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MICROBIOLOGY

Screening of antimicrobial activity of *Ilex paraguariensis* St. Hil. leaf extracts against carbapenemase-producing bacteria

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Abstract: *I. paraguariensis* St. Hil. is a south American species of agronomic interest with studies supporting its medicinal properties. As the investigation of active ingredients with antimicrobial effect from medicinal plants is a suitable approach to the current antibacterial resistance problem, the aim of the present study was to determine the antibacterial activity of yerba mate ethanolic extracts against carbapenemase-producing gram-negative bacteria (reference strains and clinical isolates). Extracts showed antibacterial activity against *Klebsiella pneumoniae* ATCC® BAA-2342™ (KPC producing), *Providencia rettgeri* (NDM producing), *Pseudomonas aeruginosa* (MBL producing) and *P. aeruginosa* (VIM producing) at the concentrations tested. The Minimal-Inhibitory-Concentration and Minimal-Bactericidal-Concentration values ranged between 1 and 32 $mg.ml^{-1}$ for the reference strains, and between 0.125 and 1 $mg.ml^{-1}$ for the clinical isolates. The MBC/MIC index characterized the extracts as bactericidal. The combinations of commercial antibiotics and extracts showed a synergistic action on the reference strains studied. The lethal concentration 50 obtained using the *Artemia salina* toxicity assay were higher than 1 $mg.ml^{-1}$ for all the extracts, indicating a low toxicity. The *in vitro* activity and low toxicity suggest that ethanolic *I. paraguariensis* leaf extracts constitute an outstanding source for new antibacterial compounds, and further studies should be carried out to understand their mechanism of action.

Key words: Antibacterial activity, *I. paraguariensis St. Hil.,* MIC, MBC, Plant Extracts, Synergism.

INTRODUCTION

The increase in antimicrobials production, availability, and prescription over the past three decades has led to the emergence of antimicrobial resistance amongst pathogens (Zilberberg & Shorr 2010). This originates failure of currently available treatments for infectious diseases, including last-line antibiotics, promoting a problem for public health and global economies (Antonanzas 2015, Tasneem 2022). Nowadays we are faced with the emergence and spread of Carbapenemase-Producing Enterobacteriaceae (CPE), bacteria that produce a family of broad-spectrum enzymes that hydrolyze most β-lactam antibiotics, including carbapenems, and resist the action of traditional β-lactamase inhibitors such as clavulanic acid. This has led carbapenem resistance to be classified as high priority as they are the last-resort drugs available for the treatment of bacterial infections, especially Enterobacterales (Iovleva & Doi 2017).

Carbapenemase coding genes offer a stable and transferable form of resistance, allowing propagation through clonal expansion

or horizontal gene transfer to naive bacteria (Bonomo et al. 2018). Both clonal propagation and plasmid-mediated transmission contribute to the continuous increase in the incidence of these bacteria and alarms have recently been issued about the appearance of strains producing combinations of these enzymes.

Infections caused by carbapenemase-producing bacteria are often identified in patients with underlying diseases and co-morbidities, contributing to high mortality rates (Stewardson et al. 2019). Besides, they contribute to poor patient health outcomes, increased healthcare requirements, prolonged hospital admissions and significant economic burden (Bartsch et al. 2017). In recent times, these bacteria are not only associated with health centers; sporadic outbreaks are also reported in the community. The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have recently classified CPE as one of the most urgent antimicrobial-resistant threats (Köhler 2018, Vink et al. 2020). Carbapenemases defy geographic boundaries, making CPE prevention a major public health problem that requires international coordination to be contained. In this sense, the WHO (2017) has published a priority list for developing new and effective antibiotic treatments. The list was intended to set research and development priorities related to new antibiotics and identify new effective drug combinations.

The current approach to the antibacterial resistance problem is oriented towards the search and identification of new active principles with antimicrobial effect from medicinal plants (Tirupathi et al. 2011). Plants produce a wide diversity of secondary metabolites that serve as defense compounds against pathogens. They are important sources for the discovery of natural products that interfere with bacterial virulence through different mechanisms of action. In this sense, the investigation of active ingredients from plants of autochthonous origin with antimicrobial capacity is among the most reliable approaches to face this problem (WHO 2017, Nolte 2014).

Argentina is characterized by a great biological diversity due to its extension and its wide climatic variety. The Misiones jungle has enormous diversity of plant species of pharmaceutical importance to humans (Alonso & Desmarchelier 2015). *Ilex paraguariensis* St. Hil. (yerba mate) is an autochthonous species of agronomic interest for the province of Misiones, Argentina. It is a tree whose green, dried, and shredded leaves are used to prepare a drink known as "mate". There are studies supporting the medicinal properties and antimicrobial effects of this plant (Alonso & Desmarchelier 2015, Gawron-Gzella et al. 2021) with potential applications in the pharmaceutical industry (Gawron-Gzella et al. 2021).

