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In vitro organo-protective and red cells membrane stability effect of *Theobroma cacao* (sterculiaceae) extracts and its derivative

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Abstract: Sickle cell anaemia induced free radicals formation which destabilize red blood cell membrane. This study aims to investigate the protective potential of *Theobroma cacao* bean extracts against ion toxicity in some organs as well as on the red blood cell membrane stability. Cocoa beans from *Obala*, *Santchou* and dark chocolate (EON, ESN, CN) were extracted in a mixture of water/ethanol. The protective activity of extracts against stress in rat liver, kidney and brain was estimated by measuring antioxidant enzymes (superoxide dismustase (SOD), catalase (CAT)), malondialdehyde (MDA) and reduced glutathione (GSH). RBC membrane fragility of Sickle cell patients and correlations between protective parameters were also performed. Extracts increased antioxidant capacity, regulated MDA by lowering its concentration in different organs and protect the erythrocyte membrane from haemolysis. Moreover, significant correlations exist between protective parameters. Cocoa bean extracts especially EON could help in protecting organs and stabilizing RBC.

Key words: *organs, red blood cells, Free radical, oxidative stress, antioxidants, Theobroma cacao*

I. Introduction

Excessive free radicals are generated in body due to unbalanced oxidants and antioxidants ratio which results into oxidative stress. Free radicals could be a consequence of some fundamental life functions such as enzymatic catalyzed reactions rely on metal ions [3]. Metal ions are indispensable for cells metabolism in trace; however, in a higher quantity, they could be toxic for human. Chronic exposure to toxic metals leads to their accumulation in the living system and becomes a health threat. For example ion toxicity have been implicated in multiple pathologies, Alzheimer's disease, Parkinson's disease, and cancer A common mechanism of toxicity involves redox-active metals via the generation of ROS through a Fenton reaction [3]. Transition

metal ions are important in the production of radical species. The ability of these ions to move electrons is the basis for the formation and propagation of many of the most toxic radical reactions [4]. For example, superoxide anion is relatively nonreactive in aqueous solution, but in the presence of hydrogen peroxide and a transition metal such as iron, the extremely reactive hydroxyl radical may be generated. This pathway, known as the iron-catalyzed Haber-Weiss reaction called Fenton reaction has been extensively studied [4]. Liver is a major organ attacked by ROS [5]. Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. Moreover, Kupffer cells, hepatic stellate cells and endothelial cells are potentially more exposed or sensitive to oxidative stress-related molecules. Liver disease can also cause damage to extra-hepatic organs, such as brain impairment and kidney failure [6]. Sickle cell anaemia (SCA) is an inherited disease which produce Sickled hemoglobin (HbS) also called pro-oxidant machine. HbS can then create reactive oxygen species (ROS), altering antioxidants profile. Due to the adverse effects of HbS, Sickle cells are unstable and need more antioxidant molecules.

Molecules called antioxidants scavenge and control the formation of free radicals thereby preventing oxidative damage to cellular components arising as a consequence of chemical reactions involving reactive oxygen species (ROS) [1,2]. Humans have developed some complex antioxidant systems both enzymatic (glutathione peroxidase, catalase, and superoxide dismutase) and nonenzymatic (vitamins E and C, thiol antioxidants). These systems work in synergistic way to protect the red cells and organ systems against free radical damage by quenching free radicals and/or chelating redox metals at physiologically related levels [7, 8]. Antioxidant based drugs and formulations have appeared during last three decades [9]. Synthetic antioxidants like butylated hydroxytoluene (BHT), tertiary butylated hydroquinon and gallic acid esters have been suspected to cause or prompt negative health effects. Hence, some restrictions have been imposed on their application.

Previous and recent studies showed that some plants such as *Hypodaphnis Zenkeri***,** *Xylopia aethiopica, T. tetraptera,* and *Syzygium guineense* are rich in polyphenols content and may act as protectors against oxidative mediated ion toxicity [10,11,12,13,14], showing the close relationship between the antioxidant capacity and the polyphenols contents of plant extracts. For instance *Harungana madagascariensis* possesses a great antioxidant capacity in relation with its polyphenol content and are implicated in protecting the red blood cell membrane [15,16]. Moreover, The fruits of *Zanthophyllum heitzii* traditionally used as spice in Cameroon and for the management of sickle cell disorder have demonstrated the role of polyphenolic compounds as implicated in the antioxidant and anti-sickling activity [17].

T. cacao (Sterculiaceae) can be found in several countries around the world. In Cameroon it is mainly cultivated in 7 of the ten regions of the country including Centre, South, East, Littoral, Southwest, West and Northwest regions. Recent study demonstrated that, cocoa extracts have antisickling and antioxidant activities [18].

The phytochemical analysis of *T. cocoa* beans from 5 localities in different regions of Cameroon (Mbalmayo/Ebolowa, Santchou, Obala, Penja and Bertoua) demonstrated the presence of higher concentrations of many classes of polyphenols [19]. These compounds present in high quantity in cocoa beans have beneficial effects against oxidative stress and diseases associated with oxidative stress such as cancer, by increasing the activities of antioxidant enzymes, the level of liver thiols, reducing the level of oxidative damage and increasing the resistance to hydrogen peroxide[20]. *T. cocoa* beans are used in different forms including cocoa powder, chocolate, capsules, tea and cocoa butter. Chocolate remains the main derivative product of cocoa[21]. Dark chocolate (CN) is loaded with nutrients that can positively affect health and is one of the best sources of antioxidants on the planet. Previous studies showed that because of the presence flavonoids content, CN increases the resistance of DNA faced with oxidative stress[22].

