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In Vivo Studies of Biochemical Effects of Root Bark Extract of *Theobroma cacao* (Linn.) on the Anti-oxidative Status of Rats Fed with High Salt-Diet

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ABSTRACT: *In vitro* antioxidant potential and membrane stabilizing effects of aqueous root-bark extract of *Theobroma cacao*, (L) and its fractions on erythrocyte of bovine (*Bos indicus*) exposed to heat and hypotonic induced lyses as well as its effects on oxidative enzyme status (GSH, GPx, MDA, catalase, SOD) of rats fed with high salt-diet were investigated. Rats were grouped into normal- treated, salt-treated and untreated; then placed on 4 % salt-diet and 1% salt-water for 21 days; after which they were orally exposed to 250 and 500 mg/kg body weight of flavonoid fraction. Phytochemical analysis of aqueous extract (AqE) revealed the presence of alkaloids, flavonoids, saponins, cardiac glycosides, tannins, steroids/phytosterols, xanthoproteins and triterpenes. The phenolic contents of AqE was 133.79 ± 0.00 mg and its fractions ranged between 0.82 ± 0.00 and 50.93 ± 0.00 mg TAE (tannic acid equivalent), while the flavonoid contents of AqE was 304.13 ± 0.01 mg and its fractions ranged between 8.36 ± 0.00 mg and 458.25 ± 0.01 mg RE/g (rutin equivalent). The fractions exhibited potent and appreciable DPPH-radical scavenging activity, ranged between 25.76 ± 0.39 % to 78.79 ± 1.12 %, ferric reducing antioxidant power between 11.92 ± 0.92 to 74.52 ± 2.37 µg AAE/ml (ascorbic acid equivalent) and compared favourably with the standard antioxidant compound. The fractions exhibited appreciable anti-inflammatory properties, with maximum membrane stability of 31.1 ± 4.14 % to 83.20 ± 2.05 %. Furthermore, the extract exhibited a biphasic response at all the concentrations tested. The activity of the extract compared favourably with that of acetaminophen (reference drug). The normal-treated, salt-treated and untreated groups of rats had increased ($P<0.05$) liver GSH level. The salt group, treated with 250 mg/kg body weight of extract had increased ($P<0.05$) liver GPx activity, while the normal-treated and untreated groups had decreased liver GPx activity. The normal group treated with 500 mg/kg body weight and salt group; treated with 250 mg/kg body weight of extract had decreased liver MDA level. There was no significant difference ($P>0.05$) between the activities of catalase and SOD, as well as the protein concentrations of the salt-treated and untreated animals. Slight decrease in body weights were observed in the salt-treated groups as against the slight increase in the untreated animals. Histologically, the extract protected the kidney tissues from renal dysfunction. The findings indicated that the root of cocoa possessed large spectrum of bioactive compounds that could be beneficial for the management of oxidative and inflammatory related conditions.

KEYWORDS: *Theobroma cacao*, Antioxidant potential, Oxidative enzymes, Bovine (*Bos indicus*), Erythrocytes, Hypotonic lyses, Membrane stabilization, Salt-diet

1.0 Introduction

The role of medicinal plants in disease prevention or control has been attributed to the antioxidant properties of their phytochemicals, usually associated with wide range of amphipathic molecules broadly termed polyphenolic compounds (Demiry *et al*., 2009). Plant polyphenols are known to have multifunctional properties such as ability to act as reducing agents, and singlet oxygen quenchers, in addition to their hydrogen donating properties (Michalak 2006; Aberoumand and Deokule 2008). Oxidative stress can initiate cellular tissue damage by modifying lipids, proteins and DNA; leading to cell death (Mikulikova and Popes 2001; Halliwell *et al*., 2002; Sian 2003). Despite the harmful cellular damaging effects, free radical reactions are also involved in beneficial physiological response when produced in low levels, such as mediating cytotoxicity of polymorphonuclear leukocytes, macrophages and monocytes during the respiratory burst, regulation of the tone of smooth muscle cells

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and up-regulation of redox sensitive transcription factors such as nuclear factor-κB and activator proteins (Scherck *et al*., 1990; Parihar and Taruna, 2003). Free radicals can be scavenged by several metalloenzymes called antioxidant enzymes as well as by non-enzymatic defenses (Sies 1993; Bojana *et al*., 2004).

Studies on the biological activities of *Theobroma cacao* revealed that the herbal preparations are capable of enhancing the activities of liver and heart tissue antioxidant enzymes, stabilizing erythrocyte membranes exposed to hypotonic or heat induced lyses or stress, and can be employed as a nerve tonic and blood supplement (Oyedapo *et al*., 2004, Falade *et al*., 2005, Noori *et al*., 2009). Common salt (sodium chloride) is a commonly used food ingredient which provides flavour enhancement, preservation and texture modification (Hutton 2002). Excess sodium chloride consumption which is associated with western diet has also been linked to numerous negative health effects such as decreased bone mineral density (Devine *et al*., 1995), cancer (Tsugane *et al*., 2004), obesity (He *et al*., 2008) and possibly hypertension (He and MacGregor 2010). Earlier studies have reported the bioactivity of root-bark extracts of *T. cacao* (Oyedapo *et al*., 2004; Falade *et al*., 2005). The present study was designed to evaluate the *in vitro* antioxidant potentials and membrane stabilizing activity of the aqueous root extract and fractions of *T. cacao* as well as the effects of its flavonoid fraction on the liver oxidative enzyme status of rats fed with high salt-diet.

