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BIOTECHNOLOGICAL APPLICATIONS OF THE MEDICINAL PLANT *PSEUDOBRICKELLIA BRASILIENSIS* AND ITS ISOLATED ENDOPHYTIC BACTERIA

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Abbreviated running headline: *P. brasiliensis* and its endophytic bacteria

Abstract

Aim: This study aimed to isolate *Pseudobrickellia brasiliensis* endophytic bacteria and evaluate the production of hydrolytic enzymes and antibiotics by these bacteria strains. The study also measured the antibacterial activity of *P. brasiliensis.*

Methods and Results: Thirteen endophytic bacteria strains were isolated from stem and leaf fragments of *P. brasiliensis*. Extracellular enzyme production by the isolated endophytic bacteria was evaluated in an agar plate-based assay. The highest protease production was achieved by *Bacillus subtilis* P4 in alkaline medium. Antimicrobial activity of endophytic bacteria and *P. brasiliensis* extracts was investigated using microbroth dilution. An MIC value of 1000 $\mu g.mL^{-1}$ against *Pseudomonas aeruginosa* was found for *Bacillus subtilis* P3, *Bacillus subtilis* P5, *Pseudomonas* sp. P8 and *Pseudomonas* sp. P12. Leaf extract of *P. brasiliensis* showed the highest antibacterial activity against *P. aeruginosa*, with an MIC value of 0.781 $mg.mL^{-1}$.

Conclusions: *P. brasiliensis* is a source of bacterial endophytes, which can produce antibacterial compounds and enzymes. This work also demonstrated the antibacterial potential of *P. brasiliensis.*

Significance and Impact of the Study: This is the first study that revealed the antibacterial activity of *P. brasiliensis* and bioactive metabolite production by *P. brasiliensis* endophytic bacteria.

Keywords: *Pseudobrickellia brasiliensis,* endophytic bacteria, antibacterial activity, minimum inhibitory concentration, hydrolytic enzymes, protease.

INTRODUCTION

Pseudobrickellia brasiliensis (Spreng.) R.M. King & H. Rob. popularly known as "*arnica-do-campo*" or "*arnica-do-mato*", is a native species of the Brazilian Cerrado (Nakajima and Semir, 2016) known for its medicinal properties. It is a shrub or subshrub species, belonging to the Eupatorieae tribe, Asteraceae family (Amorim et al., 2016). The alcoholic extract obtained from

the leaves of that species is used as a topical anti-inflammatory, wound healing, and analgesic agent. *P. brasiliensis* aqueous extract presents anti-inflammatory activity *in vitro* (Almeida et al., 2017). Previous studies with aqueous and organic extracts and with essential oil from aerial parts of *P. brasiliensis* indicated the presence of secondary metabolites of the classes of flavonoids, terpenoids (monoterpenes, sesquiterpenes, diterpenes and triterpenes) and phytosterols (Bohlmann et al., 1984; Amorim et al., 2016; Almeida et al., 2017). In histochemical tests performed on the leaves, the presence of chemotaxonomic markers of the Asteraceae family was detected, especially sesquiterpene lactones and phenolic compounds (Athayde et al., 2019). Despite its medicinal use, the antibacterial potential of the *P. brasiliensis* medicinal plant has not been studied until now.

Some medicinal properties and biological activities initially attributed to plant species were later found to be due to the secondary metabolites produced by their endophytic microorganisms (Kettering et al., 2004). Endophytes are microorganisms that inhabit the internal tissues of plants without causing apparent harm to the plant. The microorganisms residing within plants are increasingly becoming the object of research efforts, especially when the source plant is traditionally used for healing (Martinez-Klimova et al., 2017). Nearly 300,000 plant species that exist on earth are thought to be a host to one or more endophytes (Ryan et al., 2008). These endophytes can be both fungi and bacteria (Reinhold-Hurek and Hurek, 2011; Singh et al., 2011). It is well known that plant-associated microorganisms produce a variety of metabolites with novel structures and interesting biological activities (Martinez-Klimova et al., 2017; De-Silva et al., 2019). Endophytes secrete antibiotics or hydrolytic enzymes to prevent colonization of microbial plant pathogens (Alvin et al., 2014). The novel antimicrobial metabolites from endophytes are now becoming an alternative to overcome the increasing levels of drug resistance (Ferlay et al., 2010; Taechowisan et al., 2012).

