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Research Article

Microwave Pretreatment of Presoaked Bambara Groundnut Seeds Enhances the Functionality and Phenolics-Related Antioxidant Properties of the Resultant Flour

Anton Venter, Mohammad Naushad Emmambux, and Kwaku Gyebi Duodu

University of Pretoria, Department of Consumer and Food Sciences, Private Bag X20, Hatfield 0028, South Africa

Correspondence should be addressed to Kwaku Gyebi Duodu; gyebi.duodu@up.ac.za

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This study investigated the effect of microwave pretreatment on cooking time of presoaked Bambara groundnut seeds, functional properties of resultant flours, and their phenolic composition and antioxidant properties. Microwave treatment significantly decreased cooking time and resulted in decreased water solubility index (WSI) (by up to 66%) and nitrogen solubility index (NSI) (up to 81%) of the resultant flours, as well as a decrease in pasting viscosity and enthalpy compared to raw flour. However, the 1200 W treatment resulted in higher pasting viscosity, WSI, and NSI than the 900 W treatment. There were differences in type of flavonoids and phenolic acids between the two types of Bambara groundnut seeds, while microwave treatment caused certain increase and decrease in individual flavonoids, such as catechin, quercetin-3-*O*-glucoside, and hesperidin. The flours maintained good radical scavenging antioxidant activity and protected plasmid DNA from oxidative damage. Overall, the study suggests that microwave pretreatment shows potential in alleviating the hard-to-cook Bambara groundnut seeds in producing flour with functional and antioxidant properties.

1. Introduction

Bambara groundnut is an underutilized grain legume [1] and considered to be among some of the most significant African legumes [2]. Bambara groundnuts are consumed in a variety of ways, especially in sub-Saharan African countries and constitute an important source of protein, essential amino acids, and other nutrients [3–5]. The increased consumption of plant foods in the form of grain legumes, such as cowpeas and Bambara groundnuts, has been associated with a reduced risk of noncommunicable diseases (NCDs), such as cardiovascular disease and cancer [6, 7]. It is hypothesized that this is due to the presence and activity of bioactive components such as phenolic compounds [8] mainly as a result of their antioxidant properties [9]. There is therefore increasing interest in the bioactive phenolic composition of grain legumes such as Bambara groundnuts and how their health-promoting properties are affected during processing. Muchuweti et al. [10] reported that phenolic compounds in fresh and dried Bambara groundnut seeds exhibited free radical scavenging antioxidant activity.

For the production of legume flours, the seeds are usually subjected to thermal treatments for purposes such as reduction in cooking time of the legume seeds and enhancing functional properties of the resultant flours. Mokatso [11] used microwave processing for pretreatment of cowpeas for processing into flour. However, as reported by Mokatso [11], such microwave pretreatment processes can affect the phenolic content and antioxidant properties of the legume.

Microwave processing is a thermal treatment that allows for decreased cooking time and energy costs compared to conventional cooking methods. Microwaves have wavelengths in the range of 1 mm to 1 m with frequencies of 300 MHz and 300 GHz. Microwaves have the advantage of penetrating food materials and thus deliver heat energy through molecular interaction with electromagnetic field. Microwave penetrates the material accumulating energy, which allows generation of heat throughout the volume of

the material [12]. Energy transfer in microwave processing does not depend on diffusion but rather the conversion of electromagnetic energy to thermal energy in the material, which allows for fast and uniform heating [12].

Although some research has been done on the application of thermal treatments such as microwave roasting of peanuts (*Arachis hypogaea* L.) [13], γ-irradiation [14], and infrared treatment to Bambara groundnut [15], limited research exists on the application of microwave treatment to Bambara groundnut seeds and its effect on the cooking time of the seeds and functional properties of resultant flours. The use of γ-irradiation of Bambara groundnut seeds significantly reduced its cooking time in all cultivars as reported by Falade et al. [14]. γ-irradiation also significantly decreased the pasting properties of Bambara groundnut flours but increased other functional properties such as water absorption capacity [14]. Recently, Mukwevho and Emmambux [15] showed a decrease in functional properties, such as nitrogen solubility, water solubility index, and pasting viscosity after infrared and microwave alone and in combination treatment of Bambara groundnut seeds.

Until now, no research has been conducted on the phenolic composition and antioxidant activity of Bambara groundnut seeds after microwave pretreatment. Thus, the objective of this study was to determine the effect of microwave pretreatment on the alleviation of the HTC defect, phenolic composition, and antioxidant health-promoting properties of presoaked Bambara groundnut seeds and the functional properties of the resultant flours.

2. Materials and Methods

2.1. Materials. Bambara groundnut (brown and red types) was procured from Triotrade CC (Pretoria, South Africa). Pure standard phenolic compounds such as *p*-coumaric acid, ferulic acid, caffeic acid, (+)-catechin, quercetin, kaempferol, myricetin, and rutin were all purchased from Sigma-Aldrich. Folin–Ciocalteu's phenol reagent, methanol, hydrochloric acid, and potassium persulfate were purchased from Merck. 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma-Aldrich. Formic acid and ethyl acetate solvents were purchased from Merck and Sigma-Aldrich.

2.2. Methods

2.2.1. Hydration of Bambara Groundnut Seeds. Hydration of Bambara groundnuts to 40% moisture content was done as described by Sibanda [16] with modifications. The Bambara groundnuts (50 g) were placed in a polyethylene ziplock bag. The weight of water (16.5 g) required was calculated and put inside the ziplock bag. The bag was placed in an oven set to 37°C and left until all the water was soaked up by the Bambara groundnuts (24 h).

2.2.2. Microwave Treatment. The microwave treatments were done based on the method of Mokatso [11] with some modifications using a fluidized bed microwave oven (Delphius, Centurion, South Africa). The microwave oven was first allowed to preheat to an air temperature of 130°C with an electric heater set at 1.2 kW maximum. Approximately 50 g of presoaked and hydrated Bambara groundnut samples was treated with microwave power of 900 and 1200 W for 5 and 8 min. After allowing to cool, the seeds were dried to a moisture content of ≤10% in an oven at 40°C overnight and milled to a flour using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) with a 500 μm sieve. After milling, the flours were stored in air-tight ziplock bags at 4°C until analysis. Microwave treatments were done in triplicate.

2.2.3. Preparation and Fractionation of Phenolic Extracts. Phenolic extracts from the Bambara groundnut samples were prepared as described by Kayitesi et al. [17] with modifications. The extracts were prepared in three phases as follows: 10 ml of 1% v/v HCl-methanol was added to 3 g and 1 g for extraction sample in an Erlenmeyer flask, stirred for 3 h, and centrifuged at 8217 ×g for 10 min at ambient temperature (25°C) and supernatant decanted. The pellet was collected and washed with 10 ml of solvent (1% v/v HCl-methanol), stirred for 20 min, and centrifuged as above described. The washing step was repeated twice. The supernatants were pooled and placed in air-tight plastic tubes wrapped in aluminum foil and stored at −20°C until analysis.

