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MICROBIOLOGY

Phytochemical screening of *Podocarpus lambertii* Klotzch ex Endl. leaf extracts and potential antimicrobial, antioxidant and antibiofilm activity

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Abstract: Species of the genus *Podocarpus* L'Hér. ex Pers.present biological activities, such as analgesic, antioxidant, antifungal, acting in the fight against anemia, depurative and fortifying. *Podocarpus lambertii* Klotzch ex Endl. is a Brazilian native species popularly known as maritime pine and lacks information about its phytochemical profile and possible biological activities. The study was conducted to determine the phytochemical composition of soluble plant extracts of acetone (EA), ethyl acetate (EAE) and hexane (HE) from leaves of *P. lambertii*; evaluate the antimicrobial potential by the broth microdilution technique; antioxidant potential by the DPPH method, as well as to evaluate the biofilm inhibition capacity by the crystal violet assay and reduction of the yellow tetrazolium salt (MTT). Phytochemical screening detected the presence of flavonoids, triterpenoids, steroids, tannins, alkaloids and saponins. All extracts showed antimicrobial activity on the microorganisms tested, and the EA showed the best results. High free radical scavenging potential was observed only in EAE (96.35%). The antibiofilm potential was observed in the EAE extract. The results contribute to the knowledge of the species and indicate the potential of *P. lambertii* extracts as a source of plant bioactives for the development of new alternative strategies to control resistant microorganisms.

Key words: Antibiofilm, antioxidant, biological activities, phenolic compounds, plant extract.

INTRODUCTION

In many regions, especially in undeveloped or developing countries, the use of medicinal plants for the treatment of the most diverse clinical conditions is based on cultural traditions and beliefs in these alternative methods, still representing one of the main supports for health care and maintenance (Santos et al. 2021).

Brazil holds the greatest genetic diversity in the world, with estimates of 350,000 to 550,000 existing species, of which 55,000 plant species have already been recognized and cataloged, and only 1% of the flora has been investigated due to this popular knowledge (Silva Filho 2009, Carneiro et al. 2014). Sectors such as industrial, pharmaceutical, environmental already highlight the bioactive compounds (secondary metabolites) of plant extracts as: antimicrobial agents (Weber et al. 2014, Santos et al. 2021), antioxidants (Santos et al. 2021), pesticides, larvicides (Santana et al. 2018), sedatives (Mello & Zacharias 2019), aromas (Baser & Buchbauer 2015), among others.

The Podocarpaceae family comprises 18 genera and approximately 173 species spread

around the world (Klock et al. 2005, Souza 2015). Among the species, *P. lambertii*, known as maritime pine, stands out as a native and endemic species of the southern region of Brazil (Embrapa 2004, Iganci & Dorneles 2019). The study of this species is still incipient and, as a result, little is known from a scientific point of view about the pharmacological and chemical potential of this plant, justifying its academic exploration.

Taking into account the numerous biological activities already described in species within the Podocarpaceae family, further studies with the species *P. lambertii* are essential. The possibility of discovering new natural bioactives that can be employed in public health, industry and countless other application possibilities makes these studies increasingly relevant, allowing the validation of ethnobotanical knowledge and valorization of native flora.

Therefore, the aim of this study was to determine the main groups of secondary metabolites from the phytochemical screening of plant extracts from *P. lambertii* leaves, investigating the antimicrobial, antioxidant, and antibiofilm potential against pathogenic microorganisms.

MATERIALS AND METHODS

Collection and identification of *P. lambertii*

P. lambertii leaves were collected in the Paulo Gorski Ecological Park, located in the municipality of Cascavel, western region of the state of Paraná (24°57'51.61 "S and 53°26'14.80 "O). The collections were performed on random days, between 08:00 am (morning) and 12:00 pm (noon), in both rainy and dry periods, between August 2019 and March 2020. An exsiccate of the plant was delivered to the Herbarium of the Western Paraná State University (UNOP) for botanical identification and registration UNOP10730.

Chemical and reagents

Preparation of plant extracts

Plant extracts were prepared according to the methodology proposed by Pandini et al. (2015). The dried and ground leaves were added in the proportion of 10 g for each 100 mL of acetone P.A (AE), ethyl acetate P.A. (EAE) or hexane P.A (HE) and kept in rotary shaker at 220 rpm for 24 hours. After this period, the solution was filtered using Whatman No. 1 filter paper and centrifuged in conical tubes at 3800 rpm for 15 minutes. Then, the supernatant of the extracts was submitted to rotoevaporation for total elimination of solvents. The crude extracts were stored protected from light in a freezer at 4°C. The yield was calculated as a function of the extract mass and the raw material mass, in grams of material used.

