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Hydroethanolic extract of *Theobroma cacao* beans is non toxic and attenuates oxydative stress induced by Naphtalene in *Wistar* rats

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ABSTRACT

Background: Oxidative stress is a situation where the cell no longer controls the excessive presence of toxic oxygen radicals. Many human diseases have a strong relationship with oxidative stress due to an imbalance between antioxidants and pro-oxidants. The objective of this study is to evaluate the *in vivo* antioxidant capacity of *Theobroma cacao (T.cacao)* beans extract and its acute toxicity. **Methods**: *T. cacao* beans were collected in the Obala locality (Center Cameroon) and then subjected to hydroethanolic extraction (70:30) at pH 3. The *in vivo* oxidative stress induction was done using naphthalene at 110 mg / kg and different doses of extracts (50 mg / kg, 100 mg / kg, and 200 mg / kg) were orally administered to rats. Some oxidative stress parameters helped to evaluate the antioxidant potential of the extract (superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA)). In addition, the acute toxicity of *T. cacao* was evaluated by the methods recommended by the ODCE. Test groups received respectively the extract at different doses (5000 mg / kg and 2000 mg / kg) against 10% of DMSO and distilled water as neutral controls. Hepatic function was assessed using transaminase assays (ASAT, ALAT), proteins and histological sections. Also the blood count allowed to explore the haematological function. **Results**: The administration of different doses of extracts or vitamin C as standard significantly increased GSH levels as well as antioxidant enzymes (SOD, CAT) and a significant decrease in MDA in studied organs and serum of animals compared to pro-oxidant control. ALAT and ASAT activities did not significantly vary in rats compared to neutral controls. No deaths and hepatic injuries were observed at different doses of the extracts. **Conclusion**: The extract of *T. cacao* beans possess *in vivo* antioxidant capacities capable of protecting tissues against oxidative stress and toxicity in rats at 2000 mg / kg and 5000 mg / kg.

Keywords: toxicity, oxidative stress, antioxidant, *Theobroma cacao*

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Introduction

In recent years, the world of biological and medical sciences is invaded by a new concept, that of "oxidative stress", which is a situation where the cell no longer controls the excessive presence of toxic oxygen radicals. Many human diseases have a strong relationship with oxidative stress due to an imbalance between antioxidants and pro-oxidants (1). Indeed, oxidative stress plays a significant role in the emergence and physiopathology of many diseases such as cancers, diabetes, neurodegenerative diseases, atherosclerosis, sickle cell disease (2).

These mechanisms related to reactive oxygenated species (ROS) causing oxidative stress can explain the toxic effects of a substance. Indeed, this relationship is explained through several mechanisms such as lipid oxidative damage, mainly by lipid peroxidation; protein oxidative damage and oxidative damage to mitochondrial or nuclear DNA , the H_2O_2 and O_2^- generated OH radical being directly responsible for various alterations in DNA (3). Other previous studies have clearly demonstrated a close relationship between oxidative stress and toxicity induced by naphtalene. Naphthalène is an illustration of such toxic substance which induces toxicity through its ability to increase lipid peroxidation, cytochrome c reduction and hydroxyl radical production (4). Previous studies identified 1,2-dihydrodiol naphthalene and 1-naphthol as the major metabolites of naphthalene in rats (4). 1,2-naphthalenediol, one of the metabolites derived from glucuronic conjugation of naphthalene, can be oxidized to 1,2-naphthoquinone and hydrogen peroxide, which can form covalent bonds with macromolecules and cause important oxidative stress in cells. These metabolites are neutralized by GSH, the first antiradical defense system, which causes an increased depletion of its intracellular content (4). These authors found that elevated liver transaminases are associated with increased oxidative stress. In fact, an elevated TBARS (thiobarbituric-acid-reacting substances) which measure membrane lipid peroxidations was associated with hepatocellular injury in preterm infants showed by a statistically significant relationship between urinary TBARS and elevated liver transaminases (ALAT and ASAT) (5).

According to the World Health Organization, about 80% of the world's population in developing countries, because of poverty and lack of access to modern medicine, depends on traditional medicinal plants for their primary health care. Many medicinal plants have become an important source of antioxidant properties. The role of medicinal plants in the prevention or control of diseases has been attributed to the antioxidant properties they contain through a large number of polyphenolic compounds such as flavonoids, saponins and tannins (6,7). The use of synthetic antioxidant molecules is currently being questioned because of potential toxicological risks. Toxicity studies are therefore of great interest in enhancing the strengths of therapeutic substances based on medicinal plants. This obsession shown for herbal medicine is increasingly taking into account the limit doses of consumption to avoid toxic effects. Thus, high doses of the ethanolic extract of *Carica papaya* Linn have shown signs of liver and kidney toxicity in *Wistar* rats (8). Also, based on the acute and subacute toxicity assessment of the ethanol extract of *Entada pursaetha*, *Toddalia aculeata*, and *Ziziphus mauritiana* in *Wistar* rats, the authors observed some changes although not significant in the liver after 28 days (9).

Cocoa (*Theobroma cacao*) is an excellent source of antioxidant compounds that can boost the health of people around the world. It belongs to the streculiaceae family and grows in the humid tropics. In the world, *T.cacao* polyphenols are used as hypotensives. They are also used to reduce the chances of developing type 2 diabetes (10). Previous studies on *T.cacao* have demonstrated the

presence of higher concentrations of several classes of polyphenols responsible for their beneficial properties. Thus, Nguelewou, (11,6) have shown that extracts of cocoa beans (*Theoboma cacao)* from Obala have excellent anti-sickling and anti-oxidant properties. Only few data are known about the lethal dose of these extracts as well as their *in vivo* antioxidant capacities. This has raised an important question: what is the *in vivo* antioxidant potential of Obala's cocoa bean extracts and what could be its limit dose of use. Based on the assumptions that cocoa extract possesses bioactive substances capable of reducing oxidative stress induced by naphtalene and presenting no toxic effect on different organs, the main objective of this study is to evaluate the *in vivo* antioxidant capacity of the extract of *Theobroma cocoa* beans followed by its acute toxicity in rats .

