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Research Article

IN VITRO ANTISICKLING AND ANTIOXIDANT EFFECTS OF HYDRO-ETHANOLIC EXTRACTS OF *THEOBROMA CACAO* (STERCULIACEAE) FROM WEST AND CENTRE REGIONS IN CAMEROON

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ABSTRACT

Sickle cell anaemia (SCA) is a genetic disorder characterised by the presence of haemoglobin S in red blood cells. Current antioxidant treatments are expensive and long-term toxic effects can follow. The present study aimed at evaluating the antisicking and antioxidant properties of *Theobroma cacao* beans extracts in order to contribute to the management of SCA patients through new therapies based on natural substances. Cocoa bean extracts (from Santchou and Obala) were macerated in a mixture of water/ethanol (pH=3). The anti-sickling activities of both extracts followed by their effects on osmotic fragility of erythrocytes were assessed. Their antioxidant and scavenging activities on free radicals such as 2,2-Diphenyl-1-picrylhydrazyl, 2,2'-azino-bis-[3-ethylbenz-thiazolone-6-sulfonic acid (ABTS) were also investigated as well as their phenolic contents. The cocoa extracts from Obala presented the best antisickling activity with 64.34% sickle cell reduction after 24 h. The extracts from Santchou did not present any sickling reduction after the above-mentioned time. A decrease in the haemolytic percentage was observed while the concentration of saline and extracts were increasing. Cocoa beans extracts revealed a high content in polyphenols, flavonols as well as a high antioxidant potential and scavenged free radicals depending on the test sample concentration, with IC_{50} ranging between 1.93 and 2.34 µg/mL; 3.85 and 4.46 µg/mL and 3.53 and 4.61 µg/mL, on the free radicals DPPH, ABTS and NO, respectively. *Theobroma cacao* from Obala exhibited the best *in vitro* activity and constitute a preliminary step to the development of a future treatment against SCA.

Key words: Sickle cell anaemia, Sickling, Antioxidants, *Theobroma cacao*, Cameroon.

INTRODUCTION

Sickle-cell disease or sickle-cell anemia (SCA) is a genetic disease characterized by the presence of haemoglobin S (HbS) in red blood cells. This HbS is an abnormal form of haemoglobin A (HbA), a normal protein for the transport of oxygen[1]. Each year, over 500,000 children are born with a sickle-cell disease in the world, including 300 000 in Africa[2]. The prevalence of sickle-cell trait varies depending on the country. It is 15 to 25% in Central and West Africa, 10 to 12% for black Americans and between 1 to 15% in the Mediterranean regions[2]. SCA is a major public health problem in black Africa [2]. Since 2009, the management/treatment of sickle-cell disease is one of the priorities of the World Health Organization (WHO) for Africa and SCA is ranking fourth of the priorities of the global health after cancer**,** acquired immunodeficiency syndrome (AIDS) and malaria[3]. In Cameroon, 4,000 children are born each year with the sickle-cell disease, all age groups are affected but the youths of 10 to 29 represent about 89.2% of patients. According to WHO [4], the prevalence of SCA in this country is around 2-3%. Cameroon alone has 2 of the 50 million sickle-cell patients identified worldwide and 50% of the patients die before the age of five [5]. The physiopathology of sickle-cell disease shows that a number of free radicals such as the hydroxyl radical (HO•), peroxide radical (ROO•) and superoxide radical (O_2•) are produced which promote oxidative stress [6]. Several regimens of SCA have been investigated.

Allogenic bone marrow transplantation is the only available cure, but this procedure is limited to a minority of patients because of its high cost, rejection cases due to none histocompatibility (10-15%) and mortality[7]. Many drugs used for the management of sickle-cell anaemia possess antioxidant properties with the mechanisms aiming at reducing or alleviating oxidative stress or free radicals produced during the disease. These include hydroxyurea which stimulates the induction of haemoglobin F (Hb F) that will compensate the malfunctioning of haemoglobin S (Hb S) and also acts as a protector of the body against oxidative stress by increasing the amount of reduced glutathione [7,8]. Piracetam drug reduces the incidence of sickling crises [9] by reversing the sickling process of erythrocytes. However, the use of hydroxyurea in a long period can be toxic and produces some sides effects such as leg ulcer, mouth ulcer and squamous cell carcinoma [10]. A continuous use of Paracetamol in a higher dose has been shown to produce toxic effects through oxidative stress by destroying cell membranes when it is taken over a long period[11,12]. Furthermore these drugs are not only expensive but also have incompatibility problems[11]. Therefore, many researchers investigate on phytomedicines that have known therapeutic properties since many centuries to improve the management of the diseases concerned. Several single or mixture

of plant extracts have been investigated and different mechanisms of their *in vitro* antisickling properties demonstrated such as, the leaves of *Carica papaya*, the fruits of *Zanthozyllum heitzii*, *Piper guineensis*, *Pterocarpaosun*, *Eugenia caryophyllata*, *Sorghum bicolorcan*, *Sorghum bicolorcan*, *Pterocarpus santolinoides* and *Aloe vera* [13,14,15]; or *in vivo* such as Niprasin (a product from the extracts of four different kinds of plants: the seeds of *Piper guineensis*, the stems of *Pterocapus osun*, the fruits of *Eugenia caryophyllum* and the leaves of *Sorghum bicolor*). The *in vitro* antisickling properties of a mixture of six plant extracts including *Theobroma cacao* (*T. cacao*) have been previously demonstrated [16]. In most of these studies, the antisickling properties of plant extracts have been correlated with their antioxidant activities due to the presence of high polyphenol content. Also, previous studies showed that resveratrol, a natural polyphenol and antioxidant molecule from green tea revealed antisickling and Hb F induction properties[17].