2.0 Materials and Methods

2.1 Plant Materials

Fresh roots of *Theobroma cacao* (L.) were collected from a Cocoa Plantation at Babajakan Village, Ayedaade Local Government Area, Osun State, Nigeria, and authenticated (IFE 172418) at IFE Herbarium, Department of Botany, Obafemi Awolowo University.

2.2. Reagents and Chemicals

The reagents used were of analytical grade. Ethyl acetate, glacial acetic acid and tri-sodium citrate were obtained from British Drug House (BDH), Poole, England. Glutathione, hydrogen peroxide and epinephrine were obtained from Sigma-Aldrich Laboratories, Switzerland. Acetaminophen (Reference anti-inflammatory drug) was obtained from Drug Research and Production Unit (D.R.P.U), Obafemi Awolowo University, Ile-Ife. All the solutions, buffers and reagents were prepared using glass distilled water.

2.3 Experimental Animals

Thirty, healthy white albino rats were purchased from the Faculty of Health Sciences, Obafemi Awolowo University, Ile-Ife. Rats were acclimatized for two weeks, with access to standard rat Chow (Ladokun Feeds Limited, Ibadan) and clean water *ad libitium.*

2.4 Preparation of Extract

The roots were washed and thereafter the root-bark was peeled and cut into pieces. The aqueous root extract of *T. cacao* was prepared according to the earlier procedure described by Oyedapo *et al* (2004), with slight modification. Typically, 750 g of fresh root was boiled in 6.0 L of distilled water for 30 minutes. The infusion was allowed to settle for 24 hours at 25^0C, after which it was filtered. The filtrate was evaporated to dryness under reduced pressure (40^0C) on Edward High Vacuum Pump (Crawley, England) to yield a flaky brown residue.

2.5 Fractionation of Extract

Aqueous extract (20.53 g) was dissolved in hot distilled water (150 ml) in a separating funnel, followed by partitioning with hexane (100 ml x 5) to remove lipid materials. The aqueous layer was collected and partitioned with ethyl acetate (100 ml x 10). The ethyl acetate layer was evaporated to dryness to yield ethyl acetate fraction (EAF). The aqueous layer was again collected and partitioned with n-butanol (100 ml x 10) and then evaporated to give butanol fraction (n-BF). Finally, the aqueous layer was evaporated to give aqueous fraction (AqF). The extraction was carried out seven

times to afford enough samples for analysis. The solid residues were weighed and kept in air-tight container for the biological assays.

2.6 Phytochemical Screening

The aqueous root extract of *T. cacao* was screened for the presence of secondary metabolites such as alkaloids, flavonoids, tannins, cardiac glycosides, triterpenes, steroids/phytosterols, saponins, xanthoproteins and phlobatanins using standard procedures (Oyedapo *et al.*, 2004; Sofowora, 2006).

2.7 Determination of Total Phenolic Content (TPC)

The TPC of the crude/fractions of the extract was determined according to the procedure of Singleton *et al* (1999). The reaction mixture contained 0.5 ml (1mg/ml) of extract, 0.5 ml of distilled water and 1.5 ml of Folin- Ciocalteu's reagent (10 %). The mixture was incubated for 5 minutes at 25^0C, after which 1.5 ml of $NaHCO_3$ (10 % w/v) was added. The mixture was further incubated for 90 minutes at 25^0C. The absorbance was read at 725 nm, against the reagent blank. Mean of three readings were obtained. The total content of phenol was expressed in milligram tannic acid equivalent (TAE)/g of extract from the calibration curve ($y = 33.06x + 0.0093$, $R^2 = 0.9969$).

2.8 Determination of Total Flavonoid Content (TFC)

The TFC of the crude/fractions of the extract was determined according to the method of Singh *et al*. (2010). The reaction mixture consisted of 1.0 ml (1mg/ml) of extract, distilled water (1.9 ml), 0.3 ml of $NaNO_3$ (5% w/v), 0.3 ml of $AlCl_3$ (10% w/v) and 2.0 ml of NaOH (4% w/v). The reaction mixture was incubated for 10 minutes at 25^0C, after which the absorbance was read at 500 nm, against the reagent blank. The mean of three readings were obtained. The total flavonoid content was expressed in milligram rutin equivalent (RE)/g extract from the calibration curve ($y = 1.3366x + 0.0195$, $R^2 = 0.9742$).

2.9 Determination of Ferric Reducing Antioxidant Power (FRAP)

The estimation of FRAP of the crude/fractions were carried out according to the procedure of Benzie and Strain (1999). The reaction mixture consisted of 0.05 ml (0.1 mg/ml) of extract, 1.0 ml ofFRAP reagent (sodium acetate buffer, 300 mM, pH 3.6; 20 mM $FeCl_3.6H_20$ and 1N NaOH in ratio 10:1:1). The mixtures were incubated at 37^0C for 10 minutes. The increase in absorbance was read at 593 nm against the reagent blank (methanol in place of extract). The mean of three readings were obtained and the activity was expressed in microgram ascorbic acid equivalent (µgAAE)/ml extract from the calibration curve ($y = 0.0042x + 0.1142$, $R^2 = 0.9276$).

