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In vitro Evaluation of Antioxidant and Anti-sickling properties of *Theobroma Cacao* (Sterculiaceae) extracts from East and South regions in Cameroon

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Abstract

Background: Sickle cell anaemia (SCA) is a genetic disease associated with increased mortality in Africa. It generates polymerization of haemoglobin, free radicals proliferation and antioxidants deficiencies. SCA management still needs new and safe drugs. The aim of this study is to evaluate the antioxidant and anti-sickling properties of cocoa extracts from two localities of Cameroon.

Methods: *T. cacao* beans were collected in Bertoua and Mbalmayo/Ebolowa. They were dried, ground and then extracted with a mixture of ethanol/water (70/30), pH 3. The antioxidant activity of Mbalmayo/Ebolowa cocoa bean extract (EFCM) *and B*ertoua cocoa bean extract (EFCB) were determine by measuring some phenolic group content, the DPPH°, NO°, OH° scavenging property and the total antioxidant capacity (FRAP). Moreover, the antisickling and the red blood cells (RBC) stabilization of extracts was performed.

Results: EFCM and EFCB extracts showed a high level of total polyphenols, flavonols respectively, an increase ferric ions reducing antioxidant power (FRAP), a strong radical scavenging properties, with IC_{50s} between 4.17 and 6.75 µg/mL. EFCM extract showed a better ability to reduce RBC sickling and to protect the erythrocyte membrane from haemolysis.

Conclusions: These results suggest that cocoa beans extracts from these two regions, mainly EFCM, could be used in the management of sickle cell anaemia.

Keywords: Sickle cell disease; oxidative stress, antioxidants; *Theobroma cacao.*

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Background

Sickle cell anaemia (SCA) is a genetic disease caused by a single mutation on the 6th codon of the β-globin chain where a hydrophilic amino acid (glutamic acid) is replaced by a hydrophobic amino acid (valine). This mutation is responsible for the synthesis of a particular heamoglobin, heamoglobin S [1]. At low oxygen pressure, the deoxy-HbS polymerizes and organizes into large fibers inside the red blood cell, which deforms and weakens it [2]. According to statistics from the World Health Organization [3], the prevalence of the disease is 2 to 3% in Cameroon. In addition 4,000 annual births of sickle cell children [4], all age groups are affected and young people aged 10 to 29 accounts for 89.2% of patients in Cameroon [4]. So it's a public health problem.

Indeed, there is no treatment of sickle cell disease up today, many treatments based on antioxidants or not have been used: hydroxyurea reduces vaso-occlusive attacks, stimulates the induction of Hb F, thus compensating for the poor functioning of Hb S [5]. However, this compound can also lead to membrane erythrocytes damage causing the destruction of red blood cells [5]. Piracetam, which reduces the incidence of sickling attacks, is toxic because taking it over a long period of time usually causes oxidative stress by destroying cell membranes [6]. Repeated blood transfusions used to correct acute anaemia unfortunately have many disadvantages such as alloimmunization, iron overload, transfusion contaminations. Allogeneic hematopoietic stem cell transplantation is nowadays the only curative therapy for sickle cell disease but is extremely expensive and requires a heterozygous or trait free donor [2]. Due to these facts, new strategies, including the use of plant extracts promoted by WHO, have emerged.

HbS from sickle cells is considered as a pro-oxidant machine that can generate increased production of free radicals. These radicals generate a state of oxidative stress, increased both consumption and a lack of antioxidants [7]. Thus, new therapeutic agents against SCA should highly contain antioxidant molecules.

Many natural plants or molecules have demonstrated anti-sickling properties correlated with their antioxidant properties. Thus, it has been shown that *Camellia sinensis* rich in resveratrol, a powerful antioxidant, possessed anti-ulcerative properties and allowed the induction of haemoglobin F in sickle cell patients [8]. Angelo and Nanfack [9, 10] showed through their work the anti sickling and the antioxidant activity of *Pterospartum tridentatum* and *Zanthoxyllum heitzii* extracts respectively. Moreover, Gbadamosi [11] working on two plant recipes in Nigeria, the first being a mixture of six plants *(Detarium microcarpum*, *Harungana madagascariensis*, *Sorghum bicolor*, *Tetracera potatoria* and *Theobroma cacao*) and the second is the recipe for a single plant (*Phyllanthus amarus*), demonstrated that these plants had anti-cancer and antisickling activities. Previous study on cocoa beans from five production areas in Cameroon showed high quantity of polyphenol content [12]. The aim of this study is to evaluate the antioxidant and anti-sickling properties of cocoa extracts from two localities of Cameroon.

