BABT
Brazilian Archives of **Biology**
and **Technology**

Article - Food/Feed Science and Technology

Protein Concentrate Made from *Pereskia aculeata* Leaves

Thiago Teixeira Costa[1]
https://orcid.org/0009-0005-7634-1551

Thiago de Melo Costa Pereira [2,3]
https://orcid.org/0000-0003-4053-5061

Thaynara Regina de Souza Costa[2]
https://orcid.org/0000-0002-4203-7253

Hildegardo Seibert França[1,2*]
https://orcid.org/0000-0001-6129-8793

Daniela Batista de Castro[3]
https://orcid.org/0009-0009-9797-1260

[1]Universidade Federal do Espírito Santo (UFES), Departamento de Ciências Biológicas, Programa de Pós-graduação em Biologia Vegetal, Vitória, Espírito Santo, Brasil; [2]Instituto Federal do Espírito Santo, Vila Velha, Espírito Santo, Brasil; [3]Universidade de Vila Velha (UVV), Biopráticas, Vila Velha, Espírito Santo, Brasil.

*Correspondence: hildegardo.franca@ifes.edu.br; Tel.: +55-27-999837581 (H.S.F.).

HIGHLIGHTS

- The *Pereskia aculeata* leaves displayed potential to generate a protein product.

- The alkaline extraction with ultrasound in 1:20 (plant/solvent) dissolution was the best treatment.

- The use of pH 4 to aid protein recovery accelerates the process and reduces its cost.

Abstract: *Pereskia aculeata,* Miller is a Cactaceae originally from the American continent, and despite its notable nutritional value, this species is little consumed as food. The aim was a comparison between two techniques: saline extraction and alkaline extraction for produce a protein concentrate through inexpensive and easily reproduced techniques. For the saline extractions, two salts, sodium chloride and sodium carbonate, were used with 0.1 M, 0.5 M, and 1 M concentration rates. The alkaline extraction was done isolated at pH 10 and combined with ultrasound in 1:10 and 1:20 plant/solvent dissolutions. The treatments have been through isoelectric precipitation with different pH rates and then were centrifuged. The proteins resulted from precipitation were quantified through the Bradford method. Moreover, the purity level of the recovered protein products was established, and the characterization of the proteins molecular weight was done with polyacrylamide gel at 12%. The results revealed that the alkaline extraction with ultrasound in 1:20 dissolution was the best treatment. The positive effects of the highest dissolution combined with the alkaline extraction with ultrasound were evident with 27.42% (w/w) average percentage of extracted protein. The electrophoresis gel revealed two key bands of 68 kDa and 168 kDa. It is concluded that this plant's leaves have the potential to become a low-cost and industrially feasible protein product.

Keywords: Leafy cactus; Barbados gooseberry; Non-Conventional Food Plants; Supplement.

INTRODUCTION

Brazil is the country with the highest plant diversity in the world. In this territory, 50.091 species of Brazilian flora are recognized, and the vast majority, a total of 35.626, belong to the Angiosperms group [1]. However, this high diversity is not necessarily reflected by the eating habits of the country's population. In Brazil, vegetable consumption favors exotic products and culture [2]. The consumption of Non-Conventional Food Plants (NCFP) can lead to numerous nutritional advantages, including meeting the daily protein needs. Economically, NCFP can increase family farmers' income if there is a policy to promote the consumption of these plants. The nutritional properties of these plants can be explored by the food industry in several ways, and they also have great potential for the pharmaceutical industry [3].

Promoting the consumption of vegetable proteins, especially those derived from NCFP, has environmental, social, and economical advantages. Regarding the environmental aspect, the impacts caused by the production of animal protein can be highlighted since cattle raising is the leading cause of environmental harm to the Amazon Forest, particularly due to deforestation to establish pasture lands [4,5]. Another problem is the need for big areas for food production to develop and fatten the cattle. According to World Wildlife Fund Brazil [6], 79% of the soy produced in the world has this purpose. A recent study indicates that, from 2011 to 2015, the total deforestation of tropical forests was estimated at between 6.5 Mha and 9.5 Mha by year, and agricultural and cattle raising are responsible, directly or indirectly, for 90% to 99% of the total deforestation [5].

Considering the social aspect, the consumption of vegetable proteins can help socially underprivileged people to meet their daily protein needs [7,8]. *Pereskia aculeata*, Miller, popularly known in Brazil as Ora pro nobis (OPN), blade-apple cactus, rose cactus or lemon vine and outside Brazil known as Barbados gooseberry, is a shrub and leafy cactus native to the American continent. It is classified as a NCFP and, as the term suggests, it is disregarded as a food item for most of the Brazilian population; except in a few regions of the state of Minas Gerais, where it is cultivated in backyards and gardens [2].