2.10 DPPH Free Radical Scavenging Assay

The assay of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the fractions were carried out according to the procedure described by Bode and Oyedapo (2012) with slight modification. Standard and extract (1 mg/ml) were serially diluted (50 - 3.125 µg/ml) with 10 mM acetate buffer, pH 5.5; to make 1.0 ml, then 1.0 ml of DPPH (0.3 mM) was added. The reaction mixtures were incubated in the dark for 30 minutes. The absorbance of the sample/standard was read at 517 nm against the reagent blank (1.0 ml buffer in place of extract). Ascorbic acid was used as the standard. The percentage scavenging activity was determined using the expression

$$\% \text{ Scavenging Activity} = \frac{\text{Abs.}_{\text{Control}} - \text{Abs.}_{\text{Test}} \times 100}{\text{Abs.}_{\text{Control}}}$$

$IC_{50,}$ the amount of extract necessary to cause 50% inhibition of the initial concentration of DPPH was derived from the percentage inhibition versus concentration plot of the fractions.

2.11 Membrane Stabilizing Activity Assay

Assay of membrane stabilizing activity was carried out according to the procedures of Oyedapo *et al*. (2004) and Bode and Oyedapo (2012) with slight modification. The assay mixture consisted of 1.0 ml of hyposaline (0.25% (w/v) NaCl), 0.15 M Sodium phosphate buffer, pH 7.6 (0.5 ml), varying

concentrations of extracts/fractions (50 - 350 µg/ml), varying volumes of isosaline (0.85 % w/v) and 0.5 ml of erythrocyte suspension (2%) to give a total volume of 3 ml. The control was prepared as above except the extract or drug was replaced with isosaline, while the extract or drug control lacked erythrocyte suspension. The reference anti-inflammatory drug for the assay was acetaminophen (0.5 mg/ml). The reaction mixtures were incubated at 56^0C for 30 minutes, followed by centrifugation at 4000 rpm on bench centrifuge for 10 minutes at 25^0C. The absorbance of the haemoglobin released was read at 560 nm, against the control. The percentage membrane stability was estimated from the expression:

$$\% \text{ Membrane Stability} = \frac{\text{Abs.}_{\text{Test}} - \text{Abs.}_{\text{Control}} \times 100}{\text{Abs.}_{\text{Blood Control}}}$$

2.12 Preparation and Processing of Diet

The rat diet was prepared as follows: 96 g of standard rat chow was mixed with 4 g of finely ground sodium chloride to make 4% (w/w) salt-diet. The drinking water also contained 1 % of salt (freshly prepared). The rats were allowed free access to feed and water for 21 days. The body weights of the rats were recorded before, during and after the treatments.

2.13 Preparation and Administration of Extract

The appropriate weights of the extract was dissolved in distilled water and diluted to give final concentrations of 250 and 500 mg/kg body weight. The extracts were given orally to the experimental animals for a period of 21 days; during which the animals were also fed with the salt-diet and salt water regularly. The rats were divided into six groups, comprising of five animals in each group (n = 5) as follows:

I: Rats + salt free diet/water (control)
II: Rats + salt free diet/water + 250 mg/kg body weight of the extract.
III: Rats + salt free diet/water + 500 mg/kg body weight of the extract.
IV: Rats+ 4% salt-diet/1% salt water + 250 mg/kg body weight of the extract.
V: Rats+ 4 % salt-diet/1 % salt water + 500 mg/ kg body weight of the extract.
VI: Rats + 4 % salt-diet/ 1 % salt water

2.14 Animal Sacrifice and Preparation of Plasma and Liver Homogenates

On the 22nd day, the rats were put to sleep by chloroform anesthesia. Blood was collected by cardiac puncture into tubes containing anticoagulant (3.8 % tri-sodium citrate). The liver and kidneys were surgically removed and immediately perfused in heparinized saline (0.85 %) to remove blood, wrapped with aluminum foil paper and stored frozen. The blood was centrifuged at 3000 rpm for 10 minutes at 25^0C; the plasma (supernatant) was carefully collected and used for the assay of aminotransferases (AST and ALT) and total protein. Liver (1g) was thoroughly homogenized in 10 ml of freshly prepared 100 mM phosphate buffer, pH 6.8 and then centrifuged at 3000 rpm for 10 minutes at 25^0 C. The supernatants were transferred into vial bottles and used for the assay of antioxidant and hepatic marker enzymes.

2.15 Biochemical Assays

The determination of plasma and liver marker enzymes (AST and ALT) activities were carried out according to the method of Reitman and Frankel (1957), using Randox diagnostic kits. The determination of total protein concentrations of the plasma and liver homogenates were carried out according to the method described by Tietz (1990), using Randox diagnostic kits.

2.15.1 Determination of GSH

The liver glutathione level was determined according to the procedure of Moron *et al* (1979). The reaction mixture consisted of 1 ml of the homogenate and 4 ml of 5 % (w/v) trichloroacetic acid. This was thoroughly mixed and centrifuged at 4000 rpm for 10 minutes at 25^0C. The supernatant was collected after which the residue was discarded. To 0.1 ml of the supernatant, 0.9 ml of 0.2 M sodium phosphate buffer, pH 8.0. and 2 ml of freshly prepared 5, 5′-dithiobios-2-nitrobenzoic acid (DTNB) (0.6 mM in 0.2 M of the buffer) were added. The intensity of the yellow colour was read at 412 nm after 10 min, against the blank. The activity of GSH was extrapolated from the glutathione calibration curve (1 mg/ml) and expressed in µgGSH/ ml sample.

