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Full Length Research Paper

Characterization of the proteins fractions extracted from leaves of *Amaranthus dubius* (*Amaranthus* spp.)

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Amaranth is an ancient plant belonging to the Amaranthaceae family, which is believed to have originated in Central and South America. The high nutritional quality of the amaranth seed protein is one of the main factors that has attracted the attention of the researchers. However, till date, the protein profile of the leaves of the most researched varieties has not been well studied. Moreover, the nutritional profile of *Amaranthus dubius* remains unknown. Therefore, it would be interesting to study it. Protein from *A. dubius* leaves has a high nutritional value due to its balanced amino acid composition. The concentrations of albumins, globulins, prolamins and glutelins are 73.42, 6.60, 6.47 and 6.11%, respectively. It was found that the best agent for extraction of globulins was Na_2HPO_4 and for glutelins, it was NaOH. The highest amino acid content was found in the albumins fraction and the lowest one in the glutelins fraction. The chemical score of the essential amino acids from proteins of the leaves of *A. dubius* flour was 92.83%; the flour has only one limiting essential amino acid: Leucine (Leu). The leaves of *A. dubius* can be used as complement dietary for rice, wheat and corn proteins.

Key words: Amaranth, amino acids, protein content, protein fractions.

INTRODUCTION

Plants from the Amaranth genus are attracting researchers' attention mainly because of their high nutritional value. Due to their high nutritive and nutraceutical characteristics, they have excellent agronomic features (Breene, 1991; Saunders et al., 1984; Barba et al., 2009; Acevedo et al., 2007; Bressani, 2003). *Amaranthus dubius* is a species of fanerogams commonly known as "pira dulce", "bledo" or "bleo". The plant is considered to be a morphologically deviant alloploid, but quite close genetically to the other *Amaranthus* species (Pal and Khoshoo, 1974). Despite it being the most common specie of the *Amaranthus* genus growing at Venezuela, its crop is wild and sometime is considered as weeds in rice crops (Acevedo et al., 2007). Since each part of the amaranths plants has a high nutritional value, it can be useful in different ways like as grain, vegetable and forage. Indeed, the elaboration of flour from its leaves could be a way to preserve it. Moreover, from its flour, protein concentrates and hydrolysates could be obtained. Since the leaves of *A. dubius* are eaten as vegetable in Venezuela, its crops should be industrialized (Arocha, 1999). But for it to be industrialized, a research has to be done, especially on the identification and validation of its protein.

It has been reported that *Amaranthus* spp. has high grains (Teutonico and Knorr, 1985; Martínez and Añón, 1997; Gorinstein et al., 2001; Gamel et al., 2005), high protein contents, with high biological value. For example,

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the analysis of seeds *of Amaranthus hybridus* shows a high amount of protein (13.1%) with a high biological value.

There exists a method based on the solubility of fractionation (Osborne, 1924) that has quite been useful in order to characterize the protein from the grain; consequently, this method could be applied in order to fractionate the protein from the leaf. The goals of the study are to extract solubility of the protein from the *A. dubius* leaf flour, and to characterize each one of its protein fractions.

MATERIALS AND METHODS

Leaves from *A. dubius* and *A. dubius* flour

The leaves from *A. dubius* were gathered from plants for six weeks, growing from the crop of the Miranda State, Venezuela.

Methods

A. dubius flour

The gathered leaves were cleaned, and dried by liofilization (Stokes EA-94120) for 12 h. The dried leaves were milled (Alpine Augsburg 160Z) and sieved to obtain a flour of 60 mesh.

Determination of moisture, protein content and particle size of the leaves

A. dubius flour

The moisture and crude protein contents, and the particle size of the *A. dubius* flour were evaluated using the methodologies described by AACC (2003) (N° 44-16, 46-12 and 55-30, respectively).

