The African Journal of Plant Science and Biotechnology ©2013 Global Science Books

Determination of Profiles, Antioxidant Activity and Quantity of Phenolic Compounds in Bambara Nut (*Vigna subterranea*) Varieties Found in Zimbabwe

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ABSTRACT

Five (fresh and dried) varieties of *Vigna subterranean* nuts commonly known as bambara nuts were categorized according to their skin and helium color. The nuts were separated into groups namely maroon, brown, variegated, light and dark helium and were assayed for total phenolic content, free radical scavenging, reducing power, inhibition of phospholipids peroxidation and tannin content. The highest total phenolic content was found in brown nuts with (580 mg/100 g) for fresh and (347 mg/100 g) for the dried nuts. The least total phenolic content was in dark helium nut, with (185 mg/100 g) for the fresh ones and (120 mg/100 g) for the dried. The vanillin HCl assay was found to be more sensitive as it gave much higher tannin content than the butanol-HCl assay. In the vanillin HCl, highest tannin content was in the brown nut, with (1.7 g/100 g) for fresh and (1.12 g/100 g) for dry and the least in dark helium with (0.46 g/100 g) for fresh and (0.06 g/100 g) for dried nuts. Butanol-HCl values were much lower than those for vanillin-HCl, with brown nut recording the highest tannin content of (0.0058 g/100 g). Free radical scavenging activity was tested using DPPH assay. Fresh nuts exhibited a higher radical scavenging potential than the dried nuts with the brown and maroon being the most powerful scavengers. Only dried nuts were assayed for reducing power, inhibition of phospholipids peroxidation which the results were positive.

Keywords: free radical scavenging activity, phospholipid peroxidation, reducing power, tannin content, total phenolic content

INTRODUCTION

Indigenous crops are no longer consumed in Zimbabwe as often as they were long ago. Modern diet is mainly comprised of processed foods. Indigenous crops are now referred to as "poor man's food" and as a result chronic diseases like cancer, heart attack and strokes are on the increase.

Bambara nut (*Vigna subterranea* (L.) Verdc) is one of the indigenous geocarpic leguminous crops grown in sub Saharan Africa. Its leaves are trifoliate with relatively long petioles, while its flowers are yellow. During growth, the plant's inflorescence are near the soil surface, but after fertilization, the developing pods are buried underground the soil surface until they are ripe and ready for harvest. The seeds grow round and smooth with a relative diameter of 1 cm. When dried, they are very hard and have varying colours ranging from cream to brown, red, mottled or black eyed. Bambara nuts have a high adaptability quotient which is well suited for hot, dry and challenging regions where many other crops fail to survive due to lack of water and nutrients (Masawe *et al.* 2002).

Insignificant investigation has been carried out on Bambara nuts. So far, soya bean is the most investigated legume, with its health benefits clearly laid out (Wiseman *et al.* 2002). That is why soybean is well known and marketable throughout the world. Bambara nut being a crop that mainly thrives in Africa, very little is known about it in the developed countries (Gomez, 1989). One contributing factor could be the stigmatisation of the nut as "poor man's crop" and as a result, the scientific community has turned a blind eye on the indigenous crop.

The seed makes a complete food with sufficient nutrient quantities comprising of carbohydrates 63.0%, proteins 17-24.6%, fat 5.3-7.8%, energy 367-414 Kcal/100 g (Mbata *et al.* 2009). Bambara nut is also contains essential amino acids lysine and tryptophan. This gives the nut a beneficial complementary effect when consumed with cereals that are low in essential amino acids (Masawe *et al.* 2005).

