Antibacterial synergism of *Echeveria subrigida* (B. L. Rob & Seaton) and commercial antibiotics against multidrug resistant *Escherichia coli* and *Staphylococcus aureus*

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**ABSTRACT**

**Introduction:** Multidrug-resistant (MDR) bacteria is a public health threat, which requires the development of new therapeutic options. The combined use of plant extracts with commercial antibiotics is an alternative against infections by MDR bacteria. *Echeveria subrigida* is a Crassulaceae plant with very good activity against some bacteria. This study aims to evaluate the synergistic effect of the methanol extract of *Echeveria subrigida* leaves (ME-ES) with commercial antibiotics against MDR isolates of *Escherichia coli* and *Staphylococcus aureus*.

**Methods:** Six antibiotics, *E. coli* (4 MDR isolates and ATCC 25922) and *S. aureus* (2 MDR isolates and ATCC 29213) were used. ATCC strains were susceptible to the corresponding antibiotics and used as controls. The minimal inhibitory (MIC) and bactericidal (MBC) concentrations were evaluated by the microdilution broth assay, and the synergistic effect by the checkerboard and the time-kill curve methods. **Results:** The MDR of the bacterial isolates was corroborated, they were resistant to at least two families of antibiotics. The activity of ME-ES was good against one MDR *E. coli* isolate (MIC = 250 μg/mL) and the *S. aureus* strains (MIC = 250–1000 μg/mL), which was better than those registered for some commercial antibiotics. Synergism against *S. aureus* was found for the combinations ME-ES with carbencillin and ME-ES with methicillin (FICI = 0.28 to 0.5).

**Conclusions:** ME-ES was active against *S. aureus* and increased its activity when combined with betalactamic antibiotics. ME-ES can contribute to providing a best treatment for infectious diseases caused by MDR *S. aureus*.

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1. Introduction

In recent decades, most illness-causing bacteria have developed resistance to more than one family of antibiotics [1]. These multidrug-resistant (MDR) strains are a serious threat to human health. Currently, most nosocomial and community-acquired infections and 13 million deaths occurring in the world are due to the emergence of new infections or re-emergence of previously controlled diseases, this phenomena is clearly associated to the MDR bacteria [2,3]. In 2013, at least two million illnesses and 23000 deaths per year in USA were caused by microbial antibiotic resistance [4]. In particular, over 50% of *Staphylococcus aureus* isolates from 83% of the world’s regions are resistant to methicillin (MRSA), whereas the isolation of *Escherichia coli* resistant to both third-generation cephalosporins and fluoroquinolones is common. Specifically in USA, 80461 invasive MRSA infections and 11285 related deaths occurred in 2011, and approximately 1400 infections and 90 deaths were attributable to carbapenem-resistant *E. coli* each year [4]. In Mexico for the period 2000–2007, 30% of *S. aureus* isolates were resistant to methicillin; while for 2004–2010, up to 68.3% of *E. coli* isolates were third-generation cephalosporin resistant [5]. The high impact of MDR pathogens in human health implies the need of better therapeutical alternatives than the currently available. Accordingly, plants

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are still the main reservoir of bioactive compounds because they produce a great diversity of secondary metabolites. The combination of antibiotics with extracts or pure compounds isolated from various natural sources reduces the minimal inhibitory concentrations (MIC) of antibiotics; consequently, these combinations could be alternative treatments of infectious diseases caused by MDR bacteria [6–14]. The genus Echeveria belongs to the Crassulaceae family, and about 83% of the Echeveria species are endemic to Mexico. There are reports of biological activities (e.g. antifungal, antiparasitic and antibacterial) for different Echeveria species, highlighting their low antibacterial MIC values [15,16]. The methanol extract of E. subrigida (ME-ES), which is a native plant from Sinaloa, have showed good antibacterial activity mainly against Gram positive bacteria [15]. Moreover, the chromatographic profile of fractions of the ME-ES shows components with reported antibacterial activity [17]. This paper analyzes the antibacterial activity of the ME-ES in combination with commonly used antibiotics against MDR strains of Escherichia coli and Staphylococcus aureus, which are important pathogens of humans and other animals.