Materials and Methods

Material

Plant material

Cocoa beans from Obala (Center region in Cameroon) were collected during December 2015. They were identified under reference number *60071 / HNC* at the National Herbarium of Yaoundé-Cameroon.

Experimental animals

Thirty-six (36) *Wistar* albino rats (18 males and 18 females, 7–8 weeks old) weighing 130-250 g and 9 females *Wistar* albino rats, (8 - 9 weeks old), weighing 150-200 g were used for antioxidant test and acute toxicity studies respectively. These animals were bred in the animal house of the University of Dschang and housed in plastic cages. They were fed with standard diet (12). All experimental animals were given Food and water *ad libitum*. They were handled according to standard protocols for the use of laboratory animals (13).

Methods

Preparation of plant extract

The modified Benhammou (14) method was used for the extraction. Briefly, 250 g of cocoa beans were crushed. The powder obtained was macerated in the ethanol-water mixture (70/30), pH 3. The mixture was stirred several times to maximize extraction. After 48 h, the mixture was filtered using whatman paper No. 4 followed by No. 1. Then, the solvent was evaporated to near dryness under reduced pressure in a rotary evaporator at 40 °C and then kept in an oven for 24 h at a temperature of 39 °C until dryness.

Evaluation of the *in vivo* antioxidant activities of *Theobroma cacao* extracts

Experiments were performed on six groups of six rats each. 2nd to 6th groups were given simultaneously naphthalene solution (110 mg/kg) and vitamin C (3 mg/kg) or plant extract at doses of 50, 100 and 200 mg/kg. The first group has received an equal volume of the vehicle (sunflower oil) orally. All treatment was performed within a period of 15 days (15). 24 h after the last treatment, all animals were sacrificed under chloroform anesthesia and their blood samples were collected. Afterward, serum was quickly separated by centrifugation for 15 min at 3000 g, and kept at -4 ºC for biochemical assays. The liver and kidneys were also immediately removed, washed in chilled saline solution and then 15% homogenates were prepared in phosphate buffer for each tissue as previously described (16). Superoxide dismutase (SOD, malondialdéhyde (MDA), and catalase (CAT) were determined using the method described by (17) slightly modified by Kodjio (18). The peroxidase activity (POD) and reduced glutathione were evaluated using the method of Habbu (19) and Ellman (20) respectively. Absorbances were read using Schimadzu 1501 Japan spectrophotometer.

The determination of Superoxide dismustase activity

To 150 µL of each homogenate (serum and tissues) were added 1650 µL of phosphate buffer (pH 7.2) and then 200 µL of 0.3 mM adrenaline. The auto-oxidation of adrenaline

was then measured by reading the DO at 480 nm 30 s and 1 min after. SOD activity expressed as percentage inhibition was calculated as follows:

% inhibition = 100- (ΔDO sample) / (ΔDO white) X 100

with ΔDO (min) = DO_{60s}-DO_{30s}. Knowing that 50% inhibition corresponds to a unit of activity, it is shown that $A = \frac{X\%}{50\% \; X \; V} \; X \; F$

A: specific activity of SOD (in units / ml / mg of protein);

X%: is the percentage inhibition of N unit;

V: volume used;

F: dilution factor.

The determination of Catalase activity

A total of 25 µL of samples (serum and tissues) was added to tubes containing 375 µL of phosphate buffer (pH 7.4) and 100 µL of 50 mmol/L H_2O_2. After one min of incubation at room temperature, 1 mL of dichromate was added. The mixture was homogenized and incubated at 100 °C for 10 min. The mixture was then cooled in ice bath and the absorbance was recorded at 570 nm (Schimadzu 1501 spectrophotometer, Japan). One unit of activity is equal to one mmol/L of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

The determination of Peroxidase activity

Briefly, to 0.5 mL of tested samples (serum and tissues) were added 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate. The absorbance of potassium iodide was read at 353 nm, which indicates the amount of peroxidase. Then 20 µL of H_2O_2 (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of Peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit per min. The specific activity was expressed in terms of unit per mg of proteins.

Reduced Glutathione level

In different test tubes, 3 mL of Ellman's reagent (0.1M phosphate buffer, pH 7.4 and 2,2-dithio-5,5'-dibenzoic acid) was added to 20 µL of each test solution or control freshly prepared. The optical density was read at 412 nm against the blank and the glutathione concentration was calculated and expressed in micromol / L according to the following formula. **DO = ε C1.** ε of glutathione = 13600 and l the optical part.

Evaluation of lipid peroxidation

Tissues and serum peroxidation were assessed by measuring the level of malondialdehyde (MDA) according to the described method of Fodouop (17) with some modifications. Briefly, 0.5 mL of 1% orthophosphoric acid and 0.5 mL of precipitating mixture (1% thiobarbituric acid, 1% acetic acid) was added to 0.1 mL of tested sample. The mixture was homogenized and heated in boiling water for 15 min and cooled immediately. It was subsequently centrifuged at 3500 g for 10 min and the absorbance of the supernatant was recorded at 532 nm (Schimadzu 1501 spectrophotometer, Japan). The peroxidation in the tissues was calculated based on the molar extinction coefficient of malondialdehyde (MDA) (153 mM-1cm-1), and expressed in terms of micromoles of MDA/g of tissue or /mL serum.

