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EFFECT OF SOAKING AND GERMINATION ON THE ANTINUTRITIONAL FACTORS OF LIMA BEAN SEED FLOUR

[1]ADEBAYO, S. F, [2]OKOLI, E. C, [2]ABRAHAM, F. M

[1*]Department of Food Technology, Federal Polytechnic, Ado-Ekiti, Ekiti State Nigeria
[2]Department of Food Science and Technology, Ebonyi State University, Abakaliki, Nigeria
*e-mail: adebayo.stella@yahoo.com

Abstract: **The effect of soaking and germination time on the anti-nutritional properties of lima bean seed was investigated. The seeds were soaked (6-24hrs) and germinated (24-120hrs), dehulled, dried milled into flour. The flour samples were analyzed for anti-nutrients such as phenol content, phytate, tannin, saponin, trypsin inhibitors, and cyanogenic glycosides. Response surface methodology was used to model the reduction in the anti-nutritional factors; the study evaluated the optimum condition for reduction of the anti-nutrients from 6-12 hrs soaking and 24-120hrs of germination. The result showed that the soaking and germination decreases significantly ($p<0.05$) the phenolic content from 28.26 to 18.53mg/g; phytate from 1.53mg/100g to 0.75mg/100g; alkaloids from 26.62mg/g to 8.47mg/g; oxalate from 0.91mg /100g to 0.08mg/100g; trypsin inhibitors from 5.41TIU/mg to 2.40TIU/mg; saponin from 17.34% to 7.06% and cyanogenic glycosides from 10.45mg/kg to 5.82mg/kg. The model is significant in evaluating the optimum condition of reducing the anti-nutrients.**

Keywords: **lima bean, soaking, germination, anti-nutrients, response surface methodology (RSM).**

1. INTRODUCTION

Legume grains have been playing an important role in the traditional diets of human beings throughout the world and are used as therapeutic agents in the old traditional medicine. In Mediterranean regions, the grain legumes have traditionally been consumed for centuries and constitute an element of the Mediterranean diet (Guarrera, 2005). Legumes are good sources of proteins, ion chelators, free radical scavenger, and thus prevent oxidative damage to biomolecules, such as DNA, lipids, proteins (Rice-Evans *et al.,* 1996). It has been shown that the consumption of dietary antioxidants, as found in legume seed proteins provided protective effects for several chronic diseases like cardiovascular diseases, cancer, obesity and diabetes, hypercholesterolemia (Troszynska *et al*., 2002). There are several different health dietary fibers, low glycemic indexes, low levels of fat (2-5%), and high amounts of carbohydrates (55-60%) (Rochfort and Panozzo, 2007). Legume protein can act as free radical reducing agents, metal benefits associated with the regular consumption of legumes; apart from the fact that it constitutes one of the most abundant and least expensive sources of protein in the (human/animal diet. Some Legumes have been used in the reduction of cholesterol levels in the blood (Xiong, 2010), some serve in regulating the levels of blood glucose for diabetics, preventing cancer and reducing its risks, lowering of blood pressure, improving the function of the colon (Makinen *et al*., 2012). Based on this functional role of a leguminous seed plant, efforts are geared towards maximizing the potential of these seed for maximum output. Despite the functional role served by legumes, its function is limited to the anti-nutritional factors content, bitterness as well as astringency.

Lima bean (*Phaseolus lunatus* L) sometimes called butter beans, Chad beans and Pakala, by the Yoruba tribe of Nigeria are flat –shaped creamy white- colored beans with the potential for good yields in the tropics. Lima bean has a desirable

agronomic, and nutritional characteristic (Oyawoye and Ogunkule, 1998). According to NAS (1979) and Akinmutimi (2001), Lima bean is widely available and can thrive in lowland tropical rain forests and on poor soils where most crops cannot grow well. Lima bean has a crude protein content of about 22% which yields between 3000kg and 5000kg of seeds per hectare (NAS, 1979). The farmers in the area of cultivation commonly consume the crop where it contributes to the protein intake of the consumers.

