Anti-nutritional factors and "*in vitro*" digestibility of leaves of *Pereskia aculeata* Miller

Fatores antinutricionais e digestibilidade "*in vitro*" de folhas de *Pereskia aculeata* Miller

Títulos abreviados:
Antinutritional factors of *Pereskia aculeata* leaves

Fatores Antinutricionais de Folhas de *Pereskia aculeata*

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ABSTRACT
Humans need amino acids, especially the essential ones, which are obtained from the proteins ingested in the diet. With the rampant growth of the world population, it is necessary to increase the protein supplies, especially those of plant origin, which now play a major role as a food source. Leaves of ora-pro-nobis (*Pereskia aculeata* Miller) are a high quality protein source and have been used in the traditional cuisine of Minas Gerais for decades. However, it is important to analyze the anti-nutritional and/or toxic factors to consider the use of the plant leaves as an alternative source of nutrients. This work aimed to study the presence of proteic anti-nutritional factors, such as protease inhibitors and lectins, and to evaluate the "*in vitro*" digestibility throughout Tricine SDS-PAGE of these factors extracted from ora-pro-nobis (*Pereskia aculeata* Miller) leaves. The extraction of protease inhibitors and lectins from ora-pro-nobis leaves was performed in phosphate buffer. The partial purification of the fractions presenting activity of trypsin inhibitor and lectins was performed by ammonium sulfate precipitation and gel-filtration chromatography. The "*in vitro*" activity of the fractions with protease inhibitor and lectins, with or without heat treatment in the presence of digestive enzymes, showed that the cooking time of 1 minute was sufficient for protease inhibitors degradation. However, this time was not satisfactory for lectins, which remained resistant even after heating. This work shows that with proper cooking, ora-pro-nobis leaves can be used in the diet as a source of aminoacids.

Keywords: *Pereskia aculeata Miller*, anti-nutritional factors, digestibility.

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RESUMO
O homem necessita na sua alimentação de aminoácidos, especialmente os essenciais, os quais são obtidos a partir das proteínas ingeridas na alimentação, que podem ser tanto de origem animal quanto vegetal. Com o crescimento desenfreado da população mundial será necessário multiplicar o suprimento de proteínas, principalmente aquelas de origem vegetal, que passam a desempenhar um papel de grande importância como fonte de alimento. As folhas de ora-pro-nobis (*Pereskia aculeata* Miller) são uma fonte protéica de alta qualidade e vem sendo utilizada na culinária mineira há décadas. Porém, para a utilização de folhas vegetais como fonte alternativa de nutrientes, é necessário o conhecimento da presença de fatores antinutricionais e/ou tóxicos que possam afetar o valor nutricional dos alimentos. Esse trabalho teve como objetivo realizar o estudo de fatores antinutricionais, inibidores de protease e lectinas, e avaliar a digestibilidade "*in vitro*" em SDS-PAGE de Tricina desses fatores extraídos das folhas de ora-pro-nobis (*Pereskia aculeata* Miller). Foi realizada a extração dos inibidores de protease e lectinas das folhas de ora-pro-nobis em solução tampão fosfato, seguida de precipitação de sulfato de amônia e parcial purificação das frações com atividade de inibidor em tripsina e lectinas, utilizando a coluna de separação por exclusão molecular. A digestibilidade "*in vitro*" das frações com atividade de inibição de protease e lectinas, com e sem aquecimento, na presença de enzimas do trato digestivo demonstrou que o tempo de cozimento de 1 minuto foi suficiente para a degradação dos inibidores de protease, ao contrário do que ocorreu com as lectinas, que se mantiveram resistentes mesmo após o tratamento térmico. Com o cozimento adequado as folhas de orapronobis podem ser utilizadas na dieta.

Palavras-chave: *Pereskia aculeata* Miller, fatores antinutricionais, digestibilidade.

DOI 10.5433/2316-5200.2014v3n1p1

INTRODUCTION

With the demographic explosion, 12.5% of the population is still in the malnutrition range, according to the UN Food and Agriculture Organization (FAO/WFP/IFAD, 2012). To obtain the nutritional balance needed for a healthy condition, it is fundamental the access to food containing an adequate quality of nutrients, as well as the providence of essential amino acids for the production and maintenance of about 25,000 proteins encoded in the human genome (WHO/FAO/UNU, 2002).

