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Anti-inflammatory and antioxidant effects of peptides released from germinated amaranth during *in vitro* simulated gastrointestinal digestion

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ABSTRACT

Amaranth (*Amaranthus hypochondriacus*) is an ancestral nutritional grain and good source of bioactive compounds as peptides. In this study, the effect of *in vitro* simulated gastrointestinal digestion (SGD) of germinated amaranth on the release of antioxidant and anti-inflammatory peptides was evaluated. The germinated amaranth peptides generated during SGD were released after 90 min of incubation with pancreatin and fractioned to F1 (> 10 kDa), F2 (3–10 kDa), and F3 (< 3 kDa). Among germinated amaranth peptides fractions tested, F2 had the highest antioxidant activity, while F1 and F2 exhibited a high anti-inflammatory response caused by lipopolysaccharide-induced in RAW 264.7 macrophages. A total of 11 peptides sequences were identified in the fractions evaluated, and they exhibit potential biological activity against non-communicable diseases. The findings from this study showed first time report on bioactive peptides, especially anti-inflammatory, from germinated amaranth released by *in vitro* gastrointestinal digestion.

1. Introduction

Amaranth (*Amaranthus hypochondriacus*) is an ancestral grain from Mexico, which has regained relevance owing to its nutritional value. Besides, it is known and verified gluten free ingredient, increasing the interest of its characterization and food application. This pseudocereal is a high source of lipids, dietary fiber, vitamins, antioxidants, and protein with excellent quality due to the balance of essential amino acids (Chauhan, Saxena, & Singh, 2015; Reyes-Moreno, Cuevas-Rodríguez, & Reyes-Fernández, 2019; Taniya, Reshma, Shanimol, Gayatri, & Priya, 2020). Bioactive compounds, such as flavonoids, phenolic acids, anthocyanins, tannins, and phytosterols, are present in the amaranth grain (Martinez-Lopez, Millan-Linares, Rodriguez-Martin, Millan, & Montserrat-de la Paz, 2020). These bioactive compounds have been associated with health benefits in the control and or prevention of chronic non-communicable diseases such as cancer, cardiovascular diseases, diabetes, and hypercholesterolemia (Caselato-Sousa & Amaya-Farfan, 2012; Martinez-Lopez et al., 2020).

Germination is an affordable and efficient technology for processing amaranth that improved the bioactivities and bioavailability of phytochemical composites aside from developing the bioactive peptides, consequently, conducting functional food by enhanced health valuable properties (Aphalo, Martínez, & Añón, 2015; Chauhan et al., 2015; Ozuna et al., 2018; Sandoval-Sicairos et al., 2020).

Bioactive peptides from amaranth have been associated with various biological activities such as antioxidant antihypertensive, anti-atherosclerotic, and anti-inflammatory (Barba de la Rosa et al., 2010; Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014; Montoya-Rodríguez, Milán-Carrillo, Dia, Reyes-Moreno, & González de Mejía, 2014; Moronta, Smaldini, Docena, & Añón, 2016; Orsini-Delgado et al., 2016; Tironi & Añón, 2010). Amaranth peptides are mostly derived from the enzymatic hydrolysis, prepared throughout digestive enzymes such as pepsin, pancreatin, and alcalase, has been the preferred tool for obtaining unprocessed amaranth proteins hydrolysates with specific bioactivities (Moronta et al., 2016; Orsini-Delgado et al., 2016; Silva-Sánchez et al., 2008). Although some studies were carried out using processed amaranth protein by extrusion cooking, which generate better anti-inflammatory effect compared to

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unprocessed amaranth hydrolysates. However, the antioxidant capacity is higher in unprocessed hydrolysates (Montoya-Rodríguez, de Mejía et al., 2014). Contrarily, the antioxidant capacity in germinated amaranth hydrolysates are improved compared to unprocessed amaranth hydrolysates, this effect is may be related to the release of phenolic compounds and peptides by both germination process and enzymatic hydrolysis (Sandoval-Sicairos et al., 2020). Moreover, other researchers have been assayed the liberation of anti-inflammatory peptides by enzymatic hydrolysis in germinated seeds, as chickpea, soybean, Brazilian soybean and black beans (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018; López-Barrios, Antunes-Ricardo, & Gutiérrez-Uribe, 2016; Milán-Noris, Gutiérrez-Uribe, Santacruz, Serna-Saldívar, & Martínez-Villaluenga, 2018; Vernaza, Dia, Gonzalez de Mejia, & Chang, 2012).

During the inflammation progression, nitric oxide (NO) produced in large amounts by the action of the enzyme inducible nitric oxide synthase (iNOS), which in turn can contribute to several acute and chronic diseases; consequently, there is a numerous relevance to generate food ingredients that control oxidative and inflammatory actions (Majumder, Mine, & Wu, 2016). We hypothesized that germination and enzymatic hydrolysis of amaranth would produce bioactive peptides with antioxidant and anti-inflammatory action. However, there are no previous reports about the anti-inflammatory activity of bioactive compounds as peptides in germinated amaranth after enzymatic hydrolysis. Hence, this study aimed to determine the antioxidant and anti-inflammatory actions of peptides released from germinated amaranth by *in vitro* simulated gastrointestinal digestion.

2. Materials and methods

2.1. Chemicals and reagents

Murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) and penicillin/streptomycin (10,000 U/mL) were acquired from Thermo Fisher Scientific (Grand Island, NY, USA). Fetal bovine serum was obtained from biowest (Mexico origin). Cell Titer 96® Aqueous One Solution Proliferation Assay kit was supplied from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Materials

Amaranth grain (*Amaranthus hypochondriacus*) was grown and harvested in 2017 in Temoac, Morelos, Mexico. The grains were cleaned and stored in containers under refrigeration (4 °C) until analysis.

2.3. Amaranth germination

The germinated amaranth flour (GAF) was obtained, as previously reported by Sandoval-Sicairos et al. (2020). Amaranth seeds were germinated at 30 °C in light/dark photoperiod (12/12 h) for 78 h at 80% of relative humidity. The amaranth sprouts were freeze-dried. The germinated material was milled (UD Cyclone Sample Mill, UD Corp, Boulder, CO, USA) until passing through 80 mesh (0.180 mm), the GAF was packed in polyethylene bags and kept at 4 °C until analysis.

