In vitro evaluation of the antioxidant and anti-sickling potential of cocoa (Theobroma cacao) from two regions in Cameroon (South west and Littoral)

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ABSTRACT

Sickle cell anaemia is a genetic disorder which causes the expression of defective haemoglobin resulting to irregular and shapeless red blood cells, known as “sickle cells”. Sickle cells cause problems in the body, often blocking blood flow, causing painful attacks, stroke and constitute a main cause of oxidative stress process. Many treatments have been developed to improve the management of this disease. Moreover, the high cost of these therapies and several other disadvantages does not allow the patients to benefit from them. The present study aims to evaluate the anti-sickling potential of Theobroma cacao (T. cacao) extract from South West / Littoral regions of Cameroon. T. cacao was collected from Penja and Manfe and then subjected to both hydroethanolic extraction at pH 3 and water, following extracts (PH, PA, MH and MA). The antioxidant potential of the extract was evaluated by measuring free radical scavenging potential (DPPH and ABTS), the ability of iron reduction (FRAP) as well as, the quantification of total polyphenols and flavonoids. Moreover, the determination of the activities of catalase, SOD, glutathione peroxidase, reduced glutathione and malondialdehyde help in studying the protective property of T. cacao against oxidative stress. The anti-sickling activity, the osmotic fragility tests of these extracts and the principal component analysis (PCA) was furthermore studied. PH, PA, MH and MA showed a high content of polyphenols and flavonoids. Moreover, these extracts excerpt a very strong radical scavenging activity with respect to the DPPH and ABTS radicals, with IC_{50}s between 3.24 and 6.35 µg/ml and between 4.87 and 19.29 µg/ml respectively, with an accent when cocoa extract from Manfe. The PCA revealed that the extract of Manfe showed a better ability to reduce sickling, to protect erythrocyte membrane from haemolysis and to regulate the enzyme activities following the stress induced. These results suggest that cocoa bean extracts from these localities particularly from Manfe could be used against sickling. More studies such as toxicological, in vivo antioxidant and antisickling are needed to complete these data for the use of cocoa in the management of sickle cell anaemia.

Keywords: Sickle cell anaemia, oxidative stress, antioxidant, Theobroma cacao.

INTRODUCTION

Sickle cell anaemia (SCA) is an illness which results from a mutation in a genetic code such that a single amino acid (glutamic acid) is replaced by another (valin) in the beta globin chain of haemoglobin (Hb). This substitution transforms normal adult haemoglobin (Hb A) into abnormal haemoglobin (Hb S). Under conditions of low oxygen tension, Hb S molecules undergo aggregation and polymerize and the red blood cells acquire a “sickle” or
“holly leaf” shape which tend to block blood flow in the blood vessels. Hb S is considered as a pro-oxidant machine responsible for free radical formations which cause an imbalance between the free radical and antioxidants and then an oxidative stress in the body (WHO, 2010).

SCA is predominantly found among African people, Mediterranean, Arabic and Asian in relation to the survival advantage against malaria (Mabiala et al., 2005). Studies also indicate that approximately 1 in 12 African-Americans are heterozygous for the disorder, and approximately 1 in 500 African-American newborns are diagnosed positive annually (Boyd et al., 2005). The life expectancy of people living with this pathology in developing countries is less than 50 years of age. Annually, about 300,000 children are born with it and about 75% of these cases occur (Makani et al., 2011). In Cameroon particularly, with respect to the statistic given by World Health Organization and in conformity with the national prevalence, 4000 children are born annually with SCA and youths of about 10 to 29 years of age represent 89.2% of those suffering from this illness. This pathology leads to 4000 death each year in Cameroon (DREPAVIE, 2013). Owing to the high rate at which this disease is affecting the world, researchers are still trying to investigate strategies in order to limiting the crisis state, complications as well as, to definitely cure it. This led to a series of treatment such as the use of hydroxyurea which protects the body against oxidative stress by increasing the amount of reduced glutathione, stimulates the induction of haemoglobin F (Hb F) in order to reduce Hb S formation and its effects (Flaurentino et al., 2011).

