Effect of Germination Time, Light & Hydrolysis on Antioxidants and Antioxidant Activities of Soya Beans (*Glycine max*)

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***Abstract:*-Soybean is a high protein, easily grown, affordable, accessible and culturally acceptable legume, commonly consumed in most LMIC. Its protein content are said to be degraded into smaller beneficial peptides with germination time and some other conditions in most climes. Such peptides have antioxidant properties that explain its health benefits.**

In this study, locally grown soya bean was germinated with and without light for days 1 - 6, and the flour divided into four treatment groups (SBUD, SBHD, SBUL, & SBHL), that was evaluated for antioxidants and antioxidant properties and the results compared.

The results showed differences in TFC, TPC, RPA, GPX, MDA, CAT & DPPH, with those germinated under light and hydrolyzed, showing better antioxidants and properties. The optimal germination time defined by DPPH activity of 61% with excellent correlation with MDA (r value = 0.827) and good correlation with CAT (r value = 0.607) was observed in Day 2 SBHL. It can therefore be said that sprouted soya bean exhibits better antioxidant profile especially when hydrolyzed and germinated with light than raw seeds.

Keywords: **Germination, hydrolysis, Antioxidant activities, Soya bean**

Abbreviations: **SOD = Superoxide dismutase; CAT = Catalase; RPA = Reducing power assay; GPx = Glutathione Perioxidase; MDA = Malondialdehyde; DPPH = Diphenyl-1-Picrylhydrazyl; TFC = Total Flavonoid content; TPC = Total Phenolic content; TP = Total Protein;**

I. INTRODUCTION

Soya bean is a major source of protein in the eastern world and most Low and medium income countries (LMIC), with Asians consuming an average of 20 - 30g of soy foods daily (1). Soya bean protein are made up of bioactive peptides (BAPs) and phytochemicals including isoflavones (2). It is stated that soya bean is the richest source of isoflavones (3). Other phytochemicals contained in soya bean are saponins (4), protease inhibitors (5) and Inositol hexaphosphate (6). Legumes like soya bean also contain several phenolic compounds (7).These compounds have been found to increase with germination time in soybean and some other legumes (8), with some studies pegging the peak concentration at 48hrs (9, 10), though the flavonoids was seen to be highest in 72hrs of germination (11).

Germination influences the distribution of the metabolites, since secondary metabolites are produced during the process, by the mobilization of protein reserves from stored protein bodies (12). It also affects amino acid types and levels in soya beans (13) and production of relatively smaller peptides (14). Besides germination time, other factors that influence the chemical composition of germinating soya bean are temperature, moisture, aeration and light (15) and therefore these factors should be taken into consideration during germination of legumes.

In plants like soya bean, Bioactive peptides (BAPs) exist as inactive amino acid sequences encrypted in the primary structures. To make them active, germination, fermentation, food processing, invitro enzyme catalysis or enzymatic actions in the digestive system are employed (16). Soya bean proteins existing as BAPs have been found to neutralize oxidants and so posses antioxidant properties (17). Since they mop up free radicals in the system, such properties are evaluated invitro using DPPH, reducing power, malonaldehyde, hydroxyl ion scavenging or superioxide anion scavenging activity. These free radical scavenging activity of soya bean, is the basis of the reported chemoprotective actions on cancers and other health benefits (18).

In LMIC, the re-evaluation of the content and actions of locally grown and sourced soya bean, will help in addressing several public health issues since micronutrient deficiencies and chronic diseases like cancers are assuming increasing proportion. In this work, soya bean will be subjected to different germination time, with and without light, and the protein isolates treated with combined acid and enzymes hydrolysis and the results of the antioxidant activities, total phenolic content, total flavonoids content and phytochemistry, compared. Germination time is believed to influence the activation of endogenous enzymes which then mobilize seed reserves to form smaller molecular weight peptides that are biologically active (13,14,19). This composition is affected by presence or absence of light during the germination time (20).

Similarly, hydrolysis (acids, enzymes or both) frees the encrypted inactive bioactive peptides into very active smaller molecular weight compounds, thereby improving the antioxidant content and activities (21). However, these effects, varies with the type of cultivar as well as other endogenous and environmental factors. Assessing the outcome of germination time, light and hydrolysis on common locally available soybean, will also be of great importance, especially in view of the growing interests on the health benefits.