Some investigators revealed the antibacterial efficacy of extracts obtained from *I. paraguariensis* St. Hil. on gram-positive and gram-negative bacteria. In this sense, De Biasi et al. (2009) showed the activity of hydro-alcoholic extracts against gram-negative bacilli and gram-positive cocci. Similarly, Prado Martin et al. (2013) proved the antibacterial activity of methanolic and ethanolic extracts against reference strains. On the other hand, Noureddine et al. (2018) demonstrated the efficacy of aqueous extracts, while Oliveira Penteado et al. (2016) revealed the antibacterial activity of *I. paraguariensis* St. Hil. extracted with water and with hexane. Besides the activity against reference strains, there is also evidence that the presence of resistance mechanisms, such as the production of β-lactamases, does not affect the antibacterial activity of *I. paraguariensis* St. Hil. (Noureddine et al. 2018, Burris et al. 2011, Gawron-Gzella et al. 2021, Rempe et al. 2015, Paluch et al. 2021).

The extraction method influences the bioactive molecules present, both in type and quantity (Paluch et al. 2021, Bhebhe et al. 2016), so choosing an appropriate solvent is key. Girolometto et al. (2009) demonstrated the best antibacterial activity of ethanolic *I. paraguariensis* St. Hil. extracts against reference gram-positive cocci and gram-negative bacilli strains, compared to aqueous extracts. An advantage of ethanol when compared to other solvents (i.e., dimethyl sulfoxide, hexane, etc.) is its low toxicity, so that it needs not to be removed from the extracts for their use in humans (Bhebhe et al. 2016). Advantages of the ethanolic extracts against the aqueous ones include the fact that sugars are more readily extractable with water (Paluch et al. 2021) and their presence can stimulate microbial growth, leading to contamination of the aqueous extracts; also, since ethanol is more volatile, it evaporates faster during the concentration step in the extract obtention. Moreover, our working group has studied the efficacy of both the aqueous and ethanolic extracts against gram-positive and gram-negative reference bacterial strains; although both types of extracts showed antibacterial activity, ethanolic extracts presented lower Minimum Inhibitory Dose (MID) values and were easier to obtain, so they became optimal candidates for further studies (Kachuk et al. 2019, Kachuk et al. 2021, Onetto et al. 2022). Hence, the aim of the present study was to determine the antibacterial activity of ethanolic extracts of *I. paraguariensis* St. Hil. against carbapenemase-producing gram-negative bacterial strains.

MATERIALS AND METHODS

Collection and identification of *I. paraguariensis* St. Hil.

Aerial parts (leaves and branches) of adult *I. paraguariensis* St. Hil. plants from Alberto Roth Foundation, Santo Pipó, Misiones Province, Argentina (27° 09'44.7"S; 55° 21'43.9"W) were collected in the month of July of 2021. The plant material was promptly delivered to the laboratory and was taxonomically identified by the Cátedra de Farmacobotánica of the Facultad de Ciencias Exactas, Químicas y Naturales (FCEQyN) of the Universidad Nacional de Misiones (UNaM).

Extraction

Aerial parts were scalded at 100 °C for 30 seconds and quickly cooled in water as described by Holowaty et al. (2018). Plant material was separated in five groups, each one treated at a different time and temperature in a laboratory oven: without treatment (room temperature); oven at 50 °C for 30 min (50° 30'); 50 °C for 60 min (50° 60'), 80 °C for 30 min (80° 30'); and 80 °C for 60 min (80° 60'). Samples were hung to dry at room temperature for eight days. The dry leaves were separated from the branches and crushed in a Wiley-type blade mill (BroBender OH6 Duisburg N° 242 mod W1247). The powder was sieved through a nominal mesh aperture of 1.4 mm with W.S. Tyler™ O-TAP Sieve Shaker RX-29 (WSTyler, Ohio, USA). Extracts were obtained by digestion (Argentine Pharmacopoeia 2013) with a 96 % hydroalcoholic solution (ethanolic alcohol), concentrated with a rotary evaporator Laborota 4000-Efficient (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and left to completely dry at 35 ± 2 °C for approximately one week. The extracts obtained were kept as a powder in amber glass containers at -20 °C.