Africa, being the worst continent affected by Acquired Immune Deficiency Syndrome (AIDS) and the world food crisis, the antioxidant properties suspected in the nut could help in reducing the progression of the pandemic (Congressional Research Service 2006). The presence of antioxidants in the cytosol prevents reactive oxygen species from activating the transcription factors, nuclear factor kappa Beta (NFκβ) to induce the replication and expression of Human Immunodeficiency Virus (HIV) (Yao *et al.* 2004). This therefore reduces the chances of contracting full blown AIDS (Bagchi *et al.* 2000; Chun *et al.* 2005) An investigation of this locally available crop's antioxidant properties could assist the poverty stricken communities, which are the worst affected by HIV since they have very limited access to drugs that reduce viral replication.

Bambara groundnuts are may contain some useful non nutrient phytochemicals, the phenolic compounds that have antioxidant activity. In order to address this suspicion it was deemed necessary to conduct this study on the evaluation of the phenolic compounds and antioxidant properties in different bambara nut varieties. Since phenolic compounds have been associated with health benefits (Schwarz *et al.* 2006), an evaluation of these compounds in the nut could lift up the low perception majority has on the nut, boost food security, improve nutrition, and foster rural development. Hence, the nut would serve as a food and medicine.

A thorough investigation in the total phenolic content and antioxidant properties in bambara nuts could also help make it a more marketable food resource worldwide. To add

Received: 2 February, 2012. Accepted: 17 March, 2012.

Research Note

Fig. 1 Samples of different peeled bambara nut varieties.

more value to the nut we sat out to evaluate the non nutrient secondary metabolites, mainly the phenolic compounds that could be of health benefit to the human society.

MATERIALS AND METHODS

Sources of samples and chemicals

All 5 nut varieties (**Fig. 1**) were purchased from a local supermarket in Harare, Zimbabwe. The chemicals were all of high purity grade and were all from Sigma-Aldrich Chemie (Steinheim, Germany).

Solvent extraction

Total phenolic compounds were extracted from the sample as described by Makkar (1999). Ground sample (0.5 g) was mixed with 5 ml of 50% methanol and vortexed for 1 min. The sample-solvent mixture was then sonicated for 10 min after which centrifugation at 3000 rpm for 10 min, was done. The supernatant was used for the analysis.

Folin-Ciocalteau assay for total phenolics

The Folin Ciocalteau method for determination of total phenolic compounds was carried out following the method described by Singleton and Rossi (1965). Distilled water (950 µl) was added to samples (50 µl) to make up to 1 ml. An amount of 2.5 ml of 2% sodium carbonate was added followed by 500 µl of Folin-Ciocalteau reagent. Gallic acid (0.5 mg/ml) was used as standard in different concentrations. The mixture was then incubated for 40 min. Absorbance at 725 nm was measured on a Spectronic 20 Genesys spectrophotometer.

1, 1 Diphenyl-2-picryl hyrdrazyl (DPPH) assay

The radical scavenging activity was determined following method of Kuda *et al*. (2005). 2.980 ml of methanolic solution of DPPH (0.0012 g/100 ml) was placed into a cuvette and 20 µl of sample was added. Absorbance at 517 nm was read on a spectronic 20 Genesys spectrophotometer over 40 min. Ascorbic acid (0.5 mg/ml) and gallic acid (0.5 mg/ml) were used as standards.

Determination of proanthocyanidin using the butanol-HCl assay

Proanthocyanidin content was determined using a method described by Makkar (1999). Preparation of butanol-HCl reagent (butanol: HCl, 95: 5, v/v) 95 ml of butanol were mixed with 5 ml of HCl concentrated Ferric reagent (2% of ferric ammonium sulfate dissolved in 2N HCl). In making 2N HCl, about 16.6 ml of concentrated HCl was made up to 100 ml with distilled water. Therefore, 2 g of ferric ammonium sulfate was dissolved in 100 ml of 2N HCl to make the ferric reagent. 0.5 ml of sample extract was added into test tubes followed by 3 ml of butanol-HCl reagent and then 0.1 ml of ferric reagent. The tubes were vortexed, covered with a glass marble and heated in a water bath at (90-100°C) for 1 h. Tubes were then cooled and absorbances read at 550 nm on a Genesys spectrometer.

