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Morphological, proximal composition, and bioactive compounds
coloration of wild and cultivated amaranth (*Amaranthus*
spp.) species

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**Running title**: Characterization of wild and cultivated amaranth seeds

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ABSTRACT

Amaranth seeds have gained renewed interest due to the presence of encrypted peptides with several biological functions, among which the inhibition of dipeptidyl peptidase IV (DPPIV) and angiotensin converting enzyme (ACE) stands out. Amaranth seeds also contain an oily fraction rich in squalene, an unsaturated hydrocarbon, which have been attributed diverse beneficial health effects. Up to date, only cultivated species have been analyzed and no information exists about bioactive peptides and lipid composition of wild amaranths. The aim of this work was to carry out the morphological and biochemical comparison of cultivated species A. hypochondriacus (waxy and non-waxy cultivars) and A. cruentus with wild species A. powellii and A. hybridus. The highest protein and fat contents were observed in A. powellii, but A. cruentus showed the highest squalene content. The electrophoretic protein profile showed differences in protein accumulation among species. In vitro gastrointestinal digestion was used to release the encrypted peptides and their inhibitory action against DPPIV and ACE activities was evaluated. Our results showed that peptides from A. hypochondriacus cv Opaca presented the highest inhibition against both DPPIV and ACE activities. This information is valuable in order to design strategies to obtain new amaranth varieties with higher nutraceutical quality.

Keywords: ACE; biopeptides; DPPIV; cultivated and wild species; squalene
1. Introduction

The world’s human population is predicted to reach over 9.3 billion by the year 2050, which will increase the demand of food. At the same time, climate change and soil deterioration represents an increasingly serious threat for food production (Lobell et al., 2011). To face these challenges, crop improvements must intend not only to ensure food availability, but crops must meet higher nutritious food standards (McCouch et al., 2013; Muñoz et al., 2017). For this reason, a sustainable diet supply, that means not only foods with high nutritional value but also foods containing bioactive compounds, which could represent additional benefits for health, are increasingly important for present and future generations (Siró et al., 2008).

With an increasing interest in new food sources, the need to better explore untapped plant biodiversity has gained importance, including wild relatives of our current crops (Muñoz et al., 2017). Wild species have survived several climate changes and therefore, they are valuable resources of genetic information for improving crops in the face of climate change (Lobell et al., 2011).

Because populations of many wild relatives are under threat from habitat loss and degradation, there is an urgent need to collect and preserve their germplasm. Such wild relatives need to be characterized to take advantage of them in the development of strategies to solve current and future agricultural challenges (McCouch et al., 2013). Another essential component to support the rediscovery of ancient species for food diversity is the marketing of these crops and their products (McCouch et al., 2013; Muñoz et al., 2017), for example, the
gluten-free food market worth almost $1.6 billion in 2011. This resurgent interest
is expressed in re-discovering ancestral crops as functional foods, which may
offer an important alternative for people affected by celiac disease (Cooper,
2015).

Amaranth is one of the oldest cultivated plants, which had great importance for
the Aztec, Mayan, and Incas. Amaranth was grown as staple crop together with
corn, but was banned during the Spanish Conquest. Since the 70’s amaranth
resurged as an alternative crop not only due to its high nutritional value (high
lysine and methionine content) but also because amaranth prolams content is
negligible (Huerta-Ocampo and Barba de la Rosa, 2011), which are the seed
storage proteins responsible for the manifestation of celiac disease and
cerebropathias. In this new century amaranth gained renewed importance due to
its nutraceutical properties; amaranth proteins contain encrypted peptides
amongst the most studied are those with antihypertensive action (Huerta-
Ocampo and Barba de la Rosa, 2011). Furthermore, the inhibitory peptides in
amaranth seed proteins against dipeptidyl peptidase IV (DPPIV) activity have
been identified and characterized (Velarde-Salcedo et al., 2013). The oily fraction
of amaranth seeds is rich in squalene, an unsaturated hydrocarbon to which has
been attributed hypocholesterolaemic properties (Chaturvedi et al., 1993).

In addition to nutritional characteristics, amaranth plants have attractive
agronomic features; they grow where cereals and vegetables cannot such as dry
soils, high altitudes, and high temperatures (Huerta-Ocampo and Barba de la
Rosa et al., 2011). Amaranth cultivation has increased and breeders produced a
large number of new varieties adapted to different environments, however, some
of these new varieties are only new names for old varieties or landraces, hence
the use of wild amaranth species with remarkable tolerance to several abiotic
stresses such as *A. powellii* and *A. hybridus* are of great interest. Although these
wild species are proposed as the ancestors of the main cultivated species used
for seed production such as *A. hypochondriacus* and *A. cruentus*, still its
molecular relationships have not been established. The aim of the present work
was to compare the morphological characteristics and bioactive compounds
content of cultivated and wild amaranth species.

