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Biotechnological potential of actinobacteria isolated from rhizosphere of the medicinal plant, *Ipomoea pes-caprae* (L.) R. Br.

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The aim of the present study was to evaluate the antimicrobial activity and the production of L-asparaginase by actinobacteria isolated from the rhizosphere of the medicinal plant, *Ipomoea pes-caprae* (L.) R. Br., and to conduct a comparative chemical characterization of the methanolic extract from leaves and the most active micro-organism. After isolation, the antimicrobial activity of actinobacteria from rhizosphere were evaluated against *Candida albicans* UFPEDA 1007, *Bacillus subtilis* UFPEDA 86, *Escherichia coli* UFPEDA 224, *Staphylococcus aureus* UFPEDA 02 and clinically isolated *S. aureus* UFPEDA 705. The chemical characterization was conducted by thin layer chromatography for the following groups: triterpenes, steroids, mono- and sesquiterpenes, alkaloids, saponin, coumarin, flavonoids, phenylpropanoids, cinnamic acid derivatives. Finally, a qualitative assay was carried out to evaluate the production of L asparaginase. Among all isolated actinobacteria, the strain *Nocardia* sp. A94 was the most active against Gram positive bacteria, including the clinical isolate (inhibition diameter zone of 23 mm). Additionally, mono/sesquiterpene groups were detected in its methanolic extract, as well in the extract from *I. pes-caprae*. On the other hand, the production of the L-asparaginase was confirmed in 55.17% of tested actinobacteria. The results show the biotechnological potential of actinobacteria from the rhizosphere of *I. pes-caprae* as producers of antimicrobial compounds and L-asparaginase, both activities can be explored for pharmaceutical, cosmetic and food industries.

Key words: Actinobacteria, monoterpene, *Norcadia* sp., rhizosphere, L-asparaginase.

INTRODUCTION

Brazil is home to 20% of the world's biodiversity and is a varied source of bioactive materials in various fields (Azuma, 2002; Silva et al., 2013a). The rhizosphere is a region distinct from the soil, in which there is a diverse and complex interaction between soil, roots and organisms (Ambardar and Vakhlu, 2013). Through this relation, micro-organisms find the substrates necessary for their proliferation and each plant root exudates has a selective effect on its surrounding microbial population (Hartmann et al., 2009). Medicinal plants may thus host a diverse range of micro-organisms that produce bioactive substances, such as antibiotics, anti-tumor agents, immunosuppressants and enzymes (Li et al., 2008, Zhu et al., 2009).

Rhizospheric micro-organisms include actinobacteria, which make up a considerable proportion of the micro-organisms in the soil (10^4 to 10^6 spores per gram of earth) and display a great variety of morphologies (Raju et al., 2010). This group of bacteria is especially important due its capacity to produce bioactive compounds, especially antimicrobials (Bérdy, 2005). Another example is the production of the L-asparaginase enzyme, which converts L-asparagine into L-aspartic acid and ammonia and it has been used as chemotherapy drug to treat acute lymphoblastic leukemia (Dejong, 1972, Sarquis et al., 2004).

The medicinal plant *Ipomoea pes-caprae* (Convolvulaceae) is commonly known as 'morning glory' and it is native to the *restingas* of sandy dunes on the coast of Africa, Asia and Brazil (Wasuwat, 1970; Lorenzi and Matos, 2002). Its use in folk medicine comes from the habits of the Australian aborigines who used to heat the leaves and apply them on boils (Wasuwat, 1970). The microbial environment of the rhizosphere soil habitat of *I. pes-caprae* has not been the subject of much research and it is therefore interesting to attempt characterization and identification of the micro-organisms present in this rhizosphere and their biotechnological applications.

The aim of this present study was to evaluate the antimicrobial activity and the production of L-asparaginase by actinobacteria isolated from the rhizosphere of *I. pes-caprae*, and to conduct a comparative chemical characterization of the methanolic extract from leaves and the most active micro-organism (*Nocardia* sp. A94).

MATERIALS AND METHODS

Sample collection

The rhizosphere and leaves of *I. pes-caprae* were collected in January 2012 on Forte Orange Beach, in the city of Itamaracá (7°48'38''S, 34°50'27''W), in the Brazilian State of Pernambuco. A sample of *I. pes-caprae* (leaves and stem) was deposited in the herbarium of the Agronomic Institute of Pernambuco (Instituto Agronômico de Pernambuco, Brazil; voucher number 85782) and the remaining leaves were washed and subsequently divided up and prepared for the extraction process.

