

Antioxidant and antibacterial activities of the stem bark of *Aspidosperma spruceanum* Benth

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Abstract— The pharmacological activity of plants of the Apocynaceae family is mainly attributed to the presence of alkaloids. *Aspidosperma spruceanum* is a representative of this family, whose wood has been largely used in the construction industry. However, reports of its pharmacological and chemical properties are incipient. Therefore, this work aimed to investigate the antioxidant and antibacterial properties of extracts and fractions of this plant. After plant collection and identification, methanolic extract and fractions of the stem bark were prepared. Antioxidant activity was determined by •DPPH radical scavenging assay and total phenolics were estimated by reaction with Folin-Ciocalteu reagent. Minimum inhibitory and minimum bactericidal concentrations were estimated by the broth microdilution method against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The samples were also investigated for hemolytic activity. The phytochemical profile was determined by classical phytochemical tests. The methanolic extract of the bark showed bactericidal activity against *S. aureus* and bacteriostatic activity against *P. aeruginosa*. The hexane fraction obtained from crude extract fractionation acted as a bactericide against both strains. The ethanolic fractions of crude extract and dichloromethane and ethyl acetate fractions obtained from acid-base fractionation contained higher concentration of phenolics and, consequently, higher antioxidant activity *in vitro*. The analysis of the phytochemical profile indicated the presence of alkaloids, triterpenes and steroids in the samples. In conclusion, this article brings the first description of the antioxidant and antibacterial properties of *A. spruceanum* and preliminary chemical composition, and also values the components of the biodiversity of the state of Bahia not yet studied.

Keywords— *apocynaceae*; *A. spruceanum*; *antioxidant*; *anti-bacterial*.

I.

INTRODUCTION

The family Apocynaceae has between 3700 and 5100 species, classified in 250 to 550 genera, and is commonly found in the tropics and subtropics. In Brazil, this family is represented by more than 400 species distributed in 41 genera, present in all biomes, including Caatinga, Cerrado and forests (Pereira, 2007; Gomes, 2011).

The genus *Aspidosperma* is one of the most important in the Apocynaceae family. Ethnopharmacological studies have shown that species of this genus can be used to treat malaria, leishmaniasis, uterine and ovarian inflammation, fever, diabetes, impotence, disorders, cancer, rheumatism,

among others (Oliveira, 2008; Aguiar, 2010; Dolabela, 2012).

The pharmacological properties of species of the Apocynaceae family have been mainly attributed to the presence of alkaloids. In the genus *Aspidosperma* alone, more than 247 indole alkaloids with a great structural diversity have been identified through phytochemical studies. For this reason, these alkaloids are considered chemotaxonomic markers of *Aspidosperma* species (Pereira, 2007; Gomes, 2011; Henrique, 2010; Teugwa et al; 2013).

Aspidosperma spruceanum is an important representative of the genus and a native tree of the Atlantic Forest, being 5 to 20 m tall and presenting a round crown.

Its trunk, 30 to 40 cm in diameter, is covered by a thick layer of cork, and is an excellent source of wood used in the construction industry. As typical of the genus, this plant is also rich in indole alkaloids, being some of them exclusive to the species (Fumagali, 2008).

The few reports of the pharmacological properties of *A. spruceanum* attribute to their leaves and stems a moderate to high antispasmodic and antiparasitic activity against *Trypanosoma cruzi* and *Leishmania infantum*, which justifies the need for more systematic studies aimed at investigating the biological potential of this plant (Paula, 2014; Reina et al., 2014).

Thus, in view of the need for new therapeutic strategies for the treatment of inflammatory and infectious diseases (Rabêlo and Rodrigues et al., 2014), this study aimed to investigate the antioxidant and antibacterial properties of extracts and fractions of *A. spruceanum*. In addition, it is expected to contribute to the still incipient scientific knowledge about the regional flora.

II. MATERIAL AND METHODS

A. Plant material

Plant material from *Aspidosperma spruceanum* Benth. ex Müll. Arg. were collected in June 28th of 2012, in a forest fragment in Brejo Novo Farm (13°56'41"S and 40°06'33.9"W) between 617 m and 755 m of altitude at 9 km from Jequié, Bahia State, Brazil. The botanic identification was performed by comparison with a voucher (HUESB 2418) deposited in the Herbarium of the State University of Southwest of Bahia.

