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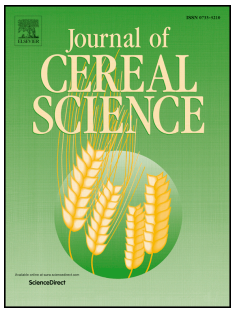

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PII: S0733-5210(18)30399-0

DOI: 10.1016/j.jcs.2018.09.004

Reference: YJCRS 2635

To appear in: *Journal of Cereal Science*

Received Date: 18 May 2018

Revised Date: 4 September 2018

Accepted Date: 6 September 2018

Please cite this article as: Bojórquez-Velázquez, Esaú., Velarde-Salcedo, A.J., De Léon-Rodríguez, A., Jimenez-Islas, H., Pérez-Torres, J.L., Herrera-Estrella, A., Espitia-Rangel, E., Barba de la Rosa, A.P., Morphological, proximal composition, and bioactive compounds characterization of wild and cultivated amaranth (*Amaranthus* spp.) species, *Journal of Cereal Science* (2018), doi: 10.1016/j.jcs.2018.09.004.

Morphological, proximal composition, and bioactive compounds characterization 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

77 gluten-free food market worth almost $1.6 billion in 2011. This resurgent interest
78 is expressed in re-discovering ancestral crops as functional foods, which may
79 offer an important alternative for people affected by celiac disease (Cooper,
80 2015).

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

96 In addition to nutritional characteristics, amaranth plants have attractive
97 agronomic features; they grow where cereals and vegetables cannot such as dry
98 soils, high altitudes, and high temperatures (Huerta-Ocampo and Barba de la
99 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 character by six generations of single seed descendent and were collected from Atzitzintla, Tlaxcala, Mexico.

2.2. Morphological and structural seeds characterization

122 Seed weight was calculated by weighing 100 seeds on an electronic balance
123 DV215CD Discovery (Ohaus, Parsippany, NJ, USA) with 0.01/0.1 mg accuracy.
124 The weight of 100 seeds was extrapolated to 1000 seeds. Seed dimensions
125 (diameter and width) were taken with the SteREO Discovery V8 (Carl-Zeiss,
126 Oberkochen, GE). All measurements were done in triplicates.
127 Images of whole seeds were obtained with the SteREO Discovery V8 (Carl-
128 Zeiss). Paradermal sections were visualized by scanning electron microscopy
129 captured with an ESEM model Quanta 200 (FEI, Hillsboro, OR, USA) from the
130 National Laboratory of Nanosciences and Nanotechnology Research (LINAN)
131 IPICYT.
132
133 *2.3. Amaranth flours proximate composition*
134 Seeds were cleaned and milled in liquid nitrogen using a KRUPS GX4100
135 (Solingen, GE) mill to obtain fine flour. Flour samples were stored in plastic tubes
136 at -80 ^{o}C until analysis. Total nitrogen content was determined by micro-Kjeldahl
137 method (AOAC, 2007, method 12.960.52), and total protein content was
138 calculated using a 5.85 factor. Fat content was determined by Soxhlet method
139 (AOAC, 2007, 996.01 method). Crude fibre and ash contents were obtained
140 according to AOAC (2007) methods 991.43 and 900.02, respectively. All
141 determinations were made at least in triplicates.
142
143 *2.4. Protein extraction and electrophoretic profile of amaranth seeds proteins*

