JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIBACTERIAL ACTIVITY AND TOXICITY OF THE ETHANOLIC EXTRACT *OF EUGENIA UNIFLORA* L. LEAVES ON *PSEUDOMONAS AERUGINOSA*

Fernando J. Bobadilla[*1,3]; *Marina G. Novosak* [1,3]; *Daniana L. Winnik*[2,3]; *Analía V. Kachuk* [2]; *Margarita E. Laczeski*[1,3]; *Marina I. Quiroga*[2,3]

Address(es):
[1]National University of Misiones (UNaM). Faculty of Exact, Chemical and Natural Sciences (FCEQyN). Department of Microbiology. National Council of Scientific and Technical Research (CONICET), Mariano Moreno Av. PC 3300, Posadas, Argentina. +54 376 4811100
[2]National University of Misiones (UNaM). Faculty of Exact, Chemical and Natural Sciences (FCEQyN). Department of Microbiology. Mariano Moreno Av. PC 3300, Posadas, Argentina.
[3]National University of Misiones (UNaM). Faculty of Exact, Chemical and Natural Sciences (FCEQyN). Biotechnology Institute of Misiones (InBioMis). Route 12 km 7,5. PC 3300, Posadas, Argentina.

*Corresponding author: ferjabobadilla@yahoo.com.ar

doi: 10.15414/jmbfs.2018.8.2.842-846

ARTICLE INFO

Received 1. 6. 2018
Revised 17. 9. 2018
Accepted 18. 9. 2018
Published 1. 10. 2018

Regular article

OPEN ACCESS

ABSTRACT

Pseudomonas aeruginosa is one of the main causes of nosocomial infections worldwide, with great potential for multi-resistance development. The World Health Organization (WHO) considers the antimicrobial resistance as an emerging public health problem that makes new therapies an extremely urgent issue. The aim of this work was to study the antibacterial capacity of the ethanolic extract of *Eugenia uniflora* L. leaves on *Pseudomonas aeruginosa*, its possible synergistic action with commercial antibiotics and to carry out general toxicity tests using the crustacean *Artemia salina*. The extract had a Minimum Inhibitory Dose (MID) of 0.5 mg.disc^{-1}. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC) for *P. aeruginosa* PAO1 were 1.33 mg.ml^{-1} and 13.33 mg.ml^{-1}. For the clinical isolations were 0.83 mg.ml^{-1} and 8.33 mg.ml^{-1}. The MBC/MIC ratio (MICI) qualified the action of the extract as bacteriostatic. The Time-killing curve showed that the total loss of viability of the bacteria occurs at 24 h to 16 mg.ml^{-1} and at 8 h to 32 mg.ml^{-1}. The combination of meropenem with the extract results in a decrease in its inhibitory action on *P. aeruginosa* PAO1. Meanwhile, the combination with piperacycline or ceftazidime produces a synergistic action. For ciprofloxacin, amicacin and colistin the combination is indifferent. The LC_{50} obtained using *A. salina* was 0.61 mg.ml^{-1}; indicating that the extract has a moderate toxicity. These results suggest that this species would be a potential source of antimicrobial agents to be applied in the treatment of infections caused by *P. aeruginosa*.

Keywords: : plant extracts, antimicrobial resistance, *Artemia salina* test

INTRODUCTION

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections worldwide, especially in lungs, blood and urinary tract. Its metabolic versatility contributes to its wide ecological adaptability and distribution, ability to acquire and disseminate resistance vertically and horizontally in hospital environment and its tendency to remain viable in animate and inanimate objects, including antiseptic solutions **(Sachdeva *et al.*, 2017)** In addition, due to its considerable potential to acquire resistance to many antibiotics, multi-resistant strains are found more frequently **(Mandell *et al.*, 2006)**.

Resistance to antimicrobials is one of the most serious problems we face today, with an alarming increase in bacteria whose infections have become non-treatable. The failure of the treatments is responsible for the increase in morbidity and mortality associated with bacterial infections and the increase in health care costs. The World Health Organization **(WHO, 2017)** lists the resistance generated by the abuse of antibiotics as an emerging public health problem that makes new antibiotic therapies a very urgent issue.

Natural products, such as plant extracts, provide unlimited opportunities for the discovery of new drugs, due to the great chemical diversity **(Cos *et al.*, 2006; Vandeputte *et al.*, 2010)**. According to the WHO, more than 80 % of the population of developing countries depends on traditional medicine for its primary health care needs. In addition, modern pharmacopoeias still contain at least 25 % of drugs derived from plants and many others are synthetic analogues made from prototype compounds derived from plants **(FAO, 1997)**.