2. Materials and Methods

2.1. Amaranth genotypes

Amaranth seeds of wild (*A. hybridus* and *A. powellii*) species as well as the
most cultivated and studied species, *A. hypochondriacus* cv Nutrisol, and *A.
cruentus* cv Amaranteca, were provided by the National Institute for Forestry,
Agriculture and Livestock Research (INIFAP), Mexico. Two more cultivars of *A.
hypochondriacus* were included in the study; Cristalina (non-waxy type) and
Opaca (waxy type), which are derived from a heterozygous plant for this
current by six generations of single seed descendant and were collected from
Atzitzintla, Tlaxcala, Mexico.

2.2. Morphological and structural seeds characterization
Seed weight was calculated by weighing 100 seeds on an electronic balance DV215CD Discovery (Ohaus, Parsippany, NJ, USA) with 0.01/0.1 mg accuracy. The weight of 100 seeds was extrapolated to 1000 seeds. Seed dimensions (diameter and width) were taken with the SteREO Discovery V8 (Carl-Zeiss, Oberkochen, GE). All measurements were done in triplicates.

Images of whole seeds were obtained with the SteREO Discovery V8 (Carl-Zeiss). Paradermal sections were visualized by scanning electron microscopy captured with an ESEM model Quanta 200 (FEI, Hillsboro, OR, USA) from the National Laboratory of Nanosciences and Nanotechnology Research (LINAN) IPICYT.

2.3. Amaranth flours proximate composition

Seeds were cleaned and milled in liquid nitrogen using a KRUPS GX4100 (Solingen, GE) mill to obtain fine flour. Flour samples were stored in plastic tubes at -80 °C until analysis. Total nitrogen content was determined by micro-Kjeldahl method (AOAC, 2007, method 12.960.52), and total protein content was calculated using a 5.85 factor. Fat content was determined by Soxhlet method (AOAC, 2007, 996.01 method). Crude fibre and ash contents were obtained according to AOAC (2007) methods 991.43 and 900.02, respectively. All determinations were made at least in triplicates.

2.4. Protein extraction and electrophoretic profile of amaranth seeds proteins
Total protein extracts, from the six amaranth species studied, were obtained by mixing 0.1 g flours with 2 ml of a solution containing 7 M urea, 2 M thiourea, 2% (w/v) Triton X-100 and 0.05 M DTT. Suspensions were mixed by vortexing for 15 min at 4 °C and centrifuged at 17,000×g at 20 °C, supernatants were recovered and protein quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Each sample was analysed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous Tris-glycine system. The stacking and resolving gels were 4% and 13.5%, respectively. Protein (15 µg) was loaded onto the gel and separated in a Mini-Protean III system (Bio-Rad), gel was run at 10 mA/gel for 30 min followed by 25 mA/gel until bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were stained with a 0.05% Coomassie blue R-250 (USB Corporation, Cleveland, OH, USA) in 40% methanolic solution containing 10% acetic acid.

2.5. In-gel digestion and LC-MS/MS protein identification

Protein bands were manually excised from gel, destained, reduced with 10 mM dithiothreitol and alkylated with 55 mM idoacetamide. Protein digestion was carried out with sequencing-grade trypsin (Promega, Madison, WI, U.S.A.). Tryptic peptides were analyzed with a nanoACQUITY UPLC System (Waters, Milford, MA, U.S.A.) coupled to a SYNAPT-HDMS Q-TOF (Waters) mass spectrometer. MS/MS spectra data sets were used to generate PKL files using Protein Lynx Global Server v2.4 (PLGS, Waters). Proteins were then identified using the MASCOT search engine v2.5 (Matrix Science, London, U.K.).
Searches were conducted against the *Viridiplantae* subset of the NCBI-nr protein database (6 686 534 sequences, May 2018). Trypsin was used as the specific protease, and one missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 50 ppm and 0.1 Da, respectively. Carbamidomethyl cysteine was set as fixed modification and oxidation of methionine was specified as variable modification. The protein identification criteria included at least two MS/MS spectra matched at 99% level of confidence, and identifications were considered successful when significant MASCOT scores >50 were obtained, indicating the identity or extensive homology at p<0.01 and the presence of a consecutive y ion series of more than three amino acids.