144 Total protein extracts, from the six amaranth species studied, were obtained
145 by mixing 0.1 g flours with 2 ml of a solution containing 7 M urea, 2 M thiourea,
146 2% (w/v) Triton X-100 and 0.05 M DTT. Suspensions were mixed by vortexing for
147 15 min at 4 °C and centrifuged at 17,000×g at 20 °C, supernatants were
148 recovered and protein quantified using the Bradford protein assay (Bio-Rad,
149 Hercules, CA, USA). Each sample was analysed by denaturing polyacrylamide
150 gel electrophoresis (SDS-PAGE) in a discontinuous Tris-glycine system. The
151 stacking and resolving gels were 4% and 13.5%, respectively. Protein (15 μg)
152 was loaded onto the gel and separated in a Mini-Protean III system (Bio-Rad),
153 gel was run at 10 mA/gel for 30 min followed by 25 mA/gel until bromophenol
154 blue reached the bottom of the gel. After electrophoresis, the gels were stained
155 with a 0.05% Coomassie blue R-250 (USB Corporation, Cleveland, OH, USA) in
156 40% methanolic solution containing 10% acetic acid.
157
158 *2.5. In-gel digestion and LC-MS/MS protein identification*
159 Protein bands were manually excised from gel, distained, reduced with 10
160 mM dithiothreitol and alkylated with 55 mM idodoacetamide. Protein digestion
161 was carried out with sequencing-grade trypsin (Promega, Madison, WI, U.S.A.).
162 Tryptic peptides were analyzed with a nanoACQUITY UPLC System (Waters,
163 Milford, MA, U.S.A.) coupled to a SYNAPT-HDMS Q-TOF (Waters) mass
164 spectrometer. MS/MS spectra data sets were used to generate PKL files using
165 Protein Lynx Global Server v2.4 (PLGS, Waters). Proteins were then identified
166 using the MASCOT search engine v2.5 (Matrix Science, London, U.K.).

Searches were conducted against the *Viridiplantae* subset of the NCBInr 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 ^{o}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 ^{o}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 $NaHCO_3$), 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,

190 trypsin and pancreatin). Digestion was stopped by heating the suspensions at 75
191 ^{o}C for 20 min and centrifuged at 13,000xg for 30 min. Peptides were ultra filtrated
192 through 10 kDa filters (Amicon Ultra-10 centrifugal filters, Sigma-Aldrich). The
193 peptides concentration was determined by the Lowry-based DC Protein Assay
194 (Bio-Rad) using BSA as a standard, and then stored at -20 ^{o}C until analysis.
195
196 *2.6.1. Inhibition of dipeptidyl peptidase IV (DPPIV) activity*
197 DPPIV activity was measured using the chromogenic substrate Gly-Pro-
198 pNitroanilide (Sigma-Aldrich) as previously reported (Velarde-Salcedo, et al.,
199 2013). Briefly, 10 µl of 100 ng/ml of dipeptidyl peptidase IV (Sigma-Aldrich) were
200 added to 40 µl amaranth peptides dissolved in 100 mM Tris pH 8 and 50 µl of 1
201 mM Gly-Pro-pNitroanilide dissolved in Tris buffer. Mix was incubated at 37 ºC for
202 1 h. Absorbance was measured at 415 nm in a Multiskan Go plate reader
203 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Results were
204 expressed as µmol of nitroaniline/min based on a p-nitroaniline (Sigma-Aldrich)
205 standard curve.
206
207 *2.6.2. Inhibition of Angiotensin Converting Enzyme (ACE) activity*
208 Peptides with inhibitory activity against ACE were measured by
209 spectrophotometric assay. Briefly 20 µl of sample was added to 0.1 ml of 0.1 M
210 potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM hippuryl–
211 histidyl–leucine (HHL, Sigma). ACE (5 mU) (EC 3.4.15.1, 5.1 U/mg, Sigma) was
212 added and the reaction mixture was incubated at 37 ^{o}C for 30 min. The reaction

213 was terminated by the addition of 0.1 ml of 1 M HCl. The hippuric acid formed
214 was extracted with ethyl acetate, heat-evaporated at 95 ^{o}C for 10 min, dissolved
215 in distilled water and measured spectrophotometrically at 228 nm. The activity of
216 each sample was tested in triplicate. Captopril was used as a positive control.
217 The IC_{50} value was defined as the peptide concentration (mg/ml) needed to
218 inhibit 50% ACE activity; it was calculated by an ACE inhibition (%) vs. log
219 peptide concentration (mg/ml) linear regression.
220
221 *2.7. Amaranth lipid extraction, GC-MS analysis, and squalene quantification*
222 Lipids from amaranth seeds were extracted by mixing 0.1 g of amaranth flour
223 with 1.75 ml of hexane, in constant agitation at room temperature by 5 h.
224 Samples were centrifuged (17,000×g, 20 min, 25 °C) and supernatants
225 transferred into new tubes. Extracts were analysed by GC-MS using a
226 7820A/5977E System (Agilent Technologies, Santa Clara, California, USA), with
227 a HP-5ms capillary column (Agilent Technologies) of 30 m length, 250 µm of
228 inner diameter and 0.25 µm-film thickness. Samples, 3 µl, were injected in
229 splitless mode. The column was held at 80 °C for 1 min after injection, the
230 temperature programmed at 20 °C/min to 210 °C and held for 10 min more, then
231 10 °C/min to 280 °C and held for 35 min. Helium was used as carrier gas, at a
232 constant column flow rate of 1 ml/min. The injector temperature was 250 °C and
233 the detector temperature was 230 °C. The mass spectrometer was operated
234 under Electron Impact Ionization at 70 eV with a mass range from 30-500 amu.
235 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 *v*12.3 (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

