

Omega 3 and *Azadirachta Indica* J. Seed Oil Inhibit Hemoglobin Polymerization and Modulate Erythrocyte Membrane ATPases in Sick Cell Disease (SCD)

Nantchouang NAL¹, Kotue TC^{1*}, Djouhou FM¹, Ngo SF², Pieme AC³, Kansci G¹, Fokou E¹

¹Laboratory for Food Science and Metabolism - Department of Biochemistry – Faculty of Science – University of Yaounde1, Cameroon

²Hematological Service at the Central Hospital, Yaounde, Cameroon

³Laboratory of Biochemistry, Physiology and Pharmacology - Faculty of Medicine and Biomedical Science / UHC– University of Yaounde 1, Cameroon

Received: 24 March, 2020; Accepted: 07 May, 2020; Published: 18 May, 2020

***Corresponding author:** Laboratory for Food Science and Metabolism - Department of Biochemistry – Faculty of Science – University of Yaounde1, Cameroon, E-mail: ctkotue_bio@yahoo.fr / ckotue@uy1.uninet.cm

Abstract

Back ground: Functional foods/Nutraceuticals efficacy depends on both its composition and the physicochemical properties of the components that used in their formulation. Vegetable oil can play an important role. Several studies are shown that omega 3 (0.2%) and *Azadirachta indica* J. seed oil (0.4%) have antisickling, antioxidant and anti-haemolytic properties.

Objective: The purpose of this present study was conducted to determine effects of omega 3 and *Azadirachta indica* J. seed oil on hemoglobin polymerization and erythrocyte membrane ATPases in Sick Cell Disease (SCD).

Method: The ability for the omega 3 and *Azadirachta indica* J. seed oil to inhibit sickle cell hemoglobin polymerization and improve the Fe²⁺/Fe³⁺ ratio of HbS blood were assed using spectroscopic method. The activities of three ATPases (Na⁺/K⁺; Ca²⁺; and Mg²⁺-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.

Results: The lowest potential was obtained with *Azadirachta indica* J. seed oil (AO: 0.4% v/v) corresponding significantly (p <0.05) to the highest potential of inhibition of polymerization (83.11 ± 8%) compared to that obtained with omega 3 (ω3 0.2% v/v) estimated at 68.15±7%. The greatest Fe²⁺/Fe³⁺ ratio was obtained (6.65±1.68) with AO (0.4% v/v) corresponding significantly (p<0.05) to the greatest rate of increase of oxyhemoglobin (79.05±3%) compared to that obtained with ω3 (0.2% v/v) estimated at 70.58±5%. As for the activities of Na⁺/K⁺, Ca²⁺ and Mg²⁺-ATPases expressed in μmole Pi/mg protein/hour x 10⁻³, there was an increase in the activities of Na⁺/K⁺ and Ca²⁺-ATPases (203.82±41 and 190.48±32) with AO (0.4% v/v), but without any significant difference (p>0.05) compared to those obtained with ω3 (0.2% v/v) (188.66±9 and 235.61±52). However, for Mg²⁺-ATPase, there was rather a significant decrease (p<0.05) in these activities ranging from 181.9±32 for ω3 (0.2% v/v) to 124.00±21 for AO (0.4% v/v).

Conclusion: The *Azadirachta indica* J. seed oil at a concentration of 0.4% v/v offered the best potential inhibition of hemoglobin S polymerization, oxyhemoglobin increase rate and the best ATPase activities.

Keywords: Omega 3 - *Azadirachta indica* J. seed oil - sickle cell disease - polymerization - Fe²⁺/Fe³⁺ ratio - ATPase activities.

Introduction

Vegetable oil is a rich source of nutraceuticals in addition to essential fatty acids. Triglyceride is the major component of the vegetable oil, and its fatty acids play a vital role in human health and nutrition. Minor components of vegetable oil such as lecithin, tocopherols and tocotrienols, phytosterols and phytostanols, oryzanol, policosanols, squalene, carotenoids, and lignans also play a significant role in human. Results from epidemiologic and clinical studies have indicated that the consumption of omega-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosasaenoic acid (DHA) have positive effects in decreasing risk factors of cardiovascular diseases [1]. The

findings from a previous retrospective and cross-sectional study suggest that omega-3 fatty acids might be beneficial in Sick Cell Disease (SCD) [2, 3]. Omega-3 fatty acids EPA/DHA at 0.2% have antisickling, anti-haemolytic and antioxidant properties [4]. Several studies have reported that tiger nut oil (*Cyperus esculentus*) and black seed oil (*Nigella sativa*) are natural products resulting to considerable antisickling effects [5]. In addition, tiger nut and black seed oils increase the antioxidant capacity of HbS (sickle) blood samples [6]. A recent investigation has shown that *Azadirachta indica* J. seed oil used to manage Sick Cell Disease (SCD) in North Cameroon at 0.4% has the best antisickling, anti-haemolytic and antioxidant properties [7, 8]. The omega-3 (linolenic acid) and vitamin E are in the composition of this oil

