Chemical composition, antioxidant and antibacterial activities and evaluation of cytotoxicity of the fractions obtained from Selaginella convoluta (Arn.) Spring (Selaginellaceae)

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ABSTRACT
The aim of this study was to evaluate the chemical composition, antioxidant and antibacterial activities and cytotoxicity of fractions from Selaginella convoluta, obtained by liquid–liquid extraction using hexane (Sc-Hex), chloroform (Sc-CHCl3) and ethyl acetate (Sc-AcOEt). The phenolic and flavonoid contents were measured by the Folin-Ciocalteu and aluminium chloride methods, respectively. Antioxidant activities were evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and β-carotene-linoleic acid bleaching test. The antibacterial effect was evaluated by the method of microdilution and the cytotoxicity analysis in HCT-116 (colon), OVCAR-8 (ovarian) and SF-295 (brain) cells were carried out for MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) test. The fractions were positive for the presence of anthracene derivatives, flavonoids, lignans, naphthoquinones, steroids and triterpenoids. The Sc-Hex and Sc-AcOEt showed good antioxidant activities. The fractions of S. convoluta demonstrated antibacterial activity and showed weak cytotoxicity. These activities were correlated with presence of phenolic compounds in active fractions.

Introduction
Oxidative stress is characterized by a deregulation between the production of reactive oxygen species and antioxidant defense activity. It is associated with many chronic diseases such as cancer, neurodegenerative, cardiovascular disease and diabetes [1]. Consumption of fruits, vegetables and other derived plant products, because of the presence of bioactive molecules, such as phenolic compounds, flavonoids and tannins, has been associated with a lower incidence of diseases related to oxidative stress [2–4]. These substances are secondary metabolites, biosynthesized by plants to prevent pathogen attack, ultraviolet stress or to attract pollinator insects [5].

Flavonoids and phenolic acids are a major group of phytochemicals that exhibit strong antioxidant, cytotoxic and antibacterial activities [6–8]. The growing multidrug resistance (MDR) in cancer chemotherapy and antibiotic therapy used in clinical practice has stimulated extensive research for the development of new antitumour and antimicrobial agents from plants extracts [9–11].

A source of such extracts is the Selaginellaceae family, which includes the genus Selaginella, and comprises about 700–800 species [12–15]. Approximately 270 species of Selaginella are found in America, of which 61 occur in Brazil, and the genus is widely distributed throughout America, Africa and Europe [14,16]. Many species of Selaginella have demonstrated medicinal potential and are sources of bioactive compounds, mainly biflavonoids [17–22]. In the Caatinga, the genus is represented by Selaginella convoluta, a medicinal plant commonly known as ‘jeríco’, ‘mão-de-sapo’ and ‘mão-fechada.’ S. convoluta is used in folk medicine as antidepresant, aphrodisiac, diuretic, analgesic and anti-inflammatory agent as well as to increase the female fertility [23–25]. Previous pharmacological studies performed with extracts of this species demonstrated antinociceptive and antimicrobial activity [26–29]. In our continuing search of the Brazilian Caatinga medicinal plants to combine biodiversity conservation with drug discovery, the aim of this study was to evaluate the antioxidant,
antibacterial and cytotoxic activity of the fractions obtained by partition of the crude ethanolic extract from *S. convoluta* using in vitro models.

Materials and methods

Plant material

The plant material of *Selaginella convoluta* was collected in the city of Petrolina (Coordinates: S 09°03'54"; W 40°19’12"), State of Pernambuco, Brazil, in March of 2012. A sample was identified by the botanist André Pavioiti Fontana, and a voucher specimen (#19203) was deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley (UNIVASF).

Extraction

The dried and pulverized plant (1935 g) was macerated with ethanol 95% at room temperature for 72 h. The extractive solution was filtered and concentrated under reduced pressure in a rotatory evaporator oven at 50 °C, producing 146.55 g of crude ethanol extract (CEE). The CEE was suspended in a mixture of MeOH:H2O (3:7) and extracted successively with hexane, chloroform and ethyl acetate in ascending order of polarity to obtain the hexane (Sc-Hex – 21.26 g), chloroform (Sc-CHCl3 – 15.14 g) and ethyl acetate (Sc-AcOEt – 5.18 g) fractions.

