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RESEARCH ARTICLE

HPLC PROFILING, *IN VITRO* ANTISICKLING AND ANTIOXIDANT ACTIVITIES OF AMINO ACIDS FROM BLACK BEAN SEEDS *(PHASEOLUS VULGARUS* L.*)* USED IN THE MANAGEMENT OF SICKLE CELL DISEASE (SCD) IN THE WEST REGION OF CAMEROON

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ABSTRACT

Background*:* Sickle Cell Disease (SCD) is a chronic, debilitating disorder with a myriad of symptoms that make disease treatment challenging. The use of food and nutrition in managing SCD is gaining increasing attention because some nutrients from food could serve as a source of the antisickling new lead compounds. ***Objective****:* It was to profile; to investigate *in vitro* antisickling properties, membrane stability effect and antioxidant potentials of amino acids extract from black bean seeds (*Phaseolus vulgarus* L.) used in the management of SCD in the West Region of Cameroon. ***Methods****:* Amino acids extract was carried up on a high performance liquid chromatography (HPLC). The sickling of red blood cells (RBCs) was induced using sodium metabisulfite (2%) followed by treatment with amino acids at different concentrations. The evaluation of the rates of inhibition of induced sickling and that of acquired reversibility was performed using microscopic enumeration. The evaluation of membrane stability effect; Ferric Reducting Antioxydant Power (FRAP) assay; 2,2-DiPhenyl Picryl Hydrazyl (DPPH°) and radical hydroxyle (OH°) was determined using colorimetric method. ***Results****:* Total 16 amino acids were found in the black bean seeds, alanine is the major constituent and followed by cysteine, asparagines and threonine. The results obtained show that 12mg/mL was the best concentration because gave the highest rates of inhibition and reversibility of sickling of red blood cells (92.06±3.52% and 86.74±2.1%). Hemolysis also decreased at different extract concentrations showing the stability effect on the membranes of erythrocytes. However the concentration of 12mg/mL has showed the best activity (from 100 to 20.12%). Amino acids extract from black bean seeds exhibited antioxidant potentials at 23.12±0.116 mg FeII/100g of black bean seeds after carrying out FRAP. It also showed a inhibitory activity on free radicals of $DPPH^{o}$ and HO^{o} at IC_{50} 10.76±1.57mg/mL and 2.04±0.029mg/mL respectively. ***Conclusion****:* Amino acids extract from black bean seeds used to manage sickle cell disease in the west region of Cameroon have antisickling, anti-hemolytic and antioxidant properties. These results may justify the use of black bean seeds by sickle cell patients.

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INTRODUCTION

Amino acids play several roles in the body (Meletis et Barker, 2005); they are essential in the synthesis of proteins and precursors in the formation of secondary metabolism molecules (Moran-Palacio *et al.,* 2014). In humans, amino acids participate in various physiological processes, such as skeletal muscle function, atrophic conditions, sarcopenia, and cancer. They play key roles in cell signalling, homeostasis, gene expression, synthesis of hormones, phosphorylation of proteins and also possess antioxidant abilities (Moran-Palacio *et al.,* 2014; Wu, 2009). The therapeutic use of amino acids presents a viable and important option for natural medicine. The use of amino acids in medicine today continues to be explored using clinical research and applications. Some of the most prominent areas of therapeutic applications of amino acids are for treatment of brain metabolism and neurotransmission imbalances. Other areas in which amino acids also find key applications are immune function, cardiovascular and gastrointestinal (GI) health (Meletis et Barker, 2005), treatment of liver diseases, fatigue, skeletal muscle damage, cancer prevention, burn, trauma and sepsis, maple urine disease and diabetes (Tamanna et Mahmood,

2014). Several researchers reported that amino acids prevent sickling, stabilize erythrocyte membrane and prevent oxidative stress in patients suffering of Sickle cell disease (Iwu *et al.,* 1988; Rumen, 1975). In fact Sickle cell disease (SCD) is a group of hereditary illnesses affecting the red cell hemoglobin (Bunn, 1997). The substitution of β6 glutamic acid by valine into the gene encoding the human β-globin subunit causes a drastic reduction in the solubility of sickle cell hemoglobin (HbS) when deoxygenated. Under these conditions, the HbS molecules polymerize to form intracellular fibers which are responsible for the deformation of the biconcave disc shaped erythrocyte into a sickle shape (Acquaye *et al.,* 1982). Substitution increases the level of reactive oxygen species (O^2, H_2O_2, •OH), leading to the development of oxidative stress (Aslan *et al.,* 2000) which causes damage in the system and amplifies conditions like inflammation and immunologic disorders (Malika, 2002). Sickle cell disease is a serious problem, management or treatment of which still possesses difficult challenges to medical practitioners worldwide. As a genetic hereditary disease, therapeutic treatment is complex and very expensive. Bone marrow transplantation is the only sure treatment but very expensive; with lack of technical equipment; unavailability of necessary expertise and problems of finding suitable donors constitute a major setback to this approach in developing countries (Mpiana *et al.,* 2008). Macromolecules from foods and edible plants have been used in the management of sickle cell crisis associated morbidities among the less privileged class of the society. Therefore, research has been ongoing in sub-Saharan Africa to investigate natural plant products that could be of profound benefit to the sickler. Foods and edible plants demonstrated their importance as nutraceuticals. Many plants constituents have been investigated for their antisickling and antioxidant properties (Iwu *et al.,* 1988). Several studies have shown that amino acids from leguminous plant *S. stemocarpa*, *M. myristica* and *M. sloanei* revealed antisickling properties (Ojiako *et al.,* 2010). Ojioka *et al*, (2012) also found antisickling amino acids in ground seeds of *Lagenariasphaerica*, *Mucunapruriens* and *Cucurbita pepo Var styriaca.* Black bean seed (*Phaseolus vulgarus*) is usually used to manage sickle cell disease in the West Region of Cameron. This was revealed following an ethnobotanical investigation carried out in this region (Kotue *et al.,* 2016). In fact *Phaseolus vulgarus* L. is a legume with an important source of amino acids for humans. Nutritionally, the high quality and quantity of amino acids found in *Phaseolus vulgarus* L. can be of profound benefit to the sicklier (Audu and Aremu, 2011). The present study aims to profiling, to investigate *in vitro* antisickling and antioxidant effects of amino acids extract from black bean seeds (*Phaseolus vulgarus* L.) used in the management of Sickle Cell Disease (SCD) in the West Region of Cameroon.

