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Simulated digestion of the pigmented legumes' (black chickpea (Cicer arietinum L.) and brown lentil (Lens culinaris Medikus)) phenolics to estimate their bioavailability

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Abstract

This study simulated the gastrointestinal digestions of black chickpea (BC) and brown lentil (BL). BC phenolics were better retained (an increase of 35.1%) than the BL phenolics (a loss of 7.4%) after cooking. In contrast, the remaining flavonoids after cooking were higher in BL (about 47%). TAA (Total antioxidant activity) of free fractions increased in BC and BL samples by cooking. After *in vitro* GID, moderate levels of flavonoids were detected in colon (OUT) fractions. TAA levels correlated well with total flavonoids. TAA by the CUPRAC assay was higher in cooked and *in vitro* GID BC and BL samples. Catechin was the most abundant compound detected in BC samples, while gallic acid was for BL. BC and BL, have unique and superior benefits for health when compared with the conventional legumes. The possible interactions between their remaining phenolics and other bioactives in the colon are promising for their widespread consumption.

Introduction

Legumes are among the most important food groups in the world with their rich nutrient profile and low price. Although the animal proteins are still the primer protein source for most of the world population, changes in consumers' nutrition trends led to the pursuit for new sources/alternative proteins for human and animal consumption. In this regard, legumes received considerable critical attention as they are significant dietary plant protein sources with their high-quality proteins and peptides, in addition to their well-balanced essential amino acids Moreover, legume flours are becoming popular as gluten-free alternatives.

Their total dietary fiber, resistant starch, vitamins, minerals and bioactive components are important contributors to their benefits in controlling and preventing various metabolic diseases including diabetes [1]. Numerous studies revealed that the consumption of legumes is effective on decreased levels of chronic diseases such as; obesity, coronary heart disease, type 2 diabetes mellitus, cardiovascular diseases, cancer as well as aging and the gut health [2, 3]. Among their bioactive components, phenolic compounds are significant contributors to the positive health benefits of legumes with their anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. Particularly in the pigmented legumes, phenolics contribute also to the seed color and sensory characteristics of the seed [4].

Chickpeas are the third mostly cultivated pulses after dry beans (*Phaseolus vulgaris* L.) and peas (*Piston sativum* L.). According to FAO; chickpea (*Cicer arietinum* L.) production was 15 million tons in 2020, while the total lentil production was more than 6.5 million tons [5]. Global legumes market valued at about 45 billion US dollars in 2017, and it is projected to reach 75.8 billion US dollars by 2025 [6].

The coat color and size of the seed separate lentils into two main groups: 1) green lentil (brown, yellow, Chilean, Continental or Macrosperma lentils) has green-brown seed coat and yellow cotyledon and 2) red lentil (Microsperma or Persian lentils) has a pale grey-dark seed coat and a red cotyledon [7]. Brown lentil, *Lens culinaris* Medikus is smaller and more circular in comparison to green lentil. Lentil carbohydrates are

beneficial in psychological effects, such as controlling and prevention of diabetes mellitus, coronary heart diseases and cancer [7]. The primary polyphenolic compounds in lentil seed coats are listed as: tannins, phenolic acids (mainly ferulic acid) and flavonoids such as flavonol glycosides, anthocyanin and tannins [1, 8]. The polyphenolic compounds are significant protectors against chronic diseases in humans by their antioxidant activity that balances the oxidative stress. The oxidative stress is among the main causes of different human diseases like cancer, diabetes, atherosclerosis and neurodegenerative diseases as it is basically the excess production of reactive oxygen species (ROS) that damages the cellular functionality by harming lipids, proteins and DNA [9].

Chickpeas are generally grouped into two types: desi and kabuli, being differing seeds according to their nutritional and phytochemical components. Pigmented black chickpeas belong to the desi type which has different seed coating ranging from light to black rather than white to cream as in kabuli type (Heiras-Palazuelos et al., 2013). Desi-type chickpea seeds also have a thicker and irregularly shaped seed coat [11]. Besides the unique nutritional value highlighted with proteins, chickpeas also have high variety of bioactive compounds especially phenolic compounds. Among them, flavonoids of formononetin, biochanin A and their corresponding glycosides are mainly in concern [10, 12]. Darker seed coat colour in legumes generally correlates with the presence of different polyphenols. For instance, the attractive color of the seed coat of lentils is abundantly related with flavonoids such as flavonol glycosides, anththocyanins and tannins [1, 8].

The bioavailability is generally used to refer the extent of absorption and transportation of nutrients to body tissues. In that sense, it is considered as one of the major factors for health effect evaluation of foods. Cooking is the anticipated treatment for legume consumption and most legumes are consumed only after cooking. Although some knowledge has been acquired about the phenolic profiles of different legumes and the effect of cooking/heat treatments on the bioaccessibility of polyphenols in some commonly consumed varieties [4, 8, 10, 13], studies on the bioavailability of pigmented variants are very limited [14–16].

Therefore, this study aims to determine the effects of the simulated *in vitro* digestion on the phenolic acids, flavonoids and the antioxidant activity of pigmented and relatively uncommon legume types such as black chickpea (*Cicer arietinum* L.) and brown lentil (*Lens culinaris* Medikus) and depict further health benefits.

Materials And Methods

Samples

Black chickpea (BC) samples were originated from Malatya, Turkey and supplied from a local market and brown lentil (BL) samples were supplied from Duru Bulgur Ltd. (Turkey). They were stored at room temperature until analysis.