Isolation of Actinobacteria

A sample (10 g) of soil was mixed with 90 mL of 0.9% (w/v) NaCl. The pH (5.6) of this solution was measured and it was agitated mechanically for 20 min and then heated in a water bath to 50°C (Marroni and Germani, 2011). Portions (1 mL) of soil suspensions were transferred to 9 mL of sterile water (diluted 10^{-1}) and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (King et al., 1954). Shortly thereafter, 200 µL of the dilutions was inoculated in Petri dishes containing 20 mL of culture medium: Tryptone yeast extract - ISP_2 (Pridham and Gottilieb, 1948) and Czapek Dox (CD) Agar medium (sucrose 30.0 g, $NaNO_3$ 3.0 g, K_2HPO_4 1.0 g, $MgSO_4$ x $7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, Agar 15.0 g, H_20 1000 mL, pH 6.6), with added antifungal agent Cyclohexamide 50 µg/mL. The test was carried out in triplicate for each culture medium. One triplicate was prepared with distilled water and in another the medium was enriched with 0.3% NaCl, producing saline Czapek Dox Agar medium. After inoculation, the dishes were incubated in a bacteriological dryer at 37°C for seven days.

Antimicrobial assays

Primary test of antimicrobial activity: "Gelose block"

The test of antimicrobial activity was carried out in accordance with Ichikawa et al. (1971), using micro-organisms from the UFPEDA collection: *Candida albicans* UFPEDA 1007, *Bacillus subtilis* UFPEDA 86 (ATCC 6633), *Escherichia coli* UFPEDA 224 (ATCC 25922), *Staphylococcus aureus* UFPEDA 02 (ATCC 6538) and the clinical isolate *S. aureus* UFPEDA 705.

Semi-solid fermentation of Actinobacteria

The strain which showed the best antimicrobial activity was identified by macro- and micro morphological analysis using the technique described by Shirling and Gottlieb (1966). This strain (*Nocardia* sp. A94) was submitted to semi-solid fermentation using sterile parboiled rice as substrate (Marinho et al., 2005). Twenty small plugs of ISP-2 medium containing aerial mycelium of the *Nocardia* sp. A94 strain were transferred to the twenty-one 500 mL Erlenmeyer flaks containing the solid rice medium (90 g of parboiled rice and 90 mL of distilled water with 0.3% NaCl) and incubated in B.O.D. at 30°C for 21 days (Borges and Pupo, 2006).

Then, cold methanol (150 mL) was added to the cultures of *Nocardia* sp. A94. After 24 h, the extract was filtered in a vacuum. Then a further 100 mL of cold solvent was added to the cultures and they were filtered again. The filtrates were concentrated in a rotary evaporator at 45°C (Borges and Pupo, 2006).

Preparation of plant extract

After drying at room temperature for one week, the sample (leaves) was reduced to small fragments. A total of 5 g of dried leaves was submitted to methanolic extraction for 24 h and filtered with Whatman no.1 paper. The solvent was then removed under reduced pressure in a rotary evaporator at 45°C.

Chemical prospection

The extracts obtained were analyzed using thin layer chromatography (TLC) (Kieselgel 60, 0.2 mm, Merck), with adequate systems and markers for each metabolite group (Harborne; 1998, Wagner and Bladt. 1996) (Table 1).

Qualitative test of production of antitumor L-asparaginase enzyme

The experiment was carried out using the rapid plate test to track

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Table 1. Chromatographic conditions of chemical prospection of methanolic extracts of *Nocardia* sp. A94 and *I. pes-caprae*.

Metabolite	Mobile phase	Marker	Standard	Reference
Triterpenes and steroids	Toluene and ethyl acetate	Lieberman-Burchard	β - sitosterol	Sharma and Dawra, 1991
Monoterpenes and sesquiterpenes	Toluene and ethyl acetate	H_2SO_4, Sulfuric Vanillin	Thymol	Wagner and Bladt, 1996
Alkaloids	Ethyl acetate, formic acid, acetic acid and water	Dragendorff	Pilocarpine	Wagner and Bladt, 1996
Coumarin, flavonoids, phenylpropanoids and cinnamic derivatives	Ethyl acetate, acetic acid, formic acid and water	NEU	Rutin	Wagner and Bladt, 1996