MATERIALS AND METHODS

The seeds of black beans sample (figure 1) were obtained from sickle cell patients families and authenticated as PNN, a wild variety at the Agricultural Institute of Research for the Development of Foumbot station, Cameroon. In Agro-processing and Natural Product Division of CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Kerala-India, the raw whole bean seeds (2 kg) were weighted (Sartorius 15DGA balance) and pulverized using an electrical grinding machine (Microfluidics M-110P). The powdered material was passed through a 40-mesh sieve to a uniform size (1.5µm) and conserved in cold room at 6°C for further use.

Figure 1. Photograph of black bean seeds (*Phaseolus vulgaris* L.) PNN (wild variety) collected from local sickle cell patient's families of west region of Cameroon

Amino acids extraction: Some fractions of 400 g of bean flour were defatted overnight in a horizontal shaker using hexane (1:10 g/ml) at room temperature. After this step, the mixture was filtered using Whatman paper N°1. The defatted flour was left in a chapel of exhaust gases at room temperature for solvent evaporation.

Extraction of free amino acids of sample: The free amino acids content in 3.5 g of delipidate powdered material was extracted in distiller water (100 mL) using soxhlet dispositive for 8 hours. The free amino acids content in distiller water were keeping in order adding later with amino acids from proteins hydrolyzed.

Extraction amino acids of sample proteins: The total proteins was extracted from 3.5 g delipidate powder used to extract free amino acids following the method described in Akbar *et al*. (2012) with 1250 µL of protein extraction [0.5M Tris HCl (pH 8), 5 M Urea, 0.5 % SDS, 1 % 2-Mercaptoethanol and few drops of Bromophenol blue]. After sufficient grinding, the seed flour suspension was vortexed vigorously for homogenization and then centrifuged at 13 000 rpm for 15 min at room temperature. The supernatant containing the protein was hydrolyzed using 10 mL of 6 N HCl in an oven at 110°C for 24 h (Baxter, 1996). After hydrolysis, $CaCO_3$, 2M was added to neutralize the reaction till to attend pH 6.9. The whole was then centrifuged at 13 000 rpm for 15 min and the resulting supernatant was completed to 25 ml with de-ionized water and mixed on free amino acids collected before. After filtration through 0.22 µm syringe filter, the resulting solution was lyophilized and then the residue was kept at 6°C for antisickling and antioxidant properties. For the determination of the amino acid composition by high-performance liquid chromatography (HPLC), on 5 µL of resulting solution obtained before lyophilization were added 25 µL of borate buffer 0.4 N, pH_{10} and 320 µL of distilled water. 20 µL of this last solution were used for injection. A standard solution of each pure amino acid in appropriate solvent/buffer was created to 0.1mg/mL.

HPLC derivatization procedure: Chromatography was carried out at a constant temperature of 30°C using a gradient elution with mobile phase A (acetone/methanol/distiller water (46:44:10)) and B (40 mM sodium phosphate buffer, pH 7.8). The HPLC equipment consisted of a Spectra Physics. HPLC

apparatus Schimazu comprising an 8700 XR ternary pump, a 20-µL Rheodyne injection loop, an agilent AAA IIas_16-12-2015.lcm column, a UFLC fluorescence detector 348nm - 450nm, and a 4290 integrator linked via Labnet to a computer-disk E- amino acid analyzer – method fils. For separation, a 15 × 4.6mm column was used. Accurate spectrum peak show sets of spectra, retention time and area of the each amino acid are obtained through advanced spectrum processing and state-of-the-act correction and quantification algorithms using computer-disk E- amino acid analyzer – method fils. The peak area corresponding based on the retention time was the detection limit of each amino acid. Amino acid concentration was calculated with the peak area equated in the standard using dilution factor. For a sample, total amino acids contain was obtained by addition of free amino acids and amino acids from protein hydrolysis. The result was expressed as mg of amino acid /g dry sample.

Blood samples collection: After receiving an ethical clearance with number 0675/CRERSHC/2017, consent from all blood donors was read and signed by all the patients participating in the study after being informed of the research objectives. The blood samples used in the evaluation of the antisickling activity of the amino acids extracts in this study were taken from confirmed sickle cell patients between 17 and 43 years old known to have sickle cell disease, attending monthly Hematology outpatient clinic at the Yaounde Central Hospital. 4.5 mL of blood samples were collected in the sodium EDTA tubes and stored for the experiment.

***In vitro* Antisickling activity**

Induction of sickling

100 µL of SS blood cell suspensions were mixed with 100 µL of 2% sodium metabisulfite solution ($Na_2S_2O_5$) and incubated at 37°C. The time course of the sickling of SS erythrocytes was analyzed microscopically according to the method described by Joppa et *al*. (2008). The number of cell was counted every one hour after take 10 µL of the mixture diluted 200 times using Marcano liquid. The number of cell was counted every one hour and the percentage of sickling cells was calculated using the formula:

$$F(\%) = \frac{\text{Number of sickled red blood cells x 100}}{\text{Total number of red blood cells}}$$

A curve of percentage of sickling in function of time was realized. This permitted the deduction of maximum time necessary to obtain maximum sickling.

In vitro antisickling activity of the amino acids extract: A serial of different concentrations of amino acids were prepared in the saline solution. For the assay, 100 µL of SS-RBC pre-incubated with $Na_2S_2O_5$, 2% was added to 100 µl of solution of different extracts for final concentration of 250, 500 and 1000 µg/mL. Each mixture was incubated at 37°C for 3 h (time necessary to obtain maximum sickling). After incubation, 10 µL of the mixture was diluted 200 times using Marcano liquid. 10 µL of each sample was examined under the light microscope and both sickled cells and total cells were counted from five different fields of view across the slide of Malassez cell. For the negative control, the solution containing the extract was replaced by the saline solution. The positive control was constituted of phenylalanine with the same concentrations as the extract.

The percentage of sickling was calculated using the formula:

$$\text{Inhibition rate (\%)} = \frac{(f0 - fn)x100}{fo}$$

(**f0**) is the % of sickling of the mixture SS blood and $Na_2S_2O_5$, 2%.
(**fn**) is the % of sickling of the mixture SS blood, $Na_2S_2O_5$, 2% and amino acids extract with each concentrations.

Reversibility assay of the amino acids extract

For the reversibility assay, freshly collected HbSS blood was diluted in 1:1 ratio with 0.9% normal saline (negative control) and test solution containing different amino acids extract concentration. The experiment was followed as mentioned above. Calculation was done after every 1 hour till maximum reversibility of sickling was attained. These percentages permitted to calculate the rate of reversibility of sickling according to the following formula.

$$R(\%) = \frac{(R0-Rn)}{R0} \text{ x } 100$$

(**R**) is the reversibility rate (%)

(**R0**) is the initial percentage of sickling and (**Rn**) is the maximum percentage of sickling obtained with different concentrations of amino acids extract.

Phenylalanine was used as the positive control at the same concentrations with the extract.