The levels of essential amino-acids present in *P. aculeata* leaves are higher than the minimum to meet human needs as recommended by the Food and Agriculture Organization of the United Nations (FAO) [9]. According to Barreira and coauthors [10], Barbados gooseberry is "important to nutritional contribution and a fundamental food species for strategies of nourishment security of family groups whose eating and nutritional habits are directly related to the consumption of this species". In addition, this plant is described as having high concentrations of iron, calcium, copper, potassium, manganese, selenium, fiber and vitamin A [10]. The interest of industries' in Barbados gooseberry has been growing significantly; however, there is still a lack of research on isolation and processing of the plant's components and their incorporation into foods [3].

Pereskia aculeata has a high nutritional value and extracting and isolating its proteins can generate a protein supplement to be used in eating diets. Therefore, this study proposed the production of a protein pool made from Barbados gooseberry leaves by using simple and inexpensive methods of extraction and isolation, this way these procedures can be industrially replicated if there is interest in it.

MATERIAL AND METHODS

Plants material preparation

Pereskia aculeata leaves were extracted on November 9th of 2020 from a specimen located in Guarapari, a city in the state of Espírito Santo – Brasil (20º 62' 65'' S 40º 44' 52'' O). As soon as this plant bloomed, one branch was collected, identified and registered at the herbarium at UFES, Goiabeiras campus, as VIES 53710.

The Barbados gooseberry (OPN) leaves were dehydrated at 45°C for 72 hours in an oven [8,11]. As the leaves were completely dried, an industrial blender was used to grind them into a fine powder, which was named, in this text, OPN powder. After this step, 10 grams of OPN powder was weighed and rehydrated with distilled water until it reached 100 ml of volume (1:10 (w:v) solution). The entire experimental process is represented by Figure 1.
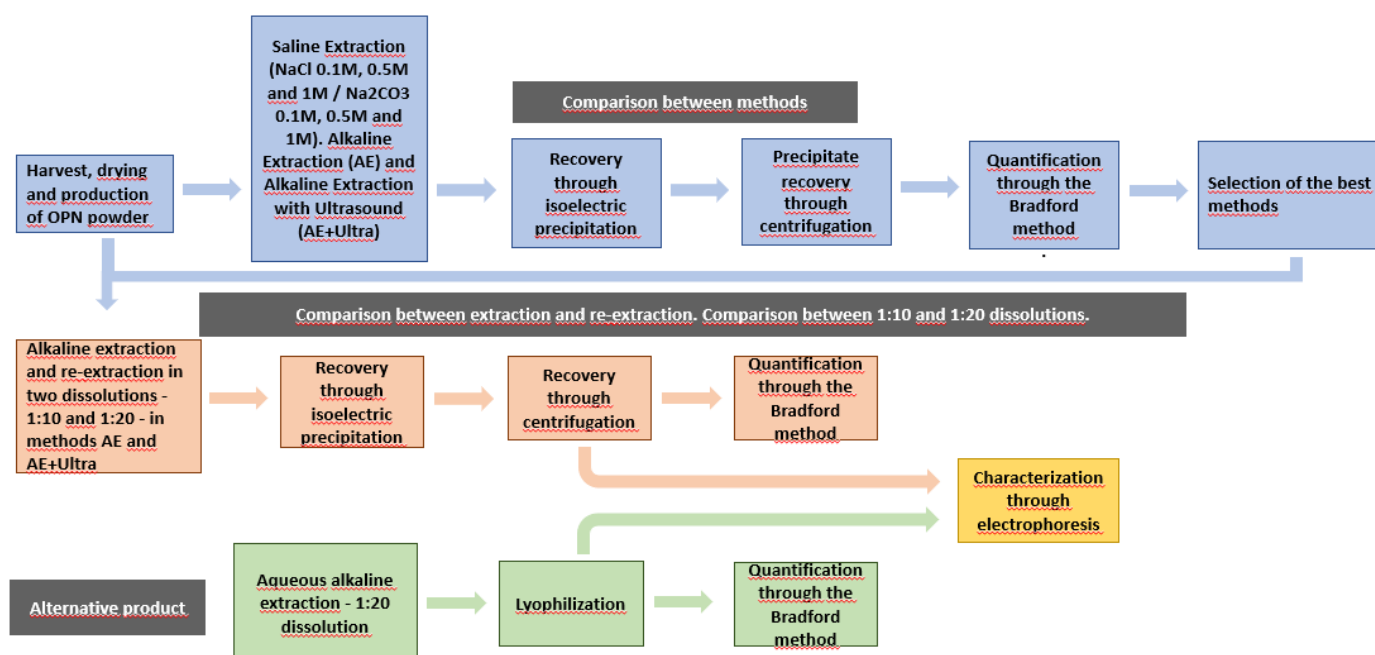
Figure 1. Experiment phases scheme.

Protein extraction

Two methodologies that are widely used in literature were chosen for this process: aqueous saline extraction (SE) and aqueous alkaline extraction (AE). Aqueous alkaline extraction also was tested combined with ultrasound assisted extraction (AE+Ultra).

