

# OPTIMISATION OF STEEPING AND GERMINATION TIMES ON THE ANTIOXIDANT POTENTIALS OF PIGEON PEA (CAJANUS CAJAN)

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## Abstract

In an attempt to maximize the antioxidant potentials of pigeon peas, the effect of steeping and germination times on the antioxidant content and radical scavenging activities of pigeon pea was investigated using response surface methodology. Using the central composite rotatable design (CCRD), pigeon pea seeds were soaked in water (2-28 h) and germinated (9-111 h). Thirteen samples were generated and analyzed for phenols, flavonoids, reducing power, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power (FRAP). Results revealed that the quadratic effects of both steeping and germination times were significant for predicting the phenols, flavonoids, reducing power, and FRAP while only the steeping time had a significant quadratic effect on DPPH. Significant correlation coefficients (r) were found to exist between the antioxidants and most of the antioxidant activities. The optimum steeping and germination times obtained were 15 and 96 hours respectively; predicted values for phenols, flavonoids, reducing power, and FRAP were 50.09 mg/100g, 26.56mg/100 g, 0.75 µg/ml, and 69.30 µmol/g respectively.

**Keywords:** Antioxidants; Flavonoids; Germination; Optimization; Phenolics

## Introduction

Pigeon pea (*Cajanus cajan*) is a key legume crop of the tropics and sub-tropics that is grown substantially in about fifty Asian countries, Africa and the Americas predominantly together with cereals (ICRISAT, 2008). The plant is used in a variety of ways. The matured dry seeds are shelled and split open as dhal (dry split pea), then boiled and served as a pulse just like similar edible dried beans. Saxena (2017) strongly stated that pigeon pea flour

can also be employed to improve the nutritive value of noodles production and other baked products. Apart from its nutritional properties, pigeon pea has different medicinal properties due to the presence of antioxidants (Asma and Mohammad, 2015). Antioxidants slowed down the oxidation process by preventing the oxidizing chain reactions (Sowndhararajan *et al.*, 2011). Moulisha *et al.* (2016) reported that they are radical scavengers that

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eliminate free radicals from the body because their presence can result in pathological conditions like asthma, anaemia, the neuro-degeneration ageing process, arthritis, inflammation, and where possible dementia. Vitamin C, N-acetyl cysteine, vitamin E, carotenoids, beta-carotene, selenium, lycopene, flavonoids, saponins, phenolic acids, etc are popularly known either as dietary or nutritive antioxidants (Ahmad, 2020). David *et al.* (2004) reported that bean seeds have potentials of antioxidant activity. Lopez *et al.* (2006) also stated that in addition to phenolic compounds, legumes contain a wide variety of bioactive compounds such as vitamins and carotenoids in varying amounts. Antioxidants such as flavonoids are present in pigeon peas (Saxena, 2017).

Germination is considered an effective way of enriching the nutritional content of legumes by reducing the anti-nutritive compounds and making the carbohydrates, free amino acids, and dietary fibre accessible (Sharma *et al.*, 2019). Germination has also been observed to increase the viability of the seeds owing to the resulting rise in antioxidants (Do *et al.*, 2016). Germination is affected by a variety of grains and other external factors such as germination and steeping times (Castiglioni *et al.*, 2015).

Response Surface Methodology (RSM) is an arrangement of mathematical techniques and a research method used in process analysis and processes optimization. In the view of Myers *et al.* (2009), it has significant applications in the concept, development, design and improvement of novel and existing products designs. In the industrial setting, RSM finds its wide application in operations where

the input variable influences some measure of performance or the quality of the product or process. Although pigeon pea is known to be enriched with many materials with antioxidant potentials, few works of literature exist on the effect of germination and steeping time and its resultant effect on the antioxidant properties of pigeon pea seeds.

This work, therefore, determined the effect of steeping and germination times on the antioxidant activities and content of pigeon pea using response surface methodology.

## Materials and Methods

### Materials

Dry pigeon peas (*Cajanus cajan* - white variety) were procured from the major open market in Enugu State, Nigeria (Ogbete Main Market). Dirt and extraneous materials were removed and the cleaned seeds stored in air-proof packaging material at 4°C until needed for analysis.

### Methods

#### *Experimental Design for The Steeping and Germination of Pigeon Pea*

The second-order central composite rotatable design (CCRD) with two independent variables (steeping and germination time) using five levels was utilized in determining how the independent variables affected the responses. Thirteen experiments (four factorial, four axial and five centre points) were conducted in this study. The randomized combination of the coded levels of variables and their actual levels for input as determined by the central composite rotatable design is shown in Table 1.