2.6. Gastrointestinal digestion in vitro

A simulated gastrointestinal digestion *in vitro* model was carried out as reported before (Velarde-Salcedo et al., 2013). Briefly, 1 g of amaranth defatted flour was resuspended in 20 ml of 0.03 M NaCl pH 2.0. In order to inactivate proteases, the suspensions were heated in a water bath at 80 °C for 5 min and allowed to cool down at room temperature. Porcine pepsin (Sigma–Aldrich, St. Louis, MI, USA) previously dissolved in 0.03 M NaCl pH 2.0 was added in a 1:20 ratio (w/w enzyme to substrate). Samples were digested at constant pH for 3 h at 37 °C and pH was then adjusted to 7.5. A mixture of trypsin (Sigma–Aldrich) and pancreatin (Sigma–Aldrich) was prepared (1:1 w/w trypsin:pancreatin ratio in 0.1 N NaHCO₃), added to the digestive solution and incubated at constant pH for an additional 3 h period (1:20 w/w enzyme to substrate ratio for both the enzymes,
trypsin and pancreatin). Digestion was stopped by heating the suspensions at 75 °C for 20 min and centrifuged at 13,000xg for 30 min. Peptides were ultra filtrated through 10 kDa filters (Amicon Ultra-10 centrifugal filters, Sigma-Aldrich). The peptides concentration was determined by the Lowry-based DC Protein Assay (Bio-Rad) using BSA as a standard, and then stored at -20 °C until analysis.

2.6.1. Inhibition of dipeptidyl peptidase IV (DPPIV) activity

DPPIV activity was measured using the chromogenic substrate Gly-Pro-pNitroanilide (Sigma-Aldrich) as previously reported (Velarde-Salcedo, et al., 2013). Briefly, 10 µl of 100 ng/ml of dipeptidyl peptidase IV (Sigma-Aldrich) were added to 40 µl amaranth peptides dissolved in 100 mM Tris pH 8 and 50 µl of 1 mM Gly-Pro-pNitroanilide dissolved in Tris buffer. Mix was incubated at 37 ºC for 1 h. Absorbance was measured at 415 nm in a Multiskan Go plate reader (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Results were expressed as µmol of nitroaniline/min based on a p-nitroaniline (Sigma-Aldrich) standard curve.

2.6.2. Inhibition of Angiotensin Converting Enzyme (ACE) activity

Peptides with inhibitory activity against ACE were measured by spectrophotometric assay. Briefly 20 µl of sample was added to 0.1 ml of 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM hippuryl–histidyl–leucine (HHL, Sigma). ACE (5 mU) (EC 3.4.15.1, 5.1 U/mg, Sigma) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction
was terminated by the addition of 0.1 ml of 1 M HCl. The hippuric acid formed
was extracted with ethyl acetate, heat-evaporated at 95 °C for 10 min, dissolved
in distilled water and measured spectrophotometrically at 228 nm. The activity of
each sample was tested in triplicate. Captopril was used as a positive control.
The IC₅₀ value was defined as the peptide concentration (mg/ml) needed to
inhibit 50% ACE activity; it was calculated by an ACE inhibition (%) vs. log
peptide concentration (mg/ml) linear regression.

2.7. Amaranth lipid extraction, GC-MS analysis, and squalene quantification
Lipids from amaranth seeds were extracted by mixing 0.1 g of amaranth flour
with 1.75 ml of hexane, in constant agitation at room temperature by 5 h. Samples were centrifuged (17,000×g, 20 min, 25 °C) and supernatants
transferred into new tubes. Extracts were analysed by GC-MS using a
7820A/5977E System (Agilent Technologies, Santa Clara, California, USA), with
a HP-5ms capillary column (Agilent Technologies) of 30 m length, 250 µm of
inner diameter and 0.25 µm-film thickness. Samples, 3 µl, were injected in
splitless mode. The column was held at 80 °C for 1 min after injection, the
temperature programmed at 20 °C/min to 210 °C and held for 10 min more, then
10 °C/min to 280 °C and held for 35 min. Helium was used as carrier gas, at a
constant column flow rate of 1 ml/min. The injector temperature was 250 °C and
the detector temperature was 230 °C. The mass spectrometer was operated
under Electron Impact Ionization at 70 eV with a mass range from 30-500 amu.
Lipids were identified comparing their retention times and the mass spectra
against NIST Mass Spectral Library v2.2. Results were expressed as the individual relative percentage of each lipid present in the sample. For squalene absolute quantification, an analytical standard (Sigma) was used to construct a calibration curve from 1-10 mg/l ($r^2=0.997$).

2.8. Statistical Analysis

A variance analysis (ANOVA) was carried out with Holm-Sidak test using the Sigma Plot software analysis v12.3 (Systat Software, Inc., San Jose, CA, USA) for paired analysis and considering $p<0.05$ for statistically significant differences.