2.4. Simulated gastrointestinal digestion of germinated amaranth

The *in vitro* simulated gastrointestinal digestion (SGD) of GAF was performed following the procedure by Montoya-Rodríguez, de Mejía et al. (2014). Briefly, GAF was suspended in water (1.10 w/v), and the sequential enzyme hydrolysis was carried out with pepsin (enzyme/substrate ratio, 1:20; pH 2.0) and incubated at 37 °C for 180 min. Subsequently, pancreatin (enzyme/substrate ratio, 1:20; pH 7.5) was added, and the reaction mixture was incubated for at 37 °C for 90 min. The germinated amaranth gastrointestinal digestion (GAD) aliquots (30 mL) were collected at 10, 25, 60, and 90 min during pancreatin hydrolysis. The digestion was terminated by heating at 75 °C for 20 min. The digests of all samples were centrifuged at 20,000*g* 4 °C for 15 min; the supernatants were freeze-dried and kept at −20 °C until analysis.

2.5. Ultrafiltration fractionation of GAD digests

The GAD at 90 min was washed twice with 80% aqueous methanol solution as previously reported by Milán-Noris et al. (2018), in order to eliminate other phytochemicals in the digest, obtaining the germinated amaranth gastrointestinal digest washed (GADW), then this was fractionated by ultrafiltration using a 10 and 3 kDa cut-off hydrophilic membranes (Millipore, Darmstadt, Germany), three peptides fractions (F1: > 10 kDa, F2: 3–10 kDa and F3: < 3 kDa) were obtained. All fractions were freeze-dried and stored at −20 °C until analysis.

2.6. Soluble protein quantification

The total soluble protein was quantified by the Protein DC assay Protocol (Bio-Rad, Hercules, CA, USA). The amounts at soluble protein were calculated using bovine serum albumin, a standard curve.

2.7. SDS-PAGE of amaranth hydrolysates

SDS-PAGE electrophoresis of amaranth protein samples was analyzed using a Mini-Protean Tetra Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). Gels consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). Samples were diluted with Laemmli Sample Buffer and loaded onto gels. The electrophoresis was carried out at 110 V for 120 min in the tris-glycine buffer. The gels were then removed and stained overnight with Coomassie blue, and subsequently washed with a washing solution for 2 h. The gel image was obtained using a Gel Doc™ XR + Gel Documentation System of Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.8. Total phenolic compounds and antioxidant capacity

Total phenolics of amaranth digests and fractions were determined using the Folin-Ciocalteu colorimetric method, as described by Singleton, Orthofer, and Lamuela-Raventos (1999). A calibration curve was prepared using Gallic acid as standard prepared similarly as the extracts. All results were expressed as milligram of gallic acid equivalents per 100 g of the dry weight basis (mg GAE/100 g sample). The antioxidant capacity (AOC) of the amaranth digests and fractions were evaluated using the oxygen radical absorption capacity (ORAC) method, according to Ou, Hampsch-Woodill, and Prior (2001). The amaranth digest and fractions (dissolved in PBS) were used for antioxidant capacity, using fluorescein (substrate) and Trolox (standard). The results were expressed as millimoles of Trolox Equivalents (mmolTE)/100 g of sample.

2.9. Anti-inflammatory activity

2.9.1. Macrophage cell culture

The anti-inflammatory activity from amaranth digests was examined through determining nitric oxide (NO) production using an inflammation-activated RAW 264.7 cell system. Murine macrophage RAW 264.7 cell line was cultured in DMEM growth medium supplemented with 10% FBS and 1% penicillin/streptomycin. RAW 264.7 cells were plated at densities 1×10^6 cells in 75 cm^2 tissue culture flasks to grow to confluence overnight in a humidified incubator at 37 °C and 5% CO_2 atmosphere.

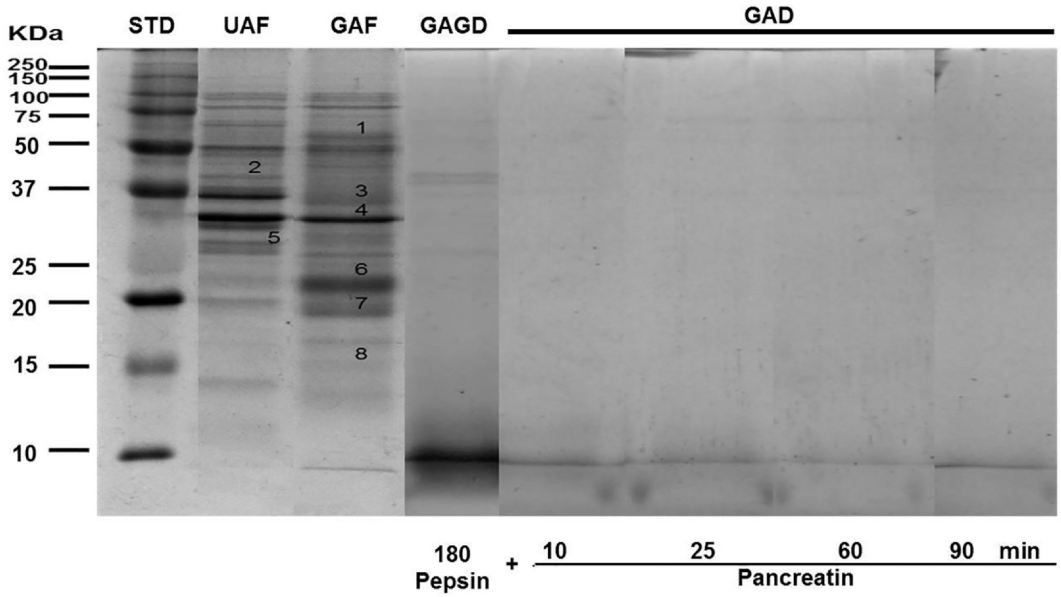

Fig. 1. SDS-PAGE electrophoresis protein profile of amaranth flours at different digestion times. 1: Globulin 11S; 2: Glutelin; 3: Amaranth albumin 1; 4: Globulin 7S; 5: Albumin; 6: Prosystemin; 7: RING Zinc finger protein; 8: Superoxide dismutase [Cu-Zn]. STD: standard. UAF: unprocessed amaranth flour. GAF: germinated amaranth flour. GAGD: germinated amaranth flour gastric digest. GAD: germinated amaranth flour gastrointestinal digest.

2.9.2. Cell viability assay

All amaranth samples were assayed for decreases in cell viability. Cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and allowed to grow to confluence overnight in a humidified incubator at 37 °C and 5% CO_2 atmosphere. Cells were exposed for 16 h to amaranth digest (0.25 to 3 mg/mL) and peptide fractions (0.25 to 1 mg/mL) dissolved in serum-free medium. After treatment, the medium was removed, and cell viability was determined using the Cell Titer 96® Aqueous One Solution Proliferation Assay kit. Briefly, 20 µL of Cell Titer 96® solution was added, followed by 100 µL of serum-free DMEM. After 45 min of incubation, absorbance was read at 490 nm in a Synergy MX microplate reader (BioTek Instruments, Winooski, VT, USA). The viability was calculated considering controls (non-treated cells) as 100% viable. All experiments were performed in three independent trials with three replicates per trial.