Mechanical fractionation of the leaves *A. dubius* flour: Three batches of 100 g of the flour sample were put on the top of a series of Tyler U.S Standard sieves from 20 mesh (841 µm) to 325 mesh (44 µm). The whole series of sieve was inserted on the stir machine (Ro-tap C Tyler), and strongly stirred for 10 minutes. The flour obtained on each sieve from 40 mesh to 120 mesh was weighted, packed, and stored for further crude protein analysis. A micro-Kjeldahl method was used to determine protein content (AACC, 2003 N° 46-12) of each fraction obtained.

Chemical fractionation of the leaves *A. dubius* flour: The sequential extraction of the proteins was carried out according to the following methods (AACC, 2003; Konishi et al., 1991; Barba et al., 1992; Segura et al., 1992), with some modifications (Figure 1). Each extraction step was performed in two stages at room temperature with ratio of 30 mL of solvent/g of meal. All solvents contained 0.1 mM phenylmethylsulfonylfluoride (PMSF), a protease inhibitor, to prevent proteolysis and 2% of NaN_3 (as antimicrobial agent). Between the stages, the extraction residue was separated by centrifugation at 10 000 g for 20 min at 4°C. The protein content in the supernatants was measured by the method of Bradford (1976).

Albumin: this was extracted with water and precipitated by adjusting to pH 3 with 2 N HCl. Extractions were evaluated at 2, 4 and 14 h. The precipitated was re-suspended in water, neutralized and free-dried.

Globulins: These were extracted from the albumin-free pellet. To compare their efficiencies as protein solvent, the following extracting agents were assayed: (a) 0.1 M NaCl, 0.01 M K_2HPO_4 (pH 7.5), 0.001 M EDTA) and (b) 0.1 M Na_2HPO_4. After being centrifuged, the supernatant was adjusted to pH 3 with 2 N HCl, and globulins were precipitated. The precipitate was neutralized and free-dried.

Prolamin: A subsample of the freeze-dried residue resulting from globulin extraction was used from the extraction prolamin with 70% aqueous 2-propanol. Supernatants were pooled, microfiltered (Gyrosep ^{TM} 300, Techmate Ltd, Milton Keynes, MK12 5WL, UK) using membrane Sepraphore (Intersep filtration system, Wokingham, RG41 2WY, UK) and freeze-dried.

Glutelin: After prolaminas were extracted, subsample of the freeze-dried residue was removed for glutelin extraction. Three extracting agents were tested: a) 0.1 M NaOH; (b) 0.1 M $Na_2B_4O_7$ (pH 10) and (c) 0.1 M $Na_2B_4O_7$ + 1% of SDS (pH 10).

Amino acid composition and chemical score: Amino acid analysis of each protein fraction was done according to procedure (Gorinstein et al., 200; Gorinstein et al., 2002), with some modifications. Samples were hydrolysed with 6 M HCl in a microwave system (ETHOS I, Advance microwave Digestion System, Millestone) for 20 min at 160°C. The power was set at 1000W for the first 5 min and 500 W for the remaining 15 min. The vacuum-dried samples were dissolved in 100 µL of 20 mM HCl and filtered through a 0.45 µm filter. Derivatisation was done with *o*-phthalaldehyde (OPA) and thiofluor. The sample was injected into a multi-pump gradient HPLC system (Perkin Elmer Series 200) with column C-18. A software TC navigator (Total Chrom Workstation Ver6.2, Perkin Elmer) was used to evaluate the amino acids profile. The scanning fluorescence detector was used at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Results are given as milligram per gram protein. The amino acid chemical score was calculated as the analysed essential amino acid content of the sample divided by the essential amino acid content of the FAO/WHO references pattern (FAO/WHO, 1991).

