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Antibacterial activity of medicinal plants against *Streptococcus agalactiae*

Marina Gisel Novosak[1,✉], Daniana Lilian Winnik[1], Margarita Ester Laczeski[1,2], Marina Inés Quiroga[1]

[1]*Department of Microbiology, Faculty of Exact Chemical and Natural Sciences, National University of Misiones, CONICET, Misiones, Argentina*
[2]*Molecular Biotechnology Laboratory, Misiones Institute of Biotechnology, Faculty of Exact Chemical and Natural Sciences, National University of Misiones, CONICET, "Dra. María Ebe Reca", Misiones, Argentina*

✉ *Corresponding author: marinanovosak2008@gmail.com*

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Abstract

Streptococcus agalactiae, group B *Streptococcus* (GBS), infects and causes severe diseases in humans and numerous animal species, including fish, given its ability to cross the host-specific barrier. The emergence of antibiotic resistant GBS strains makes it necessary to look for alternatives to treat and prevent infections that it produces. The aim of the present study was to determine the antibacterial activity of ethanolic and aqueous extracts of medicinal plants from Misiones province, northeast Argentina, against GBS from humans and fish. We used human *Streptococcus agalactiae* ATCC® BAA-611™ and tilapia *Streptococcus agalactiae* ATCC® 51487™ strains. Minimum Inhibitory Dose (MID) was determined by the disc diffusion method. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), MBC/MIC ratio, drug synergism with commercial antibiotics, and resistance assays were determined with extracts that showed antibacterial activity. Medium Lethal Dose 50 (LD_{50}) was determined by the *Artemia salina* assay. For ethanolic and aqueous *Eugenia uniflora* L. extracts, we obtained a MID = 0.5 mg.disc^{-1}. For both extracts of *Eugenia uniflora* L., the MIC and MBC values were 1 mg.mL^{-1} and 5 mg.mL^{-1}, respectively. The MICI (MBC/MIC ratio) = 5 qualified the action of these extracts as bacteriostatic. The drug synergism assay with ampicillin, erythromycin, and clindamycin combination and extracts showed indifference. The LD_{50} of the aqueous extract was 0.82 mg.mL^{-1} indicating moderate toxicity. This work is a first step to identify chemical compounds in native medicinal plants of Misiones, Argentina, that could mean an alternative for the treatment of *Streptococcus agalactiae* infections.

Introduction

Streptococcus agalactiae, Group B *Streptococcus* (GBS), is a commensal bacterium of human gastrointestinal and genitourinary tracts (Tan *et al.* 2017). It is the primary cause of life-threatening infections in newborns and infants, such as sepsis, meningitis, and pneumonia. Its transmission by GBS-colonized pregnant women is the main route of neonatal infestation (Raabe 2019). Infections by GBS in newborns decreased with the detection of this microorganism in pregnant women between 35 – 37 gestation weeks and the implementation of intrapartum antimicrobial prophylaxis. However, the rate of invasive disease in older adults and people with underlying chronic disease is increasing (Sendi *et al*. 2016).

Streptococcus agalactiae infects a wide variety of animal species due to its ability to cross the host-specific barrier. Authors have shown that some human GBS strains come from fish and other aquatic animals (Skov Sorensen *et al*. 2019). Since GBS is the leading cause of septicemia and meningoencephalitis outbreaks in freshwater fish, it is problematic in aquaculture with a significant economic impact on the fish industry (Mian *et al*. 2009; Tavares *et al*. 2019). *Streptococcus agalactiae* is the most important pathogen of Nile tilapia (*Niloticus oreochromis*). The tilapia farming industry has recently been hampered by streptococcosis outbreaks associated with high fish densities. In Brazil, it causes up to 90 % of deaths in tilapia farms (Banrie 2012). Similar problems are expected to manifest themselves in Misiones province, Argentina, where there are more than 4,000 aquaculture producers recorded through provincial development programs (Ministry of agriculture, livestock, and fisheries 2020). Several authors have identified virulence genes from human GBS strains in fish GBS strains (Delannoy *et al*. 2013; Liu *et al*. 2013) and suggested that colonization with GBS serotypes Ia and Ib in humans is associated with the consumption of infected fish (Foxman *et al*. 2007). This shows that human and fish GBS strains are closely related.