Cocoa (*Theobroma cacao*) is a plant of the family Sterculiaceae. This plant can be found in several countries in South America and sub-sahara Africa. In Cameroon *T. cacao* is cultivated in seven of the ten regions of the country which include (Centre, South, East, Littoral, Southwest, West and Northwest regions). The phytochemical analysis of the fruits of *T. cocoa* from 5 regions of Cameroon (Mbalmayo, Santchou, Obala, Penja and Bertoua) showed the presence of higher concentrations of theobromine and many classes of polyphenols[18]. Studies have shown that polyphenols, flavonols and tannins present in cocoa have beneficial effects against oxidative stress and diseases associated with oxidative stress such as cancer [19], by increasing the activities of antioxidative enzymes [20], the level of liver thiols, reducing the level of oxidative damage [21] and increasing the resistance to hydrogen peroxide[22]. The present study was performed to investigate the antisickling and antioxidant properties of fruits extracts of *Theobroma cacao* harvested in Obala and Santchou in the Centre and west regions of Cameroon respectively.

MATERIAL AND METHODS

Plant: Beans of *Theobroma cacao* (Sterculiaceae) collected at Obala and Santchou, two localities respectively in the Centre and West regions of Cameroon.

Blood samples: The blood samples used to determine the antisickling activity of the plant extracts in this study were given by 05 homozygote patients SS aged between 4 and 22 years regularly consulted in the Yaoundé Central Hospital. According to the patient age, a written informed consent was signed by either child parents or adult patients participating in the study. All the research procedures received the approval of the Regional Ethics Committee for Research on human health in the reference centre number 0282/CRERSHC/2016.

Plant collection: Beans of *Theobroma cacao* (Sterculiaceae) were collected on the 13th of August 2013 and identified under the reference number 60071/H.N.C at the National Herbarium of Yaoundé, Cameroon.

Preparation of extract: After fermentation and drying (3 days of fermentation and dried for one week under the sun for the Santchou cacao beans, 4 days of fermentation and two weeks of drying out of the sun for the Obala cacao beans). They were crushed and an aliquot of 197 g was extracted by maceration for 48 h in a mixture of water/ethanol (30V/70V; pH=3). The mixture was then filtered using Buchner funnel and Whatman No. 1 filter paper. This process was repeated once on the residue after 24 h. The filtrate was concentrated using a rotary evaporator and the extracts were dried in an oven at 55 °C for two days. Each crude extract obtained was labelled and kept in the freezer.

Antisickling Activity

Antisickling assay

Five millilitres of blood samples obtained from patients were centrifuged at 3,000 rpm for 5 min and the plasma removed. Red blood cells (RBC) obtained were washed twice in 0.9% saline. The washed RBCs of sickling induction as well as antisickling properties of extracts/standard, followed a previously described method [23] with some modifications. Briefly, in three cryo-tubes containing 200 µL of different concentrations of extracts; 500, 1000 and 2000 µg/mL; 200 µL of SS blood (previously diluted in NaCl: 1/12) were added. In two other tubes constituting the positive and negative controls where the extracts were replaced by quercetin 1000 µg/mL and 0.85% NaCl respectively, 5 µL of each mixture were fixed on the slide and sealed with nail polish and a cover slip. After mounting the slide under an optical microscope, the number of red blood cells was counted in 5 fields, at different periods of time 1 h, 2 h, 3 h, 4 h, 6 h and 24 h. The curves of sickling percentage at various concentrations over time were plotted. The percentage of sickle cells was calculated using the formula:

(%) Sickling = Number of sickle cells × 100/total cells.

Erythrocyte membrane stability activity

The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress/hypotonic lysis incubation. We used the method described by [24]. Nine solutions of concentrations varying between 0% and 0.85% were prepared from 1% NaCl. From each extract solution, dilutions were made to obtain 500, 1000 and 2000 µg/mL. For each test tube, 800 µL of the NaCl solution at various concentrations and 200 µL of extracts at different concentrations were introduced. Then 10 µL of blood were added. The mixture was allowed to stand for 24 h. The supernatant was recovered with a micropipette and the optical density was read at 540 nm against the blank containing NaCl at various concentrations instead of the extract. This wavelength gives the difference between intact cells and lysed cells.

Antioxidant Properties of the Extracts

Total polyphenol content (TP)

The Total polyphenol content assay was carried out for the evaluation of the antioxidant activity[25]. We introduced 200 µL of each extract (1 mg/mL) into a test tube, added 800 µL of the Folin Ciocalteu reagent (1/10 dilution) and 2 mL of sodium carbonate solution (7.5%). After stirring, the mixture was kept for 2 h in the dark and the absorbance was measured at 765 nm. The total polyphenol content was determined from a standard curve plotted with quercetin in a concentration range of 0-300 µg/mL and expressed in mg equivalence of quercetin per gram of dry extract (mg eq QE/g of extract).