Erythrocyte membrane stability activity of amino acids extract: The evaluation of the erythrocyte membrane stability effect of amino acids extract was done using a method proposed by Jaja et *al*. (2000). The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress/hypotonic lysis incubation. To 10 mL reaction vessel containing 4 mL of different concentrations (0.00 -0.85%) of buffered saline with pH of 7.4; 1 ml of each extract (250 µg/mL) and 0.05 ml SS-RBC blood were added. The mixture was incubated at 37°C for 24 h and then centrifuged at 3000 rpm for 15 min. The optical density of the supernatant was read at 540 nm against blank made of 0.85% buffered saline concentration. The percentage of hemolysis was calculated using the formula below:

$$\text{Percentage of hemolysis (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \text{ x } 100$$

Results were presented graphically as percent hemolysis plotted against the concentration of NaCl.

Antioxidant activity of amino acids extract: The antioxidant activity of the amino acids extract was examined based on Ferric reducing antioxidant power as well as the scavenging effect of the DPPH and hydroxyl free radical.

Total antioxidant activity by ferric reducing antioxidant power assay (FRAP): The FRAP method was used to determine the total antioxidant activity which measures the reduction of ferric ion to the ferrous form in the presence of antioxidant compounds (Benzie and Strain, 1996). The fresh FRAP reagent consist of 500 mL of acetate buffer (300 mM pH 3, 6), 50 mL of 2, 4, 6- Tri (2- pyridyl)-s-triazin (TPTZ) (10 mM), and 50 mL of FeCl3 · 6H2O (50 mM). For the assay,

to complete this evaluation, extract (0.1mL) in 3 test tubes was mixed with 3mL of freshly prepared FRAP reagent. After incubation (up to 5minutes) in darkness at room temperature (~25 °C), absorbance was read against a suitable blank at 593 nm. The test was carried out in triplets. The concentration of the amino acids extract was calculated using the standard equation obtained by using standard $FeSO_4$:

$$\boldsymbol{DO = 0.0248C + 0.0619}, \; R = 0.9847$$

where C was the Concentration. The final results were expressed in mg of Fe (II)/100 g of black bean seeds. Phenylalanine and gallic acid were used as control.

Scavenging Activity of DPPH Radical: The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity (Jain *et al.,* 1995). Briefly, in 3 mL of each diluted extract, 1 ml of methanol solution of DPPH 0.1 mM was added. The mixture was kept in the dark at room temperature for 30 min and the absorbance was measured at 517 nm against a blank. Gallic acid was used as the control for the comparison of antiradical efficacy. The results were expressed in percentage of inhibition of free radicals using the formula:

$$\text{DPPH radical inhibition percentage (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The inhibition percentages calculated permitted the realization of curves, percentage inhibition in function of extract concentration. The test was carried out in triplets. A curve of % DPPH bleaching activity versus concentration was plotted using OriginPro 8 Software to determine IC_{50} concentration that account for 50% inhibition.

Hydroxyl radical scavenging activity

The scavenging activity of the extract on hydroxyl radical was measured according to a previously described method (Yu and Rao, 1990). In 1.5mL of each diluted extract, 60 µL of $FeCl_3$ (1mM), 90 µL of 1,10- Phenanthroline (1mM), 2.4 ml of 0.2 M phosphate buffer, pH 7.8 and 150 µL of H_2O_2 (0.17 M) were added respectively. The mixture was then homogenized and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The antiradical activities different extracts were expressed in percentage of inhibition of the hydroxyl radical was determined following the formula:

$$\text{OH radical inhibition percentage (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The inhibition percentages calculated permitted the realization of curves percentage inhibition in function of amino acids extract concentrations using OriginPro 8 Software. These curves were used to determine IC_{50} concentration that account for 50% inhibition. The test was carried out in triplets.

Statistical analysis: The results were expressed as mean ± standard deviation. Data was analyzed using Analysis of Variance (ANOVA) of Kruskall-Wallis with the software Sigma Start version 3.01A analysis software. Statistical data were considered significantly different at 95% confidence interval ($p < 0.05$).

RESULTS

Amino acids profile: Figures 2 showed the typical chromatograms with the amino acids peaks detected in black bean seeds and their concentration in mg/g of bean. The main amino acids corresponding to these peaks were aspartic acid (1.447 mg/g); glutamic acid (2.913 mg/g); asparagine (41.693 mg/g); threonine (22.02 mg/g); arginine (0.937 mg/g); methionine (5.77 mg/g); phenylalanine (8.81 mg/g); histidine (0.219 mg/g); tyrosine (0.802 mg/g); alanine (130.846 mg/g); glutamine (0.019 mg/g); tryptophan (0.991 mg/g); lysine (0.263 mg/g) (Figure 3). Alanine is the major constituent and followed by cysteine, asparagines and threonine.

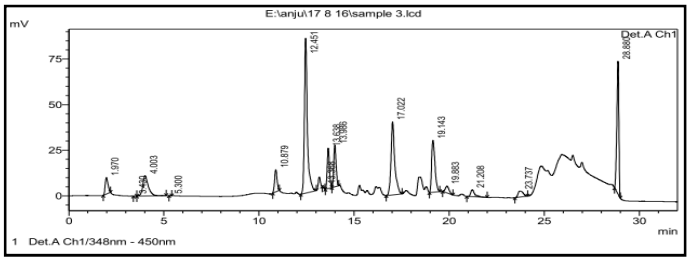

Figure 2. Typical chromatogram showing the amino acids in black bean seeds.

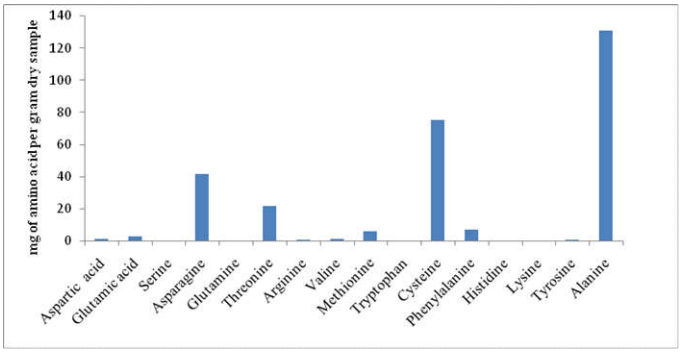

Figure 3. Amino acids content in black bean seeds (mg/g)

In vitro **induction of sickling with sodium metabisulfite 2%:** Sodium metabisulfite 2%, added to sicklier red blood cells at equal volumes provoked sickling of red blood cells (Figure 4). At time 0 hour, sickling percentage was 28.4% and after 3 hours of incubation sickling increased on average from 28.4±2.4% to 80.67±3.1% and remained constant with time. The maximum number of sickled cells was obtained after 3 hours, suggesting that this is the time necessary to obtain maximum sickling.