However, like other tropical legumes, lima beans seeds contain some anti-nutritional factors which affect its utilization. These factors which include phytins, and tannins (Kay, 1979; Akinmutimi, 2001), hydrogen cyanide and trypsin inhibitors (Ologhobo and fetuga, 1983), has limited their nutritional quality and their application in human food systems. These underscore the need to explore ways of improving the nutritional value of lima bean as well as enhancing its therapeutic potential and one out of such processing method is soaking and germination. The objective of this work is to combine the effect of soaking and germination in reducing the anti-nutrients of lima bean seed to enhance its utilization.

2. MATERIALS AND METHODS

2.1 Sample preparation

The brown variety of lima beans (*Phaseolus lunatus*) used for this work was purchased from Ikere-Ekiti, Ekiti State Nigeria. Lima beans seeds were cleaned to remove immature grains and foreign materials. The sorted grains were washed in clean tap water to remove dust and stone, steeped and germinated in a germinating container in accordance with Table 1 using Design Expert Software version 8.0.3. The lima bean seeds were germinated by soaking the cleaned seeds in distilled water (1:5w/v) based on the conditions as generated in Table 1, at room temperature (30±2)^{o}C. The water was drained off and imbibe seeds were germinated by layering them over a moistened cotton wool to keep moisture constant in a single layer of the seed, germination was carried out at 24-120hrs with soaking at 6-24hrs respectively. After germination each batch was dehulled and sprouts washed and dried at 60±2^{o}C for 08hrs the dried sample were ground to pass through a sieve mesh of 0.75-1mm and then packaged in air-tight jars kept in the refrigerator at 4^{o}C until used for analysis.

2.2 Analysis of anti-nutritional factors

2.2.1 Determination of alkaloids

The determination of alkaloids was carried out by alkaline precipitation through Gravimetric method as described by AOAC, (2005). Two gramme of the sample was soaked in 20ml of 10% Ethanolic acetic acid. The mixture was allowed to stand for 4hrs, at room temperature, and filtered through Whatman filter paper no. 40. The filtrate was concentrated by evaporation over a steam bath to ¼ of its original volume. Alkaloids were precipitated by adding drops of ammonia and in excess to the extract and the resulting alkaloids precipitate recovered by filtration. The filtrate was washed with 1 % ammonia solution and dried in the air oven at 60^{o}C for 30min, cooked in a desiccator and reweighed. The weight of the alkaloids was determined by difference and expressed as a percentage of the weight of the sample analyzed as

$$\% \text{ alkaloids} = \frac{W2-W1}{Weight\ of\ sample} \times 100$$

2.2.2 Determination of Saponin

Saponin content of the sample was determined according to the method of AOAC (2005). About two grammes of the sample was mixed with 50ml of 20% aqueous ethanol solution. The mixture was incubated at 55^{o}C in a water bath with periodic agitation for 90 minutes. It was then filtered through Whatman filter paper No 40. The residue was extracted with the 50ml portion of the 20% aqueous ethanol and both extract were pooled together. 40ml 0f the extract was transferred into a separating funnel at 90^{0}C with 40ml of diethyl ether and shaken vigorously. The separation was by partition during which the ether layer was discarded while the aqueous layer was reserved. Re-extraction by partition was done repeatedly until the aqueous layer became clear in color. The saponin were extracted with 60ml of normal butanol solution and evaporated to dryness in a pre-weighed evaporating dish at 60^{0}C for 30min in the oven (to remove any residual solvent), cooled and re-weighed. The experiment was repeated twice to get the average. The saponin content was determined by difference and calculated as a percentage of the original sample as follows.

$$\% \text{ Saponin} = \frac{W2-W1}{Wt\ of\ sample} \times \frac{100}{1}$$

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2.2.3 Determination of hydrocyanic acids

This was determined by the alkaline picrate colorimetric method of Balagopalam, *et al.*,(1988).

