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In vitro Antisickling properties, Free radicals quenching potential, protective properties against oxidative mediated ion toxicity of combinations of *Theobroma cocoa* beans (sterculaceae)

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Abstract: Sickle cell anaemia induces an abnormal haemoglobin synthesis and reactive oxygen species. The present work aims to determine the antioxidant and antisickling activities of *Theobroma cocoa* extracts combination. Cocoa beans Extracts combinations of Santchou/Obala (CSOH), Bertoua/Mbalmayo (CMBH) were prepared using water/ethanol mixture. The antioxidant capacity of extracts was determined by measuring FRAP, DPPH° and ABTS°. The protective activity of extracts was estimated by evaluating some oxidative stress parameters. Osmotic fragility resistance and antisickling properties of the combination were investigated. CSOH and CMBH respectively revealed a high content of polyphenols, flavonoids. Extracts scavenged DPPH° and ABTS° radicals, respectively with IC_{50} values between 4.07 µg/mL and 13.15 µg/mL. CMBH showed the best antisickling reduction activity (54.56% and 70.07% after 2h30 and 24h respectively). The haemolysis percentage of erythrocytes decreased with an increase of the extract concentrations. Extracts combinations decreased and increased respectively the MDA and GSH levels as well as normalizing the enzymatic activities. Extracts combinations of cocoa have antioxidants and antisickling properties and could be used as a potential agent against sickle cell anaemia.

***Key words:** Sickle cell anaemia, oxidative stress, Cocoa extract combination*

I. INTRODUCTION

Sickle cell aneamia (SCA) is an inherited disease caused by an abnormal synthesis of haemoglobin, resulting from a substitution of valine for glutamic acid at the level of β chain

structure genes on chromosome 11. This substitution leads to formation of haemoglobin S [1]. The main characteristic of haemoglobin S lies in its ability to polymerize under certain conditions such as hypoxia. The time course of the polymerization-depolymerization cycles leads to the modification of the physical characteristics of the red blood cell with a sickle-shaped appearance which is the phenomenon of sickling [2]. This pathology is a "real machine" for the production of reactive oxygen species due to the instability of haemoglobin S [3]. Sickle cell disease is the most widespread genetic disease in the world affecting more than 50 million people [1]. Each year, more than 500,000 sickle cell children are born worldwide, including 300,000 in Africa and half of these children die in Africa before the age of five [4]. In Black Africa, the prevalence can reach 1 birth out of 30 [1]. In Cameroon, according to WHO statistics in accordance with national prevalence, 4000 children are born with sickle cell anaemia every year. All age groups of population are affected and young people aged 10 to 29 represent 89.2% of patients. Currently, Cameroon alone counts 2 million sickle cell patients count among the 50 million recorded worldwide [5]. Several modes of care have been considered, with a view to relieving patients and limiting seizures and complications. In addition, blood transfusion aims to correct acute anaemia, but has many disadvantages such as alloimmunization, iron overload and transfusion contaminations [4]; Allogenic bone marrow transplantation from donors offers the possibility of hematopoietic cells replacement but a problem of compatibility and high cost limited its utilization [6]. Hydroxyurea (HU), which reduces sickling and stimulates the induction of Hb F, thus compensating for the poor functioning of Hb A, but has certain drawbacks that can produce adverse effects on the body [7, 8]. The proposed therapies, in addition to being very expensive for most African populations, have undesirable side effects. Therefore, the African poor population and other countries around the world use medicinal plants to treat certain diseases [9]. The interest in medicinal plants for the management of sickle cell anaemia has grown in recent decades. Studies demonstrated the use of some medicinal plants for the management of SCA through their anti-sickling activity in relation with their antioxidant activity. Among them was *Zanthoxyllum heitzii*, which has anti-sickling properties and allows a better membrane stability of erythrocytes [10]. *Cajanuscajan* and *Adansonia digitata* have anti-sickling properties [11, 9]. In addition, recent studies indicate that the polyphenols, flavonoids and nutrients contained in *Theobroma cacao* have a beneficial effect against oxidative stress and associated diseases [8]. Recent studies showed that cocoa beans extracts in several areas of Cameroon possess antioxidant and anti-sickling activities [12, 13]. However, studies on the cocoa extract mixture from those regions are inexistentand it is well known in pharmacology that therapeutic response could come from some positive drug interactions such as synergistic and potentiating effects. In the present work, we report the results of *in vitro* study of the antioxidant and anti-sickling properties of the combinations of beans extracts of *Theobroma cacao* from four regions of Cameroon.

II. MATERIAL AND METHODS

II.1 Plant collection and authentification

Theobroma cacao beans were harvested in four towns in Cameroon: Santchou, Obala, Mbalmayo, Bertoua in December 2016 and identified at the National Herbarium under the reference number 60071/HNC where the voucher specimen was deposited there. Beans were fermented and dried by specific methods according to each locality. Once dried, they were sorted, pulped and crushed in a blender to obtain the cocoa powder. The obtained powder was directly extracted.

