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Bioactive proteins from *Plectranthus barbatus* and detection by RP-HPLC-PDA

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Abstract
Plectranthus barbatus Andrews (Lamiaceae) is a popular medicinal plant used to treat gastrointestinal and hepatic ailments. In African countries, *P. barbatus* is widely used as an herbal remedy to manage HIV/AIDS and related conditions. This work describes the extracts phytochemical analysis to extraction of proteins and evaluation of their cytotoxic activity against A549 e RAW264.7 cancer cells, NO production and cell viability from crude extracts and protein fractions as well as detecting the presence of these proteins by RP-HPLC-PDA and spectrophotometry. The presence of proteins was analyzed by electrophoresis in SDS-polyacrylamide gel PAGE12% and detected by HPLC-PDA and spectrophotometry. In the cell viability assay, it can be classified as non-cytotoxic. The aqueous extract from *P. barbatus* leaves presented cytotoxic action against lung carcinoma tumor cell lines and the aqueous extract did not increase the NO production in relation to the control. These results may contribute to improve the use of this plant by the population in the prevention of diseases, since this species does not present significant toxicity and showed cytotoxicity against lung carcinoma cells.

Keywords: *Plectranthus barbatus*, protein, cytotoxicity, RP-HPLC-PDA

Introduction
Plants are important sources of biologically active molecules that differ in structure and biological properties. *P. barbatus* is popularly used for treatments of gastric disorders and their leaves are the most commonly used, administered orally by the population (Gerhardt *et al*., 2013) [1]. Kapewangolo *et al.* (2013) [2] demonstrated that in vitro anti-HIV-1 potential of *P. barbatus* including direct activity as well as through the stimulation of protective immune and inflammatory responses. The low cytotoxicity of the extract is also in agreement with the vast anecdotal use of this plant in treating various ailments with no reported side-effects. This work describe the analysis protein by electrophoresis and their detection through HPLC-PDA and spectrophotometry from aqueous extract of *P. barbatus* and evaluation of its pharmacological potential on RAW 264.7 and A549 cytotoxic cell lines, cell viability and nitric oxide production.

Materials and Methods
Plant Material
The plant was cultivated at ICET-UFVJM (Institute of Sciences, Engineering and Technology) and its exsiccate was deposited in the Jeanine Felfili-HDJF Dendrological Herbarium from Forestry Engineering Department of the UFVJM under number HDJF3358.

Crude extracts preparation
200g of dried plant material (leaf, flower and stem) was milled and ground in a blender and extracted with 10% w/v distilled water. The material was heated for 30 minutes at 55 °C under constant stirring and rested in an ice bath for 2 hours. The resultant from this treatment was filtered in cotton to remove the insoluble fibers present and the liquid subjected to vacuum filtration in Buchner's funnel with the aid of a filter paper and lyophilized (LS 3000, Terroni, São Paulo). The material was stored -80 °C for further.

Protein Extraction from extracts of *Plectranthus barbatus*
In order to isolate and detect the proteins present in the samples, the following protocols were used: A second plant material (*Glycine max*) has been used for comparative purpose (standard to extraction of proteins). The Protein extraction was performed, based on the methodology

proposed by Yavelow *et al*. (1985) [6] and adapted. The selected soy beans (100g) were comminuted in a blender and delipidated with 10 volumes of pure acetone. The proteins were extracted by adding 10% w/v 60% ethanol at 55°C with constant stirring for 1 hour and then cooled in an ice bath and allowed to stand for 24 hours. After extraction, both materials were gassed and maintained at pH 5.3. 2 volumes of acetone were added and then centrifuged at 4724 x g for 30 minutes and the resulting precipitate was resuspended in distilled water and stored at -80 °C.