A measured weight of each sample (0.5g) was dispensed in 200mls of distilled water in a conical

Flask and mixed properly. A strip of alkaline picrate paper was suspended over the mixture with the aid of a rubber stopper in a way that the paper did not touch the surface of the mixture. The set up was incubated for 18 hours at room temperature and the end of the incubation period; the picrate paper was carefully removed and eluted in 60ml of distilled water. Meanwhile, a standard cyanide solution (KCN) was prepared and treated as described above. The absorbance of these elutes from the standard and the samples were measured in a spectrophotometer at 540nm with the reagent blank at zero. The cyanide content (HCN mg/kg) was calculated using the formula below:

$$\text{HCN (mg/kg)} = \frac{100}{1} \times \frac{Au}{As} \times C \times D$$

2.2.4 Determination of Total Phenolics

The total phenol content of the sample was determined using the method of Makkar *et al.*, (1993). The sample (50 µL) was put in test tubes and the volume made up to 500 µL using distilled water. Then, 250 µL of Folin-Ciocalteu reagent was added into the test tube followed by 1.25 ml of 20 % sodium carbonate solution. The tube was vortexed before incubated in the dark for 40 minutes. Absorbance read at 725 nm using a spectrophotometer.

2.2.5 Trypsin Inhibitors

Trypsin inhibitors were determined according to the procedure as described by Arntified *et al.*, (1985). 0.5g of the sample was dispensed in 50mls of 0.5M NacL solution and shaken for 30min at room temperature. The mixture was centrifuged and the supernatant was used as the extract. Assay for trypsin inhibitor activity involving mixing a portion (1ml) of the extract with 90mls of 0.03% Trypsin substrate (BAPA) in a test tube containing 1ml of 0.6% trypsin enzyme solution. After mixing, the mixture was allowed to stand for 15min before absorbance was read at 410nm in a spectrophotometer. A control that consists of a 1ml enzyme solution in 9mls of trypsin substrate (BAPA) but no extract was set up as describe and its absorbance was measured. Trypsin inhibitors activity was calculated using the formula below

$$\text{TUI/mg} = \frac{absorbance\ of\ sample}{absorbance\ of\ standard} \times 0.01\text{F}$$

2.2.6 Tannin

The tannin content was determined using the method as described by Makkar *et al.*, (1993) for non-tannin phenolics, 100mg PVPP was weighed in to test tubes before being added with 1.0ml distilled water and 1.0ml of the extracted sample. The tubes were vortexed and kept at 4^0 C for 15min. The tubes were vortexed again and centrifuged at 3000 rpm for 10min. The supernatant was collected and measured for absorbance at 725 nm using a spectrophotometer. The tannin content was calculated as

Total phenolics- non tannin phenolics = tannin.

Total phenolic and tannin content was expressed as gallic acid equivalents through the calibration curve of gallic acid with the concentration range of 0-100mg/ml.

2.2.7 Determination of Phytates

The phytic acid content of the sample was determined using the method Hang and Lantzseh (1983). One g of the sample was extracted with 100mls of 0.2HCl solution. The extraction was done by shaking the mixture at room temperature for 30min and filtering with Whatman No 40 filter paper to obtain the extract. 0.5ml of the extract was mixed with 1ml of standard $FeCl_3$ solution in a test tube. The test tube was covered and boiled for 30min and cooled in ice for 15min before allowing to it attain room temperature. Then it was centrifuge at 3000 x g for 30min. Then 1ml of portion the of the supernatant was treated with 1.5ml bipyridine solution. Meanwhile, the standard phytic acid solution was prepared. 1ml of it and 1ml distilled water were put in separate test tubes and treated as discussed above. The second as standard and

