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Anti-hypertensive and Antioxidant Potentials of Wheat Substituted with Two Underutilized Seed Flours

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Abstract: The effect of the addition of *Moringa oleifera* seed and African oil bean (AOB) seed flours on the minerals, antinutritional compositions, antioxidants and Angiotensin -1 converting enzyme (ACE) inhibition potentials on wheat based composite flour was investigated. The minerals composition of the composite flour was optimized using optimal mixture design of response surface methodology (RSM) by varying the independent variables as; wheat flour (50-90 g/100g), Moringa seed flour (5-25 g/100g) and AOB flour (5-25 g/100g). The results of the optmisation indicated that run 11 (50% Wheat/25% *Moringa* seed/25% AOB flours) had overall best Fe, Ca and Mg contents while run 1 (81.20% Wheat/8.68% *Moringa* seed flour/10.12% AOB seed flour) had the best Zn, P and Na contents. However, Na/K ratios of the optimum blends were less than 0.130. Run 11 had overall best antioxidant characteristics, closely followed by run 1 while the control (100% wheat flour) had the least antioxidant contents. The ACE inhibition property of cake produced from run 1 (with the least antinutrients contents and second best antioxidants contents) showed that addition of Moringa and AOB flours had positive effect on ACE inhibition potential of Wheat flour, which increased with increasing concentration.

Keywords: ACE Inhibition, Antioxidants, Moringa Seed Flour, Na/K, Optimization, Wheat Flour

1. Introduction

The increasing prevalence of hypertension globally is the major reason for the current trend in the development of functional food products with antihypertensive effects [1]. Intake of food with reduced sodium contents, enriched bioactive compounds such as ACE and antioxidant peptides have been shown to be potential solution to the reduction of high blood pressure [1]. High blood pressure is a chronic disease which resulted in several cardiovascular diseases like stroke, renal disease and diabetes. The inhibition of ACE activity is a major target in the prevention of hypertension.

Wheat is a major source of inexpensive, readily available food products widely produced and consumed. It provides needed calorie intake as a source of staple food commodity in developing countries [2]. Bread produced from wheat had been shown to possess low ACE inhibition and low amounts of bioactive compounds. It has been reported that a 100% wheat bread had ACE inhibition capacity as low as 10.29%, whereas low sodium wheat soughdough bread had ACE inhibition potential of 68% [1]. This result clearly indicates that the ACE inhibition and hence, antihypertensive potential of wheat can be improved. African oil bean and Moringa seed are nutritious food crops with promising antioxidants potentials [3, 4]. They can therefore be added to wheat flour in order to improve the antioxidant and hence ACE inhibition potentials of wheat flour in the production of wheat based baked products.

The optimal mixture design of RSM had been applied in the experimental design, statistical analyses and optmisation of composite flours [5, 6]. In order to determine blends with the best antioxidant and ACE inhibition capacities in addition to the least antinutrients contents, the optimal mixture design of RSM was applied in the experimental design, statistical analyses and optmisation of the mineral content [7]. The antinutritional and antioxidant properties of the optimum blends (comprising the least sodium but high potassium contents) were evaluated, and subsequently the blend with best antinutritional and antioxidant properties was used for cake production. The ACE inhibition capacities of the cake were evaluated.

2. Materials and Methods

2.1. Sample Collection

African oil bean seeds were purchased from Owena market, Osun State, Nigeria. Dried *Moringa* seeds were freshly harvested from Akure, Ondo State, Nigeria. Wheat flour and other baking ingredients were purchased from Oba market in Akure while all chemicals were of analytical grades.

2.2. Preparation of African Oil Bean Seed Flour

The seeds were manually dehulled, cotyledons sliced and dried for 8 h at 95°C in a thermostated oven (Model: MC-1959K, China). The seed was milled using a laboratory type hammer mill into fine particle size (210 µm) and then stored in an air tight jars at -4°C for subsequent processing [8].

2.3. Preparation of Moringa Seed Flour

Cleaned, dried *Moringa* seed kernels were comminuted into fine powder (210 µm) using Moulinex domestic food blender. The *Moringa* powder was stored in a sealed polythene at room temperature for subsequent analyses [9].

2.4. Experimental Design for Composite Flour

Optimal Mixture model of response surface methodology (RSM) (Design expert 8.0.3.1 trial version) was used for the experimental design. The variables were wheat flour (50-90%), *Moringa* seed flour (5-25%) and African oil bean (5-25%). The dependent variables were the zinc, iron, calcium, magnesium, sodium, potassium and phosphorus compositions.

2.5. Mineral Analyses of Composite Flours

Determination of zinc, iron, calcium, magnesium, sodium, potassium and phosphorus were done according to the method of AOAC [10]. The analysis of sodium and potassium were carried out using flame photometer. Calcium, magnesium, iron and zinc were analyzed using Atomic Absorption Spectrometer (Buck Scientific, Model 210VGP). Phosphorus was determined by using phosphor-vanado molybdate method.

2.6. Antioxidant Analyses of Composite Flours

2.6.1. Total Phenols

About 0.2 ml of the extract was mixed with 2.5 ml of 10% Folin ciocalteau's reagent and 2 ml of 7.5% sodium carbonate. The reaction mixture was subsequently incubated at 45°C for 40 mins and the absorbance was measured at 700 nm in the spectrophotometer, garlic acid was used as standard phenol [11].