Acute toxicity study

The method of OCDE (13) was used for this study. Briefly, nine rats were randomly allocated into three groups of three females animals. Test groups (I and II) were given doses of 5000 and 2000 mg/kg of *T. cacao* extract respectively through gastric intubation. 10 % (v/v) DMSO/tween 80) was administered orally to the control group as a vehicle. Extracts were prepared using 10 % (v/v) DMSO/tween 80 and the volume administered was not more than 1 mL as a unique administration. Experimental animals were deprived of food for 12 h prior to the extract administration. After this period, animals were given food and water *ad libitum and* monitored daily for 14 days for changes in body weight, food and water

consumptions. Food intake and weight gain were recorded every two days during the experimental time. After sacrifice on the 14th day, heparinized blood sample was collected and used for haematological analysis (hematocrit, total red cell (RBCs), total white blood cell (WBCs), lymphocytes, neutrophils, monocytes, eosinophils and basophils). Non-heparinized blood samples were allowed for complete clotting and then centrifuged at 3000 g for 15 min. Serums were collected and frozen at -4°C for the measurement of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and total proteins using commercial kits SGM (Italia). Different organs (liver, lungs, heart, kidneys, and spleen) were isolated and weighed using an electronic balance (Mettler PE 160, France). 15% homogenate of each organ was prepared in 0.9% NaCl solution and one section of each organ was used for estimation of protein concentration using Biuret method (21) and other for the histological section

Histopathological study

The method described by Di Fiore (22) was used for this study. Briefly, Vascular perfusion of each organ was performed followed by tissue sections (5-micron thickness). These tissues were further fixed in 10% formalin and then, embedded in paraffin for histological analysis. They were routinely stained with haematoxylin and eosin (H & E), and examined under a light microscope (Olympus CH02). Any alterations compared to the normal structures were registered.

Statistical analysis

Results were expressed as mean value ± standard deviation (S.E.M.). Within the group, comparisons were performed by the analysis of variance using ANOVA test. Significant difference between control and experimental groups were assessed by *Waller Duncan*-test.

Results

In vivo antioxidant activities of *Theobroma cacao* extracts

Food intake and weight gain

Figure 1 shows the evolution of food intake and the weight gain in both male and female rats. With regard to the food consumption (figure 1 a and b). It appears that the administration of naphthalene results in a significant decrease ($p < 0.05$) of food consumption in negative control animals (from 26 ± 2.21 to 19.14 ± 1.01 in male and from 30 ± 3.13 to 24.61 ± 1.46 in female). When compared to the neutral control, it is clear that administration of the extract or vitamin C increased this parameter in both sexes (from 24 ± 3.11 to 30 ± 4.10 in male and from 16 ± 0.20 to 26.62 ± 5.03 in female). Food intake values increased with dose-dependent in male rats. Same observations were made with weight gain values (figure 1 c and d) which increased in animal groups treated at different doses of *T. cacao.* Extract (from - 4.33 ± 0.03 to 16.66 ± 3.57 in male and from - 1.66 ± 0.02 to 4 ± 0.05 in female)

Effect of the hydroethanolic extract of T. cocoa on the activity of catalase

The serum and tissue catalase activity after the simultaneous administration of the prooxidant (naphthalene) and different doses of the extract is presented in table 1. The administration of naphthalene (negative control) resulted in a significant decrease ($p<0.05$) of serum catalase activity in both sexes compared to that of neutral controls. However, administration of the extract or vitamin C (positive controls) generally induced a significant increase ($p<0.05$) in catalase activity compared with negative controls in males and females serum, liver and kidneys. Furthermore, the increase in this activity was dose-dependent excepted with the serum sample at the 100 mg / kg dose in males.

Effect of the hydroethanolic extract of Theobroma cocoa on the peroxidase activity

Table 2 shows serum and tissue peroxidase activity. The administration of naphthalene prooxidant (negative control) resulted in a significant decrease ($p<0.05$) of peroxidase

activity in both sexes compared to neutral control. The administration of the extract at different doses or vitamin C (positive control) induced a significant increase ($p<0.05$) in serum and tissue peroxidase activity compared to the negative control.

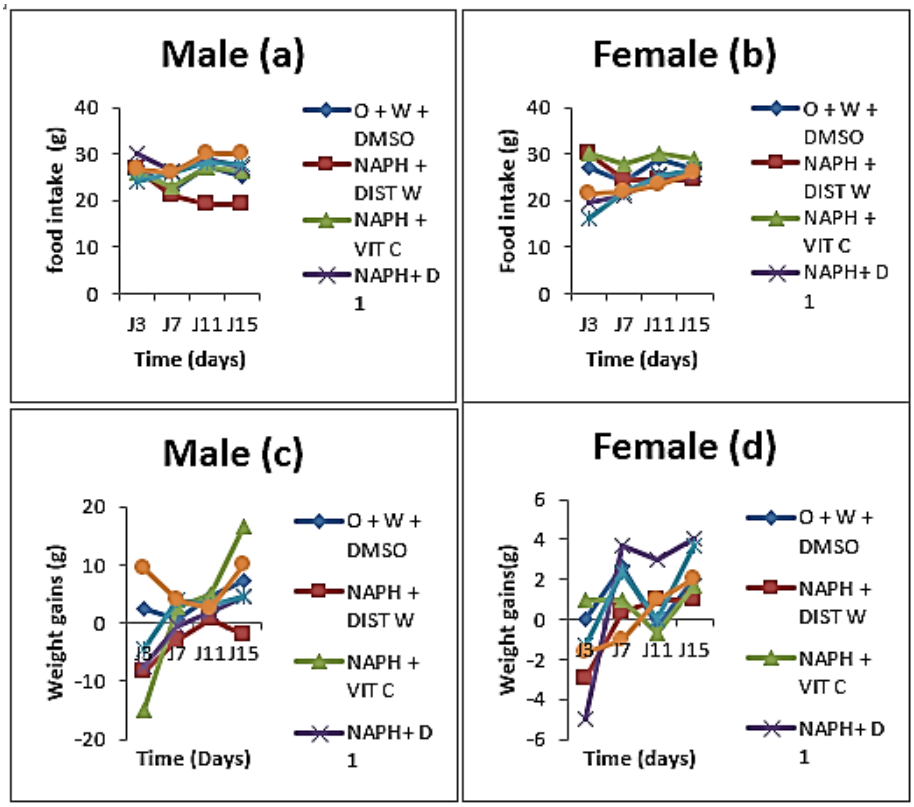

Figure1: **Effect of *T. cacao* on food consumption (a and b) and weight gain (c and d)**