Penicillin, a beta-lactam family antibiotic, is recommended as first-line therapy against GBS infections. Macrolides (erythromycin) and lincosamides (clindamycin) are second-line antibiotics. These are usually prescribed for beta-lactams allergy patients (Le Doare *et al*. 2017). Also, penicillin is commonly used in animal farming and aquaculture for prophylactic or treatment purposes (Ibrahim *et al*. 2020). The number of human clinical GBS isolates with reduced penicillin susceptibility, and a multidrug resistance tendency increased between 2005 – 2006 and 2012 – 2013 in Japan (Seki *et al*. 2015). Furthermore, a study in China described the appearance of GBS strains resistant to penicillin in human lesions and tilapia (Nagano *et al*. 2019). The appearance of multidrug resistance and GBS not susceptible to penicillin inside and outside health centers is a concern (Nagano *et al*. 2019). Also, resistances to erythromycin (14.5 to 70 %) and clindamycin (8.2 to 70 %) have been reported worldwide (Nagano *et al*. 2012). In Misiones, 6 % resistance to erythromycin and 5 % resistance to clindamycin were reported in human GBS strains, respectively (Novosak *et al*. 2020).

To date, in Argentina and Brazil, isolates show susceptibility to ceftriaxone, penicillin, and vancomycin. However, researchers reported strains with reduced susceptibility to these antimicrobial agents in Japan, the USA, the UK, and Canada (Bonofiglio *et al*. 2018; Li *et al*. 2020). The streptococcosis treatment is performed with fish food administration supplemented with antibiotics. However, the intensive antibiotic use in fish farms could lead to resistant strains emergence (Amal *et al*. 2011). In this context, it is necessary to search for alternatives for streptococcosis treatment.

Medicinal plants have been used to treat several human diseases worldwide for thousands of years. The World Health Organization (WHO) has recorded more than 20,000 species of medicinal plants with a variety of potential uses (Silva *et al*. 2021). Also, their products are used as alternatives to antibiotics and chemotherapy to prevent and control diseases in aquaculture (Cheesman *et al*. 2017). Medicinal plants are sources of nutrients and can be used in whole, in part or as an extract, alone or in combination, by aquatic route or food supplement, or even mixed with other bioactive compounds (Doan *et al*. 2019). Moreover, medicinal plants usually have stimulating and immunostimulating properties of fish growth (Awad *et al*. 2017). These have a wide range of bioactive compounds that are generally cheaper, safer, and more accessible than their synthetic equivalents (Cheesman *et al*. 2017), such as phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectin, and polypeptide compounds (Abuajah *et al*. 2015).

At least 93 species of native medicinal plants belonging to 21 families have been described in Misiones, including *Apocynaceae*, *Asteraceae*, *Lythraceae*, and *Myrtaceae*. Previous studies (Guida *et al*. 2003; Jerke *et al*. 2008; Bargardi *et al*. 2021) suggest that these possess metabolites able to inhibit the growth of human and animal pathogenic bacteria. However, the effect of many of these plants against GBS isolates has not been described so far. The aim of the present study was to determine the antibacterial activity of the ethanolic

and aqueous extracts of medicinal plants from Misiones province, northeast Argentina, against GBS from humans and fish.

Experimental

Plants collection and identification

The leaves of *Psidium guajava* L., *Eugenia uniflora* L., *Tabernaemontana catharinensis* A.DC., *Baccharis crispa* Spreng., *Cecropia pachystachya* Trécul., and *Acanthospermum australe* (Loefl.) Kuntze were collected in different Misiones province locations. The taxonomic identification was carried out in the chair of Pharmacobotany of the Faculty of Exact, Chemical and Natural Sciences (FCEQyN).