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The present study aims to investigate the protective potential of *T. cacao* bean extracts against ion toxicity in some organs as well as on the red blood cell membrane stability.

II. Materials and Methods

II.1. Plant materials and collection

Theobroma cacao beans (Sterculiaceae) were collected in the Centre (Obala) and West (Santchou) regions, two localities of Cameroon during August 2013. The sample has been identified under the reference number 60071/H.N.C at the National Herbarium of Yaoundé–Cameroon by Mr. NANA. A dark chocolate brand CHOCOCAM (Chocolatary and Confectionery of Cameroon) was bought from a supermarket in "Yaoundé" city on the 17 of October 2015. The derivatives of cocoa products were considered here as extracts that did undergo roasting.

II.2. Plant Extraction

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

II.3. Protective properties of the plant against oxidative damage

II.3.1. Animals

Male albino *Wistar* rats weighing 200 - 250 g were used in this study. The rats were maintained at room temperature under lab conditions and were fed with standard diet and water *ad libitum*. Livers were collected after dissection of the rats under mild ether anesthesia on overnight fasted rats. This study was carried out with approval from the animal Ethics Committee of university of Yaoundé I.

II.3.2. Preparation of liver homogenate

The organs were weighed and 10% (w/v) homogenate was prepared in phosphate buffer (0.1 M, pH 7.4 having 0.15 M KCl) using the homogenizer at 4 °C [23]. The homogenate was centrifuged at 3000 rpm for 15 min and the clear cell-free supernatant obtained was used for the study.

II.3.3. Preparation of the pro-oxidative solution

The oxidant solution was prepared, directly before its use by adding a solution of ferric chloride 100 mM to H_2O_2 0.50 % prepared in phosphate buffer (0.1 M, pH 7.4). This solution was used for the investigation of the protective assays on liver enzymes.

II.3.4. Total protein content

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The total protein content of the liver mixture was measured according to the protein kit supplier method (Human Kit-Hu102536, Boehringer Ingelheim, Germany). This result was used to express the activities of the different enzymes per gram of organs.

II.3.5. In vitro lipid peroxidation assay

Lipid peroxidation assay was performed by a formerly described protocol [24]. 0.58 mL of Phosphate buffer (0.1 M; PH 7.4), 200 µL of sample, 200 µL of liver homogenate and 20 µL of ferric chloride (100 mM) were combined to form a mixture ~~a~~ which was placed in a shaking water bath for 1 hour at 37° C. The reaction was completed by adding 1 mL of TCA (10 %) and 1 mL of TBA (0.67 %) to all the tubes and these were placed in a boiling water bath for 20 min. After this, the test tubes were shifted to crushed ice bath and were centrifuged at 3000 trs/rpm for 10 min. The absorbance of the supernatant was measured at 535 nm and evaluated as nM of MDA tissue using the molar extinction coefficient of 1.56×10^5 /M.cm.

II.3.6. Determination of peroxidase activity

Peroxidase activity was determined by the peroxidase kit method (CAS Number 7722-84-1, Sigma Aldrich) supplier with modifications. A solution containing the mixture of 1 mL of the oxidant solution ($FeCl_3$, 100 mM) and the extract or vitamin C (standard) for a final concentration of 100 µg/mL was incubated for 1 hr in a water bath at 37° C. An aliquot of PBS (0.1 mL), hydrogen peroxide (50 µL), and pyrogallol solution (110 µL) were added to 625 µL of distilled water that was earlier poured into an Eppendorf tube. 75 µL of the plant extract from the mixture was thereafter added. For the blank, the control oxidant solution and vitamin C used as the standard constituted the mixture. The same reagents were used except that, the extract was replaced by distilled water (75 µL). The composition was homogenized and incubated for at least 10 min. The solution containing 100 mM, pH 6.0 PBS (40 µL) and 0.002 % (v/v) of diluted liver homogenate (40 µL) was added to the blank and test mixtures respectively. These were homogenized and the increase in absorbance at 420 nm was measured after every 10 s for 3 min using a spectrophotometer (BioMate 3S UV-Visible, Thermo Scientific™ Manufacturer, Wohlen, Switzerland). One unit of peroxidase was defined as the change in absorbance/ seconds/mg of protein at 420 nm using molar extinction coefficient of 12 /M.cm.

