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Amino Acids Extract from Black Bean Seeds (*Phaseolus Vulgaris* L.) Inhibit Hemoglobin Polymerization and Modulate Erythrocyte Membrane Atpases In Sickle Cell Disease (SCD)

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Abstract

Back ground: The basic extracts involved in the formulation of functional foods/nutraceutics must first be the subject of preclinical and clinical trials. The several studies were shown that amino acids extract from black bean seeds (*Phaseolus vulgaris L.*) have antisickling, antioxidant and anti-haemolitic properties.

Objective: The objective of this study is to investigate the effect of amino acids extract from black bean seeds (*Phaseolus vulgaris L.*) on hemoglobin polymerization and erythrocytes membrane ATPases activities in Sickle Cell Disease (SCD).

Method: The ability for the amino acids extract to inhibit sickle cell hemoglobin polymerization and improve the Fe^{2+}/Fe^{3+} ratio of HbSS blood were assed using spectroscopic method. The activities of three ATPases (Na^+/K^+; Ca^{2+}; and Mg^{2+}-ATPases) were investigated by the kinetic method following the release of inorganic phosphate (Pi) by ATP taking into account the determination of the total membrane proteins of erythrocytes by Bradford's method.

Result: The lowest potential of inhibition was obtained (39.98±2%) with the amino acids extract at 12 mg/mL corresponding significantly ($p < 0.05$) to the highest potential of inhibition of polymerization (71.16±2%). The greatest Fe^{2+}/Fe^{3+} ratio was obtained (3.94±2%) with the amino acids extract at 12 mg/mL corresponding significantly ($p < 0.05$) to the best rates increase of oxy-hemoglobin (48.73±2%). The red blood cells membranes ATPase activity were estimated and expressed as µmol of Pi/mg of protein/hour x 10^{-3} there was an increase in the activities of Na+/K+ and Ca^{2+}-ATPases (255.98±62 and 282.98±36 respectively) at the concentration of 12 mg/mL of extract compared to the negative control (167.36±60 and 236.91±62 respectively). However, for Mg^{2+}-ATPase, there is rather a significant reduction ($p<0.05$) in this activity (286.71±51 against 146.53±35) obtained in the presence of the amino acid extract at 12 mg/mL).

Conclusion: The amino acids extract of black bean seeds (*Phaseolus vulgaris L.*) at a concentration of 12 mg/mL offered the best inhibition potentials of hemoglobin S polymerization, increase rates of oxyhemoglobin and the best ATPasic activities.

Keywords: Amino acid extracts; black bean seeds; sickle cell disease; polymerization; Fe^{2+}/Fe^{3+} ratio; ATPase activities

Introduction

Functional foods/nutraceutics are defined as a food or part of food that provides health benefits, including the prevention and/or treatment of disease. They are of broad terms which describes any substance extracted from food sources with additional health benefits along with the basic nutritional value already present in them [1]. Since years, functional foods/nutraceutics have played an important role in the overall well-being of humans. Several bioactive molecules are being identified to possess health benefits which continue to garner research interest so that safe and cost-effective molecules can be discovered for oral administration. These can be grouped into the following three broad categories: 1- substances with established nutritional functions, such as vitamins, minerals, amino acids and fatty acids – Nutrients / 2- Herbs or botanical products as concentrates and extracts - Herbals / 3- Reagents derived from other sources (e.g. pyruvate, chondroitin sulphate, steroid hormone precursors) serving specific functions, such as sports nutrition, weight-loss supplements and meal replacements-dietary supplements [2]. The consumption of functional foods/nutraceutics play a pivotal role as curative and preventive measure of some chronically disease like sickle cell disease (SCD). In fact, several functional foods/nutraceutics have been studied in SCD therapies. This is the case of Nicosan [formerly known as Nipris (Nix-0699)], an anti-sickling phytomedicine