The nutritional value of a protein source depends on aspects related to the overall amino acid composition, digestibility, method of preparation, bioavailability of its essential amino acids and absence of toxicity and anti-nutritional factors. With the exception of breast milk for newborns, other foods are relatively incomplete to meet the nutritional needs of the human species (LAJOLO; GENEVOSE, 2002).

The presence of less digestible fractions of protein in diets of developing countries populations, such as high concentration of insoluble fiber and presence of anti-nutritional factors, which are based on less refined cereals and grain legumes as major sources of protein, are responsible for poor protein absorption. There is lower digestibility of protein in traditional diets in countries as India, Guatemala and Brazil than the typical American diet (54-78 versus 88-94%) (GILANI et al., 2005).

Thus, the digestibility is an important factor that defines the nutritional quality of proteins. The study of "*in vitro*" digestibility has been used by many research centers because it is a quick and practical method and shows good correlation with in vivo digestibility values found for humans. The "*in vitro*" evaluation has been performed in seeds of legumes (WATI et al., 2010), cereals (ZHANG et al., 2010) and fruit (HIANE et al., 2006), despite the few studies on leaves (CHANDRAN et al., 2013).

Studies by the FAO/WHO (1991) identified several factors that contribute to lower protein digestibility of plant foods in relation to animal proteins. In plant food interactions that may decrease the digestibility of proteins and their bioavailability occur due to the presence of phenolic, aldehydic and ketonic substances and anti-nutritional proteins. Among the anti-nutritional factors, we highlight the lectins, protease inhibitors, phytic acid and tannins.

Lectins are a large and heterogeneous group of which have the ability to bind reversibly to monosaccharides and oligosaccharides. They can be defined as a class of structurally diverse proteins or glycoproteins, and consequently with different biological functions. These proteins are widely distributed in nature, being found in microorganisms (SINGH et al., 2011), animals (ELIFIO-ESPOSITO et al., 2011) and plants (KABIR et al., 2012). Generally ingested lectins cause degenerative effects on cell membranes, (VASCONCELOS; OLIVEIRA, 2004), interfering with the absorption of nutrients (SGARBIERI, 1987), causing hyperplasia of the small intestine (PUSZTAI et al., 1998), growth reduction, decrease in body weight, decrease in skeletal muscle, increase in lipid catabolism, as well as significant insulin levels reduction in blood and pancreas (BARDOCZ et al., 1996), changes in small intestine, spleen and thymus (REYNOSO-CAMACHO et al., 2003) and being even lethal (ANTUNES et al., 1995; SANTORO et al., 1997). When lectins are ingested inappropriately they interact with the intestinal mucosa, causing inflammation and interfering with nutrient uptake by mucosal injury. In the liver, hepatic lipidosis and necrosis occur and moreover, it causes local or systemic immune system hypersensitivity and direct tissue injury (LIENER, 1995).

Many food products, including cereals, vegetables, potatoes and tomatoes contain enzyme inhibitors, which inhibits enzymes such as trypsin, chymotrypsin, carboxypeptidases, α-amylase and elastase

(FRIEDMAN et al., 2001). Protease inhibitors are generally presented as seed storage proteins. They act by linking their reactive sites (where important cationic residues are present) to anionic residues in the catalytic sites of several proteases. This interaction alters the conformation of proteases, leading to inhibition of its proteolytic activity, thereby modulating its activity (SILVA et al., 2001).

Trypsin and chymotrypsin inhibitors are the most studied families and can be found in legume seeds (FANG et al., 2012), cereals (LIN et al., 2006) and legumes leaves (CHEVREUIL et al., 2011). Exposure to trypsin inhibitors results in increased synthesis and secretion of proteases (such as trypsin, chymotrypsin and elastase) and pancreatic hypertrophy and hyperplasia in animal models (FRIEDMAN et al., 2001). Trypsin and chymotrypsin are particularly rich in sulfur-containing amino acids. The effect of a hyperactive pancreatic trypsin and quimotrypsin production deviates these amino acids from the synthesis of tissue proteins which are then lost in the stool (FRIEDMAN et al., 2001).

