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Research Article

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Phytochemical and Biological Screening of *Plectranthus barbatus* (Lamiaceae)

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Abstract *Plectranthus barbatus* Andrews (Lamiaceae) is a popular medicinal plant used to treat gastrointestinal and hepatic ailments. This work describes extracts phytochemical analysis to extraction of proteins and evaluation of inhibitory action of trypsin and nitric oxide production of crude extracts and protein fractions as well as detecting the presence of these proteins by HPLC-PDA and spectrophotometry. Samples of the extraction procedures from leaves, flowers and stem of *P. barbatus* were subject to quantification of protein and detected by electrophoresis in SDS-polyacrylamide gel PAGE12% and detected by HPLC-PDA and spectrophotometry. Protein semi-purified extracts of stem and leaves did not present an inhibitory action of trypsin activity in comparison with purified soybean extract and the production of nitric oxide showed higher inhibition rates in higher concentration from protein extracts and all concentrations showed a significant difference in relation to the control group. The results may contribute to improve the use of this plant by population in the diseases prevention and treatment.

Keywords *Plectranthus barbatus*, Protein, Inhibitory action of trypsin, Medicinal Plants

Introduction

Plectranthus species (Lamiaceae family) is distributed worldwide and has numerous ethnomedicinal uses such as in antibacterial, antiviral, anti-malaria and antifungal phytotherapy and contains about 300 species with 45 of these being used ethnobotanically on the African continent [1].

Plectranthus species in Clade 1 are frequently used as medicines to treat a range of ailments, especially digestive, skin, infective and respiratory problems as well used as foods, flavours, fodder and materials. The higher incidence of study may be as a result of the higher diversity of uses and the fact that species in Clade 1, such as *Plectranthus barbatus, Plectranthus amboinicus* and *Plectranthus mollis*. About the Chemistry from *Plectranthus* genus, are found monoterpenoids, sesquiterpenoids, diterpenoids and phenolics [2].

Plectranthus barabatus Andr. is regarded as one of the most important medicinal species of the genus *Plectranthus*. This species is originated from north-eastern Africa and is found in sub-Saharan Africa as an invasive species [3].

Studies performed by Maioli et al. (2010) [1] showed that the phytochemical extract from *P. barbatus* protected mitochondria against Fe^{2}+/citrate-mediated swelling and malondialdehyde production, a property that persisted even after simulation of its passage through the digestive tract. These properties could be attributed to the phenolic molecules such as nepetoidin and caffeic acid esters identified in the extract. These effects can be probably by chelation of iron, revealing potential applicability as a therapeutic source of molecules against diseases involving mitochondrial iron overload.

Analysis *in vitro* metabolism from *P. barbatus* leaves, by the gastrointestinal tract showed that there was no appreciable degradation and that the activity was kept constant after gastric juice digestion and the action of Caco-2 cells on the extract showed that neither rosmarinic acid (main substance present in the extract) nor the other compounds present in minute quantities were metabolised by the intact cells. Rosmarinic acid could be found inside Caco-2 cells although in trace amount. Glucoronidase from *E. coli* was able to hydrolyse the flavonoid derivatives, thus the aglycones were formed and permeate the Caco-2 cells [4].

P. barbatus organic extract showed chemosuppressive activities of 81.45% and 78.69%. This antimalarial activity was not significantly different from that of chloroquine (Po0.05) [5].

Studies performed by Figueiredo et al. (2010) [6] showed that leaves aqueous extracts of *P. barbatus, P. ecklonii* and rosmarinic acid may prevent dental caries, since they demonstrate significant inhibition of water insoluble glucans synthesis and reduce biofilm production, critical factor for dental plaque formation. The results also show that this activity may be related to the presence of rosmarinic acid present in the extracts. According the authors, taking into account the low toxicity and potent antioxidant properties of both extracts found in our previous studies, together with the results obtained in the present work, these teas may be useful in the prevention or controlling several oral diseases, even while being consumed as a hot beverage.

Materials and Methods

Plant Material

This work was carried out at the Laboratory of Biochemistry and Immunology from UFVJM-Mucuri Campus. The plant material (leaves and stem) of *P. barbatu*s was collected at the ICET-UFVJM (Institute of Sciences, Engineering and Technology). The collected samples were duly pressed in wood presses and dried in greenhouses at an average temperature of 40°C. The species was taxonomically identified 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 (leaves 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 of this treatment was filtered in cotton to remove the insoluble fibers present and the liquid subjected to a second 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: The soybean (*Glycine max*) was used as the standard to extraction of proteins. Protein extraction was performed, based on the methodology proposed by Yavelow et al. (1985) [8]. 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.