Methods

Plant materials and collection

Cocoa beans from *Mbalmayo / Ebolowa* (South region) and *Bertoua* the (East region) in Cameroon were harvested between October and December 2015. They were subsequently identified under reference number *60071 / HNC* at the National Herbarium of Yaoundé-Cameroon.

Fermentation and plant extraction

Fermentation of the beans was done according to the process used in each locality.

In general, the external pods of cocoa beans have been removed, and beans have been placed on banana leaves in the shade for the fermentation process (5 and 4 days for Mbalmayo / Ebolowa and 4 Bertoua respectively). Cocoa beans were spread out on local fabrication racks under which an heating system was set until complete drying for about 4-5 days (Mbalmayo / Ebolowa) and were spread on concrete floors for a‘‘sun drying method’’ during 1 to 2 weeks. After drying, beans were packaged and stored under vacuum at room temperature until the time of analysis.

The modified Benhammou method [13] was used for the extraction. Briefly, 195 g of the plant material (cocoa beans) was crushed. The powder obtained was macerated in the ethanol-water mixture (70/30), pH 3. The mixture was stirred several times to maximize extraction. After 48 h, the whole was filtered using whatman paper No. 4 followed by No. 1. Then, the solvent was evaporated to near dryness under reduced pressure in a rotary evaporator at 40 °C and then kept in an oven for 24 h at a temperature of 39 °C until dryness.

Collection of blood samples

After obtaining an ethical clearance approval issued by the Regional Committee for Ethics Research for Human Health Center (number N ° 0282-CRERSHC / 2016), five confirmed sickle cell blood samples have been recruited including three women and two men between the ages of 16 and 40 who had been attending routine consultations in the Hematology Department of Central Hospital, Yaoundé.

Antioxidant Properties of the Extracts

Total polyphenol content (TP)

The method of Singleton [14] was used to determine the total phenol content as follows, 100 µL of each extract (1 mg / mL) was added to different tubes. Then, 400 µL of the Folin-Ciocalteu reagent diluted 1/10 and 1 mL of sodium carbonate solution (7.5%) were added subsequently. After stirring, the mixture was kept for 2 h in the dark and the absorbance measured at 765 nm against the blank. The total content of polyphenols was determined from the standard curve and expressed in mg equivalent of caffeic acid / g extract (EAC / g E).

Total flavonols content (TF)

Previous protocol with slight modifications was used [15]. Briefly, 2 mL of each extract were introduced into different tubes, followed by 2 mL of 2% ethanol solution of aluminum chloride (EtOH-$ALCl_3$) and 3 mL of 50% sodium acetate (CH3COONa). After homogenization, the tubes were incubated for 2 h and 30 min in the dark and the absorbance read at 440 nm against the blank. The total content of flavonols was determined by extrapolation on a standard curve and is expressed in mg equivalent of standard /g of extract (mgES / g E).

Total antioxidant activity by ferric reducing antioxidant power assay (FRAP)

The method of Benzie and Strain [16] was used for the FRAP assay. Seventy-five (75) µL of each 1mg / mL concentration extract was added to different test tubes into which 2 mL of FRAP reagent was added. After homogenization, the mixture was incubated for 12 minutes and the absorbance read at 593 nm against the blank. Vitamin C was used as a standard, and the result expressed in mg of vitamin C equivalence per g of extract (mg eq vit C / g of E).

DPPH Scavenger activity of extracts

The DPPH scavenging activity was determined according to the method described previously [17]. Three milliliters of extracts at different concentrations (0 - 300 µg/mL) were added to different test tubes, and then 300 mL of a 0.1 mM DPPH solution was added. Ascorbic acid was used as a standard and prepared at at the same range of concentrations. After homogenization, the mixture was placed in the dark for 30 min at room temperature. Absorbance was measured at 517 nm against blank prepared as above, but replacing the extract with the water-ethanol solution (8v:2v). The antioxidant activity of the extracts expressed as percentage inhibition is calculated with the following formula:

$$\% \text{ Inhibition} = [(DO〗 \text{ control} - 〖DO〗 \text{ sample}) * 100 / 〖DO〗 \text{ control}]$$