II. RESULTS & DISCUSSION

Germination, light and hydrolysis were observed in this study to have effect on both the levels of antioxidant activities and the antioxidants in soybean. The various treatment groups in this study, viz: soybean germinated with light but not hydrolyzed (SBUL); soybean germinated without light but not hydrolyzed (SBUD); soybean germinated with light and hydrolyzed (SBHL) and soybean germinated without light and hydrolyzed (SBHD), showed differences in antioxidant levels and activities. In terms of protein and total flavonoid content, SBUD had higher median values of 81.24g/l and 0.04mg/ml respectively when compared to SBUL values (77.35g/l & -0.018mg/ml respectively), meaning that the presence of light most probably energized the proteinacious enzymes increasing the rate of protein breakdown in the germination process, thereby justifying the initial decrease of the protein content as sprouting starts. This is consistent with the findings of different investigators that documented the improvement of the quality of legumes by germination (10,12) and that such improvement is affected by light (22). It is suggested that germination leads to degradation of seed reserves, the products of which are used for respiration and new cell development (23). Germination affects the type and level of different amino acids (13) and increases the presence of relatively smaller peptides (14, 24).

Phenolic compounds which are also known constituents of legumes (7) have not been documented by studies to have a consistent pattern of change during germination (25). However, changes in phenolic compounds of legumes like soya bean have been found to be dependent on seed type, germination time, presence of light and other process conditions, with an overall increase noted during germination (26). This is consistent with the findings in this study where the raw un-germinated soybean seed with TPC of 0.233mmol/ml, had this content increased to a median value of 0.36mmol/ml to 0.67 mmol/ml for SBUD (0.23 - 0.73 mmol/ml) and SBUL (0.32 - 0.83mmol/ml) respectively. Generally, the high values of TPC in this study were found in soybean germinated with light (Table 1) confirming earlier studies that light improves the constituents of phenolic compounds during germination of soybean (25, 26). Some of the phenolic compounds like hydroxycinnamic compounds are constituents of cell wall bonded to arabinoxylans and lignin, which are polysaccharides. It is believed that during germination, endogenous enzymes of the seeds like esterases are probably activated by temperature, light and other endogenous factors to break such bond linkages (19). This explains why phenolic compounds initially not detected in the raw seeds were detected in germinated ones.

Flavonoids also show interesting trend in this study. While the un-germinated soybean did not show presence of flavonoids (Median value -0.018 mg/ml), same compounds were detected when same seeds were germinated, showing a median value of 0.04 mg/ml in SBUD (-0.05 to 0.25 mg/ml) to 0.11 in SBUL (-0.19 to 0.41mg/ml), Table 1. The increase in the median value of total flavonoid content (TFC) can be explained by the degradation of seed reserves during germination (27), resulting in the changes in the biochemical parameters of soybean. This initial absence of other glycosides (flavonoid glycosides) in the un-germinated seeds and their subsequent appearance with germination, lends credence to the endogenous enzyme activation theory. Depending on the seed type and constitution, these enzymes which also include hydrolases and polyphenoloxydase, exhibit higher activity with germination even though the rate varies (28).

Table 1: Antioxidant levels in light, dark, hydrolyzed and unhydrolyzed preparations of soya bean

	SOD	CAT	RPA	GPx	MDA	DPPH	TPC	TFC	TP
Unit	U/ml	μMH_20_2 consumed/min /mg of protein	µg/ml	10^5 mg/min	10^{-5}mg/ml	%	mmol/ml	mg/ml	g/L
In dark-unhydrolysed: **median value** (CI of median)	**4.14** (3.26-6.32)	**0.07** (0.06-0.17)	**7.15** (-27.80-20.74)	**1.50** (1.09-1.77)	**23.87** (6.45-51.61)	**23.00** (9.00-45.00)	**0.36** (0.23-0.73)	**0.04** (-0.05-0.25)	**81.24** (53.29-106.10)
In Light-unhydrolysed: median value (CI of median)	**6.02** (2.51-6.59)	**0.06** (0.06-0.14)	**5.11** (-13.18 – 18.89)	**1.14** (1.11-1.59)	**19.35** (6.45 -33.55)	**21.00** (8.00-36.00)	**0.67** (0.23-0.83)	**-0.018** (-0.19-0.41)	**77.35** (48.93-106.10)