2.6.2. Total Flavonoids

Colorimeter assay method was used [12]. The sample extract (0.2 ml) was added to 0.3 ml of 5% $NaNO_3$. After 5 mins, 0.6 ml of 10% $AlCl_3$ was added and thereafter, another 2 ml of 1 M NaOH was added to the mixture after 6 min, followed by 2.1 ml distilled water. Absorbance was measured at 510 nm while the flavonoid content was expressed as mg rutin equivalent.

2.6.3. 1,1-diphenyl–2-Picrylhydrazyl (DPPH) Scavenging Ability

The method described by Gyamfi *et al.* (1999) [13] with slight modifications was used. Various concentrations of the sample extract were mixed with 1 ml of 0.4 M DPPH in methanol, the mixture was left in the dark for 30 min and the absorbance measured at 516 nm. The DPPH scavenging ability was subsequently calculated.

2.6.4. 2,2'-Azino-Bis (3-Ethylbenzthiazoline-6-Sulphonic Acid (ABTS) Scavenging Ability

The ABTS was prepared by oxidation of ABTS (7 mmol/L) aqueous solution with $K_2S_2O_8$ (2.45 mmol/L) and left in the dark for 16 h while the absorbance was adjusted to 0.700 at 734 nm with ethanol. About 0.2 ml of the extract dilution was added to 2.0 ml ABTS solution; the absorbance were measured at 734 nm after 15 min. ABTS was calculated as trolox equivalent antioxidant capacity (TEAC) [14].

2.7. Anti-nutritional Analyses of Composite Flour

2.7.1. Tannin Determination

Finely milled raw samples (200 mg) were mixed with 10 ml of 70% aqueous acetone for the extraction process which lasted for 2 h at 30°C in water bath. Pigments and fats were first removed from the samples by extracting with diethyl ether containing 1% acetic acid. A 0.05 ml aliquot in test tubes was made up to 1.0 ml by adding distilled water, followed by the addition of 0.5 ml of the Folin ciocalteau reagent (Sigma Chemicals) and then 2.5 ml of sodium carbonate solution in order to evaluate the tannic equivalent.. The tubes were vortexed and absorbance recorded at 725 nm after 40 min. The amount of total polyphenols was then calculated from the standard curve [15].

2.7.2. Phytate Determination

Phytate was determined using the method of AOAC (2005) [16]. Phytate was extracted using dilute HCl and the extract mixed with Na_2EDTA–NaOH solution, and placed in an ion-exchange column. The extracted phytate was diluted with 0.7 ml NaCl solution and wet-digested with H_2SO_4 / HNO_3 mixture to release phosphate which was measured colorimetrically after reacting with ammonium molybdate solution. The amount of phytate in original sample was obtained as hexaphosphate equivalent.

2.7.3. Oxalate Determination

One gram each of finely ground samples were mixed with 7.5 ml of 1.5N H_2SO_4. The solution was carefully stirred intermittently with a magnetic stirrer for about 1 h after which the solution was filtered using a Whatman number 1 filter paper. Exactly 25 ml of the filterate was then collected and titrated hot (80-90°C) against 0.1 N potassium permanganate solution to a point where faint pink colour appeared and persisted for about 30 mins [17].

2.8. Cake Production

The proportion of ingredients used were the optimum composite flour from run 1 (the least antinutrients and second best antioxidants) (100 g), sugar (62.5 g), margarine (62.5 g), baking powder (5.7 g) and vanilla essence (three drops). The margarine and sugar were creamed manually for 5 min in a bowl until soft and fluffy. The egg was beaten for 3 min, added to the mixture and mixed manually for 5 min. Flour samples from various composite blends were separately sieved, and baking powder was added and mixed thoroughly manually until soft dough was formed. The dough was transferred to a greased baking pan and baked in a preheated oven at 200°C for 30 min [18].

2.9. Angiotensin I Converting Enzyme (ACE) Inhibition Assay

Different concentrations of the sample extracts and 50 µl of rabbit lungs ACE (EC 3.4.15.1) solution (4 mU/ml) were pre-incubated at 37°C for 15 min. About 150 µl of 8.33 mM ACE substrate [hippuryl-l-histidyl-l-leucine (HHL)] in 125 mM Tris–HCl buffer (pH 8.3) was added to the reaction mixture and incubated at 37°C for 30 min in order to initiate the enzymatic reaction. About 250 µL of 1 M HCl was later added in order to halt the reaction. Hippuric acid (Bz-Gly) produced was extracted using 1.5 mL ethyl acetate; the mixture centrifuged in order to separate the ethyl acetate layer. About 1 mL of the ethyl acetate layer was later transferred into a clean test tube, evaporated to dryness, the residue was re-dissolved in distilled water and its absorbance was measured at 228 nm [19]. The experiment was carried out in triplicates while the average concentration value was used to calculate the ACE inhibition and expressed as percentage inhibition using Eq. (1)

$$\%\, Inhibition = \left[\left(Abs_{ref} - Abs_{sam}/Abs_{ref}\right)\right] \times 100 \tag{1}$$

Where Abs_{ref} is the absorbance without the essential oil and Abs_{sam} is the absorbance of the extracts.