II.2 Preparation of plant extracts and combination

The modified method of Benhammou [14] was used for the extraction. 195 g of the powdered of cocoa beans 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 a day to maximize extraction. After 48 h the mixture was filtered using wattman paper $N^{o.}4$ and 1, and then the filtrate dried in an oven for 24 h at 45 °C. The same procedure was repeated with each cocoa powder and the mixture. The combination of cocoa bean extracts were prepared by mixing the same proportion 1:1 for two extract from different regions. A serial of concentration of extract of extract were prepared (500, 1000 and 1500 µg / mL) using distilled water.

II.3 Blood sample collection

The blood samples used in this study were obtained from homozygote SS patients between 10 and 28 years old, of the Central hospital of Yaoundé. The blood samples were collected in the sodium EDTA tubes and stored for the experiment. A written informed consent was read and signed by all the patients participating in the study. All the research procedures have received the approval of Research Ethics for Human Health (CRERSH /Ce) under the reference number 00255 / CRERSHC / 2017.

II.4 Determination of antioxidant activity of combinations of *T. cocoa* 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 $FeCl_3$ (10 mM) in the proportion 10:1:1. 75 µL of plant extract of concentration 1mg/mL is 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 MD); using the regression equation of the calibration curve [15].

II.4.1 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-picrylhydrazil ($DPPH^{\bullet}$) radical. To 03 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 dark and at room temperature for 30 min. The absorbance was measured at 517 nm against the blank [16]. The percentage of inhibition of the DPPH radical by the extract was calculated according to the following formula:

$$\% \ \mathbf{Inhibition} = [\,(\mathbf{DO_{control}} - \ \mathbf{DO_{test}}) \,/\, \mathbf{DO_{control}}]\,\mathbf{x100}$$

The IC_{50} values were calculated from the graphs representing the variations of the percentage of inhibition according to a function of the different concentrations.

II.4.2 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 $ABTS^{\bullet+}$, from blue to green. Adding an oxidant to this mixture, will reduce the radical and cause discoloration. The $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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 [17]. The percentage inhibition of the ABTS radical using extracts was calculated according to the following formula:

$$\% = [(- _{test}) / _{ol}]$$

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

II.5 Determination of anti-sickling activity of combinations of bean extracts of *Theobroma cocoa*

II.5.1 In Vitro Evaluation of Inhibitory Activity of Combinations of Theobroma cocoa Beans Extracts on Sickle Cells

The evaluation of the anti-sickling activity of the *Theobroma cacao* extracts on the cells of red blood cells (RBCs) is done according to the N'Draman-donou *et al*. (2015) [11] protocol, slightly modified. We used a computing cell and performed the dilution at 1:250 blood/ physiological saline.

II.5.2 Induction of sickling by the Emmel test

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. 15µL of the mixture was placed on a computing cells covered by a cover slip and observed under light microscope to determine the percentage of sickle cell.

II.5.3 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) was added 50 µL of each extract at different concentrations (500, 1000 and 1500 µg / mL) and 50 µL of sodium metabisulfite 2%. The microscopic observation of 15 µL of this mixture was done under light microscope after 30 min, 1h, 1h30 min, 2 h and 2h30min. The percentage of sickle cells has been obtained. A control group was performed in the same way but the extract was replaced by phenylalanine (standard).

II.5.4 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, 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. After 24 hours of incubation, the supernatant from the mixture was red at 540 nm against the blank made up of NaCl [18] and the heamolysis expressed as follows:

$$\%\ \text{Heamolysis} = \frac{\text{DO}_{\text{test}}}{\text{DO}_{\text{control}}} \times 100$$

II.6 Evaluation of the protective properties of the plant against oxidative stress

II.6.1 Preparation of the liver homogenate

Liver, kidneys, and heart of 2 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, then 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.

II.6.2 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 then absorbance was obtained at 546 nm [19]. The concentrations of the samples were determined using the following formula:

C = [DO test / DO standard] × n (standard concentration)

II.6.3 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 ($FeCl_3$) in hydrogen chloride (1N) and 0.16 M nitriloacetic disodium while R2 containing 0.2 M hydrogen peroxide (H_2O_2). The resulting mixture was used as oxidant to determine the protective properties of the plant against oxidative stress.

II.6.4 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*** [20], r***educed glutathione*** [21]; ***catalase activity*** [22]; ***glutathione peroxidase activity*** (kit method: CAS Number 7722-84-1, Sigma Aldrich) and ***superoxide dismutase (SOD) activity*** [23].