Total flavonols content (TF)

Total flavonols content assay was carried out for the evaluation of the antioxidant activity using the standard method [26] with slight modifications. In different tubes, we introduced 2 mL of each extract and 2 mL of aluminium chloride (2%) and 3 mL of sodium acetate of 50 g/L concentration were added to each tube and the mixture was homogenized. The mixture was incubated for 2 h at a temperature of 20 °C and the absorbance was read at 440 nm. The total flavonols content was calculated using the equation derived from the calibration curve with quercetin as standard and

the value was expressed in mg equivalence of quercetin per gram of dry extract (mg eq QE/g of extract).

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 iron to the ferrous form in the presence of antioxidant compounds [27]. For each extract (1 mg/mL concentration), 75 µL were introduced into different test tubes and then 2 mL of FRAP reagent were added. The mixture was incubated for 12 min and the absorbance was read at 593 nm. Vitamin C was used as standard and the results were expressed in mg equivalence of vitamin C per gram of dry extract (mg eq vit C/g of extract)

Scavenging Activity of DPPH Radical

The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity [28]. Briefly, in 3 mL of each diluted extract / positive control (quercetin), 1 mL of methanol solution of 0.1 mM DPPH 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. The following equation was used to determine the percentage of the radical scavenging activity of each extracts:

Percentage DPPH radical scavenging activity = [(OD control - OD sample)/OD control] × 100.

The concentration of extracts and positive control which scavenged 50% of DPPH radicals was calculated and represented as IC_{50} (µg/mL).

Scavenging activity of NO radical

The scavenging activity of the extracts on nitric oxide radical was measured according to the method described by Garrat (1964) [29] using the Griess Illosvoy reaction. 0.5 mL of extracts / positive control (quercetin) were introduced at various concentrations in the test tubes. Then were added respectively, 2 mL of SNP (sodium nitroprusside 10 mM), 0.5 mL of phosphate buffer (0.5 M) and Griess reagent 1 mL. The pink chromophore generated was measured by spectrophotometry at 540 nm against the blank.

Percentage of radical scavenging activity = [(OD control – OD sample)/OD control] × 100.

The concentration of extracts or quercetin which scavenged 50% of NO radicals was calculated and represented as IC_{50} (µg/mL).

Scavenging activity of ABTS radical

The scavenging activity of the extract on ABTS radical was measured according to a described method[30]. To 75 µL of extract / positive control (quercetin) at different concentrations introduced into the test tubes was added 2 mL of $ABTS^{+}$, the mixture was homogenized and incubated for 6 min in the dark and the absorbance was measured at 734 nm against blank.

Percentage ABTS radical scavenging activity= [(OD control - OD sample)/OD control] × 100.

The concentration of extracts or quercetin which scavenged 50% (IC_{50} (µg/mL) of ABTS radicals was calculated.

Statistical analysis

Each test was performed in triplicates and the results were expressed as mean ± standard deviation. The *Kruskal-Wallis* test was used, followed by post-hoc *Dunnett* to analyze the antioxidant potential and antiradical activity of each plant extract, in order to determine significant differences ($p < 0.05$). The Mixed Linear Effect Model helped to study the interactions between factors (extracts, concentration and the repeated time factor) after data restructure. The Spearman correlation enabled us to establish correlations between plant extracts and various anti-radical methods. IC_{50} were determined by the use of multiple regression analyses. The software SPSS version 16.0 for Windows 7 was used for statistical analysis.

RESULTS

Antisickling properties

The results of sickling induction showed an increase in sickling cells percentage from 4.52% to 41.50% after 24 h depending on the RBC used. Figure 1 shows the results of the effects of extracts on the sickle cells. The percentage of sickle cells between 0 h and 24 h increased significantly ($P < 0.05$) from 4.52% to 41.50% in the control group. The sickle cell percentage on Figure 1 gives information on the rate of sickle cell inhibition percentage. Then, the sickle cell percentage less than 5% with both Obala and Santhou extracts (EON, EON), means at least 95% of sickle cell inhibition. It is noticeable that EON started losing its inhibition property after 4 h while ESN inhibition activity still increases up to 24 h.

Figure 2 shows the effects of time and concentration on different extracts on sickling reduction percentage. For each extract, the sickling reduction is time and concentration dependent excepted for ESN which presented its highest activity at 4 h. After 2 h, 4 h and 24 h at all the concentrations, quercetin as positive control, had the highest percentage reduction followed by EON.

The osmotic fragility of the membrane of RBC was assessed at different concentrations of extracts and salts (NaCl) and the results are represented in the Figure 3. These results demonstrate that the percentage of haemolysis varied depending on the type and concentration of extracts as well as the concentration of salts. The haemolysis percentage of all extracts and control decreased by increasing the salt concentrations. When fixing each extract concentration, all extracts decreased significantly the red blood cell haemolysis with salt concentration dependency from $S_{0.35}$ to $S_{0.85}$ compared to S_0. The extracts of "Obala" decreased considerably the red blood cell haemolysis compared to other extracts (Figure 3).

The interaction between extracts and salts concentrations revealed that, EON has the lowest percentage of haemolysis with extracts and salt concentrations dependency with a significant difference ($P < 0.05$) between 500 µg/mL and 1000 µg/mL at S_0 and $S_{0.15}$ (Figure 3).

Antioxidant properties

The results of total phenol content and antioxidant properties of extracts from cocoa are shown in the Table 1. The total polyphenols and flavonols contents of the extracts of *T. cacao* as well as their antioxidant capacities especially those from EON were higher compared to ESN.