Extracts for LC-MS analysis were prepared as described by Ojwang, Dykes, and Awika [18] with some modifications. Approximately 5 g of raw and microwaved Bambara groundnut flour samples was weighed into a beaker and soaked in 15 ml of 70% acetone acidified with 1% formic acid for 12 h at 4°C. The mixtures were then stirred with a magnetic stirrer for 2 h at room temperature. The extracts were centrifuged at 135837 ×g for 10 min at room temperature. The pellets were collected and washed twice with 10 ml of extraction solvent for 30 min. The previous two steps were repeated. The supernatants were combined and concentrated under reduced pressure using a Buchmann R110 Rotavapor (Westbury, NY, USA).

The extracts were purified and fractionated using Sep-Pak Solid Phase Octadecylsilane (C18) cartridges (Sigma, USA) as described by Prior et al. [19] and Monagas et al. [20] with some modifications. The C18 cartridges were briefly conditioned with 25 ml methanol : water (1 : 1) for 1 h and washed with 50 ml distilled water. At least 5 ml of the concentrated extracts was deposited into cartridges and washed with 5 ml distilled water to remove the sugars. Catechins, oligomeric proanthocyanidins (PA), and other smaller phenolic compounds were eluted with 15 ml ethyl acetate. The ethyl acetate fraction was dried under vacuum and redissolved in 2 ml of methanol : water (50 : 50) containing 0.05% formic acid. Flavonols, isoflavonols, and anthocyanin pigments were then eluted with 15 ml methanol acidified with 0.1% formic acid. The sequential elution procedure was done to reduce the number of compounds in each fraction injected into the mass spectrometer to enable proper characterization. Before

chromatographic analysis, the extract fractions were filtered through 0.45 μm PTFE membrane filters and deposited into amber vials.

3. Analytical Methods

3.1. Physical Properties

3.1.1. Cooking Time. The cooking time of untreated (raw presoaked) and treated presoaked Bambara groundnut seeds was determined using a Mattson bean cooker as described by Mwangwela et al. [21] with some modifications. For treated and untreated samples, 25 Bambara groundnut seeds were placed in the perforations of the cooker with the pins placed on the surface of each seed. The cooker was placed in a stainless steel pot containing 1500 ml of deionized water and cooked. The cooking time was recorded as the moment when 80% of the pins had fallen through the seeds.

3.1.2. Water Solubility Index (WSI). The WSI of flours from microwave-pretreated Bambara groundnut samples was determined using the method described by Adebowale and Lawal [22] with modification by Ocloo et al. [23].

3.1.3. Nitrogen Solubility Index (NSI). The NSI of flours from the raw and microwaved pretreated Bambara groundnut seeds was determined using the AACC method 46-23 [24] with modifications.

3.1.4. Pasting Properties. The pasting properties of flours from raw and microwaved Bambara groundnut seeds were determined using a Physica MCR 101 Rheometer (Anton Paar, Ostfildern, Austria) based on a method by Wokadala et al. [25] with modifications. The pasting cycle began with an initial stirring speed of 960 rpm at 50°C for 30 s and then stirred at 160 rpm for the remaining period. The temperature was increased at a rate of 5.5°C/min to 91°C, and this temperature was held for 5 min for short pasting. The pastes were then cooled to 50°C at a rate of 5.5°C/min.

3.1.5. Thermal Properties. The thermal properties of flours from the microwave-pretreated Bambara groundnut seed samples were determined as described by Wokadala et al. [25] using a high-pressure DSC system with STARe® software (HPDSC-827, Mettler Toledo, Greifensee, Switzerland) with modifications. Indium (Tp = 156°C, heat endothermic flow = −28.6 J/g) was used to calibrate the instrument with regard to temperature and enthalpy. A weight of 10 mg (db) of treated and untreated flour samples was added to 30 ml of distilled water in a sealed aluminum pan. The samples were allowed to equilibrate at ambient temperature overnight. Scanning of samples was done at 40°C–150°C at a rate of 10°C/min and at 40 ± 0.01 MPa with a flow rate of 60 ml/min. An empty pan was used as reference. Melting enthalpy (ΔH in J/g), onset (To), peak (T_p), and endset (Tc) temperatures of endothermic peaks were measured. Measurements were done in duplicate.

3.1.6. Light Microscopy (LM). A small amount of flour was placed on a slide with the addition of glycerol. Iodine staining was done by placing a drop of iodine on the flour. The suspension was then mixed. The slides were viewed using a Nikon Optiphot transmitted light microscope (Tokyo, Japan) with appropriate illumination sources and filters. Pictures were captured using a Nikon digital camera DXM1200 (Tokyo, Japan).

3.2. Total Phenolic Content (TPC) and Antioxidant Activity. The total phenolic content (TPC) was determined using the Folin–Ciocalteu assay modified for the use of a 96-well microplate as described by Ainsworth and Gillespie [26].

The ABTS radical scavenging activity assay was performed according to the method described by Awika et al. [27] with some modifications for use in a 96-well microplate.

3.3. UPLC-MS Analysis. The chromatographic analysis was performed as described by Stander et al. [28]. A Waters Synapt G2 Quadrupole time-of-flight (QToF) mass spectrometer (MS) connected to a Waters Acquity ultraperformance liquid chromatography (Waters, Milford, MA, USA) was used for high-resolution UPLC-MS analysis. Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275°C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for best resolution and sensitivity. Data were acquired by scanning from m/z 150 to 1500 m/z in resolution mode as well as in MS^E mode. In MS^E mode, two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40–100 V) to obtain fragmentation data as well. The instrument was calibrated with sodium formate. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 μm column. An injection volume of 2 μL was used, and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B.

Gradient elution was done according to the following programs: 100% A from 0 to 0.5 min; 74% A and 26% B from 0.5 min to 6 min; 56% A and 44% B from 6 min to 15 min; and 0% A and 100% B from 15 min to 17.5 min. Ionization was in negative mode with a capillary voltage of 3 kV and cone voltage of 15 V. Identification was done by comparing chromatograms and retention times of phenolic constituents in the extracts with external phenolic acid and flavonoid standards as well as comparison of MS/MS fragmentation data and UV spectra with those of phenolic compounds reported in literature. Quantification was done by comparing integrated peak areas of phenolic compounds in extracts with that of standards and from generated calibration curves.

3.4. Inhibition of Oxidative DNA Damage Assay. Inhibition of AAPH-induced oxidative DNA damage was determined by a method described by Apea-Bah et al. [29] with some modifications.

3.5. Statistical Analysis. Experiments were conducted in triplicate. Multifactorial analysis of variance (MANOVA) was conducted using SPSS statistical software. Significant differences between means were determined at $p \leq 0.05$ using the least significant difference (LSD) test. Dependent variables were the cooking time, functional properties, TPC, ABTS, flavonoid content, and phenolic acid content. Independent variables were microwave parameters (time and power).