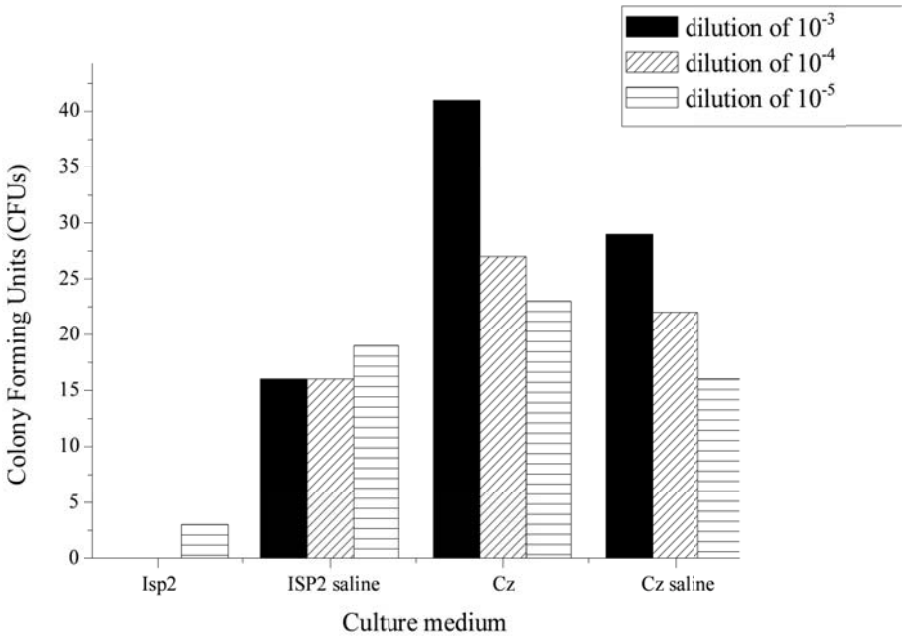

Figure 1. Report of colony forming units of isolated actinobacteria in different culture media ISP_2, ISP_2 saline, Czapek and saline Czapek.

for *in vitro* L-asparaginase production where a modified M-9 medium was prepared, produced with g/L of: $NaHPO_4.2H_2O$ (6.0); KH_2PO_4 (3.0); NaCl (0.5); $CaCl_2.2H_2O$ (0.014); $MgSO_4.7H_2O$ (0.5); glucose (2.0); L-asparagine (5.0); 0.25% ethanolic solution of phenol red (0.09) and agar (17). Phenol red coloring was used in the culture medium as an indicator of changes in pH caused by cleavage of L-asparagine into aspartic acid, changing the color from orange to pink (Gulati et al., 1997). The actinobacteria were incubated at 30°C in a Biological Oxygen Demand (BOD) dryer for seven days.

RESULTS AND DISCUSSION

Isolation and antimicrobial activity of Actinobacteria strains

It was possible to quantify 212 colony forming units (CFUs) in the rhizosphere of *I. pes-caprae* at dilutions of 10^{-3}, 10^{-4} and 10^{-5}. Of these, 81 were isolated. Czapek Dox Agar culture medium was the best for isolating colonies at all dilutions (Figure 1), with the highest number of colonies (41 at a dilution of 10^{-3} = 41 x 10^3) as shown in Figure 1. Of these 81 actinobacteria, only 29 grew with aerial mycelium after isolation.

The rhizosphere is a region of the soil that is influenced by the roots of plants and is characterized by a high level of microbial activity (Hartmann et al., 2009; Lin et al., 2010). The presence of actinobacteria has also been reported for rhizosphere of some medicinal plants such as *Rumex patientia* (Qi et al., 2012), *Crocus sativus* (Hainan and Vakhlu, 2012) and some Bangladesh medicinal plants (Ara et al., 2013). For example, a huge population of actinobacteria (168 x 10^3 CFU per gram of soil) where found in these Bangladesh medicinal plants. Similarity, a total of 400 strains of actinobacteria was