Piracetam drug reduces the incidence of sickling crises by reversing the sickling process of erythrocytes (Al Hayeri et al., 2011). However, the use of hydroxyurea in a long period can be toxic and produces some side effects such as leg ulcer, mouth ulcer and squamous cell carcinoma (Flaurentino et al., 2011). Stem cell transplant is another opportunity to manage SCA complications but the cost is very high and it faces also a problem of compatibility. Due to the aforementioned limitation and inconvenience, new and safe therapeutics need to be checked. Most researchers have now turned on plant for their numerous life saving and therapeutic properties. This is the case of Fagara zanthoxyloides (Sofowora et al., 1971), Terminalia catappa (Mgbemene et al., 1999), Carica papaya (Ogunyemi et al., 2008), Pterocarpus santolinoides and Aloe vera which are traditionally used against SCA. These plants revealed both anti-sickling and antioxidant properties (Ugbor, 2006). Most a time the anti-sickling activity of plant extracts are related to their antioxidant capacity due to their polyphenol content (Nanfack et al., 2013).

Cocoa (Theobroma cacao) is one of the richest dietary sources of polyphenols such as caffeic acid and epicatechin which quantity is directly linked to its geographic distribution (Caprioli et al., 2016). High antioxidant properties of cocoa confer to its numerous therapeutic properties (NUTRA NEWS, 2008). Is the relationship between cocoa compounds and the biological activity state extended in the case of sickle cell anaemia? The hypothesis of the present study is that T. cacao extract possesses antioxidant and anti-sickling properties. The general objective is to evaluate the anti-sickling potential of T. cacao extracts from South West / Littoral regions of Cameroon in order to contribute to the new and safe therapeutic strategy against sickle cell anaemia. More specifically, it is to evaluate the antioxidant activity of cocoa extract from two different regions of Cameroon (Littoral and South West), to determine anti-sickling and the anti-osmotic fragility potential of extracts as well as, to demonstrate the protective effect of extracts against the induced oxidative stress.

MATERIALS AND METHODS

Plant collection and authentification

T. cacao beans were harvested in two towns in Cameroon: Manfe and Penja in February, 2017 and identified at the National Herbarium under the reference number 60071/HNC where the voucher specimen was deposited. Beans were fermented and dried by specific methods according to each locality process. Once dried, they were sorted, pulped and crushed in a blender to obtain the cocoa powder. The obtained powder was directly extracted.

Preparation of T. cacao extracts

The modified method of Benhammou et al. (2008) was used for the extraction. 195 g of the cocoa beans powder were macerated in the mixture of ethanol-water (70v/30v) for 48 h at pH 3 by the addition of a few drops of acetic acid. The mixture was stirred several times daily to maximize extraction. After 48 h, the mixture was filtered using Whatmann paper No. 4 and 1. The obtained residue was re-extracted as earlier mentioned and the total filtrate dried in an oven for 24 h at 45°C.

Blood sample collection

The blood samples used in this study were obtained from homozygote SS patients between 18 and 30 years old, in the Central hospital of Yaoundé. The blood samples were collected in the sodium EDTA tubes and stored at 4°C not more than 24 h for the experiment. A written informed consent was read and signed by all the patients participating in the study. All the research procedures received the approval of Research Ethics for Human Health at the center (CRERSH / Ce) under the reference number 00255 / CRERSHC / 2017.
Determination of antioxidant activity of *T. cacao* extracts

**Determination of the total polyphenols and flavonoids content**

The total phenol content was assessed using Folin-Ciocalteu colorimetric method. Briefly to 0.1 ml of plant extract (1 mg/ml), was added 0.4 ml of the folin-Ciocalteu reagent ten times diluted and 1 ml of sodium carbonate Na₂CO₃ (7.5%). The mixture was homogenized and incubated at room temperature in the obscurity for 2 h (Singleton et al., 1999). The absorbance was measured at 765 nm and the total polyphenol content determined from the standard curve and was expressed in mg equivalent of caffeic acid / g of extract (ECA/g E).