Sample Preparation for Extraction

The free and bound phenolics of the samples were extracted according to a previous method with some modifications [17]. Sample (1 g) was homogenized in methanol: acetic acid (85:15; v/v) solution (7 ml), then sonicated (30 minutes) and diluted to 10 ml with distilled water and filtered. For bound phenolics, 12 ml distilled water and 5 ml 10 M NaOH were added on to the sample. Tubes were sealed and stirred overnight on magnetic stirrer at 20ºC. pH was adjusted to 2 with HCl and phenolics were extracted in triplicate using 15 ml diethyl ether/ethyl acetate (1:1; v/v). Then supernatants were collected, evaporated to dryness and dissolved in 10 ml methanol. After alkaline hydrolysis, acid hydrolysis was performed by adding 2.5 ml HCl and incubating in ultrasonic bath at 80ºC for 30 minutes. The samples were cooled and steps after alkaline hydrolysis were performed once more.

Domestic Processing (Soaking and Cooking) of Samples

Legume samples (50 g) were soaked in 250 ml of tap water at room temperature for 4 hours [18]. Presoaked samples were fully cooked in steam cooker (Arzum, Turkey). After cooking, samples were stored at -80ºC for one day, grinded under liquid nitrogen and freeze dried (Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany).

The degree of cooking was determined using the thermal analysis by Differential Scanning Calorimeter (DSC) and Universal Analysis 2000 Version 4.5A software (TA Instruments Inc., New Castle, DE, USA). The transition onset temperature (T_o), transition peak temperature (T_p), and transition enthalpy (ΔH) were determined. Samples (3 mg) were weighed into aluminum pans and 12 µl of distilled water were added by using a micro syringe. The hermetically sealed pans were equilibrated for 2 hours at room temperature followed by an heating from 20 to 180ºC at a rate of 100ºC/min. Gelatinization was evaluated by observing the loss of the thermal transition around 60ºC which is observed for pure starch systems at the same temperature range [19].

In vitro Gastrointestinal Digestion

Bioavailability was tested using *in vitro* digestion procedure by McDougall et al. (2005) with slight modifications [20]. This method comprises of two steps such as gastric fraction and small intestine fraction. Gastric conditions were simulated by weighing the sample (5 g) and adjusting the pH to 1.7 using HCl (5M) and incubating with pepsin (1.5 ml, 315 units/ml) with a mild shaking at 100 rpm for 2 h at 37ºC in a shaking water bath (Memmert GmbH, Schwabach Germany). After gastric digestion, 2 ml sample was taken as the post gastric (PG) fraction (representing the part of food leaving the stomach). For the next phase, 4.5 ml pancreatin (4 mg/ml) and 4.5 ml bile salt (25 mg/ml) were mixed into the remaining solution. A piece of cellulose dialysis tube as washed with distilled water and the bottom of the tube was linked tightly. After filling the dialysis tubing with 20 ml $NaHCO_3$ (1 M) solution to neutralize acidity of the sample, the top of the tube was tied. Dialysis tubing was put into a beaker, was sealed with a parafilm and placed in the heated water bath at 37ºC for 2 h with continuous shaking (simulation of the small intestine). After digestion, the solution in the dialysis tubing was taken as the IN fraction representing the constituents that entered the serum, while the rest of the solution that diffused out of the

dialysis tubing and did not penetrate into the serum, was collected as the OUT fraction. Afterwards, PG, IN and OUT fractions were centrifuged at 18000 rpm at 4ºC for 5 minutes and were stored at -20ºC until analysis. *In vitro* digestion procedure was applied to all products in duplicate.

Determination of Total Phenolic Content

Total phenolics in samples were determined using the Folin–Ciocalteu's reagent. Absorbance of the clear supernatants was measured at 725 nm using a spectrophotometer (Shimadzu UV-1700 UV-Vis). The results were expressed as mg of gallic acid equivalent per 100 mg of dry sample (mg GAE/100 mg DW) and reported as mean value ± SD.

Determination of Total Flavonoid Content

Total flavonoids contents were analyzed using the method by Dewanto et al. (2002). The absorbance measurements were performed at 510 nm. The results were given as milligrams of rutin equivalent per 100 g dry weight (mg RE/100 g DW) and reported as mean value ± SD.

Determination of Total Antioxidant Activity

Total antioxidant activities of the samples were analyzed using three different methods; ABTS radical scavenging activity [21], CUPRAC (Cupric reducing antioxidant capacity) method [22] and DPPH radical scavenging method (Kumaran & Joel Karunakaran, 2006). The results were given as mg TROLOX equivalents per 100 g of dry weight of samples (mg TEAC/100 g DW) and reported as mean value ± SD.

Detection of Major Phenolic Compounds

The major phenolic compounds in samples were detected using HPLC (High Performance Liquid Chromatography) (Waters, W600 HPLC system with PDA (photodiode array) detector. Luna C18 column (150 x 4.60 mm pore size 100 Å, particle size 5 µm, Phenomenex, Torrance, CA, USA) was used as the stationary phase The mobile phase was included solvent A, Milli-Q water with 0.1% (v/v) TFA and solvent B, acetonitrile with 0.1% (v/v) TFA [24]. A Linear gradient was used as follows: at 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returns to initial conditions. External standard calibration curves were used for quantifications. All of the samples and calibration solutions were filtered through a 0.45-µm membrane filter and 2 ml of the filtered sample was placed into vials. The flow rate was 1 ml/min. Detections were done at 280, 312, 360 and 512 nm wavelengths.