Antiradical activity by trapping nitric oxide radical (NO°)

The antiradical activity of nitric oxide was determined from the reaction of Griess Illosvoy [18]. To 0.5 mL of extracts at different concentrations were added 2 mL of 10 mM sodium nitroprusside (SNP); 0.5 mL of phosphate buffer (0.5 M) and 1 mL of Griess reagent. The generated pink chromophore was measured at 540 nm against blank. The antiradical activity of nitric oxide expressed as percentage inhibition was calculated as follow:

$$\% \text{ Inhibition} = [(DO〗 \text{ control} - 〖DO〗 \text{ sample}) * 100 / 〖DO〗 \text{ control}]$$

Anti-radical activity by scavenging of hydroxyl radical (OH°)

The antiradical activity of the OH° radical was determined using the method of Kunchandy and Rao [19]. Briefly 1.5 mL of extracts at different concentrations were introduced into the test tubes. Then were added 2.4 mL of phosphate buffer (0.2 M), 0.06 mL of iron trichloride, followed by 0.09 mL of 1,10- phenanthroline 1mM and 0.15 mL of 0.17 M H_2O_2. After homogenization, all the tubes were incubated for 5 min at room temperature. Absorbance was read at 560 nm against blank. The antiradical activity of the extract was expressed as percentage (%) of inhibition of the hydroxyl radical and was calculated according to the following formula:

$$\% \text{ Inhibition} = [(〖DO〗 \text{ control} - 〖DO〗 \text{ sample}) * 100 / 〖DO〗 \text{ control}]$$

Red blood cell purification, sickling induction and antisickling activity

Red blood cell purification and sickling induction

Pure red blood cell was obtained by pre-washing the whole blood using 0.9% NaCl solution followed by a centrifugation for 5 minutes at 3000 rpm. This process was repeated twice to completely liberate RBCs from impurities. The antisickling activity was

done according to the protocol of N'draman-Donou [20] slightly modified. Under the condition of hypoxia (absence of oxygen), the normal red blood cells are transformed into sickle cells which are identified and counted using a microscope. The induction of sickling was done using the EMMEL test; a small drop of blood (sampled on EDTA) was deposited on the slide; then a drop of 2% sodium metabisulfite was added in ratio 1/1 (v/v); the mixture was carefully mixed and covered with a coverslip avoiding the formation of air bubbles. In order to prevent drying of the preparation, slide was conserved on humidified sticks on cotton in a petri-dish.

Evaluation of in vitro cell sickling inhibitory activity of Theobroma cacao Extracts

Different concentrations (500, 1000 and 2000 µg/mL) of extracts were prepared by diluting in a solution of NaCl 0.85%. Phenylalanine and quercetin helped as standards and sodium metabisulfite solution diluted as above was used as sickling inducer. The control slide was constituted as follows: 5 µL of washed blood on the slide + 5 µL of 2% sodium metabisulphite. Red blood cells were counted under light microscope after 30 min, 1h, 1h30, 2h and 2h30 min using the objective 40. Subsequently, the test slides were formed with 5 µL of washed blood + 5 µL of 2% sodium metabisulphite, + 5 µL of extracts at different concentrations, the same count was made in different fields. The sickling percentage was calculated as follow:

$$\%\ \mathbf{sickling} = \frac{\mathbf{Sickling\ cells}}{\mathbf{Total\ red\ blood\ cells}} \times\ \mathbf{100}$$

Osmotic fragility test of erythrocytes

The osmotic fragility test was performed using the method of Jaja [21]. Nine solutions of a range of saline solution concentration (0% - 0.85%) were prepared. The EFCM and EFCB extracts were prepared at concentrations of 500, 1000 and 2000 mg/mL. At 800 µL of NaCl of different concentrations, were added 200 µL of extracts and 10 µL of washed blood; the mixture was left to rest for 24 h; the supernatant was recovered using a micropipette and the optical density was read at 540 nm against blank. Haemolysis expressed as a percentage is calculated by the ratio of absorbances:

$$\%\ \mathbf{Hemolysis} = \frac{\mathbf{DO_{sample}}}{\mathbf{DO_{control}}} \times \mathbf{100}$$