Protein dosages and SDS-PAGE

The protein dosages of *Glycine max* and *P. barbatus* were determined by colorimetric methods, using Bradford reagents (1976), in the absorption of the Coomassie Brilliant Blue G-250 reagent, using bovine serum albumin (BSA) in solutions of 20 to 100 mg/mL to obtain the standard curve, as an analytical standard at 1 mg/mL concentration. The quantifying protein by directly measuring absorbance was performed in a spectrophotometer at the wavelength of 595 nanometers (UV-Vis Cary 50, Varian). Samples from the procedures used to carry out isolation of proteins were identified and characterized by polyacrylamide gel electrophoresis in the batch system, the electrophoretic supports being 12% polyacrylamide gels of 5% concentration, prepared under conditions Denaturants (SDS), according to the method Laemmli (1970). Samples were thawed at room temperature, and thereafter a spin of one minute was given at 2800xg, 40 µl of the supernatant was withdrawn for dilution in 20 µl of 1M Tris sample buffer pH 6.8; 20% w/w SDS, 20% v/v glycerol, 0.5M EDTA, 0.1% w/v bromophenol blue, β-mercaptoethanol (Sigma-Aldrich Company) and distilled water q.s. and subjected to the boiling water bath for 5 minutes for total protein denaturation. A mixture of isoforms from protein Concavalina A (ConA SIGMA) of molecular masses 10 to 250 kDa was used as molecular mass standard (Pageruler Plus Prestained Protein Ladder, Thermo Scientific).

The 25 mMTris-HCl buffer containing 0.19 M glycine and 0.1% w/v SDS was used as the running buffer. Electrophoresis was performed under a constant electric current of 120 V for approximately 180 minutes (vert-i10, loccus, São Paulo). After the end of the electrophoresis, the presence of the protein bands was detected by immersing the gels in 0.025% Coomassie Blue R-250 solution in 25% methanol and 5% acetic acid under gentle agitation for 2 hours. The gels were then bleached in 40% methanol solution and 10% acetic acid until full visualization of the bands.

Wavelength Scan and Protein detection by RP-HPLC-PDA analysis

The supernatants from protein extracts were diluted (10x, 100x, 1000x) in distilled water to spectrophotometric analysis (UV-Vis Cary 50) of wavelength scan for analysis of UV spectra and better absorption for further analysis in HPLC-PDA. The data were generated by software Scan version 3.0 (Interscience) and analyzed by the program Originpro 8. Samples of protein fractions and crude extract were subjected to RP-HPLC-PDA for purity analysis and proteins detection. Analyzes were performed using C18 column Supelcosil Sigma-Aldrich 25cmx4.6mm, 5um (particle size) on a ProStarVarian HPLC chromatograph using the Galaxie software and data were analyzed in the original pro program 8. Elution was monitored at 190 to 300 nm. 30 µL of each sample was injected. The analysis was performed on a gradient of ACN (A: H2O + 0.1% TFA, B: ACN + 0.08% TFA, gradient elution: 0 to 100% B in 45 minutes).

Bioactivity of protein fractions from plant material of *P. barbatus*

Cell culture assay: RAW264.7 macrophages and adenocarcinomic human alveolar basal epithelial cells (A549) were maintained in culture bottles containing RPMI-1640 medium (LONZA) supplemented with 1% non-essential amino acids, 100 µg.mL-1 streptomycin and penicillin, and 5% serum fetal bovine (SIGMA) in humid atmosphere of 5% CO2 at 37ºC. After reaching confluence the bottles were scraped and the cells plated in 96-well plates at the concentration of 5x10 4 cells.mL-1.

For the cell viability assay, macrophage lineage (RAW264.7) or human lung fibroblast (A549) lineage were incubated in 96-well plates at a concentration of 5x104 cells.mL-1 in a humidified atmosphere of 5% CO2 at 37°C in the presence of the protein fractions and crude extracts at concentrations of 100, 10 and 1 µg.mL-1 or with aqueous extract at the concentrations of 200, 50 and 10 µg.mL-1 for 48 hours.