[8]. In fact, the sickle cell haemoglobin (HbS) variant is caused by a point mutation affecting the coding sequence of the β -globin gene resulting in a substitution of glutamic acid by valine at the sixth position of β -globin chains [9,10]. Under low oxygen tension, deoxyHbS molecules polymerize into microfibrils parallel to each other with concomitant reduced solubility (forms gel) and resultant erythrocyte membrane deformity and damage [11]. The unidirectional active transport of ions such as Na^+ , K^+ and Ca^{2+} , has been reported to play major roles in maintaining the stability of the erythrocyte membranes [12,13]. Indeed, the presence of three different adenosine triphosphatases (Na^+/K^+ , Ca^{2+} and Mg^{2+} -ATPases) in human erythrocyte membranes had been reported by various workers [14, 15, 16]. 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 sickle cell erythrocytes, while Mg^{2+} -ATPase was significantly higher than for normal erythrocytes [16, 17]. The new therapeutic approaches for the treatment and management of sickle cell disease include the usage of natural products that interact either non-covalently or covalently with HbS molecules to retard or inhibit hemoglobin aggregation and polymerization and/or modulate the activities of the three membrane-bound ATPases (increases for Na^+/K^+ and Ca^{2+} -ATPases, and decrease for Mg^{2+} -ATPase) [18, 19, 20]. The aim of this study, therefore, is to investigate the effects of omega 3 and *Azadirachta indica* J. seed oil on hemoglobin polymerization and erythrocyte membrane ATPases in sickle cell disease (SCD).

Methodology

The Omega-3 Fatty Acids EPA/DHA and *Azadirachta Indica* J. Seed Oil Concentrations

The omega-3 fatty acids EPA/DHA pure fish oil capsules were bought in a pharmacy. Each capsule contains 750 μL in proportion EPA/DHA (3v/2v). The following concentrations of 0.2% v/v for omega-3; 0.2% v/v and 0.4% v/v of the *Azadirachta indica* J. seed oil used to manage sickle cell disease (SCD) in North Cameroon were obtained by diluting the oils in normal saline diluted ethanol (1:4 in normal saline).

Preparation of Blood Samples

After receiving an ethical clearance with number CEN° 00650/CRERSHC/2019, consent from all blood donors was read and signed by all the patients participating to the study after being informed of the research objectives. Blood samples were collected from 19 confirmed HbS (homozygous) sickle cell patients (9 males and 10 females) between mean aged 26.52 ± 9 known to have sickle cell disease, attending monthly Hematology outpatient clinic at the Yaounde Central Hospital, Cameroon. Four mL of blood samples were collected into sodium EDTA tubes and stored at the 6°C for the experiment.

Sickle Cell Hemoglobin Polymerization Experiment

Erythrocytes were isolated from 1200 μL 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 by repeated inversion suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was freeze thawed at 4°C 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 [21]. 0.5 mL of 2% solution of sodium metabisulphite ($\text{Na}_2\text{S}_2\text{O}_3$), 0.5 mL normal saline and 0.1 mL 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 omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v) and 0.1 mL 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 potential of inhibition 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 [22].

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

The $\text{Fe}^{2+}/\text{Fe}^{3+}$ rate of omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v) on the sickle cell blood was determined by the methods of [23] and reported by [24]. The procedure involves the lysing of 100 μL of whole blood from each patient in 2.5 mL of distilled and deionized water in the presence of the omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v), using 0.9% NaCl and Phenylalanine at 4 mg/mL as negative and positive controls respectively and then determining the absorbance of hemoglobin and methemoglobin at their characteristic wavelengths of 540 nm and 630 nm, respectively.

Effects of the Omega-3 and *Azadirachta Indica* J. Seed Oil on the Activities of Membrane ATPases of Sickle Cell Erythrocytes

Preparation of Erythrocyte Membrane

This followed the method described by [25] reported by [20]. Briefly, the whole blood was centrifuged at 5,000 g for 10 minutes and the resultant precipitate washed with 0.15 M NaCl (pH 7.4). This 'washing' process was repeated thrice, the final precipitate lysed by swirling in 5 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (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 distilled water. The isolated membranes were stored at 4°C and used within 12 hours of collection of blood samples.