Phytochemical analysis

The preliminary phytochemical screening, in order to establish the possible chemical nature of the compounds, was carried out with the fractions obtained by partitioning the CEE of *S. convoluta*. The tests were performed according to the methodology described by Wagner and Bladt [30] seeking to highlight the main groups of secondary metabolites.

Determination of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu reagent, which is based on the method reported by Slinkard and Singleton [31]. Aliquots (40 μL) of suitably diluted Sc-Hex, Sc-CHCl3 and Sc-AcOEt extracts were added to 3.16 mL of distilled water and 200 μL of the Folin-Ciocalteu reagent, and were mixed well. The mixture was shaken and allowed to stand for 6 min, before adding 600 μL of sodium carbonate solution, and was shaken to mix. The solutions were left at 20 °C for 2 h and the absorbance of each solution was determined at 765 nm against the blank and plot absorbance vs. concentration. The total phenolic content of the extracts (three replicates per treatment) was expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) through a calibration curve with gallic acid. The calibration curve range was 50–1000 mg/L ($R^2 = 0.9938$). All samples were performed in triplicates.

In vitro antioxidant activity

DPPH radical assay

The free-radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay [33]. Sample stock solutions (1.0 mg/mL) of extracts were diluted to final concentrations of 243, 81, 27, 9, 3 and 1 μg/mL, in ethanol. One millilitre of a 50 μg/mL DPPH ethanol solution was added to 2.5 mL of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min, the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: $AA\% = ([A_c - A_t]/A_c) \times 100$, where $A_c$ is the absorbance of the control and $A_t$ is the absorbance of the sample at 518 nm. Ethanol (1.0 mL) plus plant extracts solutions (2.5 mL) were used as a blank sample. DPPH solution (1.0 mL) plus ethanol (2.5 mL) was used as a negative control. The positive controls were ascorbic acid, butyl-hydroxyanisole (BHA) and butyl-hydroxytoluene (BHT) standard solutions. Assays were carried out in triplicate. The half maximal inhibitory concentration (IC$_{50}$) values were calculated by linear regression using GraphPad Prism 5.0.
β-Carotene bleaching assay

β-Carotene (2 mg) was dissolved in 10 mL chloroform and to 2 mL of this solution, linoleic acid (40 μL) and Tween 40 (400 μL) were added. Chloroform was evaporated under vacuum at 40 °C and 100 mL of distilled water was added; then the emulsion was vigorously shaken for 2 min. Reference compounds (ascorbic acid, BHA and BHT) and sample extracts were prepared in ethanol. The emulsion (3.0 mL) was added to a tube containing 0.12 mL of 1 mg/mL solutions of reference compounds and sample extracts. The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 °C for 120 min, when the absorbance was measured again. Ascorbic acid, BHA and BHT were used as positive control. In the negative control, the extracts were substituted with an equal volume of ethanol. The antioxidant activity (%) was evaluated in terms of the bleaching of β-carotene, using the following formula: % Antioxidant activity = [1 – (A₀ – A₁)/ (A₀ᴬ – A₀)] × 100; where A₀ is the initial absorbance and A₁ is the final absorbance measured for the test sample, A₀ᴬ is the initial absorbance and A₁ is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The studied microorganisms included strains of *Bacillus cereus* ATCC 11 778, *Enterococcus faecalis* ATCC 19 433, *Escherichia coli* ATCC 25 922, *Klebsiella pneumoniae* ATCC 13 883, *Salmonella enterica* ATCC 10 708, *Serratia marcescens* ATCC 13 880, *Shigella Flexneri* ATCC 12 022 and *Staphylococcus aureus* ATCC 25 923 obtained from the National Institute of Quality Control in Health (INCQS/ FIOCRUZ, Rio de Janeiro, Brazil).