The province of Misiones, stands out in the Argentine Republic for its rich biodiversity and the use of medicinal plants as a therapeutic alternative is strongly rooted in the community. At least 93 species of autochthonous medicinal plants belonging to 21 families have been described. Among them are the *Apocynaceae*, *Asteraceae*, *Lythraceae* and *Myrtaceae* families **(Keller *et al.*;2006)**. *Eugenia uniflora* L. belongs to the latter, a species popularly known as pitanga. *Eugenia uniflora* L. is a small tree 3-7 meters tall, native to subtropical America. Ethnomedicinal uses of leaf infusion include: antimicrobial, antidiarrheal, antifebrile, astringent, stimulant and antirheumatic tonic. Its essential oil is also used as a digestive, eupeptic and carminative **(Alonso *et al.*; 2005)**.

In *E. uniflora* L. leaves metabolites belonging to the groups of flavonoids, including quercitrin, quercetin, miricitrin and merecetin have been found **(Schmeda-Hirschmann *et al.*, 1987)** and tannins such as gallocatechin, oenothein B, eugeniflorins D1 and D2 **(Lee *et al.*, 2000)**. In the essential oils of the leaves have been found mainly isoflurane-germacrene and germacra-3,7,9, -trien-6-one **(Pereira *et al.*, 2017)** selin-1,3,7(11)-trien-8-one, atractilone y furanodiene **(Adebajo *et al.*, 1989)**.

The aim of this work was to study the antimicrobial properties of the metabolites of *Eugenia uniflora* L. ethanolic leaf extract on *Pseudomonas aeruginosa*, its possible synergistic action with antibiotics used in the treatment of infections by this microorganism, as well as to perform a general toxicity test using the crustacean *Artemia salina*.

MATERIALS AND METHODS

Microorganisms

Pseudomonas aeruginosa PAO1 was kindly donated by Dr. Claudio Valverde from the Laboratory of Biochemistry, Microbiology and Biological Interactions in Soil (LBMIBS) of the National University of Quilmes (UNQ). The clinical isolations L2417 (sputum), L3135 (abscess) and L3220 (eschar), used in the

determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC), were assigned by Biochemist Karina Acosta from the High Complexity Laboratory of Misiones (LACMI). All the microorganisms were stored in Cathedra of Bacteriology of the Faculty of Exact, Chemical and Natural Sciences (FCEQyN) of the National University of Misiones (UNaM).

Plant material

Samples of *Eugenia uniflora* L. were collected in December 2016 in Oberá, Misiones Province, Argentina (27°540514 S; 55°168381 W). The plant material was taxonomically classified by Dr. Marta Yajía from Cathedra of Pharmacobotany and stored for its conservation in the Herbarium of the FCEQyN-UNaM (access number MNESF n° 4545).

Extraction procedure

Collected samples were dried at room temperature for 8 days, stirring sporadically. Subsequently they were subjected to grinding by using a Wiley-type blade mill (BroBender OH6 Duisburg n° 242 mod W1247). The resulting powder used in the extraction was sieved through the 14 mesh (*American Society of Testing Methods* - ASTM).
The extract was obtained by digestion: for each gram of powder, 10 ml of a 96 % hydro-alcoholic solution (commercial alcohol) was added. The vessel with the extracted medium was placed on a shaker (ROSI 100^{TM} Thermolyne) at 80 rpm and 37 °C, for 48 h. The extract was filtered under vacuum with qualitative filter paper. The solvent was partially removed and the extract concentrated using a rotary evaporator (Laborota 4000-Efficient), then placed on a glass plate which was incubated in oven at 37 °C until the extract got dried. The resulting powder was stored in glass jars, inside a desiccator until its use.

Minimum Inhibitory Doses (MID)

It was determined by the disk diffusion method on *P. aeruginosa* PAO1, according to the protocol described by de **Seyyednejad *et al.* (2014)** with some modifications. A bacterial suspension, equivalent to McFarland 0.5 was inoculated on Müeller-Hinton agar (MHA) plate (Britania Argentina S.A.) using a sterile swab. Seven extract solutions (267; 133; 67; 33; 25; 17 and 8 mg.ml^{-1}) were prepared using Dimethyl Sulfoxide (DMSO-Biopack Argentina®) as solvent. 30 µl of these solutions were impregnated on sterile 6 mm diameter filter paper discs. The effective dose of each disc was 8; 4; 2; 1; 0.75; 0.5 and 0.25 mg. Discs were allowed to dry and placed on the surfaces of inoculated plates, which were incubated at 35-37 °C for 24 h and the inhibition diameters (ID) were measured. MIDs and IDs associated with each dose were determined. An inhibitory effect was considered to be the visible presence of a halo without bacterial development. 30 µg amicacin discs (Britania Argentina S.A.) and filter paper discs with 30 µl of DMSO were used as positive and negative controls, respectively.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