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

The assays of the enzyme activities followed the procedure

of [26], reported by [20] and monitored the inorganic phosphate released from ATP. Enzyme activities were expressed as $\mu\text{mol Pi/mg protein/hour} \times 10^{-3}$. The concentration of phosphate in 1 mL of the supernatant was measured by the method described by [27] reported by [20]. 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 colour development. The absorbance of the final mixture was measured at 725 nm using spectrophotometer.

Protein Determination

The protein content of the membranes was determined by the method of [28], reported by [20] using Bovine Serum Albumin (BSA) as the protein standard.

Omega-3 and Azadirachta Indica J. Seed Oil 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 omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) to produce the desired final concentrations in the total reaction mixture of 1 mL. 0.9% NaCl and Phenylalanine at 4 mg/mL as negative and positive controls respectively.

Statistical Analysis

The results were expressed as mean $\pm$ standard deviation. Data was analyzed using Analysis of Variance (ANOVA) of Kruskal-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

The results illustrated in table I shows booth the evaluation of the potential of hemoglobin polymerization (PP) and inhibition of hemoglobin polymerization (PIP) without (NE) and in the presence of omega 3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) and the phenylalanine (Phe 4: mg/mL) used as positive control. Comparatively to negative control (56.5 $\pm$ 3%), phenylalanine (Phe 4: mg/mL); omega 3 ($\omega 3$: 0.2% v/v) and *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) inhibited significantly ($p < 0.05$) the HbS polymerization. *Azadirachta indica* J. seed oil at 0.4% v/v has show the significantly higher value ($p < 0.05$) of potential of inhibition of hemoglobin polymerization (83.11 $\pm$ 8%).

Table II shows the effect of oils on the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio and increase $\text{Fe}^{2+}/\text{Fe}^{3+}$ without (NE) and in the presence of the omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) and the phenylalanine (Phe 4: mg/mL) used as positive control. Comparatively to negative control, phenylalanine (Phe 4: mg/mL); omega 3 ($\omega 3$: 0.2% v/v) and *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) improved significantly ($p < 0.05$) the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio of the sickle cell blood. The *Azadirachta indica* J. seed oil at 0.4% v/v has

showed the significantly higher value ($p < 0.05$) increase $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio(79.05 $\pm$ 3%).

The modulation of Na^+/K^+ ; Ca^{2+} and Mg^{2+} -ATPases activities in the absence (negative control) and presence of the omega 3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v) and the phenylalanine (Phe: 4 mg/mL) used as positive control are shown by the figures 1; 2 and 3 respectively.

Figure 1 below shows the activity of the Na^+/K^+ -ATPase erythrocyte membrane in the absence of the oil samples, of its modulation with the positive control (Phe: 4 mg / mL) and the oil samples omega 3 ($\omega 3$: 0.2%); AO (0.2%), then AO (0.4%) samples compared to the negative control. This figure shows a significant increase ($p < 0.05$) in the activities of this enzyme expressed in $\mu\text{mol Pi/mg proteins/hour} \times 10^{-3}$. For the control (-) this activity was 123.30 ± 33 . In the presence of the positive control (Phe: 4 mg/mL), it increases considerably to 209.35 ± 27 . In addition, these activities were respectively 188.66 ± 9 , 196.84 ± 16 and 203.82 ± 41 for omega 3 ($\omega 3$: 0.2%); AO (0.2%) and AO (0.4%). However, no significant difference ($p > 0.05$) was observed between the different values obtained despite the high activity recorded with phenylalanine 4 mg/mL. The greatest activity was obtained with AO (0.4%).

Figure 2 bellow shows the activity of Ca^{2+} -ATPase membrane of sickle cell erythrocytes in the absence of phenylalanine (Phe: 4 mg/mL) and omega 3 ($\omega 3$: 0.2%); HA (0.2%), AO (0.4%) samples and its modulations with the latter. A modulation of the activity of Ca^{2+} -ATPase expressed in $\mu\text{mol Pi/mg Proteins/hour} \times 10^{-3}$ was observed compared to the negative control. The greatest activity was obtained with Phe (4 mg/ml) used as a positive control (275.96 ± 73), followed by omega-3 ($\omega 3$: 0.2%) (235.61 ± 52); by AO: 0.4% (190.48 ± 32) and finally by AO: 0.2% (178.21 ± 39). However, a significant difference ($p < 0.05$) was observed between the different values obtained compared to the negative control.

