Manuscript Number: FOODCHEM-D-18-01296R1

Title: Peptides and isoflavones in gastrointestinal digests contribute to the anti-inflammatory potential of cooked or germinated desi and kabuli chickpea (*Cicer arietinum* L.)

Article Type: Research Article (max 7,500 words)

Keywords: chickpea, germination, boiling, peptides, isoflavone, anti-inflammatory, gastrointestinal digestion

Corresponding Author: Dr. Cristina Martinez-Vilalluenga, PhD

Corresponding Author's Institution: Institute of Food Science, Technology and Nutrition

First Author: Keila Milan, PhD

Order of Authors: Keila Milan, PhD; Janet A Gutierrez-Urbe, PhD; Arlette Santacruz, PhD; Sergio O Serna-Saldivar, PhD; Cristina Martinez-Vilalluenga, PhD

Abstract: It is largely unknown how processing affects bioactive potential of chickpea proteins to prevent bowel inflammatory diseases. The aim was to investigate the anti-inflammatory activity of protein concentrates from germinated and cooked chickpeas (GC and CC, respectively) and its relationship with protein and isoflavone composition before and after in vitro gastrointestinal digestion and absorption. Anti-inflammatory activity of GC digests was almost 2-fold higher than CC digests ($p < 0.05$), which was associated to greater content of peptides, formononetin and biochanin A ($p < 0.05$). Anti-inflammatory activity of phenolic fraction in digests was 7-fold higher than the protein fraction ($p < 0.05$). The most active peptide fraction from GC digest ($IC_{50} = 93 \mu g/mL$) contained a total of 24 peptides derived from legumin and vicilin. In conclusion, this study stands out the potential of germinated chickpea proteins concentrates to exert anti-inflammatory effects in the lower gut which may contribute to the prevention of bowel inflammatory diseases.

Highlights

1. Sprouted vs. cooked chickpea concentrate showed higher anti-inflammatory potential
2. Sprouted chickpea digests showed more abundance of peptides and isoflavones
3. Chickpea phenolics showed higher inhibition of inflammation *in vitro* than peptides
4. Anti-inflammatory peptides in sprouted chickpea digest were identified

Peptides and isoflavones in gastrointestinal digests contribute to the anti-inflammatory potential of cooked or germinated desi and kabuli chickpea (*Cicer arietinum* L.)

Ada Keila Milán-Noris^a, Janet Alejandra Gutiérrez-Uribe^a, Arlette Santacruz^a, Sergio O. Serna-Saldívar^a, Cristina Martínez-Villaluenga^{b,*}.

^aTecnologico de Monterrey, Centro de Biotecnología-FEMSA. Escuela de Ingeniería y Ciencias, Av. Eugenio Garza Sada 2501 Sur, CP 64849, Monterrey, N.L. México.

^bInstitute of Food Science, Technology and Nutrition (ICTAN-CSIC), Juan de la Cierva 3, 28006, Madrid.

*Corresponding author:

E-mail address: c.m.villaluenga@csic.es (C. Martínez-Villaluenga)

1 **Abstract**

2 It is largely unknown how processing affects bioactive potential of chickpea proteins
3 to prevent bowel inflammatory diseases. The aim was to investigate the anti-inflammatory
4 activity of protein concentrates from germinated and cooked chickpeas (GC and CC,
5 respectively) and its relationship with protein and isoflavone composition before and after *in*
6 *vitro* gastrointestinal digestion and absorption. Anti-inflammatory activity of GC digests was
7 almost 2-fold higher than CC digests ($p<0.05$), which was associated to greater content of
8 peptides, formononetin and biochanin A ($p<0.05$). Anti-inflammatory activity of phenolic
9 fraction in digests was 7-fold higher than the protein fraction ($p<0.05$). The most active
10 peptide fraction from GC digest ($IC_{50}=93 \mu\text{g/mL}$) contained a total of 24 peptides derived
11 from legumin and vicilin. In conclusion, this study stands out the potential of germinated
12 chickpea proteins concentrates to exert anti-inflammatory effects in the lower gut which
13 may contribute to the prevention of bowel inflammatory diseases.

14 **Keywords:** chickpea, germination, boiling, peptides, isoflavone, anti-inflammatory,
15 gastrointestinal digestion.

16 **Chemical compounds studied in this article:**

17 biochanin A (Pubchem CID: 5280373) and formononetin (Pubchem CID: 52800378)

18

1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important legume in the world (FAOSTAT, 2014). Because of its nutritional value (80% of dry seed is comprised of digestible carbohydrates, fiber and high quality protein) and agronomic potential this pulse crop may help facing environmental and food challenges such as climate change, malnutrition, obesity, demographic expansion, etc (Bar-El Dadon, Abbo, & Reifen, 2017). Besides its high nutritional value, chickpea also contains a diverse profile of bioactive compounds including, but not limited to, phenolic compounds. Isoflavonoids (formononetin, biochanin A and their corresponding glycosides) are the main phenolic group in chickpea (Wu et al., 2012). There are two main varieties of chickpea: the light seeded Kabuli type and the smaller dark Desi type that differ in nutritional and phytochemical composition (Heiras-Palazuelos et al., 2013; Segev et al., 2010).

Chickpea is also gaining importance as its consumption provides health benefits preventing the onset of many gut-associated diseases such as colon cancer and inflammatory bowel disease among others (Gupta et al., 2017; Jukanti, Gaur, Gowda, & Chibbar, 2012). Recent animal studies have demonstrated that chickpea consumption enhances gut health through inhibition of cancer cell proliferation, attenuation of inflammation, modulating microbiome composition and activity, promoting epithelial barrier integrity, mucus production and antimicrobial defenses (Chino et al., 2017; Monk et al., 2017). Most of these beneficial effects are attributed to its bioactive compounds that after gastrointestinal digestion remain bioaccessible in the unabsorbed fraction of the digesta. Fermentable fiber and the resulting fermentation products are most likely responsible for the gut health promoting effects; however, the contribution of isoflavonoids and other phenolic compounds to gut health cannot be excluded (Monk et al., 2017). Specifically, isoflavones such as biochanin A have been shown to improve gut health exerting antioxidant and anti-

44 inflammatory effects (Kole, Giri, Manna, Pal, & Ghosh, 2011; Piegholdt et al., 2014).
45 Proteins are one of the main components of chickpea (15 to 30%) and a notable source of
46 peptides with antioxidant and anti-proliferative activity against colon cancer cells (Jamdar,
47 Deshpande, & Marathe, 2017; Xue et al., 2015). It is, as yet, largely unknown whether these
48 bioactive peptides are released and become bioaccessible during gastrointestinal digestion.
49 They escape small intestine permeation in the upper gut and reach the colon wherein they
50 exert beneficial physiological effects.

51 Prior to consumption, chickpea generally undergoes different types of processing to
52 increase its palatability and nutritional value. Upon processing, phenolic composition of
53 chickpea is modified and affect its bioactivity (Singh, Singh, Kaur, & Singh, 2017). For
54 instance, domestic cooking significantly decreases phenolic compounds and antioxidant
55 activity of chickpea (Aguilera et al., 2011). On the contrary, germination increases total and
56 free phenolic content and isoflavones what enhances its antioxidant activity (Hithamani &
57 Srinivasan, 2014; Wu et al., 2012). Similarly, chickpea germination and domestic cooking
58 cause biochemical modifications of proteins, which have shown to impact chickpea
59 angiotensin converting enzyme inhibitory and antioxidant activities (Jamdar et al., 2017;
60 Mamilla & Mishra, 2017). However, it is largely unknown how processing followed by
61 gastrointestinal digestion and absorption affects phenolic compounds and peptides with
62 relevant bioactivity for health maintenance in the gut.

63 The aim was to investigate the anti-inflammatory activity of protein concentrates
64 from germinated and cooked chickpeas (GC and CC, respectively) and its relationship with
65 protein and isoflavone composition before and after *in vitro* gastrointestinal digestion and
66 absorption. The individual contribution of peptides and isoflavones to the anti-inflammatory
67 effect of chickpea protein digests was examined. Finally, bioassay-guided fractionation of

protein digests was performed to purify and identify potential anti-inflammatory peptides of chickpea.

