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Antioxidant Activities of Bambara Groundnuts as Assessed by FRAP and DPPH Assays

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Abstract There is a growing interest in legumes and legume based foods because of the health claims associated with their consumption. The aim of the current study was to explore the nutraceutical potential of bambara groundnuts (*Vigna subterranea* L. Verdc) based on the antioxidant properties. Two market classes of bambara groundnuts (red and brown) were screened. The study employed 1,1-diphenyl-2- picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) *in vitro* assays to screen for antioxidant properties. Bambara groundnuts were found to possess antioxidant activities. Brown bambara groundnuts exhibited the highest DPPH free radical scavenging activity with EC_{50} = 347 ± 4.2 µg dried extract / ml compared to 495 ± 12 µg dried extract / ml for the red bambara groundnuts. Again FRAP derived total antioxidant power was higher in the brown (6.00 ± 0.21 mmole Fe^{2+} / 100 g DW) compared to (5.00 ± 0.13 mmole Fe^{2+} / 100 g DW) in the red. Antioxidant activities demonstrated by bambara groundnuts suggest that it could be very useful as a source of antioxidants besides the commonly consumed legumes. This in itself presents an opportunity for diversification of food resources considering that bambara groundnuts are currently very much under-utilized.

Keywords: *Bambara groundnuts, antioxidant activities, nutraceuticals*

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1. Introduction

Consumers are increasingly aware of the health benefits of foods and pay particular attention to the potential disease preventing and health promoting compounds that a given food contains. This awareness, coupled with a well-known understanding of how diet affects our health, is motivating a quest for natural health products. There is a mounting market demand for natural products from traditional foods primarily due to the increasing consumer awareness of the role of food in health promotion and disease prevention (Liu,2009). Legumes and legume based foods are becoming popular as nutraceutical targets because of the health claims associated with their consumption. A number of studies on the nutraceutical value of well known legumes such as common beans, lentils, peas and soyabeans have been conducted. However, a wide gap exists regarding the nutraceutical potential of underutilized legumes such as bambara groundnuts. Exploring underutilized legume resources is of great importance considering their rich nutraceutical value and the presence of bioactive compounds (Bhat and Karim, 2009). Bambara groundnuts (*Vignea subterranean* L. verdc) have a long history of cultivation and are predominantly grown in drier areas with short inconsistent rainfall in the Sub-Saharan Africa (FAO, 2001). They are grown for their seeds which are used for dietary purposes. The pods develop underground and may attain a length of up to 3.7 cm, depending on the number of seeds they contain (Egoli, 1995). Bambara groundnuts have the ability to do well in poor soils and harsh climatic conditions. They adapt to a wide range of soils and perform better on poor soils than common peanuts (Tweneboah, 2000). The seeds are nutritionally rich as they contain proteins, carbohydrates and fats in adequate proportions. Ripe seeds contain 16 – 21% proteins, 4.5 - 6.5% fats, and 50 – 60% carbohydrates (Purseglove 1992; Brough 1992; Brough *et al*., 1993). Whilst the nutritional value of bambara groundnuts is well known, their potential as nutraceuticals has not been exploited. Studies on the nutraceutical properties of bambara groundnuts are essential to establish their potential for use in the functional foods and nutraceutical industry. Additionally, this information is necessary for strategies aimed at promoting consumption of bambara groundnuts, which are slowly drifting into the category of "neglected species" or "forgotten crops" of Africa. Hence, this study was undertaken to explore the nutraceutical antioxidant potential of bambara groundnuts using the FRAP and DPPH *in vitro* antioxidant assays.

2. Materials and Methods

2.1. Sample Collection

Popularly cultivated market classes of bambara groundnuts (red and brown) were procured directly from the farmers in the Eastern region of Zambia immediately after harvest. In order to make the samples representative, an attempt was made to collect the seeds of each market class from 15 farmers in the area with not less than 0.5 kg per farmer. Both market classes are landraces that have become well adapted to the local climate and soils, and indigenous knowledge about the germplasm is well preserved in the communities.