The antibacterial effect was evaluated by the method of microdilution [34] as recommended by The National Committee for Clinical Laboratory Standards [35]. Initially, a stock solution of 25 mg/mL of extracts was prepared using an aqueous solution of 20% dimethyl sulfoxide (DMSO) (v/v). Then, 200 μL of this dilution were transferred to the microplate containing 200 μL of Müller-Hinton broth. Serial dilutions were performed resulting in concentrations of 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.195 mg/mL. The inoculum containing 5 × 10⁵ CFU/mL (0.5 in McFarland scale) was added to each well. Some wells of the plates were reserved for growth control (without inoculants), for negative control (without antimicrobial agent) and for positive control (gentamicin). For gentamicin, an initial concentration of 1.6 mg/mL was used, which was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 μg/mL. The microplates were incubated under aerobic conditions for 18–24 h at 37 °C when 10 μL of 2,3,5-triphenyl-tetrazolium (CTT) 2% were added to each well to detect the colour change of the CTT (colourless) to red, reflecting the bacterial metabolic activity. The MIC was defined as the lowest concentration of the extracts that visibly inhibited the bacterial growth.

To determine the MBC, aliquots of 10 μL were withdrawn from each well containing the extracts and transferred to Petri dishes containing Müller–Hinton agar. The plates were incubated for 24 h at 37 °C. The appearance of bacterial colony for a given concentration indicates that it was not able to kill 99.9% or more of the cells in the bacterial inoculum used. Assays were performed in triplicate. The density of the extracts was employed to convert μL/mL units into mg/mL, the latter being used to express the MIC and MBC.

Cell line and cell culture

Human tumour cell lines, including OVCAR-8 (ovarian), SF-295 (brain) and HCT-116 (colon) were obtained from the National Cancer Institute (Bethesda, MD, USA). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C with 5% CO₂.

Determination of cytotoxicity

All fractions were tested for cytotoxic activity against three tumour cell lines. For all experiments, cells were plated in 96-well plates: OVCAR-8 (0.1 × 10⁶ cells/mL), SF-295 (0.1 × 10⁵ cells/mL) and HCT-116 (0.7 × 10⁵ cells/mL). After 24 h, all fractions (50 μg/mL) dissolved in 1% DMSO were added to each well using a high-throughput screening system (Biomek 3000 – Beckman Coulter, Inc. Fullerton, CA, USA), and the cultures were incubated for 72 h. The control groups received the same amount of DMSO. The general viability of cultured cells was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product. At the end of the incubation, the plates were centrifuged and the medium was replaced with fresh medium (150 μL) containing MTT (0.5 mg/mL). Three hours later, the plates were centrifuged, the MTT formazan product was dissolved in 150 μL DMSO, and the absorbance was measured using a multilreade reader (Spectra Count, Packard, Ontario, Canada).

The extract effect was quantified as the percentage of the control absorbance of the reduced dye at 595 nm. All absorbance values were converted into cell growth
inhibition percentage (GI%) by the following formula: GI% = 100 – [(T/C) × 100%], where C is the absorbance of the negative control and T is the absorbance in the presence of the tested extracts. The intensity scale was used to assess the cytotoxic potential of tested samples: samples without activity (SA), with little activity (PA, cell growth inhibition ranging from 1% to 50%) with moderate activity (MO, inhibition of cell growth ranging from 50% to 75%) and very potent activity (MA, cell growth inhibition ranging from 75% to 100%).

Data analysis

All determinations were conducted in triplicates, and the data are expressed as mean values with standard deviation (± SD). The IC_{50} values were obtained by interpolation from linear regression analysis with 95% confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of the maximum effect.

Results and discussion

Preliminary phytochemical screening showed that the Sc-Hex and the Sc-CHCl_3 were positive for the presence of flavonoids, anthracene derivatives, quinones, triterpenes and steroids. In Sc-AcOEt, we only detected the presence of flavonoids, lignans and naphthoquinones. Presence of alkaloids was not detected in any of the phases (Table 1).

The content of total phenolics determined by the Folin-Ciocalteu method was 27.81 ± 14.18, 40.03 ± 1.27 and 268.36 ± 8.55 mg GAE/g for Sc-Hex, CHCl_3 and Sc-AcOEt, respectively. The quantitative determination of total flavonoids expressed in catechin equivalents (CE) in mg/g of plant extract was 276.70 ± 2.94 (Sc-Hex), 109.80 ± 1.48 (Sc-CHCl_3) and 44.88 ± 2.69 (Sc-AcOEt). These results are presented in Table 2.