Vanillin HCl assay

To 500 µl of sample 2.5 ml of (HCl-methanol, 25:25, v/v) was added followed by 2.5 ml of vanillin reagent (0.5 g/50 ml). The mixture was incubated at room temperature for 20 min. Absorbance was read at 500 nm on a Genesys spectrometer. The concentration of flavonoids was expressed as catechin (4 mg/ml distilled water) equivalents, following the Porter *et al*. (1986) method.

Reducing power effects assay

Reducing power effects were determined following the method of Kuda *et al*. (2005). Up to 80 µl sample of ascorbic acid control solution was mixed with phosphate buffer (0.2 ml 0.2M pH 7.2) and 1% potassium ferricyanide (0.2 ml). The mixture was incubated at 50°C for 20 min. Tricarboxylic acid (TCA) (0.2 ml, 10%) was added. After transferring an aliquot of the mixture (0.125 ml) into a microtitre plate, distilled water (2855 µl) and $FeCl_3$ (0.02 ml 0.1%) was added. The absorbance at 655 nm was measured on a Genesys spectrometer.

Inhibition of phospholipid peroxidation

Female Sprague Dawley rats (*Rattus norvegious*) were obtained from the animal house, University of Zimbabwe. An animal license was obtained from the Livestock and veterinary services and ethical approval was sought from the Joint Parirenyatwa hospital and University of Zimbabwe College of Health Sciences Ethical Committee, to use the animals for experiments. The rats were dissected in the Biochemistry laboratory to obtain the rat brain and liver. The rat brain and liver were stored at -85°C until used. Homogenisation of the rat brain (2 g) was done in a chloroform methanol mixture (10 ml) (2:1, v/v) followed by centrifugation at 3000 g for 5 min. The supernatant was collected and used as the source of phospholipids. The blank contained the phospholipid solution (50 µl) mixed with distilled water instead of sample and 50% methanol (0.2 ml). The test run contained the phospholipid solution (50 µl) the tea extract up to 80 µl, 50% methanol (0.2 ml) and ferrous sulphate (0.5 ml). Ascorbic acid was used as the positive control. Incubation at 37°C was followed by addition of thiobarbituric acid (TBA) (0.5 ml, 1%) and trichloroacetic acid TCA (4 ml, 10%) and the solution was then heated in a boiling water bath for 15 min. After cooling on ice the absorbance was read at 532 nm on a Spectronic20® Genesys™ spectrophotometer.

Statistical analyses

Samples were analysed in triplicate and the results reported in this study are given as means ± standard error.

RESULTS

Folin Ciocalteau for fresh and dry nuts

A comparison between the fresh and dry nut total phenolic content is shown in **Fig. 2**. The brown, maroon, variegated, light helium and dark helium varieties of the nuts are abbreviated B, M, V, LH and DH, respectively. From each pair, the bar on the left represents the fresh bambara nut and the bar on the right is for the dried nuts. Among fresh samples, the brown nut had the highest total phenolic content of

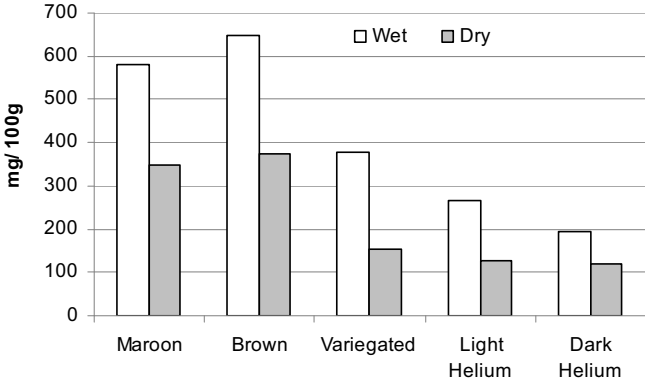

Fig. 2 Total phenolic content between fresh and dry nuts. M - maroon, B - brown, V - variegated, LH - light helium, DH - dark helium. The results are presented as mean ± standard deviation of three independent measurements.