Statistical analysis

Each analysis was performed in triplicate and the means and standard deviation were calculated. The data collected were analyzed by one and two-way ANOVA followed by the Duncan test, using the Statgraphics® software version 6.0 (Manugistics, 1992), with the significance level set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Characterization of the leaf *A. dubius* flour

The moisture content of *A. dubius* flour was 10.74%. This value is in agreement with those suggested for a high shelf life of conventional flours. The protein content of the flour measured by macroKjedhal was 21.53% on dry basis.

The particle size of the flour statistically ($p \leq 0.05$) varied from 20 mesh to -325 µm, being the representative population between 80 mesh (177 µm)

Table 1. Particle size of the *A. dubius* leaves flour.

Sieve (N° mesh)	% Retention
20	0.20±0.28[a]
30	0.83±0.21[a]
40	2.57±0.07[b]
50	7.33±0.14[c]
60	5.77±0.21[d]
70	2.93±0.00[e]
80	18.97±4.31[f]
100	14.63±5.30[g]
120	12.63±1.20[g]
140	13.30±0.07[g]
170	11.37±0.23[h]
200	6.20±0.00[i]
230	3.53±3.68[j]
270	1.17±0.64[j]
325	0.57±0.07j[a]
-325	1.13±0.28[j]

Values (average of three determinations ± standard deviation) in a column followed by the same letter are not significantly different ($p \leq 0.05$).

to 140 mesh (105 µm) with a 59.53% (Table 1). Its results are indicative of a small particle size that shall improve the protein extraction, due to its size offering a high contact surface with the extraction agents.

Mechanical fractionation of the leaf *A. dubius* flour

Figure 2 shows the particle size and crude protein content of each sieve fraction from 20 mesh (841 µm) to 120 mesh (125 µm). In the Figure 2, below 40 mesh, the weight of the retained flour fraction was insignificant. Beyond this point (40 mesh) the crude protein is increasing to reach 27.34%; above this point, the crude protein content starts to decrease. The fine flour (less than 80 mesh) retained at 120 mesh was chosen for fractioning, because it (Figure 2) is the faction shows the higher crude protein content than the other fractions.

Chemical fractionation and identification of each protein fraction

In order to perform the chemical fractionation, the flour retained at the 120 mesh was chosen.

Albumins extraction

Figure 3a shows the albumins contents as a function of the extraction time. As can be seen the albumins contents increase with the extraction time. After 2 h with a second extraction, it gets to 50%. The maximum albumins content was obtained using two extractions at 14 h. However, as can be seen in the Figure 3a, the increment in albumins content from 2 h (second extraction) to 14 h with same procedure was 10.09%. It also was determined, that at the aforementioned two steps of extraction, the albumins contents decreased by a dilution effect.

The extraction of the albumins is represented in Figure 1. The flour/solvent ratio used in this study (1 g/ 30 ml) was higher compared to that in literature (Konishi and Yoshimoto, 1989) (1 g/10 ml). Because Amaranth leaves contain more fiber (8.68%) than grain (3.2 to 7.2%, according to Teutonico et al. (1985), water absorption was higher in the leaves flour suspension as compared to those used by Konishi and Yoshimoto (1989) for grains. On the other hand, some other authors like Konishi et al. (1991), Castellani et al. (1999) and Martínez et al. (1997) had used three phases (60, 30 and 30 min) in a water systems (1 flour: 10 water) to perform the albumins extraction from *A. cruentus* and *A. hypochondriacus* grains, but in this study two phases were used (12 and 2 h). These authors found a 30 to 40% of albumins of the total protein of the grains of *A. cruentus* and *A. hypochondriacus*. The albumins content found in this study was 73.42% of the total protein content (Figure 3a). These results suggest that the albumins are representing the main protein fraction of the leaves of A. *dubius*. It has been reported a 51.00% of albumins content in the protein fraction of the grains of *A. hypochondriacus* (Konishi et al., 1991; Segura et al., 1992) and 61.90% in those of *A. cruentus* (Soriano et al., 1992). This suggests a difference in the proportion of different species of Amaranth protein fractions, as well as between parts of the plant.