In dark-hydrolysed **median value** (CI of median)	**0.62** (0.18-0.09)	**0.09** (0.06-0.09)	**569.50** (501.20-643.10)	**521.80** (0.68-966.50)	**0.72** (0.62-0.95)	**34** (22.00-46.00)	**0.45** (0.32-0.73)	**0.17** (-0.04-0.88)	**1.30** (0.60-2.30)
In Light-hydrolysed: **median value** (CI of median)	**0.71** (0.44-0.94)	**0.09** (0.06-0.12)	**624.10** (555.20-687.40)	**649.10** (182.20-1230.00)	**0.71** (0.65-0.83)	**36.00** (14.00-61.00)	**0.44** (0.30-0.78)	**0.31** (0.06-0.94)	**1.70** (0.40-5.90)
Kruskal Wallis test value	20.56	0.57	20.51	16.03	20.33	7.24	0.94	10.00	20.71
P value	**0.0001**	**0.9028**	**0.0001**	**0.0011**	**0.0001**	**0.0647**	**0.8158**	**0.0186**	**0.0001**
*UL vs. UD	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
UL vs. HL	0.0121	>0.9999	0.0030	0.0052	0.0120	0.2236	>0.9999	0.0674	0.0380
UL vs. HD	0.0024	>0.9999	0.0150	0.1314	0.0077	0.1423	>0.9999	0.3185	0.0069
UD vs. HL	0.0282	>0.9999	0.0049	0.0091	0.0097	0.9413	>0.9999	0.0847	0.0109
UD vs. HD	0.0062	>0.9999	0.0229	0.1993	0.0061	0.6648	>0.9999	0.3833	0.0016
HL vs. HD	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
UL is unhydrolysed light preparation, UD is unhydrolysed dark preparation; HL is hydrolysed light preparation and HD is hydrolysed dark preparation. * Dunn's multiple comparison is used as post-hoc test, following Kruskal Wallis test. Adjusted P value was reported									

Similarly, the protein content of the raw un-germinated soybean which was 106.09 g/L, showed a decrease during germination. This decrease is likely due to the degradation of protein reserves from stored protein bodies by proteases, to form bioactive peptides (14). Though such protein breakdown is weak during germination, the effect of enzyme hydrolysis enhances this (21). As germination progressed, protein content slightly increased, though still lower than the raw seed value, due to the degradation of other seed components for respiration (27).

The presence of phenolic compounds and flavonoids result in antioxidant activities due to the free radical scavenging properties of the shorter chain peptides. These activities are demonstrated by the SOD, CAT, RPA, GPX, MDA & DPPH (Table 1). There appear to be variation in antioxidant activities among soybean seeds germinated with light and those without light, with the latter showing better activities in almost all the parameters. These activities were found to be highest in sprouted soybean (SOD: Day3 SUBL = 6.59u/ml; CAT: Day5 SUBL = 0.142umH_2O_2consumed/min/mg Protein; RPA: Day5 SUBL = 18.89ug/ml; DPPH: Day3 SUBL = 36%; MDA: Day1 SUBL = 33.548 x10^{-5}mg/ml and GPX: Day2 SUBL = 1.588 x10^5/min) when compared to the values of un-sprouted seeds. Similar trend was noticed in seeds germinated without light (Table 1). These findings are consistent with previous studies that observed that sprouting increases free radical scavenging by many folds (8, 15). Sprouting also modifies the phytochemical content by introducing other antioxidants (29, 30). In this study, saponins, an antioxidant in soybean, detected from the second to third day of germination, was an additional antioxidant mix to flavonoids, as shown in the result of the qualitative phytochemical screening assay.

In vitro hydrolysis like germination increases significantly both the antioxidant activities and the antioxidants in this study. For hydrolysis to be effective, it must be extensive, with degree of hydrolysis, DH > 10% (31). The increase is due to the release of inactive amino acids and bioactive compounds by enzyme-catalyzed proteolysis (16). Hydrolysis caused a significant increase in RPA and GPx levels in both dark and light soybean flours, with SBUL, showing better activity (Fig. 1). The excellent correlation of Day 2 SBHL with MDA (r value = 0.827), the good correlation with CAT (r value = 0.607) and the weak correlation with TP (r value = 0.214), made it the optimal germination for soybean (Fig. 1D), unlike SBHD (Fig. 1C). This is in agreement with similar studies which put the optimal germination effect for soybean at 2-3 days (9, 10, 11). Though there was no statistical difference among the four treatment groups in terms of DPPH activity (P value = 0.067), optimal DPPH activity was obtained in soybean treated with hydrolysis and germinated with light (fig. 2). The RPA, MDA, DPPH & the total protein levels show better correlation in soybean preparation with the highest DPPH level (61%), observed in Day 2 SBHL (Fig. 3).