Saline extraction

In the SE method the substrate is diluted in a saline solution in the desired ionic strength. Salting in (solution with salt concentration lower than 0.15 M) and salting out (solution with salt concentration higher than 0.15 M) processes were done [12]. Two salts (NaCl and Na_2CO_3) were used in three different concentration levels (0.1 M, 0.5 M and 1 M), which resulted in six treatments. The solutions were in constant stirring for 30 minutes at 45° C and subsequently they were filtered using a funnel and cotton. Afterwards, the solutions were centrifuged at 3400 rpm for 2 minutes to remove residues that may have passed in the filtration process.

Alkaline extraction and alkaline extraction combined with ultrasound

AE and AE+Ultra were prepared by adding sodium hydroxide (NaOH 0.25 M) to the 1:10 solution (described above) until it reached pH 10. These solutions were in constant stirring for 30 minutes at 45° C; the EA+Ultra solution, specifically, was, under those conditions, kept in a beaker inside an ultrasonic sonicator with 40 khz. Then, the solutions were filtered using a funnel and cotton [12]. These two methods were compared to extraction by salt. All extractions were done according to the 1:10 OPN weigh/solvent (w:p) ratio. After the methods were compared and the two best ones were defined (EA and EA+Ultra), new extractions were carried out to measure the effect of dissolution and re-extraction.

For that purpose, extractions with OPN powder/solvent in 1:10 (w:v) and 1:20 (w:v) ratios were executed using the methodology previously described. This generated other four treatments: alkaline extraction with 1:10 concentration (AE 1:10), alkaline extraction with 1:20 concentration (AE 1:20), alkaline extraction with ultrasound with 1:10 concentration (AE+Ultra 1:10) and alkaline extraction with ultrasound with 1:20 concentration (AE+Ultra 1:20). After the filtration of AE and AE+Ultra, the OPN powder residues, which would have been discarded, were subjected to the same methodological processes as described previously. This way, four other treatments were obtained: alkaline re-extraction with 1:10 concentration (AR 1:10), alkaline re-extraction with 1:20 concentration (AR 1:20), alkaline re-extraction with ultrasound with 1:10 concentration (AR+Ultra 1:10) and alkaline re-extraction with ultrasound with 1:20 ultrasound (AR+Ultra 1:20). All treatments were performed three times.

Recovery of extracted proteins

The extracted proteins were recovered through isoelectric precipitation, followed by centrifugation at 3400 rpm for 25 minutes. The solution's pH was adjusted to 6 using a 1 M hydrochloric acid solution, and then the first centrifugation process was performed in 50 ml Falcon tubes. In their weighed and identified respective Falcon tubes, the precipitates were put in a dryer and the supernatant's pH was adjusted to 5. After this, the centrifugation process was repeated, and this entire process was performed again until the dehydrated precipitate obtained pH 2. The weight of the precipitates was obtained by measuring the difference in weight between the empty Falcon tubes and those filled with the dehydrated precipitate.

Aqueous lyophilized extract

At last, 50 grams of OPN powder that was rehydrated until it reached 1000 ml of volume, that is, an aqueous alkaline extraction 1:20 (w:p), was weighted. The objective was to produce an alternative product, the aqueous lyophilized extract (ALE). This extract was filtered and centrifuged for 2 minutes at 3400 rpm to remove any residues that may have passed in the filtration process. However, this extract did not go through isoelectric precipitation and, instead, it was lyophilized.

The effectively recovered volume of the extracts were measured after the extract filtration was done in all different treatments. The percentage of recovered volume was measured through a simple rule of three, as equation 01 displays:

$$\% \, Rec.Vol. = \frac{Rec.Vol. * 100\%}{Total \, Volume \, of \, Extract}$$

Protein quantification

The protein quantification was performed through the Bradford method [13]. ALE samples and precipitates in different treatment samples were rehydrated and solubilized with the aid of an ultrasonic sonicator at 5% and 10% dissolutions. The calibration curve was done with human albumin at 10%, 20%, 40% and 80%. ELISA readings were done three times at 595 nm.

The percentage of extracted proteins was calculated by relating the weight of the OPN powder used in each treatment with the total of extracted proteins, as displayed in equation 02:

$$\% \, Prot.Extracted = \frac{Total \, Prot.Ext. * 100\%}{OPN \, powder \, weight}$$

As described previously, the precipitates' weights were registered just as the recovered extracts' volumes, therefore allowing the calculation of percentage of the precipitates' purity through equation 03:

$$\% \, Purity \, of \, Precipitate = \frac{Total \, Prot.Ext. * \% \, Rec.Vol.}{Precipitate \, weight}$$

Characterization of molecular weight of the proteins

The proteins' molecular weight was characterized by polyacrylamide lyophilized extract (*SDS-Page*) at 12%, using the method described by Laemmli [14].

The precipitated samples were rehydrated and solubilized into 10 mg/ml solutions with the aid of an ultrasonic sonicator. 20 µL of each sample was transferred to a previously identified vial and another 20 µL from the sample buffer was added. The vials with the samples were properly shut and submerged in water for 5 minutes at 95° C to perform the complete denaturation of the protein. The tracer *GE Healthcare LMW SDS*'s sample went through the same treatment.