281 less protein content (15.8%) in comparison with cvs Opaca and Cristalina
282 (16.7%).
283
284 *3.3. Electrophoretic pattern and protein identification*
285 The amount of protein quantified by Bradford (**Supplementary Figure S1**)
286 correlated with the values obtained by Kjeldahl method (**Table 1**), which indicates
287 that total protein extracted was almost the total protein present in seeds. Total
288 proteins were analysed by SDS-PAGE (**Figure 3**). In all species and cultivars
289 were observed the bands located at 35-37 kDa and 18-20 kDa, which represent
290 the acidic and basic subunits of the canonical 11S globulins. The most
291 remarkable differences among species and cultivars analysed were observed in
292 the range of 50 to 70 kDa. Both wild species as well as *A. hypochondriacus* cv
293 Cristalina have a band around 65 kDa. *A. powellii* and *A. cruentus* share a band
294 of 60 kDa. *A. hybridus* and all *A. hypochondriacus* cultivars showed a 55 kDa
295 band. These three bands were cut from gel, analysed by LC-MS/MS and
296 identified as a Granule Bound Starch Synthase I or GBSSI (**Supplementary**
297 **Table S3 and Supplementary Figure S2**).
298
299 *3.4. Amaranth peptides with inhibitory activity against DPPIV and ACE*
300 A simulated gastrointestinal digestion *in vitro* method was used to release the
301 encrypted peptides from all amaranth samples. The capacity of released
302 amaranth peptides to inhibit both DPPIV and ACE enzymes was measured.
303 DDPIV inhibition increased in a dose-response relationship (**Figure 4A),** higher

304 inhibition activity was detected at the highest tested concentration (3.2 mg/ml). At
305 this concentration, *A. hypochondriacus* cv Opaca rendered the highest inhibitory
306 activity reaching a 60% of DPPIV with an IC_{50} of 1.6 mg/ml. *A. hypochondriacus*
307 cv Cristalina and *A. powellii* showed the least DPPIV inhibition reaching only 40%
308 at the highest tested peptide concentration (3.2 mg/ml). A similar ACE inhibitory
309 activity profile was observed (**Figure 4B**). *A. hypochondriacus* cv Opaca peptides
310 presented the highest activity reaching of 80% inhibition at 3.2 mg/ml with an IC_{50}
311 of 0.6 mg/ml. *A. hypochondriacus* cv Nutrisol, *A. hybridus* and *A. cruentus*
312 showed an IC_{50} of 1.5 mg/ml while *A. hypochondriacus* cv Cristalina and *A.*
313 *powellii* the IC_{50} was of 2.5 mg/ml.
314
315 *3.5. Characterization of lipids in amaranth seeds*
316 The lipid composition analysed by GC-MS showed the presence of palmitic
317 and linoleic acids in all species. Linoleic ethyl esters was present only in *A.*
318 *hybridus* and *A. hypochondriacus* cvs Cristalina and Nutrisol, while oleic acid
319 ethyl ester was only present in *A. hypochondriacus* cvs Cristalina and Nutrisol.
320 Butyl ester of palmitic and stearic acid were detected in all samples analysed.
321 Stigmasterol, an important phytosterol, was detected in higher abundance in the
322 wild species *A. powellii*, followed by *A. hypochondriacus* cvs Cristalina*, and A.*
323 *hybridus* (**Supplementary Table S4**). Squalene, an unsaturated hydrocarbon,
324 was detected in all samples but interestingly the highest abundance was
325 detected in *A. cruentus* (**Supplementary Table S4 and Supplementary Figure**
326 **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. powellii* 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 α- and γ-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 μm), which gives functional characteristics of great interest in food applications (Kong et al., 2009). Carbohydrates were very