For the NO dosage, the macrophages were incubated in humidified atmosphere of 5% CO2 at 37ºC in the presence of IP enriched extract at the concentrations of 100, 10 and 1 µg.mL-1 or with aqueous extract at concentrations of 200, 50 and 10 µg.mL-1 for one hour and subsequently stimulated with LPS (10 µg.mL-1) and IFN-γ (9ng.mL-1) at 10% of the culture volume. After 48 hours of culture, supernatant was collected for further analysis.

Cell viability assay: Cell viability was measured using the MTT [{3-(4, 5-dimethylthiazol-2yl) -2,5-diphenyltetrazolium bromide] assay in culture of unstimulated cells. After 48 hours of culture, the supernatant was removed and the cells were incubated with 100µL of supplemented RPMI and 10µL of MTT (5mg / mL-1) for 4 hours in a humidified atmosphere of 5% CO2 at 37°C. After this time, the plates were centrifuged for 5 minutes at 1500 rpm. The supernatant was removed from the wells without any change in the precipitate. The formed formazan crystals were then dissolved by the addition of 100µL of DMSO in each well. The complete solubilization was done by gentle agitation of the plates. Optical density was measured at the wavelength of 560nm (EZ Read 2000, Biochrom).

Nitric Oxide dosage (NO): The 48 hour supernatants from the stimulated cultures were analyzed to quantification of nitrites by the Griess method. Aliquots of supernatants were plated with equal volumes of 1% sulfanilamide and 0.1% N-(1-naphthyl) (ethylenediamine). The NO production was quantified by comparison to a standard curve with different concentrations of NaNO2. Optical density was measured at the wavelength of 540nm (EZ Read 2000, Biochrom).

The results presented are representative of at least 3 independent experiments and are shown as mean ± standard deviation. The difference significance was analyzed using Student's t-test and Mann-Whitney test where appropriate. Differences are considered significant when $p < 0.05$.

Results and Discussion

Detection of proteins by electrophoresis

Based on data from the quantification of proteins present in the extracts, they were subjected to polyacrylamide gel detection. Figure S2 shows the electrophoretic profile from extracts semi-purified and prepared from the soybean grains.

According to Leal (2010) [4], the result proves the presence of proteins, which demonstrates the efficiency of the purification process used to extract proteins/protease inhibitors according to the methodology described by Yavelow *et al*. (1985) [6]. Indicative of protein with apparent molecular mass of 13, 23 and 30 KDa.

In the analysis of the electrophoretic profile (12% SDS-PAGE) of the protein extracts a band at the height of 14 kDa in the soybean extract was observed, being able to be related to the molecular weight of the inhibitors of trypsin and Bowman-Birk (BBI). *Glycine max* has wide distribution of protease inhibitors in its seeds, besides the lectin that presents molecular mass around 30 kDa.

Polyacrylamide gel of the protein extracts of stem and leaves from *P. barbatus* demonstrate the presence of proteins of molecular mass around 28 kDa. In the stem of the *P. barbatus* was also found a band around 90 kDa. In the extract of the leaves from "Boldo do chile", another species, and flowers from *P. barbatus* ("Boldo da terra"), no band was detected (Figure S2).

According to Sharon and Lis (2001) [5], these results showed in the figure S2 may suggest the presence of lectins, which usually consist of two or four subunits, with molecular mass ranging from 25kDa to 30KDa. Lectins are widely distributed in nature.

Thus, from the satisfactory results to detection of proteins of the extracts from *P. barbatus* leaves by means of the electrophoretic profiles, they were subjected to a wavelength scan to obtain spectra of UV tests prove the presence of amino acid units and to identify the best absorption of these constituents for HPLC-PDA analysis.

Results from quantification of protein extracts and the standard of 595 nm absorbance soy showed similar values to leaves, stem and flowers of *P. barbatus* (figure S1).