2.10. Statistical Analyses

All analyses were carried out in triplicates and the results of the triplicates were expressed as mean of triplicate determinations ± standard deviations. The SPSS 16.0 for windows computer software package was used for one way analysis of variance (ANOVA) and the Pearson correlation coefficients. Significance of the differences was ascribed at $p \leq 0.05$ for ANOVA. The difference in means was compared using the Duncan's new Multiple Range test. In addition, statistical analyses using RSM was carried out using the ANOVA, R^2 value, Adj R^2 values, p-value ($p \leq 0.05$), and lack of fit.

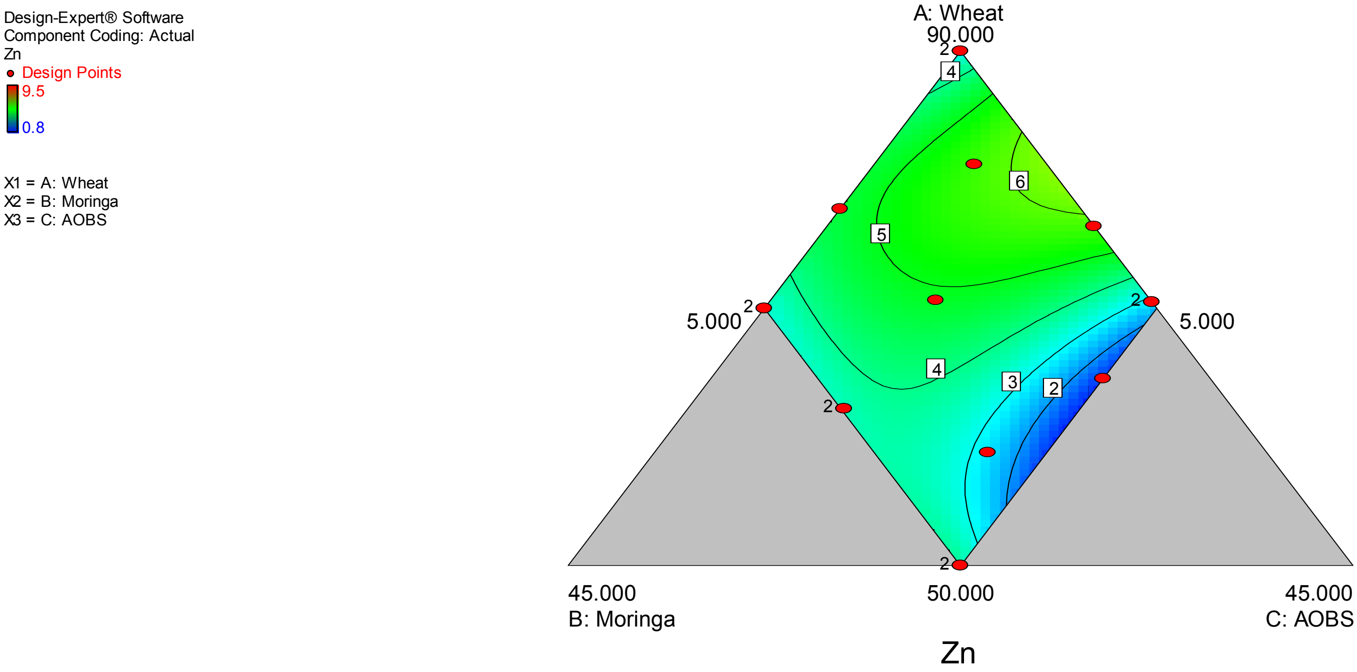


Figure 1. Contour plot showing the interactions between the variables with the response (zinc).

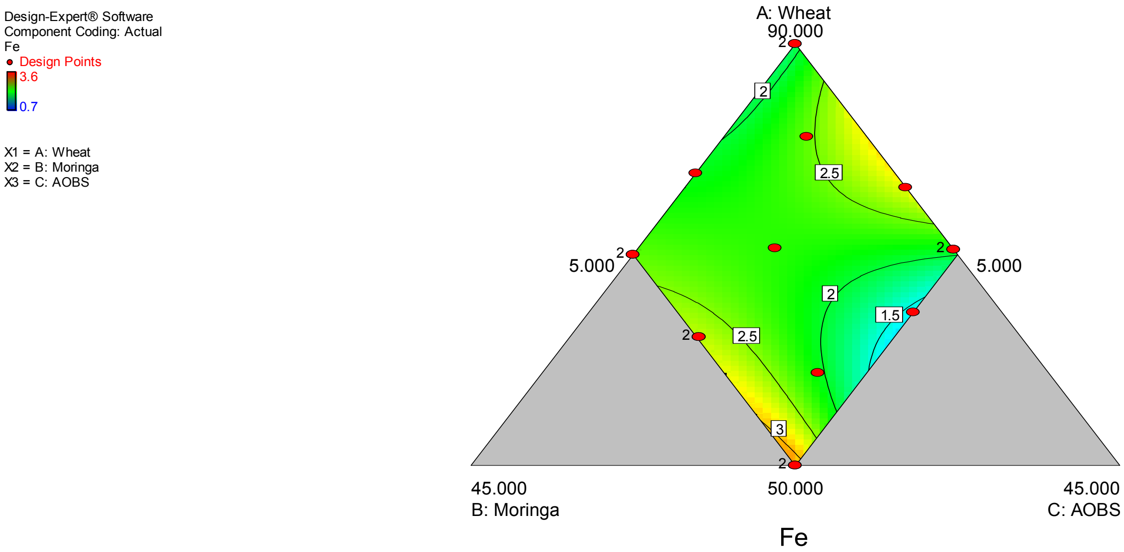


Figure 2. *Contour plot showing the interactions between the variables with the response (iron).*