O: Oil; W: Water; NAPH: Naphtalene; DIST W: Distilled Water; VIT C: Vitamin C; D1: Dose 1; D2: Dose 2; D3: Dose 3

Table 1: Effect of the hydroethanolic extract of *Theobroma cocoa* on the activity of catalase

Groups and doses	serum	liver	kidney
Catalase activity (µmol / min / g tissue or µmol / min / ml serum)			
Male			
Positive control	0.350 ± 0.01^{e}	5.303 ± 1.93^{ab}	13.712 ± 1.27^{c}
Neutral control	0.307 ± 0.06^{c}	5.261 ± 1.61^{ab}	18.680 ± 3.90^{d}
Negative control	0.286 ± 0.10^{a}	4.799 ± 0.71^{a}	4.201 ± 0.62^{a}
50 mg/ kg	0.302 ± 0.03^{b}	5.197 ± 1.49^{ab}	9.110 ± 1.22^{b}
100 mg/ kg	0.305 ± 0.09^{b}	5.801 ± 1.48^{ab}	11.219 ± 1.57^{bc}
200 mg/kg	0.337 ± 0.04^{c}	6.241 ± 1.39^{b}	12.768 ± 1.03^{bc}
Female			
Positive control	2.307 ± 1.37^{a}	9.041 ± 1.53^{b}	9.041 ± 1.53^{b}
Neutral control	0.343 ± 0.01^{d}	6.224 ± 1.13^{b}	8.447 ± 1.84^{b}
Negative control	0.253 ± 0.06^{a}	2.194 ± 1.83^{a}	5.368 ± 1.91^{a}
50 mg/ kg	0.261 ± 0.05^{a}	2.569 ± 1.10^{a}	5.634 ± 1.13^{a}
100 mg/ kg	0.280 ± 0.08^{b}	4.398 ± 1.06^{ab}	6.648 ± 1.75^{a}
200 mg/kg	0.311 ± 0.04^{c}	5.991 ± 1.45^{ab}	14.112 ± 1.99^{c}

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, values with same letters are significantly different ($p < 0.05$) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Table 2: Effect of the hydroethanolic extract of *Theobroma cocoa* on the peroxidase activity

Groups and doses	serum	liver	kidney
Peroxidase activity (µmol / min / g tissue or µmol / min / mL * 10^{-2} serum)			
Male			
Positive control	6.502±0.037^{c}	1.811±0.083^{d}	1.357±0.047^{d}
Neutral control	3.308±0.105^{a}	0.913±0.019^{a}	0.408±0.001^{a}
Negative control	4.491±0.128^{ab}	1.548±0.047^{c}	1.538±0.050^{e}
50 mg/ kg	4.513±0.103^{ab}	1.566±0.098^{b}	0.660±0.065^{b}
100 mg/ kg	4.537±0.164^{ab}	1.906±0.016^{d}	0.705±0.090^{b}
200 mg/kg	5.155±0.186^{b}	2.105±0.029^{e}	1.106±0.057^{c}
Female			
Positive control	3.312±0.007^{a}	0.791±0.020^{a}	0.598±0.082^{a}
Neutral control	4.142±0.007^{b}	1.606±0.055^{e}	1.046±0.815^{b}
Negative control	4.003±0.009^{c}	1.053±0.051^{b}	1.011±0.075^{c}
50 mg/ kg	4.534±0.053^{d}	1.284±0.009^{c}	1.698±0.051^{d}
100 mg/ kg	4.614±0.010^{e}	1.490±0.048^{d}	1.707±0.050^{e}
200 mg/kg	4.624±0.046^{e}	1.820±0.021^{f}	1.794±0.001^{f}

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, values with same letters are significantly different (p < 0.05) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Table 3: Effect of the hydroethanolic extract of *Theobroma cocoa* on the activity of superoxide dismustase (SOD)

Groups and doses	Serum	liver	kidney
SOD activity (µmol / min / g tissue or µmol / min / ml * 10^{-2} serum)			
Male			
Positive control	1.302±0.0007^{a}	1.299±0.027^{a}	1.289±0.033^{a}
Neutral control	1.301±0.0007^{a}	1.275±0.022^{a}	1.288±0.007^{a}
Negative control	1.331±0.001^{b}	1.294±0.008^{a}	1.301±0.001^{b}
50 mg/ kg	1.328±0.0007^{a}	1.298±0.0007^{a}	1.331±0.001^{c}
100 mg/ kg	1.301±0.020^{a}	1.330±0.002^{c}	1.302±0.000^{b}
00 mg/kg	1.330±0.001^{b}	1.331±0.0008^{c}	1.332±0.001^{c}
Female			
Positive control	1.309±0.002^{a}	1.210±0.097^{a}	1.231±0.002^{a}
Neutral control	1.310±0.001^{a}	1.227±0.008^{a}	1.231±0.0007^{a}
Negative control	1.330±0.001^{a}	1.332±0.0007^{c}	1.299±0.049^{b}
50 mg/ kg	1.329±0.004^{a}	1.230±0.001^{b}	1.332±0.0004^{bc}
100 mg/ kg	1.332±0.0004^{a}	1.243±0.010^{b}	1.298±0.057^{b}
200 mg/kg	1.329±0.018^{a}	1.304±0.038^{bc}	1.360±0.0007^{c}

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, values with same letters are significantly different (p < 0.05) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Effect of the hydroethanolic extract of *Theobroma cocoa* on the activity of superoxide dismutase (SOD)

Tissue and serum superoxide dismutase activity after simultaneous administration of naphthalene and *Theobroma cacao* extract is shown in table 3. This table reveals that the administration of naphthalene (negative control) induced an increase in SOD activity in serum and organs (liver, kidneys). The presence of extract or vitamin C results in an increase of this parameter in both sexes.