II.3.7. Determination of catalase activity

Prior to the test, a solution containing a mixture of 1 mL total volume of the oxidant solution and extract or vitamin C (standard) for a final concentration of 100 µg/mL was incubated for 1h in a water bath at 37 °C. The catalase activity of the liver homogenate was assayed as previously described by [25] with modifications. An aliquot of hydrogen peroxide (0.8 mL) was introduced into an Eppendorf tube. Phosphate buffer (1.0 mL), extracted sample/Vitamin C/oxidant solution (75 µL) and (0.002 % v/v) diluted homogenate (125 µL) were added. 0.5 mL of the reaction mixture were introduced into1.0 mL of dichromate reagent (5 %) and the mixture shaken vigorously. The mixture was heated in a Clifton water bath for 10 min, and allowed to cool at room temperature. The absorbance at 570 nm was taken using the spectrophotometer (BioMate 3S UV-Visible, Thermo Scientific™ Manufacturer, Wohlen, Switzerland). The absorbance obtained was extrapolated from the following standard curve y = 0.0028x + 0.0132. The catalase activity was thereafter expressed as Unit/min/mg of protein (UI/mg Prot.)

$$CAT\left(\frac{unit}{mg}protein\right) = (Abs/min \times 30000\, units)/(40cm/M \times mgprotein) \times df$$

Where: df = dilution factor, Abs= absorbance

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II.2.8. Superoxide dismutase (SOD) activity

The measurement of total SOD activity was performed according to the method the of Misra and Fridovich with some slight modifications [26]. The principle of this method is based on the inhibition of epinephrine autoxidation. 0.2 mL distilled water and 2.5 mL of sodium carbonate buffer (00.05 M and pH 10.2) were added into 0.3 mL epinephrine buffer to initiate the reaction. The absorbance at 480 nm was read for 150 s at 30 s intervals against a blank made up of 2.5 mL buffer, 0.3 mL epinephrine and 0.2 mL distilled water. The following equation allowed the calculation of the SOD activity:

$$SOD(unit/mg\ protein) = SOD(units/mL/min)/protein(mg/mL\ ) \times df$$

where: df = dilution factor,
The SOD activity was there after expressed as Unit/min/mg of protein (UI/mg Prot.)

II.4. Erythrocyte membrane stability test

II.4.1. Blood collection and red blood cell washing

The ethical clearance approval issued by the Regional Committee for Ethics Research for Human Health Center (0282/CRERSHC/2016) was obtained. Prior to the blood collection, a written informed consent was read and signed by all the patients participating in the study. confirmed sickle cell patient blood samples between the ages of 16 and 40 who had been attending routine consultations in the Hematology Department of Central Hospital, *Yaoundé* have been collected**.** Plasma was isolate after the first centrifugation (5 min at 3000 rpm) of the whole blood. Then red blood cells was washed using 0.9% NaCl solution followed by a centrifugation (5 min at 3000 rpm). This process was repeated twice to completely liberate RBCs from impurities.

II.4.2. Osmotic fragility test

The osmotic fragility test was performed using previous method [27]. Nine solutions of a range of concentration (0% - 0.85%) of were prepared. The EFCM and EFCB extracts were prepared at concentrations of 500, 1000 and 2000 mg/mL. At 800 µL of NaCl of different concentrations, were added 200 µL of extracts and 10 µL of washed blood; the mixture was incubated at room temperature during for 30 min and then centrifuged at 2000 rpm for 15 min. The supernatant was collected and read at 540 nm against blank (containing NaCl solution at various concentrations instead of the extract). The supernatant was recovered using a micropipette and the optical density was read at 540 nm against blank. Haemolysis expressed as a percentage is calculated as follow:

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

II.4.3. Statistical analysis

The results were presented as mean ± SD of triplicate assays. The Mixed Linear Effect Model helped to study the interactions between factors (extracts and concentrations). The *Kruskal wallis* test and *Dunnett*'s multiple test (SPSS program version 18.0 for Windows, IBM

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Corporation, New York, NY, USA) was used, followed by a *Dunnet* post-hoc to analyze the osmotic fragility test of each plant extract. Spearman correlation between protective parameters was done using the software XLstat version 7 (Addinsoft, New York, NY, USA) and the best extract was revealed with the principal analysis component (PCA). The differences were considered as significant at $p < 0.05$.

III. Results

III.1. Properties of T. cacao extracts on Catalase activity.

Figure 1 shows the effects of *T. cacao* extracts on CAT activity. In general, the evolution profiles of CAT activity in relation with extracts and control groups effects are similar in each organ. The positive control group (oxidant), has a significant (p < 0.05) lower CAT activity (between 0.024 ± 0.0003 and 0.047 ± 0.001 UI / mg prot.) compared to the normal control (without any treatment) (between 0.16 ± 0.006 and 0.47 ± 0.005 UI/ mg prot.) in studied organs (brain, liver and kidney). Among the tested samples, a significant (p < 0.05) increase of the CAT activity was noted with quercetin and vitamin C respectively (between 0. 39 ± 0.002 and 0. 55 ± 0.003 UI / mg prot.; and between 0. 31 ± 0.0005 and 0. 54 ± 0.0001 UI / mg prot.) in studied organs. Moreover, in those organs, cacao extracts increased SOD activities, especially when using ESN (between 0.38 ± 0.002 to 0.67 ± 0.004 UI / mg prot.) followed by EON and CN respectively (between 0.23 ± 0.0003 to 0.58 ± 0.007 UI / mg prot.; and between 0.12 ± 0.001 to 0.19 ± 0.002 UI / mg prot.) in studied organs. Increasing of activities is most most noted in brain followed by liver and kidney.

