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Effect of different treatments on *in vitro* protein digestibility, antinutrients, antioxidant properties and mineral composition of *Amaranthus viridis* seed

Babatunde T. Olawoye^{1*} and Saka O. Gbadamosi¹

Abstract: In recent times, only the leaves of *Amaranthus viridis* are been utilized, the seeds are usually discarded. Processing this seed will enhance its utilization as food or functional food. *A. viridis* seed with protein, fat, ash and carbohydrate content of 11.06, 7.30, 2.94, and 65.01%, respectively was subjected to autoclaving, fermentation, blanching, defatting, and germination. Changes in *in vitro* protein digestibility, antinutrients, minerals and antioxidant properties were investigated. All the processing methods increase the *in vitro* protein digestibility of the amaranths seed. There was significant reduction in tannin (13–56.5%), oxalate (10–46%), saponin (15–41%). Fermentation and germination significantly ($p < 0.05$) increase antioxidant activity (DPPH) and metal chelating activity. In contrast, thermal processing (autoclaving and blanching) significantly reduce the total phenolic compound and FRAP of the amaranth seed. In addition, the minerals composition of the seed was greatly affected by the processing methods. The study showed that germination provided a new approach to further develop *A. viridis* seed as a functional food for human consumption.

Subjects: Food Science & Technology; Food Additives & Ingredients; Food Chemistry

Keywords: *Amaranthus viridis*; processing methods; intro protein digestibility; antinutrients; antioxidant

1. Introduction

Amaranthus spp. is a fast-growing herb mainly cultivated in Asia, Africa, Latin America (Amin, Norazaidah, & Hainida, 2006). At the present time, 60 species of *Amaranthus* are documented with *Amaranthus viridis* popularly known as “tete” in Yoruba being the most widely cultivated vegetable

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PUBLIC INTEREST STATEMENT

Plants from the Amaranth genus are attracting researchers' attention mainly because of their high nutritional value and nutraceutical characteristics. *Amaranthus viridis* seed have remarkable nutritional composition, not only from its protein content (13–14%), but also from its high amino acid balance. However, for this seed to be fully utilized as food or functional foods, the nutritional as well anti-nutritional properties of the seed need to be examine. Researchand development into underutilized pseudo-cereal crop like *A. viridis* with good macro and micronutrient profile, can solve malnutrition problems particularly in lowland middle-income countries.

in Nigeria. Being resistant to drought, hot climate and pests, and with little requirements for its cultivation, this pseudocereal has attracted much attention as an important food commodity (Saxena, Venkaiah, Anitha, Venu, & Raghunath, 2007). Recently, interests have developed on Amaranth because its leaves offer high nutritional quality when utilized as a vegetable and its grains possess high and quality protein content when compared to cereals plants. In the last decade, the use of amaranth has expanded not only in the common diet but also in the diet of people with celiac disease or allergies to typical cereals (Berti, Riso, Brusamolino, & Porrini, 2005; Olawoye & Kadiri, 2016). The risk imposed by the consumption of free radicals and oxidation products could be lowered by the intake of dietary phenolics against various forms of cancer and cardiovascular disease (Sharma, Gujral, & Singh, 2012). Interest in search for new natural antioxidants has grown over the past few years because of their preventive role in protecting from oxidative stress related chronic diseases (Halvorsen et al., 2002). *A. viridis* has a high nutrient content but bioavailability is low, inherently due to the presence of antinutritional factors, such as phytic acid, oxalate, polyphenols, and tannins. Germination, fermentation and heat processing (autoclaving and blanching) are one of those processes known to reduce these antinutrients and also improve minerals and antioxidant properties. The aim of this present study was to investigate changes occurring in minerals composition, antioxidant properties, antinutritional factors and the *in vitro* protein digestibility during germination, fermentation and heat processing of *A. viridis* seed.

2. Materials and methods

2.1. Materials procurement

A. viridis grains were obtained from Ondo town central market, Ondo state, Nigeria. The species of the grain was confirmed at the herbarium of the Department of Botany, Obafemi Awolowo University Ile-Ife, Nigeria. The chemicals used for analyses were of analytical grade and purchased from Sigma Chemical Company, St. Louis, MO, USA. The chemical analyses of the amaranth seed flour samples were carried out in the Food Science and technology laboratory, Obafemi Awolowo University Ile-Ife, Nigeria. The samples were divided into six batches of 1 kg each. These constituted samples for germination, fermentation, defatting, autoclaving and blanching while one batch that was untreated constituted the control.

2.2. Raw amaranth flour

One kilogram of Amaranth grains was weighed sorted to remove dirt and stones. The fermented sample was then oven dried at 50°C for 8 h and the grains were milled using Marlex Excella grinder (OB 308, United Kingdom) and passed through 200 µm sieve. It was stored in air tight container and put in refrigeration at 4°C for analyses.

