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Effect of simulated gastrointestinal digestion on the phenolic compound content and in vitro antioxidant capacity of processed Cowpea (V. unguiculata) cultivars

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ABSTRACT

The aim of this study was to analyze the phenolic content and antioxidant activity of five cowpea cultivars after processing and in vitro digestion. Raw cowpea samples showed a significant decrease in total phenolic content when compared with the processed samples, however an increase was subsequently observed in digested samples. The antioxidant activity determined using DPPH, ABTS, ferric reducing antioxidant power (FRAP) assay, and total peroxyl radical-trapping antioxidant parameter showed a similar trend to the phenolic content with a significant decrease in activity upon processing and an increase after digestion. In conclusion, all cowpea cultivars showed a high TPC content as well as an increased antioxidant activity after digestion indicating the potential health benefits which cowpea could provide to consumers. Therefore, this study shows that in vitro digestion improves the digestion and absorption of beneficial components of processed cowpea at the intestinal level.

Introduction

The world today is encountering concerns that relate to poor nourishment and particularly the coexistence of under- and overnutrition. This leads to a dual problem regarding health that includes infectious and noncommunicable diseases. Amongst the many food sources that may possibly be consumed, based on traditional knowledge, in order to establish improved health is cowpea (Vigna unguiculata). Cowpea is one of the most nutritive, versatile, and widely adapted of the grain legumes and has been classified as an indigenous and underutilized food security crop in South Africa. The Agricultural Research Council has characterized and selected different cowpea genotypes for yield and yield-related traits to improve the genetic potential of this crop that will improve the food security and increase the income of the communities within rural areas. Bressani (1985) noted that the nutritional profile of cowpea is similar to that of common beans; however, cowpea has lower levels of anti-nutritional factors. According to Knekt et al. (2002), this legume is a good source of phenolic compounds and has the potential to protect the body against chronic diseases. This is also in agreement with the results from a study by Wang, Melnyk, Tsao, and Marcone (2011) where the majority of plant-based foods (including cowpea) were identified as having a high content of phytochemical antioxidants and exhibit cardio-health promotion properties. The awareness of consumers with regard to the health benefits associated with legumes has increased in recent years resulting in an increase in the demand for convenient, ready-to-eat whole grain cereal, and leguminous products. However, processing methods have been shown to affect both the quality and quantity of available phytochemicals. Previous studies have reported a decrease in the anthocyanin and total phenolic content (TPC) in thermally processed corn, but the thermal processing may have been responsible for the release of bound phenolic...
acids (Dewanto, Wu, Adom, & Liu, 2002). Other studies have also shown thermal processing to significantly decrease the TPC, anthocyanin content, and antioxidant activity (Hiemori, Koh, & Mitchell, 2009). TPC compounds are known to act as antioxidants, delaying the formation of free radicals or reducing reactive oxygen species, which may lead to deterioration of biological molecules (Fereidoon Shahidi, 1997). Bermúdez-Soto, Tomás-Barberán, and García-Conesa (2007) found that digestion might be responsible for an alteration to the composition and levels of TPC. This amplifies the importance to investigate the effect of digestion on the antioxidant capacity of cowpea. The effect of simulated gastrointestinal digestion on TPC and antioxidant capacity of cooked cowpea varieties was investigated by Hachibamba, Dykes, Awika, Minnaar, and Duodu (2013), who found that the TPC and radical scavenging property of cowpea was reduced upon cooking, but increased with simulated enzyme digestion. From previous literature, it can be concluded that in vitro digestion affects TPC and antioxidant activity. In a study by Fallier, Fialho, and Liu (2012), the TPC and flavonoid content in feijoada (beef and pork stewed with beans) were unaffected by digestion, however the antioxidant activity was higher before digestion than afterwards. In vitro digestion of white-bread samples was also found to increase antiradical activity (Gawlik-Dziki et al., 2013). In the present study, an in vitro model of the gastrointestinal tract was used to simulate the digestion process to assess any changes in the antioxidant activity of extracts from raw and processed cowpea samples, in order to evaluate the impact of digestion on the TPC and antioxidant capacity.