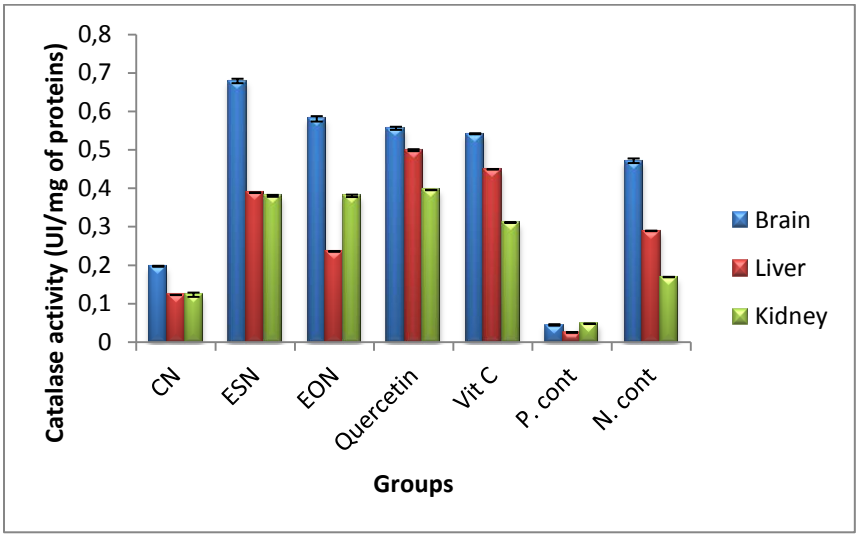

Figure 1: *Protective effect of Theobroma cacao on CAT activity. Kruskal-Wallis Statistical tests and the post-hoc Dunnet. Values are expressed as mean ± SD of three replicates. The values affected with different letter are significantly different at P < 00.05. CN = Dark Chocolate, ESN = Cocoa Extract from Santchou, EON = Theobroma Cacao Extract from Obala, Quercetin = standard, Vit C = Vitamin C (standard), P. cont= oxidant (positive) control), N. cont = Normal control.*

III.2. Properties of T. cacao extracts on SOD activity.

The effects of *T. cacao* extracts on SOD activity is presented in figure 2. The same activity profile as above is noted in this case. The positive control group (oxidant), has a significant (p < 0.05) lower activity in both three organs (between 1.78 ± 0.00 and 5.19 ± 0.00 UI*10^{-5} / mg prot.) compared to the normal control (without any treatment) (between 8.20 ± 0.00 and 20 ±

0.00 UI*10^{-5} / mg prot.). In different studied organs, increasing in SOD activities is both organ and extract dependent. In the liver, brain and kidney respectively, EON is the most SOD activity decreasing agent (2.03 ± 0.00 UI*10^{-4} / mg prot. in brain; 7.38 ± 0.00 UI*10^{-5} / mg prot. in liver and 10.05 ± 0.00 UI*10^{-4} in kidney), followed by ESN (1.52 ± 0.00 UI*10^{-4} / mg prot. in brain; 5.26 ± 0.00 UI*10^{-5} / mg prot. in liver and 9.53 ± 0.00 UI*10^{-5} in kidney). CN remains at the position with 5.55 ± 0.00 UI*10^{-5} / mg prot. in brain; 4.43 ± 0.00 UI*10^{-5} / mg prot. in liver and 3.91 ± 0.00 UI*10^{-5} in kidney.

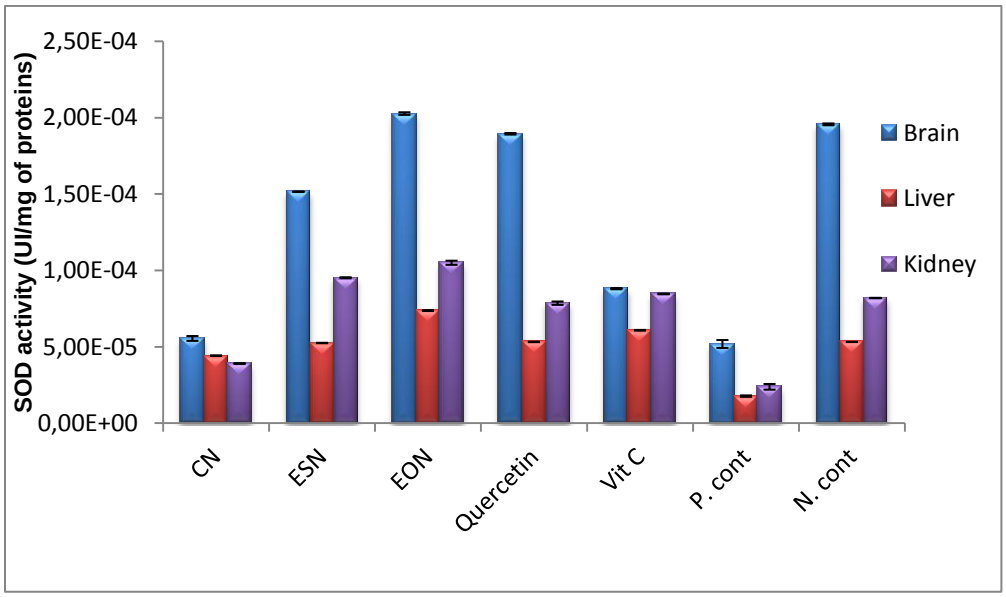

***Figure 2**: Protective properties of T. cacao on SOD activity. Kruskal-Wallis Statistical tests and the post-hoc Dunnet. Values are expressed as mean ± SD of three replicates. The values affected with different letter are significantly different at P < 00.05. others without any letters are not significantly different. CN = Dark Chocolate, ESN = Cocoa Extract from Santchou, EON = Theobroma Cacao Extract from Obala, Quercetin = standard, Vit C = Vitamin C (standard), P. cont= oxidant (positive) control,), N. cont = Normal control.*

III.3. Properties of T. cacao extracts on reduced glutathione.

