Abstract

Bambara groundnut (*Vigna subterranea*) an indigenous legume proclaimed to have medicinal properties within rural areas. Therefore, this study aimed to identify possible medicinal properties of Bambara groundnut (BGN). Flavonoids and tannins were highly concentrated in the red and brown BGN hulls. Among the flavonoid compounds rutin was observed in highest concentrations in brown hull (24.458 ± 0.234 mg g⁻¹) and myricetin (1.800 ± 0.771 mg g⁻¹). While among tannin compounds chlorogenic acid was found in highest concentrations (0.115 ± 0.199 mg g⁻¹) and ellagic acid in red hull (0.105 ± 0.082 mg g⁻¹). The form and colour of the BGN were all important factors to optimize the best extraction yield of phytochemicals. Overall the hulls of the BGN were the optimum source of flavonoids and tannins.

Keywords: Food science, Food analysis, Nutrition, Food technology
1. Introduction

Bambara groundnut (*Vigna subterrenea*, BGN) is an indigenous African crop that is grown across the continent from Senegal to Kenya and from the Sahara to South Africa (Mpotokwane et al., 2008; Eltayeb et al., 2011; Jideani and Diedericks, 2014; Nyau et al., 2014). The colour of the seed varies from black, brown or red and may be mottled with various colours (Onimawo et al., 1998; Jideani and Diedericks, 2014). Before consumption, the hulls are removed and generally regarded as waste. However, BGN hulls have been reported to have quantities of phenolic compounds. In addition, the hulls are an inexpensive source of nutraceuticals and functional ingredients (Klompong and Benjakul, 2015).

The medicinal use of BGN is based on information obtained from communities in several parts of Africa, where this crop is reportedly used for the treatment of various ailments. The water boiled from the maize and pulse mixture is ingested to treat diarrhoea. Anonymous (2011) reported that raw BGN can be chewed and swallowed to treat nausea suffered by pregnant women, while in Kenya, the Luo tribe use BGN to cure diarrhoea (Mkandawire, 2007).

The traditional utilization of BGN to treat several ailments is notable and therefore creates an opportunity for detailed scientific study on the nutraceutical value of the crop (Mølgaard et al., 2011). According to Mbagwu et al. (2011) the phytochemical screening carried out on whole BGN (sourced from Idemili in Anambra State, Nigeria) showed the presence of valuable phytochemicals such as flavonoids and alkoloids. Flavonoids have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity (Cushnie and Lamb, 2005).

Furthermore, Nyau et al. (2015) reported that whole BGN from Lusaka in Zambia possessed antioxidant activities. These findings point toward BGN having the potential as a functional food. However, there is limited research with regards to phytochemical properties on whole, dehulled (endosperm) and hulls of BGN for different seed colours (black, black-eye, brown, brown-eye and red) from South Africa. Therefore, in the present study the aim was to evaluate the phytochemical properties of BGN.

2. Materials and methods

2.1. Source of BGN, chemical reagents

Bambara groundnuts were purchased from Mpumalanga Province, South Africa. The BGN seeds were sorted into their respective colours: black, black-eye, brown, brown-eye and red.
The chemicals used in this study were of HPLC and analytical grade. Reagents were prepared according to standard analytical procedures. All prepared reagents were stored at 18–20 °C in a dark environment to prevent deterioration or contamination.

2.2. Preparation of whole, dehulled and hull Bambara groundnut flour

Upon receiving the BGN, it was sorted into its respective colours. The black, black-eye, brown, brown-eye and red colours of whole BGN were milled separately into flour (sieve size of 250 μm) using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) and packed and sealed in clear plastic bags. The whole BGN flour each variety was stored in a refrigerator at 4–6 °C until further analysis. The whole BGN seeds were dried at 40 °C in an industrial oven dryer (Geiger and Klotzebuecher, Cape Town, South Africa) for 48 h. A Corona® manual grain was used to mill BGN to allow easier manual removal of hulls to produce dehulled BGN. The black, black-eye, brown, brown-eye and red dehulled BGN and their respective hulls were milled into flour (250 μm sieve size) using a hammer mill (Perten Mill, Perten Instruments AB, Sweden), packed and sealed in clear plastic bags. The dehulled BGN flour of each BGN seed colour was stored in a refrigerator at 4–6 °C until further analysis.