II.6.5 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 of concentration (1 mg/mL) was added 0.4 mL of the folin-Ciocalteu reagent ten times diluted and 1 mL of sodium carbonate Na_2CO_3 (7.5%). The mixture was homogenized and incubated at room temperature in the dark for 2 h [24]. The absorbance was measured at 765 nm and the total polyphenol content was 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 Zhishen [25] 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%) is 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 are 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 dry matter (mg ES / g E).

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 *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 analyzes. The software SPSS version 16 for Windows 7 was used for statistical analysis.

III. RESULTS

III.1 Antioxidant activity of the *T. Cacao* extracts combinations

III.1.1 Antioxidant potential of combinations of T. cacao extracts by Ferric Reducing Antioxidant Power (FRAP) method

The total antioxidant capacity of the CMBH (*Combination Mbalmayo* + *Bertoua*) extract is significantly higher (114.33 mg equivalent of ascorbic acid (MEAA)/g of extract) than that of CSOH (*Combination Santchou* + *Obala*) which is 119.83 MEAA/g of extract.

III.1.2 Radical scavenging potential of the *T. Cacao* extract combinations

Figure 1 shows the DPPH° and ABTS° scavenging activity. The inhibition is proportional to the concentration of extract and is highest with quercetine followed by CMBH and CSOH extracts (figure 1 A and B). In general, a significant difference ($P <0.05$) exists between quercetin and extracts at each extract concentration. It also appears a slight increase of inhibition when considering CMBH. The IC_{50} of the CSOH and CMBH combinations with DPPH° are

respectively 4.232 µg/mL and 4.072 µg/mL while that of ABTS are respectively 12.341 µg/mL and 13.156 µg/mL.

III.1.3 Protective properties of *T. cocoa* against oxidative stress

As far as figure 2 is concerned, it presents the enzymatic antioxidant potential (B, C, D, E) and the lipid peroxidation status (A) as the protective effect of *T. cacao* extracts combinations on studied organs (heart liver and kidney). It appears in general that the stress condition (Cpos or oxidant) increased significantly ($P < 0.05$) MDA concentration in heart, liver and kidney respectively (0.27±0.00, 0.29±0.00 and 0.39±0.00 µmol/L) compare to normal control (0.22±0.00, 0.19±0.00 and 0.15±0.00 µmol/L). These concentrations decreased significantly ($P < 0.05$) after administration of *Theobroma cacao* extracts combinations in each studied organ. The oppose observations have been made with Glutathion concentrations (figure 2 B). Relative to enzymatic antioxidant potential (figure 2 C,D and E), it is well observed that positive control group or oxidant group presented a significant lower ($P < 0.05$) activity of all enzymes and organs compare to negative (normal) control. Equally, the extract combinations administration reversed the situation by increasing each enzyme activity in each studied organ.

III.1.4 Total content of polyphenols and flavonoids

The total polyphenol and flavonoid content of Mbalmayo + Bertoua cocoa extract are respectively higher (289 ± 2.00 and 4 ± 0.2) significantly ($P < 0.05$) than that of Santchou + Obala (274 ± 5.1and 3.8 ± 0.1) (table 1).

III.2 Evaluation of anti-sickling properties of *T. Cacao* extracts combinations

III.2.1 Sickling induction rate with sodium metabisulfite 2%

After induction of sickling with 2% sodium metabisulphite, the sickling percentages increase and vary with time and range from 7.5% to 77.77% depending on the blood samples; the maximum being obtained at 2h30 min. The induction rate is between 28.2 and 70.27% for all blood samples in the interval 30 min to 2h30 min.

III.2.2 Effect of combinations of the T. Cacao extract combinations on cell sickling after 2h30 min of induction

Figure 3 below revealed that phenylalanine and cocoa combinations reduce sickling for all blood samples used with time and extract concentration dependent. In general, there is a significant difference ($P < 0.05$) between control (positive control) and all combinations of extracts at different concentrations. At each extract concentration, there is a significant decrease ($P < 0.05$) of sickling percentage with salt concentration dependent. Also at each concentration of salt, there are significant differences ($P < 0.05$) between control (positive control) and extracts when considering each extract concentration.

The sickling induction rate obtained varied between 28.2% and 70.27% after 2h30 min of induction with an average of 41.66%.

III.2.3 Sickling reduction capacity of the T. Cacao extract combinations at different concentrations after 30min, 2h30 and 24 h

It appears that the sickling reduction percentage is proportional to the extract concentration and time (figure 4). In fact, the highest percentages of reduction are obtained after 24 h for all the extracts, phenylalanine having the best activity followed by the combination of Mbalmayo + Bertoua at the concentration of 1000 µg/mL.
Combinations of extracts reduce the rate of sickling over the time. Overall, the better sickling reduction was after 24 h at 1000 µg/ml of extract, the best combination being CMBH with a 70.07% sickling reduction percentage.