The codes for the independent variables-steeping time ( $x_1$ ) and germination time ( $x_2$ ) were obtained using the following equations: (Chakraborty *et al.*, 2011)

$$x_1 = \frac{\text{Steeping time} - 15}{9} \quad (1)$$

$$x_2 = \frac{\text{Germination time} - 60}{9} \quad (2)$$

Where  $x_1$  and  $x_2$  are the coded variables.

### Sample Preparation

A portion of the seeds were sterilized by soaking in 1% sodium hypochlorite for 20 min prior to steeping. The grains were thoroughly washed and steeped in water for 2, 6, 15, 24 and 28 hr according to the experimental design (Table 1). The steeped grains were spread on wet jute bags, covered with a

**Table 1. Showing Experimental design for the experiment in their coded and actual values**

| Experimental runs | Independent variables in the coded form |           | Experimental variables in their actual values |           |
|-------------------|-----------------------------------------|-----------|-----------------------------------------------|-----------|
|                   | ( $X_1$ )                               | ( $X_2$ ) | ( $X_1$ )                                     | ( $X_2$ ) |
| 1                 | 0                                       | -1.41     | 15                                            | 9         |
| 2                 | -1                                      | -1        | 6                                             | 24        |
| 3                 | 0                                       | 0         | 15                                            | 60        |
| 4                 | 0                                       | 0         | 15                                            | 60        |
| 5                 | 0                                       | 0         | 15                                            | 60        |
| 6                 | +1                                      | +1        | 24                                            | 96        |
| 7                 | +1.41                                   | 0         | 28                                            | 60        |
| 8                 | +1                                      | -1        | 24                                            | 24        |
| 9                 | -1.41                                   | 0         | 2                                             | 60        |
| 10                | 0                                       | 0         | 15                                            | 60        |
| 11                | -1                                      | +1        | 6                                             | 96        |
| 12                | 0                                       | +1.41     | 15                                            | 111       |
| 13                | 0                                       | 0         | 15                                            | 60        |

$X_1$  = Steeping time;  $X_2$  = Germination time

Code '0' = centre point of the parameter range investigated,  $\pm 1$  = factorial points,  $\pm 1.41$  = axial points

cotton cloth and left to sprout at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 9, 24, 60, 96, 111 hrs (Table 1).

The sprouted seeds were dried for 24 hours in a Gallenkamp oven (BS model OV - 160, Manchester, UK) at  $50^\circ\text{C}$ . The sprouts were removed from the grain via abrasion by rubbing in between the palms before milling and sieving through a 100 mm mesh sieve. Ungerminated grain ground into finest particles served as the control. Ten grams of each of the samples obtained from the steeping and germination process were extracted by stirring in methanol (100 ml) at ambient temperature for 24 hours at 150 rpm after which it was filtered using Whatman filter paper No. 4. The filtrate was extracted twice using the same process outlined above. The methanolic extracts were combined and concentrated at  $40^\circ\text{C}$  until dry and redissolved in methanol (50 mg/ml concentration) and kept at  $4^\circ\text{C}$  in airtight bottles before use (Ferreira *et al.*, 2007).

#### Total Phenol Content

The method of Khatiwora *et al.* (2010) was used in determining the total phenolic content. A 50  $\mu\text{L}$  sample was placed in a test tube and using distilled water, its volume was increased to 500  $\mu\text{L}$ . Folin-Ciocalteu standard reagent (250  $\mu\text{L}$ ) and 20% sodium carbonate solution (1.25 ml) were sequentially added into a test tube. The vortexed tubes were set to incubate for 40 minutes in the dark. A 725 nm absorbance was observed.

#### Total Flavonoid Content

The method adopted by Chang *et al.* (2002), in using aluminium chloride colourimetry was employed in this study to ascertain the total flavonoid content. All the methanol extracts (0.5 ml of 1:10 g/ml) were mixed individually with parts of 0.1 ml of 1 M potassium acetate combined with 1.5 ml methanol, 0.1 ml of 10% aluminium chloride and 2.8 ml of clean water. The mixture was kept for 30 minutes at ambient temperature. Double beam Perkin Elmer UV/Visible spectrophotometer (USA) was used to measure the reaction mixture for absorbance at 415 nm. To determine the calibration curve, quercetin solutions were prepared at concentrations of 12.5 to 100  $\text{gml}^{-1}$  in methanol.