Phytochemical screening of extracts

The qualitative phytochemical screening for secondary metabolites present in the extracts followed the methodology of Matos (1997) and Weber et al. (2014), with modifications. Colorimetric visualization tests and/or precipitate formation after addition of specific reagents were performed. The classes of secondary metabolites identified were: saponins through reaction with distilled water and hydrochloric acid P.A.; steroids and triterpenoids through Liebermann-Burchard reaction; tannins through reaction with ferric chloride, and coumarins through fluorescence reaction with potassium hydroxide; anthocyanins, anthocyanidins, aurones, chalcones, flavanonols, flavones, flavonols and xanthones (flavonoids) from pH changes in the medium; alkaloids using Dragendorff reagent.

Quantitative estimation of secondary metabolite from *P. lambertii* leaves

Total Phenolic Content (TPC)

The TPC of the extract was determined according to the Folin-Ciocalteu method of Slinkard & Singleton (1977) and Tabasum et al. (2016), with some modifications. In summary 1.0 mL of extract (1.0 $mg.mL^{-1}$) was mixed with 2.5 mL Folin–Ciocalteu reagent 10% (w/v). After 5 min, 2.0 mL of Na_2CO_3 (75%) was added to the mixture and incubated at 50 °C for 10 min with intermittent stirring. The absorbance of the samples was measured at 765 nm against the blank. Gallic acid was used as a calibration substance using 1.0 $mg.mL^{-1}$ as a standard solution with different dilutions for the calibration curve (y = ax + b). The amount of TPC was calculated as mg of gallic acid equivalents in milligrams per gram (mg GAE/g) of extract and calculated as mean value ± SD (n = 3).

Total Flavonoid Content (TFC)

TFC was determined using Arvouet-Grand et al. (1994). A 1.0 mL aliquot of the extract was mixed with 0.2 mL of 10% (w/v) AlCl3 solution in methanol, 0.2 mL (1 M) of potassium acetate (CH_3CO_2K) and 5.6 mL of distilled water. The mixture was incubated for 30 min at room temperature, followed by measuring the absorbance at 415 nm against the blank. Quercetin was used as a calibration substance using 1.0 $mg.mL^{-1}$ as a standard solution with different dilutions for the calibration curve (y = ax + b). TFC quantity was expressed as mg/g of quercetin equivalents in milligrams per gram (mg QE/g) of extract and calculated as mean value ± SD (n = 3).

Total Tannin Content (TTC)

TTC was determined using Folin-Ciocalteu phenol reagent with tannic acid as standard, adapted from the method reported by Son et al. (2013). 1.0 mL of extracts was added to 1.0 mL of 0.2 M Folin-Ciocalteu phenol reagent in a test tube and incubated for 4 min at room temperature. Then 800 µL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The reactions were incubated in a dark chamber at room temperature for 2 h followed by measuring the absorbance at 725 nm against the blank. Tannic acid was used as calibration substance using 1.0 $mg.mL^{-1}$ as standard solution with different dilutions for the calibration curve (y = ax + b). TTC quantity was expressed as mg of tannic acid equivalent per gram (mg TAE/g) of extract and calculated as mean value ± SD (n = 3).

Total Alkaloid Content (TAC)

TAC was also quantified by the spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green (BCG), adapted from the method reported by Tabasum et al. (2016). The plant extract (1.0 $mg.mL^{-1}$) was dissolved in 2 N HCl and then filtered. The pH of the phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1.0 mL of this solution was transferred to a separating funnel, and then 5 mL of BCG solution along with 5 mL of phosphate buffer were added. The mixture was stirred and the complex formed was extracted with chloroform by vigorous stirring. The extract was collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. TAC quantity was expressed as mg of atropine equivalent per gram (mg AE/g) of extract and calculated as mean value ± SD (n = 3).

Bacterial strains

The extracts were tested against different microorganisms from the *American Type Culture Collection* (ATCC) being three Gram

negative strains: *Escherichia coli* (ATCC 25922), *Salmonella enterica* Enteritidis (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 27853), four Gram- positive: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 19433), *Staphylococcus epidermidis* (ATCC 12228) *Listeria monocytogenes* (ATCC 1911), and one yeast *Candida albicans* (ATCC 10231).

Evaluation antimicrobial activity

Sutitle inoculums preparation

For the antimicrobial activity test, the microorganisms were recovered in Brain Heart Infusion (BHI) enrichment broth and incubated for 24 h at 35±2°C. After this period, the strains were repotted onto Mueller Hinton Agar (MHA) medium and standardized in saline solution (0.85%) resulting in a final concentration of 1×10^{5} CFU. mL^{-1} for bacteria and 1×10^{6} CFU. mL^{-1} for yeast, according to the McFarland scale.