3. Results

3.1. Morphological characterization

$A. powellii$ showed the lowest value for thousand seeds weight (TSW) with only 0.45 g and $A. hypochondriacus$ cv Cristalina presented the highest TWS of 0.90 g (Supplementary Table S1). $A. hypochondriacus$ cv Nutrisol have the smallest seed length 1.19 mm and the smallest width was observed in $A. powellii$ (0.88 mm). The largest seeds were those of $A. hybridus$ and $A. hypochondriacus$ cv Cristalina with dimensions of 1.32x1.15 mm and 1.33x1.13 mm, respectively. Although different in length and width, most species conserve the same diameter/width ratio, with exception of $A. hypochondriacus$ cv Cristalina, which present more oval seeds (1.17 D/W ratio) and $A. cruentus$ with the most rounded seeds (1.11 D/W ratio).
Phenotypic characteristics of wild and cultivated seeds are shown in Figure 1. The wild species are bright black in colour, while seeds of cultivated species are cream light colour. Different A. hypochondriacus cultivars are distinguished due to the translucent (vitreous) or opaque characteristics; the cv Nutrisol is the most opaque while cv Cristalina, as its name indicates, is a translucent bright seed. Vitreous characteristic has been related with the type, degree of cross-linking, and molecular weight distribution of proteins in seeds.

SEM analyses of paradermal cuts showed that wild species A. hybridus and A. powellii as well as A. hypochondriacus cv Cristalina contain polyhedral defined structures in the vitreous perisperm, structures that are not observed in A. cruentus, A. hypochondriacus cvs Nutrisol and Opaca, which perisperm is not vitreous (Figure 2).

3.2. Proximal composition of amaranth seeds flours

The flours proximal composition from wild and cultivated amaranth seeds is shown in Table 1 and Supplementary Table S2. Although A. powellii is the smallest seeds, is the species with the highest protein (17.8%) and fat (8.1%) contents. A. cruentus is the species with the lowest protein content (14.8%), but the highest starch content (73.0%). On the other hand, A. hybridus and A. hypochondriacus cv Cristalina, with the largest seeds, are the species with the lowest fat content (5.9 and 5.7%, respectively). Interestingly, A. hybridus has the highest crude fibre (6.1%) and ash (3.7%) contents. It is interesting that among the A. hypochondriacus species, the most commercial cultivar, Nutrisol, showed
less protein content (15.8%) in comparison with cvs Opaca and Cristalina (16.7%).

3.3. Electrophoretic pattern and protein identification

The amount of protein quantified by Bradford (Supplementary Figure S1) correlated with the values obtained by Kjeldahl method (Table 1), which indicates that total protein extracted was almost the total protein present in seeds. Total proteins were analysed by SDS-PAGE (Figure 3). In all species and cultivars were observed the bands located at 35-37 kDa and 18-20 kDa, which represent the acidic and basic subunits of the canonical 11S globulins. The most remarkable differences among species and cultivars analysed were observed in the range of 50 to 70 kDa. Both wild species as well as A. hypochondriacus cv Cristalina have a band around 65 kDa. A. powellii and A. cruentus share a band of 60 kDa. A. hybridus and all A. hypochondriacus cultivars showed a 55 kDa band. These three bands were cut from gel, analysed by LC-MS/MS and identified as a Granule Bound Starch Synthase I or GBSSI (Supplementary Table S3 and Supplementary Figure S2).

3.4. Amaranth peptides with inhibitory activity against DPPIV and ACE

A simulated gastrointestinal digestion in vitro method was used to release the encrypted peptides from all amaranth samples. The capacity of released amaranth peptides to inhibit both DPPIV and ACE enzymes was measured. DDPIV inhibition increased in a dose-response relationship (Figure 4A), higher
inhibition activity was detected at the highest tested concentration (3.2 mg/ml). At this concentration, *A. hypochondriacus* cv Opaca rendered the highest inhibitory activity reaching a 60% of DPPIV with an IC\textsubscript{50} of 1.6 mg/ml. *A. hypochondriacus* cv Cristalina and *A. powellii* showed the least DPPIV inhibition reaching only 40% at the highest tested peptide concentration (3.2 mg/ml). A similar ACE inhibitory activity profile was observed (Figure 4B). *A. hypochondriacus* cv Opaca peptides presented the highest activity reaching of 80% inhibition at 3.2 mg/ml with an IC\textsubscript{50} of 0.6 mg/ml. *A. hypochondriacus* cv Nutrisol, *A. hybridus* and *A. cruentus* showed an IC\textsubscript{50} of 1.5 mg/ml while *A. hypochondriacus* cv Cristalina and *A. powellii* the IC\textsubscript{50} was of 2.5 mg/ml.