Statistical Analyses

The results were analyzed statistically by IBM SPSS (21th version) by using one-way analysis of variance (ANOVA) at 95% significance level and Duncan's New Multiple Range Test was applied as post-hoc test. For testing the effect of cooking on each sample, the differences between the raw and cooked values were statistically analyzed by paired t-test for each sample ($p<0.05$). Correlations among the antioxidant

activity methods and total phenolic contents were calculated by using the Pearson's correlation coefficient (R^2). Each analysis was performed in triplicate. The results were reported as mean value ± standard deviation.

Results And Discussion

Total Phenolic Content (TPC)

Phenolics are significant contributors to the health benefits of legumes. Cooking is a significant treatment for chickpea and lentil samples, since most of the legumes are consumed after cooking.

TPC in black chickpea and brown lentil samples are given in Table 1. TPC in bound fractions were higher in BC and BL samples.

BC samples had the TPC value of 105.01 mg GAE/100 mg DW (44.41 mg GAE/100 mg DW free and 60.60 mg GAE/100 mg DW bound). Cooking increased the TPC in all fractions ($p>0.05$). The total increase was measured as 35.1%, in total phenolics content.

For BL samples, cooking had negative effects. The TPC was found to be 143.26 mg GAE/100 mg DW (55.91 mg GAE/100 mg DW free and 87.35 mg GAE/100 mg DW bound). TPC decreased about 7.4% after cooking. This decrease was due to the slight decrease in phenolics especially in the bound fraction (68.83 mg GAE/100 mg DW).

When the raw and cooked BC and BL samples were compared, the differences between the total phenolic contents in free, bound and total fractions of both samples were insignificant ($p>0.05$). Therefore, steam cooking made no significant changes on different fractions of black chickpea and brown lentil samples ($p<0.05$).

Current findings were in agreement with the previous studies, since TPC of free fraction extracts from six different pigmented chickpea samples from desi and kabuli cultivars were reported to range between 14.0 and 37.0 mg GAE/100 g DW while bound fraction extracts were between 43.0 and 123.0 mg GAE/100 g DW. Similar results were found to our findings, for desi cultivars (between 123-151 mg GAE/100 g DW) for TPC in the mentioned study [10]. Another study revealed that, TPC was between 147.0-183.0 mg GAE/100 g DW for chickpeas. Desi varieties have been reported to contain relatively higher content of total phenolics than kabuli types due to the darker seed coat and smaller seed size [10, 11]. Phenolic contents of different lentils and chickpeas were between 147.0-183.0 for chickpeas and 109.8-159.4 mg GAE/100 g for lentils [25]. Although according to the findings from another group, TPC of different green lentil cultivars ranged between 456–834 mg GAE/ 100 g, being higher than the current findings [26]. Moreover, black chickpeas and brown lentils had significantly higher amount of phenolics when compared to whiter chickpeas and green lentils as their more common counterparts, both for raw and cooked samples [27].

Similar to the reported findings from literature [27], lentils have slightly higher phenolics when compared to the raw chickpeas .

According to the literature, different processes such as cooking or steam cooking have different effects on the measured phenolic contents of legumes. Previously, domestic cooking has been reported to decrease the total phenolic content of chickpea [28, 29] and lentils [30] significantly. In contrast, other studies reported no decrease in the TPC of chickpeas after steaming [11], or significant increases in TPC of faba beans, soybeans, lentils and peas after cooking [27] and colored bean varieties from Mexico [14]. Either decreases (for whole Medium green, Red and French green) and increases (in whole Spanish brown and Large green) in TPC after cooking were detected in the same study [27]. There may be two main factors affecting the extent of phenolic compound loss or increase in legumes; 1) The type of legume and 2) Two-sided effect of cooking; either decreases in the amount of heat sensitive phenolic components or release of some complex/bound phenolics [15, 27]. Other reports emphasized the simultaneously occurring events of partial release of free phenolics, the effect of thermal degradation and the increase in the amount of physically bound fractions due to the interactions of phenolic acids with macromolecules of the food matrix during cooking and cell disruptions [27].

Total Flavonoid Content (TFC)

Flavonoids are the largest group of secondary metabolites in plants. The flavonoids in lentils and chickpea, are among their important bioactive compounds. Isoflavonoids (formononetin, biochanin A and their corresponding glycosides) have been reported as the main phenolic group in chickpeas. For lentils, flavonoids in addition to phenolic acids and procyanidins have been reported as the most dominant phenolic compounds [26]. Since both of lentils and chickpeas are processed before consumption to increase the palatability and nutritional value, flavonoids are also affected from these processes. TFC results are given in Table 1. TFC in bound fractions for BC and BL samples were slightly higher than in free fractions.

TF of the samples ranged between 335.88 mg RE/100 g DW for the BL bound and 1342.76 mg RE/100 g DW for BC. Before cooking BC samples had higher TF contents than the BL samples. Steam cooking decreased TFC in all BC and BL fractions. The most dramatic decreases were measured in the bound BC fraction (55%) and accordingly in TF of BC (72.9%), being statistically significant ($p<0.05$). In BL samples, similar trend was evident, since the steam cooking decreased the TF content both in free and bound fractions ($p>0.05$).