419 important during amaranth domestication that is thought to have occurred during
420 the prehispanic times. Aztecs used sticky grain amaranths to make cakes as part
421 of religious ceremonies (Sauer, 1967). The sticky grain selection was the origin
422 of so called waxy varieties of cereals and other starch-producing crops (Hunt et
423 al., 2010). Amaranth waxy types were selected and nowadays are the cultivated
424 species (*A. cruentus*, *A. hypochondriacus*). Sticky starch type is characterized by
425 very low content of amylose, a character that modifies the glycaemic index. Waxy
426 starch types generally have a higher glycaemic loads, its consumption is related
427 with a better physical performance and quicker recovery associated with intense
428 physical activity and with the reloading of glycogen storages after exercise
429 (Wright, 2005). Therefore, higher-glycaemic cereal grains, which may have been
430 an advantage and better tasting, treat in past cultures, today may impose a
431 disadvantage for modern civilization where exercise and physical activity has
432 decreased and glycaemic loads are related with type-2 diabetes risk (WHO,
433 2016). So non-waxy varieties should be reconsidered for the generation of new
434 amaranth cultivars and wild species are an important source of non-waxy
435 starches and high protein contents.

436 The waxy and non-waxy amaranth types are related with the morphological
437 observation, the wild species showed a well-defined polyhedral perisperm
438 (**Figure 2**), but also with the differential accumulation of proteins (**Figure 3**). The
439 differentially accumulated proteins were identified as GBSSI (**Supplementary**
440 **Table S3 and Supplementary Figure S2**), enzyme responsible for amylose
441 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 insulinotropic 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

488 blood cholesterol levels reducing the risk of atherosclerosis and heart attack
489 (Reddy and Couvreur, 2009).

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

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

501

502 **Conclusions**

503 Based on these results, we propose *A. powellii* as an interesting option to
504 generate amaranth cultivars with higher protein contents in their grains. *A.*
505 *hybridus* showed the highest crude fibre content (6.1%), while *A. cruentus* and *A.*
506 *hypochondriacus* cv Nutrisol had the highest starch content. Wild species and *A.*
507 *hypochondriacus* cv Cristalina presented a perisperm with polyhedral well-
508 defined structures and share the presence of a 65 kDa band corresponding to
509 GBSSI; while *A. hypochondriacus* cvs Nutrisol and Opaca and *A. cruentus* cv
510 Amaranteca showed a starch with low or no amylose content. The higher

511 inhibition of DPPIV activity was detected at the highest concentration of peptides
512 (3.2 mg/ml). *A. hypochondriacus* cv Opaca rendered the highest activity reaching
513 until 60% of DPPIV inhibition with an IC_{50} of 1.6 mg/ml. Regarding ACE inhibitory
514 activity, also *A. hypochondriacus* cv Opaca showed the highest activity reaching
515 of 80% inhibition at 3.2 mg/ml with an IC_{50} of 0.6 mg/ml. Lipids in amaranth
516 varied among species and cultivars, squalene highest concentrations were
517 detected in *A. cruentus* followed by *A. hybridus*.

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

521

522 **Acknowledgments**

523 EBV thanks to CONACYT fellowship 298096 and we thank to CONACYT-
524 Problemas Nacionales “Amaranto en la Soberania Alimentaria Grant No.
525 248415”. JLPT thanks to support from CONACYT. Thanks to Dr. O.A. Patrón-
526 Soberano for her technical assistance in microscopic techniques and Dr. V.E.
527 Balderas-Hernández for his assistance with GC-MS.