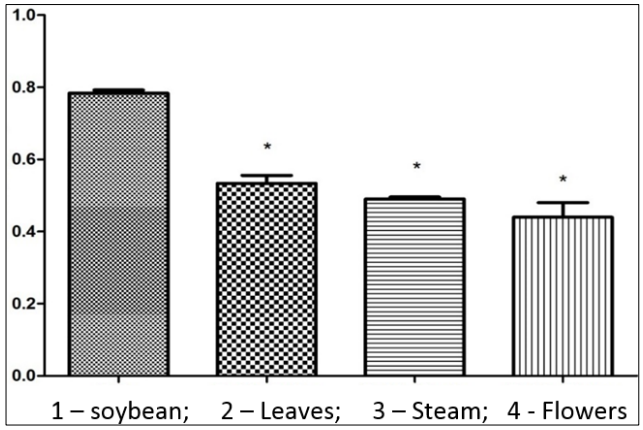

Fig S1: Quantification of protein extracts from plant material of *P. barbatus* and standard soy (595 nm absorbance). The differences were considered significant in relation to soybeans when * p 0.05.

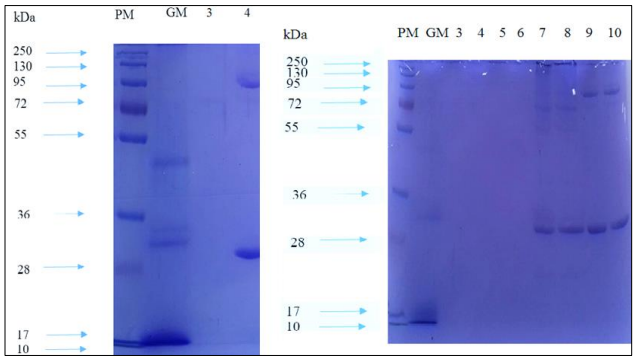

Fig S2: Electrophoretic profile (12% SDS-PAGE) of stem, leaves and flower protein extracts obtained by the method described by Yavelow *et al*. (1985) [6]. PM: Molecular weight from 10 to 250kDa, GM: extract based on G. max grain (standard protease inhibitors). 1-2-3: flower extracts; 4-5-6: leaves extracts; 7-8-9: stem extracts

Wavelength scan and RP-HPLC-PDA analysis of protein extracts

The result shows the presence of two bands at 230nm and 280 280nm for the stem extract and 230 nm for leaves extract.

Protein extracts were subjected to RP-HPLC-PDA analysis for purity analysis. The analysis presented constituents that could be detected in absorbance of 215 to 400 nm. Therefore, having the chromatographic profile presented two peaks at retention times of 16.39 and 27.45 minutes for stem extract and 12 and 13 min for leaves extract as shown in figure S3. The peak around 27 min, possibly represent lectins such as already characterized by Leal (2010) [4] in soybean seeds. The analysis by HPLC-PDA was able to detect protein in extracts from *P. barbatus*.

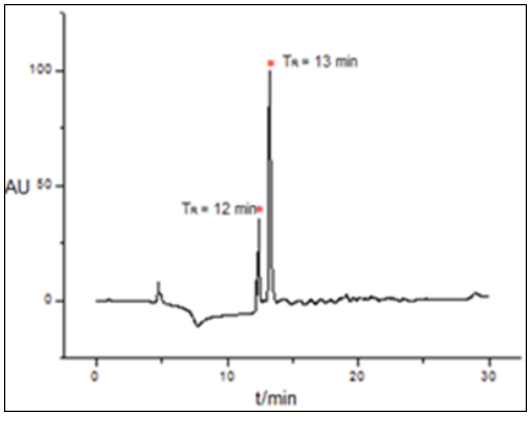

Fig S3: Chromatogram of leaves extract of *P. barbatus* in 254 nm obtained by analysis in RP-HPLC-PDA

Cell viability and NO production of crude extracts from leaves and stem

RAW 264.7 cells were treated at different concentrations of protein extracts from leaves and stem from *P. barbatus*. The result demonstrates a small reduction in cell viability (Figure S4). Therefore, it was concluded that the cells in the presence of the proteins when compared to the control had a low cytotoxic effect at the concentrations evaluated.