In the literature, the findings on total amount of flavonoids depict significant changes and different values have been attributed to the different extraction methodologies, and genotypes [26]. In different cultivars of desi type of chickpeas, TFC in free fractions ranged between 15 and 24 mg CAE/100 g DW which is in agreement with current findings, the TFC in bound fraction was higher (changing between 27 and 68 mg CAE/100 g DW) [10]. In contrast, the flavonoid content in brown chickpeas was 23.5 mg RE/100 g, as being significantly lower than the present findings [8]. Their flavonoid findings were generally lower for all legume samples, and these results might be related with the experimental differences among two

studies [8]. Previously, it has been reported that desi type of chickpeas had a higher flavonoid content when compared to Kabuli types, which was related to the presence of glycosides of luteolin, myricetin and quercetin [4, 28].

According to one of the previous findings, the cooking treatment caused an average of 60% of decrease in TFC among 6 different varieties of commercial lentils (brown, red, dark green, French green, Beluga, dehulled and split, red, and dehulled yellow) [30]. This finding was in similar to the current findings.

Total Antioxidant Activity (TAA)

TAA results measured by 3 different methods are given in Table 2. For BC samples before cooking, TAA in the bound fractions (65.2-89.0%) were higher than in free fractions. The similar trend was also evident for bound fractions of BL samples, TAA being in the range of 72.5-73.6%. CUPRAC assay gave the highest TAA among all three assays (1559.27 mg TEAC/100 g DW for TAA of BC).

According to the results, cooking treatment increased TAA in free fraction for both BC and BL samples (increases in BC being statistically significant ($p<0.05$). All three assays performed (ABTS, CUPRAC and DPPH) were in agreement to reveal the increases in the TAA of the free fractions of BC and BL samples which were steam-cooked. In contrast, TAA in bound fractions and the sum of the two fractions (free and total) were in agreement between CUPRAC and DPPH assays, but not for ABTS assay. According to these methods, TAA in bound fractions decreased about 12.5-29.7% after cooking. The total reductions in TAA after cooking ranged between 13.2-24.4% for BC and 9.4-16.3 for BL, by CUPRAC and DPPH assays, respectively. TAA results of DPPH and CUPRAC assays also correlated positively and significantly ($p<0.01$) with TPC results (0.856 and 0.648, respectively). As expected, a significant correlation (0.793) was detected between CUPRAC and DPPH assays ($p<0.01$). However in the literature, moderate levels of correlations among the antioxidant activity and total phenolic content of different lentil cultivars has been related with the role of other bioactive compounds such as flavonols and flavanols, as other significant contributors to total antioxidant activities [26].

According to the findings in literature, 20 different lentil cultivars have been reported to have TAA ranging between 596.4 and 876.7 mg TEAC/100 g DW [26] detected by DPPH assays. These results were higher than the present findings and this might be related to different cultivars and methodology used in those studies. Stronger antioxidant activity is usually observed for colored chickpea lines and Desi type of pigmented chickpeas, which have been reported to have a higher antioxidant activity than the Kabuli type [11].

Different from the colored varieties used in this current study, a common green lentil variety depicted an increased TAA levels after cooking, whereas for the common white chickpeas TAA was almost constant after cooking measured by DPPH assay [27]. Although domestic cooking has beenreported to decrease the antioxidant activity of chickpea significantly in another study[28], These decreases in TAA detected in our study, might be related with the losses in different flavonoids found in colored seed coats of the lentil

and chickpea samples used [1, 8]. The significantly high correlations ($p<0.01$) between TF and DPPH and CUPRAC assays (0.618 and 0.611, respectively) support this discussion.

Changes During *In Vitro* Gastrointestinal Digestion (GID)

Effect of *In Vitro* Digestion on TPC

In vitro GID aims to mimic the physiological environment of gastrointestinal tract to estimate the bioavailability of bioactive components (Zhang et al., 2017). *In vitro* digestion was applied directly to raw and cooked samples at each fractions; PG, IN, OUT to measure the total phenolic and total flavonoid contents. The methanolic extracts of raw samples for total free and bound forms was accepted as 100%.

Changes in TPC of BC and BL samples after *in vitro* gastrointestinal digestion are summarized in **Table 3.** *In vitro* GI digestion was applied directly to BC and BL samples (both for raw and cooked) and TPC was measured.

TPC of *in vitro* digested samples changed between 51.43 and 295.60 mg GAE/100 mg DW. The highest TPC was measured for the colon fraction (OUT) of BL raw sample (295.60 mg GAE/100 mg DW). For all samples TPC increased after *in vitro* gastric digestion phase, PG ranging from 140.1 to 195.5%. The highest increase was measured for cooked BC-Raw and BL-Cooked (around 2-fold increases) samples.

The increase in TPC after gastric digestion was in agreement with the findings in the other studies and might be explained by the breakdown of chemical bonds, the activity of digestive enzymes and the consequent release of phenolics [32]. The findings were in accordance with the previous studies, as TPC after digestion were higher than the uncooked and cooked samples of pigmented bean varieties of Mexico [14] and 6 different unpigmented pulses [27].

After the colon digestion, TPC values of the samples declined to either half or one third of the initial values ($p<0.05$) except for BL Raw sample (maintained TPC as in the PG fraction). The decreased amounts of TPC in IN fraction (absorption in small intestine) were in parallel in accordance with the previous literature which revealed that only small fractions of the phenolics in raw legume matrix were detected in the serum [15]. One possible explanation might be related with the alkaline conditions of the small intestine and bile salt secretion that may have caused changes in chemical structures and their degradation or formation of new compounds [32]. Compared to initial values, increases in OUT fractions were observed for all samples ($p<0.05$). This increase may be explained by the limited bioavailability in the serum (IN) to make these phenolics available for the microbial metabolism in large intestine (OUT) exerting beneficial effects on gut and systemic health through modulation of gut microbiota metabolism [33]. The fibers from beans and lentils were reported to contain associated hydroxybenzoic and hydroxycinnamic compounds, flavan-3-ols, procyanidins, flavonols and flavones [34]. Therefore, interactions of phenolic compounds with the indigestive polysaccharides may end up with the formation of hydrogen and hydrophobic linkages to decrease the bioaccessibility after intestinal digestion by

restricting the diffusion of the enzymes to their substrates as an entrapping matrix [24]. Therefore these commodities may only be absorbed after the activity of microorganisms in the intestinal lumen [27, 35].