2.3. Preparation of whole, dehulled and hull Bambara groundnut extracts

The 70% methanol extracts were prepared from whole BGN, dehulled BGN and BGN hull flour. For this process, an Ultrasound-Assisted Extraction (UAE) method was used. Approximately 15 g of each BGN flour (whole, dehulled and hull) was added separately to 150 mL of 70% methanol, and was sonicated for 30 minutes at 25 °C using the Lasec SA 2510 Branson ultrasound bath 42 kHz ± 6%, USA. After extraction, the mixture was centrifuged at a speed of 15316 x g for 15 min at 4 °C (Beckman Coulter Avanti J-E centrifuge, USA). The resulting supernatant was concentrated to 30 mL by evaporation under pressure in a rotary evaporator (Buchi RE 011 model, Switzerland) at 40 °C to remove residual methanol (Nyau et al., 2014). The extracts of whole, dehulled and hull BGN were frozen at −80 °C and freeze dried (BenchTop Pro with Omnitronics freeze dryer, United Scientific, Germany) to obtain a powdered extract. The freeze-dried extracts were stored at −4 °C and subjected to phytochemical screening.

2.4. Qualitative phytochemical analysis

The method consisted of adding 1 mL of freshly prepared 10% (m v⁻¹) KOH solution to 1 g of individual extracts of whole, dehulled and hull. If an opaque precipitate appeared, it indicated the presence of tannins (Santosh et al., 2013).
As described by Bhandary et al. (2012) 1 g of extracts from whole, dehulled and hull BGN was treated with a few drops of ferric chloride solution (40% w/v, ferric chloride crystals dissolved in milli-Q water). Blackish red colour indicated the presence of flavonoids.

2.5. Flavonoid analysis on BGN seed extracts

Four flavonoid standards were used as external standards, namely quercetin, kaempferol, rutin and myricetin (Sigma Aldrich, Germany). The standard solutions were prepared as described by Kaliyaperumal et al. (2013). All standards were stored at −20 °C for a maximum of 2 weeks. The stock solutions of the four flavonoids standards were prepared by dissolving 1 mg into 10 mL HPLC grade methanol (Merck).

The HPLC separations of the standards and samples were performed using Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany). The HPLC system consists of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment set at 30 °C, an autosampler and a G1315C Diode Array Detector (DAD) set at 190 nm−600 nm. The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) integrated peak areas was used to record and store data.

A reversed phase analytical column (ZORBAX SB-C18 3.5 μm, 4.6 × 150 mm, Agilent, USA) was used with a reversed-phase C18 guard column; analysis was performed at 40 °C. The whole, dehulled and hull BGN extract samples were individually injected at a concentration of 20 μg mL−1. The guard cartridge was replaced after every 150 injections. The isocratic mobile phase methanol:acetonitrile:water:acetic acid in the ratio of 40:20:39:1 (v/v/v/v) with a flow rate of 0.8 mL min−1, the peaks were simultaneously identified using UV absorbance at 350 nm for kaempferol and 254 nm for rutin, myricetin and quercetin (Kaliyaperumal et al., 2013).

The chromatographic peaks of the extracts were confirmed by comparing their retention time and UV spectra with those of the pure standards. The utilisation of the calibration function of the Agilent Chemstation software to interpret the calibration curve and quantification of the flavonoid levels of samples were performed by the external standard method.

2.6. Thiamine (vitamin B₁) and riboflavin (vitamin B₂) analysis of BGN seed extracts

Two vitamin standards were used as external standards, namely riboflavin and thiamine (Sigma-Aldrich, Germany). The standard solutions were prepared as described by Otemuyiwa and Adewusi (2013). All standards were stored at −20 °C for a
maximum of 2 weeks. The mobile phases consisted of phase A and mobile phase B at a ratio of 50:50 v/v. Mobile phase A was made up of a 20 mM di-potassium hydrogen orthophosphate anhydrous (Unilab) solution in 500 mL of milli-Q water and adjusted to a pH of 6 using ortho-phosphoric acid 85% (Merck); once pH was adjusted, the remaining volume was made up to 1 L. Mobile phase B consisted of milli-Q water only. Both mobile phases were sonicated for 30 min before usage. The stock standard solutions of thiamine and riboflavin were prepared as described by Otemuyiwa and Adewusi (2013).