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reagent blank respectively. The absorbance of each was measured at 519nm in a spectrophotometer with the reagent blank at zero. The phytic acid content was calculated as

$$\% \text{ phytic acid} = \frac{100}{W} \times \frac{au}{as} \times C \frac{Vf}{Va} \times D$$

2.2.8. Determination of oxalates

Oxalate was determined using the method as described by Sanchez-Alonso and Lachica, (1987), about one gramme of the sample was placed in 250cm^3 volumetric flasks,90ml of distilled water and 10cm^3 of 6M HCl was added. The mixture was then warmed in a water bath at 90^{o}C for 4hrs and the digested sample centrifuged at 2000rpm for 5mins. The supernatant was then diluted to 250cm^3, three 50cm^3 aliquots of the supernatant was evaporated to 25cm^3, the brown precipitates were filtered and washed. The combined solution and washings were then titrated with colour of methyl orange changed to yellow. The solution was then heated in a water bath to 90^{o}C and then centrifuged. Precipitates were washed with hot 25% H_2SO_4 diluted to 125ml with distilled water and titrated against 0.05M $KMnO_4$. 1ml 0.05M $KMnO_4$ = 2.2mg Oxalate

2.3 Experimental Design.

Variation effects in soaking time and germination time were analyzed using Response surface methodology with a central composite design. The independent variables studied were germination time (24-120hrs) and soaking time (6-24hrs). Symbols and coded factors levels for these variables are presented below in Table 1

Table 1: Experimental layout

Independent variables		Levels of combinations				
Coded	Real	-2	-1	0	1	2
X_1	soaking time	2.27	6	15	24	27.73
X_2	germination time	4.12	24		72	120
		139.88				

2.4 Statistical analysis

The analysis was performed using Design Expert (version 8.0.3, State-Ease, Inc. Minneapolis 2010) Software. Model significance ($p<0.05$), lack of fit, and adjusted regression coefficient (R^2) which indicate the model fitness were determined from the analysis. The generalized regression model was $\gamma = b_o + AX_1 + BX_2$; where γ= predicted response; X_1 and X_2 are the coded variables and b_o as regression coefficients. The resulting models were tested for significance using analysis of variance (ANOVA) and R^2 regression coefficient calculated at 5 % significant level.

3. RESULTS AND DISCUSSION.

The effect of soaking and germination on the anti-nutritional qualities of lima bean is presented in Table 2. The total phenol content in the raw seed was 16.93 mg/g, Soaking and germination time bring about a significant ($p<0.05$) increase in the phenolic content of lima bean seed flour. The highest value of (28.26 mg/g) was observed in lima bean seed soaked and germinated for 24/24 hrs respectively. However, as the germination time increases there is a significantly ($p<0.05$) decreased in the value of the processed seed. The linear and quadratic effects of the soaking and germination time were significant (Table 2). The response surface plot for polyphenol showed that as the germination time increased with increasing soaking time the bottom of the ridge descended (fall), showing a reduction in the phenol content. The result also shows that the model is significant with an F-value of (<0.05). The regression coefficient R^2 and adjusted R^2 values are 96.86 and 94.60 % respectively (fig.1), as recorded. According to Mugendi *et al.*, (2010), high content of polyphenol is undesirable for human consumption, because they interfere with digestion and absorption of dietary protein in the body therefore, lower levels of polyphenol exhibited in Lima seeds during germination of the seed could have been attributed to leaching out of the phenolics from the seeds under the influence of adsorbed solution (Vijayakumani *et al.*, 1998) would make the germinated seed beneficial in nutrition and health.

