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## Article

# Antibacterial and Antibiofilm Potential of Ethanolic Extracts of *Duguetia vallicola* (Annonaceae) against in-Hospital Isolates of *Pseudomonas aeruginosa*

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogen, especially dominant in people with cystic fibrosis; the drug resistance expressed by this pathogen and its capacity for adaptation generate a great challenge for its treatment and control, increasing morbidity and mortality rates globally. In this sense, the search for new treatment alternatives is imminent today, with products of plant origin being an excellent alternative to be used. The objective of this research was to evaluate the antibacterial and antibiofilm potential and to explore the possible effect of ethanolic extracts of wood and bark of *Duguetia vallicola* on the cell membrane. Microdilution assays showed inhibition of bacterial growth by more than 50%, with the lowest concentration evaluated (62.5 µg/mL) of both extracts. Furthermore, we report the ability of both extracts to inhibit mature biofilms, with inhibition percentages between 48.4% and 93.7%. Intracellular material leakage experiments (260/280 nm), extracellular pH measurement, and fluorescence microscopy with acridine orange (AO) and ethidium bromide (EB) showed cell membrane damage. This indicates that the antibacterial action of ethanolic extracts of *D. vallicola* is associated with damage to the integrity of the cell membrane and consequent death of these pathogens. These results serve as a reference for future studies in establishing the mechanisms of action of these extracts.

**Keywords:** Ethanolic extracts; *Duguetia vallicola*; antibacterial activity; antibiofilm; *Pseudomonas aeruginosa*

## 1. Introduction

*Pseudomonas aeruginosa* is a gram-negative pathogen that causes illness and death in people with immunosuppressive and chronic conditions [1,2]. It is dominant in people with cystic fibrosis [3–5], burns or who have acquired this pathogen at the surgical site, causing coinfections and generalized sepsis [6]; its high intrinsic resistance to antiseptics and antibiotics [7,8], make it a pathogen of great clinical relevance due to the high rates of morbidity and mortality, especially in immunocompromised people [9]. According to the World Health Organization (WHO), these pathogens represent between 7 and 12% of isolates in healthcare-associated infections (HAIs) [10]. The pathogenicity, adaptability and flexibility of *P. aeruginosa* is due to the expression of various virulence factors [1,3,7], highlighting the production of powerful biofilms [4,11], that facilitate their multi-drug resistance and evasion of the immune system [12]. Thus, *P. aeruginosa* infections represent a serious public health problem at a global level [13]. Therefore, the search for therapeutic alternatives that help mitigate the problem generated by this pathogen is important today.

In this context, compounds of natural origin, especially plants, are an excellent alternative to be used, since they are an invaluable source of metabolites with recognized medicinal properties [14,15]. Compounds obtained from members of the Annonaceae family are well known for their outstanding biological activity [16–21]. The genus *Duguetia* is one of the most abundant of this family, from which various compounds have been obtained that include: terpenes, lignans, flavonoids, aromatic compounds and mainly it has been documented with a high content of alkaloids [22]. Alkaloids have been widely reported for their important biological activities such as: antitumor [23,24],

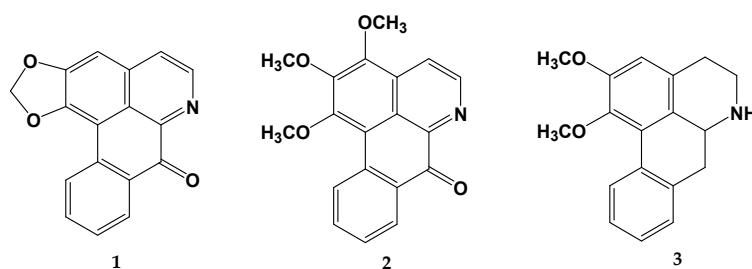
antimicrobials [25–28], trypanocidal, leishmanicidal [23], antiplasmodial [17], antivirals [29], antioxidant, anti-inflammatory and antinociceptive, among other [30–33].

Particularly, alkaloids such as N-methylaurotetanine and its O-methylated and O-acetylated derivatives have been isolated and characterized from *Duguetia vallicola*, which have been documented with antioxidant activity [30]; glaziovine, with neuropharmacological properties [34] and isoquinoline alkaloids (duguevalline, cleistopholine, O-methylmoschatoline, (-)-oliveroline and (-)-oliveridine) with antiplasmodial activity against *Plasmodium falciparum* [17]. However, there are no reports on the antibacterial potential of *D. vallicola* extracts against clinical isolates of *P. aeruginosa*. We hypothesize that its bark and wood extracts have an antibacterial effect against *P. aeruginosa*. This research aimed to evaluate the antibacterial and antibiofilm potential of ethanolic extracts of wood and bark of *D. vallicola*, as well as to explore its mechanism of action, targeting the cell membrane. The results of this research contribute to the search for molecules and compounds of plant origin with antimicrobial potential against *P. aeruginosa*.