For the total flavonoids determination, the method of Zhishen et al. (1999) was used. To 500 μl of the extracts (100 μg/ml), were added 300 μl of distilled water and 30 μl of sodium nitrite (5%). After incubating for 5 min at room temperature, 30 μl of aluminum trichloride (10%) were added to the mixture. After 1 min of incubation at ambient temperature, 200 μl of sodium hydroxide (1 mM) and 1000 μl of distilled water were added. The absorbance of the solution was determined at 510 nm. A calibration curve was performed in parallel under the same operating conditions using quercetin as a positive control. The total flavonoid content of the extracts was determined using a standard curve and expressed in milligram (mg) standard equivalent / g of extract (mg ES/g E).

**Determination of antioxidant potential of the extracts by the method of Ferric Reducing Antioxidant Power (FRAP)**

This method measures the ability of samples to reduce iron at pH 3.6 in the presence of an antioxidant. The FRAP reagent is a mixture of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM) and FeCl₃ (10 mM) in the proportion 10:1:1. 75 μl of plant extract of 1 mg/ml concentration was added to 1000 μl of FRAP reagent. The mixture was homogenized and incubated for 12 min, and then the absorbance read at 539 nm. Ascorbic acid concentration (100 μg/ml) was used as the standard and the final results were expressed as mg equivalence of ascorbic acid per g of dry matter (mg EAA/g E), using the regression equation of the calibration curve (Benzie and Strain, 1996).

**Determination of the scavenging activity of the DPPH radical of plant extracts**

This method is based on measuring the ability of antioxidants to trap the 2,2-diphenyl-1,1-picrylhydrazil (DPPH*) radical. To 0.3 ml of extract of a series of concentrations (0, 10, 15, 25, 75 and 100 μg/ml) was added 0.3 ml of (DPPH 0.1 mM) solution. The control consists of 0.3 ml of the DPPH solution and 3 ml of distilled water. Ascorbic acid was used as standard and prepared at the same concentration. After homogenization they were incubated in the obscurity at room temperature for 30 min. The absorbance was measured at 517 nm against the blank (Molyneux, 2004). The percentage of inhibition of the DPPH radical by the extract was calculated according to the formula:

\[
\% \text{ DPPH radical scavenging activity} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100
\]

The IC₅₀ values were calculated from the graphs representing the variations of the percentage of inhibition according to a function of the different concentrations.

**Determination of free radical scavenging activity of 2,2’-azino-bis- [3-ethylbenz-thiazolone-6-sulfonic acid] (ABTS *)**

By reacting with a strong oxidant such as potassium permanganate (KMnO₄) or potassium persulfate, ABTS forms the radical ABTS**, from blue to green. Adding an antioxidant to this mixture will reduce the radical and cause discoloration. The ABTS** stock solution consisted of 200 ml of ABTS (7 mM) and 200 ml of ammonium persulfate (2.45 mM). The reaction mixture was incubated in the dark for 12 h at room temperature. To 40 μl of extracts at 6 different concentrations (0, 25, 75, 100, 150 and 300 μg/ml) were added 2000 μl of the ABTS** solution. The control consisted of 40 μl of distilled water and 2000 μl of ABTS* solution. Ascorbic acid was used as standard and prepared at the same concentration. After homogenization, the whole was incubated at room temperature for 6 min. Absorbance was measured at 734 nm against the blank (Re et al., 1999). The percentage inhibition of the ABTS radical using extracts was calculated according to the formula:

\[
\% \text{ ABTS radical scavenging activity} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100
\]

The IC₅₀ value will be defined as the concentration of the extract that causes the loss of 50% of the ABTS activity.