4. Results and Discussion

4.1. Techno-Functional Properties of Flours from Microwave Pretreated Bambara Groundnut Seeds

4.1.1. Cooking Time of Microwave Pretreated Bambara Groundnut Seeds. The effects of microwave time and power on the cooking time of presoaked Bambara groundnut seeds are shown in Table 1. The microwave time and power interaction caused a significant reduction ($p \leq 0.05$) in the cooking time of Bambara groundnut seeds as compared with the raw seeds. The Bambara groundnut type had no significant ($p \leq 0.05$) effect on the cooking time of the seeds. The biggest decrease in cooking time for brown Bambara groundnut seeds was by the 1200 W microwave treatment for 5 minutes, which had a cooking time of 45.33 minutes. The cooking time of untreated red Bambara groundnut seeds was 369 minutes. The red Bambara groundnut seeds microwaved at 900 W decreased cooking time up to 6 times, whereas 1200 W treatment reduced the cooking time of red Bambara groundnut seeds up to 12 times. There were no significant ($p \leq 0.05$) differences between cooking time of Bambara groundnut seeds at 1200 W and 900 W treatment.

Ogundele and Emmambux [30] reported a reduction in cooking time from 162 minutes for raw seeds to 83, 75, and 62 minutes after micronization at 5, 10, and 15 minutes, respectively. The reduction in cooking time of legumes has been attributed to parenchyma cell separation along the middle lamella [31] caused by β-elimination reactions of pectic substances [32] and gelatinization of starch in the parenchyma cells of the cotyledon.

4.2. Pasting Properties of Flours from Microwave Pretreated Bambara Groundnuts. Figure 1 shows the effect of microwave pretreatment on the pasting properties of flours from presoaked Bambara groundnut seeds. Bambara groundnut type had no ($p \leq 0.05$) significant effect on the pasting viscosity of the resulting flours. The pasting viscosity of flours from microwaved brown and red Bambara groundnut seeds was significantly ($p \leq 0.05$) lower than that of the raw untreated flour. The maximum, setback, and final viscosity of flours from presoaked microwaved Bambara groundnut seeds at all processing parameters were significantly ($p \leq 0.05$) lower than that of the untreated flours.

Microwave time of 8 minutes caused a significant ($p \leq 0.05$) decrease in pasting viscosities as compared to the 5-minute treatment for both microwave powers for flour from red Bambara groundnut seeds. On the other hand, no significant ($p \leq 0.05$) difference in pasting viscosity of flours from presoaked red Bambara groundnut seeds was observed between microwave powers of 900 W and 1200 W. For the red Bambara groundnut flour, pasting viscosity decreased significantly ($p \leq 0.05$) with an increase in microwave power and time as compared to the untreated seeds.

The pasting viscosities of flours from microwaved brown Bambara groundnut seeds decreased with a decrease in microwave power. The pasting viscosity of flours from presoaked brown Bambara groundnut seeds, and to an extent, the red type microwaved at 1200 W for 5 minutes was significantly ($p \leq 0.05$) higher than that of seeds microwaved at 900 W for both microwave times. This is unusual as it is expected that a higher power would result in a bigger change in the molecular structure of starch and protein leading to a decrease in pasting viscosity as seen in previous studies by Makatso [11] with flour of microwaved cowpea seeds.

Ogundele [33] and Mwangwela et al. [34] reported reduction in pasting viscosities of flours of micronized Bambara groundnut and cowpea, respectively. Mokatso [11] found that flours from microwaved cowpea seeds had reduced pasting viscosities compared to flours from tempered-only cowpea seeds. Ogundele [33] proposed that the decrease in pasting viscosities of thermally treated flours could be due to protein denaturation resulting in exposure of hydrophobic sites, which could limit water access to starch granules surrounding protein matrix. According to Ogundele [33], the hydrophobic protein surrounding the starch granules prevents starch from swelling and leaching out to form entangled networks called junction zones and thus resulting in reduced pasting viscosities. Recently, Mukwevho and Emmambux [15] reported similar reduction in pasting viscosities in flour from microwave, infrared, and combination treatments of presoaked Bambara groundnut seeds. They assigned the reduced pasting viscosities to the aggregation and formation of adherent starch-protein complex network. The denatured proteins that surround the starch granules restrict their expansion thus resulting in lower pasting viscosities.

4.2.1. Water Solubility Index (WSI) and Nitrogen Solubility Index (NSI) of Flours from Microwave Pretreated Bambara Groundnut Seeds. The effects of microwave time and power on water solubility index and nitrogen solubility index of flours from presoaked Bambara groundnut seeds are shown in Table 2. The microwave time and temperature interaction caused a significant ($p \leq 0.05$) reduction in WSI of Bambara groundnut flours.

The Bambara groundnut type and heating temperature had no significant ($p \leq 0.05$) effect on the WSI and NSI of the seed flours. The WSI and NSI of flours from both brown and red Bambara groundnut seeds microwaved at 900 W were significantly ($p \leq 0.05$) lower than that of seeds microwaved at 1200 W, respectively, for 5 minutes of microwave time. This corresponds with the lower pasting properties for flours from seeds microwaved at 900 W for 5 minutes compared to the 1200 W for 5 minutes treatment (Figure 1 A).

Similar results in reduction in WSI with increase in microwave power and time were reported for cowpea flour

Table 1: Effect of microwave time and power on the cooking time of presoaked Bambara groundnut seeds.

Samples	Microwave power (Watt)	Microwave time (min)	Cooking time (min)
Brown Bambara groundnut*	0	0	$219^{a} \pm 8.6$
	900	5	$60^{b} \pm 2.9$
		8	$50^{b} \pm 3.6$
	1200	5	$45^{b} \pm 5.7$
		8	$55^{b} \pm 7.7$
Red Bambara groundnut*	0	0	$369^{a} \pm 4.0$
	900	5	$54^{b} \pm 5.3$
		8	$29^{b} \pm 6.4$
	1200	5	$31^{b} \pm 0.7$
		8	$29^{b} \pm 3.8$

Values are means of triplicates ± Standard deviations. For each Bambara groundnut type, mean values with different superscript letters in columns are significantly different ($p \leq 0.05$) from each other. *no significant difference ($p \leq 0.05$) between Bambara groundnut types.