S. convoluta was evaluated for its antioxidant capacity by the use of methods based on scavenging the free radical DPPH and inhibition of the auto-oxidation of β-carotene. The antioxidant activity was analysed according to the IC_{50} values, where lower values indicate high antioxidant activity (Table 3).

The DPPH assay showed that Sc-AcOEt exhibited the strongest free-radical scavenging activity among the tested extracts, with an IC_{50} value of 47.17 ± 1.19 mg/mL. Sc-Hex and Sc-CHCl_3 showed low antioxidant activity, with IC_{50} values equal to 289.50 ± 19.05 and 107.00 ± 07.05 mg/mL, respectively. Among the

| Sample       | TP (mg GAE/g) | TF (mg CE/g) | DPPH (IC_{50}, μg/mL) | β-Carotene (% AA) |
|--------------|---------------|--------------|-----------------------|-------------------|
| Sc-Hex       | 27.81 ± 14.18 | 276.70 ± 2.94| 289.50 ± 19.05        | 58.99 ± 0.65      |
| Sc-CHCl_3    | 40.03 ± 1.27  | 109.80 ± 1.48| 107.00 ± 5.07         | 44.99 ± 0.70      |
| Sc-AcOEt     | 268.36 ± 8.55 | 44.88 ± 2.69 | 47.17 ± 1.19          | 58.91 ± 10.24     |
| Ascorbic acid| –             | –            | 5.91 ± 0.22           | –                 |
| BHA          | –             | –            | 6.84 ± 0.29           | 91.21 ± 0.65      |
| BHT          | –             | –            | 32.66 ± 3.25          | 95.04 ± 3.22      |

Note: Due to the nature of the methods used, it is possible that other substances, such as non-phenolic antioxidants and glycosylated derivatives, might interfere with the determination of the TP and TF content. However, all analyses were performed in triplicate and no precipitate formation or turbidity was observed in the samples, which often occurs when there is presence of glycosylated derivatives.

| Microorganisms | MIC (mg/mL) | MBC (mg/mL) | MIC (mg/mL) | MBC (mg/mL) | MIC (mg/mL) | MBC (mg/mL) |
|----------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S. convoluta   |             |             |             |             |             |             |
| Sc-Hex         |             |             |             |             |             |             |
| Sc-CHCl_3      |             |             |             |             |             |             |
| Sc-AcOEt       |             |             |             |             |             |             |

Table 1. Phytochemical characterization of extracts from the leaves of S. convoluta.

Table 2. Total phenolics (TP), total flavonoids (TF) and antioxidant activity of extracts from S. convoluta.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the S. convoluta extract fractions against Gram-negative strains.
standards, ascorbic acid was the most effective antioxidant, with an IC50 value equal to 5.91 ± 0.22 µg/mL. Based on the inhibition of the autoxidation of β-carotene, the results were expressed as percentage antioxidant activity. In this assay, all the extracts showed moderate antioxidant activity: 58.99 ± 0.65 (Sc-Hex), 44.99 ± 0.70 (Sc-CHCl3) and 58.91 ± 10.24 (Sc-AcOEt). In this test, the results were compared with BHT and BHA, with potential antioxidant activity.

The results for the evaluation of the antibacterial activity of the S. convoluta extracts are shown in Table 3 and are expressed as MIC and MBC. According to the MIC values, the data showed that the strains were sensitive to the ethyl acetate fraction. The treatments showed potential bacteriostatic effect for all cases. However, the results were more promising for Ent. faecalis, E. coli and S. marcescens, with a MIC value of 0.39 mg/mL for the Sc-AcOEt fraction. Sc-CHCl3 showed moderate activity against K. pneumoniae (MIC 0.78 mg/mL). The hexane fraction had weak effect on bacterial growth: the MIC values were higher than 1.56 mg/mL. In the MBC test, the fractions showed weak bactericidal activity, and only the Sc-AcOEt fraction showed moderate activity against the B. cereus strain (0.78 mg/mL).

The S. convoluta fractions did not demonstrate cytotoxic activity at the tested concentration, except for the chloroformic fraction, which showed moderate activity, inhibiting human glioblastoma cell growth in 62.23% (Table 4).