Materials and methods

Sample preparation

Samples of five cowpea cultivars (Veg Cowpea 2, Veg Cowpea 3, Makhatini, Embu buff, and Glenda) were obtained from the Agricultural Research Council-Vegetable and Ornamental Plant Institute (ARC-VOPI), Pretoria, South Africa. Plants were grown at the Research Farm of ARC-VOPI [25.6045 28.345E], during the 2014/2015 cropping seasons at an altitude of 1168 m above sea level. The location received approximately 610 mm of rain during the growing period with a minimum and maximum recorded temperature of 9.11°C and 36.37°C, respectively, during the growth period. Legumes from each of the cultivars were subjected to two different processing methods viz. boiling and pressure-cooking. Pretreatment involved soaking 50 g of each of the respective cultivars for 24 h at 25°C in a ratio of 1:10 w/v, the water was then decanted and the cowpea samples subjected to the different processing techniques according to Sagratini et al. (2013); Boiling: Pretreated cowpea samples were weighed and boiled for 30 min in distilled water (1:20 w/v) in a pot. The cooking liquids and cowpea were separated by filtration for simulated gastric digestion.

Simulated gastrointestinal digestion model

Simulated gastrointestinal digestion was carried out according to methods defined by Gil-Izquierdo, Zafrilla, and Tomás-Barberán (2002) with minor modifications. The process involves three consecutive phases: the first two phases are saliva and gastric digestion, to mimic the mouth and the gastric conditions, respectively, followed by digestion with bile salts and pancreatin, which mimics the intestinal digestion process. For the first two digestion phases, 10 g of cowpea sample (raw and processed, respectively) was mixed with 6 ml of synthetic saliva comprising KCl (89.6 g/L), KSCN (20 g/L), NaH₂PO₄ (88.8 g/L), Na₂SO₄ (57 g/L), NaCl (175.3 g/L), NaHCO₃ (84.7 g/L), urea (25 g/L), and 290 mg of α-amylase [Sigma-Aldrich (5 kU)]. The pH of this solution was adjusted to 6.8 with 0.1 N NaOH. The cowpea and synthetic saliva mixture were then placed in stomach bags containing 40 ml of distilled water and homogenized using a stomacher for 30 s. To this mixture, 0.5 g of pepsin [Sigma-Aldrich (250 U/mg)] dissolved in 25 mL of 0.1 N HCl was added. The mixture was then adjusted to a pH of 2 using 6 N HCl, and incubated in a 37°C orbital shaker at 250 rpm for 2 h. Following this procedure the intestinal digestion phase was simulated. The pH was increased to 6.5 using 0.5 N NaHCO₃, and then 5 mL of (1:1 v/v) pancreatin (8 mg/mL) [Sigma-Aldrich] and bile salts (50 mg/mL), dissolved in 20 mL of water, were added and incubated in a 37°C orbital shaker at 250 rpm for 2 h.

Determination of total phenolic content (TPC)

TPC was determined using the Folin–Ciocalteau assay as noted by Hachibamba et al. (2013), briefly 1 ml of the sample (1, 20, 40, 60, 80, 100, 250, and 500 μg/ml respective sample concentrations) was reacted with 0.4 ml Folin–Ciocalteau reagent and 0.9 ml of 0.5 M ethanolamine for 20 min at 25°C. The absorbance was measured at 765 nm against a reagent blank (70% aqueous acetone). A calibration curve was generated using a standard gallic acid solution (R² = 0.9913). The TPC values were expressed as milligrams of gallic acid equivalents (GAE)/g of cowpea sample (dry basis).

Determination of antioxidant capacity

Free radical scavenging capacity (DPPH)

The DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging capacity was determined by using a method by Oboh (2006). 1 ml aliquots of samples (1, 20, 40, 60, 80, 100, 250, and 500 μg/ml) were mixed with 1 ml, 0.3 mM methanol solution containing 1.5 mM DPPH solution. The mixture was left in the dark at 25°C for 30 min before determining the absorbance at 516 nm. Rutin (1 mM) was used as a positive control.

DPPH scavenging capacity (%) = (absorbance sample/absorbance control) x 100.