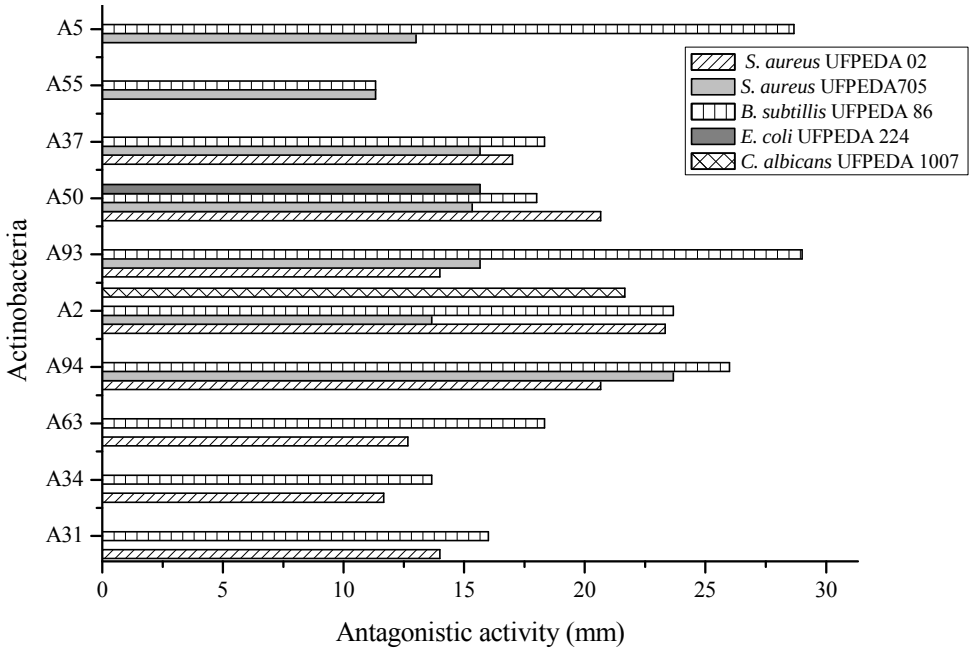

Figure 2. Antimicrobial activity of actinobacteria from rhizosfere of *I. pes-caprae* (Forte Orange, Itamaracá-PE, Brazil).

isolated from 12 soil samples of medicinal plant collected from tropical botanical garden in Danzhou city (Xiaolong et al., 2012).

Although these actinobacteria are from the habitat of the rhizosphere of *I. pes-caprae*, a plant found in dunes on the Brazilian coast (Wasuwat, 1970; Lorenzi and Matos, 2002), the highest number of isolates were found in a non-saline culture medium, suggesting that these actinobacteria are halotolerant (Carro et al., 2013). After purification of strains of actinobacteria from the rhizosphere, only 29 grew with a sufficient number of secondary hyphae to have antimicrobial properties. Sixty-two percent presented activity against at least one test micro-organism. The A2, A50 and A94 actinobacteria, identified as species of the *Nocardia* genus, presented antibacterial activity with inhibition diameter zone (IDZ) of 23.33, 20.66 and 20.66 mm for *S. aureus* UFPEDA 02; 13.66, 23.66 and 15.33 mm for *S. aureus* UFPEDA 705; and 23.66, 18.00 and 26.00 mm against *B. subtilis* UFPEDA 86, as shown in Figure 2. The A2 strain was the only one that presented antifungal activity against *C. albicans* (21.66 mm).

Similar antimicrobial activity was reported in a study of *Streptomyces* (BT-408) isolated from the sediment of the Bay of Bengal in the Indian Ocean, aiming to evaluate its activity against methicillin-resistant *S. aureus* (MRSA). The authors reported that this actinobacteria presented a IDZ of 20 mm (Sujatha et al., 2005) and a study of actinobacteria isolated from the Visakhapatnam region found that, after fermentation and isolation of compounds, the B01 fraction presented moderate activity against *E. coli*, *B. subtilis*, *S. aureus*, *P. aeroginosa* and *B. cereus* (Rao et al., 2012).

The search for secondary metabolites that are active against species of the genus *Staphylococcus* is important, especially to combat *S. aureus* (Da Silva et al., 2013a, b), owing to its high incidence in both hospital and community acquired infections, linked to its patterns of resistance and factor virulence expression (Gould et al., 2012). This problem is compounded when faced with multi-resistant strains of the *S. aureus* (Davis et al., 2013).

Identification and chemical characterization of methanolic extract of strain A-94

Amongst all tested bacteria (29), the strain A-94 showed the best antimicrobial activity against Gram-positive pathogens, especially *S. aureus*; therefore, the strain A-94 was selected to be partially identified and chemical characterized. The microculture technique identified the A-94 strain at the genus level as *Nocardia*. The strain A-94 exhibited well-developed vegetative hyphae with irregular branches penetrating the agar and bearing white aerial sparse hyphae. At a late stage of their growth, the filaments fragment into rod-shaped elements characteristic of the family Nocardiaceae and the genus *Nocardia* (Hoshino et al., 2004).

A chemical characterization was conducted for the MeOH extract of the biomass of *Nocardia* sp., and the MeOH exctract of *I. pes-caprae*. Mono- and sesquiterpenes were found both in the extract of the micro-organism and that of the plant. The other

Table 2. Evaluation of chemical characterization of MeOH extracts of *Nocardia* sp. when compared with MeOH extracts of *I. pes-caprae*.