Effect of the hydroethanolic extract of Theobroma cocoa on the level of Malondialdéhyde (MDA)

The evolution of the malondialdehyde level in both tissue and serum after simultaneous administration of naphthalene and extract is shown in table 4. The analysis of this table shows that the administration of naphthalene (negative control) led to a significant increase ($p < 0.05$) of MDA compared to neutral controls except in the serum in males. However, the administration of the extract at 100 and 200 mg / kg decreased it significantly ($p < 0.05$). When comparing the test doses to the neutral controls, it is found that the administration of the extracts resulted in the regulation of the MDA level.

Effect of Theobroma cocoa on the reduced glutathione level

Tissue and serum GSH levels is presented in table 5. The administration of naphthalene decreased GSH concentrations. In the contrary, the presence of the extract or vitamin C increased significantly ($p < 0.05$) GSH concentrations compared to the negative control in both sexes.

Table 4: Effect of the hydroethanolic extract of *Theobroma cocoa* on the level of Malondialdéhyde

Groups and doses	serum	liver	kidneys
MDA (µmol / g tissue or µmol / mL serum)			
Male			
Positive control	0.012±0.004^{a}	0.015±0.003^{a}	0.002±0.001^{ab}
Neutral control	0.012±0.005^{a}	0.019±0.004^{a}	0.001±0.000 ^{a}
Negative control	0.013±0.010^{a}	0.023±0.009^{b}	0.004±0.001^{b}
50 mg/ kg	0.011±0.004^{a}	0.023±0.005^{b}	0.003±0.001^{b}
100 mg/ kg	0.007±0.001^{a}	0.013±0.003^{a}	0.001±0.000^{a}
200 mg/kg	0.010±0.005^{a}	0.016±0.008^{a}	0.001±0.000^{a}
Female			
Positive control	0.003±0.002^{a}	0.015±0.009^{b}	0.001±0.000^{a}
Neutral control	0.009±0.003^{b}	0.029±0.008^{c}	0.004±0.001^{b}
Negative control	0.004±0.001^{a}	0.017±0.002^{b}	0.002±0.001^{a}
50 mg/ kg	0.008±0.004^{a}	0.024±0.010^{c}	0.003±0.001^{ab}
100 mg/ kg	0.007±0.003^{a}	0.008±0.003^{a}	0.001±0.000^{a}
200 mg/kg	0.006±0.001^{a}	0.005±0.001^{a}	0.001±0.000^{a}

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, values with same letters are significantly different ($p < 0.05$) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Table 5: Effect of *Theobroma cocoa* on the reduced glutathion level

Groups and doses	serum	liver	kidney
GSH (µmol / g tissue or µmol / ml serum)			
Male			
Positive control	130.318±13.204^{b}	1.273±0.421^{a}	1.075±0.136^{a}
Neutral control	110.833±8.564^{a}	1.094±0.240^{a}	0.736±0.268^{a}
Negative control	105.980±9.319^{a}	1.188±0.132^{a}	0.786±0.268^{a}
50 mg/ kg	124.702±14.446^{b}	1.357±0.283^{a}	1.146±0.268^{ab}
100 mg/ kg	130.343±23.192^{b}	1.591±0.251^{b}	1.175±0.136^{b}
200 mg/kg	142.886±28.323^{b}	1.682±0.242^{c}	1.191±0.163^{c}
Female			
Positive control	121.410±16.101^{ab}	1.102±0.109^{ab}	0.746±0.052^{a}
Neutral control	86.126±27.760^{a}	0.910±0.240^{a}	0.609±0.449^{a}
Negative control	117.446±22.512^{ab}	1.036±0.224^{a}	0.682±0.545^{a}
50 mg/ kg	124.023±21.855^{ab}	1.164±0.278^{ab}	0.846±0.052^{ab}
100 mg/ kg	135.540±7.051^{ab}	1.340±0.563^{b}	0.989±0.449^{ab}
200 mg/kg	138.476±6.261^{b}	1.687±0.490^{c}	1.037±0.598^{b}

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column for each sex, values with same letters are significantly different (p < 0.05) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Acute oral toxicity

Effect of *Theobroma cocoa* on the weight gain variation in acute toxicity study

Figure 3 shows the effect of single administration of *Theobroma cocoa* extract at different doses on the body weight of animals. From this Figure, it appears that the single administration of this extract induced a gradual increase in the weight growth during the first ten days in all animals. Afterward, growth becomes more pronounced in rats treated at 5000 mg / kg compared to controls.