Ora-pro-nobis (*Pereskia aculeata* Miller) leaves have been used in Minas Gerais traditional cuisine as an alternative source of protein from plant. The species is a native cactaceous that can reach 10m in height, with long branches and thorns in the armpit leaves elliptical and fleshy, with a high content of mucilage, and protein and it is used in food and pharmaceutical industry. The plant can be found in tropical America, from the southern United States (Florida) to southern Brazil, being widely distributed among the states of Bahia and Rio Grande do Sul (DUARTE; HAYASHI, 2005). The presence of this plant was highlighted in preparations such as flours, salads**,** stews, pies. In food industry, a mass of macaroni noodle added dehydrated ora-pro-nobis has already been developed and approved with an index greater than 70% of acceptability (ROCHA, 2008).

The purpose of this study was to identify the anti-nutritional factors, protease inhibitors and lectins, of *Pereskia aculeata* leaves and perform the "*in vitro*" digestibility study using SDS -PAGE.

MATERIAL AND METHODS

Material

The leaves of *P. aculeata* were collected in a private residence according to geographic location at 20°6'14''S 44°58'27''O. Leaves were selected according to maturity and integrity; they were fully expanded and in good phytosanitary state, free of necrosis or chlorosis. The research group has permission for Access and Shipment of Genetic Component granted by CNPQ (National Council for Scientific and Technological Development) with the permission number 010545/2012-3.

Extraction of the leaves

The collected leaves had petioles removed and were dried at 40°C during 7 days. Subsequently, they were crushed in an analytical mill to obtain finely powdered material. The pulverized material (26g) was homogenized in 260ml of 0.1M phosphate buffer (10% w/v) pH 7.0 under light stirring for 2h at room temperature (25 ± 3 °C). The suspension was centrifuged at 3600 rpm for 20 min and the supernatant was dialyzed against distillated water for 48h. After drying, the material was used in later steps.

Precipitation with ammonium sulfate and partial purification

The protein crude extract was submitted to precipitation with ammonium sulfate, 0-30, 30-60 and 60-90% (w/v). The fraction with the lowest proportion of ammonium was chromatographed in gel-filtration Sephadex G-50 resin in a FPLC system with 0.1M phosphate buffer pH 7.0

under a flow of 0.5 ml/min (7.5 ml/tube) and the resulting peaks lyophilized.

Determination of inhibitory activity on trypsin

The inhibitory activity against trypsin was determined toward the substrate 0.1M N-benzoyl-arginine-p-nitroanilide (BAPNA) in acetate buffer pH 8.0 at 37oC in 30min (SILVA et al., 2001). With 500mL acetic acid 30% (v/v) the reaction was stopped and the substrate hydrolysis followed by absorbance at 405nm. All assays were done in triplicate. One trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance units at 410nm per ml of reaction medium. Results were expressed as units of trypsin inhibitory (TI) per mg protein, one inhibitory unit responsible for the inhibition of one unit of trypsin.

Determination of hemagglutination activity

The hemagglutinating activity (HA) was tested on intact proteins and trypsinized human type O erythrocytes, kept in Alsever solution (2.05g glucose, 0.80g sodium citrate and 0.42g of sodium chloride, 100mL of distilled water, pH 6.1 adjusted with citric acid) (SILVA et al., 2007). Intact or trypsinized erythrocytes solution (2 %, w/v) were serially diluted in 0.15M NaCl to a final volume of 100mL and then incubated at 37°C for 30min. The HA titer is defined as the highest dilution that allows you to check the macroscopic agglutination of cells. HA was run in triplicate.

Protein Determination

The relative concentration of proteins in plant extracts was estimated according to the method described by Bradford (1976) using bovine albumin serum (BSA) as standard. 50mL of plant extract was incubated with 2.5ml of Bradford reagent for 5min at room temperature (25°C ± 3), protein concentration was estimated by reading the absorbance at 590nm. All samples were run in triplicate.

Polyacrylamide-tricine gel

The polyacrylamide gel was prepared from a stock solution of acrylamide-bisacrylamide (49.5%T, 3% C) gel and the buffer was composed of 3M Tris, 0.3% SDS pH 8:43. The sample buffer was composed of 4.0g of 89% glycerol in 10ml of gel buffer, 0.025% PSA (100mg/mL), 6.1mL of stock solution and 0.05% TEMED in a final volume of 30ml for each gel. The separating gel was prepared with 10ml of gel buffer, 10mL acrylamide stock solution to a final volume of 30ml with ultrapure water. The upper gel was prepared using 1.0ml acrylamide stock solution in 3.1mL of buffer gel, 100mL PSA (100mg/mL) and 0.1% TEMED in a final volume of 12.5ml. The electrophoresis was carried out at room temperature in cathode buffer 0.1M Tris-HCl/0.1% SDS pH 8, and the anode buffer 0.2M Tris-HCl pH 8,9. After the run the gel was stained with Coomassie blue R-250 0.25% in methanol, acetic acid and water (40:10:50 v:v). The discoloration of the gel was done by washing it in methanol, acetic acid and water (40:10:50 v:v).