Bacterial strains

The bacterial strains used in the assays were *Klebsiella pneumoniae* ATCC® BAA-2342™ (resistant to carbapenem antibiotics), *K. pneumoniae* ATCC® 700 603™ (a strain producing extended-spectrum *β*-lactamases), and three reference carbapenemase-producing strains belonging to the culture collection of the Cátedra de Microbiología General of the Universidad Nacional de Buenos Aires (UBA): NDM metallo-*β*-lactamase producing *Providencia rettgeri* (hereinafter referred to as "*P. rettgeri* NDM"), MBL metallo-*β*-lactamase producing *Pseudomonas aeruginosa* (hereinafter referred to as "*P. aeruginosa* MBL") and VIM metallo-*β*-lactamase producing *P. aeruginosa* (hereinafter referred to as "*P. aeruginosa* VIM"). Thirty carbapenem-resistant clinical isolates were also assayed: *K. pneumoniae* (n = 18), *Enterobacter cloacae* (n = 1), *Enterobacter aerogenes* (n = 1), *Citrobacter freundii* (n = 1), *Serratia* spp. (n = 1), *Escherichia coli* (n = 4), *Proteus mirabilis* (n = 3) and *Proteus vulgaris* (n = 1). Clinical isolates were part of the bacterial collection of the Laboratorio of the Cátedra de Bacteriología (FCEQyN, UNaM).

Antibacterial activity evaluation by Minimum Inhibitory Dose (MID) assay

Minimum Inhibitory Doses (MID) of the extracts for each strain were measured by Kirby-Bauer Disk Diffusion Susceptibility Test (Clinical and Laboratory Standards Institute 2012) with modifications. Bacterial suspensions, equivalent to the optical density of the 0.5 McFarland standard were inoculated on Mueller-Hinton Agar plates (MHA, Britania S.A., Argentina®) using sterile swabs. Room temperature, 50° 30', 50° 60', 80° 30' and 80° 60' extracts solutions were prepared using dimethyl sulfoxide (DMSO, Biopack Argentina®) as solvent. Sterile 6 mm filter paper discs were impregnated with 25 µL of solutions of different concentrations of the extracts, and the solvent was left to evaporate for 24 h. The effective dose of each disc was 8; 4; 2; 1; 0.75; 0.5 and 0.25 mg (Seyyednejad et al. 2014). Standard antibiotics including Cefoxitin 30 µg (Britania S.A., Argentina®), Colistin 10 µg (BD BBL, USA®), and Trimethoprim-Sulfamethoxazole 25 µg (Thermo Scientific, USA®) were used as positive controls, while discs impregnated with 25 µL of DMSO were used as negative control.

Agar plates were incubated at 35 ± 2 °C for 24 h. After incubation, the diameter of the zone of bacterial growth inhibition around each disc was measured and recorded in millimeters. MID was considered the minimum quantity of the extract included in a paper disc able to show a visual inhibition of microbial growth. Only extracts with antibacterial activity were used in the subsequent assays.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays

A dilution method on Mueller-Hinton Broth ("MHB, Britania S.A., Argentina®") was carried out following the Clinical and Laboratory Standards Institute (CLSI 2016) and Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC 2016) recommendations. Sterile 96-well microplates were used, where one row (12 wells) was used to evaluate the MIC of one extract against one strain. Two-fold serial dilutions of the extracts were made in a range from 64 to 0.125 $mg.ml^{-1}$. One well was reserved as a sterility control of the dilutions, another as a control of viability of the strain (MHB without extract or solvent), and a third one was reserved as a viability control for the solvent (MHB with DMSO at a concentration equal to that of the most concentrated dilution, 1.5 %). The volume of culture medium added to each well was 100 µL. Extracts were initially dissolved in a volume of 6 µL of DMSO, to which 94 µL of sterile MHB

was added. Then 100 µL of this solution was added to the first well, and two-fold dilutions were made up to the tenth well (sterility control of the dilutions). Wells were inoculated with 100 µL of a suspension of 10^6 CFU.ml^{-1}, thus obtaining a final concentration of 5.10^5 CFU.ml^{-1} in each well. Inoculated microplates were incubated at 35 ± 2 °C for 24 h, after which the bacterial development was evaluated by the presence of turbidity in the broth (Navarro et al. 2011).

The MIC was defined as the lowest concentration of extract that restricted visible bacterial growth. To establish the MBC, one loopful (5 µL) of broth was transferred from each well without visible development to nutritive agar plates (Britania S.A., Argentina®). After 24 h of incubation, the MBC was determined as the lowest concentration of extract in which no colony formation occurred (Seyyednejad et al. 2014).

The MBC/MIC index was calculated as the ratio between MBC and MIC. Extracts showing a MBC/MIC ratio ≤ 2 was considered to have a bactericidal effect against the evaluated strain, while a ratio between 2 and 16 was considered bacteriostatic, and values ≥ 16 were deemed ineffective, according to the criteria of Shanmughapriya et al. (2008).