#### In-Vitro Antioxidant Activity

##### Reducing Power Assay

The method of Yang *et al.* (2008) was adopted in determining the reducing power of the sample extracts. A 0.2 M phosphate buffer at a pH of 6.6 containing 2.5 ml of sample extracts were added to 2.5 ml of potassium ferricyanide at 10mg/ml. The mixture was incubated for 20 min at  $50^\circ\text{C}$ . A 2.5 ml of 100 mg/ml solution of trichloroacetic acid was incorporated into the blend, vortexed and

centrifuged for 10 min at 1000 rpm. A 2.5 ml of the resultant supernatant was combined with the same volume of distilled water and ferric chloride (0.5 ml) was added at 1 mg/ml mixture. Spectrophotometrically the absorbance was calculated at 700 nm against the ascorbic acid standard and if the sample had a higher absorbance, it indicated more reducing power.

##### Ferric Reducing Antioxidant Power (FRAP) Assay

This study adopted the FRAP determination method advocated by Benzie and Strain (1996). Freshly prepared FRAP reagent (900  $\mu\text{L}$ ) was set to incubate at  $37^\circ\text{C}$  and mixed with clean water (900  $\mu\text{L}$ ) and seed extract (30  $\mu\text{L}$ ). The reagent (blank) and seed extracts were allowed to incubate for 30 minutes in a water bath set at  $37^\circ\text{C}$ . The sample designated for testing in the reaction mixture was diluted to  $\frac{1}{34}$ . The test reagent contained 2.5 ml at 20 mmol/l 2,4,6-tripyridyl-triazine solution in 40 mmol/l HCl in addition of 2.5 ml of 0.3 mmol/l acetate buffer at pH 3.6. It was then incubated for about 6 minutes at ambient temperature, a decrease of 2,4,6-tripyridyl-triazine to the ferrous complex turned to a blue colour. It was then measured at a wavelength of 593 nm. The standard used was  $\text{FeSO}_4$ .

##### 1,1, Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The method as described by Blois (1958) was adopted in the determination of scavenging activity of 1, 1, diphenyl-2-picrylhydrazyl. Two hundred and fifty (250)  $\mu\text{g/ml}$  of sample extract with methanol was dissolved in DMSO (dimethyl sulphoxide). Five milliliters of a 0.1M methanol solution of DPPH was introduced to the test tube and shaken very well. The mixture was kept for 20 minutes at  $35^\circ\text{C}$ . The sample containing no extract was used as a control. In carrying out baseline corrections in absorbance (OD) of the sample, methanol was used and measured at 517 nm.

#### Statistical Analysis

A one-way ANOVA was conducted on the data obtained from phenols, flavonoid and antioxidant activities. This was followed by Tukey's test to identify the differences in the means. A correlation between the antioxidants and antioxidant activities was determined using Pearson's coefficient. The analysis was carried out using SPSS (v. 20 for Windows, SPSS Inc., Illinois, USA). Response surface methodology was applied to experimental data obtained using Design-Expert - version 8.0.3. A quadratic polynomial regression model was employed to predict individual responses

(Gacula and Singh, 1984). For each response, the model was:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 + e$$

Where  $b_0$  = intercept,  $b_1$ ,  $b_2$  are linear,  $b_{11}$ ,  $b_{22}$  are quadratic and  $b_{12}$  are interaction regression coefficient terms.  $X_1$  and  $X_2$  are the linear,  $X_1^2$ ,  $X_2^2$  is quadratic and  $X_1X_2$  are interaction terms of steeping and germination times.

The dependent variables (Y) as identified in the study were: total phenol, flavonoid, reducing power, ferric reducing antioxidant power and DPPH radical scavenging activity. The regression model was tested for adequacy by the adjusted  $R^2$  above 0.75 (Faridah and Widjanarko, 2013). The coefficients of regression were tested for significance by F-test using  $P < 0.05$ . A statistical tool was employed to determine the surface plots of each response.

### Optimization

Simultaneous optimization of the responses was carried out using the numerical optimization

technique of Design-Expert Software. The desired goal was to maximize all the responses.