3.5. Characterization of lipids in amaranth seeds

The lipid composition analysed by GC-MS showed the presence of palmitic and linoleic acids in all species. Linoleic ethyl esters was present only in *A. hybridus* and *A. hypochondriacus* cvs Cristalina and Nutrisol, while oleic acid ethyl ester was only present in *A. hypochondriacus* cvs Cristalina and Nutrisol. Butyl ester of palmitic and stearic acid were detected in all samples analysed. Stigmasterol, an important phytosterol, was detected in higher abundance in the wild species *A. powellii*, followed by *A. hypochondriacus* cvs Cristalina, and *A. hybridus* (Supplementary Table S4). Squalene, an unsaturated hydrocarbon, was detected in all samples but interestingly the highest abundance was detected in *A. cruentus* (Supplementary Table S4 and Supplementary Figure S3). Because the importance of squalene and relative abundance do not reflect
the real quantity present in samples, squalene was quantified. Results showed that squalene concentration ranged from 0.197 to 0.335 g/100 g of seeds and this values in relation to oil content ranged from 2.85 to 4.86 g/100 g oil (Table 2).

4. Discussion

Wild ancestors of common cereals, such as rice and wheat, have been used as resources for quality improvement of cultivated grains (Cooper, 2015). However, despite the potential of a several seeds progenitors to face the challenges of modern agriculture, there are few collections of wild relatives and even the available wild genetic resources are still under-utilized (McCouch et al., 2013).

Mexico is rich in genetic diversity of amaranth species such as A. powellii and A. hybridus (Espitia-Rangel et al., 2012). These wild accessions have been considered as the ancestors of the cultivated species A. hypochondriacus and A. cruentus (Sauer et al., 1967), but concerns have been raised about the hypothesis of amaranths ancestors. Hence, that morphological and molecular analysis of wild and cultivated species could help validate the amaranth phylogeny and evolutionary relationships (Espitia-Rangel et al., 2012).

For years, plant wild species have survived to abiotic and biotic stresses. Seeds have used dark or bright colours, as a signal of toxic materials, as protective action against predators (Lev-Yadun, 2016). These pigmentations are due to polyphenols, plant metabolites that play a role in the protection of plants
against ultraviolet radiation, pathogens, and herbivores (Alvarez-Jubete et al., 2010). The absence of these pigments in cultivated amaranths is considered as a trait of domestication.

However, there are black seeds such as *Pisum humile* and *P. fulvum*, which are highly edible but mimic various toxic seeds of legumes that grow in the same region such as *Lathyrus ochrus* (Lev-Yadun, 2016). Wild black seeded amaranths (a hybrid between *A. hypochondriacus* and *A. hybridus*), are grown in Michoacán-Mexico to make special black tamales (Sauer et al., 1967). Another characteristic of wild seeds is the hardness of their testa as protective tissues for mechanical defences against granivores attacks. However, a light color seed with soft testa has been the target for domestication. This also can be observed in amaranth, light seeds with soft testa were selected for cultivation and domestication (Figure 1).

Seed size is another characteristic related to the profitability of agricultural operations. Selection of big seeds, in terms of genetic changes, is related to breakdown of seed dispersal and seed dormancy (Fernández-Marín, 2014). The reported size for amaranth seeds is 0.9-1.7 mm diameter and TSW ranged from 0.6-1.0 g (Assad et al., 2017), values that agree with our results, except for *A. powelli* that have the smallest diameter and TSW of 0.88 mm 0.45 g, respectively. Interestingly, the cultivated species *A. hypochondriacus* cv Cristalina, *A. cruentus*, and the wild *A. hybridus* bear the largest seeds. Genotypes with small seeds are correlated with low seed quality, since larger seed size is probably advantageous because of their better standability under
agricultural conditions, and because of the greater plantlets size arising from them, and particularly important for crops with edible seeds (Lush and Wien, 1980; Espitia-Rangel et al., 2012). The weight has been also widely used as characteristic for seeds improvement and increased yield. Our results showed that the cultivated species *A. hypochondriacus* cvs Cristalina and Opaca, as well *A. cruentus* are the cultivars that presented the highest values of TSW.

Although several morphological characteristics have been studied in relation to seeds breeding, less attention has been paid and it is poorly understood how agricultural selection and cultivation affected the nutritional quality of seeds, which are important traits for crop improvement and meet with Food Sovereignty (Muñoz et al., 2017).

The scarce literature available on cultivars and their wild relatives are focused on protein and amino acid contents and very little information exists on other important nutritional traits such oils or starch. In general, a decrease in protein, fibre, and minerals is related with the increase in carbohydrates. Seeds with higher carbohydrate content are bigger and with higher TSW. This is in agreement with our results, *A. powellii*, wild species with the smallest size and TSW presented the highest protein and lowest carbohydrate contents (17.8% and 64.5%, respectively). Among the cultivated species, which are bigger seeds, *A. cruentus* showed the lowest protein but highest carbohydrate content (14.8 and 73.9%, respectively).