Extraction

Leaves were dried at room temperature for ten days, stirring periodically. Then, dry leaves were crushed in a Numak F 100 460 W blade mill (Instrumentación Científica S. A., Buenos Aires, Argentina). 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 water and 96 % hydroalcoholic solution (commercial alcohol) and concentrated with a rotary evaporator Laborota 4000-Efficient (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). Powder was stored in a dark and dry place.

Bacterial strains

The bacterial strains used in the assays were *Streptococcus agalactiae* Lehmann and Neumann (ATCC® BAA-611™, AATC, Manassas, USA) (Clinical specimen–human) and *Streptococcus agalactiae* (ATCC® 51487™, AATC, Manassas, USA) (*Tilapia* sp. brain, Israel).

Minimum Inhibitory Dose (MID) assay

Minimum Inhibitory Dose (MID) was determined by the agar diffusion assay according to Seyyednejad *et al*. (2014) with modifications. Extracts effective doses were 0.5, 1, 5, 10, and 15 mg. Extract solutions were prepared using dimethyl sulfoxide (DMSO) as a solvent for ethanolic extracts and sterile distilled water for aqueous extracts. Thirty microliters of these solutions were impregnated on filter paper discs. Ampicillin discs (10 µg) (Laboratorios Britania S.A., Buenos Aires, Argentina) were used as positive controls. Discs impregnated with DMSO were used as negative control for the ethanolic extracts, whereas discs impregnated with sterile distilled water were used as negative control for aqueous extracts. The bacterial suspension equivalent to McFarland 0.5 was inoculated on Müeller-Hinton agar (MHA) plates (Laboratorios Britania S.A., Buenos Aires, Argentina) supplemented with 5 % sheep blood using a sterile swab. Discs were placed on the surface of inoculated plates. Plates were incubated at 35 – 37 °C for 24 h for the subsequent measurement of inhibition diameters (ID). 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 assays that follow. The percentage of inhibitory effect was obtained by the following expression (Eq. 1; Martinez *et al*. 1996):

$$\text{Percentage of inhibitory effect} = \frac{\text{Average diameter of inhibition halo (mm)}}{\text{Average diameter of inhibition halo of positive control (mm)}} \times 100\ \% \quad (1)$$

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays, and MBC / MIC ratio

MIC and MBC assays were carried out following Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI 2015). The concentration range tested for each selected extract was from 20 to 0.0625 $mg.mL^{-1}$. MIC was defined as the lowest concentration without visible development. To determine MBC, MIC cultures that did not show visible growth were seeded in MHA with 5 % sheep blood and incubated for 24 h at 35 – 37 °C in a 10 % CO_2 atmosphere. MBC was defined as the lowest concentration of the extract capable of totally inhibiting microbial growth (Procedures in Clinical Microbiology 2000). MIC and MBC assays were performed in triplicate. When

MBC/MIC ratio was ≤2 the effect of the active fraction was considered bactericidal, >2 and <16 bacteriostatic, and ≥16 ineffective (Shanmughapriya *et al*. 2008).

Induction of resistance assay

GBS strains were sub-cultured in sub-MIC concentration for ten consecutive days to investigate their ability to develop drug resistance (Su *et al*. 2015). Tubes with MH broth and 0.5 mg.ml^{-1} of extract were prepared and stored in tubes at -20 ºC. A tube was used each day. On the first day of an assay, a tube was inoculated with 0.5 McFarland of *Streptococcus agalactiae* ATCC® BAA-611™ and incubated in an oven at 35 – 37 °C for 24 h. The next day, 50 µL from the first tube was inoculated into a second tube with the extract. In addition, each day the broth was plated on a nutritive agar plate supplemented with 5 % of sheep blood and incubated at 35 – 37 ºC for 24 h to evaluate possible contamination. The assay was repeated until the tenth day. MIC was determined on test day 11. The same assay as in the *Streptococcus agalactiae* ATCC® BAA-611™ was carried out with *Streptococcus agalactiae* ATCC® 51487™. Assays were carried out in triplicate.