III.2.4 Osmotic fragility test of erythrocytes

The percentage of haemolysis depending on the salt concentration and different concentrations of extracts (figure 5). This percentage decreases with the increase of concentration of the extracts (500, 1000 and 1500 µg/mL) and the increase of salt concentration for the different blood samples tested. In addition, when fixing the extract concentration, each extract influenced in decreasing significantly ($P < 0.05$) the haemolysis percentage with salt concentration dependent. Moreover at each concentration of salt, there are some significant differences ($P < 0.05$) between control (positive control) and extracts at each extract concentration.

IV. DISCUSSION

Determination of total polyphenols and flavonoids showed that combinations of *T. cacao* are rich in these compounds. Mbalmayo + Bertoua Combination possessed more of these compounds than that of Santchou + Obala. The variability of polyphenol contents is due to the phenolic composition of the extracts depending to some conditions such as season, climate and temperature [26, 27].

Antioxidant tests revealed that combinations of *T. cacao* extracts presented a good total antioxidant capacity and the studied extracts scavenged the DPPHº ABTSº radical in a dose dependent manner with an accent on CMBH . The inhibitory effect of these extracts may be attributed to the presence of significant elevated levels of phenolic compounds found in *T. cacao* extracts. In fact, phenolic compounds can complex metals and seize the free radical chain of oxidation and form stable free radicals [28, 29].
A positive and significant correlation at $P<0.05$ between radical scavenging properties, FRAP, and polyphenol, flavonoid content has been found; which could explain the relation between the antioxidant activity of the extract combinations and their phenolic compound content. These results corroborate those of Nanfack[10] who obtained a positive correlation between the presence of polyphenols in *Zanthoxyllum hetzii* and their ability to reduce iron; also several other works report that any plant having high contents in phenolic groups, would have a good antioxidant activity [30, 31, 32, 33]. IC_{50} values of DPPH radical are between 4.232 and 4.072 µg/mL followed by that of ABTS° radical (between 12.341 and 13.156 µg/mL) with a best value affected to CMBH. This implies that the most potent combination is CMBH which may be useful for the treatment of the radical-related pathological damages such as SCA [34].
The protective effects of *T. cacao* extracts were investigated on some organ antioxidant enzymes namely, SOD, catalase and peroxidases and non-enzyme (GHS and MDA). The results obtained showed that the tested extracts protected efficiently these macromolecules and then the studied organs against induced oxidative stress. The extracts, although acting less than the standard (Quercetin) tend to protect our organs against oxidative stress, the combination of Mbalmayo + Bertoua extract having a better aptitude than that of Santchou + Obala. This activity could be attributed to the total phenol content found in that extract combination. These results are in agreement with those obtained previously [32], which showed

that the protective effect of ginger with respect to oxidative stress is positively associated with the phenol content.

High oxidative stress in sickle cell patients is initially due to sickling of red blood cells, which is a repeated process that can lead to multiple complications. The induction of sickling cells by 2% metabisulphite on sickle red blood cells shows an increase in sickle cell size over time and which vary from patient to patient. The sickling induction rate obtained varied between 28.2% and 70.27% after 2h30 min of induction with an average of 41.66%. This result is similar to that of Nguelewou [13], (2016) who obtained an induction rate of 41.5% after 24 h of induction with nail polish. this result is lower than those of Nanfack [10], whose induction rate value was 52.08% after 2 h of 2% sodium metabisulphite induction and greater than that of Yembeau [13], (2017) which obtained a value of 32.2% after 2 h of 2% sodium metabisulfite induction. The variation in the sickling percentage after induction could be explained by genetic variability related to patient polymorphism and environmental conditions [10]. Indeed, the variation of induced sickling depends on the individual and his initial sickling rate.

Combinations of extracts reduce the rate of sickling over the time. Overall, the better sickling reduction was after 24 h at 1000 µg/ml of extract, the best combination being CMBH with a 70.07% sickling reduction percentage. This action could be assign to phenolic compounds, more precisely the flavonoids contained in our extracts. According to Seck [35], flavonoids are involved in the inhibition of the deleterious effects of reactive oxygen species produced during sickle cell disease. These results corroborate those of Egunyomi [36] having obtained 63.4% inhibition with a mixture of 28 plants and Gbadamosi [8]having obtained 71.6% inhibition with a mixture of 05 plants containing *Theobroma cacao.*

With regard to the osmotic fragility test of erythrocytes, results are extract and saline solution concentration dependent. The decrease in the percentage of haemolysis observed is related to the presence of phenolic compounds in extracts of *T. cacao.* Indeed, according to Ngbolua [37], certain phenolic compounds would not only reduce the polymerization of HbS, but also stabilize the erythrocyte membrane. These results are in agreement with those obtained by Nanfack [10] on extracts of *Zanthoxylum heitzii*.