2.3. Fermentation

The method of Igbabul, Idikwu, and Inyang (2012) was employed. Amaranth grains were rinsed and soaked for 24 h with warm (45°C) water. The warm water was changed every 6 h interval for 24 h. The Soaked seeds were then transferred into different calabash pots, lined uniformly with banana leaves (up to 5 layers) and allowed to ferment inside the incubator (30°C) for 96 h. The fermented sample was then oven dried at 50°C for 8 h to terminate the fermentation process and was milled using Marlex Excella grinder (OB 308, United Kingdom). The finely ground flour obtained was stored in an air-tight plastic container and put in a refrigeration at 4°C until the time of use.

2.4. Germination

A. viridis seeds were germinated according to the method of Elkhalfa and Bernhardt (2010). Briefly, 1,000 g of *A. viridis* seed was steeped in distilled water overnight with two changes of water during the day to remove dirt and husk. The wet grain was subsequently soaked in 1–2 volumes of 0.2% formaldehyde solution for 30 min to prevent the growth of mold during germination. The soaked grains were then washed with distilled water several times and subsequently soaked in water for 20 min to remove residual formaldehyde. The wet *A. viridis* grains were thinly spread on jute bag and placed inside locally fabricated germination chamber for 72 h at 28 ± 2°C. The amaranth grains were

then dried in Gallenkamp oven (OVB 305, United Kingdom) at 50°C for 8 h. After that the rootlets of the grains were removed manually, milled into fine flour and passed through 200 µm sieve. It was stored in air tight container and put in refrigeration at 4°C until used for further analyses.

2.5. Autoclaving

The whole *A. viridis* seeds were heat treated (autoclaving at 121°C for 15 min), then dried in hot air oven at 50°C for 8 h. The samples were milled using Marlex Excella grinder and passed through 200 µm sieve. It was stored in air tight container and put in refrigeration at 4°C until used for further analyses.

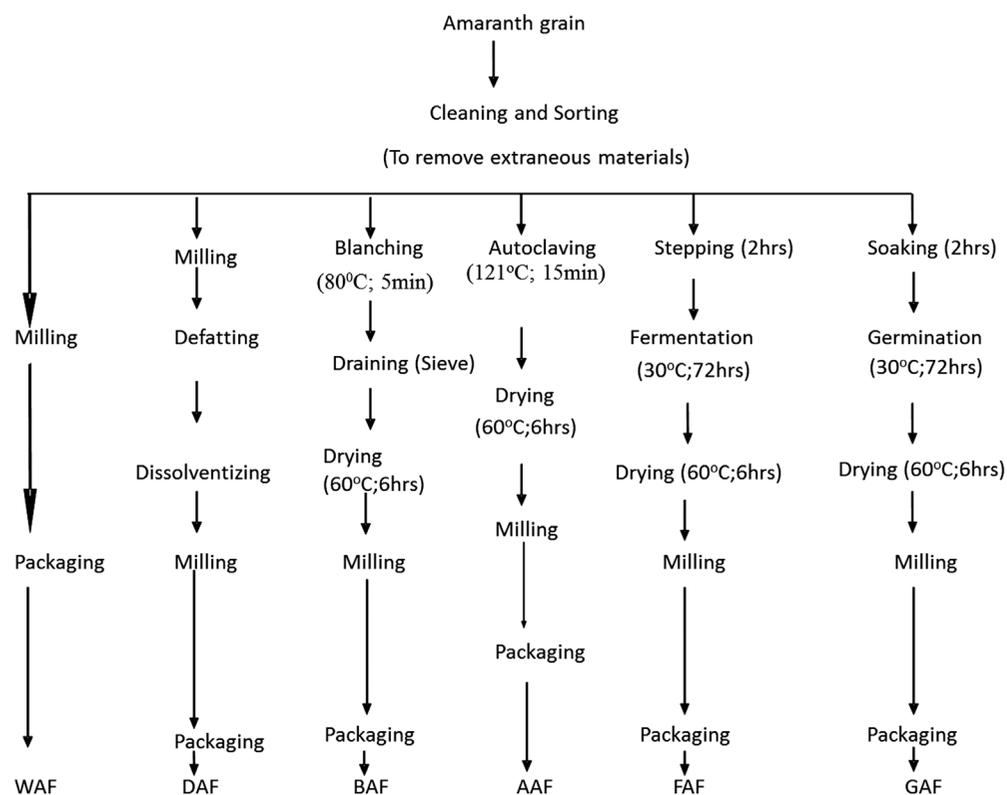
2.6. Blanching

The Amaranth grains were blanched in hot water at 75°C for five minutes. The blanched grains were drained using muslin cloth and dried using a hot air oven at 50°C for 8 h. The dried grains were milled using Marlex Excella grinder, stored in airtight container and put in refrigeration at 4°C before the time of use.

2.7. Defatting

The method described Fasasi, Eleyinmi, and Oyarekua (2007) was used. The whole *A. viridis* seed flour was defatted using cold (4°C) extraction method with acetone as the solvent (flour to solvent ratio 1:5 w/v) stirred over a magnetic stirrer for 4 h. The slurry was then filtered through a Whatman No. 1 filter paper and re-extracted twice in a similar fashion. The defatted flour was desolventized by drying in Gallenkamp oven (OVB 305, United Kingdom) at 40°C for 8 h and the dried flours was finely ground in a Marlex Excella grinder set at high speed to obtain homogenous defatted flours. The defatted flours obtained was stored in airtight container and put in refrigeration at 4°C before the time of use. The flow chart for the production of fermented, germinated, blanched, cooked and whole Amaranth grain flour is shown in Figure 1.