2.9.3. Nitric oxide (NO) quantification in macrophages culture medium

All amaranth samples were assayed for potential anti-inflammatory activity. Macrophages were seeded in 96-well plates at a density of 5×10^4 cells/well and allowed to grow to confluence overnight in a humidified incubator at 37 °C and 5% CO_2 atmosphere. The cells were pre-treated for 24 h with amaranth digest (0.25 to 3 mg/mL), and peptide fractions (0.25 to 1 mg/mL) dissolved in serum-free medium, then elicited with polysaccharide (LPS, from Escherichia coli B5:O55) at 1 µg/mL for an additional 24 h. After LPS elicitation, anti-inflammatory activity was investigated through the determination of inhibition of NO production. Nitrite accumulation, an indicator of NO synthesis, was measured in the macrophages culture medium by the Griess reaction according to a previously described method (Martinez-Villaluenga, Dia, Berhow, Bringe, & Gonzalez de Mejia, 2009). Briefly, 100 µL of medium were plated in 96-well plate and an equal amount of the Griess reagent constituted of 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-(naphthyl) ethylenediamine-diHCl in 2.5% (v/v) H_3PO_4, was added. The plate was incubated for 15 min and the absorbance measured at 550 nm in a Synergy MX microplate reader (BioTek Instruments, Winooski, VT, USA). The amount of NO was calculated using a sodium nitrite standard curve (0–10 µg/mL). All experiments were performed in three independent trials with three replicates per trial.

2.10. Peptides characterization

Although the GADW 90 was fractioned in F1: > 10 kDa, F2: 3–10 kDa and F3: < 3 kDa, the identification of amaranth peptides sequences was carried out up to 2 KDa. The fractions were analyzed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) using a Q-ToF Ultima mass spectrometer (Waters, Milford, MA, USA). A gradient system was used (A: 95% water, 5% of acetonitrile, and 0.1% of formic acid; B: 95% of acetonitrile, 5% of water, and 0.1% of formic acid). The volume of injection was 10 µL, and the samples were passed through a PDA detector wavelength at 280 nm. The results were analyzed in PepSeqTN de novo software (Waters Corp., Milford, MA, USA), and the sequence of amino acids was identified based on the accurate mass measurements and tandem MS fragmentation. The identification of bioactive peptides sequences was conducted using the peptide database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). Also, the PepDraw tool was used to get the physicochemical properties and peptides sequences structures.

2.11. Statistical analysis

Data represent the mean and standard deviation of three replicates, unless other specified. Data were subjected by two-way or one-way analysis of variance (ANOVA) to compared experimental values using JMP 14 software from SAS Institute (Cary, NC, USA). A comparison between means was performed using Tukeý́s test, and differences were considered significant at p-value < 0.05.

3. Results and discussion

3.1. Effect of gastrointestinal digestion on the amaranth proteins profile

The germination process modified the protein profile of germinated amaranth flour (GAF) concerning unprocessed amaranth flour (UAF) as previously described (Sandoval-Sicairos et al., 2020). At the end of gastric digestion with pepsin (GAGD 180), the disappearance of most of the high molecular weight bands is observed, however, bands between 28 and 40 kDa can still be observed, corresponding to albumin and glutelins proteins, respectively. After 10 min of gastrointestinal digestion with pancreatin (+180 min with pepsin) (GAD 10) and until the

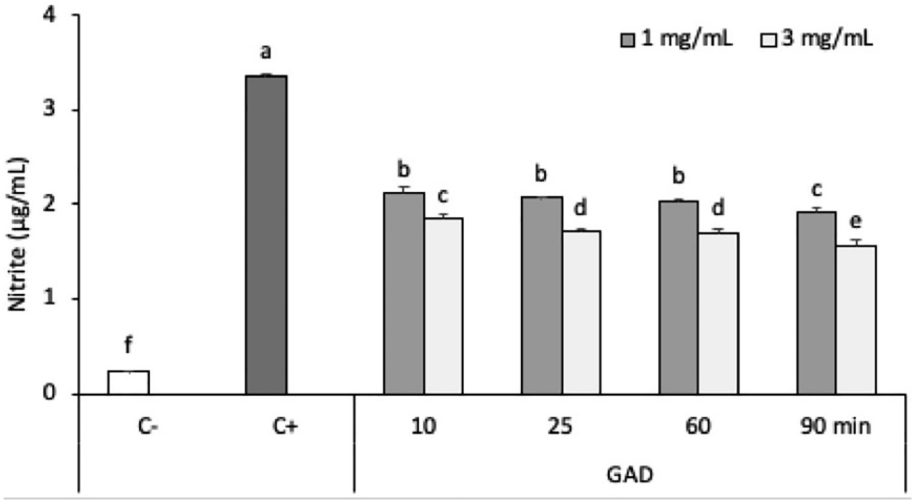

Fig. 2. *In vitro* anti-inflammatory activity of germinated amaranth flour gastrointestinal digest (GAD) with different digestion times (10 to 90 min). Raw 264.7 macrophages were treated with samples and inflammatory response was induced with 1 µg/mL LPS for 24 h. C−: negative control. C+: positive control. Values are means ± standard deviation. Means with different letter are significantly different by Doses × Time of hydrolysis ($p < 0.05$).