Compare to the previous works on individual cocoa source [12, 13], it appears that the combination of cocoa gives better properties as antioxidant capacity for the red blood cell protection as well as the reduction of cell sickling.

***Table 1**: Total polyphenol and flavonoid content of different combinations of T. cacao extracts.*

Extracts	**Flavonoids content** (mg Eq of caffeic acid/g of extract)	**Polyphenol content** (mg Eq of Quercetin/g of extract)
CSOH	3,8 ± 0,1	274,6 ± 5,1
CMBH	4 ± 0,2[a]	289 ± 2[a]

Independent T-test.The values affected with letters are significantly different at p<0.05. CSOH = Combination Santchou + Obala, CMBH = Combination Mbalmayo + bertoua

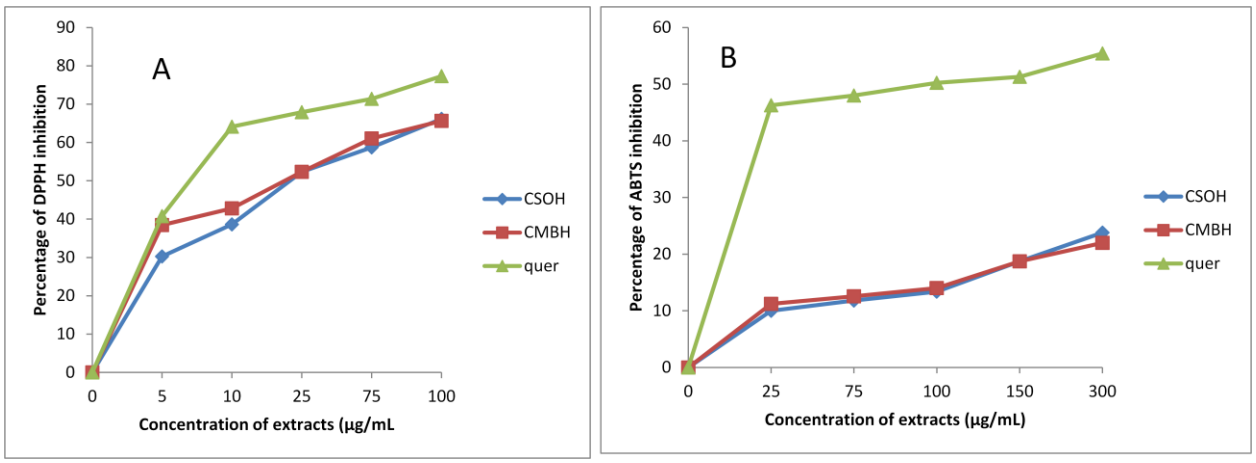

Figure 1: *2,2-diphenyl-1-picrylhydrazil (DPPH°) (A) and 2,2'-azino-bis-[3-ethylbenzo-thiazolone-6-sulfonic acid] (ABTS) (B) scavenging activity of different extract combinations.*
kruskal-Wallis test was used, followed by a Dunnet post-hoc. CSOH = Combination Santchou + Obala, CMBH = Combination Mbalmayo + Bertoua, quer = quercetin.