Figure 1. Flow chart for the production of *A. viridis* seed flour.



2.8. In vitro protein digestibility

In vitro protein digestibility of samples was measured according to the method described by Chavan, Shahidi, and Naczki (2001). Two hundred and fifty milligrams of the sample were suspended in 15 ml of 0.1 M HCl containing 1.5 mg pepsin, followed by gentle shaking for 1 h at room temperature. The resultant suspension was neutralized using pH meter with 0.5 M NaOH and treated with 7.5 ml of phosphate buffer (0.2 M, pH 8.0) containing 4.0 mg pancreatin. The mixture was shaken with a shaker for 24 h at room temperature. The mixture was then filtered using Whatman No 1 filter paper and the residue was washed with distilled water, air-dried and used for protein determination using Kjeldahl procedure (Association of Analytical Chemists International [AOAC], 2005).

Protein digestibility was obtained by using the following equation:

$$\text{Protein digestibility} = \frac{(I - F)}{I} \times 100 \quad (1)$$

where I is protein content of sample before digestion and F is protein content of sample after digestion.

2.9. Tannin content

Qualitative estimation of tannin was carried out using the modified vanillin-HCl method according to Price, Van Scoyoc, and Butler (1978). Amaranth seed flour was extracted separately with 10 ml of 1.0% (v/v) HCl-MeOH. The extraction time was 1 h with continuous shaking. The mixture was filtered and made up to 10 ml mark with an extracting solvent. Filtrate (1.0 ml) was reacted with 5.0 ml vanillin-HCl reagent and another with 5.0 ml of 4% (v/v) HCl-MeOH solution to serve as blank. The mixture was left to stand for 20 min before the absorbance was taken at 500 nm. Catechin was used as a standard.

$$\text{Tannin(mg/g)} = \frac{x(\text{mg/ml}) \times 10 \text{ ml}}{0.2 \text{ (g)}} = 50 \times (\text{mg/g})$$

where x = value obtained from standard catechin graph.

2.10. Oxalate

Oxalate was determined using the titrimetric method described by Day and Underwood (1986). One gram of the sample was weighed into 100 ml conical flask. Seventy five milliliters of 1.5 M H_2SO_4 was added and the solution was carefully stirred intermittently with a magnetic stirrer for about 1 h and then filtered using Whatman No 1 filter paper. About 25 ml of sample extract (filtrate) was collected and titrated hot (80–90°C) against 0.05 M KMnO_4 solution to the point when a faint pink colour appeared that persisted for at least 30 s.

The oxalate was calculated as the sodium oxalate equivalent.

One milliliters of 0.05 M KMnO_4 = 2 mg sodium oxalate equivalent/g of sample.

2.11. Saponin

The spectrophotometric method of Brunner (1984) was used for saponin analysis. 1 g of the finely ground sample was weighed into a 250 ml beaker and 100 ml of isobutyl alcohol was added. The mixture was shaken for 2 h to ensure uniform mixing. Thereafter the mixture was filtered through a Whatman No. 1 filter paper into a 100 ml beaker, 20 ml of 40% saturated solution of magnesium carbonate was added and the mixture made up to 250 ml in a 250 ml standard flask. The mixture obtained with saturated MgCO_3 was again filtered through a whatman No. 1 filter paper to obtain a clear colourless solution. One milliliters of the colourless solution was pipette into a 50 ml volumetric flask and 2 ml of 5% FeCl_3 solution was added and made up to mark with distilled water. It was allowed to stand for 30 min for blood red colour to develop. 0–10 ppm standard saponin was prepared from saponin stock solution. The standard solutions were treated similarly with 2 ml of 5%

FeCl₃. The absorbance of the sample, as well as standard saponin solution, was read after colour development on a spectrophotometer at a wavelength of 380 nm.

$$\text{Saponin} = \frac{\text{Absorbance of sample} \times \text{dil. factor} \times \text{gradient of standard graph}}{\text{sample weight} \times 10,000} \text{ (mg/g)}$$

2.12. Total phenolic content

The total phenolic content (TPC) was determined by following the Folin–Ciocalteu spectrophotometric method described by Makkar, Francis, Kerem, and Becker (2007). Aliquot of extract (1,000 μl) was transferred to a test tube and 1.5 ml freshly diluted (10-fold) Folin–Ciocalteu reagent was added. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml of sodium carbonate solution (20% w/v). After incubation in dark at room temperature (25°C) for 90 min, the absorbance of the mixture was read at 750 nm with UV–visible spectrophotometer (INESA 752 N, England). Methanol was used as a blank. The results were expressed as mg of gallic acid equivalents (GAE)/g of flour.