Drug synergism assay between extracts and commercial antibiotics

It was performed on *Streptococcus agalactiae* ATCC® BAA-611™ and *Streptococcus agalactiae* ATCC® 51487™ by the double-disc assay described by Sachdeva *et al*. (2017) with modifications. Commercial antibiotics used for the treatment of GBS were used: ampicillin (10 µg), erythromycin (15 µg), and clindamycin (2 µg) discs (Laboratorios Britania S.A., Argentina). Bacterial suspensions equivalent to McFarland 0.5 were inoculated with a sterile swab on MHA supplemented with 5 % sheep blood. A disc with 1 mg of *Eugenia uniflora* L. extract was placed in the center of the plate. Commercial antibiotic discs were placed on the sides 2 cm from center to center. The plates were incubated at 35 – 37 ºC for 24 h. The increased inhibition halo in the proximity of discs was interpreted as drug synergism. Assays were carried out in triplicate.

Toxicity assay

The *Artemia salina* larvae assay was used to determine the extract toxicity (Meyer *et al.* 1982). Approximately 0.1 g of *Artemia salina* cysts (Aquagreen®, Argentina) was added to one liter of a saline solution (containing 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 Submersible Pump BL-200 (Baojie, Zhejiang, China) and constant illumination. After 24 – 48 h the cysts hatched, and the larvae were taken in groups of ten to submit them to different extract concentrations in a 96-well plastic microplate. Each well was filled with 200 µL of saline solution (containing 10 *Artemia salina* larvae), 12.5 µL of *Eugenia uniflora* L. aqueous extract dissolved in DMSO and the final volume of 250 µL was completed with the same saline solution. Concentrations tested were 0.0625, 0.125, 0.25, 0.5, 0.75, 1, 2 mg.mL^{-1}. The microplate was incubated under illumination in a previously saturated glass container (humid atmosphere) at 28 – 30 °C and for 24 h. Then, the number of surviving larvae in each well is counted with a stereoscopic magnifying glass Nikon SMZ 445 (Nikon Corp. Tokyo, Japan). Two controls were carried out: a growth control containing only the larvae in saline solution and a DMSO control, which contained larvae, saline solution, and 12.5 µL of DMSO without extract. The larvae death was established by the total lack of movement during 10 seconds of observation (Vanhaecke *et al.* 1984). The lethality percentage in each well was calculated by the following equation (Eq. 2):

$$\text{Percentage of lethality} = \frac{\text{Number of alive larvae control - Number of alive larvae test}}{\text{Number of alive larvae control}} \times 100\ \% \qquad (2)$$

The lethal concentration 50 (LC_{50}) was determined by graphic estimation, representing the lethality percentage of the larvae depending on the extract concentration. The LC_{50} value was obtained by the linear regression method using the software Statgraphics Centurion XVII (Statgraphics Technologies, Inc., The Plains, USA).

Results

Minimum Inhibitory Dose (MID) assay

Ethanolic and aqueous extracts of *Eugenia uniflora* L. presented antibacterial activity against *Streptococcus agalactiae* ATCC® BAA-611™ and *Streptococcus agalactiae* ATCC® 51487™ at the concentrations tested The MID was 0.5 mg.disc^{-1} for both extracts (Table 1 and Fig. 1). While *Psidium guajava* L., *Tabernaemontana catharinensis* A.DC., *Baccharis crispa* Spreng., *Cecropia pachystachya* Trécul. and *Acanthospermum australe* (Loefl.) Kuntze did not show antibacterial activity. Thus, following assays were conducted only with aqueous and ethanolic extracts of *Eugenia uniflora* L.

Table 1. Inhibition zone diameters and relative inhibitory effect percentages for ethanolic and aqueous *Eugenia uniflora* L. extracts by agar diffusion assay against *Streptococcus agalactiae* ATCC® BAA-611™ and *Streptococcus agalactiae* ATCC® 51487™.