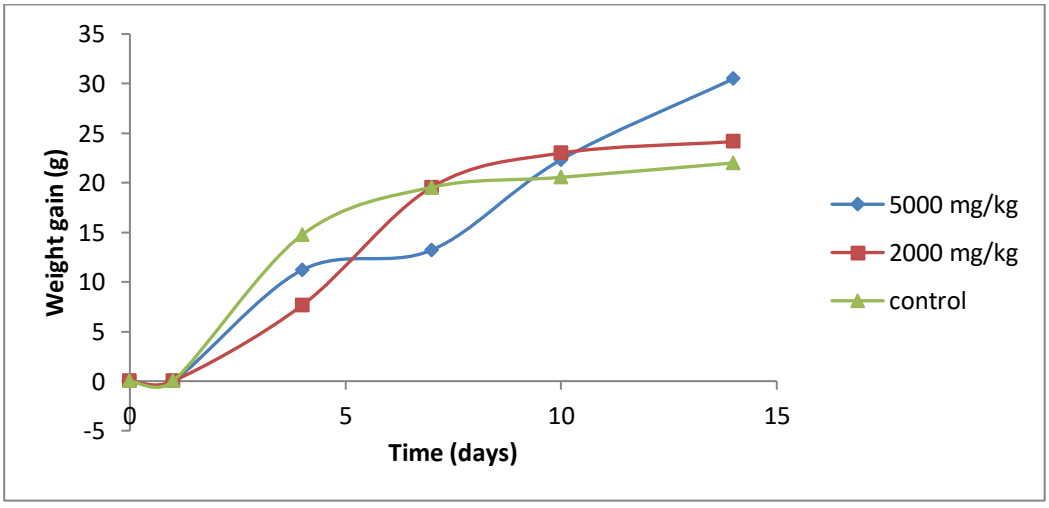

Figure 2: Effect of *T. cacao* on the weight gain variation in acute toxicity

Waller-Duncan

Table 6 shows total proteins concentrations (serum, tissues), transaminase activity (serum) as well as the relative weight of studied organs after the single administration of the hydroethanolic extract of *Theobroma cacao*. It appears that the administration of the extract did not induce any significant variation in both concentrations (serum, tissues), transaminase activity (serum) as well as the relative weight of studied organs compared to the control.

Table 6: Effect of *T. cacao* on the total proteins, transaminases and relative organ weights

Groups	**serum**	**liver**	**kidneys**
Total proteins (g / dL serum)			
5000 mg/kg	4.337±0.633[a]	0.331±0.186[a]	0.513±0.235[a]
5000 mg/kg	4.914±0.157[a]	0.537±0.038[a]	0.498±0.041[a]
Control	5.045±0.302[a]	0.647±0.221[a]	0.427±0.092[a]
Transaminase (UI/L)			
	ALAT	ASAT	
5000 mg/kg	8,29±1,30[a]	53,544±3,670[a]	
5000 mg/kg	7,85±1,74[a]	43,276±2,417[a]	
Control	6,54±1,30[a]	70,342±3,477[a]	
Relative weight gain (g)			
5000 mg/kg	3.679±0.099[a]	0.667±0.023[a]	
5000 mg/kg	3.502±0.664[a]	0.666±0.060[a]	
Control	3.723±0.354[a]	0.680±0.055[a]	

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column, values with same letters are significantly different (p < 0.05) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO

Table 7 shows the variation of hematological parameters. It appears that, in spite of the average platelet volume (MPV), no significant difference was observed between groups relative to the all studied hematological parameters. Excepted at 5000 mg / kg where a significant ($p<0.05$) increase in MPV was observed compared to control. However, no significant variation of this data was noticed between the two extract doses used.

Table 7: Effect of extracts on hematological parameters in acute toxicity study

	RBC (X 10^3 / mL)	**Hb (g/dL)**	**Hct (%)**	**MCV (fl)**	**MCH (Pg)**	**MCHC (g/dl)**
5000 mg/kg	2,06±0,36[a]	11,53±3,57[a]	16,66±5,72[a]	79,83±17,09[a]	46,06±1,54[a]	70,10±1,19[a]
2000 mg/kg	2,67±1,31[a]	11,73±2,04[a]	19,13±3,11[a]	61,76±16,08[a]	43,06±1,21[a]	72,50±1,78[a]
Control	3,47±2,77[a]	12,56±2,54[a]	21,40±1,57[a]	61,9±10,26[a]	48,33±2,90[a]	80,26±4,50[a]
	WBC (X 10^3 / mL)	**Lcyte (%)**	**Mcyte (%)**	**Gcyte (%)**	**Platelet (X 10^3 µl)**	**MPV (fl)**
5000 mg/kg	11,30±2,93[a]	51,90±6,91[a]	15,26±4,35[a]	32,83±3,26[a]	698,50±2,50[a]	12,80±1,90[a]
2000 mg/kg	12,73±2,61[a]	49,56±9,30[a]	18,26±2,28[a]	32,16±3,44[a]	718,33±1,23[a]	11,25±0,05[a]
Control	13,70±8,45[a]	58,10±7,79[a]	13,20±5,41[a]	28,70±6,52[a]	670,50±38,50[a]	9,73±2,55[a]

Waller Duncan test. Assay done in 3 replicates. a, b, c, d: In the same column, values with same letters are significantly different (p < 0.05) with Positive control: Naphthalene + Vitamin C; Negative control : Naphthalene + distilled water; Neutral control: Oil + distilled water + DMSO. RBC: red blood cell, Lcyte: lymphocyte, Mcyte: monocyte, Hct: hematocrit, MCH: mean corpuscular hemoglobin, MCV: mean cell volume, MCHC: mean corpuscular haemoglobin concentration, WBC: white blood cell, MPV: mean platelet volume, fl: fentoliter