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(bioactive non-nutrient plant compounds), is reported to inhibit the polymerization of hemoglobin S. As reported earlier, it is a cocktail of four medicinal plants, *Piper guineense, Pterocarpus osun, Eugenia Caryophyllusand Sorghum bicolor* as components and is currently being marketed in Nigeria in encapsulated [3]. There is also Ciklavit. It is a plant extract preparation available for the management of the sickle cell anemia condition. It contains primarily extracts of the plant Cajanus cajan proteins (essential amino acids), vitamins such as vitamin C (ascorbic acid), and minerals such as zinc. Ciklavit (*Cajanus cajan extract*) has been reported to have antisickling properties and to improve well-being of sicklers [4]. The role played by other components in Ciklavit (*besides Cajanus cajan*) is basically nutritional. Ciklavit may cause a reduction in bone pains (painful crises) and may ameliorate the adverse effect of sickle cell anemia on the liver [5].Earlier reports of the antisickling constituent of *Cajanus cajan* suggested phenylalanine and several other amino acids [6]. This study shows that amino acids play a key role in the management of sickle cell disease. Otherwise, black bean seed (*Phaseolus vulgaris*) 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 [7]. In fact *Phaseolus vulgaris* 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 vulgaris* L. can be of profound benefit to the sicklier [8]. Several studies reveled that a total of 16 amino acids were found in extract from black bean seeds used to manage sickle cell disease in the west region of Cameroon and have shown that this amino acid extract have antisickling, antioxidant and anti-haemolitic properties [9]. The objective of this study was to assess the effect of the extract of these amino acids on the polymerization, improving the Fe^{2+}/Fe^{3+} ratio of hemoglobin S and on the ATPases activities of sickle cell patient's erythrocytes membrane.

Methodology

Amino Acids Extraction

The Amino acids extraction was carried out at the Agroprocessing and Natural Product Division of CSIR-National Institute for Interdisciplinary Science and Technology (CSIR/ NIIST), Kerala-India [9].

Preparation of Blood Samples

Blood samples were collected from 19 confirmed HbS (homozygous) sickle cell patients (10 males and 9 females) with a mean aged 25.39±6 known to have sickle cell disease, attending monthly Hematology outpatient clinic at the Yaounde Central Hospital, Cameroon. Permission for the use of blood was granted by the bio-ethics committee number 8512/CRERSHC/2019. Portions of HbS blood were collected into EDTA tubes.

Sickle Cell Hemoglobin Polymerization Experiment

Erythrocytes were isolated from 1200µL HbS blood samples by centrifugation at a gravitational force of 1500x g for 15 minutes using the bench centrifuge (5804R Centrifuge). Following careful siphoning of the plasma with Pasteur pipette, the erythrocytes were obtained by inversion suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was freeze thawed at 4^0C to produce a hemolyzate for the hemoglobin polymerization experiment. HbS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2% Sodium metabisulphite as reductant or deoxygenating agent [4]. 0.5 mL of 2% solution of sodium metabisulphite ($Na_2S_2O_3$), 0.5 mL normal saline and 0.1mL of hemoglobin solution (HbS) were pipetted into a cuvette shaken and the absorbance read at 700 nm at 30s, then at 180s. This represented the negative control. Distilled water was used as blank in all assays. In the main assay, 0.5 mL of 2% solution of sodium metabisulphite, 0.5 mL of amino acids extract (10 and 12 mg/mL) and 0.1mL of hemoglobin solution (HbS) were pipetted into a cuvette and the optical density reading taken as above. Phenylalanine at 4 mg/mL was used as positive control. The rates of the inhibition potentials of hemoglobin polymerization (PIP) in percentage were estimated after calculated the potential of hemoglobin polymerization (PP) from the formula of average change in optical density/absorbance against time in seconds [10].

Determination of the Fe^{2+}/Fe^{3+} Rate of Sickle Cell Blood

The Fe^{2+}/Fe^{3+} rate amino acids extract on the sickle cell blood was determined by the methods of and reported by [11,12]. The procedure involves the lysing of 100µL of whole blood from each patient in 2.5mL of distilled and deionized water in the presence of the amino acids extract, using 0.9% NaCl and Phenylalanine at 4 mg/mL as negative and positive controls respectively and then determining the absorbance of oxy-hemoglobin and methemoglobin at their characteristic wavelengths of 540nm and 630nm, respectively.