In the absence of the positive control (Phe: 4 mg/mL) and of the various oil samples, the membrane Mg^{2+} -ATPase of sickle cell erythrocytes showed an activity ($\mu\text{mol Pi/mg proteins/hour} \times 10^{-3}$) of 213.30 ± 58 . In the presence of Phe (4 mg/mL), this activity was 176.50 ± 56 while in the presence of oil samples the activities were 181.90 ± 32 ; 192.52 ± 37 and 124 ± 21 corresponding respectively to omega 3 ($\omega 3$: 0.2%); AO (0.2%) and AO (0.4%). However, apart from AO (0.2%), all values obtained with the positive control and the other oil samples are significantly ($p < 0.05$) lower than the activity of the negative control. This reflects an inhibition of the activity of of the Mg^{2+} -ATPaseby the different tests.

Table I: Potential of hemoglobin polymerization (PP) and potential of inhibition of hemoglobin polymerization (PIP) without (NE) and in the presence of omega 3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v). Phenylalanine (Phe 4: mg/mL) was used as positive control

Test	NE	Phe (4mg/mL)	$\omega 3$ (0.2% v/v)	AO (0.2% v/v)	AO (0.4% v/v)
Potential of hemoglobin polymerization (%)	56.5 $\pm$ 3 ^a	23.59 $\pm$ 7 ^b	17.99 $\pm$ 7 ^c	17.72 $\pm$ 5 ^c	9.54 $\pm$ 8 ^d
Potential of inhibition of hemoglobin polymerization (%)	0.0 ^a	58.24 $\pm$ 8 ^b	68.15 $\pm$ 7 ^c	68.63 $\pm$ 5 ^c	83.11 $\pm$ 8 ^d

Values followed by different superscripts along the rows are significantly different (p<0.05)

Table II: Effect of oils on the Fe²⁺/Fe³⁺ ratio and increase Fe²⁺/Fe³⁺ without (NE) and in the presence of the omega-3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v). Phenylalanine (Phe 4: mg/mL) was used as positive control

Test	NE	Phe (4mg/mL)	$\omega 3$ (0.2% v/v)	AO (0.2% v/v)	AO (0.4% v/v)
Fe ²⁺ /Fe ³⁺ ratio	1.34 $\pm$ 0.2 ^a	3.7 $\pm$ 0.5 ^b	4.67 $\pm$ 0.6 ^c	4.8 $\pm$ 1 ^c	6.65 $\pm$ 1.68 ^d
Increase Fe ²⁺ /Fe ³⁺ ratio (%)	0.0 ^a	63.1 $\pm$ 8 ^b	70.58 $\pm$ 5 ^b	70.99 $\pm$ 6 ^b	79.05 $\pm$ 3 ^c

Values followed by different superscripts along the rows are significantly different (p<0.05)

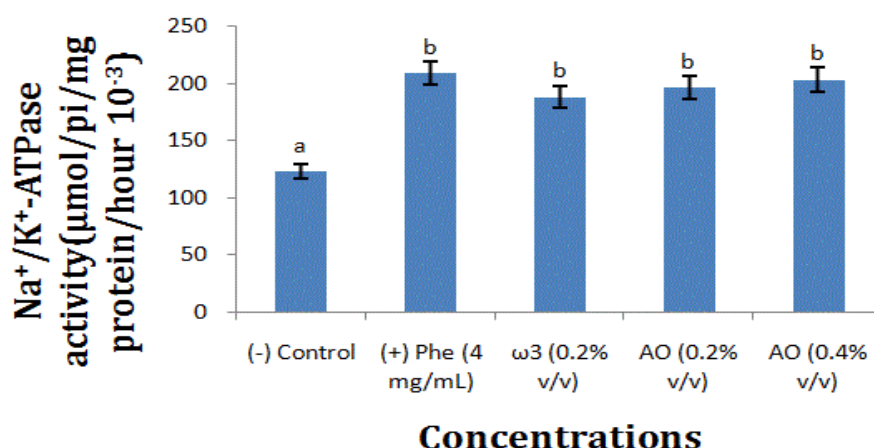


Figure 1: Effect of oils on Na⁺/K⁺-ATPase activity without (negative control) and in the presence of the omega 3 ($\omega 3$: 0.2% v/v), *Azadirachta indica* J. seed oil (AO: 0.2% and AO: 0.4% v/v). Phenylalanine (Phe 4: mg/mL) was used as positive control. Values followed by different superscripts are significantly different (p<0.05)

Discussion

Research on formulations based on extracts from foods and edible plants is the new trend in the management of genetic diseases like sickle cell with a view to finding a cheaper alternative which sickle cell patients can access immediately. For this, different strategies have been considered to explain the modes of action of the omega-3 and the *Azadirachta indica* J. oil seed used by certain sickle cell families in the region of North Cameroon. They directly targeted polymerization, the fundamental basis of sickle cell formation and certain alterations in sickle cell red

blood cells such as the oxidation of ferrous iron (Fe²⁺) to ferric iron (Fe³⁺) and the destabilization of the membrane ATPases activities. Indeed, the polymerization of hemoglobin S also called "gelation" is a physicochemical process based on non-covalent interactions governed by kinetic and thermodynamic factors. It is a reversible phenomenon [29].