In contrast, Zhang et al. (2017) depicted that although TPC after *in vitro* gastric digestion has decreased (22%), the intestinally digested amount of phenolics has increased to 51% in the cooked Canadian green lentils (cultivar Greenland). But they have not mentioned about the fraction remaining in the GI tract [3]. According to Lafarga et al. (2019), TPC was significantly higher after the gastric and intestinal phases of digestion of white chickpea and green lentil samples when compared to the initial stage ($p < 0.05$)[27].

Therefore, the digestion and bioavailability properties of the colored lentil and chickpea varieties may be considered as quite different than that of the common pulses.

Effect of *In Vitro* Digestion on TF

The effect of the *in vitro* GID on the TF is given in Table 3. For all samples TF decreased significantly after the gastric digestion ($p<0.05$). The drop during the gastric digestion (PG) was relatively limited in the BC cooked sample (57.7%) when compared with the remaining raw and cooked samples (around 3.4-5.3% of the initial TF). Therefore, cooking was significantly preserved the TF content during the gastric digestion in BC (around 40%). This was not evident for the brown lentils. However, during the intestinal digestion no such effect was detected, since TF remaining in all samples changed between 0.6-2.0% of the initial levels. In the colon fraction (OUT) TF contents were better retained in both of the cooked BC and BL samples (32-39.6%) when compared to their uncooked counterparts (7.5-9.6%).

In literature, the green lentils had a contradicting trend. According to the findings of Zhang et al (2015), TFC after the *in vitro* gastric digestion was 0.48 mg CE/g DW, which accounted for 31% in terms of bioavailability. Further intestinal digestion increased TFC values to 1.02 mg CE/g DW, resulting in a final bioavailability of 67%, respectively, after the gastrointestinal digestion [26].

However, a previous study on beans revealed that, TFC was not detected after *in vitro* digestion of common beans, for the Pinto beans the *in vitro* bioavailability was around 1.26 to 4.39 %, being similar to the current results except for the BC raw samples (23.6%)[13].

Low amount of *in vitro* bioavailability during digestion (in oral, stomach, and small intestine digestion), but relatively higher amount detected in the large intestine (OUT) by the microbial metabolism was parallel to the previous study on three different legumes (soybean, vicia faba, and kidney bean) [36].

Effect of *In Vitro* Digestion on TAA

TAA were measured at each fraction of the GID (PG, IN, OUT). The initial extracts were accepted as 100% and the results of the three different assays are given in Table 4.

TAA of the samples during the GID ranged between 2.33 and 927.38 mg TEAC/100 g DW, CUPRAC results being the highest among all three methods. According to the results from ABTS, CUPRAC and DPPH

assays, gastric digestion (PG) significantly decreased TAA contents in all samples (cooked and raw) ($p<0.05$). The most dramatic decreases were measured for ABTS assay (to 8.0-13.9 % of the initial TAA levels, higher in the cooked samples). A general evaluation of the changes during in vitro GID reveals that TAA decreases in PG, were opposite to the TP results but being more in accordance with the TF results. Among the PG results cooking was shown as protective on TAA of both BC and BL samples according to the DPPH assay (with around 20% higher TAA in cooked BC and BL than the raw counterparts). The other assays depicted no significant effects of cooking during gastric digestion. This might be related with the sensitivity of DPPH assay to the acidic condition of the gastric digestion [37].

According to ABTS and DPPH assays, further decreases ($p<0.05$) were detected in the serum (IN) of BC and BL samples, depicting no significant differences due to cooking. However according to the CUPRAC assay, significant increases ($p<0.05$) in the TAA of both of the cooked BC (to 36.6%) and BL (to 74.2%) were shown, although significant decreases were evident in the uncooked samples (to 4.7 % and 8.8%, respectively for the uncooked BC and BL). This difference might be related to the differences in the radical scavenger activity of polyphenols by different assays.

Literature findings present that the CUPRAC assay, working at pH of 7.0, might work better to make a more sensitive evaluation of the intestinal digesta [24]. Therefore, TAA increases due to the digestion in the cooked BC and BL samples were better detected. The OUT results by CUPRAC assay also support this finding since TAA was higher in the uncooked samples (59.5% and 75.7 % in BC and BL, respectively). The other two assays were not as effective, or sensitive to exert significant differences among PG and OUT fractions.

According to the literature, the significant drop in the measured TAA after the *in vitro* digestion of Pinto beans by DPPH assay, has been related with the presence of the insoluble indigestible fractions of polyphenols such as proanthocyanidins that can be fermented in the colon by microorganisms rather than the gastrointestinal digestion. However according to their findings, such digestion conditions were not able to make them soluble, and therefore colon fractions had also lower TAA, when measured by the DPPH assay [13]. In contrast, Zhu et. al (2020) more recently, reported that different bound phenolics were extremely low in *in vitro* digestion, have been released during the colonic whereas colonic fermentation according to the FRAP assay. Moreover, they highlighted significant correlations with the TFC and TAA, rather than the TPC. Therefore, our findings show that some flavonoids that are found in the pigmented BC and BL samples might have become more bioavailable by cooking to increase TAA in the OUT fraction. Although this activity is evident with only one of TAA assays, FRAP; it is important to emphasize the use of different TAA assays during studies.