The HPLC separations of the standards and samples were performed using an Agilent 1100 HPLC system. The HPLC system consisted of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment, an autosampler, a G1315C Diode Array Detector (DAD), and a Fluorescence Detector (FLD). The Agilent Chemstation software (Agilent Technologies, Waldbronn, Germany) integrated peak areas were used to record and store data.

The chromatographic separation column consisted of a Phenomenex Luna C18 column (4.6 × 150 mm, 3 μm) and column temperature was set at 30 °C. The HPLC diode array detector was set at wavelengths 360 nm for riboflavin (vitamin B1) and 254 nm for thiamine (vitamin B2) to monitor the elution. The whole, dehulled and hull BGN extract samples were individually injected at a concentration of 20 μg mL⁻¹. The elution was isocratic with mobile phases of 50:50 v/v ratio (milli-Q water and 20 mM phosphate buffer, pH = 6) with a flow rate of 0.60 mL min⁻¹ (Otemuyiwa and Adewusi, 2013).

2.7. Tannin analysis on BGN seed extracts

Five phenolic compounds were used as external standards, namely gallic acid (Sigma Aldrich), catechin (Sigma Aldrich), methyl gallate (Industrial analytics), chlorogenic acid (Industrial analytics) and ellagic acid (Sigma Aldrich). The standard solutions were prepared as described by Møller et al. (2009). The stock solution of the five phenolic compounds was prepared to a concentration of 0.5 mg mL⁻¹ in a solvent consisting of 95% methanol and 5% water. From the stock solution five calibration standards were prepared by diluting with 50% (v/v) methanol-water with the following dilutions 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mg mL⁻¹.

HPLC was performed using the equipment previously described. As described by Møller et al. (2009), the chromatographic separation was achieved with a Phenomenex Luna C18 column (250 × 4.6 mm i.d.; 5 μm). The diode array detector was set a wavelength from 245 nm –360 nm. The HPLC-DAD/MS system used a programmed gradient mobile phase A [methanol, water, and formic acid (5:95:0.1) (v/v/v)] and mobile phase B [methanol and formic acid (100:0.1) (v/v)] in the
following gradient sequence: 0 min, 5% B; 7 min, 18% B; 11 min, 18% B; 15 min, 25% B; 20 min, 50% B; 25 min, 75% B; 26 min, 100% B; 29 min, 100% B; and 30 min, 5% B. The whole, dehulled and hull BGN extract samples were individually injected at a concentration of 20 μg mL⁻¹. The flow rate was 1.0 mL min⁻¹ and column temperature was maintained at 40 °C.

2.8. Data analysis

Multivariate analysis of variance was used to establish mean difference between treatments. Results expressed as mean ± standard deviation of triplicate measurements. Duncan multiple range test was used to separate means where difference existed (IBM - SPSS, 2015).

3. Results and discussion

3.1. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity, limit of detection (LOD) and limit of quantification (LOQ) for tannins consisted of five tannin compounds interpreted over a concentration range between 0.01 and 50 μg mL⁻¹. The multiple correlation coefficients (R²) of the five tannin compounds ranged between 0.997 and 0.999 and the correlation coefficients (R) ranged between 0.998 and 0.999. The LOD for gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid were between 0.04 and 0.49 μg mL⁻¹, while the LOQ was between 0.13 and 1.64 μg mL⁻¹.

For flavonoids, the linearity curves of the calibration mixture consisting of four flavonoid compounds interpreted over a concentration range between 0.50 and 50 μg mL⁻¹. The multiple correlation coefficients (R²) of the four flavonoid compounds ranged between 0.996 and 0.997 and the correlation coefficients (R) ranged between 0.99639 and 0.99893. The LOD for rutin, myricetin, quercetin and kaempferol were between 0.05 and 0.65 μg mL⁻¹, while the LOQ was between 0.19 and 2.17 μg mL⁻¹.