TABLE 1: PHENOL CONTENT AND ANTIOXIDANT PROPERTIES OF EXTRACTS

Extracts	Flavonol (mg Eq of Quercetin/g of extract)	Phenol content (mg Eq of Quercetin/g of extract)	FRAP (mg Eq of Vit C/g of extract)
ESN	61.72 ± 2.4^{a,b}	486.5 ± 22.1^{b}	91.4 ± 9.5^{a,b}
EON	73.72 ± 6.2^{b}	487.33 ± 20.4^{b}	106.86 ± 19.2^{b}

Kruskal-Wallis and post-hoc Dunnett. The extracts with different letters have values that differ significantly at $P < 0.05$. Each value assigned with different letters for each extract concentration differs significantly at $P< 0.05$. ESN = Santchou cocoa extract, EON = Obala cocoa extract.

Table 2 presents the 50% inhibitory concentration (IC_{50}) of extracts which varied depending on the extracts and the radical used. These values ranged between 1.93 and 5.9 µg/mL. Quercetin had the lowest IC_{50} in all the three radicals tested (ABTS, DPPH and NO radicals), followed by EON in ABTS and NO radicals.

TABLE 2: INHIBITION PERCENTAGE AND INHIBITORY CONCENTRATIONS 50 (IC_{50}) OF THE DPPH, NO AND ABTS RADICALS BY THE EXTRACTS OF *THEOBROMA CACAO* AT DIFFERENT CONCENTRATIONS

		Concentrations of samples (µg/mL)			
		25	50	75	150
	IC_{50} (µg/mL)	**ABTS Inhibition percentage (%)**			
ESN	4.46	30.10 ± 3.62^{a}	30.44 ± 3.07^{a}	37.22 ± 5.52^{a}	48.14 ± 3.70^{b}
EON	4.33	33.40 ± 2.04^{a}	38.32 ± 1.47a	47.62 ± 3.26^{a,b}	49.40 ± 3.21^{b}
Quercetin	3.85	41.2 ± 5.1^{b}	45.7 ± 2.31^{b}	50.5 ± 4.25^{b}	55.1 ± 6.27^{b}
		DPPH Inhibition percentage (%)			
ESN	2.12	74.63 ± 0.91^{a}	79.65 ± 2.62^{a}	83.63 ± 3.65^{b}	96.67 ± 0.10^{b}
EON	2.34	75.26 ± 4.34^{a}	72.79 ± 6.14^{a}	72.92 ± 0.24^{a}	81.55 ± 0.47^{b}
Quercetin	1.93	80.2 ± 3.2	85.75 ± 5.31	88.5 ± 7.1^{b}	100.1 ± 5.3^{b}

Kruskal-Wallis and post-hoc Dunnett. Values are expressed as mean ± SD of three replicates. Each value assigned with different letters for each extract concentration differs significantly at P < 0.05.

Kruskal-Wallis and post-hoc Dunnett. Values are expressed as mean ± SD of three replicates. Each value assigned with different letters for each extract concentration differs significantly at P<0.05. CN = Dark chocolate, ESN = Santchou cocoa extract, EON = Obala cocoa extract. IC50 = Inhibitory Concentrations 50

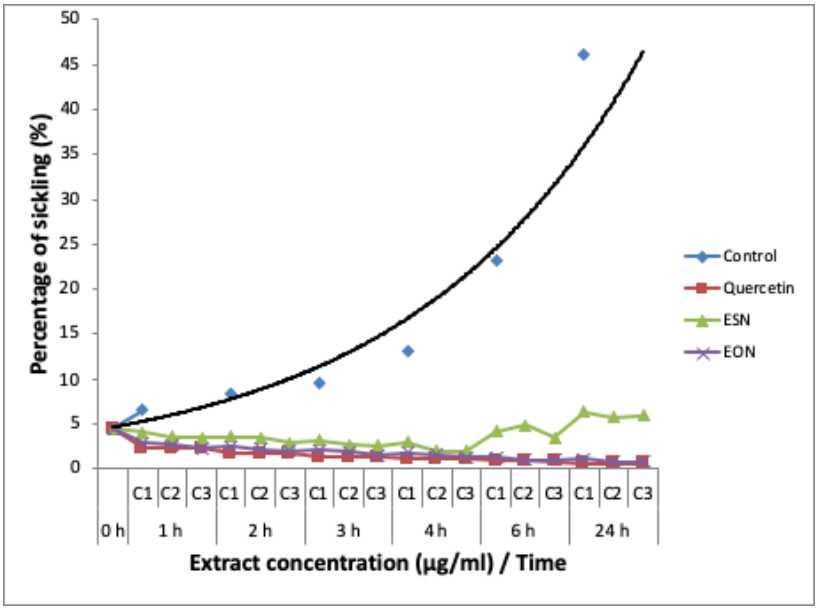

Figure 1: Effect of cocoa extracts on sickling as a function of extract concentration and time

Mixed Linear Effect Model helped studying interactions between factors (extracts, concentration and the repeated time factor) after data restructure. C1= concentration 500 µg/ml. C2= concentration 1000 µg/ml. C3= concentration 2000 µg/ml. Control = Negative control (Blood that did not under any treatment), Quercetin = Positive control, ESN = Santchou cocoa extract, EON = Obala cocoa extract, CN = Dark chocolate. Ex conc: concentration of extracts