The scavenging effect of the samples was expressed as 50% effective concentration (EC₅₀), which represented the concentration of sample having 50% DPPH radical scavenging effect.
ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging assay

The total antioxidant activity of cowpea extracts was measured by the ABTS radical cation decolorization assay containing preformed ABTS radical cation, according to methods by Siddhuraju (2006). ABTS was generated by mixing 1000 ml of 1.7 mM ABTS with 18 ml of 47 mM potassium per sulfate in the dark at 25°C for 16 h. The solution was diluted to a ratio of 1:15 (deionized water) and the absorbance was measured at 734 nm. The ABTS radical cation scavenging activity of the samples was then assessed by mixing 3 ml ABTS solution (absorbance of 0.7 ± 0.05) with 1 ml of sample (1, 20, 40, 60, 80, 100, 250, and 500 µg/ml). Ascorbic acid (1 µg/ml) was used as a positive control. Scavenging capability was calculated by the following formula: % of scavenging = ((A₀−A₁)/ A₀) × 100, where A₀ is absorbance of control and A₁ is absorbance of sample.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was performed as described by Benzie and Szeto (1999). The FRAP reagent was prepared as follows: A – Acetate buffer 300 mM (pH 3.6); B – TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in 40 mM HCl; and C – FeCl₃ 6H₂O (M.W. 270.30) 20 mM. The working FRAP reagent was prepared by mixing solutions A, B, and C in the ratio of 10:1:1 at the time of use. To measure the FRAP, 1 ml of the sample (1, 20, 40, 60, 80, 100, 250, and 500 µg/ml) was mixed with 3 ml of working FRAP reagent and the absorbance (593 nm) was measured at 0 min after mixing. Thereafter, a sample was placed at 37°C in water bath for 4 min and the absorbance was again measured. Ascorbic acid (1000 µM) was used as a positive control.

Total peroxyl radical-trapping antioxidant parameter (TRAP)

The radical-trapping antioxidant parameter (total TRAP) was determined according to the methods by Cao, Alessio, and Cutler (1993) and Ioannone et al. (2015). Briefly, the final reaction mixture of 100 ml for the assay contained 0.43 mg R-phycocerythrin (target probe) and 0.3 g AAPH (peroxyl radicals) in 50 mM phosphate buffer (pH = 7.4). A final volume of 2 ml was used with 1 ml diluted sample (1, 20, 40, 60, 80, 100, 250, and 500 µg/ml of cowpea sample). Once the sample was mixed, the reaction mixture was incubated at 37°C for 5 min. Fluorescence was measured in a quartz cuvette at the emission of 565 nm and excitation of 540 nm using a fluorescence spectrophotometer [Varian (Cary Eclipse) Fluorescence Spectrophotometer]. Ascorbic acid was used to develop a standard curve and TRAP values were calculated using a calibration curve obtained from increasing concentrations of Trolox. TRAP results were expressed as µmol of Trolox equivalents per g (dry weight).

Statistical analysis

All experiments were carried out in triplicate and data expressed as mean ± standard deviation (SD). Experimental data were analyzed using two-way analysis of variance and Tukey’s multiple comparison tests of means. A level of p ≤ 0.05 was considered significant. Statistical computations and analyses were carried out using GraphPad Prism.

Results and discussion

An in vitro gastrointestinal model was used in this study to mimic the in vivo physiological environment. TPC was found to be high in the raw samples of all the V. unguiculata cultivars, however after processing a significant reduction was observed. This may be attributed to the fact that the raw cowpea samples had the full variety of the phenolic compounds found in the cultivars as noted in a study by Nderitu, Dykes, Awika, Minnaar, and Duodu (2013). The reduction in TPC observed in the cowpea cultivars after processing may be due to the leaching of phenolic compounds into the cooking water which was subsequently discarded. In a study by Siddhuraju and Manian (2007), it was found that during processing phenolic compounds had the ability to form complexes with proteins as well as carbohydrates consequently becoming unextractable. Oxidation of other phenolic compounds during cooking may have also occurred since different compounds have different levels of susceptibility to oxidation due to their chemistry. There was no significant difference observed in the TPC of boiled against the pressure-cooked samples across all the cultivars. With regard to samples that underwent simulated digestion, the TPC of all digested samples was increased in both the processed samples and those subjected to simulated digestion (Figure 1). However, the difference was only significant in pressure-cooked vs. pressure-cooked digested samples (p ≤ 0.05). There was no significant difference that was observed in TPC between pressure-cooked samples and those subjected to simulated digestion across all the cultivars with the exception of Veg Cowpea 3. The conditions generated in the course of simulated gastrointestinal digestion due to the enzymes used as well as the pH conditions encourage macromolecules to undergo hydrolysis, these macromolecules include proteins, which may be bound to phenolic compounds. This binding of phenolic compounds with macromolecules has the ability to be liberated therefore becoming more extractable in the enzyme digestion (Hachibamba et al., 2013). Components of the food matrix may also contribute significantly to improvement of the stability of the phenolic compounds in various pH conditions of simulated gastrointestinal digestion. In a study by Ortega, Macià, Romero, Reguant, and Motilva (2011) on carob flour, it was reported that the soluble dietary fiber as well as the lipids, were found to bring about an improvement in the stability of phenolic compounds in the course of in vitro duodenal digestion. Overall, there was a trend in TPC, which displayed a directly proportional result with regard to the antioxidant assays of all the cultivars. Results show that readings were high on the raw samples and subsequently decreased upon processing, however an increase was observed after samples were subjected to simulated digestion. This trend may be attributed to the fact that phenolic compounds are mainly responsible for the antioxidant activity of the samples, therefore the higher the TPC, the higher the antioxidant activity (Brewer, 2011; Shahidi, 2006).