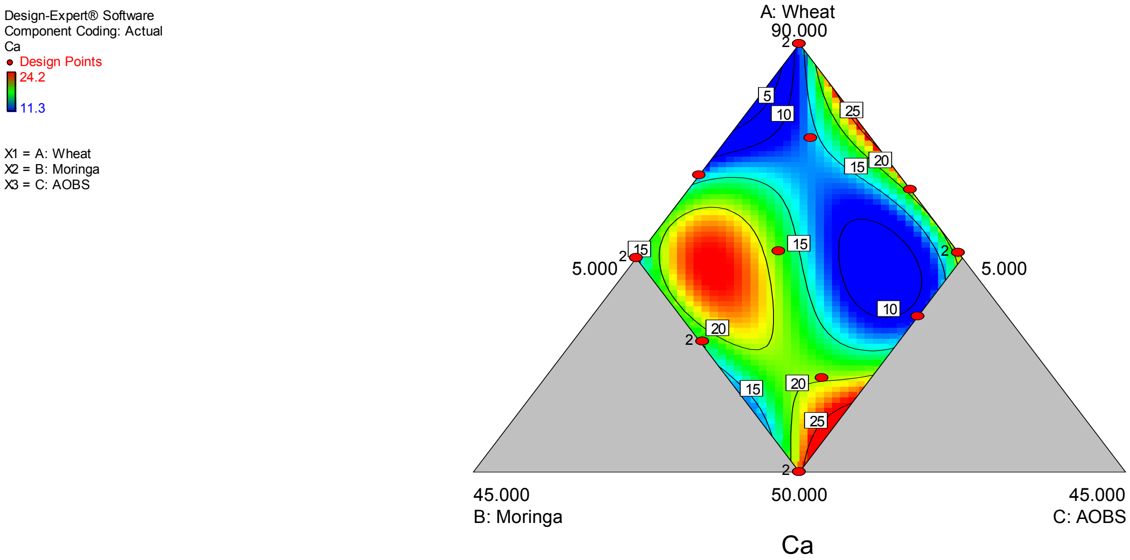


Figure 3. *Contour plot showing the interactions between the variables with the response (calcium).*

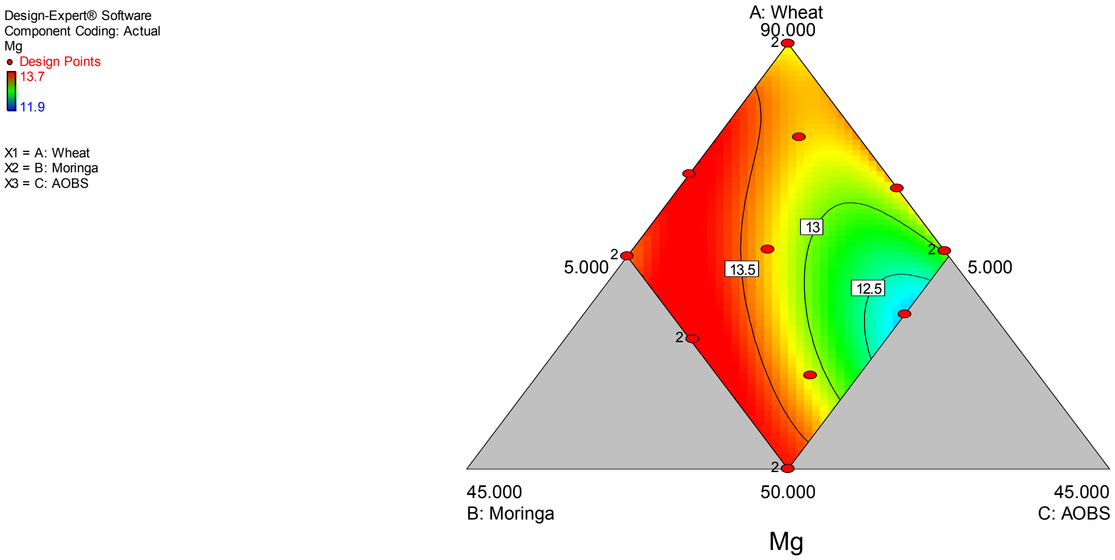


Figure 4. *Contour plot showing the interactions between the variables with the response (magnesium).*