Determination of minimum inhibitory concentrations (MIC's) / minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the plants extract

The antimicrobial activity of *P. lambertii* extracts was evaluated following the *Clinical and Laboratory Standards Institute* (CLSI 2018) and Scur et al. (2014) standards with modifications, and the Minimum Inhibitory Concentration (MIC) was performed by broth microdilution method. AE, EAE and HE were solubilized in methanol (P.A), filtered and diluted in Mueller Hinton Broth (MHB). In 96-well flat-bottomed microplates, 150 µL of MHB was dispensed in all wells, 150 µL of the plant extract was added in the first well, with serial dilutions at concentrations of 200 - 0.09 $mg.mL^{-1}$ for the extracts. Then, a 20 µl aliquot with microorganisms was added at $1x10^{5}$ CFU. mL^{-1} in each well and the plates were incubated at 35±2°C for 24 h. For the positive control, the commercial antibiotic gentamicin (bacteria) and the antifungal nystatin (*C. albicans*) were used at the same concentrations as tested in experiments. As a negative control, inoculum was added to MHB without the presence of extract to verify the viability of the tested microorganism. As colorimetric developer, 20 µL of 0.5% triphenyl tetrazolium chloride (2,3,5-tryphenyl-2H-tetrazolium chloride) (TTC) solution was used in each well of the plate. The presence of red coloration was interpreted as negative evidence of the inhibitory effect. The MIC assay was performed in triplicate, allowing to determine the lowest concentration of the extracts capable of inhibiting microbial growth. After incubation, turbidity was observed and each well received a 20 µl aliquot of TTC to reveal wheter or not bacteria were inhibited. After performing the MIC assay, before the addition of TTC, a 2 µl aliquot was removed and inoculated in MHA for MBC/MFC determination and the plates were incubated for 24 h at 35±2°C, observing bacterial growth.

The MIC and MBC/MFC were classified according to Araújo (2011) and Pandini et al. (2015), with the activity in 4 classes: high (<12.5 $mg.mL^{-1}$), moderate (12.5 to 25 $mg.mL^{-1}$), low (50 to 100 $mg.mL^{-1}$) and very low (>100 $mg.mL^{-1}$) .

Evaluation of antioxidant activity

The antioxidant activity was analyzed according to the methodologies proposed by Weber et al. (2014) with modifications, by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical reduction method. For the determination of the percent antioxidant activity (%AA), the sample was prepared with 1 mg of extract and 1 mL of methanol P.A. in 1.5 mL conical tube, homogenized in a tube shaker for 30 seconds. A 0.1 mL aliquot was transferred to 3.9 mL of 0.2 mM DPPH methanolic solution and homogenized. The readings were measured in a spectrophotometer

(Femto, 600plus) with absorbance of 515 nm and the reduction was monitored every minute until complete stabilization, making up the pre-test assay. The final absorbance reading for the calculation was expressed by: $AA\%: \frac{|0|-|1|}{|0|} x100$, where Abs0 is the absorbance of the control and Abs1 is the absorbance of the sample.

After obtaining the %AA of the extracts, the concentrations used to obtain the IC_{50} (amount of antioxidant substance needed to reduce the initial DPPH concentration by 50%) were defined. The concentrations were defined based on the %AA values obtained in the pre-test assay; when this was higher than 80%, the following concentrations were used: 0.1, 0.25, 0.5, 0.75, 1 $mg.mL^{-1}$; below 80%, the concentrations were 1, 2.5, 5.0, 7.5 and 10 $mg.mL^{-1}$. The readings of all reactions were performed using methanol as blank, free radical reagent (DPPH) and methanol as negative control, and butylated hydroxytoluene (BHT) as positive control at concentrations of (0.05, 0.025, 0.01, 0.005 and 0.0025 $mg.mL^{-1}$). The spectrophotometer calibration was performed with methanol. The tests were performed in triplicate. The data obtained by calculations of the DPPH radical scavenging capacity and IC_{50} were evaluated using the Tukey 5% test ($p<0.05$), using the Sisvar software. The experiments were performed in triplicate of samples and assays.

Activity of plant extracts on the development of preformed biofilms

The potential of AE, EAE and HE extracts was evaluated on biofilm development (24 h - irreversible fixation). In summary a 20 µL aliquot of inoculum at McFarland's concentration of 0.5 ($1x10^6$ $CFU.mL^{-1}$) of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) was added to 130 µL of Brain Heart Infusion (BHI) medium supplemented with 1% glucose, added to 96-well flat-bottomed microplates and incubated at 37° C for 24 h without shaking (irreversible fixation). The medium was then removed and aliquots of 150 µL at concentrations of 100 to 0.09 $mg.mL^{-1}$ for extracts and then incubated again at 37° C for 24 h without shaking. Untreated biofilm controls (BHI supplemented with 1% glucose and inoculum), positive control (Gentamicin) and inoculum and color interference control (BHI and the concentrations of the extract diluted in DMSO without inoculum) were used. Biomass was quantified using the crystal violet (CV) staining method and cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetazolium bromide (MTT) reduction assay.