B. Preparation of extracts

The plant material was dried in Tecnal drying oven (TE 394\2 Model) at 40 °C for 48 h and submitted to cold maceration with methanol for prepare of the bark extract from *A. spruceanum* (ME). The extract solution was filtered and concentrated under vacuum in rotary evaporator (Fisatom, 801 Model) at 50 °C (Serafin et al., 2007).

C. Extraction fractionation

The methanolic extract (ME) (3.6 g) was initially dissolved in methanol and subsequently fractionated by silica gel 60 column chromatography (Silva et al., 2014). Hexane, ethyl acetate, ethanol solvents were used as the mobile phase. Finally, the hexane fraction (HF), ethyl acetate fraction (EAF), and ethanolic fraction (EF) of the stem bark were concentrated in a rotary evaporator.

D. Isolation of total alkaloid fraction

The ethyl acetate fraction (EAF) (0.94 g) was dissolved in methanol before acid-base fractionation. HCl (1%) was then added until the pH of the suspension was 2.0. Extraction with dichloromethane (3x50 mL) was made in a

separation funnel. After separation of the dichloromethane phase (DF1) from the acidic aqueous phase (AAF), aqueous NH₄OH (5%) was added to the AAF to approximately pH 10 to obtain the basic aqueous phase 1 (BAF1). Then, in a separation funnel, alkaloids (lower polarity) were extracted from BAF1 with dichloromethane (3x30 mL) to result in the dichloromethane phase 2 (DF2) and basic aqueous phase 2 (BAF2). The most polar alkaloids were extracted from BAF2 with ethyl acetate (3x30 mL) by partition to obtain the ethyl acetate fraction 2 (EAF2) and the basic aqueous phase 3 (BAF3). Finally, BAF3 was extracted with butanol to obtain the butanol fraction (BuF) and the basic aqueous phase 4 (BAF4). All organic fractions obtained were concentrated in a rotary evaporator (Matos, 2009).

E. Detection of alkaloids by Dragendorff test

The extract and fractions were analyzed by Thin Layer Chromatography on 60 GF₂₅₄ silica gel plates (dimensions 5 x 10 cm), eluted with a mixture of chloroform and methanol (9:1). After elution, the plates were dried and observed under ultraviolet (UV) light. Finally, the Dragendorff reagent was sprayed onto the plate, and the appearance of orange bands suggests the presence of alkaloids (Costa et al., 2008).

F. Detection of triterpenes and steroids by Liebermann-Burchard assay

About 10 mg of the hexane fraction of *A. spruceanum* bark was dissolved in chloroform. Then 5 drops of Liebermann-Burchard reagent were added. After a few minutes, the change in the color of the solution was inspected, until a greenish color appeared, suggesting the presence of triterpenes and/or steroids (Burke et al., 1974).

G. Minimum Inhibitory Concentration (CIM) and Minimum Bactericidal Concentration (CBM)

Staphylococcus aureus (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 2785) suspensions were diluted in BHI broth to 0.1 absorbance at 625 nm. Then extracts or fractions were added at varying concentrations (25 to 1000 µg/mL). The antibiotics ceftriaxone (50 µg/mL) and tetracycline (50 µg/mL) were used as positive control for gram negative and gram positive bacteria, respectively. After incubation at 37°C for 24 h, 60 µL of the resazurin dye (0.01%) was added in 200 µL of the bacterial suspension. MIC corresponded to the lowest concentration at which no visible bacterial growth (blue color) was seen after additional incubation at 37°C for 30 min. For determination of MBC, 10 µL of the bacterial suspension preincubated with extracts or fractions at different concentrations (25 to 1000 µg/mL) were plated on Muller-Hinton Agar at 37°C for 24 h. MBC corresponded to the concentration at which no evidence of

bacterial growth on the surface of the culture medium was seen (Santos et al., 2017).

H. Phosphomolybdenum complex reduction method

The concentration of total phenolic compounds present in the samples was determined by the Folin-Ciocalteu spectrophotometric assay (Singleton et al., 1992). Samples were diluted in their solvents to a final concentration of 1 mg/mL. An extract aliquot of 125 μ L was mixed with 125 μ L of Folin-Ciocalteu reagent and 1 mL of distilled water. After 3 min, 125 μ L of saturated Na₂CO₃ solution was added and incubated for 30 min at 37°C. At the end of incubation, the absorbance of the sample was measured spectrophotometrically at 750 nm. A standard gallic acid curve (0.5 to 25 μ g) was constructed to quantify the total phenolics in the samples ($y = 0.366x + 0.0721$, $R^2 = 0.9932$), which were expressed as μ g gallic acid equivalents per milligram sample (μ g GAE/mg).