2.15.2 Assay of Glutathione Peroxidase (GPX) Activity

The determination of liver GPx activity was carried out according to the procedure of Rotruck *et al* (1973). The assay mixture consisted of 0.5 ml of 0.2 M phosphate buffer, pH 8.0, 0.1 ml of hydrogen peroxide (2.5 mM), 0.1 ml of homogenate and distilled water (1.0 ml). The mixture was incubated at 37^0C for 3 minutes and 0.5 ml TCA (10 %) was added and centrifuged at 3000 rpm for 10 minutes at 25^0C. Disodium hydrogen phosphate (0.9 ml, 0.3 M) and 1.0 ml of freshly prepared DTNB (0.6 mM in the buffer) were added to 0.1 ml of homogenate. The absorbance was read at 412 nm against the blank (buffer in place of homogenate). The activity (µmol/mg protein) was calculated from the glutathione calibration curve, using the expression:

$$GPx = \frac{Abs_{412nm} \times \text{Total Assay Vol.} \times \text{Dilution Factor}}{\text{Extinction Coef } (6.22 \times 10^3) \times \text{Enzyme Volume}}$$

2.15.3 Assay of Superoxide Dismutase (SOD) Activity

The catalytic activity of SOD was assayed according to the method described by Misra and Fridovich (1972). Homogenate, 0.2 ml (20 %), 0.05 M carbonate buffer, pH 10.2 (2.5 ml) and 0.3ml of freshly prepared epinephrine (0.3 mM) were pipetted into the test cuvette. The increase in absorbance was monitored at 480 nm for 2 min. at 30 seconds intervals, against the blank (buffer in place of homogenate). One unit of SOD activity was expressed as the amount of enzyme that caused 50 % inhibition of the oxidation of epinephrine, under the assay condition, using the expressions:

$$\text{Increase in Abs. substrate/min.} = \frac{A_3 - A_o}{2 \text{ min}}$$

A_3 = Absorbance at 120 sec.
A_0 = Absorbance at 30 sec.

$$\% \text{ Inhibition} = \frac{\text{Increase in Abs. of Substrate}}{\text{Increase in Abs. of Blank}}$$

2.15.4 Assay of Catalase (CAT) Activity

The assay of CAT activity was carried out according to the method described by Sinha (1972). Briefly, the reaction mixture consisted of 2.5 ml phosphate buffer (0.01 M, pH 7.0), 0.2 ml hydrogen peroxide (0.2 M), 2 ml distilled water and 0.1 ml of homogenate (10%). The reaction mixture (1.0 ml) was quickly added to 2 ml of potassium dichromate (5%, w/v) in acetic acid and then boiled at 100^0C for 10 minutes. The decrease in absorbance was monitored at 570 nm at 15 seconds interval for 2 minutes. The activity was calculated from the CAT calibration curve ($y = 0.017\ x + 0.0646$, $R^2 = 0.994$), using the expression:

$$H_2O_2 \text{ Consumed } (\mu\text{mol}/\frac{\text{min}}{\text{mg}} \text{ protein}) =$$

Initial H_2O_2 (200µmol) – H_2O_2 remaining

Activity (µmol/min/mg protein) = H_2O_2 Consumed.

2.16 Histological Examination

The kidneys were sliced into approximately 0.3 cm thick and placed in neutral buffered 10 % formalin for fixation, after which they were dehydrated, mounted, sectioned and stained with hematoxylin and eosin (Prophet *et al*., 1992).

2.17 Statistical Analysis

The data was expressed as mean of ± SEM, n= 5 readings. Differences between the control and test values were evaluated by One-way ANOVA, using graph pad (instat and prism). The values of $P < 0.05$ were considered statistically significant.

3.0 Results and Discussion

The antioxidant potential and membrane stabilizing activity of aqueous root bark extract/fractions of *T. cacao* on bovine erythrocyte exposed to hypotonic lyses/stress were investigated; as well as the effects of its flavonoid fraction on liver oxidative enzyme status of rats fed on high salt-diet. This was with the view to provide scientific evidence that could ascertain the use of *T. cacao* root-bark extract/fractions as blood supplement and nerve tonic in inflammatory and oxidative related

conditions. The phytochemical screening revealed that the phytoconstituents of extract/fraction of *T. cacao* include alkaloids, flavonoids, cardiac glycosides, saponins, triterpenes, tannins, steroids/phytosterols and xanthoproteins. Phlobatannins were absent; confirming the earlier observations (Oyedapo *et al*., 2004 and Falade *et al*., 2005).

The total phenol and flavonoid concentrations of the aqueous root-bark extract of *T. cacao* and its fractions is presented in Table 1. Natural polyphenolic compounds exert their beneficial health effects mainly through their antioxidant activity by decreasing reactive oxygen species (ROS), binding metal ion catalysts, decomposing primary products of oxidation of non-radical species and preventing abstraction of hydrogen atom from biomolecules (Xu and Chang 2007). The ethyl acetate fraction (EAF) of *T. cacao* root-bark exhibited the highest total phenol and flavonoid contents, probably due to greater extraction of these antioxidant compounds. Flavonoids and its subclasses have been reported to be the most common and widely distributed group of plant phenolics (Pietta, 2000).

Iron is an essential metal for normal cellular physiology and plays a catalytic role in the inhibition of free radical reactions (Ferrati *et al*., 1992). Halliwell *et al* (2002) has shown that increased cytosolic and mitochondrial iron loading is associated with oxidative damage by increasing superoxide production. The ferric reducing antioxidant power (FRAP) of *T. cacao* extract/fractions is associated with the actions of reductants, which exerts antioxidant activity by free radical chain breaking via donating electron, hence reducing Fe^{3+}/ferricyanide complex to a ferrous state (Fe^{2+}). The higher the FRAP value, the greater the antioxidant activity (Table 1).