Amaranth lipid content varies from 5.7% to 8.1% (Table 1), values that are in the range of reported values from 6 to 20% (Assad et al., 2017). In soybean, oil
content is higher in domesticated seeds as compared with its wild counterpart (Zong et al., 2017), however in amaranth this relationship is not clear. The wild A. *powellii*, is the species with the highest fat content but A. *hybridus*, also a wild species, showed the lowest fat content. Our results are in agreement with Fernández-Marín et al. (2014) who reported that in some legumes such as soybean, peanut, lens, among others, the domestication caused a decreased in total carotenoid content, especially a reduction of $\alpha$- and $\gamma$-tocopherol was detected as domestication increased. In amaranth was observed a reduction in abundance of stigmasterol from 3.17% presented in the wild species A. *powellii*, while in the most domesticated species, A. *hypochondriacus* cv Nutrisol, the value was 1.44% (Supplementary Table S4).

Amaranth grain is considered as a good source of crude fibre, content that is higher than in rice, sorghum, oat, barley, and potato (USDA, ndb.nal.usda.gov/ndb/foods/list). A. *hybridus* showed the highest crude fibre content (6.1%) and the lowest content was found in A. *cruentus* and A. *hypochondriacus* cv Nutrisol, species with the highest starch content. Fibre is an important part of human nutrition; sufficient fibre intake is related with prevention of colon cancer. It has been reported that fibre in amaranth could be responsible for the control of blood cholesterol level preventing the development of atherosclerosis and its complications (Caselato-Souza et al., 2014).

Carbohydrates in amaranth are of especial attention due to the very small size of the starch granules (0.5 - 2 $\mu$m), which gives functional characteristics of great interest in food applications (Kong et al., 2009). Carbohydrates were very
important during amaranth domestication that is thought to have occurred during the prehispanic times. Aztecs used sticky grain amaranths to make cakes as part of religious ceremonies (Sauer, 1967). The sticky grain selection was the origin of so called waxy varieties of cereals and other starch-producing crops (Hunt et al., 2010). Amaranth waxy types were selected and nowadays are the cultivated species (A. cruentus, A. hypochondriacus). Sticky starch type is characterized by very low content of amylose, a character that modifies the glycaemic index. Waxy starch types generally have a higher glycaemic loads, its consumption is related with a better physical performance and quicker recovery associated with intense physical activity and with the reloading of glycogen storages after exercise (Wright, 2005). Therefore, higher-glycaemic cereal grains, which may have been an advantage and better tasting, treat in past cultures, today may impose a disadvantage for modern civilization where exercise and physical activity has decreased and glycaemic loads are related with type-2 diabetes risk (WHO, 2016). So non-waxy varieties should be reconsidered for the generation of new amaranth cultivars and wild species are an important source of non-waxy starches and high protein contents.

The waxy and non-waxy amaranth types are related with the morphological observation, the wild species showed a well-defined polyhedral perisperm (Figure 2), but also with the differential accumulation of proteins (Figure 3). The differentially accumulated proteins were identified as GBSSI (Supplementary Table S3 and Supplementary Figure S2), enzyme responsible for amylose synthesis. It is important to mention that only the species that presented
polyhedral structures observed by SEM (Figure 2), have the GBSSI band at 65 kDa (Figure 3). GBSSI isoforms of 60 and 55 kDa could be not functional leading to the synthesis of starches with different ratios of amylose/amylopectin, and therefore different rheological and physicochemical characteristics, which may be highly valued in the food and beverage science and technology industry.

In relation to nutraceutical characteristic, although limited data are available in this sense, it has been reported that ancient wheat are not really healthier than modern wheat (Shewry and He, 2015). In this work we have shown that one cultivated species, A. hypochondriacus cv Opaca showed the highest inhibition against the DPPIV and ACE activities.