Crystal violet staining test

The assay was carried out according to Christensen et al. (1982) adapted by Bandeira et al. (2022). The 96-well flat-bottomed microplates were washed three times with 1X phosphate buffered saline (PBS) 1X pH 7.2. Then, for cell fixation, 150 µL methanol P.A. was added to the wells (Neon) for 20 minutes. After methanol removal, 150 µL of 1% crystal violet (Scientific Exodus) was added for 15 min. The wells were then washed under running water to remove the unabsorbed dye. At this point, biofilms were observed as purple rings next to the well. A 150 µL aliquot of 95% ethanol was added and left in contact for 30 min for biofilm decolorization. After this time, the contents of the microplates were transferred to a new sterile plate and the absorbance (OD) was measured at 570 nm using Epoch model microplate reader. The data were calculated in overall mean absorbance by Microsoft Excel 2010 program, and the percentage of biofilm inhibition was determined using the following equation:

$$\% \text{ growth inhibition} = [(Ac-At)/Ac]*100$$

Where Ac: mean absorbance of the untreated control; At: mean absorbance of the test with extract.

The result was classified according to Famuyide et al. (2019) where: < 50% indicate low antibiofilm activity; > 50% indicate high antibiofilm activity. Negative values indicate increase in mature biofilm biomass.

Testing the cell viability of the biofilms formed

Metabolic viability analysis of mature biofilms was assessed by MTT reduction at a concentration of 0.5%. After methanol removal, an aliquot of 180 µL and 20 µL of MTT were added to each well, and the plates were incubated in the dark at 37 °C for 2.5 h. After this period, the medium was removed and the dye (MTT) was resolubilized with 150 µL of DMSO for 15 min. The reading was performed using a microplate reader at a wavelength of 570 nm. Cell viability data of the mature biofilms were expressed as overall mean optical density (OD) and the percent cell viability (CV%) was determined by the equation: experimental OD / untreated control OD x 100 (Jia et al. 2010, Bandeira et al. 2022, Laskoski et al. 2022) and classified into: Percent cell viability values: < 50% indicate low cell activity; > 50% indicate high cell activity (Famuyide et al. 2019).

The activity of the extracts and oil on the mature biofilm was measured by the reduction (%) of the absorbance value when compared to the untreated control, since the

tested sample is a biofilm producer. As a criterion for expressing the results of the antibiofilm potential of AE, EAE and HE of *P. lambertii,* both biofilm biomass by crystal violet assay and cell viability by MTT assay were determined from the MIC, 2X MIC and 4X MIC concentrations of each bacterial strain tested.

Statistical analysis

The experimental results were expressed as mean ± standard deviation. The experiments were performed in triplicate of samples and assays. The data obtained were evaluated by Analysis of Variance (ANOVA) and Tukey 5% test ($p<0.05$).

RESULTS AND DISCUSSION

Extract yields

The following yields were obtained from the preparation of *P. lambertii* plant extracts with different solvents: AE (6.76%), EAE (7.60%) and HE (4.51%). A better yield of our extracts was obtained when compared with the results of Abdillahi et al. (2008), who evaluated four different *Podocarpus* species, and acetone (average 3%) and hexane (average 2%) extracts. Many variables influence the yield of metabolites in plant extracts, from temperature, extraction time, solid-solvent ratio, among others. However, the most important factor is solvent selection, which due to its complex chemical characteristics, such as polarity and solubility, directly influence the yield of extracts (Cabana et al. 2013, Fernández-Agulló et al. 2013, Dirar et al. 2019, Kong et al. 2020).

Phytochemical screening of the extracts (Qualitative and Quantitative)

After obtaining AE, HE and EAE extracts from *P. lambertii* leaves, the phytochemical screening was performed. Five groups of compounds were verified: tannins, alkaloids, flavonoids, steroids, and saponins. Coumarins, anthocyanins, anthocyanidins and triterpenoids were not detected in the extracts (Table I).

The AE showed the greatest diversity of secondary metabolites, with the presence of five different classes, followed by HE and EAE extracts, which presented four classes.

Table I. Phytochemical screening of secondary metabolites present in *Podocarpus lambertii* leaf extracts.

Classes of Metabolites	Hexanic (EH)	Acetonic (EA)	Ethyl Acetate (EAE)
Tannins	-	+	-
Alkaloids	+	+	+
Cumarins	-	-	-
Anthocyanins	-	-	-
Flavonoids	+	+	+
Triterpenoids	-	-	-
Steroids	+	+	+
Saponins	+	+	+
Anthocyanidins	-	-	-

(+) presence; (-) absence.