Considering that only a small proportion of the existing endophytic bacteria have been studied, especially those growing in tropical plants from Brazil, this paper focused on the investigation of the endophytic bacteria living in the tissues of *P. brasiliensis* as source of hydrolytic enzymes and antibiotics. In the present study, the antibacterial activity of the medicinal plant *P. brasiliensis* was also investigated.

MATERIALS AND METHODS

Plant sampling and study area

Ten individuals of the medicinal plant *P. brasiliensis* were selected for isolation of the endophytic bacteria. Samples were collected at the Juscelino Kubitschek (JK) Campus, belonging to the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM). Campus JK is located in Diamantina, Minas Gerais, Brazil and is inserted in *Serra do Espinhaço*. The native vegetation cover is made up of Cerrado, Campos Rupestres and Matas de Galeria. The collection was carried out from June 28 to September 10, 2013. Leaves and stems of each individual were collected in three locations, situated in the following geographical coordinates obtained by the Global Positioning System, GPS: *I)* S 18º 12029 'W 43º 34607 '; *II)* S 18 ° 12156 ° W 43 ° 34245 °; *III)* S 18 ° 12 152 ° W 43 ° 34 532 °. The samples were transported in properly identified plastic bags and kept under refrigeration (2 °C to 8 °C) until processing. A representative exsiccate of the plant material was deposited (HDJF-2817) at the UFVJM Jeanine Felfili Dendrological Herbarium (HDJF), where the species was identified. The use of *P. brasiliensis* was authorized by the Brazilian Genetic Patrimony Management Council (CGEN) under the registration number A17AB0D.

Isolation of endophytic bacteria

The leaves and stems were washed with neutral detergent and sterile distilled water. Three stem and leaf fragments (8 mm) were removed from each individual plant with the aid of scissors and forceps. These fragments were disinfected by immersion in 70% alcohol (2 minutes), 2% sodium hypochlorite (3 minutes) and sterile distilled water (2 minutes). The effect of surface sterilization was checked by spreading the final rinse water (200 μL) onto TSA plates and culturing at 35 °C for 48 h. After disinfection, the fragments were plated onto tryptic soy agar (TSA) plates and incubated at 35 °C for 3 days. Different colonies were selected and streaked on TSA plates to check the purity and preserved at -20 °C in nutrient broth with 30% (v/v) glycerol.

Molecular identification of the endophytic bacteria

Classification of the bacteria that produced bioactive compounds was based on the analysis of 16S rRNA gene sequencing using the universal primers 27 F/1492R (Penido et al., 2018). For DNA extraction, a loopful of freshly grown bacterial cells was dissolved in tubes with 100 μL of Tris–EDTA (TE). Then, 100 μL of phenol–chloroform–isoamyl alcohol (25:24:1) and approximately 0.3 g of glass beads were added. This mixture was vortexed for 3 minutes and spun down for 5 minutes at 18,928 *g*. The upper aqueous phase was transferred to a new tube, and (v/v) of 960