Effects of the Amino Acids Extract on the Activities of Membrane Atpases of Sickle Cell Erythrocytes

Preparation of Erythrocyte Membrane

This followed the method described, reported by [13, 14]. Briefly, the whole blood was centrifuged at 5000 g for 10 minutes and the resultant precipitate washed with 0.15 M NaCl (pH 7.4). This 'washing' process was repeated three time, the final precipitate lysed by swirling in 5 mM $NaH_2PO_4.2H_2O$ (pH 7.7), and then centrifuged at 5,000 g for 10 minutes. The resultant precipitate was 'washed' with 10 mM Tris-HCl (pH 7.7) and suspended in 3 mL of distilled water. The isolated membranes were stored at 4^oC and used within 12 hours of collection of blood samples.

Na^+/K^+; Ca^{2+} And Mg^{2+}-ATPases Activities Assays

The assays of the enzyme activities followed the procedure of [14], reported by [15] and monitored the inorganic phosphate released from ATP. Enzyme activities were expressed as µmole Pi/mg protein/hour x 10^{-3}. The concentration of phosphate in 1

ml of the supernatant was measured by the method described by, reported by [14, 16]. For this, 1.0 ml of 2.5% ammonium molybdate was added and after 10 minutes, the addition of 0.1 mL of 2% ascorbic acid followed. The mixture was kept at room temperature for 20 minutes for color development. The absorbance of the final mixture was measured at 725 nm using spectrophotometer (JENWAY 6015).

Protein Determination

The protein content of the membranes was determined by the method of Bradford, reported by [17, 14] using Bovine Serum Albumin (BSA) as the protein standard.

Amino Acids Extract Assays on the Different Atpases Activities

The reaction tubes, in addition to the contents the various ATPases, contained 0.5 mL of different concentrations of the amino acids extract to produce the desired final concentrations in the total reaction mixture of 1 mL 0.9% NaCl and Phenylalanine at 4 mg/mL were used as negative and positive controls respectively.

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

Table I below shows the potential of hemoglobin polymerization (PP) and inhibition potentials of hemoglobin polymerization (PIP) without (NE) and in the presence of amino acids extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control. Comparatively to negative control without amino acids extract, all the concentrations of amino acids extract and phenylalanine significantly ($p<0.05$) inhibited HbS polymerization compare to negative control. (E2: 12 mg/mL) has show the significantly higher value ($p<0.05$) of potential of inhibition of hemoglobin polymerization (71.16±2%).

Table II shows the effect of amino acids extract on the Fe^{2+}/Fe^{3+} ratio and increase oxy-hemoglobin rates without extract (NE) and in the presence of amino acids extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control. Comparatively to negative control without amino acids extract, all concentrations and phenylalanine improved significantly ($p<0.05$) the Fe^{2+}/Fe^{3+} ratio of the sickle cell blood. (E2: 12 mg/mL) has show the significantly higher value ($p<0.05$) increase Fe^{2+}/Fe^{3+} ratio (48.73±2).

The levels of Na^+/K^+, Ca^{2+} and Mg^{2+}-ATPases in the absence (negative control) and presence of varying concentrations of amino acids extract (E1: 10 and E2: 12 mg/mL) and Phenylalanine (Phe 4: mg/mL) used as positive control are shown by the Figures 1, 2, 3 respectively. For Na^+/K^+-ATPase (Figure 1), the enzyme activities (µmole Pi/mg protein/hour x 10^{-3}) in the absence of the amino acids extract was 167.36±60. The activities in the presence of amino acids extract showed increases with increasing concentration of the extract 226.74±75 for 10mg/mL and 255.98±62 for 12 mg/mL. For Phenylalanine (Phe 4: mg/mL) used as positive control, this activity was 183.57±75. A statistically significant increase ($p<0.05$) in comparison with activities in the absence (negative control), the positive control and the two concentrations of amino acids extract was noted.

For Ca^{2+}-ATPase (Figure 2), enzyme activities in the absence of amino acids extract was 236.36±62. In the presence of varying concentrations of the amino acids extract (E1: 10 and E2: 12 mg/mL), the activities showed dose dependent increases (274.19±37 and 282.98±36 respectively). ($p<0.05$) when compared with values in the absence of the amino acid extract and Phenylalanine (Phe 4: mg/mL) used as positive control (269.52±22).