Following the treatment with various extracts, results related to the reduced glutathione (GSH) level are presented in figure 3. the concentration of reduced glutathione varies according to each organs and extracts. In general extracts act in increasing the level of reduced glutathione compared to the P. (oxidant) control where GSH decreased although not significant. EON is the best extract which mainly increased GSH level in the liver, brain and kidney (9 ± 0.10; 1.71 ± 0.008 and 5.98 ± 0.04 µmol / l respectively).

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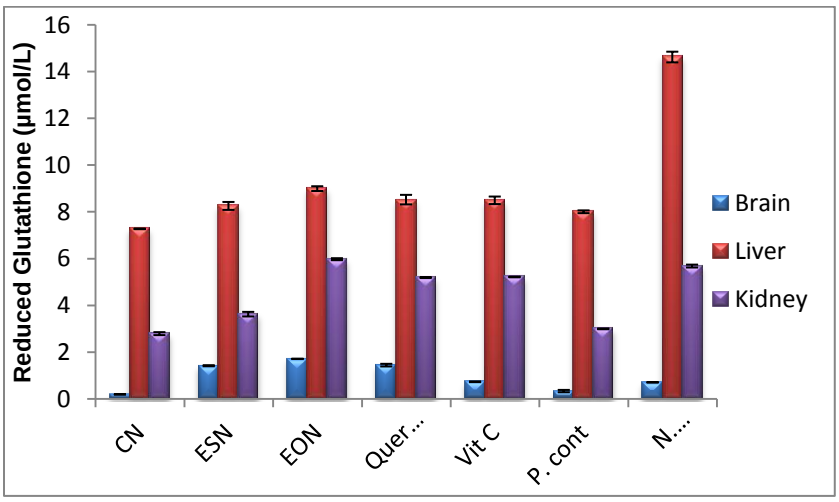

Figure 3: Effects of T. cacao extracts on GSH activity. *Kruskal-Wallis Statistical tests and the post-hoc Dunnet. Values are expressed as mean ± SD of three replicates. The values affected with different letter are significantly different at P < 00.05. others without any letters are not significantly different. CN = Dark Chocolate, ESN = Cocoa Extract from Santchou, EON = Theobroma Cacao Extract from Obala, Quercetin = standard, Vit C = Vitamin C (standard), P. cont= oxidant (positive) control,), N. cont = Normal control*

III.4. Properties of T. cacao extracts on lipid peroxidation.

Figure 4 reveals the variation of MDA concentration in liver, brain and kidney due to the effects of extracts. In the P. (oxidant) control group, MDA level significantly ($p < 0.05$) increased in brain, liver and kidney respectively (0.3 ± 0.00, 0.31 ± 0.00 and 0.38 ± 0.00 µmol*10^{-7}/L) compared to other groups. The lowest concentration of MDA in all studied organs is observed when using ESN in kidney and quercetin in liver (standard) (from 0.03 ± 0.01 to 0.07 ± 0.01 µmol*10^{-7} / L respectively). In the liver, among *T. cacao* extracts, EON mostly decreased the MDA level (0.08 ± 0.00 µmol*10^{-7}/L), followed by ESN (0.10 ± 0.001 µmol*10^{-7}/L) and CN (0.20 ± 0.002 µmol*10^{-7}/L).

In order to well understanding the relationship between protective parameters in different organs, following results have been obtained

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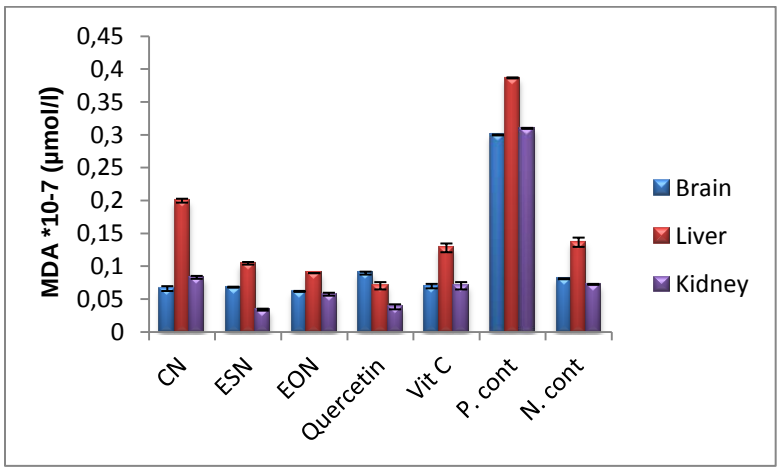