mL.L^{-1}ethanol was added. The samples were again centrifuged at 18,928 *g* for 5 minutes, and the supernatant was discarded. The pellets were dried overnight at room temperature and then eluted in 100 µL of TE buffer. The concentration of genomic DNA was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000; NanoDrop™, USA). The amplification of the 16s RNA gene was done using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Total DNA (50-500 ng) was added to the PCR mix (50 µL), which contained 5 µL of 10× buffer, 1 µL of 0.05 mmol dNTPs L^{-1}, 3 µL of 1.5 mmol $MgCl_2$ L^{-1}, 1 µL of each primer, 0.2 µL of 1 U Taq DNA polymerase and 1 µL of DNA in a total volume of 50 µL. This reaction was carried out with the following conditions: one cycle of 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 55° C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplified DNA products were separated by agarose gel electrophoresis, stained with GelRedTM solution (Biotium, USA) and visualized under UV-light. The amplified DNA was concentrated, cleaned and sequenced by capillary electrophoresis in ABI3130 equipment using POP7 polymer and BigDye v3.1. The sequences were assembled, edited, and aligned using the program MEGA7 (Kumar et al., 2016) and were compared with those in the GenBank database using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the sequence homology with closely related organisms (Altschul et al., 1997). In the present study, the microorganisms with high level of identity (100%) were selected as the closest match, and all bacterial isolates were respectively classified to the genus level according to the information of the closest microbes.

Screening for endophytic bacteria enzyme production

All endophytic bacterial isolates were characterized for hydrolytic enzyme production, such as protease, cellulase, amylase, lipase and esterase, which were detected on respective agar plates with variable substrates. For protease, cellulase, amylase, lipase and esterase, the substrates, namely, casein (Frazier and Rup, 1928), carboxy methyl cellulose (Kasana et al., 2008), starch, tween 20 (Hankin and Anagnostakis, 1975) and tween 80 (Sierra, 1975), respectively, were used. A volume of 10 µL of each bacterial isolate (that had been cultured in sterile distilled water for 48 hours) was plated on specific agar plates that were incubated at 30 °C for 48 h. Development of the halo zone around the bacterial colonies was considered as enzyme production for protease, lipase or esterase. Cellulase production was confirmed through staining with 0.1% Congo red

solution and destaining with 1 mol NaCl L^{-1} for 15 min (Stamford et al., 1998; Vijayalakshmi, et al., 2016). Amylase agar plates were flooded with 1% iodine in 2% potassium iodide to confirm (Vijayalakshmi, et al., 2016). The enzymatic index (IE), which is obtained through the relation between the diameter of the halo of degradation and the diameter of the colony, was used for the quantification of the extracellular enzymatic activity.

Screening for endophytic bacteria protease production

Endophytic bacterial isolates that showed an enzymatic index ≥ 1 for protease production were tested by the cup plate method according to Dingle and Solomons (1953) to discover the alkaline, acidic or neutral character of the protease produced in an induction medium. Endophytic bacterial isolates were standardized on the McFarland scale 3 to obtain a final inoculum of 9 x 10^8 cells.mL^{-1}. A volume of 50 µL of the standardized bacterial solution was put into test tubes containing 5 mL of induction medium containing casein 1.0 g.L^{-1}, ammonium phosphate 7.0 g.L^{-1}, bibasic potassium phosphate 1.5 g.L^{-1}, magnesium sulfate 0.5 g.L^{-1}, calcium chloride 0.3 g.L^{-1}, trace element solution 2.5 mL.L^{-1} (ferrous sulfate 0.01 g.L^{-1}, manganous chloride 0.1 g.L^{-1}, zinc sulfate 0.1 g.L^{-1}), pH 7.0 at 28 °C and 150 rpm, for 48 h (Wang et al., 2008 with modifications). A volume of 1 mL of the culture in induction medium was centrifuged at 11,400 g for 10 minutes at 4 °C for separation of the biomass, and 150 µl of the supernatant was transferred to 6 mm diameter cup plates drilled on the surface of solid culture medium (containing the similar composition of the induction medium, added with 2% agar), calibrated to a pH 5.0, 7.0 or 9.0. Plates were incubated at 37 °C for 24 h. Development of the halo zone around the application indicated protease production (Wang et al., 2008). All tests were performed in triplicate.