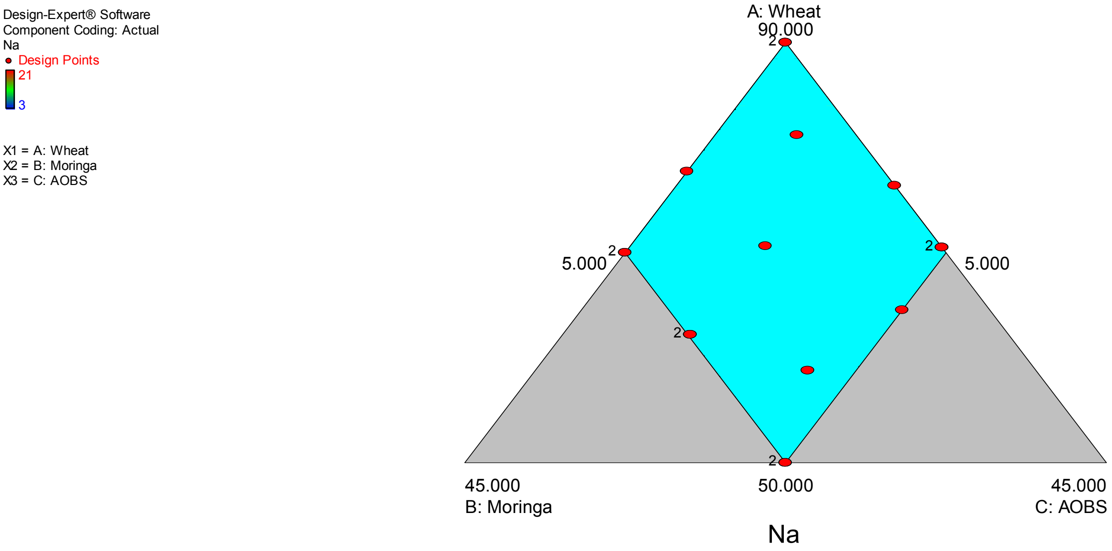


Figure 5. *Contour plot showing the interactions between the variables with the response (sodium).*

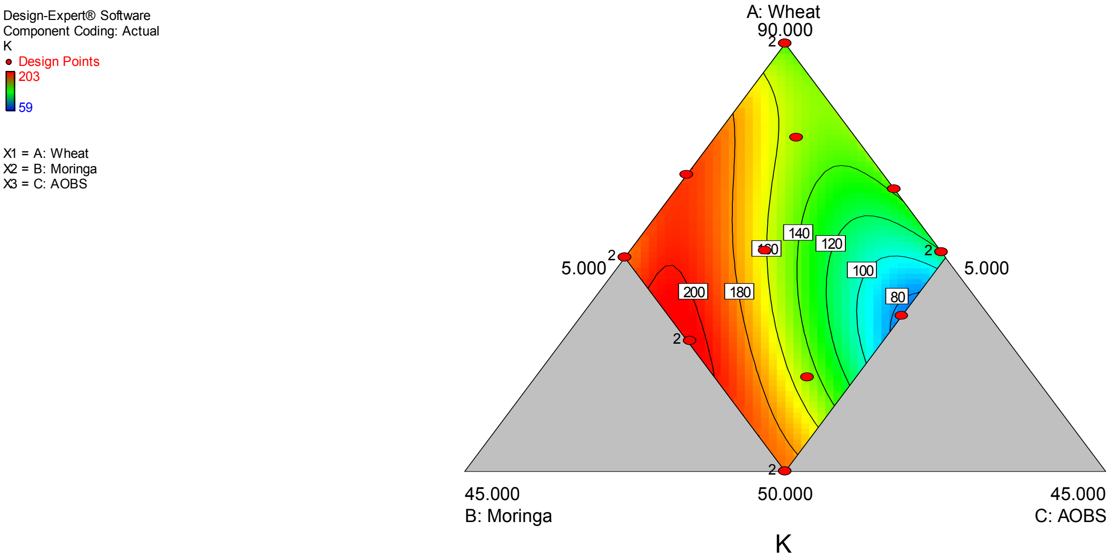


Figure 6. *Contour plot showing the interactions between the variables with the response (potassium).*

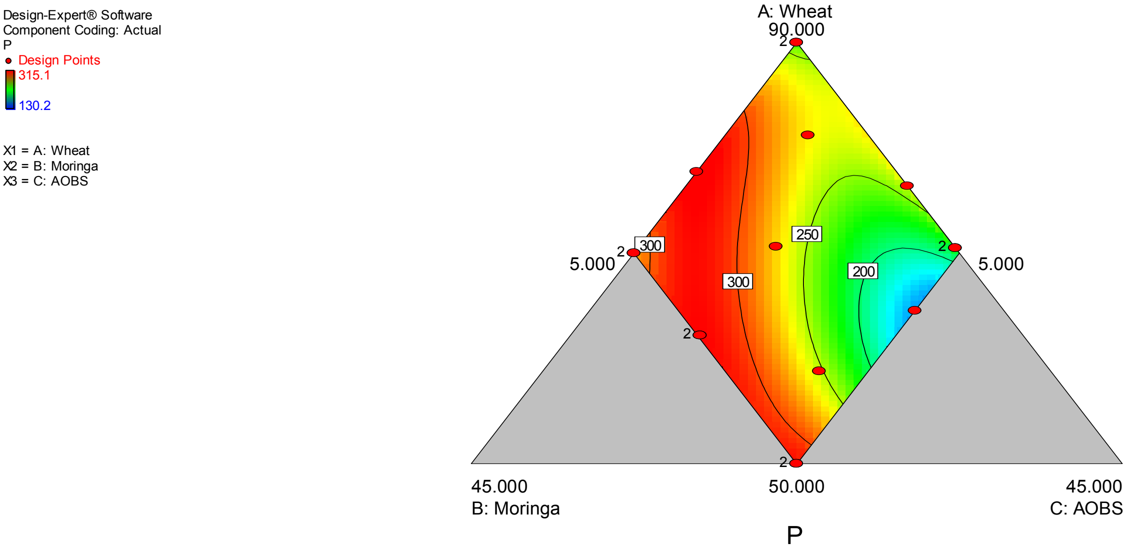


Figure 7. *Contour plot showing the interactions between the variables with the response (phosphorus).*