DPPH assay of an extract measures its ability to donate hydrogen electron to DPPH-radical to become paramagnetic molecule. This results in bleaching of the DPPH solution, after dark incubation and is reflected by subsequent decrease in absorbance at 517 nm with a lower $1C_{50}$ value. The bleaching action of a compound is a direct measure of its antioxidant activity. The IC_{50} values of the fractions which were 26.93 ± 0.86 (ascorbic acid), 29.61 ± 0.42 (EAF), 49.94 ± 4.12 (n-BF), 76.21 ± 6.76 (AqF) and 112.84 ± 2.02 (n-HF) µg/ml respectively implied that the *T. cacao* root bark exhibited higher DPPH-radical- scavenging activity (Figure 1). Previous studies have shown that aqueous and ethanolic extracts of *Icacina tricantha* as well as the extract and fractions of *Lantana camara* protected red blood cells against both heat and hypotonic induced lyses and also exhibited appreciable DPPH-radical scavenging and anti-oxidant activities (Egemole *et al*., 2007; Oyedapo *et al*., 2010).

Inflammation is the response of living tissues to injury. Acute and chronic inflammations are complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair (Halliwell *et al*., 2002, Michalak, 2006). The viability of cells depends on the integrity of their membranes. The exposure of erythrocytes to injurious substances such as hypotonic medium and heat resulted in lyses of its membrane due to turgidity (and haemolysis) and oxidation of haemoglobin (Sadique *et al*., 1989). Such injury to RBC membrane would further render the cell more susceptible to secondary damage through free radical lipid peroxidation (Sadique *et al*., 1989; Roome *et al*., 2008). Anti-inflammatory agents exert their effects through a variety of mechanisms such as inhibition of platelet granulation, uncoupling of oxidative phosphorylation, inhibition of denaturation of protein, erythrocyte and lysosome membrane stabilization, inactivation of ATPase, inhibition of proteases and cyclooxygenases (Dorfman and Dorfman, 1968; Kalyanpur *et al*., 1968; Kumar and Sadique 1987; Ahmed *et al*., 1993; Oyedapo *et al*., 1999).

The stabilization of erythrocyte membrane exposed to both heat and hypotonic induced lyses by fraction of *T. cacao* root-bark was investigated. The fractions exhibited a dose dependent membrane stabilizing activity over ranges of concentration tested, with maximum stability of 83.2 % (EAF), 74.4% (n-BF), 68.3 % (AqF) and 31.1 % (n-HF). The response of the fractions on RBC was biphasic; confirming the earlier observations (Oyedapo *et al*., 2004; Falade *et al*., 2005). The result compared

favourably with the activity of acetaminophen, a non-steroidal anti-inflammatory standard drug used, which exerted maximum percentage stability of 48.1 % (Figure 2).

The liver is an organ that performs many essential metabolic activities such as protein synthesis, carbohydrate and lipid metabolism, detoxification of various metabolic products

Table 1: Concentrations of Total Phenol, Total Flavonoid and Ferric Reducing Antioxidant Power

Fractions	TPC (mg TAE/g)	TFC (mg RE/g)	FRAP (µg AAE/ml)
Crude	133.79 ± 0.00	304.13 ± 0.01	31.78 ± 0.53
n-HF	0.82 ± 0.00	8.36 ± 0.00	2.98 ± 0.93
EAF	50.93 ± 0.00	458.25 ± 0.01	74.52 ± 2.37
n-BF	35.00 ± 0.00	186.44 ± 0.00	30.86 ± 0.87
AqF	5.01 ± 0.00	31.50 ± 0.00	11.92 ± 0.92

Each value represented the Mean ± SEM, n = 3 readings. Total Phenol Contents (TPC) were expressed as mg TAE/g (tannic acid equivalent), Total Flavonoid Contents (TFC) as mg RE/g (rutin equivalent) and Ferric Reducing Antioxidant Power (FRAP) as µg AAE/ml (microgram ascorbic acid equivalent) extract respectively. n-HF (hexane fraction); EAF (ethyl acetate); n-BF (butanol fraction) and AqF (aqueous fraction).

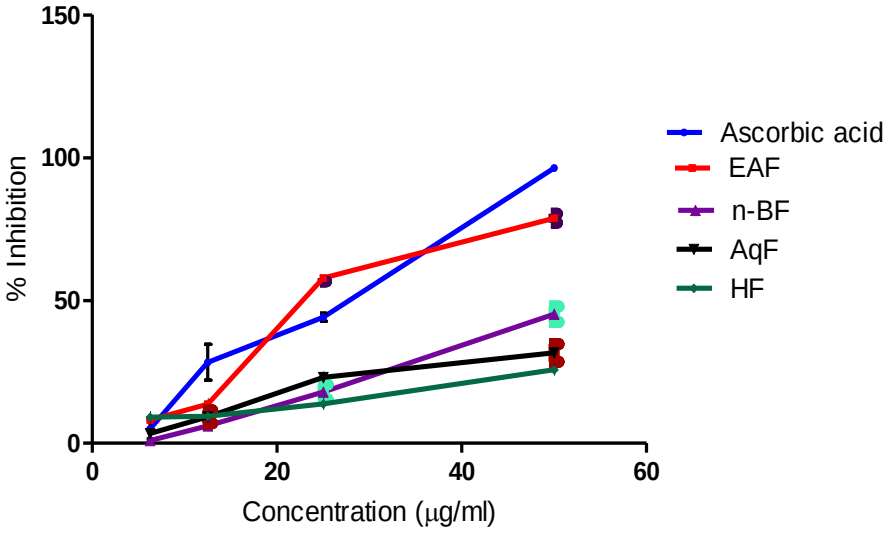


Fig. 1: DPPH-radical scavenging activity
Each value represented Mean ± SEM, n = 3 readings.
EAF (ethyl acetate fraction); n-BF (n butanol fraction); AqF (aqueous fraction) and HF (hexane fraction.)