When the gel is completely polymerized, the electrophoretic tank was assembled, and the running buffer was added. 30 µL of each sample was pipetted into different holes. The precipitated samples were from the AE+Ultra 1:20 treatment with pH 2, 3, 4, 5 and 6, as well as ALE treatment samples and a gross extract (GE), which is a sample of the AE+Ultra 1:20 treatment before isoelectric precipitation. The equipment was programmed to run with 130 volts for 3 hours.

The measurement of the band masses and the graphic was made on Microsoft Excel, and the gel was analyzed with the aid of ImageJ, an image-processing program.

Statistical analysis

To verify if significant differences occurred between the different treatments, analyses of variance (ANOVA) tests were done with a significance level $p = 0.05$. P values equal to or lower than 0.05 lead to rejection of the null hypothesis, which proposes that all treatments are statistically identical, and by accepting the alternative hypothesis as real, it is assumed that there are significant differences between the treatments. ANOVA, however, only provides information regarding similarities and differences between treatments. To know which treatments were statistically different among the others and which were more efficient, it was necessary to perform Tukey tests. These analyses were done by using a software named Rstudio.

RESULTS AND DISCUSSIONS

Comparison between SE, AE, AE + Ultra.

The extraction methods were compared regarding protein extraction efficiency, relating the triplicate treatments to the concentration of proteins in mg/mL, and the ANOVA result was $p = 6.02-16$. The preliminary comparison of the methods displayed differences in the protein extraction from the leaves of Pereskia aculeata, where higher amounts of extracted proteins were obtained in more alkaline media, which is in accordance with the studies of Potin and coauthors [15].

According to the results of the Tukey test, the most efficient treatment of protein extraction was AE+Ultra, followed by AE (Figure 2). Extractions with salts had much inferior performances when compared to treatments with alkalinized solutions. This is because proteins become more charged in more alkaline pH environments, resulting in repulsion among the molecules and, consequently, increasing solubility [16]. In addition, the increase of solubilized proteins combined with ultrasound is supported by literature [17-19], probably due to the mechanic effects of ultrasound, such as the release of compounds by breaking the cell wall and stirring the solution, which allows higher interactions between the solvent and the proteins [20].
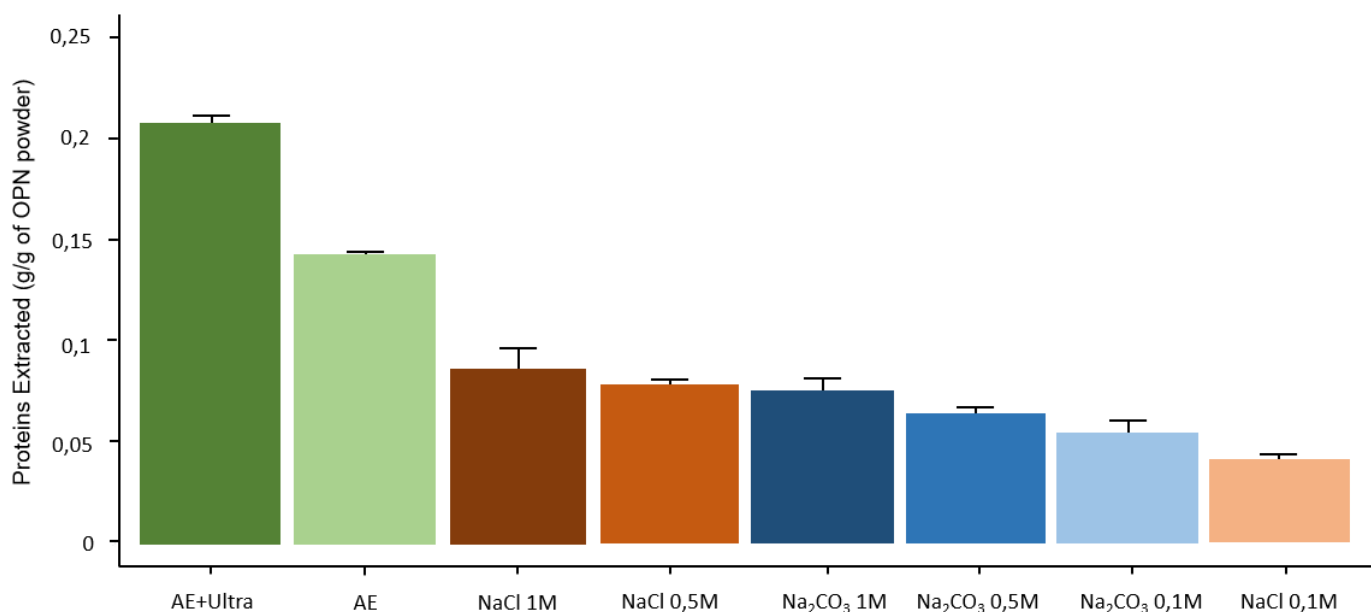

Figure 2. Quantity of proteins extracted in each treatment.