Table 1. *Antioxidant Properties of Optimized Flour Blend.*

Samples	Total flavonoids (mg/g)	Total phenols (mg/g)	DPPH (%)	ABTS (mol/g)
A	0.12 ± 0.01^{d}	1.21 ± 0.01^{d}	16.06 ± 0.00^{d}	7.36 ± 0.21^{d}
Run 1	3.64 ± 0.14^{b}	3.70 ± 0.06^{b}	42.10 ± 0.68^{b}	16.39 ± 0.12^{b}
Run 11	4.61 ± 0.03^{a}	5.05 ± 0.01^{a}	48.68 ± 3.63^{a}	19.45 ± 0.10^{a}
Run 15	1.59 ± 0.01^{c}	2.58 ± 0.27^{c}	36.71 ± 1.08^{c}	14.25 ± 0.19^{c}

*Means of triplicate determinations ± S.D. Means with different superscripts on the same column are significantly different at $p \leq 0.05$
*Control (A) = 100%Wheat flour
*Run 1 = 81.20%Wheat flour/8.68% Moringa seed flour/10.12% African oil bean seed flour
*Run 11 = 50%Wheat flour/25% Moringa seed flour/25%African oil bean seed flour
*Run 15 = 90%Wheat flour/5% Moringa seed flour/5%African oil bean seed flour

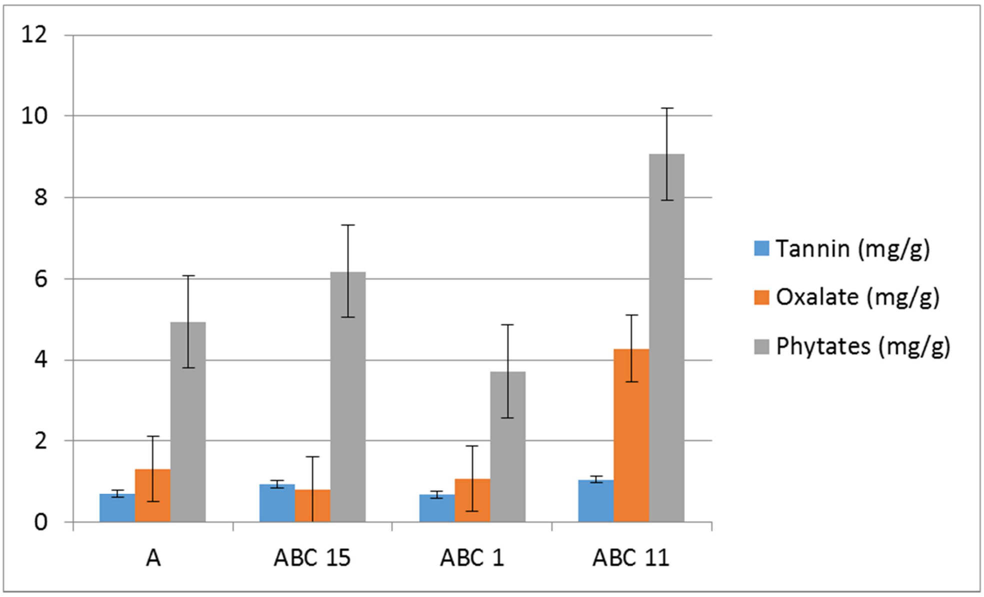
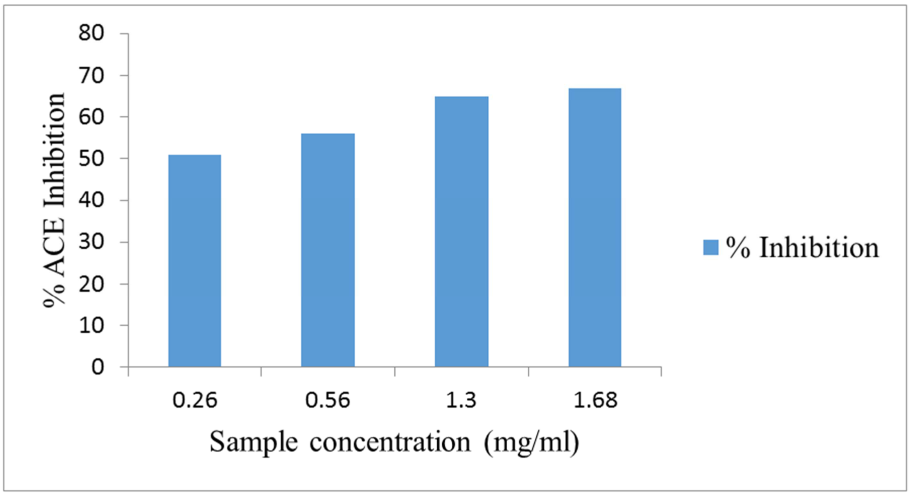


Figure 8. *Anti-nutritional properties of optimized flour blends.*

*Control (A) = 100%Wheat flour
*Run 1 = 81.20%Wheat flour/8.68% *Moringa* seed flour/10.12% African oil bean seed flour
*Run 11 = 50%Wheat flour/25% *Moringa* seed flour/25%African oil bean seed flour
*Run 15 = 90%Wheat flour/5% *Moringa* seed flour/5%African oil bean seed flour