2. Materials and methods

2.1. Reagents and solvents

The reagents and solvents were analytical grade. The Minimal Inhibitory (MIC) and Minimal Bactericidal (MBC) concentrations and synergism were determined for selected antibiotics commonly used in medical therapy; Carbencillin (CAR), ampicillin (AMP), sulfamethoxazole/trimethoprim (SXT), nalidixic acid (NAL), methicillin (MET) and gentamicin (GN) (Sigma-Aldrich, USA). Culture media were tryptic soy agar (TSA), Mueller Hinton Broth, MacConkey agar and blood agar.

2.2. Plant material

Echeveria subrigida (B. L. Rob & Seaton) leaves were collected in the area nearby the town “El Palmito” Concordia, Sinaloa (2000 masl, 230°34′06″N, 105°50′53″W). A specimen (number 11742) was deposited in the Herbarium of the Agronomy School, Autonomous University of Sinaloa, and the collector was Vega-Aviña R.

2.3. Bacterial strains

Four multidrug-resistant (MDR) Escherichia coli and two MDR Staphylococcus aureus isolates were obtained from clinical samples of children with E. coli 1–3) and without diarrhea (E. coli 4), and from apparently healthy children in child care centers (S. aureus 1–2). These bacteria were from our culture collection. The resistance profile (Table 1) of the bacterial isolates, which was previously obtained by the Kirby-Bauer method, was corroborated by the microdilution method. The methicillin resistance phenotype of S. aureus 1–2 was previously confirmed by PCR amplification of the meca gen (unpublished information), which confers resistance to beta-lactams. The bacterial isolates were of children who lived in the municipality of Culiacan, Sinaloa at the time of sampling. The strains Escherichia coli ATCC® 25922™ and Staphylococcus aureus ATCC® 29213™ were used as controls for the antimicrobial susceptibility testing, as recommended by the Clinical and Laboratory Standards Institute [18,19].

2.4. Preparation of methanol extract of Echeveria subrigida (ME-ES)

Echeveria subrigida leaves were lyophilized, ground in a blender, and the obtained flour was passed through a mesh no. 40. The flour was extracted with methanol (1:10 w/v) for three consecutive days at room temperature. The mixture was every day filtered and retained solids were added with fresh solvent; the three days filtrates were mixed and concentrated on a rotary evaporator (40 °C) (BÜCHI Labortechnik AG, Switzerland) to obtain the methanol extract of E. subrigida (ME-ES). The ME-ES was stored at −20 °C in the dark until use [20].

2.5. Determination of the minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations

The MIC values were determined by the microdilution method in 96-well plates [18]. The strains were initially grown on TSA plates (37 °C/18–20 h), an aliquot of this culture was suspended in Mueller Hinton broth adjusting the turbidity to 0.5 McFarland, and later diluted to 105 CFU/ml (CFU, colony forming units). Fifty microliters of this bacterial suspension was added to each well containing 50 µl of antibiotic solution (NAL, 0.5–4096 µg/ml; MET, 0.1–2048 µg/ml; AMP, 0.125–2048 µg/ml; CAR, 0.25–2048 µg/ml; SXT, 0.5/0.026–380/20 µg/ml) or extract (7.812–1000 µg/ml), and the 96-well plates were incubated for 20 h at 37 °C. Gentamicin (0.125–16 µg/ml) was used as positive control, whereas the negative controls were inoculum without antibiotic solution or plant extract. The MIC value is the minimal concentration at which no turbidity or button of growth was observed in the well. To determine the MBC value, samples of those wells without growth, including that corresponding to the MIC value, were plated on MacConkey agar (Gram negative) or blood agar (Gram positive). The plates were incubated for 18–20 h at 37 °C, and the MBC value was the lowest concentration of antibiotic

Table 1

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Antibiotic resistance profile</th>
<th>Antibioticsa,b,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 1</td>
<td>bqs</td>
<td>AMP, CAR, PRL, TIM, NAL and SXT</td>
</tr>
<tr>
<td>E. coli 2</td>
<td>bs</td>
<td>GN, AMP, CAR, CXX, PRL, TIM, CIP, NAL, OFX and SXT</td>
</tr>
<tr>
<td>E. coli 3</td>
<td>abqs</td>
<td>GN, TDB, AMP, CAR, CXX, PRL, TIM, CIP, NAL, NOR, OFX and SXT</td>
</tr>
<tr>
<td>E. coli 4</td>
<td>abqs</td>
<td>GN, AMP, CAR, CXX, PRL, TIM, CIP, NAL, ORX, OFX and SXT</td>
</tr>
<tr>
<td>S. aureus 1</td>
<td>bcmst</td>
<td>AMP, P, DC, FOX, SX, TE, E, and CTX</td>
</tr>
<tr>
<td>S. aureus 2</td>
<td>bmqit</td>
<td>AMP, P, DC, FOX, SX, TE, E and LEV</td>
</tr>
</tbody>
</table>