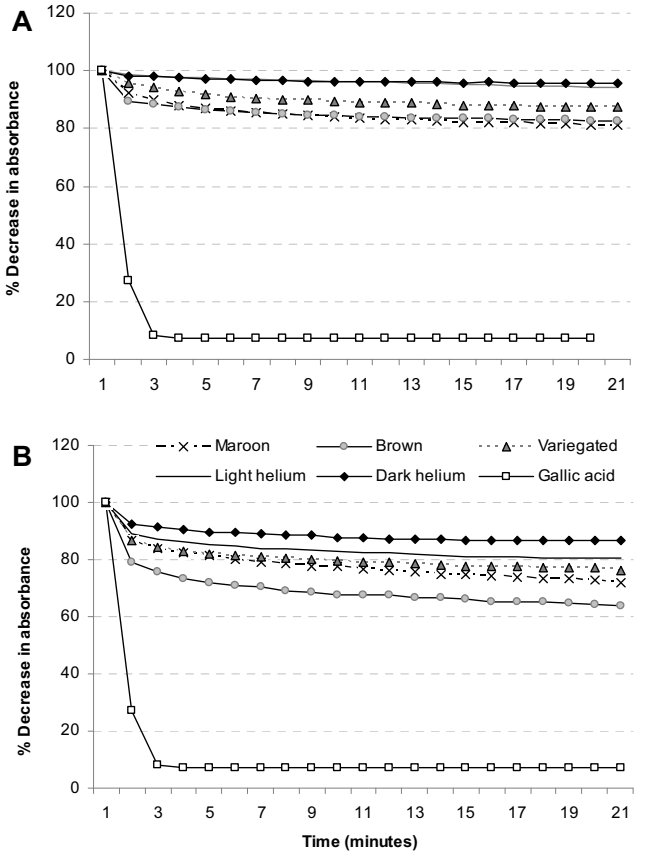

Fig. 3 Free radical scavenging activity of phenolic compounds in dried (A) and fresh (B) bambara nuts. The results are presented as mean ± standard deviation of three independent measurements.

approximately 647 mg/100 g of sample followed by maroon, variegated, light helium and then lastly, dark helium with around 194 mg/100 g of crude sample. The same trend is observed amongst the dry samples in the order B > M > V > LH > DH. In all the assays, the dry samples had less phenolic content than fresh samples and this may be because, when cells die, there is loss of cellular integrity. During senescence phenolic compounds come into contact with oxidising enzymes that they are not normally exposed to in living cells (Türkoğlu *et al.* 2007).

DPPH radical scavenging activity

Free radical scavenging activity of the fresh and dry nuts is shown in **Fig. 3A** and **3B**, respectively. Among dried nuts, free radical scavenging activity was in the order: variegated > maroon > brown > dark helium > light helium whilst

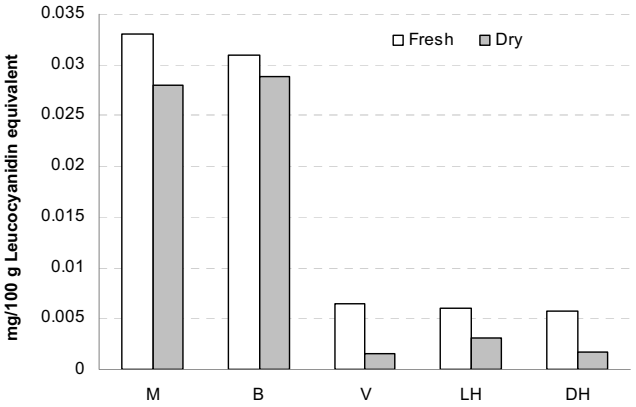

Fig. 4 Total content of tannins between fresh and dried bambara nuts. M - maroon, B - brown, V - variegated, LH - light helium, DH - dark helium, CE - catechin equivalent. The results are presented as mean ± standard deviation of three independent measurements.