When HbS was treated with phenylalanine 4 mg/mL (used as a positive control) and with Omega 3 ($\omega 3$: 0.2%); AO (0.2%) and AO (0.4%) samples, a marked change in the polymerization was observed. This reflects the direct influence of these substances

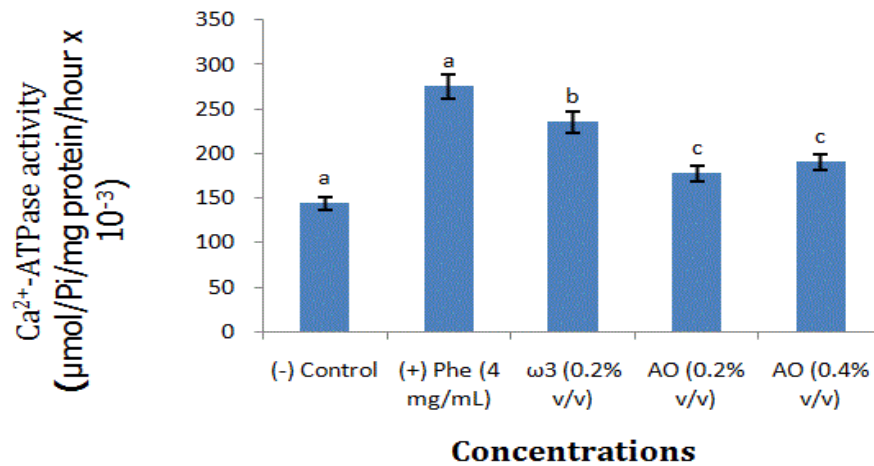


Figure 2: Effect of oils on Ca²⁺-ATPase activity without (negative control) and in the presence of the omega-3 (ω3: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v). Phenylalanine (Phe 4: mg/mL) was used as positive control. Values followed by different superscripts are significantly different (p<0.05)

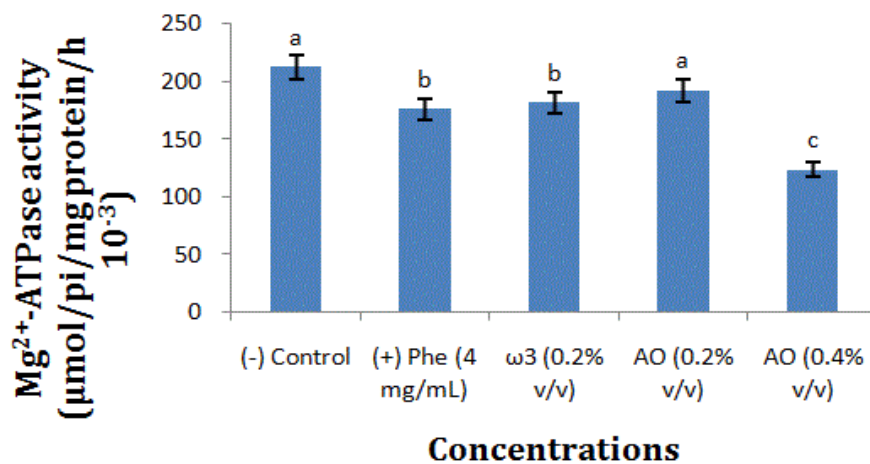


Figure 3: Effect of oils on Mg²⁺-ATPase activity without (negative control) and in the presence of the omega 3 (ω3: 0.2% v/v), *Azadirachta indica* J. seed oil(AO: 0.2% and AO: 0.4% v/v). Phenylalanine (Phe 4: mg/mL) was used as positive control. Values followed by different superscripts are significantly different (p<0.05)

on the gelation of HbS. With phenylalanine used as a positive control, the polymerization inhibition potential was $58.24 \pm 8\%$. Furthermore, the greatest potential for polymerization inhibition ($83.11 \pm 8\%$) was obtained with AO (0.4%). This value is less than $94.17 \pm 0.1\%$ and close to the $84 \pm 0.12\%$ obtained respectively by [30] with the lipid fraction of *Telferia occidentalis* seed and by [31] with the lipid fraction of *Mucuna sloanei* seed. This PIP is greater than 72.08% and 77.31% obtained by [32] respectively with the lipid fractions of *Xylopia aethiopica* and *Monoda myristica* seeds. This inhibition may be due to the greater influence of HA (0.4%) on the balance between the relaxed (R) and tense (T) forms of the hemoglobin tetramer, which translates into an

increase in the percentage of oxygen saturation of HbS. The same is true for omega 3 (ω3: 0.2%). The inhibition of polymerization by AO (0.4%) and omega 3 (ω3: 0.2%) seems to justify the mode of action by which these oils inhibit sickling as shown by [4, 8].