I. 2,2-Diphenyl-1-picrylhydrazyl (*DPPH) radical scavenging method

Antioxidant activity was assessed by the ability of the extract and fraction to react with the free radical *DPPH (Brand-Wiliams, 1995; Duarte-Almeida, 2006). Samples were diluted in ethanol to a final concentration of 1 mg/mL. Aliquots of 12.5, 25, 50, 100, 200, and 300 μ L of samples were diluted in 1700 μ L of *DPPH-ethanol solution (50 μ M). Ethanol was used to complete the reaction volume to 2 mL. Reaction mixtures were incubated for 20 min at 20°C, and the residual absorbance of *DPPH was determined at 517 nm. The effective concentration capable of reducing 50% of free radical concentration (EC₅₀) was determined by nonlinear adjustment of the data in the GraphPad Prism 6.0 software.

J. Determination of cytotoxicity

The *in vitro* hemolytic activity of extracts and fractions was evaluated as described by Farmacopeia and Homen, with some modifications (Homem, 2015; Farmacopeia Brasileira, 2010). Citrate blood was collected from apparently healthy donors, washed three times with saline (0.9%) and resuspended in PBS (phosphate buffer) at the final hematocrit concentration of 2%. Extracts and fractions (1 mg/mL) were added to the suspension of erythrocytes and incubated at 37°C for 3 h. Then, the reaction mixtures were centrifuged at 3000 rpm for 5 min. To quantify hemolysis, supernatants were transferred to an ELISA plate and supernatants were observed and classified into: total hemolysis (+++), moderate hemolysis (++) , low hemolysis (+) and no hemolysis (-). Triton X-100 (1%) was used as positive cell lysis control (+++). Ethanol was used as negative control (-). These procedures were approved by the Research Ethics Committee under number 46135315.4.0000.0055.

K. Statistical analysis

All tests were performed at least in triplicate and expressed as mean \pm standard deviation. Data normality was verified by the Shapiro-Wilk test ($p > 0.05$). Data were analyzed using one-way ANOVA and Dunnett as post-test. The correlation was made according to the Pearson's correlation coefficient. All tests were performed using the GraphPad Prism (6.0) software. A significance level of 5% ($p < 0.05$) was adopted.

III. RESULTS AND DISCUSSION

Extracts and fractions were initially evaluated for their ability to inhibit the growth of *S. aureus* and *P. aeruginosa* strains. ME and HF presented MIC and MBC of 25 and 50 μ g/mL for *S. aureus*, respectively. In the case of *P. aeruginosa*, ME and HF presented MIC of 50 and 500 μ g/mL and MBC of 1000 and 500 μ g/mL, respectively (Table 1).

According to Tanaka et al. (2005), antimicrobial activity can be classified as: inactive (MIC $>$ 1000 μ g/mL); weak (500 $<$ MIC $<$ 1000 μ g/mL); moderate (100 $<$ MIC $<$ 500 μ g/mL), and good (MIC $<$ 100 μ g/mL). When the MBC/MIC ratio of any compound is between 1:1 and 2:1, the substance can be considered bactericidal. On the other hand, if the ratio is greater than or equal to 2:1, the most likely mechanism of action of the compound is bacteriostatic (Faria, 2012). Thus, ME has the characteristic of an antibacterial agent, with bactericidal mechanism on *S. aureus* strains (MBC/MIC = 2:1) and bacteriostatic mechanism on *P. aeruginosa* (MBC/MIC = 20:1). The ME fraction, on the other hand, possibly acted as a bactericide in the case of both *S. aureus* and *P. aeruginosa*, with MBC/MIC ratios of 2:1 and 1:1, respectively. EAF presented MIC and MBC greater than 1000 μ g/mL for *S. aureus*, but could not be tested for *P. aeruginosa* due to a sample limitation. The literature indicates that indole alkaloids often present in species of the Apocynaceae family may be responsible for the antibacterial activity of plants in this family (Teugwa et al., 2013). In a previous study with the alkaloid fraction of *A. pyrifolium* bark, it was observed that this plant inhibited the growth of *S. aureus* and *Bacillus subtilis* with MIC of 125 and 250 μ g/mL, respectively (Pessini, 2015). *A. olivaceum* extracts were also active against *B. subtilis* (Oliveira et al., 2009).