2. Materials and Methods

2.1 Materials

Chickpea cultivar “ICC5613” (Desi-type, green) was grown at experimental station of INIFAP (National Research Institute for Forestry, Agriculture and Livestock), in Culiacan, Sinaloa, Mexico. Commercial chickpea cultivar Blanco Sinaloa (Kabuli-type, cream) was grown at Evora Region, Sinaloa, Mexico. The chickpea seeds were harvested in April-May 2016, cleaned and stored at 4 °C until analysis. Murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). High-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) and penicillin/streptomycin (10,000 U/mL) were purchased from Lonza Group Ltd. (Madrid, Spain). Fetal bovine serum (FBS) was obtained from Hyclone (GE Healthcare, Logan, UT, USA). Cell Titer 96® AQueous One Solution Proliferation Assay kit was supplied from Promega (Madison, WI, USA). Quantitative colorimetric peptide assay kit was from PierceTM (Rockford, IL, USA). Cell culture flasks and plates were obtained from Sarstedt (Nümbrecht, Germany). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.2. Chickpea processing and protein concentration

Germinated or sprouted chickpeas were obtained as previously reported by (Guardado-Félix, Serna-Saldivar, Cuevas-Rodríguez, Jacobo-Velázquez, & Gutiérrez-Urbe, 2017). Briefly, seeds were disinfected with 2 volumes of 0.12% sodium hypochlorite solution for 3 min and then washed 4 times with distilled water until pH 6 was reached. Subsequently, the seeds were hydrated in 0.85 volumes of distilled water for 5 h at 25 °C

with constant agitation. The resulting soaked seeds were transferred onto plastic trays and germinated at 24 °C in darkness conditions for 5 days at 80% of relative humidity. Resulting sprouted seeds were freeze-dried, ground to pass through a 60 US sieve and stored at -20 °C until analysis. Previous to protein concentration, the germinated chickpea flour was defatted with hexane (1:4, w/v) in agitation at 500 rpm for 4 h, and the defatted cake dried overnight at 25 °C. The defatted cake (300 g) was suspended in water (1:10, w/v) and blended for 1 min. Proteins were extracted at pH 8.5 using a solution 1 M NaOH and a continuous agitation speed of 500 rpm for 2 h. Flour suspension was centrifuged at 3,000 x g for 10 min and the pellet extracted again using the same conditions. For protein precipitation, the clarified supernatants were pooled and adjusted to pH 4.5 using 1 M HCl. Protein precipitate was centrifuged at 3,000 x g for 10 min, and the pellet was freeze-dried and stored at -20 °C until analysis.

Cooked chickpeas were prepared by soaking the seeds in 10 volumes of distilled water at room temperature for 12 h. Then, excess water was drained and seeds were cooked in 3 volumes of boiling water for 30 min (Sagrati et al., 2013). Boiled chickpeas were freeze-dried, ground to pass through a 60 US sieve and stored at -20 °C until analysis. Prior to protein isolation, the gelatinized starch of boiled chickpeas was hydrolysed with thermoresistant α -amylase from *Bacillus subtilis* (Megazyme, Wicklow, Ireland). First, a flour (500 g) suspension (1:6, flour to water ratio) was mixed at 500 rpm for 30 min. Then α -amylase (10 units/g sample) was added and heated for 20 min at 95 °C. Then, the sample was incubated at 60 °C at 500 rpm for 12 h. Finally, 2 volumes of ethanol were added and shaken at 500 rpm for 20 min for enzyme inactivation. The resulting sample was centrifuged at 3,000 x g for 10 min and the pellet was used as feedstock for protein extraction as previously described for germinated chickpeas. The protein concentrates were freeze-dried and stored at -20 °C until analysis.

2.3. Simulated gastrointestinal digestion of protein concentrate

The chickpea concentrates from processed samples were *in vitro* digested according to the method reported by (Mosele, Macià, Romero, & Motilva, 2016). The method consists in a simulated digestion process mimicking physiological conditions of mouth, stomach and small intestine. Briefly, protein concentrate (3 g) suspended in 100 mL of phosphate buffer solution (pH 6.9) containing 10 U/mL of α -amylase from porcine pancreas (EC 3.2.1.1.) was incubated for 5 min at 37 °C at 200 rpm. For gastric digestion, the pH was lowered to 2 using 1 M HCl. Subsequently, 5 mL of pepsin from porcine gastric mucose (EC 3.4.32.1) solution (2250 U/mL in 0.01 M HCl) was added and reaction mixture was incubated for 1 h at 37 °C at 200 rpm. For the intestinal digestion the gastric digest (adjusted to pH 6.5) and 5 mL of duodenal juice (200 mg of bile salts with 1980 U/mL of pancreatin from porcine pancreas) were added in a dialysis membranes with a weight cut-off of 3.5 kDa and dialyzed for 2 h at 37 °C at 200 rpm immersed in 5 mM phosphate buffer solution (pH 7.4). After intestinal digestion, two fractions (IN and OUT) were collected and freeze dried. IN fraction (dialysis membrane) is the non-absorbable fraction that reaches the colon whereas OUT (phosphate buffer) represents the absorbable fraction. In this study, only the IN fraction was analyzed. The digestions fractions were freeze-dried and stored at -20 °C until analysis.

2.4. Preparation of methanolic extracts

Extractions were carried out as previously reported by Luthria et al. (2007) with some modifications. Briefly, freeze-dried chickpea sample (0.5 g) was extracted twice in 10 mL of 80% aqueous methanol and mixed for 1 min. The sample suspension was sonicated at 40 kHz, 135 W for 15 min and centrifuged at 8,000 x g at 4 °C. Supernatant was collected and residue was extracted in the same conditions. The two extracts were combined and evaporated under reduced pressure at 45 °C to remove excess solvent. Finally, the resulting residue was reconstituted in 1 mL 80% aqueous methanol.

2.5. Sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis

SDS-PAGE analysis of protein extracts of chickpea samples was performed loading 20 µg of protein/well on NuPAGE® Novex 4-12% Bis-Tris Gels (Invitrogen, Madrid, Spain). Gels were placed in an XCell-sure lock Mini-Cell and run at 200 V for 35 min under reducing conditions. NuPAGE® MES-SDS and NuPAGE® LDS (Invitrogen, Madrid, Spain) were used as running and sample buffers, respectively. Gels were stained with SimplyBlue SafeStain (Invitrogen, Madrid, Spain) for 1 h and destained in deionized water for 2 h. After destaining, an image of the gel was taken using a Chemdoc® XRS+ Imaging system (BioRad, Hercules, CA, USA). The molecular weight of poly- and oligopeptides was determined by comparison with the molecular weight marker Novex® Sharp Prestained Protein Standard (20-260 kDa) (Invitrogen, Madrid, Spain).

2.6. Protein and peptide content

Protein and peptide content were determined following methods previously described elsewhere (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018). Briefly, soluble protein quantification was carried out in duplicate using the Detergent compatible (DC) Protein Assay (Biorad, Hercules, CA, USA). Bovine serum albumin was used as standard at a concentration range from 0.1 to 1 mg/mL. Peptide concentration was measured by the Quantitative Colorimetric Peptide Assay kit from Pierce™ (Rockford, IL, USA). Results were expressed as mg/g freeze-dried sample.

2.7. Total phenolic content

Total phenolics were determined using the Folin-Ciocalteu method described previously (Vinson, Proch, & Bose, 2001). Briefly, 100 µL of diluted extract was mixed with 625 µL of distilled water, 250 µL 7.5% (w/v) Na₂CO₃ and 25 µL Folin-Ciocalteu reagent. Samples were vortexed and incubated for 2 h at room temperature in darkness. The

absorbance was measured at 739 nm using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). Total phenolics were quantified by external calibration using gallic acid (4.5-225 µg/mL). Samples were analyzed in triplicate and results expressed as mg of gallic acid equivalents (GAE) per g of freeze-dried sample (mg GAE/g).

2.8. Quantification and identification of flavonoids

The quantification of flavonoids in chickpea samples was performed, as previously reported (Guardado-Félix et al., 2017), by high performance liquid chromatography (HPLC) with diode array detection (Agilent technologies, Santa Clara, CA, USA). The separation was achieved using an Eclipse XDB C18 column (3 mm x 150 mm, 3.5 µm; Agilent technologies, Santa Clara, CA, USA) at flow rate of 0.4 mL/min and column temperature of 30 °C. The injection volume was 2 µL and detection was recorded at 260 nm. The mobile phase consisted of 0.1% of formic acid in water (solvent A) and acetonitrile (solvent B). The gradient profile was set as follows: 0%-10% solvent B for 8 min, 10%-35% solvent B for 8 min, 35%-90% solvent B for 10 min and 90%-100% solvent B for 10 min.

Isoflavonoids were measured using Biochanin-A and formononetin standards to quantify these aglycones or their conjugates and values were expressed as µg/g of freeze-dried sample. The concentration of the other isoflavonoids was calculated as biochanin-A equivalents (µg/g of freeze-dried sample). Myricitin was used as internal standard. Analysis were performed in triplicate. The method showed a linearity ranges of 10-100 µg/mL and 5-100 µg/mL, correlation coefficient (R^2) of 0.9994 and 0.9999, recovery (%) of 97.6 and 95.8, limit of quantification (LOQ) of 10.27 and 1.11 µg/mL, limit of detection (LOD) of 3.37 and 0.36 µg/mL, relative standard deviation (RDS%) of 2.76 and 1.93, for formononetin and Biochanin-A, respectively.

The identification of flavonoids in chickpea samples was performed by HPLC coupled to time of flight mass spectrometry (1100 series, Agilent technologies, Santa Clara,

CA, USA) using the chromatographic conditions described above. Ionization was carried out using a electrospray source at 350 °C and 2500 V capillary temperature and voltage nebulizer pressure at 345 kPa and nitrogen gas flow rate at 13 mL/min. Range for mass scan covered from m/z 150 to 2000 and data was acquired in positive mode. Mass Hunter workstation was used to extract the experimental m/z at different retention times. The identity of compounds was confirmed by UV absorption, mass spectra and retention time as previously reported isoflavonoids in chickpea (Guardado-Félix et al., 2017).