***Figure 4**: Protective properties of T. cacao extracts against lipid peroxidation. Kruskal-Wallis Statistical tests and the post-hoc Dunnet. Values are expressed as mean ± SD of three replicates. The values affected with different letter are significantly different at P < 00.05. others without any letters are not significantly different. CN = Dark Chocolate, ESN = Cocoa Extract from Santchou, EON = Theobroma Cacao Extract from Obala, Quercetin = standard, Vit C = Vitamin C (standard), P. cont= oxidant (positive) control,), N. cont = Normal control*

III.5. Correlation between protective parameters

Table 1 revealed that there are some positive and negative significant correlation between protective parameters in different studied organs with coefficient values between 0.7 and 1.
Out of 66.45% and 14.38% respectively on F1 and F2 axis, the contribution of each test (figure 6 a) is as followed: 10.28%, 9.17%, 9.14%, 9.96%. 1.76%, 8.95%, 10.88%, 9.27%, 10.69% for respectively CATr, MDAr, SODr, CATc, MDAc, SODf, GSHc, SODc and MDAf for F1 axis. F2 axis showed a contribution of 13.70% (MDAr), 17.47% (GSHr), 9.15% (CATf), 21.34% (GSHr).
According to the percentages of contributions of the extracts, Based on the contribution of the extracts on the axes F1 and F2, it appears from figure 5 (b) that the distribution of different test parameters is linked to extracts. Then contribution percentages ranks EON as the best (16.90%) followed by ESN (13.65%), and dark chocolate (CN) (4%).

***Table 1:** Correlation matrix between protective parameters in different studied organs*

Variables	SODf	GSHf	MDAf	CATf	SODc	GSHc	MDAc	CATc	SODr	GSHr	MDAr	CATr
SODf	**1**											
GSHf	0.757	**1**										
MDAf	-0.685	-0.536	**1**									
CATf	0.523	0.429	-0.750	**1**								
SODc	**0.775**	**0.893**	-0.714	0.393	**1**							
GSHc	0.739	0.607	**-0.929**	0.571	0.750	**1**						
MDAc	-0.414	0.000	0.250	0.107	-0.357	-0.250	**1**					
CATc	0.577	0.393	**-0.857**	0.607	0.643	**0.857**	-0.464	**1**				
SODr	**0.775**	0.536	-0.643	0.393	0.714	0.750	-0.643	**0.857**	**1**			
GSHr	**0.901**	**0.929**	-0.536	0.357	**0.857**	0.679	-0.214	0.464	0.714	**1**		
MDAr	-0.487	-0.357	**0.893**	-0.750	-0.571	**-0.821**	0.286	**-0.964**	-0.714	-0.357	**1**	
CATr	0.618	0.487	**-0.991**	**0.793**	0.667	**0.901**	-0.216	**0.883**	0.631	0.468	**-0.937**	**1**

Bold: Significant values $p < 0.05$ (bilateral test). Spearman correlation. Values in bold are different (P<0.05)

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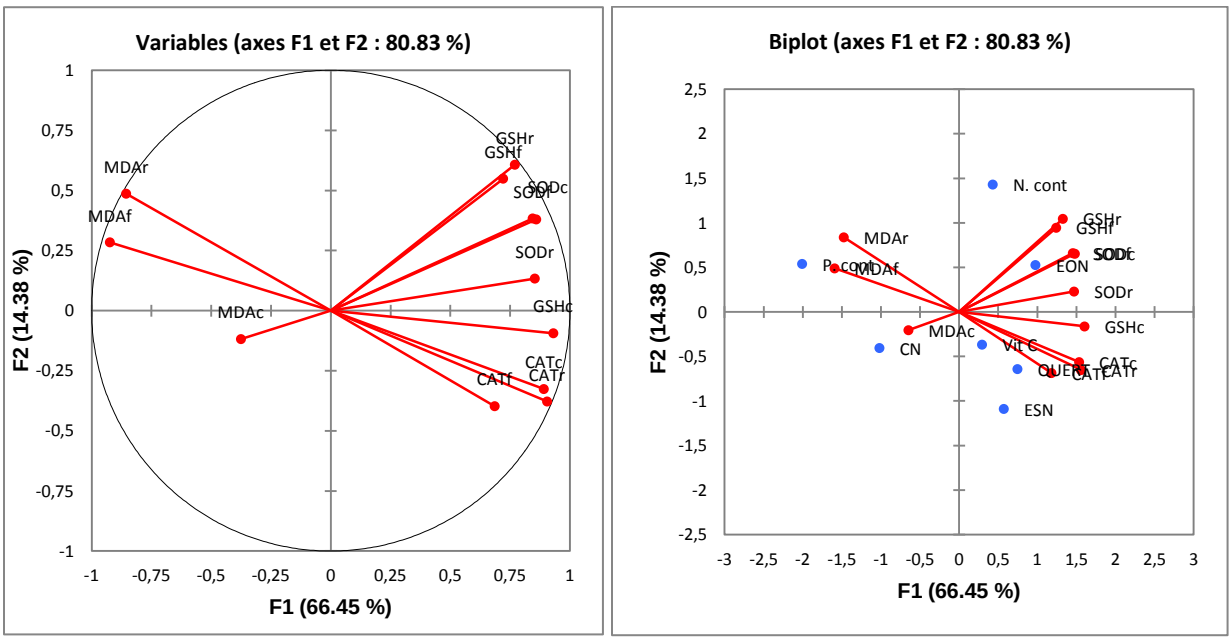