The effect of compounds on the production NO was performed by means of the Griess reagent. The most common screening methods for testing compounds involve pre-treatment with the compound before stimulating the response. After treatment with protein extracts of stem and leaves, the production of nitric oxide showed higher inhibition rates in the extracts with higher concentration. All concentrations showed a significant difference in relation to the control group.

The results presented from the MTT colorimetric test of protein extracts from leaves and stem were tested on carcinoma cells (A549). The tests did not present significant difference between the different concentrations, presenting viability superior to 80%, allowing to classify it as non-cytotoxic, according to ISO 10993-5.

Many authors indicate to be approved in *in vitro* cytotoxicity test, a product should not cause cell death or affect its cellular functions. Thus, in the present study, viability tests were also carried out using RAW 264.7 macrophages from RAW 264.7 mice, to evaluate the cell viability by the MTT method with their exposure at different concentrations. In this assay, it can be inferred that the aqueous extracts from leaves of *P. barbatus*, as shown in figure S5, showed a higher percentage of cell death in the highest concentrations of the extract.

Nitric oxide plays a role in the regulatory and cytotoxicity functions, therefore, after exposing the aqueous extracts, the presented results allowed to observe that the aqueous extract did not increase the NO production in relation to the control.

It was also evaluated the cytotoxicity of the aqueous extract of leaves from *P. barbatus* through the analysis of cell viability, it is verified that the A549 cells presented an indicative for cytotoxic action in the lung carcinoma tumor cell lines. The results presented for the extracts in higher concentration cause a decrease in cell viability.

Similarities and differences between results obtained from different extracts in bioactivity tests show its potential bioactive and confirm its use as a medicinal plant for various purposes.