Alkaloids, flavonoids, steroids and saponins were common in all extracts tested. Tannins were detected only in AE (Table II). The literature demonstrates that plant extracts commonly rich in phytoconstituents mainly use acetone and ethyl acetate as extracting solvents (Santana et al. 2022).

The differences observed in the composition of the extracts can be explained by the abundance of metabolites present, the yield during the extraction process, and the polarity characteristics of the solvent used (Fernández-Agulló et al. 2013, Pimentel et al. 2013).

No studies were found in the literature related to the presence of phytochemicals in *P. lambertii*, but our results corroborate studies in species of the genus *Podocarpus* from New Zealand, such as *P. elongatus* (Aiton) L'Hér. ex Pers., *P. falcatus*, *P. henkelii* Stapf ex Dallim. & A. B. Jacksque, which found the presence of several types of flavonoids, such as monoflavonoids, biflavonoids and flavonoid glycosides (Abdillahi et al. 2010).

Flavonoids, alkaloids, steroids and saponins are classes of biologically active compounds found in all *P. lambertii* leaf extracts and exert antimicrobial, pharmacological, and important ecological functions (Silva & Paiva 2012, Takshak & Agrawal 2019, Guimarães et al. 2019, Reddy et al. 2020, Fakhri et al. 2020), as well as surfactant (Ribeiro et al. 2013), anticancer (Nadaraia et al. 2019), and immunomodulatory (Orczyk et al. 2020) capacity.

Tannins present in greater quantities only in AE are water-soluble polyphenols and also exhibit an antimicrobial action mechanism that constitutes substrate deprivation and enzyme inhibition (Sharma 2019, Reddy et al. 2020). The efficacy of these secondary compounds has already been proven in several other plant species, thus confirming the potential of *P. lambertii* as a raw material for the extraction of bioactive compounds (Nicácio et al. 2017, Sharma 2019, Reddy et al. 2020).

There are no reports in the literature regarding the presence of phytochemicals in *P. lambertii*. Our results of phytochemical quantification revealed a high total phenolic content in EAE (111.76 mg GAE/g). Previous studies showed that the total phenolic content in leaves of *P. elongatus* and *P. henkelli* is 6.94 mg GAE/g and 6.85 mg GAE/g, respectively (Abdillahi

Table II. Total phenolic, flavonoid, tannin, and alkaloid content of *Podocarpus lambertii* leaf extracts.

Phytochemical constituents (µg/mL)	AE	HE	EAE
Total Phenolic mg GAE/g	62.95 ± 0.003 b	25.15 ± 0.003 b	111.76 ± 0.003 a
Total Flavonoids mg QE/g	33.93 ± 0.002 b	21.43 ± 0.005 b	140.50 ± 0.005 a
Total Tannins mg TAE/g	64.60 ± 0.005 a	3.15 ± 0.002 b	8.61 ± 0.006 b
Total Alkaloids mg AT/g	113.59 ± 0.003 b	82.85 ± 0.003 b	322.94 ± 0.004 a

Values followed by the same letter, on the same line do not differ by Tukey's test (p-value <0.05); mean ± standard deviation; AE=acetone extract; HE= hexane extract; EAE = Ethyl Acetate Extract. GAE= gallic acid; QE= quercetinic acid; TAE= tannic acid; AT= atopine; Source: authors.

et al. 2011). In the present study, the maximum flavonoid and alkaloid levels were observed in EAE (140.50 mg QE/g and 322.94mg AT/g) (Table II). These results corroborate with studies in other species of the genus *Podocarpus*, which found the presence of different types of flavonoids, biflavonoids, glycosides, encompassing a wide variety of steroidal and terpenic compounds (Abdilahi et al. 2010). Extracts with high phenolic content do not always present a high amount of condensed tannin, as was evident with the leaf extract of *P. elongatus* in the study conducted by Abdillahi et al. (2011). This result was also identified in our study with *P. lambertii*.

Antimicrobial activity

In the broth microdilution assay, all *P. lambertii* leaf extracts were tested for their ability to inhibit the growth (MIC) or cause death (MBC/MFC) of microorganisms (Table III).

The activity of the extracts varied according to the extracting solvent and the microorganism tested. AE, HE and EAE extracts showed antimicrobial potential against the 12 standard strains tested and AE showed high antimicrobial activity (12.5 $mg.mL^{-1}$). Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Staphylococcus epidermidis* were more susceptible than gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*.

HE and EAE extracts showed antimicrobial activity ranging from 12.5 to 100 $mg.mL^{-1}$, classified from high to very low. These extracts show similar antimicrobial properties and phytochemical compounds. HE and EAE extracts showed alkaloids, flavonoids, steroids and saponins, while AE showed a high amount of tannins in its composition (Table II), which may explain the better performance in antimicrobial action (high activity).