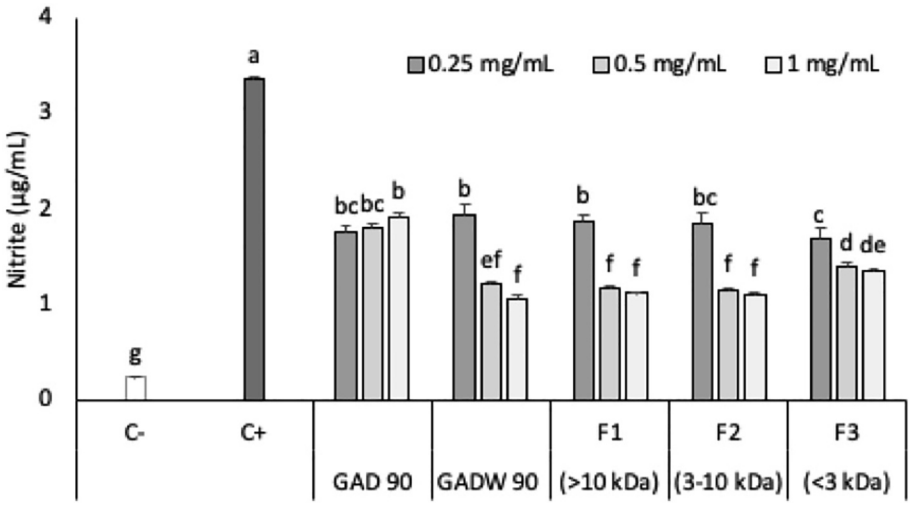

Fig. 3. *In vitro* anti-inflammatory activity of peptides from GAD90 min and its fractions. Raw 267.4 macrophages were treated with samples and inflammatory response was induced with 1 µg/mL LPS for 24 h. GAD: germinated amaranth flour gastrointestinal digest. GADW: germinated amaranth flour gastrointestinal digest washed. C−: negative control. C+: positive control. Values are means ± standard deviation. Means with different letter are significantly different by Doses × Treatment ($p < 0.05$).

end of the digestion time (GAD90), the high molecular weight bands disappear, and molecular weight bands smaller than 10 kDa appear (Fig. 1), which indicates that the flour underwent complete hydrolysis of the proteins releasing bioactive peptides of size < 10 kDa; small peptides according to previous studies, present biological activities as antioxidants and anti-inflammatory (Ruiz Ruiz, Segura Campos, Betancur Ancona, & Chel Guerrero, 2013; Montoya-Rodríguez, de Mejía et al., 2014; González-Montoya et al., 2018).

3.2. Effect of GAD and its peptide fractions on the inflammatory response.

During the inflammation process, large amounts of nitric oxide (NO) at µM levels are produced by the action of the enzyme inducible nitric oxide synthase (iNOS), which in turn can cause tissue damage at the site of inflammation, organs dysfunction and tumorigenesis related to the inflammation process (Conforti & Menichini, 2011). The effect of GAD at different digestion times on NO production was determined by measuring the level of nitrite accumulation in LPS-induced RAW 264.7 macrophage cells (Fig. 2). The statistical data using two-way ANOVA is given in a Supplementary Table 1. Therefore, NO production is determined as a biomarker of inflammation response. An exploration of the cytotoxicity of GAD was assayed in the highest concentrations (1 or 3 mg/mL); the samples were considered not cytotoxic since they not reduced cell viability below 85% compared with test control (data not shown).

The LPS stimulation on macrophage increased significantly ($p < 0.05$) NO production from 0.24 (C−) to 3.35 µg/mL (C+). The production of NO in macrophages treated with 1 and 3 mg/mL of GAD had significant and diminutions in the earlier stage of SGD (10 to 60 min) compared to C+, followed by the biggest significant ($p < 0.05$) decrease up to the lowest values (1.92 and 1.57 µg/mL) at 90 min, respectively. These values were 42.0 and 53.0% lower compared to C+. The germinated flour gastrointestinal digest at 90 min (GAD90) showed higher anti-inflammatory activity compared with its counterparts GAD (10 to 60 min) digests. Similar to previously reported, this sample also showed a higher antioxidant capacity with a positive correlation with soluble protein content (Sandoval-Sicairos et al., 2020). Consequently, it could be inferring that GAD90 contains a higher quantity of peptides than their counterparts (Fig. 1). Nevertheless, during digestion, phenolics compounds were also released (Sandoval-Sicairos et al., 2020), so the biological effects could be a synergy among phenolics and peptides.

In order to only observe the biological activity of amaranth peptides, we performed a solvent extraction in GAD90 to attain peptides free of phenolic compounds; after that, the peptide sample was fractionated by ultrafiltration method with membranes (10 and 3 kDa) to obtain a total of three amaranth peptide fractions F1 (> 10 kDa), F2 (3–10 kDa), and F3 (< 3 kDa). As can be seen from Fig. 3, treatments in LPS-stimulated macrophages with the amaranth peptide fractions at concentrations (0.25–1.0 mg/mL) all led to a significant diminution of NO when compared to the positive control. The F values of two-way ANOVA is depicted in a Supplementary Table 1. Besides, among

amaranth peptide fractions tested, fractions F1 and F2 showed significantly ($p < 0.05$) stronger reduced the NO production, especially treatments of macrophages with dose (0.5 and 1.0 mg/mL) than F3. However, there was not significantly ($p > 0.05$) difference between F1 and F2. Both fractions, F1 and F2 at 0.5 mg/mL decrease NO production with 65.2 and 66.6%, respectively, while at 1.0 mg/mL both peptides fractions reduction NO production with 65.5 and 67.1%, respectively. These results indicate that the peptides in amaranth have better anti-inflammatory effect than its synergy with phenolics compounds. Montoya-Rodríguez and de Mejía (2015) reported that pure peptides from extruded amaranth flour had better biological activity compared to other studies where those peptides were in a synergic environment with other compounds. Correspondingly, pepsin/pancreatin digests from amaranth, soybean, and chickpea grains reduce the inflammatory response on LPS-stimulated human and mouse macrophages (Montoya-Rodríguez, de Mejía et al., 2014; González-Montoya et al., 2018; Milán-Noris et al., 2018). The detected decrease in the production of NO may be described by the capability of amaranth peptides formed during hydrolysis to impede the expression of enzymes reliable for their synthesis (Dia, Bringe, & de Mejia, 2014). Overall, it was observed that amaranth peptides fractions showed a potential NO production inhibition in LPS-induced RAW 264.7 macrophages, which makes germinated amaranth a potential source of anti-inflammatory peptides released throughout gastrointestinal digestion.

3.3. Effect of GAD fractionation on total phenolic compounds, soluble protein, and antioxidant capacity

Digests were assessing for the total phenolics content (TPC), soluble protein (SP), and antioxidant capacity (AOC) of peptides liberated during SGD (Table 1). The statistical data using one-way ANOVA is given in a Supplementary Table 1. As expected, the TPC was not detected in GADW 90 neither in all amaranth peptide fractions, the TPC in amaranth samples were only detected in GAD90 (374.23 mg GAE/100 g), which indicates that methanolic solution effectively removed to traces the TPC in the consequent samples.

