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 antimicrobials. 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.
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], leishmanicidal [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 sub- stances 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 potentiating 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. aureus), 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®, 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.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Solvent A *</th>
<th>% Solvent B **</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0–8.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>8.1–10.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10.1–12.0</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

*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 50–1200 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 HPS-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 MRSA and 13 MSSA) 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 dis-appear 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 Müller–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 combination with ten classical antibiotics (oxacillin, erythromycin, clindamycin, chloramphenicol, tetracycline, gentamicin, ciprofloxacin, vancomycin, penicillin and ceftoxitin) plus one association of antimicrobials (sulfamethoxazole + trimethoprim – cotrimoxazole) in S. aureus isolates [22,32].
**Table 2.** S. aureus isolates used in tests of synergistic antibacterial activity of ethanolic extracts with antimicrobial drugs.

<table>
<thead>
<tr>
<th>S. aureus isolates Type</th>
<th>S. aureus isolates Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 43300 MRSA</td>
<td>11202024 MRSA</td>
</tr>
<tr>
<td>ATCC 29213 MSSA</td>
<td>11161831 MSSA</td>
</tr>
<tr>
<td>11188308 MRSA</td>
<td>11140905 MSSA</td>
</tr>
<tr>
<td>11149669 MRSA</td>
<td>11175830 MSSA</td>
</tr>
<tr>
<td>11145131 MSSA</td>
<td>11175921 MSSA</td>
</tr>
<tr>
<td>11172486 MRSA</td>
<td>11171695 MSSA</td>
</tr>
<tr>
<td>111722853 MRSA</td>
<td>11147733 MSSA</td>
</tr>
<tr>
<td>11176032 MRSA</td>
<td>11140902 MSSA</td>
</tr>
<tr>
<td>11173791 MRSA</td>
<td>811641 MSSA</td>
</tr>
<tr>
<td>11183712 MRSA</td>
<td>11180960 MSSA</td>
</tr>
<tr>
<td>11190401 MRSA</td>
<td>11187226 MSSA</td>
</tr>
<tr>
<td>11189367 MRSA</td>
<td>11198474 MSSA</td>
</tr>
<tr>
<td>11196262 MRSA</td>
<td></td>
</tr>
</tbody>
</table>

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

The inoculum was prepared with fresh cultures of *S. aureus* with turbidity compared to the 0.5 scale of the McFarland standard (10⁸ CFU/mL) in BHI broth.

Acrylic petri dishes (90 × 15 mm) containing 19.5 mL of Müller–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 Müller–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 Müller–Hinton broth in triplicates. Using a Steers replicator, the isolates were incorporated into growth dishes containing only Müller–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 Müller–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.

**Figure 1.**

Figure 1. Phytochemical profiles using UHPLC-MS: (A) *B. dracunculifolia* DC. ethanolic extract (BDEE); (B) green propolis ethanolic extract (PVEE).
Several phenolic compounds and diterpenes were identified by UHPLC-MS, as described in Table 3.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>B. dracunculifolia</em> DC. Ethanolic Extract (BDEE)</th>
<th>Green Propolis Ethanolic Extract (PVEE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>p</em>-coumaric acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dicafeoylquinic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Propol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Prenyl-<em>p</em>-coumaric acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3,4-Dihydroxy-5-prenylcinnamic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>nq</td>
<td>+</td>
</tr>
<tr>
<td>Kaempferide</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nerolidol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Betuletol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3,5-diprenyl-<em>p</em>-coumaric acid (Artepillin C)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>nq</td>
<td>+</td>
</tr>
</tbody>
</table>

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](image-url)
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 Ethanol 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).

**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.** Chemical structure of Artepillin C (3,3-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 southeast 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 dicafeoylquinic acid, 3-prenyl-p-coumaric acid, 3,4-dihydroxy-5-prenyl cinnamic acid, kaempferol, kaempferide and betulol, 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, dicafeoylquinic acid, 3-prenyl-p-coumaric acid, 3,4-dihydroxy-5-prenyl cinnamic acid, kaempferide 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 southeast. In this study, there was an even higher proportion of
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 membrane permeability or overexpression of efflux pumps [36].

According to [37], antimicrobial associations can be evaluated for their ability to suppress 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 com-pound) 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 gentamicin. 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 permeability 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. dracunculifolía 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 cytoplasm, 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.
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.

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