2.13. Antioxidant activity using DPPH

Antioxidant activity was measured using a modified version of the method explained by Brand-Williams, Cuvelier, and Berset (1995). This involved the use of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in the methanol. Ground amaranth seed samples (250 mg) were extracted with 10 ml methanol for 4 h using a magnetic stirrer (Cenco, Netherland) and centrifuged at 3,000 rpm for 10 min. The different concentrations of methanolic extracts (200, 400, 600, 800 and 1,000 μg/ml of the flour) were reacted with 3.9 ml of 0.3 mM DPPH in methanol. The mixture was mixed and incubated in the dark for 30 min after which the absorbance was read at 517 nm against a DPPH control containing only 1 ml methanol in place of the extract.

The percent of inhibition was calculated from the following equation:

$$\text{Inhibition (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \quad (2)$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound, and A_{sample} is the absorbance of the test compound. IC₅₀ values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radical formation (percentage inhibition DPPH was assayed). Synthetic antioxidant L-ascorbic acid was used as a positive control and all tests were carried out in triplicates.

2.14. Metal chelating (Fe²⁺) activity

The chelating of ferrous ions by seed methanolic extracts of *A. viridis* was estimated by the method of Singh and Rajini (2004). The different concentrations of methanolic extracts (200, 400, 600, 800 and 1,000 μg/ml of the flour) were mixed with 100 μl of 2 mM ferrous sulphate solution and 300 μl of 5 mM ferrozine. The mixture was incubated at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. Ethylene diamine tetra acetate (EDTA) was used as a standard. All the tests were performed in triplicate and percentage of inhibition was calculated by using this formula:

$$\text{Percentage of inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.15. Reducing power activity

Reducing power assay was determined according to the method of Yildirim, Mavi, and Kara (2001). A 300 mmol/L acetate buffer of pH 3.6, 10 mmol/L 2,4,6-tri-(2-pyridyl)-1,3,5-triazine and 20 mmol/L FeCl₃·6H₂O were mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent.

A 50 µl aliquot of the extract at concentration (0.0, 0.2, 0.4, 0.0.6, 0.8 and 1 mg/ml) and 50 µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100 µg/ml) was added to 1 ml of FRAP reagent. Absorbance measurement was taken at 593 nm exactly 10 min after mixing against reagent blank containing 50 µl of distilled water and 1 ml of FRAP reagent. All the tests were performed in triplicates and ascorbic acid was used as reference standard.

2.16. Minerals

The analyses for mineral elements were carried out by the atomic absorption spectrophotometric method described by Fashakin, Ilori, and Olarenwaju (1991). The sample (0.5 g) was weighed into 75 ml digestion flask and 20 ml digestion mixture (10 ml HNO₃ and 10 ml HCl) was added and was digested for 15 min in a hood. After 15 min, the digested sample was cooled at room temperature and subsequently filtered and the filtrate was made up to the mark in 50 ml standard flask with distilled water. Calcium, magnesium, and iron were determined using Atomic Absorption Spectrophotometer (Perkin-Elmer, model 402) while sodium and potassium were determined by flame photometer.

2.17. Statistical analysis

Each sample analysis was performed in triplicate. Experimental results recorded were means ± standard deviation (SD). The results were analysed by One-way analysis of variance and the least significant differences were calculated by Duncan multiple range test using IBM SPSS version 23 software (IBM Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$ levels.

3. Results and discussion

The results of *in vitro* protein digestibility are presented in Table 1. The result revealed that germination, fermentation and the heating process of autoclaving and blanching had a significant effect on the *in vitro* digestibility of the processed amaranth flour. Germination having the most profound effect on the protein digestibility. Protein digestibility of legumes and cereals had been reported to increase as a result of germination and fermentation (Chavan, Chavan, & Kadam, 1988; Taylor & Taylor, 2002). Taylor and Taylor (2002) proposed that, during fermentation, insoluble proteins (prolamine and glutelin) undergo structural changes which enable them to be more accessible to pepsin attack, rather than being broken down into smaller sub-units. These changes are likely to have a profound effect on the digestibility of the seed protein and could be partly responsible for the increased protein digestibility observed in this study in fermented amaranth seed. This also agrees with Mohiedeen, Tinay, Elkhalya, Babiker, and Mallasiy (2010) who reported that fermentation was found to improve the IVPD of two maize cultivars and this was attributed to the partial degradation of complex storage proteins into more simple and soluble products.

Table 1. *In vitro* protein digestibility and antinutritional properties of the processed *A. viridis* seed flour

Sample	<i>In vitro</i> protein digestibility (%)	Tannin (mg/100 g)	Oxalate (mg/100 g)	Saponin (mg/100 g)
AAF	57.65 ± 0.91 ^{ab}	1.504 ± 0.32 ^c	3.727 ± 1.23 ^c	2.949 ± 0.42 ^c
GAF	65.21 ± 0.50 ^a	2.260 ± 0.73 ^b	5.109 ± 1.05 ^b	3.872 ± 0.37 ^b
FAF	63.09 ± 0.52 ^a	2.550 ± 1.03 ^b	5.427 ± 0.62 ^b	3.669 ± 0.71 ^b
BAF	53.70 ± 0.06 ^b	1.930 ± 0.23 ^{bc}	3.904 ± 0.32 ^c	3.401 ± 0.06 ^{bc}
WAF	35.84 ± 0.60 ^d	3.459 ± 0.09 ^a	6.841 ± 1.34 ^a	4.962 ± 0.54 ^a
DAF	42.48 ± 0.97 ^c	3.008 ± 0.87 ^a	6.135 ± 0.21 ^a	4.231 ± 0.82 ^{ab}

Notes: AAF: autoclaved amaranth flour; GAF: germinated amaranth flour; FAF: fermented amaranth seed; BAF: blanched amaranth flour; WAF: whole amaranth flour; DAF: defatted amaranth flour.