**Determination of anti-sickling activity of *T. cacao* bean extracts**

*In vitro evaluation of inhibitory activity of extracts on sickle cells*

The evaluation of the anti-sickling activity of the *T. cacao*
extracts was done according to the N'Draman-donou et al. (2015) protocol, slightly modified. The Malassez cell helped counting RBCs. A dilution of 1:250 (blood / physiological saline) was performed. In order to obtain pure RBCs, plasma was isolated from the whole blood by pre-washing them using 0.9% NaCl solution followed by a centrifugation for 5 min at 3000 rpm. This process was repeated twice to completely liberate RBCs from impurities. Each extract stock solution was prepared by mixing each plant extract in 0.85% NaCl solution for a final concentration of 10 mg / ml. From these, different dilutions helped preparing corresponding concentrations (500, 1000 and 2000 μg / ml).

**Study of the anti-sickling activity of the extract**

50 μl of washed SS blood previously diluted with physiological saline (1:250 blood/physiological saline) and 50 μl of 2% sodium metabisulphite solution were mixed in an Eppendorf tube. 15 μl of the mixture was placed on a Malassez cell covered by a cover slip and observed under light microscope to determine the percentage of sickle cell.

**Study of the anti-sickling activity of the extract**

To 50 μl of washed SS blood previously diluted with physiological saline (1:250 blood/physiological saline), were added 50 μl of each extract at different concentrations (500, 1000 and 2000 μg/ml) and 50 μl of sodium metabisulphite 2%. The microscopic observation of 15 μl of this mixture was done using Malassez cell under light microscope (40X objective) after 30 min, 1 h, 1 h 30 min, 2 h and 2 h 30 min. The percentage of sickle cells was then obtained. A control group was performed in the same way but the extract was replaced by phenylalanine (standard).

**Osmotic fragility test of erythrocytes**

The osmotic fragility of the erythrocytes is based on the measurement of the stabilizing effect of their membrane after 24 h of incubation with the extract. Cell lysis is determined by observing the turbidity shift using spectrophotometric method at 540 nm. Briefly, a serial concentration of extracts (500, 1000 and 2000 μg/ml) were diluted in NaCl (0.85%). In 800 μl of different NaCl concentrations, were added respectively 200 μl of extracts and 10 μl of blood. The supernatant absorbance from the mixture was read at 540 nm against the blank made up of NaCl after 24 h of incubation (Jaja et al, 2000). Hemolysis was expressed as:

\[
\% \text{ Haemolysis} = \frac{DO_{\text{sample}}}{DO_{\text{control}}} \times 100
\]

**Evaluation of the protective properties of the plant against oxidative stress**

**Preparation of the homogenate of organs**

Liver, kidneys and heart of two normal Wistar albino rats were isolated and then lodged in phosphate buffer (0.1 M, pH 7). They were weighed and milled until a paste was obtained and thereafter, homogenized in the same phosphate buffer. The organ homogenate of 10% (w/v) obtained was centrifuged at 3000 rpm for 10 min. The supernatant was kept at ~20°C until use.

**Determination of total protein**

The total protein was determined using Biuret method. Briefly 20 μl of each homogenate were added 1000 μl of the Biuret reagent and incubated at room temperature for 30 min. The standard protein (70 g/L) was used. The contents were homogenized and absorbance obtained at 546 nm (Gornall et al, 1949). The concentrations of the samples were determined using the formula:

\[
C = \frac{[DO \text{ test} / DO \text{ standard}]}{n} \times (\text{standard concentration})
\]

**Preparation of the pro-oxidative solution**

The pro-oxidative solution prepared consisted of mixing in an equimolar volume two solutions R1 and R2. R1 containing 0.14 M iron trichloride (FeCl₃) in hydrogen chloride (1N) and 0.16 M nitriloacetic disodium while R2 containing 0.2 M hydrogen peroxide (H₂O₂). The resulting mixture was used as oxidant to determine the protective properties of the plant against oxidative stress.