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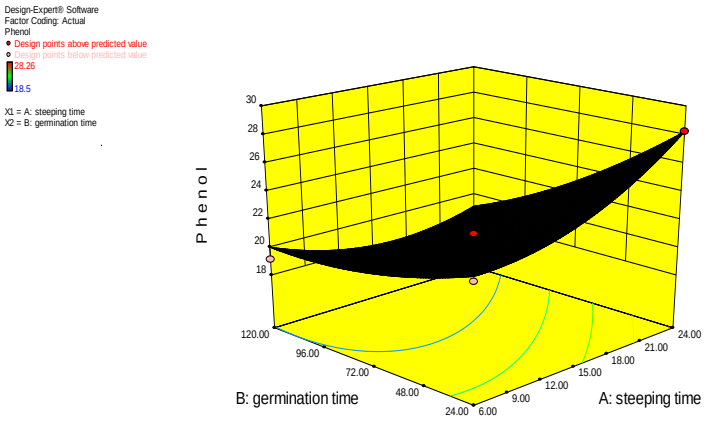

Figure 1. Response surface plot for total phenol content in lima bean seed

The alkaloid content of the raw seed was 29.03 mg/g Table 2. Soaking and germination significantly ($p<0.05$) reduce most of the alkaloids present in lima beans seed. This was revealed in the sample soaked for 2.27hrs and germinated for 72hrs (26.62mg/g). Longer soaking time of 27.73hrs and 72hrs of germination reduced the alkaloid content to 8.47mg/g, similarly, 24hrs of soaking and 120hrs of germination had 9.78 mg/g alkaloid contents. This shows that alkaloids are water-soluble anti-nutrients which easily hydrated in water. The finding is in agreements with the report of Philips and Abbey (1989) that water soluble anti-nutrients such as glycosides, tannin, alkaloids, oligosaccharides, and phytates leach out in the water during soaking. The model was not significant. The F- value was 0.1399, the R^2 0.3252 adjusted R^2 0.3252, and the non-significant lack of fit was significant.

The oxalate composition in the raw Lima bean flour was 1.02mg/100g and after processing ranged between 0.08- 0.86 mg/100g. The quantity of oxalate present in the flour was small and soaking and germination for 6/120hrs significantly ($p<0.05$) reduced the oxalate content to 0.08mg/100g. The decrease in oxalate during germination of lima bean is similar to the report of Pal *et al*., (2016) that reduction in oxalate is due to the activation of oxalate oxidase which breakdown oxalic acid into carbon dioxide and hydrogen dioxide to release calcium. The surface linear model was significant ($p<0.05$) with soaking and germination conditions. The F-value less than 0.05, with a non-significant lack of fit showing the adequacy of the model, the values for R^2 , and adjusted R^2 were 0.6610 and 0.5932. The response surface plot show a decline in the ridge as germination increases with time (fig. 2), to give a fall in the ridge signifies a reduction in oxalate content of the soaked and germinated lima bean.

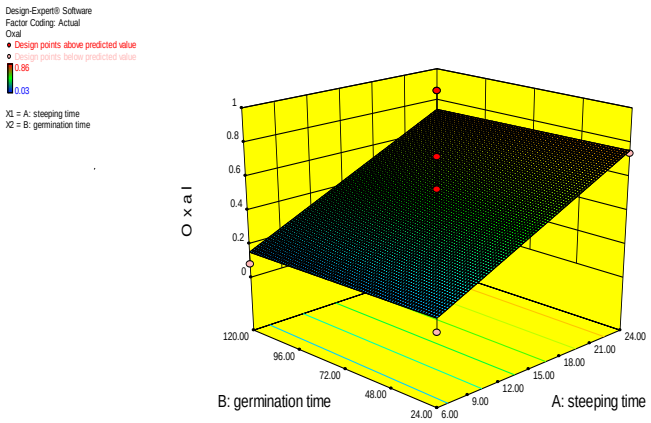

Figure 2. Response surface plot for oxalate content in lima bean seed.

The saponin content of the raw lima bean seed was 18.31 %, soaking and germination have significantly ($p<0.05$) reduced saponin from 18.31 % to 7.06 %. Soaking for 27.73hrs and germination for 72hrs reduced the saponin content considerably to 7.06 %. Similarly, soaking for 2.27hrs and germination for 72hrs was 16.30 %. The surface linear model

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was significant with an F-value of less than 0.05, the values for R^2 and adjusted R^2 as 0.5537 and 0.4644 the processing parameter was effective in reducing saponin content in lima bean seed flour, signifying that a high level of reduction is achieved with soaking.