## Results and Discussion

### Effect of Steeping and Germination Times on the Phenol, Flavonoid and Antioxidant Activities of Pigeon Pea Flour

Table 2 shows the content of phenol, flavonoid, reducing power, ferric reducing antioxidant power (FRAP) and DPPH (1,1, Diphenyl-2-picrylhydrazyl).

The results of the analysis revealed that steeping of pigeon pea grain and its germination significantly ( $P < 0.05$ ) increased the content of phenol and flavonoid in it and its antioxidant activities.

The regression analysis by its result is displayed in Table 3 shows the coefficient estimates, model significance and adjusted coefficients of determination ( $\text{adj}R^2$ ) for phenols, flavonoids, and the antioxidant activities.

**Table 2. Showing Phenol, flavonoid and antioxidant activity of steeped and germinated pigeon pea flour**

|         |                  |                     | Phenol<br>(mg/100g dry<br>weight) | Flavonoids<br>(mg QE/mg) | Reducing power<br>( $\mu\text{g/ml}$ ) | Frap<br>( $\mu\text{mol/g}$ ) | DPPH<br>( $\mu\text{g/ml}$ ) |
|---------|------------------|---------------------|-----------------------------------|--------------------------|----------------------------------------|-------------------------------|------------------------------|
|         | Steeping<br>time | Germination<br>time |                                   |                          |                                        |                               |                              |
| Control | 0                | 0                   | 19.62 <sup>f</sup> ±0.53          | 9.78 <sup>a</sup> ±0.25  | 0.20 <sup>f</sup> ±0.01                | 49.51 <sup>f</sup> ±0.68      | 48.70 <sup>a</sup> ±1.04     |
|         | 15               | 9                   | 23.05 <sup>e</sup> ±2.57          | 24.84 <sup>b</sup> ±2.46 | 0.60 <sup>e</sup> ±0.05                | 54.99 <sup>d</sup> ±0.47      | 60.44 <sup>d</sup> ±0.58     |
|         | 2                | 60                  | 28.55 <sup>d</sup> ±0.55          | 15.00 <sup>f</sup> ±0.52 | 0.32 <sup>e</sup> ±0.01                | 60.71 <sup>c</sup> ±0.39      | 45.88 <sup>b</sup> ±2.86     |
|         | 6                | 24                  | 31.22 <sup>c</sup> ±0.74          | 20.34 <sup>c</sup> ±1.16 | 0.73 <sup>b</sup> ±0.02                | 68.79 <sup>b</sup> ±0.49      | 70.32 <sup>a</sup> ±0.48     |
|         | 6                | 96                  | 38.62 <sup>b</sup> ±0.47          | 9.71 <sup>a</sup> ±0.60  | 0.22 <sup>f</sup> ±0.01                | 40.12 <sup>a</sup> ±1.23      | 63.48 <sup>c</sup> ±1.19     |
|         | 15               | 60                  | 51.13 <sup>a</sup> ±0.81          | 16.00 <sup>f</sup> ±1.51 | 0.44 <sup>d</sup> ±0.01                | 68.41 <sup>b</sup> ±0.71      | 65.02 <sup>b</sup> ±1.10     |
|         | 15               | 60                  | 51.13 <sup>a</sup> ±0.81          | 16.00 <sup>f</sup> ±1.51 | 0.44 <sup>d</sup> ±0.01                | 68.41 <sup>b</sup> ±0.71      | 65.02 <sup>b</sup> ±1.10     |
|         | 15               | 60                  | 51.13 <sup>a</sup> ±0.81          | 16.00 <sup>f</sup> ±1.51 | 0.44 <sup>d</sup> ±0.01                | 68.41 <sup>b</sup> ±0.71      | 65.02 <sup>b</sup> ±1.10     |
|         | 15               | 60                  | 51.13 <sup>a</sup> ±0.81          | 16.00 <sup>f</sup> ±1.51 | 0.44 <sup>d</sup> ±0.01                | 68.41 <sup>b</sup> ±0.71      | 65.02 <sup>b</sup> ±1.10     |
|         | 15               | 60                  | 51.13 <sup>a</sup> ±0.81          | 16.00 <sup>f</sup> ±1.51 | 0.44 <sup>d</sup> ±0.01                | 68.41 <sup>b</sup> ±0.71      | 65.02 <sup>b</sup> ±1.10     |
|         | 24               | 24                  | 29.62 <sup>d</sup> ±0.46          | 18.81 <sup>d</sup> ±0.98 | 0.30 <sup>e</sup> ±0.01                | 49.99 <sup>f</sup> ±0.68      | 50.00 <sup>f</sup> ±0.89     |
|         | 24               | 96                  | 39.81 <sup>b</sup> ±0.33          | 16.49 <sup>e</sup> ±1.85 | 0.81 <sup>a</sup> ±0.02                | 70.48 <sup>a</sup> ±0.66      | 60.12 <sup>d</sup> ±0.50     |
|         | 28               | 60                  | 31.08 <sup>c</sup> ±1.34          | 26.67 <sup>a</sup> ±0.60 | 0.74 <sup>b</sup> ±0.01                | 49.81 <sup>f</sup> ±0.44      | 51.33 <sup>f</sup> ±0.27     |
|         | 15               | 111                 | 29.68 <sup>cd</sup> ±1.73         | 11.23 <sup>a</sup> ±0.27 | 0.42 <sup>d</sup> ±0.03                | 51.79 <sup>e</sup> ±0.95      | 52.90 <sup>e</sup> ±2.00     |