***Eugenia uniflora* L. extract concentrations [mg]**	***Streptococcus agalactiae* ATCC® BAA-611™**				***Streptococcus agalactiae* ATCC® 514871™**			
	EE		**AE**		**EE**		**AE**	
	Inhibition zone diameters [mm]	**Relative inhibitory effect [%]**	**Inhibition zone diameters [mm]**	**Relative inhibitory effect [%]**	**Inhibition zone diameters [mm]**	**Relative inhibitory effect [%]**	**Inhibition zone diameters [mm]**	**Relative inhibitory effect [%]**
0.5	9.0 ± 0.0	28.12	9.0 ± 0.0	28.12	9.0 ± 0.0	28.12	9.0 ± 0.0	28.90
1	10.66 ± 0.57	33.33	11.0 ± 0.0	34.37	10.66 ± 0.57	33.33	11.33 ± 0.57	35.41
5	12.0 ± 0.0	37.5	11.66 ± 0.57	36.45	12.33 ± 0.57	38.54	12.33 ± 0.57	38.54
10	13.33 ± 0.57	41.66	13.33 ± 0.57	41.66	14.0 ± 0.0	43.75	13.66 ± 0.57	42.70
15	14.66 ± 0.57	45.83	14.0 ± 0.0	43.75	15.33 ± 0.57	47.91	15.0 ± 0.0	46.87

EE – Ethanolic extract; AE – Aqueous extract.

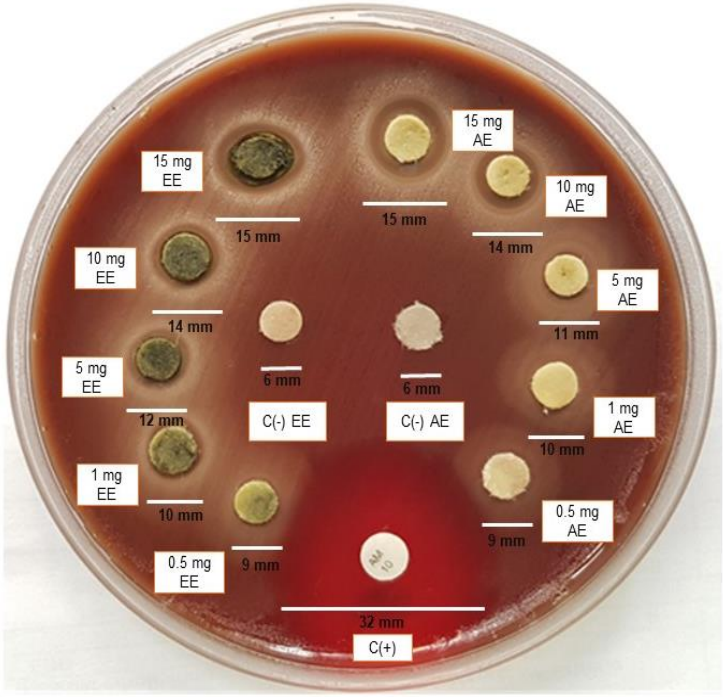

Fig. 1. Minimal Inhibitory Dose (MID) – Agar diffusion assay of aqueous and ethanolic extract of *Eugenia uniflora* L. AE – Aqueous extract; EE – Ethanolic extract; C (+)– Positive control; C (-) AE – Negative control for the aqueous extract; C (-) EE – Negative control for the ethanolic extract; mm – millimeter.

MIC and MBC assays, and MBC / MIC ratio

MIC and MBC obtained for ethanolic and aqueous *Eugenia uniflora* L. extracts were 1 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$, respectively. The MBC/MIC ratio was 5 for both extracts. The action of extracts was bacteriostatic according to Shanmughapriya *et al.* (2008) criteria.

Induction of resistance assay

The MIC value was 1 $mg.mL^{-1}$ after GBS exposure to sub MIC extract concentrations, suggesting that the microorganism would not develop resistance to the active principles of *Eugenia uniflora* L.

Drug synergism assay between extracts and commercial antibiotics

Drug synergism trial showed indifference by the double-disc method.