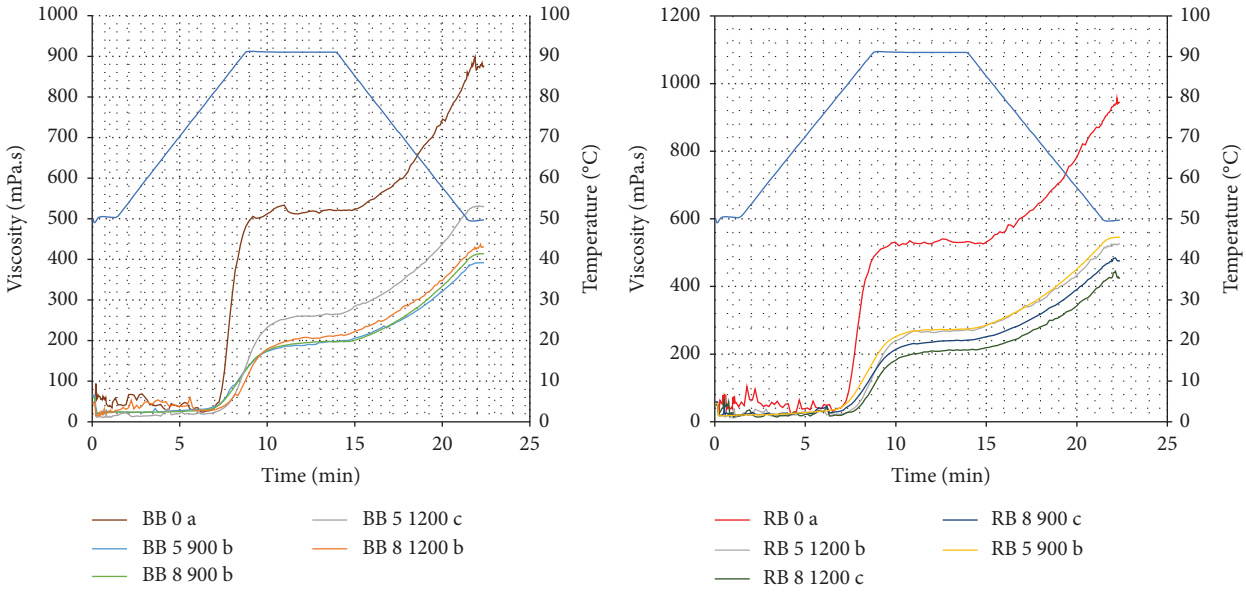

Figure 1: Effect of microwave pretreatment on the pasting properties of presoaked brown and red Bambara groundnuts.

by Mokatso [11]. Ogundele [33] found a decrease in water solubility index with an increase in micronization time and power in flours from whole and dehulled Bambara groundnut seeds. The reduction in WSI could be attributed to the denaturation of legume proteins during thermal treatment resulting in a decrease in protein solubility [34, 35]. To confirm that the reduction in water solubility index was due to a reduction of protein solubility, the nitrogen solubility index was determined.

The results for WSI and NSI support our statement that the 1200 W treatment of brown Bambara groundnut seeds showed flour pasting viscosities significantly higher than that of the lower power of 900 W. These results are further explored by looking at the flours under a polarized light microscope with iodine staining.

Bellido et al. [36] reported a decrease in solubility of protein in navy and black beans after micronization treatment as compared to their nonprocessed counterparts. The NSI for the 1200 W treatments was higher than those of the 900 W treatments, which corresponds to the results for WSI. Reference [37] reported the reduction of NSI of flours from infrared-treated Bambara groundnut seeds with increase in micronization time of 5, 10, and 15 minutes. The decrease in protein solubility or NSI of flours after heat treatment was attributed to the denaturation of protein resulting in exposure of hydrophobic sites, thus making the protein less soluble [37]. Mukwevho and Emmambux [15] also found a reduction in NSI and WSI indices, which was attributed to the mechanism as previously explained with the pasting properties. The lower NSI also suggests an increase in protein hydrophobicity due to denaturation during the heat treatment.

4.3. Thermal Properties of Flours from Microwave Pretreated Bambara Groundnut Seeds. The effect of microwave time and power on the thermal properties of flours from Bambara groundnut seeds is shown in Figure 2. A single endothermic

Table 2: Effect of microwave time and power on nitrogen solubility index (NSI) and water solubility index (WSI) of flours from presoaked Bambara groundnut seeds.

Samples	Heating temperature (°C)	Microwave power (Watt)	Microwave time (min)	WSI (%)	*NSI (%)
Brown Bambara groundnut	50	0	0	$51^{a} \pm 4.0$	$78^{a} \pm 1.2$
		900	5	$18^{ab} \pm 1.0$	$19^{b} \pm 0.3$
			8	$20^{ab} \pm 0.7$	$18^{b} \pm 0.4$
		1200	5	$38^{ab} \pm 4.5$	$26^{b} \pm 5.3$
			8	$17^{ab} \pm 0.6$	$22^{b} \pm 2.1$
	95	0	0	$62^{a} \pm 3.4$	
		900	5	$26^{ab} \pm 1.4$	
			8	$25^{ab} \pm 0.9$	
		1200	5	$31^{ab} \pm 2.9$	
			8	$22^{ab} \pm 1.5$	
Red Bambara groundnut	50	0	0	$33^{a} \pm 2.2$	$87^{a} \pm 4.2$
		900	5	$20^{ab} \pm 2.2$	$16^{b} \pm 0.4$
			8	$19^{ab} \pm 0.5$	$19^{b} \pm 5.4$
		1200	5	$17^{ab} \pm 1.0$	$19^{b} \pm 0.8$
			8	$17^{ab} \pm 1.2$	$19^{b} \pm 1.0$
	95	0	0	$74^{a} \pm 1.6$	
		900	5	$26^{ab} \pm 0.2$	
			8	$25^{ab} \pm 0.9$	
		1200	5	$25^{ab} \pm 1.1$	
			8	$25^{ab} \pm 0.9$	

*NSI done at 30°C only.

peak was observed in raw untreated flours from both brown and red Bambara groundnut seeds. Microwave pretreatment of Bambara groundnut seeds caused a significant ($p \leq 0.05$) reduction in endothermic peaks and transition enthalpy as compared to the flour from raw seeds. Very small endothermic peaks were observed for flours from all microwaved brown Bambara groundnut seeds. Similar endothermic peaks were observed for all flours from microwaved red Bambara groundnut seeds.

The endothermic peaks observed for raw Bambara groundnut flours may be due to starch gelatinization and protein denaturation. The small endothermic peaks observed for microwaved samples might explain the partially gelatinized but intact "Maltese crosses" in the starch granules observed under light microscopy after microwave treatment indicating partially gelatinized starch (Figure 3). The low transition enthalpy values may indicate the presence of crystalline regions in the partially gelatinized starch granules after microwave pretreatment. Sirivongpaisal [38] showed that Bambara groundnut contains A-type crystalline starch through X-ray diffraction pattern according to Gidley [39], which is the most thermodynamically stable form.

Mwangwela et al. [34] also reported a decrease in endothermic peaks and enthalpy for flours from micronized cowpea seeds. Ogundele [33] found a decrease in endothermic peaks and enthalpy of flours from dehulled and whole micronized Bambara groundnut seeds. Ogundele [33] attributed the reduction in endothermic peaks and enthalpy to the partial gelatinization of starch molecules.

4.4. Light Microscopy Analysis of Flours from Microwave Pretreated Bambara Groundnut Seeds. Figure 3 shows polarized and iodine-stained light microscopy images of flour from raw and microwaved Bambara groundnut seeds. In the raw untreated flour of both brown and red Bambara groundnut seeds, the polarized light microscopy images showed intact starch granules as evident by the "Maltese cross" birefringence (indicated with arrows). With increase in microwave power and time, it could be seen that there was a decrease in "Maltese cross" birefringence, which could explain the reduction in endothermic peak for microwaved flours as compared to raw samples.

Iodine staining showed an increase in aggregation and swelling of black-blue starch (orange arrows) as microwave power and time increased, which indicates partially gelatinized starch and cross-linking with protein. The starch granules (black) (blue arrow) embedded in a protein matrix (yellow) (black arrow) could be seen in the microscope image of flour from red Bambara groundnut treated for 5 minutes at 1200 W. The protein matrix surrounding the starch granules could be the reason for the lower WSI, NSI, and pasting viscosities of the flour from microwave-treated Bambara groundnut seeds. The hydrophobic protein matrix could restrict water access to starch granules, which reduces starch swelling and leaching out.