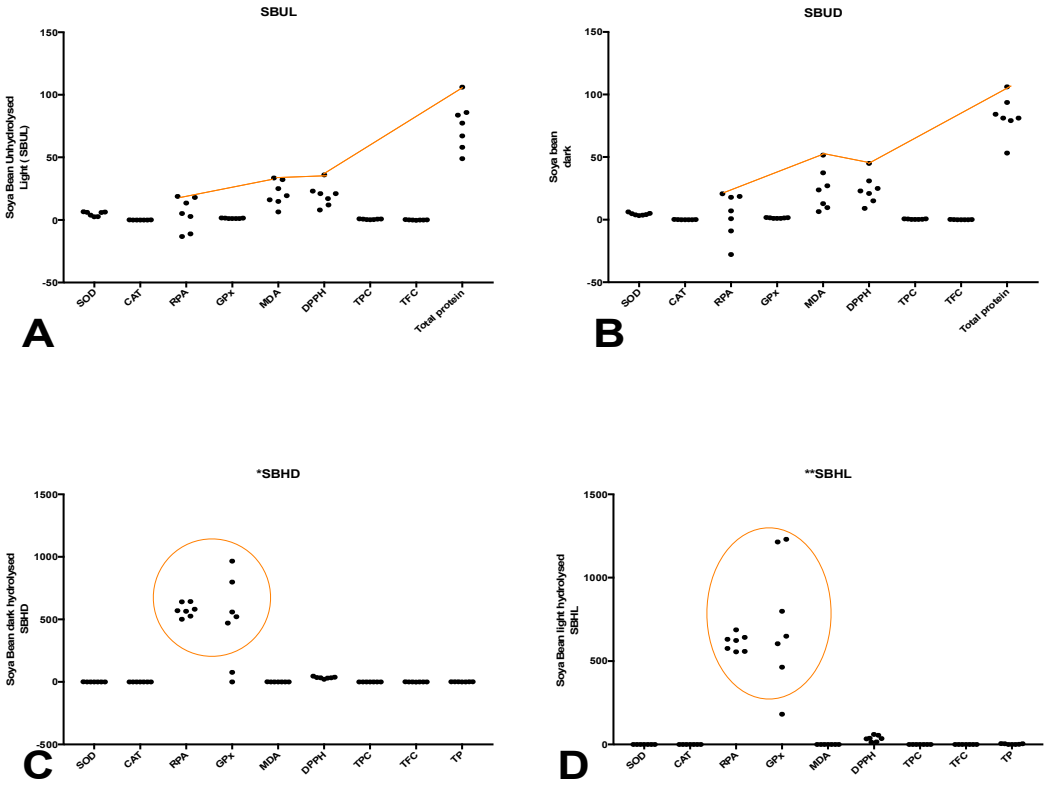

Figure 1: Levels of anti-oxidants in light and dark preparations of soya beans.
A)Soya bean light preparation unhydrolysed. B) Soya bean dark preparation unhydrolysed, C) Soya bean dark preparation hydrolysed and D) soya bean light preparation hydrolysed. Hydrolysis caused a significant increase in RPA and DPx levels

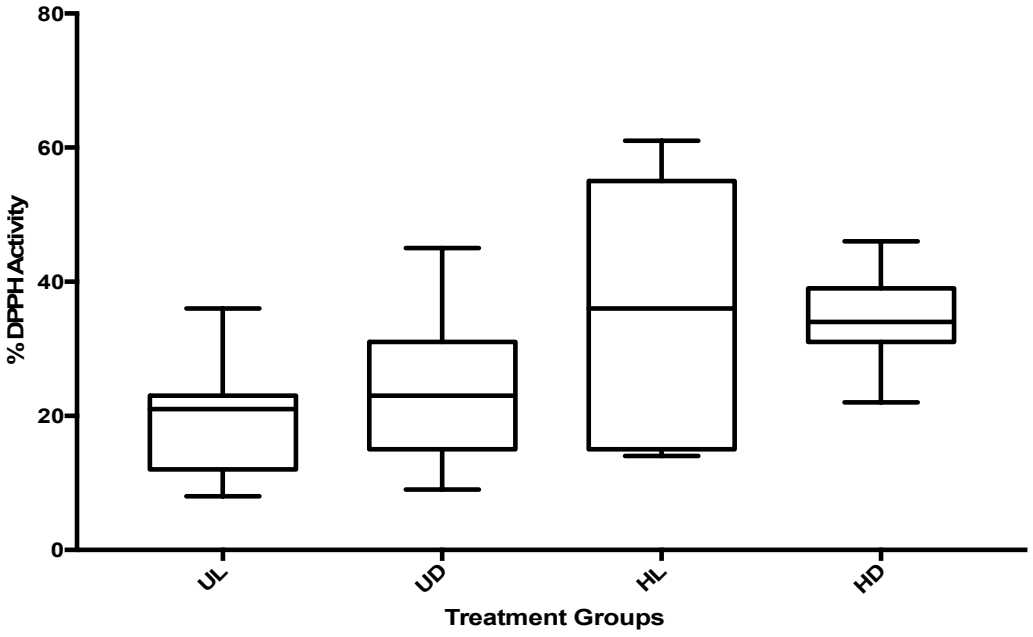

Figure 2: DPPH activity in various treatments of soya bean
UL is germinated in light and unhydrolysed; UD is germinated in darkness and unhydrolysed; HL is germinated in light and hydrolysed while HD is germinated in darkness and hydrolysed.