**Determination of antioxidant parameters**

An aliquot containing 0.58 ml of phosphate buffer, 200 μl of each extract or standard, 200 μl of liver, heart or kidney homogenate and 20 μl of oxidizing solution were introduced into different tubes and the mixture obtained was then incubated for 1 h at 37°C to form the test solutions. Three other tubes were prepared under the same conditions to serve as normal, negative and positive control; in these tubes, the extracts were respectively replaced by the extraction, solvent and quercetin. These test solutions and control were used to determine enzymatic and non-enzymatic parameters such as malondialdehyde (Wilbur et al, 1949), reduced glutathione (Ellman, 1959); catalase activity (Sinha, 1972); glutathione peroxidase activity (Sigma-Aldrich, 2016) and superoxide dismutase (SOD) activity (Misra and Fridovich, 1972).
Table 1: Total polyphenol content, flavonoids and ferric reducing antioxidant power (FRAP) of different extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Polyphenol content (mg Eq of Quercetin/g of extract)</th>
<th>Flavonoids content (mg Eq of caffeic acid/g of extract)</th>
<th>FRAP (mg equivalent of ascorbic acid /g of extract)</th>
<th>50% Inhibitory concentration (IC50 in µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>MH</td>
<td>200.2 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.01 ± 1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 ± 1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.16</td>
</tr>
<tr>
<td>PH</td>
<td>130 ± 2.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 ± 1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.51</td>
</tr>
<tr>
<td>MA</td>
<td>150.2 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01 ± 0.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>90 ± 1.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.35</td>
</tr>
<tr>
<td>PA</td>
<td>95 ± 2.10&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.01 ± 0.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>50 ± 1.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.98</td>
</tr>
</tbody>
</table>

The statistical analysis includes Kruskal-Wallis followed by Dunnet. Values with different letter represent significant difference at 0.05. PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract.

### Statistical analysis

Results were expressed as mean ± standard deviation and each experiment was performed in triplicates. The Kruskal-Wallis test was used, followed by post-hoc Dunnet 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 antiradical methods. IC50 were determined by the use of multiple regression analyzes. The software SPSS version 16 for Windows 7 was used for statistical analysis.

### RESULTS

#### Antioxidant activity of the various extracts of *T. cacao*

**Total polyphenol content, Flavonoids, ferric reducing antioxidant power (FRAP) and IC50 of different extracts**

Table 1 shows results relative to the total polyphenol content, flavonoids and Ferric Reducing Antioxidant Power (FRAP) of different extracts. In general, results revealed that all extracts (MH, PH, MA and PA) possess high polyphenol content and antioxidant power. Polyphenol content, flavonoids and FRAP values vary significantly (P<0.05) and ranged between 95 and 200.2 mg Eq of Quercetin/g of extract, 1 and 10 mg Eq of caffeic acid/g of extract and between 50 and 110 mg equivalent of ascorbic acid /g of extract respectively. MH presented the best potential of both three studied parameters. IC50 is between 4.16 and 6.35 for DPPH, 5.17 and 9.27 for ABTS. The IC50 of Manfe extract is lower than that of Penja.

**Radical scavenging activity of different extracts**

Figure 1 shows the radical scavenging activity of different extracts. In general, all the studied extracts scavenged significantly (P<0.05) DPPH and ABTS radicals with extract concentrations dependent. The inhibition is most accentuated when using quercetin (standard).

**Properties of the *T. cacao* extracts against red blood cell membrane fragility**

Figure 2 shows the percentage of haemolysis as a function of the salt concentration and the concentrations of various extracts. In general, it is clear that the haemolysis percentage decreases with both increase in the extract concentration (500, 1000 and 1500 µg/ml) and the salt concentrations. Results also revealed that there are some significant differences (P<0.05) between control (positive control) and extracts at each extract concentration from 0.35% salt concentration (MH, PH, and MA), from 0.35% salt concentration (PA) and at each salt concentration for the standard (PHE). Furthermore, when fixing the concentration of extracts, each extract influenced in decreasing significantly (P<0.05) the haemolysis percentage with salt concentration dependent. MH and MA seem to present the best activity.