The broth dilution method on Müeller-Hinton (MHB) **(ESCMID, 2003; Wiegand *et al.*, 2008)** was used to establish MIC for *P. aeruginosa* PAO1 and three clinical isolations: L2417, L3135 and L3220. A serial dilution of the extract was made in a range of 32; 16; 8; 4; 2; 1; 0.5; 0.25; 0.125 and 0.0625 mg.ml^{-1}. One tube was reserved as a control for sterility and another as a control of viability (without extract). The final volume of the culture medium in each tube was 2 ml. The extract was initially dissolved in a volume of 100 µl of DMSO, to which 900 µL of MHB was added. Finally, each tube was inoculated with 1 ml of a suspension of 10^6 CFU.ml^{-1}, thus obtaining an initial inoculum of 5.10^5 CFU. ml^{-1}. These tubes were incubated at 35-37 °C and after 24 h the bacterial development was verified by the presence of turbidity in the broth.
The MIC was defined as the lowest concentration of extract that restricted visible bacterial growth. To establish the MBC, one loop was transferred (~ 5 µl) from each tube without visible development to nutritive agar plates. 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 MIC index (MICI) was calculated as the ratio between MBC and MIC (MBC/MIC). It was interpreted according to the criteria of **Shanmughapriya *et al.*, (2008)** as follows: MICI ≤2 considered bactericidal, > 2 but <16 bacteriostatic and ≥16 without effect.

Time-Killing Curve

The assay was carried out according to the protocol described by **Su *et al.*, (2015)** with some modifications. Five tubes were prepared, as for the determination of MIC and MBC, at the highest concentrations: 2; 4; 8; 16 and 32 mg.ml^{-1}. An initial inoculum of 5.10^5 CFU.ml^{-1} of *P. aeruginosa* PAO1 was used. Aliquots of 10 µl of broth were taken at 0; 4; 8; 12 and 24 h. These were diluted in 990 µl of sterile broth, from the resulting dilutions 10 µl were taken and plated on nutritive agar. After 18-24 h of incubation, the colonies were counted and multiplied by the dilution factor (10,000) to determine the total number of viable bacteria in broths at the time the aliquots were taken. Broth with DMSO without extract was used as control.

Resistance analysis

P. aeruginosa PAO1 was cultured in MH broth at a sub-inhibitory concentration (0.5 mg.ml^{-1}) for 10 consecutive days to evaluate its ability to develop resistance to the extract. Daily, 100 µl of broth with the microorganism was sub-cultured in 1.9 ml of broth with extract (final concentration of 0.5 mg.ml^{-1}). The CIM was determined on day 11, according to the methodology previously described.

Synergism

Six commercial antibiotics to be tested were selected, which represent the groups commonly used in the treatment of infections caused *by P. aeruginosa*: amikacin (AMK-aminoglycoside), meropenem (MER-carbapeneme), ciprofloxacin (CIP-fluoroquinolone), ceftazidime (CAZ-cephalosporin), piperacycline (PIP-ureidopenicillin) and colistin (COL- polymyxin).

A) Double disc test

Carried out based on the protocol described by **Sachdeva *et al.* (2017)** with modifications. A suspension of *P. aeruginosa* PAO1 equivalent to McFarland 0.5 was inoculated with swab on MH agar. A disc containing 1 mg of *E. uniflora* L. ethanolic extract was placed in the center of the plate. The commercial antibiotic discs were placed on the sides in three directions, at varying distances from center to center with respect to the extract disc (according to the inhibition diameters presented on the same strain in previous tests). After 24 h of incubation at 37 °C, synergistic action is considered to expand the halo of inhibition of the commercial antibiotic to the disc containing the extract.

B) Disc diffusion method

Modification of the Kirby-Bauer method used by **Abreu *et al.* (2014)**. Briefly, the extract was added to the molten MH agar at a final concentration of 0.5 mg.ml^{-1}. 20 ml of the medium was poured into 90 mm plates. The suspension of *P. aeruginosa* PAO1 was adjusted to McFarland 0.5 and seeded on the agar with swab. The commercial antibiotic discs were placed on the surface of the agar. Plates without extract were used as a positive control, to which the microorganism was inoculated and the commercial discs were placed. Discs impregnated only with DMSO were used as a negative control. After 24 h of incubation at 37 °C, values of the inhibition diameters were recorded. The increase in the diameter of the inhibition zones was considered to be a synergistic effect in the tests carried out with the extract in the culture medium respect to the diameters presented by the positive controls.