Type-2 diabetes is a chronic metabolic disorder considered as one of the major global health problems (WHO, 2016). The actual therapies to lower the hyperglycaemic state in patients with diabetes are based on the inhibition of DPPIV, enzyme responsible for degradation and inactivation of glucose-dependent insulino-tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP and GLP are incretin hormones, which have a function to induce insulin secretion in the β-pancreatic cells. Synthetic DPPIV inhibitors are used as drugs therapies, however, there is a risk of side effects, DPPIV has several other functions than incretins inhibition (Matteucci and Giampietro, 2011) and natural DPPIV inhibitors are of great interest as therapy to promote a healthy life (Siró et al., 2008). The possible mechanisms of action of amaranth peptides with inhibitory activity against DPPIV have been described (Velarde-Salcedo et al., 2013).
On the other hand, hypertension is one of the main risk factors for cardiovascular diseases, hypertension might affect as many as 1 billion individuals worldwide. ACE plays an important role in the regulation of blood pressure by catalysing the production of the vasoconstrictor Angiotensin II and inactivating the vasodilator Bradykinin. Thus, ACE-inhibitory drugs are commonly used to control high blood pressure in hypertensive subjects. It is reported that the main source of ACE inhibitory peptides is fermented milk with IC$_{50}$ values ranged from 0.47 to 1.70 mg/ml (Gonzalez-Gonzalez et al., 2011). Amaranth IC$_{50}$ value was 0.6 mg/ml, which is in the range of milk peptides. Peptides YP, LPP, LRP, VPP, and IKP peptides have been detected in amaranth seed protein hydrolyzates, the IKP peptide has been described as one of the most potent inhibitor of ACE activity (Huerta-Ocampo and Barba de la Rosa, 2011).

Interestingly, although cultivated amaranth species presented higher carbohydrates content, the amount of fat was no dramatically decreased (5.7 to 8.1%). We found that the oil composition in amaranth was characterized for the high levels of squalene, reaching values from 2.85 g/100g oil in $A. hypochondriacus$ cv Nutrisol and up to 4.86 g/100g oil in $A. cruentus$ (Table 2). He and Corke (2003) reported values from 1.0% to 7.3% squalene/oil, depending on the amaranth cultivar/specie analysed, while D’Amico and Schoenlechner (2017) reported concentrations from 2.26 to 11.19%.

Amaranth has the ability to modulate cholesterol levels in serum, which is due to its content of squalene (Chaturvedi et al., 1993; D’Amico and Schoenlechner, 2017). The recommended squalene intake (0.25 to 0.5 mg a day) may lower
blood cholesterol levels reducing the risk of atherosclerosis and heart attack (Reddy and Couvreur, 2009).

Palmitic, linoleic, and cis-octadecanoic acids were detected (Supplementary Table S4). The ethyl esters of linoleic and oleic acids as butyl esters of palmitic and stearic acids were detected as minor components. In this regard, it was reported that in carob (Ceratonia siliqua L.) seeds, the most abundant fatty acids were the methyl-esters of oleic acid (C18:1), linoleic acid (C18:2n6), palmitic acid (C16:0), and stearic acid (C18:0) (Gubbuk et al., 2018).

Several clinical studies have shown that a high trans-fatty acid diet causes adverse changes in the plasma lipoprotein profile, with an increase in LDL and a decrease in HDL (Siddhuraju and Becker, 2001). In the present study, no trans-fatty acids such as elaidic and linolelaidic, myristic, behenic, eruci and lignoceric acids were detected.

Conclusions

Based on these results, we propose A. powellii as an interesting option to generate amaranth cultivars with higher protein contents in their grains. A. hybridus showed the highest crude fibre content (6.1%), while A. cruentus and A. hypochondriacus cv Nutrisol had the highest starch content. Wild species and A. hypochondriacus cv Cristalina presented a perisperm with polyhedral well-defined structures and share the presence of a 65 kDa band corresponding to GBSSI; while A. hypochondriacus cvs Nutrisol and Opaca and A. cruentus cv Amaranteca showed a starch with low or no amylose content. The higher
inhibition of DPPIV activity was detected at the highest concentration of peptides (3.2 mg/ml). *A. hypochondriacus* cv Opaca rendered the highest activity reaching until 60% of DPPIV inhibition with an IC$_{50}$ of 1.6 mg/ml. Regarding ACE inhibitory activity, also *A. hypochondriacus* cv Opaca showed the highest activity reaching of 80% inhibition at 3.2 mg/ml with an IC$_{50}$ of 0.6 mg/ml. Lipids in amaranth varied among species and cultivars, squalene highest concentrations were detected in *A. cruentus* followed by *A. hybridus*.

Further efforts are needed in order to improve amaranth phenotyping with special focus on food quality and health-promoting compounds, hence wild species rediscovery will provide more information to support amaranth breeding.

### Acknowledgments

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### References


Figure Legends

Figure 1. Morphological characteristics of amaranth wild and cultivated species

Figure 2. Scanning Electron Microscopy (SEM) micrograph of transversal cuts of amaranth seeds

Figure 3. 1-DE electrophoretic pattern of total proteins extracted from amaranth seed. Lanes: M=molecular weight marker; 1=A. hybridus, 2=A. powellii, 3=A. cruentus cv Amaranteca, 4=A. hypochondriacus cv Opaca (waxy), 5=A. hypochondriacus cv Cristalina (non-waxy), and 6=A. hypochondriacus cv Nutrisol.