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 (Fe²⁺) to ferric iron (Fe³⁺) unable to fix oxygen [33]. In the absence of any treatment with HbS, the Fe²⁺/Fe³⁺ ratio was 1.34 ± 0.2 and 3.7 ± 0.52 for phenylalanine (4 mg/mL) used as a positive control. At concentrations of 0.2% and 0.4%, the oil extracted from the

seeds of *Azadirachta indica* J. significantly improves ($p < 0.05$) this ratio, to 4.8 ± 1 and 6.65 ± 1.68 respectively, corresponding to the oxy-hemoglobin increase rate (TAO) of $70.99 \pm 6\%$ and $79.05 \pm 3\%$. As for omega 3 ($\omega 3$: 0.2%), its TAO ($70.58 \pm 5\%$) is not significantly different ($p > 0.05$) from that of AO (0.2%). These values are greater than $50.04 \pm 4\%$ obtained by [30] with the lipid fraction of *Telferia occidentalis* seed. This increase may be due to the fact that these oil samples would prevent the oxidation of ferrous iron (Fe^{2+}) and thus increase the affinity of hemoglobin S for oxygen. Indeed, it has been shown that AO (0.4%) and omega 3 ($\omega 3$: 0.2%) had large chelating activities [4, 8]. It may also be due to vitamin E recognized as a powerful antioxidant. Indeed, it has been shown that this oil contains $486.51 \pm 0.7 \mu\text{g/g}$ of vitamin E [8].

Membrane transporters such as Ca^{2+} -activated K^+ channel and K^+Cl^- cotransporter contribute to dehydration [34]. In the erythrocyte membrane, ATPases drive energy dependent transport of electrolytes an activity that is abnormally high in sickle cell anaemia resulting to sickling of the red cells [35]. All the ATPase activities of the erythrocyte membranes of sickle cells expressed in $\mu\text{mol Pi/mg proteins/hour} \times 10^{-3}$ have been studied by treating the hemolysates of the erythrocyte membranes with Phe: 4 mg/mL and omega 3 ($\omega 3$: 0, 2%); AO (0.2%) and AO (0.4%). 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 HbS erythrocytes membrane preparations.

In the absence of oil samples, the activity of Na^+/K^+ -ATPase was 123.30 ± 33 . The greatest activity (209.35 ± 27) was obtained in the presence of phenylalanine (Phe: 4 mg/mL) used as a positive control. With oil samples, the greatest activity (203.82 ± 41) was obtained with AO (0.4%). This value which is significantly higher ($p < 0.05$) than that of the negative control is less than 220.1 ± 0.9 obtained by [20] with the aqueous extract of *Zantoxylum macrophylla* and tends towards the reference range [272.4-276.6].

In the absence of oil samples, the activity of Ca^{2+} -ATPase was 144.26 ± 61 . The greatest activity (275.96 ± 73) was obtained in the presence of phenylalanine (Phe: 4 mg/mL) used as a positive control. With the oil samples, the greatest activity (235.61 ± 52) was obtained with omega 3 ($\omega 3$: 0.2%) followed by AO (0.4%) (190.48 ± 32). These values which are significantly greater ($p < 0.05$) than the value of the negative control are less than 258.8 ± 0.8 obtained by [20] with the aqueous extract *Zantoxylum macrophylla*, but nevertheless tends towards the reference range [290.5 - 296.7].

As for the Mg^{2+} -ATPase from the erythrocyte membrane, phenylalanine (4 mg/mL), omega 3 ($\omega 3$: 0.2%); AO (0.2%) and AO (0.4%) significantly decrease ($p < 0.05$) its activities compared to the negative control. The lowest activity (124.00 ± 21) was obtained with AO (0.4%) which tends more to restore the normal activity of Mg^{2+} -ATPase. This value is less than 135.4 ± 1.9 obtained by [20] with the aqueous extract *Zantoxylum macrophylla* and tends towards the reference range [101.2 - 105.8].