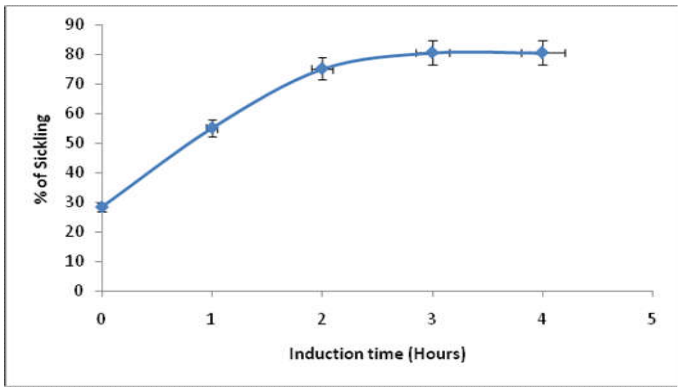

Figure 4. Percentage of sickling as a function of time after induction of red blood cells with MBS (2%). At time 0 hour, MBS 2% was replaced by NaCl 0.9%

Figure 5 illustrates the different morphological states of the SS red cells observed under an optical microscope (objective 40x/0.65) during the different hours until the maximum sickling.

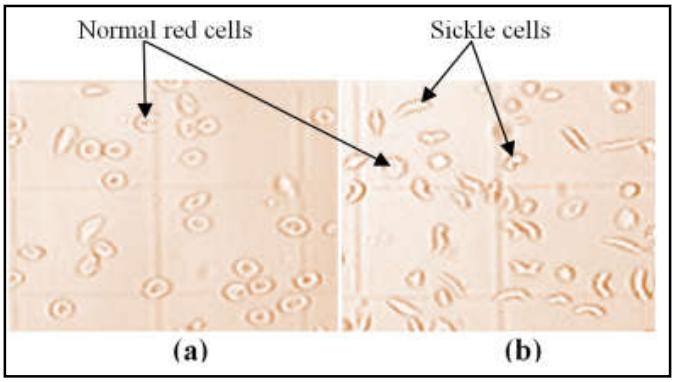

Figure 5. Morphological states of patients red blood cells observed under the optical microscope (40x/0.65) according to time. (a) before induction (t = 0 hour); (b) after induction with MBS 2% (t = 3 hours)

Inhibitory activity of amino acids extract from black bean seeds on sickling: After calculating the percentages of inhibition of sickling it was realized that amino acids from black bean seeds significantly inhibited sickling ($p<0,05$) giving inhibition rates of 70.82±3.4%; 74.93±3.48%; 77.82±3.5%; 92.06±3.52% and 92±3.1 for amino acids extract concentrations of 6mg/mL; 8mg/mL; 10mg/mL; 12mg/mL and 14 mg/mL respectively. These rates are higher than those obtained with phenylalanine, used as the positive control which gave inhibition rates of 29.71±2.3; 34.96± 2.4, 48.96±2.6; 67.96±2.8 and 68.56±2.6 with the same concentrations as the extract (Figure 6). This result shows that, sickling is dependent on the concentration of amino acids extract (6mg/mL; 8mg/mL; 10mg/mL and 12mg/mL) representing C1, C2, C3 and C4, with a significant difference ($p<0, 05$). The increase rate of inhibition is significantly different from that of phenylalanine.

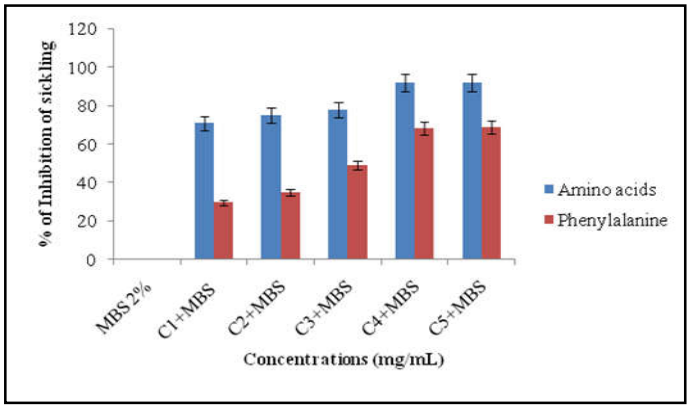

Figure 6. Rate of inhibition of amino acids extract from black bean seeds at different concentrations (6mg/mL; 8mg/mL; 10mg/mL; 12mg/mL and 14 mg/mL) representing C1, C2, C3, C4 and C5 respectively

Reversibility effect of the amino acids extract from black bean seeds: After calculating rates of reversibility, we noticed a significant reversal effects ($p<0, 05$) with amino acids extract from black bean seeds giving rates of 47.9 ± 3.5% 69±38% 79.99±4% 86.74±4.3% and 73.9±3.8 % at concentrations of 6mg/mL; 8mg/mL; 10mg/mL 12mg/mL and 14 mg/mL respectively. This effect is higher than that obtained with phenylalanine used as the positive control with the same concentrations (Figure 7).

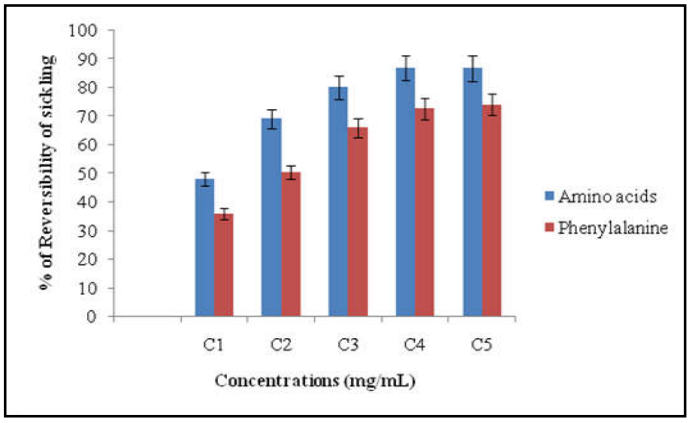

Figure 7. Reversibility rate of amino acids extract from black bean seeds at different concentrations (6mg/mL; 8mg/mL; 10mg/mL; 12mg/mL and 14 mg/mL) representing C1, C2, C3, C4 and C5 respectively

Membrane stability effect of the extract

The influence of the amino acids extract on the red blood cell fragility is given as the percentage of cell haemolysis as a function of the salt concentration and the concentrations of various extracts (Figure 8). Based on 100% initial total haemolysis, amino acids extract reduced significantly ($P < 0.05$) the cell fragility compared with the control. Hemolysis decreased for all concentrations in the presence of sodium chloride (0.85%) until 20.12% for C5 (12mg/mL) concentrations.