Dissolution and re-extraction effect

The solvent ratio 1:20 (w:p) was the best one at extracting proteins from *Pereskia aculeata* leaves. This may be related to the fact that the 1:10 solution is closer to the saturation point of the medium. Two factors highlighted the influence of the solvent ratio: (i) the treatments that extracted higher quantities of proteins had 1:20 solvent ratio and (ii) the statistics analyses suggested differences between the AE 1:20 and AE 1:10 treatments and between the AE+Ultra 1:20 and AE+Ultra 1:10 treatments (Table 01). The statistical equivalence between AE 1:20 and AE+Ultra 1:10 treatments indicates that higher dissolution favors protein extraction, similarly to the same combination treatment but with lower dissolution. That is, dissolution and ultrasound both enhanced protein extraction in the same manner.

Table 1: Extracted proteins (g/g of OPN powder), percentage of proteins extracted from OPN powder and precipitates' purity in each treatment.

Treatment	Average of total extracted proteins (g/g of OPN powder)	Average percentage of extracted protein	Average percentage of the precipitate's purity
AE+Ultra 1:20	0.2742 ±35.9 a	27.42%	72.1% ± 0.785 b
AE 1:20	0.2166 ±136 b	21.66%	60.8% ± 5.87 c
AE+Ultra 1:10	0.2054 ±73.5 b	20.54%	60.8% ± 2.98 c
AE 1:10	0.1502 ±73.2 c	15.02%	53.9% ± 1.26 d
AR 1:10	0.0584 ±16.3 d	5.84%	82.8% ± 0.123 a
AR+Ultra 1:10	0.0579 ±41.2 d	5.79%	83.6% ± 0.747 a
AR 1:20	0.0461 ±23.3 d	4.61%	86.4% ± 1.42 a
AR+Ultra 1:20	0.0130 ±19.1 e	1.3%	84.5% ± 1.31 a
ALE	0.2099 ±79,3 b	20.99%	24.6% ± 0.894 e

The use of the same letters indicate that the treatments are statistically equal.

Moreover, the statistics analyses also revealed the influence of the combination of the method with ultrasound since the AE+Ultra 1:20 treatment best performed protein extraction and the AE+Ultra 1:10 treatment was significantly more efficient than the extraction performed by the AE 1:10 treatment (Figure 3). The combination of the best dissolution, AE and the AE+Ultra 1:20 resulted in an average of 27.42% proteins extracted from Barbados Gooseberry leaves; these results are similar to those found by Takeiti and coauthors [9].

Regarding re-extractions, the AR+Ultra 1:20 treatment had the worst performance (Figure 3); this suggests that the OPN powder's soluble proteins may have been nearly exhausted since the treatment was significantly different from the other re-extractions. Furthermore, its extraction (AE+Ultra 1:20 treatment) was more efficient, being able to extract the OPN powder's soluble proteins almost entirely. It is also clear that, due to the small quantity of extracted proteins, re-extraction processes may not be interesting to the industry.

The spot where the biggest quantity of proteins was covered was pH 4, which is in accordance with many studies [21-24] and indicates that this is the isoelectric point of the *Pereskia aculeata* leaves' proteins. In most cases, protein recovery through the isoelectric precipitation method results in a darkened product due to the precipitation of chlorophyll and polyphenol molecules that were co-extracted along with the proteins. When this happens, it is common for them to exhibit an unpleasant bitter taste, which is undesired [25].
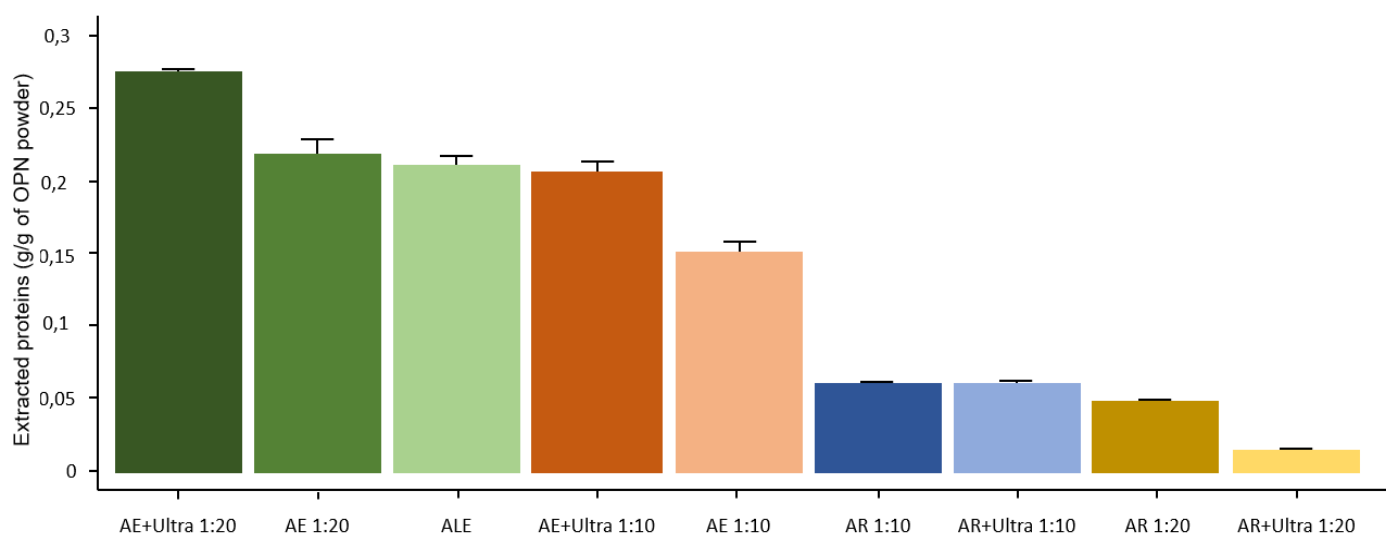
Figure 3. Quantity of proteins extracted by grams of OPN powder.