The calibration procedures for tannins and flavonoids were executed according to the AOAC method (Anonymous, 2002). High multiple correlation coefficients (R²) and correlation coefficients (R) of >0.98 were evidence of a good linear fit (Anonymous, 2002). The correlation coefficient and multiple correlation coefficients for tannins, flavonoids and vitamin B₁ and vitamin B₂ ranged between 0.98 and 0.99, indicating a noteworthy linear relationship between the concentration of the analytical standards and the response, while the proximity to one of the regression coefficients shows that the regression line fits the data. The HPLC method was, therefore, adequately sensitive and can be used as an analytical tool.
3.1.1. Peak identification and peak purity

The identification of tannins, flavonoids, and vitamin B$_1$ and B$_2$ were achieved by means of the retention time ($R_t$), and the quantification was performed by measuring the peak area of the samples relative to that of the standards (Anonymous, 2002). The retention times for all compounds were as follows: gallic acid (6.19 min), catechin (12.42 min), methyl gallate (14.25 min), chlorogenic acid (15.55 min) and ellagic acid (24.50 min) (Fig. 1); rutin (1.96 min), myricetin (2.23 min), quercetin (2.68 min) and kaempferol (3.36 min) (Fig. 2), and for the vitamins: thiamine (2.31 min) and riboflavin (14.99 min).

In addition, purity tests were performed using the “Check purity” option of the Chemstation system. This option was used to determine the purity factor of peaks which were within the threshold limit and therefore can be used to calculate the tannin, flavonoid and vitamin B$_1$ and vitamin B$_2$ concentrations. The purity factors were $>990$, which indicated an excellent peak.

Fig. 1. Chromatogram of a standard mixture containing five tannins at their respective retention times ($R_t$), namely gallic acid (6.196), catechin (12.426), methyl gallate (14.250), chlorogenic acid (15.555) and ellagic acid (24.508).

Fig. 2. Chromatogram of a standard mixture containing four flavonoids at their respective retention times ($R_t$), namely rutin (1.966), myricetin (2.232), quercetin (2.681) and kaempferol (3.360).
3.2. Flavonoid characteristics of BGN extracts

Flavonoids of BGN extracted with 70% methanol solvent are summarized in Table 1. Brown BGN was significantly (p ≤ 0.05) higher in rutin than black, brown-eye, black-eye and red. Brown-eye and black were significantly (p ≤ 0.05) lower in myricetin, whereas red was significantly (p ≤ 0.05) higher in myricetin for all samples.

The different forms of BGN (hulls, whole, dehulled) did not differ in their quercetin and kaempferol content. Rutin was better extracted with 70% methanol from black, brown-eye, black-eye and red BGN. The red BGN had the highest concentration of myricetin when extracted with 70% methanol. When comparing the three forms of BGN for utilization, preference should go to whole BGN, as it contains both the endosperm and the hulls. Reason being, the endosperm or dehulled BGN contained the protein of BGN and the hulls include high flavonoid concentration whereby providing overall nutritional benefits.

The darker colours in BGN hulls namely, red and brown have higher concentration of flavonoids than the black-eye and brown-eye counterparts. In summary, the brown and red hulls had the highest concentration of flavonoids compared to whole and dehulled, with the highest flavonoid concentration being rutin at 24.46 mg g⁻¹ found in brown hulls and myricetin at 1.80 mg g⁻¹ found in red hulls.

Table 1. Flavonoid characteristics of BGN extracted with 70% methanol solvent.¹ ²