Major Phenolic Compounds

According to the present findings 5 main compounds for BC (gallic acid, catechin, rutin, kaempherol and quercetin dihydrate) and 6 main compounds for BL (gallic acid, catechin, epicatechin, coumaric acid, rutin and quercetin dihydrate) were identified and they are shown in Table 5.

Catechin was the most abundant compound detected in BC samples. The highest amount was measured in BC Cooked-OUT fraction as 579.80 mg/100 g. Cooking decreased the catechin content of free fraction, whereas liberating the bound form. Although gastric digestion caused further increases to total 348.27 mg/100 g, no Catechin was detected at the end of GID. Catechin (a member of flavan-3-ols) has been reported to a low GID bioavailability [38]. However, in the colon (OUT) it was measured as 66% more than in the cooked sample. Gallic acid was also high, particularly the cooking almost doubled gallic acid in both of the free and bound fractions, although having a restricted *in vitro* bioavailability after GID. But in the colon (OUT) it was measured as around 30% of the total cooked fragment. Rutin was relatively bioavailable, although its total amount was lower.

The highest amount of phenolic compound was gallic acid (431.37 mg/100 g) in BL- Cooked Bound samples. However only a limited amount (around 4%) was bioavailable after GID, while a significant amount (30.8 %) was in the colon (OUT). Catechin also for the BL samples, in BL-Cooked OUT (210 mg/100 g). Catechin content became measureable by cooking and only around one fourth bioavailable after *in vitro* GID. A considerable amount of catechinwas detected in the OUT fraction. Although their contents were lower, epicatechin and coumaric acid were found as bioavailable after the *in vitro* GID as much as 64 and 39% of the total cooked BL, respectively.

A poor selectivity of the HPLC method for gallic acid and catechin has been mentioned in a previous study and this finding has been correlated with the interferences from polar substances coming from the enzymes and/or the simulated digestion fluids used in the simulated digestion model might have affected the bioavailability findings some other phenolics [15]. Moreover, different pH levels during the GID have been reported to cause changes in the structures of the polyphenols such as catechin to affect their bioavailability [39]. The hydroxybenzoics, hydroxycinnamics, flavonols, flavones, and flavanones besides to isoflavones have been detected as the main phenolic compounds in two chickpea varieties (Sinaloa and Castellano) [28]. The major phenolic acids in desi type chickpeas (BC) is scarce with only limited number of studies. In one of these studies, the major common phenolic acids chickpea samples of five Kabuli and one Desi (var. Elmo) chickpeas were p-hydroxybenzoic and gentisic acids, whereas syringic acid was found only in the Desi chickpea. Desi contained comprising glycosides of luteolin, myricetin and quercetin as flavonoids [4]. Rutin and quercetin has been detected in the soaking water of black chickpeas, although not identified in the raw samples [15]. The same study revealed that delphinidin 3-glucoside in black chickpeas was detected only in the soaking water. Similar to the present findings the gallic acid was predominant with also 4-hydroxybenzoic acid, syringic acid, delphinidin 3-glucoside, rutin, qurcetin, and kaempferol 3-glucoside. In a previous study gallic acid was detected in raw black lentils (10 mg/100 g dry weight) but not in the soaking or boiling water [15]. Although the detected amount was lower, present methodology to measure both of the free and bound fractions might have been helpful. Moreover, the destruction of the structural integrity of the vegetal tissue during cooking might have caused the measured increase by cooking [27]. Among 20 different lentil cultivars from Canada, kaempferol glycosides dominated the phenolic profile of lentils, followed by some catechin derivatives (catechin glucoside, catechin gallate, epicatechin glucoside). Trans-p-coumaric acid and p-hydroxybenzoic acid were the main free phenolic acids found in all lentils, but in much lower amounts.

These results suggest that the majority of extractable phenolics in tested lentils are flavonoid glycosides [26]. Similarly, p-hydroxy-benzoic acid, syringic acid, trans-p-coumaric acid, epicatechin gallate, quercetin-3-xyloside, quercetin-3-glucoside, and kaempferol-3-glucoside were detected as the main phenolic compounds from a typical green lentil variety [2]. According to another study comprising French green whole lentil, red whole lentil and green whole lentil, kaempherol, myricetin and lutein (these two flavonoids being only in the French green variety) have been the only detected flavonoids in lentils [40].

Conclusion

This study demonstrated the simulated digestion of the pigmented and relatively uncommon legumes of black chickpea (*Cicer arietinum* L.) and brown lentil (*Lens culinaris* Medikus). These legumes may play a key role to improve the colon health, particularly via their flavonoids. Black chickpeas and brown lentils should be more commonly consumed for their specific health benefits. Further studies are necessary for the in-depth investigation of their bioactives and their synergistic effects with other components.

Declarations

Author's Contributions

D.N.E. created the concept. B.E.K conducted the analyses. Z.T.C. helped in the analyses and methodology. Z.T.C. and B.E.K wrote the main manuscript text and Z.T.C. prepared the Tables. All authors reviewed the manuscript.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

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Conflict of Interest Authors state no conflict of interests with respect to the objective, interpretation, and presentation of the results in this study.