In Abdillahi et al. (2008) study, the acetonic extracts obtained from the leaves of four *Podocarpus* species exerted better antimicrobial activity on *Candida albicans* when compared to the other tested extracts (ethanolic, dichloromethane, petroleum ether), through microdilution test. This result corroborates our study, in which *P. lambertii* species also showed better results with the acetonic extract compared to the other extracts tested.

Due to the proven antimicrobial properties of secondary metabolites in plants, it is suggested that the antimicrobial potential of *P. lambertii* plant extracts is related to its phytochemical profile. The flavonoids present in the three extracts tested are hydroxylated phenolic substances with proven antimicrobial activity in the literature. These in turn act in the formation of complexes with extracellular and soluble proteins, which bind to the bacterial cell wall causing irreversible damage to the cell (Samy & Gopalakrishanakone 2010, Toledo 2023).

The tannins present in AE also belong to the group of phenolic compounds. They are

Table III. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of plant extracts obtained from *Podocarpus lambertii* leaves against the pathogenic microorganisms tested.

Microorganism	Extracts MIC/MBC		
Gram negative	**Hexane (HE)**	**Acetone (AE)**	**Ethyl Acetate (EAE)**
S. Enteritidis ATCC 13076	25/100	12.5/25	25/50
S. Typhimurium ATCC 14028	25/25	12.5/25	25/50
Escherichia coli ATCC25922	25/50	3.12/3.12	25/50
P. aeruginosa ATCC 27853	25/25	6.25/6.25	12.5/100
P. mirabilis ATCC 25933	25/50	6.25/200	25/100
K. pneumoniae ATCC13883	25/50	6.25/12.5	12.5/100
S. Abaetetuba ATCC 35640	25/25	12.5/50	25/50
Gram positive			
S. aureus ATCC 25923	6.25/25	1.56/6.25	12.5/25
E. faecalis ATCC 19433	25/50	1.56/6.25	25/100
S. epidermidis ATCC 12228	25/50	1.56/6.25	25/100
B. subtillis CCCD B005	25/50	3.12/6.25	12.5/100
Yeast			
C. albicans ATCC 10231	25/50	12.5/25	25/100

Activity High (≤12.5 mg.mL^{-1}); Moderate (25 mg.mL^{-1}); Low (50 mg.mL^{-1}); Very low (100 to 200 mg.mL^{-1}); Source: authors.

characterized by their astringent properties, inhibiting Gram-positive bacteria that cause food spoilage (*B. subtilis*), contaminated foodborne pathogens (*S. aureus*), as well as Gram-negative bacteria (*E. coli*) (Samy & Gopalakrishnakone 2010, Gyawali & Ibrahim 2014). Their action mode may be related to the formation of complexes with microbial enzymes and proteins, inactivating their functions (Samy & Gopalakrishnakone 2010, Mendez et al. 2012, Gyawali & Ibrahim 2014). Saponins found in AE, HE and EAE have been reported for their antimicrobial potential, acting on the bacterial cell membrane and increasing its permeability (Simões et al. 2004, Gyawali & Ibrahim 2014).

As a rule, all extracts inhibited the growth or caused the death of the pathogenic strains, suggesting that the compounds present in these extracts, especially the phenolic compounds (tannins), play an important antimicrobial role against the tested strains.

Antioxidant activity

The antioxidant capacity of *P. lambertii* extracts was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Table IV). This assay is a direct and reliable method for measuring the anti-radical capacity of plant extracts (Cabana et al. 2013).

EAE extract showed higher DPPH radical scavenging at the concentration of 1 $mg.mL^{-1}$, obtaining an antioxidant percentage of 96.36%, and IC_{50} values considered significantly equal when compared to the commercial antioxidant BHT ($p<0.05$).

The antioxidant activity of EAE is probably associated with the presence of phenolic compounds, since they were found in high amounts as shown in Table II, highlighting alkaloids (322.94 mg AT/g) and flavonoids (140.50 mg QE/g),which have recognized antioxidant activity (Ali et al. 2011). The efficiency of these compounds is linked to hydrogen transfers that neutralize the action of free radicals (Brewer 2011). Flavonoids act as metal chelators and singlet oxygen deactivators, consequently reducing free radicals (Mello & Filho 2002, Boligon 2014).

It is noteworthy that IC_{50} values are inversely proportional to the percentage of DPPH scavenging, and the higher the percentage of scavenging, the lower the IC_{50} value. Therefore, HE and AE extracts require relatively high concentrations to reach the maximum antioxidant potential (80%), thus making their use unfeasible.

AE and HE extracts, despite also having phenolic compounds, except for the tannins present only in AE, did not show significant antioxidant activity, which may be related to their amount of phenolic compounds and/or their type of action to interrupt the free radical chain (Lai et al. 1991).