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634 **Figure Legends**

635 **Figure 1**. Morphological characteristics of amaranth wild and cultivated species

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

638 **Figure 3**. 1-DE electrophoretic pattern of total proteins extracted from amaranth
639 seed. Lanes: M=molecular weight marker; 1=*A. hybridus*, 2=*A. powellii*,
640 3=*A. cruentus* cv Amaranteca, 4=*A. hypochondriacus* cv Opaca (waxy),
641 5=*A. hypochondriacus* cv Cristalina (non-waxy), and 6=*A.*
642 *hypochondriacus* cv Nutrisol.

643 **Figure 4**. Inhibitory activity of amaranth peptides released by gastrointestinal
644 simulated digestion *in vitro* against A) DPPIV and B) ACE. Peptides
645 bigger than 10 kDa were removed by ultrafiltration and ACE activity was
646 measured at different peptides concentrations.

647

Table 1
Proximate composition of wild and cultivated amaranth species (%db)

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

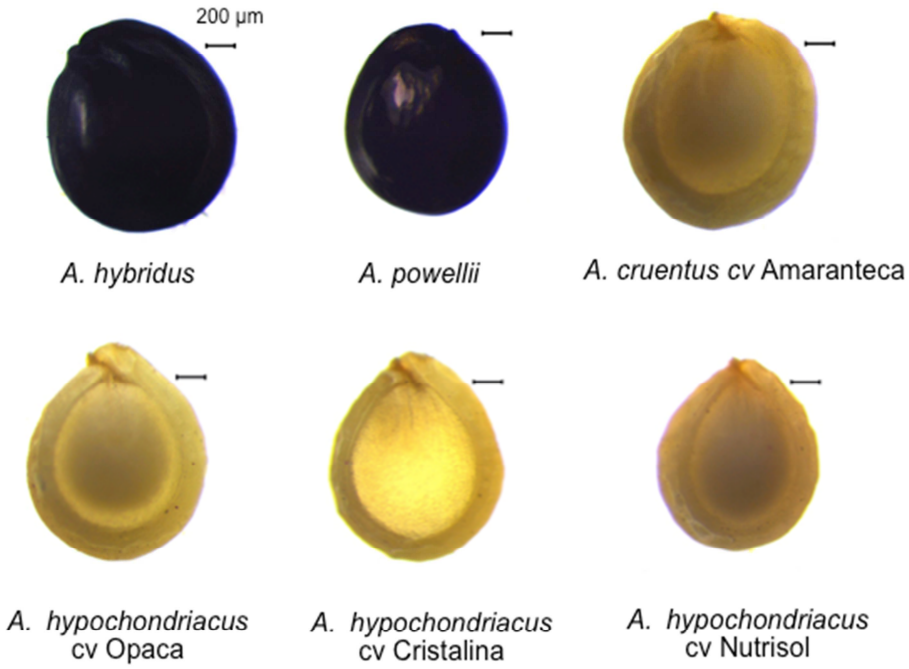
[1]N×5.85; [2]As difference; Mean values of three replicates ± standard deviation; different superscript letters by column indicate statistically significant differences at $p<0.05$.

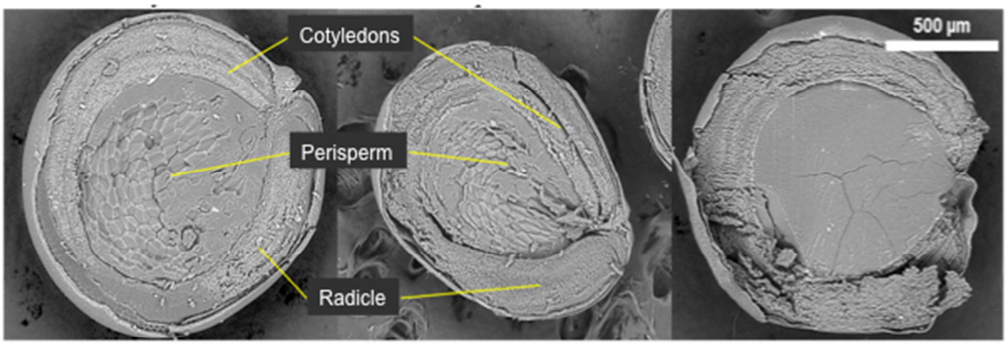
659 **Table 2**
660 Squalene quantification by GG-MS in wild and cultivated amaranth species