The extraction of the proteins from leaves and stem of *P. barbatus* was performed by adapted methodology Yavelow [8]: The leaves and stem were ground in a blender, 100g of vegetable material was used for each preparation, adding 10% w/v 60% ethanol, heated for 1 h at 55 °C with constant stirring 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 min, 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).

Samples were thawed at room temperature, and thereafter a spin of 1 minute was given at 2800 x g, 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 (PagerulerPlusPrestained Protein Ladder, ThermoScientific).

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: The supernatants of the 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.

Protein analysis by HPLC-PDA

Samples of protein fractions and crude extract were subjected to HPLC-PDA for purity analysis and proteins detection. Analyzes were performed using C18 column Supelcosil Sigma-Aldrich (5ìm particles, 25cmx4.6mm) on a ProStarVarian HPLC chromatograph using the Galaxie software and data were analyzed in the original pro program 8. The column chromatography was previously (H_2O) (system A), and eluted in a gradient of 5 to 100% solvent acetonitrile (ACN) + 0.08% TFA (solvent B), under a flow rate of 0.8 mL/min for 45 minutes. 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: H_2O + 0.1% TFA, B: ACN + 0.08% TFA, gradient elution: 0 to 100% B in 45 minutes).

Inhibition of trypsin activity

For the determination of enzymatic activity of the supernatants, in the presence or absence of proteins, was used the method proposed by Erlanger et al. (1961) [7], in which the proteolytic activity of the bovine trypsin was measured using as synthetic substrate the D, L -BAOLA. The concentration of the n-nitroaniline product, released from the enzymatic hydrolysis of D, L-BañNA, which was measured by spectrophotometric analysis at 410nm.

For control blank, 60% acetic acid (v/v) was added prior to D, L-BANNA. The percentage of trypsin inhibition was calculated from the control readings from enzyme and the samples tested. The calculation of the trypsin inhibitor activity (TIA) was done by converting the results obtained into milligrams of inhibited pure trypsin per gram of sample, as suggested by Rackiset al., 1996, as indicated: mg of inhibited trypsin/g sample = (AxB)/(Cx1000xP) where A (410nm)=absorbance of the standard- absorbance of the sample (corrected for absorbance of sample blank); B=sample dilution; C=trypsin factor, i.e. the product of the action of 1 ìg of active trypsin on the D substrate, L-BAñNA provides an absorbance reading at 410 nm under the assay conditions; P = weight of the sample in g.

Dosage of Nitric Oxide (NO)

Supernatants from the stimulated cultures were analyzed for quantification of nitrites by the Griess method. Aliquots of the supernatants were plated with equal volumes of 1% sulfanilamide and 0.1% N- (1-naphthyl) (ethylenediamine). The nitrite production was quantified by comparison to a standard curve with different concentrations of $NaNO_2$. Optical density was measured at the wavelength of 540nm (EZ Read 200, Biochrom).

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 2 shows the electrophoretic profile from extracts purified and prepared from the soybean grains. 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. [8]. Indicative of protein with apparent molecular mass of 13, 17, 28 and 36 KDa.

In the analysis of the electrophoretic profile (12% SDS-PAGE) of the protein extracts a band at the height of 14 kDa (figure 1) in the soybean extract was observed, being able to be related to the molecular weight of the inhibitors of trypsin and Bowman-Birk (BBI). In the present study, it is possible to determine the presence of BBI inhibitors in the presence of the inhibitor dimer in a variety from leguminous plants [8].

Glycine max has wide distribution of protease inhibitors in its seeds, besides the lectin that presents molecular mass around 30 kDa. They are resistant to the heat treatment employed for extraction of protease inhibitors, the heating temperature being not sufficient to denature them which makes their expected presence in the polyacrylamide gel [9].

Polyacrylamide gel from the protein purified extracts and the *P. barbatus* leaves demonstrate the presence of proteins of molecular mass around 28 kDa (Figure 1). In the stem from *P. barbatus*, a band around 90 kDa was also found (Figure 1). In the extract of the leaves of the "boldo of chile" and of the flowers *P. barbatus* no band was detected (Figure 1).

