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Antibacterial activity of five Peruvian medicinal plants against *Pseudomonas aeruginosa*

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ABSTRACT

Objective: To evaluate the susceptibility of *Pseudomonas aeruginosa* (*P. aeruginosa*) *in vitro* to the ethanolic extracts obtained from five different Peruvian medicinal plants.
Methods: The plants were chopped and soaked in absolute ethanol (1:2, w/v). The antibacterial activity of compounds against *P. aeruginosa* was evaluated using the cup-plate agar diffusion method.
Results: The extracts from *Maytenus macrocarpa* ("Chuchuhuasi"), *Dracontium loretense* Krause ("Jergon Sacha"), *Tabebuia impetiginosa* ("Tahuari"), *Eucalyptus camaldulensis* Dehn (eucalyptus), *Uncaria tomentosa* ("Uña de gato") exhibited favorable antibacterial activity against *P. aeruginosa*. The inhibitory effect of the extracts on the strains of *P. aeruginosa* tested demonstrated that *Tabebuia impetiginosa* and *Maytenus macrocarpa* possess higher antibacterial activity.
Conclusions: The results of the present study scientifically validate the inhibitory capacity of the five medicinal plants attributed by their common use in folk medicine and contribute towards the development of new treatment options based on natural products.

1. Introduction

In 2014, the World Health Organization released its first report on surveillance of antimicrobial resistance, revealing that this is an increasing global threat and putting our capacity to treat common nosocomial or community-acquired infection at risk [1].

This growing problem was characterized by the infectious diseases caused by multidrug-resistant Gram-negative bacteria that challenge the public health policies worldwide at the point of being known as the ESKAPE pathogens [*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacter* spp.]. This term represents their escape from the effects of the antibacterial agents or the nonexistence of newer and more effective antibiotics [2].

P. aeruginosa is the most important toxigenic pathogen within the genus *Pseudomonas* because of the quantity and types of invasive infections it produces, as well as the noteworthy morbidity and mortality associated [3]. This Gram-negative bacterium has the ability to survive in adverse environments and develop multiple antibiotic resistance mechanisms. Among them, the most representative are the expression of chromosomal-encoded AmpC β-lactamase, the reduction of porin channels, the production of extended-spectrum β-lactamase and the mutation of topoisomerase II and IV [4]. It must be considered that several resistant mechanisms can coexist in one strain and just one of them can be effective against numerous antimicrobials [5].

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The resistant mechanisms in *P. aeruginosa* are related to enhancement of the mortality rate of patients infected with this pathogen [6]. Furthermore, this rate is higher among patients infected with resistant strains and received inappropriate initial empirical treatment [4]. Additionally, the rising indiscriminate use of antimicrobials in health centers or by people who practice self-medication could lead susceptible patients to get infected by multidrug-resistant microorganisms [7,8]. The emergence of antibiotic resistance and related toxicity issues limit the use of these drugs, and generate a renaissance in phytotherapy research [9]. To address this challenge, there is growing interest in identifying and evaluating antimicrobial compounds in extracts of medicinal plants as a new source of drugs and alternative treatment approach [10].

Of all the regions in the world with a diverse flora that can naturally provide medicinal plants, Peru is a privileged one. It possesses approximately 20 000 plant species, which is equivalent to 8% of the total number of plants in the world. Most of them are native plants or grow in the Peruvian Amazon. Nevertheless, probably less than 1% of the species have been studied to determine their phytochemicals with potential medicinal value [11,12].

The aim of present study was to evaluate the antibacterial capacity of five traditionally used Peruvian plants against *P. aeruginosa* in order to validate scientifically the inhibitory activity attributed by their popular use and to propose new sources of antimicrobial agents.

2. Materials and methods

2.1. Collection of plant materials

The plants *Maytenus macrocarpa* (*M. macrocarpa*) (common name: "Chuchuhuasi"), *Dracontium loretense* Krause (*D. loretense*) (common name: "Jergon Sacha"), *Tabebuia impetiginosa* (*T. impetiginosa*) (common name: "Tahuari"), *Eucalyptus camaldulensis* Dehn (*E. camaldulensis*) (common name: eucalyptus), *Uncaria tomentosa* (*U. tomentosa*) (common name: "Uña de gato") and *Allium sativum* (*A. sativum*) (common name: garlic) used in this study were purchased from naturist houses and six of them had sanitary registration.

2.2. Preparation of plant extracts

The plants were chopped and soaked in absolute ethanol (1:2, w/v) under shade for 10 days at room temperature. The mixtures were filtered through a Whatman No. 4 filter paper and the filtrates were evaporated at 50 °C [13]. All extracts were stored at 4 °C until use.