Amaranth species	Squalene (g/100g)	
	in seeds	**in oil**
A. hybridus	0.246 ±0.03^{b}	4.17 ±0.45^{b}
A. powellii	0.252 ±0.02^{b}	3.12 ±0.27^{c}
A. cruentus	0.335 ±0.02^{a}	4.86 ±0.31^{a}
A. hypochondriacus cv Opaca	0.271 ±0.01^{b}	3.93 ±0.08^{b}
A. hypochondriacus cv Cristalina	0.217 ±0.00^{c}	3.80 ±0.05^{b}
A. hypochondriacus cv Nutrisol	0.197 ±0.01^{d}	2.85 ±0.12^{c}

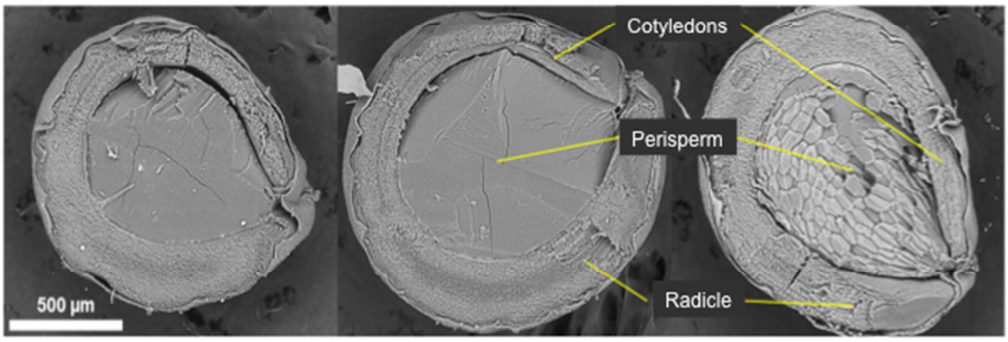
Values are the mean ± SD of three determinations.

661

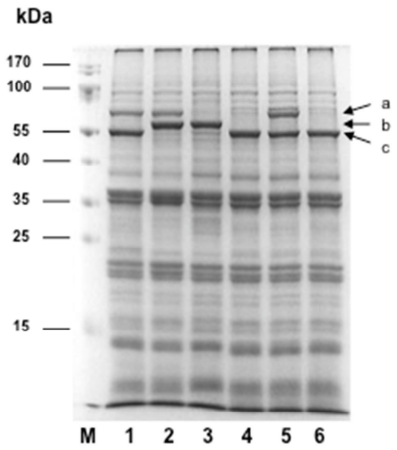




A. hybridus *A. powellii* *A. cruentus cv* Amaranteca



A. hypochondriacus cv Nutrisol *A. hypochondriacus* cv Opaca *A. hypochondriacus* cv Cristalina



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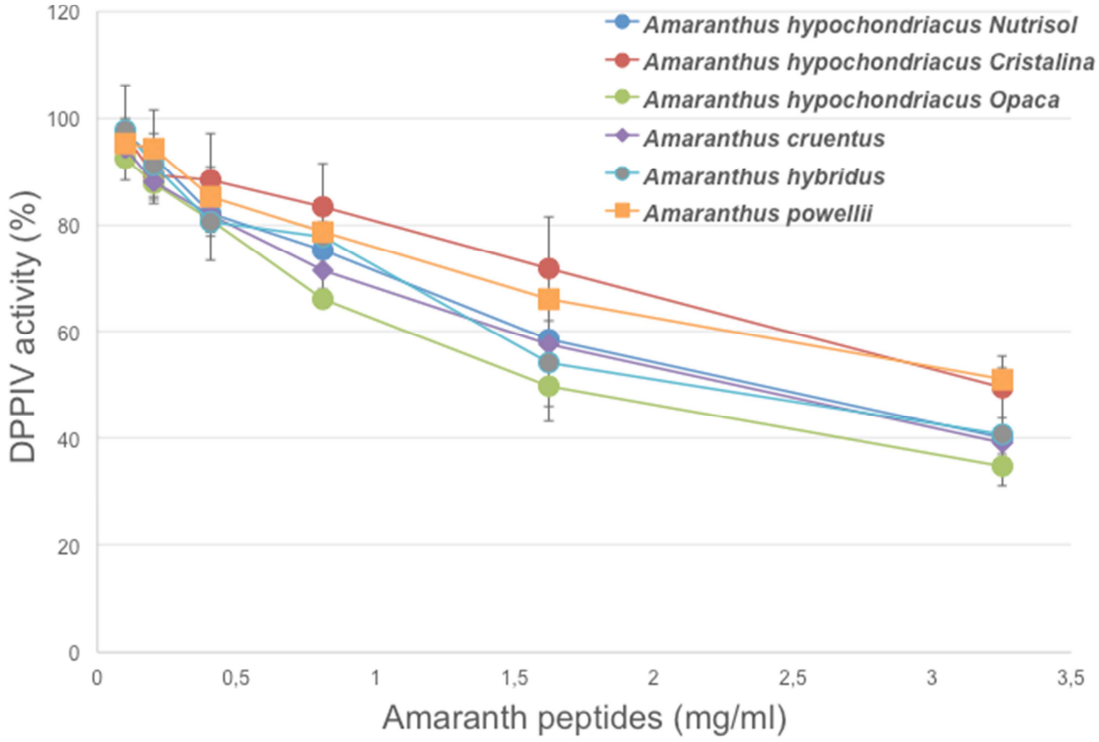