Antibacterial activity of endophytic bacteria

Cultivation and extraction of endophytic bacterial metabolites

Thirteen isolates of endophytic bacteria from the leaves and stems of *P. brasiliensis* that were previously cultured on TSA at 30 °C for 48 hours were used to perform the fermentation. A loopful of freshly grown bacterial cells from each bacterial isolate was inoculated into 100 mL tryptic soy broth (TSB), followed by incubation for 7 days at 30 °C and shaking at 125 rpm. Extraction of secondary metabolites was performed using the method described by Campos et al. (2015), with modifications. After 7 days of fermentation, 100 mL of ethyl acetate was added to extract the metabolites. This procedure was performed three times to obtain maximum extraction.

The samples were then evaporated and dried in a circulating air oven at 40 °C. After drying, the residues were dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 20 mg. mL^{-1}.

Determination of the minimum inhibitory concentration of endophytic bacterial extracts

Antimicrobial activity was evaluated using the following microorganisms: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213. Bacteria were grown on TSA plates at 35 °C for 24 h, and their inocula were adjusted in saline solution to obtain a concentration of 1-2 x 10^8 colony-forming units (CFU).mL^{-1} according to the spectrophotometric method prescribed by CLSI M7-A6 (NCCLS 2003). The bacteria inoculum was obtained by diluting the bacterial saline suspension with Mueller Hinton Broth (Himedia, India) to a 1 x 10^7 CFU.mL^{-1} concentration.

The minimum inhibitory concentration (MIC) of endophytic bacteria extracts was determined using the microbroth dilution method following the CLSI M7-A6 (NCCLS 2003, Campos et al., 2015). Three serial dilutions (500, 1000 and 2000 µg.mL^{-1}) of bacterial endophytic extracts were prepared using Mueller-Hinton broth (Himedia, India) as the diluent. For each dilution, aliquots of 100 µL were distributed in the microplates. At the end of the test, a concentration of 250 µg.mL^{-1}, 500 µg.mL^{-1} and 1000 µg.mL^{-1} of the extracts was obtained because 100 µL of inoculum was added to each well. For growth and sterility control, media alone with inocula and media with extract were used, respectively. As a control for solvent toxicity, a culture with 1% (v/v) DMSO (Sigma, St. Louise, MO, USA) was made. Chloramphenicol (Sigma-Aldrich) (15 µg.mL^{-1}) was used as the positive antibacterial control. Microplates were incubated at 37 °C for 24 h, and the MIC was recorded as the least concentration of endophytic bacteria extract that showed no visible bacterial growth which was detected by comparing the growth in the sample wells to the growth in extract-free control wells. MIC measurements are expressed in µg.mL^{-1}.

Antibacterial activity of *P. brasiliensis*

Preparation of *P. brasiliensis* alcoholic extracts

After collection, the aerial parts of the plant were dried in a circulating air oven at 40 °C to a constant weight. The leaves were separated from the stems, and then the dried plant materials were milled into powder using a knife mill (Marconi ®, Piracicaba, Brazil). Each powdered aerial part of the plant was macerated in ethanol (Dinâmica ®, Diadema, Brazil) at a ratio of 1/10 w/v, for 72 h. Stem (SE) and leaf (LE) extracts were filtered through cotton wool, concentrated in a rotary

evaporator (Fisatom ®, São Paulo, Brazil) at 40–42 °C under reduced pressure and stored in glass vials. For the evaluation of antibacterial activity, SE and LE were dissolved in sterile DMSO (Sigma, St. Louise, MO, USA) at a stock concentration of 50 $mg.mL^{-1}$.