"*In vitro*" digestibility of anti-nutritional factors

Fractions with inhibitory and hemagglutinating activity were subjected to hydrolysis by simulated intestinal fluid and gastric fluid. Each fraction was assessed in both native condition and denatured by heat (100ºC for 30 minutes), simulating the time of preparation of seed for consumption. The protein fractions were solubilized in 10mM phosphate buffer pH 6.0 (when evaluate with pepsin) or 0.1M phosphate buffer saline 0.1 M, pH 7.6 (evaluation with trypsin/chymotrypsin) at a concentration of 0.5 mg/ml. Subsequently, 250ul of this sample solution were incubated with 10ul of enzyme solution (50 mM HCl with pepsin and trypsin /chymotrypsin in saline phosphate buffer at concentration of 0.25 mg/ml) at different

reaction times (0.5h, 1h, 2h, 4h and 8h). The reactions were stopped by heating at 100°C for 1 minute, time used to prepare leaves of *P. aculeata* in Minas Gerais traditional cuisine.

RESULTS AND DISCUSSION

The determination of the specific activity of the precipitates and supernatants by ammonium sulfate precipitation (0-30, 30-60 and 60-90%) are shown in Table 1. It was detected that the greatest inhibitory activity was found in the 30% supernatant followed by 60% supernatant and reduced inhibitory activity was found in the precipitate fraction of 90%.

The precipitate from 30%, obtained in the previous step was then applied on a gel-filtration column containing Sephadex G-50 resin, and the resulting chromatographic profile revealed the separation of the precipitate into three main fractions, as shown (Figure 1).

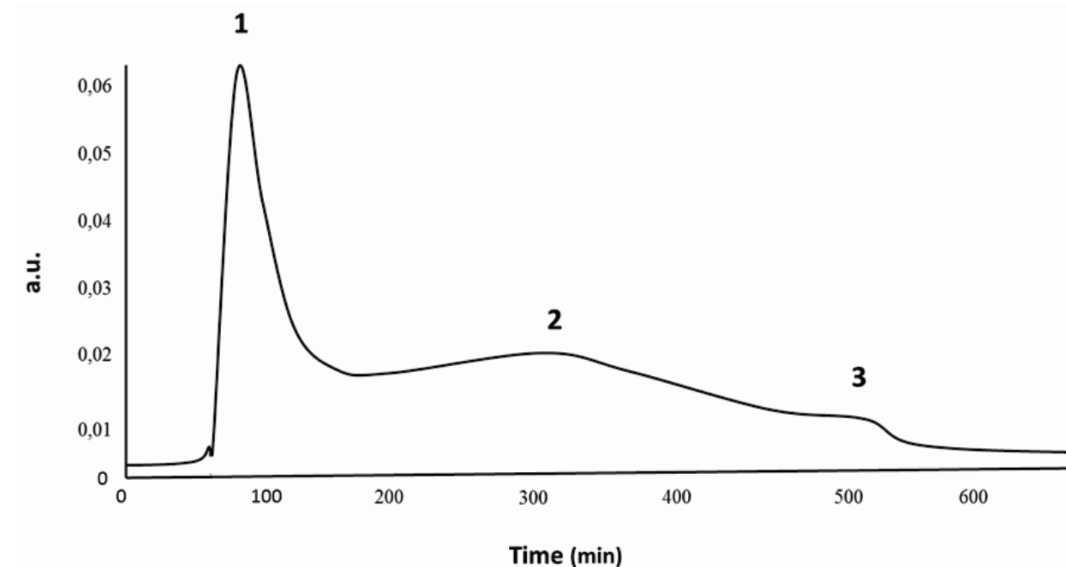
Table 1 Ammonium sulfate fractionation stages.