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within the same column are significantly ($p < 0.05$) different.

3.1. Tannin content

The tannin content of the amaranth seed flour expressed as catechin equivalents (CE), is shown in Table 1. The tannin content was found to varied between 1.50 and 3.46 mgCE/100 g with autoclaving having the most significant ($p < 0.05$) on the tannin content. The reduction in tannin content of the autoclaved amaranth flour could be attributed to high temperature in which the seed was subjected to during the autoclaving process. This finding conformed to the report of Khattab and Arntfield (2009) who reported highest reduction of tannins content with boiling followed by autoclaving and microwave cooking in some legumes such as cowpea, pea, and kidney bean. The reduction of tannins after blanching and autoclaving is mainly due to the fact that those compounds, in addition to their predominance in seed coats are water-soluble and consequently leach into the liquid medium (Kumar, Sinha, Makkar, & Becker, 2010; Reddy & Pierson, 1994). This decrease could also be related to the fact that these compounds are heat labile and degrade upon heat treatment. These results are also in line with those of Rehman, Israr, Srivastava, Bansal, and Abdin (2003) who stated that tannin content of quinoa, buckwheat, a similar pseudo-cereal significantly reduced after ordinary cooking and pressure cooking at 121°C for 20 min, respectively. Tannin is known to have a dietary effect. The mechanism of dietary effects may be understood by their ability to form complex with dietary proteins and may bind and inhibit the endogenous protein, such as digestive enzyme (e.g. amylase, trypsin, chymotrypsin, and lipase) (Liener, 1995). Tannins have been reported to affect nutritive value of food products by chelating metals such as iron and zinc and reduce the absorption of these nutrients and also forming complex with protein thereby inhibiting their digestion and absorption (Oboh, 2006).

3.2. Oxalate

The result in Table 1. also show the oxalate content of the processed amaranth seed flour which ranged from 3.73 to 6.81 mg/100 g. There was a significant difference ($p < 0.05$) in the oxalate value of the processed amaranth seed flour. Whole amaranth flour (WAF) had the highest oxalate content (6.81 mg/100 g). This value was significantly reduced when the seed was subjected to processing operation, an evidence which was seen in AAF, GAF, FAF and BAF where the oxalate values were reduced by 45.45, 25.29, 20.76 and 42.98% respectively. The values obtained in this work are lower than 0.585 mg/g reported for wheat flour (Ijarotimi, 2006). It's well known that oxalic acid and its salts can have a deleterious effect on human nutrition and health, particularly by decreasing calcium absorption and aiding the formation of kidney stones (Noonan & Savage, 1999).

3.3. Saponin

The saponin content of the processed amaranth flour ranged from 2.94 to 4.962 mg/100 g with a significant difference between the highest value and the lowest value. However, there is no significant difference amongst ($p < 0.05$) GAF, FAF, and DAF. The range of values of saponin observed in this study, however, fall below the value reported for quinoa (9.2 mg/g) by Belitz, Grosch, and Schieberle (2004). Saponins are glycoside containing a polycyclic aglyconic moiety. They are found naturally in plants especially in dessert plants (Miller, Youngs, & Oplinger, 1980). They have the characteristics bitter taste, foaming properties and can cause injuries to the digestive mucosa and haemocytic changes in blood.

3.4. Total phenolic compound

Changes in the total phenolic compound of amaranth seed as affected by different treatments are presented in Table 2. Autoclaving and blanching were found to decrease the total phenolic compound of the amaranth seed. The decrease observed on the total phenolic compound may be due to some degradation of phenolic compound by thermal processing. The data reported by Zhou and Yu (2004) had shown that the contents of TPC in cereal grains were affected by the extraction solvents in the following descending order: acetone > ethanol > methanol, which can be used to explain lower amounts of phenols present in this amaranth (Table 1). According to Zielinski and Troszynska (2000), extraction duration and extraction method greatly affect the TPC in rye, so it is very difficult to compare the results in our work with those reported by others. Generally, it is difficult to compare our data with other literature data due to different methods used for extraction and antioxidant activity determination, and different