The concentration of SP of peptide fractions varied from 70.34 to 129.34 mg/g. There was not significant difference in SP content between GAD90 and F2 of amaranth samples. However, a significant amount of SP remained at the end of SGD in F1 (63.7%), and F3 (53.8%) digests compared with GAD90. The increase on SP content is associated to small peptides generated during the digestion process (Montoya-Rodríguez, de Mejía et al., 2014), and as it can be observed in Table 2, the average of molecular mass between fraction is smaller in F2 (687.98 Da), followed by F1 and F3 (712.60 Da and 721.83 Da, respectively).

Moreover, the GAD90 showed the highest AOC compared with the other amaranth samples that could be due to synergistic effect among phenolics and peptides, compared to the washed sample and their fractions (Table 1). Regarding the amaranth peptide fractions, F2 had the highest ORAC value with 33.6 mmol TE/100 g, followed by F3 and F1 fractions, with ORAC values of 14.4 and 13.6 mmol TE/100 g, respectively. Interestingly, another finding of the present study was that amaranth peptide fractions F1 and F2, which had the smaller peptides (Table 2), showed the highest peroxyl radical scavenging capacity, which meant 64.9% of the activity shown by the GAD90. This result suggests that smaller peptides of amaranth released by SGD could be the cause of the antioxidative activity seen in this study. Similarly, Vilcacundo, Martínez-Villaluenga, Miralles, and Hernández-Ledesma (2019) also reported that the antioxidant activity of amaranth peptides depends on their molecular weight, as well as on other aspects such as their amino acid composition, structure, and hydrophobic character, which determines their mechanism and efficiency.

Table 1
Total phenolic compounds, soluble protein, and antioxidant capacity on germinated amaranth flour gastrointestinal digest (GAD) and its fractions.

Sample	TPC (mg GAE/100 g)	SP (mg/g)	AOC (mmol TE/100 g)
GAD 90	374.23 ± 28.13	130.78 ± 4.57^{a}	74.00 ± 0.69^{a}
GADW 90	< LOQ	60.22 ± 5.28^{c}	13.60 ± 0.69^{d}
F_1 (> 10 kDa)	< LOQ	83.28 ± 4.17^{b}	16.26 ± 0.92^{c}
F_2 (3–10 kDa)	< LOQ	129.34 ± 7.31^{a}	33.60 ± 0.80^{b}
F_3 (< 3 kDa)	< LOQ	70.34 ± 0.64^{bc}	14.40 ± 0.40 ^{cd}

TPC: Total phenolic compounds. SP: soluble protein. AOC: antioxidant capacity. GAE: gallic acid equivalents. TE: Trolox equivalents. < LOQ: limit of quantification less than 5 μg/mL. GADW: germinated amaranth flour gastrointestinal digest washed. Values are means ± standard. Means with different letter are significantly different ($p > 0.05$).

3.4. Identification of potential bioactive peptides

The peptide profile in amaranth fractions was examined by LC-MS/MS method; all of the peptides identified were smaller than 1.2 KDa. In our study, 11 peptides were identified, which four were present in F1, three in F2 and four in F3. The fractions size (F1: > 10 kDa, F2: 3–10 kDa and F3: < 3 kDa) were determined using cut-off hydrophilic membranes, however the identification of peptides sequences was carried out up to 2 KDa, due to equipment/method limitations, so the peptides size in each fraction correspond to the residual peptides present herein and they not correspond to the size proposed originally. The physicochemical properties of each peptide release of GAD during *in vitro* SGD were predicted by the PepDraw database (Table 2). Hydrophobicity of the peptides ranged from +4.64 (GLLVSLIS) to +23.88 kcal/mol (PQQEHSGGEHQ). The isoelectric point (pI) of peptides ranged from the acidic 3.02 (DIFAM) to alkaline 12.49 (AITGQVPRR). Moreover, the peptides showed neutral (6), negative (3) and positive (3) charge (Table 2).

The bioactivity of amaranth peptides was explored using the analysis database BIOPEP to determine sequences with known biological activities. Table 2 and Fig. 4 depicts the amaranth peptides identified in all fractions that contribute with their anti-inflammatory and antioxidant activities as well as with some sequences of these peptides that have been related with biological activities such as dipeptidyl peptidase IV (DPP-IV) inhibitors, dipeptidyl peptidase III (DPP-III) inhibitors, angiotensin-converting enzyme (ACE) inhibitors, neuropeptide, stimulant glucose uptake, among others.

Biopeptides originated from amaranth, soy, beans, and chickpea have shown anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages; among their mechanisms of action, suggested that presence of glutamine (Q) and poly-glutamine in these peptides may exert its anti-inflammatory activity through the inhibition on NF-kB pathway (González-Montoya et al., 2018; Lozano-Ojalvo & López-Fandiño, 2017; Milán-Noris et al., 2018; Moronta et al., 2016). In this study, peptides sequences of GAD: QDMK, RFQDQHQ, AITGQVPRR, and PQQEHSGEHQ showed from 1 to 3 glutamine residues, which presence of these amino acids could be responsible for improving the anti-inflammatory activity. Also, a peptide sequence of F3, SEPFG, is imbedded in a peptide sequence (HGSEPFGPR) of an anti-inflammatory peptide that was reported previously in extruded amaranth (Montoya-Rodríguez, de Mejía et al., 2014).

Host Defense Peptides (HDP), also recognized as antimicrobial peptides, are essential constituents of the innate immune system and own broad-spectrum antibacterial, antiviral, and immunomodulatory actions. They may provide the inhibition of pro-inflammatory mediators such as lipopolysaccharide, and by modulating the inflammatory response to infection. Most HDP are small (< 100 amino acids) and contain a high proportion of arginine and lysine residues that promote their positive charge (López-Abarrategui, del Monte-Martinez, Reyes-Acosta, Franco, & Otero-González, 2013). In our study, HDPs such as positively charged peptides AITGQVPRR (F1), and GRFREF (F2) might

Table 2
Sequences and physicochemical properties of bioactive peptides identified by MS/MS in the fractions of GAD collected after gastrointestinal digestion.