a Antibiotic resistance profile was determined in a previous research by the Kirby-Bauer method.
b β, beta lactamases; q, quinolones; s, sulfaamides; a, aminoglycosides; c, cephalosporins; m, macrolides; t, tetracyclines.
c Antibiotics: AMP, Ampicillin; CAR, Carbencillin; PRL, Piperacillin; TIM, Ticarcillin; NAL, Nalidixic acid; SXT, Sulfamethoxazole/Trimethoprim; CXX, Cefuroxime; CIP, Ciprofloxacin; OFX, Ofloxacin; NOR, Norfloxacin; GN, Gentamicin; TOB, Tobramycin; P, Penicillin; DC, Dicloxacillin; FOX, Cefoxitin; OX, Oxacillin; TE, Tetracycline; E, Erythromycin; CTX, Cefotaxime; LEV, Levofloxacin.
or extract where no growth was observed. All analyses were performed in triplicate.

2.6. Evaluation of synergism

2.6.1. Checkerboard method

Assays including two dimensional arrays of antibiotic (2 MIC-1/8 MIC) and extract (2 MIC-1/32 MIC) concentrations in 96-well plates were carried out. Other controls were included in the microplate: blank (antibiotic, extract and solvent), MIC (50 μL of inoculum and 50 μL of extract or antibiotic), and bacterial growth (50 μL of inoculum and 50 μL of solvent). The microplate was incubated for 18–20 h at 37 °C, and the MIC value determined. To evaluate the type of interaction between the antibiotic and the extract, the Fractional Inhibitory Concentration Index (FICI) was calculated as

\[ FICI = \frac{MIC_{A} + MIC_{B} + \min(MIC_{A}, MIC_{B})}{MIC_{A}} \]

where, \( FICI_{A} = \frac{MIC_{A} \text{ of the component A in combination}}{MIC_{A}} \) and \( FICI_{B} = \frac{MIC_{B} \text{ of the component B in combination}}{MIC_{B}} \). The FICI value was interpreted as synergist (FICI < 0.5), additive (FICI = 0.5–1), indifferent (FICI > 1–4), and antagonist (FICI > 4) [21]. All analyses were performed in triplicate.

2.6.2. Time-kill curves

The macrodilution method was used. Curves were determined for the two combinations (extract with antibiotic) with the lowest FICI values, which were obtained by the checkerboard method, for each strain. Mixtures were prepared in five tubes: growth control (0.8 mL of water); MIC evaluation for the antibiotic (0.8 mL) and for the extract (0.8 mL); and two more for the respective combinations (0.4 mL of antibiotic and 0.4 mL of ME-ES). All tubes were inoculated with 0.8 mL of 1 × 10^8 CFU/mL. Bacteria other five tubes with Mueller-Hinton broth were used as blank. The tubes were incubated for 18–20 h at 37 °C in shaking water bath. Aliquots of 10 μL were collected at 0, 1, 3, 6, 9, 12 and 24 h; each aliquot was diluted from 10^-1 (0 h) to 10^-8 (24 h), adjusting the bacterial counting, and plated on Mueller-Hinton agar in duplicate. Plates were incubated for 18–20 h at 37 °C, and the average number of CFU/mL was determined using an automatic bacterial colony counter (UVP Colony Doc-It Imaging Station Fisher Scientific). The growth (log10 CFU/mL) was plotted against time (0–24 h), and the change in bacterial concentration was assessed.

To identify the type of interaction, the change in the bacterial concentration at 24 h for the combination treatment (antibiotic with ME-ES) was contrasted to that of the most active component (antibiotic or ME-ES). Interaction was synergistic (≥ 2 log_{10} CFU/mL decrease), additive (1–2 log_{10} CFU/mL decrease), antagonist (> 1 log_{10} CFU/mL increase) or indifferent (within 1 log_{10} CFU/mL increase or decrease) [22,23].

