# Effect of simulated *in vitro* upper gut digestion of processed cowpea beans on phenolic composition, antioxidant properties and cellular protection.

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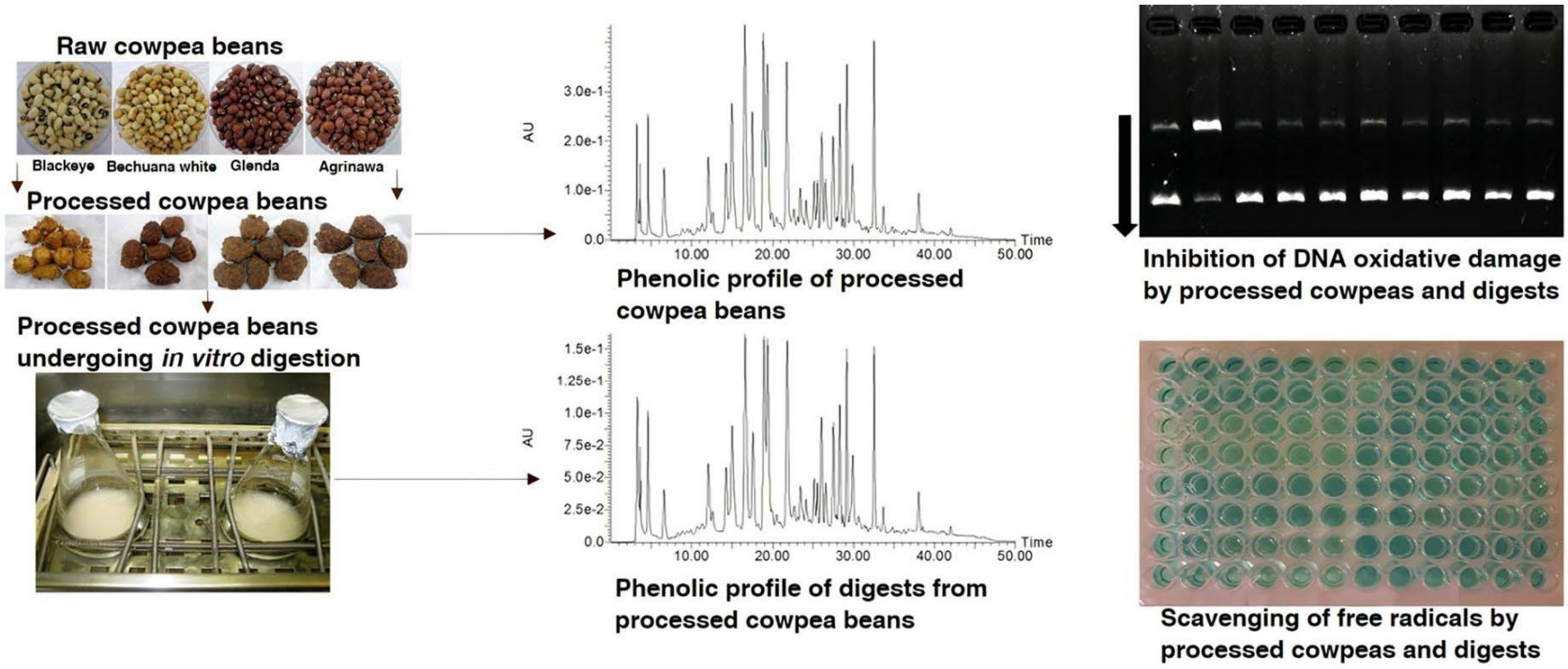
**Graphical abstract**



**Abstract**

The effect of simulated *in vitro* upper gut digestion on the phenolic composition and antioxidant properties of processed cowpea beans was studied. The samples comprised four cowpea cultivars: a cream, brownish-cream and two reddish-brown cultivars. Dry cowpea seeds were soaked in water, blended into paste and deep-fried in vegetable oil. The fried samples were taken through *in vitro* upper gut digestion followed by freeze-drying of the supernatant. Phenolic composition of extracts from the supernatants were determined using HPLC-MS. Radical scavenging activities were documented using the TEAC, ORAC and nitric oxide (NO) assays. *In vitro* digestion of the processed cowpeas resulted in phenolic-peptide complexes that were identified for the first time, and decreased extractable phenolic compounds. However, the radical scavenging activities increased. The processed cowpeas and their digests inhibited cellular NO production, and oxidative DNA and cellular damage. In conclusion, deep-fried cowpeas when consumed, could potentially help alleviate oxidative stress-related conditions.

**Keywords**

*Vigna unguiculata*, digests, phenolic acids, flavonoids, phenolic-peptide interactions, murine fibroblasts, DNA protection, antioxidants, HPLC-MS.

## 1.0 Introduction

Cowpeas (*Vigna unguiculata*) are pulses with excellent nutritional composition, since they are a good and inexpensive source of plant proteins, carbohydrates (such as starch), dietary fibre (Devi, Kushwaha, & Kumar, 2015; Madodé, Houssou, Linnemann, Hounhouigan, Nout, & Van Boekel, 2011) and water-soluble vitamins (Nnanna & Phillips, 1989). Therefore they provide nourishment to millions of consumers in the Southern Hemisphere (Li, Yang, Liu, Redden, Maalouf, & Zong, 2017; Nedumaran, Abinaya, Jyosthnaa, Shraavya, Rao, & Bantilan, 2015).

Cowpeas are also known to contain phenolic compounds that include flavonoids, phenolic acids (Dueñas, Fernandez, Hernandez, Estrella, & Muñoz, 2005), proanthocyanidins (Ojwang, Yang, Dykes, & Awika, 2013) and anthocyanins (Ojwang, Dykes, & Awika, 2012). These phenolic compounds are hypothesized to contribute to antioxidant health benefits of cowpea.

Cowpea seeds are cooked for consumption either by washing and boiling in water (Hachibamba, Dykes, Awika, Minnaar, & Duodu, 2013) or by soaking in water prior to boiling (Mtolo, Gerrano & Mellem, 2017). Soaking softens the seeds and reduces the cooking time. Pressure cooking has also been reported for cowpea seeds (Mtolo et al., 2017). Another cooking method involves washing and soaking the seeds in water, wet milling into a paste and deep-frying the paste in oil (Apea-Bah, Serem, Bester, & Duodu, 2017). The deep-fried cowpea products are usually consumed as side dish or snack (Madodé et al., 2011). While many different cowpea cultivars varying in seed coat colour are known, the lighter seed coat-coloured cultivar (*Blackeye*) is the most preferred for deep frying because they produce an attractive golden brown-coloured product. Darker cowpea cultivars, on the other hand, produce darker products with less consumer appeal (Apea-Bah et al., 2017).

The effects of boiling cowpea seeds in water and simulated *in vitro* gastrointestinal digestion on their phenolic composition and antioxidant capacities have been reported (Hachibamba et al., 2013). While some dietary phenolic constituents are thermally labile, others appear not to be affected by boiling (Nderitu, Dykes, Awika, Minnaar, & Duodu, 2013). Boiling decreased the total quantified phenolic compounds content in *Agrigold* (a golden-yellow cowpea cultivar) by 20% but increased the value in *Glenda* (a reddish-brown cowpea cultivar) by 25% (Hachibamba et al., 2013). In both cases, however, their total phenolic content and radical scavenging activities were unaffected by boiling. Nderitu et al. (2013), on the other hand, reported

boiling to decrease the total quantified phenolics content by 29% in *Agrinawa* (a reddish-brown cowpea cultivar) while the value for *Blackeye* (a cream cowpea cultivar) was unaffected. When the boiled cowpeas were subjected to simulated *in vitro* upper gut digestion, the total quantified phenolic compounds in *Agrigold* and *Glenda* decreased by 64% and 82%, respectively (Hachibamba et al., 2013), while the levels in *Agrinawa* and *Blackeye* remained unaffected (Nderitu et al., 2013).

Our previous study on the processed cowpea beans investigated the effect that deep-frying as a cooking method, had on the composition of phenolic compounds and antioxidant health-promoting properties of cowpea, and confirmed that phenolic compounds in different cowpea cultivars respond to thermal processing differently (Apea-Bah et al., 2017). The study reported deep-frying to cause a 1.5 fold decrease in total quantified phenolic acids in *Blackeye* cowpea type while the levels in three other cultivars increased by 1.7-2.7 folds. Also, while deep-frying had no effect on the total quantified flavonoids in *Blackeye*, it decreased the levels by 6-22 folds in the three other cultivars (Apea-Bah et al., 2017). To date, no work has been reported on how *in vitro* upper gut digestion affects the constituent phenolic compounds and antioxidant capacities of processed cowpea beans. It is hypothesized that *in vitro* upper gut digestion will increase the total quantified phenolic compounds and their antioxidant capacities in the processed cowpea beans due to enzymatic hydrolysis of macromolecular nutrients such as proteins that interact with some of the phenolic compounds, thereby enhancing their extractability. The objective of the study therefore, was to determine the effect of simulated *in vitro* upper gut digestion on the phenolic composition and antioxidant properties of deep-fried cowpea beans. This study is important since it will provide information about the potential phenolic-antioxidant health benefits of processed cowpea beans, which are breakfast side dish and snack to millions of consumers in developing

nations. Again, it will provide an alternative preparation method for cowpea thereby enhancing its utilization.