Determination of the minimum inhibitory concentration of *P. brasiliensis* extracts

The MIC of SE and LE was determined using CLSI guidelines of M7-A6 (NCCLS 2003). Solutions of the extracts were prepared by mixing the plant extract DMSO stock concentration of 50 $mg.mL^{-1}$ with Muller-Hinton broth (Sigma-Aldrich, Steinheim, Germany). Serial dilutions were prepared using the corresponding media as the diluents. For each dilution, aliquots of 100 µL were distributed in the microplates. The concentrations of the plant extracts used were 0.78, 1.0, 1.56, 3.12, 6.25, 12.5 and 25 $mg.mL^{-1}$. In addition, 100 µL of *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 inoculum were added separately to each well at 1×10^7 $CFU.mL^{-1}$. The same controls used in the antibacterial activity of endophytic bacteria test were used in this test. The microplates were then incubated at 37 °C for 24 h. After that, 10 µL of 0.01% sodium resazurin was added to each well. The presence of growth after one hour was detected visually; the blue colour characterized the bacterial inactivity and the red characterized the bacterial activity.

Statistical analysis

The samples were tested in triplicate. Values represent the mean of three replicates. One-way analysis of variance (ANOVA) was performed, and Tukey's multiple comparisons of means test was used to identify differences between the means. The statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), version 7.03. Statistical significance was set at $p < 0.05$.

RESULTS

Isolation and molecular identification of endophytic bacteria from *P. brasiliensis*

Thirteen endophytic bacteria strains were isolated from leaves and stems of *P. brasiliensis.* From the thirteen endophytic bacteria isolates, we identified those that presented antimicrobial activity and greater production of enzymes (Table 1). Based on 16S rRNA gene sequences, the closest related species were achieved by BLAST analysis. Bacteria endophyte isolates P3, P4 and P5 were

identified as *Bacillus subtilis* P3, *B. subtilis* P4 *and B. subtilis* P5, respectively. Endophyte isolates P8 and P12 were identified as *Pseudomonas* sp. P8 and *Pseudomonas* sp. P12, respectively. The sequences obtained in this study were deposited in GenBank with accessions MK883236 - MK883239 and MN308428 (Table 1).

Enzymatic activities of endophytic bacteria

The thirteen isolated bacteria from *P. brasiliensis* were tested for protease, cellulase, esterase, amylase and lipase production. Only 15% of the isolates produced cellulase or lipase in agar plate-based assay (Table 2); 61% produced protease, 69% produced esterase and none of the isolated bacteria produced amylase. Most of the isolates (53%) produced protease and esterase.

The enzymatic index (IE) was used for the quantification of the extracellular enzymatic activity in solid medium. It was considered a moderate reaction when the extracellular enzyme ratio was less than 2 but greater than 1 and weak reaction when the ratio was equal to or less than 1. Six bacteria isolates (P2, P4, P6, P9, P10 and P13) showed moderate protease production, with enzyme production ratios of 1.30, 1.17, 1.62, 1.29, 1.31 and 1.50, respectively (Table 2). One bacterial isolate (P7) presented moderate esterase production with a value of 1.43, and only two bacteria isolates produced cellulase and lipase with weak enzyme production. The other isolates showed weak enzymatic reactions (Table 2).

Most of the isolates that presented a moderate enzyme production (with an extracellular enzyme ratio between 1 and 2) produced proteases (85%). Therefore, six isolates that presented moderate protease production were tested by the cup plate technique in solid medium after the induction of protease production in liquid medium containing casein. Of those six isolates tested, only one bacterial isolate (P4) produced proteases on casein solid-state medium at pH 5.0, 7.0 and 9.0 (Table 3), and this bacteria was identified as *Bacillus subtilis* P4. The other four bacteria isolates tested showed no protease production in solid medium containing casein at the different pH values tested. Protease production by *Bacillus subtilis* P4 increases with an increase in pH (Table 3). The clear zone diameter varies from 1.9 cm (in the medium with pH of 5.0) to 3.0 cm (in the medium with pH of 9.0).