among the fresh nuts free radical scavenging activity followed the order: brown > maroon > variegated > light helium > dark helium. Among the fresh varieties, brown had higher free radical scavenging activity than the variegated. In the dry samples, light helium and dark helium had almost similar radical scavenging activities, but in fresh samples, their activities were distinct, with light helium exhibiting more percentage decay of DPPH. Generally, fresh nuts exhibited more aggressive free radical scavenging activity than dry nuts. This is also shown by the high percentage of DPPH that decayed when exposed to fresh sample extract in a shorter period of time. Notably are the extremes of both the fresh and dry nuts. For the dry samples, the highest percentage of DPPH that was bleached was around 19% by brown and the lowest 4% by dark helium. As for the fresh nuts, the highest percentage of DPPH bleached was 36% by the brown nut and the least being 13% by dark helium nut. This shows that more DPPH was reduced by fresh bambara nut extracts as compared with dry ones. Polyphenol oxidase, an enzyme which catalyses the oxidation of phenolic compounds, is activated when cell integrity is disrupted and when contents of plastids and vacuole are mixed. Upon oxidation, phenolics are converted to quinones and then a polymerisation reaction would follow. *O*-quinones may also oxidise other phenolic compounds of lower redox potential such as anthocyanins, to colourless products (Friedman 1996). When oxidation occurs, the quality of phenolic compounds is lowered resulting in antioxidant activity and reducing power also being lowered (Makkar 1999).

Vanillin-HCl for fresh and dry samples

A comparison of tannin content between fresh and dry bambara nuts is shown in **Fig. 4**. From each pair of bars in the graph, the bar on the left represents fresh nuts while the one on the right is for the dry ones. Tannin content among fresh samples was in the order B > M > V > LH > DH. An almost similar trend was followed among dry samples however with the maroon nut exhibiting the highest tannin content followed by the brown one. In all cases of comparison, the fresh nuts had more tannins than the dry nuts. The tannin content between fresh and dry nuts recorded using the butanol HCl assay is shown in **Fig. 5**. Much lower tannin content was recorded from this assay as compared with the vanillin HCl method. However, the trend is almost the same, with fresher samples exhibiting higher tannin content than dry samples and maroon and brown nuts continuing to dominate in their phenolic content while the dark helium and light helium nuts show the least phenolic content.

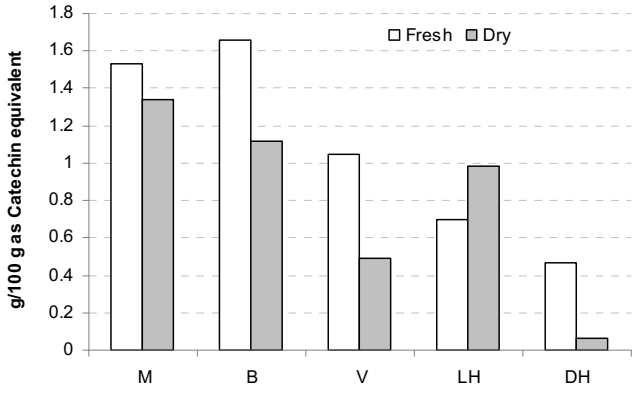

Fig. 5 Total tannin content between fresh and dried bambara nuts. M - maroon, B - brown, V - variegated, LH - light helium, DH - dark helium. The results are presented as mean ± standard deviation of three independent measurements.