The iodine-stained flours of both brown and red Bambara groundnut seeds treated for 8 minutes 1200 W showed a swelled-up and slight disappearance of the black color of starch granules showing a distinct purple color (Figure 3) The intense blue color can be due to amylose that leached (red arrow) out, while the purple color (amylopectin) (yellow arrow) remains as an insoluble irregular mass after heating. This is evidence of some regions of flour not having formed a protein matrix around partially gelatinized starch granules and could be the reason for the lower pasting viscosities and increase in WSI and NSI of the 1200 W treatments compared to that of 900 W.

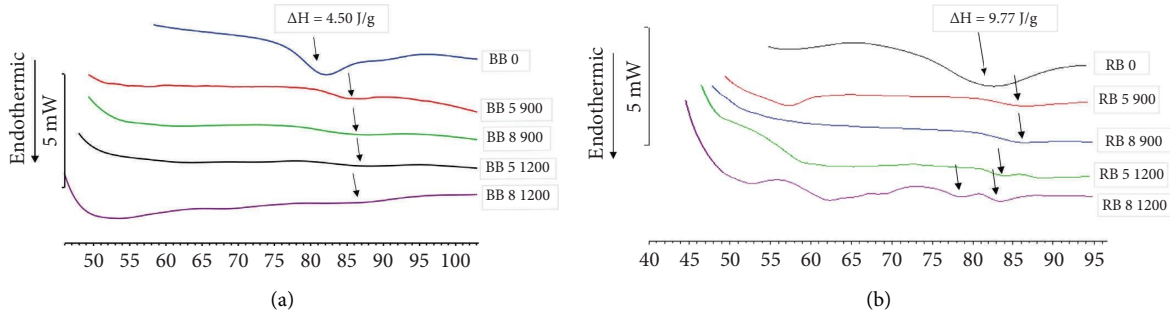

FIGURE 2: Effect of microwave pretreatment on the thermal properties of flours from brown (a) and red (b) Bambara groundnut seeds.

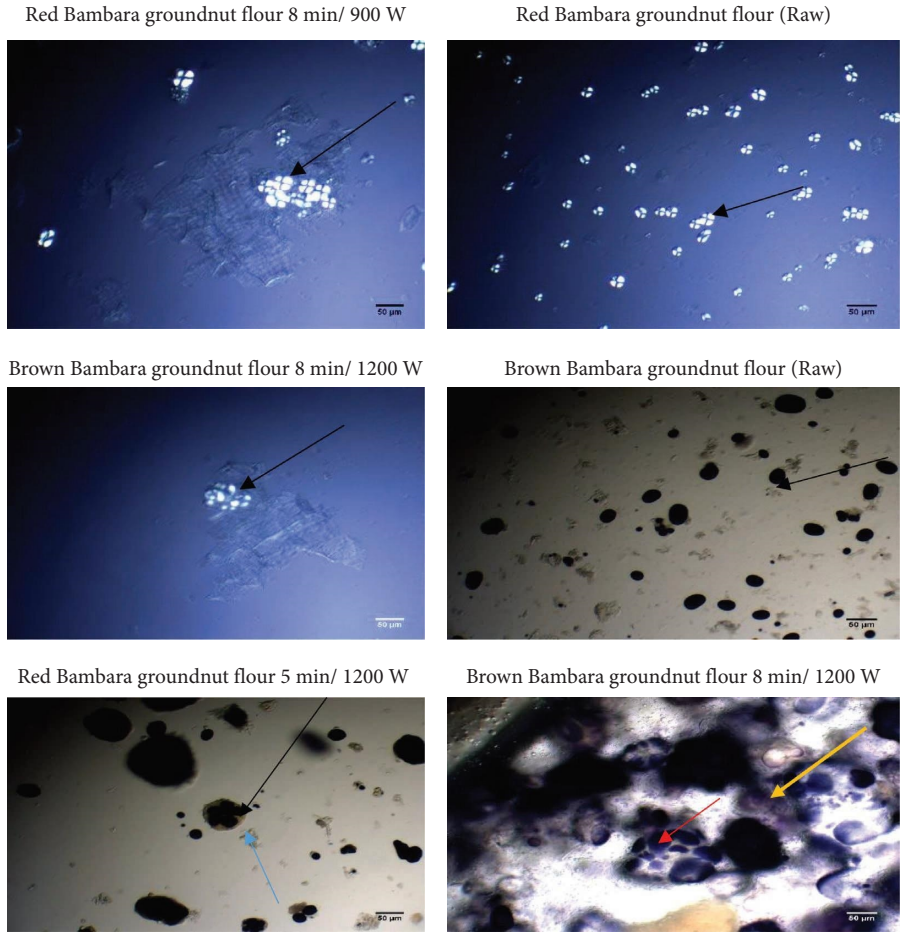

FIGURE 3: Polarized light microscopy images of flour from raw and microwaved Bambara groundnut seeds with and without iodine staining. Bambara groundnut flours from raw presoaked and microwaved (900 W and 1200 W for 5 and 8 min) seeds were viewed under polarized light for loss of birefringence. Bar = 50 μm. Arrows indicate aggregates of partially gelatinized starch granules and birefringence of starch granules in flours from raw untreated and microwave-treated samples. Black arrows show iodine-stained swelled starch granules, and light blue arrows indicate hydrophobic protein layer surrounding starch granules. Orange and red arrows show aggregation and swelling of starch granules in microwaved and starch granules in raw untreated samples. Dark blue arrows indicate the purple insoluble irregular mass of starch in microwaved samples.

4.5. Total Phenolic Content (TPC) and Antioxidant Activity of Microwave Pretreated Bambara Groundnut Seed Flours. The effect of microwave pretreatment on the total phenolic content (TPC) and ABTS radical scavenging activity of flours from presoaked brown and red Bambara groundnut seeds is shown in Table 3. The total phenolic content of flours from raw untreated brown and red Bambara groundnut seeds was 3.04 mg CE/g and 2.47 mg CE/g, respectively. These are similar to the TPC value of 2.54 mg CE/g reported by Ogundele [33] for Bambara groundnut seeds.

Table 3: Effect of microwave pretreatment of presoaked Bambara groundnut seeds on the total phenolic content (TPC) (mg CE/g db) and ABTS radical scavenging activity (μmol TE/g db) of the resultant flours.

		Microwave pretreatment				
		Raw	5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W
Brown Bambara groundnut seeds	TPC	$3^{a} \pm 0.4$	$3^{as} \pm 0.1$	$3^{a} \pm 0.1$	$3^{a} \pm 0.2$	$3^{a} \pm 0.0$
Red Bambara groundnut seeds		$2^{a} \pm 0.4$	$3^{a} \pm 0.3$	$3^{a} \pm 0.4$	$3^{a} \pm 0.1$	$3^{a} \pm 0.0$
Brown Bambara groundnut seeds	ABTS	$48^{a} \pm 5.8$	$40^{a} \pm 0.4$	$39^{a} \pm 9.6$	$31^{a} \pm 3.0$	$31^{a} \pm 0.2$
Red Bambara groundnut seeds		$31^{a} \pm 9.6$	$44^{a} \pm 1.3$	$42^{a} \pm 0.3$	$38^{a} \pm 1.6$	$43^{a} \pm 1.7$

ABTS: 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; TE: Trolox equivalents; db: dry basis; CE: catechin equivalents; db: dry basis. a,b,c: numbers with different superscripts differ significantly ($p \leq 0.05$) within rows.