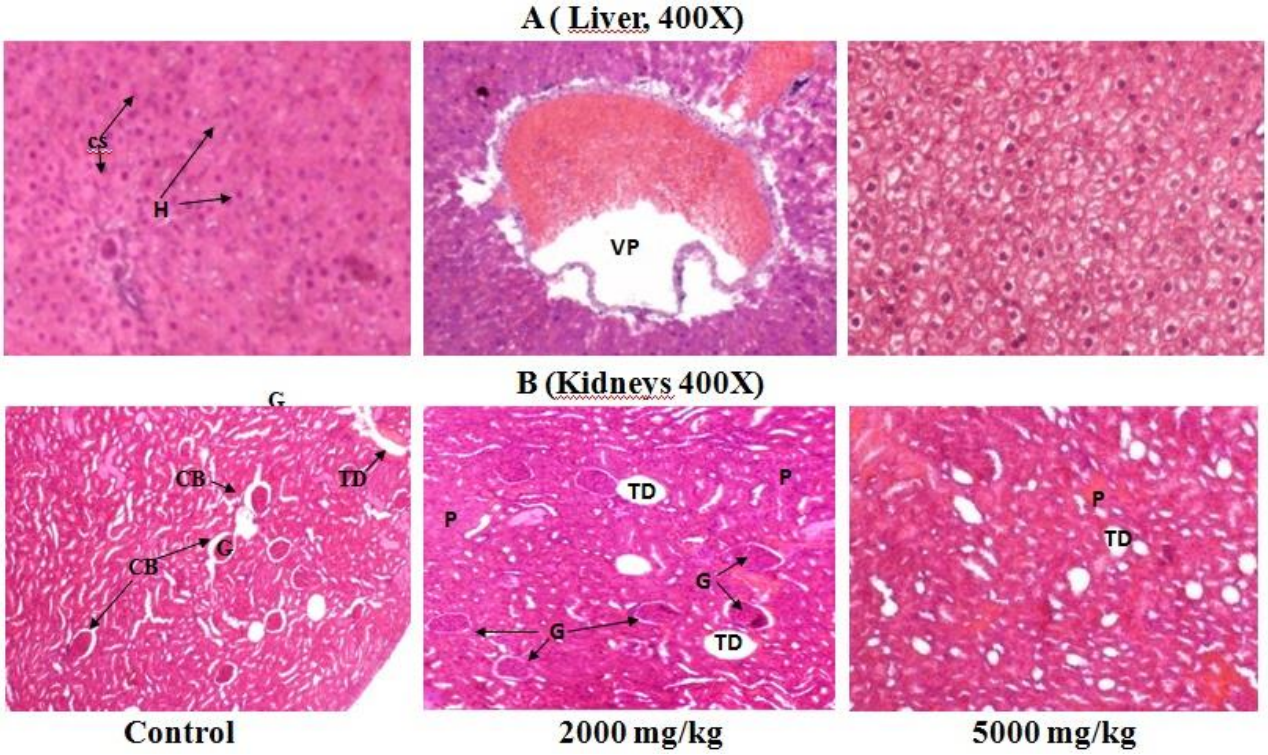

Figure 3: Histological sections of liver and kidneys

Hepatocytes (H); Normal portal vein (VP); Capillary sinusoidal (CS), Bowman's capsule (CB), Distal convoluted tubule (TD), Proximal tubes (P), Glomerules (G). Haematoxylin-eosin coloration

Histopathology analysis

The single administration of the *Theobroma cocoa* extract and monitoring of treated animals for 14 days showed no adverse effects on the rat liver and kidneys tissues (Figure 3).

Discussion

Enzymatic and non-enzymatic systems are used by living organisms to fight free radicals, produced during stress (Kumar *et al.*, 2013). Oxidative stress of a substance explains its toxicity capacity. These authors found that elevated liver transaminases are associated with increased oxidative stress. In fact an elevated TBARS (thiobarbituric-acid-reacting substances) which measure membrane lipid peroxidations was associated with hepatocellular injury in preterm infants showed by a statistically significant relationship between urinary TBARS and elevated liver transaminases (ALAT and ASAT) (5). Previous studies have shown that nano-particles-mediated oxidative stress is another illustration explaning the role of oxidative stress leading to the toxicity (23). In fact, cells and tissues respond to increasing levels of oxidative stress via antioxidant enzyme systems upon Nano-particles exposure. During conditions of mild oxidative stress, transcriptional activation of phase II antioxidant enzymes occurs via nuclear factor (erythroid- derived 2)-like 2 (Nrf2) induction. At an intermediate level, redox-sensitive mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain enhancer of activated B cell cascades. The extreme toxic effect of Oxidative stress result in mitochondrial membrane damage and electron chain dysfunction leading to cell death. Then it become important to found substances which could protect against toxicity through it ability to fight aginst oxidative stress (24).

In this study, elevated levels of MDA in the liver and kidneys of animals receiving 110 mg / kg of naphthalene compared to those receiving concomitant naphthalene and different doses of extracts could be due to the hyperoxidation of these tissues due to the naphthalene administration. In fact naphthalène is an

aromatic compounds used in various domestic and commercial applications.(lavatory scent disks, moth bails…). This compounds induce toxicity through its ability to increase lipid peroxidation, cytochrome c reduction and hydroxyl radical production (25). Previous studies identified 1,2-dihydrodiol naphthalene and 1-naphthol as the major metabolites of naphthalene in rats (4). 1,2-naphthalenediol, one of the metabolites derived from glucuronic conjugation of naphthalene, can be oxidized to 1,2-naphthoquinone and hydrogen peroxide, which can form covalent bonds with macromolecules and cause stress. These metabolites are neutralized by GSH, the first antiradical defense system, which causes an increased depletion of its intracellar content (4).

This study showed a significant decrease in the MDA concentration in the presence of different doses of the extract. It has been previously shown that cocoa extract contains high level of flavonoids as revealed by the phytochemical analysis (11, 6, 26). Indeed, the flavonoids (polyphenols) are cellular chemoprotective compounds according to their antiperoxidant capacity, playing a screen effect against the deleterious effect of the free radicals, which could explain the capacity of this extract to regulate the MDA level. The protective effect of this extract against oxidative stress is therefore related to their high total polyphenol content (11). Our results are in agreement with the study conducted by Andújar (27), which in their review, have listed two similar works (28, 29) showing that cocoa polyphenols increase the body's resistance to oxidative stress and thus protect it against diseases where oxidative stress is involved.