2.2. Preparation of the Extracts

Raw dry bambara groundnuts and common beans were ground into powder of the same consistency using a coffee grinder (Braun, Mexico). Crude aqueous and 70% methanol extracts were obtained using Ultrasound-Assisted Extraction (UAE) from the seed flour (Dobiáš *et al*., 2010). Approximately 15 g of seed powder in 150 ml of either water or 70% methanol was sonicated for 30 minutes at 25°C using the Eumax UD500SH 40 kHz ultrasonic bath. After extraction, the mixture was centrifuged at a speed of 10,000 rpm for 15 minute in Beckman Coulter JE centrifuge. The supernatant of the water extraction was frozen at -80°C and freeze dried to get a powdered aqueous extract. The supernatant of the 70% methanol extraction, however, was first concentrated to 30 ml by evaporation under reduced pressure in a rotary evaporator (Buchi R-210 model, Switzerland) to remove methanol. The extract was then frozen at -80°C and freeze dried to obtain a powdered methanolic extract using the Telstar LyoQuest -85 freeze dryer. The freeze dried aqueous and methanolic extracts were stored at -4°C until further analysis.

2.3. Determination of Free Radical Scavenging Activity of the Bambara Groundnuts

The free radical scavenging reaction between DPPH and an antioxidant (Y-H) can be written as:

$$(\mathrm{DPPH}) + (\mathrm{Y-H}) \rightarrow \mathrm{DPPH-H} + (\mathrm{Y})$$

$$(\mathrm{Purple}) \qquad (\mathrm{Yellow})$$

(Oktay *et al*., 2003)

DPPH stable free radicals are reduced to DPPH-H leading to discoloration from purple to yellow and consequently a decrease in absorbance. The degree of discoloration indicates the scavenging potential of the antioxidant compounds (Oktay *et al*., 2003; Pal *et al*., 2008). This assay therefore involves the measurement of hydrogen atom transfer or electron donation from a potential antioxidant to free radical molecules (Becker *et al*., 2004). First, it was important to study the kinetic behaviour of the extracts towards DPPH free radicals when the freeze dried extracts from each market class were added at the same concentration. The knowledge of the kinetics of atom transfer is important because free radicals in the organism are short-lived species, implying that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals (Villañ*o et al*., 2007). The free radical scavenging kinetic determinations were adapted from (Villañ*o et al*., 2007). Under the experimental conditions used, the DPPH concentration was in large excess with respect to that of the extracts in order to follow pseudo first-order kinetics. This was done to exhaust the hydrogen donating capacity of the extracts. The excess concentration of DPPH (200 mM) was determined to be the optimum concentration after performing a number of runs with the extracts. This was the only way the excess DPPH concentration could be determined since it was not posible to work it out based on the DPPH: antioxidant molar ratios as the antioxidants in the extracts were not pure compounds. In the assessement of the kinetic behaviour, 2 ml of the extracts were added at the same concentration (400 µg / ml) to 2 ml of DPPH radical solution (200 mM) prepared in 95% methanol. The reaction was run at room temperature within a time period of 80 minutes. The absorbances of the mixture were automatically measured every 10 seconds using the spectrophotometer at 517 nm connected to a computer and the output was displayed using SWIFT 1000 software (Ultraspec 1000 model, England). From the reaction between an antioxidant and DPPH;

(DPPH) + (Y-H) → DPPH-H + (Y), it can be deduced that:

$$-\frac{d[DPPH]}{dt} = k[DPPH][Y-H] \qquad (1)$$

Considering that DPPH was in excess and therefore the experiment was under pseudo first-order conditions, one can say:

$$InA = InA_o - kt \qquad (2)$$

Where A_0 is the absorbance of the reaction mixture (DPPH and the extract) at $t = 0$; A is the absorbance of the reaction mixture (DPPH and extract) at time t.

The pseudo first order rate constant 'k' for the reaction of the antioxidants in the extracts and DPPH in the first seconds of the reaction was calculated from the slopes of *InA* versus time plots.

The percentage of DPPH remaining at any time t can be determined as:

$$\%DPPH_{remaining} = \frac{A_t}{A_0} X100 \qquad (3)$$

(Villañ*o et al*., 2007)

Where A_0 is the initial absorbance and A_t is the absorbance at time = t, both measured at 517 nm respectively. Plots of percentage DPPH versus time were constructed to show the disappearance pattern of the DPPH with time in the presence of each extract.

After studying the kinetic behaviour of the extracts towards the DPPH free radicals, it was necessary to evaluate the Effective Concentration (EC_{50}) for each extract. The extracts were assayed with the DPPH and incubated in the dark at room temperature for 30 minutes. The degree of free radical scavenging activity in the presence of different concentration of extracts and their absorbance were measured using spectrophotometer at 517 nm (Ultrospec 1000 model, England). The degree of free radical scavenging activity was expressed as:

DPPH free radical scavenging (%) =

$$\frac{A_{Control} - A_{Sample}}{A_{Control}} X100 \quad (4)$$

$A_{Control}$ = Absorbance of DPPH alone, A_{Sample} = Absorbance of DPPH in the presence of different concentrations of the extracts.

