Optimal germination condition impacts on the antioxidant activity and phenolic acids profile in pigmented desi chickpea (*Cicer arietinum* L.) seeds

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Abstract Legume sprouts are considered natural, healthy products that provide a source of bioactive compounds to fight against chronic diseases. This study aims to identify the optimal germination temperature (GT) and germination time (Gt) to maximize total phenolic and flavonoid contents (TPC, FC), and antioxidant activity (AoxA) of desi chickpea. Response surface methodology was used as an optimization tool. An experimental design with two factors (GT and Gt) and five levels was used (13 treatments). The sprouts from each treatment were lyophilized, tempered, and milled to obtain germinated chickpea flours (GCF). To predict the phytochemicals composition and AoxA in GCF, regression models were developed. Maximum TPC, FC, and AoxA were attained during germination 33.7 °C for 171 h. Optimized germinated chickpea flour produced applying the optimal germination conditions resulted in an increase of protein and total dietary fibre content, TPC, FC, phenolic acids profile, and AoxA. Germination at optimal conditions also increased the level of coumaric, ferulic,

synapic, ellagic, and syringic acids. This study demonstrated that germination carried out under optimal conditions enhanced the nutraceutical value of desi chickpea seeds.

Keywords Desi chickpea · Germination · Antioxidant activity · Phenolic acids

Introduction

Legumes have high nutritional value and play a major role in traditional diets throughout the world. Epidemiological studies have confirmed that regular consumption of legumes can increase lifespan by preventing chronic diseases, including cardiovascular disease, diabetes, cancer, and obesity, as well as improving gut health (Curran 2012). Legume sprouts are extensively accepted since they are considered healthy products from natural origin and a source of bioactive compounds (Megat et al. 2016). Germination is seen as a simple and cost-effective process that improves the nutritional value of grains by changing the structure of protein and starch, and thus increasing protein digestibility, and the content of free amino acids and other components (Ghavidel and Prakash 2007; Ghumman et al. 2016). It also improves the functionality of the grains by increasing the number of bioactive compounds and their associated antioxidant activity (Wu et al. 2012). Examples of these bioactive compounds are the polyphenols, which have been linked to DNA-damage protection and prevention of risk of degenerative diseases due to their antioxidant potential (Nderitu et al. 2013).

Epidemiological studies showed that the consumption of high phenolic content-foods is correlated with the reduction of different pathologies, such as cardiovascular and

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inflammatory diseases, and cancer (Shams-Ardekani et al. 2011). Chickpeas (*Cicer arietinum* L.) are the second-most important legume crop in the world (after dry beans) with a worldwide production of 13.12 Mt in 2013 (FAOSTAT 2015). Chickpeas are an excellent source of protein (17–25%, dw), carbohydrates, minerals (Ca, Mg, Zn, K, Fe, P), vitamins (such as thiamine and niacin), and unsaturated fatty acids such as oleic and linolenic acids (Heiras-Palazuelos et al. 2013). Dry chickpea seeds contain different phenolic compounds, among them, phenolic acids such as gallic, caffeic, vanillic, coumaric, and ferulic. These compounds are biologically active and have been associated with anti-diabetic, and anti-carcinogenic properties (Vinayagam et al. 2015; Parmar et al. 2016).

Chickpea seeds are classified into two categories: desi and kabuli (Fig. 1). Kabuli-type seeds are bigger than the desi-type (the 100-seed weight varies from 28 to 70 g), have a thin seed coat, and their colors vary from white to cream (Segev et al. 2012). The desi-type seeds are small (the 100-seed weight is less than 28 g), have a thick, irregular shaped seed coat, and their colors vary from light to black. Also, Heiras-Palazuelos et al. (2013) reported significant differences among these chickpea varieties, regarding other properties such as water absorption capacity, cooking time, hardness-to-cook, proximate composition, protein digestibility, total phenolic content, and antioxidant activity. Some researchers concluded that the dark-colored and pigmented pulses (including chickpea) tend to have higher phenolic content compared to the light-colored varieties and the content of phenolics change during storage and processing (Segev et al. 2010; Parmar et al. 2017; Singh 2017) of the grains. Other investigators found that chickpea seeds with a colorful coating exhibited higher levels of antioxidant activity, making them more attractive as functional foods (Segev et al. 2010; Singh 2017). To the best of our knowledge, the impact of

germination conditions on the phenolic content of pigmented chickpea seeds has not been studied. The purpose of this work was to find the optimal germination temperature (GT) and time (Gt) to maximize total phenolic and flavonoid content (TPC, FC), and antioxidant activity (AoxA) of germinated desi chickpea.