3. Results

3.1. Minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC)

The four clinical E. coli strains showed resistance principally to betalactamics, quinolones and sulfamides, and their MDR profiles were different (Table 1). Escherichia coli isolates of children with (E. coli 1–3) and without diarrhea (E. coli 4) showed high MIC values; however, those for E. coli 4 were the highest for GN, AMP and NAL. Interestingly, only E. coli 4 was susceptible to ME-ES (MIC = 250 μg/mL) but merely with bacteriostatic activity evaluated up to 1000 μg/mL (Table 2). In general, the S. aureus strains were resistant to betalactamics, macrolides, sulfamides and tetracyclines, but they were sensitive to ME-ES (MIC = 250–1000 μg/mL).

The MIC values for GN, positive control, against the reference strains E. coli ATCC 25922 and S. aureus ATCC 29213 corresponded with the established by the CLSI. Overall, ATCC strains had the smallest MIC values for the tested antimicrobials corroborating their sensitivity (Table 2).

3.2. Synergic effect of E. subrigida

The synergism was assessed for all strains with MIC value for both antibiotic and extract. Moreover, although the MIC value for AMP against S. aureus 2 was not found, synergism was evaluated at the maximum tested concentration (1024 μg/mL). According to the fractional inhibitory concentration index (FICI) and the checkerboard method, three combinations (antibiotic with ME-ES) were synergists: S. aureus ATCC 29213 with CAR (FICI = 0.28) and MET (FICI = 0.5), and S. aureus 2 with CAR (FICI = 0.28). In the checkerboard method was identified an additive effect (FICI = 0.75) for the combination of AMP with ME-ES against E. coli 4. Notably, the MIC value for AMP with ME-ES against S. aureus 2 was found (MIC = 1024 μg/mL), and the MIC value for the ME-ES was reduced to 1/16 MIC. Synergy experiments showed reductions in the MIC values for antibiotics from 1/4 MIC (CAR with S. aureus 2) to 1/32 MIC (MET with S. aureus 2) (Table 3). Of the tested combinations, 20% were synergistic, 40% indifferent, 6.66% additive, and the type of interaction for the rest (33.33%) was undefined (Table 3), as growth was registered to the highest concentrations of antibiotic and ME-ES.

### Table 2

Minimal Inhibitory Concentration (μg/mL) (MIC) and Minimal Bactericidal Concentration (MBC) for antibiotics and methanol extract of Echeveria subrigida (ME-ES) against multidrug-resistant Escherichia coli and Staphylococcus aureus strains.

<table>
<thead>
<tr>
<th>Antibiotic/extract</th>
<th>Escherichia coli ATCC 25922</th>
<th></th>
<th></th>
<th></th>
<th>Staphylococcus aureus ATCC 29213</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
</tr>
<tr>
<td>GN</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>AMP</td>
<td>128</td>
<td>256</td>
<td>–</td>
<td>–</td>
<td>1024</td>
<td>1024</td>
<td>32</td>
</tr>
<tr>
<td>CAR</td>
<td>32</td>
<td>32</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>NAL</td>
<td>2</td>
<td>2</td>
<td>512</td>
<td>512</td>
<td>128</td>
<td>1024</td>
<td>4096</td>
</tr>
<tr>
<td>MET</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>SXT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4/0.21</td>
</tr>
<tr>
<td>ME-ES</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>250</td>
<td>–</td>
<td>250</td>
</tr>
</tbody>
</table>

* – MIC and MBC values were not found up to the maximum evaluated concentration (μg/mL): GN (16); AMP (2048); CAR (2048); NAL (4096); MET (2048); SXT (380–20); ME-ES (1000). The bacteria presented intrinsic resistance to the antibiotic. Escherichia coli strains (1–3) were isolated from children with diarrhea, and E. coli 4 was from a child without diarrhea. Staphylococcus aureus strains (1 and 2) were isolated from children in child-care centers.