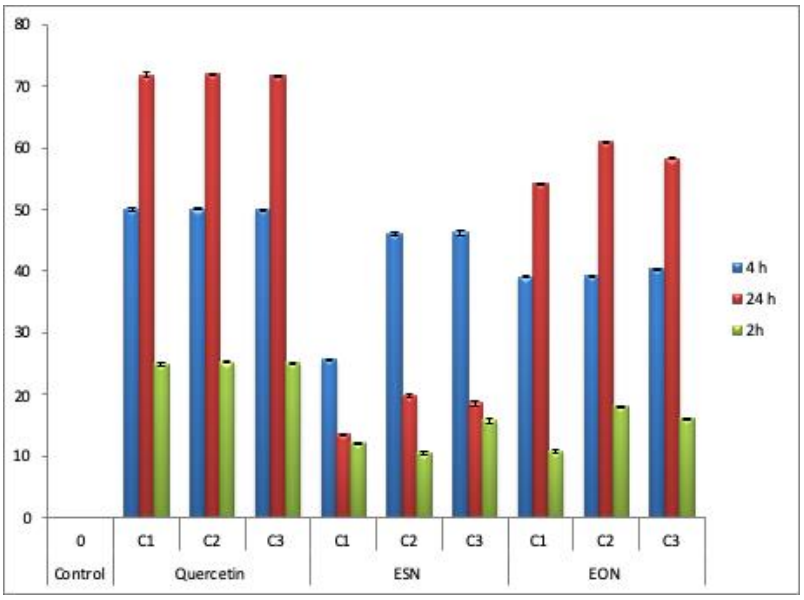

Figure 2: Reduction percentage of sickling by different extracts at different concentrations after 2, 4 and 24 h of induction
Mixed Linear Effect Model helped studying interactions between factors (extracts, concentration and the repeated time factor) after data restructure. C1= concentration 500 µg/ml. C2= concentration 1000 µg/ml. C3= concentration 2000 µg/ml. Control = Negative control (Blood that does not undergo any treatment), Quercetin = Positive control, ESN = Santchou cocoa extract, EON = Obala cocoa extract, CN = Dark chocolate.

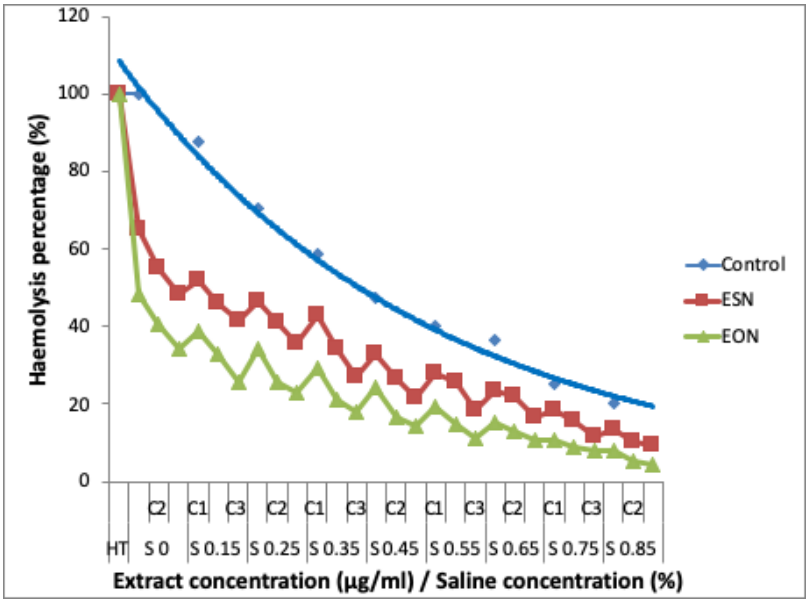

Figure 3: Haemolysis Percentage as a function of saline and extract concentrations
Mixed Linear Effect Model helped studying interactions between factors (extracts, concentration and the repeated saline % factor) after data restructure. C1= concentration 500 µg/ml. C2= concentration 1000 µg/ml. C3= concentration 2000 µg/ml. Blank = tube containing the blood sample that did not undergo any treatment, ESN = Santchou cocoa extract, EON = Obala cocoa extract, CN = Dark chocolate, S = Sample, HT= total haemolysis, Ex conc= concentration of extract

DISCUSSION

The variation of the percentage of sickling cells in this study may be explained by either the difference of age but also by gender, genetic and environmental conditions which are different between the patients who accepted to give their blood. The average of the initial percentages of sickling cells was 4.52% before induction. This value increased to 41.50% after 24 h of induction and is lower than that obtained by Nanfack *et al.* [15] and Joppa *et al.*[23] which were 52.08% and 96.5% after 2h and 3h respectively using sodium metabisulphite (2%) for induction. This is explained by the fact that the creation of hypoxia conditions by sealing the slides with nail polish induces sickling of cells very slowly compared to induction with sodium metabisulphite (2%).