Materials and methods

Plant materials

The desi chickpea cultivar (Black ICC3761) from the *Core Collection/World Germplasm Bank of the International Crops Research Institute for the Semi-Arid Tropics* (ICRISAT) was grown outdoors from December 2014 to April 2015 at *Culiacan Experimental Station of the National Research Institute for Forestry, Agriculture and Livestock* (INIFAP), Sinaloa, México. Four irrigations (pre-sowing, the start of flowering, full bloom, and grain filling) and 90 kg/ha of nitrogenous fertilizer were used. Temperature and amount of rain were within the average seasonal range. After harvesting the seeds (April 2015), a yield of 2214 kg/ha and 100 kg of chickpea seeds were cleaned and stored in tightly sealed containers at 4 °C until use.

Production of germinated chickpea flours

Germinated chickpea flours were prepared according to the methods by Perales-Sánchez et al. (2014) with some modifications. Desi chickpea seeds (200 g) were washed with purified water, sterilized (0.1% sodium hypochlorite solution) for 10 min, drained, and rinsed with sterile distilled water. Chickpea seeds were soaked with distilled water (25 °C/8 h) and placed in germination plates. The plates were placed in a germination chamber (relative

Fig. 1 Desi and kabuli chickpea

humidity = 80–90%) and germination was conducted using different combinations of GT/Gt (13 treatments) (Table 1). The seeds were germinated under conditions of 12 h of light and 12 h of darkness daily. The resulting bioprocessed seeds from each treatment were lyophilized (Virtis Company, INC, Gardiner, NY, USA) and milled (80-US mesh = 0.180 mm) to obtain germinated chickpea flours (GCF). Additionally, whole raw chickpea seeds were milled (80-US mesh = 0.180 mm) to produce unprocessed chickpea flour (UCF) to use as a control. GCF and UCF were packed in plastic bags under vacuum and stored (4 °C) in tightly sealed containers.

Extraction of free and bound phenolic compounds

The extraction of free phenolic compounds was done as previously reported by Heiras-Palazuelos et al. (2013). One gram of OGCF was stirred in 10 mL of ethanol–water (80:20, v/v) for ten minutes in a rotator tube at 50 rpm. Then, the supernatant was recovered by centrifugation (3000xg/25 °C/10 min) (Sorvall RC5C, Sorvall Instruments, Dupont, Wilmington, DE, USA). The extracts were concentrated to a volume of 2 mL using a vacuum

evaporator at 45 °C (Savant SC250 DDA Speed Vac Plus centrifugal, Holbrook, NY, USA) and stored at – 20 °C until use. The extraction of bound phenolic compounds was carried out according to the methodology reported by Adom and Liu (2002). Briefly, the residues from the free phenolic-extraction were hydrolyzed with 10 mL of 2 M NaOH at 95 °C, and then at 25 °C for 60 min. The hydrolyzate was neutralized with HCl before removing the lipids with hexane. The final solution was extracted five times with 10 mL of ethyl acetate and the pool evaporated to dryness. Bound phenolic compounds were reconstituted in 2 mL of 50% methanol and kept at – 20 °C until use all extractions were made in triplicate.

Total phenolic and flavonoid content

TPC and FC of free and bound phenolic extracts from UCF and GCF were analyzed following the colorimetric assays of Singleton et al. (1999) and Ou et al. (2001), respectively. Absorbance was measured using a Microplate Reader (Synergy™ HT Multi-Detection, BioTek Inc, Winooski, VT, USA). TPC was expressed as gallic acid equivalents (mg GAE)/100 g dw, while FC as of quercetin equivalents (mg QE)/100 g dw. All measurements were made in triplicate.