For brown Bambara groundnut seeds, compared with the raw seeds, microwave treatment at 900 W increased TPC by up to 5%, while microwave treatment at 1200 W decreased TPC by up to 5%. For red Bambara groundnut seeds, microwave treatment at both 900 W and 1200 W increased TPC by up to 26%. Unlike for TPC, microwave treatment at both 900 W and 1200 W decreased ABTS radical scavenging activity of brown Bambara groundnut seeds by up to 39%. The trends in ABTS radical scavenging activity of red Bambara groundnut seeds after microwave treatment were similar to the TPC with increases in radical scavenging activity by up to 26%. Overall, these results indicate that microwave treatment of Bambara groundnut seeds could lead to either increases or decreases in TPC and radical scavenging activity, although not significant ($p \leq 0.05$).

The observed decrease in TPC and radical scavenging activity of flour from Bambara groundnut seeds after microwave treatment could be due to thermally induced reaction of phenolic compounds with proteins [40] making them less extractable. Another possible reason could be due to thermal oxidation or degradation of phenolic compounds as reported by Luthria and Pastor-Corrales [41].

On the other hand, we observed increases in TPC and radical scavenging activity after microwave treatment may be attributed to the release of phenolic compounds bound to cell wall components as a result of thermal-induced changes in structural integrity of the seeds [42]. This makes the phenolic compounds more extractable and this is reflected as increases in TPC and antioxidant activity. Maillard reaction products produced as a result of the thermal treatment are known to have reducing properties [43] and could also contribute to increases in TPC and antioxidant activity. Similar results have been reported by Nyembwe et al. [44] who observed an increase in total phenolic content of marama beans after roasting.

4.6. Identification of Phenolic Compounds in Microwave Pretreated Bambara Groundnut Seeds. Table 4 shows the chromatographic and mass spectral data of phenolic acids and flavonoids identified in flour extracts from the raw and microwave-pretreated Bambara groundnut seeds.

4.6.1. Phenolic Acids and Derivatives. Peak 1 was identified as gallic acid. It produced a fragment at m/z 125 possibly due to loss of a CO_2 (−44 amu) from the carboxylate group [45]. Peak 2 with retention time of 4.98 min, molecular ion at m/z 153, and UV-vis absorption wavelength 220 and 266 nm was identified as protocatechuic acid. It produced an ionic fragment at m/z 109, which corresponds to loss of a CO_2 (−44 amu) moiety from the carboxylate group [45]. Peak 3 was identified as vanillic acid. It produced fragments at m/z 152 [due to loss of a methyl group (CH_3; −15 amu)], m/z 123 [due to loss of a carbon dioxide (CO_2; −44 amu)], and m/z 108 [due to loss of both CH_3 (−15 amu) and CO_2 (−44 amu)] [46].

Peak 4 was tentatively identified as coumaric acid isomer. Fragmentation produced an ion at m/z 119, which corresponds to the loss of a CO_2 unit (−44 amu) [45]. Peak 5 (t_R = 9.41, λ_{max} = 282) with molecular ion at m/z 253 was tentatively identified as caffeoyl glycerol. Fragmentation produced an ion at m/z 179 corresponding to the loss of a glycerol molecule (−74 amu) [47]. Peak 6 was tentatively identified as ferulic acid hexoside. Fragmentation produced an ionic fragment at m/z 193 corresponding to the loss of a hexose unit (−162 amu) [48].

4.7. Flavonoids and Derivatives. Peak 7 was identified as procyanidin B 2 dimer. It produced fragments at m/z 407, 289, and 125. Retro-Diels-Alder (RDA) fragmentation of the C ring at positions 1 and 3 of one of the flavan-3-ol monomeric units produces an m/z 425 fragment, which can then undergo loss of a water (H_2O) molecule (−18 amu) to produce the m/z 407 fragment. Cleavage of the interflavan linkage produces the flavan-3-ol monomeric fragment at m/z 289 [49]. RDA cleavage of the C ring of one of the monomeric flavan-3-ol fragments at positions 1 and 3 produces a trihydroxy phenol moiety (^{1,3}A) at m/z 125. Peak 8 was identified as procyanidin C 2 trimer. Its fragments are produced from cleavage of the interflavan bond to produce the dimeric (m/z 577) and monomeric (m/z 289) procyanidin species [50,51].

Peak 9 was identified as catechin. Fragmentation produced ionic fragments at m/z 271, 245, and 205. The m/z 271 fragment corresponds to the loss of a H_2O (−18 amu) molecule. The m/z 245 fragment corresponds to either the loss of a CO_2 (−44 amu) residue [52] or loss of a CH=C-OH group (−42 amu) most likely from the A ring [53] together with hydrogen (H_2) molecule (−2 amu). The m/z 205 fragment corresponds to the loss of the A ring (−84 amu) [52]. Peak 10 was tentatively identified as a glucoside derivative of catechin. Fragmentation produced an ionic fragment at m/z 289. The fragment at m/z 289 is the resulting catechin aglycone after loss of the glucose moiety (−162 amu) [54].

Table 4: Retention time (t_R), UV-visible absorption maxima, and mass spectral characteristics of phenolic compounds identified in ethyl acetate fraction of extracts from raw and microwaved Bambara groundnut seeds.

Peak number	t_R (min)	λ_{maz} (nm)	[M-H]$^-$	MS/MS fragments	Proposed compound ID
Hydroxybenzoic acid derivatives					
1	4.04	271, 289	169	125 (71)	Gallic acid
2	4.98	220, 266	153	109 (57)	Protocatechuic acid
3	4.51	220, 343	167	152 (20), 123 (20), 108 (26)	Vanillic acid
Hydroxycinnamic acid derivatives					
4	10.17	348, 311	163	119 (80)	Coumaric acid isomer
5	9.41	282	253	179 (18)	Caffeoyl glycerol
6	1.50	265	355	193 (23)	Ferulic acid hexoside
Flavan-3-ols					
7	5.26	276	577	407 (58), 289 (66), 125 (84)	Procyanidin B2 dimer
8	5.59	279	865	577 (13), 289 (53)	Procyanidin C2 trimer
9	5.71	280, 348	289	271 (5), 245 (24), 205 (21)	Catechin
10	4.77	277	451	289 (100)	Catechin glucoside
Flavonols					
11	9.42	341, 280	285	243 (9), 151 (17), 145 (25), 109 (11)	Kaempferol
12	7.24	353, 279	463	301 (75), 179 (5.4), 151 (20)	Quercetin-3-*O*-glucoside
13	10.33	281	317	317 (100)	Myricetin
Flavonol					
14	14.41	281	303	125 (5)	Taxifolin
Flavones					
15	11.38	348, 341	269	151 (46), 117 (8)	Apigenin
Flavanones					
16	6.46	348, 275	609	301 (75), 286 (6), 258 (2), 151 (14)	Hesperidin
17	9.88	368, 341	301	177 (5), 151 (54)	Hesperetin
18	11.21	348, 281	271	151 (20), 119 (50)	Naringenin
19	13.49	281	287	151 (2), 135 (10), 125 (10)	Eriodictyol
20	6.20	282	449	287 (35), 151 (29)	Eriodictyol-7-*O*-*β*-D-glucoside

Peak 11 was identified as kaempferol. Fragmentation produced ionic fragments at m/z 243, 151, 125, and 109. The m/z 243 fragment could be due to loss of a CH=C-OH group (−42 amu) most likely from the A ring. RDA fragmentation of the C ring at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151 [55]. Similarly, RDA fragmentation of the C ring at positions 1 and 4 produces a $^{1,4}A^-$ fragment (trihydroxy benzene molecule) at m/z 125 [56]. Loss of a CH=C-OH group (−42 amu) from the A ring of the m/z 151 fragment produces the m/z 109 fragment [47].