Trypsin inhibitors content of raw lima bean seed flour was 6.50 TIU/mg, soaking and germination has significantly ($p<0.05$) decreased the value from between 5.41TIU/mg to 2.40 TIU/mg. This was in range with the report of Egbe and Akinyele, (1989) for lima bean seed flour. Trypsin inhibitors were reduced significantly ($p<0.05$) at a longer soaking time of 24hrs and 120hrs of germination (2.40TIU/mg) and the highest value (5.41 TIU/mg) at a shorter germination period of 24hrs/24hrs. The model was significant at ($p<0.05$). The values for R^2 and adjusted R^2 are 0.9273 and 0.8753 respectively (fig. 3). The response surface plot for trypsin inhibitors showed a decline in the graphic range signifying a decrease as germination time increases.

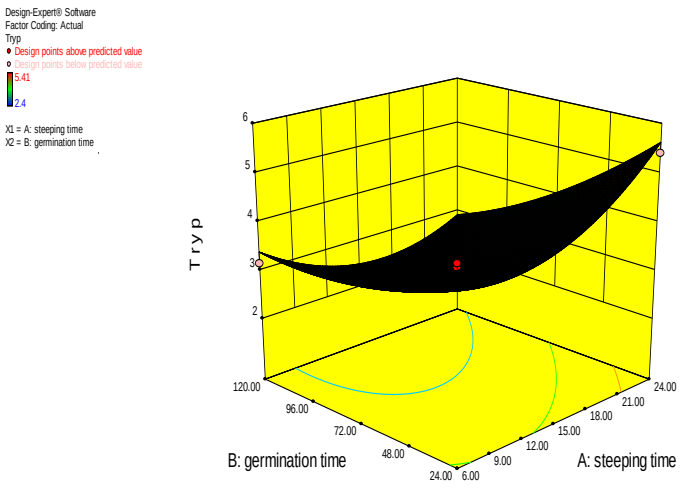

Figure 3. Response surface plot for Trypsin inhibitors in lima bean seed

The phytate composition of the raw sample was 2.30 mg/100g; this was reduced considerably upon soaking and germination from 1.53mg/100g to 0.75mg/100g. The composition was higher in 24hrs /24hrs (1.53mg/100g) and least in 24hrs/120hrs. This shows germination for a longer period has reduced the phytate content of the bean seed. In this case germination was a significant model term ($p<0.05$). The R^2 and adjusted R^2 values are 0.5183 and 0.4219. A decrease in phytate has been attributed to leaching out effect during hydration (Belevia *et al*., 1993). Removal of phytate from legumes during germination is attributed to enzymatic (phytase) hydrolysis of phytate followed by diffusion (Bau *et al*., 1997).