Antioxidant activity

Oxygen radical absorbance capacity (ORAC)

The hydrophilic antioxidant activity of free and bound phenolics was measured using the oxygen radical absorbance capacity (ORAC) assay (Ou et al. 2001). This assay measures the antioxidant's capacity to prevent fluorescein (FL) oxidation by scavenging free radicals derived from the degradation of 2,20-azobis(2-methyl-propionamide)di-hydrochloride (AAPH). Aliquots of 25 µL of free and bound phytochemical extracts of OGCF and UCF were placed into 96-plate wells and loaded into the plate holder of a Microplate Reader (Synergy™ HT Multi-Detection, BioTek Inc.). 150 µL of fluorescein (0.1 µmol/l) was dispensed, mixed and incubated at 37 °C for 30 min before adding 25 mL of AAPH. The reaction was conducted at 37 °C and the fluorescence (485 nm for excitation and 538 nm for emission) was measured at 2 min intervals for 60 min. The antioxidant activity of the samples and control was calculated from the areas under the fluorescence decay curves (AUC) [AUC_{sample}–AUC_{control}]. The results were expressed as micromoles of Trolox-equivalents (µmol TE)/100 g sample, dw (Ou et al. 2001).

Table 1 Experimental design^a used to obtain different combinations of germination temperature/time to produce chickpea flours and experimental results for the response variables

Assay ^b	Process variables		Response variables		
	GT (°C)	Gt (h)	TPC	FC	AoxA
1	20	125	167	99	9157
2	22.2	43.6	149	84	7110
3	22.2	206.3	188	130	12,626
4	27.5	10	97	54	5707
5	27.5	125	180	107	13,033
6	27.5	125	192	122	12,430
7	27.5	125	201	112	13,016
8	27.5	125	183	107	11,487
9	27.5	125	182	115	12,070
10	27.5	240	189	93	13,008
11	32.8	43.6	135	78	10,494
12	32.8	206.3	178	123	14,361
13	35	125	179	124	13,698

GT germination temperature, Gt Germination time, TPC total polyphenols content [mg gallic acid equivalents (GAE)/100 g sample dw], FC flavonoid content [mg quercetin equivalents (QE)/100 g sample dw], AoxA antioxidant activity [µmol Trolox equivalents (TE)/100 g dw]

^aCentral composite rotatable design with two factors and five levels; 13 assays

^bDoes not correspond to order of processing

ABTS

This assay is based on the reduction of the ABTS cation radical (dissolved in phosphate buffered saline) by free and bound phytochemical extracts of OGCF and UCF. The ABTS radical cation (ABTS•1) was produced by the combining the ABTS solution with 2.45 mM potassium persulfate and letting the mixture in the dark at room temperature for 12–16 h before use. 50 µL of the as mixed with 6 mL of the ABTS.+ solution ($A_{734\text{nm}} = 0.700$) and after 6 min the absorbance was measured at 734 nm. The results were expressed as µmol of Trolox equivalents (TE)/100 g of dry weight (dw) sample (Re et al. 1999). All measurements were made in triplicate.

Chemical composition

The AOAC (1999) standard methods were utilized to analyze the chemical composition of the flours: (1) drying at 130 °C to determine moisture (method 925.09 B); (2) defatting in a Soxhlet apparatus with petroleum ether, for lipid analyses (method 920.39 C); and (3) micro-Kjeldahl assay for protein quantification (Nx6.25) (method 960.52). Soluble and insoluble dietary fibre (SDF/IDF) were both determined using the total dietary fibre assay kit [Sigma-Aldrich (TDF 100 A)] according to the manufacturer instructions.

HPLC analysis of phenolic acids

The chromatographic separation of free and bound phenolic extracts was carried out on a High-Performance Liquid Chromatographer (HPLC) (Perkin Elmer series 1100, Inc., Germany) equipped with a YMC-Pack ODS-AM-303 column (250 mm × 4.6 mm, 5 µm) and a DAD detector. The mobile phase used two solvents: (A) 100% methanol and (B) 99:1 v/v (water/trifluoroacetic acid). The flow rate was constant at 1 mL/min, with a step gradient of 90, 80, 75, 65, 35 and 90% of solvent B at 0, 5, 10, 20, 30, 45 min, respectively. The column temperature was set at 25 °C and the injection volume at 20 µL. The phenolic detection was carried out at three different wavelengths: 260 nm (for *p*-hydroxybenzoic, vanillic, protocatechuic and ellagic acids), 275 nm (for gallic and syringic acids), and 320 nm (for caffeic, *p*-coumaric, ferulic and sinapic acids). Detector signals were acquired and integrated into the Chromera software (PerkinElmer Inc, Germany). 10 µL of three different concentrations (0.5, 1 and 25 mg/mL) of external standards were injected. Phenolics acids were quantified from the peak areas recorded, retention times and UV spectra of their respective standards. All samples were analyzed in triplicate.