Antimicrobial activity of endophytic bacteria

The ethyl acetate extracts of 13 bacteria isolates from *P. brasiliensis* were tested by an *in vitro* biological assay to predict their antimicrobial activity. *Bacillus subtilis* P5 showed antibacterial

activity against Gram-positive (*S. aureus*) and Gram-negative bacterial species (*E. coli* and *P. aeruginosa*), with an MIC value of 1000 μg.mL^{-1} (Table 4). *Pseudomonas* sp. P12, *Bacillus subtilis* P3 and *Pseudomonas* sp. P8 showed an MIC value of 1000 μg.mL^{-1} against *P. aeruginosa.* Of the four isolates that showed antibacterial activity, only *Pseudomonas* sp. P12 produced one of the enzymes tested, producing esterase.

Antimicrobial activity of *P. brasiliensis* extracts

The alcoholic extracts from stems (SE) and leaves (LE) of *P. brasiliensis* showed antibacterial activity against all tested organisms, such as *E. coli*, *P. aeruginosa* and *S. aureus*. An MIC value of 12.5 mg.mL^{-1} was found for the stem and leaf alcoholic extracts of *P. brasiliensis* against the Gram-negative bacteria *E. coli* (Table 5). *P. aeruginosa* ATCC 27853 was more susceptible to the leaf extract of *P. brasiliensis* (MIC = 0.781 mg.mL^{-1}) than to the stem extract (with MIC of 6.25 mg.mL^{-1}). Both extracts had activity against the Gram-positive bacteria *S. aureus*, with MICs of 6.25 mg.mL^{-1}.

DISCUSSION

Endophytic bacteria were isolated from collected *P. brasiliensis*, individually. According to Partida-Martínez and Heil (2011), endophytic bacteria have been found in every plant species that has been studied, as occurred in this study. The type of endophytic community of a plant is strongly influenced by the nature of the plant host species (Ding and Melcher, 2016).

Endophytic bacterial diversity has been reported for several plant species (Miliute et al., 2015; Santoyo et al., 2016). The most common isolated bacterial genera are *Bacillus, Burkholderia, Microbacterium, Micrococcus, Pantoea, Pseudomonas* and *Stenotrophomonas* (Hallmann et al., 1997; Chaturvedi et al., 2016). Among the thirteen endophytic bacteria that presented antibacterial activity and greater enzyme production, three isolated bacteria belonged to the *Bacillus subtilis* species and two species to *Pseudomonas* spp. Studies have reported that *Bacillus* spp. and *Pseudomonas* spp. are the major endophytic bacteria found associated in most plants (Hallmann et al., 1997). The dominant status of *Bacillus* species might be due their inherent capacity of production of stable endospores, which persist for long periods in many microenvironments associated with plants (Chen et al., 2014; Hu et al., 2014; Zhao et al., 2014). *Pseudomonas* species

are ubiquitously found in plants, and members of this species have a broad metabolic versatility. *Pseudomonas* spp. strains with plant growth-promoting traits have been identified among the endophytes of different herbaceous and woody plants (Campisano et al., 2015; Wu et al., 2016).

All the endophytic bacteria isolated from *P. brasiliensis* produced at least one of the studied enzymes, except for *Bacillus subtilis* P3, *Bacillus subtilis* P5 and *Pseudomonas* sp. P8. Hydrolases, the extracellular enzymes produced by endophytic bacteria, help the plants to establish systemic resistance against pathogen invasion (Singh et al., 2017).

Bacillus subtilis P4 produced proteases at pH 9.0, and this may be indicative that the proteases produced have better activity at alkaline pH. These results are in accordance with several previous reports showing optimal activity at alkaline pH (between 8 and 12) for alkaline proteases from microorganisms (Nadeem et al., 2013). Dorra et al (2018) identified *Bacillus halotolerans*, a protease-producing strain CT2 isolated from Tunisian potatoes, which exhibits an optimal protease activity at pH 9.0. Microbial proteases have attracted considerable attention and account for approximately 60% of the total enzyme market (Cheng et al., 2010). Proteases are classified into acid, neutral and alkaline considering their optimal pH, and alkaline proteases alone constitute approximately 89% of the total protease sales (Ibrahim et al., 2015).

