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Research paper

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 carbenicillin 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*.

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 of 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: Carbenicillin (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 *mec*A 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 Labortechnick 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 10^6 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 and 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
Resistance profile of the bacterial isolates used in this study.[a]

Bacterial isolates	Antibiotic resistance profile	
	Families of antibiotics[b]	Antibiotics[c]
E. coli 1	bqs	AMP, CAR, PRL, TIM, NAL and SXT
E. coli 2	bs	GN, AMP, CAR, CXM, PRL, TIM, CIP, NAL, OFX and SXT
E. coli 3	abqs	GN, TOB, AMP, CAR, CXM, PRL, TIM, CIP, NAL, NOR, OFX and SXT
E. coli 4	abqs	GN, AMP, CAR, CXM, PRL, TIM, CIP, NAL, NOR, OFX and SXT
S. aureus 1	bcmst	AMP, P, DC, FOX, OX, SXT, TE, E, and CTX
S. aureus 2	bmqst	AMP, P, DC, FOX, SXT, TE, E and LEV

[a] Antibiotic resistance profile was determined in a previous research by the Kirby-Bauer method.
[b] b, betalactamics; q, quinolones; s, sulfamides; a, aminoglycosides; c, cephalosporins; m, macrolides; t, tetracyclines.
[c] Antibiotics: AMP, Ampicillin; CAR, Carbenicillin; PRL, Piperacillin; TIM, Ticarcillin; NAL, Nalidixic acid; SXT, Sulfamethoxazole/Trimethoprim; CXM, Cefuroxime; CIP, Ciprofloxacin; OFX, Ofloxacin; NOR, Norfloxacin; GN, Gentamicin; TOB, Tobramycin; P, Penicillin; DC, Dicloxacillin; FOX, Cefoxitin; OX, Oxacilline; TE, Tetracycline; E, Erythromycin; CTX, Cefotaxime; LEV, Levofloxacin.