Table 2. Antioxidant properties of methanolic extract of *A. viridis* seed flour

Sample	DPPH IC ₅₀ (mg/ml)	MC 1C ₅₀ (mg/ml)	FRAP (AAE µg/g)	TPC (GAE µg/g)
AAF	1.69 ± 0.78 ^a	1.67 ± 0.15 ^a	0.946 ± 1.32 ^d	0.259 ± 0.98 ^d
GAF	1.57 ± 0.26 ^b	1.64 ± 0.63 ^c	1.639 ± 0.90 ^a	0.732 ± 0.25 ^a
FAF	1.56 ± 0.68 ^b	1.56 ± 0.72 ^c	1.533 ± 03.36 ^{ab}	0.533 ± 0.34 ^b
BAF	1.71 ± 0.48 ^a	1.81 ± 0.91 ^b	1.247 ± 0.74 ^c	0.450 ± 0.15 ^c
WAF	1.843 ± 0.72 ^a	2.11 ± 0.64 ^b	1.458 ± 0.83 ^b	0.450 ± 0.94 ^c
DAF	1.790 ± 0.83 ^b	1.83 ± 0.85 ^{bc}	1.729 ± 0.94 ^a	0.560 ± 0.97 ^b
Ascorbic acid	1.16 ± 0.90 ^c			
ETDA		1.24 ± 0.81 ^d		

Notes: AAF: autoclaved amaranth flour; GAF: germinated amaranth flour; FAF: fermented amaranth seed; BAF: blanched amaranth flour; WAF: whole amaranth flour; DAF: defatted amaranth flour.

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within the same column are significantly ($p < 0.05$) different.

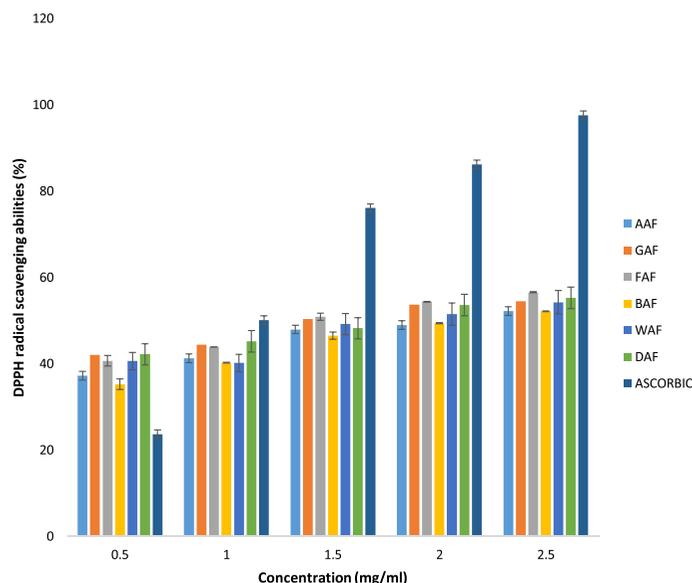
interpretation of data applied by other authors. Germination affects the total phenolic compound of the amaranth seed by increasing from 4.50 mg GAE/g in raw amaranth flour to 7.3 mg GAE/g in germinated amaranth seed. The higher TPC of germinated amaranth flours compared to non-germinated amaranth flours could be due to the biosynthesis of phenolic compounds caused by enzyme hydrolysis during germination (He, Han, Yao, Shen, & Yang, 2011). This increase could also be attributed to the production of β-glucosidase during germination as reported by Reyes-Bastidas et al. (2010). These researchers suggest that β-glucosidase, produced by fungi, catalyse the release of aglycones from the bean substrate and thereby increases their TPC. Phenolic compounds are most significant contributors to the antioxidant capacity of cereal grains and play an important role in the prevention and control of degenerative diseases (Zielinski, Kozłowska, & Lewczuk, 2001).

3.5. Antioxidant activity using DPPH

DPPH is a stable free-radical compound widely used to test the free-radical scavenging ability of various samples (Sakanaka, Tachibana, & Okada, 2005). It is a stable organic free radical with an adsorption peak at 517 nm. Adsorption disappears when accepting an electron or a free radical species, which results in a noticeable discoloration from purple to yellow (Liu, Du, Zeng, Chen, & Niu, 2009). The scavenging effect of DPPH radicals assay showed concentration-dependent activity, as shown in Figure 2. Ascorbic acid showed the highest DPPH radical scavenging activity, while the scavenging activity of processed *A. viridis* seed flour samples were significantly lower compared to that of ascorbic acid. The scavenging effect of plant extracts using the highest sample concentration (2.5 mg/ml), as shown in Figure 2, was weak for WAF, with only 34% DPPH radical inhibition. Stronger scavenging effects on the DPPH radical were found for autoclaving, germination, fermentation and blanching (62, 64, 66 and 62%, respectively). The increased in DPPH radical inhibition exhibited by the thermal processing (autoclaving and blanching) may be due to the extractability of other bioactive antioxidants or it caused the formation of compounds such as the Maillard reaction products with strong antioxidant activity (Guibing, Tao, & Fuli, 2014). Fermentation had a positive effect on the DPPH inhibitory effect in the amaranth seed. This could be attributed to the synthesis of phenolic acid such as vanillic acid (hydroxybenzoic acid derivative) and ferulic acid (hydroxycinnamic acid derivative) during the fermentation process as reported by Silva et al. (2014). This phenolic acid had been reported to be efficient antioxidant in scavenging radicals due to a longer distance of carboxyl group to phenyl ring, which has a positive influence on their H-donating ability (Chaovanalikit et al., 2012; Kadiri & Olawoye, 2016). As shown in Table 2, DPPH radical scavenging activity was also expressed in terms of IC₅₀ which is the concentration of antioxidant compound required to reduce the initial DPPH* concentration by 50% at steady-state. Lower IC₅₀ value indicates higher radical scavenging activity of a sample. Furthermore, the high antioxidant activity could be due to the increased in hydroxyl groups or amino groups in antioxidant compounds found particularly in the FAF extract.