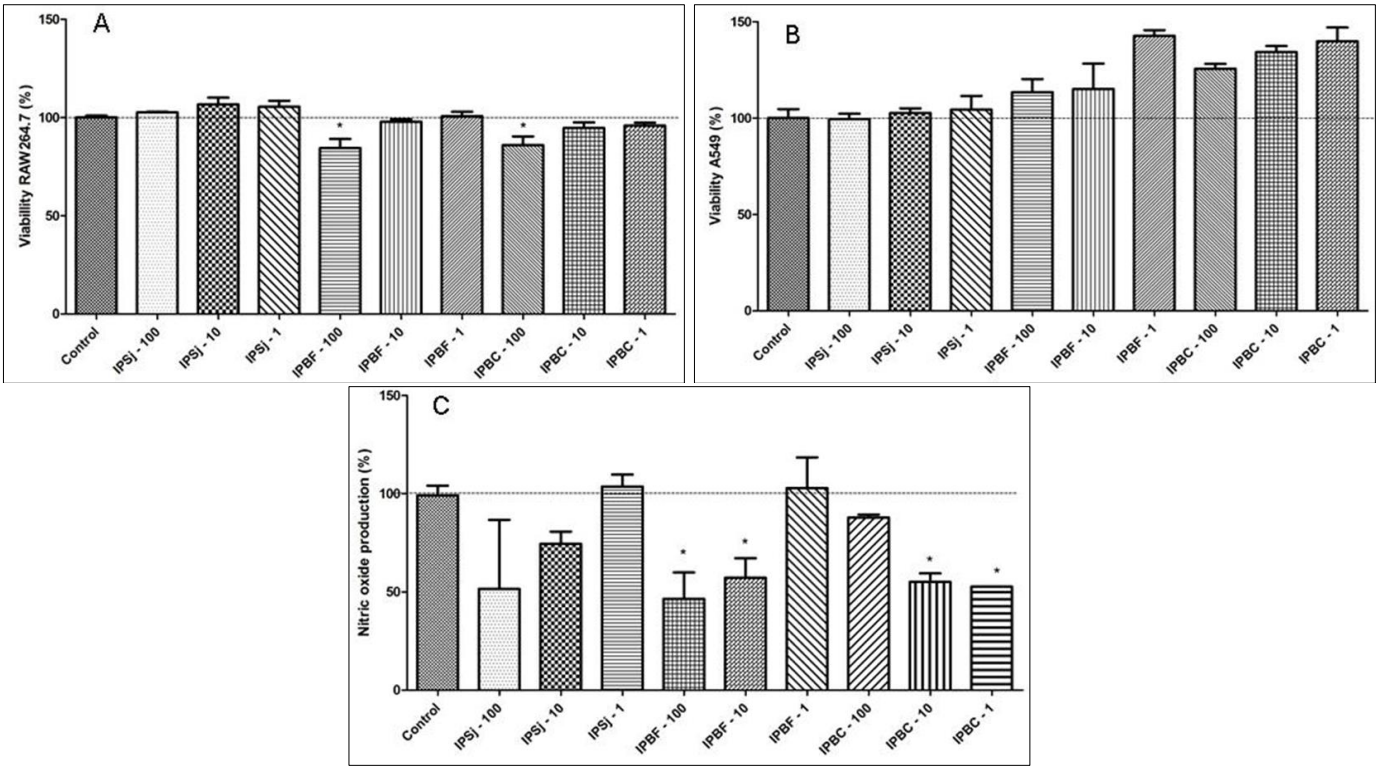

Fig S4: Proteins activity. Cellular viability of RAW264.7 (A) and A549 (B), and nitric oxide production (C). A549 and RAW264.7 were treated by 48hs with dilutions (100, 10, 1 µg/ml) of P. barbatus proteins extracted of leaves (IPBF) or stem (IPBC), and proteins of G. max seeds (IPSJ) and the cellular viability (A,B) were determined by MTT assay. In another experiment, RAW264.7 were stimulated with 10 µg/ml of LPS plus 9 ng/ml of IFN-γ and cultured by 48hs in the presence of dilutions (100, 10, 1 µg/ml) of P. barbatus proteins extracted of leaves (IPBF) or stem (IPBC), and proteins of G. max seeds (IPSJ) and the supernatants were collected to determine the nitric oxide production (C). Each bar represents Mean±SEM. Control=cells not treated with proteins. *p<0.05 in comparison to control. Data are representative of three independent experiments