*Run 1 = 81.20%Wheat flour/8.68% *Moringa* seed flour/10.12% African oil bean seed flour

Figure 9. *ACE inhibition of cake from run 1 as affected by increase in sample concentration.*

3. Results and Discussion

3.1. Minerals Composition of Composite Flour

The zinc content ranged from 0.8 – 9.5 mg/100g. The contour plot (Figure 1) showing the interactions between the variables and zinc content indicated that wheat flour had highest positive effect on the zinc content, followed by Moringa flour. It has been found that wheat flour contained 13.08 mg/100g zinc content [20]. The model (special cubic) and model terms (linear mixture, AC and BC) were significant ($P \leq 0.05$) while the R^2 and the adjusted R^2 values were 0.4514 and 0.0856 respectively. Specifically the adjusted R^2 value showed that the composite flour is not a good source of zinc. This could be as a result of low content of zinc in *Moringa* flour. The final equation representing the effect of the variables on zinc is given in Eq. (2). The equation showed that *Moringa* (B) and African oil bean (C) had negative coefficients.

$$Zinc = 3.15A - 8.23B - 27.07C + 23.56AB + 57.40AC + 85.65BC - 111.44ABC \tag{2}$$

Where A = wheat flour, B = *Moringa* seed flour, C = African oil bean seed flour

Iron content ranged between 0.7 and 3.6 mg/100g. The contour plot (Figure 2) showed that Moringa flour had the highest positive effect on the iron content. The special cubic model and model terms (linear mixture, AC) were significant ($P \leq 0.05$) while the R^2 and the adjusted R^2 values were 0.6207 and 0.3678 respectively. Moringa seed had been shown to contain considerate iron content of about 3.10 mg/100g [21]. On the other hand, African oil bean had 0.013 mg/100g iron content [8] while wheat had 1.95 mg/100g iron content [22]. The final equation (Eq. 3) indicated that while Wheat and Moringa flours had positive coefficients, AOB flour had negative coefficient.

$$Iron = 1.89A + 3.86B - 6.04C - 1.90AB + 16.34AC + 17.10BC - 33.41ABC \tag{3}$$

Where A = wheat flour, B = *Moringa* seed flour, C = African oil bean seed flour

The calcium contents varied from 11.3 to 24.2 mg/100g. Moringa flour was the best source of calcium, and then wheat flour (Figure 3). The design model (special cubic) and model terms (linear mixture, AB, BC, ABC, AB(A-B), AC(A-C), BC(B-C)) were significant ($P \leq 0.05$) while the R^2 and the adjusted R^2 values were 0.9807 and 0.9517 respectively. These as well as the final equation (Eq. 4) were indication that the composite flour is a good source of calcium.

Magnesium content ranged from 11.9 to 13.7 mg/100g. Wheat and Moringa flours are rich in magnesium (Figure 4). A magnesium content of 26.33 mg/g had been reported for Moringa flour [23]. A magnesium content of 0.47 mg/100g for AOB has also been reported. The R^2 and the adjusted R^2 values were 0.8206 and 0.6156 respectively. This in effect indicated that the magnesium content of the composite flour is above average. In accordance with the final equation (Eq. 5), all the composite flour components (A, B and C) had positive coefficients.

$$Calcium = 12.57A - 427.58B + 295.40C + 885.60AB - 525.43AC + 358.81BC - 605.19ABC - 638.84AB(A-B) + 427.69AC(A-C) + 900.89BC(B-C) \tag{4}$$

$$Magnesium = 13.18A + 10.64B + 9.78C + 6.31AB + 6.01AC + 13.84BC - 48.74A^2BC + 52.83AB^2C - 77.90ABC^2 \tag{5}$$

Where A = wheat flour, B = *Moringa* seed flour, C = African oil bean seed flour

Sodium content ranged from 3.00 – 21.00 mg/100g. A sodium content of 3.5 mg/100g had been reported for wheat flour [24]. The result of the ANOVA showed there were no significant model terms, indicating the raw materials were not good sources of sodium. A low-sodium containing diet would be useful as an antihypertensive food. This is because sodium contributes to high blood pressure. In addition, the R^2 and the adjusted R^2 values were 0.0000 each; which further buttressed the negative effect of the composite flour on sodium content. The contour plot (Figure 5) showing the effects of raw materials on sodium content also indicated that the raw materials were not good sources of sodium. The final equation representing the effect of the variables on sodium is given in Eq. (6). All the composite flour components (A, B and C) had no coefficient, showing that they do not support sodium at either the linear, quadratic or higher models.

$$Sodium = 7.44 \tag{6}$$

Potassium values ranged from 59 – 203 mg/100g. The special quartic model and its terms (linear mixture, BC and ABC^2) were significant ($P \leq 0.05$). The results of R-squared (0.9318), adjusted R-squared (0.8539) values and contour plot (Figure 6) indicated that wheat and *Moringa* flours were good sources of potassium. Potassium helps control heart rate and blood pressure by countering negative effects of sodium [25]. The final equation given in Eq. (7) showed that wheat and potassium flours had positive coefficients.