There is lack of order in the antioxidant values with germination time in this study, in contrast with the initial increase steadily to a peak and then a decrease, observed elsewhere (10, 20). The reason may be partly due to local variety / cultivar, harvest time and structure of the seeds (32) and partly as a result of the environmental factors and endogenous factors including enzyme issues, since all these affect germination and bioactive peptide availability (19, 26).

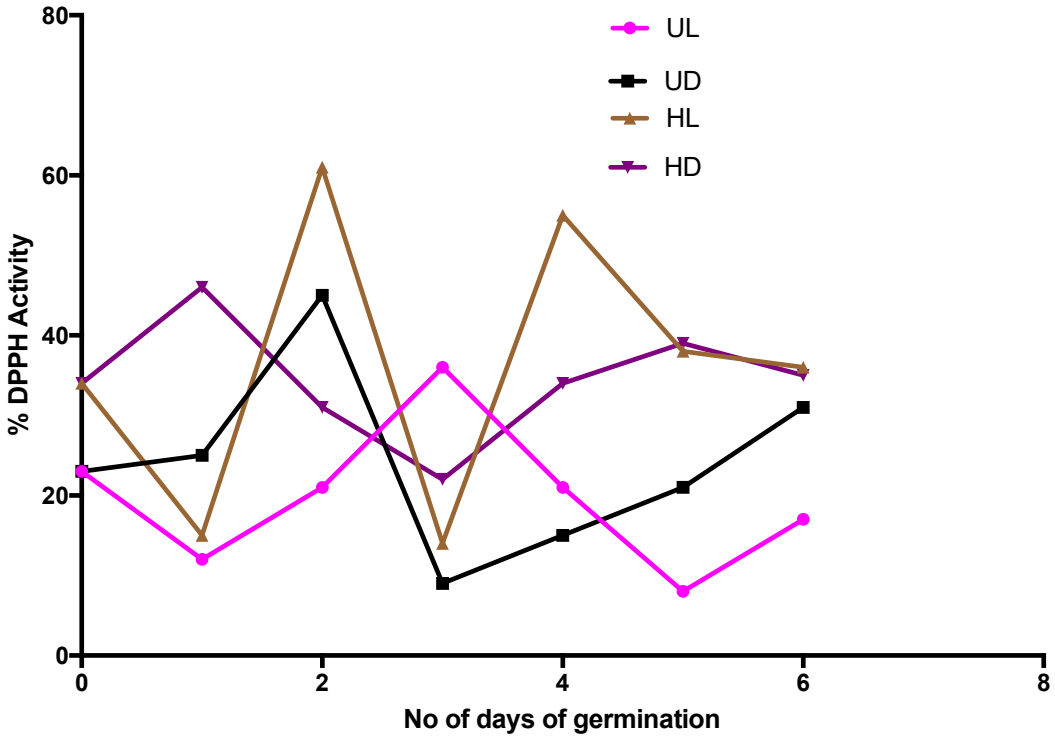

Figure 3: DPPH activity over days of germination.
Day 2 of light germination followed by hydrolysis treatment produced the maximum % DPPH activity of 61 %.

The increased antioxidants (TPC, TFC & Saponins) and the improved antioxidant properties in this study implies that the locally available soybean seeds in Nigeria, exhibits similar properties as observed in other climes, when subjected to germination and hydrolysis (29). The protective role of these antioxidants against chronic diseases like cancers are well documented (33, 34). These antioxidant activities are due to the chemical structure of the compounds that enables them to scavenge free radicals including the chelation of pro-oxidant metal ions (35, 36). These findings have important public health implication, especially in developing countries like Nigeria, where access to and availability of quality health care and routine medical checkup for early detection of chronic diseases is a luxury. In low/medium income countries, LMIC, cancers of the cervix for instance accounts for 80% with 85 - 88% of deaths also recorded of the global prevalence (37). With the growing economic recession, these figures are bound to worsen and complicated by other disease burden. This makes consumption of antioxidant-rich legumes like soybean a good preventive approach if one must reverse the unacceptable high prevalence of cancer morbidity and mortality as well as freeing the productive population, which is the population at risk, thereby improving both the household, community and national economic growth in third world countries. Incidentally, soybean is a cheap, easily processed and commonly grown & consumed and therefore available high protein food.

III. MATERIALS AND METHODS

The seeds of soya bean (*Glycine max*) were purchased from a retail outlet in Abakaliki, Ebonyi State, South East Nigeria. The reagents used include 2mM hydrogen peroxide in phosphate buffer, Trichloroacetic acid (10% w/v), Folin-Ciocalteu`s reagent, 1.0% Potassium ferricyanide, 0.1% (w/v) Ferric Chloride, 0.2mM/l DPPH in 95% ethanol, Superoxide dismutase from Randox laboratories, UK and Glutathione Peroxidase from Randox laboratories, UK. All reagents used were of analytical grades.