Toxicity assay

LD_{50} obtained for the aqueous extract was 0.82 $mg.mL^{1}$, indicating moderate toxicity. The larval control group maintained 100 % viability and showed no behavioral changes during the test.

Discussion

The results of current research revealed the antibacterial activity of *Eugenia uniflora* L. against *Streptococcus agalactiae* ATCC® BAA-611™ and *Streptococcus agalactiae* ATCC® 51487™. *Psidium guajava* L., *Tabernaemontana catharinensis* A.DC., *Baccharis crispa* Spreng., *Cecropia pachystachya* Trécul., and *Acanthospermum australe* (Loefl.) Kuntze did not show any antibacterial activity at the different concentrations assayed.

Psidium guajava L. is used in the treatment of diarrhea, dysentery, menstrual disorders, vertigo, anorexia, digestive problems, gastric insufficiency, inflamed mucous membrane, laryngitis, skin problems, ulcers, vaginal discharge, and cough (Díaz de Cerio *et al.* 2017). Silva *et al.* (2016) informed a low activity of aqueous and ethanolic extracts of *Psidium guajava* L. against GBS but high on other bacterial species. Sivananthan *et al.* (2013) mentioned that *Psidium guajava* L. leaves extract showed antibacterial activity against *Staphylococcus aureus* and *Streptococcus agalactiae*. But the solvent used for the extraction procedure was chloroform. Other authors informed the activity of ethanolic and aqueous P*sidium guajava* L. extract against *Escherichia coli*, *Pseudomonas aeruginosa*, *Saphylococcus aureus*, *Klebsiella pneumoniae* y *Streptococcus pneumoniae* (Ifeanyichukwu *et al.* 2015; Kenneth *et al.* 2017).

The genus *Tabernaemontana* has an important biological activity for the treatment and prevention of diseases, such as sore throat, hypertension, abdominal pain, and pulmonary disease (Naidoo *et al.* 2021). Richard *et al.* (2021) suggested that the crude latex of *Tabernaemontana catharinensis* A. DC. displays an antimicrobial effect against *Alicyclobacillus*, with potential for application in the food industry. Goncalves *et al.* (2011) informed the in vitro antimicrobial activity of the *Tabernaemontana catharinensis* A. DC. extract against *Staphyloccocus aureus* and *Pseudomonas aeruginosa*. However, no similar studies were found with GBS.

Baccharis crispa Spreng. is used in infusion or decoction, as hepatic, diuretic, as a drying agent of ulcers and antiseptic in external use (Rodriguez *et al.* 2008). Palacios *et al.* (1983) informed antibacterial activity of *Bacharis crispa* Spreng. against *Bacillus subtilis* and *Micrococcus luteus* but no activity against *Staphylococcus aureus*. No similar studies against GBS were reported.

Cecropia pachystachya Trécul. is used in traditional medicine to treat respiratory disorders, renal diseases, and for its anti-inflammatory, diuretic, anti-hypertensive, and anti-diabetic properties, antidepressant-like, cardiotonic, sedative, and antimalarial effects (Machado *et al.* 2021). de Andrade *et al.* (2021) informed antibacterial activity of ethanolic extract of *Cecropia pachystachya* Trécul. against *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25925, *Streptococcus pyogenes* 1033B with halos that ranged from 12 mm to 18 mm.

Acanthospermum australe (Loefl.) Kuntze is used in folk medicine for the treatment of various

conditions such as diarrhea, skin diseases, blennorrhagia, dyspepsia, parasitic worms, and malaria. Mallman *et al*. (2018) studied the efficacy of aqueous and hydroalcoholic extracts of *Acanthospermum australe* (Loefl.) Kuntze against diarrhea-inducing bacteria. The hydroalcoholic root extract was unique in presenting a bactericidal effect against *Shigella dysenteriae*. None of the extracts showed bacteriostatic or bactericidal activities against *Yersinia enterocolitica* and *Enterococcus faecalis*. Similar works with extracts of *Cecropia pachystachya* Trécul. and *Acanthospermum australe* (Loefl.) Kuntze against GBS strains were not reported.