Experimental design

Response surface methodology (RSM) was applied to determine the optimal conditions to obtain enhanced germinated flours from desi chickpea. Data from the literature and preliminary trials were taken into account to select the number and variation level of the process variables. A central composite experimental design with two factors (germination temperature *GT* and germination time *Gt*) and five levels (two factorials, two axial, and one central) was selected (Khuri and Cornell 1987). The independent process variables considered in this study were germination temperature ($X_1 = GT$, 20–35 °C) and germination time ($X_2 = Gt$, 10–240 h). The dependent response variables chosen were: (1) total phenolic content (TPC), (2) flavonoid content (FC), and (3) antioxidant activity (AoxA). To estimate the variables response, the following second order polynomial equation was applied (1):

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^2 \beta_{ij} X_i X_j + \epsilon \quad (1)$$

In Eq. (1), *Y* is the estimated response. β_0 , β_i , β_{ii} and β_{ij} represent the constant and coefficients of linear, quadratic and interactive effects, respectively. X_i , X_i^2 , and $X_i X_j$ represent the linear, quadratic, and interactive effects of the independent variables, respectively. Finally, ϵ represents the error, to account for the inability to determine the true model. Applying the stepwise regression procedure, non-significant terms ($p \leq 0.1$) were deleted from the second-order polynomial equation, and a new polynomial term was calculated to obtain a predictive model for each response variable (Khuri and Cornell 1987). The STAT-EASE software Design Expert (2007) was used for analysis and evaluation.

Optimization

A conventional graphical method was applied as an optimization technique to obtain maximum values for TPC, FC, and AoxA in germinated chickpea flours. Predictive models (Table 2) were used to graphically represent the system using the STAT-EASE software Design Expert (2007) (Fig. 2a–c). The contour plots of each of the response variables were superimposed to obtain a new contour plot (Fig. 2d) to select the best combination of *GT*/*Gt* to produce optimized germinated chickpea flour (OGCF).

Statistical analysis

Results obtained from the chemical composition analyses and the phenolic acid profiles of OGCF and UCF were

Table 2 Germinated pigmented desi chickpea flours

Coefficient	TPC (Y_{TPC})	FC (Y_{FC})	AoxA (Y_{AoxA})
Intercept			
β_0	183.72***	114.65***	12,197.78***
Lineal			
β_1	NS	NS	1442.62***
β_2	26.40***	17.77***	2463.52***
Quadratic			
β_{11}	NS	NS	NS
β_{22}	– 20.20***	– 17.68***	– 1296.77***
Interactions			
β_{12}	NS	NS	NS
P	< 0.0001	< 0.0001	< 0.0001
R^2	0.8838	0.8667	0.9449

Regression coefficients and analyses of variance of the second-order polynomial models showing the relationships among the response variables (Y_K) and the coded process variables (X)

TPC total phenolic content, FC flavonoid content, AoxA antioxidant activity

Significant at * $p \leq 0.10$; ** $p \leq 0.05$; *** $p \leq 0.01$. NS not Significant ($p > 0.10$)

expressed as mean $\pm$ standard deviations for at least one triplicate. The ANOVA was used for the analysis of the experimental results using Minitab Software 2013 version. Differences among means were compared by Duncan's multiple range tests at $p < 0.05$.