Drug synergy assay

The preliminary detection of positive interactions between *I. paraguariensis* St. Hil. extracts and commercially available antibiotics was assessed by the double-disc test, performed according to the CLSI guidelines with modifications (2016). Bacterial suspensions equivalent to the 0.5 McFarland standard were inoculated with sterile swabs on MHA plates. Paper discs prepared as described for the MID assay with an effective dose of 8 mg were placed at a 2 mm distance (centre to centre) of commercial antibiotic discs. Antibiotics evaluated were selected based on the commercial availability and latest use recommendations for treating infections against carbapenemase-producing microorganisms (Imipenem 10 µg, Meropenem 10 ug, Colistin 10 ug, Tigecycline 15 µg, Aztreonam 30 µg, Ceftazidime-Avibactam 14 µg, Fosfomycin 200 µg, Amikacin 30 µg, Minocycline 30 µg, Ceftazidime 30 µg) (Britania S.A., Argentina®). After 24 h of incubation at 35 ± 2 °C, the enhancement of the inhibition zones between the discs indicated a synergy between the tested antibiotics and the extracts. Only the reference strains were evaluated in this assay.

Toxicity assay

To assess the safety for their use in higher organisms, extracts were tested using a toxicity bioassay on *Artemia salina*. This crustacean, considered a standard test organism, is widely used for the initial screening of the toxicity of potential new drugs and plant extracts since the assay is low cost, easy to perform, and correlates well with the acute toxicity in animals such as mice (Nitulescu et al. 2013, Guţu et al. 2015). The *A. salina* larvae assay according to Meyer et al. (1986) was used. Briefly, extracts were previously dissolved in DMSO and added to the wells of a 96-well microplate containing saline solution (1 g.L^{-1}). Final concentrations of the extract tested were 0.25, 0.5, 1, and 2 mg.ml^{-1} and the working volume was 250 µL. Final DMSO concentrations were below 5% w/v, which is innocuous for the larvae as determined in previous works of our group (Novosak et al. 2022). Saline solution alone, and saline solution with 12.5 µL of DMSO without extract were used as growth controls, and the bioassays were performed in triplicate.

Approximately 0.1 g of *Artemia salina* cysts (Aquagreen®, Argentina) were hydrated in artificial saltwater containing NaCl (1 g.L^{-1}; pH 8 ± 0.5). The container was kept at room temperature (28 ± 1 °C) and constant illumination. Aeration

was maintained by an aquarium air pump (Sumersible Pump BL – 200) until the cysts hatched (after 24-48 h). Ten larvae were added to each well. The microplate was incubated under illumination in a humid atmosphere (glass container) at room temperature for 24 h. The number of surviving larvae in each well was determined under a stereomicroscope (Nikon SMZ 445). The endpoint (immobility/death) was assessed by the total lack of larvae movement during 10 seconds of observation (Vanhaecke et al. 1981). Larvae were not fed during bioassays.

The lethality percentage in each well was calculated by the following equation:

$$\text{Percentage of lethality} = \frac{\text{Number of alive larvae control} - \text{Number of alive larvae test}}{\text{Number of alive larvae control}} \times 100\%$$

The lethal concentration 50 (LC_{50}) was determined by regression analysis (linear regression) using the software Statgraphics Centurion XVII; this value represents the concentration of each extract that would render a mortality of 50 % of the larvae. The toxicity criterion adopted considered values of LC_{50} higher than 1 $mg.ml^{-1}$ to be of low toxicity, those lower than 1 $mg.ml^{-1}$ but greater than 0.5 $mg.ml^{-1}$ moderately toxic, and less than 0.2 $mg.ml^{-1}$ high toxicity (Leos-Rivas et al. 2016).

RESULTS

Antibacterial activity evaluation by Minimum Inhibitory Dose (MID) assay

I. paraguariensis St. Hil. extracts presented antibacterial activity at the concentrations tested against reference strains *K. pneumoniae* ATCC® BAA-2342™ (80° 30' extract: MIC= 2.00 $mg.disc^{-1}$), *P. rettgeri* NDM (room temperature and 80° 60' extracts: MIC= 8.00 $mg.disc^{-1}$; 50° 30' and 80° 30' extracts: MIC= 4.00 $mg.disc^{-1}$; and 50° 60' extract: 2.00 $mg.disc^{-1}$), *P. aeruginosa* MBL (room temperature, 50° 30', and 80° 60' extracts: MIC= 8.00 $mg.disc^{-1}$) and *P. aeruginosa* VIM (50° 30', 50° 60', and 80° 30' extracts: MIC= 8.00 $mg.disc^{-1}$). Antibacterial activity was also detected against two of the clinical isolates coded as *K. pneumoniae* CPKPC7 (50° 30' and 80° 60' extracts: MIC= 8.00 $mg.disc^{-1}$; 80° 30' extract: MIC= 4.00 $mg.disc^{-1}$) and *P. vulgaris* CPMBL22 (room temperature extract: MIC= 4.00 $mg.disc^{-1}$; 50° 30', 50° 60', 80° 30', 80° 60' extracts: MIC= 2.00 $mg.disc^{-1}$). The inhibitory activity of the different effective loads of the discs are shown in Figure 1.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays

The MIC value for *K. pneumoniae* ATCC® BAA-2342™ against the 80° 30' extract was 16 $mg.ml^{-1}$ (MBC/MIC=2). For *P. rettgeri* NDM, room temperature, 50° 60' and 80° 60' extracts had MIC and MBC values of 2 $mg.ml^{-1}$ (MBC/MIC=1.00), while 50° 30' and 80° 60' extracts had MIC

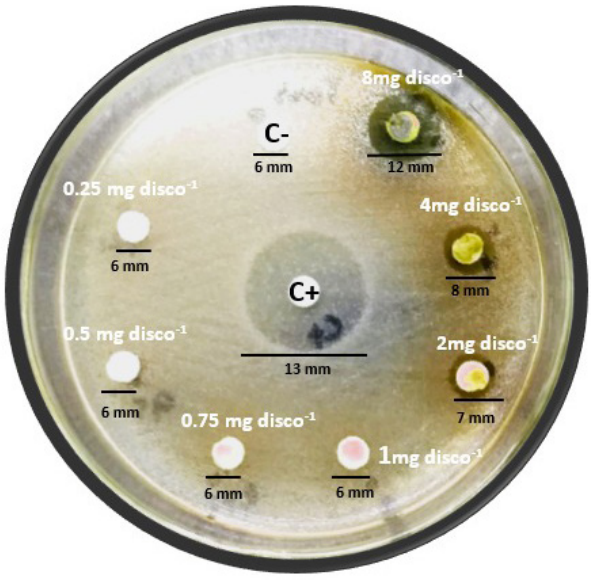

Figure 1. Minimal Inhibitory Dose (MID) disc diffusion test of 80° 30' extract of *Ilex paraguariensis* St. Hil. against carbapenemases-producing *K. pneumoniae* CPKPC 7. The discs contained doses from 0.25 to 8 mg. A disc impregnated with DMSO was used as negative control (C-) and a disc of Fosfomycin 200 µg (Britania S.A., Argentina®) as positive control (C+). Diameters of the inhibition zones are shown in millimeters (mm).

and MBC values of 1 mg.ml^{-1} (MBC/MIC=1.00). *P. aeruginosa* MBL had MIC and MBC values of 16 mg.ml^{-1} for the 50° 30' and 80° 60' extracts (MBC/MIC=1), and a MIC value of 8 mg.ml^{-1} and MBC value of 16 mg.ml^{-1} for the room temperature extract (MBC/MIC=2.00). Similarly, *P. aeruginosa* VIM had MIC and MBC values of 16 mg.ml^{-1} for the 50° 30', 50° 60', and 80° 30' extracts (MBC/MIC=1.00).

The clinical isolates showed smaller values than the reference strains, with *K. pneumoniae* CPKPC7 having MIC and CBM values of 1 mg.ml^{-1} for the 50° 60' extract (CBM/MIC=1.00), MIC and CBM values of 0.5 mg.ml^{-1} for the 80° 30' extract (CBM/MIC=1.00), and CIM and CBM values of 0.125 mg.ml^{-1} for the 80° 60' extract (CBM/MIC=1.00). Strain *P. vulgaris* CPMBL22 had MIC and CBM values of 0.5 mg.ml^{-1} for the 50° 60' extract (CBM/MIC=1.00), and MIC and CBM values of 0.125 mg.ml^{-1} for all the other extracts (CBM/MIC=1.00). According to the criterion presented by Shanmughapriya et al. (2008), the activity was interpreted as bactericidal for all extracts.

Drug synergy assay

Results for the synergy assay are presented in Table I, which shows the combination of antibiotics and extracts for which synergies were detected. Most of the synergies were detected for the strains *K. pneumoniae* ATCC® BAA-2342™ and *P. rettgeri* NDM. Figure 2 shows the assay (double-disc test) of different *I. paraguariensis* St. Hil. extracts against the reference strains tested.

Table I. Synergies detected with different *Ilex paraguariensis* St. Hil. extracts against commercially available antibiotics.

Bacterial strain	Antibiotic	*I. paraguariensis* St. Hil.
***Klebisella pneumoniae* ATCC® BAA-2342™**	Aztreonam 30 ug Ceftazidime-Avibactam 14 ug Colistin 10 ug Fosfomycin 200 ug Imipenem 10 ug Meropenem 10 ug	50° 30', 80° 60' Room temperature, 50° 60', 80° 30' Room temperature All extracts Room temperature Room temperature
***Klebsiella pneumoniae* ATCC® 700 603™**	Cefotaxime 30 ug Imipenem 10 ug Meropenem 10 ug	50° 60' 50° 30' 50º 30', 80° 30', 80° 60'
***Providencia rettgeri* NDM**	Amikacin 30 ug Aztreonam 30 ug Ceftazidime 30 ug Ceftazidime-Avibactam 14 ug Colistin 10 ug Fosfomycin 200 ug Imipenem 10 ug	All extracts 50° 30' All extracts 50° 30', 50° 60', 80° 30' All extracts 50° 60' 50° 60', 80° 30', 80° 60'
Pseudomonas aeruginosa MBL	Aztreonam 30 ug Meropenem 10 ug	All extracts Room temperature
***Pseudomonas aeruginosa* VIM**	Aztreonam 30 ug Ceftazidime 30 ug	Room temperature, 50° 60' 50° 30', 50° 60, 80° 60'