The endophytic bacteria belonging to genera *Bacillus* (P3) and *Pseudomonas* (P8 and P12) showed antimicrobial activity against *P. aeruginosa* ATCC 27853. Furthermore, *Bacillus subtilis* P5 presented antimicrobial activity against the three bacteria species tested (*Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213). In recent years, *Bacillus* spp. have received much attention because they are well-known antibiotic producers, and various *Bacillus* species have been found to control diverse phytopathogenic fungi and bacteria, such as *Fusarium graminearum* (Dunlap et al., 2013; Zhao et al., 2014), *S. sclerotiorum* (Chen et al., 2014; Hu et al., 2014; Sun et al., 2017), and *Xanthomonas oryzae* (Lin et al., 2001). The endophytic *Pseudomonas* of plant origin has shown promising broad spectrum of activity against several soil borne pathogens. There is evidence that clearly indicates that endophytes are a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential (Azevedo et al. 2000; Ryan et al. 2008).

The alcoholic extracts from stem and leaf of *P. brasiliensis* showed antibacterial activity, as occurred with its endophytic bacteria. *Chaptalia nutans*, another species in the Asteraceae family, likewise showed antibacterial activity against Gram-positive bacteria (Truiti et al, 2003). The methanol extract and the isolated compounds of *Solidago microglossa* (Asteraceae) had inhibitory

activity (MIC > 1 mg.mL^{-1}) against *S. aureus, S. epidermidis, Klebsiella pneumoniae, E. coli, Salmonella setubal, B. subtilis, P. aeruginosa, Saccharomyces cerevisiae* and *Candida albicans*. Furthermore, its essential oil effectively inhibited the growth of all of these organisms (Morel et al., 2006).

P. brasiliensis leaf alcoholic extracts (LE) had a lower MIC value against *P. aeruginosa* than the ethyl acetate extracts of *P. brasiliensis* endophytic bacteria. However, the endophytic bacteria extracts showed lower MIC values against *E. coli* and *S. aureus* than *P. brasiliensis* alcoholic extracts. Janso and Carter (2010) suggested that it is possible that horizontal gene transfer occurs between the cells of a plant and the microorganisms that inhabit the plant. Thus, both the endophytic microorganism and its host plant can produce substances that have antibacterial activity, as occurred in this study.

The chemical constituents present in the extracts of *P. brasiliensis* belong to classes of secondary metabolites that have reported antimicrobial activity (Silva and Fernandes, 2010; Compean and Ynalvez, 2014). Thus, the antimicrobial results observed in this study may be related, at least in part, to the presence of phenolic compounds and terpenoids.

The present work is the first report that demonstrates *in vitro* antibacterial activities of the medicinal plant *P. brasiliensis.* In this study, the antibacterial property of *P. brasiliensis* seems to have justified its use for the treatment of infected wounds.

Scientific discoveries contribute to enhance the value of biodiversity: new bioactive drugs and new microorganisms are waiting to be discovered. When ethnobotanical knowledge is taken seriously at the time of selecting a medicinal plant to collect, the reward frequently is the isolation of endophytes and plants that produce bioactive compounds.

All observations in this study showed that the medicinal plant *P. brasiliensis* is a source of bacterial endophytes, which can produce bioactive compounds with antibacterial properties and enzymes. This work also demonstrated the antibacterial potential of the medicinal plant *P. brasiliensis* for the first time. It has been reported that bacterial endophytes may produce the same or similar bioactive compounds as their host plant. The present findings corroborate the statements that microorganisms that inhabit the interior of medicinal plants, especially those used for pain, such as *P. brasiliensis*, produce bioactive metabolites secreting antibiotics and hydrolytic enzymes. The antibacterial compounds obtained from *P. brasiliensis* and *P. brasiliensis* bacterial endophytes can be used for the development of new drugs.