b GN, Gentamicin; AMP, Amoxicillin; CAR, Carbencillin; NAL, Nalidixic Acid; MET, Methicillin; SXT, Sulfamethoxazole/Trimethoprim.
Table 3
Synergy for the combination of antibiotics and methanol extract of Echeveria subrigida (ME-ES) against multidrug-resistant Escherichia coli and Staphylococcus aureus establishing the Fractional Inhibitory Concentration Index (FICI) and by using two analytic methods (checkerboard and time-kill curve).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Bacteria</th>
<th>Inhibitory concentrations</th>
<th>Synergistic result</th>
<th>Time-kill curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;a&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;b&lt;/sub&gt;</td>
<td>FICI</td>
</tr>
<tr>
<td>CAR</td>
<td>S. aureus ATCC 29213</td>
<td>2</td>
<td>7.8</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>S. aureus 1</td>
<td>1024</td>
<td>31.25</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>S. aureus 2</td>
<td>64</td>
<td>15.75</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>E. coli 4</td>
<td>4</td>
<td>0.25</td>
<td>3</td>
</tr>
<tr>
<td>AMP</td>
<td>S. aureus ATCC 29213</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>S. aureus 1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>S. aureus 2</td>
<td>1024</td>
<td>15.75</td>
<td>1.03</td>
</tr>
<tr>
<td>SXT</td>
<td>S. aureus ATCC 29213</td>
<td>4</td>
<td>7.8</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>S. aureus 1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>S. aureus 2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MET</td>
<td>S. aureus ATCC 29213</td>
<td>2</td>
<td>62.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>S. aureus 1</td>
<td>64</td>
<td>31.25</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>S. aureus 2</td>
<td>8</td>
<td>15.75</td>
<td>1.03</td>
</tr>
<tr>
<td>NAL</td>
<td>E. coli 4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> CAR, Carbenicillin; AMP, Ampicillin; SXT, Sulfamethoxazole/Trimethoprim; MET, Methicillin; NAL, Naldixic Acid.

<sup>b</sup> Escherichia coli 4 was an isolated from a child without diarrhea. Staphylococcus aureus 1 and 2 were isolated from children in child-care centers.

<sup>c</sup> MIC<sub>a</sub>: Minimal Inhibitory Concentration of the antibiotic in combination; MIC<sub>b</sub>: Minimal Inhibitory Concentration of the methanol extract of Echeveria subrigida in combination. – Growth was observed in all the combinations evaluated. *Time-kill curves were made only for synergistic combinations in checkerboard method.

3.3. Time-kill curves

The time-kill curves were evaluated for the combinations that proved to be synergistic by the checkerboard method. Results against S. aureus ATCC 29213 showed: synergy by combining MET 1/4 MIC = 2 μg/mL and ME-ES 1/8 MIC = 31.25 μg/mL (Fig. 1A); additive effects for MET 1/8 MIC = 1 μg/mL with ME-ES 1/8 MIC = 31.25 μg/mL (Fig. 1B); and for CAR 1/8 MIC = 1 μg/mL with ME-ES 1/32 MIC = 7.87 μg/mL (Fig. 1C). The reduction in bacterial growth was from 1.44 log<sub>10</sub> (Fig. 1A) to 2.28 log<sub>10</sub> (Fig. 1A). Combinations of CAR and ME-ES against S. aureus 1 (1/4 MIC = 64 μg/mL and 1/32 MIC = 15.755 μg/mL, respectively) and S. aureus ATCC 29213 (1/4 MIC = 2 μg/mL and 1/32 MIC = 7.87 μg/mL, respectively) showed an indifferent result; the growth of S. aureus 1 decreased by 0.2 log<sub>10</sub>, whereas it increased 0.76 log<sub>10</sub> for S. aureus ATCC 29213. Finally, an antagonist result was obtained with S. aureus 2 for the combination CAR 1/8 MIC = 32 μg/mL and ME-ES 1/32 MIC = 15.755 μg/mL; the lowest bacterial growth was with CAR, whereas the growth increase for the combination CAR with ME-ES was higher than 2.7 log<sub>10</sub>.

According to Fig. 1, the growth rate of S. aureus ATCC 29213 treated with a high concentration of MET (1/4 MIC, panel A) was greater at longer incubation times (12–24 h) than at shorter times, under 6 log<sub>10</sub> CFU/mL from 0 to 12 h. On the other hand, bacteria at the lower MET concentration (1/8 MIC, panel B) showed approximately the same colony count at 24 h than that obtained for 1/4 MIC but with an increased growth rate (above 9 log<sub>10</sub> CFU/mL from 6 to 12 h). Thus, the antimicrobial effect of MET on S. aureus at 1/4 MIC was stronger from 0 to 12 h in comparison with the effect of MET at 1/8 MIC from 6 to 12 h (panel B).