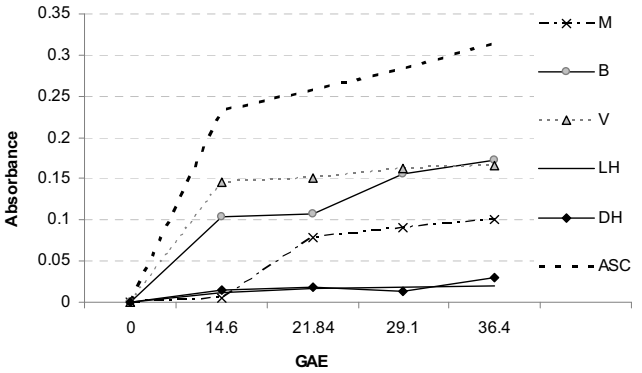

Fig. 6 Reducing power activity of dried samples. M - maroon, B - brown, V - variegated, LH - light helium, DH - dark helium, ASC - ascorbic acid, GAE - gallic acid equivalent. The results are presented as mean ± standard deviation of three independent measurements.

Reducing power for dry samples

The results show a concentration-dependent reducing power (**Fig. 6**). The more phenolic compound extracts were added, the more Fe(III) was reduced to Fe(II). This is shown by the rise in absorbance on increasing volume of sample extract. In this assay, when Fe(III) was reduced to Fe(II) a Prussian blue colour was formed. The blue colouring is the one that caused the increase in absorbance. The more reduction, the darker the blue colour and the higher the absorbance.

Phospholipid peroxidation for dry samples

There was a concentration-dependent increase in antioxidant activity of the plant extracts (**Fig. 7**). This is shown by the general decreasing trend in absorbance as the volume of plant extract was increased. As more plant extract was added, lesser MDA was formed, and so lesser of it could react with TBA to form a chromophore that would give a high absorbance. In this case, the brown nut had the most potent antioxidant activity, since it had the lowest absorbance. Weaker antioxidants are seen by the high absorbance values that they exhibited. Antioxidant potential was in the order M > B > V > LH > DH.

DISCUSSION

Phenolic content and antioxidant activity in bambara nuts varied from landrace to landrace. The maroon and brown coloured seeds had a higher phenolic content and antioxidant activity compared to the light pigmented varieties. The dried samples of maroon and brown seeds had a total phenolic content just above 300 mg/100 g while the light variegated, light and dark helium varieties had less than 200

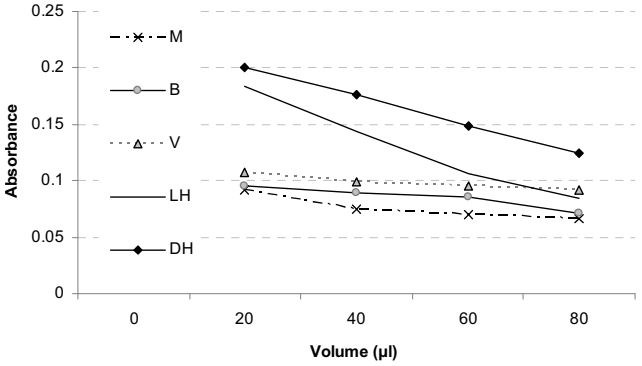

Fig. 7 Effect of increasing sample extract volume on ability to inhibit phospholipid peroxidation. M - maroon, B - brown, V - variegated, LH - light helium, DH - dark helium.

mg/100 g. Since all the assays were conducted on bambara nuts, one would have expected to have an almost equivalent total phenolic content for all the nuts, but this was not so. Instead, the nuts had varying phenolic contents and antioxidant activities. The varying characteristics amongst the bambara nut species may have been as a result of genetic polymorphism.

Phenolic compound content is influenced by varietal and genetic differences, maturity stage, climate and growing conditions (Dalis *et al*. 1997). Experimental evidence by Hoek and colleagues (2000) identified varietal and growing location effects on isoflavone content in six soybean cultivars grown at eight locations for two years. Furthermore, Fluery and Magnolato (1990) noted that growing location induces a greater difference in isoflavone content than varietal type. Since the bambara nuts under investigation were grown in different locations and conditions, this could be the reason why they exhibited such significant differences ($P < 0.05$) in phenolic content. The different colours exhibited by the nuts suggest differences in their genetic makeup. In immature soybean seeds harvested at 80% maturity, it was shown that, depending on the genotype, they may contain higher contents of isoflavones than mature samples. This suggests that the regulation of isoflavone synthesis may be influenced by genotype (Simmone *et al*. 2000).