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CONFLICT OF INTEREST:

No conflicts of interest declared.

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Table 1- Identification of endophytic bacteria isolated from *P. brasiliensis* (Asteraceae) by sequence analysis of the 16S rRNA gene using the primers 27F and 1492R

WC*	Closest related species	Similarity (%)	Base pairs analysed (n)	Identification and GenBank accessions
P4	*Bacillus subtilis*	100	364	*B. subtilis* [MN308428]
P3	*Bacillus subtilis*	100	511	*B. subtilis* [MK883236]

P5	*Bacillus subtilis*	100	388	*B. subtilis* [MK883237]
P8	*Pseudomonas* sp.	100	526	*Pseudomonas* sp. [MK883238]
P12	*Pseudomonas* sp.	100	464	*Pseudomonas* sp. [MK883239]

*WC: working code

Table 2- Extracellular enzyme production by the isolated endophytic bacteria from *P. brasiliensis* in agar plate-based assay

Endophytic bacteria	**Enzyme production ratio**				
	Protease	**Cellulase**	**Esterase**	**Amylase**	**Lipase**
P1	0.59	0.80	0.39	-	0.66
P2	1.30	-	0.28	-	-
Bacillus subtilis P3	-	-	-	-	-
Bacillus subtilis P4	1.17	-	0.21	-	-
Bacillus subtilis P5	-	-	-	-	-
P6	1.62	-	-	-	-
P7	-	0.13	1.43	-	-
Pseudomonas sp. P8	-	-	-	-	-
P9	1.29	-	1.00	-	-

P10	1.31	-	0.50	-	-
P11	0.48	-	0.48	-	0.33
Pseudomonas sp. P12	-	-	0.42	-	-
P13	1.50	-	0.46	-	-

All values are means of three replicates. Enzyme production ratio = the ratio of clear zone diameter to that of colony diameter. The extracellular enzymatic reactions were classified into the following four types: (i) strong reaction, the extracellular enzyme ratio was greater than or equal to 2; (ii) intermediate reaction, the extracellular enzyme ratio was less than 2 but greater than 1; (iii) weak reaction, the extracellular enzyme ratio was equal to or less than 1; and (iv) no reaction, there was no reaction at all or the enzyme ratio was 0.

Table 3- *Bacillus subtilis* P4 protease production in solid medium containing casein at different pH values.

pH	**Clear zone diameter (cm)**
5.0	$1.9 \pm 0.4^{a,b}$
7.0	$2.6 \pm 0.5^{b,c}$
9.0	3.0 ± 0.0^{c}

The values are the average of three replicates ± standard deviation.

Different letters indicate significant differences ($p < 0.05$, ANOVA with Tukey`s test).

Table 4- *In vitro* antimicrobial activities of extracts of endophytic bacteria from *P. brasiliensis* (Asteraceae).

Bacteria isolate	**Microorganisms**		
	Minimal inhibitory concentration (MIC) ($\mu g.mL^{-1}$)		
	E. coli	***P. aeruginosa***	***S. aureus***
Bacillus subtilis P5	1000	1000	1000
Pseudomonas sp. P12	-	1000	-
Bacillus subtilis P3	-	1000	-
Pseudomonas sp. P8	-	1000	-
Control			
Chloramphenicol	15	15	15

-: inactive

Table 5– Minimum inhibitory concentration (MIC) of the alcoholic extracts of *P. brasiliensis* against tested organisms.

Part used	MIC ($mg.mL^{-1}$)		
	E. coli	***P. aeruginosa***	***S. aureus***
Stems (SE)	12.5	6.25	6.25
Leaves (LE)	12.5	0.781	6.25
Control			
Chloramphenicol	0.015	0.015	0.015