may represent important therapeutic alternatives in the treatment of infections.

In a previous study [38], nerolidol, bisabolol and apritone (sesquiterpene compounds) increased the antibacterial activity of six antibiotic drugs (ciprofloxacin, clindamycin, tetracycline, erythromycin, gentamicin and vancomycin) tested in *S. aureus* strains. The largest relative increases in the bacterial growth inhibition zone sizes were recorded for each sesquiterpene tested with gentamycin. The increase in the antibiotic activity was observed even at low concentrations of these sesquiterpenes (0.5 mM). There was an increase in the antibiotic activity as the concentrations of the sesquiterpenes (1.0 and 2.0 mM) increased. Nerolidol demonstrated the greatest antibiotic potential against

S. aureus, followed by bisabolol and apritone [38]. The antimicrobial effects of the volatile compounds in the essential oils are associated with hydrophobicity, which enables them to penetrate through the lipid layer of the cell membrane and mitochondria (in eukaryotes) of the microorganism, causing cytoplasmic membrane disturbance an increased per- meability to K^+ and H^+ ions, which consequently leads to lysis and cell death [39,40].

Diverse mechanisms may have been present in the synergistic antibacterial activities observed in the associations of the *B.dracunculifolia* DC. and green propolis ethanolic extracts (BDEE and PVEE, respectively) with the classic antibiotic drugs used in this study (Figures 3 and 4). It is possible that the prenylated phenolic compounds and nerolidol present in BDEE and PVEE also contributed to increasing the cell membrane permeability of MSSA and MRSA isolates, since the antibacterial activity of phytochemicals, in general, occurs through the synergism of phenolic compounds and hydroxylated compounds, with several other compounds present in smaller proportions, thus triggering various mechanisms to reach the sensitive cells. The main effects are damage to the cytoplasmic membrane, cell wall degradation, protein membrane damage, coagulation of the cyto- plasm, extravasation or leakage of the cell contents, depletion of the cell and depletion of the proton motive force.

As well as the results of the antibacterial activity evaluation for the ethanolic extracts (BDEE and PVEE) in association with classical antibiotics and the possible synergistic mechanisms of antibacterial activity presented in this discussion, we observed more significant synergistic antibacterial activities for these extracts with gentamicin and vancomycin in both MSSA and MRSA isolates in the presence of sub-doses (1/4 of CIM90) of BDEE and PVEE.

In conclusion, BDEE showed better general performance than PVEE, which was possibly associated with the higher concentration of nerolidol in BDEE, since this sesquiterpene is already consecrated as a promoter of synergism in the antibacterial activity of ciprofloxacin, clindamycin, tetracycline, erythromycin, gentamicin and vancomycin *S. aureus* isolates [38]; however, further studies are needed.

Author Contributions: R.D.S.V., C.B.D.S., S.M.S., A.N., G.M., G.T.M., E.T.F.: antimicrobial studies; synesgistic studies and manuscript writing. M.C.M., C.L.Q. and A.C.H.F.S.: chemical analysis; chemical composition by HPLC, UPLC-MS and GC-MS; C.P.G.: computational studies and M.J.M.: supervisor, manuscript correction. All authors have read and agreed to the published version of the manuscript.

Funding: Faculty of Medical Sciences of Santa Casa de São Paulo, Sao Paulo, Brazil.

Institutional Review Board Statement: Faculty of Medical Sciences of Santa Casa de São Paulo, Sao Paulo, Brazil.

Funding: Faculty of Medical Sciences of Santa Casa de São Paulo, Sao Paulo, Brazil.

Institutional Review Board Statement: Faculty of Medical Sciences of Santa Casa de São Paulo, Sao Paulo, Brazil.

Informed Consent Statement: The research did not involve humans or animals. All studies were conducted through *in vitro* tests with microorganisms.

Data Availability Statement: The data are available in open access in the data repository of the Faculty of Medical Sciences of Santa Casa de São Paulo, Sao Paulo, Brazil. The basic metadata

are identified (DOI, title, description, authors, funding agencies) and may be located by third parties with the help of the metasearch of research data of the universities. The data will be available one year after the associated publication, respecting the policy of access to scientific journal data.

Conflicts of Interest: The authors declare no conflicts of interest.

RFERENCES

1. Casey, A.L.; Lambert, P.A.; Elliott, T.S.J. Staphylococci. Int. J. Antimicrob. Agents 2009, 29, 23–32.

2. Gordon, J.R.; Lowy, F.D. Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin. Infect. Dis. 2008, 46, 350–359.

3. Bächi, B.B.; Rohrer, S. Factors influencing methicillin resistance in Staphylococci. Arch. Microbiol. 2002, 178, 165–171.