Globulins extraction

Figure 3b shows the globulins content of the fraction isolated from the amaranth leaves with a solution of 0.1M of Na_2HPO_4 and K_2HPO_4 0.01M; EDTA 0.001M; NaCl 0.1M at extraction time of 2, 4 and 14 h. As can be seen in Figure 3b, the globulins quantities isolated with the solution of 0.1 M of Na_2HPO_4, at all extraction time were higher than those isolated with the solution of K_2HPO_4 0.01 M; EDTA 0.001 M; NaCl 0.1 M. The statistics shows that the interaction of the time and reagent type has the best effect on the globulins isolation. The highest value obtained was at 14 hours with solution 0.1 M of Na_2HPO_4. Barba et al. (1992), had used a 0.1 M solution of Na_2PHO_4 for grain globulins extraction of *A. hypochondriacus* with an extraction process of two steps and took less time than that used in this study. The author had reported 51.60 and 66.10% of albumins + globulins fraction for 1 h and 2 h, respectively. In this study, the result of albumins + globulins contents of the fraction isolated with a 0.1 M

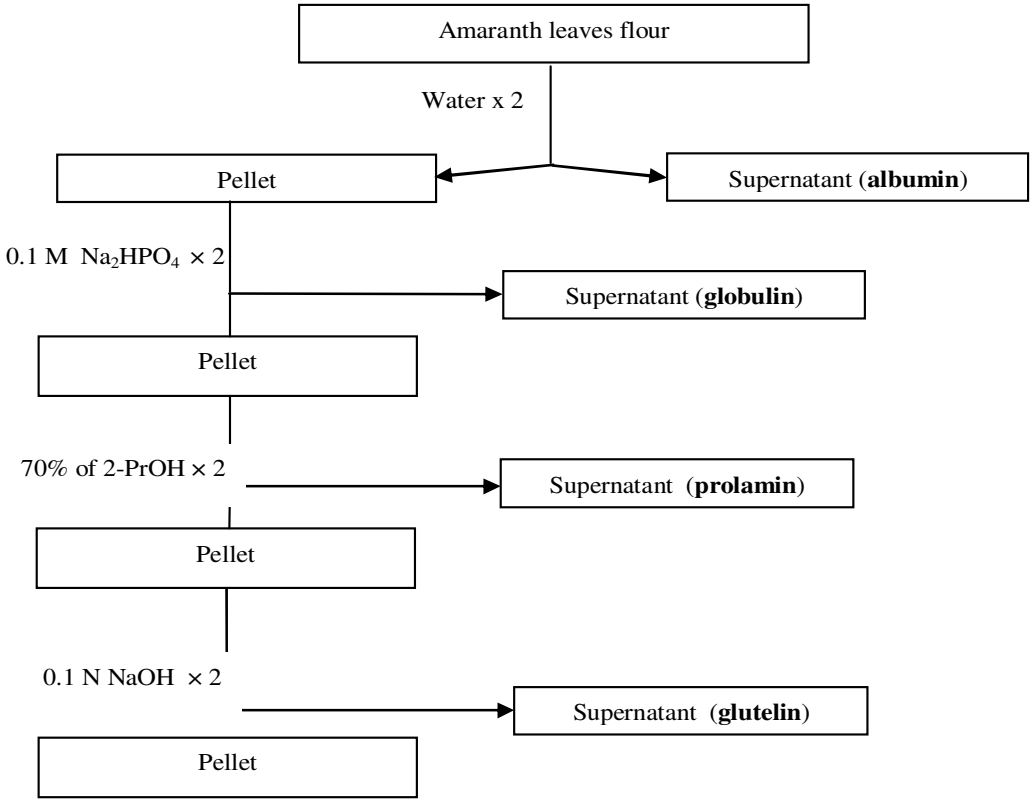

Figure 1. Schematic representation of the procedure used to isolate amaranth leaves protein fractions.