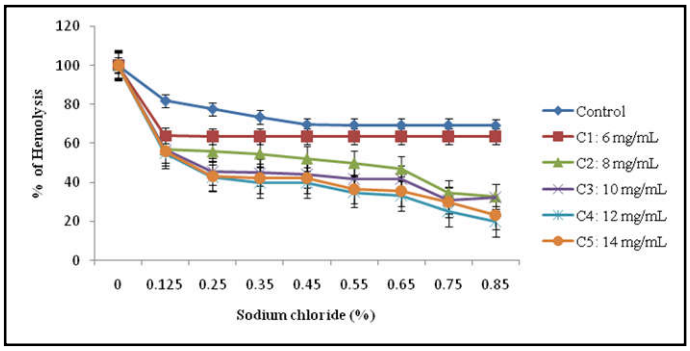

Figure 9. Effects of amino acids extract from black bean seeds on hemolysis

Antioxidant properties of the amino acids extract: Table 1 show the evaluation of the antioxidant properties of amino acids extract using gallic acid as references. amino acids extract exhibited antioxidant potentials and an average reducing power at 10.76±1.5 mg FeII/100g black bean seeds after carrying out FRAP but significantly ($p<0.05$) lower compared to phenylalanine (6.47±0.0mg FeII/100g) and gallic acid (9.6078±0.1mg FeII/100g). For DPPH radical, phenylalanine and gallic acid showed scavenging activities at 6.47±0.01mg/mL and 11.59±0.23mg/mL respectively, higher than Amino acids which showed a scavenging activity at 12.22±0.19mg/mL. The scavenging activity of different compounds (Amino acids, phenylalanine and gallic acid) differ significantly *($p<0.05$)*. For hydroxyl radical, the activity was maximal at 1.21±0.368mg/mL, 2.04±0.029mg/mL 6.58±0.385mg/mL for phenylalanine, amino acids and gallic acid respectively. This activity is very high for phenylalanine

and amino acids. Amino acids extract exhibited an appreciable scavenging activity for hydroxyl radicals compared to standard gallic acid. But lower than phenylalanine.

Table 1. Evaluation of the scavenging and reduction properties of Amino acids using phenylalanine and gallic acid as references

Compounds	Amino acids extract	Phenylalanine	Gallic acid
DPPH (IC_{50}mg/mL)	10.76±1.5^{c}	6.47±0.0^{a}	9.6078±0.1^{b}
OH (IC_{50}mg/mL)	2.04±0.1^{a}	1.28±0.3^{a}	6.58±0.3^{b}
FRAP (mgFeII/100g)	23.12±0.1^{c}	26.08±0.3^{b}	4.29±0.1^{a}

* Mean values from triplicate measurements ± standard deviation. Values in the same row followed by different
Superscripts are significantly different ($p<0.05$).

DISCUSSION

Nutritionally, the essential amino acids in the black bean seeds were higher in phenylalanine, methionine, tryptophan, threonine, lysine and histidine compared with FAO/WHO (1991) recommended daily allowances (RDA) (2.8 mg/100g; 2.2 mg/100g; 1.1 mg/100g; 3.4 mg/100g; 5.8 mg/100g and 1.9 mg/100g respectively). The role of some amino acids in the inhibition of polymerization of sickle cell blood had been documented. Current research focuses on the additive or synergistic effect of some amino acids in the management of SCD. Certain amino acids such as phenylalanine, alanine, lysine, arginine and serine have exhibited proven ability to inhibit sickle cell hemoglobin polymerization, and reduce the LDH activity levels in the sera of SCD patients (Ekeke and Shode, 1990; Ekeke *et al.,* 2001). L-Arginine supplementation of transgenic sickle cell mice resulted in inhibition of erythrocyte Gardos channel activity and amelioration of red cell dehydration (De Franceschi and Corroche, 2004). A phase II study to test the effect of arginine supplementation have shown no major effects on Gardos channel function and erythrocyte hydration in patients with sickle cell disease (Morris *et al.,* 2005). An *in vivo* study on SCD patients supplemented with L-glutamate to increase GSH and glutamate levels has shown some improvement of chronic pain (Morris *et al.,* 2008). To verify the antisickling activity of these amino acids detected in black bean seeds, an *in vitro* bioassay was performed. MBS 2% added to sickle cell blood at equal volumes significantly *($p<0.05$)* induced sickling from 28.4±2.4% to 80.67±3.1% after 3 hours of incubation. These results are in accordance with those obtained by Elekwa et *al*. (2005) after 1h of incubation. However, this value is lower than that obtained by Kotue *et al.,* (2014) (87.61%) and Joppa et *al*.,(2008) (96.5%) but it seems to be higher than 52.08% found by Nanfack *et al.,* (2013) after 2 hours of incubation. Sickle cell hemoglobin (HbS) is a product of a defective genetic code of hemoglobin molecule. Sodium metabisulfite creates hypoxic conditions leading to the loss of the morphology and sickled erythrocytes. *In vitro* deoxygenation of RBC by sodium metabisulfite causes progressive aggregation and polymerization of the individual hemoglobin molecules (Steinberg, 1993; Glactéros, 2001). The process of gelation (polymerization) of hemoglobin molecules increases the formation of sickling cells. After treatment of red blood cells with amino acids extract from black bean seeds at different concentrations in the presence of sodium metabisulfite (2%), a significant *($p<0.05$)* increase in inhibition of induced sickling was observed. Inhibition by amino acids from black bean seeds was significant for all concentrations compared to phenylalanine used as the positive control. The inhibition rates of induced sickling were 70.82±3.4%, 74.93±3.48%, 77.82±3.5% and 92.06±3.52% for amino acid concentrations of 6mg/mL; 8mg/mL; 10mg/mL and 12mg/mL. For phenylalanine, inhibition rates were 29.71±2.3, 34.96± 2.4, 48.96±2.6 and 67.96±2.8 with the same concentrations as the extract. Inhibition was maximal at 12mg/mL with inhibition percentage 92.06% for amino acids and 67.96±2.8 for phenylalanine used as the control. These rates of inhibition are lower than 99.10%, 96.30% and 94% obtained by Ojioka et *al*. (2010) based on certain amino acids in extracts of *S. sternocarpa, M.myristica* and *M. myristic* seeds respectively. The rates of inhibition obtained in this study are also higher than those estimated by Nwaoguikpe and Uwakwe, (2005) whoobtained from two indigenous spices of *Xylopia aethiopica* and *Monodora myristica*, sickling inhibition percentage rates of 87.66% and 90.93% respectively based on the amino acid content. Innocent et *al.* (2014) attained 66.7 % with amino acids investigated *in E. polysperma* extract, and Ojioka et *al*. (2012) also attained 65% with amino acids detected in *Cucurbita pepo.* The differences in inhibition percentages with the various extracts presented by these authors could be due to a possible variation in the quality and/or quantity of antisickling amino acids.