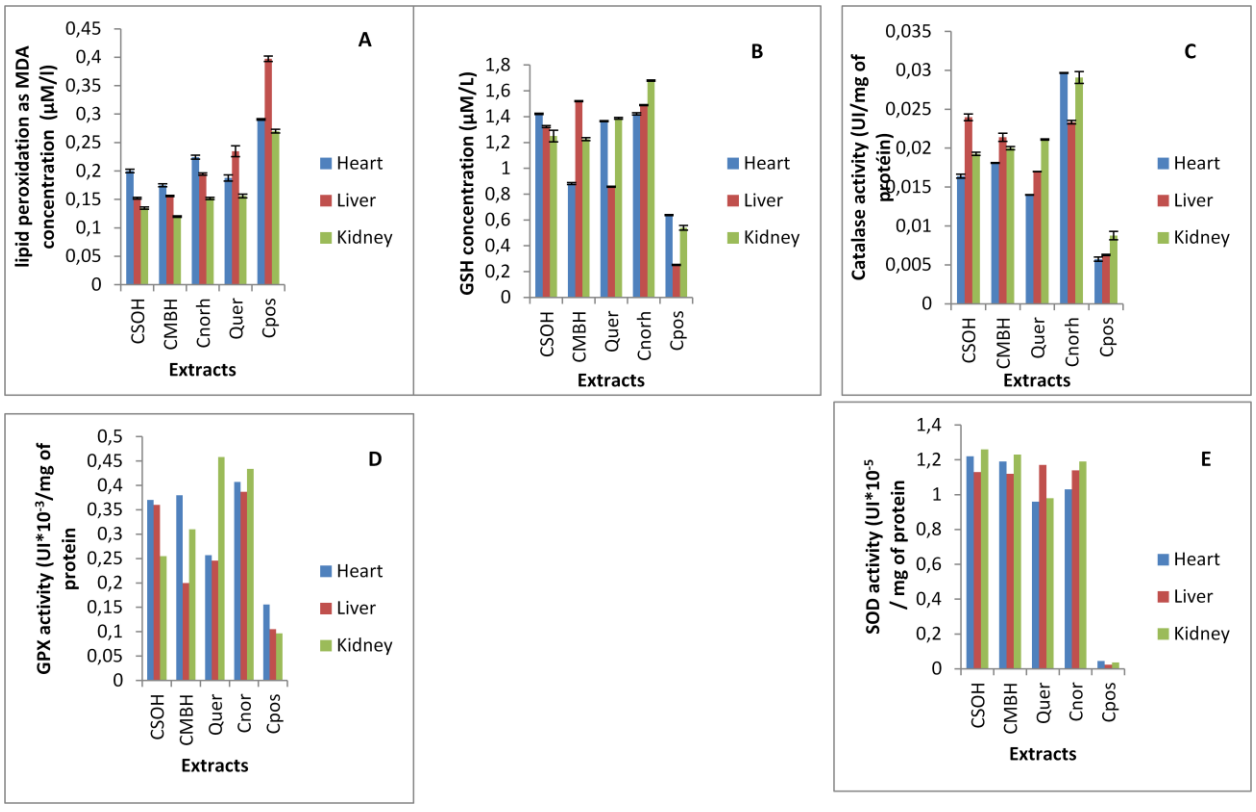

Figure 2*: Enzymatic and non enzymatic antioxidant potential as well as the lipid peroxidation status as the protective effect of T. cacao extracts combinations on red blood cell membrane*

kruskal-Wallis test was used, followed by a Dunnet post-hoc . CSOH = Combination Santchou + Obala. CMBH = Combination Mbalmayo + bertoua. C.pos = Positive control (under stress without treatment). C.norh = Normal control (no stress, no treatment). Quer = quercetin (Standard). MDA= malonedialdehyde. SOD= superoxide dismustase. GPx= gluthation peroxidase. GSH= reduced gluthation

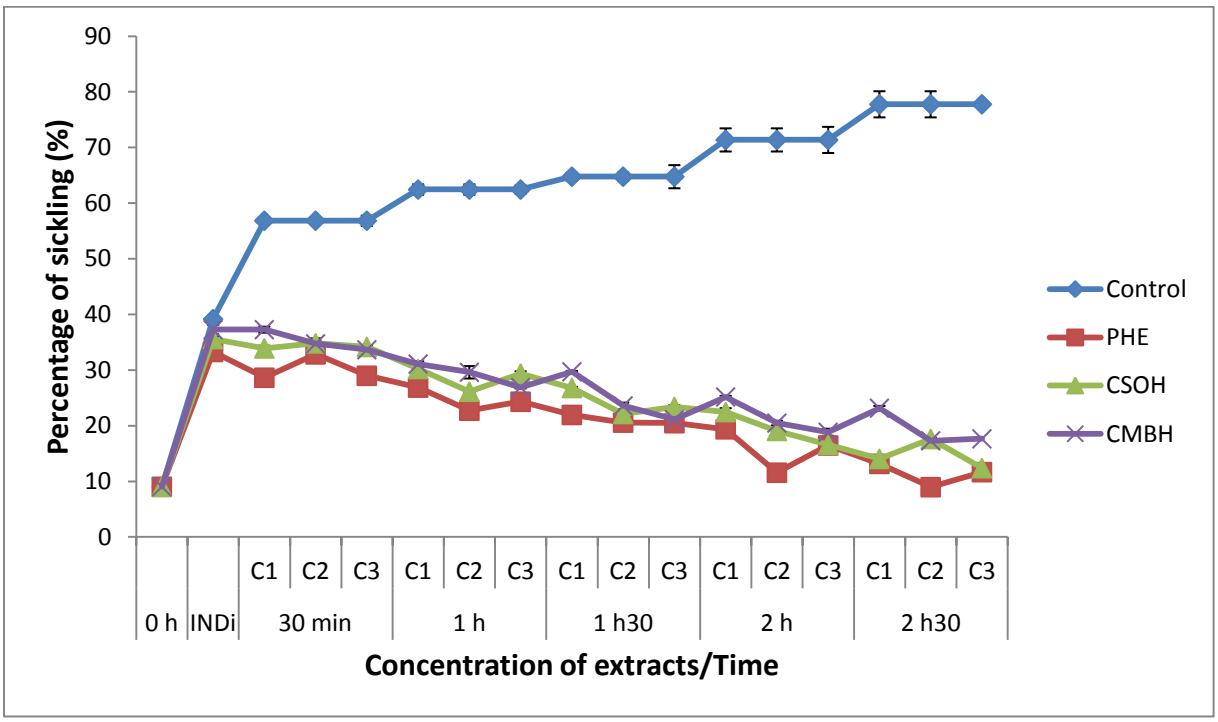

***Figure 3**: Sickling percentage as a function of the different combinations of extracts and concentrations as well as time*