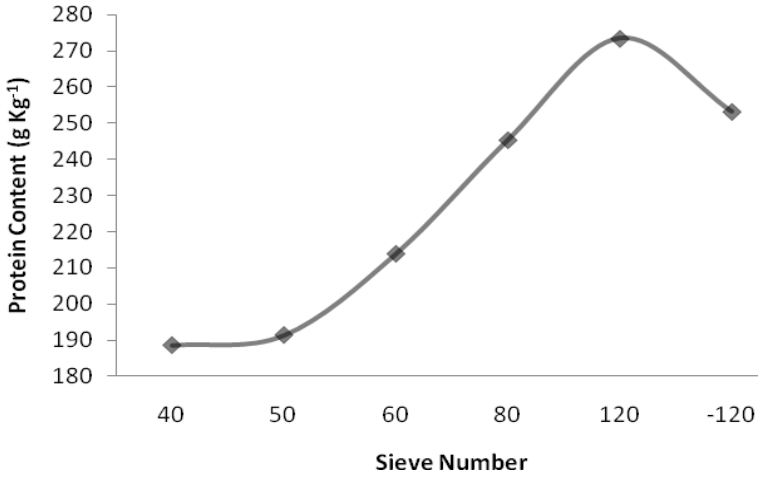

Figure 2. Protein content of each fraction sieve.

solution of Na_2PHO_4 at 2 h of extraction was 67.52% (62.43% of albumins and 5.09% of globulins), being higher than those reported in the literature (Barba et al., 1992). The globulins content here reported is lower than those shown by others authors for grains; for example, Segura et al. (1992) reported a content of 15.90% in *A. hypochondriacus* Azteca type. Some other authors (Soriano et al., 1992; Búcaro and Bressani, 2002) have reported 18.30 and 21.64%, respectively for protein isolated from A. *cruentus* grains.

Prolamins extraction

The isolation of the prolamins was performed on the pellet of the globulins extraction. Figure 3c shows the