For the germination process, 500g of the seeds were soaked in 1500mL of distilled water for 16hr at room temperature. The distilled water was changed every 6hrs, to avoid growth of microorganisms. The hydrated seeds were placed on two sets of six wet jute bags, with one set on the laboratory table and

the other inside a dark laboratory cupboard representing germination with light and without light respectively. Both of them were covered with jute bag, that was sprinkled with water every 3hr, to maintain humidity. They were then allowed to germinate for 1,2,3,4,5 and 6 days. The germinated seeds were sun-dried, ground to pass 20µm sieve and stored in air-tight container in a refrigerator for the analysis.

I. Catalase activity assay

This was performed using Luke`s method (38), in which to prepare the enzyme extract, 20 g of sample flour was added into a tube containing 100 ml of phosphate buffer and the homogenate mixed very well and centrifuge for 10 min at 5000 rpm. This gave the supernatant for the enzyme assay. Then the reagent was prepared by adding 32 µl of H_2O_2 into a measuring cylinder containing 499.68 ml of phosphate buffer. This gave 2mM H_2O_2 in phosphate buffer. 1.5 ml of H_2O_2-phosphate buffer was pipette into a cuvette and shaken gently. 250 µl of the enzyme extract was added into the cuvette and mixed thoroughly. This mixture was read at 240nm using spectrophotometer and the time taken to decrease the absorbance mixture from 0.45 to 0.40 was noted as ΔT. H_2O_2-free phosphate buffer was used as control. The activity of the enzyme was calculated as unit of the assay mixture = $17/\Delta T$, where ΔT was the time in seconds. The activity of the enzyme was expressed as µmole of H_2O_2 consumed /min/Mg protein.

II. Malonaldehyde (MDA) assay

This was done using the Thiobarbituric Acid Reactive Substance (TBARS) method (35). The reagent was prepared by dissolving 10 g of Tricholoacetic acid (TCA) in 100 ml of distilled water (dH_2O) and filtered with Whatman paper, while the sample was prepared by dissolving 2.5 g of the flour in a beaker containing 50 ml of methanol. This was allowed to stand overnight and then filtered with filter paper. Then 2.5 ml of TCA was pipette into a tube containing 0.5 ml of the prepared sample and cooled to room temperature. This was then centrifuged at 1000 x g for 10 min. 2 ml of the supernatant was transferred to a new tube and 1 ml of thiobarbituric acid (TBA) solution added. The tube was placed in boiling water for 15 min and then allowed to cool to room temperature, after which, the absorbance was read at 532 nm and then at 600 nm. Two controls, the negative and positive control were used in place of the sample by using phosphate buffer and tetraethoxypropane (TEP) respectively.

The concentration of MDA (mg/ml) = (A532 - A600) / 155,

where 155 is the Extinction-coefficient of MDA - TBA at 532 nm in $mM^{-1}cm^{-1}$

III. Total phenolic content

This was determined using the Singleton and Rossi method (39). To Folin Ciocalteu`s reagent was added 5 ml 85% phosphoric acid and 10 ml concentrated hydrochloric acid. This was reflux for 10 hr and then 15 g lithium sulfate, 5 ml water and one drop of bromine solution, were added. This was reflux for 15 min, cooled to room temperature and made up to 100 ml with water. The enzyme extract was prepared by adding 100 mg of sample flour into a tube containing 4 ml of 70% aqueous ethanol with 0.1% acetic acid. A calibration curve was prepared using 10 mM of Gallic acid as a working solution with 5 dilution points (1.0, 0.8, 0.6, 0.4 and 0.2 mM) in duplicates, with water as negative control. 100 µl of sample was put in a tube containing 100 µl of Folin Coicalteu`s reagent with a pipette. This was mixed well and allowed to stand for 3 min, after which 100 µl of saturated sodium carbonate solution and 700 µl of distilled water were added. The reaction was allowed to stand for about 90 min in the dark and the absorbance read at 725 nm using spectrophotometer. The results are calculated by extrapolation using Gallic Acid curve.

IV. Reducing power activity

The reducing power of the sample was determined using Oyaizu method (40). 1.0% Potassium Ferricyanide, 10% TCA and 0.1% of Ferric chloride were prepared as the reagents, while the sample was prepared by dissolving 5 g of the flour in a beaker containing 100 ml methanol and allowed to stand overnight and filtered with filter paper. 2.5 ml of the prepared sample was pipette into a 20 ml tube and 2.5 ml of phosphate buffer added. Also added was 2.5 ml of 10% Potassium ferricyanide and the mixture incubated at 50^0C for 20 min. The reaction was stopped at incubation by adding 2.5 ml of TCA, centrifuged for 10 min at 5000 g. From the upper layer of the centrifuged mixture was taken 2.5 ml into a new tube, to which 2.5 ml of distilled water was added. Finally, 0.5 ml of 0.1 % ferric chloride was added and the absorbance of the mixture, read at 700 nm. Butylated hydroxyl toluene (BHT), 20 µg/ml was used as standard. The reducing power (RP) is calculated as follows:

Increase in RP (%) = (A test - A blank / A blank) x 100.