2.3. Bacterial test strain and growth conditions

For this study, a strain of *P. aeruginosa* (ATCC 55925) was used and provided by the Microbiology Laboratory of the Institute of Nutritional Research. The cultivation medium was trypticase soy agar (trypticase soy broth) (Oxoid, Hampshire, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37 °C. For antibacterial activity assay, three or four isolated colonies were inoculated in 3 mL of brain heart infusion (BHI) broth and incubated without agitation for 24 h at 37 °C. The cultures were later diluted with fresh medium to approximate the density of 0.5 McFarland standard, which represented an estimated concentration of 1.5×10^8 CFU/mL.

The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the concentration specified before [14].

2.4. Antibacterial screening of the ethanolic extracts

2.4.1. Determination of antibacterial activity

To determine the antibacterial activity of studied extracts, the cup-plate agar diffusion method was used [15]. BHI agar was autoclaved for 15 min at 121 °C and cooled to about 40–42 °C. The medium was then inoculated with 0.1 mL of the prepared bacterial suspension, mixed gently and finally poured into sterile Petri dishes. These agar plates were incubated under sterile conditions for 8 h at room temperature. Three wells per plate of 6 mm in diameter and 4 mm in depth were made with the help of a sterile cork borer, preserving a distance of 3 cm between them. The wells were filled with 100 μL of the corresponding ethanolic extract. The extract from *A. sativum* (control of the extraction process) and ciprofloxacin (32 g/mL) (commercial control) were used as positive controls [8,16]. The Petri dishes were incubated under the same growth conditions as mentioned above. At the end of the period, the inhibition zones formed were measured in millimeters using the vernier. The inhibition zones less than 12 mm in diameter were not considered for the antibacterial activity analysis. For each extract, 12 replicates were assayed.

2.4.2. Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum bacteriostatic concentration (MBSC)

The MIC was determined using the microdilution method as described by Jayaraman *et al.* [17] and Clinical and Laboratory Standards Institute [18]. Serial two-fold dilutions of all the extracts were prepared with sterile saline in a 96-well microtiter plate, obtaining a concentration range from 100 to 1.56 mg/mL. Then, 5 μL of *P. aeruginosa* suspension (optical density at 550 nm = 0.6) were added to the wells containing the dilutions. Each dose was assayed in quadruplicate. Uninoculated wells containing sterile saline or saline and extract were used as controls. After incubation for 24 h at 37 °C, the samples were observed. MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity.

To estimate the MBSC, an aliquot of each well that did not show microbial growth in the prior tests was swabbed on the entire surface of BHI agar plates and then incubated under the growth conditions described before. Subsequently, the lowest concentration of extract at which there was growth after subculturing was considered as the MBSC. In contrast, the lowest concentration that prevented the bacterial growth was registered as MBC.

2.5. Statistical analysis

All data were analyzed using SPSS package (version 21.0.0) for statistical analysis. For further inferential statistical analysis, Levene's and Welch's tests were carried out.

3. Results

3.1. Antibacterial activity of the plant extracts

The ability of the selected ethanolic plant extracts to inhibit *P. aeruginosa* growth was determined in this study. The results revealed that 5 extracts exert antibacterial activity against this microorganism. However, the extracts from *M. macrocarpa* and *T. impetiginosa* exhibited higher inhibitory activity ($P \leq 0.05$) than the extracts from *U. tomentosa*, *E. camaldulensis* and *D. loretense*. Maximum *in vitro* inhibition was scored by *M. macrocarpa*, followed by *T. impetiginosa*, and *U. tomentosa*, *E. camaldulensis* and *D. loretense*, which presented inhibition zones of (22.47 ± 5.22) mm, (20.83 ± 3.03) mm, (19.32 ± 2.81) mm, (18.20 ± 3.45) mm and (17.21 ± 2.76) mm, respectively. In the case of the positive controls, ciprofloxacin and *A. sativum* extract possess a clear anti-*P. aeruginosa* effect [16], which presented inhibition zones of (25.89 ± 0.52) mm and (22.56 ± 1.94) mm respectively.

3.2. Minimum inhibitory and bacteriostatic concentration

MIC values ranged from 25 to 50 mg/mL. The ethanolic extracts from *T. impetiginosa* displayed the minimum activity against the evaluated bacteria strain with a MIC value of 50 mg/mL. In the case of *M. macrocarpa*, *D. loretense*, *U. tomentosa* and *E. camaldulensis*, the MIC value was 25 mg/mL, the same as the one scored by *A. sativum* (positive control).