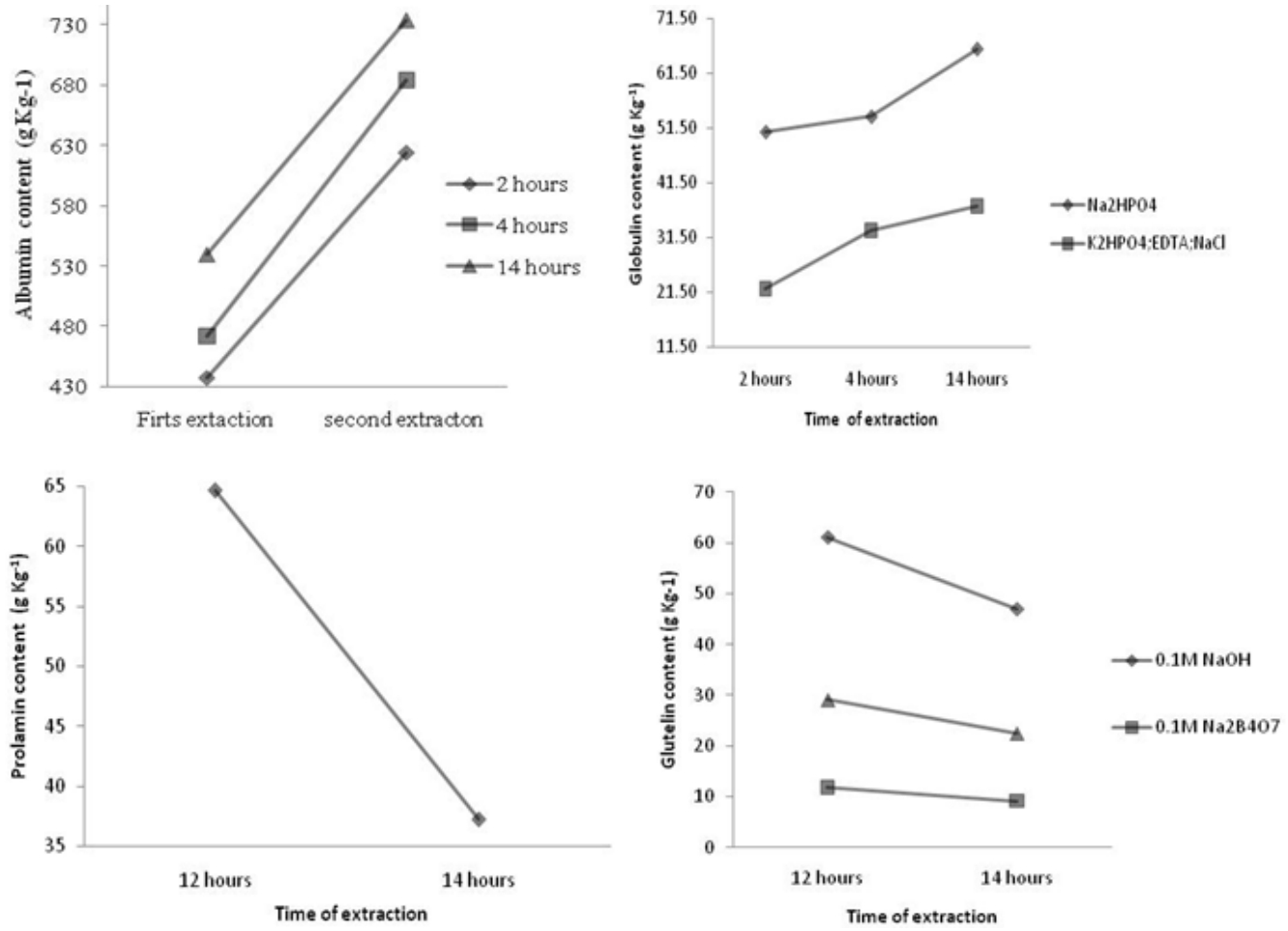

Figure 3. Chemical fractionation of the leaves *A. dubius* flour: (a). Albumins, (b) Globulins, (c). Prolamins and (d). Glutelins.

prolamins contents at different extraction times using a solution of 70% of 2-propanol. As can be seen in Figure 3c, the prolamins content at 12 h of extraction was statistically ($p \leq 0.05$) higher than the content of prolamins extracted at 14 h. As discussed prteviously, it must be attributed to the dilution effect. The results of this research are higher (6.47 and 3.72% for 12 and 14 h, respectively) than the ones reported by Segura et al. (1992) and Barba et al. (1992) (ranging from 7 and 2%) for *A. Hypochondriacus* grain proteins. But they are lower than those reported by Soriano et al. (1992) (15.5%) and similar to those reported by Bucaro and Bressani (2003) (4.14%) for proteins from *A. cruentus* grains.

Glutelins extraction

The statistical analysis ($p \leq 0.05$) shows an interaction of the solvent type and the extraction time on the glutelins content. As can be seen in Figure 3d, the highest glutelins contents were found when solution of 0.1N NaOH was used, followed by the extraction with solution of 0.1 M of $Na_2B_4O_7$ + 10 g Kg^{-1} of SDS; and finally with 0.1 M of $Na_2B_4O_7$. 12 h was the best extraction time (Figure 3d); increasing it decreases the glutelins contents. Also, it can be noted that this time is superior to the procedure of 1 h reported in other researches (Barba et al., 1992). Data here reported are lower than those reported by Barba et al. (1992) (22.5 to 41.2%), Segura et al. (1992) (31.10%), for protein isolated from *A. hypochondriacus* grains and Bucaro and Bressani (2003) (25.85%) for protein isolated from *A. cruentus* grains.