Figure 2. DPPH radical scavenging activities of processed *Amaranthus virides* seed flour at different concentrations.

Notes: AAF: autoclaved amaranth flour; GAF: germinated amaranth flour; FAF: fermented amaranth seed; BAF: blanched amaranth flour; WAF: whole amaranth flour; DAF: defatted amaranth flour.

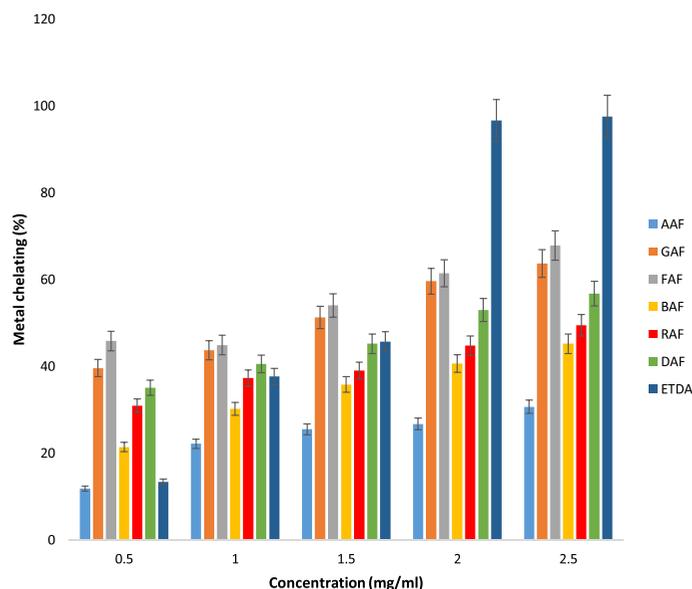


3.6. Metal chelating (Fe^{2+}) activity

Free iron is a potential enhancer of reactive oxygen species (ROS) formation as it leads to reduction of H_2O_2 and generation of the highly aggressive hydroxyl radical (Singh & Rajini, 2004). Metal chelating activity (MC) is the most commonly used method for the evaluation of antioxidant activities. Figure 3 shows the metal chelating ability of the processed *A. virides* seed flour in the concentration range of 0.5–2.5 mg/ml. The metal chelating activity of amaranth seed as affected by processing treatment varied significantly ($p < 0.05$) and ranged from 49.46 to 67.83%. Randhir, Kwon, and Shetty (2008) reported that the increase in metal chelation after thermal processing may be attributed to alteration of phenolic structure and/or degradation of phenolic compounds to different Maillard reaction products like melanoid which could also act as antioxidants. The high metal chelating ability of fermented amaranth flour could be attributed to the synthesis of β -glucosidase produced by fungi which catalyse the release of aglycones from the amaranth substrate thereby increases its metal chelating ability (Reyes-Bastidas et al., 2010). In Table 2, the metal chelating (MC) ability of the processed seed flour extracts revealed antioxidant potency based on the IC_{50} values when compared with EDTA. A low IC_{50} value indicates a higher metal chelating activity. The metal

Figure 3. Metal chelating ability of processed *Amaranthus virides* seed flour at different concentrations.

Notes: AAF: autoclaved amaranth flour; GAF: germinated amaranth flour; FAF: fermented amaranth seed; BAF: blanched amaranth flour; WAF: whole amaranth flour; DAF: defatted amaranth flour.



chelating activities of FAF, GAF and AAF (IC_{50} value 1.56, 1.64 and 1.67 mg extract/ml, respectively) were higher than DAF, WAF, and BAF (1.84, 2.12, and 1.82 mg extract/ml, respectively) but both were lower when compared to that of EDTA (1.25 mg extract/ml). The result revealed that samples FAF, AAF and GAF had better chelating properties than samples DAF, WAF, and BAF.

3.7. Reducing power activity

The FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyl triazine (Fe^{3+} -TPTZ) complex to produce a coloured ferrous tripyridyl triazine (Benzie & Strain, 1996). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free-radical chain by donating a hydrogen atom. The reduction of the Fe^{3+} -TPTZ complex to a blue-coloured Fe^{2+} -TPTZ occurs at low pH (Benzie & Strain, 1996). The fermentation of the amaranth seed significantly ($p < 0.05$) increased the reducing power. Data also revealed that increasing the concentration of extract led to a significant increase in reducing power in amaranth sample. The increase in reducing power of fermented sample may be attributed the synthesis or enzymatic transformation of various bioactive compounds may occur during fermentation processes (Katina et al., 2007; Priefert, Rabenhorst, & Steinbüchel, 2001).