Peak 12 was identified as quercetin-3-*O*-glucoside. The compound produced fragments at m/z 301, 179, and 151. The m/z 301 fragment corresponds to quercetin aglycone after the loss of a glucose molecule (−162 amu) [57]. The m/z 179 fragment represents the $^{1,2}A^-$ ion resulting from fragmentation of the C ring of quercetin aglycone at positions 1 and 2 [55]. RDA fragmentation of the C ring of quercetin aglycone at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151 [55]. Peak 13 was identified as myricetin based on the molecular ion at m/z 317.

Peak 14 was identified as taxifolin. RDA fragmentation of the C ring at positions 1 and 4 produces a $^{1,4}A^-$ fragment (trihydroxy benzene molecule) at m/z 125 [56]. Peak 15 was identified as apigenin. The compound produced fragments at m/z 151 and 117. RDA fragmentation of the C ring at positions 1 and 3 produces a $^{1,3}A^-$ (m/z 151) and $^{1,3}B^-$ fragment (m/z 117) [55].

Peak 16 was identified as hesperidin. The compound produced fragments at m/z 301, 286, 258, and 151. Loss of the diglucoside moiety (−308 amu) produces the m/z 301 fragment [58], which is hesperetin (the aglycone form of hesperidin). Loss of a methyl group (CH_3, −15 amu) from the hesperetin aglycone produces the fragment at m/z 286, which can further lose a carbonyl group (CO, −28 amu) to produce the m/z 258 fragment. RDA cleavage of the C ring of the hesperetin aglycone at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151. Peak 17 was identified as hesperetin. Fragmentation produced ions at m/z 177 and 151. Cleavage of the bond joining the B ring and carbon 2 of the C ring leads to loss of the B ring moiety and produces the fragment at m/z 177. The RDA cleavage of the C ring of hesperetin at positions 1 and 3 produces the $^{1,3}A^-$ fragment at m/z 151. Peak 18 was identified as naringenin. The compound produced fragments at m/z 151 and 119, which correspond to the $^{1,3}A$- and $^{1,3}B$-fragments after RDA cleavage of the C ring at positions 1 and 3, respectively [55].

Peak 19 was identified as eriodictyol. Fragmentation produced ionic fragments at m/z 151, 135, and 125. RDA cleavage of the C ring at positions 1 and 3 produces the $^{1,3}A$-fragment (m/z 151) and the $^{1,3}B$-fragment (m/z 135) [55, 56]. RDA cleavage of the C ring at positions 1 and 4 produces a trihydroxy benzene moiety, which is the $^{1,4}A$-fragment at m/z 125 [56]. Peak 20 was tentatively identified as eriodictyol-7-*O*-*β*-D-glucoside. Fragmentation produced ions at

Table 5: Effects of microwave pretreatment on the flavonoid and phenolic acid content (μg/g sample dry basis) of extracts from flours of Bambara groundnut seeds.

	Raw Bambara groundnut seeds		Microwaved Bambara groundnut seeds							
			Brown				Red			
	Brown	Red	5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W	5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W
Catechin	$48^{ab} \pm 4.6$	$29.3^{a} \pm 1.5$	$164^{de} \pm 8.2$	$55^{abc} \pm 19.4$	$28^{a} \pm 10.0$	$324^{f} \pm 35.1$	$116^{cd} \pm 10.5$	$102^{bc} \pm 19.3$	$206^{e} \pm 38.2$	$218^{e} \pm 0.07$
Kaempferol	$10^{bcd} \pm 1.2$	$11.2^{cd} \pm 4.0$	ND	$3^{ab} \pm 0.1$	$10^{cd} \pm 0.4$	$19^{d} \pm 2.4$	$18^{de} \pm 0.4$	$8^{bc} \pm 0.02$	$11^{cd} \pm 5.0$	$20^{d} \pm 2.9$
Quercetin-3-*O*-glucoside	$17^{a} \pm 1.6$	$117.3^{b} \pm 36.3$	$3^{a} \pm 0.1$	$9^{a} \pm 5.0$	$23^{a} \pm 2.6$	$16^{a} \pm 5.3$	$128^{b} \pm 14.2$	$128^{b} \pm 14.3$	$188^{c} \pm 7.0$	$179^{c} \pm 8.3$
Naringenin	ND	$3.9^{a} \pm 0.3$	ND	ND	ND	$33^{d} \pm 0.6$	$17^{c} \pm 0.4$	$8^{b} \pm 0.5$	ND	$60^{e} \pm 1.8$
Hesperidin	$71^{b} \pm 1.6$	$364.5^{c} \pm 87.8$	ND	$33^{a} \pm 1.7$	$53^{a} \pm 9.0$	$42^{a} \pm 25.2$	$351^{c} \pm 11.1$	$217^{c} \pm 61.2$	$677^{d} \pm 92.9$	$750^{d} \pm 57.4$
Apigenin	$16^{ab} \pm 6.7$	$22.9^{ab} \pm 0.4$	ND	ND	$24^{ab} \pm 15.1$	$37^{b} \pm 12.9$	$8^{a} \pm 0.5$	$10^{a} \pm 0.6$	$34^{ab} \pm 0.8$	$14^{ab} \pm 8.4$
Hesperetin	ND	$7.2^{a} \pm 0.1$	ND	ND	ND	ND	$39^{a} \pm 1.6$	$56^{ab} \pm 6.4$	$181^{b} \pm 67.9$	$100^{ab} \pm 47.0$
Total flavonoids	163	556	167	101	139	508	676	529	1297	1340
Gallic acid	ND	$4^{a} \pm 0.6$	ND	ND	ND	ND	$10^{b} \pm 0.6$	$2^{a} \pm 0.3$	$9^{b} \pm 2.3$	$15^{b} \pm 6.8$
Protocatechuic acid	$4^{a} \pm 0.4$	$24^{ab} \pm 3.1$	ND	ND	$18^{ab} \pm 6.5$	ND	$25^{ab} \pm 2.3$	$20^{ab} \pm 13.2$	$63^{ab} \pm 13.4$	$84^{b} \pm 10.6$
Total phenolic acids	4	28	ND	ND	18	ND	35	22	72	98

Values = mean ± SD; nd = not detected; a,b: values in a row with different superscripts differ significantly ($p \leq 0.05$) from each other.