Amino acids profile

Figure 4 is a summary of the amino acids (aa) profile of each protein fractions, as compared to those of leaves flour. There are statistical differences of the aa content among the studied samples. The highest amino acid content is in the albumins fraction (g amino acid Kg^{-1} protein) and the lowest is in the glutelins fractions (Table 2). The aa of the albumins was also higher than of the flour and the aa most representatives were Asp, Glu and

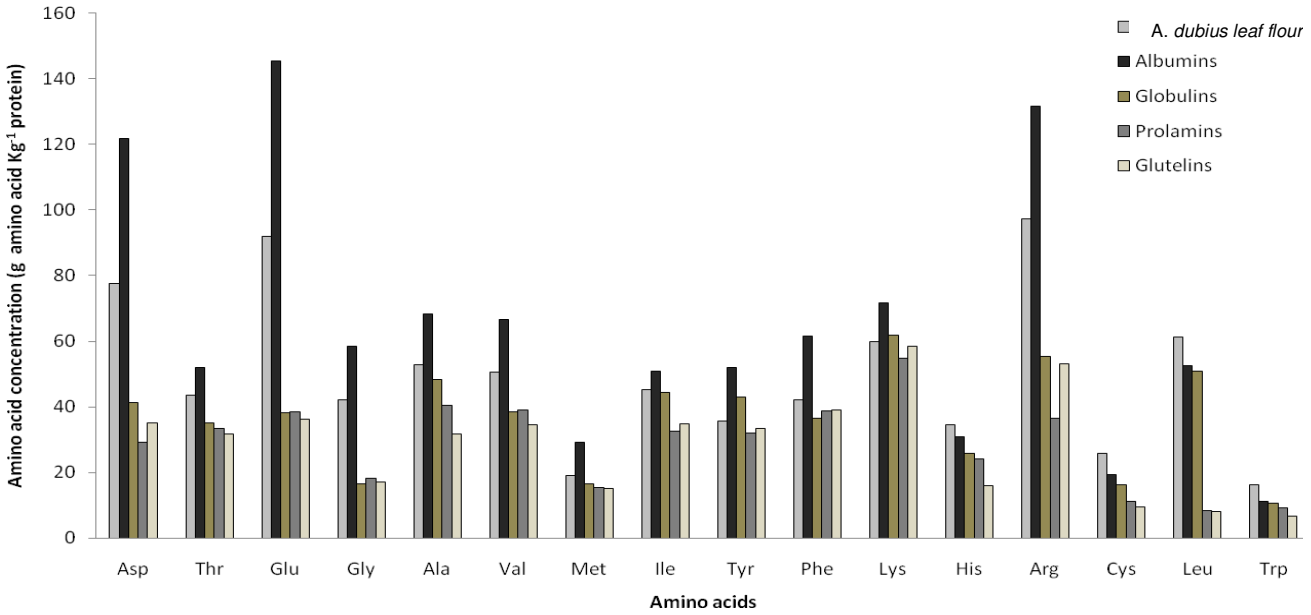

Figure 4. Amino acid composition of leaves flour *A. dubius* and proteins fractions.

Table 3. Essential amino acid composition (g amino acid Kg^{-1} protein) of leaves *A. dubius* flour compared with the grain of another species of *Amaranthus.*

Species	Amino acid							
	Trp	Cys+Met	Thr	Ile	Val	Lys	Tyr+Phe	Leu
A. *dubius*	16.19	44.73	43.57	45.25	50.57	59.85	77.83	61.27
A. *cruentus*[a,b]	9-20	40-63	27-45	28-40	33-45	49-61	60-85	44-62
A. *hypochondriacus*[a,c]	12-15	40-41	28-30	30-60	34-63	34-49	55-103	47-106
A. *caudatus*[a,b]	18	28-62	28-42	18-31	12- 41	40-57	31-91	32-58
A. *hibridus*[a]	-	7-15	27-37	30-37	-	45-63	152-182	60-71
A. *edulis*[a]	11	40	38-40	40-41	45- 7	59-64	81-86	61-63

a. Teutonico and Knorr, 1985; b. Gamel et al., 2005; Gorinstein et al., 2001.