In the stem extract we observed protein with molecular mass around 30 KDa and 100 KDa.

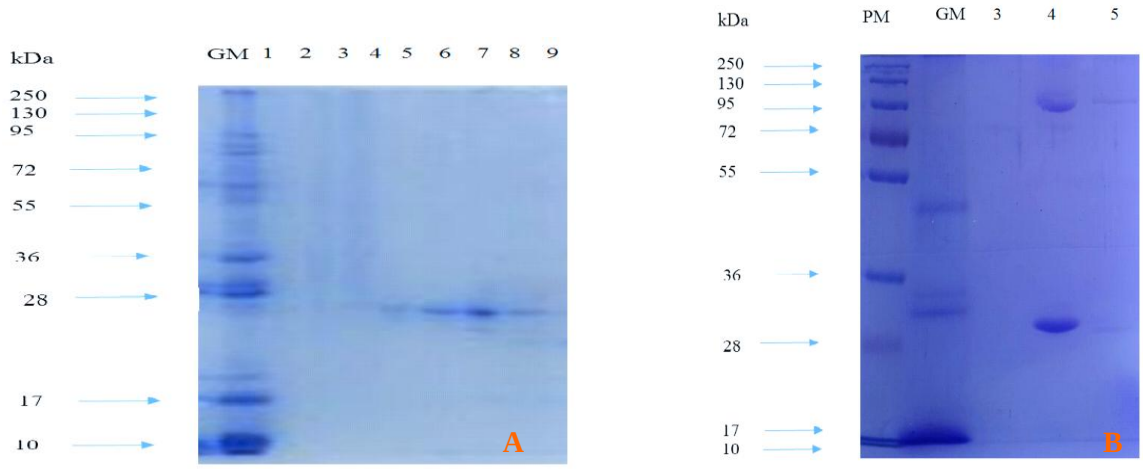

Figure 1: Electrophoretic profile (12% SDS-PAGE) from protein extract of *P. barbatus* obtained by the method described by Yavelow et al. [8]. PM: Molecular weight from 10 to 250 kDa, GM: extract based on *G. max* grain (protein standard). Columns 1-2-3A represents the flower extracts of the *P. barbatus*. Columns 4-5-6 indicate extracts based on *P. barbatus* leaves. Further, columns 7-8-9A are the results for the stem extracts of *P. barbatus*. 50 mg / mL of protein was applied to each well. Gel stained with Coomassie Blue R-250. 3B-flowers extract, 4B- stem extract, 5B- leaves extract.

50 mg/mL of protein extract was applied to each well. Coomassie Blue R-250 Color Gel. Column 3 represents the flower-based extracts of *P. barbatus*. The column 4 for the extracts of the stem from *P. barbatus* and the column 5 for the leaves extracts from *P. barbatus*. 50 mg/mL of protein was applied to each well. Gel stained with Coomassie Blue R-250.

According to Sharon and Lis [10], these results 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 and can

be found in plant tissues such as: root, leaf, stem, pod, fruit, flowers and even bark. The largest amount of plant lectins is found in the stock organs, depending on the plant, the stock organs have different locations [10].

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.

The result shows the presence of a maximum peak at 230nm for stem and to leaves extracts (Figure 2) and a band around 280nm for the stem extract.

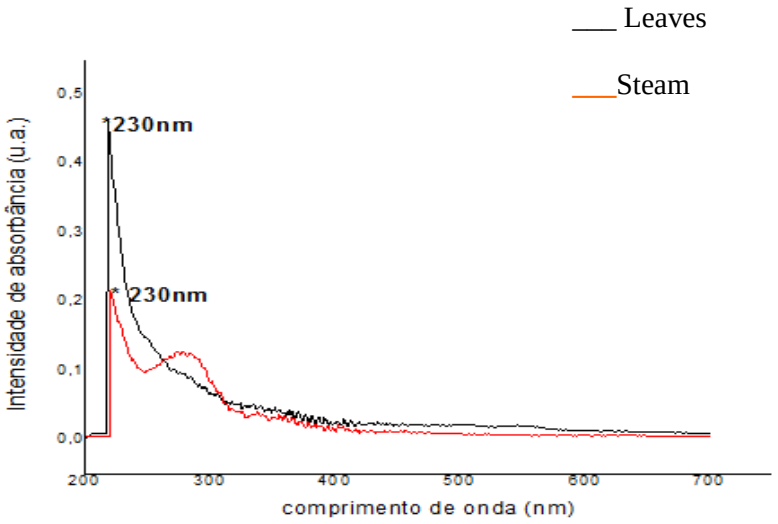

Figure 2- Molecular absorption spectrum of proteins from extracts of leaves and stems. The samples presented analytical curves 230 nm absorption for the extracts, being monitored from 200 to 700 nm