4. Wertheim, H.F.; Melles, D.C.; Vos, M.C.; Van Leeuwen, W.; Van Belcum, A.; Verbrugh, H.A.; Nouwen, J.C. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect. Dis. 2005, 5, 751–762.

5. Liu, G.Y. Molecular Pathogenesis of Staphylococcus Aureus Infection. Pediatric Res. 2009, 65, 71–77.

6. Mimica, M.J.L.; Carvalho, R.L.; Berezin, E.N.; Damaceno, N.; Caiaffa Filho, H.H. Comparison of five methods for oxacilin susceptibily. testing of Staphylococcus aureus isolates from cystic fibrosis patients. Rev. Inst. Med. Trop. Sao Paulo 2012, 54, 305–306.

7. Von Eiff, C.; Becker, K.; Machka, K.; Stammer, H.; Peters, G. Nasal carriage as a source of S.aureus bacteremia. N. Engl. J. Med. 2001, 344, 11–16.

8. Zomenhan, L.C.P.; Pamplona, B.C.; Silva,C.B.; Marcucci, M.C.; Mimica, L.M.J. Evaluation of the in vitro antimicrobial activity of an ethanol extract of Brazilian classified propolis on strains of S. aureus. Braz. J. Microbiol. 2011, 42, 12591264.

9. Fukuda, M.; Ohkoshi, E.; Makino, M.; Fujimoto, Y. Studies on the constituents of the leaves of Baccharis dracunculifolia (Asteraceae) and their cytotoxic activity. Chem. Pharm. Bull. 2006, 54, 1465–1468.

10. Verdi, L.G.; Brighente, I.M.C.; Pizzolatti, M.G. Gênero Baccharis (Asteraceae): Aspectos químicos, econômicos e biológicos. Quím Nova 2005, 28, 85–94.

11. Rozza, A.L.; Pellizzon, C.H. Essential oils from medicinal and aromatic plants: A review of the gastroprotective and ulcer-healing activities. Fundam. Clin. Pharmacol. 2013, 27, 51–63.

12. Canton, M.; Onofre, S.B. Interferência de extratos da Baccharis dracunculifolia DC.; Asteraceae, sobre a atividade de antibióticos usados na clínica. Braz. J. Pharmacogn. 2010, 20, 348–354.

13. Bankova, V.S.; Castro, S.L.; Marcucci, M.C. Propolis: Recent advances in chemistry and plant origin. Apidology 2001, 31, 3–15.

14. Missima, F.; Filho, A.A.D.S.; Nunes, G.A.; Bueno, P.C.P.; De Sousa, J.P.B.; Bastos, J.K.; Sforcin, J.M. Effect of Baccharis dracunculifolia D.C. (Asteraceae) extracts and its isolated compounds on macrophage activation. J. Pharm. 2007, 59, 463–468.

15. Koru, O.; Toksoy, F.; Acikel, C.H.; Tunca, Y.M.; Baysallar, M.; Guclu, A.U.; Akca, E.; Tuylu, A.O.; Sorkun, K.; Tanyuksel, M.; et al. In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens. Anaerobe 2007, 13, 140–145.

16. Ayres, D.C.; Marcucci, M.C.; Giorgio, S. Effects of Brazilian propolis on Leishmania amazonensis. Mem. Inst. Oswaldo Cruz 2007, 102, 215–220.

17. Tavares, D.C.; Barcelos, G.R.M.; Silva, L.F.; Tonin, C.C.C.; Bastos, J.K. Propolis induced genotoxicity and antigenotoxicity in Chinese hamster ovary cells. Toxicol. In Vitro 2006, 20, 1154–1158.

18. Simões, L.M.C.; Gregório, L.E.; Da Silva Filho, A.A.; De Souza, M.L.; Azzolini, A.E.C.S.; Bastos, J.K.; Lucisano-Valim, Y.M. Effect of Brazilian green propolis on the production of reactive oxygen species by stimulated neutrophils. J. Ethnopharmacol. 2004, 94, 59–65.

19. Veiga, R.S.; 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. Artepillin C and phenolic compounds responsible for antimicrobial and antioxidante activity of green propolis and Baccharis dracunculifolia DC. J. Appl. Microbiol. 2017, 122, 911–920.

20. Banskota, A.H.; Tezuka, Y.; Adnyana, I.K.; Ishii, E.; Midorikawa, K.; Matsushige, K.; Kadota, S. Hepatoprotective and anti-Helicobater pylori activities of constituents from Brazilian propolis. Phytomedicine 2001, 8, 16–23.

21. Bankova, V.S. Recent trends and important developments in propolis research. Evid. Based Complement. Alternat. Med. 2005, 2, 29–32.