The EC_{50} value required for 50% of the DPPH free radicals scavenging by the extracts was obtained from a series of dose-response data (extract concentrations and DPPH free radical scavenging (%). Using an x-y plot, the data was fitted with a linear regression line (Iranshahi *et al*., 2009, and the EC_{50} was estimated using the following relationship:

$$Y = a*X + b, \quad (5)$$

$$EC_{50} = (0.5 - b)/a \quad (6)$$

2.4. Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was used to determine the ferric reducing antioxidant power of the bambara groundnuts (Benzie, 1996). The method measures the ferric reducing ability of the antioxidants compounds in the extracts. At low pH, ferric-2,4,6-tri-2-pyridyl-*s*-triazine (TPTZ) complex (Fe^{3+} TPTZ) is reduced to the ferrous form Fe^{2+} in the presence of the antioxidant producing an intense blue colour with an absorption maximum at 593 nm. Powdered sample of bambara groundnuts (5 g) in 50 ml of 70 % methanol or water was sonicated for 30 minutes at 25°C followed by centrifugation at 10,000 rpm for 15 minutes at 4°C to obtain a clear supernatant. Working FRAP reagent was prepared by mixing 25 ml of acetate buffer (300 mM, pH 3.6); 2.5 ml ferric chloride solution (prepared by dissolving 54 mg ferric chloride in 10 ml distilled water) and 2.5 ml TPTZ solution (prepared by dissolving 31 mg TPTZ in 40 mM HCl at 50°C). The mixture was placed in a water bath at 37°C for 10 minutes. The assay was performed as follows: 1 ml of water and 80 µl of the test sample were pipetted into a cuvette. About 600 µl of the incubated FRAP reagent was added to the cuvette and mixed by inversion. A reagent blank was prepared as above with 80 µl water added instead of the test sample. The change in absorbance was recorded at 593 nm using a spectrophotometer after exactly 4 minutes (Ultrospec 1000 model, England). The amount of Fe^{2+} produced from the reduction of Fe^{3+} by the extract was calculated from the standard curve prepared from ferrous sulphate solution and results were expressed as mg Fe^{2+} / 100 g dry sample. The experiment was conducted three times and all measurements were performed intriplicate.

3. Results and Discussion

3.1. DDPH Free Radical Scavenging Activities

Figure 1 presents the disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts within a time period of 80 minute. The free radical scavenging pattern is biphasic, characterised by the fast initial decay, followed by the subsquent slower step in which degradation of by products may be involved. The fast initial decay is attributed to reactions (i) and (ii):

$$DPPH + (Y-H) \rightarrow DPPH-H + Y \quad (i)$$

$$Y + DPPH \rightarrow \text{products} \quad (ii)$$

while the subsequent decay is attributed to secondary slow reactions from the products of dimerization (or disproportionation) of A or from the products of reaction (ii). From the decrease in absorbance versus time in the first few seconds of the reaction, information about the pseudo first-order rate constant for reaction (i) can be acquired (Villaño *et al*., 2007).

Employing equations (1) and (2), and the plot of InA versus time, the pseudo first-order rate constant K of the aqueous and methanol extracts of bambara groundnuts were obtained. The pseudo first-order rate constants (K) for the bambara groundnuts extracts in a DPPH reaction are presented in Table 1. The K values for the brown bambara groundnuts were higher than the red one in both the aqueous and methanol extracts. The results would indicate that the antioxidants from the brown market class have faster reaction kinetics than the antioxidants from the red one. The slower kinetics for the red bambara groundnuts can also be observed by looking at the amount of DPPH scavenged after 80 minutes incubation time (Table 1). By the end of 80 minutes, over half of the DPPH was scavenged by the brown bambara groundnuts antioxidants in both the aqueous and methanol extracts, whereas 39 and 43 % DPPH was scavenged by the red bambara antioxidants in the aqueous and methanol extracts respectively. Trolox showed a very fast initial decay with the higest K value 1.55 (min^{-1}) and the amount of DPPH scavenged (85.5%). The free radical scavenging ability of bambara groundnuts antioxidants was found to be moderate as compared to Trolox. This, however, may not be very surprising because Trolox is a pure compound and is considered to be a powerful antioxidant (Madhavi *et al*., 1996). These observations have positive implications on the potential of bambara groundnuts as a dietary source of antioxidants.