Means of triplicate determinations  $\pm$  Standard Deviation

Means within a column with the same superscript (a-g) are not significantly different ( $p > 0.05$ )

FRAP= Ferric Reducing Antioxidant Power

DPPH = 1,1, Diphenyl-2-picrylhydrazyl

**Table 3. Showing Phenol, flavonoid and antioxidant activity of steeped and germinated pigeon pea flour**

| Variables      | Phenols | Flavonoids | Reducing power         | FRAP   | DPPH  |
|----------------|---------|------------|------------------------|--------|-------|
| Intercept      | -6.65   | 12.57      | 0.70                   | 68.50  | 57.04 |
| $X_1$          | 3.73    | 1.31       | n.s                    | n.s    | n.s   |
| $X_2$          | 0.87    | n.s        | n.s                    | n.s    | n.s   |
| $X_1X_2$       | n.s     | n.s        | 0.0008                 | 0.04   | n.s   |
| $X_1^2$        | -0.124  | -0.048     | -0.002                 | -0.08  | -0.08 |
| $X_2^2$        | -0.007  | -0.003     | $-7.80 \times 10^{-5}$ | -0.006 | n.s   |
| Model (Prob>F) | 0.003   | 0.003      | <0.0001                | 0.001  | 0.18  |
| Adj. $R^2$     | 0.81    | 0.81       | 0.94                   | 0.86   | 0.32  |

### Phenol Content

The increase in phenols after steeping and germination is similar to the findings of Lopez-Amoros *et al.* (2006), who revealed that the nature and amount of phenolic compounds in legumes can be changed by the germination process. Tian *et al.* (2010) and Gharachorloo *et al.* (2012) also observed that the total phenols increased after steeping and germinating oat and Lentil and this may lead to an increase in viability of the seeds (Do *et al.*, 2016).

The regression model for the phenols in steeped and germinated pigeon pea was obtained as:

$$\text{Phenol} = -6.65 + 3.73 X_1 + 0.87X_2 - 0.124 X_1^2 - 0.007 X_2^2.$$

The results showed that the quadratic regression model was found to be significant in predicting the phenol content of the samples. Analysis of variance showed the linear and quadratic factors of the time it takes for steeping, and germination of the seeds were considered significant model terms. The regression model that predicts the phenol content could explain 81% of the observed variations. This means that 19% of the variation observed could be attributed to factors which were not reflected in the model. Okpala and Okoli (2012) reported that the positive nature of the coefficients in the predictive equation is suggestive that the response value increased directly with the variable while the negative coefficient revealed an antagonistic effect of the variable on the response value. It goes to say concerning this study that only the linear terms of the time it takes for steeping and germination can significantly affect the phenol content while the quadratic terms negatively affect the phenol content. The equation also showed that increasing the steeping time will greatly affect the phenol content than increasing the germination time.

### Flavonoid Content

An increase in flavonoid was documented by Luthia *et al.* (2014) for black gram, chickpea, cowpea and yellow mustard. Lopez-Amoros *et al.* (2006) also observed a rise in flavonoid levels for beans and peas after germination. This rise in flavonoid content after steeping and germination may be because of the biochemical metabolism of seeds constituents during steeping and germination which resulted to the production of secondary plant metabolites such as flavonoids, for example, anthocyanins and flavanols (Alvarez-Jubete *et al.*, 2004). The present results revealed that germinated pigeon pea is a good source of flavonoid, the bioactive compound with health-promoting antioxidants.