## 2. Results

### 2.1. Identification of Alkaloids in the Wood Extract.

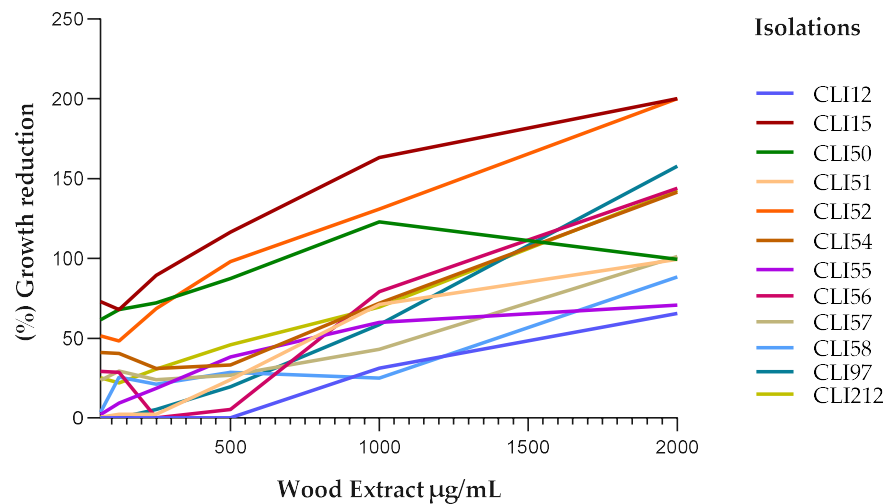
Two alkaloids with an oxoaporphin nucleus known as liriodenine (**1**) and O-methylmoschatoline (**2**) were obtained from the *D. vallicola* wood extract, along with an aporphinic alkaloid known as nornuciferine (**3**). Alkaloids **1** and **3** are reported for the first time in *D. vallicola*. Their structures (Figure 1), were established by comparison of their physical and spectral data with those published in the literature. The <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, HMQC and HMBC data for compounds **1**, **2** and **3** are presented in Tables S1, S2 and S3, respectively. The <sup>1</sup>H-NMR spectra are presented in Figures S1, S2, S3, S4 and S5.



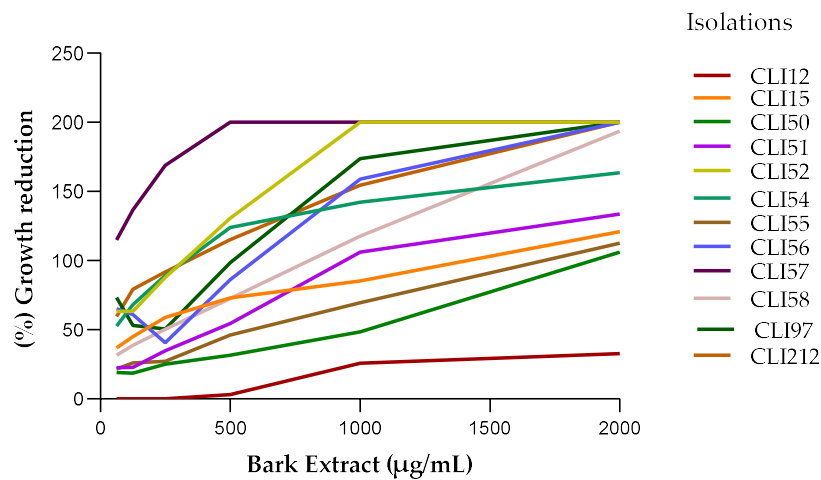
**Figure 1.** Structures of liriodenine (**1**), O-methylmoschatoline (**2**) and nornuciferine (**3**), isolated from *Duguetia vallicola* wood extract.

### 2.2. Antibacterial Susceptibility Testing

The ethanolic extracts of wood and bark of *D. vallicola* showed antibacterial activity against all the clinical isolates of *P. aeruginosa* studied; we observed a reduction in the percentage of growth of bacteria treated with the extracts, compared to the untreated isolates used as a control. Figures 2 and 3 show the similar tendency among isolates to increase the percentage of growth reduction as the concentration of wood and bark extracts increases, respectively. The wood extract, at the lowest concentration evaluated (62.5µg/mL), achieved more than 50% growth inhibition of the CLI15, CLI50 and CLI52 isolates. With the bark extract (62.5µg/mL), more than 50% and 100% growth reduction was achieved in the isolates CLI52, CLI54, CLI56, CLI57, CLI97, CLI212.



**Figure 2.** Reduction in the growth of *P. aeruginosa* isolates exposed to ethanolic wood extract. A positive linear relationship was observed between the concentration of the extract and the percentage of inhibition of bacterial growth. The higher the concentration of the extract, the greater the percentage reduction in bacterial growth; this is consistent with the Pearson correlation coefficient ( $0.84 < r < 0.98$ ) for all isolates. Furthermore, the hypothesis test on the correlation coefficient yields a  $p$  value  $< 0.05$ , which indicates that, with 95% confidence, there is a significant linear relationship.



**Figure 3.** Reduction in growth of *P. aeruginosa* isolates exposed to ethanolic bark extract. A positive linear relationship was observed between the concentration of the extract and the percentage of inhibition of bacterial growth.