Table 1. Pseudo-first order rate constant of antiradical (Y-H) in bambara groundnuts extracts and the amount of DPPH scavenged after 80 minutes of incubation

Market class	Pseudo-first order rate constant (K) [min^{-1}]		Amount $DPPH^{\bullet}$ quenched [%] after 80 minutes incubation	
	Aqueous extract	70% Methanol extract	Aqueous extract	70% Methanol extract
Brown	0.056	0.053	57.1 ± 3.1	53.7 ± 2.6
Red	0.034	0.041	38.9 ± 1.8	43.3 ± 4.6

The EC_{50} values were obtained from the full dose-response curves fitted with the regression line (see example in Figure 2). The EC_{50} is inversely related to the antioxidant capacity of the compound. The lower the EC_{50}, the higher the antioxidant activity of the compound is (Villaño *et al*., 2007). The EC_{50} of bambara groundnuts extracts and Trolox, which was used as a positive reference standard, are presented in Table 2. Generally, in comparison with the positive reference standard, all extracts displayed moderate scavenging activity. Among the extracts, the aqueous extract from the brown bambara groundnuts was the most efficient scavenger with the lowest EC_{50} value (347 µg dried extract / ml). The lowest

scavenging effect was displayed by the methanol extract of the red bambara groundnuts (EC_{50} = 525.5 µg dried extract / ml). Both the aqueous and the methanol extracts of the brown bambara groundnuts showed higher scavenging activity than both extracts from the red one.

The DPPH free radical scavenging ability of five legumes that included chickpea, lentil, mung beans, mash beans and peas extracted in aqueous methanolic mixture (80:20 v/v) have been investigated previously (Zia-Ul-Haq *et al*., 2012). The following EC_{50} values were reported: Desi chickpea (367.2 µg dried extract / ml), Kabuli chickpea (432.1 µg dried extract / ml), lentil (465.5 µg dried extract / ml), mung beans (389.2 µg dried extract / ml), mash beans (401.4 µg dried extract / ml) and peas (457.1 µg dried extract / ml) respectively. The DPPH free radical scavenging of the four legumes was found to be moderate compared to ascorbic acid (192 µg / ml) used as a positive reference standard. The results on bambara groundnuts in the present study are somewhat comparable to these findings. In other studies, the DPPH radical scavenging capacity of the *Lupinus albus*, *Lens culinaris* and *Phaseolus vulgaris* seeds were investigated and EC_{50} values ranging from 2300 to 7600 µg dried extract / ml were reported (Spanou *et al*., 2007). Comparing these findings to the present study, bambara groundnuts with EC_{50} ranging from 347 to 525 µg dried extract / ml can be said to be more potent scavengers than *Lupinus albus*, *Lens culinaris* and *Phaseolus vulgaris* seeds. The DPPH free radical scavenging of bambara groundnuts indicates that they contain compounds capable of scavenging free radicals.