2.9. Anti-inflammatory activity

2.9.1. Macrophages cell culture

The anti-inflammatory potential of chickpea samples was evaluated using an inflammation-activated RAW264.7 cell system and based on nitric oxide (NO) analysis produced from the cell reaction. Murine macrophage RAW 264.7 cell line was maintained at a subconfluent density in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated at densities of 1×10^6 cells in 75 cm² tissue culture flasks and incubated in a humidified incubator at 37 °C and 5% CO₂ atmosphere. The culture medium was changed every 2 days.

2.9.2. Cell viability assay

All chickpea 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% CO₂ atmosphere. Cells were exposed for 16 h to chickpea protein isolates (0.5-5 mg/mL), digests (0.5-5 mg/mL) and phenolic extracts (0.1-0.5 mg/mL) dissolved in serum-free medium. After treatment, 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 chickpea 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% CO₂ atmosphere. The cells were pre-treated for 20 h with chickpea protein isolates (0.5-5 mg/mL), digests (0.5-5 mg/mL) and phenolic extracts (0.1-0.5 mg/mL) dissolved in serum-free medium, then elicited with polysaccharide (LPS, from *Escherichia coli* B5:O55) at 1 µg/mL for an additional 20 h. After LPS elicitation, anti-inflammatory activity was investigated through 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) H₃PO₄, 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. Fractionation of chickpea peptides by preparative reverse phase liquid chromatography coupled to mass spectrometry (RP-LC-MS)

Further purification of potential anti-inflammatory peptides from the most active chickpea sample was performed by preparative RP-LC-MS. Peptides separation was performed on a

HPLC system (Waters, Mildford, MA, USA) equipped with four pumps, a Binary Gradient Module 2545, a System Fluidic Organizer (5015 HPLC Pump), a Sample Manager 2767a diode array detector (module 2998v) and a 3100 Mass Detector. The data-processing software was MassLynx (Waters, Mildford, MA, USA). A SunFire C18 reverse phase column (150 x 19 mm, 5 μ m particle size, Waters, Mildford, MA, USA) was used. The peptide fractions were dissolved in distilled water at concentration of 100 mg/mL, and the injection volume was 1500 μ L. The mobile phase flow rate was 17 mL/min using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Fractions were eluted in isocratic mode using solvent A for 10 min followed by a linear gradient profile set from 2%-100% solvent B for 15 min. Each chromatographic run was repeated 3 times and the fractions were collected automatically using a Sample Manager 2767 (Waters, Mildford, MA, USA). The collection times for each fraction were: F1 (1.5-4.5 min), F2 (7.5-8.5 min) and F3 (18-21 min). A total of 3 fractions were collected for each time frame and collected fractions were pooled, freeze-dried and stored at -20 $^{\circ}$ C until further analysis. Quantification of peptides in each fraction was performed by a colorimetric assay using the PierceTM Peptide Colorimetric assay kit according to the manufacturer's protocol.

2.11. Peptide identification by nanoUPLC–ESI-MS/MS

In the most active fraction, a peptidomic analysis was performed to identify potential anti-inflammatory peptides present in the undigested fraction of germinated chickpea. All peptide separations were carried out on an Easy-nLC 1000 nano system (Thermo Scientific, Madrid, Spain) as previously reported with minor modifications (Mas et al., 2017). For each analysis, the sample was loaded into a precolumn Acclaim PepMap 100 (Thermo Scientific, Madrid, Spain) and eluted in a RSLC PepMap C18 (150 mm long, 75 μ m inner diameter and 3 μ m particle size; Thermo Scientific, Madrid, Spain). The mobile phase flow rate was 300 nL/min using 0.1% formic acid in water (solvent A) and 0.1% formic acid and 100%

acetonitrile (solvent B). The gradient profile was set as follows: 5%–35% solvent B for 100 min, 35%–100% solvent B for 10 min, 100% solvent B for 20 min. Four microliters of each sample were injected. MS analysis was performed using a Q Exactive mass spectrometer (Thermo Scientific, Madrid, Spain). For ionization, 2000 V of liquid junction voltage and 270 °C capillary temperature was used. The full scan method employed a m/z 400–1500 mass selection, an Orbitrap resolution of 70,000 (at m/z 200), a target automatic gain control (AGC) value of $3e6$, and maximum injection times of 100 ms. After the survey scan, the 15 most intense precursor ions were selected for MS/MS fragmentation. Fragmentation was performed with a normalized collision energy of 27 eV and MS/MS scans were acquired with a starting mass of m/z 100, AGC target was $2e5$, resolution of 17,500 (at m/z 200), intensity threshold of $8e3$, isolation window of 2 m/z units and maximum IT was 100 ms. Charge state screening was enabled to reject unassigned, singly charged, and equal or more than seven protonated ions. A dynamic exclusion time of 20 s was used to discriminate against previously selected ions.

Mass spectrometry data were analyzed with Proteome Discoverer (version 1.4.1.14) (Thermo Scientific, Madrid, Spain) using standardized workflows. Mass spectra raw files were searched against files in Uniprot *Cicer arietinum* database (29535 sequences protein entries) using SEQUEST search engine. Precursor and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively, allowing 2 missed cleavages, carbamidomethylation of cysteines as a fixed modification, methionine oxidation as a variable modification. Identified peptides were filtered using Percolator algorithm 9 with a q -value threshold of 0.01.

2.12. Statistical analysis

Data was reported as the mean $\pm$ standard deviation of three replicates. Data were subjected by one-way analysis of variance (ANOVA) followed by Tukey's test to detect differences among chickpea samples. A p -value <0.05 was considered significant. All statistical

analyses were performed with the use of JMP 13 software from SAS institute (Cary, NC, USA).

3. Results and Discussion

3.1 Effect of processing and digestion on the protein profile of chickpea

Fig. 1. shows the SDS-PAGE profile in denaturing and non-reducing conditions of flours, protein concentrates and digests from cooked (CC) and germinated (GC) Kabuli and Desi chickpea varieties. Proteins with molecular weights (MW) in the range of 15 to 120 kDa were found for both raw chickpea (RC) cultivars which is consistent to previous study (Wang et al., 2010). Major chickpea proteins with MW of 19, 33-35, 51 and 70 kDa corresponded to vicilin subunits and 10, 22-23 and 40-39 kDa were assigned to legumin subunits according to literature (Chang, Alli, Molina, Konishi, & Boye, 2012). After germination, some proteins were hydrolysed as indicated by intensity reduction of bands with MW of 20, ~50, 70, 80 and 120 kDa in both Kabuli and Desi varieties. This results are comparable to the study of Mamilla & Mishra (Mamilla & Mishra, 2017) who reported that proteins with MW of 78-83, 95-99 and 110-120 kDa are preferentially metabolized during Kabuli type chickpea germination either at 30 °C or 40 °C. But unlike this latter study, SDS-PAGE results did not clearly showed herein the generation of low MW polypeptides after chickpea germination. This fact could be explained by intra-varietal variability in the type of proteases and total protease activity of chickpea seeds (Bewley, Bradford, Hilhorst, & Nonogaki, 2013).

SDS-PAGE pattern of protein concentrate produced from CC showed a reduced intensity in most of the vicilin and legumin subunits and the appearance of new bands that ranged from 40 to 50 kDa (**Fig. 1**). These observations are consistent with previous studies showing the effect of different thermal processing methods such as canning, pressure-cooking and roasting on protein profile of chickpeas (Parmar, Singh, Kaur, Viridi, & Thakur,

2016; Xu et al., 2017). Banding pattern of CC was the result of thermal-induced protein denaturation, unfolding and concomitantly aggregation of unfolded protein molecules. Minor differences were observed in the relative intensity of protein bands (10-15 and 20-22 kDa) between protein concentrates from Kabuli and Desi varieties. Protein concentrates produced from GC showed similar protein profiles to their corresponding flours, only losses of two minor protein bands in the range of 80-110 kDa were found.

To evaluate the effect of the processing treatments on the digestion of chickpea proteins and to identify digestion-resistant peptides, protein concentrates were subjected to an *in vitro* digestion method that mimics the mouth, stomach and small intestine physiological conditions and absorption of low molecular weight nutrients. Proteins were readily hydrolysed during digestion to produce low MW polypeptides < 15 kDa that remained in CC and GC gastroduodenal digests (**Fig. 1**). A portion of the proteins were not resolved being accumulated at the bottom of the gel. There were differences in the apparent MW of polypeptides resultant from digestion of cooked and germinated chickpea proteins. CC digests showed 12 and 3.5 kDa bands whereas in GC digests apparent MW of polypeptides ranged from 3.5 to 10 kDa. Similarly, an earlier study showed that sequential pepsin-trypsin hydrolysis of Kabuli and Desi varieties for a total of 240 min produced low MW polypeptides <18.4 kDa (Wang et al., 2010). More recently, Ribeiro et al. (Ribeiro et al., 2017) demonstrated that storage proteins and albumins were able to resist digestion being these effect more pronounced in raw than cooked chickpea.