| BGN form | BGN sample | Quercetin (mg g⁻¹) | Kaempferol (mg g⁻¹) | Rutin (mg g⁻¹) | Myricetin (mg g⁻¹) |
|----------|------------|--------------------|---------------------|---------------|-------------------|
| Whole    | Brown      | n.d                | 0.052 ± 0.070*-     | 0.645 ± 0.013*- | 0.062 ± 0.006ab*  |
|          | Black      | 0.006 ± 0.010*     | 0.131 ± 0.034*-     | 1.427 ± 0.458* | 0.123 ± 0.062*    |
|          | Brown-eye  | 0.005 ± 0.008*     | 0.105 ± 0.013*      | 0.417 ± 0.337* | 0.040 ± 0.038*    |
|          | Black-eye  | n.d                 | 0.544 ± 0.097*      | 0.064 ± 0.006ab* |
|          | Red        | 0.007 ± 0.012*     | 0.096 ± 0.085*      | 0.878 ± 0.858* | 0.135 ± 0.151b    |
| Dehulled | Brown      | 0.021 ± 0.026*     | 0.129 ± 0.007*      | 0.881 ± 0.251* | 0.070 ± 0.017ab*  |
|          | Brown-eye  | 0.007 ± 0.011*     | 0.168 ± 0.062*      | 0.837 ± 0.190* | 0.065 ± 0.008*    |
|          | Black-eye  | 0.004 ± 0.007*     | 0.077 ± 0.067*      | 0.476 ± 0.430* | 0.042 ± 0.036ab*  |
|          | Red        | 0.004 ± 0.007*     | 0.119 ± 0.007*      | 0.729 ± 0.149* | 0.061 ± 0.010b    |
| Hull     | Brown      | 0.077 ± 0.123*     | 0.297 ± 0.094*      | 24.458 ± 0.234* | 0.932 ± 0.550b    |
|          | Brown-eye  | 0.062 ± 0.041*     | 0.219 ± 0.090*      | 3.977 ± 0.447b | 0.622 ± 0.279*    |
|          | Black-eye  | 0.057 ± 0.054*     | 0.224 ± 0.008*      | 6.233 ± 1.251b | 0.986 ± 0.432ab*  |
|          | Red        | 0.070 ± 0.043*     | 0.159 ± 0.129*      | 7.236 ± 4.101b | 1.800 ± 0.771b    |

¹ Results are mean ± standard deviation. Means with different letter superscripts representing BGN sample within each BGN form column are significantly different (p ≤ 0.05).
² n.d. = not detected.
3.3. Tannin characteristics of BGN extracts

The tannins of the different BGN varieties extracted with 70% methanol solvent are summarized in Fig. 3. Red BGN was significantly (p ≤ 0.05) higher in ellagic acid especially within the hulls when compared to other BGN samples i.e. brown, black, brown-eye and black-eye. Tannins reported by Salawu (2016) for whole BGN were, gallic acid (1.03 ± 0.01 mg g⁻¹), catechin (2.34 ± 0.03 mg g⁻¹), chlorogenic acid (2.37 ± 0.02 mg g⁻¹) and ellagic acid (1.09 ± 0.02 mg g⁻¹). These results reported by Salawu (2016) are significantly higher than the present study. This could be due to a lower pH of the solvent (acidified methanol) used by Salawu (2016), thus changing the characteristic of the solvent and potentially leading to better extraction of phenolic compounds whereas the present study used non-acidified 70% methanol.

Furthermore, the extraction time used in the Salawu (2016) method was 24 h using plant tissue extraction procedure, whereas in the present study the extraction time was only 30 min using sonication according to the method described by Nyau et al. (2014). This also could be a contributing factor as the phytochemicals have a longer period to interact with solvent, thereby possibly yielding better extraction and resulting in higher amounts of phenolic compounds (Tiwari et al., 2011). The levels of tannins found in BGN are far below the recommended proanthocyanidins (condensed tannins) levels. Recent research has also indicated that condensed tannins in low concentrations have beneficial effects in animal and human nutrition and health (Tibe et al., 2007).

Fig. 3. Tannins of BGN extracted with 70% methanol solvent from A = Whole, B = Dehulled & C = Hull.
4. Conclusion

The form and colour of BGN were all important factors to optimize the best extraction yield of phytochemicals. Overall the hulls of the BGN were the optimum source of flavonoids and tannins: the brown and red hulls had the highest concentration of flavonoids compared to whole and dehulled, with the highest flavonoid concentration being rutin at 24.46 mg g$^{-1}$ found in brown hulls and myricetin at 1.80 mg g$^{-1}$ found in red hulls. Lastly, formulating products with higher concentration of BGN hulls could potentially result in a product with higher phytochemical content.

Declarations

**Author contribution statement**

Taahir Harris: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Victoria Jideani: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Marilize Le Roes-Hill: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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