Results and discussion

Prediction models for total phenolic content, total flavonoid content, and antioxidant activity

The predictive regression equation models were computed for total phenolic content (TPC), flavonoid content (FC) and antioxidant activity (AoxA). TPC, FC, and AoxA of the germinated desi chickpea flours varied from 97 to 201 mg GAE/100 g sample (dw), 54–130 mg QE/100 g sample (dw), and 5707–14,361 $\mu\text{mol TE}/100$ g sample (dw), respectively (Table 1). Table 2 summarizes the significant coefficient levels of the second-order polynomial equation. ANOVA showed that TPC, FC, and AoxA were significantly ($p < 0.001$) dependent on linear and quadratic terms of germination time (Gt). AoxA was the only variable dependent on linear terms of germination temperature GT. The prediction models using un-coded variables for the response variables (TPC, FC, and AoxA) were:

$$\text{TPC} = 95.41 + 1.08(\text{Gt}) - 3.05E - 003(\text{Gt})^2$$

$$\text{FC} = 45.54 + 0.887(\text{Gt}) - 2.67E - 003(\text{Gt})^2$$

$$\text{AoxA} = -2133.94 + 272.02(\text{GT}) + 79.32(\text{Gt}) - 0.1961(\text{Gt})^2$$

The regression models explained 88, 87 and 94% of the total variability ($p < 0.0001$) in TPC, FC, and AoxA, respectively (Table 2). For all the response variables (TPC, FC, and AoxA), the lack-of-fit analysis was not significant ($p > 0.05$), and the coefficient of variation was found to be $< 10\%$. These values indicate the adequacy and reproducibility of the model used for optimizing the germination conditions of desi chickpea seeds. TPC and FC increased with Gt, showing the highest values from 125 to 215 h (Fig. 2a, b) after that, these responses decreased. In general, the AoxA of germinated chickpea flours increased with GT and Gt until reaching maximum values at around 27.5–35 °C and 125–240 h (Fig. 2c). Miglani and Sharma (2016) reported that germination time and temperature significantly influenced the phenolic constituents in soybeans. Likewise, the antioxidant activity of beans was not only dependent upon the quantitative but also the qualitative characteristics of phenolics, and not all phenolic compounds in soybeans exhibit antioxidant activity (Haung et al. 2014).

Optimization of the germination process

A numerical multi-response optimization technique of RSM was applied to determine the optimal combination of time and temperature for the production of germinated chickpea flour with the highest TPC, FC, and AoxA values. The superimposed contour plots (Fig. 2a–c) were utilized to determine the best combinations (Fig. 2d) of process variables. The optimal combination of the germination variables was $\text{GT} = 33.7$ °C/ $\text{Gt} = 171$ h. The predicted values for TPC, FC, and AoxA using optimal germination conditions for each response variable were: 192.04 mg GAE/100 g sample (dw), 119.03 mg QE/100 g sample (dw), and 14,877 $\mu\text{mol TE}/100$ g sample (dw), respectively (Fig. 2d). The OGCF was produced using the optimal combination of variables for the germination process. The experimental values of TPC, FC, and AoxA for OGCF (Table 3) were similar to those predicted by the model indicating that the optimal conditions used were appropriate and reproducible.

Chemical composition and nutraceutical properties of optimized germinated chickpea flours

Table 3 shows the chemical composition of raw and bio-processed flours. Applying an optimal combination of temperature and time during germination (33.7 °C for

Fig. 2 Contour plots show the effect of germination temperature and time on **a** total phenolic compounds, **b** total flavonoids, and **c** antioxidant activity of pigmented desi chickpeas. **d** Region of the

171 h) a significant increase (+ 16.4%, $p < 0.05$) in chickpea protein content was observed compared to the raw flours. Consistent with our results, an increase of 8–9% in protein content after germination of chickpea seeds has been reported by El-Adawy (2002). The protein increase observed after this process could be attributed to dry matter losses, especially carbohydrates, throughout the seeds respiration during the germination (Mbithi-Mwikya et al. 2000).

Chickpea seeds are rich in unsaturated fatty-acids such as linoleic and oleic acids. Also, important sterols as β -sitosterol, campesterol, and stigmasterol are present in the chickpea oil (Jukantil et al. 2012). The lipid content of

best combination of process variables (*GT* germination temperature, *Gt* germination time) to produce optimized germinated chickpea flours (OGCF)

OGCF significantly declined (– 37% vs. raw, $p < 0.05$) compared to the UCF (Table 3). This reduction is probably due to the use of lipids as an energy source during the germination process. Consistent with our results, Fouad and Rehab (2015) reported that during the germination of lentil seeds (3–6 days/25 °C in dark conditions) the lipid content showed a decrease compared with raw seeds.