The free radical scavenging capacity values were found to be significantly high for all the raw samples (Figure 2(a)). The percentage DPPH values in the raw samples of ranged from concentrations 60–500 µg/ml
showing no significant difference between cultivars or concentrations, however at concentrations 1–40 µg/ml there was a significant difference between both the cultivars and the concentrations ($p \leq 0.05$). The percentage radical scavenging capacity of processed samples (Figure 2(b and c)) was reduced when compared with the raw samples (Figure 2(a)). Although there was no significant difference that was found between the pressure-cooked and boiled samples across all the cultivars, there was a significant difference between the raw and pressure-cooked samples ($p \leq 0.05$) apart from Veg Cowpea 2. However, only two cultivars (Glenda and Makathini) displayed significant difference between raw and boiled samples ($p \leq 0.05$) with respect to the samples that underwent simulated digestion, the percentage radical scavenging capacity of all digested samples increased in both pressure-cooked and boiled samples as shown in Figure 2(d and e). The increase in the DPPH values of digested samples was such that there was no significant difference from those found in the raw samples across all the cultivars. Both pressure-cooked vs. pressure-cooked (digested) and boiled vs. boiled (digested) showed a significant difference ($p \leq 0.05$). The DPPH values of pressure-cooked (digested) and boiled (digested) were found not to be significantly different ($p \leq 0.0001$) (Figure 2(d and e)). However, these values were increased after the samples were subjected to simulated digestion post processing. When comparing processed and raw samples, the unprocessed samples had a higher free radical scavenging capacity which may be attributed to a high amount of free radical reducing phenolic compound present which are found not to be stable upon cooking due to the heat treatment i.e. pressure-cooking as well boiling. The DPPH values in the raw samples amongst different concentrations (60–500 µg/ml) had high values and did not vary significantly for concentrations ranging from 1–40 µg/ml. This lack of variation may be due to raw samples having a high amount of reactive compounds that react with DPPH radical even in low concentrations, resulting in high readings. In previous studies, compound such as carotenoids, have been found to interfere with test.
results (Rajamanikandan et al., 2011). The percentage DPPH values of processed samples (Figure 2(b and c)) were reduced when compared with the raw samples (Figure 2(a)). This may be attributed to a decrease in reducing agents that had high availability of atoms, which can donate electrons therefore reacting with DPPH free radicals and consequently causing a conversion of these free radicals into stable compounds that will end the radical chain activity (Rajamanikandan et al., 2011). After simulated digestion, the percentage DPPH values of all digested samples increased in both pressure-cooked and boiled samples exposed to simulated digestion (Figure 2(d and e)). This may be attributed to the DPPH color being lost through radical reaction or reduction, as well as other unrelated reactions that might have also occurred. Simulated digestion may also have increased the steric accessibility for the DPPH to react with the compounds (Pyrzynska & Pękal, 2013). This could have occurred by decreasing the size of molecules into smaller portions (due to enzymatic action or pH conditions), since small molecules are found to have better access to the radical site of DPPH.