3.2 Effect of processing and digestion on the soluble protein and peptide content of chickpea protein concentrates

Table 1 shows soluble protein and peptide contents in flours, protein concentrates and digests from CC and GC Kabuli and Desi varieties. The soluble protein content of RC and GC flours ranged from 120.8-250.7 mg/g freeze-dried weight and low molecular weight

peptides (<10 kDa) were not detected. There was no significant difference in soluble protein content between GC and RC flours of Kabuli and Desi varieties. Although it is important to take note that flours from RC and GC Desi variety had a higher soluble protein content than the counterpart obtained from Kabuli variety ($p<0.05$). This could be explained by larger total protein content reported for Desi varieties (Heiras-Palazuelos et al., 2013). In consistency with this observation CC and GC protein concentrates from Desi variety had significantly greater soluble protein content than their Kabuli counterpart ($p< 0.05$). Less than 10% of the soluble protein in concentrates either produced from CC and GC of Desi and Kabuli chickpea varieties had peptides < 10 kDa.

A significant amount of soluble proteins remained at the end of gastrointestinal digestion either in CC and GC digests (**Table 1**). Moreover, it was observed that soluble protein in CC and GC digests was composed of 75% and 79% of low molecular weight peptides, respectively, that escaped permeation during gastroduodenal digestion. Interestingly, CC protein digests of Kabuli and Desi varieties showed lower soluble protein content compared to GC counterparts ($p<0.05$). These results indicate that a larger proportion of cooked protein digestion products permeated during gastrointestinal digestion. This observation is consistent with Bar-El Dadon et al. (2017) who recently reviewed the effect of different processing methods on chickpea protein digestibility showing that thermal treatments resulted in higher protein digestibility than germination. A potential explanation is that cooking induces protein denaturation and formation of aggregates of unfolded proteins which is related to increased protein digestibility and greater extent of hydrolysis to produce amino acids and small peptides that could easily permeate during digestion (Laguna, Picouet, Guàrdia, Renard, & Sarkar, 2017). Additionally, cooking is considered more effective than germination for the removal of antinutrient compounds that interfere with protein digestion (Patterson, Curran, & Der, 2017). These effects are consistent with

the lower peptide content that remained in CC digest (195.6 and 345.7 mg/g freeze-dried weight) compared to GC digest (441.2 and 469.3 mg/g freeze-dried weight) in Kabuli and Desi correspondingly ($p<0.05$). Interestingly, another finding of the present study was that CC digest produced from Desi variety showed the lowest soluble protein (262.5 mg/g freeze-dried weight) and peptide contents (195.6 mg/g freeze-dried weight), which suggest that once cooked, proteins of this variety are more readily digested and bioavailable.

3.3. Effect of processing and digestion on the isoflavone profile and total phenolic content of chickpea protein concentrates

The isoflavonoid profile, total isoflavone content (TIC) and total phenolic content (TPC) of flours, protein concentrates and digests from CC and GC Kabuli and Desi varieties are depicted in **Table 2**. The representative chromatograms at 260 nm of chickpea samples are shown in **Supplementary Fig. S1**. Previous studies on chickpea seeds and sprouts have shown that isoflavones are the major phenolic compounds (Aguilera et al., 2011). Biochanin A and its glycosides were detected as the major isoflavonoids in Kabuli and Desi RC flours at concentrations within ranges reported by earlier studies (Aguilera et al., 2011; Wu et al., 2012). After germination, Kabuli and Desi flours contained between 50- and 77-fold and 6- and 9-fold higher TIC and TPC, respectively than RC flours (**Table 2**, $p<0.05$). Similar observations were reported previously when the effect of germination on phenolic and isoflavone contents of chickpea were examined (Wu et al., 2012). Germination activates the central phenylpropanoid pathway and the specific isoflavonoid branch pathways in legumes (Wu et al., 2012). As such, in the present study chickpea germination increased isoflavonoid diversity as a total of seven isoflavonoids were identified (isoformononetin glycoside, formononetin malonyl glycoside, biochanin A glycoside, biochanin A malonyl glycoside, pseudobaptigenin, formononetin, biochanin A) (**Supplementary Fig. S1, Table 2**).

Interestingly, RC and GC flours obtained from Desi variety had larger TIC and TPC than the mean value found in the corresponding Kabuli flours (**Table 2**), which is in agreement to earlier studies reporting greater TPC and total flavonoid content for Desi varieties (Heiras-Palazuelos et al., 2013; Segev et al., 2010). These results were attributed to the variability in the seed coat percentages of total phenolic compounds between Desi and Kabuli cultivars (seed coat contributes to 75% of total phenolic compounds). Moreover, formononetin and biochanin A malonyl glycosides were the dominant isoflavonoids in Kabuli sprouts (**Supplementary Fig. 1**) whereas formononetin and biochanin A aglycones were the most abundant isoflavonoids found in Desi variety (**Table 2**).

Protein concentration by isoelectric precipitation allowed the recovery of isoflavonoids and other phenolic compounds (**Table 2**). The isoflavone profiles in GC and CC protein concentrates were similar for both varieties. As such, formononetin and biochanin-A were the most abundant compounds in all protein concentrates. Similarly, Megías et al. (Megías, Cortés-Giraldo, Alaiz, Vioque, & Girón-Calle, 2016) also reported that preparation of concentrates by isoelectric precipitation from raw chickpea flour releases mostly biochanin A. The present study showed that not only biochanin A but also formononetin was recovered to a greater extent in GC and CC protein concentrates. In contrast, biochanin A and formononetin glycosides that were predominant compounds in RC and GC flours were not detected in concentrates (**Table 2**). Therefore, these glycosides were hydrolyzed at alkaline pH and co-precipitate with proteins at pH 4.5, as previously observed and the resulting less hydrophilic aglycones could bind into globulin fraction in the protein concentrates (Megías et al., 2016).

For both varieties, the protein concentrates produced from GC showed greater TIC and TPC than the ones produced from CC probably due to a decrease in the percentage of phenolics during soaking and cooking that are known to cause leaching and thermal

degradation of these compounds, respectively (Parmar et al., 2016). In addition, thermal treatments favor phenolic-protein interactions reducing their extractability. TIC of GC concentrates produced either for Kabuli or Desi varieties (3431 and 3479 $\mu\text{g/g}$ freeze-dried weight, respectively) was much higher than raw chickpea (2000 $\mu\text{g/g}$ freeze-dried weight) and soy (1353 $\mu\text{g/g}$ freeze-dried weight) concentrates (Megías et al., 2016) which suggest that the former is a better source of health-promoting compounds. The effect of the chickpea variety was also studied, and it was observed that Kabuli CC protein concentrate had larger TIC than the Desi counterpart ($p<0.05$) whereas opposed effects were observed for TPC ($p<0.05$). In the case of GC protein concentrates, similar TIC content was observed for varieties and only a slightly lower content of TPC was found for the Desi protein concentrate ($p<0.05$).

Isoflavone profiles of protein digests were similar to protein concentrates in which formononetin and biochanin A were the main isoflavonoids (**Table 2**). CC and GC digests from Kabuli variety contained higher TIC (80 and 7196 $\mu\text{g/g}$ freeze-dried weight) and TPC (7.4 and 11.7 mg GAE/g freeze-dried weight) than Desi protein digests ($p<0.05$). This results are consistent with greater soluble protein and peptide content of the digests (**Table 1**) and the higher relative content of trypsin inhibitors reported for protein concentrates produced from this chickpea variety (Mondor et al., 2009). Protein digests had larger TIC and TPC than protein concentrates for both varieties ($p<0.05$) indicating that these compounds remained bound to protein digestion products and were not bioaccessible for absorption. Some researchers have suggested that 5 to 20 % of TPC in legumes can be absorbed (Chen et al., 2015). The only exception was CC protein digest for which digestion resulted in lower TIC compared to the CC protein concentrate produced from Kabuli and Desi varieties. These results suggest that higher amounts of isoflavonoids present in CC protein concentrates would have permeated the dialysis membrane used in the digestion

setup of the present study. In fact, this hypothesis is consistent with the lower soluble protein and peptide content of CC protein digest observed in **Table 1**. Taken all together, it seems that physicochemical changes in chickpea proteins (denaturation and unfolding) caused by cooking enhanced not only protein digestibility but also isoflavone intestinal absorption *in vitro* as polyphenols had less affinity for small size peptides. Contrasting results were found in GC digests for both varieties which showed the highest TIC and TPC retentions ($p<0.05$). It is known that native proteins of GC have lower protein digestibility than proteins from CC. Lower extent of proteolysis produced GC digests enriched in high MW peptides as shown in **Fig. 1** that could have greater binding affinity to phenolic compounds. All these observations are consistent with a previous study focused on the effect of domestic processing (sprouting and cooking) on polyphenols bioaccessibility of wheat, sorghum, green gram and chickpea (Hithamani & Srinivasan, 2014). This study concluded that germination drastically reduced polyphenols bioaccessibility in chickpea unlike the positive effects reported in phenolics bioaccessibility for pressure-cooking, open-pan boiling and microwave heating.