Statistical analyzis

Each test was performed in triplicate and the results were expressed as mean ± standard deviation. The Mixed Linear Effect Model helped to study the interactions between factors (extracts, concentration and the repeated time factor) after data restructure. The *kruskal-Wallis* test was used, followed by a *Dunnet* post-hoc to analyze the antioxidant potential, the antiradical activity, anti-sickling and osmotic fragility test of each plant extract, in order to establish the significant differences ($p < 0.05$). Spearman's test established correlations between plant extracts and different antiradical methods. Principal Component Analysis (PCA) was used to determine the best extract. IC_{50} were determined by the use of multiple regression analyzes. The SPSS software version 16 and X-L stat version 17 for Windows 7 was used for this purpose.

Results

The results of total polyphenol, flavonol content are presented in Table 1 below. Total polyphenol content and flavonols are significantly ($P < 0.05$) high in EFCM extract than that of EFCB. It also possesses a greater total ferric reducing antioxidant capacity (FRAP) compare to that of EFCB. In addition, the IC_{50} (inhibitor concentration 50) are between 3.87 and 5.16 for DPPH°; 2.88 to 6.75 for the NO° and 1.23 to 3.25 for the OH°. The IC_{50} of quercetin was the lowest followed by EFCM and EFCB (Table 1).

Induction of sickling with 2% sodium metabisulfite

The induction of sickling revealed that the percentages of sickling varied between 28% to 70% depending of the blood sample used. After 30 min of sickling induction, a slight little increase of sickling percentages was noted. After two hours this value up to 96%. The induction rate was between 21 and 66% for all blood samples. The induction of cell sickling was very rapid and varied from one patient to another.

Figure 1 represents the percentage of sickling in presence or not of extracts. it reveals that standards (quercetin and phenylalanine) and cocoa extracts reduce cell sickling of blood samples in general. Also, the percentage of sickling decreased significantly ($P < 0.05$) with the concentration of each extract and time. Then at 500 µg/mL (C1) of each extract with time dependent, a significant difference ($P < 0.05$) between control (negative control), standards (phenylalanine and quercetin) and extracts as well as between phenylalanine and Mbalmayo cocoa extract have been noted. At 1000 µg/mL (C2) of extract, there is a significant difference between phenylalanin and extracts at 1h and 1h30 min while at 2000 µg/mL (C3), a significant difference is observed between phenylalanine and extracts (1h, 1h30, 2h) and between phenyalanine and Bertoua cocoa extract.

Table1. Antioxidant activity of the different extracts of *Theobroma cacao* and their IC_{50}

Extracts	Polyphenols content (Quercetin mg Eq / g extract	Flavonols (Quercetin mg Eq / g extract	FRAP (mg Eq de Vit C/g extract)
EFCM	479.33 ± 9.38[a]	69.72 ± 2.52[a]	197.1 ± 0.1[a]
EFCB	269.41 ± 13.99[b]	57.05 ± 4.89[b]	187.13 ± 0.31[b]
	IC_{50} DPPH (µg/mL)	**IC_{50} NO° (µg/mL)**	**IC_{50} OH° (µg/mL)**
Quercetin	3.87 ± 0.308	2.88 ± 0.332	1.23 ± 0.552
EFCM	4.17 ± 0.512	4.65 ± 0.114	1.97 ± 0.343
EFCB	5.16 ± 0.288	6.75 ± 0.197	3.25 ± 0.589

Kruskal-Wallis followed by Dunnet. Values affected with different letters differ significantly at P <0.05. EFCM = Mbalmayo cocoa bean extract, EFCB = Bertoua cocoa bean extract.