Figure 5: *Principal component analysis between antioxidant tests and extracts. CN = Dark Chocolate, ESN = Santchou Cocoa Extract, EON = Obala Cocoa Extract. MDAc: MDA from brain, MDAr: MDA from kidney, MDAf: MDA from liver. (a) distribution of tests around the F1 and F2 axes; (b) Pojection of the heads and extracts around the F1 and F2 axes.*

III.6. Erythrocyte membrane stability activity

The effect of extracts on the erythrocyte membrane stability through the osmotic fragility test of the membrane of RBC is showed in the figure 6. In general, the percentage of haemolysis varied depending on the type and concentration of extracts as well as the concentration of salts. All extracts and control decreased the haemolysis percentage with the increasing of saline solution concentrations. When fixing each extract concentration, all extracts decreased significantly ($P < 0.05$) the red blood cell haemolysis with salt concentration dependency from $S_{0.35}$ to $S_{0.85}$ compared to S_0. EON decreased considerably the red blod cell haemolysis compared to other extracts.

IV. Discussion

Metals cause deleterious effects on human health by producing various kinds of metabolic disorders and even chronic diseases. The toxicity of ions may be mediated by the induction of oxidative stress which causes damage to cellular components, particularly DNA, resulting in genomic instability [28].

In the present study, Lipid peroxidation (LPO) was induced with the iron complex of the chelating agent nitrilotriacetic acid (Fe3+- NTA). Results show that Fe3+- NTA (oxidant) led to a significant increase of lipid peroxidation associated with SOD, catalase, and glutathione peroxidase activity depletion in all tissues assayed (brain, kidney and liver) compared to the normal control. These results corroborate those of [29] which revealed that Fe3+-NTA was nephrotoxic and hepatotoxic. Indeed, Fe3+- NTA may act through the generation of free radicals with simultaneous decrease in antioxidant defenses. Hydroxyl radicals generated from Fenton reaction on deoxy-ribose produce malondialdehyde (MDA) and similar substances [30]. The biologic damage by hydroxyl radical is characterized by its capacity to stimulate lipid peroxidation (LPO), which occurs when OH radical is generated close to membranes. LPO is a

natural metabolic process under normal aerobic conditions and it is one of the most investigated consequences of ROS action on membrane structure and function. Polyunsaturated fatty acids (PUFA), the main components of membrane lipids, are susceptible to per-oxidation. Hydroxyl radicals and singlet oxygen can react with the methylene groups of PUFA forming conjugated dienes, lipid peroxy radicals (LOOP) and hydro-peroxides (LOOH) [31]. The inhibition of lipid peroxidation by a compound is a crucial property that makes it possible to reduce the induction and / or propagation of oxidative stress in relation with its polyphnols content [31]. This confirm the role of *T. cacao* extracts in protecting tissue membranes against oxidative stress. Moreover, this corroborate previous work on *T. cacao* showing its hability to reduce the red blood cell membrane damage and the hemoglobin sickling due to its polyphenols content and then its antioxidant properties [18]. The high activity of either EON or ESN compared to the dark chocolate (CN) could be linked to their high polyphenols content and then their antioxidant properties as observed previously [18].

Enzymatic and non-enzymatic systems are used by living organisms to fight free radicals produced during stress [32]. In this study, the enzymatic system consists of the activity of SOD and CAT while the non enzymatic system consists of GSH assay and lipid peroxidation (MDA assay). For enzymatic systems (SOD and CAT), results show that *T. cacao* extracts regulated enzymes activities by increasing SOD and CAT activities compared to positive (oxidant) control. The function of antioxidant enzymes such as CAT and SOD is to protect cells from toxic reactive oxygen species[33]. SOD is one of the important enzymes which scavenges the superoxide radicals by converting them to H2 O2 and molecular oxygen. CAT are involved in the elimination of H_2O_2. The pre-administration of the extract stabilizes the activities of CAT and SOD as compared to the normal control receiving. This result could reinforce the protective property of *T. cacao* extracts on tissue membranes and corroborates with previous works showing that pre-treatment of experimental animals with *Harungana madagascariensis* extract prevented changes in the CAT and SOD activity [16]. The protective effect of extracts against oxidative stress is therefore linked to their content in polyphenols. Furthermore, previous study showed that cocoa polyphenols increase the body's resistance to oxidative stress and thus protect it against diseases [34]. Moreover, it has been shown that *Sizygium guineense* var macrocarpum, *Hypodaphnis Zenkeri*, *Xylopia aethiopica, Tetrapleura tetraptera* exhibited significant antioxidant and protective effects in relation with their polyphenols content, in kidney, brain and liver homogenates [10,11,12,13].

For non enzymatic systems, GSH is used as a cofactor by multiple enzymes such as peroxidase, glutathione S- transferase enzymes. The present study demonstrated that reduced glutathione levels are increased when administrating different cocoa extracts and derived product in all the organ homogenates. This result furthermore confirms the aptitude of these extracts to protect vital organs from deleterious effects of ROS. Similar results have been found previously [10,11,12,13].