Toxicity assay

Estimated LC_{50} were 1.13 (confidence interval=0.91-1.27 mg.ml^{-1}; R=79.02); 1.02 (CI= 0.85-1.16 mg.ml^{-1}; R=83.83); 1.12 (CI=0.99-1.21 mg.ml^{-1}; R=92.97); 1.22 (CI=1.01-1.34 mg.ml^{-1}; R=84.11); and 1.36 (CI=1.02-1.47 mg.ml^{-1}; R=73.23) for the room temperature, 50° 30', 50° 60', 80° 30', and 80° 60' extracts, respectively. Since the estimated LC_{50} were higher than 1 mg.ml^{-1} for all extracts, they were categorized as of low toxicity. The control groups showed 100 % viability.

DISCUSSION

Ilex paraguariensis St. Hil. has gained attention recently (with over 8.710 articles listed in Google Scholar in the last 5 years) for the increase in its consumption (Instituto Nacional de la Yerba Mate 2022) and scientific reports of its biological activity (Heck & de Mejia 2007, Dellacassa et al. 2007, Croge et al. 2020).

Bhebhe et al. (2016) indicate that the selection of a solvent for obtaining the extracts determines the amount and type of phenolic compounds present, which are proposed to be responsible, at least in part, for the antibacterial activity of *I. paraguariensis* St. Hil. (Rempe et al. 2015, Prado Martin et al. 2013). Bastos et al. (2007) found that the composition in both quality and quantity of phenolics was similar between the aqueous and ethanolic *I. paraguariensis* St. Hil. extracts, although slightly higher in the latter. These authors reported that the main phenolic compounds were caffeic acid, caffeoyl glucose, caffeoylquinic acid, feruloylquinic acid, dicaffeoylquinic acid and rutin. Rempe et al. (2015) indicate that in addition to those, other phenolics present in *I. paraguariensis* St. Hil. are caffeoylquinates, caffeoylshikimates, kaempferol, and quercetin. Other compounds could also contribute to the antimicrobial effect, like chlorogenic acid, flavonoids, and saponins (Prado Martin et al. 2013, Paluch et al. 2021).

The results of the present research revealed the antibacterial activity of all the *I. paraguariensis* St. Hil. ethanolic extracts (room temperature, 50° 30', 50° 60', 80° 30', and 80° 60') against *P. rettgeri* NDM at the concentrations tested. Most of the extracts also showed antibacterial activity against *P. aeruginosa* MBL (room temperature, 50° 30', and 80° 60') and *P. aeruginosa* VIM (5° 30', 50° 60', and 80° 30'), while only the 80° 30' ethanolic extract showed antibacterial activity against *K. pneumoniae* ATCC® BAA-2342™. None of the extracts showed any antibacterial activity against *K. pneumoniae* ATCC® 700 603™ at the concentrations assayed. The MIC and MBC values in this study ranged between 1 mg.ml^{-1} and 32 mg.ml^{-1} for the reference strains studied, and between 0.125 mg.ml^{-1} and 1 mg.ml^{-1} for the clinical isolates. According to the criteria presented by Shanmughapriya et al. (2008), all the extracts tested here had a bactericidal activity.

Limited studies were done to evaluate the effect of yerba mate ethanolic extracts on gram-negative bacteria. Regarding reference bacterial strains, Noureddine et al. (2018) showed that the extract of the stems and leaves of *I. paraguariensis* St. Hil. extracted at 70 °C with water have a significant antibacterial activity against *P. aeruginosa* ATCC® 27853™ (MIC=1.875 mg.ml^{-1} and MBC=1.875mg.ml^{-1}), *E. coli* ATCC® 25922™ (MIC=1.875 mg.ml^{-1} and MBC=3.75 mg.ml^{-1}), *Staphylococcus aureus* ATCC® 29213™ and *Acinetobacter baumannii* ATCC® 17978™ and clinical isolates of *K. pneumoniae*, *Enterococcus faecalis*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, *Serratia marcesens*, *E. coli*, *S. aureus* and *P. mirabilis*. As is the case for these authors, there are many reports in the literature of the composition and antibacterial activity of *Ilex paraguariensis* St. Hil. extracts obtained with