For Mg^{2+}-ATPase, the enzyme activities in the absence of the amino acids extract was 286.71±51. A dose dependent decrease in activity was obtained in the presence of varying concentrations (E1: 10 and E2: 12 mg/mL) of the amino acids extract for this enzyme (193.80±62 and 146.53±35 respectively). For Phenylalanine (Phe 4: mg/mL) used as positive control, this activity was 178.62±71. The decreased obtained for the two concentration and phenylalanine were significant ($p<0.05$) when compared with values in the absence of the amino acids extract.

Discussion

The substitution of glutamic acid in position 6 by a valine in the β chain of hemoglobin causes its precipitation in red blood cells with sickling, promoting the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) unable to fix oxygen [18]. Hence the drop in the Fe^{2+}/Fe^{3+} ratio (drop in the erythrocyte concentration of oxyhemoglobin (Fe^{2+}) and increase that in methemoglobin (Fe^{3+}).

It has been reported that, an ideal anti-sickling agent would affect the oxygen affinity of the hemoglobin molecule which can be measured by determining the effect of the agent on Fe^{2+}/Fe^{3+} ratio of sickle cell blood [19]. Table I shows that the phenylalanine (Phe 4: mg/mL) used as positive control, the two concentration of the amino acids extract (E1: 10 and E2: 12 mg/mL) improved the increasing of Fe^{2+}/Fe^{3+} ratio significantly ($p<0.05$).

The amino acid extract of black bean grains (*Phaseolus vulgaris L.*) at the concentrations 10 mg/mL and 12mg/mL significantly improves ($p < 0.05$) this ratio which goes from 2.02 ± 0 in the absence of extract at 2.99 ± 1 and 3.94 ± 2 respectively, corresponding to an oxy-hemoglobin increase rate of 32.44 ± 1% and 48.73 ± 2% respectively. These values are greater than 17.82 ± 0.0 and 31.73 ± 0.0 obtained by respectively with phenylalanine and Lysine[20].

In fact, several studies are shown that the total of 16 amino acids were found in the amino acids extract corresponding to aspartic

Table I: Potential of hemoglobin polymerization (PP) and potential of inhibition of hemoglobin polymerization (PIP) without extract (NE) and in the presence of amino acids extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control

Test	NE	Phe (4mg/mL)	E1 (10mg/mL)	E2 (12mg/mL)
Potential of hemoglobin polymerization (%)	68.43 ± 6^{a}	46.56 ± 5^{b}	41.95 ± 6^{c}	39.98 ± 2^{d}
Potential of inhibition of hemoglobin polymerization (%)	0.0^{a}	46.97 ± 5^{b}	63.12 ± 6^{c}	71.16 ± 2^{d}

Values with the same superscripts are statistically significant at $p \leq 0.05$ along the rows

Table II: Effect of amino acids extract on the Fe^{2+}/Fe^{3+} ratio and increase Fe^{2+}/Fe^{3+} ratio without (NE) and in the presence of amino acids extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control

Test	NE	Phe (4mg/mL)	E1 (10mg/mL)	E2 (12mg/mL)
Fe^{2+}/Fe^{3+} ratio	2.02 ± 0^{a}	2.47 ± 0^{b}	2.99 ± 1^{c}	3.94 ± 2^{d}
Increase Fe^{2+}/Fe^{3+} ratio (%)	0.0^{a}	18.22 ± 0^{b}	32.44 ± 1^{c}	48.73 ± 2^{d}

Values with the same superscripts are statistically significant at $p \leq 0.05$ along the rows