The effect of germination on dietary fibre content in desi chickpea is provided in Table 3. Total dietary (+ 15%), soluble (+ 26%) and insoluble (+ 15%) fibre content in chickpea seeds increased significantly ($p < 0.05$) after germination compared to the UCF. According to Martín-Cabrejas et al. (2003), the germination of peas carried out

Table 3 Chemical composition, phytochemicals contents, and antioxidant activity of desi chickpea flours

Property	UCF	OGCF
Chemical composition (%dw)		
Proteins	20.22 ± 0.15 ^B	23.54 ± 0.21 ^A
Lipids	5.3 ± 0.04 ^A	3.29 ± 0.03 ^B
Dietary fibre		
Soluble fibre	1.003 ± 0.01 ^B	1.26 ± 0.02 ^A
Insoluble fibre	28.29 ± 0.43 ^B	32.53 ± 0.37 ^A
Total fibre	29.29 ± 0.41 ^B	33.79 ± 0.39 ^A
Phenolic content ^a		
Free	14.07 ± 0.06 ^B	97.43 ± 1.63 ^A
Bound	96.28 ± 2.01 ^B	120.21 ± 1.77 ^A
Total	110.17 ± 1.59 ^B	217.22 ± 2.07 ^A
Flavonoid content ^b		
Free	18.49 ± 0.09 ^B	30.05 ± 0.09 ^A
Bound	40.35 ± 1.11 ^B	92.55 ± 1.27 ^A
Total	58.77 ± 0.83 ^B	122.63 ± 1.44 ^A
Antioxidant activity ORAC ^c		
Free	2329 ± 119 ^B	8266 ± 210 ^A
Bound	3127 ± 123 ^B	6876 ± 187 ^A
Total	5456 ± 179 ^B	15,143 ± 225 ^A
Antioxidant activity ABTS ^c		
Free	2783 ± 133 ^B	7854 ± 197 ^A
Bound	3088 ± 121 ^B	6582 ± 174 ^A
Total	5871 ± 221 ^B	14,435 ± 230 ^A

Data are expressed as mean ± SD. Means with different superscripts (A, B) in the same row are significantly different (Duncan, $p \leq 0.05$)

UCF unprocessed chickpea flour, OGCF optimized germinated chickpea flour

^amg gallic acid equivalents (GAE)/100 g sample (dw)

^bmg quercetin equivalents (QE)/100 g sample (dw)

^cμmol Trolox equivalents (TE)/100 g (dw)

at 20 °C for 2–6 days under light/dark conditions, significantly ($p < 0.05$) increased the total dietary fibre content (insoluble and soluble fractions) of the seeds compared to the unprocessed sample. The germination process can change the structure of the cell wall polysaccharides of the seeds, and produce cell wall biosynthesis, as well as new dietary fibre. Megat et al. (2016) studied the influence of germination on dietary fibre in legumes (kidney beans, mung beans, soybeans, peanuts). They found that in all germinated seeds, the total dietary fibre (soluble and insoluble) increased significantly ($p < 0.05$) compared with the non-germinated seeds. Dietary fibre is associated with the prevention of different diseases, and it is fundamental for the maintenance of intestinal health (Liu 2007). Thus, germination could be used as a tool for increasing the content of fibre in seeds, improving their health benefits.

Total phenolic content

As shown in Table 3, the optimized germination also resulted in greater ($p < 0.05$) in free from 14.07 to 97.43 mg gallic acid equivalents (GAE)/100 g sample dw (+ 593%), bound from 96.28 to 120.21 mg gallic acid equivalents (GAE)/100 g sample dw (+ 25%), and total from 110.17 to 217.22 mg gallic acid equivalents (GAE)/100 g sample dw (+ 97%) phenolic content in the OGCF compared with the UCF. Dueñas et al. (2016) reported that the improvement in the phytochemical and nutritional quality of germinated legumes was mainly due to the changes in phenolic compound composition and dietary fibre observed.

Most of the phenolic compounds associated with whole grains are in the insoluble bound form (Adom and Liu 2002). Bound phenolics are covalently bound to cell wall structures such as cellulose, hemicellulose (e.g. arabinoxylans), lignin, pectin and structural proteins (Wong 2006). Phenolics acids, such as hydroxycinnamic and hydroxybenzoic acids, form ether linkages with lignin through their hydroxyl groups in the aromatic ring and ester linkages with structural carbohydrates and proteins through their carboxylic group (Liu 2007; Liyana-Pathirana and Shahidi 2006).