## 2.0 Materials and Methods

### *2.1 Chemicals*

Flavonoid standards: (+)-catechin and quercetin, as well as porcine pepsin A, porcine pancreatin, porcine bile extract, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), Dulbecco's Modified Eagle's Medium (DMEM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2',7'-dichlorofluorescein diacetate, fluorescein disodium salt and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). pBR322 plasmid DNA was procured from Promega (Madison, WI, USA). L929 murine fibroblast cells was obtained from Cellonex, and supplied by Separations (Johannesburg, Gauteng, South Africa). All other reagents and solvents were obtained from ThermoFisher Scientific (West Palm Beach, FL, USA) or Merck Chemical Co. (Johannesburg, Gauteng, South Africa).

### *2.2 Raw Materials*

Four cowpea cultivars were used in this study and they comprise the following: a) *Glenda* cultivar with a reddish-brown seed coat colour was obtained from Agricol (Potchefstroom, South Africa); b) *Agrinawa* cultivar with reddish-brown seed coat colour was obtained from Premier Seed International (Pretoria, South Africa); c) *Bechuana white* cultivar with brownish-cream seed coat was obtained from Agricol (Potchefstroom, South Africa); d) *Blackeye* cultivar with a cream seed coat colour was purchased from a grocery shop in Pretoria, South Africa. The *Blackeye*

cultivar is preferred for deep-fried cowpea products due to its lighter seed coat colour. It was therefore chosen as reference sample for the purpose of comparison.

### *2.3 Preparation of samples*

The deep-fried products were prepared according to the method of Apea-Bah et al. (2017) with slight modification. The modification involved blending 100 g of soaked cowpea seeds in approximately 120 ml water. The resulting products were pulverised and submitted to simulated *in vitro* upper gut digestion.

### *2.4 Simulated in vitro upper gut digestion*

The processed cowpea beans were digested using the method of Apea-Bah, Minnaar, Bester, and Duodu (2016) with modification. The modification involved using a simulated gastric juice consisting of 0.94% (w/v) porcine pepsin A in 0.1 M HCl-KCl buffer (pH 1.5), and simulated duodenal juice comprising 2% (w/v) porcine pancreatin and 12.5% (w/v) porcine bile extract dissolved in 0.1 M aqueous $NaHCO_3$ (Nderitu et al., 2013). After digestion, the undigested (control) and digested (test) suspensions were centrifuged at 2810 × *g* for 30 min at 4$^{o}$C and the supernatants retained. The residues were rinsed twice with 10 ml distilled water, centrifuged and the supernatants collected. The two supernatants were combined and represented the bioaccessible portions of the digests and undigested samples. The combined supernatants were frozen at -20$^{o}$C, freeze-dried, defatted with hexane, air dried in fume cupboard and milled into powder with particle size less than 500 μm. They were stored in zip-lock polyethylene bags at -20$^{o}$C for antioxidant studies. For the phenolic composition study, 50 g of the processed products were similarly digested with corresponding volumes of digestive enzymes, and the supernatants defatted and freeze-dried for analysis.

### *2.5 Phenolic extraction*

Phenolic compounds in the undigested and digested samples were extracted according to the method of Qiu, Liu, and Beta (2009) with modification. The modification involved extracting 1 g of each freeze-dried sample with 20 ml of the solvent (acetone: water: formic acid – 70:29:1, respectively) under sonication for 1 h. The suspension was centrifuged at 7000 × *g*, for 10 min at 10$^{o}$C. The supernatant obtained was filtered through 0.22 µm Millex-GV syringe filter (EMD Millipore Corp., Billerica MA, USA) and stored at -20$^{o}$C for analyses.

### *2.6 Phenolic profile*

The phenolic profile of the samples was determined using a Waters Alliance 2695 HPLC (Waters, Milford, MA, USA) equipped with a quaternary pump, a Waters 2996 PDA detector, a Waters 717 Plus autosampler, and coupled to a Micromass Q-TOF Micro$^{TM}$ mass spectrometer. The HPLC-MS analysis was based on the method of Qiu et al. (2009) with a slight modification involving using formic acid as acidulant for the mobile phases instead of acetic acid.

The compounds were identified by comparing their retention times, UV and mass spectral characteristics with that of authentic standards where available, and compounds reported in literature for cowpeas. Catechin 3-*O*-glucoside and taxifolin glucoside were quantified based on their peak areas at 280 nm wavelength. Their results were expressed as microgram catechin equivalent per gram of freeze-dried samples on dry weight basis for catechin 3-*O*-glucoside (µg CE/g DW), and microgram quercetin equivalent per gram of freeze-dried samples on dry weight basis for taxifolin glucoside (µg QE/g DW).

All hydroxycinnamic acid derivatives and all other flavonoids were quantified based on their peak areas at 320 nm. Concentrations of coumaroylaldaric acids were expressed as microgram *p*-coumaric acid equivalents per gram of freeze-dried samples on dry weight basis (µg CAE/g DW), while concentrations of feruloylaldaric acids, feruloyl methylaldaric acids and 1,3-

coumaroyl-feruloyl-glycerol were expressed as ferulic acid equivalents (µg FAE/g freeze-dried sample dry weight basis). Concentrations of quercetin and quercetin glycosides were expressed as quercetin equivalents (µg QE/g freeze-dried sample dry weight basis). MS settings and calibration to obtain $[M-H]^-$ and MS/MS data were done based on the method of Qiu et al. (2009). MassLynx v. 4.1 software (Waters, Milford, MA, USA) was used for data acquisition.

### *2.7 Total phenolic content*

The 96-well microplate method of Apea-Bah et al. (2016) was used to measure total phenolic content (TPC). The method involved adding 36.4 µL of 10% (v/v) aqueous Folin Ciocalteu reagent and 145.4 µL of 700 mM $Na_2CO_3$ to 18.2 µL of appropriately diluted extracts. The reaction mixture was incubated in the dark for 2 h, and the absorbance was read at 750 nm. The results were expressed as milligram catechin equivalents per gram processed product, on dry weight basis (mg CE/g).

### *2.8 Radical scavenging activities*

Radical scavenging activities of appropriately diluted samples were measured using the Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and nitric oxide (NO) radical scavenging activity methods described by Apea-Bah et al. (2017). The results for TEAC and ORAC were presented as micromole Trolox equivalents per gram of processed product on dry weight basis (µmol TE/g DW), and µmol $NaNO_2$ equivalents per gram of processed product on dry weight basis, for NO radical scavenging activity.

### *2.9 Inhibition of nitric oxide production by endotoxin-stimulated cells*

#### *2.9.1 Preparation of Escherichia coli*

The ability of the processed cowpea beans and their digests to inhibit NO production under *Escherichia coli* (*E. coli*) endotoxin stimulation was tested using L929 murine fibroblasts that were

between passages 50 – 55. Two to three colonies of overnight-activated (37ºC) *E. coli* (ATCC strain 25922), were grown in 10 ml of Luria Bertani (LB) broth overnight at 37˚C, ensuring that the bacteria reached stationary phase. The bacteria were fixed with formaldehyde (final concentration 2%) for 1 h at room temperature, then washed twice with sterile PBS. They were then re-suspended in 10 ml LB, sonicated for 1 h and aliquots were stored at -80˚C until used.

### *2.9.2 NO production in L929 cells under E. coli endotoxin stimulation*

This assay was based on the method of Raso, Meli, Di Carlo, Pacilio, and Di Carlo (2001) with modification. An 80 µL volume of L929 cells were plated at a concentration of $1.25\times10^6$ cells/ml in a 96-well plate. To this, 10 µL of 10 times diluted extracts from the undigested and digested samples and 10 µL of fixed *E. coli* were added, yielding final cell concentration of $1\times10^6$ cells/ml. After 24 h incubation at 37ºC and 5% $CO_2$, 50 µL of supernatant was collected to which 50 µL Griess reagent (1% sulphanilamide + 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) was added and then the absorbance was read at 570 nm. NO produced was quantified as µM $NO_2$ (using $NaNO_2$ standard at final concentrations between 0 – 0.05 mM) and further converted to % NO scavenged. To the remaining 50 µL in the 96-well plate containing L929 cells, 5 µL MTT was added to determine cell viability, to confirm that a decrease in NO produced was actually due to cell response to sample, and not due to cell death.

A 5 µL volume of 1 mg/ml MTT in PBS was added to the wells containing cells, at final concentration of 0.1 mg/ml and incubated for 3 hours. The dye and medium were removed and the plate left to dry. The purple insoluble formazan product was then solubilised using 25% DMSO in ethanol and the absorbance read at 570 nm. Results were reported as percentage cell death, compared to control (cells not exposed to samples).

## *2.10 Inhibition of oxidative cellular damage*

The protective capacity of the samples and their digests against peroxyl radical-induced oxidative damage to body cells was measured using L929 murine fibroblasts as described by Apea-Bah et al. (2017).