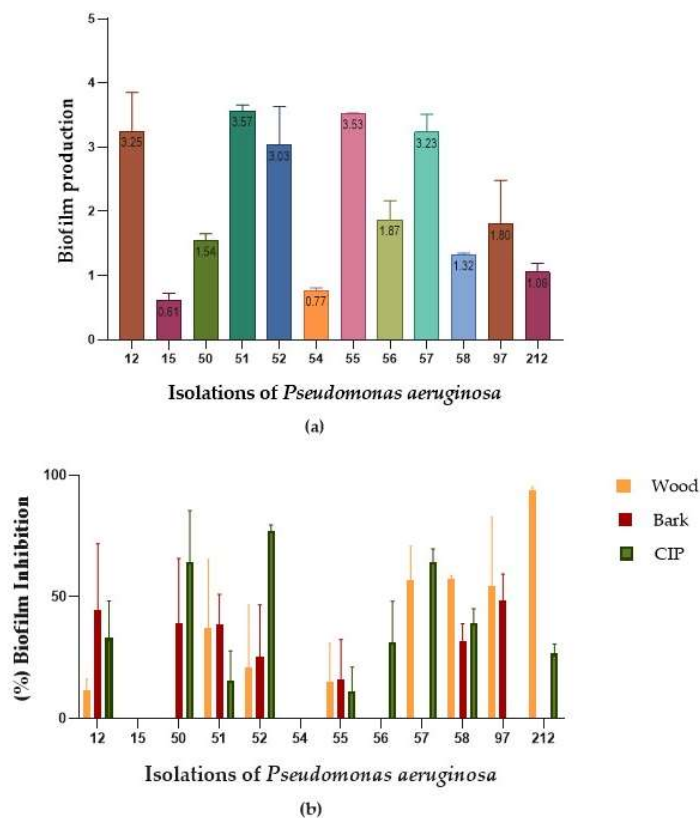
Table 1 shows the MIC<sub>90</sub> values of the extracts for each of the clinical isolates evaluated. The effect of the extracts on the inhibition of *P. aeruginosa* varied between each isolate. The lowest MIC<sub>90</sub> of the wood extracts (180 µg/mL) and bark (62.5 µg/mL) were observed with the isolates CLI15 and CLI57 respectively, while the highest MIC<sub>90</sub> of the wood extracts (3800 µg/mL) and bark (6300 µg/mL), were observed with the CLI12 isolate.

**Table 1.** Minimum inhibitory concentration (MIC<sub>90</sub>) values of ethanolic extracts of wood and bark (µg/mL), against clinical isolates of *P. aeruginosa*..

<i>P. aeruginosa</i>	Wood Extract MIC <sub>90</sub>	Bark Extract MIC <sub>90</sub>
CLI 12	3800	6300
CLI 15	180	1460
CLI 50	500	2200
CLI 51	3000	1600
CLI 52	490	73
CLI 54	1270	300
CLI 55	3120	2000
CLI 56	1480	392
CLI 57	2600	62.5
CLI 58	2790	794
CLI 97	1560	250
CLI 212	1430	100

2.3. Biofilm Reduction

All *P. aeruginosa* isolates produced biofilms in different proportions. The isolates CLI12, CLI51, CLI52, CLI55, and CLI57 were strong producers of biofilms in polystyrene microplates, while the rest of the isolates were weak and moderate producers of biofilms (Figure 4a). The effect of bark and wood extracts against *P. aeruginosa* biofilms varied between each isolate. Table 2 shows the inhibition percentages when the MIC<sub>90</sub> of the extracts was added to the biofilms formed in each isolate; a percentage of biofilm biomass reduction was obtained between 11.5% and 93.7% with the wood extract and between 16.0% and 48.4% with the bark extract (Figure 4b), after 1 h of exposure; while the percentage of biofilm biomass reduction in cells treated with ciprofloxacin (CIP) ranged between 0.00% and 77.19%. The Kruskal Wallis test with a value of  $p < 0.64$  and the Dunnett test ( $0.99 > P < 0.70$ ) for the wood and bark extracts respectively, indicated that there are no statistically significant differences between the effect of CIP and the extracts evaluated. -two in the inhibition of mature biofilms in *P. aeruginosa*.



**Figure 4.** Effect of bark and wood extracts on *P. aeruginosa*. (a) Biofilm formation at 37 °C for 24 hours. (b) Percentage reduction of mature biofilms, after 1 h of treatment with the MIC<sub>90</sub> of each extract and CIP (2µg/mL).

**Table 2.** Percentages of inhibition of mature biofilms of ethanolic extracts of wood and bark *vs.* CIP in *P. aeruginosa*.

<i>P. aeruginosa</i>	Wood	Bark	CIP
CLI12	11.51	44.79	33.03
CLI15	0.00	0.00	0.00
CLI50	0.00	38.97	64.09
CLI51	37.09	38.85	15.49
CLI52	20.91	25.51	77.19
CLI54	0.00	0.00	0.00
CLI55	15.20	16.07	11.28
CLI56	0.00	0.00	31.41
CLI57	56.79	0.00	64.19
CLI58	57.58	31.80	39.07
CLI97	54.31	48.46	0.00
CLI212	93.74	0.00	26.63

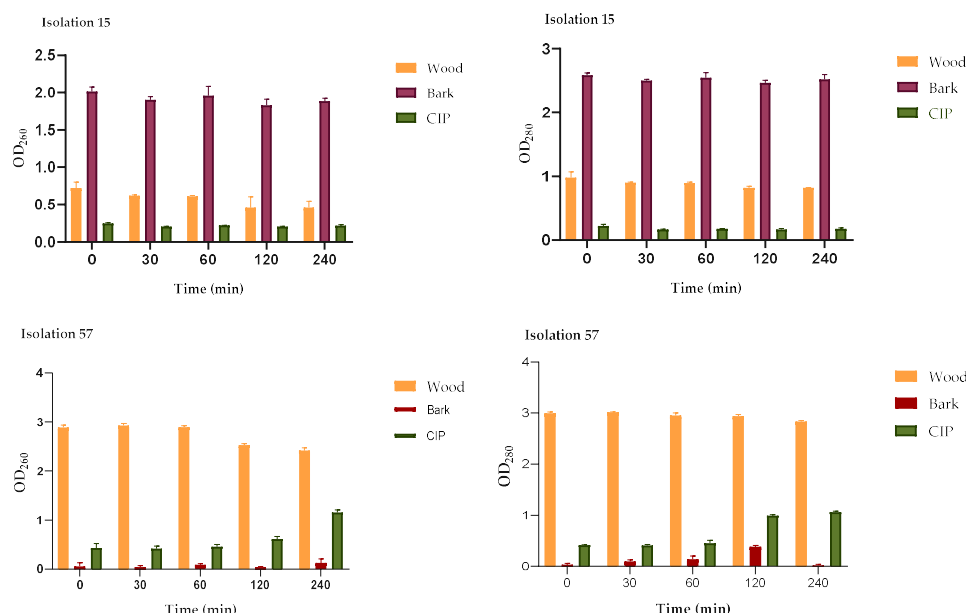
2.4. Effect of Extracts on Cell Membrane Integrity