Fraction	Peptide Sequence	MW (Da)	Bioactive Sequence	Activity[1]	pI	Net charge	Hydrophobicity (Kcal/mol)
F1	SPSS	376.15	SP, PS	DPP-IV inhibitor	5.38	0	9.42
	QDMK	520.23	QD, MK	DPP-IV inhibitor	6.47	0	14.44
	RFQDQHQ	957.44	RF, FQ	ACE inhibitor	7.52	0	16.28
			FQ, QD, QH	DPP-IV inhibitor			
			AI, TG, GQ, VP, PR, RR	ACE inhibitor			
	AITGQVPRR	996.58	TG, QV, VP, RR	DPP-IV inhibitor	12.49	2	12.75
			GQ	Neuropeptide			
			PR, RR	DPP-III inhibitor			
F2	ISYNY	658.29	SY, YN	ACE inhibitor	5.43	0	6.67
			SY, YN	DPP-IV inhibitor			
	GRFREF	810.41	GR, FR, RF	ACE inhibitor	10.98	1	12.88
			RF, FR	DPP-III inhibitor			
			FR	DPP-IV inhibitor			
			EF	CaMPDE inhibitor			
	DIFAM	595.26	IF	ACE inhibitor	3.02	−1	8.54
			FA	DPP-III inhibitor			
			FA	DPP-IV inhibitor			
F3	PQQEHSGEHQ	1175.49	PQ, SG, GE	ACE inhibitor	5.06	−2	23.88
			PQ, QQ, QE, EH, HS, GE	DPP-IV inhibitor			
			GE	DPP-III inhibitor			
	GLLVSLIS	800.49	GL	ACE inhibitor	5.46	0	4.64
			GL, LL, SL, LI	DPP-IV inhibitor			
			LL, LV, LI	Glucose uptake stimulating			
	SEPFG	535.22	SEPFG	Anti-inflammatory[2]	3.21	−1	11.57
			SE	Stimulating vasoactive substance release			
			EP, PF	DPP-IV inhibitor			
			PF	DPP-III inhibitor			
			FG	ACE inhibitor			
	SPSS	376.15	SP, PS	DPP-IV inhibitor	5.38	0	9.42

[1] Identification of bioactive peptides was conducted using http://www.edu.pl/biochemia/index.php/en/biopep.
[2] Montoya-Rodríguez, de Mejía et al. (2014). Physicochemical properties were obtained from Pepdraw. ACE Inhibitor: Angiotensin converting enzyme inhibitor; DPP-IV inhibitor: dipeptidyl peptidase IV inhibitor; DPP-III inhibitor: Dipeptidyl peptidase III inhibitor; CaMPDE inhibitor: calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitor; MW: molecular weight; pI: isoelectric point.

also be considered as multifunctional peptides (Table 2).

Additionally, some of the reported antioxidant amino acids are the aromatic (tryptophan, and phenylalanine), the sulfur-containing (methionine and cysteine), and histidine. Predominantly, the histidine exhibits vigorous radical scavenging activity due to its imidazole ring (Udenigwe & Aluko, 2011). Our case, amaranth peptides with diverse molecular mass (375 to 1200 Da) were detected (Table 2), similar as previously reported to antioxidant peptides (Gallego, Mora, Hayes, Reig, & Toldrá, 2017; Vilcacundo et al., 2019). In this study, the amino acid profiles of the sequences ISYNY, GRFREF, and DIFAM from amaranth peptide (F2) released by SGD containing aromatic amino acids as tyrosine and phenylalanine, could be responsible to the prominent antioxidant activity observed. Besides, the peptides sequences in this study, RFQDQHQ, PQQEHSGEHQ, and SEPFG, were within the sequences previously reported in *Amaranthus spp* with antioxidant, anti-inflammatory and antithrombotic activity (Montoya-Rodríguez, de Mejía et al., 2014; Orsini-Delgado et al., 2016; Sabbione, Nardo, Añón, & Scilingo, 2016).

The above results suggest that germinated amaranth peptides released under SGD possess antioxidant and anti-inflammatory actions. Thus, the consumption of amaranth-based foods could lead to enhanced human health conditions, particularly those produced by oxidative stress.

4. Conclusion

Our study reports the earliest antioxidant and anti-inflammatory activities of peptides derived during the *in vitro* gastrointestinal digestion from germinated amaranth hydrolysate (GAD). The peptide fractions to F1 (> 10 kDa), F2 (3–10 kDa), and F3 (< 3 kDa) were obtained by ultrafiltration of GAD after incubation with pancreatin during 90 min. The results showed that peptide fraction F2 had the more potent antioxidant capacity and higher soluble protein content after the SGD, while peptide fractions F1 and F2 exhibited a high anti-inflammatory response in lipopolysaccharide-induced RAW 264.7 macrophages. Moreover, the eleven sequences of identified peptides have been associated with biological activities such as anti-inflammatory, antioxidant, enzyme inhibitors, among others. Further research is needed to identify the other phytochemicals released during digestion and bioaccessibility.

CRediT authorship contribution statement

Eslim Sugey Sandoval-Sicairos: Writing - original draft, Investigation. **Ada Keila Milán-Noris:** Writing - original draft, Formal analysis, Conceptualization. **Diego Armando Luna-Vital:** Writing - original draft, Formal analysis. **Jorge Milán-Carrillo:** Writing, Reviewing and Editing Manuscript, Funding acquisition. **Alvaro Montoya-Rodríguez:** Writing, Reviewing and Editing Manuscript, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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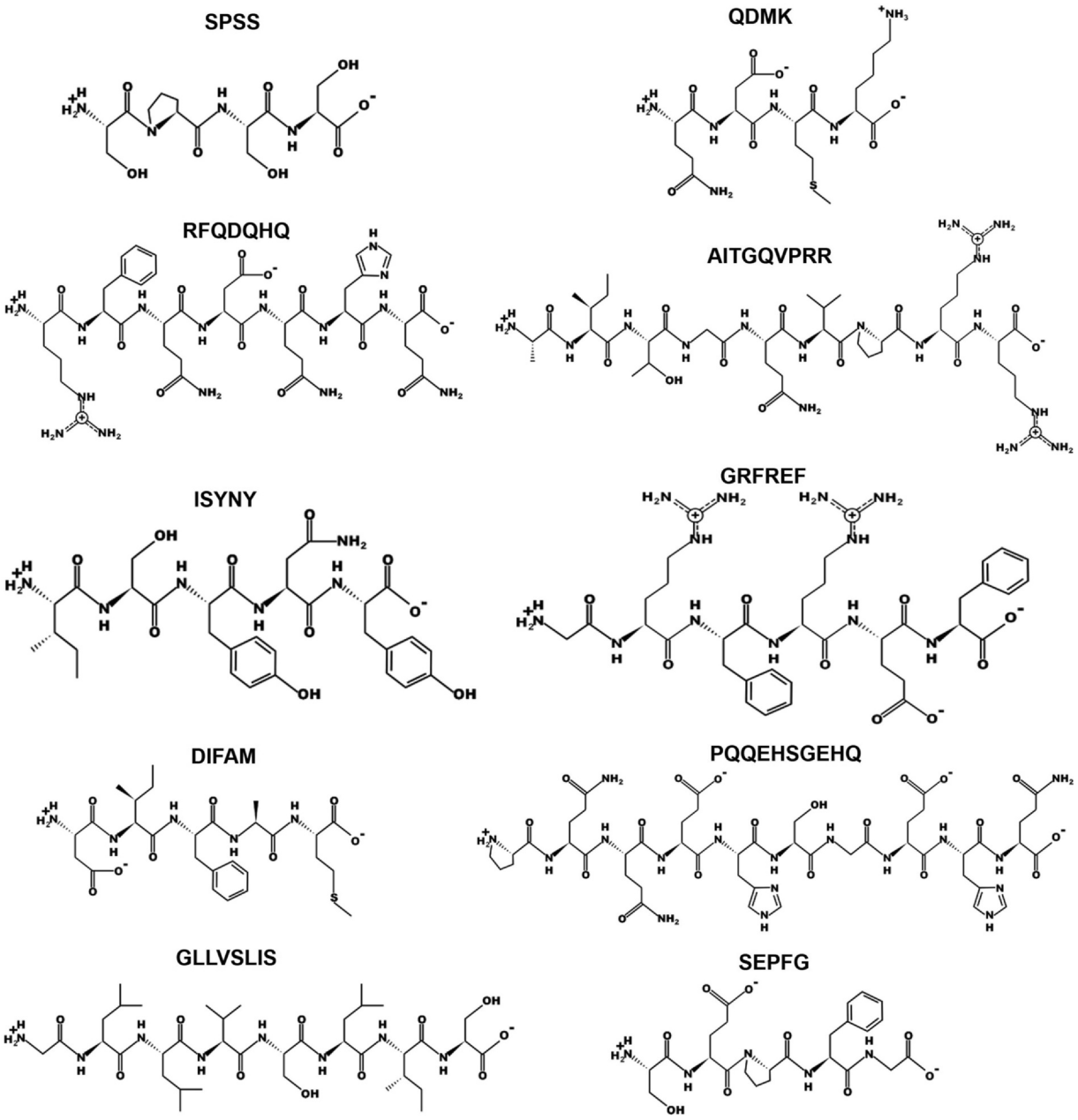