After the germination process, we observed several changes in the total phenolic content. These changes may be attributed the biosynthesis and release of phenolic compounds that occur during the process. During seed germination, the enzyme phenylalanine ammonia lyase (PAL) is highly active and could promote the biosynthesis of phenolic compounds (Duodu 2014). Additionally, the seed respiratory system is active during germination, and different proteolytic, amylolytic, and cell wall degradation enzymes that contributed to the release of bound phenolic compounds could be produced (Acosta-Estrada et al. 2014). On the other hand, considerable variability in the analysis of phenolic compounds was also observed. Previous reports have indicated the difficulty to identify and quantify the free and bound phenolic acids in grains. In this study, the main free and bound phenolic acids were separated and characterized using a methodology that enhances the liberation of free and bound phenolics through alkaline hydrolysis (Adom and Liu 2002). Thus, our results showed that germination is an efficient bioprocess for enhancing the concentration of phenolic compounds in chickpea seeds.

Phenolic acids profile

Table 4 shows the HPLC analyses (A2 and A3) of phenolic acids in ethanolic chickpea seed extracts. A total of ten phenolic acids were identified: 4 hydroxycinnamic acids

Table 4 Effect of germination on phenolic acids content in desi chickpea flours

Phenolic acid ^a	UCF			OGCF		
	Free	Bound	Total	Free	Bound	Total
Hydroxycinnamic						
Caffeic	0.020	0.090	0.110 ^B	0.280	0.001	0.281 ^A
Coumaric	0.020	0.090	0.110 ^B	0.320	0.860	1.180 ^A
Ferulic	0.020	0.200	0.220 ^B	0.390	0.770	1.160 ^A
Sinapic	0.010	0.110	0.120 ^B	0.370	0.010	0.380 ^A
Hydroxybenzoic						
Ellagic	0.090	0.340	0.430 ^B	10.03	0.100	10.130 ^A
Gallic	1.39	3.270	0.466 ^A	2.340	1.400	3.740 ^B
<i>p</i> -Hydroxybenzoic	0.030	0.050	0.080 ^B	0.040	0.050	0.090 ^A
Protocatechuic	0.240	0.270	0.510 ^B	1.210	0.110	1.320 ^A
Syringic	0.290	0.340	0.630 ^B	1.130	0.970	2.100 ^A
Vanillic	0.290	0.050	0.340 ^B	0.610	0.170	0.780 ^A

Means with different superscripts (A, B) in the same row are significantly different (Duncan, $p \leq 0.05$)

UCF unprocessed chickpea flour, OGCF optimized germinated chickpea flour

^amg/100 g of sample (dw)

(caffeic, coumaric, ferulic, sinapic), and 6 hydroxybenzoic acids (gallic, ellagic, hydroxybenzoic, protocatechuic, syringic, vanillic).

The HPLC analyses revealed that several acids significantly ($p > 0.05$) increased during germination. For instance, sinapic acid increased from 0.12 to 0.380 mg/100 g (+ 217% vs. the UCF), syringic acid from 0.630 to 2.1 mg/100 g (+ 233% vs. the UCF), ferulic acid from 0.22 to 1.16 mg/100 g (+ 427% vs. the UCF), coumaric acid from 0.11 to 1.18 mg/100 g (+ 972% vs. the UCF), and ellagic acid from 0.43 to 10.13 mg/100 g (+ 2256% vs. the UCF) (Table 4).

Earlier an increase in hydroxycinnamic and hydroxybenzoic acids-derivatives during germination has been reported (Dueñas et al. 2016; Xiang et al. 2017). The variation of phenolic compounds (content and profile) in germinated seeds could be explained by a gene expression mechanism. PAL plays an important role in the first reaction of the phenolic biosynthesis pathway, catalyzing phenylalanine to cinnamic acid. The following step requires the action of the cinnamate-4-hydroxylase (C4H) enzyme to generate *p*-coumaric acid, a precursor of phenolics and flavonoids. Likewise, the expression of genes related to the synthesis of secondary metabolites depends on factors such as light exposure, climatic conditions, and biotic stress (Xiang et al. 2017).