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Tables

Table 1. Total phenolic and flavonoid contents of raw and cooked samples

	TPC%	TPC (mg GAE / 100 g DW)	TFC%	TFC (mg RE/100 g DW)
BC- Raw Samples				
Free	**42.3**	44.41±10.33	**27.5**	368.75±30.42
Bound	**57.7**	60.60±10.33	**72.5**	974.01±8.66
Total	**100**	**105.01±17.43**	**100.0**	**1342.76±187.56**
BC- Steam Cooked Samples				
Free	65.6 ↑	68.84±7.62	9.7 ↓	130.12±25.49
Bound	69.5 ↑	73.01±3.71	17.5 ↓	234.43±33.17*
Total	135.1 ↑	**141.86±9.98**	27.1 ↓	**364.55±36.69***
BL- Raw Samples				
Free	**39.0**	55.91±4.59	**53.0**	402.53±39.34
Bound	**61.0**	87.35±0.46	**47.0**	335.88±8.54
Total	**100.0**	**143.26±19.82**	**100.0**	**757.14±32.41**
Steam Cooked Samples				
BL- Steam Cooked Samples				
Free	44.6 ↑	63.87±5.52	13.3 ↓	100.33±35.44
Bound	48.0 ↓	68.83±17.48	29.7 ↓	225.22±57.39
Total	92.6 ↓	**132.70±19.12**	43.0 ↓	**325.55±71.94**

Table 2. TAA of raw and cooked samples

Total Antioxidant Activity	TAA%	ABTS (mg TEAC / 100 g DW)	TAA%	CUPRAC (mg TEAC /100 g DW)	TAA%	DPPH (mg TEAC/ 100 g DW)
BC- Raw						
Free	**34.8**	60.22±1.45	**11.0**	171.56±12.21	**19.7**	12.85±1.57
Bound	**65.2**	112.58±7.23	**89.0**	1387.71±22.47	**80.3**	52.44±1.19
Total	**100.0**	**172.80±6.84**	**100.0**	**1559.27±39.20**	**100.0**	**65.29±1.89**
BC- Steam Cooked						
Free	**47.3** ↑	81.78±7.83*	**14.1** ↑	220.70±6.82*	**25.0** ↑	16.33±1.43*
Bound	**71.3** ↑	123.21±13.87	**76.5** ↓	1192.32±55.27	**50.6** ↓	33.04±2.83*
Total	**118.6** ↑	**204.99±19.29**	**90.6** ↓	**1413.02±78.28**	**75.6** ↓	**49.38±4.84**
BL-Raw						
Free	**27.5**	57.73±1.57	**26.4**	247.72±17.19	**27.5**	20.29±6.14
Bound	**72.5**	151.98±5.59	**73.6**	692.08±55.44	**72.5**	53.39±4.83
Total	**100**	**209.71±6.81**	**100**	**939.81±60.57**	**100**	**73. 68±4.49**
BL- Steam Cooked						
Free	**36.6** ↑	76.70±1.71	**26.9** ↑	252.43±14.51	**31.7** ↑	23.38±2.16
Bound	**73.3** ↑	153.67±11.30	**59.9** ↓	563.13±153.99	**52.0** ↓	38.29±2.97*
Total	**109.9** ↑	**230.37±11.14**	**86.8** ↓	**815.57±155.01**	**83.7** ↓	**61.67±7.77**

*Values are means of triplicate measurements ± standard deviations. Different letters in the same column represent significant difference at $p<0.05$.

Table 3. The effect of *in vitro* digestion on total phenolic and flavonoid contents

Total Phenolics (TPC) (mg GAE/100 mg DW)	BC-Raw	BC-Steam Cooked	BL-Raw	BL-Steam Cooked
Initial	105.01±17.43^{b} **(100%)**	141.86±9.98^{b} **(100%)**	143.26±19.82^{b} **(100%)**	132.70±19.12^{b} **(100%)**
PG	214.76±16.58^{a} **(204.4%)**	198.74±7.14^{a} **(140.1%)**	223.52±56.31^{ab} **(156.0%)**	259.44±11.40^{a} **(195.5%)**
IN	52.77±2.53^{c} **(50.3%)**	51.43±6.99^{c} **(36.3%)**	229.03±14.70^{ab} **(159.9%)**	58.09±10.19^{c} **(32.3%)**
OUT	217.92±24.87^{a} **(207.5%)**	196.96±25.75^{a} **(138.8%)**	295.60±18.60^{a} **(206.3%)**	221.95±6.99^{a} **(167.3%)**
Total Flavonoid Content (TFC) (mg RE/100 g DW)	BC-Raw	BC-Steam Cooked	BL-Raw	BL-Steam Cooked
Initial	1342.76±187.56^{a} **(100%)**	364.55±36.69^{a} **(100%)**	1074.29±238.29^{a} **(100%)**	325.55±71.94^{a} **(100%)**
PG	71.38±5.53^{c} **(5.3%)**	154.12±45.94^{b} **(42.3%)**	36.62±6.25^{c} **(3.4%)**	12.69±18.89^{c} **(3.9%)**
IN	316.50±12.76^{b} **(23.6%)**	2.28±1.29^{c} **(0.6%)**	16.65±0.52^{c} **(1.6%)**	6.63±3.78^{c} **(2.0%)**
OUT	100.35±8.11^{a} **(7.5%)**	144.36±32.75^{b} **(39.6%)**	138.70±37.87^{b} **(12.9%)**	104.22±22.02^{b} **(32.0%)**

*Values are means of triplicate measurements ± standard deviations. Different letters in the same column represent significant difference at $p<0.05$.