Step	Total Inhibitory Units[a]	Total Protein [a]	Specific Activity [a]
Crude Extract	233.32 (1.13)	263.24 (1.23)	0.88
Supernatant 30%	213.956 (1.7)	240.39 (1.14)	0.89
Precipitate 30%	29.196 (0.06)	16.78 (0.56)	1.74
Supernatant 60%	175.2 (1.15)	195.43 (2.7)	0.89
Precipitate 60%	33.9 (3.3)	40.23 (0.67)	0.84
Supernatant 90%	110.2 (2.7)	152.76 (2.48)	0.72
Precipitate 90%	52.47 (0.89)	41.82 (1.2)	1.25

[a] Values reported are the means of triplicate ± the standard error of the mean

The peaks were analyzed individually for the presence of protease inhibitors and lectins. Only peak 2 had inhibitory activity against trypsin enzyme. In figure 2A is shown the inhibition curve obtained from peak 2 of the sample. The molar ratio of protease inhibitor relative to trypsin of 1:1 showed enzymatic activity of 50%, whereas molar ratio of 1:5, trypsin had only 35% of residual activity. The non-linear extrapolation on the x-axis indicated that inhibitors of *P.aculeata* Miller obtained from leaves bind to the enzyme at a molar ratio of 1:1 (one mol of inhibitor is required to inhibit the activity of one mol of enzyme). This proportion was observed in protease inhibitors derived from seeds of Cajanus cajan (PRASAD et al., 2010).

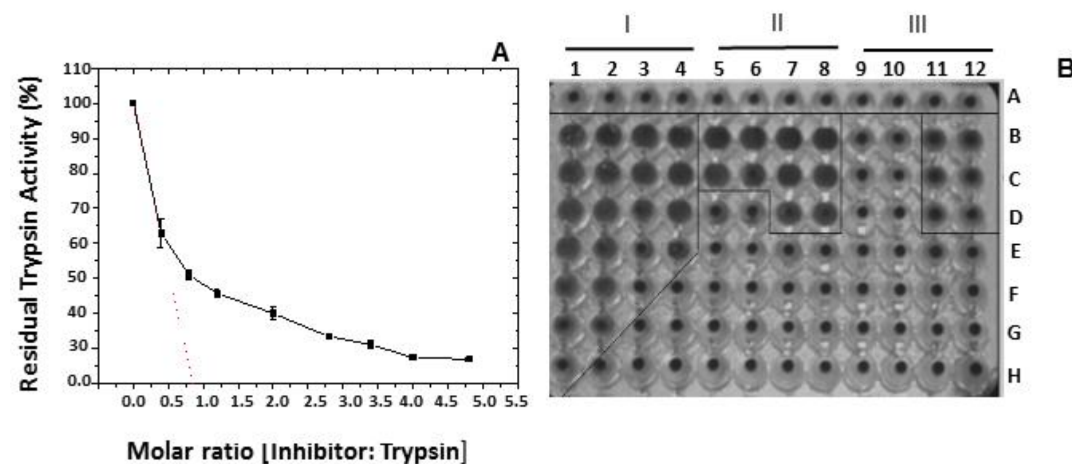
Figure 1 Chromatography on Sephadex G-50 using FPLC system. The column was equilibrated with 0.2M Ambic buffer, under a flow of 0.5 ml/min (7.5ml/tube). The numbers correspond to the 3 fractions obtained.



The samples from peak 1, 2 and 3 (Figure 1) were diluted and tested against blood type O, intact or trypsinized (Figure 2B). The hemagglutination occurred for the 3 peaks when the blood was trypsinized.

The Peak 1, with a higher hemagglutinating activity (up to the H line) followed by peak 2 (until line D). No hemagglutination was detected only in rows 9 and 10, corresponding to peak 3 in the intact blood. Lectins possess the ability to form dimers and tetramers, so they are expected to be eluted as first molecules on the gel-filtration chromatography, explaining the higher concentration of lectin in peak 1. Hemagglutinating activity was also observed in leaves of Neoregelia flandria (YAGI et al., 1996), however, in this hemagglutination was observed only on rabbit erythrocytes, but not with human erythrocyte.