V. DPPH radical-scavenging activity

This was measured based on the method described by Sefatie *et al.,* (10). The reaction mixture was made up of 1 ml of sample, 1 ml of 0.02 mol/L phosphate buffered saline, and 1 ml of 0.2 mM DPPH in 95% ethanol. This mixture was shaken and allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was read at 517 nm against the blank, using a spectrophotometer. The experiment was conducted in duplicate and the percentage scavenging effect was evaluated as follows:

Scavenging rate = $[1 - (A_1 - A_2) / A_0] X 100$

where,

A_0 = the absorbance of the negative control

A_1 = the absorbance of the sample.

A_2 = the absorbance without DPPH

VI. Superoxide Dismutase Assay

The sample extract was prepared by placing 100 mg of the flour in a test tube containing 4.0 ml of 70% aqueous ethanol with 0.1% acetic acid at room temperature, extracted for one hour with constant agitation using shaker at 250 rpm**.** 0.05 ml of sample was mixed with 1.7 ml of reconstituted mixed substrate, R1. R1 was composed of 1 vial of R1a (0.05 mmol/l xanthine & 0.025 mmol/l I.N.T) and 20 ml of R1b, buffer, (40 mmol/l, PH 10.2 CAPs & 0.94 mmol/l EDTA). The mixture was read 505 nm after 30 sec (initial absorbance, $A_{1)}$, and at final absorbance, A_2, after 3 min. The negative and positive control were also read at the same wavelength after mixing 0.05 ml of sample diluent with 1.7 ml of R1, and 0.05 ml of standard with 1.7 ml of R1 respectively. The percentage inhibition was calculated as follows:

$A_2 - A_1 / 3 = \Delta A$/min of standard or sample.

Sample diluent rate (SI rate) = rate of uninhibited reaction.

% inhibition = $100 - (\Delta A_{std/min} \times 100) / (\Delta A_{SI/min})$

% inhibition = $100 - (\Delta A_{sample/min} \times 100) / (\Delta A_{sI/min})$

The percentage inhibition for each standard was plotted against Log_{10} and the percentage inhibition of sample was used to obtain the units of SOD from standard curve.

VII. Glutathione peroxidase (GPX) assay

The method used was that according to Paglia and Valentine (41). The mixture of diluted sample and reagent blank, {(0.05 mL diluted sample, 2.50 mL of reagent R1 and 0.10 mL, Cumene R2) and (0.05 mL distilled water, 2.50 mL reagent R1 and 0.10 mL Cumene R2)} respectively, was read after 1 min at 340 nm (A1). The timer was started simultaneously and read at 340 nm after 1 and 2 min (A2 & A3) respectively. The value of the reagent blank was subtracted from the value of the sample. The concentration of GPX (U/L) = 8412 X ΔA 340 nm/min.

VIII. Total flavonoid Content.

This was conducted according to the method described by Chia-chi *et al.,* (42). 100 µl of the sample (prepared by mixing 100 mg of the flour with 4 ml 70% aqueous ethanol with 0.1% acetic acid) was mixed in a test tube with 300 µl of 95% ethanol, 20 µl of 10% aluminum chloride, 20 µl of 1M potassium acetate and 560 µl of distilled water. The mixture was allowed to stand for 30 min at room temperature and read at 415 nm against a blank using spectrophotometer. A graph-pad prism was used to calculate the results.

IX. Qualitative Phytochemistry assay

This was a qualitative screening, carried out for alkaloids, steroids, tannins resins, saponins, other glycosides, anthraquinones, anthracenes, acidic compounds, starch, reducing sugar and cardiac glycosides. To test for alkaloids, the alcoholic extract of the flour was mixed with water and 2% hydrochloric acid, heated to boiling point, cooled and filtered and the filtrate added few drops of Mayer's reagents. The observation of turbidity or yellow precipitate indicated presence of alkaloids.

For the steroid test, concentrated sulphurc acid solution was slowly added to a mixture of 20 mg of the extract, 2.5 ml of acetic acid and 2.5 ml of chloroform and the presence of red violet colour signified terpenoids while green bluish colour indicated steroids.

The tannins presence was checked according to the method by Ukoha *et al,* (43), by adding 1 ml of water and 1 drop of ferric chloride solution to 0.5 ml of the extract. A blue colour and green colour indicated the presence of gallic tannin and catecholic tannins respectively.

For the resins, 15 ml of distilled water added to the methanol extract and the mixture observed for turbidity.