The effects of *T. cocoa* extracts to prevent the induction of sickling of cells varied depending on the nature of the extracts, its concentration and the region where the plant was harvested. Compared to ESN, EON showed the best inhibitory activity with an average percentage of sickling reduction of 64.34% after 24 h. This could be explained by the fact that, the presence of phenolic components in the extracts inhibited the polymerization of haemoglobin S *in vitro*. It could be possible that this extract acts

by increasing the latent period prior to polymerization of haemoglobin and makes haemoglobin more soluble [31,32]. This activity could be attributed to flavonoids (flavonols), the flavonoids that are involved in inhibiting the deleterious effects of reactive oxygen species of oxygen produced during sickle cell anaemia [33]. This result is comparable to that of Egunyomi *et al.* [34] who obtained a sickling reduction of 63.8% with their first plant mixture. It is higher than that obtained by Nanfack *et al.* [15] who obtained 39.5% for the fruit extracts of *Zanthoxylum heitzii*, lower than that obtained by Gbadamosi *et al.* [16] showing 71.6% with their first plant recipe that contained *T. cocoa*. Joppa *et al.* [23] obtained a sickling reduction of 81% with the extracts of *Morinda lucida*.

The Santchou extracts had the second-best inhibitory activity. The sickling reduction percentage average after 4 h of induction was 39.25% and 0.00% after 24 h of induction. Nanfack *et al.* [15] obtained a comparable result with 39.5% inhibition using the fruit extracts of *Zanthoxylum heitzii*. We noticed that this extracts from Santchou loosed their activity after 4 h to have almost no activity at 24 h. Since there is a relationship between the antisickling and the antioxidant potentials of most extracts [15]; It is well known that the antioxidant potential is kinetic dependent[35]. The lost of ESN anti-sickling activity after 4 h could be explained by its rapid antioxidant kinetic. Further research needs to be done in order to clarify this assertion.

However, we observed a decrease in activity at 2000 µg/mL concentration for sample 3 (blood) with respect to the two extracts at 24 h of induction and sample 5 (blood) at 4 h and 24 h of induction for the extracts of Cocoa from Obala. This could be explained by the genetic variability associated with the polymorphism and the initial foetal haemoglobin rate of each patient [36].

The best activity of the cocoa extracts from Obala compared to the cocoa extracts from Santchou is explained by the fact that, these cocoas are grown in different regions, the climate and the composition of the soils not being the same, and also by the fact that fermentation and drying methods are specific to each region.

Regarding the osmotic fragility test of erythrocytes, there was a considerable reduction in the percentage of haemolysis generally with increase in the concentration of the saline solution and the concentration of extracts. For our two extracts, the percentages remained lower than that of the standard. For the five blood samples used, the Obala cocoa extracts had the best protective effect on the erythrocyte membrane. The decrease in the haemolysis percentage observed is related to the appreciable protective effect of the extracts of *T. cacao* on the erythrocyte membrane, hence their resistance to haemolysis. Indeed, the extracts would prevent the generation of prooxidants and reduce the damages caused to the erythrocyte membrane, the adhesion to phagocytosis of the oxidized erythrocytes and consequently reduce haemolysis [37]. These results are similar to those obtained by Nanfack *et al.* [15] on extracts of *Zanthoxylum heitzii.*

Determination of total polyphenols and flavonols contents revealed higher contents of total polyphenols and flavonols in the extracts. This content being higher in the cocoa extract of Obala especially as regards the content of flavonols would probably explain its strong antisickling and antioxidant activities. This is in agreement with the studies made by Andújar *et al.* [19] which show that flavonols and procyanidins are the main polyphenols contained in cocoa.

However, the ability of the Obala cocoa extracts to reduce the iron was greater than that of the Santchou cocoa extracts. According to the results obtained, we can suggest here that it would be the phenolic compounds (flavonols) present in our extracts acting as reducing agents by converting the free radicals into more stable compounds [38]. Oxidation of haemoglobin (Fe^{2+}) to methemohaemoglobin (Fe^{3+}) in sickle cell anemia is due to the important oxidative stress in sickle red blood cells [39, 40]. Hence our extracts, which were able to reduce Fe^{3+} to Fe^{2+}, would have an antioxidant effect on sickle red cells and would therefore be useful in the management of sickle cell anaemia.

The trapping test of the DPPH radical is widely used in the investigation of the antioxidant activity of several natural compounds, thus making it possible to test their ability to act as free radical scavengers or hydrogen donors [41]. It is well known that nitric oxide (NO) plays an important role in various processes of inflammation. The maintenance of a high level of this radical is directly toxic to the tissues and leads to a vascular collapse [42] consequently, the ability of a compound to trap this radical would make it a good antioxidant. The ABTS trapping test is known to be one of the fastest methods for determining the antioxidant activity of compounds and can be a useful tool for screening samples to obtain a large number of natural antioxidants effects in food [43]. In general, the extracts inhibited the DPPH°, NO° and ABTS° radicals. This ability to trap free radicals was much more important EON. The ability of these to trap the free radicals is due to their high content in total polyphenols (flavonols), which corroborates the works of Schinella *et al.* [44] who studied the *in-vitro* antioxidant activity of cocoa extracts and showed that this activity was directly proportional to their polyphenol content. We also corroborate the study with those of Chin *et al.* [45], which shows that the antioxidant activity of cocoa and its ability to trap free radicals is linked to its flavonols. The antioxidant activity of the extracts was better on the DPPH° radical (IC_{50} between 2.12 and 3.48 µg/mL) followed by the ABTS° radical (IC_{50} between 4.33 and 5.79 µg/mL) and the NO° radical (IC_{50} between 4.33 and 5.86 µg/mL).