Toxicity test with *Artemia salina*

Approximately 0.1 g of *Artemia salina* cysts (Aquagreen®, Argentina) was added to one liter of saline solution prepared by dissolving 10 g of NaCl per liter of distilled water. The container was kept at room temperature (28-30 °C), with air supply through a pump and constant illumination with a 60 W bulb. After 24-48 h the cysts hatched, and the larvae (nauplii) were taken in groups of ten to submit them to different concentrations of the extract. A 96-well plastic microplate was used, each well was filled with 200 µl of saline solution (containing 10 *A. salina* nauplii), 12.5 µl of the ethanolic extract of *E. uniflora* L. dissolved in DMSO and the final volume of 250 µl was reached with the same saline solution. The concentrations tested were 2; 1; 0.75; 0.5; 0.25; 0.125 and 0.0625 mg.ml^{-1}. The microplate was incubated in a previously saturated glass container (humid atmosphere), illuminated by a 60 W bulb, at 28-30 °C and for 24 h. Subsequently, the number of surviving nauplii in each well is counted with a stereoscopic magnifying glass. In parallel, 2 controls were carried out: a growth control containing only the nauplii in saline and a DMSO control, which contained nauplii, saline and 12.5 µL of DMSO without extract. The death was established by the total lack of movement during 10 seconds of observation. The lethality percentage in each well was calculated by the following equation:

$$\% \text{ Letality} = \left(\frac{\text{N alive nauplii control} - \text{N alive nauplii test}}{\text{N alive nauplii control}}\right) \times 100$$

The determination of the lethal concentration 50 (LC_{50}) was made by graphic estimation, representing the percentage of lethality of the nauplii depending on the concentration of the extract, the LC_{50} value was obtained by the linear regression method using the software STATGRAPHICS Centurion XV II. The toxicity criterion is the one established by **Déciga-Campos *et al.* (2007)**, which considers values of LC_{50} higher than 1 mg.ml^{-1} as low toxicity, those lower than 1 but greater than 0.5 mg.ml^{-1} moderately toxic, and less than 0.5 mg.ml^{-1} toxic.
All tests were performed in triplicate.

RESULTS

Minimum inhibitory dose (MID)

The extract presented a MID value of 0.5 mg, the associated inhibition diameter (ID) had an average of 7.3 +/- 0.3 mm. The inhibition diameters corresponding to the different doses tested were found in a range between 6 and 13.7 mm. Data is summarized in **table 1**. The inhibitory activity of the different effective loads of the discs can be seen in **figure 1**.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

The MIC values for *P. aeruginosa* PAO1 were between 1 and 2 mg.ml^{-1}, mean of 1.33 mg.ml^{-1}. For the clinical isolations, values were 0.5 and 1 mg.ml^{-1}, mean of 0.83 mg.ml^{-1}. The values of MBC showed a wider range, from 8 to 16 mg.ml^{-1} for *P. aeruginosa* PAO1 (mean 13.33 mg.ml^{-1}) and 1 to 16 mg.ml^{-1} for clinical isolations (mean 8.33 mg.ml^{-1}). The MBC/MIC ratio (CIMI) had an average of 10.67 for *P. aeruginosa* PAO1 and 8.67 for the clinical isolations. According to **Shanmughapriya *et al.* (2008)** criteria previously described, its action is interpreted as bacteriostatic on *P. aeruginosa* PAO1 and the clinical isolations of this species. Values and interpretations can be seen in **table 2**.

Table 1 inhibition diameters in disc diffusion test for the different loads (doses) of the discs

Doses (mg)	ID (mm)	standard error (mm)
0.25	6.0	0.0
0.50	7.3	0.3
0.75	8.7	0.3
1.00	10.0	0.6
2.00	11.0	0.6
4.00	12.3	0.9
8.00	13.7	1.3

ID, inhibition diameter.

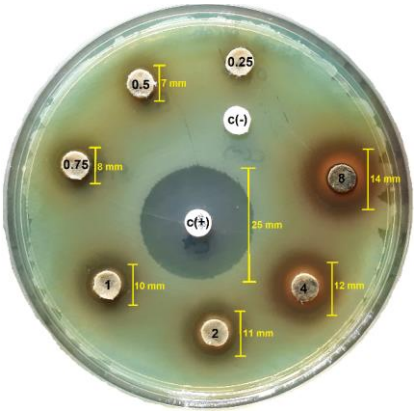

Figure 1 disc diffusion test. The discs contain doses from 0.25 to 8 mg. A disc impregnated with DMSO is used as negative control C (-) and another of amicacin as positive control C (+).

Time-killing curve

The antimicrobial capacity of *E. uniflora* L. ethanolic extract was also determined by the time-killing curve. **Figure 2** shows the development of the test for the negative control, medium with DMSO without extract, in which it is appreciated that the colony count increases as time passes, indicating that DMSO has no antimicrobial activity on *P. aeruginosa* PAO1. At concentrations higher than MIC (> 1.33 mg.ml^{-1}) but lower than 8 mg.ml^{-1}, the bacteria keeps viability and in a count equal to the negative control. At 8 mg.ml^{-1} the count remains constant over time, similar to the initial inoculum. Meanwhile, the total loss of viability occurs at 24 h at 16 mg.ml^{-1} and at 8 h at a concentration of 32 mg.ml^{-1}.

Resistance analysis

The resistance induced by the extract was determined by sub-culture of the microorganism at a sub-MIC concentration (0.5 mg.ml^{-1}). On day 11, the MIC of *P. aeruginosa* PAO1 was determined. The resulting value was 1.33 mg.ml^{-1}, what means there was no change. It would not be expected that the microorganism develop resistance to the active substance of the extract.