Metabolite	*Nocardia* sp. extract	Plant extract
Triterpenes and steroids	Negative	Negative
Monoterpenes and sesquiterpenes	Positive	Positive
Alkaloids	Negative	Negative
Coumarin, flavonoids, phenylpropanoids e and cinnamic derivatives	Negative	Positive (flavonoid)

Table 3. Evaluation of production of L-asparaginase by actinobacteria isolated from the rhizosphere of *I. pes-caprae* oriunda from Forte Orange Beach, Itamaracá - PE.

Actinobacteria	Qualitative Test	Actinobacteria	Qualitative Test
A31	+++	A208	+++
A34	+++	A119	ND
A63	+++	A180	++
*A94	+++	A101	++
A2	+++	A222	+++
A93	ND	A210	ND
A50	+++	A207	++
A37	+++	A209	ND
A55	+++	A196	+++
A5	+++	A231	ND
A65	ND	A114	+++
A122	+++	A5.5	+++
A202	+++	A201	++
A120	++	A118	+
UFPEDA 224	+++	UFPEDA 224	+++

ND = Not degraded; + = low level of degradation of L-asparagine in 48 h; ++ = medium level of degradation of L -asparagine in 48 h; +++ = high level of degradation of L-asparagine in 48 h. **Nocardia* sp. A9.

secondary metabolites under study were not found, as shown in Table 2. The presence of sesquiterpenes and other secondary metabolites in the extract of *I. pes-caprae* has previously been reported (Pongprayoon, 1991, 1992). Both monoterpenes and sesquiterpenes are associated with antibacterial, antifungal and antitumor activity (Schwab, Fuchs and Huang, 2013). These compounds are used as components in fragrances, cosmetics, cleaning products, disinfectants, food additives and medicines, owing to their pleasant smell and antimicrobial properties (Schwab, Fuchs and Huang, 2013). Although chemical prospection is generally carried out with extracts from different parts of the plant, such as leaves and roots, it is important to bear in mind that, in the rhizosphere, there are inter- and intra-species relations between micro-organisms and the environment, which causes it to produce similar compounds (Kent and Triplett, 2002).

Qualitative test of production of L-asparaginase enzyme

Additionally, a qualitative evaluation of the L-asparaginase production was performed with all strains isolated from rhizosphere of *I. pes-caprae*. The result was recorded as positive according to a change of color in the M-9 medium. The coloration is the result of degradation of the asparagine in the growth medium caused by the breakdown of L-asparagine into L-aspartic acid and ammonia, which changes the color from orange to pink (Amena et al., 2010). In the screening, 55.17% of strains tested presented an area of degradation of asparagine similar to the positive control, *E. coli* UFPEDA 224, and 20.68 % presented an area of degradation with weaker coloration in comparison with the positive control (Table 3).

L-asparaginase is an enzyme that possesses antineoplastic properties against acute lymphoblastic leukemia and can be produced by a variety of micro-organisms, such as bacteria, actinobacteria and fungi (Dejong, 1972; Moura et al., 2004). *E. coli* and *Erwinia chrysanthemi* are two well-known producers of L-asparaginase, nevertheless, there is a need to search for micro-organisms able to produce high amounts of L-asparaginase, with more therapeutic efficacy and, if

possible, with fewer side effects (Nagarethinam et al., 2012). It is established that preparations from each kind of species differ on their pharmacological properties (therapeutic efficacy, bioavailability and induction of side effects) structural characteristics and physico-chemical kinetics (Labrou et al., 2010). In fact, different habitats have been explored to obtain these micro-organisms as soil (Shukla et al., 2014), marine (Basha et al., 2009), leaves (endophytic) (Chow and Ting, 2014) and rhizosphere (Khamna et al., 2009). For example, actinobacteria strains isolated from 16 samples of rhizosphere of medicinal plants from Thailand and, of 445 isolated, 30 were found to produce this enzyme (Khamna et al., 2009).

Conclusion

The present study suggests that the micro-organisms present in the rhizosphere of *I. pes-caprae* possess biotechnological properties since they are producers of antimicrobial compounds and L-asparaginase. Both activities are of interest to different industries such as pharmaceutical, food, cosmetic and cleaning industries. Further studies on the molecular characterization of each active isolate and purification of the bioactive compounds are in progress.

Conflict of Interests

The authors have not declared any conflict of interests.

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