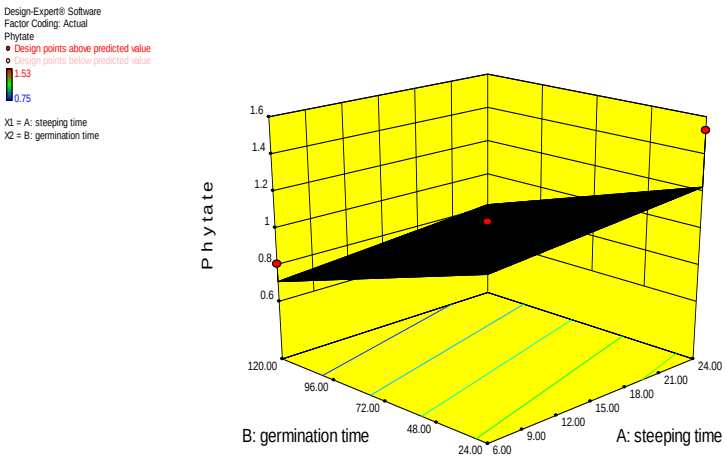

Figure 4. Response surface plot for Phytate in lima bean seed

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The tannin content of raw lima bean flour was 3.05 mg/100g, and the processed flour ranged from 0.31 to 2.11mg/100g, in which case germination for 24hrs and soaking for the hour recorded the highest tannin value (2.11mg/100g) and least value with longer germination period (24hrs/120hrs), this is in agreement with Sagronis and Machado, (2007). At 24hrs of germination of lima bean seed, sprouts or rootlet formation were scant or none, this shows that long time of germination favored the reduction in the anti-nutrients as observed for polyphenol, phytate, tannin, and trypsin inhibitors respectively. The processing condition for tannin reduction was significant ($p<0.05$) and the values of R^2 and adjusted R^2 are 0.8695 and 0.8260 respectively. It has been documented that enzymatic hydrolysis by poly-phenolase causes loss of tannin in grain during germination (Reddy *et al*., 1985). The response surface plot shows a falling ridge signify as germination time increases the ridge falls to bring about a decrease the tannin content (Fig. 5).

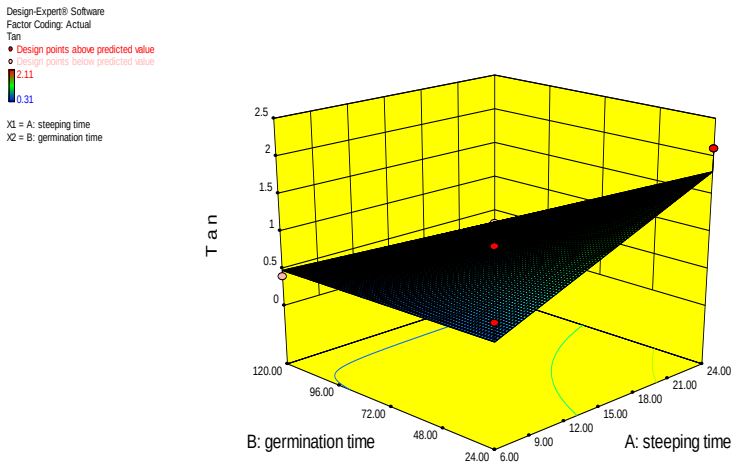

Figure 5. Response surface plot for tannin in lima bean seed

The cyanogenic glycoside content was reduced from 10.45 to 5.82mg/kg. A high concentration of glycoside (10.45mg/kg) was observed with steeping and germination condition of 24/24hrs and the least value at 24/120hrs condition. This show that long steeping and germination time significantly ($p<0.05$) reduce glycosides. On the overall, the result obtained showed that raw lima bean had the highest anti-nutrients when compared with the treated sample except for polyphenol. The result is in agreement with Nwosu, (2010) who reported that steeping hydrates the grains and induces the leaching out of water-soluble anti-nutrients.

4. CONCLUSION

Response surface methodology has been used to determine the variation effect of the anti-nutrients in lima bean seed. The decrease in antinutrient content observed during germination could pose lima beans healthy foods. The result suggests that an optimum preprocess condition of soaking at 17hrs and germination 120hrs for antinutrients reduction would make lima foods acceptable in food formulation.

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Table 2. Effect of steeping and germination condition on the anti-nutrients of lima beans seed