2.4.1. Leakage of Nucleic Acids and Proteins Through the Cell Membrane

The action of wood and bark extracts on the integrity of *P. aeruginosa* membranes was evaluated by release assays of intracellular constituents that absorb at 260/280 nm, such as nucleic acids and proteins. These tests were carried out at 0, 30, 60, 120 and 240 minutes after treatment with the MIC of the extracts for each isolate. As seen in Figure 5, the effect of the extracts on the integrity of the cell



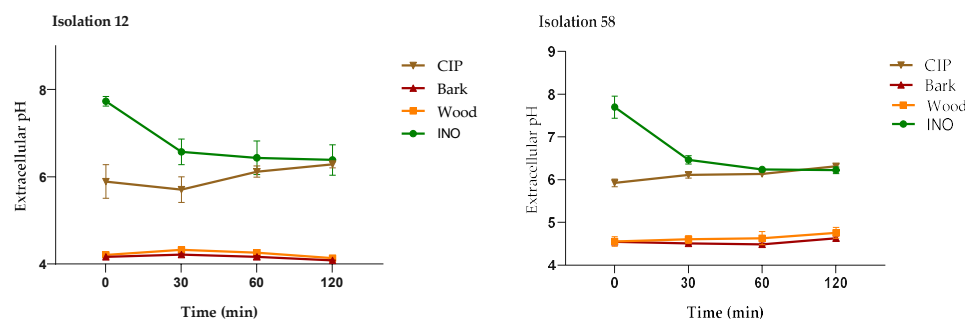
membrane varied between the isolates, but in all cases leakage of intracellular material was observed. In the CLI15 isolate, the  $OD_{260}/OD_{280}$  values in the groups treated with the extracts are significantly higher from time zero, compared to the groups treated with CIP; a greater effect of the bark extract is highlighted, while for the CLI57 isolate a greater effect is observed with the wood extract. These results suggest alteration in the integrity of the cell membrane of *P. aeruginosa* caused by the extracts.

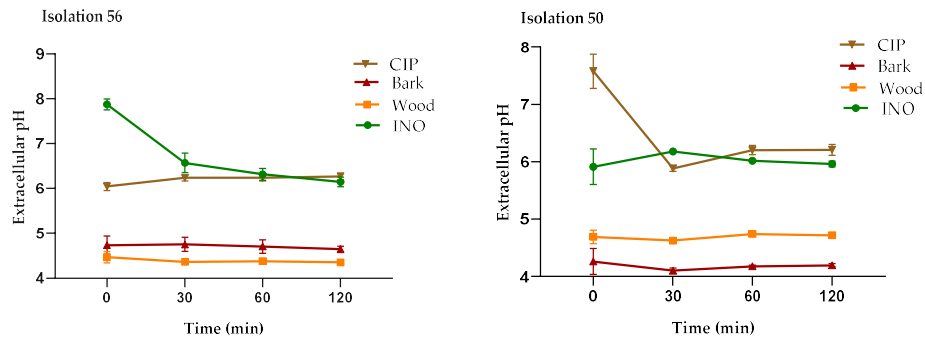


**Figure 5.** Release of intracellular content at 260/280 nm versus time of *P. aeruginosa* treated with wood and bark extracts (MIC  $\mu\text{g/mL}$ ) and CIP (2  $\mu\text{g/mL}$ ). The figure shows the  $OD_{260}/OD_{280}$  values of the CLI15 and CLI57 isolates treated with the extracts and CIP at different times. The results are expressed as the absorbance of the sample (treated with extracts) minus the absorbance of the control (samples without extracts).

#### 2.4.2. Measurement of Extracellular pH

The extracellular pH measurements of *P. aeruginosa* treated with the extracts, CIP and untreated cells are shown in Figure 6. As seen in the four isolates, the cells treated with the MIC of the bark and wood extracts showed a significant decrease in extracellular pH, compared to untreated cells (INO) and cells treated with CIP (2  $\mu\text{g/mL}$ ); this behavior was similar in all *P. aeruginosa* isolates. These results support the alteration in the integrity of the cell membrane caused by treatment with *D. vallicola* extracts.