**Anti-sickling properties of the *T. cacao* extracts**

Figure 3 shows the percentage of anti-sickling activity of various extracts at different concentrations as a function of time. This study revealed that the simple induction of cell sickling without other any treatment (2% metabisulfite control) increased significantly (P<0.05) the sickling with time dependent (between 22.05 and 56.67%). The presence of different extracts lowered
Figure 1: DPPH and ABTS scavenging activity of various extracts. Kruskal-Wallis followed by Dunnet. PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract.

Figure 2: Percentage of haemolysis as a function of the salt concentration and the concentration of the various extracts. The statistical analysis include Kruskal-Wallis followed by Dunnet. Values with different letter represent significant difference at 0.05. PHE: phenylalanine (standard). PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract. HT: total haemolysis. S0 – 0.85%: salt concentration. Control: red blood cell treated only with salt solution at different concentration.
significant (P<0.05) the aforementioned observations as a function of time and the extract concentrations compared to the 2% metabisulfite control. It is noticeable that from all the studied extracts, cocoa extracts from Manfe-Cameroon presented best values of anti-sickling activity (between 22.05 and 15.04).

Protective properties of *T. cacao* extracts on some organ homogenates

Figure 4A, B, C and D shows both enzymatic and non-enzymatic antioxidant potential as well as the lipid peroxidation status as the protective effect of *T. cacao* extracts on heart, liver and kidney homogenates. Figure 5A, B and C represent the enzymatic antioxidant potential (Catalase (CAT), superoxide dismutase (SOD) and Glutathion peroxidase (GPX) respectively). In general, compared to normal control, these enzyme activities decreased significantly (P<0.05) in all studied organs when treated only with the pro-oxidative solution (positive control). Enzyme activities significantly increased (P<0.05) as compared to positive control in all studied organs when extracts were administered. Among extracts, MH and PH presented the activity especially in liver homogenate. As far as, the GSH is concerned (Figure 4D), the same observation as earlier mentioned is presented in this case. GSH level decreased in the positive control group whereas the administration of various extracts increased the GSH concentration in all studied organ homogenates. MH showed the best potential to increase GSH followed by PA as the aforementioned increase in GSH is more pronounced in liver homogenate. The reverse situation is observable in Figure 4E where lipid peroxidation (MDA) increased significantly (P<0.05) in positive control compared to the normal control. As earlier mentioned, the extract administration regulated the situation in decreasing the MDA level with significant difference compared to the

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**Figure 3:** Anti-sickling percentage of different extracts. The statistical analysis include Kruskal-wallis followed by Dunnet. Values with different letter represent significant difference at 0.05. PHE: phenylalanine (standard). PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract. HT: total haemolysis. C1 – C3: concentration of extracts (C1=500, C2=1000 and C3=2000 µg/ml). Control: red blood cell treated only with 2% sodium metabisulfite. 0 h: before sickling induction.
**Figure 4:** Enzymatic and non enzymatic antioxidant potential as well as, the lipid peroxidation status and the protective effect of *T. cacao* extracts on studied organs homogenates. Mixed Linear Effect Model helped studying interactions between factors (extracts, organs) after data restructure. Kruskal wallis and Dunnett when fixing factors. C.Pos = Positive Control (oxidant). Cnor: normal control. PHE: phenylalanine (standard). PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract.

**Variables (Axes: F1 and F2: 64.50%)**

**Figure 5:** Principal component analysis between protective tests. PH: Penja hydroethanolic cocoa extract. MH: Manfe cocoa hydroethanolic extract. PA: Penja cocoa aqueous extract. MA: Manfe cocoa aqueous extract. MDA: malondialdehyde, GSH: reduced glutathion, GPX: Glutathion peroxidase, SOD: superoxide dismutase, CAT: Catalase, H: from heart, L: from liver, K: from kidney. DPPH: 2,2-diphenyl-dipicryl phenyl hydrazine, ABTS: 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), FRAP: ferric reducing antioxidant power. (a) distribution of tests around the $F_1$ and $F_2$ axes; (b) Projection of the heads and extracts around the $F_1$ and $F_2$ axes.
positive control.