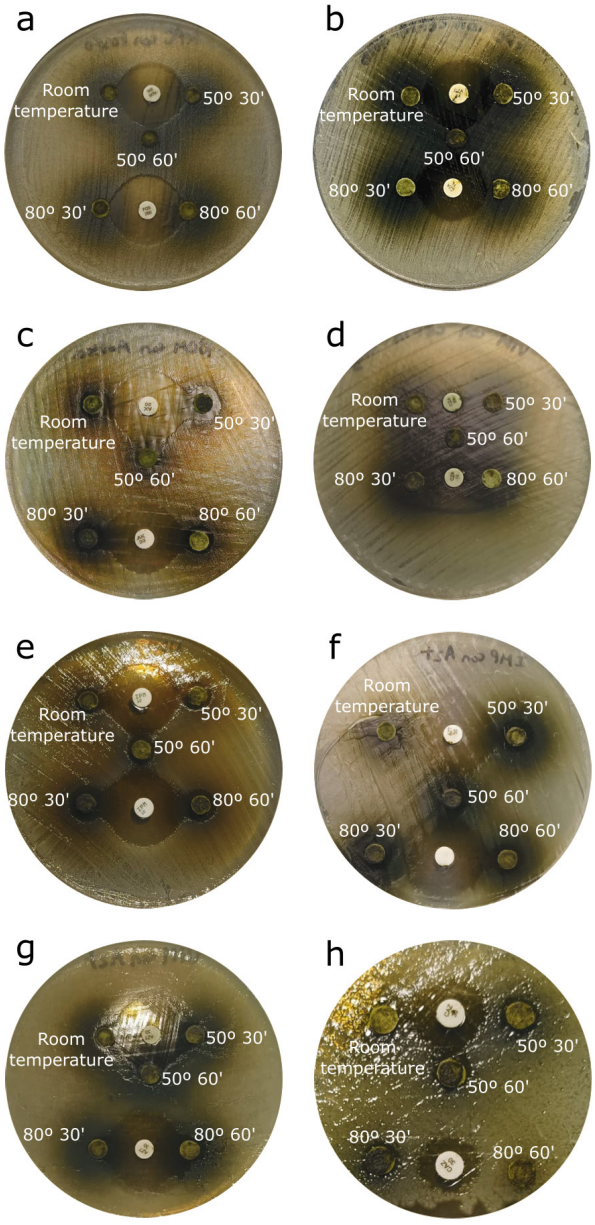

Figure 2. Drug Synergism assay (double-disc test) of *I. paraguariensis* St. Hil. extracts with different pre-treatments (room temperature, 50° 30', 50° 60', 80° 30', and 80° 60'). a: Extracts against commercial Fosfomycin 200 µg discs (FOS) for *K. pneumoniae* ATCC® BAA-2342™. b: Extracts against commercial Ceftazidime-Avibactam 14 µg discs (CZA) for *K. pneumoniae* ATCC® BAA-2342™. c: Extracts against commercial Amikacin 30 µg discs (AK) for *P. rettgeri* NDM. d: Extracts against commercial Ceftazidime-Avibactam 14 µg discs (CZA) for *P. rettgeri* NDM. e: Extracts against commercial Imipenem 10 µg disc (IPM) for *P. rettgeri* NDM. f: Extracts against commercial Aztreonam 30 µg (AZT) for *P. aeruginosa* MBL. g: Extracts against Aztreonam 30 µg (AZT) for *P. aeruginosa* VIM. h: Extracts against Aztreonam 30 µg (AZT) for *P. aeruginosa* VIM.

different solvents (i.e., water, hexane, methanol, supercritical CO_2, etc.), but there is little about the efficacy of ethanolic extracts, particularly against gram-negative bacteria with resistance mechanisms, which is the focus of the present work. However, since water and ethanol have similar polarities, it should be expected that the compounds extracted with both be similar and hence their efficacy be comparable.

On the other hand, when considering clinical isolates, in our work, MIC and MBC values ranged between 0.125 - 1 $mg.ml^{-1}$ and 0.125 - 0.5 for clinical carbapenemase-producing *K. pneumoniae* CPKPC7 and *P. vulgaris* CPMBL22, respectively. These results differ from the ones presented by Noureddine et al. (2018) who found that MIC and MBC ranged between 1.875 and 15 $mg.ml^{-1}$ against clinical strains of *K. pneumoniae* of different resistance profiles, and MIC=3.75 $mg.ml^{-1}$ and MBC=7.5 $mg.ml^{-1}$ against *P. mirabilis*. They concluded that the antibacterial activity of their extract did not show any correlation with the resistance profile of the tested bacteria (β-lactamase production). The resistance to β-lactams is usually increased with the number of beta-lactamases found in a specific strain, however, this was not observed with the *I. paraguariensis* St. Hil. extract.