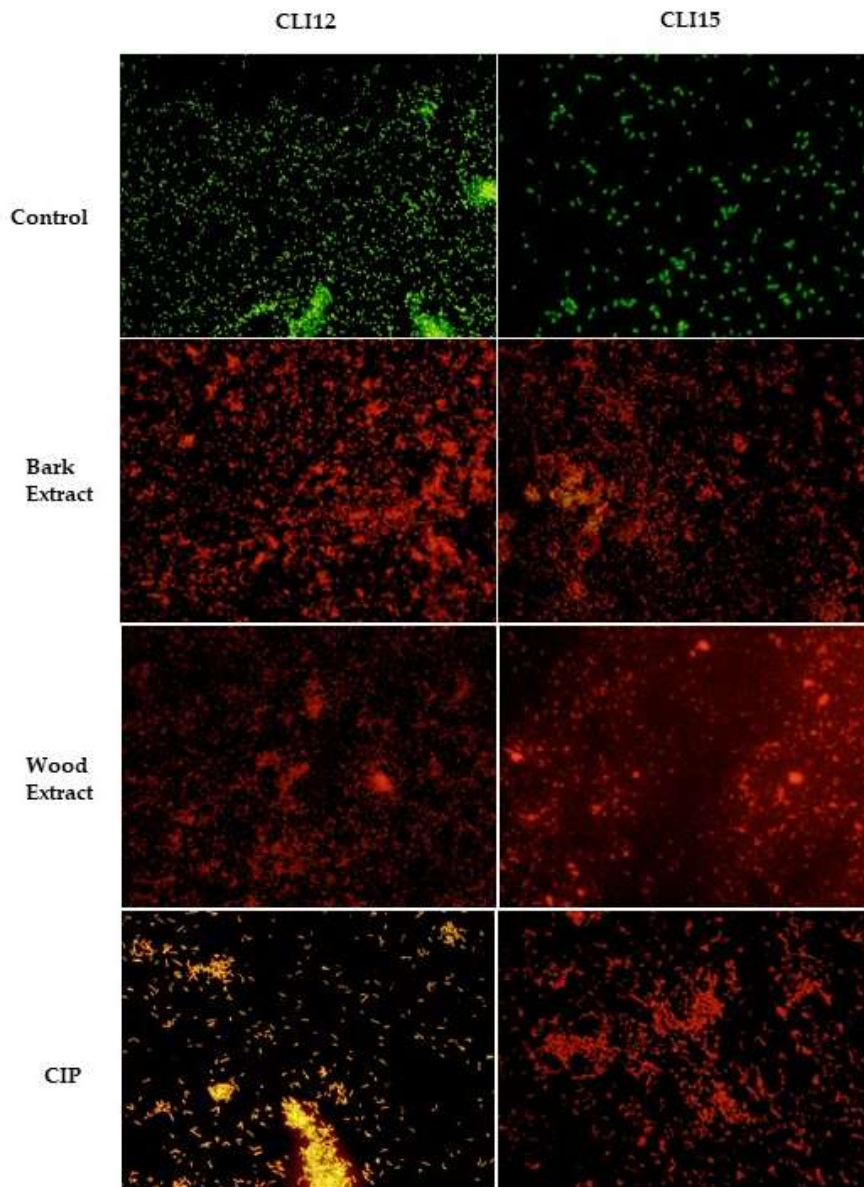


**Figure 6.** Measurement of extracellular pH of *P. aeruginosa* treated with *D. vallicola* extracts, untreated cells (INO) and CIP (2  $\mu\text{g/mL}$ ). The results of the ANOVA indicate that there are significant differences between the extracts compared to the INO control ( $p < 0.0001$ ) for all isolates. In cells treated with CIP, there are no significant differences with respect to cells without treatment (control). The Dunnett test with a confidence level of 95% indicates that there are significant differences between the effect of the extracts on extracellular pH values compared to the effect of CIP.

#### 2.4.3. LIVE/DEAD Assays

Isolates of *P. aeruginosa* treated with the extracts of *D. vallicola*, CIP and without treatment were observed under fluorescence microscopy. Acridine orange (AO) diffuses through intact cytoplasmic membranes in living cells where it interacts with DNA, emitting bright green fluorescence; in contrast, ethidium bromide (EB) penetrates only cells with damaged membranes and cell walls in dead cells, intercalates with DNA and emits orange-red fluorescence [35]. As seen in Figure 7, untreated cells (control), with completely green fluorescence, grew well after 24 h; while dead cells, with red fluorescence, were observed massively in the groups treated with the extracts; similar results to the group treated with CIP.





**Figure 7.** Fluorescence microscopy of *P. aeruginosa* without treatment (control), treated with the bark and wood extracts, and treated with CIP after 24 h. Live cells with intact membranes appear green, while dead cells with damaged membranes appear red and orange.

### 3. Discussion

*Pseudomonas aeruginosa* is a common cause of healthcare-associated infections, particularly pneumonia and in patients with structural lung disease such as cystic fibrosis. The increasing trend of resistance to antimicrobials (including multidrug-resistant (MDR) isolates) in recent years, and the presence of various virulence mechanisms that increase their ability to cause serious infections [36–39], represent a great challenge in the treatment of this pathogen. This awakens our interest in the search for compounds and new therapeutic options for the control of *P. aeruginosa*, with products of plant origin being an excellent alternative.

In this research, we report for the first time the antibacterial activity of ethanolic extracts of bark and wood of *D. vallicola*, against intrahospital isolates of *P. aeruginosa*. The effect of the extracts was different between the isolates and in all cases it was concentration dependent. These results coincide with those reported by Sousa [40], who found differences in the efficiency of essential oils extracted from the bark of *D. lanceolata* against the strains *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *P. aeruginosa* and *Candida albicans*; since the antimicrobial effect varies depending on

the concentration and the microorganism evaluated. Studies carried out with extracts from other species of the genus, *D. gardneriana*, *D. moricandiana* and *D. quitensis* have also reported antibacterial activity against *S. aureus*, *S. pyogenes* and *E. coli*, attributing this effect to the content of terpenes present in the essential oils evaluated [41].