A difference in phenolic composition and free radical scavenging activity was also noted between fresh and dry samples with the fresh nuts recording superior antioxidant activity and higher phenolic content. Excessive drying can lead to inactivation of phenolics and to some extent decrease their extractability in solvents. Lower phenolic content levels may be recorded as a result (Gordana *et al*. 2004). Lower extractability due to drying may be another reason why the phenolic content was lower in the dry nuts than the fresh ones. The dried light helium nuts had one of the lowest radical scavenging activities, almost equal to those of the dark helium nuts, yet they exhibited a much greater and distinct scavenging potential from the dark helium in their fresh form. Most of the phenolics in the dried light helium nut may have already been oxidised to low quantities comparable to those in the dark helium nut, thereby exhibiting an almost equivalent free radical scavenging activity. However in their fresh state, there was a clear and distinct difference in the radical scavenging potentials of the nuts, with the light helium nut exhibiting a higher free radical scavenging activity than the dark helium nut. The fresh samples exhibited a higher total phenolic content because most of the phenolics present in the fresh form had not yet been oxidised by processes that follow during senescence.

Total phenolic content in dry bambara nuts was found to be comparable with that of soybean flour. From the assays on dry nuts, total phenolic content ranged between 120 mg/100 g and 350 mg/100 g and literature evidence shows that soybean flour has about 455 mg/100 g (Nackz *et al*. 1986). The findings on soybean flower were however lower

than those in fresh bambara nuts with the highest fresh brown nut recording 647 mg/100 g. Therefore, these experimental findings indicate that bambara nuts are competitive sources of phenolics and antioxidants in as much as as other "popular" legumes like soybeans.

Despite the differences in phytochemical properties within the bambara nut landraces, these nuts are usually mixed up together and served in that state of mixture and so phenolic content and antioxidant activity would be an average of all the varieties present within that particular mixture. This could be one of the many reasons why the phenolic content recorded by other authors was lower than the ones we recorded. Some examples of the findings by other authors are highlighted below.

Oboh *et al*. (2009) extracted dry bambara nuts samples from Nigeria with 80% acetone and expressed total phenolic content as tannic acid equivalents with a record of 60 mg/100 g. This value was relatively lower than any of the yields we extracted, possibly in this case because of the differences in solvents used for extraction and environmental factors of growth surrounding each landrace. Oboh *et al*. also found that bambara nut (*Vigna subterranea*) and three other underutilised legumes pigeon pea (*Cajanus cajan*), African yam bean (*Sphenostylis stenocarpa*), and kidney bean (*Phaseolus vulgaris*) exhibited free radical scavenging activity, reducing power and inhibited peroxidation of the phospholipids *in vitro*. Mune *et al*. (2011) tested a bambara bean concentrate for total phenolic content extracted in 70% aqueous acetone and expressed as gallic acid equivalent and found out that the sample had about 232 mg/100 g. Ademilui and Oboh (2009) also recorded a total phenolic content expressed as gallic acid equivalents of 211 mg/100 g in dried bambara nuts after extracting with cold water. Malenčić *et al*. (2007) in Serbia studied several cultivars of soya beans. They extracted the phenolic compounds with 70% acetone and expressed total phenolic content as catechins equivalents. The highest total phenolic content from the soya beans they analysed was recorded in the Chinese cultivar (LN92-7369) 466 mg/100 g while the lowest was from the Tara cultivar 270 mg/100 g. Such experimental findings indicate that bambara nuts are competitive sources of phenolics and antioxidants as much as other "popular" legumes like soybeans.