Table 2 values of MIC, MBC, CIMI and their corresponding interpretations for the strains tested

Strains	MIC mg ml^{-1}	MBC mg ml^{-1}	CIMI	Interpretation
P. aeruginosa PAO1	$\overline{x}$= 1.33 +/- 0.33	$\overline{x}$= 13.33 +/-2.67	$\overline{x}$=10.67 +/-2.67	bacteriostatic
L2417	0.5	1.0	2.0	bacteriostatic
L3135	1.0	8.0	8.0	bacteriostatic
L3220	1.0	16.0	16.0	bacteriostatic
Clinical isolations	0.83 +/- 0.17	8.33 +/- 4.33	8.67 +/- 4.05	bacteriostatic

MIC, minimum inhibitory concentration. MBC, minimum bactericide concentration. CIMI, minimum inhibitory concentration index.

Synergism

A) Double disc test

None of the commercial antibiotics showed evident expansion of their inhibition halos towards the disc containing the extract.

B) Disc diffusion method

Table 3 shows the inhibition zone diameters produced by the antibiotics alone and in combination with the extract in the culture medium. The combination of the antibiotic meropenem (MER) with *E. uniflora* L. extract results in a decrease in its inhibitory action on *P. aeruginosa* PAO1 (negative interaction). In both, combination of piperacycline (PIP) and ceftazidime (CAZ) with the extract produces a synergistic action of these antibiotics. For ciprofloxacin (CIP), amikacin (AMK) and colistin (COL) the combination is indifferent.

Toxicity test with *Artemia salina*

There was 100 % viability of the control group, no alterations in mobility or behavior changes were evident. The estimated LC_{50} was 0.61 [0.51 -0.74] mg.ml^{-1}. Thus, it is considered as moderate toxicity, according to the criteria of **Déciga-Campos *et al.* (2007)**.

DISCUSSION

This work has registered there are metabolites in *Eugenia uniflora* L. leaves, extracted with hydro-alcoholic solvent, with antibiotic activity on *P. aeruginosa*. Establishing that the load or minimum dose to demonstrate such effect in the disc diffusion test is 0.5 mg. MIC (0.83-1.33 mg.ml^{-1}), MBC (8.33-13.33 mg.ml^{-1}) and MICI values (8.67-10.67) were also established. Which gives an approximation of the type of antimicrobial activity this extract presents (bacteriostatic). The time-killing curve allowed to know that the effect of the extract is bacteriostatic up to a concentration of 8 mg.ml^{-1}, that the total loss of viability occurs at 24 h at 16 mg.ml^{-1} and at 8 h at 32 mg.ml^{-1}. The resistance test established that *P. aeruginosa* PAO1 did not develop tolerance to the extract within a period of 10 days of exposure to it at a sub-inhibitory concentration. The existence of synergism between the extract and commercial antibiotics PIP and CAZ, the negative interaction of the combination of MER with the extract and the indifferent effect of the combination of the extract with CIP, AMK and COL were also demonstrated.

Other authors have studied and demonstrated the antimicrobial activity of *Eugenia uniflora* L. extracts on a wide variety of microorganisms. **Adebajo *et al.* (1989)** recorded in vitro antimicrobial effects of the essential oil of leaves of this plant through several tests and microorganisms. Similar results to those of the present work were found by authors such as **Falcão *et al.* (2018)** who obtained MIC values of 1.25 mg.ml^{-1} for the crude extract of a mixture of acetone: water 7:3 (v/v), 1.875 mg.ml^{-1} for the ethyl acetate fraction and 2.5 mg.ml^{-1} for the aqueous fraction on *P. aeruginoisa* ATCC 27853. While **Auricchio *et al.* (2007)** obtained a MIC of 0.4 mg.ml^{-1} for the 70 % hydro-alcoholic extract of this plant on *P. aeruginosa* ATCC 15442. The same strain was used by **Holetz *et al.* (2002)**

who obtained MIC values greater than 1 mg.ml^{-1} with a 90 % hydro-alcoholic extract of leaves. On the other hand, **Fiúza *et al.* (2008)** differed considerably, since they obtained a greater value of inhibition diameter, which turned out to be 25 mm for an effective dose of 3.3 mg of the ethanolic extract of leaves on *P. aeruginosa* ATCC 9027. However, their MIC values are higher; 4.375 mg.ml^{-1} for ATCC 9027 and 8.75 mg.ml^{-1} for ATCC 27853. In general, these studies conclude that extracts of *E. uniflora* L. have higher activity against gram-positive bacteria. This may be due to the outer membrane of gram-negative bacteria that represents a barrier for the penetration of numerous antibiotic molecules, and that the periplasmic space contains enzymes capable of cleaving foreign molecules **(Duffy *et al*., 2001)**.