The extracts of *D. vallicola* have been little studied; the presence of various types of isoquinoline alkaloids has been demonstrated, including pseudopalmatine, isoboldine, isocorydine, N-methylaurotetanine, oliveridine, oliveroline, among others [22], however, studies of antimicrobial activity of its extracts or compounds have not been reported. Alkaloids are a large and structurally diverse group of compounds that have served as scaffolds for important antibacterial drugs such as metronidazole and quinolones. They have been widely studied for their potential, not only antibacterial, but also antibiotic enhancers; furthermore, the effects of these compounds on virulence gene regulatory systems, such as quorum sensing and virulence factors, have also been reported [42–44]. Isoquinoline alkaloids of plant origin comprise a broad source of multimodal agents with unique structural diversity and a varied range of pharmacological activities [45], they are of great interest due to their promising biological activity [46], highlighting their antibacterial, antifungal, antiviral and antiparasitic activity, among others [47]. Likewise, studies reported by [48,49], have documented the antibacterial structure-activity relationship of isoquinoline alkaloids of the type protoberberine, protopine, benzophenanthridine, aporphine and bisbenzylisoquinoline, evidencing damage to the integrity of the membrane and cell wall, inhibition of efflux pumps and related enzymes, damage in bacterial DNA and protein synthesis, as mechanisms of antibacterial action of these compounds. Taking into account the great antibacterial potential reported for isoquinoline alkaloids and the presence of a diverse group of these alkaloids in *D. vallicola*, we could suggest their contribution to the inhibitory activity of ethanolic extracts of wood and bark against *P. aeruginosa*.

The notorious persistence of *P. aeruginosa* in clinical environments is attributed to its ability to form antibiotic-resistant biofilms; these function as a framework to enclose bacteria on surfaces and protect them from environmental stress, preventing phagocytosis and therefore, giving them the capacity for colonization, persistence and contributing to their multi-drug resistance [50–52]. We report the ability of ethanolic extracts of bark and wood to inhibit mature biofilms in *P. aeruginosa* (48.4% up to 93.7%), this effect being similar to that observed in cells treated with CIP in some cases. The antibiofilm activity of species of the genus *Duguetia* had not been reported.

Inhibition of biofilm formation by berberine in *P. aeruginosa* [53] and *S. epidermidis* [54] has been reported. Likewise, the action of sanguinarine, a benzophenanthridine alkaloid of plant origin, to inhibit bacterial biofilms has been documented [55] and the action against fungal biofilms of the chelerythrine-sanguinarine combination has also been demonstrated [56]. Taking into account the content of isoquinolinic alkaloids present in *D. vallicola* extracts [22], these could, at least in part, be responsible for the antibiofilm action evidenced in this research. These results are the first report of the antibiofilm action of *D. vallicola* extracts on *P. aeruginosa*.

On the other hand, the experiments of leakage of intracellular material (260/280), measurement of extracellular pH and fluorescent staining with AO and EB, showed alteration in the integrity of the cell membrane, indicating that the ethanolic extracts of *D. vallicola*, alter the integrity of the membrane of these pathogens, as an antibacterial mechanism, causing cell death. Our results provide new and important insights into the antibacterial and antibiofilm potential of *D. vallicola* extracts against *P. aeruginosa*; besides, we show the cell membrane as a possible target of its action; however, more studies are required to elucidate the mechanisms involved in the antimicrobial activity of this plant species. This research provides a reference for future research on the mechanisms of antimicrobial action against pathogenic bacteria of intrahospital origin.

## Materials and Methods

### 4.1. Reagents

Mueller-Hinton broth (MHB) (Sigma, Mendota Heights, MN, USA) was used for the determination of MIC and cultures of bacterial isolates. Tryptic Soy Agar (TSA) and Tryptic Soy Broth

(TSB) (Becton, Dickinson and Company, San Diego, CA, USA), Mueller-Hinton agar (MHA) (Sigma, Mendota Heights, MN, USA), and Brain Heart Infusion (BHI) broth (Sigma-Aldrich, St. Louis, MO, USA) were also used for bacterial cultures. Dimethyl

sulfoxide (DMSO), phosphate-buffered saline (PBS), crystal violet (CV), acridine orange (AO), ethidium bromide, (EB) and antibiotic ciprofloxacin (CIP), used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA; ammonium hydroxide (NH<sub>4</sub>OH), dichloromethane (DCM), hydrochloric acid (HCl), methanol (MeOH) and glacial acetic acid were obtained from Carlo Erba Reagents, Milano, Italy.

#### 4.2. Obtaining the Extracts

The wood and bark of *Duguetia vallicola* were collected from a specimen located in the El Corozo village (8° 33' 39"N, 75°45'30"W) municipality of Montería Córdoba, a specimen rests in the Herbarium of the Botanical Garden Joaquín Antonio Uribe, from the city of Medellín under collection number JAUM 037841. The plant material, free of impurities, was dried at room temperature and pulverized in a knife mill. The dried and ground material (250 g of bark and 300 g of wood) were extracted separately by percolation with 96% ethanol until exhaustion. Each extract was concentrated in a rotary evaporator (Hei-VAP Core, Wood Dale IL, USA), until obtaining 32g of ethanolic bark extract and 11.4g of ethanolic wood extract.