### *2.11 Protection of DNA against oxidative damage*

The protective capacity of the processed products and their digests against peroxyl radical-induced oxidative damage to plasmid DNA, was measured based on the agarose gel electrophoresis method of Apea-Bah et al. (2017). All the *Blackeye* samples (both undigested and digested) and all the undigested samples from all cultivars were diluted 5 times with PBS while all digests, excluding those from the *Blackeye* cultivar, were diluted 10 times. Percent DNA damage or DNA protection by a sample, was calculated by comparing the quantified band intensity of the sample reaction mixture with that of the negative control (which contained $H_2O$ in place of AAPH and PBS in place of the sample, 100% protection) and the positive control (which contained AAPH and PBS in place of the sample, 0% protection), using the following equations:

$$\%\ DNA\ damage = \frac{[(In - Is) \times 100\%]}{(In - Ip)};\ Equation - 1$$

$$\%\ DNA\ protection = 100 - \%\ DNA\ damage; Equation - 2$$

Where, $In$ = band intensity of negative control; $Is$ = band intensity of sample; $Ip$ = band intensity of positive control.

### *2.12 Statistical Analysis*

Results from all analyses were presented as means ± standard deviations of at least triplicates. The effects of sample type (processed products and their digests) and replication on the response variables were determined by two-way analysis of variance. Fisher's least significant difference was used to compare means that significantly differed from each other at a 5%

significance level ($p<0.05$). All statistical analyses were performed using Statistica 8 (StatSoft Inc., Tulsa, OK, USA).

## 3.0 Results and Discussion

### *3.1 Phenolic profile of the processed cowpea beans and their digests*

A sample chromatogram of compounds identified in the processed cowpea beans is presented in Fig. 1. Generally, esters of hydroxycinnamic acids and flavonoids (flavan-3-ols, flavonols and flavanonol) were the main compounds identified and quantified in the processed cowpea beans (Table 1). The hydroxycinnamic acids were mostly conjugated to other compounds and included feruloylaldaric acids, feruloyl methylaldaric acids, coumaroylaldaric acids, coumaroylaldaric acid dirhamnoside, and 1,3-coumaroyl-feruloyl-glycerol. These compounds have been reported in cowpeas by other researchers (Apea-Bah et al., 2017; Dueñas et al., 2005; Hachibamba et al., 2013; Nderitu et al., 2013).

Aldaric acids are dicarboxylic acid derivatives of the 6-carbon sugars aldohexoses, with the carboxylic groups at the C1 and C6 positions. There are eight different stereoisomers of aldohexoses, each having a D-isomer and an L-isomer, based on the position of hydroxyl (OH) group on the penultimate (C5) carbon atom, yielding sixteen isomers (Sakuta & Nakamura 2019). The aldohexoses include allose, altrose, glucose, gulose, idose, mannose, galactose and talose. Oxidation of the C1-aldehydic and C6-hydroxyl groups of these compounds result in the following corresponding aldaric acids: allaric, altraric, glucaric, idaric, mannaric and galactaric acids. The

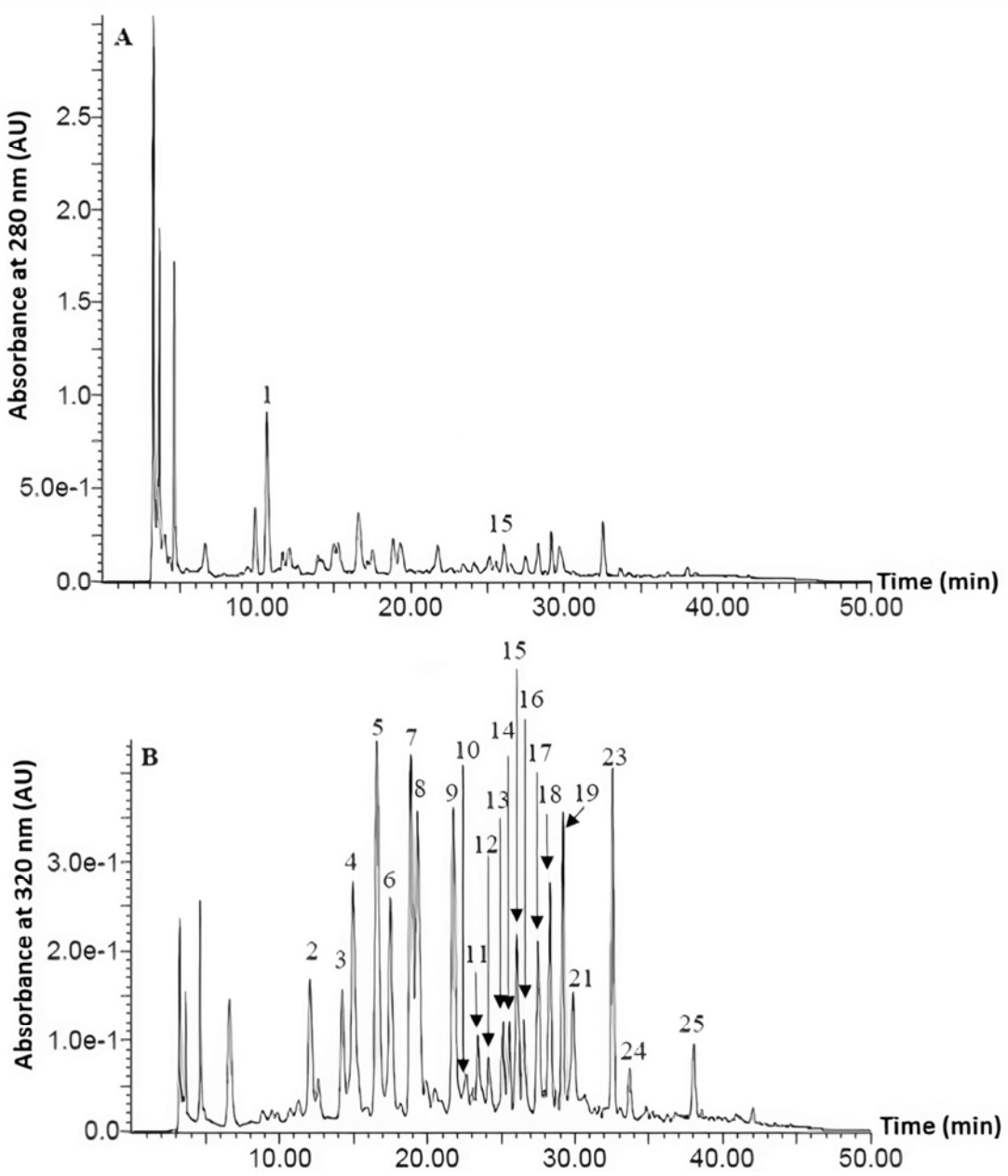



**Fig. 1**. A sample chromatogram of phenolic extract from undigested processed cowpea bean at 280 nm (A) and 320 nm (B).