Furthermore, decreased activity of catalase and total peroxidase in animals receiving naphthalene alone is thought to be due to an inhibition of cell catalase activity by an excess of its substrate (hydrogen peroxide). The increase of the activity of these enzymes in the animals simultaneously receiving the naphthalene and the extracts could be the result of a rise of the hydrogen peroxide inhibition under the antioxidant effect of the cocoa extract which stimulates the catalase activity in the host cell (16).

Reduced glutathione is a biological antioxidant most often found in the liver. It protects cell constituents (proteins) against reactive oxygen species following increased production of pro-oxidants (30). The decrease in GSH concentration is associated with an increase in lipid peroxidation which is confirmed in this study following the administration of naphthalene. The increase of GSH in the animals having received different doses of the extract is due to the fact that the free radicals were neutralized by this extract. The extract of *T. cacao* would have reactivated the activity of GSH reductase which would have led to the inhibition of lipid peroxidation. These results corroborate those of (31). Furthermore other previous studies demonstrated that radical scavenging activity is due to the presence of flavonoids, anthraquinones and phenolic compounds present extracts which have the ability to stabilize free electron atom which becomes in pair (32). Moreover, this finding corroborates the study of Chin (33) and Nguelewou (11), which showed that the antioxidant activity of cocoa and its ability to trap free radicals is related to its content of flavonols.

Tissue toxicity was studied by assaying some biochemical parameters and histopathological analysis of the kidney and liver. ALAT is a cytosolic enzyme secreted in liver cells from which it is released into the blood in case of hepatic cell necrosis (34). It is a liver-specific enzyme, making it an important and very sensitive indicator of hepatotoxicity (35). ASAT, an enzyme found at the mitochondrial level, is also an indicator of hepatocyte destruction, although in addition to the liver it is found in the heart, skeletal muscles, lungs and kidneys (34). ALAT and AST levels rise rapidly when the liver is damaged for a variety of reasons including hepatic cell necrosis, hepatitis, cirrhosis, and

hepatotoxicity of certain drugs (34,35). In the present study, no significant variation in ASAT and ALAT activity was observed in animals treated with 2000 mg / kg and 5000 mg / kg. Significant reductions in ASAT levels have already been obtained by Akpanabiatu (36) and Mukinda and Syce (37) respectively with aqueous leaf extracts of *Eleophorbia drupifera* and *Artemisia afra* in rats. The results of this study showed in contrary, the hepatoprotective effect of *T. cacao* in rats. Similarly, this hypothesis was further confirmed by the work of Ramasami (38). These authors have demonstrated that the ethanolic extract of the fruits of *Passiflora foetida* exerts a hepatoprotective effect in the case of hepatotoxicity induced by carbon tetrachloride (CCl4).

Following the single administration of *Theobroma cocoa* extract in acute toxicity, no deaths were recorded at the 2000 mg / kg dose and at the 5000 mg / kg dose after successive administration of the extracts in 3 rats per dose; which shows that the LD_{50} is greater than 5000 mg / kg. Thus, it appears that the extract is not toxic or relatively harmless according to the classification scale of Hodge and Steiner (39). These results corroborate those of Kotué (40) who studied the acute and subacute toxicity of the extract "Hemodya": a combination of three medicinal plants and concluded that its LD_{50} was greater than 5000 mg / kg.

The results of the acute toxicity study showed an increase in the weight of treated animals compared to controls, whatever the dose. This increase in weight could be related to a stimulation of the appetite of the animals by the extract. Indeed, we observed an increase in food intake in animals after administration of extracts. This result is in agreement with those of Pieme (41) who showed that studied organs (kidneys, liver, heart, spleen, lungs) did not experience any significant variation in their weight after administrated the tested plant extracts. Also, Gandhare (42) have found that *Ceiba pentandra* root bark extract did not cause any deleterious macroscopic signs on the organs of the rats when they received the methanolic extract until 750 mg / kg dose.

Histological analysis of the liver and kidneys in this study revealed no evidence of toxicity in these organs. this could be explained by the capacity of the extract to protect tissues against damages. Tis is confirmed by the above results related to the non-significant variations of SAT and ALAT. Moreover, Kotué (40) have demonstrated during the acute and subacute toxicity study that the aqueous extract of "Hemodya" which, a combination of three medicinal plants used to fight against sickle cell disease in Albino rats did not induce any damage to the liver, kidneys and pancreas of rats after histological analysis of these organs.

The present study revealed no significant changes in the Haematological parameters analysis at different doses of the extract (2000 mg / kg and 5000 mg / kg). this could be explained by the capacity of the extract to regulate these parameters against damages. Similar results have been found by Suriyavadhana and Pakutharivu, (9) with the ethanolic extracts of *Entada pursaetha*, *Toddalia aculeata*, and *Ziziphus mauritiana*.

Conclusion

At the end of this study which main objective was to evaluate both the *in vivo* antioxidant capacity of *Theobroma cacao* and its acute toxicity on *Wistar* rats, it is clear that this extract has high *in vivo* antioxidant and is non-toxic. These findings suggest that *Theobroma cacao* could be a real hope in the management of sickle cell patients

Authors' contributions

Feudjio AF conducted the assays. Kodjio N, Yembeau NL, Kengne FC, Kwikeu NP assisted in conducting the assays. Biapa NPC designed, followed the assays as well as statistical data analysis. Pieme CA co-directed the research work; Telefo PB supervised the research. All the authors read and approved the final manuscript.

Abbreviations

ASAT: aspartate amino transferase

ALAT: alanine amino transferase

NFS: Blood count formular

DNA: desoxyribonucleic acid

DO: Optic density

SOD: superoxide dismutase

DMSO: Dimethylsulfooxide

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