3.4. Effect of processing and digestion on the anti-inflammatory potential of chickpea protein concentrate

While NO levels are generally low in macrophages, they are increased dramatically by acute inflammatory responses due to newly synthesis by inducible nitric oxide synthase (Elisia et al., 2017). Therefore, analysis of NO levels in RAW264.7 macrophage cell line has been performed to examine bioactivity and support molecular pathways related with anti-inflammatory effect of active phytochemicals. To study the individual contribution of protein/peptides and phenolic compounds to the anti-inflammatory activity, screening and potency determination was performed for soluble protein/peptide (**Fig. 2a-c**) and phenolic (**Fig. 2d-f**) extracts from each chickpea sample.

3.4.1. Cytotoxic effect of treatments

Phenolic and protein fractions were evaluated for cytotoxicity at the highest concentration tested (0.5 mg/mL and 5 mg/mL, respectively). None of the samples reduced cell viability below 85% of the control and thus phenolic and protein/peptide fractions were not considered cytotoxic at concentrations varying from 0.5 and 5 mg/mL (data not shown).

3.4.2. Anti-inflammatory effects of chickpea protein/peptides

RC and GC protein fractions did not significantly reduce NO levels at 5 mg/mL as compared to control cells (data not shown), therefore, results indicate that native proteins from RC and GC did not have the ability to exert anti-inflammatory effects. Protein fraction from CC concentrates could not be evaluated due to its poor solubility and tendency to precipitate. Protein/peptide fractions from GC concentrates slightly reduced NO production with 33.64% and 25.60% for Kabuli and Desi varieties, respectively at 5 mg/mL (**Fig. 2a**), which indicated their limited anti-inflammatory capabilities. Interestingly, protein digests from CC and GC were more effective to inhibit NO production ($p<0.05$) at 5 mg/mL. To ascertain the breadth of the anti-inflammatory effect of CC and GC protein digests, their activity was confirmed at lower doses (**Fig. 2b**). GC protein digests showed higher potency (average $IC_{50} = 2.01$ mg/mL) than CC counterparts (average $IC_{50} = 3.55$ mg/mL) without significant difference between varieties (**Fig. 2c**). This observation was probably related to peptide content of protein digests. To confirm this hypothesis a correlation analysis was performed resulting in a significant positive correlation between inhibition of NO production and peptides content of chickpea samples ($r=0.9490$, $p=0.0001$).

Peptides derived from *in vitro* digestion of raw chickpea proteins have shown antioxidant activity in previous reports (Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015). However, results from the present study provide evidence for the first time that remaining chickpea peptides in gastrointestinal digests show anti-inflammatory potential

what would be beneficial for health maintenance in the gut. This finding is potentially disruptive, as most studies showing beneficial effects of chickpea consumption in the gut have focused on fiber and polyphenols as the principal bioactive species (Chino et al., 2017; Monk et al., 2017). In the present study it was evaluated the anti-inflammatory potential of protein concentrates from germinated chickpea. Protein concentrates from germinated chickpea could have many applications as nutritional supplements or ingredients in beverages and foods that would not require thermal treatment. For these applications, protein concentrate from chickpea sprouts show promise as a better source of non-digestible peptides than thermal treated concentrate to contribute to the prevention of gut-inflammatory diseases. In fact, the anti-inflammatory potency of GC protein digests almost 5-fold higher than the one reported for germinated soybean protein digests (González-Montoya et al., 2018). These findings warrant future *in vivo* studies related to gut health benefits of diets supplemented with GC protein concentrates.

3.4.2 Anti-inflammatory effect of chickpea isoflavones

Phenolic extracts of chickpea samples inhibited NO production from 10% to 72% at 0.5 mg/ml (**Fig. 2a**). Chickpea samples from Desi variety either germinated or cooked had higher anti-inflammatory activity than Kabuli counterparts. The only exception was GC protein concentrates and digests in which lower or similar anti-inflammatory activity were found for Desi variety at 0.5 mg/mL. In general, anti-inflammatory activity of phenolic extracts was RC flour $\leq$ CC protein concentrate $\leq$ GC flour $<$ GC protein concentrate $\leq$ CC protein digest $<$ GC digest (**Fig. 2a**). Phenolic extracts from CC and GC protein digests inhibited much more effectively (**Fig. 2a**) and dose-dependently the production of NO in LPS-challenged macrophages (**Fig. 2b**). IC₅₀ values for phenolic extracts from CC digest were greater (average 0.5 mg/mL) than GC digests (average 0.2 mg/mL) and there was no significant difference between varieties. These results indicated that phenolic compounds

remaining after *in vitro* digestion of GC protein concentrate could attenuate more effectively inflammation in the lower gut than the ones in CC. Moreover, as compared to protein/peptide fractions, phenolic compounds were most potent anti-inflammatory compounds in CC and GC protein digests.

Correlation analysis between inhibition of NO production and total phenolic compounds, formononetin and biochanin A concentrations of each phenolic extract was performed. Significant positive correlations were obtained for TPC ($r=0.8358$, $p<0.0001$), formononetin ($r=0.5935$, $p<0.0419$), and biochanin A ($r=0.6742$, $p<0.0162$). These results agree with previous research showing the anti-inflammatory effects of raw chickpea phenolic extracts and isolated isoflavones. Phenolic compounds extracted from Egyptian chickpea cultivar Giza 1 lowered the hepatic tumor necrosis factor α in an *in vivo* model of acute toxicity (Mekky et al., 2016). Biochanin-A attenuated the NO and pro-inflammatory cytokines production in LPS-stimulated RAW 264.7 macrophages (Kole et al., 2011). The mechanisms involved in these effects were attributed to downregulation of inducible nitric oxide synthase (iNOS) gene expression, the enzyme involved in NO synthesis. Likewise, formononetin has shown anti-inflammatory effects on LPS-induced RAW267.4 macrophages reducing in a greater extent prostaglandin E_2 and D_2 production (Shin, Peng, Kang, & Choi, 2016). Moreover, isoflavones and other phenolic compounds are known to suppress intestinal inflammation (Shimizu, 2017).

3.5 Identification of potential anti-inflammatory peptides

In order to identify which peptides were the main contributors to the anti-inflammatory effects of GC protein digests from Kabuli and Desi varieties, both samples were fractionated by RP-LC-MS. Chromatographic profiles were similar for both varieties **Fig. 3a** shows as an example the representative chromatogram and sub-fractions collected from the protein digest of Kabuli variety. The resulting separation yielded 3 sub-fractions for each variety

named F1, F2 and F3. The three sub-fractions were collected and analyzed for peptides contents and anti-inflammatory effects (**Fig. 3b**). The isolated sub-fractions were re-evaluated utilizing the same inflammatory *in vitro* cell system at concentrations ranging from 0.05-1 mg/mL. F1 fraction represented 79% and 87% of the total weight of protein digests for Desi and Kabuli varieties, respectively. Although this fraction effectively reduced NO production in LPS-activated macrophages (average IC_{50} =160 μ g/g) its peptide purity was low (only 16-17% of F1 was composed of peptides). F2 fraction represented less than 5% of the total weight of protein digest in which peptide concentration varied between 18% and 30% for Kabuli and Desi varieties. In addition, F2 did not show anti-inflammatory effects. Contrasting results were observed for F3, which represented 8 and 18% of the parental digest for Kabuli and Desi varieties, respectively. This particular fraction showed the highest anti-inflammatory potential (average IC_{50} =93 μ g/g) and purity (98% of F3 was composed of peptides). F3 of Kabuli and Desi varieties, was further analyzed by LC-MS/MS. Peptide profile was similar for both varieties and results are summarized in **Table 3**. Peptides contributing to the anti-inflammatory effect of GC protein digest were identified for the first time which is one of the merits of this study. A total of 24 different peptide sequences were successfully identified as fragments from storage (legumins, vicilin-like proteins, globulin-1-S allele like), structural (P24 oleosin isoform A), nutrient reservoir (sucrose-binding protein) and catabolic (adenosylhomocysteinase) proteins. Legumins and vicilin-like proteins of raw and soaked+cooked chickpeas are known to resist *in vitro* digestion (Ribeiro et al., 2017; Torres-Fuentes et al., 2015) due to very stable compact β -barrel structural motif that could make protease cleavage sites inaccessible (Mills, Jenkins, Alcocer, & Shewry, 2004) which is in agreement to the findings showed herein.

A number of food-derived peptides, particularly from soy, fish and shell fish have shown anti-inflammatory properties on LPS-stimulated murine RAW 264.7 cells, as

estimated by different markers associated with beneficial effects against inflammation and oxidative stress. To date, the structure-activity relationship for anti-inflammatory peptides is still unclear, although it was found some similarities within the amino acid sequence of peptides identified in F3 and previous identified food-derived anti-inflammatory peptides. Glutamine- and poly-glutamine-containing peptides from wheat, soy and fish proteins have shown anti-inflammatory effects in LPS-treated RAW 264.7 macrophages reducing the production of pro-inflammatory mediators through a mechanism involving the inhibition of nuclear factor κ B (NF- κ B) activation (González-Montoya et al., 2018; Lozano-Ojalvo & López-Fandiño, 2017). This is, for instance, the case of fragments LHQNIGSSSSPDIYNPQAGR, LHQNIGSSSSPDIYNPQAGRIK, QQSQETDVIVK, VLLEEQEQQPK, DVGQKTKEVGQDIQAK that showed at least two glutamine residues.