Arg. Globulins are represented by Lys, Arg and Leu. Prolamins have shown a high content of Lys, ala and Val, while the glutelins has Lys, Arg and Glu as the main amino acids.

Table 3 is a summary of the essential amino acid composition (g amino acid Kg^{-1} protein) of *A. dubius* leaves flour in comparison with the amino acid composition of the protein from the grain of another species of *Amaranthus* reported in the literature. The Cys+Met, Thr and Val contents from the *A. dubius* leaves are higher than those found in the grain proteins of the other species evaluated. The contents of Trp, Ile, Leu, and Tyr+Phe identified in leaves of *A. dubius* are in the ranges reported in the literature (Teutonico and Knorr, 1985; Gorinstein et al., 2001; Gamel et al., 2005). Given that albumins are rich in Lys, Val, Phe, Tyr, Leu and Ile (Figure 4), and they are main fraction of the total protein from the leaves (73.42%), they have higher essential amino acids contents than the rest of protein fraction, and the leaves of amaranth flour.

The chemical score of the essential amino acids from proteins of *A. dubius* flour is shown in Table 4. The essential amino acids composition of the proteins isolated from the leaves of *A. dubius* were compared with those from the standard pattern suggested by the FAO/WHO (1991) (suggested requirements for children 2 to 5 years old) in order to evaluate the biological quality of the proteins for all ages, except those suggested for youngest infants below 1 year old. In Table 4 it can be seen that the proteins of the leaves of *A. dubius* flour have only one essential amino acid limiting (Leu), which shows the lower percentage calculated (92.83%). This value is similar to those reported in the literature for different species of grain amaranth. Mujica et al. (1997) pointed out that Leu is the limiting amino acid in the protein of the grains of

Table 4. Chemical score (%) of essential amino acids of leaves *A. dubius* flour.

Amino acid	Standard pattern [a]	Leaves *A. dubius* flour [b]	Chemical score (%)
Ile	28	45.25	161.61
Leu	66	61.27	92.83[c]
Lys	58	59.85	103.19
Met + Cys	25	44.73	178.92
Phe + Tyr	63	77.83	123.54
Thr	34	43.57	128.15
Trp	11	16.19	147.18
Val	35	50.57	144.49
His	19	34.61	182.16

[a]FAO/WHO (1991) suggested requirements (children 2 to 5 years old). [b] g aa Kg^{-1} protein; [c], limiting amino acid.

A. caudatus, A. hypochondriacus and *A. cruentus*. The chemical scores for these amino acids are 70, 86 y 77%, respectively. When compared with those found in this study they are low. In addition to Leu as limiting essential amino acid, Lys was found in minor concentration in the proteins of grain of A. *cruentus* (94%) and *A. caudatus* (96%) (Gamel et al., 2004).

The concentration of the limiting essential amino acids ought to diminish the net utilization of food proteins; for example, only 70% of the protein total of *A. caudatus* is available (Mujica et al., 1997), and it is established by the first limiting essential amino acid (Leu). Bearing in mind the postulation before, the proteins from the leaves of *A. dubius* will be used in a 92.83%. That means, they have a higher biological value than those published for wheat proteins (73%) and soy beans (74%) (Mujica et al., 1997). Unlike those found by Gamel et al. (2004) at the present study Lys is not the limiting amino acid for the proteins from the leaves of *A. dubius*; in contrast, it is found in quantities, which can be used as complement for rice, wheat and corn proteins. Data reported in this research pointed out that the proteins from the leaves of *A. dubius* can cover all requirements of the essential amino acids of elementary students (9 to 12 years old) and adults. Except for Leu, this protein should supply the majorities of the essential amino acids, in the pre-school population.

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