Sample codes	Polyphenol (mg/g)	Alkaloids (mg/g)	Oxalates (mg/100g)	Saponin (%)	Trypsin inhibitors (TIU/mg)	Phytate (mg/100g)	Cyanogenic glycosides (mg/kg)	Tannin (mg/100)
15/72	19.9 ± 0.19^{e}	11.57 ± 0.05^{e}	0.43 ± 0^{d}	13.35 ± 0.15^{d}	3.07 ± 0.04^{e}	0.85 ± 0.04^{def}	7.05 ± 0.04^{de}	0.61 ± 0.01^{ef}
15/72	19.6 ± 0.01^{f}	12.10 ± 0.01^{d}	0.53 ± 0.01^{c}	14.55 ± 0.05^{a}	2.97 ± 0.0^{f}	0.84 ± 0.01^{efg}	7.15 ± 0.0^{d}	0.72 ± 0.01^{de}
15/72	21.02 ± 0.01^{d}	12.30 ± 0.10^{cd}	0.41 ± 0.01^{d}	13.75 ± 0.05^{c}	3.18 ± 0^{d}	0.79 ± 0.01^{gh}	6.86 ± 0.02^{fg}	0.60 ± 0.01^{de}
15/72	20.13 ± 0.04^{e}	11.64 ± 0.14^{e}	0.31 ± 0^{e}	13.97 ± 0.12^{b}	3.16 ± 0^{d}	0.90 ± 0^{c}	6.96 ± 0.06^{ef}	0.76 ± 0.02^{d}

15/72	20.16± 0.02^{e}	12.26 ± 0.04^{d}	0.72 ±0.01^{b}	15.37 ±0.14^{a}	2.82±0.05^{g}	0.87±0.01^{cde}	6.90±0.05^{fg}	0.81±0.01^{d}
6/120	19.15± 0.02^{e}	13.04 ± 0.16^{b}	0.08 ±0.01^{g}	12.40 ± 0.1^{e}	3.16± 0.01^{d}	0.81± 0^{fg}	7.40 ± 0^{c}	0.40±0.01^{fg}
2.27/72	22.18± 0.06^{c}	26.62 ± 0.02^{a}	0.71± 0.01^{f}	16.30 ± 0.1^{a}	4.05 ± 0^{c}	0.92 ± 0^{b}	10.12 ± 0^{a}	0.51±0.01^{ef}
6/24	22.25± 0.04^{c}	15.66 ± 0.36^{f}	0.91 ±0.02^{g}	14.05± 0.01^{b}	4.12± 0.01^{b}	1.37± 0.01^{a}	10.45±0.01^{a}	0.76 ±0.01^{d}
15/139	18.53± 0.00^{h}	12.44 ± 0.01^{c}	0.48 ±0.02^{d}	11.01± 0.01^{f}	2.86± 0^{g}	0.81 ± 0^{fg}	6.82 ± 0^{g}	0.41±0.01^{fg}
24/120	18.50± 0.14^{h}	9.78 ± 0.32^{g}	0.86 ± 0^{a}	10.66 ±0.09^{g}	2.40± 0.01^{h}	0.75 ± 0^{h}	5.82±0.06^{h}	0.31 ±0.02^{g}
15/4.12	26.02± 0.01^{a}	25.36 ± 0.05^{a}	0.60 ±0.02^{c}	17.34 ±0.14^{a}	4.82± 0.09^{a}	1.02± 0.01^{a}	9.82 ±0.03^{a}	1.04 ±0.01^{c}
24/24	28.26± 0.05^{a}	20.40 ± 0.10^{a}	0.74 ±0.02^{b}	16.80 ±0.01^{a}	5.41± 0^{a}	1.53± 0.01^{a}	10.15±0.02^{a}	2.11 ±0.01^{a}
27.73/72	24.97± 0.14^{b}	8.47 ± 0.04^{h}	0.86 ±0.01^{a}	7.06 ± 0.04^{h}	5.20± 0^{a}	1.16 ± 0^{a}	9.15±0.01^{b}	1.21 ±0.01^{b}
Raw	16.93± 0.05^{i}	29.03 ± 0.27	1.02 ± 0.03	18.31 ± 0.11	6.50± 0.01	2.30 ±0.01	12.70± 0.01	3.05 ± 0.01

Means in the same column with varying superscripts differ ($p<0.05$) significantly. Where sample codes indicate steeping and germination condition time.

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