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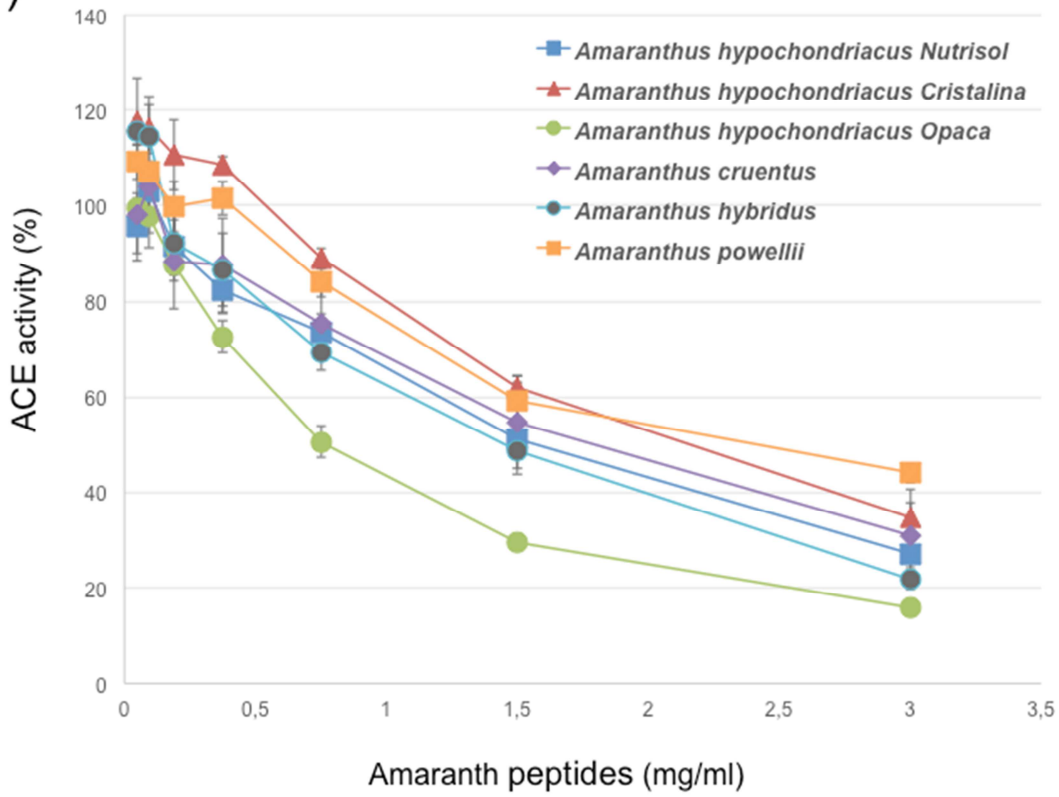

Highlights

- *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