The curve of bacterial survival for the combination of MET with ME-ES confirmed the synergistic effect. Compared with the curve for the higher MET concentration (1/4 MIC) (Fig. 1A), that for the lower MET concentration (1/8 MIC) (Fig. 1B) registered a higher survival rate up to 12 h. Additionally, after 12 h the bacterial count for the combination (ME-ES with MET) declined, being lower than only with MET and reflecting the additive effect of the combination.

4. Discussion

The antibacterial activity of the ME-ES was good (MIC < 1000 μg/mL) (Table 2) [24]. Early reports suggest the ME-ES have components with good antibacterial activity (MIC = 20–125 μg/mL) [15–17,25]. We showed that the MIC values for the ME-ES (250–1000 μg/mL) (Table 2) were higher than the reported, as expected for our MDR bacteria. Specifically for the methanol extract of E. subrigida, a MIC value of 62.5 μg/mL against S. aureus ATCC 29213 is reported [15], a value that differs from our result.
(MIC = 250 µg/mL), whereas for E. coli ATCC 25922 activity is not reported up to 1000 µg/mL, a finding consistent with this paper. The variations among our results and those reported could be associated with differences in the strain characteristics, extract composition, and in addition even with standard strains, a variation range is permissible. On the other hand, methanol extracts of other species of the Crassulaceae family have shown similar antibacterial activity [26–28], suggesting that compound(s) of similar nature, which may be common in this family, are associated with such activity. The ME-ES contains flavonoids, coumarins and tannins [15], all with potential antibacterial activity: flavonoids can reduce the transport of materials in the cell and cause lysis and death [29]; tannins have been associated with inactivation of bacterial adhesins and enzymes, and interact with cell membrane components and specifically with transport proteins; whereas coumarins interact with DNA [30]. According to our results and those reported, Gram positive bacteria are more sensitive to the methanol extracts of Echeveria species, which may be attributed to the absence of the outer membrane that in Gram negative bacteria provides greater protection [31,32].

All E. coli isolates showed very high antibacterial MIC values, accordingly to the CLSI classification, and were resistant to SXT and NAL (Table 2). The MIC value with NAL of E. coli ATCC 25922 coincided with that reported (MIC = 1.953 µg/mL), and NAL was a good internal control of our antibacterial evaluations [11]. For AMP, the MIC values for the E. coli isolates were highly variable, but the lowest value was still high; that is, strains were resistant [19]. It must be emphasized that all bacterial isolates were MDR, highlighting the resistance of E. coli 3 to sulfonamides, betalactamics, aminoglycosides and quinolones (Table 2). Moreover, the seriousness of the bacterial resistance problem was also observed in that E. coli 4 showed higher MIC values than E. coli 2, strains obtained from children without and with diarrhea, respectively. Thus, the circulation of MDR strains within healthy individuals is a potential risk when patients with an immunocompromised system are exposed to such microorganisms. On the other hand, both S. aureus isolates showed high MIC values for CAR and SXT and were MDR; moreover, S. aureus was also resistant to AMP. The two S. aureus isolates were classified as methicillin-resistant (MRSA) with MIC values greater than 8 µg/mL (Table 2) [19]. These results were consistent with the presence in these strains of the mecA gene, which has been associated with resistance to methicillin and other β-lactamic antibiotics. Thus, our results were of great importance since the activity of ME-ES against E. coli 4 and S. aureus 2 (MIC = 250–500 µg/mL) was 2–4 times better than with AMP (MIC ≥ 1024 µg/mL). Remarkably, ME-ES up to 500 µg/plate is neither toxic nor mutagenic in the Ames assay [17], and ME-ES (up to 2 g/L) is innocuous in the Artemia salina assay. Moreover, an ethanol extract of E. subrigida is nontoxic in the Larke assay in mice up to 5 g/kg of body weight (unpublished data). Thus, ME-ES could be an alternative or complementary treatment of bacterial infections.