The structure of phenolic compounds is a key determinant of radical scavenging potential (Nandita and Rajini 2003). A more hydroxylated phenolic compound has higher antioxidant activity. Synergy is also a key factor in antioxidant activity. When hydroxyl groups are exposed, the chances of interacting with radicals and quenching them are high, and so antioxidant activity will be high (Gordana *et al.* 2004). Reduced steric hindrance could be a possible explanation why the brown and maroon nuts exhibited stronger radical scavenging activity and reducing power. Another possible reason why the brown and maroon nuts were good antioxidants is that they had a high total phenolic content. However, antioxidant activity is not determined by quantity, but by quality of phenolic compounds. The phenolics may be abundant, but it is the hydroxyl groups available to quench radicals, that make them good antioxidants (Pena-Neira *et al*. 2000). Muchuweti *et al*. (2006) reported that different phenolic compounds show different colorimetric responses the Folin–Ciocalteau assay from the DPPH free radical scavenging assay because of the differences on the chemical structures of the compounds involved and the oxidation conditions. Thus the antioxidant activity of an extract cannot be predicted on the basis of its phenolic content. However, a contrasting result was published by Malenčić *et al*. (2007) who investigated phenolic content and antioxidant activity of soya beans and found that antioxidant activity increased proportionally to the phenolic content and that a linear relationship between DPPH-radical scavenging activity and total phenolics was established.

Higher levels of tannins were recorded in the vanillin-HCl assay compared to butanol-HCl. It is likely that the tannins in the vanillin-HCl assay are overestimated since a monomer; catechin was used as a standard. Norton (2000) reported that the standard (catechin) used in the vanillin HCl assay has no tannin properties, and so using it results in and overestimation of condensed tannins. The standard used in the vanillin-HCl assay also gives a less steep standard curve, which when used to evaluate the tannins would give a higher catechin equivalent. Moreover, some false positive results have been recorded using the vanillin-HCl assay. A red color developed in the presence of HC1 with or without vanillin would indicate the false positive result (Walton *et al.* 1983). Therefore, some of the red colour formed may not have been from the tannins. Since a more intense red colour gives a higher absorbance, it would give high tannin content.

However, comparing tannin content between bambara nuts and rapeseed using the vanillin-HCl method, rapeseed had almost twice as much more tannins (3 g/100 g) than bambara nuts.

In contrast to the vanillin HCl method, the butanol-HCl assay does not rely on the standard catechin but on the formation of cyanidin from the depolymerisation of the tannin molecule (Porter et al., 1986). Absorbance is linearly related to the number of monomers released from tannins irrespective of whether they are biologically active or not. Evaluation is directly based on how much tannin is hydrolysed by butanol-HCl to give monomers which would then produce a chromophore (Wina *et al*. 1999). The intensity of that chromophore gives absorbance which is then used to calculate tannin content expressed as leucocyanidin equivalent).

The phenolic compounds in bambara nuts were good inhibitors of phospholipid peroxidation. This was shown by the capability of the nuts to prevent peroxidation of lipids in rat brain *in-vitro*. A diet rich in phenolics from these nuts may therefore protect cell membranes form peroxidation by free radicals. Pivochit *et al*. (2010) reported a positive correlation between total phenolic content and anti-lipid peroxidation activity in several Thai medicinal plant extracts as measured by the TBARS test.

Bambara nuts were also found to be good sources of reducing agents as shown by their ability to reduce iron III to iron II in the reducing power assay. Brown and maroon nuts are the greatest sources of reducing power.

CONCLUSIONS

Bambara nuts contain considerable quantities of phenolic compounds. The phenolic compounds in the nut exhibit antioxidant activity. This is evidenced by their ability to scavenge free radicals, reduce iron III to iron II and inhibit phospholipid peroxidation. Fresh nuts have higher amounts of phenolic compounds and more potent antioxidant activity than dried nuts. Condensed tannins are also found in both the fresh and dry nuts.

ACKNOWLEDGEMENTS

The authors thank Dr. Jaime A. Teixeira da Silva for significant improvements to style and figures.

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