The regression model significantly predicts the flavonoid content. It was observed that the linear factor of steeping time and the quadratic factors of the time it takes for steeping, and germination were the significant model terms. The adjusted coefficient of determination ( $\text{adj. } R^2$ ) was high (81%). The regression equation was given as:

$$\text{Flavonoids} = 12.57 + 1.31 X_1 - 0.048X_1^2 - 0.003 X_2^2$$

The equation showed the steeping time positively affects the flavonoid content while the other terms in the equation had a significant reducing effect.

### Reducing Power

The higher reducing power of treated samples indicates that they will have a higher capability to donate electron which was associated with the antioxidant activity. Pei and His (2006) observed a similar trend where germinated black soybean had higher reducing power than the ungerminated black soybean.

The results obtained reveal that the quadratic regression model significantly predicts the reducing power. Neither the linear factor of steeping time nor the germination time significantly predicts the reducing power. The interaction of the time it takes for germination and likewise, the time it takes for steeping with the quadratic factors of germination was the significant model terms. However, it was only the interactive effect that positively affects reducing power. The  $\text{adj. } R^2$  was 94% meaning that only about 6% of the variation was due to factors not found in the model. The regression equation was given as:

$$\text{Reducing power} = 0.70 + 0.0008X_1X_2 - 0.002X_1^2 - 7.8 \times 10^{-5}X_2^2$$

### Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing capacity of the pigeon pea extracts used in this study revealed every one of them gave well FRAP activity. FRAP assay was used by several authors for the evaluation of the antioxidant activity of various food samples (Guo *et al.*, 2003; Halvorsen *et al.*, 2006). Halvorsen *et al.* (2006) proposed that most of the secondary metabolites are redox-active compounds that will be picked up by the FRAP assay.

The results show that the interaction model was significant ( $p=0.02$ ) but not as significant as a quadratic model ( $p=0.001$ ) in predicting the FRAP. ANOVA of the quadratic model indicated that the linear factors were not significant but the interaction factor with the quadratic factors of the time it takes

for steeping, and germination were significant model terms. The regression model explained about 86% of the variation in the FRAP. This means 14% of the variation cannot be explained by factors within the scope of this model. The equation shows that the interactive effect of steeping and germination times had a positive effect on the FRAP. The predictive equation for the response variable is given below as:

$$\text{FRAP} = 68.5 + 0.04X_1X_2 - 0.08X_1^2 - 0.006X_2^2.$$

#### DPPH (1,1, Diphenyl-2-picrylhydrazyl)

The DPPH, steady nitrogen targeted free radical, has been utilized to assess the antioxidant activity of natural products by way of measuring the radical quenching potential in a moderately brief time frame (Sanchez-Moreno, 2002). The higher values obtained in steeped and germinated pigeon pea is following the works carried out by Lopez-Amoros *et al.* (2006); Fernandez *et al.* (2006) where they observed a rise in antioxidant activity of legumes after germination.

None of the model terms were significant in predicting the DPPH. The p-value for the F-test was not significant ( $P > 0.05$ ) and the  $\text{adj}R^2$  was very low (below 0.75). The low  $\text{adj}R^2$  suggests that the DPPH could explain only 32% of the observed variation, thus as high as 68% of the variation can be explained as being caused by other factors exclusive in the model. Owing to the non-significance of the model, a predictive equation for DPPH could not be generated.

#### Correlation Between Antioxidants and Antioxidant Activities

Table 4 revealed the correlation existing between the antioxidants and antioxidant activities. It was observed that there were significant correlations between total phenol and DPPH ( $r = 0.678$ ;  $P < 0.01$ ) as well as total phenol and FRAP ( $r = 0.639$ ;  $P < 0.01$ ). Total phenol does not have a significant relationship with reducing power. The flavonoid had a significant correlation only with the reducing power. The relationship with DPPH and FRAP was not significant ( $P > 0.01$ ).