Extracts and fractions of *A. spruceanum* were monitored by thin layer chromatography (TLC) for the presence of alkaloids. The results showed that the ME presents metabolites that react positively on Dragendorff's reagent, suggesting the presence of alkaloids (Costa et al., 2008). In fact, the alkaloids aspidoalbina, *N*-acetyl-*N*-

despropionylaspidoalbine (*N*-acetyl aspidoalbine), des-*O*-methyl-aspidolimidine, spruceanumine A, and spruceanumine B have already been isolated from *A. spruceanum* (Oliveira, 2008). Since TLC demonstrated

that EAF had the highest concentration of alkaloids (Figure 1), this fraction was subjected to extraction of total alkaloids.

Table 1. Determination of minimum inhibitory and minimum bactericidal concentrations of *A. spruceanum* extracts and fractions

Sample	MIC (µg/mL)		MBC (µg/mL)		MBC/MIC	
	<i>S. Aureus</i>	<i>P. aeruginosa</i>	<i>S. Aureus</i>	<i>P. Aeruginosa</i>	<i>S. aureus</i>	<i>P. Aeruginosa</i>
ME	<25	<25	50	1000	>2:1	>40:1
EAF	>1000	>1000	>1000	>1000	-	-
HF	< 25	500	50	500	>2:1	1:1
DF2	1000	1000	1000	1000	1:1	1:1
BuF	>1000	>1000	>1000	>1000	-	-
Tetracycline	50	-	50	-	-	-
Ceftriaxone	-	50	-	50	-	-

(-): Not tested. *The amount of the ethyl acetate fraction was not sufficient for the assay. ME: Methanolic extract of the stem bark of *A. spruceanum*. EAF: Ethyl acetate fraction of the methanolic extract of the stem bark of *A. spruceanum*. HF: Hexane fraction of the methanolic extract of the stem bark of *A. spruceanum*. D2F: Dichloromethane fraction 2 of the stem bark of *A. spruceanum*. BuF: Butanolic fraction of the stem bark of *A. spruceanum*. **p* < 0.05 when compared to tetracycline and ceftriaxone by ANOVA with Dunnet's post-test.

Additionally, the Liebermann-Burchard qualitative assay indicated the presence of triterpenes and/or steroids in the hexane fraction of the stem bark of *A. spruceanum*. It is noteworthy that these compounds can be partitioned into the

hexane fraction due to their non-polarity, being equally important from a biological point of view due to the myriad properties, including antibacterial properties (Virtuoso et al., 2005; Barbosa et al., 2010; Bannwart et al., 2013).

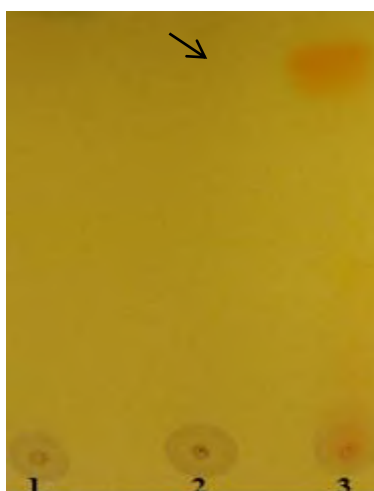


Fig.1: Detection of alkaloids in *A. spruceanum* extracts by plate chromatography.

The arrow indicates the spot containing alkaloids. A- Alkaloid detection. 1) ME: Hexane fraction of the stem bark of *A. spruceanum*; 2) EAF: Ethyl acetate fraction of the stem bark of *A. spruceanum*; 3) EF: Ethanolic fraction of the stem bark of *A. spruceanum*. Stationary phase: silica gel layer 1.0 mm thick. Mobile phase: chloroform and methanol. Dragendorff's reagent was used for staining alkaloid molecules.