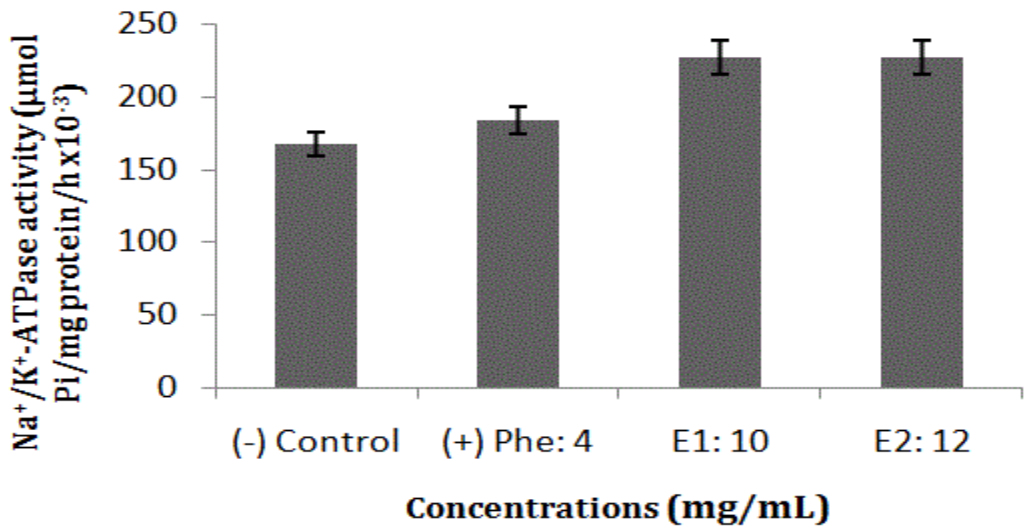

Figure 1: Effect of amino acids extract on Na^{+}/K^{+}-ATPase activity without (negative control) and in the presence of amino acid extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control

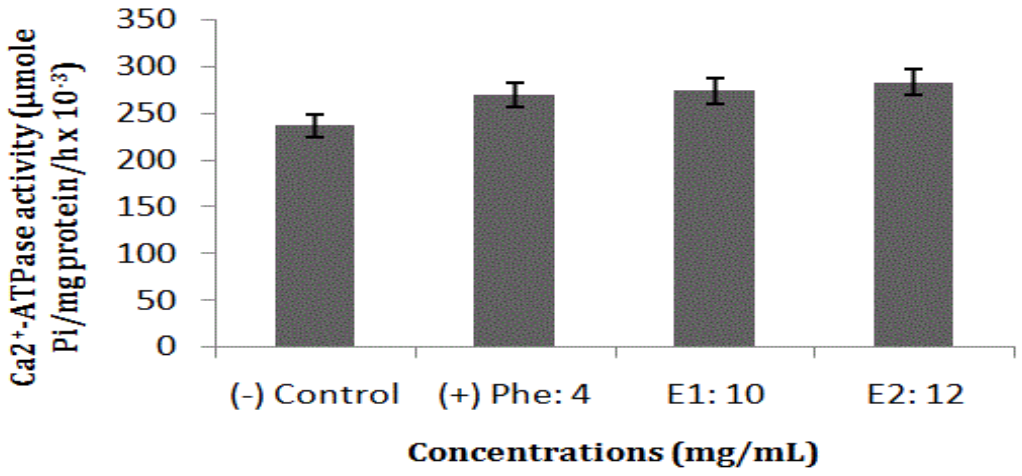

Figure 2: Effect of amino acids extract Ca^{2+}-ATPase activity without (negative control) and in the presence of amino acid extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control

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) [9].

The antioxidant activity of this amino acid extract has been proven. By this mechanism, we can explain why it would prevent the oxidation of ferrous iron and thus increase the affinity of HbS for oxygen in sickle cell patients.

Red blood cells which contain sickle haemoglobin (HbS) under hypoxic conditions assume a characteristic sickle shape due to polymerisation of deoxygenated HbS molecules which aggregate to form elongated rod-like polymers. The presence of these polymers leads to drepanocytes, red blood cells transformed by haemoglobin polymerisation into rigid, inflexible cells with at least one pointed projection [21]. The phenylalanine (Phe 4: mg/mL) used as positive control, the two concentration of the amino acids extract (E1: 10 and E2: 12 mg/mL) inhibited polymerisation significantly ($p<0.05$). (E2: 12 mg/mL) that has the higher value increase Fe^{2+}/Fe^{3+} ratio (48.73±2) is respectively lower than the values found with phenylalanine (83.30 ± 0.0%) and lysine (84.80 ± 0.1%). This may be due to their different concentrations in the extract. Since an ideal anti-sickling agent would inhibit polymerisation of the abnormal hemoglobin (HbSS), this attribute is used in evaluating a possible antisickling property of a drug. Often times, this evaluation is done with Phe as the gold standard [22]. The observed results are attributed to the polar nature of these fractions and their subsequent ability to diffuse into the haemoglobin molecule to bind at the heme pocket, thereby obstructing the protein-protein interaction necessary for gelation [23, 24].