**Correlation between protective and antioxidant parameters**

Table 2 shows correlation matrix of the protective and antioxidant parameters. The studied parameters were MDA (malondialdehyde), GSH (reduced glutathione), GPX (Glutathion peroxidase), SOD (superoxide dismutase) and CAT (Catalase) in three organs (Heart, liver and kidney). There are strong correlations between CAT (Heart, liver and kidney) and GSH (Heart), GSH from heart and liver, flavonoids and polyphenol and between ABTS and DPPH.

The Principal Component Analysis (PCA) shows both the contribution of each parameter on F1 and F2 axes (Figure 5a) as well as, the impact of each extract to that contribution (Figure 5b). MDAH, MDAL, MDAK, GSHH, GSHL, GSHK, GPXL, GPXK, SODH, SODL, SODK, CATH, CATL, CATK, DPPH, ABTS, PolyP, Flavd, FRAP correlations between the contribution of each parameter on F1 and F2 axes. It is clear from the Figure 5b that the extract contributions are as follows: MH>MA>PH>PA.

**DISCUSSION**

In Cameroon, 4000 children are given birth annually with SCA and youths of about 10 to 29 years of age represent 89.2% of those suffering from this illness. Existing treatments such as the use of hydroxyurea and piracetam, etc are still effective considering the position of studied parameters on both F1 and F2 axes.
against SCA, but recent research have shown some of their limitations as earlier mentioned in the introduction. Plants constitute a rich natural source of bioactive chemicals which are currently used to fight this disease (Williamson et al., 1992). The present study aims to evaluate the anti-sickling potential of T. cacao extract from South West and Littoral regions of Cameroon.

Results obtained from the antioxidant tests showed that T. cacao extracts from different sources (MH, MA, PH and PA) and especially the hydroethanolic extracts from Manfe followed by Penja (MH) contain high total polyphenols, flavonoids and possess a capacity to reduce ferric iron (FRAP). This could be explained either by the geographical difference of cocoa beans or by the type of extraction solvent. This result corroborates the report of Vicaș et al. (2009) on the study of Viscum album collected at different seasons which attributed the aforementioned results to environmental factors such as climate, soil and temperature. The observed antioxidant potential is directly linked to the presence of polyphenols in the studied extracts. Previous study of N’guessan et al. (2007) revealed that the different amount of polyphenols could also contribute to different level of antioxidant capacity which is beneficial to human health. The hydroethanolic solvent at pH 3 is commonly used in maximizing polyphenol extraction and could explain the aforementioned result.

The radical scavenging activity of extracts translated by the DPPH and ABTS tests increased with the extract concentration dependency. The IC_{50} of extracts varies from 4.16 at 6.35 for DPPH, 5.17 and 9.27 for ABTS. The IC_{50} of Manfe extract is lower than that of Penja. This anti-radical potential of extracts could be related to the high polyphenol content observed. Indeed, N’guessan et al. (2007) previously showed a correlation between the total phenol content and the anti-radical activity of some Ivorian plants. According to Chen and Ho (1995), the functional groups present in the phenolic compounds of green and black tea in general can easily give up an electron or a proton to neutralize the free radicals.

In this study, enzymatic and non-enzymatic parameters were measured in order to evaluate the protective effect of extracts against oxidative stress. Activities of CAT, SOD and GPX; GSH and MDA concentrations increase in the presence of extracts. Due to the high production of free radicals, the severity of a disease could be explained by the imbalance between antioxidants and pro-oxidants in favour of pro-oxidants. Thus, more antioxidants need to be either synthesized or absorbed to regulate that balance. The antioxidant molecules of plant presenting free-radical scavenging properties could be of interest as therapeutic agents in several diseases related to oxidative stress (Ramchoun et al., 2009). These results are in agreement with those of Chirico and Pialoux (2012) which showed that the increase of the activities of such parameters could explain the severity of the disease. Moreover, it has been demonstrated that decrease in SOD, CAT, and GPX activities may be attributed to the high production of ROS (Alsultan et al., 2010). The excess production of MDA has additional toxic effects leading to alterations of the proteins, including antioxidant enzymes and protein receptors (Bruno et al., 2015). Alsultan et al (2010) added that GSH is an essential cofactor for GPX activity. It was reported that GSH concentration decreased in erythrocytes of sickle cell disease (SCD) individuals due to the excess production of ROS which consume GSH leading to the reduction in the activity of GPX. Excess production of ROS may also have a serious adverse effect on RBCs membrane leading to protein and lipid peroxidation enhancing production of carbonyl and MDA.