**Table 1.** Phenolic composition (μg/g of freeze-dried sample, dry weight basis) of processed cowpea beans

| No. | Rt, min | λ, nm | $[M-H]^-$ | MS/MS | Name | *Blackeye* undigested | *Bechuana white* undigested | *Glenda* undigested | *Agrinawa* undigested |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.70 | 278 | 451 | 289 | Catechin 3-*O*-glucoside | 320.31±8.71$^{a}$ | 253.27±54.79$^{a}$ | 274.25±0.66$^{a}$ | 970.73±10.04$^{b}$ |
| 2 | 12.35 | 210, 314 | 647 | 355, 191, 163 | Coumaroylaldaric acid dirhamnoside | 31.39±1.62$^{b}$ | 10.94±2.40$^{a}$ | 28.67±0.27$^{b}$ | 28.07±2.92$^{b}$ |
| 3 | 14.70 | 216, 327 | 385 | 191, 385 | Feruloylaldaric acid | 41.36±2.95$^{c}$ | 8.65±0.30$^{a}$ | 21.27±1.81$^{b}$ | 24.85±1.84$^{b}$ |
| 4 | 15.38 | 210, 314 | 355 | 191, 209, 147, 129, 163 | Coumaroylaldaric acid | 43.98±5.7$^{b}$ | 12.86±0.37$^{a}$ | 55.71±1.55$^{b}$ | 46.27±3.06$^{b}$ |
| 5 | 16.90 | 210, 314 | 355 | 191, 209, 147, 129, 163 | Coumaroylaldaric acid | 69.18±2.13$^{b}$ | 33.82±4.65$^{a}$ | 104.94±4.90$^{c}$ | 99.15±8.83$^{c}$ |
| 6 | 17.78 | 216, 327 | 385 | 191, 147, 129, 209, 193 | Feruloylaldaric acid | 79.67±4.25$^{d}$ | 12.92±2.96$^{a}$ | 30.82±0.29$^{b}$ | 52.68±0.73$^{c}$ |
| 7 | 19.09 | 216, 327 | 385 | 191, 147, 209, 129, 134, 193 | Feruloylaldaric acid | 115.25±7.05$^{c}$ | 18.84±2.77$^{a}$ | 32.64±3.98$^{a}$ | 65.14±1.81$^{b}$ |
| 8 | 19.53 | 210, 314 | 355 | 191, 209, 147, 129, 163 | Coumaroylaldaric acid | 71.94±0.45$^{c}$ | 17.93±3.15$^{a}$ | 64.80±4.50$^{c}$ | 53.53±1.53$^{b}$ |
| 9 | 21.85 | 216, 327 | 385 | 191, 147, 209, 129, 134, 193 | Feruloylaldaric acid | 122.55±8.40$^{d}$ | 23.83±2.04$^{a}$ | 53.65±2.57$^{b}$ | 83.00±6.70$^{c}$ |
| 10 | 22.72 | 210, 326 | 399 | 205, 223, 129, 193, 134 | Feruloyl methylaldaric acid | 20.24±1.36$^{c}$ | 4.22±1.61$^{a}$ | 12.60±0.69$^{b}$ | nd |
| 11 | 23.47 | 210, 326 | 399 | 191, 147, 223, 205, 129, 399 | Feruloyl methylaldaric acid | 20.82±0.37$^{c}$ | 5.33±0.51$^{a}$ | 18.82±0.40$^{c}$ | 10.68±1.47$^{b}$ |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 12 | 24.87 | 210, 255, 347 | 625 | 300, 301, 625, 271 | Quercetin dihexoside | nd | nd | nd | 123.86±4.42 |
| 13 | 25.15 | 210, 255, 353 | 625 | 625, 301 | Quercetin dihexoside | 132.29±2.64$^{c}$ | 19.50±1.36$^{a}$ | 204.97±6.99$^{d}$ | 54.38±3.42$^{b}$ |
| 14 | 25.28 | 210, 255, 353 | 625 | 625, 301 | Quercetin dihexoside | nd | 10.29±0.41$^{a}$ | 12.91±3.91$^{a}$ | 43.49±3.76$^{b}$ |
| 15 | 26.20 | 210, 286 | 465 | 303 | Taxifolin glucoside | 101.24±1.90$^{b}$ | 38.15±1.50$^{a}$ | 143.35±13.62$^{c}$ | 110.97±14.05$^{bc}$ |
| 16 | 26.85 | 210, 326 | 399 | 191, 147, 129 | Feruloyl methylaldaric acid | 14.95±0.14$^{b}$ | nd | 24.28±4.79$^{c}$ | 7.62±1.36$^{a}$ |
| 17 | 27.38 | 261, 356 | 479 | 479, 316 | Myricetin 3-*O*-glucoside | 34.06±0.71$^{c}$ | 9.32±0.32$^{a}$ | 22.63±1.34$^{b}$ | 24.24±0.71$^{b}$ |
| 18 | 28.43 | 210, 326 | 399 | 191, 147, 129 | Feruloyl methylaldaric acid | 73.61±0.42$^{c}$ | 10.44±0.74$^{a}$ | nd | 24.88±1.96$^{b}$ |
| 19 | 29.12 | 253, 336 | 463 | 301 | Quercetin hexoside | nd | 73.12±1.64$^{a}$ | 80.37±2.52$^{a}$ | 223.16±38.08$^{b}$ |
| 20 | 29.72 | 210, 326 | 399 | 191, 147, 205, 129, 399 | Feruloyl methylaldaric acid | 37.30±4.75$^{c}$ | 9.71±0.36$^{a}$ | 20.42±0.78$^{b}$ | nd |
| 21 | 30.50 | 210, 326 | 399 | 191, 147, 205, 129, 399 | Feruloyl methylaldaric acid | 7.26±0.11$^{a}$ | nd | nd | 23.47±2.01$^{b}$ |
| 22 | 31.97 | 270, 320 | 413 | 193, 235 | 1,3-coumaroyl-feruloyl-glycerol | 6.80±0.20 | nd | nd | nd |

differences between these isomers are related to the positions of OH-groups on the chiral centers (C2, C3 and C4) (Sakuta & Nakamura 2019). The diversity of these compounds may account for the different hydroxycinnamic aldaric acid isomers that were identified at different retention times in this study (Table 1). Awika and Duodu (2017) reported feruloylgalactaric acid as the most commonly found aldaric acid in cowpea. Similarly, Lin, Harnly, Pastor-Corrales, & Luthria (2008) reported this compound and other hydroxycinnamic acid derivatives of aldaric acid in navy bean.

The flavonoids that were identified, existed mainly as glycosides and acylglycosides. The flavonol glycosides comprised quercetin hexosides, quercetin dihexoside, quercetin *O*-malonyl hexoside, quercetin 3-*O*-(6″-diacetyl)-diglucoside, and myricetin 3-*O*-glucoside (Table 1). Glucose, and to a lesser extent galactose, are the most common hexosides that have been reported as substituents to quercetin in cowpeas, and the preferred positions of attachment are mostly the C3-OH and C7-OH (Hachibamba et al., 2013; Kayitesi, 2013; Nderitu et al., 2013). The only flavan-3-ol identified was catechin 3-*O*-glucoside while the only flavanonol identified was taxifolin glucoside. Quercetin was the only flavonoid identified in its aglycone form in this study (Table 1). These compounds have previously been reported in raw and cooked cowpea beans and digests (Apea-Bah et al., 2017; Hachibamba et al., 2013; Nderitu et al., 2013; Ojwang et al., 2012).

Catechin 3-*O*-glucoside was the predominant flavonoid, making up 32-54% of the total flavonoids quantified in the processed cowpeas (Table 1). This is in agreement with the reports of Ojwang et al. (2013) for raw cowpeas, and Hachibamba et al. (2013) and Nderitu et al. (2013) for boiled cowpeas and their digests. Processed cowpeas from the *Agrinawa* (reddish-brown) cultivar

had 3-4 folds higher concentration of catechin 3-*O*-glucoside than all the other cultivars: *Blackeye* (cream cultivar), *Bechuana white* (brownish-cream cultivar) and *Glenda* (reddish-brown cultivar)), all of which had comparable values.

The composition of the hydroxycinnamic acids and flavonoids differed among the processed cowpea beans from different cultivars. When all the hydroxycinnamic acids in the processed cowpeas were summed up, their total concentrations were as follows: *Bechuana white* (178.8±16.6 µg/g) < *Glenda* (491.2±0.1 µg/g) < *Agrinawa* (543.6±3.8 µg/g) < *Blackeye* (790.4±0.6 µg/g). With regards to the flavonoids, the total concentrations in the processed cowpea bean cultivars were as follows: *Bechuana white* (495.5±57.2 µg/g) < *Blackeye* (591.6±16.5 µg/g) < *Glenda* (844.8±21.4 µg/g) < *Agrinawa* (1832.8±7.0 µg/g). It is observed from here that, processed cowpea beans from the brownish-cream cultivar (*Bechuana white*) had the lowest total hydroxycinnamic acids and flavonoids concentrations. On the other hand, the cream cultivar (*Blackeye*) and reddish-brown cultivar (*Agrinawa*) had the highest total hydroxycinnamic acid and flavonoid concentrations, respectively. With regards to the overall concentration of phenolic compounds, *Bechuana white* had the lowest overall value while *Agrinawa* had the highest overall value. *Blackeye* and *Glenda* had comparable overall phenolic composition values.

A sample chromatogram of the phenolic compounds identified in digests of the processed cowpeas is presented in Fig. 2.