4. Conclusions

This study has demonstrated that protein concentrates produced from GC had a promising potential to exert anti-inflammatory effects in the lower gut. This fact was determined by the lower protein digestibility and bioaccessibility of peptides and isoflavones in GC digest compared to CC digest. These findings provide the idea that besides non-digestible carbohydrates and phenolics, the hydrolysed protein fraction may also contribute to the anti-inflammatory activity of chickpea.

Acknowledgments

A.K. Milán-Noris, acknowledges CONACYT (Consejo Nacional de Ciencia y Tecnología) for the scholarship for PhD studies (233598) and Beca Mixta. This research was supported by the Ministry of Economy and Competitiveness, Spanish National Research Council (MINECO, Spain), FEDER program (grant numbers AGL2013-43247-R and 201670I044), CONACYT, and NutriOmics group funds from Tecnológico de Monterrey.

Conflict of interest statement

All authors of this research paper declare not conflict of interest.

References

- Aguilera, Y., Duenas, M., Estrella, I., Hernandez, T., Benitez, V., Esteban, R. M., & Martin-Cabrejas, M. A. (2011). Phenolic profile and antioxidant capacity of chickpeas (*Cicer arietinum* L.) as affected by a dehydration process. *Plant Foods for Human Nutrition*, 66(2), 187-195.
- Bar-El Dadon, S., Abbo, S., & Reifen, R. (2017). Leveraging traditional crops for better nutrition and health - The case of chickpea. *Trends in Food Science and Technology*, 64, 39-47.
- Bewley, J. D., Bradford, K. J., Hilhorst, H. W. M., & Nonogaki, H. (2013). Germination. In J. D. Bewley, K. J. Bradford, H. W. M. Hilhorst, & H. Nonogaki (Eds.), *Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition* (pp. 133-181). New York, NY: Springer New York.
- Chang, Y.-W., Alli, I., Molina, A. T., Konishi, Y., & Boye, J. I. (2012). Isolation and characterization of chickpea (*Cicer arietinum* L.) seed protein fractions. *Food and Bioprocess Technology*, 5(2), 618-625.
- Chen, P. X., Dupuis, J. H., Marcone, M. F., Pauls, P. K., Liu, R., Liu, Q., Zhang, B., Tsao, R. (2015). Physicochemical properties and in vitro digestibility of cooked regular and nondarkening cranberry beans (*Phaseolus vulgaris* L.) and their effects on bioaccessibility, phenolic composition, and antioxidant activity. *Journal of Agricultural and Food Chemistry*, 63(48), 10448-10458.
- Chino, X. M. S., Martínez, C. J., Garzón, V. R. V., González, I. Á, Treviño, S. V., Bujaidar, E. M., Ortiz, G. D., Hoyos, R. B. (2017). Cooked chickpea consumption inhibits

613 colon carcinogenesis in mice induced with azoxymethane and dextran sulfate
 614 sodium. *Journal of the American College of Nutrition*, 36(5), 391-398.

615 Elisia, I., Pae, H. B., Lam, V., Cederberg, R., Hofs, E., & Krystal, G. (2018). Comparison of
 616 RAW264.7, human whole blood and PBMC assays to screen for immunomodulators.
 617 *Journal of Immunological Methods*, 452, 26-31.

618 FAOSTAT. (2014). Chickpea world production. FAOSTAT Retrieved April, 21, 2016
 619 <http://faostat3.fao.org/home/E>

620 González-Montoya, M., Hernández-Ledesma, B., Silván, J. M., Mora-Escobedo, R., &
 621 Martínez-Villaluenga, C. (2018). Peptides derived from in vitro gastrointestinal
 622 digestion of germinated soybean proteins inhibit human colon cancer cells
 623 proliferation and inflammation. *Food Chemistry*, 242, 75-82.

624 Guardado-Félix, D., Serna-Saldivar, S. O., Cuevas-Rodríguez, E. O., Jacobo-Velázquez, D.
 625 A., & Gutiérrez-Urbe, J. A. (2017). Effect of sodium selenite on isoflavonoid
 626 contents and antioxidant capacity of chickpea (*Cicer arietinum* L.) sprouts. *Food*
 627 *Chemistry*, 226, 69-74.

628 Gupta, R. K., Gupta, K., Sharma, A., Das, M., Ansari, I. A., & Dwivedi, P. D. (2017).
 629 Health risks and benefits of chickpea (*Cicer arietinum*) consumption. *Journal of*
 630 *Agricultural and Food Chemistry*, 65(1), 6-27.

631 Heiras-Palazuelos, M. J., Ochoa-Lugo, M. I., Gutiérrez-Dorado, R., López-Valenzuela, J.
 632 A., Mora-Rochín, S., Milán-Carrillo, J., Garzon-Tiznado, J. A., Reyes-Moreno, C.
 633 (2013). Technological properties, antioxidant activity and total phenolic and
 634 flavonoid content of pigmented chickpea (*Cicer arietinum* L.) cultivars.
 635 *International Journal of Food Sciences and Nutrition*, 64(1), 69-76.

636 Hithamani, G., & Srinivasan, K. (2014). Bioaccessibility of polyphenols from wheat
 637 (*Triticum aestivum*), sorghum (*Sorghum bicolor*), green gram (*Vigna radiata*), and

638 chickpea (*Cicer arietinum*) as influenced by domestic food processing. *Journal of*
639 *Agricultural and Food Chemistry*, 62(46), 11170-11179.

640 Jamdar, S. N., Deshpande, R., & Marathe, S. A. (2017). Effect of processing conditions and
641 *in vitro* protein digestion on bioactive potentials of commonly consumed legumes.
642 *Food Bioscience*, 20, 1-11.

643 Jukanti, A. K., Gaur, P. M., Gowda, C. L. L., & Chibbar, R. N. (2012). Nutritional quality
644 and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of*
645 *Nutrition*, 108(SupplementS1), S11-S26.

646 Kole, L., Giri, B., Manna, S. K., Pal, B., & Ghosh, S. (2011). Biochanin-A, an isoflavon,
647 showed anti-proliferative and anti-inflammatory activities through the inhibition of
648 iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NFκB
649 nuclear translocation. *European Journal of Pharmacology*, 653(1), 8-15.

650 Laguna, L., Picouet, P., Guàrdia, M. D., Renard, C. M. G. C., & Sarkar, A. (2017). In vitro
651 gastrointestinal digestion of pea protein isolate as a function of pH, food matrices,
652 autoclaving, high-pressure and re-heat treatments. *LWT - Food Science and*
653 *Technology*, 84, 511-519.

654 Lozano-Ojalvo, D., & López-Fandiño, R. (2017). Immunomodulating peptides for food
655 allergy prevention and treatment. *Critical Reviews in Food Science and Nutrition*, 1-
656 21.

657 Mamilla, R. K., & Mishra, V. K. (2017). Effect of germination on antioxidant and ACE
658 inhibitory activities of legumes. *LWT - Food Science and Technology*, 75, 51-58.

659 Martinez-Villaluenga, C., Dia, V. P., Berhow, M., Bringe, N. A., & Gonzalez de Mejia, E.
660 (2009). Protein hydrolysates from beta-conglycinin enriched soybean genotypes
661 inhibit lipid accumulation and inflammation *in vitro*. *Molecular Nutrition and Food*
662 *Research*, 53(8), 1007-1018.

663 Mas, S., Oeo-Santos, C., Cuesta-Herranz, J., Díaz-Perales, A., Colás, C., Fernández, J.,
 664 Barber, D., Rodríguez, R., de los Ríos, V., Barderas, R., Villalba, M. (2017). A
 665 relevant IgE-reactive 28 kDa protein identified from *Salsola kali* pollen extract by
 666 proteomics is a natural degradation product of an integral 47kDa polygalacturonase.
 667 *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1865, 8, 1067-
 668 1076.

669 Megías, C., Cortés-Giraldo, I., Alaiz, M., Vioque, J., & Girón-Calle, J. (2016). Isoflavones
 670 in chickpea (*Cicer arietinum*) protein concentrates. *Journal of Functional Foods*, 21,
 671 186-192.

672 Mekky, R. H., Fayed, M. R., El-Gindi, M. R., Abdel-Monem, A. R., Contreras, M. M.,
 673 Segura-Carretero, A., & Abdel-Sattar, E. (2016). Hepatoprotective effect and
 674 chemical assessment of a selected Egyptian chickpea cultivar. *Frontiers in*
 675 *Pharmacology*, 7(SEP).

676 Mills, E. N. C., Jenkins, J. A., Alcocer, M. J. C., & Shewry, P. R. (2004). Structural,
 677 biological, and evolutionary relationships of plant food allergens sensitizing via the
 678 gastrointestinal tract. *Critical Reviews in Food Science and Nutrition*, 44(5), 379-
 679 407.