HPLC-PDA analysis

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 13.45 and 27.12 min for leaves extract. The peak around 27 min, possibly represent lectins such as already characterized by Leal SC (2010) [11] in soybean seeds.

Evaluation of anti-trypsin activity of extracts from P. barbatus

The extracts from leaves and stem, previously characterized in polyacrylamide gel and analyzed by RP-HPLC-PDA, were submitted to anti-trypsin activity. The results are shown in figure 3.

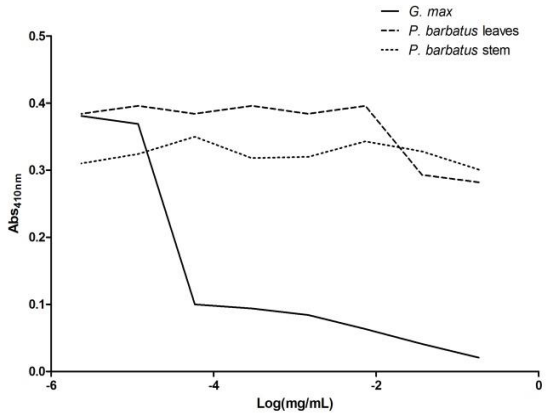

Figure 3- Anti-trypsin activity of protein extracts of soybean, leaves and stem extracts from *P. barbatus*. 20 µl serial inhibitor dilutions were added to each test tube containing 200 µl DL-BApNA (N-α-benzoyl-DL-arginyl-p-

nitroanilide) and 20 µl trypsin (0.05 mg mL -1). Quantification of the inhibitory activity was analyzed at 410 nm. The EC50 was calculated by analyzing the non-linear regression of the curve.

The results shows that the protein semi-purified extracts of stem and leaves did not present an inhibitory action of trypsin activity in comparison with purified soybean extract.

Production of nitric oxide from proteins of leaves and stem extracts

The effect of the compounds on the production of nitric oxide (NO) was performed by means of the Griess reagent. The most common screening methods for testing compounds involve pretreatment with the compound before stimulating the response. As shown in figure 4, after the treatment with enriched extracts of stem and leaf protein, the production of nitric oxide showed higher inhibition rates in extracts with higher concentration. All concentrations showed a significant difference in relation to the control group.

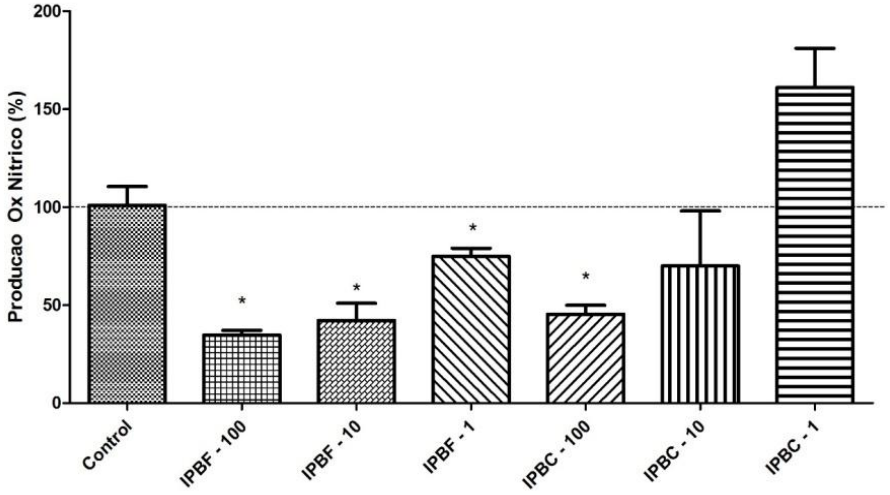

Figure 4 - Production of Nitric Oxide. RAW 264.7 were stimulated with LPS and IFN-γ and treated with 100, 10 or 1 µg / ml enriched extract of leaf protease inhibitors (IPBF) and stem (IPBC) of P. barbatus. The production of Nitric Oxide was determined by the Griess method. The columns represent Mean ± SEM. Control = stimulated and untreated cells with enriched extract of protease inhibitors.