Dark chocolate (CN) is loaded with nutrients that can positively affect health. Made from the seed of the cocoa tree, it is one of the best sources of antioxidants on the planet. In this study, CN revealed protective activities against oxidative stress in brain kidney and liver. Nevertheless it activities remained the lowest compared to *T. cacao* beans extracts. This could be due to the low quantity of antioxidant compounds (polyphenols) in relation with different modifications of the crude cocoa before it obtention. After fermentation and drying, one step from the complete fractionation process in the industry is that the beans are usually roasting. the raw beans are exposed to temperatures between 130 and 150 °C for 15–45 min [35]. This process determines the final color, aroma and flavor of the cocoa products. The changes in the organoleptic characteristics of the beans are the result of oxidation and polymerization of polyphenols, degradation of proteins [36].

The significant correlation between protective parameters in different organs properties of the extracts and dark chocolate suggests the potential role of polyphenols molecules considered as

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antioxidant previously quantified in bean cocoa extracts and derived products [18, 19]. This rich content is responsible to the protective role of extracts. Some previous works also found strong correlations between antioxidant capacity and the protective property in relation with the polyphenols content of plant extracts [10,11,12,13].
Moreover, results showed the decrease in the percentage of red blood cell membrane haemolysis. This observation could be related to the protective property of *T. cacao* extracts on the erythrocyte membrane. Previous study has shown the presence of high polyphenol content and the antioxidant potential in these extracts[18]. This opportunity together with the present result showing the protective effect of these extract in rat liver, heart and kidney could actually explain the resistance and the stability of red blood cells against haemolysis. Since the antioxidant capacity in linked to the polyphenol content, the activity observed could be then attributed to the presence of this type of compound. furthermore, previous works done on *Carica papaya* and *Zanthophyllum heitzii* have showed a significant correlation between the red blood cell membrane and their antioxidant capacity [17, 36].

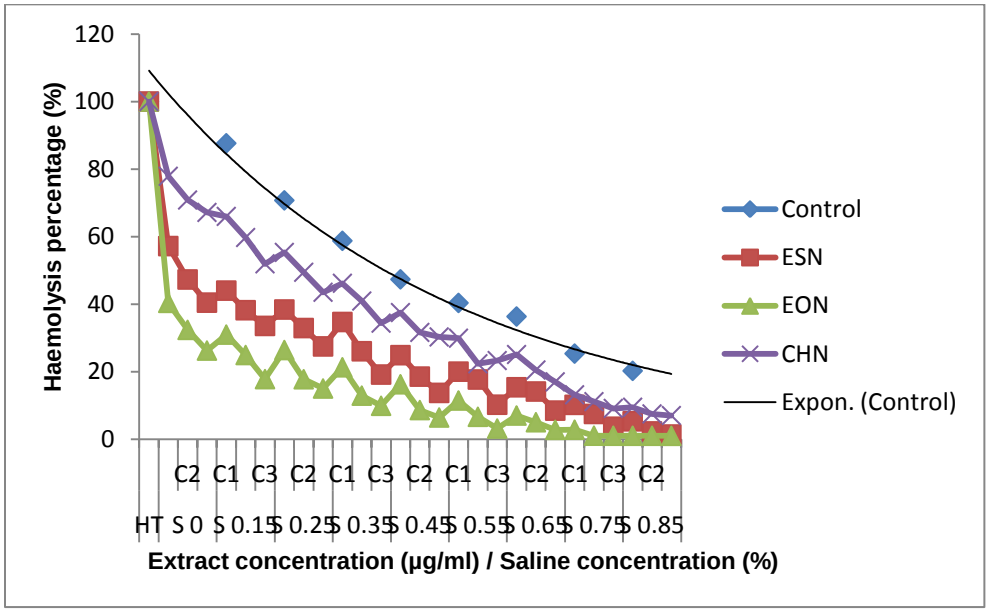

Figure 6: Effect of extracts on the erythrocyte membrane stability. *The statistical analysis include Kruskal-wallis followed by Dunnet at P<0.05. PHE: phenylalanine (standard). = Dark Chocolate, ESN = Santchou Cocoa Extract, EON = Obala Cocoa Extract. HT: total haemolysis. S0 – 0.85 %: salt concentration. Control: red blood cell treated only with salt solution at different concentration*

V. Conclusion

The aim of the present study was to investigate the protective potential of *T. cacao* bean extracts against ion toxicity in some organs as well as the red blood cell membrane stability. Cocoa bean extracts especially EON have protective properties against oxidative mediated ion toxicity in rat liver, heart and kidney as well as a capacity to stabilize red blood cell against haemolysis. In order to complete these results, further studies such as the toxicity study (search for the limit dose) and the extract fractionation (identification of chemical content responsible to their properties) should be realized.

Abreviations

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RNS: Reactive nitrogen species
ROS: Reactive oxygen species
MDA: Malondialdehyde
TBA: Thiobarbituric acid
H_2O_2: Hydrogen peroxide.

Declaration

Availability of data and material: Data and material are available

Competing interests: The authors declare that they have no competing interests.

Authors' contributions

Biapa NPC designed the research, conducted the study as well as the statistical analysis of the study; Kengne FC, Yembeau LN, Nkwikeu NPJ assisted during assays; Pieme CA directed the research work; All the authors read and approved the final manuscript.

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