CONCLUSION

Compared to the Santchou cocoa extracts, Obala's cocoa extracts have the best *in vitro* antisickling activity at all concentrations and even after 24 h of sickling induction. It is followed by the Santchou cocoa extracts, whose activity decreases after 4 h. The cocoa extracts and especially those of Obala cocoa have a better antioxidant potential. In view of all of the above, these data constitute a preliminary step to the development of a future treatment against sickle cell anaemia.

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REFERENCES

1. Cook GC, ZumLa AI. Manson's tropical diseases. 21th Ed, London: WL Saunders; 2003: 1205-1295.
2. Aubry P. Hémoglobinoses. Médecine tropicale. *In French.* http://medecinetropicale.free.fr/cours/hemoglobinoses. pdf. 2015. Accessed on the 10-12-2018
3. Deme D. Drépanocytose, maladie génétique la plus répandu dans le monde. 2013. *www.journalducameroun.com/ article.php?* Accessed on the 7th April 2015.

4. World Health Organization (WHO). WHO Sickle Cell Disease Report. 2007. *www.Blog/outil sur la drépanocytose, Genetic desease.* Accessed on the 5th April 2015.
5. Drepavie. La drépanocytose, première maladie génétique au monde. 2013. *www.lib.ohchr.org/.../drepavieuprcmr 2013drepavie.pdf.* Accessed on the 7th April 2015.
6. Sess ED, Carbonneau MA, Meité M. Marqueurs de la lipoperoxydation (LPO) membranaires et tocopherols plasmatiques dans les drépanocytoses homozygote et hétérozygote. Bulletin de la Société de pathologie exotique 1998; 91: 238-241.
7. Awa D. Prise en charge de la crise douloureuse drépanocytaire Selon Les Critères de L'OMS en Milieu pédiatrique. Thesis, university of Bamako. Mali, 2008. 115p.
8. Flaurentino PT, Romélia PG, Darcielle BD, Cleiton PA, Hemerson LF, Magalhães. Analysis of oxidative status and biochemical parameters in adult patients with sickle cell anemia treated with hydroxyurea, Ceará, Brazil. Revista Brasileira de Hematologiae Hemoterapia 2011; 33(3): 207-210.
9. Al Hajeri A, Fedorowicz Z, Omran A, Tadmouri GO. Piracetam pour réduire l'incidence des crises drépanocytaires douloureuses 2011. *www.cochrane.org/.../piracetam-pour-reduire-lincidence-des-crises-drep.* Accessed on the 9th April 2016.
10. Steels E, Heenen M, Germaux MA, Moens JP. Complications cutanées du traitement au long cours par l'hydroxyurée. Revue Médicale de Bruxelles 2006; 27: 39-41.
11. Mpiana PT, Mudogo V, Tshibangu DST, Shetonde OM, Ngbolua, Oboh G, Rocha JBT. Antioxidant and neuroprotective properties of sour tea (Hibiscus sabdariffa, calyx) and green tea (Camellia sinensis) on some prooxidant-induced lipid peroxidation in brain in vitro. Food Biophysics 2008; 3: 382-389.
12. 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 Medical Association 2010; 51(3): 230-234.
13. Imaga NOA, Ukpo GE, Owolabi MA, Shekoni AI, Gbenle GO. *In vivo* analyses of the effects of co-administration of *Carica papaya* leaf extract with ciprofloxacin. African Journal of Pharmacy and Pharmacology 2012; 6(48): 3268-3273.
14. Meena S, Varsha S, Somnath Y, Harris KK. Plant extracts with antisickling propensities: a feasible succour towards sickle cell disease management- a mini review. Journal of Phytology 2012; 4(3): 24-29.
15. Nanfack P, Biapa N, Pieme CA, Ama MV, Moukette B, Ngogang YJ. The in vitro antisickling and antioxidant effects of aqueous extracts *Zanthoxyllum heitzii* on sickle cell disorder. BMC Complementary and Alternative Medicine 2013; 13: 162.
16. Gbadamosi IT, Adeyemi SB, Adeyemi AA, Moody JO. In vitro antisickling activities of two indigenous plant recipes in Ibadan, Nigeria. International Journal of Phytomedicine 2012; 4(2): 205-211.
17. Fibach E, Prus E, Bianchi N, Zuccato C, Breveglieri G, Salvatori F, Finotti A, Lipucci di Paola M, Brognara E, Lampronti I, Borgatti M, Gambari R. Resveratrol: Antioxidant activity and induction of fetal hemoglobin in erythroid cells from normal donors and β-thalassemia patients. International Journal of Molecular Medicine. 2012; 29(6): 974-982.
18. Giovanni C, Dennis F, Filippo M, Marcello N, Massimo R, Chiara T, Biapa P, Sauro V, Gianni S. Nutritional composition, bioactive compounds and volatile profile of cocoa beans from different regions of Cameroon. 2016. International Journal Of Food Sciences and Nutrition, http://dx.doi.org/10.3109/09637486.2016.1170769.
19. Andújar I, Recio MC, Giner RM, Ríos JL. Cocoa Polyphenols and Their Potential Benefits for Human Health. Oxidative Medicine and Cellular Longevity 2012; 1–23.
20. Ramiro PE, Urpí-Sardá M, Pérez-Cano FJ, Franch A, Castellote C, Andrés-Lacueva C. Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. Journal of Agricultural and Food Chemistry 2007; 55: 6431-6438.
21. Chandranayagam C, Gayathri V, Ananthi S, Hannah RV. Restoration of arsenite induced hepato-toxicity by crude tannin rich fraction of *Theobroma cacao* in Sprague Dawley rats. Food Research International 2012; 50:46-54.
22. Martorell P, Forment JV, Llanos R. Use of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as model organisms to study the effect of cocoa polyphenols in the resistance to oxidative stress. Journal of Agricultural and Food Chemistry 2011; 59(5): 2077-2085.
23. Joppa KM, Vovor A, Eklu-Gadegbeku K, Agbonon A, Akikokou K, Gbeassor M. Effet de *Morinda lucida benth*. (Rubiaceae) et de *Newbouldia leavis p. beauv*. (bignoniaceae) sur la falciformation. Medécine Tropicale 2008; 68: 251-256.
24. Jaja SI, Kehinde MO, Gbenebitse S, Mojiminyi FB, Ogungbemi AI. Effect of vitamin C on arterial blood pressure, irreversible sickled cells and osmotic fragility in sickle cell anemia subjects. Nigerian journal of physiological sciences 2000; 16(2):14-18.
25. Singleton V, Rossi J. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. American Journal of Enology and Viticulture 1965; 16: 144-158.
26. Kumaran A, Karunakaran R. In vitro antioxidant activities of methanol extracts of Phyllanthus species from India. Lebens-Wiss Technologie 2004; 40: 344-352.
27. Benzie IF, Strain J.J. The Ferric Reducing Ability of Plasma (FRAP) as a measure of antioxidant power. The FRAP assay. Analytic Biochemistry 1996; 239: 70-76.
28. Molyneux P. The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin Journal of Science and Technology 2004; 26(2): 212-219.
29. Garratt CJ. Effect of iodination on the biological activity of insulin. Nature 1964; 201: 1324-1325.
30. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C**.** Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26: 1231-1237.
31. Efemwonkiekie WI, Ernest A, Turner, Toshio A. In vitro effects of NIPRISAN (Nix-0699): a naturally occurring, potent antisickling agent. British Journal of Haematology 2002; 118: 337-343.
32. 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. Evidence-Based Complementary and Alternative Medicine 2007. doi: 10.1093/ecam/nem/39.
33. Seck M, Sall C, Gueye PM, Seck I, Dioum MD, Lembachar Z, Gueye RS, Fall D, Fall M, Dieye TN. Etude de l'activité antifalcémiante d'extraits de racines de *Leptadenia hastata* Decne. (Asclepiadacae). International Journal of Biological and Chemical Sciences 2015; 9(3): 1375-1383.
34. Egunyomi A, Moody JO, Eletu OM. Antisickling activities of two ethnomedicinal plant recipes used for the management of sickle cell anaemia in Ibadan, Nigeria. African Journal of Biotechnology 2009; 8(1): 20-25.