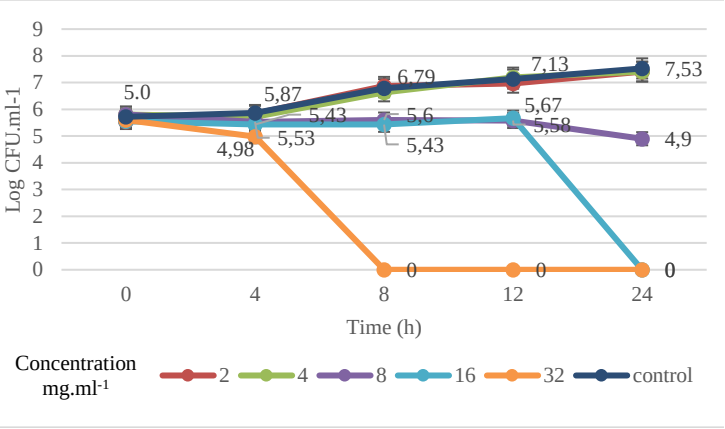

Figure 2 antimicrobial effect of *E. uniflora* L. ethanolic extract on *P. aeruginosa* PAO1. An initial inoculum of 5.10^5 CFU.ml^{-1} was cultured at different concentrations of the extract. Aliquots of the culture medium were taken at hours 0, 4, 8, 12 and 24; they were sown on agar and the colonies formed were counted.

Table 3 inhibition zones (mean +/- standardized error) produced by the antibiotics alone (no extract) and in combination with the extract (extract). The combinations that produce statistically significant differences (P <0.05) are highlighted in bold.

Antibiotic	Inhibition zone diameter (mm)	
	No extract	Extract
MER	**31.7 +/- 1.0**	**25.3 +/- 1.0**
PIP	**32.0 +/- 0.4**	**37.0 +/- 0.4**
CIP	37.0 +/- 0.6	37.3 +/- 0.6
AMK	27.7 +/- 0.2	28.0 +/- 0.2
CAZ	**31.3 +/- 0.5**	**37.0 +/- 0.5**
COL	17.0 +/- 0.5	15.7 +/- 0.5

MER, meropenem. PIP, piperacycline. CIP, ciprofloxacin. AMK, amicacin. CAZ, ceftazidime. COL, colistin.

Other plants have shown antibiotic activity against *P. aeruginosa*: **Pratiwi *et al.* (2015)** studied the antimicrobial activity of hydroalcoholic extracts of numerous plant species on *P. aeruginosa* PAO1. Among them, *P. guajava* L. belonging, as *E. uniflora* L., to *Myrtaceae* family. Its extract presented a MIC of 1 mg.ml^{-1}. They also described the extracts of *Curcuma xanthorrizha* and *Myristica fragans* Houtt. as those with greater antimicrobial activity on this strain, both with MIC values of 0.25 mg.ml^{-1}. **Hamdi *et al.* (2017)** worked with the hydro-alcoholic extract of different parts of *Asparagus albus* L. on a clinical isolation of multiresistant *P. aeruginosa*. They obtained values of 13 mm of inhibition diameter by the diffusion method with an effective load of 6 mg for the leaves and an increased activity was presented by the extract of the pericarp with an inhibition diameter of 21 mm for the same load. The MIC values were 1.56 mg.ml^{-1} for the hydro-alcoholic extract of leaves and 0.39 mg.ml^{-1} for the pericarp extract. With MBC values of 25 mg.ml^{-1} for leaves and 6.25 mg.ml^{-1} for pericarp. **Latha *et al.* (2010)** assessed the hydro-alcoholic extract of *Vernonia cinerea* for which they registered inhibition growth of clinical *P. aeruginosa* with a dose of 10; 5 and 2.5 mg and halos of 20, 15 and 10 mm, respectively, with a MIC value of 3.3 mg.ml^{-1}.

In this work it was also determined the LC_{50} of the extract, which turned out to be 0.61 [0.51-0.74] mg.ml^{-1}, interpreting it as an extract of moderate toxicity. **Pimentel *et al.* (2002)** obtained an LC_{50} higher than 1 mg.ml^{-1} for *E. uniflora* L. ethanolic extract on the crustacean *A. salina*. In contrast, the results of **Arcanjo *et al.* (2012)** showed that the aerial parts of *Eugenia uniflora* L. had an LC_{50} of 0.288 mg.ml^{-1}.

Schmeda *et al.* (1987) have advanced with more sophisticated toxicity tests, in which they demonstrated that the hydro-alcoholic extract did not produce signs of acute or subacute toxicity in rats, at doses of 4,200 mg.kg^{-1} orally and the LC_{50} of the extract was calculated at 220 mg.kg^{-1} intraparenterally in rats.

CONCLUSIONS

The results of this study proved the antibacterial activity attributed to *Eugenia uniflora* L., in this case against *Pseudomonas aeruginosa*. This suggests that this species would be a potential source of antimicrobial agents to be applied in the treatment of infections caused by this nosocomial pathogen.