22. Fernandes Junior, A.; Balestrin, E.C.; Betoni, J.E.C.; Orsi, R.O.; Cunha, M.L.R.S.; Montelli, A.C. Propolis: Anti-S. aureus activity and synergism with antimicrobial drugs. Mem. Inst. Oswaldo Cruz 2005, 100, 563–566.

23. Aslam, B.; Wang, W.; Arshad, M.I.; Khurshid, M.; Muzammil, S.; Rasool, M.H.; Nisar, M.A.; Alvi, R.F.; Aslam, M.A.; Qamar, M.U.; et al. Antibiotic resistance: A rundown of a global crisis. Infect. Drug Resist. 2018, 11, 1645–1658.

24. Bouchelaghem, S. Propolis characterization and antimicrobial activities against Staphylococcus aureus and Candida albicans: A review. Saudi J. Biol. Sci. 2022, 29, 1936–1946.

25. Stepanovic, S.; Antie, N.; Dakie, I.; Svabie-Vlahovic, M. In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. Microbiol. Res. 2003, 158, 353–357.

26. 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. Chemical composition and antimicrobial activity of two extract of propolis against isolates of Staphylococcus spp. and multiresistant bacterials Pesq. Vet. Bras. 2019, 39, 734–743.

27. Assumpção, Y.M.; Barbosa, E.V.; Pereira, R.F.A.; Rocha, L.; Penna, B. Comparative Analysis Between the in vitro

Performances of the Hydroalcoholic Extracts of Green Propolis and Baccharis dracunculifolia against Staphylococcus aureus. J. Adv. Vet. Res. 2022, 12, 68–72.

28. Carneiro, M.J.; López, B.G.C.; Lancellotti, M.; Franchi, G.C.; Nowill, A.E.; Sawaya, A.C.H.F. Evaluation of the chemical composition and biological activity of extracts of Tetragonisca angustula propolis and Schinus terebinthifolius Raddi (Anacardiaceae). J. Apic. Res. 2016, 55, 315–323.

29. Marcucci, M.C.; Ferreres, F.; Garcıa-Viguera, C.; Bankova, V.S.; De Castro, S.L.; Dantas, A.P.; Valente, P.H.M.; Paulino, N. Phenolic compounds from Brazilian propolis with pharmacological activities. J. Ethnopharmacol. 2001, 74, 105–112.

30. Adams, R.P. Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy; Allured Publishing Coorporation: 2007, Illinois, USA.

31. Navarini, A.; Martino, M.D.V.; Sasagawa, S.M.; Massaia, I.F.D.; Mimica, M.J. Accuracy of a vancomycin brain heart infusion screening plate for the screening of Staphylococcus aureus isolates with increased vancomycin minimum inhibitory concentrations. New Microbiol. 2015, 38, 423–426.

32. Darwish, R.M.; Aburjai, T.A. Effect of ethnomedicinal plants used in folkloremedicine in Jordan as antibiotic resistant inhibitors on Escherichia coli. BMC Complementary Altern. Med. 2010, 10, 9.

33. Steers, E.; Foltz, E.L.; Graves, B.S. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 1959, 9, 307–311.

34. Feresin, G. E.; Tapia, A.; Gimenez, A.; Ravelo, A.G.; Zacchino, S.; Sortino, M.; Schmeda-Hirschmann, G. Constituents of the Argentinian medicinal plant Baccharis grisebachii and their antimicrobial activity. J. Ethnopharmacol. 2003, 89, 73D80.

35. Maróstica Júnior, M.R.; Daugsch, A.; Moraes, C.S.; Queiroga, C.L.; Pastore, G.M.; Park, Y.K. Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin Baccharis dracunculifolia. Ciênc. Tecnol. Aliment. Camp. 2008, 28, 178–181.

36. Fernandez, L.; Hancock, R. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. 2012, 25, 661–681.

37. Musumeci, R. Berberis ACP Berberis aetnensis C. Presl. extracts: Antimicrobial properties and interaction with ciprofloxacin. Int. J. Antimicrob. Agents 2003, 22, 48–53.

38. Brehm-Stecher, B.F.; Johnson, E.A. Sensitization of Staphylococcus aureus and Escherichia coli to Antibiotics by the Sesquiterpenoids Nerolidol, Farnesol, Bisabolol, and Apritone. Antimicrob. Agents Chemother. 2003, 47, 3357–3360.

39. Sikkema, J.; De Bont, J.A.M.; Poolman.; B. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 1995, 59, 201–222.

40. Inoue, Y.; Shiraishi, A.; Hada, T.; Hirose, K.; Hamashima, H.; Shimada, J. The antibacterial effects of terpene alcohols on Staphylococcus aureus and their mode of action. FEMS Microbiol. Lett. 2004, 237, 325–331.