The activity for most of the antibiotics was bactericidal, and the MIC and MBC values were practically the same; however, ME-ES was only bacteriostatic. Currently there are efficient bacteriostatic drugs that boost the patient’s immune system to eliminate the bacterial pathogens [33]. Differences in the antibacterial activity may be associated with variations in the mechanism of action, chemical structures of the active components, type of bacteria, and permeability of the bacteria to active substances, among other reasons [34].

For some MDR bacteria, the high MIC values of antibiotics decreased from 4 to 16 times when combined with the ME-ES (Table 3), suggesting the synergistic effect. So far, there are no reports of previous studies regarding the antibacterial activities of combinations of antibiotics with extracts of Echeveria or other succulent plants. Antibiotics combined with extracts of other plant species have shown antibacterial synergism similar to the obtained for the ME-ES. The combination of the ethanol extract of Melissa officinalis with amoxicillin inhibits the growth of S. aureus; however, E. coli is not affected [35], corresponding with the results for the ME-ES (Table 3). The methanol extract of the pomegranate fruit (Punica granatum) combined with the β-lactamic antibiotics (ampicillin and oxacillin) shows synergistic effects against clinical isolates of resistant and sensitive S. aureus but only demonstrated by the time-kill curve method [36]. The checkerboard and time-kill curve methods measure different antibacterial activities, that is, bacteriostatic and bactercidial, respectively; thus, it is reasonable that the results obtained by these two methods do not match 100% [37], as it happened with the ME-ES. The combination of acetone extract of Helichrysum longifolium only with penicillin and amoxicillin decreases the growth of S. aureus ATCC 6538 from 3.3 to 4.5 log₁₀ [38]. Similarly, considering the time-kill curves, the ME-ES was synergistic only when combined with β-lactam antibiotics and against S. aureus ATCC 29213 (Fig. 1).

Up to date there is no explanation for the high growth rates of S. aureus ATCC 29213 from 12 to 24 h of incubation with MET (1/4 and 1/8 of MIC). It is established that sub-inhibitory concentrations of MET decrease peptidoglycan cross-linking leading to cell walls with an increased potential for lysis [39]. However, this does not explain the survival increase from 12 to 24 h when 1/4 MIC was used (Fig. 1A). Perhaps, this behavior could be due to a differential synthesis of peptidoglycan in the bacterial population that improves its survival at longer times. However, further research is needed to test this hypothesis.

The synergistic activity of antibiotics combined with plant extracts varies widely according to the nature of the evaluated extract, which should be related with its chemical composition. In our previous report, the gas chromatography-mass spectrometry analysis of ME-ES fractions shows high concentrations of phenolics (e.g. isorhamnetin and gallic acid) and terpenes/steresoids (e.g. sitosterol) [17], compounds with registered antibacterial activities. Some polyphenols (e.g. epicatechin gallate) inactivate β-lactamases produced by S. aureus strains, making them sensitive to β-lactamic antibiotics, and the ME-ES polyphenols could be contributing with the reported synergism [40]. Compounds of ME-ES in low concentration [17] are active against S. aureus (e.g. amyrin and germancol) and E. coli (e.g. lupeol) [41–43]. On the other hand, synergism against human bacterial pathogens has been reported for combinations of β-sitosterol glucopyranoside with antibiotics, particularly with ciprofloxacin, and this sterol could be associated with the synergistic activity demonstrated in this paper [13]. Regarding the phenols, isorhamnetin-3-glucose is active against Gram negative and Gram positive bacteria [25], but gallic acid show better MIC values (3.5 – 12.5 µg/mL) against methicillin resistant S. aureus (MRSA) [44].

5. Conclusions

The methanol extract of Echeveria subrigida showed better antibacterial activity against Gram positive (Staphylococcus aureus) than against Gram negative (Escherichia coli) bacteria, and better than some antibiotics on multidrug-resistant strains. Although synergism was not demonstrated for some combinations (extract with antibiotic), such combinations inhibited the growth of multidrug-resistant strains at lower concentrations than the required for individual commercial antibiotics. Thus, these mixtures could reduce the adverse side effects of antibacterial treatments that use only high antibiotic concentrations. This is the first report about synergism between extracts of Echeveria species and antibiotics, and the mixture of methanol extract of E. subrigida with β-lactamic antibiotics could be an alternative for the treatment of infections caused by multidrug-resistant S. aureus.
of clinical importance. However, future studies must demonstrate the in vivo effect of the synergistic combinations as well as their involved antibacterial mechanisms.

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