Table 4. Effect of *in vitro* digestion on TAA

Total Antioxidant Activity	Sample			
ABTS **(mg TEAC/100 g DW)**	BC-Raw	BC-Steam Cooked	BL-Raw	BL-Steam Cooked
Initial	172.80±6.84[a] **(100%)**	204.99±19.29[a] **(100%)**	209.71±6.81[a] **(100%)**	230.37±11.14[a] **(100%)**
PG	23.58±0.16[b] **(13.6%)**	16.32±3.05[b] **(8.0%)**	29.10±1.42[b] **(13.9%)**	26.48±1.18[b] **(11.5%)**
IN	11.09±0.39[c] **(6.4%)**	10.53±0.03[c] **(5.1%)**	10.32±0.03[c] **(4.9%)**	10.14±0.45[c] **(4.4%)**
OUT	ND	ND	ND	ND
CUPRAC **(mg TEAC/100 g DW)**	BC-Raw	BC-Steam Cooked	BL-Raw	BL-Steam Cooked
Initial	1559.27±39.20[a] **(100%)**	1413.02±78.28[a] **(100%)**	939.81±60.57[a] **(100%)**	815.57±15.01[a] **(100%)**
PG	372.37±63.49[c] **(23.9%)**	328.34±31.87[c] **(23.2%)**	271.06±11.85[c] **(28.8%)**	247.29±7.02[c] **(30.3%)**
IN	73.54±1.98[d] **(4.7%)**	517.03±68.00[b] **(36.6%)**	82.63±3.47[d] **(8.8%)**	604.98±13.91[b] **(74.2%)**
OUT	927.38±51.01[b] **(59.5%)**	138.93±5.34[d] **(9.7%)**	711.54±41.60[b] **(75.7%)**	108.26±15.10[d] **(13.3%)**
DPPH **(mg TEAC/100 g DW)**	BC-Raw	BC-Steam Cooked	BL-Raw	BL-Steam Cooked
Initial	65.29±1.89[a] **(100%)**	49.38±4.84[a] **(100%)**	73.68±4.49[a] **(100%)**	61.67±17.77[a] **(100%)**
PG	12.92±1.42[b] **(19.8%)**	19.59±1.51[b]	13.32±0.16[b] **(18.1%)**	18.21±1.63[b]

		(39.7%)		**(29.5%)**
IN	3.33±0.03^{c} **(5.1%)**	5.22±0.27^{c} **(10.6%)**	2.33±0.39^{c} **(3.2%)**	4.52±0.26^{c} **(7.3%)**
OUT	4.40±0.39^{c} **(6.7%)**	3.98±0.91^{c} **(8.0%)**	7.75±0.57^{bc} **(10.5%)**	5.89±0.75^{c} **(9.6%)**

*Values are means of triplicate measurements ± standard deviations. Different letters in the same column represent significant difference at $p<0.05$. The terms represent; PG, post gastric fraction leaving the stomach; IN, fraction entering the serum-dialyzable fraction; OUT, fraction remaining in the GI tract-undialyzable fraction.

ble 5. Major phenolic components that were identified in raw, steam cooked and GID samples.

Compound (mg/100 g)	Gallic acid	Catechin	Epicatechin	Coumaric acid	Rutin	Kaempherol	Quercetin dihydrate
Sample							
BC							
BC-Raw-Free	5.47± 0.44	184.47± 2.40	ND	ND	ND	1.05± 0.74	ND
BC-Raw-Bound	171.83± 36.29	ND	ND	ND	6.42± 0.08	0.54± 0.04	2.60± 0.13
BC-Cooked-Free	9.60± 1.43	133.82± 20.50	ND	ND	4.60± 0.28	ND	ND
BC-Cooked Bound	319.53± 10.30	54.28± 5.99	ND	ND	4.18± 0.28	0.53± 0.05	5.88± 0.45
BC-PG-Cooked	2.70± 0.80	348.27± 48.9	ND	ND	13.08± 0.00	ND	ND
BC-IN-Cooked	6.13± 1.61	ND	ND	ND	4.17± 0.28	ND	ND
BC-OUT-Cooked	107.50± 19.3	579.80± 34.8	ND	ND	6.07± 0.83	ND	ND
BL							
BL-Raw-Free	35.65± 9.39	ND	15.2± 3.72	5.73± 0.18	5.55± 0.00	ND	ND
BL-Raw-Bound	6.13± 0.06	ND	ND	0.98± 0.11	ND	ND	30.38± 0.84
BL-Cooked-Free	4.98± 0.46	16.28± 0.95	23.67± 0.51	8.20± 0.22	3.17± 0.11	ND	ND
BL-Cooked-Bound	431.37± 19.37	42.10± 3.73	11.20± 2.31	ND	8.55± 0.76	ND	42.88± 9.67
BL-PG-Cooked	3.12± 0.07	65.92± 2.45	76.70± 0.22	ND	7.15± 0.88	ND	ND
BL-IN-	18.75±	12.78±	22.53±	3.15±	ND	ND	2.07±

Cooked	2.86	3.57	2.90	0.82			0.26
BL-OUT-Cooked	134.38± 37.67	210.07± 9.78	ND	19.70± 0.83	ND	ND	ND

*Values are means of triplicate measurements ± standard deviations. The terms represent; PG, post gastric fraction leaving the stomach; IN, fraction entering the serum-dialyzable fraction; OUT, fraction remaining in the GI tract-undialyzable fraction