Include tannins, phenolic acid, flavonoid and other compounds which possess phenolic structure. Flavonoids that have OH in A and or B ring could be termed as phenolic groups. Flavonoids have been reported to have higher antioxidant activity than phenolic acids (Heim *et al.*, 2002). The significant correlation between total phenol and DPPH, FRAP indicated that higher total phenol content in the test samples could give higher antioxidant activity in both DPPH and FRAP assays. There was no significant correlation between total flavonoid and

DPPH, FRAP. This result means that higher total flavonoid in the sample could have little or no influence on DPPH scavenging capacities and FRAP level. Based on this data (Table 4) it could be predicted that flavonoid in the samples has no OH in ortho C3', C4', O H in C3, oxo function in C4, double bond at C2 and C3, which could influence the capacity for antioxidant activity. This could also suggest that there are other antioxidants in the samples which could be responsible for the observed antioxidant activities. Some authors (Katsube *et al.*, 2004; Djeridane *et al.*, 2006) have reported a correlation which was linear between the total phenolic compounds and their antioxidant potential, while others have observed poor linear correlation (Czapecka *et al.*, 2005; Wong *et al.*, 2006).

#### Optimisation

The numerical optimization procedure was conducted by maximizing those responses with significant models (phenols, flavonoids, reducing power and FRAP). Results showed that steeping for 15 h and germinating for 56 h resulted in pigeon pea flour with 50.09 mg/100 g phenols, 26.56 mg/100 g flavonoids, 0.75  $\mu\text{g/ml}$  reducing power and 69.30  $\mu\text{mol/g}$  FRAP (Figure 1). The desirability of this steeping and germination time combination was 0.93. Optimization involves the detection of the best combination of factors that are able to produce the expected characteristics of the final product. The use of these optimized conditions will result in pigeon peas with high antioxidant activity which can be used in the production of functional foods.

Findings of this research imply that steeping and germination increased some of the bioactive compounds like total flavonoids and total phenols with the successive increase in an antioxidant

**Table 4. Showing Correlation between total phenols, flavonoids of seed extract of germinated pigeon pea and DPPH, reducing power and FRAP**

| Antioxidant/ Antioxidant activity | R                              |
|-----------------------------------|--------------------------------|
| Phenol/DPPH                       | 0.678<br><0.01 ** Significant  |
| Phenol/reducing power             | 0.039 not significant          |
| Phenol/FRAP                       | 0.639 * Significant<br>< 0.01  |
| Flavonoid/DPPH                    | 0.064 not significant          |
| Flavonoid/ reducing Power         | 0.702<br>< 0.01 ** significant |
| Flavonoid/FRAP                    | 0.134 not significant          |

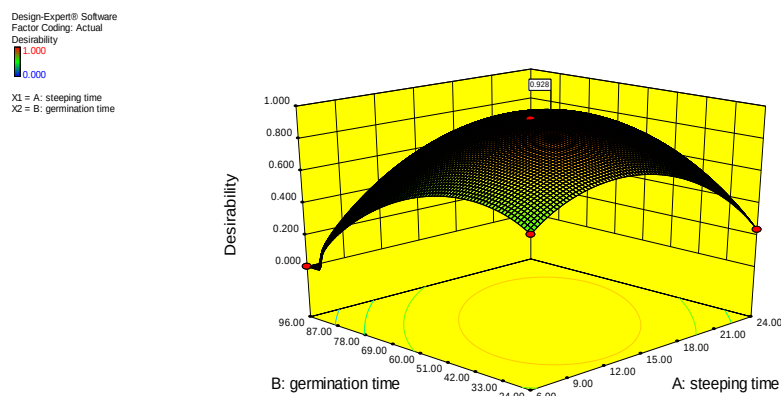
\*\* = Significant at  $P < 0.01$

\* = Significant at  $P < 0.05$

FRAP= Ferric Reducing Antioxidant Power

DPPH = 1,1, Diphenyl-2-picrylhydrazyl

R = Correlation Co-efficient



**Figure 1. Showing Three-dimension response surface plot of Phenol/Flavonoid/Reducing power/FRAP/DPPH showing optimum point for steeping and germination time**

activity like FRAP, reducing power and DPPH. Germinated pigeon pea can be used as composite flour in bakery industries and the production of puddings. It can be boiled and taken as boiled peas or use as complementary flour with cereal as a breakfast cereal. Hence, the cultivation of pigeon pea and consumption of germinated pigeon pea seed should be encouraged while research efforts should continue for increased utility value of germinated pigeon pea.

## Conclusions

This study has shown that response surface methodology can be used to verify the effects of steeping and germination times on the antioxidants and antioxidant activities of pigeon pea. Useful predictive equations were also generated in the process. Results have shown that for optimal antioxidants and antioxidant activities, the seeds should be soaked for 15 hours and germinated for 56 hours.

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