Figure 4. Inhibitory activity of amaranth peptides released by gastrointestinal simulated digestion in vitro against A) DPPIV and B) ACE. Peptides bigger than 10 kDa were removed by ultrafiltration and ACE activity was measured at different peptides concentrations.
Table 1
Proximate composition of wild and cultivated amaranth species (%db)

<table>
<thead>
<tr>
<th>Amaranth species</th>
<th>Protein 1</th>
<th>Fat</th>
<th>Crude Fibre</th>
<th>Ash</th>
<th>Carbohydrates 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hybridus</td>
<td>15.9 ±0.1 b</td>
<td>5.9 ±0.0 c</td>
<td>6.1 ±0.0 a</td>
<td>3.7 ±0.1 a</td>
<td>68.5 ±0.2 d</td>
</tr>
<tr>
<td>A. powellii</td>
<td>17.8 ±0.1 a</td>
<td>8.1 ±0.0 a</td>
<td>5.9 ±0.0 b</td>
<td>3.6 ±0.1 a</td>
<td>64.5 ±0.2 e</td>
</tr>
<tr>
<td>A. cruentus</td>
<td>14.8 ±0.4 c</td>
<td>6.9 ±0.0 b</td>
<td>2.5 ±0.0 e</td>
<td>2.8 ±0.0 b</td>
<td>73.0 ±0.5 a</td>
</tr>
<tr>
<td>A. hypochondriacus cv Opaca</td>
<td>16.7 ±0.8 b</td>
<td>6.9 ±0.0 b</td>
<td>3.5 ±0.1 d</td>
<td>3.0 ±0.0 b</td>
<td>69.9 ±0.8 c</td>
</tr>
<tr>
<td>A. hypochondriacus cv Cristalina</td>
<td>16.7 ±0.1 b</td>
<td>5.7 ±0.1 c</td>
<td>3.9 ±0.0 c</td>
<td>2.9 ±0.0 b</td>
<td>70.9 ±0.3 b</td>
</tr>
<tr>
<td>A. hypochondriacus cv Nutrisol</td>
<td>15.8 ±0.1 b</td>
<td>6.9 ±0.1 b</td>
<td>2.4 ±0.0 f</td>
<td>3.5 ±0.2 a</td>
<td>71.4 ±0.1 b</td>
</tr>
</tbody>
</table>

1 N × 5.85; 2 As difference; Mean values of three replicates ± standard deviation; different superscript letters by column indicate statistically significant differences at p < 0.05.
Table 2  
Squalene quantification by GG-MS in wild and cultivated amaranth species

<table>
<thead>
<tr>
<th>Amaranth species</th>
<th>Squalene (g/100g)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in seeds</td>
<td>in oil</td>
<td></td>
</tr>
<tr>
<td>A. hybridus</td>
<td>0.246 ±0.03</td>
<td>4.17 ±0.45</td>
<td></td>
</tr>
<tr>
<td>A. powellii</td>
<td>0.252 ±0.02</td>
<td>3.12 ±0.27</td>
<td></td>
</tr>
<tr>
<td>A. cruentus</td>
<td>0.335 ±0.02</td>
<td>4.86 ±0.31</td>
<td></td>
</tr>
<tr>
<td>A. hypochondriacus cv Opaca</td>
<td>0.271 ±0.01</td>
<td>3.93 ±0.08</td>
<td></td>
</tr>
<tr>
<td>A. hypochondriacus cv Cristalina</td>
<td>0.217 ±0.00</td>
<td>3.80 ±0.05</td>
<td></td>
</tr>
<tr>
<td>A. hypochondriacus cv Nutrisol</td>
<td>0.197 ±0.01</td>
<td>2.85 ±0.12</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± SD of three determinations.
A. hybridus

A. powellii

A. cruentus cv Amaranthea

A. hypochondriacus cv Opaca

A. hypochondriacus cv Cristalina

A. hypochondriacus cv Nutrisol
Figure B: Graph showing the ACE activity (%) against Amaranth peptides (mg/ml) for different species of Amaranthus. The species are
- Amaranthus hypochondriacus Nutrisol
- Amaranthus hypochondriacus Cristalina
- Amaranthus hypochondriacus Opaca
- Amaranthus cruentus
- Amaranthus hybridus
- Amaranthus powellii
Highlights

• *A. powellii*, a wild species, showed the higher protein content among the amaranth species analyzed

• Protein profiles of wild and cultivated amaranth species showed high polymorphism in the high molecular weight region

• *A. hypochondriacus* cv Opaca released peptides inhibited 80% of angiotensin converting enzyme activity

• Squalene content in amaranth oil extract varies from 53-94% depending of the species