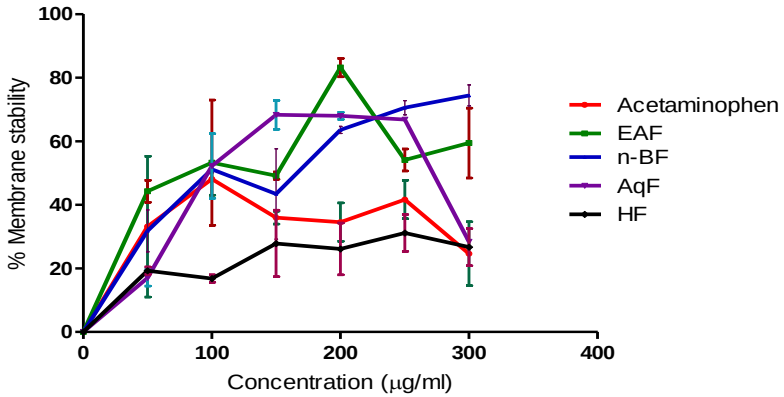


Fig. 2: Membrane Stabilizing Profile of the fractions.

Each value represented Mean ± SEM, n=3 readings.

EAF (ethyl acetate fraction); n-BF (n-butanol fraction); AqF (aqueous fraction) and HF (hexane fraction).

storage of various compounds necessary for body metabolism. Total protein concentrations as well as aminotransferases activities (ALT and AST) are good biomarkers of hepatic function. Aminotransferases catalyze the transfer of amino group from an amino acid to the oxoglutaric acid, a process that is essential in metabolism of amino acid. ALT is found in high concentration in the liver. The heart, liver, skeletal muscle and kidney are rich in AST. Increase in plasma ALT activity could be an indication of liver damage. Since AST is found in other tissues other than the liver, any increase in plasma AST activity may be attributed to necrosis and/ or inflammation of the heart, liver, kidney and skeletal muscle tissues.

The investigation revealed that the liver ALT activity in the normal treated, salt-treated and untreated rats as well as the control group increased ($P < 0.05$); while decreased ALT activity was observed in the plasma. On the other hand, the AST activity in the liver of the groups decreased. The elevated plasma AST activity could be attributed to necrosis or inflammation of the heart, kidney and/ or skeletal muscle tissues. Also, the study revealed increased liver protein concentrations in the normal treated, salt-treated and untreated rats; as well as in the control group (Figures 3- 5). This is an indication of normal hepatic function, striking a balance between the rate of protein synthesis and its degradation.

An imbalance between free radical generation and antioxidant status results in oxidative stress. Plant-based dietary antioxidants are believed to play an important role in the maintenance of human health, because human endogenous antioxidants provide insufficient protection against ROS (Halliwell *et al*., 2002). Several metalloenzymes called antioxidant enzymes (such as glutathione peroxidase, superoxide dismutase, and catalase) and non-enzymatic anti-oxidants (glutathione, selenium, vitamins C and E) are capable of scavenging ROS generated within the endogenous system. These enzymes are located within the cytoplasm, peroxisomes and other organelles, where they carry out their synergistic defense activities (Bojana *et al*., 2004). Lipid peroxidation of plasma membrane, a free radical reaction is associated with various pathological events such as atherosclerosis, inflammation and liver injury (Rice-Evans *et al*., 1996). The production of thiobarbituric acid reactive substances such as malondialdehyde (MDA) was used as a biomarker, to measure the level of oxidative stress in organisms. High MDA value is an indication of high rate of damage by free radical generation (Yen and Chen 1995; Rice-Evans *et al*., 1996).

The flavonoid fraction (250 mg) was able to decrease the level of MDA in the liver of the salt-treated rats, although at this concentration, high MDA was released in the normal-treated rats, compared to the control group. The untreated group had elevated levels of MDA (Table 2). The observation indicated that low concentration of flavonoid fraction of the extract was able to protect

the plasma membranes from free radical damage, while the consumption of high salt-diet alone could result in oxidative stress as it increased the amount of MDA generated.

The liver GSH activity of the normal- treated and salt-treated rats increased with increasing flavonoid concentration. The untreated group also had increased GSH activity, while the control group had decreased GSH activity (Table 2). This indicated that the flavonoid fraction of *T. cacao* root-bark is a potent antioxidant. The consumption of high salt-diet alone, may act as the activator of GSH, probably to create a balance between the level of free radical generation and the antioxidant status. The low GSH activity in the control group could be due to oxidative stress. The investigation confirmed the earlier observation (Noori *et al*., 2009) on the effect of cocoa seed powder on the liver GSH activity of normal rats.

Investigations of the liver glutathione peroxidase (GPx) activity of the rats revealed that normal rats treated with the extract decreased the GPx activities when compared with the control group. The salt group, treated with 250 mg of flavonoid fraction had significant ($P < 0.05$) increase in GPx activity when compared with the decreased activity observed in the untreated group (Table 2). The results indicated that the extract is a potent antioxidant as it enhanced the liver GPx activity of rats fed with high salt-diet while the consumption of high salt-diet alone could lead to oxidative stress as it decreased the enzyme activity.