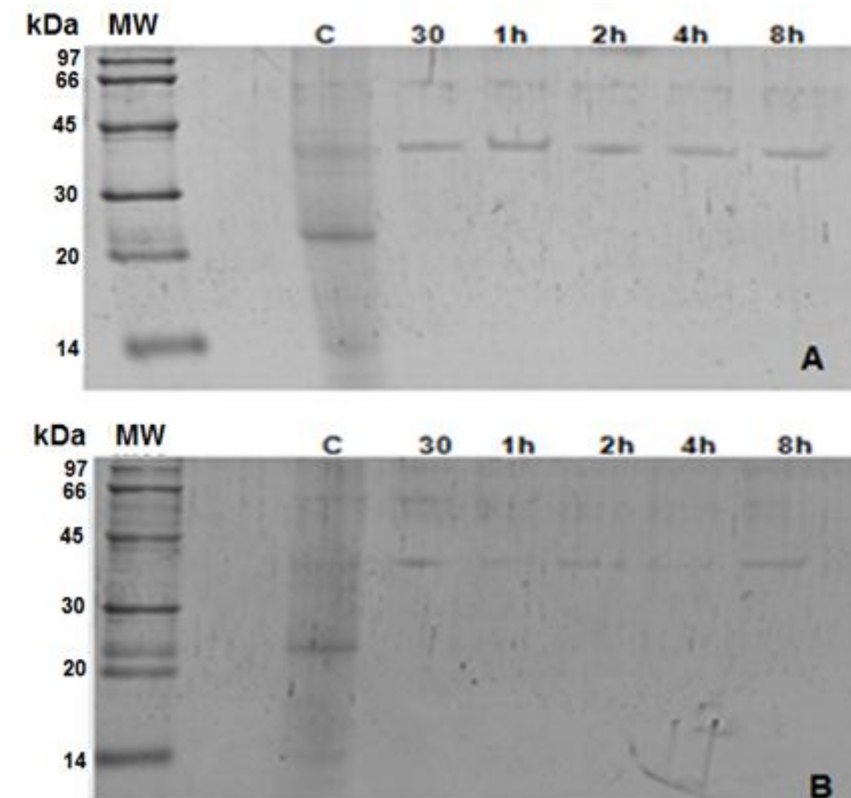
Figure 2 A) Inhibition Curve of bovine trypsin by peak II obtained from gel-filtration chromatography. The residual trypsin activity was monitored by hydrolysis of DL-BAPNA. B) Hemagglutination assay. The samples applied were obtained from gel-filtration chromatography. As the wells 1, 2, 7, 8, 11 and 12 corresponds to the trypsinized blood samples, wells 3, 4, 5, 6, 9 and 10 corresponding to the intact blood sample. Control (line A), Peak 1 (lanes 1-4), Peak 2 (lanes 5-8) , Peak 3 (lanes 9-12) . Wells delimited by the stroke are those in there hemagglutination.



"*In vitro*" Digestibility

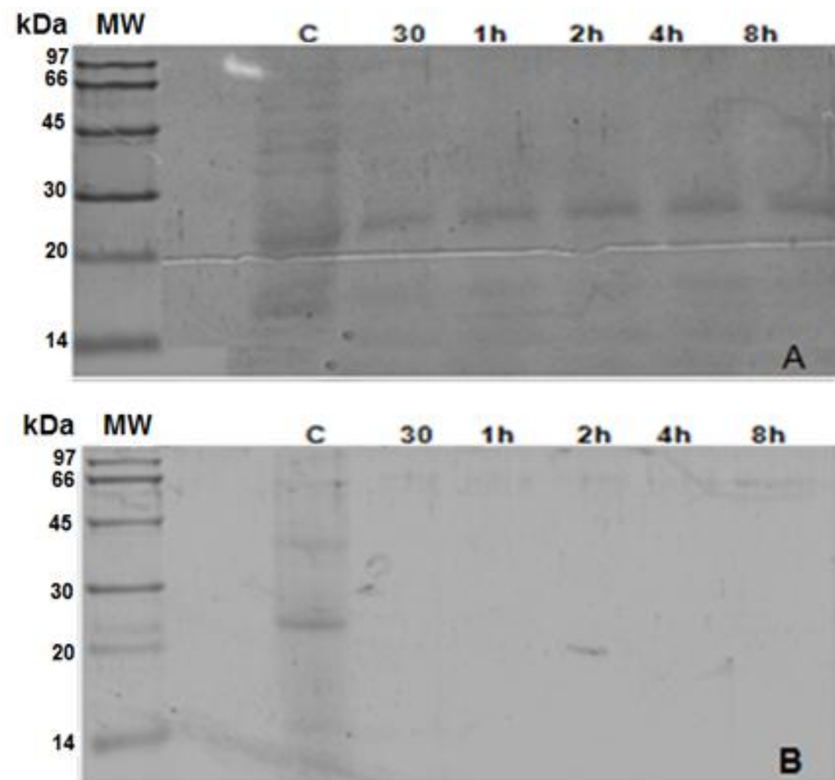
For "*in vitro*" evaluation of *Pereskia aculeata* Miller leaves, peak 2 from gel-filtration chromatography was incubated with enzymes of mammalian digestive tract (pepsin, trypsin and chymotrypsin) simulating stomach and intestine conditions.