Mixed Linear Effect Model helped studying interactions between factors (extracts, concentrations and repeated times) after data restructure. Kruskal-wallis and Dunnett when fixing factors. Control = Positive Control (Blood without any treatment), PHE = Phenylalanine, CSOH = Combination santchou + obala, CMBH = Combination mbalmayo + bertoua, INDi = initial induction rate, C1 = concentration 500µg / ml, C2 = concentration 1000 µg / ml, C3 = concentration 1500 µg / ml.

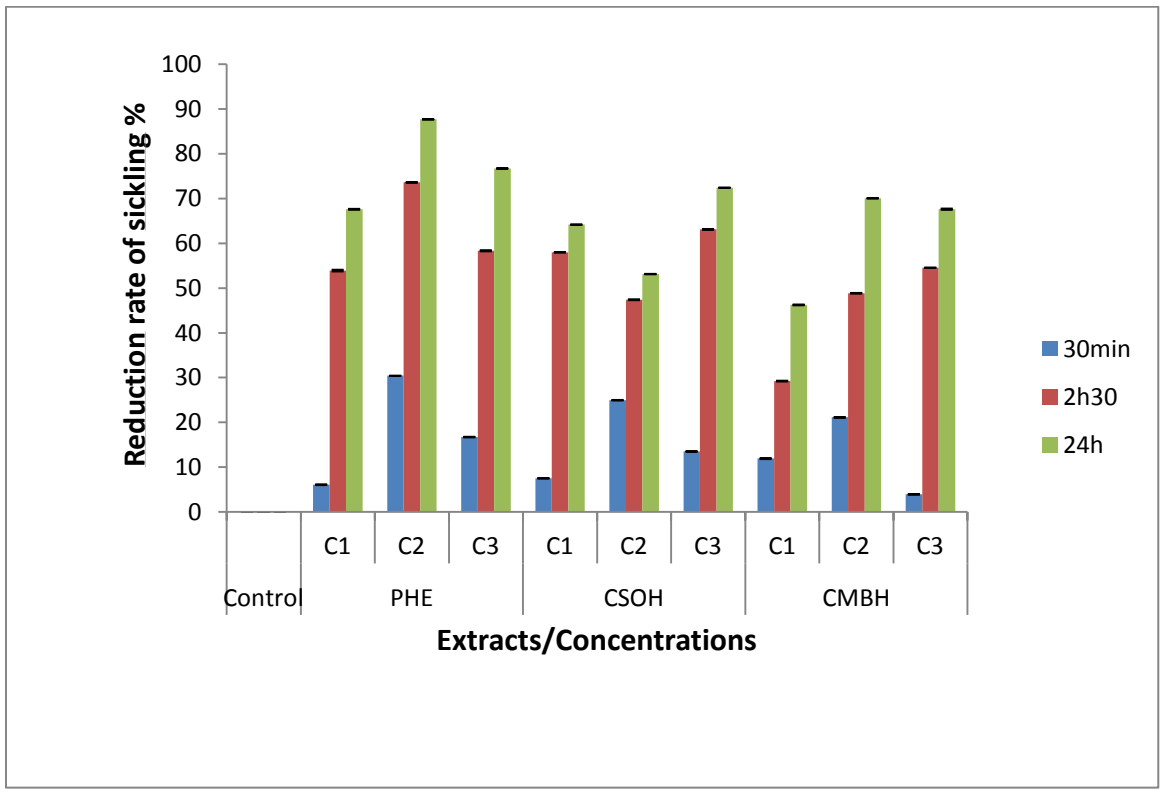

Figure 4: *Sickling Reduction rate of with combined cocoa extracts at different concentrations and time*

Mixed Linear Effect Model helped studying interactions between factors (extracts, concentrations and repeated times) after data restructure. Kruskal-wallis and Dunnett when fixing factors. Control = Positive Control (Untreated Blood), PHE = phenylalanine (Standard), CSOH = Combination Santchou + Obala, CMBH = Combination Mbalmayo + Bertoua, C1 = concentration 500µg / ml, C2 = concentration 1000µg / ml, C3 = concentration 1500µg / ml