Fig. 4. Structure of the 11 peptides sequences found on the fractions (F1-3; see Table 2) collected from gastrointestinal digestion of germinated amaranth flour (GAD) using PepDraw tool. SPSS peptide sequence appears on F1 and F2.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128394.

References

Aphalo, P., Martínez, E. N., & Añón, M. C. (2015). Amaranth Sprouts: A potential health promoting and nutritive natural food. *International Journal of Food Properties, 18*, 2688–2698. https://doi.org/10.1080/10942912.2015.1004585.

Barba de la Rosa, A. P., Barba Montoya, A., Martínez-Cuevas, P., Hernández-Ledesma, B., León-Galván, M. F., De León-Rodríguez, A., & González, C. (2010). Tryptic amaranth glutelin digests induce endothelial nitric oxide production through inhibition of ACE: Antihypertensive role of amaranth peptides. *Nitric Oxide, 23*(2), 106–111. https://doi.org/10.1016/j.niox.2010.04.006.

Caselato-Sousa, V. M., & Amaya-Farfan, J. (2012). State of knowledge on amaranth grain: A comprehensive review. *Journal of Food Science, 77*, R93–R104. https://doi.org/10.1111/j.1750-3841.2012.02645.x.

Chauhan, A., Saxena, D. C., & Singh, S. (2015). Total dietary fibre and antioxidant activity of gluten free cookies made from raw and germinated amaranth (*Amaranthus* spp.) flour. *LWT - Food Science and Technology, 63*(2), 939–945. https://doi.org/10.1016/j.lwt.2015.03.115.

Conforti, F., & Menichini, F. (2011). Foods of plant origin as source of nitric oxide production inhibitors. In S. Haugen, & S. Meijer (Eds.). *Handbook of nutritional biochemistry: Genomics, metabolomics, and food supply* (pp. 385–403). Nova Science. https://doi.org/10.2174/092986711795029690.

Dia, V. P., Bringe, N. A., & de Mejia, E. G. (2014). Peptides in pepsin–pancreatin hydrolysates from commercially available soy products that inhibit lipopolysaccharide-induced inflammation in macrophages. *Food Chemistry, 152*, 423–431. https://doi.org/10.1016/j.foodchem.2013.11.155.

Gallego, M., Mora, L., Hayes, M., Reig, M., & Toldrá, F. (2017). Effect of cooking and *in vitro* digestion on the antioxidant activity of drycured ham by-products. *Food Research International, 97*, 296–306. https://doi.org/10.1016/j.foodres.2017.04.027.

González-Montoya, M., Hernández-Ledesma, B., Silván, J. M., Mora-Escobedo, R., & Martínez-Villaluenga, C. (2018). Peptides derived from *in vitro* gastrointestinal digestion of germinated soybean proteins inhibit human colon cáncer cells proliferation and inflammation. *Food Chemistry, 242*, 75–82. https://doi.org/10.1016/j.foodchem.2017.09.035.

López-Abarrategui, C., del Monte-Martinez, A., Reyes-Acosta, O., Franco, O. L., & Otero-González, A. J. (2013). LPS in mobilization on porous and non-porous supports as an

approach for the isolation of anti-LPS host-defense peptides. *Frontiers in Microbiology, 4*, 389. https://doi.org/10.3389/fmicb.2013.00389.

López-Barrios, L., Antunes-Ricardo, M., & Gutiérrez-Uribe, J. A. (2016). Changes in antioxidant and antiinflammatory activity of black bean (*Phaseolus vulgaris* L.) protein isolates due to germination and enzymatic digestion. *Food Chemistry, 203*, 417–424. https://doi.org/10.1016/j.foodchem.2016.02.048.

Lozano-Ojalvo, D., & López-Fandiño, R. (2017). Immunomodulating peptides for food allergy prevention and treatment. *Critical Reviews in Food Science and Nutrition, 1–21*. https://doi.org/10.1080/10408398.2016.1275519.

Majumder, K., Mine, Y., & Wu, J. (2016). The potential of food protein-derived anti-inflammatory peptides against various chronic inflammatory diseases. *Journal of the Science of Food and Agriculture, 96*(7), 2303–2311. https://doi.org/10.1002/jsfa.7600.

Martinez-Lopez, A., Millan-Linares, M. C., Rodriguez-Martin, N. M., Millan, F., & Montserrat-de la Paz, S. (2020). Nutraceutical value of kiwicha (*Amaranthus caudatus* L.). *Journal of Functional Foods, 65*, Article 103735. https://doi.org/10.1016/j.jff.2019.103735.