To test for saponins, a mixture of 0.5 mg of the methanol extract and 5 ml of distilled water, shaken vigorously, gave a persistent froth which on addition of 3 drops of olive oil with vigorous shaking formed an emulsion for a saponin positive reaction.

To test for glycosides, a mixture of 0.5 ml of the methanol extract, 1 ml of glacial acetic acid that was added 1 ml of concentrated sulphuric acid gave an upper layer that was bluish green in colour indicating glycosides content.

The presence of anthraquinones, gave a pink violet / red colour when a filtrate from 5 ml chloroform and 0.5 g of the extract mixture that was shaken for 5 min, was mixed with equal volume of 10% ammonium solution and shaken. A mixture of 3 g of sample powder and 4 ml of ammonium solution, heated for 15 min and observed for a red colour, was an indication of anthracenes.

For the cardiac glycosides test was conducted by mixing 100 mg of extract with 1 ml of glacial acetic acid containing one drop of ferric chloride solution, with a brown ring at the interphase indicating a positive reaction.

The presence of acidic compound was confirmed when a water-wetted blue litmus paper dipped into a warmed filtrate of 0.1 g extract in water, turned red.

The starch was tested by adding 3 -5 drops of iodine on the test sample. The observation of a blue-black / purple colouration confirmed presence of starch.

The reducing sugar was detected if a brick red precipitate was observed when 1ml of water and 5 - 8 drops of hot Fehling`s solution were added to 0.5 ml of the extract solution.

X. Determination of total protein

This was determined using the Biuret method by Randox Laboratories, UK (41) in which a protein of 57.86g/l was used as a standard to calibrate the semi-automated randox machine. After this, 0.5ml Biuret reagent consisting of a mixture of 100 mmol/l Sodium hydroxide, 16 mmol/l Na-K-tartrate, 15

mmol/l Potassium iodide and 6 mmol/l Cupric sulphate, was mixed with 0.01 ml of the sample. The mixture was incubated for 30 mins at room temperature and the total protein value read on the screen.

XI. Preparation of the protein hydrolysates

The stored dried protein was dissolved in distilled water at the ratio 1:5 w/v. To the mixture was added 0.5 ml of 1% pepsin (pH 2.0) for 30 min. The pepsin reaction was stopped by increasing the pH to 7.0 using 0.1 mol/L NaOH, after which, 0.5 ml of 2% pancreatin (pH 7.0) at 37 ^{0}C for another 30 min. The proteases was inactivated and the hydrolysis stopped by heating the mixture in a thermostat-controlled water bath for 15 min at 95^{0}C. The hydrolysate formed was cooled to room temperature, centrifuged at 4,000 g for 10 min and the supernatant stored at -20^{0}C for separation and further analysis. The degree of hydrolysis was then determined.

XII. Determination of the Degree of Hydrolysis (DH).

The DH was determined using titration method (44) in addition to the usual Tricholoacetic acid & Biuret methods. To a test tube was a mixture of 10 ml of protein hydrolysate, and 10 ml of 20% TCA, that was mixed gently by vortex for 10 sec. The mixture was allowed to stand for 30 min at room temperature and centrifuged at 7800 g (1400 rpm) for 15 min. The supernatant (soluble peptides) was harvested and analyzed for protein content, using Biuret method. The nitrogen content in the protein hydrolysate with and without TCA treatment was determined using the titration method according to Nilsang *et al.,* (44) and calculated by the formula:

% N = 0.1 X Titration volume (ml) x 14 x 10 /sample weight (g) x 1000

% DH = Soluble N in TCA 10% (w/v) x 100 /Total N in the sample.

< 10% DH = partial hydrolysis

> 10% DH = extensive hydrolysis

13. Determination of antioxidant activities of hydrolyzed samples.

The extensively hydrolyzed samples were tested for CAT, RPA, SOD, MDA, DPPH, GPx, TPC and TFC using the methods outlined above.

Statistical Analysis

Due to the small sample size and numerous variables (nine in number), non-parametric statistical analysis method was used. Hence median, inter-quartile range or confidence interval (CI) of median could be generated and used. In this study, the median with CI, was used and significant differences among the groups determined using Dunn`s multiple comparison as post-Hoc test following Kruskal Wallis test, with SPSS 20.0 software package program (SPss Inc. USA). A P value of ≤0.05 was considered significant from the result.

Tables:

Table 1: Antioxidant levels in light, dark, hydrolyzed and unhydrolyzed preparations of soya bean

Figures

Figure 1: Levels of antioxidants in light and dark preparations of soya bean

Figure 2: DPPH activity in various treatments of soya bean

Figure 3: DPPH activity over days of germination

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CONFLICT OF INTEREST STATEMENT

There was no conflict of interest declared.

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