#### 4.3. Obtaining alkaloids from wood extract.

An 8 g fraction of the ethanolic wood extract was alkalized to pH 8-9 with 100 mL of 5% ammonium hydroxide (NH<sub>4</sub>OH) and extracted twice with 100 mL portions of dichloromethane (DCM). The organic phase was partitioned with 150 mL of 3% hydrochloric acid (HCl) and the aqueous phase obtained was alkalized with NH<sub>4</sub>OH until pH 8-9. Finally, it was extracted twice with 100 mL portions of DCM, obtaining a total alkaloid fraction of 235 mg. The fractionation of the total alkaloids was carried out by chromatography in successive columns (silica gel 60 (0.063-0.200mm) Merck®), eluting with DCM/MeOH mixtures from 99:1 to 90:10. The chromatographic monitoring was done with aluminum thin layer chromatography TLC plate, silica gel coated with fluorescent indicator F<sub>254</sub> (Merck®). 11.4 mg of liriodenine (**1**), 16.1 mg of O-methylmoschatoline (**2**) and 7.8 mg of nornuciferin (**3**) were obtained. Their structures were established by comparison of their physical and spectral data with those published in the literature, using <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, DEPT, COSY <sup>1</sup>H-<sup>1</sup>H, HMQC and HMBC spectra, performed on a 400 MHz Bruker Advance DRX spectrometer, in deuterated chloroform (CDCl<sub>3</sub>). The mass spectrum was obtained in electron impact ionization mode at 70 eV.

#### 4.4. Strains

Twelve clinical isolates of *P. aeruginosa* (CLI12, CLI15, CLI50, CLI51, CLI52, CLI54, CLI55, CLI56, CLI57, CLI58, CLI97, CLI212) were used in this study. The isolates were cultured from blood and urine culture samples from patients hospitalized at the Social Health Service S.A.S. from the city of Sincelejo, Colombia. All microorganisms were identified using standard methods: Vitek® 2 Compact. Biomerieux SA. BHI medium and cetrimide agar were used to maintain cultures until testing was performed.

#### 4.5. Antibacterial Susceptibility Testing

The minimum inhibitory concentration (MIC) of the ethanolic extracts of wood and bark of *D. vallicola* against clinical isolates was defined as the lowest concentration at which 90% (MIC<sub>90</sub>) of bacterial growth was inhibited, compared to the control (untreated cells). MIC<sub>90</sub> was determined by performing broth microdilution assays, using 96-well microtiter plates (Nunclon Delta, Thermo Fisher Scientific, Waltham, MA, USA), as described in *Clinical Laboratory Standards Institute* method M07-A9 (CLSI) [57], with minor modifications. Serial dilutions were performed in MHB to obtain final concentrations of 2000, 1000, 500, 250, 125, and 62.5 µg/mL of the extracts in each reaction well.

To carry out the experiments, a stock solution of each extract was prepared at 25000 µg/mL in 10% DMSO. Cells without treatment and cells treated with CIP 2 µg/mL were used as negative control and positive control respectively. The assays were developed at a final volume of 200 µL per well as follows: 100 µL of bacterial inoculum at a concentration of 10<sup>8</sup> CFU/mL and 100 µL of the extracts, adjusted to reach the previously described concentrations in the reaction wells final. Wells with bacterial inoculum, without extracts and with CIP (2 µg/mL) were used as growth controls and positive controls, respectively. The plates were incubated at 37 °C for 24 h. The experiments were performed in triplicate. The inhibition of bacterial growth by the extracts was determined by changes in optical density using a SYNERGY LX microplate reader (Biotek), at 600 nm, from the beginning of incubation to the end of incubation (24 h). Finally, the percentage of inhibition of bacterial growth [58] was calculated using the following equation:

$$\% \text{Inhibition} = (1 - (\text{OD}_{t24} - \text{OD}_{t0}) / (\text{OD}_{gc24} - \text{OD}_{gc0})) \times 100$$

where OD<sub>t24</sub>: optical density of the test well at 24 h post-inoculation; OD<sub>t0</sub>: optical density of the test well at 0 h post-inoculation; OD<sub>gc24</sub>: optical density of the growth control well at 24 h post-inoculation; OD<sub>gc0</sub>: optical density of the growth control well at 0 h post-inoculation.

#### 4.6. Quantitative Assessment of Biofilm Formation

The effect of *D. vallicola* wood and bark extracts on mature *P. aeruginosa* biofilms was evaluated following the protocol described by [59,60], with minor modifications. For biofilm formation, bacterial colonies from 24 h of incubation in TSA were used, standardizing the bacterial inoculum at 10<sup>8</sup> cells/mL. Then, in 96-well polystyrene microplates, 200 µL of the bacterial inoculum was discharged into each well and incubated at 37 °C for 24 h. Subsequently, the broth was removed from the microplates and 200 µL of the wood and bark extracts (evaluated separately), at the MIC concentration of each isolate, were added to TSB broth and incubated at 37 °C for 1 hour. Subsequently, the floating cells were removed and the biofilms from the bottom of the wells were washed with deionized water. Excess moisture was then removed by tapping the microplates on sterile napkins and the plates were dried for 5 minutes. Six replicates of each experiment were performed. Cultures without extracts were used as a negative control and cultures with CIP as a positive control. Biofilm reductions were quantified by staining the wells with 200 µL of 0.1% CV for 20 min. The samples were washed with deionized water until excess dye was removed; the excess water was carefully dried and then the CV was solubilized in 250 µL of 30% glacial acetic acid. Absorbance values were measured at 590 nm (OD<sub>590</sub>), using a SYNERGY LX microplate reader (Biotek). Biofilm production was grouped into the following categories: OD<sub>590</sub> < 0.1: non-producers (NP), OD<sub>590</sub> 0.1–1.0: weak producers (WP), OD<sub>590</sub> 1.1–3.0: moderate producers (MP) and OD<sub>590</sub> > 3.0: producers strong (SP). Biofilm reduction was calculated [61] using the following equation:

$$\% \text{ Biofilm reduction: } \text{AbsCO} - \text{AbsExt} / \text{AbsCO} \times 100$$

where AbsCO: absorbance of the control sample and AbsExt: absorbance of the sample treated with the extracts.