FRAP of cowpea samples were found to be significantly high for all the raw samples as shown in Figure 3(a). Across the concentration range (1–500 µg/ml), Veg Cowpea 3 was observed to have the highest percentage FRAP values in contrast to Veg Cowpea 2 which had the lowest. The percentage FRAP values of processed samples (Figure 3(b and C)) were found to be reduced when compared with the raw samples (Figure 3(a)). Although there was no significant difference found between pressure-cooked and boiled samples across all the cultivars, there was however a significant difference between raw and processed samples (pressure-cooked samples and boiled samples) (p ≤ 0.05). With regard to samples that underwent simulated digestion, FRAP values of all digested samples were increased in both pressure-cooked vs. pressure-cooked and digested; as well as boiled vs. boiled and digested as shown in Figure 3(d and e). The increase in FRAP values of digested samples was such that
there was no significant difference from those found in raw samples across cultivars. However, FRAP values were observed to increase after the samples were subjected to simulated digestion. The FRAP values in the raw samples of Glenda amongst different concentrations (1–500 µg/ml) were observed to be higher in comparison to raw samples of other cultivars. In previous studies, it was found that lipid-soluble antioxidants were responsible for total protection to the target probe (R-phycoerythrin), which may have caused the other cultivars to have a lower reading when compared to Glenda (Cao et al., 1993).

The capacity of cowpea samples to scavenge ABTS free radicals was found to be significantly high for all the raw samples as well as raw vs. boiled samples (p ≤ 0.05). With regard to samples that underwent simulated digestion, the ABTS values of all digested samples were found to increase in both pressure-cooked vs. pressure (digested) and boiled cooked vs. boiled (digested) as shown in Figure 4(d and e). The increase in ABTS values of digested samples was such that they were not significantly different from those found in raw samples across all the cultivars. Both pressure-cooked vs. pressure (digested) and boiled cooked vs. boiled (digested) were significantly different (p ≤ 0.05). The ABTS values of pressure (digested) and boiled (digested) were found not to be significantly different from each other as shown in Figure 4(d and e). However, ABTS values were observed to increase after the samples were subjected to simulated digestion post processing. The change in pH conditions during simulated digestion could be the main reason for an increase in the antioxidant capability in this test due to the electron transfer being facilitated at an acidic pH in this assay. Thermodynamics may also have had an influence since a compound could reduce ABTS if it possesses
Most phenolic compounds were found to have a redox potential, which is lower than that of ABTS; therefore can be involved in a reaction with ABTS (Rajamanikandan et al., 2011).

The potential of cowpea samples to restrict the reaction between peroxyl radicals generated by AAPH and a target probe (R-phycoerythrin) was expressed in terms of TRAP values as shown in Figure 5. The values were found to be significantly high for all the raw samples as shown in Figure 5(a) with values for the raw samples of Glenda at concentrations 1–500 µg/ml observed to be higher in comparison to other cultivars. This is similar to finding for the ABTS assay where no significant difference was found in pressure-cooked vs. boiled cooked samples across all the cultivars, however there was a significant difference in raw vs. pressure-cooked as well as raw vs. boiled cooked samples. With regards to the samples that underwent simulated digestion, the TRAP values of all digested samples increased in both pressure-cooked vs. pressure (digested) and boiled cooked vs. boiled (digested) as shown in Figure 5(d and e). In both pressure-cooked vs. pressure (digested) and boiled cooked vs. boiled (digested), there was a significant difference. Overall, the trend was similar with the observations made in the TPC determination results, FRAP assay, DPPH assay as well as the ABTS assay; the TRAP values of all the cultivars were high for raw samples and then decreased upon processing. However, these values were observed to increase after the samples were subjected to simulated digestion post processing. The reduction of TRAP values upon processing may possibly be explained by the decrease of flavanols as well as proanthocyanidins (Ioannone et al., 2015). With regard to the samples that underwent simulated digestion, the TRAP values of all digested samples increased in both pressure-cooked vs. pressure (digested) and boiled cooked vs. boiled (digested). The increase of TRAP values observed in the digested samples may be attributed to the development of proanthocyanidins, which possess high molecular weight as well as higher reducing power (Di Mattia et al., 2013).
Conclusion

In conclusion, processing and simulated digestion were found to significantly affect the TPC and the antioxidant activity for the *V. unguiculata* cultivars. The changes in the overall antioxidant activity of processed cowpea may be attributed to cumulative factors, such as the leaching of water-soluble antioxidant compounds and the formation or breakdown of antioxidants during processes. Processing reduced the TPC and antioxidant properties of the cowpea cultivars, however these parameters were found to increase upon simulated digestion. This implies that products of enzyme digestion, such as phenolics, may possess bioactivity and have the potential to scavenge reactive oxygen species thereby potentially defending the human body against chronic diseases as a result of excessive free radicals formed.

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Disclosure statement

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