Figure 3 Electrophoresis SDS-PAGE-tricine of the second peak obtained from gel-filtration chromatography on Sephadex G-50 digested with pepsin. (MW) molecular weight markers; incubation time: 30min to 8h; (C) control without enzyme. A) Correspond to samples without heating. B) samples submitted to heat treatment.



Under simulated intestinal fluid (SIF) conditions, on native state, only a proteic band of approximately 45kDa was resistant even after 8 hours, while the regions of other proteins were completely degraded after 30 minutes (Figure 3A). After the heat treatment, digestion was complete after 30 minutes (Figure 3B).

Figure 4 Electrophoresis SDS-PAGE-tricine of the second peak obtained from gel-filtration chromatography on Sephadex G-50 digested with trypsin and chimotrypsin. (MW) molecular weight markers; incubation time: 30min to 8h; (C) control without enzyme. A) Correspond to samples without heating. B) samples submitted to heat treatment.



The evaluation performed using Tricine SDS-PAGE simulating gastric fluid (SGF) conditions without heating, showed that the region corresponding to the protease inhibitor (14 and 22-kDa) was completely degraded after 30 min (Figure 4A). However, the bands corresponding to 45 and 66KDa (probably lectins) remained stable even after 8h incubation. When heated, only the protein band of 45 kDa was still resistant, even after 8 hours of incubation (Figure 4B).

A difference between the behavior of this 2 studied conditions studied was observed. The effect against lectin was greater for digestion under SIF and protease inhibitor under SGF. This suggests that the lectin present in the sample have low concentrations of amino acids such as tryptophan, phenylalanine, tyrosine, and leucine, while the protease inhibitor might have low concentrations of arginine and lysine. This is because the pepsin hydrolyzes peptide bonds that involve the carboxyl groups of the amino acids tryptophan, phenylalanine, tyrosine, and leucine, while trypsin only recognizes peptide bonds that involve carboxyl groups from arginine and lysine (NELSON et al., 2011).

After the heat treatment, there was still a resistant protein of around 45kDa. The heat treatment was more efficient compared to native conditions, but with the cooking time of only 1 minute, usual for cooking these leaves, part of the anti-nutritional factors has not suffered complete degradation, since it is possible to visualize the presence of bands protein between 45 and 66.2 kDa (Figure 4B).

According Lajolo and Genovese (2002) effective methods, such cooking even at atmospheric pressure or under pressure, are effective for the inactivation of most or even all of the activity of protease inhibitors. Akinmutimi (2004) noticed that for a good reduction in the anti-nutritional substances percentage from *Mucuna utilis* seed, the best time cooking is 100°C for 90min. Therefore, a significant detoxification of anti-nutritional factors and ensuring optimal bioavailability of nutrients, an excellent cooking time of 60 minutes at 100°C is recommended for seeds (OMOIKHOJE et al., 2009). For this reason, after heating treatment, certain proteins of ora-pro-nobis leaves were digested by digestive enzymes, indicating that the heat treatment in adequate time can improve the protein quality of ora-pro-nobis leaves.

The anti-nutritional factors protease inhibitors and lectins were detected in leaves of *P.aculeata*. The "*in vitro*" digestibility study demonstrated that

time commonly used for baking its leaves (1 minute) was not enough for the degradation of proteins related to lectin, however bands related to protease inhibitors were fully degraded thereafter heating. Proper cooking time of the leaves for preparing meals is crucial for the denaturation of these anti-nutritional factors.

ACKNOWLEDGEMENTS

FAPEMIG (CDS-APQ-02337-12). AGMP is grateful for the fellowship from CAPES.

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RECEIVED 29 Apr 2014
ACCEPTED 18 Mar 2014