680 Mondor, M., Aksay, S., Drolet, H., Roufik, S., Farnworth, E., & Boye, J. I. (2009). Influence
 681 of processing on composition and antinutritional factors of chickpea protein
 682 concentrates produced by isoelectric precipitation and ultrafiltration. *Innovative*
 683 *Food Science and Emerging Technologies*, 10(3), 342-347.

684 Monk, J. M., Lepp, D., Wu, W., Graf, D., McGillis, L. H., Hussain, A., Carey, C., Robinson
 685 L. E., Liu, R., Tsao, R., Brummer, Y., Tosh, S. M., Power, K. A. (2017). Chickpea-
 686 supplemented diet alters the gut microbiome and enhances gut barrier integrity in
 687 C57Bl/6 male mice. *Journal of Functional Foods*, 38, 663-674.

688 Mosele, J. I., Macià, A., Romero, M.-P., & Motilva, M.-J. (2016). Stability and metabolism
689 of *Arbutus unedo* bioactive compounds (phenolics and antioxidants) under in vitro
690 digestion and colonic fermentation. *Food Chemistry*, 201, 120-130.

691 Parmar, N., Singh, N., Kaur, A., Virdi, A. S., & Thakur, S. (2016). Effect of canning on
692 color, protein and phenolic profile of grains from kidney bean, field pea and
693 chickpea. *Food Research International*, 89, 526-532.

694 Patterson, C. A., Curran, J., & Der, T. (2017). Effect of processing on antinutrient
695 compounds in pulses. *Cereal Chemistry*, 94(1), 2-10.

696 Piegholdt, S., Pallauf, K., Esatbeyoglu, T., Speck, N., Reiss, K., Ruddigkeit, L., Stocker, A.,
697 Huebbe, P., Rimbach, G. (2014). Biochanin A and prunetin improve epithelial
698 barrier function in intestinal Caco-2 cells via downregulation of ERK, NF-κB, and
699 tyrosine phosphorylation. *Free Radical Biology and Medicine*, 70, 255-264.

700 Ribeiro, I. C., Leclercq, C. C., Simões, N., Toureiro, A., Duarte, I., Freire, J. B., Chaves, M.
701 M., Renaut, J., Pinheiro, C. (2017). Identification of chickpea seed proteins resistant
702 to simulated in vitro human digestion. *Journal of Proteomics*, 169, 143-152.

703 Segev, A., Badani, H., Kapulnik, Y., Shomer, I., Oren-Shamir, M., & Galili, S. (2010).
704 Determination of polyphenols, flavonoids, and antioxidant capacity in colored
705 chickpea (*Cicer arietinum* L.). *Journal of Food Science*, 75(2), S115-S119.

706 Shimizu, M. (2017). Multifunctions of dietary polyphenols in the regulation of intestinal
707 inflammation. *Journal of Food and Drug Analysis*, 25(1), 93-99.

708 Shin, J. S., Peng, L., Kang, K., & Choi, Y. (2016). Direct analysis of prostaglandin-E2 and -
709 D2 produced in an inflammatory cell reaction and its application for activity
710 screening and potency evaluation using turbulent flow chromatography liquid
711 chromatography-high resolution mass spectrometry. *Journal of Chromatography A*,
712 1463, 128-135.

713 Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2017). Phenolic composition and antioxidant
714 potential of grain legume seeds: A review. *Food Research International*, 101, 1-16.

715 Torres-Fuentes, C., Contreras, M. D. M., Recio, I., Alaiz, M., & Vioque, J. (2015).
716 Identification and characterization of antioxidant peptides from chickpea protein
717 hydrolysates. *Food Chemistry*, 180, 194-202.

718 Vinson, J. A., Proch, J., & Bose, P. (2001). Determination of quantity and quality of
719 polyphenol antioxidants in foods and beverages. *Methods in Enzymology*, 335, 103-
720 114.

721 Wang, X., Gao, W., Zhang, J., Zhang, H., Li, J., He, X., & Ma, H. (2010). Subunit, amino
722 acid composition and *in vitro* digestibility of protein isolates from Chinese kabuli
723 and desi chickpea (*Cicer arietinum* L.) cultivars. *Food Research International*,
724 43(2), 567-572.

725 Wu, Z., Song, L., Feng, S., Liu, Y., He, G., Yioe, Y., Liu, S. Q., Huang, D. (2012).
726 Germination dramatically increases isoflavonoid content and diversity in chickpea
727 (*Cicer arietinum* L.) seeds. *Journal of Agricultural and Food Chemistry*, 60(35),
728 8606-8615.

729 Xu, Y., Obielodan, M., Sismour, E., Arnett, A., Alzahrani, S., & Zhang, B. (2017).
730 Physicochemical, functional, thermal and structural properties of isolated Kabuli
731 chickpea proteins as affected by processing approaches. *International Journal of*
732 *Food Science and Technology*, 52(5), 1147-1154.

733 Xue, Z., Wen, H., Zhai, L., Yu, Y., Li, Y., Yu, W., Li, Y., Yu, W., Cheng, A., Wang, C.,
734 Kou, X. (2015). Antioxidant activity and anti-proliferative effect of a bioactive
735 peptide from chickpea (*Cicer arietinum* L.). *Food Research International*, 77, Part
736 2, 75-81.

FIGURE CAPTIONS

Fig. 1. a) SDS-PAGE protein profile of flours, protein concentrates and digests of Kabuli and Desi chickpea varieties. RC: raw chickpea, CC: cooked chickpea, GC: germinated chickpea. STD: Novex sharp unstained protein standard (Life technologies, Madrid, Spain).

Fig. 2. *In vitro* anti-inflammatory activity of protein/peptide fractions (panels a-c) and phenolic extracts (panels d-f) from Kabuli and Desi varieties as function of processing and digestion. a) Effect of protein/peptides (5 mg/mL) from flour, protein concentrates and digests on NO inhibition b) Dose-response effect of peptides in cooked (CC) and germinated chickpea (GC) protein digests on NO production c) IC₅₀ values (mg/mL) of peptides in CC and GC protein digests on NO inhibition; d) Effect of phenolic extracts (0.5 mg/mL) from flour, protein concentrates and digests on NO production; e) Dose-response effect of phenolic extracts from CC and GC protein digests on NO production; f) IC₅₀ values (mg/mL) of phenolics extracts from CC and GC protein digests on NO production; RAW 267.4 macrophages were pretreated with chickpea samples for 20 h and inflammatory response was induced with 1 µg/mL LPS for 20 h. Percentage inhibition on NO production is reported relative to untreated control cells as mean response ± standard deviation of 6 replicates (n = 6). Means with different letter are significantly different (p>0.05). RC: raw chickpea, CC: cooked chickpea, GC: germinated chickpea.

Fig. 3. Purification of anti-inflammatory peptides from germinated chickpea (GC) protein digests of Kabuli and Desi a) Representative chromatogram at 280 nm of peptides fractions collected by preparative RP-HPLC-ESI- MS; b) Recovery (% of fraction weight relative to weight of protein digest), peptide content (mg/g freeze-dried weight), and IC₅₀ values (µg/mL) for F1, F2 and F3 sub-fractions to inhibit 50% the NO production in LPS-induced

763 RAW 294.7 macrophages. RAW 267.4 macrophages were pretreated with chickpea samples
764 for 20 h and inflammatory response was induced with 1 µg/mL LPS for 20 h. Values are
765 means ± standard deviation of 6 replicates (n = 6). Lowercase letters indicate significant
766 differences among treatments for each chickpea variety ($p<0.05$). Uppercase letters indicate
767 significant differences between chickpea varieties ($p<0.05$).

768

Table 1. Soluble protein and peptide contents in flours, protein concentrates and digests from cooked (CC) and germinated (GC) Kabuli and Desi chickpea varieties

Samples	Soluble protein		Peptides	
	(mg/g freeze-dried weight)		(mg/g freeze-dried weight)	
	Kabuli	Desi	Kabuli	Desi
RC flour	187.7 ± 22.3 ^{c,A}	120.8 ± 3.4 ^{c,B}	nd	nd
GC flour	250.7 ± 21.0 ^{c,A}	152.1 ± 12.7 ^{c,B}	nd	nd
CC protein concentrate	398.1 ± 18.6 ^{b,B}	526.9 ± 33.6 ^{a,A}	26.8 ± 1.9 ^{c,A}	26.2 ± 0.4 ^{c,A}
GC protein concentrate	453.7 ± 35.3 ^{b,B}	515.9 ± 28.4 ^{a,A}	28.9 ± 1.5 ^{c,A}	25.1 ± 2.1 ^{c,A}
CC protein digest	455.8 ± 29.7 ^{b,A}	262.5 ± 20.5 ^{b,B}	345.7 ± 25.9 ^{b,A}	195.6 ± 5.8 ^{b,B}
GC protein digest	590.0 ± 56.4 ^{a,A}	564.8 ± 46.8 ^{a,A}	469.3 ± 24.4 ^{a,A}	441.2 ± 22.8 ^{a,A}

Values are means ± standard deviation of three replicates. Lowercase letters within a column indicate significant differences among treatments ($p < 0.05$). Uppercase letters within a row indicate significant differences between chickpea varieties ($p < 0.05$). nd: not detected. RC: raw chickpea; CC: cooked chickpea; GC: Germinated chickpea.