Eugenia uniflora L. is often used in folk medicine as antidiarrheal, antihypertensive, antirheumatic, anti-inflammatory, respiratory disorders, digestive disorders, and numerous infections (de Souza *et al*. 2018). Several authors reported the activity of this plant against Gram-positive and Gram-negative bacteria. Falcão *et al*. (2018) demonstrated activity against *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* INCQS 00016, *Enterococcus faecalis* ATCC 29212, *Salmonella enteritidis* INCQS 00258, and *Pseudomonas aeruginosa* ATCC. However, the extract did not show activity against methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* ATCC 25922. Also, Victoria *et al*. (2012) obtained good activity of the essential oil of the leaves of *Eugenia uniflora* L. against *Staphylococcus aureus* and *Listeria monocytogenes*. Oliveira *et al*. (2008) worked with the lectin from *Eugenia uniflora* L., obtaining antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella* sp. Although antibacterial activity has been reported for this plant, there are no published data against GBS so far.

The antibacterial action of a plant extract is considered high when its percentage of relative inhibition is > 70 %, intermediate between 50 and 70 %, and low when it is 50 % (Cruz-Carrillo *et al*. 2010). For *Eugenia uniflora* L. extracts, it was observed a relative inhibition percentage of less than 50 %, indicating low antibacterial activity. However, secondary metabolites with antibacterial activity are often found at low concentrations proportionally in the weight of dry extracts (Compean *et al*. 2005). So, the extract may contain significant antimicrobial activity but in concentrations that are not high enough to inhibit bacterial growth considerably.

According to the criteria of Avellaneda *et al*. (2005) a bacterial strain has a high susceptibility when the tested substance has a MIC less than 12.5 $mg.mL^{-1}$, moderate susceptibility, between 12.5 and 50 $mg.mL^{-1}$, and low susceptibility when the MIC is between 50 and 100 $mg.mL^{-1}$. During MIC evaluation, the growth inhibition was obtained with both *Eugenia uniflora* L. extracts to a lower concentration than 12.5 $mg.mL^{-1}$. These results indicate high activity of *Eugenia uniflora* L. extracts against the GBS strains studied. MIC is considered the fundamental parameter for comparing the bacterium susceptibility against an antibacterial (Struthers *et al*. 2005). It is the most reliable technique to determine substance antimicrobial properties. In Brazil, Lazzarotto-Figueiró *et al*. (2021) reported a MIC value of 0.87 $mg.mL^{-1}$ for the ethanolic extract of *Eugenia uniflora* L. leaves when testing against *Staphylococcus aureus* strains. They obtained higher MIC values when testing with Gram-negative bacilli (5 $mg.mL^{-1}$ for *Escherichia coli*, 20 $mg.mL^{-1}$ for *Pseudomonas aeruginosa*, and 10 $mg.mL^{-1}$ for *Salmonella typhymurium*). Borges Monteiro *et al*. (2019) obtained a MIC value of 0.128 $mg.mL^{-1}$, but they worked with methanolic extracts of *Eugenia uniflora* L. leaves and *H. pylori* strains. In this study, MBC values were lower than 20 $mg.mL^{-1}$ with both extracts, which indicates a high activity against GBS, according to the criteria of Avellaneda *et al*. (2005). The MBC/MIC ratio was 5 for both extracts, indicating the bacteriostatic extract capacity according to the criteria of Shanmughapriya *et al*. (2008).

Medicinal plant extracts with intrinsic antimicrobial properties effectively prevent or reduce antimicrobial resistance. However, a combined approach that allows for a drug synergism between plant extracts and conventional antibiotics is possibly the most effective method of combating antibacterial resistance (Inui *et al*. 2007; Cheesman *et al*. 2017). In addition, the drug synergy between the bioactive plant product and antibiotics can prevent problems of toxicity or overdose, as two lower concentrations of agents that are combined in the treatment are required. In

this study, we did not observe drug synergy or inhibition effects between *Eugenia uniflora* L. ethanolic and aqueous extracts and commercial antibiotics tested against GBS strains. Coutinho *et al*. (2010) demonstrated a pharmacological synergism between the *Eugenia uniflora* L. ethanolic extract and amikacin, gentamicin, kanamycin, neomycin, and tobramycin when testing against clinical isolates of *Staphylococcus aureus*. However, the clinical isolates of *Escherichia coli* did not show pharmacological synergism with this extract and antibiotics.