35. Biapa NPC, Oben JE, Ngogang JY. Scavenging radical kinetic and Antianaemic Screening Properties of some Medicinal Plants used in Cameroon. International Journal of Applied Research in Natural Products 2011; 4(1): 29-35.
36. Abdulmalik O, Osafa MK, Chen Q, Yang J, Brugnara C, Ohene-Frempong K, Abraham DJ, Asakura T. 5-hydroxymethyl-2-furfural modifies intracellular sickle hemoglobin and inhibits sickling of red blood cells. British Journal of Haematoly 2005; 128(4): 525-561.
37. Arruda M, Grazielle M, Celso A, Sandra S, Iara B, Maria S. Antioxidant vitamins C and E supplementation increases markers of haemolysis in sickle cell anaemia patients: arandomized, double-blind, placebo-controlled trial. British Journal of Haematology 2012; 1-13.
38. Carocho M, Ferreira I. A review on antioxidants, pro-oxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food and Chemical Toxicology 2013; 51:15-25.
39. Mpiana PT, Ngbolua KN, Bokota MT, Kasonga TK, Atibu EK, Tshibangu DS, Mudogo V. *In vitro* effects of anthocyanin extract from *Justicia secunda* Vahl on the solubility of haemoglobin S and membrane stability of sickle erythrocytes. Blood Transfus 2010; 8: 248-254.
40. Ngbolua KN. Evaluation de l'activité antidrepanocytaire et antipaludique de quelques taxons végétaux de la RD Congo et de Madagascar. Doctorate Thesis, University of Kinshasa, Kinshasa, 2012; 300p.
41. Ansari NM, Houlihan L, Hussain B, Pieroni A. Antioxidant activity of five vegetables traditionally consumed by South-Asian migrants in Bradford, Yorkshire, UK. Phytotherapy Research 2005; 19: 907-911.
42. Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of Spondias pinnata. BMC Complementary and Alternative Medicine 2008; 9: 8-63.
43. Silva S, Gomes L, Leitão F, Coelho V, Boas L. Phenolic Compounds and Antioxidant Activity of Olea europaea L. Fruits and Leaves Food Science Technology International 2006; 12: 385-396.
44. Schinella G, Mosca S, Cienfuegos-Jovellanos E. Antioxidant properties of polyphenol-rich cocoa products industrially processed. Food Research International 2010; 43(6): 1614-1623.
45. Chin E, Miller K, Payne M, Hurst W, Stuart D. Comparison of antioxidant activity and flavanol content of cacao beans processed by modern and traditional Mesoamerican methods. Heritage Science 2013; 1(9): 1-7.

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