Gelation of HbS was observed to occur rapidly with sodium metabisulphite 2%. Metabisulphite has a strong reducing power capable to remove oxygen from the immediate environment of the haemoglobin, thereby, eliciting gelation of the haemoglobin and the consequent sickling of erythrocytes. Reduction of sickling was dose-dependent with increasing concentration of extract. Indeed, in the presence of cocoa bean extracts 30 min after induction, sickling was reduced, showing that the extracts of T. cacao contain anti-sickling substances. This result is in line with previous works of Mpiana et al. (2010) and Ngbolua (2012) which revealed that some plant metabolites could interact with haemoglobin S inhibiting polymerization and thus, prevent the sickling of erythrocytes. In addition, they recently demonstrated that phenolic compounds have a great importance in fighting against SCA due to its capacity in either reducing the polymerization of HbS or stabilizing the erythrocyte membrane.

In addition, Tabassum et al. (2011) showed that flavonoids have the ability to improve endothelial function and vasodilation (by stimulation of NO production). It would appear that they also act on platelet aggregation and cytokine production (Buijsse et al., 2006).

Principal Component Analysis (PCA) showed some significant correlation between protective parameters in liver, heart and kidney and antioxidant tests. This result can furthermore be explained by the high content on bean cocoa extracts in polyphenols which in turn protects liver, heart and kidney against oxidative stress. PCA also allowed MH to be chosen as the best extract.

The effect of T. cacao on the membrane stability of RBCs can be evaluated by comparing the haemolysis rates of untreated and treated sickle RBCs with beans of cocoa. The stability test as the osmotic fragility assay is one of the possible mechanism in establishing potential anti-sickling substances (Mpiana et al., 2010). A decrease in the percentage of haemolysis as a function of the salt concentration and that of the extracts was generally noted. This decrease is related to the appreciable protective effect of the extracts on the erythrocyte membrane, hence, their resistance against haemolysis. This indicates that the cocoa beans extract...
regulated the ability of sickle cells to take up water by a mechanism of re-hydration without lysis (Mpiana et al., 2010). This stabilization effect could be explained by the fact that flavonoids reduce the effect of the high concentration of NaCl solution (Elekwa et al., 2005). In fact, the low concentration of NaCl solution (hypotonic solution) in the presence of red cells is responsible for the increased volume of the RBCs and then its haemolysis due to the entrance of water in RBCs. In contrary, because of the difference in osmotic potential caused by the high concentration of salt solution, water will diffuse out of the red cells causing them to shrink in size. In consequence, antioxidants prevent red cells dehydration in maintaining both membrane integrity (Elekwa et al., 2005), haemoglobin from oxidizing into methaemoglobin and inhibit the generation of free radicals (Ibegbum et al., 2011; Nanfack et al., 2013). These results corroborate also those obtained previously (Amujoyegbe et al., 2012), whose work on extracts of the roots and leaves of Caliandra portoricensis correlates the anti-sickling activity with their antioxidant capacity.

Conclusion

The aim of this study was to evaluate the anti-sickling potential of T. cacao bean extract from South West and Littoral regions of Cameroon. These extracts possess high content of polyphenols and flavonoids which conferred them their protective effect on red cell membrane, in liver, heart and kidney. Among these extracts, Manfe extracts showed the best activity. These results suggest that cocoa in these two regions, particularly from Manfe, could be used against sickle cell disease. In order to complete these data, other studies such as in vivo antioxidant capacity, toxicology study are needed for the use of cocoa in the management of sickle cell anaemia.

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