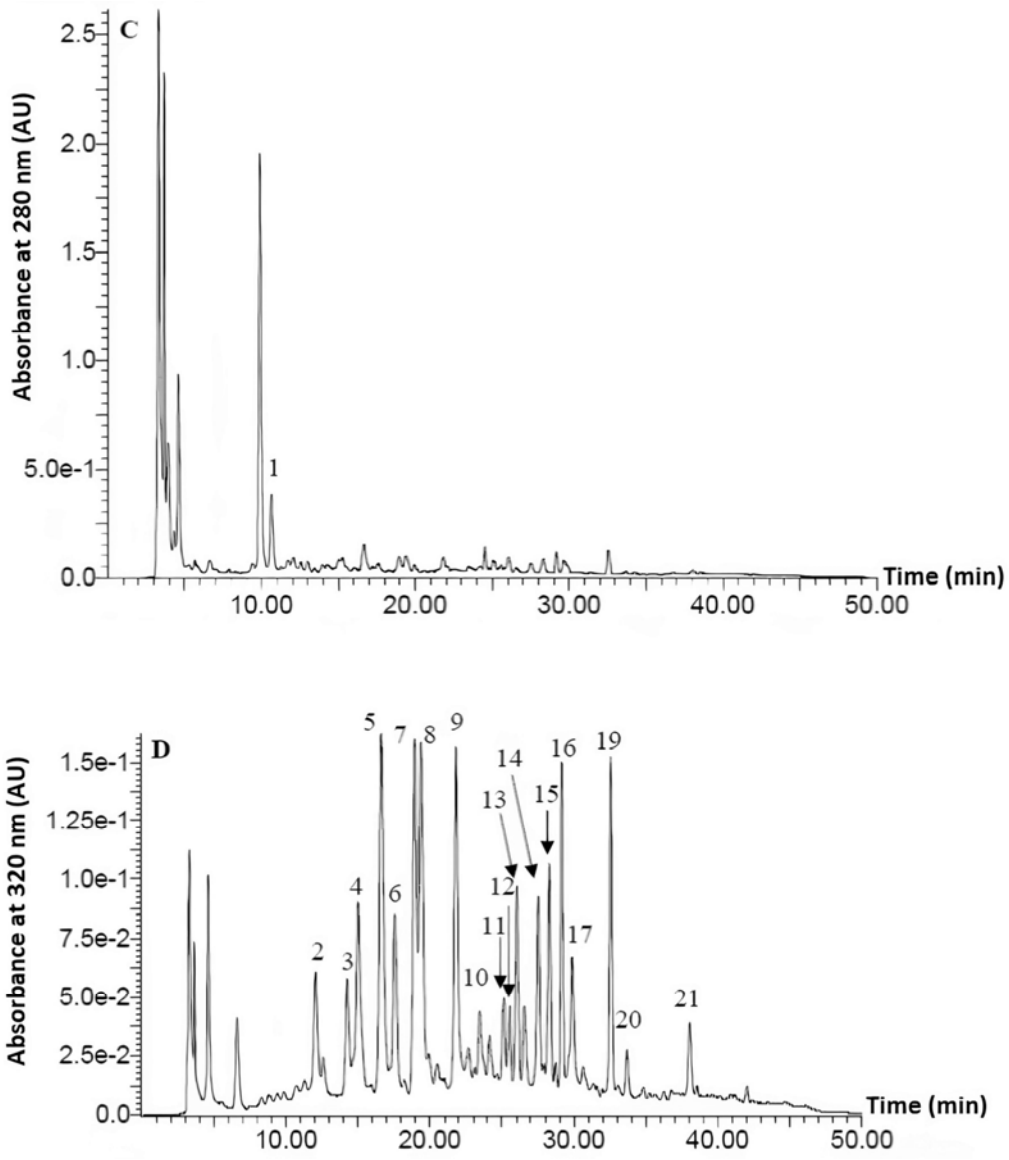



**Fig. 2.** A sample chromatogram of phenolic extract from the *in vitro* enzyme digest of processed cowpea bean at 280 nm (C) and 320 nm (D).

*In vitro* upper gut digestion had a number of effects on the phenolic composition of the processed cowpea beans such as decreasing the concentration of some of the phenolic compounds, while others became unidentifiable. Similar trends have been reported for the effect of simulated *in vitro* digestion on boiled cowpea seeds (Hachibamba et al., 2013; Nderitu et al., 2013). For example, Hachibamba et al. (2013) observed that, simulated *in vitro* gastrointestinal digestion decreased the concentrations of flavonol diglycosides to below detection limits. They attributed these losses to binding of the compounds with other food components released during digestion.

In this current study, the masses of some of the compounds were also transformed after *in vitro* upper gut digestion of their processed cowpea beans. Phenolic compounds are known to interact with nutrient macromolecules (proteins, carbohydrates and lipids) (Jakobek, 2015). However, the most reported is the interaction between phenolic compounds and proteins or peptides (Ozdal, Capanoglu, & Altay, 2013). Phenolic acids such as gallic, ferulic and chlorogenic acids have been reported to interact with different proteins including human serum albumin, bovine serum albumin, soy glycinin and lysozyme (Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 2003; Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007; Rawel, Meidtner, & Kroll, 2005). Similarly, flavonoids such as quercetin, isoquercetin, rutin, epicatechin and catechin, interact with proteins (Arts, Haenen, Voss, & Bast, 2001; Papadopoulou, Green, & Frazier, 2005; Rawel et al., 2005). Not only do proteins interact with flavonoid aglycones but they also do with flavonoid glycosides. For example, using NMR relaxation data, Martini, Bonechi, and Rossi (2008) demonstrated protein interaction with quercetin and quercetin 3-*O*-*β*-glucoside. They observed the protein to interact more strongly with the aglycone than with the glycoside.

In the present study, the molecular ion of catechin 3-*O*-glucoside (retention time 10.4-10.7) changed from m/z of 451 to 579 after simulated *in vitro* upper gut digestion of the processed

**Table 2.** Phenolic composition of *in vitro* upper gut digests from processed cowpea bean

| No. | Rt, min | λ, nm | $[M-H]^-$ | MS/MS | Name | *Blackeye* digest | *Bechuana* white digest | *Glenda* digest | *Agrinawa* digest |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.45 | 280 | 451, 579 | 451 | Glycyl-alanyl catechin 3-*O*-glucoside | nd | 155.35±4.55$^b$ | 69.26±2.43$^a$ | 357.58±16.19$^c$ |
| 2 | 12.10 | 210, 314 | 455 | 455, 355, 261 | Coumaroylaldaric acid derivative | 5.29±0.48$^a$ | 9.75±0.15$^b$ | 9.62±0.89$^b$ | 12.60±0.17$^c$ |
| 3 | 14.78 | 216, 327 | 454 | 454, 385 | Feruloylaldaric acid derivative | 6.40±0.35$^a$ | 5.93±0.55$^a$ | 5.13±0.85$^a$ | 10.06±0.23$^b$ |
| 4 | 15.56 | 216, 326 | 577 | 577 | Procyanidin dimer | 8.27±0.89$^a$ | 13.69±1.00$^b$ | 13.41±0.37$^b$ | 16.66±1.85$^b$ |
| 5 | 16.90 | 210, 312 | 642 | 642, 641, 355 | Threonyl-tryptophyl coumaroylaldaric acid | 24.88±4.18$^a$ | 32.98±2.01$^a$ | 33.21±0.96$^a$ | 44.44±7.33$^a$ |
| 6 | 18.08 | 216, 327 | 385 | 385 | Feruloylaldaric acid | 13.78±0.08$^b$ | 9.02±0.89$^a$ | 8.33±1.07$^a$ | 17.21±3.89$^b$ |
| 7 | 19.09 | 216, 327 | 385 | 385 | Feruloylaldaric acid | nd | 13.29±0.17$^a$ | 11.42±0.74$^a$ | 16.29±0.53$^b$ |
| 7 | 19.09 | 215, 326 | 553 | 387 | Alanyl-prolyl feruloyl trullixic acid | 16.88±1.70$^b$ | nd | nd | nd |
| 8 | 19.53 | 210, 314 | 483 | 355, 483, 241 | Glycyl-alanyl coumaroylaldaric acid | 10.10±0.05$^a$ | 22.51±1.15$^b$ | 23.91±2.59$^b$ | 14.17±0,73$^a$ |
| 9 | 22.04 | 216, 327 | 483 | 483, 385 | Feruloyl fumaroylaldaric acid | 28.05±2.60$^b$ | 18.91±0.10$^a$ | 16.85±0.32$^a$ | 42.27±2.90$^c$ |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 10 | 23.47 | 210, 326 | 647 | 647, 399 | Phenylalanyl-threonyl feruloyl methylaldaric acid | 4.41±1.00[a] | 3.54±0.60[a] | 4.26±0.52[a] | 5.80±0.09[a] |
| 11 | 24.12 | 210, 326 | 612 | 612 | Valinyl-asparagyl feruloyl methylaldaric acid | 2.83±0.69[a] | 3.13±0.09[a] | 4.17±1.59[a] | 5.05±1.99[a] |
| 12 | 25.05 | 210, 255, 347 | 625 | 301 | Quercetin *O*-dihexoside | 23.55±1.23[a] | 16.53±0.63[a] | 60.08±4.10[b] | 23.18±4.29[a] |
| 13 | 26.07 | 210, 286 | 465 | 303 | Taxifolin glucoside | 89.43±2.36[d] | 40.93±0.01[a] | 51.28±3.47[b] | 71.04±3.33[c] |
| 14 | 27.33 | 261, 356 | 479 | 479, 316 | Myricetin 3-*O*-glucoside | 4.78±1.04[a] | 5.51±0.86[a] | 8.83±1.43[b] | 9.86±0.60[b] |
| 15 | 28.32 | 210, 325 | 684 | 684, 399 | Arginyl-glutamyl feruloyl methylaldaric acid | 17.20±0.35[c] | 4.53±0.08[a] | 7.09±0.49[b] | 29.49±0.82[d] |
| 16 | 29.12 | 210, 337 | 463 | 463 | Quercetin *O*-hexoside | nd | nd | 26.61±3.57[a] | 102.38±10.01[b] |
| 17 | 30.02 | 210, 326 | 625 | 625, 399 | Prolyl-glutamyl feruloyl methylaldaric acid | 31.93±5.02[b] | 4.49±0.68[a] | 29.50±3.33[b] | 14.88±2.08[a] |
| 18 | 31.95 | 210, 326 | 413 | 193 | 1,3-coumaroyl-feruloyl-glycerol | 1.45±0.30 | nd | nd | nd |
| 18 | 31.95 | 210, 326 | 399 | 399 | Feruloyl methylaldaric acid | nd | 2.45±0.09 | nd | nd |

cowpea beans (Table 2). This may be due to binding of the dipeptide glycyl-alanine (m/z 128 = 146-18 amu) to catechin 3-*O*-glucoside, resulting in the new molecular ion of 579 amu.

At retention time of 16.9 min, a peak with m/z of 642 was observed in the digested samples, which did not fragment into smaller molecules after collision with argon gas. The retention time and UV-visible spectral characteristics of this compound were similar to that of coumaroylaldaric acid (m/z 355 amu) in the processed cowpea beans. We therefore postulate that this compound may have formed from binding of the dipeptide threonyl-tryptophan (m/z 305 amu) to coumaroylaldaric acid through condensation reaction (m/z 642 = 355+305-18, which is loss of $H_2O$) (Table 2). At retention time of 19.1 min, a peak with deprotonated molecular ion $[M-H]^-$ of m/z 553 was observed in the digested samples from the processed *Blackeye* cowpea beans but not in the digested samples of the other cultivars (Table 2). The compound yielded MS/MS product ion of 387 that corresponds to ferulic truxillic acid based on its mass spectral characteristic (Xiang, Apea-Bah, Ndolo, Katundu, & Beta, 2019). We propose that this compound may have been formed from binding of the dipeptide alanyl-proline (m/z 186 amu) to feruloylaldaric acid with loss of water (m/z 553 = 385+186-18, which is loss of $H_2O$) (Table 2).