Martinez-Villaluenga, C., Dia, V. P., Berhow, M., Bringe, N. A., & Gonzalez de Mejia, E. (2009). Protein hydrolysates from β-conglycinin enriched soybean genotypes inhibit lipid accumulation and inflammation *in vitro*. *Molecular Nutrition & Food Research, 53*(8), 1007–1018. https://doi.org/10.1002/mnfr.200800473.

Milán-Noris, A. K., Gutiérrez-Uribe, J. A., Santacruz, A., Serna-Saldívar, S. O., & Martínez-Villaluenga, C. (2018). Peptides and isoflavones in gastrointestinal digests contribute to the anti-inflammatory potential of cooked or germinated desi and kabuli chickpea (*Cicer arietinum* L.). *Food Chemistry, 268*, 66–76. https://doi.org/10.1016/j.foodchem.2018.06.068.

Montoya-Rodríguez, A., & de Mejía, E. G. (2015). Pure peptides from amaranth (*Amaranthus hypochondriacus*) proteins inhibit LOX-1 receptor and cellular markers associated with atherosclerosis development in vitro. *Food Research International, 77*(P2), 204–214. https://doi.org/10.1016/j.foodres.2015.06.032.

Montoya-Rodríguez, A., de Mejía, E. G., Dia, V. P., Reyes-Moreno, C., & Milán-Carrillo, J. (2014). Extrusion improved the anti-inflammatory effect of amaranth (*Amaranthus hypochondriacus*) hydrolysates in LPS-induced human THP-1 macrophage-like and mouse RAW 264.7 macrophages by preventing activation of NF-κB signaling. *Molecular Nutrition & Food Research, 58*(5), 1028–1041. https://doi.org/10.1002/mnfr.201300764.

Montoya-Rodríguez, A., Milán-Carrillo, J., Dia, V. P., Reyes-Moreno, C., & González de Mejía, E. (2014). Pepsin-pancreatin protein hydrolysates from extruded amaranth inhibit markers of atherosclerosis in LPS-induced THP-1 macrophages-like human cells by reducing expression of proteins in LOX-1 signaling pathway. *Proteome Science, 12*, 30. https://doi.org/10.1186/1477-5956-12-30.

Moronta, J., Smaldini, P. L., Docena, G. H., & Añón, M. C. (2016). Peptides of amaranth were targeted as containing sequences with potential anti-inflammatory properties. *Journal of Functional Foods, 21*, 463–473. https://doi.org/10.1016/j.jff.2015.12.022.

Orsini-Delgado, M. C., Nardo, A., Pavlovic, M., Rogniaux, H., Añón, M. C., & Tironi, V. A. (2016). Identification and characterization of antioxidant peptides obtained by gastrointestinal digestion of amaranth proteins. *Food Chemistry, 197*(Pt B), 1160–1167. https://doi.org/10.1016/j.foodchem.2015.11.092.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626. https://doi.org/10.1021/jf010586o.

Ozuna, C., Cerón-García, A., Elena Sosa-Morales, M., Salazar, J. A. G., Fabiola León-Galván, M., & Del Rosario Abraham-Juárez, M. (2018). Electrically induced changes in amaranth seed enzymatic activity and their effect on bioactive compounds content after germination. *Journal of Food Science and Technology, 55*(2), 648–657. https://doi.org/10.1007/s13197-017-2974-0.

Reyes-Moreno, C., Cuevas-Rodríguez, E. O., & Reyes-Fernández, P. C. (2019). Whole grains processing, product development, and nutritional aspects. In A. M. Shabir, M. Annamalai, & A. S. Manzoor (Eds.). *Amaranth* (pp. 1–23). Boca Raton, USA: CRC Press Inc. https://doi.org/10.1201/9781351104760.

Ruiz Ruiz, J., Segura Campos, M., Betancur Ancona, D., & Chel Guerrero, L. (2013). Proteínas y péptidos biológicamente activos con potencial nutracéutico. In M. Segura-Campos, L. Chel-Guerrero, & D. Betancur-Ancona (Eds.). *Bioactividad de péptidos derivados de proteínas alimentarias* (pp. 11–27). Barcelona: OmniaScience. https://doi.org/10.3926/oms.136.

Sabbione, A. C., Nardo, A. E., Añón, M. C., & Scilingo, A. (2016). Amaranth peptides with antithrombotic activity released by simulated gastrointestinal digestion. *Journal of Functional Foods, 20*, 204–214. https://doi.org/10.1016/j.jff.2015.10.015.

Sandoval-Sicairos, E. S., Domínguez-Rodríguez, M., Montoya-Rodríguez, A., Milán-Noris, A. K., Reyes-Moreno, C., & Milán-Carrillo, J. (2020). Phytochemical compounds and antioxidant activity modified by germination and hydrolysis in Mexican Amaranth. *Plant Foods for Human Nutrition*. https://doi.org/10.1007/s11130-020-00798-z.

Silva-Sánchez, C., De La Rosa, A. P. B., León-Galván, M. F., De Lumen, B. O., De León-Rodríguez, A., & de Mejía, E. G. (2008). Bioactive peptides in Amaranth (*Amaranthus hypochondriacus*) Seed. *Journal of Agricultural and Food Chemistry, 56*, 1233–1240. https://doi.org/10.1021/jf072911z.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, 299*, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1.

Taniya, M. S., Reshma, M. V., Shanimol, P. S., Gayatri, K., & Priya, S. (2020). Bioactive peptides from amaranth seed protein hydrolysates induced apoptosis and anti-migratory effects in breast cancer cells. *Food Bioscience, 35*, Article 100588. https://doi.org/10.1016/j.fbio.2020.100588.

Tironi, V. A., & Añón, M. C. (2010). Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis. *Food Research International, 43*, 315–322. https://doi.org/10.1016/j.foodres.2009.10.001.

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences, 12*(5), 3148–3161. https://doi.org/10.3390/ijms12053148.

Vernaza, M. G., Dia, V. P., Gonzalez de Mejia, E., & Chang, Y. K. (2012). Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry, 134*(4), 2217–2225. https://doi.org/10.1016/j.foodchem.2012.04.037.

Vilcacundo, R., Martínez-Villaluenga, C., Miralles, B., & Hernández-Ledesma, B. (2019). Release of multifunctional peptides fron kiwicha (*Amaranthus caudatus*) protein under in vitro gastrointestinal digestion. *Journal of the Science of Food and Agriculture, 99*, 1225–1232. https://doi.org/10.1002/jsfa.9294.