These different modulations may be due to the restoration of the membrane structure of sickle cell erythrocytes by these oils. Indeed, the composition and organization of membrane lipids are maintained throughout the life cycle of the cell. In the event of the polymerization, there is therefore a destabilization of the membrane structure in the event of a decrease in phospholipids [36]. 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 omega 3 and *Azadirachta indica* J. seed oil noted in this study suggest vital roles for the natural product 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 the role of the omega 3 and *Azadirachta indica* J. seed oil in reversing this sickling [7, 8] thereby supporting its use in the therapy of the sickle cell patients.

Conclusion

Omega-3 at 0.2% and *Azadirachta indica* J. seed oil used in the management of SCD in the North Region of Cameroon at 0.4% inhibit the polymerization, increase rates of oxyhemoglobin of hemoglobin S and modulate the membrane ATPase activities. These results justify the antisickling, antioxidant and membrane stability properties of these oils. Nutraceutical capsules/functional foods formulation with these oils for SCD management will be the next point of focus within this research.

References

1. Karuna MSL, Prasad RBN. Vegetable Oil-Based Nutraceutical. Plant Biology and Biotechnology. 2015;793-812.
2. Ren H, Ibegbulam GO, Okpala IE, Ghebremeskel K, Ugochukwu CC. Steady-state haemoglobin level in sickle cell anaemia increases with the proportion of erythrocyte membrane n-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids. 2005;72(6): 415-421.
3. Ren H, Ghebremeskel K, Okpala I, Ugochukwu C, Crawford M. Abnormality of erythrocyte membrane n-3 long-chain fatty acids in sickle cell haemoglobin C (HbSC) disease is not as remarkable as in sickle cell anaemia (HbSS). Prostaglandins Leukot Essent Fatty Acids. 2006;74(1):1-6.
4. Kotue TC, Djote WNB, Marlyne M, Pieme AC, Kansci G, Fokou E. Antisickling and Antioxidant Properties of Omega-3 Fatty Acids EPA/DHA. Nutri Food Sci Int J. 2019;9(1):01-6.
5. Ibraheem NK, Ahmed JH, Hassan MK. The effect of fixed oil and water extracts of *Nigella sativa* on sickle cells: an in vitro study. Singapore Med J. 2010;51(3):230-234.
6. Jagpal C. The in vitro use of natural antioxidant oils (*Cyperus esculentus*; *Nigella sativa*) to reduce cell sickling in sickle cell anaemia. Biochemical Pharmacology. 2017;139:134. p29.
7. Kotue TC, Pieme AC, Fokou E. Ethnobotanicals usages in the management of Sickle Cell Disease (SCD) in some localities of Cameroon. Pharmacophore. 2016;7(4):192-200.
8. Djote WNB, Kotue TC, Mafogang B, Ngo SF, Pieme AC, et al.