At retention time of 19.5 min, a peak with $[M-H]^-$ of m/z 483 amu, which yielded a product ion (MS/MS) of 355 amu corresponding to coumaroylaldaric acid, was observed in the digests of all the processed cowpea cultivars. We postulate that the coumaroylaldaric acid probably reacted with the dipeptide glycyl-alanine (483 = 355+146-18) with the loss of water molecule, to give

glycyl-alanyl coumaroylaldaric acid (Table 2). Similarly, another peak with $[M-H]^-$ of m/z 483 amu was observed in the digests of all the processed cowpea cultivars, at retention time of 22.0 min with a product ion of 385 amu corresponding to feruloylaldaric acid. We propose that feruloylaldaric acid may have reacted with the dicarboxylic acid, fumaric acid (molecular weight 116), which is a metabolic intermediate of the citric acid cycle, and present in abundant quantities in legumes (Chia, Yoder, Reiter, & Gibson, 2000), to form feruloyl fumaroylaldaric acid (483=385+116-18) (Table 2).

Feruloyl methylaldaric acid ($[M-H]^-$ of m/z 399 amu) may have reacted with the dipeptide phenylalanyl-threonine (m/z 266 amu) yielding a compound with m/z 647 amu (399+266-18) identified at retention time of 23.5 min in all digests of the processed cowpea cultivars. Similarly, feruloyl methylaldaric acid may have reacted with the dipeptide valinyl-asparagine (m/z 231 amu) in the digested samples from all the processed cowpea bean cultivars to yield a peak with m/z 612 amu (399+231-18) at 24.1 min (Table 2). Feruloyl methylaldaric acid (m/z 399 amu) again reacted with a dipeptide arginyl-glutamate (m/z 303 amu) resulting in a compound with m/z 684 amu (399+303-18) that gave a peak at 28.3 min for the digested samples. A peak with $[M-H]^-$ of 625 and MS/MS of 399 was observed at 30.0 min in all the digested samples. The UV spectrum was identical to that of feruloyl methylaldaric acid (m/z 399 amu). It is proposed that this compound was formed from a condensation reaction between feruloyl methylaldaric acid and the dipeptide prolyl-glutamate (625=399+244-18). Similarly, quercetin hexoside (m/z 463 amu) also probably reacted with prolyl-glutamate (m/z 244 amu) to give a peak with m/z 689 amu (463+244-18) at retention time 32.3 min (Table 2) in the digests from the reddish-brown cultivars.

The amino acid constituents of the dipeptides that have been proposed to bind to the phenolic compounds, have all been previously reported in cowpea beans (Iqbal, Khalil, Ateeq, &

Sayyar Khan, 2006; Vasconcelos et al., 2010). During the *in vitro* upper gut digestion experiment, the simulated gastric juice contained porcine pepsin while the simulated duodenal juice contained porcine pancreatin which comprised trypsin, amylase and lipase. Pepsin effectively cleaves peptide bonds between hydrophobic and aromatic amino acids such as phenylalanine, tryptophan and tyrosine (Dunn, 2001, 2002). Trypsin, on the other hand, usually cleaves peptide bonds at the carboxyl side of the amino acids lysine or arginine, with or without proline attached to these amino acids (Olsen, Ong, & Mann, 2004; Rodriguez, Gupta, Smith, & Pevzner, 2007). Since these amino acids and others have been reported in cowpea beans, it is plausible to expect their dipeptides in the *in vitro* upper gut digests of the processed cowpea beans. These dipeptides then reacted with the phenolic compounds mostly through condensation reactions as evident by the loss of $H_2O$ molecules observed in their molecular ions in this study. This is consistent with the report of Hernández-Jabalera et al. (2015) on the peptide-phenolic interactions observed in protein hydrolysates from *Brassica napus*.

The total concentration of quantified phenolic compounds in the processed cowpea beans were significantly higher than that measured in the digests, including the compounds that were bound to peptides ($p<0.05$). Putting it into perspective, the processed cowpea beans from the *Blackeye*, *Bechuana white*, *Glenda* and *Agrinawa* cultivars had, respectively, 4.6, 1.8, 3.2 and 2.5 times higher total concentration of quantified phenolic compounds than that measured in their corresponding digests. This is indicative of the susceptibility of phenolic compounds to *in vitro* upper gut digestion. Among the cowpea cultivars, the total concentration of phenolic compounds in the digests of the processed cowpeas were as follows: *Blackeye* < *Bechuana white* < *Glenda* < *Agrinawa*. Processed cowpea beans from the reddish-brown cultivars therefore produced digests containing higher amounts of phenolic compounds than the digests from processed cowpeas of

less pigmented cultivars. This may indicate differences among the matrix of the processed cowpea cultivars resulting in differences in their protective effects over constituent phenolic compounds during *in vitro* upper gut digestion.

### *3.2 TPC and radical scavenging activities*

Table 3 shows the TPC and radical scavenging activities of the processed cowpea beans and their digests. The processed cowpea beans from *Blackeye* (cream), *Bechuana white* (brownish-cream) and *Glenda* (reddish-brown) cultivars had comparable TPC. However, the TPC of processed cowpea beans from *Blackeye* and *Bechuana white* cultivars were significantly lower ($p<0.05$) than that of *Agrinawa* (reddish-brown) cultivar. The processed cowpea beans responded differently to the different radical scavenging assays, probably due to the different reaction mechanisms involved in their measurements. TEAC of processed cowpea beans from the cream cultivar (*Blackeye*) was comparable to that of the brownish-cream (*Bechuana white*) cultivar, but significantly lower ($p<0.05$) than that of the reddish-brown (*Glenda* and *Agrinawa*) cultivars. While TEAC values of processed cowpea beans from *Bechuana white* and *Glenda* were comparable, they were both significantly lower ($p<0.05$) than that of *Agrinawa*.

ORAC values for processed cowpea beans from all the cultivars were comparable. Although NO scavenging activity of processed cowpea beans from *Blackeye*, *Bechuana white* and *Glenda* were comparable, the value for processed beans from *Blackeye* was significantly lower ($p<0.05$) than that of *Agrinawa* (Table 3). Therefore processed cowpea beans from *Agrinawa* had higher TPC and radical scavenging activities (except ORAC) than that from the less pigmented

**Table 3.** Total phenolic content and radical scavenging activities of undigested processed cowpea beans and their *in vitro* digests.

| Assay | *Blackeye* | | *Bechuana white* | | *Glenda* | | *Agrinawa* | |
|---|---|---|---|---|---|---|---|---|
| | Processed cowpea bean | Processed cowpea bean digest | Processed cowpea bean | Processed cowpea bean digest | Processed cowpea bean | Processed cowpea bean digest | Processed cowpea bean | Processed cowpea bean digest |
| TPC | 2.52±0.26 [a] | 12.16±0.84 [c] | 3.53±0.30 [a] | 15.98±1.64 [d] | 4.01±0.32 [ab] | 17.42±1.08 [d] | 5.50±0.17 [b] | 23.94±0.71 [e] |
| TEAC | 38.97±2.72 [a] | 188.11±8.62 [d] | 60.12±2.75 [ab] | 276.74±14.14 [e] | 65.92±3.25 [b] | 276.77±14.10 [e] | 90.32±5.24 [c] | 324.17±17.77 [f] |
| ORAC | 188.93±4.30 [a] | 517.18±34.56 [b] | 185.78±4.83 [a] | 725.70±47.95 [c] | 202.79±7.15 [a] | 683.38±18.51 [c] | 235.89±5.25 [a] | 813.47±37.89 [d] |
| NO | 4.64±0.68 [a] | 21.07±1.10 [c] | 7.06±0.56 [ab] | 23.95±2.78 [cd] | 8.22±0.49 [ab] | 26.88±1.11 [de] | 9.31±0.91 [b] | 30.12±1.27 [e] |

Key: TPC – total phenolic content (mg CE/g processed cowpea bean DWB); TEAC – trolox equivalent antioxidant capacity (µmol TE/g processed cowpea bean DWB); ORAC – oxygen radical absorbance capacity (µmol TE/g processed cowpea bean DWB); NO – nitric oxide radical scavenging capacity (µmol $NaNO_2$/g processed cowpea bean DWB); CE – Catechin equivalents; TE – Trolox equivalents; DWB – dry weight basis. Results are means of at least triplicates ± standard error of means. Means in a row with different superscript letters are significantly different ($p<0.05$).

cultivars. This is in agreement with the total quantified phenolic compounds concentration of the samples (Table 1). The trend is also consistent with the report of Nderitu et al. (2013), who observed the raw, cooked and enzyme digests of Agrinawa cultivars, to have higher overall phenolic compounds concentration and plasmid DNA protective capacity than that of Blackeye. The observed trends in this study indicate that processed cowpea beans from *Blackeye* (cream), *Bechuana white* (brownish-cream) and *Glenda* (reddish-brown) cultivars have comparable TPC and radical scavenging activities. The results imply that not all darker cowpeas yield processed products with higher TPC and radical scavenging activities than those from less pigmented cowpeas.