Aqueous lyophilized extract

The ALE was tested as an alternative product to other treatments; however, this treatment was compared to the others in terms of efficiency in protein extraction. With $p = 1.52^{-7}$ in this variance analysis, it also rejects the null hypothesis that all treatments are statistically identical. The Tukey test displayed similarities between

ALE, AE 1:20 and AE+Ultra 1:10. The ALE was only proved to be less efficient in protein extraction when compared to AE=Ultra 1:20 (Figure 3). Therefore regarding protein concentration, ALE had an excellent performance since it is an alkaline extraction with 1:20 dissolution.

Effect of treatments on the purity of precipitates

The analyses exhibit significant differences in the treatments. The Tukey test revealed that the precipitates had the highest percentage of proteins in re-extraction treatments; these results were statistically identical in all treatments (Figure 4). Regarding the extractions, the best method was the AE+Ultra 1:20 method, followed by AE 1:20 and AE+Ultra 1:10 that were statistically identical (Figure 4).
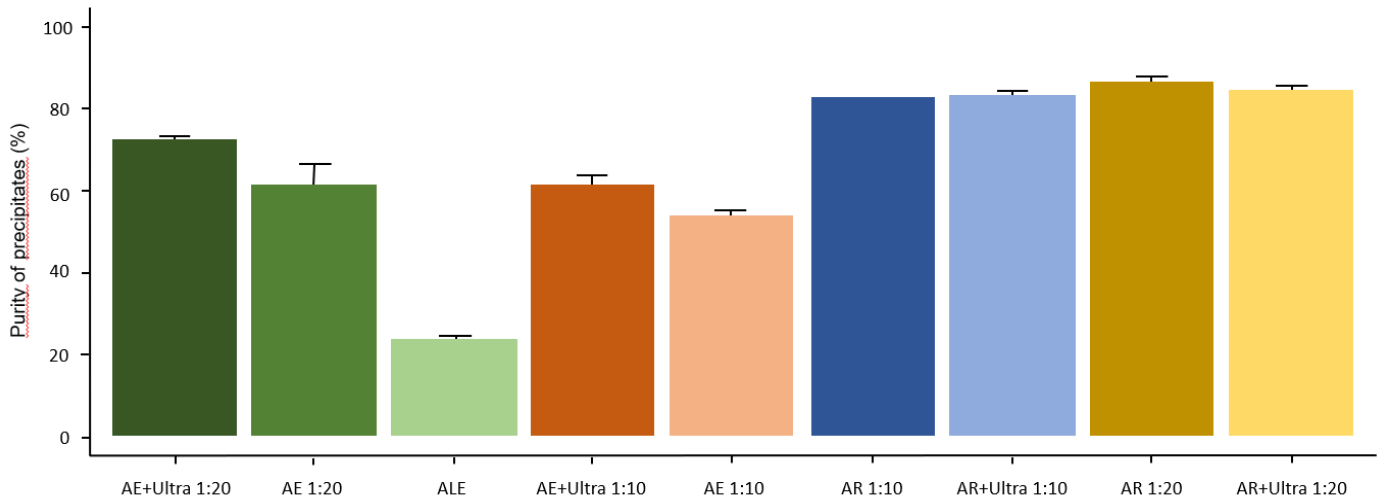

Figure 4. Percentage of purity of precipitates in each treatment.