Reversibility of sickling studied by incubation of red blood cells, in the absence of MBS 2% significantly ($p<0.05$) reversed sickling at increasing rates of 47.9±3.5%, 69±38% 79.99±4% and 86.74±4.3 % at concentrations of 6mg/mL; 8mg/mL; 10mg/mL and 12mg/mL respectively, higher than those of Phenylalanine. This rate of reversibility is higher than 50% obtained by Nwaoguikpe and Uwakwe (2005) on two spices: *Xylopia aethiopica* and *Monodora myristica.* The effect of varied concentrations (6mg/mL; 8mg/mL; 10mg/mL and 12mg/mL) of black bean seeds extracts on erythrocyte membranes at different saline concentrations was also analyzed using the osmotic fragility test. Amino acids from black bean seeds significantly (*$p<0.05$*) stabilized the membranes of erythrocytes with the lowest percentage of hemolysis obtained at 12mg/mL most appreciable than that exhibited by phenylalanine, the positive control (Nwaoguikpe and Ejele, 2010). Antisickling properties of certain amino acids such as Phe, Lys, Leu, Asp, Ser, Arg and Tyr have been revealed (Nwaoguikpe and Ejele, 2010; Ojioka *et al.,* 2012 et Nwaoguikpe and Uwakwe, 2005), they inhibited polymerization of sickled cells and stabilized erythrocyte membranes. These amino acids have also been found to inhibit the formation of dense cells of HbSS blood *in vitro* (Ohnishi and Ohinishi, 2001; Ekeke *et al.,* 2001).Phenylalanine, like other aromatic amino acids, have been suggested to have antisickling potentials (Noguchi and Schechter, 1978). The presence of these amino acids in black bean seeds (Kotue *et al.,* 2016) may explain the origin of high antisickling effectiveness with respect to HbSS polymerization inhibition and the reversion of sickled erythrocytes. This could be attributed to their ability to diffuse into the hemoglobin molecule to bind at the heme pocket, thereby obstructing the "sticky patches" of the sickle cell Hb molecules (Noguchi *et al.,* 1982). This will prevent polymerization of Hb molecules into long fibers that would have caused deformation into sickle shapes of the normal disc biconcave shape of RBCs (Iyamu *et al.,* 2003). The high antisickling effectiveness with respect to stability of erythrocytes membrane could also be attributed to protection of erythrocytes membranes of HbSS blood from oxidative injury by reactive oxygen species (ROS), thus preventing membrane deformation, hemolysis and the formation of dense cells (Ekeke *et al.,* 1990; Ekeke *et al.,*

2001; Iwu *et al.,* 1988; Noguchi and Schetcher, 1978). Due to the above activities, amino acids from black beans seeds could therefore, have immense nutritional and therapeutic importance in the management of sickle cell disease. The antioxidant activities were evaluated using DPPH, ^{o}OH antiradical scavenging and FRAP assays. The antiradical activity in this study was represented by 50% inhibitory concentration (IC_{50}). Note that the antiradical activity of an extract is high when its IC_{50} is low. For DPPH radical scavenging assay, amino acids from black bean seeds significantly ($p<0.05$) stabilized free DPPH radicals (IC_{50}=10.76±1.571). This activity remains below the positive control (Phenylalanine, IC_{50}=6.47±0.01 and gallic acid, IC_{50}=9.6078±0.134). This finding is in good agreement with that observed with amino acids in peanut (Zhang *et al.,* 2011) and soybean (Zhu *et al.,* 2008), which showed a significant increase in the DPPH scavenging effect. A high scavenging activity for DPPH by certain antioxidant amino acids like tyrosine, tryptophan, histidine, alanine, phenylalanine, isoleusine, lysine, serine and glycine found in *Vigna unguiculata L.* were documented (Maira et *al.* 2012). Aromatic amino acids (Tyrosine and Phenylalanine) exhibited effective radical scavenging activities because they could donate protons easily to electron deficient radicals (Hernández-Ledesma *et al.,* 2005). The hydroxyl radicals are the most reactive species among the oxygen radicals and severely induce damage to almost any adjacent bimolecular (Lee and Yoon, 2004). This leads to oxidative stress which is responsible for several crises in sickle cell patients. From the results obtained amino acids from black bean seeds significantly ($p<0.05$) scavenged hydroxyl radical with IC_{50}=2.04±0.029higher than gallic acid used as the standard (IC_{50}=6.58±0.385) and no significant difference with phenylalanine. This activity is higher than that estimated by Hong *et al.,* (2015) with hydrophobic amino acids produced from cotton seed meal (IC_{50}= 3.28mg/mL); IC_{50}=6.04mg/mL for fermented wheat germ peptides (Niu *et al.,* 2013) and 4.92 mg/mL for rape seed protein hydrolysates (Pan *et al.,* 2011). Reducing power of a compound is closely related to its ability to donate electrons.

Therefore, the measurement of reducing power is widely used to assess the potential antioxidant ability of natural antioxidants. In this study the reduction power was determined using ferric reducing antioxidant power assay. 23.12±0.116mg FeII/100g of amino acids from black bean seeds exhibited an appreciable reducing power ($p<0.05$), lower than that of phenylalanine (26.08±0.355) and higher than that of gallic acid (4.29±0.133) which is a well-recognized reducing agent. The results agree with the results reported by Zhang et *al*, (2008) on rape seed and yam bean seed (Ajibola *et al.,* 2011) that exhibited a strong reducing power due to high amounts of hydrophobic amino acids. Similar findings of the reducing power have been previously reported in fermented soy bean amino acids (Zhu *et al.,* 2008) and peanut amino acids extract (Zhang *et al.,* 2011). Free radicals with the major species of reactive oxygen species (ROS) are unstable, and react readily with groups or substances in the body, resulting to oxidative stress; this is one of the major problems faced by sickle cell patients. Moreover, previous study has ascribed the higher antioxidant activity of amino acids extract to an increase in the concentration of acidic (aspartic acid and glutamic acid) and basic (lysine, arginine, and histidine) amino acids (Zhang *et al.,* 2011). Specifically, histidine can neutralize radical species to form stable oxidation products due to its imidazole ring (Pan *et al.,* 2011). The scavenging activity (DPPH) of amino acids from black bean seeds in this study is attributed to the presence of antiradical amino acids which can stabilize DPPH by releasing a hydrogen atom. The highest scavenging (OH) activity of amino acids from black bean seeds is accredited to the presence of hydrophobic amino acids which accounts for the high reduction power ascribed to the ability to give out electrons (Ranathunga *et al.,* 2006).