Protein semi-purified extracts of stem and leaves did not present an inhibitory action of trypsin activity in comparison with purified soybean extract. Legume species that have properties of forming protein-protein complexes, whether reversible or not, as proteolytic enzymes, promoting the inhibition of protease activity by their catalytic site, such as soybean trip inhibitors.

Conclusion

The results demonstrated that the purified protein extract was able to inhibit the production of nitric oxide in the concentration of 10 µg/ml for leaf and stem and 100 µg/ml for leaf and also demonstrated that the semi-purified extracts do not constitute an act of inhibition from trypsin activity in relation to the purified extracts of soybean. The chromatographic and spectrophotometric analysis showed able to detect the protein present in the extracts. Therefore the results may contribute to improve the use of this vegetal resource by the population in the prevention and treatment of other types of diseases. The "boldo da terra" (*Plectranthus barbatus*) is popularly used for the treatment of gastrointestinal disorders and for liver diseases. Due to the existence of a large number of species available for research and pharmacological studies, plants are important in the progress of science, especially in the development of new drugs.

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References

1. Maioli MA, Alves LC, Campanini AL, Lima MC, Dorta DJ, Groppo M, Cavalheiro AJ, Curti C, Mingatto FE. (2010). Iron chelating-mediated antioxidant activity of *Plectranthus barbatus* extract on mitochondria. *Food Chemistry*, 122: 203–208.
2. Lukhoba CW, Simmonds MSJ, Paton AJ. (2006). *Plectranthus*: a review of ethnobotanical uses. *Journal of Ethnopharmacology*, 103: 1–24.
3. Rice LJ, Brits GJ, Potgieter CJ, Staden JV. (2011). *Plectranthus*: a plant for the future*? South African Journal of Botany*, 77: 947–959.
4. Porfírio S, Falé PLV, Madeira PJA, Florêncio MH, Ascensão L, Serralheiro MLM. (2010) Antiacetylcholinesterase and antioxidant activities of Plectranthus barbatus tea, after in vitro gastrointestinal metabolism. *Food Chemistry*, 122: 179–187.
5. Kiraithe MN, Nguta JM, Mbaria JM, Kiama SG. (2016). Evaluation of the use of *Ocimum suave* Willd. (Lamiaceae), *Plectranthus barbatus* Andrews (Lamiaceae) and *Zanthoxylum chalybeum* Engl. (Rutaceae) as antimalarial remedies in Kenyan folk medicine. Journal of Ethnopharmacology, 178: 266–271.
6. Figueiredo NL, Aguiar SRMM, Falé PL, Ascensão L, Serralheiro MLM, Lino ARL (2010). The inhibitory effect of *Plectranthus barbatus* and *Plectranthus ecklonii* leaves on the viability, glucosyltransferase activity and biofilm formation of Streptococcus sobrinus and *Streptococcus mutans*. *Food Chemistry*, 119: 664–668.
7. Erlanger BF, Kokawsky. Cohen W. (1961). The preparation and properties of two new chromogenic substances of trypsin. Arch, *Biochem.Biophys*, 95: 281-278.
8. Yavelow J, Collins M, Birk Y, Troll W, Kennedy AR. (1985). Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress X-ray induced transformation *in vitro*. *Proc. Nati. Acad. Sci*. 82: 5395-5399.
9. Kapewangolo P, Hussein AA, Meyer D. (2013). Inhibition of HIV-1 enzymes, antioxidant and anti-inflammatory activities of *Plectranthus barbatus*. *Journal of Ethnopharmacology*, 149: 184-190.
10. Sharon N, Lis H. (2001). The structural basis for carbohydrate recognition by lectins. In: Wu AM. Editors.The Molecular Immunology of Complex Carbohydrates-2. KluwerAcademic/PlenumPublishers, New York, 1.
11. Leal SC. (2010). Purificação preparativa de inibidores Bowman-Birk, quantificação em alimentos e características imunogênicas dos inibidores de *Glycine max* e da Macrotyl omaaxillare. 2010. 84f. Dissertação (Mestrado em Ciências Biológicas) – Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto.