

In Vitro Antisickling Properties, Free Radicals Quenching Potential, Protective Properties against Oxidative Mediated Ions Toxicity of Combinations of *Theobroma Cocoa* Beans (Sterculaceae)

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ABSTRACT

Background: Sickle cell anaemia induces an abnormal haemoglobin synthesis (Hb S). Hb S undergoes polymerization and causes the red blood cell (RBC) sickling with the production of reactive oxygen species (ROS). The present work aimed to determine the antioxidant and antisickling activities of the combination of *Theobroma cacao* (*T. cacao*) extracts from different regions in Cameroon.

Methods: The extract combinations (Santchou and Obala (CSOH), Bertoua and Mbalmayo (CMBH)) were prepared using water/ethanol (30v/70v; pH=3). Ferric-Reducing Antioxidant Power (FRAP), 2,2-diphenyl-1-picrylhydrazil (DPPH^o) and 2,2'-azino-bis-[3-ethylbenzothiazolone-6-sulfonic acid] (ABTS^o) scavenging properties of extracts were determined for the antioxidant capacity. The liver homogenate protective activity of extracts was estimated by measuring Superoxide dismutase (SOD), glutathione peroxidase (GPX), Malondialdehyde (MDA) and reduced glutathione (GSH). Osmotic fragility reduction and antisickling properties of the combination were also investigated.

Results: CSOH and CMBH respectively revealed a high content of polyphenols (274.6±5.1 and 289±2 mg Eq of Quercetin/g of extract), flavonoids (3.8±0.1 and 4±0.2 mg Eq of caffeic acid/g of extract) and FRAP. Extracts scavenged DPPH^o and ABTS^o radicals, respectively with IC₅₀ values between 4.07 µg/mL and 13.15 µg/mL. CMBH showed the best antisickling reduction activity (54.56% and 70.07% after 2h30min and 24h, respectively). The haemolysis percentage of erythrocytes decreased with an increase in the extract concentrations. Extracts combinations decreased and increased the MDA and GSH levels, respectively as well as normalizing the enzymatic activities.

Conclusion: Cocoa extracts combinations, especially CMBH, are antioxidant and antisickling.

Keywords: Sickle cell anaemia, oxidative stress, Cocoa extract combination

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Abbreviations: *T. cocoa*: *Theobroma cocoa*, 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).

INTRODUCTION

Sickle cell anaemia (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, half of whom die 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 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 it 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, 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 antisickling activity in relation to their antioxidant activity. Among them was *Zanthoxylum heitzii*, which has

antisickling properties and allows a better membrane stability of erythrocytes [10]. *Cajanuscajan* and *Adansonia digitata* have antisickling 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 antisickling activities [12, 13]. However, studies on the cocoa extract mixture from those regions are inexistent and 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 antisickling properties of the combinations of beans extracts of *Theobroma cacao* from four regions of Cameroon.

METHODS

Plant collection and authentication

Theobroma cacao beans were harvested in four towns in Cameroon: Santchou, Obala, Mbalmayo, and Bertoua in December 2016 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. Once dried, they were sorted, pulped

and crushed in a blender to obtain the cocoa powder. The obtained powder was directly extracted.

Preparation of plant extracts and combination

The modified method of Benhammou [14] was used for the extraction.

195 g of the powder of cocoa beans was 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^o4 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 was prepared by mixing the same proportion 1:1 for two extracts from different regions. A series of concentration of extract was prepared (500, 1000 and 1500 µg / mL) using distilled water.

Blood sample collection

The blood samples used in this study were obtained from homozygote SS patients in the age group of 10 to 28 years from 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.

Determination of antioxidant activity of combinations of T. cacao extracts

Determination of antioxidant potential of the extract combinations 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 concentration 1mg/mL was added to 1000 µL of FRAP reagent. The mixture was homogenized and incubated for 12 min, and then the absorbance was read at 539 nm. Ascorbic acid with the concentration of 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) by using the regression equation of the calibration curve [15].

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[•]) radical. To obtain 0.3 mL of the extract of a series of concentrations (0, 10, 15, 25, 75 and 100 µg / mL), 0.3 mL of the DPPH solution (0.1 mM) was added. The control consists of 0.3 mL of the DPPH solution and 3 mL of the 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:

$$\% \text{ Inhibition} = [(\text{DO}_{\text{control}} - \text{DO}_{\text{test}}) / \text{DO}_{\text{control}}] \times 100$$

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 oxidant 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 obtain 40 µL of extracts at 6 different concentrations (0, 25, 75, 100, 150 and 300 µg/mL), 2000 µL of the ABTS^{•+} solution was added. 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:

$$\% \text{ Inhibition} = \left[\frac{(\text{DO}_{\text{control}} - \text{DO}_{\text{test}})}{\text{DO}_{\text{control}}} \right] \times 100$$

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

Determination of antisickling activity of combinations of bean extracts of *Theobroma cacao* In Vitro Evaluation of Inhibitory Activity of Combinations of *Theobroma cacao* Bean Extracts on Sickle Cells

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

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 computing cells covered by a cover slip and observed under light microscope to determine the percentage of sickle cell.

Study of the antisickling activity of the extract

To obtain 50 µL of washed SS blood previously diluted with physiological saline (1:250 blood/ physiological saline), 50 µL of each extract at different concentrations (500, 1000 and 1500 µg / mL) and 50 µL of sodium metabisulfite 2% were added. 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 with 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 series of concentration of extracts (500, 1000, 2000 µg / mL) was diluted in NaCl (0.85%). In 800 µL of different NaCl concentrations, 200 µL of extracts and 10 µL of blood were added, respectively. After 24 hours of incubation, the supernatant from the mixture was read at 540 nm against the blank made up of NaCl [18] and the hemolysis was expressed as follows:

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

Evaluation of the protective properties of the plant against oxidative stress

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 and then homogenized in the same phosphate buffer. The obtained organ homogenate of 10% (w / v) 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 was added to 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:

$$C = \left[\frac{\text{DO test}}{\text{DO standard}} \right] \times n$$

(standard concentration)

Preparation of the pro-oxidative solution

The prepared pro-oxidative solution consisted of mixing in an equimolar volume with two solutions of R1 and R2. R1 contained 0.14 M iron trichloride (FeCl₃) in hydrogen chloride (1N) and 0.16 M nitriloacetic disodium, while R2 contained 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, and 200 µL of liver, heart or kidney homogenate as well as 20 µL of oxidizing solution was introduced into different tubes and the obtained mixture 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 with the extraction, solvent and quercetin. These test solutions and control were used to determine enzymatic and non-enzymatic parameters such as *malondialdehyde* [20], *reduced glutathione* [21], *catalase activity* [22], *glutathione peroxidase activity* (kit method: CAS Number 7722-84-1, Sigma Aldrich) and *superoxide dismutase (SOD) activity* [23].

Determination of the total polyphenols and flavonoids content

The total phenol content was assessed using Folin-Ciocalteu colorimetric method. Briefly, to obtain 0.1 mL of the plant extract with the concentration of 1 mg/mL, 0.4 mL of the folin-Ciocalteu reagent ten times diluted and 1 mL of sodium carbonate Na₂CO₃ (7.5%) were added. 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

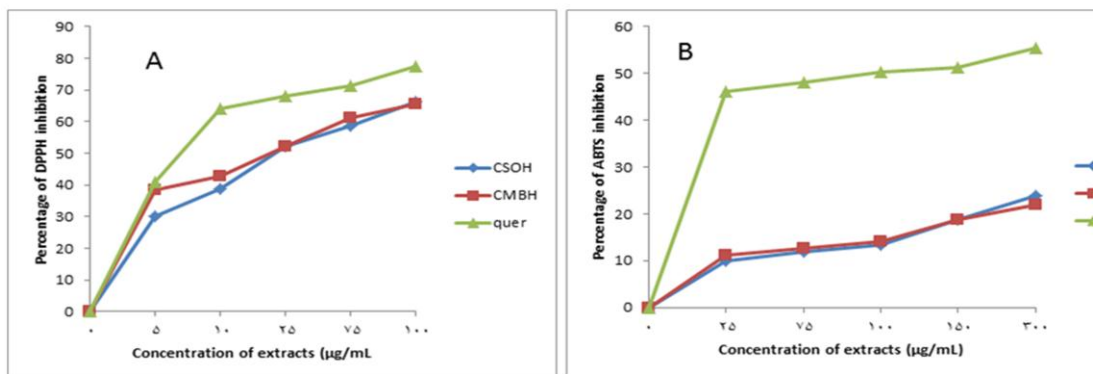


Figure 1: DPPH° (A) and ABTS (B) scavenging activity of different extract combinations

kruskal-Wallis test was used, followed by a *Dunnett* post-hoc. CSOH = *Combination Santchou* + *C*
 CMBH = *Combination Mbalmayo* + *Bertoua*, quer = *quercetin*.

polyphenol content was determined from the standard curve and expressed in mg equivalent of caffeic acid / g of the extract (ECA / g E).

For the total flavonoids determination, the Zhishen Method [25] was used.

To obtain 500 µL of the extracts (100 µg/mL), 300 µL of distilled water and 30 µL of sodium nitrite (5%) were added. After incubating for 5 min at room temperature, 30 µL of aluminum trichloride (10%) was 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 equivalence / g of the 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 be able 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 SPSS software version 16 for Windows 7 was used for statistical analysis.

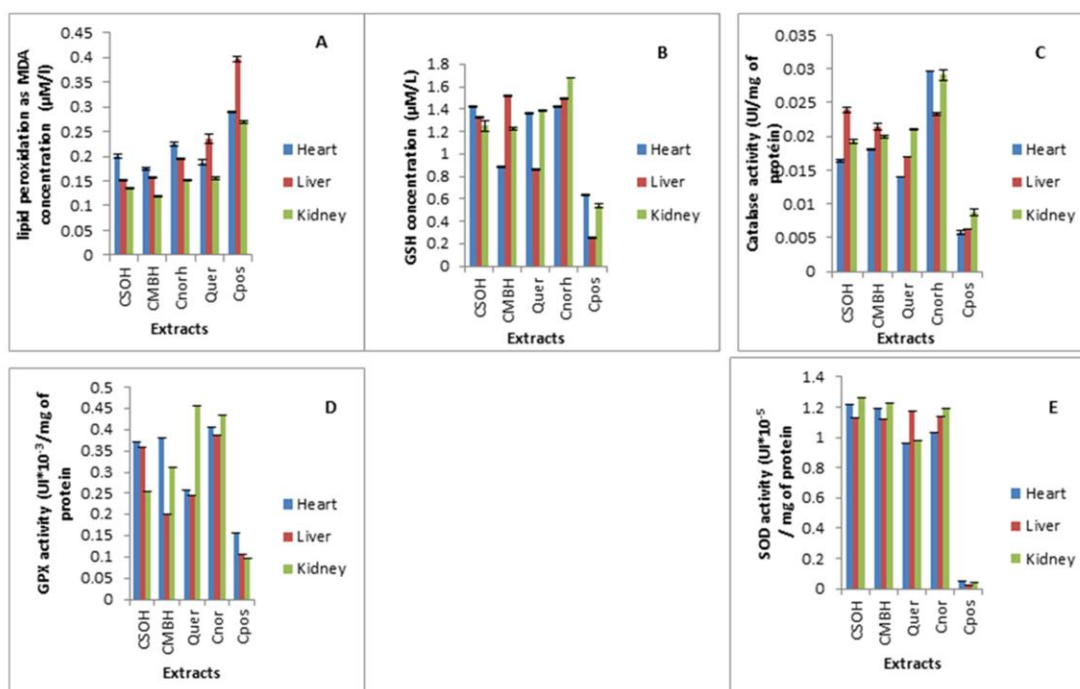


Figure 2: Enzymatic and non enzymatic antioxidant potential as well as the lipid peroxidation status as the protective effect of *T. cacao* extracts combinations on red blood cell membrane *kruskal-Wallis* test was used, followed by a *Dunnet* post-hoc. CSOH = Combination Santchou + Obala. CMBH = Combination 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 dismutase. GPx= glutathion peroxidase. GSH= reduced glutathion

RESULTS

Antioxidant activity of the *T. Cacao* extracts combinations

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 the CSOH (*Combination Santchou + Obala*), which is 119.83 MEAA/g of the extract.

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 the extract and is the

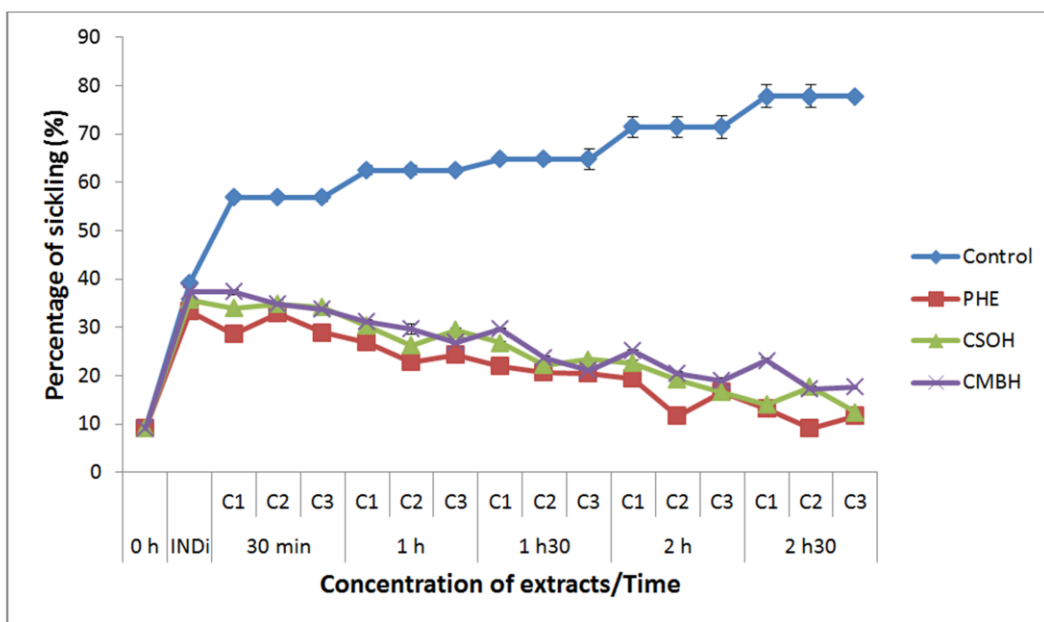


Figure 3: Sickling percentage as a function of the different combinations of extracts and concentrations as well as time

highest with quercetin followed by CMBH and CSOH extracts (figures 1 A and B). In general, a significant difference ($P < 0.05$) exists between quercetin and extracts at each extract concentration. There also appears to be a slight increase of inhibition when considering CMBH. The values of IC_{50} of the CSOH and CMBH combinations with $DPPH^{\circ}$ are respectively $4.232 \mu\text{g/mL}$ and $4.072 \mu\text{g/mL}$, while those of ABTS are respectively $12.341 \mu\text{g/mL}$ and $13.156 \mu\text{g/mL}$.