Although processed products from *Blackeye* and *Glenda* had comparable total phenolic compounds concentration, both of which were higher than values present in processed products from *Bechuana white*, their compositions were different. Products from *Bechuana white* and *Glenda* had wider variety of quercetin glycosides than that of *Blackeye*. Quercetin and its glycosides, which belong to the flavonol class of flavonoids, have higher radical scavenging properties than the hydroxycinnamic acids (Rice-Evans, Miller, & Paganga, 1996) which were most abundant in processed products from *Blackeye*. This may explain why *Blackeye* and *Bechuana white* had comparable radical scavenging activities, although *Blackeye* had higher total quantified phenolic compounds concentration than *Bechuana white*.

It is also noteworthy that thermal processing affects phenolic compounds in many different ways including thermal denaturation, oxidation and promoting phenolic reactions with other molecules in the food matrix. Depending on the food matrix, soaking and thermal processing may either increase or decrease the total phenolic content (measured by Folin Ciocalteu assay) and radical scavenging activities of cereals and legumes (Duodu, 2014; Taylor & Duodu, 2015). This

is probably because some oxidation products formed from the phenolic compounds also contributed to the TPC and radical scavenging activities even though they may not have been identified in the HPLC-MS analysis. It is worth noting that the Folin Ciocalteu assay is not specific to phenolic compounds. It measures the total reducing property of a sample since other oxidation substrates can also be tested with the assay (Ainsworth and Gillespie, 2007).

Melanoidins, which are Maillard reaction products, are produced during heat treatment of food such as deep-frying which was applied in this study. These compounds are known to contribute antioxidant properties to foods (Delgado-Andrade & Morales, 2005). It is conceivable that melanoidins produced during the deep-frying process, imparted antioxidant properties that balanced any thermal loss of naturally occurring phenolic compounds in the processed cowpea seeds (Anese, Manzocco, Nicoli, & Lerici, 1999).

All the *in vitro* upper gut digests from the processed cowpea beans had significantly higher ($p<0.05$) TPC and radical scavenging activities than that of the undigested processed samples. Similar trend has been reported in earlier studies that compared processed (boiled and pressure cooked) cowpea seeds to their corresponding digests (Hachibamba et al., 2013; Mtolo et al. 2017; Nderitu et al., 2013). Rufián-Henares & Morales (2007) reported that low molecular weight melanoidins released during gastrointestinal digestion, possess higher antioxidant properties than the corresponding melanoidins present in the undigested product. They attributed this to the release or modification of low molecular weight antioxidant compounds that were ionically bound to the melanoidins, or the formation of new more bioactive structures from the melanoidin skeleton after enzymatic treatment. This therefore compensated for the lower concentration of the total quantified phenolic compounds in the digests, giving them higher antioxidant activities than their corresponding undigested processed products. Also, digestion releases small molecules such as

reducing sugars, amino acids and peptides from complex food macromolecules such as starch and proteins. Amino acids and peptides do react with Folin Ciocalteu reagent (Everette, Bryant, Green, Abbey, Wangila, & Walker, 2010) and could contribute to the higher TPC results of the digests in comparison to the undigested processed cowpea beans.

Among the cultivars, *in vitro* digests of processed cowpea beans from *Blackeye* had the lowest TPC, TEAC and ORAC while digests from *Bechuana white* and *Glenda* had comparable values, and digest from *Agrinawa* had the highest value. Although the NO radical scavenging activity of the *Blackeye* and *Bechuana white* were comparable, *Blackeye* had lower values than that of the reddish-brown cultivars. In summary, both the processed cowpea beans and their digests from *Agrinawa* cultivar had the highest concentration of phenolic compounds and correspondingly highest TPC and radical scavenging activities.

ORAC and NO radical scavenging activity are both related to biologically relevant radicals (peroxyl and NO, respectively). While peroxyl radicals can cause lipid peroxidation (Lobo, Patil, Phatak, & Chandra, 2010), NO can damage DNA, cells and other biomolecules (Burney, Caulfield, Niles, Wishnok, & Tannenbaum, 1999) if their production is not modulated. The constituent phenolic compounds in the processed cowpea beans and their digests, have been demonstrated to scavenge physiologically relevant radicals that could otherwise cause radical-induced oxidative stress and consequent inflammation. This study therefore suggests that when processed cowpea beans are consumed and get digested, the phenolic compounds released could potentially help to alleviate peroxyl and NO radical-induced oxidative stress and inflammation.

### *3.3 Inhibition of endotoxin-stimulated cellular nitric oxide production*

Generally, cell viability of more than 97% was recorded for all samples, in the presence of the bacterial endotoxin (Table 4). Under bacterial endotoxin-stimulation, cells produce high and

sustained amounts of NO through catalytic action of the enzyme inducible nitric oxide synthase (iNOS), as an immunological response to pathogenic infection (Kim, Cheon, Kim, Kim, & Kim, 1999). The NO produced is aimed at destroying any invading pathogens, but might also oxidatively damage neighbouring host cells leading to inflammation, if not modulated. Therefore, inhibition of iNOS-induced NO production may potentially have health benefits related to preventing inflammation and septic shock (Kim et al., 1999). As seen from Table 4, all the undigested samples and their *in vitro* digests inhibited bacterial endotoxin-stimulated cellular NO production by over 50%.

Quercetin, which was identified in all the samples either as an aglycone or in the glycoside form, is reportedly one of the most potent inhibitors of bacterial lipopolysaccharide-induced NO production in cells (Raso et al., 2001). The inhibitory activity is achieved through modulation of iNOS (Raso et al., 2001). From the foregoing, it may be hypothesized that phenolic compounds released during upper gut digestion of processed cowpea products, as well as the phenolic-peptide complexes produced, can potentially contribute to preventing NO-induced cellular damage and consequent inflammation in the gastrointestinal tract. Such protection may also be imparted when the phenolic compounds are absorbed into systemic circulation.

### *3.4 Cellular protection from oxidative damage*

When the murine fibroblasts were exposed to peroxyl radicals in the presence of the processed cowpea beans and their digests, all the samples protected the cells from radical-induced oxidative damage (Table 4). Although statistically significant differences existed between the

**Table 4.** Inhibition of bacterial endotoxin-stimulated cellular nitric oxide production, and protection against peroxyl radical-induced DNA and cellular oxidative damage, by processed cowpea beans and their *in vitro* digests.

| Assay | *Blackeye* | | *Bechuana white* | | *Glenda* | | *Agrinawa* | |
|---|---|---|---|---|---|---|---|---|
| | Processed cowpea beans | Digests of processed cowpeas | Processed cowpea beans | Digests of processed cowpeas | Processed cowpea beans | Digests of processed cowpeas | Processed cowpea beans | Digests of processed cowpeas |
| % Cell viability | 102.08±2.77 | 102.58±1.24 | 103.98±1.92 | 105.39±2.10 | 98.05±5.37 | 97.48±5.31 | 105.61±1.20 | 105.61±1.20 |
| % NO scavenged | 52.95±8.03 | 58.70±0.10 | 65.59±6.60 | 51.05±5.33 | 64.26±0.66 | 86.52±4.96 | 82.74±17.64 | 69.80±24.42 |
| % Cellular protection | 105.24±0.29 $^{b}$ | 98.64±0.26 $^{a}$ | 105.92±0.42 $^{b}$ | 106.06±0.20 $^{bc}$ | 107.97±0.43 $^{d}$ | 107.52±0.60 $^{d}$ | 108.12±0.59 $^{d}$ | 107.14±0.74 $^{cd}$ |
| % DNA damage | -21.81±3.57 $^{c}$ | -27.17±0.32 $^{bc}$ | -32.99±1.02 $^{ab}$ | 2.01±0.31 $^{d}$ | 8.59±0.94 $^{d}$ | -33.88±3.93 $^{a}$ | 5.15±0.10 $^{d}$ | 3.34±1.50 $^{d}$ |
| % DNA protection | 73.85±1.56 $^{ab}$ | 100.28±3.40 $^{c}$ | 72.60±22.75 $^{ab}$ | 100.61±9.54 $^{c}$ | 67.45±12.08 $^{a}$ | 109.01±5.79 $^{c}$ | 64.04±4.37 $^{a}$ | 95.02±7.57 $^{bc}$ |

Key: NO – cellular nitric oxide. Results are means of at least triplicates ± standard error of means. Means in a row with different superscript letters are significantly different ($p<0.05$) from each other.

cellular protection of samples from the different cultivars ($p<0.05$), from a biological standpoint, all the samples totally (100%) protected the cells against radical-induced oxidative damage. Similarly, the statistically significant difference in cellular protection between processed cowpea beans from the *Blackeye* (cream) cultivar and its corresponding digest may not be of substantial biological relevance. The present study is consistent with that of Hernández-Jabalera et al. (2015) who reported peptide-phenolic complexes from protein hydrolysates of *Brassica napus* to exhibit radical scavenging activities and cellular antioxidant protection. This study therefore demonstrates the ability of processed cowpea beans and their *in vitro* digests to protect body cells from damage caused by reactive oxygen species (ROS). Such damage if not prevented, could result in necrotic or apoptotic cell death (Serem & Bester, 2012).