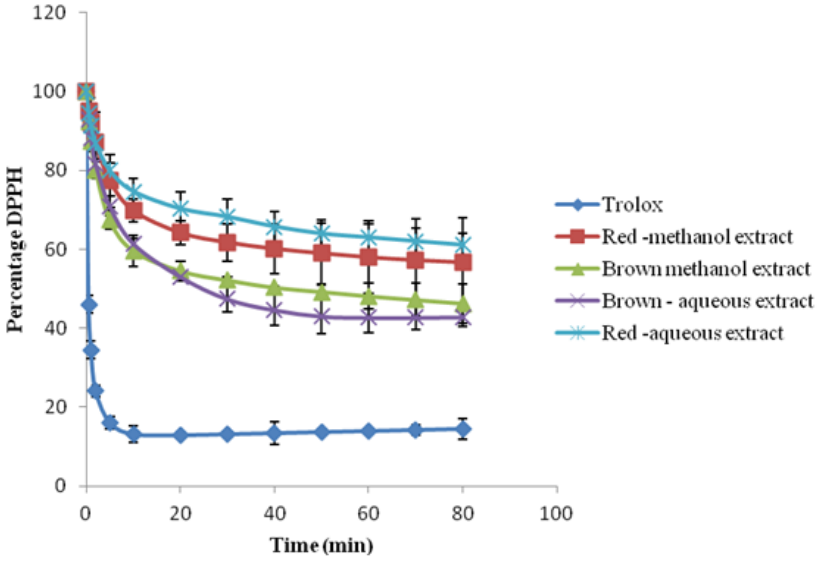
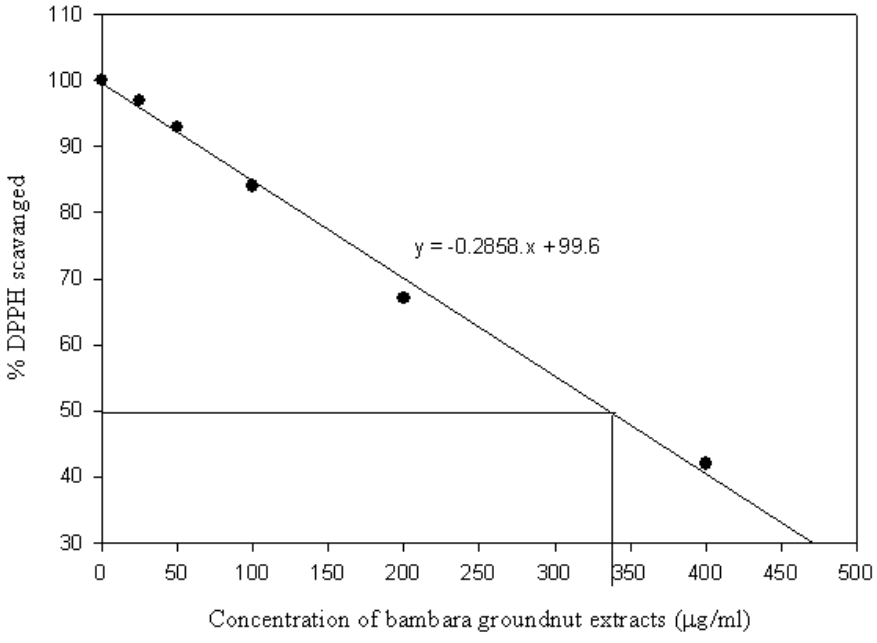


Figure 1. Disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts of bambara groundnuts



Note: y = a*x + b, and y = 0.5,
EC_{50} = (0.5 - b)/a = (0.5 - 99.6)/-0.2858 = 347.0

Figure 2. Dose-response plot [concentration of aqueous extract of brown bambara groundnuts and DPPH scavenged (%)] fitted with linear regression line

Table 2. EC_{50} values for DPPH free radical scavenging by bambara groundnut extracts after 30 minutes of incubation

Market classes of bambara groundnuts	DPPH radical scavenging EC_{50} (µg dried extract / ml)	
	70 % Methanol extract	Aqueous Extract
Brown	477.5 ± 3.5	347.0 ± 4.2
Red	525.5 ± 7.8	495.5 ± 12.0

Trolox EC_{50} = 21.0 ± 1.4

3.2. Ferric Reducing Antioxidant Power

The FRAP values of the aqueous and methanol extracts of the brown and the red market classes of bambara groundnuts are presented in Table 3. As can be seen, the methanol extract of both market classes had higher FRAP values than the aqueous extract. The results showed that the brown groundnuts had higher ferric reducing ability than the red bambara groundnuts and the difference was significant ($p < 0.05$). In a similar study, the antioxidant reducing power of different market classes of yellow peas, green peas, chickpea, lentils and common beans grown in North Dakota, Idaho and Washington regions of the United States of America were investigated and their FRAP values are presented in Table 4 (Xu *et al*., 2007). In comparison to the present study, bambara groundnuts can be considered to have stronger antioxidant reducing power than peas, but comparable to ranges reported for lentils and common beans.

Table 3. Ferric Reducing Antioxidant Power (FRAP) values for bambara groundnuts

Market classes of bambara groundnuts	FRAP value (mmole Fe^{2+} / 100 g DW)	
	70 % Methanol extract	Aqueous Extract
Brown	9.70 ± 0.07	5.65 ± 0.21
Red	8.01 ± 0.13	5.00 ± 0.13

Table 4. Ferric Reducing Antioxidant Power (FRAP) values for other legumes

Legume type	Range of FRAP value (mmole Fe^{2+} / 100 g DW)
Yellow peas	0.62 – 0.82
Green peas	0.43 – 0.86
Lentils	8.75 – 13.92
Common beans	1.27 – 9.70

Source: (Xu *et al*., 2007)

4. Conclusion

The study has shown that bambara groundnuts have antioxidant activities that are comparable to commonly consumed legumes such as lentils, common beans and chickpeas. These findings indicate that bambara groundnuts have the potential for use in the nutraceutical industry. Based on this, it is suggested that consumption of bambara groundnuts could possibly offer some health benefits.

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