The results of antioxidant activity reported in the literature are difficult to compare, as they are influenced by the method of determination. Several methods have been described to evaluate the antioxidant activity of chemical compounds present in plant extracts (Molyneux 2004, Pandini et al. 2015).

Evaluation of the activity of plant extracts on preformed bacterial biofilm (irreversible fixation)

HE, AE and EAE extracts were tested at MIC, 2X MIC and 4X MIC concentrations on each bacterial strain evaluated and as there was no statistically significant difference between concentrations, the results expressed refer to the MIC. The data from the colorimetric assays were calculated as overall averages of the optical density (OD) of the biofilm biomass quantified by crystal violet staining and the evaluation of biofilm cell viability was assessed MTT reduction (Table V-VII).

Table IV. Percentage of antioxidant activity of *Podocarpus lambertii* leaf extracts by the DPPH method.

Test solution	% reduction DPPH [1 $mg.mL^{-1}$]	Mean ± standard deviation of % DPPH reduction	IC_{50}
BHT(controle)	98.00	0.01 ± 0.003 a	1.31
EAE	96.35	0.25 ± 0.009 a	1.56
AE	19.38	0.28 ± 0.015 b	3.34
HE	2.84	0.68 ± 0.003 c	8.46

AE=acetone extract; HE= hexane extract; EAE=Ethyl Acetate Extract; mean ± standard deviation. Values followed by the same letter do not differ by Tukey's 5% test. Source: authors.

The results showed that only EAE showed high antibiofilm activity on *Pseudomonas aeruginosa* strain, which can be explained by the high content of total phenolic compounds (111.76 mg GAE/g), flavonoids (140.50 mg QE/g) and alkaloids (322.94 mg AT/g), as described in Table II. HE and EAE extracts showed low inhibition of preformed biofilm biomass (<50%) on *Escherichia coli* strains, 4.85% and 30.99% respectively, and *Pseudomonas aeruginosa* was weakly inhibited by HE (low inhibition). For *Staphylococcus aureus* strains tested against HE and EAE and all strains against AE, biofilm inhibition was not observed, indicating biomass increase.

This increase in biomass can be explained by the fact that bacteria in planktonic form are more susceptible to antimicrobial agents than cells in a biofilm system. Bioactives are not able cross the extracellular matrix of the biofilm easily (Lewis 2001, Sandasi et al. 2008). The inability of AE, HE and EAE extracts to inhibit biomass growth of all strains may be related to several factors, such as the biofilm growth pattern and the required concentration of antibacterial agents. In this state, the concentration of biofilm-producing cells can be 1000 times higher than

Table V. Antibiofilm activity of hexane extract of *Podocarpus lambertii* leaves on cell viability of preformed biofilms of standard bacteria by crystal violet and of 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium (MTT) reduction method.

HE	Violet Crystal Rehearsal			Test with MTT		
Microorganisms	**Control**	**HE**	**%I**	**Controle**	**HE**	**% VC**
Escherichia coli (ATCC 25922)	0.16±0.017	0.15±0.017	4.85	0.05±0.003	0.07±0.022	74.04
Pseudomonas aeruginosa (ATCC 27853)	1.87±0.199	1.42±0.632	24.22	0.05±0.002	0.10±0.026	51.65
Staphylococcus aureus (ATCC 25923)	0.24±0.011	0.43±0.088*	NI	0.06±0.007	0.10±0.006*	62.32

Mean OD ± standard deviation; NI= no inhibition; CV= Crystal Violet; HE = hexane extract; %I = percentage of inhibition; %CV= percentage of cell viability; NI= no inhibition. Values followed * differ by the Tukey Test (p>0.05). Percent inhibition (CV) values: < 50% indicate low antibiofilm activity; > 50% indicate high antibiofilm activity. Negative values indicate an increase in the biomass of the formed biofilm. Percent cell viability (MTT) values: < 50% indicate low cell activity; > 50% indicate high cellular activity.

Table VI. Antibiofilm activity of acetone extract of *Podocarpus lambertii* leaves on cell viability of preformed biofilms of standard bacteria by crystal violet and 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium (MTT) reduction method.

AE	Violet Crystal Rehearsal			Test with MTT		
Microorganisms	**Control**	**AE**	**%I**	**Controle**	**EA**	**% VC**
Escherichia coli (ATCC 25922)	0.16±0.018	0.85±0.214*	NI	0.05±0.003	0.06±0.002	86.83
Pseudomonas aeruginosa (ATCC 27853)	1.87±0.199	2.03±0.685*	NI	0.05±0.002	0.06±0.002*	85.65
Staphylococcus aureus (ATCC 25923)	0.24±0.011	0.51±0.140	NI	0.06±0.007	0.16±0.169	39.69

Mean OD ± standard deviation; CV= Crystal Violet; AE = acetone extract; %I = percentage of inhibition; %CV= percentage of cell viability; NI= no inhibition; Values followed * differ by the Tukey Test (p>0.05). Percent inhibition (CV) values: < 50% indicate low antibiofilm activity; > 50% indicate high antibiofilm activity. Negative values indicate an increase in the biomass of the formed biofilm. Cell viability percentage (TTC) values: < 50% indicate low cell activity; > 50% indicate high cellular activity.

that of bacteria in the planktonic state (Frank & Patel 2007, Hoiby et al. 2010, Pereira 2014, Chen et al. 2018).