- Antisickling, Antioxidant Properties, Fatty Acids and Vitamin E Compositions of *Azadirachta Indica* J. Seed Oil Used to Manage Sick Cell Disease (SCD) in North Cameroon. *J Nutr Food Lipid Sci.* 2020;1:86-93.
9. Kutlar A. Sick cell disease: A multigenic perspective of a single gene disorder Hemoglobin. 2007;31(2):209-224.
10. Bianchi N, Zuccato C, Lampronti I, Borgatti M, Gambari R. Fetal hemoglobin inducers from the natural world: A novel approach for identification of drugs for the treatment of β -thalassemia and sickle cell anemia. *eCAM.* 2009;6(2):141-151.
11. Xu X, Lockamy VL, Chen K, Zhi H, Shields H, et al. Effects of iron nitrosylation on sickle cell hemoglobin solubility. *J Biol Chem.* 2002;277:36787-36792.
12. Kyte J. Phosphorylation of a purified adenosine triphosphatase. *Biochem Biophys Res. Commun.* 1971;43(6):1259-1265.
13. Quist EE, Roufogalis BD. The relationship between changes in viscosity of human erythrocyte membrane suspensions and $(\text{Mg}^{2+} - \text{Ca}^{2+})$ -ATPase activity. *Biochem Biophys Res. Commun.* 72, 673-679.
14. Drickamer LK. The red cell membrane contains three different adenosine triphosphatases. *J Biol Chem.* 1975;250(5):1952-1954.
15. Ibeh GO, Anosike EO, Ekeke GI. Effects of age on erythrocyte membrane ATPases in normal and sickle cells. *Nig J Biochem.* 1992;7:38-46.
16. Ibeh GO and Anosike EO. Erythrocyte membrane ATPases: effect of hyperphosphatemia on ATPases. *Global J Pure & Appl Sci.* 2000;6:455-460.
17. Elekwa I, Monanu MO and Anosike EO. Studies on the effect of aqueous extracts of *Garcinia kola* seed on human erythrocyte adenosine triphosphatases of HbAA, HbAS and HbSS genotypes. *Global J of Medical Sciences.* 2003;2(2):107-114.
18. Xu ASL, Labotka RJ, London RE. Acetylation of human hemoglobin by methylacetyl phosphate: Evidence of broad regio-selectivity revealed by NMR studies. *J Biol Chem.* 1999;274:26629-26632.
19. Abdulmalik O, Safo MK, Chen Q, Yang J, Brugnara C, et al. 5-hydroxymethyl-2-furfural modifies intracellular sickle hemoglobin and inhibits sickling of red blood cells. *Br J Haematol.* 2005;128(4): 552-561.
20. Elekwa I, Monanu MO, Anosike EO. Effects of aqueous extracts of *Zanthoxylum macrophyllum* roots on membrane stability of human erythrocytes of different genotypes. *Biokemistri.* 2005;17(1):7-12.
21. Iwu MM, Igboko AO, Onwubiko H, Ndu UE. Effect of cajamimose from *Cajanus cajan* on gelation and oxygen affinity of sickle cell hemoglobin. *J Ethnopharmacol.* 1988;23(1):99-104.
22. Nwaoguikpe RN, Ekeke GI, Uwakwe AA. The effect of extracts of some foodstuffs on lactate dehydrogenase (LDH) activity and hemoglobin polymerization of sickle cell blood. PhD thesis, University of Port Harcourt, 1999; Nigeria: Pp.92-93.
23. Davidson J, Henry JB. Clinical Diagnostics by Laboratory Methods. Todd-Sanford. W.B. Saunders, Philadelphia. 1974;112:380.
24. Kotue TC, Pieme AC, Ama Moor VJ, Nanfack P and Ngogang YJ. The in vitro antisickling properties of "Hémodya". *J Biol Chem. Research.* 2014;31(2):757-768.
25. Hamlyn JM, Duffy T. Direct stimulation of human erythrocyte membrane Na^+/K^+ -ATPase, Mg^{2+} -ATPase activity in vitro by physiological concentrations of d-aldosterone. *Biochem Biophys Res Commun.* 1978;84(2):458-464.
26. Hesketh JE, London JB, Reading HW, Glen AI. The effect of lithium treatment on erythrocyte membrane ATPase activities and erythrocyte ion content. *British J Clin Pharmacol.* 1978;5:323-329.
27. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem.* 1925;68:375-400.
28. Bradford M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry.* 1976;72, p.248-254.
29. Bookchin RM, Balaz T, Wang Z, Joseph L and Lez VL. Polymer structure and solubility of desoxyhemoglobin S in the presence of high concentration of volume excluding 70 kDa dextran. Effects of non-hemoglobins and inhibitions. *J Biol Chem.* 1999; 274(10):6689-6697.
30. Nwaoguikpe RN, Ujowundu CO and Okwu GN. The Antisickling Potentials of Four Curcubits (*T. occidentalis*, *C. Maxima*; *C. sativus* and *C. lonatus*). *Sch J App Med Sci.* 2013;1(3):191-198.
31. Ojiako OA, Anugweje K, Igwe CU and Alisi CS. Evaluation of the amino acid profile and hemoglobin polymerization inhibition potential of some Nigerian legumes. *British J Pharma Res.* 2012;2(2): 80-88.
32. Uwakwe AA, Nwaoguikpe R. In vitro antisickling effects of *Xylopias aethiopica* and *Monodora myristica*. *Journal of medicinal plant research.* 2008;2(6):119-124.
33. Dahmani O, Belcaid A, Ouafa E and Hayate H. Biosynthèse et rôle Physiologique de l'hémoglobine. Ibom State, Nigeria. DCS report at national institute for interdisciplinary science and technology (CSIR-NIIST), Kerala India. 2009;36: 31-32.
34. Gibson JS, Khan A, Speake PF, Ellory JC. O₂ dependence of K⁺ Transport in Sick Cells: The Effect of Different Cell Populations and the Substituted Benzaldehyde 12C79. *FASEB J.* 2001;15: 823-832.
35. Fowles JR, Green HJ, Quyang J. Na^+/K^+ -ATPase in Rat Skeletal Muscle: Content, Isoform, and Activity Characteristics. *J Appl Physiology.* 2004;96:316-326.
36. Mohammad ZZ, Zuhair MM, Samir MK. Plasma and red blood cell membrane lipid concentration of sickle cell disease patients. *Saudi Med J.* 2003;24(4): 376-379.