Further analyses are required to identify the active components of the extract. In addition, in vitro studies of the extract effects on human cell lines as a first step to verify safety in human models.

Acknowledgment: The authors are enormously grateful to Dr. Claudio Valverde of the Laboratory of Biochemistry, Microbiology and Biological Interactions in Soil (LBMIBS) of the National University of Quilmes (UNQ) and to Bioch. Karina Acosta of the Laboratory of High Complexity of Misiones (LACMI) for the kindness of yielding the microorganisms for the development of this work. They also thank Dr. Marta Yajía (FCEQyN-UNaM) for the botanical identification of the plant material and Eng. Fernando Kramer for his assistance in the process of obtaining extracts.

REFERENCES

ABREU, A.; SERRA, S.; BORGES, A.; SAAVEDRA, M.; SALGADO, A.; SIMÕES, M. (2014). *Evaluation of the best method to assess antibiotic potentiation by phytochemicals against Staphylococcus aureus*. Diagn Microbiol Infect Dis. *79(2)*. 125-134. https://doi.org/10.1016/j.diagmicrobio.2014.03.002

ADEBAJO, A.; OLOKE, K.; ALADESANMI, A. (1989). *Antimicrobial activities and microbial transformation of volatile oils of Eugenia uniflora*. Fitoterapia. *50*. 451-455.

ALONSO, J.; DESMARCHELIER, C. (2015). *Pitanga. Plantas medicinales autóctonas de la Argentina, Bases científicas para su aplicación en atención primaria de la salud*. (1° ed. Vol 1). Ed. LOLA. p.454-459. ISBN 950-9725-80-3

ARCANJO D.; ALBUQUERQUE, A.; MELO-NETO, B.; SANTANA, L.; MEDEIROS, M. CITÓ A. (2012). *Bioactivity evaluation against Artemia salina Leach of medicinal plants used in Brazilian Northeastern folk medicine*. Braz. J. Biol., 72(3), 505-509. https://doi.org/10.1590/S1519-69842012000300013

AURICCHIO, M.; BUGNO, A.; BARROS, S.; BACCHI, E. (2007). *Actividades antimicrobiana, antioxidante e toxicidade de Eugenia uniflora*. Lat. Am. J. Pharm. 26(1). 76-81.

COS, P.; VLIETINCK, A.; BERGHE, D.; MAES, L. (2006). *Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'*. J Ethnopharmacol. 106(3). https://doi.org/10.1016/j.jep.2006.04.003

DÉCIGA-CAMPOS, M.; RIVERO-CRUZ, I.; ARRIAGA-ALBA, M.; CASTAÑEDA-CORRAL, G.; ANGELES-LÓPEZ, G.E.; NAVARRETE, A.; MATA, R. (2007). *Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine*. J Ethnopharmacol. 11(2). 334-342. https://doi.org/10.1016/j.jep.2006.10.001

DUFFY, C.; POWER, R. (2001). *Antioxidant and antimicrobial properties of some Chinese plant extracts*. Int J Antimicrob Agents. 17(6). 527-529. https://doi.org/10.1016/S0924-8579(01)00326-0

EUROPEAN COMMITTEE FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING-EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (EUCAST-ESCMID). (2003). *Determination of minimum inhibitory concentration (MICs) of antibacterial agents by broth dilution*. CMI. 9(8). 9-15. https://doi.org/10.1046/j.1469-0691.2003.00790.x

FALCÃO T.; ARAÚJO A.; LIRA SOARES, L.; MORAES RAMOS, R.; FERRAZ BEZERRA, I; ASSUNÇÃO FERREIRA, M. (2018). *Crude extract and fractions from Eugenia uniflora Linn leaves showed anti-inflammatory, antioxidant, and antibacterial activities*. BMC Complement Altern Med. 18(1). 84. https://doi.org/10.1186/s12906-018-2144-6

FAO, FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. *Medicinal plants for forest conservation and healrh care*. 1997. ISBN 92-5-104063-X.

FIÚZA, T.; SABÓIA-MORAIS, S.; PAULA J.; TRESVENZOL, L.; PIMENTA, F. (2008). *Evaluation of antimicrobial activity of the crude ethanol extract of Eugenia uniflora L. leaves*. Rev. ciênc. farm. básica apl., 29(3), 245-250. ISSN 1808-4532

HANDI, A.; JARAMILLO-CARMONA, S.; SRAIRI BEJI, R.; TEJ, R.; ZAOUI, S.; RODRÍGUEZ-ARCOS, R. (2017). *The phytochemical and bioactivity profiles of wild Asparagus albus L. plant*. Food Res Int. 720-729. 10.1016/j.foodres.2017.06.027

HOLETZ, F.; PESSINI, G.; SANCHES, N.; GARCIA CORTEZ, D.; NAKAMURA, C.; DIAS FILHO, B. (2002). *Screening of some plants used in Brazilian folk medicine for the treatment of infectious diseases*. Mem Inst Oswaldo Cruz. 97(7). 1027-1031. https://doi.org/10.1590/S0074-02762002000700017

KELLER, H.; ROMERO, H. (2006). *Plantas medicinales utilizadas por campesinos del área de influencia de la Reserva de Biósfera Yabotí (Misiones, Argentina)*. Bondplandia. 15. 3-4.