Characterization of the proteins' molecular weight

The gel analysis revealed two key bands; all treatments revealed a protein with 168 kDa and another protein with 68 kDa was verified in the pH 2, pH3, pH 4, pH 5 and gross extract (GE) precipitates. Additionally, other weaker bands that varied between 108 kDa and 217 kDa were also verified (Figure 5). The aqueous lyophilized extract revealed the highest number of bands among all treatments. The total running length was 9284 mm. The band masses that were found are plotted in Table 2.
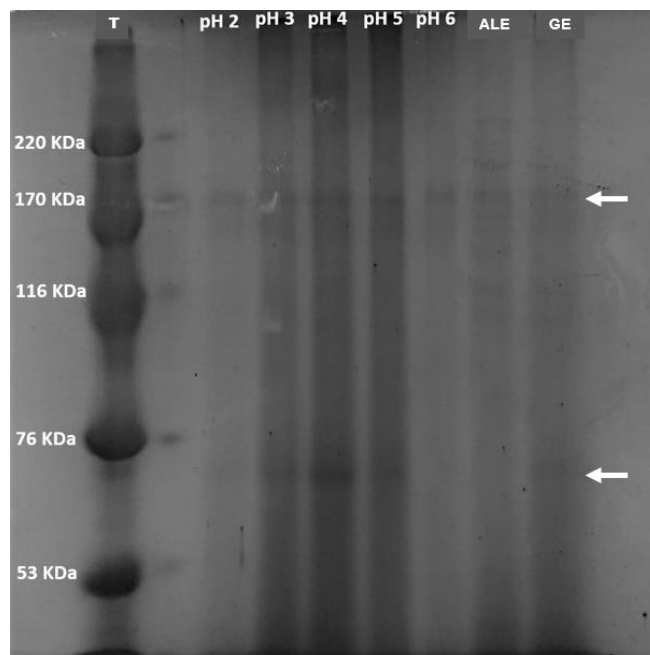

Figure 5. Profile of proteins in electrophoresis gel. T = tracer; ALE = aqueous lyophilized extract; GE = gross extract; pH2 = precipitated by pH2; pH3 = precipitated by pH3; pH4 = precipitated by pH4; pH5 = precipitated by pH5 and pH6 = precipitated by pH6.

In their studies, Lazova and Stemler [26] found a protein with 160 kDa in carbonic anhydrase activity associated with pea seeds RuBisCO, which may correspond to the protein with 168 kDa found in this study. RuBisCO is the most abundant enzyme in plants, reaching even 40% of the total weight of soluble proteins present in most leaves [27]. Therefore, the fact that the 168 kDa protein band is the most well-traced in gel may be related to the association of this band with an enzyme related to the process of photosynthesis. Takeiti and coauthors [9] found a very striking band with 61 kDa in *Pereskia aculeata* leaves that can be compared to the 68 kDa band found in the present study. In that context, the authors associated the protein at hand with the β subunit from β-conglycinin 7S of soy, a globulin.

Table 2. Molecular weight of the proteins traced in gel. T = tracer; ALE = aqueous lyophilized extract; GE = gross extract; pH2 =precipitated by pH2; pH3 =precipitated by pH3; pH4 =precipitated by pH4; pH5 =precipitated by pH5 and pH6 =precipitated by pH6.

Treatment	Running Length (mm) *	Protein Mass (kDa)
T	7920	53
T	6050	76
T	3916	116
T	2552	170
T	1628	220
ALE	1452	217
ALE and GE	2112	187
pH 2, pH 3, pH 4, pH 5, pH 6, ALE and GE	2592	168
ALE	2772	162
ALE	2992	154
ALE	3322	143
ALE	3998	124
ALE and GE	4268	116
ALE and GE	4554	108
pH 2, pH 3, pH 4, pH 5 and GE	6611	68

CONCLUSION

The *Pereskia aculeata* leaves exhibited potential to produce a protein product. The results obtained in this research displayed high concentration of proteins and the possibility to extract them through easy and affordable methods that can be industrially replicated. The method that best meet the expectations was AE+Ultra 1:20, as it achieved good yield and a high percentage of purity in the precipitate.

The study revealed the positive effect of dissolution and ultrasound in protein extraction of *Pereskia aculeata* leaves, which can be an alternative for process adjustment for industries to obtain better cost-benefits. In regions where water is widely available and energy is expensive, good yield can still be produced with increased dissolution and dismissing the ultrasound, as it found in the AE 1:20 treatment. While in regions where water is limited and solar energy is abundant, dissolution can be increased to a certain extent and the ultrasound can be used to aid the extraction process and, still, good yield in extraction can be produced, as seen in the AE+Ultra 1:10 treatment.

Despite satisfying results, the use of the protein concentrate obtained in this study still demands more research before being implemented in food diets.