### *3.5 Inhibition of DNA oxidative damage*

A number of studies have demonstrated the ability of plant-based extracts to inhibit oxidative DNA damage, using electrophoretic mobility of pBR322 plasmid DNA as a model (Falcioni et al., 2002; Nderitu et al., 2013; Verma, Shrivastava, & Kumar, 2015). This model is based on the principle that undamaged plasmid DNA exist in supercoiled conformation while damage caused by ROS can result in either unwinding of the supercoiled structure to form an open-circular conformation, or single strand breaks leading to open chain conformation. Of the three conformations, the supercoiled plasmid DNA has the highest electrophoretic mobility on an agarose gel while the damaged open circular plasmid DNA has the lowest electrophoretic mobility (Llorens, del Valle, & Puiggalí, 2014).

Two separate agarose gels (Figs. 3A and 3B) were run in triplicates to determine first, whether the samples caused damage to the plasmid DNA (Fig. 3A), and secondly whether the samples protected the plasmid DNA from AAPH-generated peroxyl radical-induced oxidative

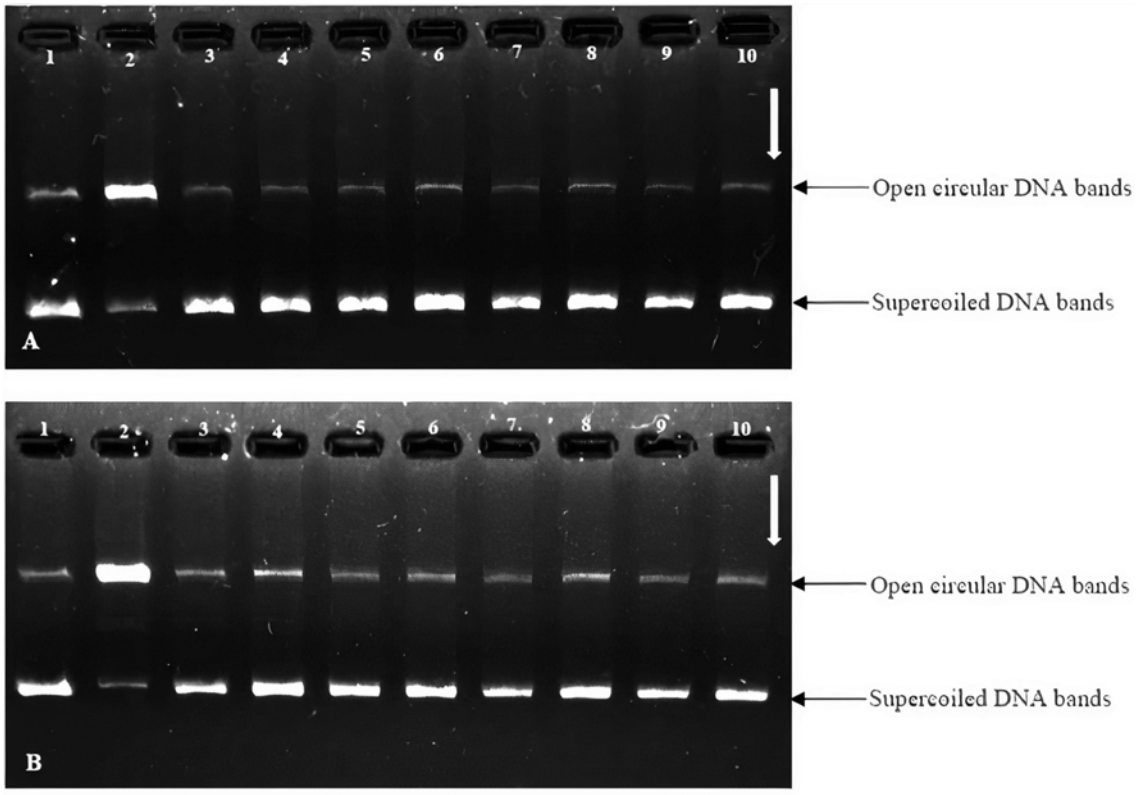



**Fig. 3.** Agarose gel electropherogram demonstrating: (A) effect of processed cowpea beans and their digests on plasmid DNA, and (B) antioxidant protection of plasmid DNA by processed cowpea beans and their digests against peroxyl radicals. Key: Agarose Gel A: Lane 1 = negative control (DNA + $H_2O$ + PBS); Lane 2 = positive control (DNA + AAPH + PBS); Lane 3 = DNA + $H_2O$ + *Blackeye* processed cowpea beans; Lane 4 = DNA + $H_2O$ + *Blackeye* digests of processed cowpea beans; Lane 5 = DNA + $H_2O$ + *Bechuana white* processed cowpea beans; Lane 6 = DNA + $H_2O$ + *Bechuana white* digests of processed cowpea beans; Lane 7 = DNA + $H_2O$ + *Glenda* processed cowpea beans; Lane 8 = DNA + $H_2O$ + *Glenda* digests of processed cowpea beans; Lane 9 = DNA + $H_2O$ + *Agrinawa* processed cowpea beans; Lane 10 = DNA + $H_2O$ + *Agrinawa* digests of processed cowpea beans. Downward arrow showing direction of DNA electrophoretic migration. Agarose Gel B: Lane 1 = negative control (DNA + $H_2O$ + PBS); Lane 2 = positive control (DNA + AAPH + PBS); Lane 3 = DNA + AAPH + *Blackeye* processed cowpea beans; Lane 4 = DNA + AAPH + *Blackeye* digests of processed cowpea beans; Lane 5 = DNA + AAPH + *Bechuana white* digests of processed cowpea beans; Lane 6 = DNA + AAPH + *Bechuana white* digests of digests of processed cowpea beans; Lane 7 = DNA + AAPH + *Glenda* digests of processed cowpea beans; Lane 8 = DNA + AAPH + *Glenda* digests of digests of processed cowpea beans; Lane 9 = DNA + AAPH + *Agrinawa* digests of processed cowpea beans; Lane 10 = DNA + AAPH + *Agrinawa* digests of digests of processed cowpea beans. Downward arrow showing direction of DNA electrophoretic migration.

damage (Fig. 3B). On both agarose gels, each reaction mixture produced two DNA bands which represented the supercoiled DNA band (higher electrophoretic mobility) and the open circular DNA band (Figs. 3A and 3B). It is noteworthy that, a batch of well-prepared plasmid DNA will have majority of its molecules in the supercoiled form. However, small amounts of open circular DNA forms may also be present either due to enzyme action or other causes, and the amount depends on the batch (Wei, Zhou, Cai, Yang, & Liu, 2006).

In both gel runs, the negative control (which contained distilled deionised water in place of AAPH and PBS in place of sample) showed an intense supercoiled band and faint open circular DNA band (Figs. 3A and 3B, lane 1). The positive control (reaction mixture containing AAPH and PBS in place of sample) on the other hand, produced an intense open circular DNA band as a result of peroxyl radical-induced oxidative damage to the supercoiled structure (Figs. 3A and 3B, lane 2). The processed cowpea beans and their *in vitro* digests, in the presence of distilled deionised water (instead of AAPH) (Fig. 3A, lanes 3-10), generally caused less than 10% damage to the plasmid DNA (Table 4).

In the presence of AAPH-generated peroxyl radicals, all the samples protected the plasmid DNA from oxidative damage to different extents (Fig. 3B, lanes 3-10). Generally, the processed beans showed between 64-76% protection against oxidative damage to the DNA. This may be due to the varying amounts of constituent phenolic compounds that scavenged the peroxyl radicals through H-atom transfer. The protective ability of the processed cowpea beans against oxidative DNA damage were comparable among the cultivars. The digested samples from all the cultivars

had higher protective ability against oxidative DNA damage than the undigested samples. As explained before for the radical scavenging activities, this may probably be due to the phenolic-peptide interactions in the digests that resulted in more available functional groups capable of scavenging the peroxyl radicals. It is expected that when processed cowpea beans are consumed and digested, their constituent phenolic compounds will contribute to DNA protection against oxidative damage in the gastrointestinal tract which could otherwise cause mutations and consequent carcinogenesis (Llorens et al., 2014).

## 4.0 Conclusion

This study demonstrates that not all darker cowpeas yield processed products with higher radical scavenging activities and better protection against oxidative damage to cells and DNA, than less pigmented cowpea cultivars. Processed cowpea beans from different cultivars, protect their phenolic compounds from thermal and enzymatic digestive effects to different extents due to their different food matrix compositions or structures. The phenolic compounds extracted from the enzymatic digests of processed cowpea beans, demonstrate antioxidant capacity by scavenging the biologically active peroxyl and nitric oxide radicals, inhibiting bacterial endotoxin-induced cellular nitric oxide production, and protecting DNA and body cells from oxidative damage. Although *in vitro* upper gut digestion decreases the amount of extractable phenolic compounds from processed cowpea beans, digestion increases their radical scavenging activities and protective ability against oxidative damage of DNA. From this research, we conclude that processed cowpea beans when consumed and digested, have potential to contribute to averting oxidative stress-related pathologies including cellular damage that results in apoptotic or necrotic cell death, and mutagenesis that could lead to cancer.

## 5.0 Declarations of interest: None

## 6.0 Acknowledgement

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