LATHA, L.; DARAH, I.; KASSIM, M.; SASIDHARAN, S. (2010). *Antibacterial activity and morphological changes of Pseudomonas aeruginosa cells after exposure to Vernonia cinerea extract*. Ultrastruct Pathol. 34(4). 219-225. https://doi.org/10.3109/01913121003651513

LEE, M.; CHIOU, J.; YEN, K.; YANG, L. (2000). *EBV DNA polymerase inhibition of tannins from Eugenia uniflora*. Cancer Lett. 154(2). 131-136. https://doi.org/10.1016/S0304-3835(00)00353-0

MANDELL, G.; BENNET, J.; DOLIN, R.; PIER, G.; REUBEN, R. (2006). Enfermedades Infeccionsas: Principios y prácticas. *Pseudomonas aeruginosa*. (6° ed., Vol. 3). ELsevier. p. 2587-2615 ISBN 84-8174-842-0

PEREIRA, N.; AQUINO, P.; JÚNIOR, J.; CRISTO, J.; FILHO, M.; MOURA, F. (2017). *In vitro evaluation of the antibacterial potential and modification of antibiotic activityof Eugenia uniflora L. essential oil in associaton with led lights*. Microb Pathog. 110. 512-518. https://doi.org/10.1016/j.micpath.2017.07.048

PIMENTEL MONTANHER, A.; PIZZOLATTI, M.; BRIGHENTE, I**.** (2002). *An Application of the Brine Shrimp Bioassay for General Screening of Brazilian Medicinal Plants*. Acta Farmacéutica Bonaerense, 21(3), 175-178.

PRATIWI, S.; LAGENDIJK, E; HERTIANI, T.; WEERT, S.; VAN DEN HONDEL, C. (2015). *Antimicrobial effects of Indonesian Medicinal Plants Extracts on Planktonic and Biofilm Growth of Pseudomonas aeruginosa and Staphylococcus aureus*. J. Horticulture. 2(119).

SACHDEVA, R.; SHARMA, B.; SHARMA, R. (2017). *Evaluation of different phenotypic tests for detection of metallo-β-lactamases in imipenem-resistant Pseudomonas aeruginosa.* J Lab Physicians. 9(4). 249-253. https://doi.org/10.4103/JLP.JLP_118_16

SCHMEDA-HIRSCHMANN, G.; THEODULOZ, C.; FRANCO, L.; FERRO, E.; DE ARIAS, A. (1987). *Preliminary pharmacological studies on Eugenia uniflora leaves: xanthine oxidase inhibitory activity*. J Ethnopharmacol. 21(2). 183-186. https://doi.org/10.1016/0378-8741(87)90128-0

SEYYEDNEJAD, SM.; MOTAMEDI, H.; VAFEI, M.; BAKHTIARI, A. (2014). The Antibacterial Activity of Cassia fistula Organic Extracts. Jundishapur J Microbiol., 7(1), e8921. https://doi.org/10.5812/jjm.8921

SHANMUGHAPRIYA, S.; MANILAL, A.; SUJITH, S.; SELVIN, J.; KIRAN, G.; NATARAJASEENIVASAN, K**.** (2008). *Antimicrobial activity of seaweeds extracts against multiresistant pathogens*. Ann Microbiol. 58(3). 435-541. https://doi.org/10.1007/BF03175554

SU, P.W.; YANG, C.; YANG, J.; SU, P.Y.; CHUANG, L. (2015). *Antibacterial Activities and Antibacterial Mechanism of Polygonum cuspidatum Extracts against Nosocomial Drug-Resistant Pathogens*. Molecules. 20(6). 11119-11130. https://doi.org/10.3390/molecules200611119

VANDEPUTTE, O.; KIENDREBEOGO, M.; RAJAONSON, S.; DIALLO, B.; MOL, A.; EL JAZIRI, M**.** (2010). *Identification of catechin as one of the flavonoids from Combretum albiflorum bark extract that reduces the production of quorum-sensing-controlled virulence factors in Pseudomonas aeruginosa PAO1*. Appl Environ Microbiol. 76(1). 243-253. https://doi.org/10.1128/AEM.01059-09

WHO, WORLD HEALTH ORGANIZATION. Antibiotic resistance. 2017. [Online]. http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/.

WIEGAND, I.; HILPERT, K.; HANCOCK, RE. (2008). *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances*. Nat Protoc. 3(2) 163-175. https://doi.org/10.1038/nprot.2007.521