REFERENCES

1. Flora e Funga do Brasil. [Botanic Garden of Rio de Janeiro] [Internet]. [place unknown: publisher unknown]; [cited 2022 Set. 04]. Available from <http://floradobrasil.jbrj.gov.br/>.
2. Kinupp VF, Lorenzi H. [Non-conventional Food Plants (NCFP) from Brazil: identification guide, nutritional aspects and illustrated recipes].1 ed. Rio de Janeiro: Instituto Plantarum de Estudos da Flora, 2014.
3. Porto FGS, Campos AD, Carreño NLV, Garcia TS. *Pereskia aculeata* leaves: properties and potentialities for the development of new products. Nat. Prod. Res, 2022;36:4827-38.
4. Darnet LAF, Poccard-Chapuis RJM, Claudino LSD, Carvalho AS. [Biophysical aptitudes and agroecological intensification of dairy farming in settlements in the Brazilian Amazon]. Geografia Ensino & Pesquisa, 2021; 25.
5. Pendril F, Gardner TA, Meyfroidt P, Persson UM, Adams J, Azevedo T, Lima MGB, et al. Disentangling the numbers behind agriculture-driven tropical deforestation. Science, 2022;377:1-11.
6. WWF Brazil. Soya. [Internet]. [cited 2020 Jul 4]. Available from:
   https://www.wwf.org.br/natureza_brasileira/reducao_de_impactos2/agricultura/agr_soja/.
7. Odhav B, Beekrum S, Baijanath H. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. Food Compos. Anal, Africa do Sul, 2007; 20:430-5.
8. Almeida MEF, Junqueira AMB, Simão AA, Corrêa AD. [Chemical characterisation of unconventional vegetables known as Ora-pro-nobis]. Biosci.J, 2014; 30(3):431-9.
9. Takeiti CY, Antonio GC, Motta EMP, Collares-Queiroz FP, Park KJ. Nutritive evaluation of a non-conventional leaf vegetable (*Pereskia aculeata*, Miller). Int. J. Food Sci. Nutr, 2009;60:148-60.
10. Barreira TF, Paula-Filho GX, Priore SE, Santos RHS, Pinheiro-Sant´Ana HM. Nutrient content in ora-pro-nóbis (*Pereskia aculeata* Mill.): unconventional vegetable of the Brazilian Atlantic Forest. Food Sci. Technol, 2021;41:47-51.
11. Torres TMS, Álvares-Rivera G, Mazzutti S, Sánches-Martínez JD, Cifuentes A, Ibañez E, et al. Neuroprotective potential of extracts from leaves of ora-pro-nobis (*Pereskia aculeata*) recovered by cleam compressed fluids. J. Supercrit. Fluids, 2022;179:01-08.
12. Boye J, Zare F, Pletch A. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. Food Res. Int, 2010; 43:414-31.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem, 1976;72:248-54.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature, 1970;227:680-5.
15. Potin F, Lubbers S, Husson F, Saureu R. Hemp (*Canabis sativa* L.) protein extraction conditions affect extraction yield and protein quality. J. Food Sci, 2019;84:3682-90.
16. Polegrine DHG, Gasparetto CA. [Study of egg white protein solubility as a function of temperature and pH]. Revista de Ciências Exatas e Naturais, 2003;5:169-78.
17. Yu X, Gouyo T, Grimi N, Bals O, Vorobiev E. Ultrasound enhanced aqueous extraction from rapeseed green biomass for polyphenol and protein valorization. Compters Rendus Chimie, 2016;19:766-77.
18. Vernès L, Albert-Vian M, El Maâtaoui M, Tao Y, Bornard I, Chemat F. Application of ultrasound for green extraction of proteins from spirulina. Mechanism, optimization, modeling, and industrial prospects. Ultrason. Sonochem, 2019;54:48-60.
19. Rahman M, Lamsal BP. Ultrasound-assisted extraction and modification of plant-based proteins: Impact on physicochemical, functional, and nutritional properties. Compr. Rev. Food Sci. Food Saf, 2021;20;1457-1480.
20. Gordalina M, Pinheiro HM, Mateus M, Fonseca MMR, Cesário MT. Microalgae as protein sources – A review of protein bioactivity, extraction, purification and characterization. Appl. Sci, 2121:11;1-22.
21. Martínez EN, Añón MC. Composition and structural characterization of amaranth protein isolates. An electrophoretic and calorimetric study. J. Agric. Food Chem, 1996;44:2523-2530.
22. Glória MM, Regitano-d´Arce MAB. [Brazil nut protein concentrate and isolate production: chemical and functional properties]. Food Sci. Technol, 2000;20(2).
23. Lawal OS. Functionality of African locust bean (*Parkia biglobossa*) protein isolate: effects of pH, ionic strength and various protein concentrations. Food Chemistry, 2004;86:345-55.
24. Reis C, Netto FM. [Influence of extraction method on yield, protein profile and solubility of amaranth protein concentrates]. Bol. Centro Pesqui. Process. Aliment, 2006;24:289-302.
25. Hadnađev MS, Hadnađev TRD, Pojić MM, Šarić BM, Mišan Č, Javanov PT, et al. Progress in vegetable proteins isolation techniques: a review. Food Feed Res, 2017;44:11-21.
26. Lazova GN, Stemler AJ, A. 160 kDa protein with carbonic anhydrase activity is complexed with rubisco on the outer surface of thylakoids. Cell Biol. Int, 2008;32:646-653.
27. Raven PH, Evert RF, Eichhorn SE. [Plant Biology] 8 ed. Rio de Janeiro, TLC 2019. 856p