DF2, rich in alkaloids, presented MIC and MBC values of 1000 µg/mL for Gram positive strains, while BuF presented values greater than 1000 µg/mL for Gram negative strains (Table 2). It is possible that these

Phenolic compounds present in plants are another class of molecules with a number of pharmacological properties including anti-inflammatory, vasodilatory, antitumor, antiulcerogenic, antiplatelet, antimicrobial, and antiviral activity. The antioxidant activity of the extracts was evaluated by the DPPH free radical reduction method. ME presented lower EC₅₀ value (28.67 µg/mL) and, consequently, the phenolic concentration was relatively high (123.28 µg EAG/mg) (Table 3). Extracts with EC₅₀ values below 50 µg/mL indicate high antioxidant activity, below 50-100 µg/mL indicate moderate antioxidant activity, below 100-200 µg/mL indicate low activity, and above 200 µg/mL are considered inactive.

The ethanolic fractions of the crude extract and dichloromethane and ethyl acetate fractions obtained from acid-base fractionation contained higher concentration of phenolic components and, consequently, higher antioxidant activity *in vitro* (Table 2). On the other hand,

molecules were not responsible for the antibacterial properties of *A. Spruceanum*, or they may be important only when acting synergistically with other substances (Betoni et al., 2006).

the antioxidant activity of the alkaloid fractions did not correlate with phenolic concentration (p = 0.761). A study with iboga alkaloids of *Peschiera affinis* (Apocynaceae) demonstrated that the antioxidant activity in these plants may be attributed to the presence of these alkaloids (Santos, 2009), which may also have occurred in the present work.

Thus, antioxidant activity in the alkaloid fractions of *A. spruceanum* can be associated not only with phenolic compounds but also with iboga alkaloids, which explains the difficulty in obtaining a correlation between the two phenomena. Taken together, the results suggest that *A. spruceanum* bark may contains bioactive molecules with important pharmacological properties, being a source of antioxidant and antibacterial substances.

Table 2. Phenolic concentration and antioxidant activity of the extracts of *A. spruceanum*

Sample	Total phenolics (µg EAG/mg)	CE ₅₀ (µg/ml)
ME	123.28±0.14	28.67±0.01*
HF	-	-
EAF	46.73± 0.014	54.23±0.02*
FMCASE	116.3±0.0014	38.66±0.03*
FBu	104.56±0.002	43.41±0.01*
EF	141.52±0.018	31.28±0.01*
DF2	173.69±0.030	39.36±0.72*
Gallic acid	-	7.11±0.23

(-): Not tested. Results expressed as mean ± standard deviation (n = 3). (-): Not tested. * ME: Methanolic extract of the stem bark of *A. spruceanum*. EAF: Ethyl acetate fraction of the methanolic extract of the stem bark of *A. spruceanum*. HF: Hexane fraction of the methanolic extract of the stem bark of *A. spruceanum*. EF: Ethanolic fraction of the stem bark of *A. spruceanum*. D2F: Dichloromethane fraction 2 of the stem bark of *A. spruceanum*. BuF: Butanolic fraction of the stem bark of *A. spruceanum*. * p < 0.05, when compared to gallic acid by ANOVA with Dunnet's post-test.

Finally, *A. spruceanum* extracts and fractions were tested for lytic activity on human erythrocytes. Erythrocytes are useful as a model for assessing the preliminary toxicity of a wide variety of substances, allowing for information on their effects on the cell membrane. The occurrence of hemolysis after exposure to the test product may be directly correlated with its cytotoxicity and used as the first step in *in vitro* toxicological screening (Souza et al., 2014). With the exception of HE, all other samples caused little or no hemolysis (Table 3).

Considering that plant bioactive substances may be responsible for the protective effect against the risks of many pathological conditions, the results described in this work stimulate the continuity of studies on the biological properties of the total alkaloid (D2F), butanolic (BuF) and hexanic fraction (HF) of *A. Spruceanum* as well as the isolation and characterization of bioactive molecules.

Table 3. *In vitro* cytotoxicity of *A. spruceanum* extracts and fractions.

Sample	Hemolysis (%)
ME	+
EAF	+
HF	++
BuF	-
Triton X-100	+++
Ethanol	-

+++ = total hemolysis; ++ = moderate hemolysis; + = low hemolysis; - = no hemolysis. Triton X-100 was used at 1% concentration. ME: Methanolic extract of *A. spruceanum* stem bark. EAF: Ethyl acetate fraction of methanolic extract of *A. spruceanum* stem bark. HF: Hexane fraction of the methanolic extract of the stem bark of *A. spruceanum*. BuF: Butanolic fraction of the stem bark of *A. spruceanum*.

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