The exposure of a bacterial population to the antimicrobial action usually produces a deleterious effect, either by inhibiting its growth or producing its death. This effect is not always observed due to the emergence of resistance mechanisms or the selection of resistant mutants (Meyer *et al*. 1982). When determining MIC after exposing GBS to sub-MIC concentrations of aqueous and ethanolic *Eugenia uniflora* L. extracts for ten consecutive days, results revealed no change in value. Therefore, GBS would not be expected to develop resistance to the active substance in the extract during treatment.

It is also necessary to evaluate the toxicity of a product which can limit its benefit (Santos *et al*. 2013). The toxicity criteria used were established by Leos-Rivas *et al*. (2016), where LD_{50}> 1 $mg.mL^{-1}$ represents low toxicity, ≥ 0.5 and ≤ 1 $mg.mL^{-1}$ moderately toxic, and < 0.2 $mg.mL^{-1}$ high toxicity. LD_{50} is defined as the substance concentration that causes 50 % of individuals to die in a study population. For LD_{50} determination, there are different methods using cell lines, laboratory animals, or *Artemia salina* larvae. The latter is fast, economical, and simple. It does not require any special equipment or training and uses a relatively small test sample (Leos-Rivas *et al*. 2016). Our research group have had previously determined that the toxicity of the ethanolic extract of *Eugenia uniflora* L. is moderate [LD_{50} 0.61 (0.51 – 0.74) $mg.mL^{-1}$] (Bobadilla *et al*. 2018). In this work, the LD_{50} value of the aqueous extract of *Eugenia uniflora* L. was 0.82 $mg.mL^{-1}$ (0.76 – 0.90 $mg.mL^{-1}$), indicating also moderate toxicity. Arcanjo *et al*. (2012) obtained an LD_{50} value of 288.46 $\mu g.mL^{-1}$ (194.24 – 433.67 $\mu g.mL^{-1}$) for the ethanolic extract of *Eugenia uniflora* L. in their tests with *Artemia salina*. However, toxicity studies carried out with this plant present wide methodological variations, making it difficult to compare the observed biological effects.

The emergence of a GBS clone with zoonotic potential produced a sepsis outbreak in humans through raw fish consumption in Singapore (Kalimuddin *et al*. 2017). The application of antibiotics and chemotherapeutics to control infectious diseases in aquaculture eradicates microflora and emerges the resistant bacteria and accumulates residues in the human body (WHO 2006). Challenges in treating infectious diseases in humans and fish have increased due to the resistance emergence. So, it is necessary to search for alternatives for antibacterial therapy development. Natural products are a good option as they are less toxic and have fewer adverse effects than synthetic products. So, the present study expands the knowledge of natural antibacterial options existing in the region.

Conclusion

Aqueous and ethanolic extracts of *Eugenia uniflora* L. have antibacterial activity against GBS of human and fish origin. These do not exhibit drug synergism with commercial antibiotics, the intra-treatment resistance would not develop, and have moderate toxicity. Aqueous and ethanolic extracts from *Psidium guajava* L., *Tabernaemontana catharinensis* A.DC., *Baccharis crispa* Spreng., *Cecropia pachystachya* Trécul. and *Acanthospermum australe* (Loefl.) Kuntze did not show antibacterial activity against GBS. More studies are required with other solvents for secondary metabolite extraction from these plant species. This work is a first step to identify chemical compounds in native medicinal plants of Misiones, Argentina, that could mean an alternative for the treatment of *Streptococcus agalactiae* infections.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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