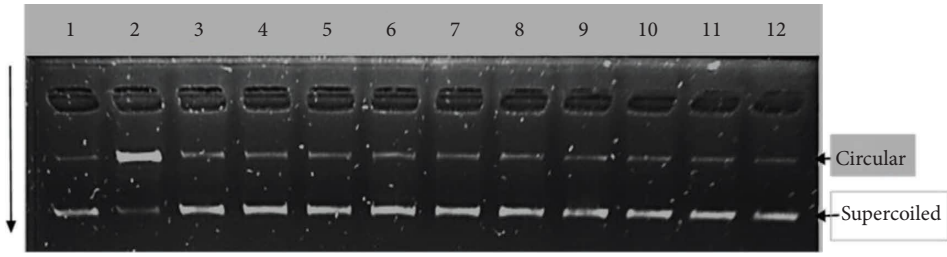

Figure 4: Agarose gel electrophoretogram showing the inhibitory effects of phenolic extracts from raw and microwaved Bambara groundnut flour against AAPH radical-induced oxidative pBR322 vector DNA damage Lane 1: negative control (DNA + H_2O).

m/z 287 and 151. The m/z 287 fragment corresponds to the eriodictyol aglycone after loss of the glucose moiety (–162 amu) [59]. The ion at m/z 151 is likely the ^{1,3}A-fragment produced through the RDA cleavage of the C ring of the eriodictyol aglycone at positions 1 and 3 [55].

Table 5 shows the effect of microwave pretreatment on the phenolic acid and flavonoid content of phenolic extracts from flours of Bambara groundnut seeds. In general, microwave pretreatment of Bambara groundnut seeds caused no significant ($p \leq 0.05$) changes in flavonoid content and phenolic acid content for the red type. There were differences in the types and levels of flavonoids in the two Bambara groundnut types. For the raw seeds, catechin and hesperidin were the major flavonoids in the brown type, while quercetin-3-*O*-glucoside and hesperidin were the major flavonoids in the red type. Naringenin and hesperetin were not detected in the brown type. Overall, the raw red Bambara groundnut type contained a higher level of total flavonoids than the brown type.

For the two Bambara groundnut types, seeds microwaved at the higher power of 1200 W generally had higher levels of flavonoids than seeds microwaved at 900 W. This means that the flavonoids (e.g., catechin, quercetin-3-*O*-glucoside, and hesperidin) were rendered more extractable when microwaved at a higher power of 1200 W. The higher microwave power may facilitate the thermal hydrolysis of these compounds from cell wall components of Bambara groundnut seeds [42] and thus increase their extractability. This observation also suggests that these compounds are relatively thermally stable at the high microwave power of 1200 W.

For the brown Bambara groundnut seeds, microwaving time generally increased extractability of flavonoids. This is evident from the observation that microwaving for 8 min at either 900 W or 1200 W mostly led to increases in levels of specific flavonoids and total flavonoids than for the shorter duration of 5 min. However, the effect of microwaving time seemed to be different for the red Bambara groundnut seeds where there were mostly decreases in levels of flavonoids after microwaving for 8 min compared to 5 min or the flavonoid levels did not change significantly. This suggests that type may play a role in determining the effect of microwaving time on flavonoids in Bambara groundnut. This hypothesis needs to be tested using a larger number of Bambara groundnut varieties.

It is important to note that in this work, only a small number of phenolic acids were detected in the Bambara groundnut samples among which only two could be quantified. This is because the phenolic extraction methodology applied is specifically designed for optimum extraction of flavonoids. Therefore, conclusions about the effect of microwave pretreatment on phenolic acids in this research can only be made to a limited extent. In agreement with the results on flavonoid levels, varietal differences were also found in the levels of phenolic acids in the two Bambara groundnut types. Gallic acid and protocatechuic acid could be detected and quantified in all the red Bambara groundnut samples. In contrast, apart from protocatechuic acid in the 5 min/1200 W treated seeds, no phenolic acids could be identified and quantified in any of the brown Bambara groundnut seeds.

4.7.1. Inhibitory Effects of Extracts from Raw and Microwaved Bambara Groundnut Seeds against Oxidative DNA Damage. Figure 4 shows the effect of extracts from raw and microwaved brown and red Bambara groundnut seeds on the inhibition of peroxyl radical-induced pBR322 vector DNA damage. AAPH radicals react with nucleotide bases in DNA molecules, which lead to damage of the DNA [60]. The oxidative reaction with AAPH radicals leads to single-strand (circular) or double-strand (linear) breaks in the pBR322 DNA vector [61]. The undamaged supercoiled form of DNA and the damaged circular or linear forms have differing mobility during electrophoresis [62].

The negative control in Lane 1 (DNA + H_2O) showed the DNA predominantly in the supercoiled form (Figure 4). Lane 1 also showed a faint DNA band in the circular form. This may suggest that either the DNA may have been slightly damaged by the hydrochloric acid [63] used in the solvent or some of the DNA as supplied by the manufacturer was already in the damaged circular form. According to Wei et al. [61] and Adarkwah-Yiadom and Duodu [64] depending on the batch, some plasmid DNA as supplied by the manufacturer may have some damaged forms. The positive control in Lane 2 (DNA + H_2O + AAPH) showed the DNA predominantly in the circular (single-strand break) form as indicated by the high-intensity band. A faint band of supercoiled DNA could still be seen in Lane 2, which suggests that the DNA was not completely damaged by the AAPH radicals [64].

Lanes 3 to 12 that contained plasmid DNA, AAPH, and phenolic extracts from the raw and microwave-treated seeds showed the DNA predominantly in the supercoiled form with high band intensity. This is an indication that the phenolic extracts exerted protective effects against damage of the DNA by the AAPH radicals. Phenolic compounds in the extracts could scavenge the AAPH radicals and thus prevent them from reacting with the DNA. Similar protective effects of phenolic extracts from whole red, brown, black, and white beans and hulls [60] and from raw and microwaved cowpeas [11] against AAPH radical-induced oxidative DNA damage have been reported.

5. Conclusion

Microwave pretreatment of Bambara groundnut seeds reduced cooking time and functional properties, such as pasting viscosity, thermal properties, WSI, and NSI of the resultant flours. The observed decreases can be attributed to microwave-induced starch-protein complex formation. The higher microwave power (1200 Watts) produced flour with higher pasting properties possibly due to increased degree of starch gelatinization and little starch-protein complex formation. Microwave pretreatment of Bambara groundnuts led to both increases and decreases in extractability of phenolic compounds depending on Bambara groundnut type, microwave power, and time. Flours from the microwave-pretreated Bambara groundnut seeds exhibited good radical scavenging antioxidant activity and protection against oxidative DNA damage. Overall, the phenolic compounds exhibited good thermal stability during the microwave treatment under the conditions used. Microwave pretreatment holds promise in its application for the alleviation of the hard-to-cook defect in Bambara groundnuts as well as the modification of microstructural components, which enhances its use as flour in food applications. The flours from the microwave-pretreated Bambara groundnut seeds also retain bioactive phenolics with enhanced health-promoting properties.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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