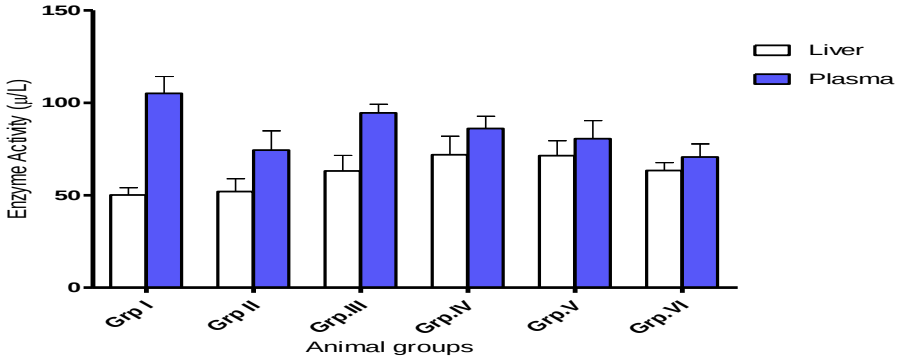


Fig. 3: The Liver and Plasma AST Activity Profile

Each value represented Mean ± SEM, n=5 replicates. Values of $P < 0.05$ were considered significantly different from the control. Bars without superscripts are non-significantly different from to the control.

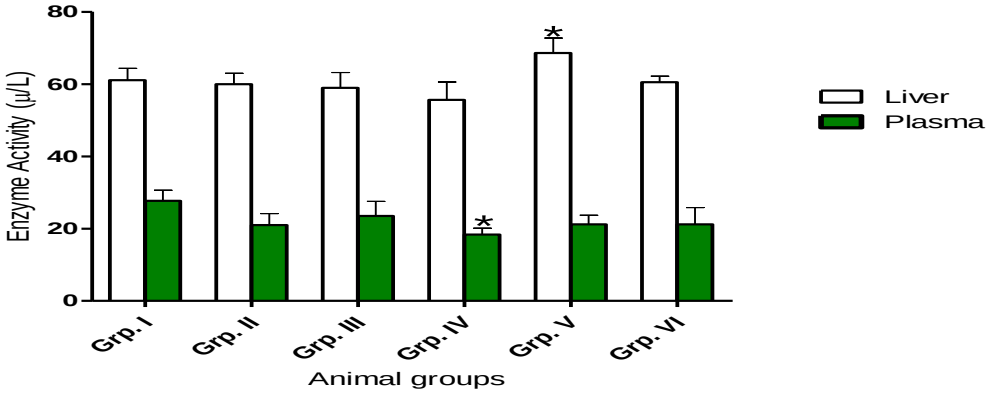


Fig. 4: The Liver and Plasma ALT Activity Profile

Each value represented Mean ± SEM, n= 5 replicates. Values of $P < 0.05$ were considered significantly different from the control. Bars with same superscripts are significantly different from the control, while those without superscripts are not significantly different.

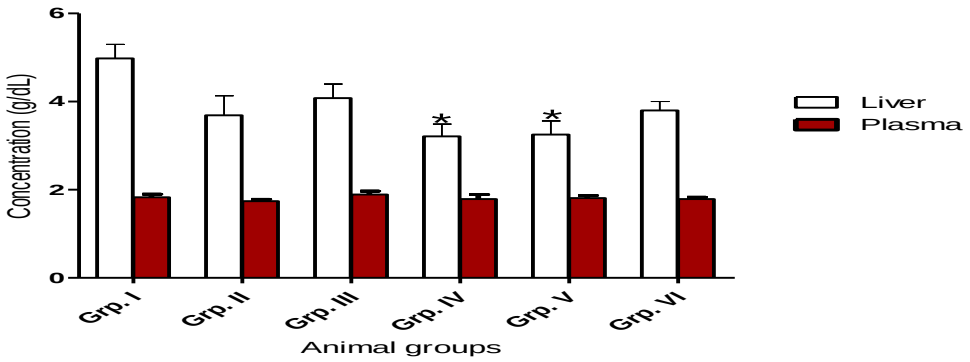


Fig .5: Liver and Plasma Protein Concentrations

Each value represented Mean ± SEM, n=5 replicates. Values of $P < 0.05$ were considered significantly different from the control. Bars with same superscripts are significantly different from the control, while those without superscripts are not significantly different from the control.

Table 2: Liver antioxidant enzymes of normal treated, salt treated and untreated rats

Group	MDA (µmol./L)	GSH (µg/ml)	GPx (µmol/mg protein)	SOD (µmol/min/mg protein)	CAT (µmol/min/mg protein
I	21.90 ± 1.56	0.61 ± 0.14	27.60 ± 1.21	3.34 ± 0.80	145.6 ± 2.8
II	214.00 ± 8.28^{a}	0.81 ± 0.10^{b}	19.60 ± 2.89^{b}	3.33 ± 0.61^{a}	153.9 ± 4.2
III	24.20 ± 1.41^{b}	1.32 ± 0.12^{a}	16.10 ± 1.70^{a}	3.71 ± 0.17^{b}	152.1 ± 3.2
IV	27.00 ± 2.33^{c}	1.47 ± 0.07^{a}	86.60 ± 1.18^{a}	4.97 ± 0.35^{c}	157.3 ± 4.4
V	230.00 ± 6.37^{a}	1.55 ± 0.01^{a}	17.30 ± 2.52^{c}	3.54 ± 0.51^{d}	154.7 ± 6.9
VI	214.00 ± 5.74^{a}	2.08 ± 0.12^{a}	2.46 ± 0.55^{a}	3.19 ± 0.07^{e}	173.1 ± 4.3^{a}

Each value represents mean ± SEM of five replicates. Values with superscripts different from the control are significantly different ($P<0.05$)

In addition, the results on the liver SOD activities revealed that there was no significant difference between the SOD activities of normal-treated, salt-treated and the untreated groups, compared to the control group. Also, there was no significant difference between the SOD activities of the salt-treated and untreated rats. This indicated that the extract and/or high salt-diet individually or in combination had no effects on the liver SOD activities of the rats (Table 2).