The unidirectional active transport of ions such as Na^+; K^+; Ca^{2+} and Mg^{2+} has been reported to play major roles in maintaining the stability of the erythrocyte membranes [25, 26]. Indeed, the presence of three different adenosine triphosphatases (Na^+/K^+-ATPase; Ca^{2+}-ATPase and Mg^{2+}-ATPase) in human erythrocyte membranes had been reported by various workers [27, 28, 29]. The determinations of the activity levels of these ATPases in different human genotypes showed that (Na^+/K^+ and Ca^{2+}-ATPases) were significantly lower in HbS erythrocytes, while Mg^{2+}-ATPase was significantly higher than for HbA erythrocytes [29, 30]. The activity of membrane ATPases were found to be significantly high in patients of sickle cell crisis in comparison to steady state ones [31].

The presence of phenylalanine and two varying concentrations of the amino acids extract from black bean seeds (*Phaseolus vulgaris L.*) had various effects on the ATPase activities. A dose-dependent activation was observed for Na^+/K^+ and Ca^{2+}-ATPases while an inhibition was noted for Mg^{2+}-ATPase The amino acid extract (12 mg/mL) may have better actions on the possible regulation of the cellular effects performed by these ATPases. Furthermore, the activations/inhibitions for HbS suggesting more 'benefits' of the effects on the ATPases for sickle cell patients. In fact, several studies are shown that the total of 16 amino acids were found in the amino acids extract corresponding to 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) [9]. However, it has been shown that, the phenylalanine, a known anti-sickling agent at 4 mg/mL, activated both Na^+/K^+ and Ca^{2+}-ATPases, but inhibited the Mg^{2+}-ATPase activity of HbS erythrocytes membrane preparations [32, 33]. The presence of the phenylalanine in this extract may justify the ATPases activities with synergistic action link to other amino acids.

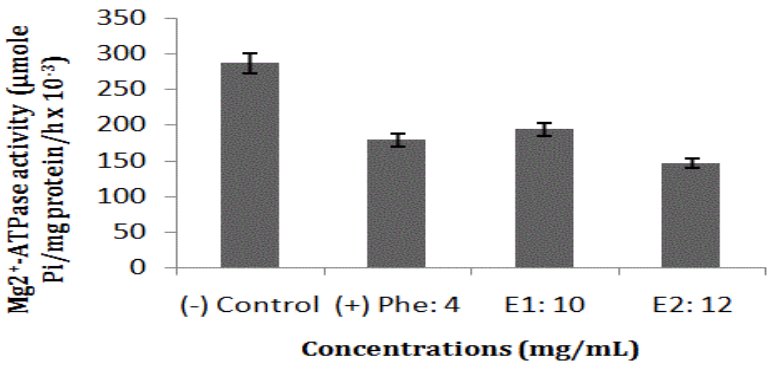

Figure 3: Effect of amino acids extract Mg^{2+}-ATPase activity without (negative control) and in the presence of amino acid extract (E1: 10 and E2: 12 mg/mL). Phenylalanine (Phe 4: mg/mL) was used as positive control

The modulation of the activities of the three membrane-bound ATPases (increases for Na^+/K^+ and Ca^{2+}-ATPases and decrease for Mg^{2+}-ATPase) by the amino acids extract noted in this study suggest vital roles for the amino acids extract from black bean seeds (*Phaseolus vulgaris* L.) in the maintenance of the shape/volume of erythrocytes. It further provides some clues at the molecular level on the possible roles of the ATPases in the sickling of erythrocytes, and of the amino acids extract from black bean seeds (*Phaseolus vulgaris* L.) in reversing this sickling thereby supporting its use in the therapy of the sickle cell patients [9].

Conclusion

The amino acid extract of black bean seeds (*Phaseolus vulgaris* L.) used in the management of SCD in the West Region of Cameroon at a concentration of 12 mg/mL have the best potential of inhibitions of polymerization, the best increase rates of oxyhemoglobin of hemoglobin S and the best ATPasic activities. Nutraceutical capsules/functional foods formulation with these amino acids extract at 12mg/mL for SCD management will be the next step of this work.

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