Protective properties of *T. cacao* 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) increases significantly ($P < 0.05$) MDA concentration in heart, liver, and kidney, respectively (0.27 ± 0.00 , 0.29 ± 0.00 and $0.39 \pm 0.00 \mu\text{mol/L}$) compared to the normal control (0.22 ± 0.00 , 0.19 ± 0.00 and $0.15 \pm 0.00 \mu\text{mol/L}$). These concentrations decreased significantly ($P < 0.05$) after administration of

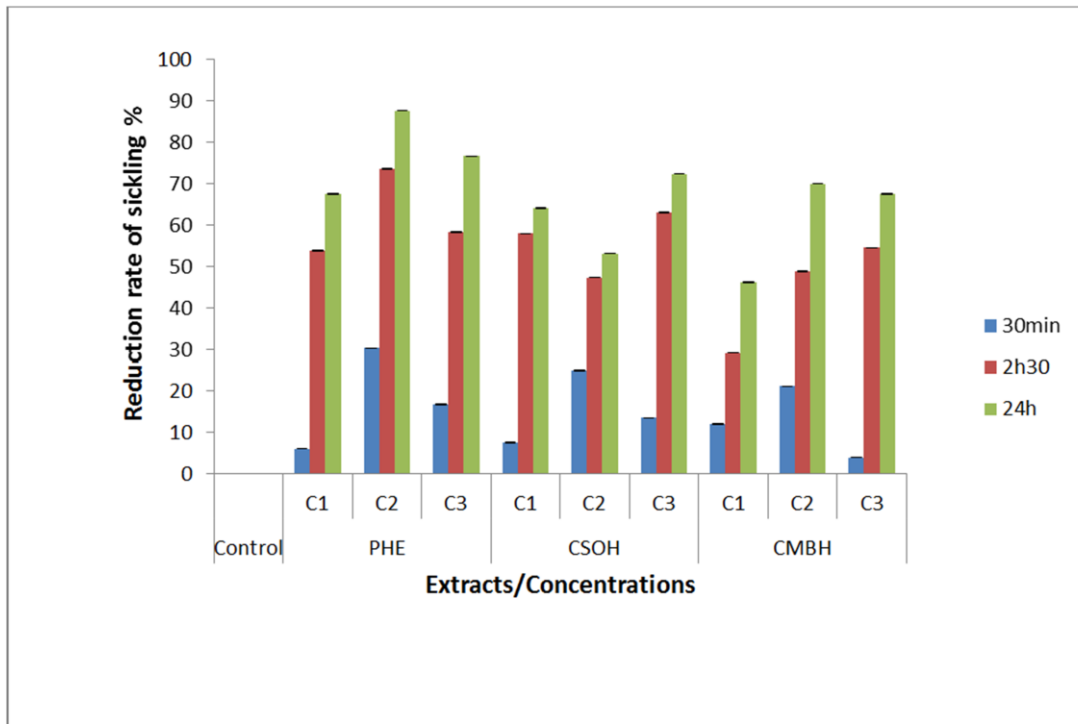


Figure 4: Sickling Reduction rate of with combined cocoa extracts at different concentrations and time

of *T. Cacao* extracts combinations 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 time interval of 30 min to 2h30 min.

which it is 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.

Mixed Linear Effect Model helped studying interactions between factors (extracts, concentrations and repeated times) after data restructure. *Kruskal-wallis* and *Dunnnett* 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.

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, with phenylalanine having the best activity followed by the combination of Mbalmayo + Bertoua at the concentration of 1000 µg/mL.

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

Osmotic fragility test of erythrocytes

The percentage of haemolysis is dependent on the salt concentration and different concentrations of extracts (figure 5). This percentage decreased with the increase of concentration of the extracts (500, 1000 and 1500 µg/mL), and also the increase of salt concentration for the different blood samples was tested. In addition, when fixing the extract concentration, each extract influenced in decreasing significantly the haemolysis percentage ($P < 0.05$) with salt concentration on which it was 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.

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

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

= concentration 500µg / ml, C2 = concentration 1000µg / ml, C3 = concentration 1500µg / ml, HT = Total hemolysis.

Total content of polyphenols and flavonoids

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

DISCUSSION

Determination of total content of polyphenols and flavonoids showed that combinations of *T. cacao* are rich in these compounds. Mbalmayo + Bertoua Combination possessed more of these compounds than Santchou + Obala. The variability of polyphenol

content is due to the phenolic composition of the extracts depending on such conditions 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 DPPH° ABTS° 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 and 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 *Zanthoxylum hetzii* and their ability to reduce iron. Besides, several other works report that any plant having high content in phenolic groups would have a good antioxidant activity [30, 31, 32, 33]. IC₅₀ 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)

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 as well as non-enzymes (GHS and MDA). The obtained results 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

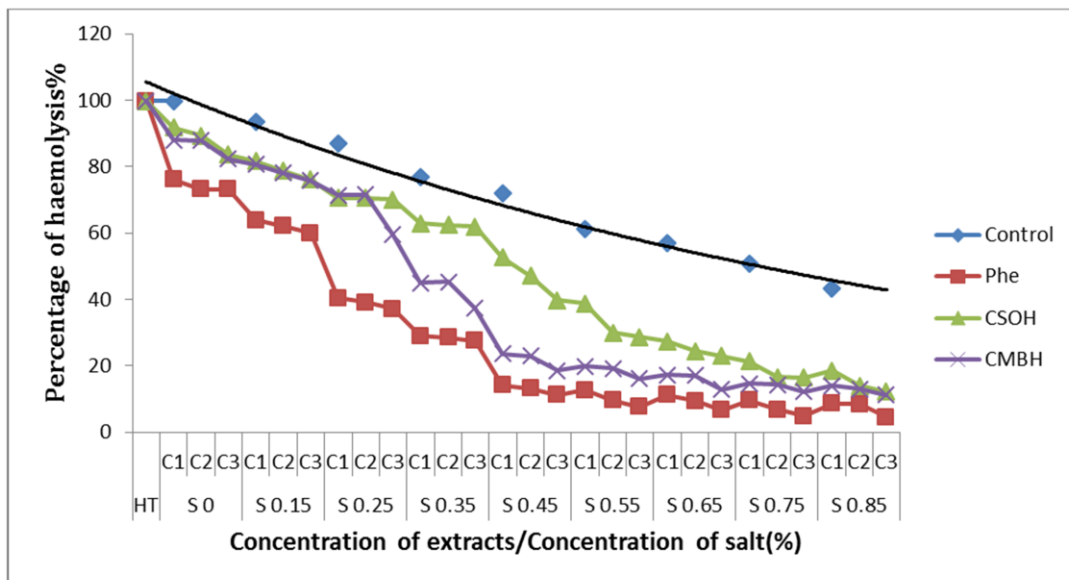


Figure 5: Percentage of haemolysis at different saline solution and *cacao* combinations concentration

with the best value affected to CMBH.

our organs against oxidative stress, with

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 may vary from patient to patient. The obtained sickling induction rate 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 (2016) [13], 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 (2017)

[13], obtaining a value of 32.2% after 2 h of 2% sodium metabisulphite 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 or her 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 better combination as CMBH was obtained with a 70.07% sickling reduction percentage. This action could be assigned 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] and Gbadamosi [8], which obtained 63.4% and 71.6% inhibition with a mixture of 28 and 05 plants containing *Theobroma cacao*, respectively.

With regard to the osmotic fragility test of erythrocytes, results are dependent on

extract and saline solution concentration. The decrease in the observed percentage of haemolysis 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*.

Compared with 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.

CONCLUSIONS

The combinations of *T. cacao* extracts from Mbalmayo + Bertoua and Santchou + Obala exhibit antioxidant, liver protection and antisickling activities, with an accent on the Mbalmayo + Bertoua combination. In order to complete this study, 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. cacao*, for the better management of sickle cell disease.

CONFLICT OF INTEREST

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.

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