$$Potassium = 140.55A + 2.76B - 91.42C + 454.55AB + 364.27AC + 925.96BC - 3057.26A^2BC + 3803.29AB^2C - 5412.29ABC^2 \tag{7}$$

Where A = wheat flour, B = *Moringa* seed flour, C = African oil bean seed flour

Phosphorus content ranged between 130.2 and 315.1 mg/100g. The contour plot (Figure 7) showing graphical interactions of the variables and phosphorus indicated that *Moringa* seeds flour was a major source of phosphorus. The result of the ANOVA also indicated that the linear mixture components were significant ($p \leq 0.05$) model terms while the R^2 and the adjusted R^2 values were 0.8315 and 0.6390 respectively. The final equation representing the effect of the variables on phosphorus as given in Eq. (8) also indicated *Moringa* flour (A) had positive coefficient while the other components had negative coefficients.

$$Phosphorus = 238.03A - 94.93B - 167.66C + 855.48AB + 731.25AC + 1781.31BC - 4970.13A^2BC + 4516.78AB^2C - 8128.29ABC^2 \quad (8)$$

Where A = wheat flour, B = *Moringa* seed flour, C = African oil bean seed flour

3.2. Optimized Blends

The optimum blends (best three), based on the minerals composition were Run 1 (81.20%Wheat/8.68% *Moringa* seed/10.12% African oil bean seed flours), Run 11 (50% Wheat / 25% *Moringa* seed / 25% African oil bean seed flours) and Run 15 (90%Wheat/5% *Moringa* seed/5%African oil bean seed flours). The results of further analyses on the optimum blends are discussed below.

3.3. Antioxidant Properties of Optimized Flour Blends

The results of the antioxidant properties of the optimized flour blends are presented in Table 1. There was gradual increase in flavonoid content from 0.12 mg/g in the control (100% wheat flour) to a range of 1.59 – 4.61 mg/g in the flour blends. This increase was as a result of the inclusion of African oil bean and *Moringa* seed flours. Flavonoids have been reported to have anti-inflammatory, anti-allergic and anti-cancer activities [26]. Total phenols, DPPH and ABTS followed the same pattern as total falavonoids, The blends with the highest moringa and African oil bean seed flours (run 11) had the best antioxidant behaviour. DPPH radical scavenging activity of 34.05% had also been reported in *Moringa* seed flour [27]. The gradual increase in antioxidant properties observed in the flour blends suggests that African oil bean seed and *Moringa* seed would be a good combination of raw materials in healthy snacks development.

3.4. Anti-nutritional Composition of Optimized Flour Blends

The results of anti-nutritional properties for selected optimized flour blends are shown in Figure 8. The tannin content of run 1 was not significantly ($p \geq 0.05$) different from the control (100% wheat). In addition, run 1 had lower oxalate and phytate contents than the control (100% wheat flour). This low values of antinutrient in run 1 (lower than 100% wheat flour) is an indication that run 1 could be used for product development while guarantee the safety of the product. Runs 11 and 15 had higher antinutrient contents than the control (100% wheat flour). Run 1 may therefore not have negative effects on nutritional value; protein and minerals availability of the food products developed from it [28].

3.5. Cake Production

Composite flour from run 1 was used to produce cake due to its lowest antinutrient contents,

though it has the second best antioxidants activity. In addition run 1 had the best zinc, phosphorus and sodium contents. The Na/K value had been shown to be lower than 1 for all the optimum blends. Run 11 actually had the overall best antioxidant properties but with antinutrients contents higher than 100% wheat flour (control).

3.6. Angiotensin-I Converting Enzyme (ACE) Inhibition of Cake Baked with the Best Minerals, Antioxidants and Antinutritional Compositions

The result of ACE inhibition of cake from run 1 is shown in Figure 9. Increase in concentration of extract within a range of 0.26 – 1.68 mg/ml resulted in a gradual increase of 51 – 67% in the percentage inhibition of ACE. This suggests that the higher the intake of cake baked from the composite flour, the higher the percentage ACE inhibition, resulting in reduction of Angiotensin II production (a major factor in induction of high blood pressure). It has been reported that consumption of fermented seed of African oil bean seed for two weeks by a hypertensive patient resulted in the reduction of blood pressure from 190/110 mm/Hg to 100/70 mm/Hg. In addition, Moringa seed and leaves have been reported to possess ACE inhibition ability [29]. The ACE inhibition for 100% wheat flour had been reported to be 10.29% [1]. Comparing the ACE inhibition of 100% wheat flour with run 1, the addition of Moringa and AOB flour must have accounted for the higher values found in run 1. Thus, the high inhibition (50% to 68%) observed in this work would be as a result of the combined effect of Moringa and African oil bean flours inclusion.

4. Conclusions

Addition of Moringa seed flour to the composite flour greatly enhanced its nutritional composition with the exception of the sodium content that was reduced. Composite flour blend consisting 81.2% wheat flour, 8.68% moringa seeds flour and 10.12% African oil bean flour showed antioxidant properties that is over 200% higher than the control (100% wheat). In addition, the anti-nutrient contents of run 1 are lesser than control (100% wheat). The ACE inhibition had a high value that is greater than 50%. So a composite flour consisting wheat, Moringa seed and AOB seed flours in ratios 81.2%:8.68%:10.12% will be suitable in the production of cake of high antioxidant characteristics with a high ACE inhibition potential which might serve as a very good antihypertensive functional food product.

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