Table 2. Isoflavones ($\mu\text{g/g}$ freeze-dried weight), total isoflavones content (TIC, $\mu\text{g/g}$ freeze-dried weight) and total phenolic content (TPC, mg GAE/g freeze-dried weight) in flours, protein concentrates and digests from cooked (CC) and germinated (GC) Kabuli and Desi chickpea varieties

	Peak										TIC	TPC
	1	2	3	4	5	6	7	8	9	10		
Kabuli RC flour	nd	nd	27 $\pm$ 0 ^{b,B}	9 $\pm$ 2 ^{b,B}	nd	nd	nd	nd	nd	13 $\pm$ 0 ^{f,A}	48 $\pm$ 1 ^{d,B}	0.26 $\pm$ 0.02 ^{c,B}
GC flour	163 $\pm$ 0	1545 $\pm$ 27 ^A	120 $\pm$ 0 ^a	1075 $\pm$ 25 ^{a,A}	nd	nd	226 $\pm$ 3 ^{c,B}	nd	nd	592 $\pm$ 2 ^{c,B}	3725 $\pm$ 59 ^{b,B}	2.5 $\pm$ 0.1 ^{d,AB}
CC protein concentrate	nd	nd	nd	nd	tr	7 $\pm$ 0 ^{c,A}	102 $\pm$ 12 ^{cd,A}	3 $\pm$ 0 ^{a,B}	tr	119 $\pm$ 23 ^{d,A}	233 $\pm$ 36 ^{d,A}	0.4 $\pm$ 0.0 ^{c,A}
GC protein concentrate	nd	nd	nd	nd	138 $\pm$ 5 ^{a,A}	119 $\pm$ 8 ^{b,A}	1366 $\pm$ 34 ^{b,A}	nd	86 $\pm$ 5 ^{b,A}	1718 $\pm$ 8 ^{b,A}	3431 $\pm$ 65 ^{c,A}	4.6 $\pm$ 0.0 ^{c,A}
CC protein digest	nd	nd	nd	nd	tr	3 $\pm$ 0 ^{c,A}	11 $\pm$ 0 ^{d,A}	3 $\pm$ 0 ^{a,B}	2 $\pm$ 0 ^{c,A}	59 $\pm$ 1 ^{de,A}	80 $\pm$ 3 ^{d,A}	7.4 $\pm$ 0.2 ^{b,A}
GC protein digest	nd	nd	nd	nd	67 $\pm$ 0 ^{b,B}	237 $\pm$ 37 ^{a,B}	3313 $\pm$ 133 ^{a,A}	nd	135 $\pm$ 10 ^{a,A}	3443 $\pm$ 43 ^{a,A}	7196 $\pm$ 225 ^{a,A}	11.7 $\pm$ 0.3 ^{a,A}
Desi RC flour	nd	nd	92 $\pm$ 5 ^A	26 $\pm$ 0 ^{b,A}	nd	nd	nd	nd	nd	17 $\pm$ 1 ^{d,A}	135 $\pm$ 4 ^{d,A}	0.5 $\pm$ 0.0 ^{c,A}
GC flour	nd	981 $\pm$ 15 ^B	nd	781 $\pm$ 55 ^{a,B}	nd	238 $\pm$ 5 ^b	3385 $\pm$ 121 ^{a,A}	nd	nd	1565 $\pm$ 54 ^{c,A}	6953 $\pm$ 140 ^{a,A}	3.1 $\pm$ 0.0 ^{d,A}
CC protein concentrate	nd	nd	nd	nd	tr	4 $\pm$ 0 ^{d,A}	19 $\pm$ 1 ^{d,B}	7 $\pm$ 0 ^{a,A}	tr	18 $\pm$ 0 ^{d,B}	50 $\pm$ 1 ^{d,B}	0.5 $\pm$ 0.0 ^{c,B}
GC protein concentrate	nd	nd	nd	nd	77 $\pm$ 2 ^{b,B}	72 $\pm$ 6 ^{c,B}	1407 $\pm$ 124 ^{c,A}	nd	73 $\pm$ 10 ^{b,A}	1841 $\pm$ 122 ^{b,A}	3479 $\pm$ 265 ^{c,A}	4.0 $\pm$ 0.0 ^{c,B}
CC protein digest	nd	nd	nd	nd	tr	4 $\pm$ 0 ^{d,A}	6 $\pm$ 0 ^{d,B}	6 $\pm$ 1 ^{a,A}	3 $\pm$ 0 ^{c,A}	12 $\pm$ 1 ^{d,B}	33 $\pm$ 3 ^{d,B}	4.5 $\pm$ 0.0 ^{b,B}
GC protein digest	nd	nd	nd	nd	126 $\pm$ 4 ^{a,A}	287 $\pm$ 13 ^{a,A}	2816 $\pm$ 54 ^{b,B}	nd	141 $\pm$ 5 ^{a,A}	3015 $\pm$ 27 ^{a,B}	6386 $\pm$ 104 ^{b,B}	9.9 $\pm$ 0.2 ^{a,B}

Values are means $\pm$ standard deviation of three replicates. Lowercase letters indicate significant differences among treatments for each chickpea variety ($p < 0.05$). Uppercase letters indicate significant differences between chickpea varieties ($p < 0.05$). Identified compounds: 1: Isoformononetin glycoside, 2: Formononetin malonyl glycoside, 3: Biochanin-A glycoside, 4: Biochanin-A malonyl glycoside, 5: isoflavonoid derivative, 6: Pseudobaptigenin, 7: Formononetin, 8: isoflavonoid derivative, 9: 5-hydroxypseudobaptigenin, 10: Biochanin-A. nd: not detected; tr: traces (detected amounts that were below the limit of quantification)

Table 3. Peptides identified by nanoUPLC-ESI-MS/MS in fraction F3 from Kabuli and Desi GC protein digests*

Source protein	Accession number	Sequence	Fragment	MH+(Da)
Legumin	A0A1S2XTK6	SEGLIETWNPSNK	47-60	1531.73
		NEDEEKGAIVKVK	238-250	1458.77
		VKGGLSIITPPEKEPR	249-264	1720.99
		GGLSIITPPEKEPR	251-264	1493.83
		GGLSIITPPEKEPRQK	251-266	1749.98
Vicilin	A0A1S2Y087	LHQNIGSSSPDIYNPQAGR	324-343	2141.03
		LHQNIGSSSPDIYNPQAGRIK	324-345	2382.21
		GRRGSEEEEGDAIVK	343-358	1718.82
		RGSEEEEGDAIVK	345-358	1505.70
		GSEEEEGDAIVK	356-358	1349.60
		SRNPIYSNKFVK	390-401	1410.75
		ISREQIEELSKNAK	221-234	1644.89
		QQSQETDVIVK	211-220	1274.66
		EQIEELSKNAK	224-234	1288.67
		ISREQIEELSKNAK	221-234	1644.89
Provicilin	Q304D4	VLEEQEQKPK	186-196	1340.74
		EQIEELSKNAK	224-234	1288.67
		SPIYSNKFVK	375-384	1140.60
P24 oleosin isoform A	A0A1S2XXJM3	DVGQKTKEVGQDIQAK	170-185	1743.92
Sucrose-binding protein-like	A0A1S2XXVJ8	IFKISKEDVHGLAPK	259-273	1681.96
Legumin A2	A0A1S3E0V2	HIVDKLQGRDEDEEK	166-180	1810.89
Globulin-1 S allele	A0A1S2YZ56	SREEETTEWEEEVAK	596-610	1851.82
Legumin J	A0A1S2XVG1	FSGNRGPLVQPR	516-527	1327.72
Adenosylhomocysteinase	A0A1S2YCQ2	IVGVSEETTTGVK	199-211	1319.70

*F3 was collected by RP-HPLC at 18-20 min from Kabuli and Desi GC protein digest

Fig. 1

Fig. 2.

Fig. 3.

a)

b)

Variety	Fraction	Recovery (%)	Peptides (mg/g freeze-dried weight)	IC ₅₀ (μg/mL)
Kabuli	F1	87.24	162.20 ± 8.23 ^{b,A}	161.40 ± 24.03 ^{a,A}
	F2	4.96	179.67 ± 12.49 ^{b,A}	>1000
	F3	7.79	969.06 ± 23.78 ^{a,A}	90.70 ± 20.21 ^{b,A}
Desi	F1	79.47	169.06 ± 5.00 ^{c,A}	158.41 ± 34.39 ^{a,A}
	F2	1.54	296.75 ± 8.86 ^{b,B}	>1000
	F3	18.98	986.16 ± 3.38 ^{a,A}	97.31 ± 19.13 ^{b,A}

Supplementary Material

[Click here to download Supplementary Material: Supplemental information.docx](#)