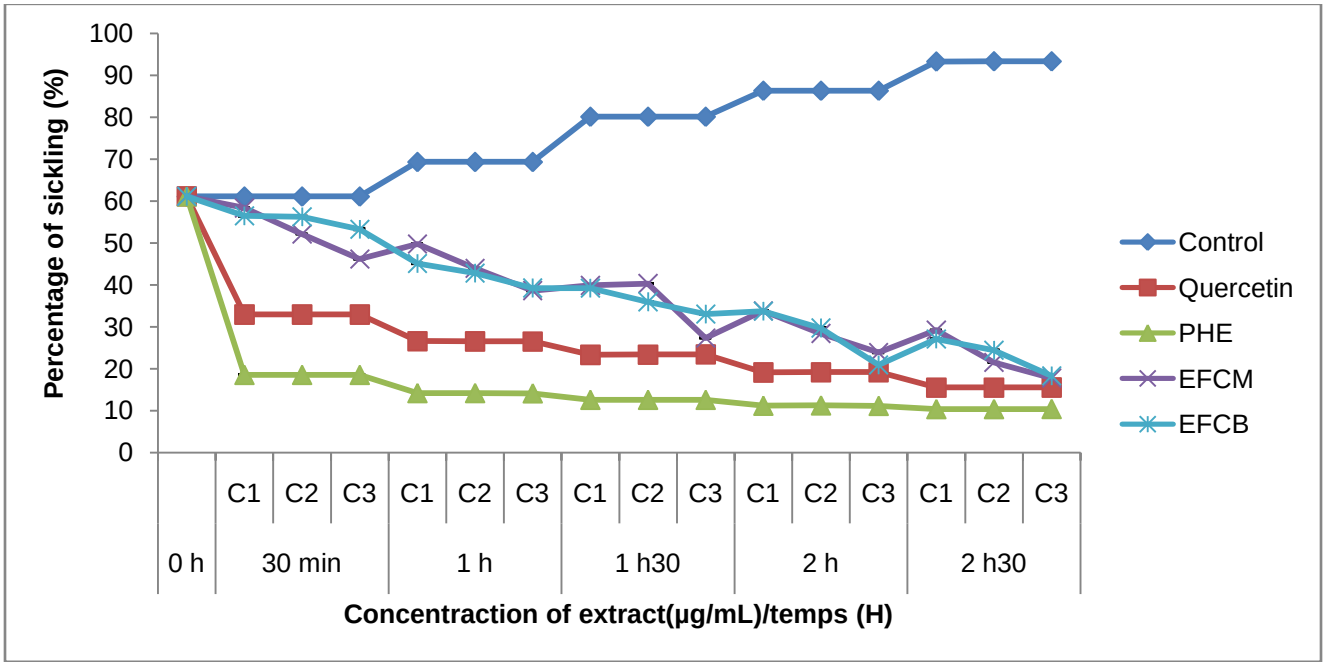

Figure 1. Antisickling activity of extracts

Kruskal-Wallis followed by Dunnet. Extracts affected with different letters have values that differ significantly at P<0.05. EFCM = Mbalmayo cocoa bean extract, EFCB = Bertoua cocoa bean extract, Phe= phenylalanine (standard), C1 = concentration 500 µg/mL, C2 = concentration 1000 µg/mL, C3 = concentration 2000 µg/mL.

Reduction of sickling at different concentration of Theobroma Cacao extracts with time dependent

The reduction percentage of sickling varies significantly with the concentration of extracts (Figure 2). Following the incubation of blood samples with different treatments (extracts, standards and blank) at different time (30 min, 1h, 1h30 min, 2h and 2h30 min), reduction of sickling has been calculated after 30 min, 2h and 2h30 min. No sickling reduction has been noted in the control group (blank). The reduction of sickling increased with extract / standard concentrations and time dependent. After 02h30 min, quercetin reduces sickling significantly more than phenylalanine. In addition, At 1000 µg/mL (C2) and 2000 µg/mL (C3), EFCM has a better reduction activity than that of EFCB after 2h and 2h30 min. compare to standards, EFCM reduced better than the cell sickling at each concentration after 2h and 2h30 min.

Osmotic fragility test of erythrocytes

Figure 3 shows the percentage of hemolysis as a function of saline concentration at different concentrations of extracts. This percentage decreases according to the concentration of the extracts (500, 1000 and 2000 µg/mL) and the salt concentration for the different blood samples tested. Indeed, the higher the salt concentration, the lower

the percentage of hemolysis. This observation becomes more effective in the presence of extracts, particularly with that of EFCM, where the percentage of hemolysis varies significantly. The standard (phenylalanine) acts more effectively against hemolysis follow by EFCM extract.

Correlation matrix between antioxidant and antisickling tests of plant extracts

The correlation between antioxidant tests and sickling presented in Figure 4 shows a positive and strong correlation between the five radicals (DPPH, OH°, NO°, FRAP, and Flav) and negative with total sickling (Fal tot). In addition, the Biplot figure revealed that EFCM and Quercetin contribute more for antioxidant tests and antisickling activity. Through these results, we can say that the best cocoa extract is that of Mbalmayo/Ebolowa.