3.8. Correlation of the antioxidants

From the above analysis, Table 3 showed the correlation matrix of the antioxidant properties. From the result, DPPH and Metal chelating activity had the highest correlation (0.9365). A similar correlation was found in sorghum (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003). TPC and ferric reducing antioxidant power (FRAP) were highly correlated ($r = 0.8673$). The close relationship between phenolic content and reducing power activity further demonstrated that phenolic accumulation was an important process in the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). There was negative correlation between TPC and DPPH. This negative correlation indicates that compounds other than phenolics donate hydroxyl groups for reducing the number of DPPH the antioxidant scavenging activity as some phenolic compounds might not have the ability to scavenge free radicals.

3.9. Minerals

The results of mineral composition of the processed *A. viridis* seed flour are presented in Table 4. Among the minerals present in the processed seed flour, sodium, potassium, and calcium were

Table 3. Pearson's correlation between DPPH radical scavenging activity, MC, FRAP, and total phenolic compound for autoclaved, germinated, fermented, blanched and defatted *A. viridis* seed

	DPPH	MC	FRAP	TPC
DPPH	1	0.9365	0.2012	-0.1376
MC		1	0.0594	-0.1990
FRAP			1	0.8672
TPC				1

Table 4. Minerals composition (mg/100 g) of the processed *A. viridis* seed flour

Sample	Mg	Na	K	Ca	Fe
AAF	88 ± 0.05 ^b	128 ± 0.13 ^d	201 ± 0.04 ^{ab}	195 ± 0.14 ^b	4.01 ± 0.14 ^b
GAF	100 ± 0.02 ^a	252 ± 0.04 ^a	183 ± 0.02 ^b	203 ± 0.22 ^a	5.09 ± 0.50 ^a
FAF	95 ± 0.02 ^a	199 ± 0.11 ^b	196 ± 0.06 ^b	157 ± 0.23 ^d	5.21 ± 0.12 ^a
BAF	102 ± 0.06 ^a	155 ± 0.06 ^c	229 ± 0.03 ^a	226 ± 0.19 ^a	7.13 ± 0.17 ^a
WAF	75 ± 0.08 ^c	119 ± 0.11 ^d	204 ± 0.13 ^{ab}	171 ± 0.36 ^c	3.98 ± 0.14 ^b
DAF	60 ± 0.02 ^b	102 ± 0.05 ^a	103 ± 0.20 ^c	103 ± 0.32	3.03 ± 0.02 ^c

Notes: AAF: autoclaved amaranth flour; GAF: germinated amaranth flour; FAF: fermented amaranth seed; BAF: blanched amaranth flour; WAF: whole amaranth flour; DAF: defatted amaranth flour.

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within the same column are significantly ($p < 0.05$) different.

shown to be present in abundance as compared to magnesium which is moderately present in the processed flour. Iron is present in the flour in moderately low amount. Of all the minerals present, sodium was the highest and GAF had the highest value. The value is significantly different ($p < 0.05$). The high sodium content of GAF can be attributed to the reduction of antinutrient such as phytate and tannin which binds with this minerals during the germination process. The calcium content of the processed seed ranged from 103 to 226 mg/100 g with blanched amaranth flour having the highest value. However, there is no significant difference ($p < 0.05$) between the calcium of blanched amaranth flour (BAF) and germinated amaranth flour. It had been reported by (Obizoba & Atii, 1994) that heat treatment (cooking, blanching, and roasting) remove a substantial amount of oxalate which is known to bind with calcium and render the mineral not available for body utilization. Also, increase in calcium content of sorghum-millet complementary food was reported by Obizoba and Atii (1994) who noted that during sprouting the activities of hydrolytic enzymes may lead to the release of more free calcium from its organic complexes. Similar observations were made by van der Riet, Wight, Cillicrs, and Datel (1987) in germinated legumes seeds. Iron is the lowest detectable minerals in the processed seed flour as it ranged from 3.03 to 7.13 mg/100 g. Heat treatment is effective in reducing the levels of antinutrients, thereby improving the bioavailability of minerals such as iron, zinc, and calcium known to be affected by these antinutrients. The detected minerals are nutritionally important because they help in maintaining the body catabolic and anabolic process. Sodium, iron, and calcium also play important role in controlling hypertension, preventing anemia, and impeding the development of osteoporosis, respectively (Choudhary & Bandyopadhyay, 1999).

4. Conclusion

The result in this study depicted that the increase or decrease in *in vitro* protein digestibility, antinutrients, minerals and antioxidant activity will depend upon the processing methods adopted to the samples. Germination, fermentation and autoclaving significantly affect the protein digestibility, antioxidant activity, and minerals of the amaranth seed. The use of germination and fermentation as a separate process can enhance the levels of many bioactive compounds in cereals and can be used to improve product properties by changing the ratio of nutritive and antinutritive components of plants. The exploitation of these amaranth seeds would help us to overcome the present scarcity of conventional cereals in the market and also it protects against free radical mediated degenerative diseases. Future studies will be conducted on identification of bioactive constituents, molecular mechanisms involved in antioxidant activity, determination of their efficacy by *in vivo* studies and demonstration of their safety and effectiveness in clinical trials.

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