Phenolic compounds, widely distributed in plants, have gained such attention, due to their antimutagenic, antitumour and antioxidant activity, presenting free-radical scavenging ability, which potentially could have beneficial effects on human health [36,37]. The effectiveness of most antioxidant agents is generally proportional to the number of hydroxyl (OH) groups present in their aromatic ring(s). Hence, the natural compounds would seem to have better antioxidant activity than the currently used synthetic antioxidants, making them particularly attractive for the food and the pharmaceutical industry [38,39].

Selaginellaceae is a family rich in biflavonoids, for example 4′-methoxy-robusta flavone and 7,4′-dime-thoxy-2′,3′-dihydroxy-robusta flavone, isolated from S. delicatula. In the literature, species from this family present antimalarial, anti-inflammatory, antibacterial, antioxidant and antiviral activities [40,41]. The results from preliminary phytochemical screening in the fractions indicated the presence of flavonoids, anthranidine derivatives, quinones, triterpenes, lignans and steroids. The results from the present study corroborate the reports of Sá et al. [42] and Sinvaraman et al. [4] about the presence of phenols, tannins, flavonoids, steroids and terpenoids in the crude ethanol extract of this species.

In the β-carotene bleaching test, the fractions presented moderate activity. This method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion [43,44]. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (for review, see [44]). In the test of DPPH free radical scavenging, the hexanic and chloroform fractions showed moderate activity. However, the ethyl acetate fraction presented potential antioxidant activity, showing IC50 values lower than those reported by Sá et al. [42] for this fraction. The DPPH reaction usually involves a hydrogen atom transfer reaction [45], but some kinetic data have also implied an electron transfer mechanism for this assay [46]. It depends on substrate polarity as well as hydrogen donation and/or radical scavenging activity, which may be conferred by the presence of multiple hydroxyls, for example. The DPPH method is generally sensitive; however, the DPPH radical has little relevance to the processes present in biological systems and living organisms. Nevertheless, this method is widely considered indicative of the ability of plant extracts to scavenge free radicals, and will refer to hydrogen atom or electron donation ability, independent of any enzymatic activity [47]. There is a positive relationship between total phenolics and flavonoid contents and antioxidant activity in many plants species [48–50], indicating that the activity in this study, particularly that of Sc-AcOEt, is due to the presence of these compounds.

Many studies are being performed with Brazilian medicinal plants in order to obtain biologically active natural products as an alternative to combat infections caused by antibiotic-resistant organisms [51]. This is the first study that shows that fractions of S. convoluta have antibacterial activity, where the ethyl acetate fraction has a low MIC value for strains of Enterococcus faecalis, Escherichia coli and Serratia marcescens. Thus, it could be considered promising as an antibacterial agent. However, another study by Sá et al. [52] with strains isolated from animals, indicated that the ethanol extract of S.
convoluta has weak antibacterial activity against Salmonella spp. and Streptococcus suis isolates. Such activity could be related to the presence of phenolic compounds such as flavonoids present in the ethyl acetate fraction, as demonstrated in phytochemical screening. Flavonoids feature antimicrobial activity, whose mechanisms of action can be classified as the inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolism [10,53].

The cytotoxicity of fractions was evaluated in HCT-116, SF-295 and OVCAR-8 at a concentration of 50 μg/mL, where the fractions showed moderate cytotoxic activity only against the SF-295 cells. Selaginellaceae species, such as S. delicatula, S. labordei and S. tamariscina, show bioflavonoids in their ethyl acetate fraction, for example robustaflavone and amentonflavone, and exhibit relatively stronger activities on Bel-7402 (hepatocellular carcinoma), HeLa and HT-29 (human colon adenocarcinoma) cells [54,55].

Conclusions

This study showed that the S. convoluta fractions have promising antioxidant activity, antibacterial activity and moderate cytotoxicity. Such activities can be justified by the major presence of phenolic compounds in this species. We suggest carrying out more detailed chemical composition analysis of this species to identify the substances involved in these biological activities and determine through pharmacological tests the mechanism of action involved in the biological response.

Disclosure statement

No potential conflict of interest was reported by the authors.

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