Discussion

Plants are natural substances for the production of chemical compounds. Many of these are used to promote disease control and are referred to as medicinal plants [22]. The procedure of fermentation and drying differ according to each locality where cocoa beans have been collected which could imply the difference in some secondary metabolite content observed. The polyphenols and flavonols content of EFCM are higher than those of EFCB. The origin of plant samples is a great factor which can influence plant metabolite content [23]. Results revealed that cocoa bean extracts had the ability to reduce iron. This antioxidant property could be due to the phenolic compounds contained in cocoa as mentioned above. This result is in agreement with that of Verdan [24] who reported that the antioxidant effects of flavonols are due in part to their ability to bind different metals (Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}). In addition, the work of Nanfack [10] demonstrated a strong correlation between the presence of polyphenols in *Zanthoxyllum hetzii* and their ability to reduce iron.

Radical scavenging activity results showed a proportionality between the increase of the concentration of extracts and the radical scavenging, attesting that the hydroethanolic extracts contain free radical scavengers which are phenolic compounds also called antioxidants. The action of these antioxidants is due to their ability to donate hydrogen atoms or electrons derived mainly from their phenolic cycle [25]. The greater amount in polyphenols and flavonols in EFCM than that of EFCB could explain its better ability to trap free radicals. Similarly, other researchers revealed that the ability of cocoa to trap radicals is directly proportional to their polyphenol content [26]. The antioxidant activity of cocoa extracts is better on the OH° radical (IC_{50} 1.97 for EFCM and 3.25 µg/mL for EFCB) followed by the DPPH° radical (IC_{50} 4.17 EFCM and 5.16 µg/mL for EFCB) and the radical NO° (IC_{50} 4.65 for EFCM and 6.75 for EFCB µg/mL).

In the presence of extracts at each concentration, a decrease in sickle cell percentages over the time was observed. This anti-sickling activity of the extracts tested could be explained by their ability to increase the production of fetal haemoglobin, thereby inhibiting the polymerization of haemoglobin S [27]. Likewise, it has been shown that herbal extracts with antioxidant capacity can increase latency before sickling [28]. According to the principal components analysis, a negative correlation between antioxidant assay and sickling was observed. This result could be explained by the fact that the secondary metabolites found in the extracts tend to inhibit sickling. This action could be linked to the phenolic compounds, specifically the flavonoids contained in extracts. Thus, Seck [29] showed that the presence of flavonoids in the root extracts of *Leptadenia hastata* reduce sickling and concluded that flavonoids are involved in the inhibition of the deleterious effects of the reactive oxygen species, produced during sickle cell disease.

As far as osmotic fragility test of erythrocytes is concerned, a considerable decrease in the percentage of haemolysis with concentration dependent of the saline solution and extracts was noted. For all the extracts tested, this percentage remains lower than that of the standard (Phenylalanine). EFCM showed the best protective effect on the erythrocyte membrane. The decrease in the percentage of haemolysis observed is related to the appreciable protective effect of *Theobroma cacao* extracts on the erythrocyte membrane, hence their resistance to haemolysis. These results corroborate some previous works on the roots and leaves of *Caliandra portoricensis* and *Carica papaya* respectively which showed a significant correlation between the antisickling activity ant their antioxidant capacity [30, 31]. Adesanya and Sofowora [32] also demonstrated this activity on the roots of other Zanthoxylum species (*Z. gilleti* and *Z. zanthoxyloides*). Indeed, this result could be also explained by the presence in the extracts of phenolic compounds such as flavonols.