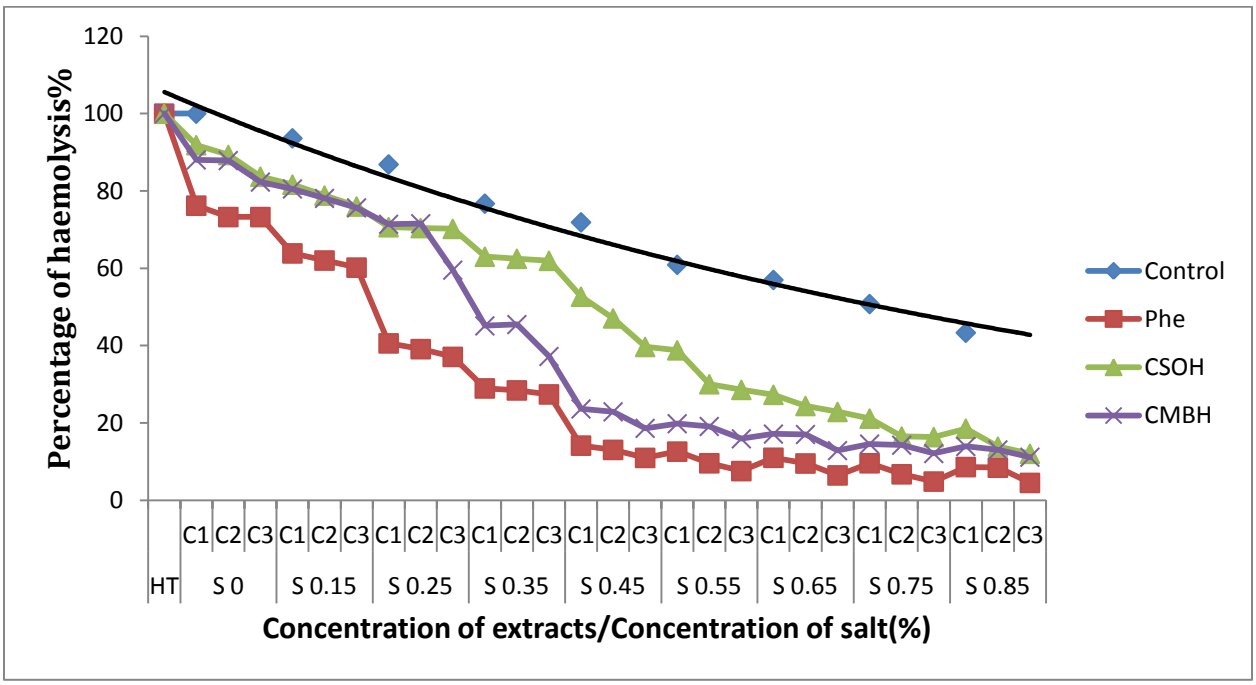

Figure 5*: Percentage of haemolysis at different saline solution and cacao combinations concentration*

Mixed Linear Effect Model helped studying interactions between factors (extracts, concentrations and repeated times) after data restructure. Kruskal-wallis and Dunnett when fixing factors. Control = Positive Control (Untreated Blood), Phe = phenylalanine (Standard), CSOH = Combination Santchou + Obala, CMBH = Combination Mbalmayo + Bertoua, C1 = concentration 500µg / ml, C2 = concentration 1000µg / ml, C3 = concentration 1500µg / ml, HT = Total haemolysis.

V. CONCLUSION

The combinations of *T. Cocoa* extracts from Mbalmayo + Bertoua and Santchou + Obala exhibits antioxidants, liver protection and antisickling activities, with an accent on the Mbalmayo + Bertoua combination. In order to complete this, further studies need to be done such as *in vivo* study of the antioxidant and antisickling properties as well as the toxicity evaluation of the best combinations of extracts of beans *T. cocoa*, for the better management of sickle cell disease.

DECLARATIONS

Ethics approval and consent to participate

All the research procedures have received the approval of Research Ethics for Human Health at the center (CRERSH/Ce) under the reference number 00255 / CRERSHC / 2017. A written informed consent was read and signed by all the patients participating in the study.

Consent for publication

Not applicable

Availability of data and material: Data and material are available

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Abbreviations: *T. cocoa: Theobroma cacao,* DPPH: 2,2-diphenyl-1-picrylhydrazil, ROS: reactive oxygen species, RBC: Red blood cell, ABTS: 2,2'-azino-bis- [3-ethylbenzo-thiazolone-6-sulfonic acid], FRAP: Ferric Reduction Antioxidant Power, Hb: Haemoglobin, MDA: Malonedialdehyde, SCA: Sickle Cell Anaemia, GPX: glutathione peroxidase, GSH: reduced glutathione, SOD: Superoxide dismutase, CMBH (extract Combination *of Mbalmayo* + *Bertoua*), CSOH (extract Combination of *Santchou* + *Obala*), MEAA: mg equivalent of ascorbic acid, Hydroxyurea (HU).

Author's contributions

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

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