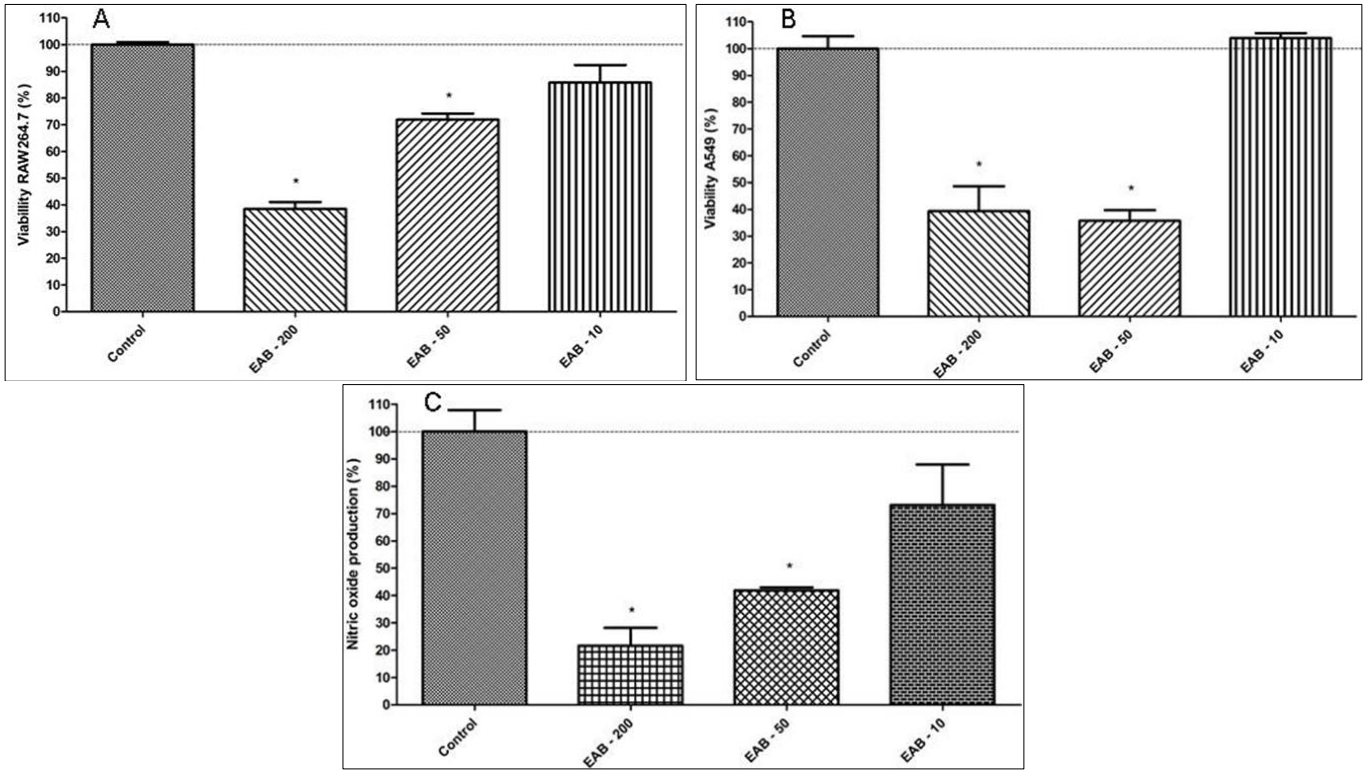

Fig S5: Aqueous extract activity. Cellular viability of RAW264.7 (A) and A549 (B), and nitric oxide production (C). A549 and RAW264.7 were treated by 48hs with dilutions (200, 50, 10 µg/ml) of P. barbatus aqueous extract of leaves (EAB) and the cellular viability (A,B) were determined by MTT assay. In another experiment, RAW264.7 were stimulated with 10 µg/ml of LPS plus 9 ng/ml of IFN-γ and cultured by 48hs in the presence of dilutions (200, 50, 10 µg/ml) of P. barbatus aqueous extract of leaves (EAB) and the supernatants were collected to determine the nitric oxide production (C). Each bar represents Mean±SEM. Control=cells not treated with aqueous extract. *$p<0.05$ in comparison to control. Data are representative of three independent experiments

Conclusion

In this work, we focus on the extracts chemical analysis to extraction of proteins and detecting the presence of these proteins by RP-HPLC-PDA and spectrophotometry and evaluation of their cytotoxic activity against A549 e RAW264.7 cancer cells, cell viability and NO production of crude extracts and protein fractions as well. According to the obtained results, the aqueous extract did not increase the NO production in relation to the control and the aqueous extract from *P. barbatus* leaves presented an indicative cytotoxic action against lung carcinoma tumor cell lines. In the cell viability assay, it can be classified as non-cytotoxic. The analysis by HPLC-PDA and spectrophotometry was able to detect the protein present in the extracts. The results may contribute to improve the use of this plant by the population in the prevention and treatment of diseases, since this species does not present significant toxicity and showed cytotoxicity against lung carcinoma cells. Considering the fact that protein of this species is not investigated at all, more studies are needed regarding protein characterization and bioactivity of its species. Similarities and differences between results obtained from different extracts in bioactivity tests show its potential bioactive and confirm its use as a medicinal plant for various purposes.

Supplementary Material

Experimental details relating to this paper are available online.

Disclosure statement

No potential conflict of interest was reported by the authors.

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