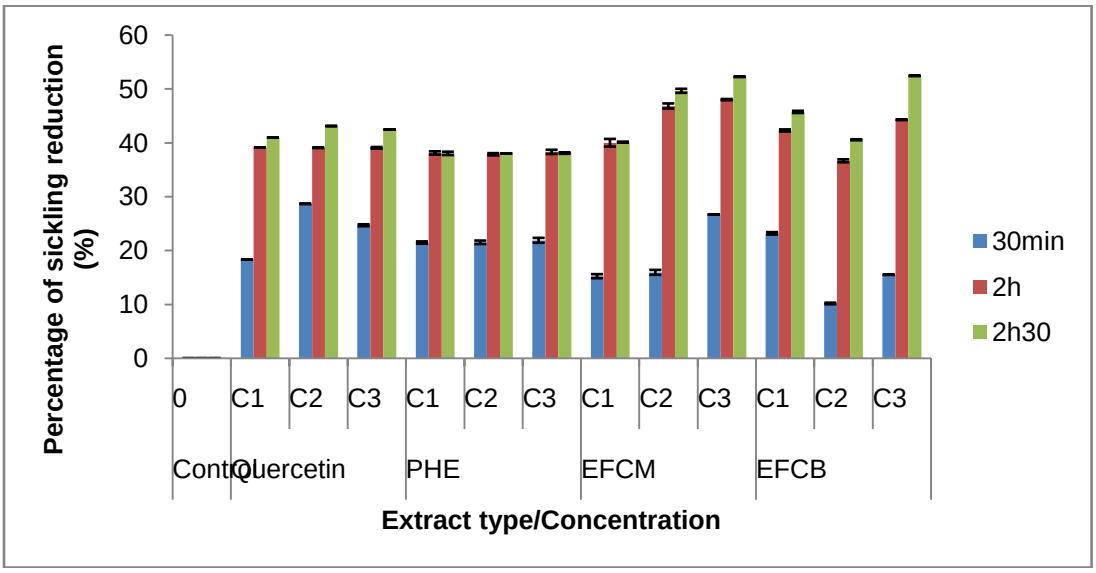

Figure 2. Reduction of sickling at different concentration of *Theobroma cacao* extracts with time dependent
Kruskal-Wallis followed by Dunnet. EFCM = Mbalmayo cocoa bean extract, EFCB = Bertoua cocoa bean extract, Phe= phenylalanine (standard). C1 = concentration 500µg/mL, C2 = concentration 1000µg/mL, C3 = concentration 2000 µg/mL.