#### 4.7. Effect of Extracts on Cell Membrane Integrity

The evaluation of the action of ethanolic extracts of wood and bark of *D. vallicola* on the cell membrane of *P. aeruginosa* was carried out through experiments of intracellular material leakage, measurement of extracellular pH and cell fluorescence microscopy treated with each of the evaluated extracts.

##### 4.7.1. Leakage of Nucleic Acids and Proteins Through the Cell Membrane

The release of intracellular material was measured according to the methodology proposed by [62], with some modifications. Cells cultured in nutrient broth were centrifuged at 3000 g for 20 minutes, washed three times and resuspended in 20 ml of PBS (pH 7.0). The cell suspension was then treated with the wood and bark extracts (MIC for each isolate; separate experiments) and incubated



at 37 °C for 0, 30, 60, and 120 min. Subsequently, 2 mL of the samples were collected and centrifuged at 3000 g for 20 min. Then, to determine the concentration of the released constituents, 2 mL of supernatant was used to measure the absorbance at 260/280 nm with a Spectroquant® Prove 300 UV/Vis spectrophotometer. Samples without extracts and samples with CIP were used as controls. All assays were performed in triplicate.

#### 4.7.2. Measurement of Extracellular pH

The measurement of the extracellular pH of *P. aeruginosa* after treatment with *D. vallicola* wood and bark extracts was determined according to [61], with some modifications. 20 mL of the bacterial suspension ( $1 \times 10^7$  CFU/mL) in nutrient broth was incubated at 37 °C for 24 h. The samples were then centrifuged at 3000 g for 20 min; the sediment was collected, resuspended and washed three times with double-distilled water, resuspending again in 20 mL of sterile double-distilled water. After the addition of the extracts (MIC of each isolate), the extracellular pH of *P. aeruginosa* was determined at 0, 30, 60 and 120 min, using a Schott® Instruments Handylab pH 11 pH meter. They were used as controls samples without extracts and samples with CIP.

#### 4.7.3. LIVE/DEAD Assays

The LIVE/DEAD assays were developed following the methodology proposed by [35]. A suspension of *P. aeruginosa* ( $10^7$  CFU/mL) was placed on sterile slides and incubated for 24 h. The cells were then washed three times with PBS. Subsequently, the MIC of the extracts of each isolate and CIP were added to the experimental groups and the bacterial inoculum in nutrient broth was used as a control. The prepared slides were incubated at 37 °C for 24 h and then washed three times with PBS. Together, AO (5 µL, 100 mg/L) and EB (5 µL, 100 mg/L) were mixed under dark conditions and added to the slides under dark conditions for 30 sec. The samples were then observed on an Olympus BX43 fluorescence microscope and photographed with a DP72 camera.

#### 4.8. Data Analysis

The results were analyzed using GraphPad Prism version 8.0 software and the Microsoft Excel program. Initially, the Shapiro Wilk test was used to determine the distribution of the data. Subsequently, Pearson's correlation coefficient was used to measure the degree of linearity, the correlation between the concentration of the extracts and the percentage reduction in bacterial growth. To compare the effect of the extracts and CIP on biofilm reduction, the Kruskal Wallis test and the Dunnett test were used; this test was also used to compare the effects of the extracts and CIP on the leakage of intracellular material through the membrane (260/280 nm) and to compare the effects of the treatments on the extracellular pH of *P. aeruginosa*.

### 5. Conclusions

In this study we investigated the antibacterial potential of ethanolic extracts of *Duguetia vallicola* wood and bark against clinical isolates of *Pseudomonas aeruginosa*, as well as their role in biofilm inhibition. Furthermore, we explored the action against the cell membrane of these pathogens. We demonstrate the antibacterial action of the extracts under study against *P. aeruginosa*, this effect being associated with damage to the integrity of the cell membrane, in addition to its action against bacterial biofilms. Additionally, we report the presence of three isoquinoline alkaloids in the wood of *D. vallicola*. It is necessary to continue these studies, with a view to elucidating the mechanisms of antibacterial action and the composition of the extracts of this species, which promises as an alternative tool for the treatment and control of intrahospital pathogens such as *P. aeruginosa*.

**Supplementary Materials:** The following supporting information can be downloaded at: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.. Table S1. NMR Spectroscopic Data ( $^1\text{H}$  400 MHz,  $^{13}\text{C}$  100 MHz) of **1** in  $\text{CDCl}_3$ . Table S2. NMR Spectroscopic Data ( $^1\text{H}$  400 MHz,  $^{13}\text{C}$  100 MHz) of **2** in  $\text{CDCl}_3$ . Table S3. NMR Spectroscopic Data ( $^1\text{H}$  400 MHz,  $^{13}\text{C}$  100 MHz) of **3** in  $\text{CDCl}_3$ . Figure S1.  $^1\text{H}$ -NMR spectrum (amplification 6.2 – 9.1 ppm) of **1**, ( $\text{CDCl}_3$ , 400 MHz). Figure S2.  $^1\text{H}$ -NMR spectrum

(amplification 4.0 – 9.2 ppm) of **2**, (CDCl<sub>3</sub>, 400 MHz). Figure S3. <sup>1</sup>H-NMR spectrum (amplification 7.5 – 9.2 ppm) of **2**, (CDCl<sub>3</sub>, 400 MHz). Figure S4. <sup>1</sup>H-NMR spectrum of **3**, (CDCl<sub>3</sub>, 400 MHz). Figure S5. <sup>1</sup>H-NMR spectrum (amplifications) of **3**, (CDCl<sub>3</sub>, 400 MHz).

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