Conclusion

Total 16 amino acids were found in black bean seeds used to manage sickle cell disease in the west region of Cameroon. Amino acids extract have antisickling, anti-hemolytic and antioxidant properties. These results may justify the use of black bean seeds by sickle cell patients. Mode of action, nutraceutical capsules/functional foods formulation with these amino acids extract at 12mg/mL for SCD management will be the next step of this work.

Conflict of interest statement: We declare that we have no conflict of interest.

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REFERENCES

Acquaye, C. T. A., Young, J. D. and. Ellory, J. C. 1982. Mode of transport and possible mechanisms of action of L-phenylalanine benzyl ester as an anti-sickling agent. *Biochimica et Biophysica Acta*, vol. 693, no. 2, pp. 407–416.

Akbar, F., Nahida, Y. and Rabbani, M. A., (2012. Study of total seed proteins pattern of Sesame (*Sesamum indicum* L.) landraces via sodium dodecyl sulfate polyacrylamide gel elecrophoresis (SDS-PAGE). *Pak. J. Bot.,* 44, 2009-2014.

Aslan, M., Thornley-Brown, D. and Freeman, B. A., (2000. Reactive species in sickle cell disease. *Annal N Y.*, 899, 375–39.

Audu, S. S. and Aremu, M. O. 2011. Nutritional composition of raw and processed pinto bean (*Phaseolus vulgaris L.*) grown in Nigeria. *Journal of Food, Agriculture & Environment*, Vol.9 (3&4), 72-80.

Baxter, J. H. 1996. Amino Acids. In: Nollet, L.M.L., Ed., Handbook of Food Analysis, Vol. I, Marcel Dekker. Inc., New York, 179-228.

Benzie, I. and Strain, J. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power: The FRAP Assay". *Analytical Biochemistry,* 239, 70-76.

Bunn, H. F. 1997. Pathogenesis and treatment of sickle cell disease. *New England Journal of Medicine*, vol. 337, no. 11, pp. 762–769.

Comfort, F., Ajibola, J. B. Fashakin, Tayo, N., Fagbemi, I. and Rotimi, E. Aluko (2011. Effect of Peptide Size on Antioxidant Properties of African Yam Bean Seed (*Sphenostylis stenocarpa*) Protein Hydrolysate Fractions. *Int. J. Mol. Sci.*, 12, 6685-6702.

De Franceschi, L., Corroche, R. 2004. Established and experimental treatments for sickle cell disease. *Haematologica*, 89, 348-356.

Elekwa, I., Monanu, M. O. and Anosike, E. O. 2005. Effects of aqueous extracts of *Zanthoxylum macrophylla* roots on membrane stability of human erythrocytes of different genotypes. *Biochemistry,* 17, 7-12.

Ekeke, G. I. and Shode, F. O. 1990. Phenylalanine is the predominant antisickling agent in Cajanus cajan seed extract. *Planta Medica*, 56, 41-43.

Ekeke, G. I, Uwakwe, A. A. and Nwaoguikpe, R. N. 2001. The action of ripe juice on haemoglobin polymerization; Fe^{2+}/Fe^{3+} ratio and Lactate dehydrogenase activity of sickle cell blood. *Nigerien Journal of Biochemistry and molecular Biology*, 16(1), 31-35.

Ekeke, G. I. 2001. Sickle cell anemia: Basic Understanding and Management. Eddy-Joe Publishers, Nigeria, PP.56-73

Ekeke, G. I. and Shode, F. O. 1990. Phenylalanine is the predominant antisickling agent in *Cajanus cajan* seed extract. *Planta Medica*, 56, 41-43.

FAO/WHO (1991. Protein quality evaluation. Roe, Italy, Food and Agricultural organization of the United Nations, pp 1–66

Galacteros, F. 2001. Bases physiopathologiques de la drépanocytose, prise en charge et actualités thérapeutiques. *Bull. Soc. Pathol. Exot*., 94, 77-99.

Hong, S., Xiaohong, Y., Xin, W., Yifei, W., Yong, L., Jiangwu, T. and Jie (2015. Chemical composition and *in vitro antioxidant* property of peptides produced from cottonseed meal by solid-state fermentation. *CyTA - Journal of Food*., 13(2), 264-272.

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., and Amigo, L. 2005. Preparation of antioxidant enzymatic hydrolysates from a-lactalbumin and b-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 53, 588-593.

Innocent, U., Iba, 1., Monday, I., Akpanabiatu,1., Oboso, E., Etim, Edet, O., Akpanyung,1. Ekaete, U. I., Etuk, 1., Udoudo, M., Ekanemesang, 1., Etim, M. and Essien (2014. Antisickling activity of *eremomastax polysperma* and its effects on serum lipid and protein profiles of albino wistar rats. *European Journal of Biology and Medical Science Research*, Vol.3, N°1, pp.57-65.

Iwu, M. M., Igboko, A. O., Onwubiko, H., andNdu, U. E. 1988. Effectof cajaminose from Cajanus cajan on gelation and oxygen affinity of sickle cell haemoglobin. *Journal of Ethnopharmacology*, vol. 23, no. 1, pp. 99–104.

Iyamu, E. W, Turner, E. A, Asakura, T. 2003. Niprisan (Nix-0699) improves the survival rates of transgenic sickle cell mice under acute severe hypoxic conditions. *Brit. J. Haematol*., 122(6), 1001-1008.

Jain, A., Soni, M., Deb, L., Jain, A., Rout, S., Gupta, V., Krishna, K. 2008. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. leaves. *J. Ethnopharmacol,* 115(1), 61-66.

Jaja, S. I., Kehinde, M. O., Gbenebitse, S., Mojiminyi, F. B. O., Ogungbemi, A. I. 2000. Effect of vitamin C on arterial blood pressure, irreversible sickled cells and osmotic fragility in sickle cell anemia subjects. Nig J Physio Sci, 16(1–2):14–18.

Joppa, K. M., Vovora, Eklu, K., Agbonon, A., Aklikokou, K. and Gbeassor, M. 2008. Effet de *Morinda Lucida Benth* (Rubiaceae) et de *NewbouldiaLeavis p. Beau v.* Bignoniaceae) sur la falciformation. *Med. Trop*., 68, 251-256.

Kotue, T. C., Pieme, A. C. and Fokou, E. 2016. Ethnobotanicals usages in the management of sickle cell disease (SDC) in some localities of Cameroon, *Pharmacophore*, Vol. 7 (4), 192-200.

Kotue, T. C., Pieme, A. C., Ama Moor, V. J., Nanfack, P. and Ngogang, Y. J. 2014. The *in vitro* antisickling properties of "Hémodya". J. *Biol. Chem. Research,* Vol. 31, N°2, 757-768.