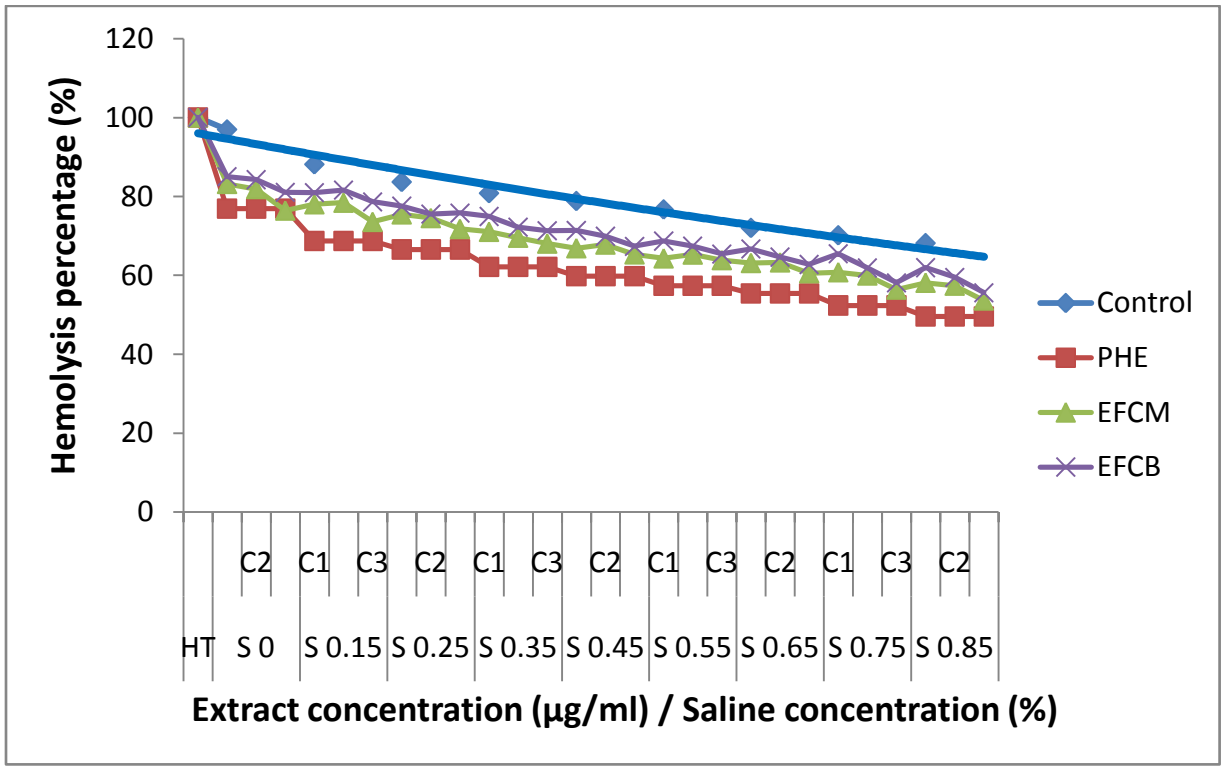

Figure 3. Osmotic fragility test of erythrocytes
Kruskal-Wallis followed by Dunnet. Phe= Phenylalanin; EFCM = Mbalmayo cocoa bean extract; EFCB = Bertoua cocoa bean extract; C1 = concentration 500µg/mL, C2 = concentration 1000µg/mL, C3 = concentration 2000µg/mL . S0-S0.85= Saline concentration from 0 up to 0.85%

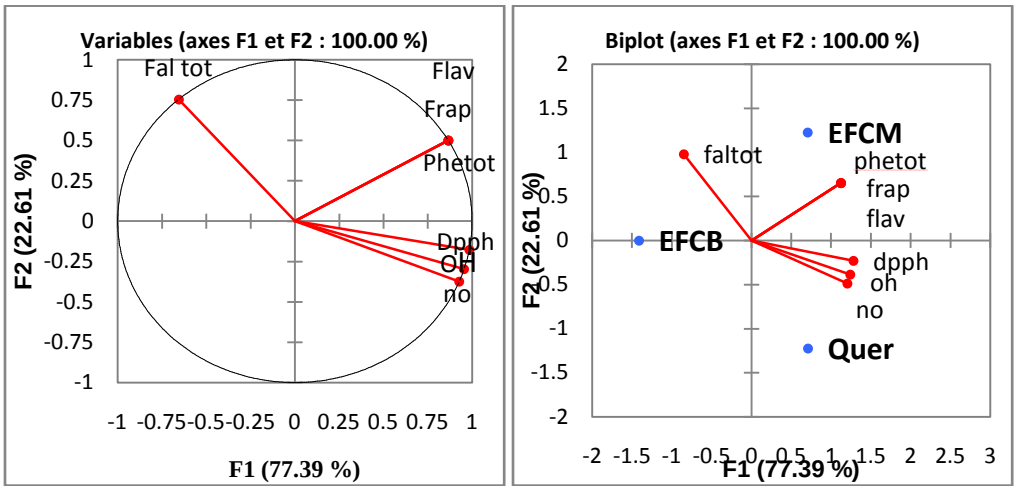

Figure 4. Correlation matrix between antioxidant and antisickling tests of plant extracts
EFCM = Mbalmayo cocoa bean extract; EFCB = Bertoua cocoa bean extract. Faltot=total sickling; phetot=total phenol; flav=flavonol; quer=quercetin

Conclusions

The present study aim was to evaluate the *in vitro* antioxidant and antisikling properties of cocoa beans from the Eastern and southern regions of Cameroon. It emerges from this study that the hydroethanolic extracts of cocoa from these localities possess secondary metabolites such as polyphenols which might be responsible to the antioxidant, antisickling and the red cells stabilization properties. These results suggest that the cocoa beans in these two regions, particularly that from Mbalmayo, could be used in the management of sickle cell anaemia.

Abbreviations

SCA: Sickle Cell Anaemia
FRAP: Ferric Reducing Antioxidant Power
Hb: Haemoglobin
MDA: Malonedialdehyde
WHO: World Health Organization
HbS: Sickle haemoglobin
FRAP: Ferric ions Reducing Antioxidant Power
EFCM: Mbalmayo cocoa bean extract
EFCB: Bertoua cocoa bean extract.
RBC: Red Blood Cells

Authors' Contribution

YL conducted the study and assays. NNPJ, NF, KFC assisted in conducting the assays. BNPC designed the research co-directed the research work as well as the statistical analysis study. CB facilitated the contact between principal investigator and SCA patients and helped explaining the importance of such research to patients. PBF and PCA supervised the research work. All the authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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