As for MBC and MBSC, *M. macrocarpa*, *D. loretense* and *T. impetiginosa* showed a bacteriostatic action at a concentration of 75 mg/mL, the same as MBSC of *A. sativum* (positive control). While for the case of *U. tomentosa* and *E. camaldulensis*, MBSC is 100 and 50 mg/mL, respectively. In contrast, a bactericidal action was observed at a concentration of 50 mg/mL in the case of *M. macrocarpa*, *D. loretense*, *T. impetiginosa* and *U. tomentosa*, which was the same as that of *A. sativum* (positive control). While in the case of *E. camaldulensis* MBC is 25 mg/mL.

4. Discussion

After the emergence of multi-drugs resistant pathogens, the research for new remedy alternatives has led to the recognition of the potential of medicinal plant extracts for treating the infections associated to these type of microorganisms [19,20]. Moreover, there is a synergistic effect of antimicrobial plant extracts with commonly used antibiotics; this effect has become the foundation of a multitargeted approach used against multi-drugs resistant bacteria [21–23].

Among the 6 major pathogens to which newer antimicrobials are urgently required, *P. aeruginosa* is recognized as the etiological agent of several community- and healthcare-associated bacterial infections difficult to eradicate [19]. This Gram-negative bacterium has developed strains with remarkable survival, disseminations and resistant mechanisms to the first election antibiotics such as β-lactam because its outer membrane functions as a barrier to several substances [24].

Therefore, this study aimed to determinate the antimicrobial activity of five endemic plants commonly used in traditional medicine in the Amazon and sierra regions of Peru in order to validate scientifically their therapeutic properties [19]. The use of these plants in Peruvian Amazon folk medicinal remedies for treating various health problems has already been reported, and the plants have been tested as antirheumatic, antidiarrheic, anticancer, antidiuretic and tonic, antidiabetic and anti-inflammatory, and proposed as novel therapy alternatives against this high-level resistant bacterium [12].

By cup-plate agar diffusion method, the ethanolic extracts from *M. macrocarpa*, *D. loretense*, *T. impetiginosa*, *E. camaldulensis* and *U. tomentosa* showed anti-*P. aeruginosa* activity, evidencing that ethanol is an efficient organic solvent to be used for the extraction of bioactive plant materials. Many of the compounds with antibiotic efficacy identified in plants are saturated organic molecules or aromatic substances accumulated as secondary metabolites in all plant cells to which ethanol is a suitable solvent [25]. This information is important because, according to Romero *et al.* [26], water is usually the main solvent used by traditional healers to prepare plant extracts, an election solvent that can affect the antibacterial activity of the analyzed extracts. The microbial growth inhibition capacity relies on the rich variety of phytochemicals including flavonoids, tannins, coumarins, terpenoids, triterpenes, alkaloids and vegetable oils (such as eucalyptol), and all compounds can be easily isolated by ethanol, which is a suitable solvent for the extraction of bioactive plant materials [27,28]. In fact, it is important to emphasize that traditional healers can easily use alcohol or aguardiente to prepare the macerations employed in phytotherapy. Many of these remedies are used to treat conditions that commonly result from bacterial infections of the gastrointestinal and urinary tracts and in wound infections [4].

Though all the ethanolic extracts possess similar antibacterial efficacy, our results demonstrated that MIC values are varied, leading to conclude that the active principle exerting the bacterial inhibition activity is different in each analyzed plant or that the potent substance can be the same but the concentration presented in each plant varies [11,16,29].

The results of the present study reinforce the importance of the analyzed plants as a source of bioactive compounds for the treatment of *P. aeruginosa* related infectious diseases. Similar results were described by Mishra and Padhy [20], Yildirim *et al.* [30], Radji *et al.* [9] and Zhang *et al.* [29] by using extracts from Indian timber-yielding plants, Turkey medicinal plants, green tea, and traditional Chinese medicinal herbs, respectively. Nevertheless, their antibacterial ability was less than that reported in our study.

Further chemical analysis of the aforementioned plant extracts should be performed to determinate their chemical composition and identify the exact phytocompounds responsible for antimicrobial activity. In addition, they should be subjected to pharmacological evaluations with the aim of assessing their *in vivo* efficacy, toxicity, potential adverse effects, interactions and contraindications.

The results proved that the extracts from *M. macrocarpa*, *D. loretense*, *T. impetiginosa*, *E. camaldulensis* and *U. tomentosa* exhibit a favorable antibacterial activity against *P. aeruginosa*. These validate scientifically their inhibitory capacity attributed by their common use in folk medicine and contribute towards the development of new treatment options based on natural products.

Conflict of interest statement

We declare that we have no conflict of interest.

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