Lee, C. H., and Yoon, J. 2004. Application of photoactivated periodate to the decolorization of reactive dye: Reaction parameters and mechanism. *J. Photochem. Photobiol. A: Chem*., 165, 35–41.

Li-Ya, N., Shao-Tong, J., and Li-Jun, P. 2013. Preparation and evaluation of antioxidant activities of peptides obtained from defatted wheat germ by fermentation. *J. Food Sci Technol*., 50(1), 53–61.

Maira, SC. , Jorge, R-R. , Luis C-G. and David B-A. 2013. Antioxidant activity of *Vigna unguiculata L*. walp and hard-to-cook *Phaseolus vulgaris L*. protein hydrolysates. CyTA - Journal of Food, 11(3. 208-215.

Malika, B., Charlotte, D., Lamia, B., Michèle, F., Etienne, V., Jacques, E., Marie-Anne, G-P. and Carole, E. 2002. Hydroxyurea corrects the dysregulated L-selectin expression and increased H_2O_2 production of polymorphonuclear neutrophils from patients with sickle cell anemia. *Blood,* 99, 2297-2303.

Meletis, C. D. and Barker, J. E. 2005. Therapeutic uses of amino acids. *Alternative and Complementary Therapies*, 11, 24-28.

Moran-Palacio, E. F., Tortoledo-Ortiz, O., Yañez-Farias, G. A., Zamora-Álvarez, L. A., Stephens-Camacho, N. A., Soñanez-, J. G., Ochoa-López, L. M., Rosas-Rodríguez, J. A. 2014. Determination of amino acids in medicinal plants from Southern Sonora, Mexico. *Tropical Journal of Pharmaceutical Research*, 13(4), 601-606.

Morris, C. R., Kato, G. J., Poljakovic, M., Wang, X. 2005. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *Jama*, 294, 81-90.

Morris, C. R., Suh, J. H., Hagar, W. 2008. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. *Blood*, 111, 402-410.

Mpiana, P.T., Mudogo, V., Tshibangu, D. S. T., Kitwa, E. K., Kanangila, A. B., Lumbu, J. B. S., Ngbolua, K. N., Atibu, E. K., Kakule, M. K. 2008. Antisickling activity of anthocyanins from Bombaxpentadrum, Ficuscapensis and Ziziphusmucronata: P*hotodegradation effect. J. Ethnopharmaco*, 2, 413–418.

Nanfack, P., Biapa, N. P. C., Pieme, C. A., Ama, M. V J., Moukette, B., and Ngogang, Y. J. 2013. The *in vitro* antisickling and antioxidant effects of aqueous extracts *Zanthoxyllum heitzii* on sickle cell disorder. *BMC Complementary and Alternative Medicine*, 13(162), 1-7.

Ngozi, A. I. 2013. Phytomedicines and Nutraceuticals: Alternative Therapeutics for Sickle Cell Anemia. *The Scientific World Journal*, Vol. 20, 269 659.

Noguchi, C. T., Ackermann, S., Dimai, J., Sciller, P. W., Schechter, A. N. 1982. The effect of phenylalanine analogs on the solubility of deoxyHbS. A model class of gelation inhibitors. *Mol. Pharm*., 23, 106-110.

Noguchi, C. T. and Schechter, A. N. 1978. The inhibition of sickle cell hemoglobin and related compounds. *Biochem*., 17, 5455-5459.

Nwaoguikpe, R. N. and Uwakwe, A. A. 2005. The antisickling effects of dried fish (Tilapia) and dried prawn (*Astacus* red. *J. Appl. Sci*., 9(3), 115-119.

Nwaoguikpe, R. N. and Ejele (2010. Amino acid profile of some anti-sickling plant extracts and their hemoglobin polymerization inhibition. *Nig J Biochem Mol Biol*., 25(2), 53-59.

Ojiako, O. A., Igwe, C. U., Agha, N. C., Ogbuji, C. A.and Onwuliri, V. A. 2010. Protein and Amino acid compositions of *Sphenostylis stenocarpa, Sesanum indicum*, *Monodora myristica* and *Afzelia africana* seeds from Nigeria. Pak. J. Nut., 9(4), 368-372.

Ojiako, O. A., Anugweje, K., Igwe, C. U., Ogbuji, C. A., Alisi, C. S. and Ujowundu, C. O. 2012. Antisickling Potential and Amino Acid Profile of Extracts from Nigerian Lagenariasphaerica, Mucunapruriens and Cucurbita pepo Var Styriaca. *Journal of Research in Biochemistry*, 90, 55.

Ohnishi, S. T. and Ohnishi, T. 2001. *In vitro* effect of aged Garlic extract and other nutritional supplements on sickled erythrocytes. *J. Nutrition*, 131, 1085-1092.

Pan, M., Jiang, T. S., and Pan, J. L. 2011. Antioxidant activities of rapeseed protein hydrolysates. *Food and Bioprocess Technology,* 4(7), 1144-1152.

Ranathunga, S., Rajapakse, N., Kim, S.K. 2006. Purification and characterization of antioxidantative peptide derived from muscle of conger eel (*Conger myriaster*. *Eur. Food Res. Technol*., 222, 310–315.

Rumen, N. M. 1975. Inhibition of sickling in erythrocytes by amino acids. *Blood*, 45, 45 - 48.

teinberg, M. H. 1993. Management of sickle cell disease. *Engl. J. Med.*, 340, 1021-1030.

Tamanna, N., and Mahmood, N. 2014. Emerging roles of branched-chain amino acid supplementation in human diseases. *International Scholarly Research Notices*, 2014, 1-8.

Wu, G. 2009. Amino acids: Metabolism, functions, and nutrition. *Amino Acids*, 37, 1-17.

Yu, L., Halley, S., Perret, J., Palma, M., Wilson, J., Qian, M. 2002. Free radical scavenging properties of wheat extracts. *Journal of Agricultural and Food Chemistry*, 50, p.1619.

Zhang, Y., Zhang, H., Wang, L., Guo, X., Qi, X., and Qian, H. 2011. Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal. *European Food Research and Technology*, 232, 941–950.

Zhang, S. B., Wang, Z., & Xu, S. Y. 2008. Antioxidant and antithrombotic activities of rapeseed peptides. *Journal of the American Oil Chemists' Society*, 85, 521–527.

Zhu, X.L., Watanabe, K., Shiraishi, K., Ueki, T., Noda, Y., Matsui, T. and Matsumoto, K. 2008. Identification of ACE-Inhibitory Peptides in Salt-Free Soy Sauce That Are Transportable across Caco-2 Cell Monolayers. *Peptides*, Vol. 29, pp.338-344.

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