Despite the low antibiofilm activity and/or increased biomass of the extracts, low cell viability was observed for the *S. aureus* strain (39.69%) in the AE extract and for the *P. aeruginosa* strain with EAE (44.13%). Probably, the phytochemical compounds were not able to break the polymer matrix of the biofilm and there was no disaggregation of the biomass when stained with crystal violet, but in the MTT assay the antibiofilm potential of these extracts was indeed observed.

The secondary compounds found in the phytochemical screening (Table I) and in the total phenolic compounds content (Table II) of EAE, HE and AE extracts are similar (except for AE, which has a higher tannin content) and have proven antibiofilm activity in the literature. However, in our study, the activity of EAE on the mature biofilm of *P. aeruginosa* strain (Cushnie & Lamb 2011, Gandhi et al. 2017, Nuño et al. 2018) was evidenced. These substances are present in different concentrations, lower or even present a synergistic effect between them, increasing the biomass of the formed biofilm, besides activating genes that will produce excess cellular matrix, suggesting a defense strategy for survival stress situations from the external environmental (Costa et al. 2015). In addition, low penetration of these compounds may occur, or even increased efflux pumps, which expel these antimicrobial agents from bacterial cells (Jamal et al. 2018).

To date there are no reports in the literature regarding the ability of plant extracts from species of the family Podocarpaceae and/or the genus *Podocarpus* to reduce and/or destroy preformed biofilms. This study is the first scientific report on this biological activity of *P. lambertii*.

In summary, the results of the antibiofilm potential tests by the crystal violet assay indicate that EAE presented activity on mature biofilm of *P. aeruginosa* and AE on *S. aureus* and *E. coli*. As for the MTT assay, only AE was effective in reducing biofilm viability for *S. aureus* and EAE for *P. aeruginosa*, causing damage to the cellular activity of the formed biofilms.

CONCLUSION

EAE, AE and HE extracts obtained from *P. lambertii* leaves revealed flavonoids, steroids, tannins, alkaloids and saponins in the phytochemical

Table VII. Antibiofilm activity of ethyl acetate extract of *Podocarpus lambertii* leaves on cell viability of preformed biofilms of standard bacteria by crystal violet and 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium (MTT) reduction method.

EAE	Violet Crystal Rehearsal			Test with MTT		
Microorganisms	**Control**	**EAE**	**%I**	**Controle**	**EAE**	**% VC**
Escherichia coli (ATCC 25922)	0.16±0.018	0.11±0.012*	30.99	0.05±0.003	0.10±0.004*	52.10
Pseudomonas aeruginosa (ATCC 27853)	1.87±0.199	0.66±0.147*	64,75	0.05±0.002	0.115±0.002*	44.13
Staphylococcus aureus (ATCC 25923)	0.24±0.011	0.41±0.142	NI	0.06±0.007	0.10±0.001*	61.28

Mean OD ± standard deviation; NI= no inhibition; CV= Crystal Violet; EAE = ethyl acetate extract; %I = percentage of inhibition; %CV= percentage of cell viability; Values followed * differ by the Tukey Test (p>0.05). Percent inhibition (CV) values: < 50% indicate low antibiofilm activity; > 50% indicate high antibiofilm activity. Negative values indicate an increase in the biomass of the formed biofilm. Cell viability percentage (TTC) values: < 50% indicate low cell activity; > 50% indicate high cellular activity.

prospecting, proving that the plant is an efficient reservoir of bioactive compounds, which add medicinal value to the species. In addition, the tested extracts have antimicrobial and fungicidal activity on the tested standard strains, highlighting the efficacy of AEon all microorganisms. EAE showed antioxidant activity with DPPH radical scavenging percentage of 96.35%, demonstrating the potential to prevent and/or control oxidative stress. The biofilm biomass formation inhibition activity was effective in EAE on *P. aeruginosa*. As for cell viability, AE reduced the metabolic activity of *S. aureus* and EAE of *P. aeruginosa*. Therefore, the results of the extracts obtained from *P. lambertii* leaves represent a source for the manufacture of natural products for the development of new alternative strategies to controlresistant microorganisms.

However, further investigations on its pharmacological properties in vitro and in vivo are needed.

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