Other authors have studied and demonstrated the antimicrobial activity of different plant extracts on a wide variety of microorganisms. Voukeng et al. (2016) obtained MIC values ranging from 64 to 1024 $µg.ml^{-1}$ for six Cameroonian medicinal plants namely *Alstonia boonei, Catharanthus roseus, Ageratum conyzoides, Croton macrostachys, Cassia obtusifolia,* and *Paullinia pinnata* against

multi-drug resistant microorganisms including *E. coli* ATCC® 10536™, *E. aerogenes* ATCC® 13048™, *K. pneumoniae* ATCC® 12296™, *Providencia stuartii* ATCC® 29916™ and multidrug-resistant clinical strains of gram-negative bacteria (*E. coli, P. aeruginosa, E. aerogenes, P. stuartii, K. pneumoniae* and *E. cloacae*). Similarly, Saeidi et al. (2015) registered a MIC of 2.5 $mg.ml^{-1}$ for the alcoholic extract of seeds of *Peganum harmala* (*Zygrophyllaceae*) collected in Iran against selected β-lactamase producing *E. coli* strains.

Our *I. paraguariensis* St. Hil. extracts did not show activity against clinical isolates of *Providencia* spp., *Enterobacter* spp., *E. coli, C. freundii*, *Serratia* spp., and *P. mirabilis* at the concentrations assayed. Unlike us, Faujdar et al. (2020), who tested the antibacterial potential of ethanolic extract of *Syzygium aromaticum* against a total of 221 gram-negative uropathogens, including bacteria producing extended spectrum β-lactamase (ESBL), AmpC cephalosporinase, and neither ESBL nor AmpC or metallo-β-lactamase producing strains, and found that *S. aromaticum* was effective against all gramnegative isolates, with the best antibacterial activity shown against *Proteus* species, with 19 mm inhibition zones, 0.39 $mg.ml^{-1}$ MIC and 0.19 $mg.ml^{-1}$ MBC.

The double-disc synergy assay is applied for preliminary detection of positive interactions between extracts and antibiotics. We detected synergies between all extracts and commercial antibiotics against *K. pneumoniae* ATCC® BAA-2342 ™, *K. pneumoniae* ATCC® 700 603 ™, *P. rettgeri* NDM, *P. aeruginosa* MBL and *P. aeruginosa* VIM. Several authors report the existence of synergies between natural products and antibiotics, but to our knowledge there are no studies reporting the positive interaction of *I. paraguariensis* St. Hil. extracts and gram-negative bacteria. Dettweiler et al. (2020) found that *Lechea mucronata* extract showed synergistic interactions and *Schinus terebinthifolia* extract was non-interactive overall against *A. baumannii*. Similarly, Ahmad & Aqil (2007), demonstrated significant synergistic interactions among alcoholic crude extracts and some fractions from 15 traditionally used Indian medicinal plants for 12 different combinations against β-lactamases producing multidrug-resistant *E. coli*. Liktor-Busa et al. (2016) demonstrated that mushroom extracts did not have synergistic effects with different antibiotics against multiresistant *A. baumannii*, *P. aeruginosa* and vancomycin-resistant *Enterococcus faecium*. Natural substances can be abundant sources of useful drug mixtures because secondary metabolites produced by living organisms do not often act alone *in vivo* (Dettweiler et al. 2020). While many of the compounds found in *I. paraguariensis* St. Hil. extracts are known and characterised (Kubo et al. 1993, Filip et al. 2001), limited information is available on which compounds might contribute to its antimicrobial activity and whether they may have additive or synergistic effects in combination. Kungel et al. (2018) determined that *I. paraguariensis* St. Hil. polysaccharides had prominent antimicrobial effects against both gram-negative (*E. cloacae, Salmonella enteritidis*, and *Salmonella typhimurium*) and gram-positive bacteria (*Bacillus cereus, Micrococcus flavus, S. aureus*, and *Listeria monocytogenes*).

Toxic effects of *I. paraguariensis* St. Hil. extracts on *A. salina* have not been reported. In this work we estimated the LC_{50} of the *I. paraguariensis St. Hil.* extracts, all of which were higher than 1 $mg.ml^{-1}$. Therefore, they are considered as of low toxicity according to the criteria of Leos-Rivas et al. (2016). Studies on the toxicity of different plants extracts against *A. salina* are amply reported in the literature. Silva et al. (2022) performed a toxicity assay for *Camellia sinensis* (herbal matcha green

tea) and found mild toxicity (LC_{50} = 0.4 $mg.ml^{-1}$) and Braguini et al. (2019) assessed the LC_{50} for aqueous extracts of leaves and flowers of *Lavandula officinalis* (lavender) with values of 0.467 and 0.724 $mg.ml^{-1}$, respectively, whereas the ethanolic extracts of leaves and flowers were 0.074 and 0.126 $mg.ml^{-1}$, respectively.

The results of this study proved the antibacterial activity and low toxicity of *I. paraguariensis St. Hil.* extracts; both characteristics add value to this plant species for medicinal purpose and demonstrate new uses that should be exploited. Further studies are needed to distinguish the contributing compounds, which may contribute to formulating new antibacterial agents needed for the management of infectious diseases caused by multi-drug resistant bacteria.

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