Free phenolic acids have shown to improve glucose and lipid plasma levels in diseases such as obesity and diabetes mellitus by increasing the uptake of glucose and regulating glycogen synthesis (Vinayagam et al. 2015). On the other hand, bound phenolics can be released and absorbed in the gastrointestinal tract where microorganism, enzymes, and

even glucose transporters are involved (Acosta-Estrada et al. 2014). Therefore, Liu (2007) proposed that health benefits of bound whole grain phenolics are more effective in the colon. Our findings revealed that pigmented desi chickpea seeds are an important source of phenolic compounds that could prevent diseases and improve human health.

Flavonoid content

After germinating in optimal conditions (33.7 °C/171 h) chickpea seeds showed a significant increase ($p < 0.05$) in flavonoid content. Free flavonoid ranged from 18.49 to 30.05 mg quercetin equivalents (QE)/100 g sample dw (+ 67%), bound flavonoid from 40.35 to 92.55 mg quercetin equivalents (QE)/100 g sample dw (+ 131%), and total flavonoid from 58.77 to 122.63 mg quercetin equivalents (QE)/100 g sample dw (+ 111%) contents when compared with the unprocessed material (Table 3).

Wu et al. (2012) reported that germination (4 days/25 °C) significantly increases flavonoid content in chickpea seeds, approximately 90 times greater than the raw chickpea. The health benefits of chickpea seeds have received very little attention. However, laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have important biological activity acting as chemopreventive and chemotherapeutic agents. According to Ren et al. (2003) flavonoids may be promising anticancer agents, not only due to their antioxidant properties but by exerting different mechanisms of action, such as cell-cycle regulation.

Antioxidant activity

In general, AoxA of chickpea seeds increased after the optimized germination (33.7 °C/171 h) (Table 3). The increase in AoxA by ORAC assay was: from 2329 to 8266 µmol TE/100 g dw (+ 255%) for free, 3127–6876 µmol TE/100 g dw (+ 120%) for bound, and 5456–15,143 µmol TE/100 g dw (+ 178%) for total phytochemicals, respectively compared to the UCF. The ABTS method showed similar results to the ORAC method. This study revealed that bound phytochemicals, which are usually minimized in the literature, significantly contributed to the total antioxidant activity. Wu et al. (2012), also found that the germination maximized the antioxidant activity of legumes, compared with the raw samples. Nevertheless, they did not determine the antioxidant activity in bound phenolic extracts.

During germination many important metabolic changes take place. One of them is the increase of AoxA, principally attributed to an increase of bioactive compounds, such as phenolic compounds, by the action of endogenous hydrolytic enzymes. Therefore, the increase in total phenolic content and antioxidant activity in chickpea seeds results in the improvement of their nutraceutical properties (Cevallos-Casals and Cisneros-Zevallos 2010). Xue et al. (2016) carried out a study on the change in bioactive compounds and antioxidant activity in three types of legumes during germination and concluded that TFC and FC made the highest contribution to the total antioxidant activity of the germinated legumes. On the other hand, Singh et al. (2016) concluded that, phenolics like sinapic acid, ferulic acid, catechin, *p*-coumaric acid, resveratrol, quercetin and luteolin were the mayor compounds in mung bean extract and were the mayor contributors to antioxidant activity. Likewise, Khang et al. (2016) detected and quantified 13 different phenolic compounds in six germinated grains, and showed a significant correlation of the phenolic compounds with antioxidant activity. Consistently, in the present study we showed that AoxA had a stronger correlation ($r = 0.835$, $p < 0.005$ and $r = 0.822$, $p < 0.007$) with TPC and FC, respectively. Our results provide important information on the nutraceutical quality of germinated desi chickpea seeds.

Conclusion

The germination bioprocess carried out under optimal conditions is an efficient alternative to increasing the antioxidant activity, bioactive compounds, protein, and dietary fiber contents of desi chickpea seeds. For the production of optimized germinated chickpea flour (OGCF) the best germination conditions to obtain the highest

antioxidant activity and the greater phenolic content were a temperature = 33.7 °C and a time = 171 h. As a result, we obtained germinated chickpea flours rich in antioxidants, protein, and dietary fiber appropriate for the formulation of functional foods. However, more research is needed to determine the impact on health of other types of germinated pigmented chickpeas.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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