In the study, the liver CAT activities of the rats were investigated. Non-significant increase in CAT activity was observed in the normal-treated groups compared to the control group. Also, non-significant increase in the activity was observed in the untreated group compared to the salt-treated groups (Table 2). It implied that the flavonoid fraction had no significant effect on the CAT activity of the rats. However, high salt-diet alone activated the metalloenzyme (CAT) activity against free radicals, either generated or from other sources, probably to maintain a balanced redox status.

The kidney performs the function of excreting waste products of metabolism, osmoregulation and maintenance of the amount of different constituents of the plasma. The functional unit of the kidney is the nephron, which is responsible for the

glomerular ultra-filtration of blood, selective reabsorption of molecules and tubular secretion of hormones. Failure in the normal function of the kidney can lead to the syndrome of acute renal failure.

Histological investigation revealed that in the kidney tissues of the normal-treated, salt-treated (250 mg extract) and the control rats, the glomerulus and glomerular spaces were retained by the visceral and parental layers and well nucleated (Plate 1), which indicated that the tissues morphology were intact. The salt-treated (500 mg extract) and the untreated rats had deranged glomerular spaces, hypercellularity of glomerular messengeal cells and scanty nucleus (Plate 1), which could be an inflammatory response that could lead to free radical generation and cell damaging. The speculated findings in this study revealed that sub-chronic administration of cocoa root-bark extract slightly reduced the body weights of salt-treated rats. There was no observable effect in the normal-treated rats, while the untreated group had slight increased body weight (Figure 6). The fluctuation in weights could be due to change in oxidative status of the liver, formation of complex between the methylxanthines of cocoa and salt ions, which could have either reduced the rate of animal food intake, increased the utilization of stored fuels or inhibit the rate of fatty acid synthesis compared to the slight increase in the body weight observed, when either the extract and/or salt-diet were independently investigated during the study.

In conclusion, the root of *T. cacao* possessed large spectrum of bioactive antioxidants that could be beneficial for the management of inflammatory and oxidative related conditions elicited by high-salt diets.

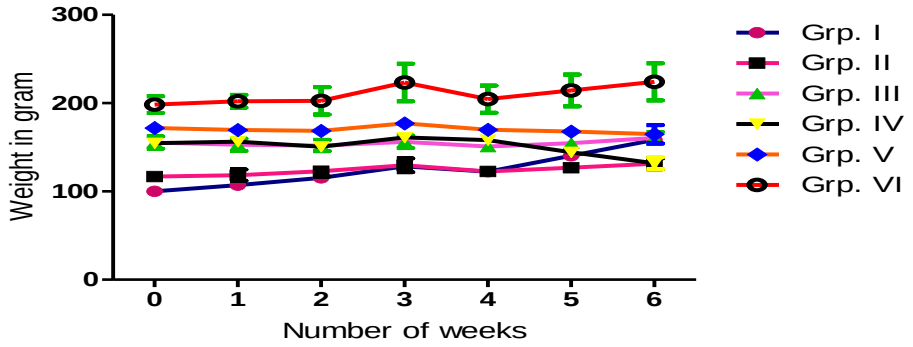
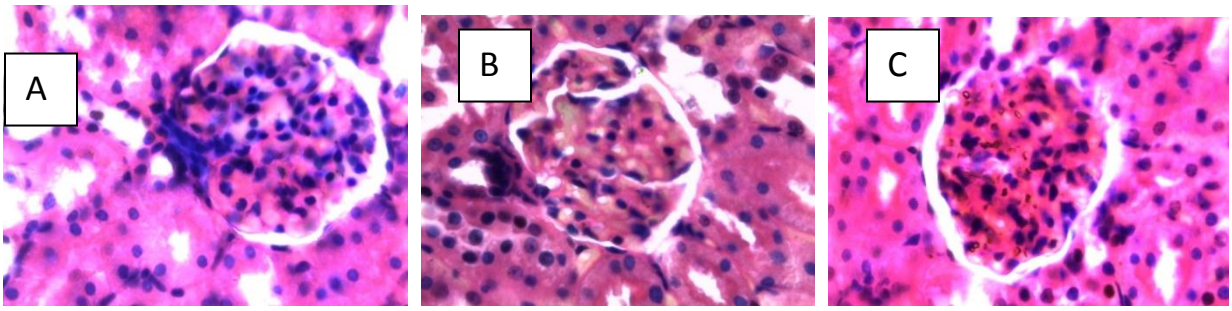


Fig .6: Weights of animals before, during and after treatment.

Week 0: (before treatment of rats). Weeks 1-3: (when groups IV, V and VI rats were treated with salt-diet/salt-water). Weeks 4 – 6: (when groups II and III were treated with the extract while groups IV and V were treated with the extract in addition to the salt-diet/salt-water and the group VI rats were constantly fed with salt-diet/salt-water only).



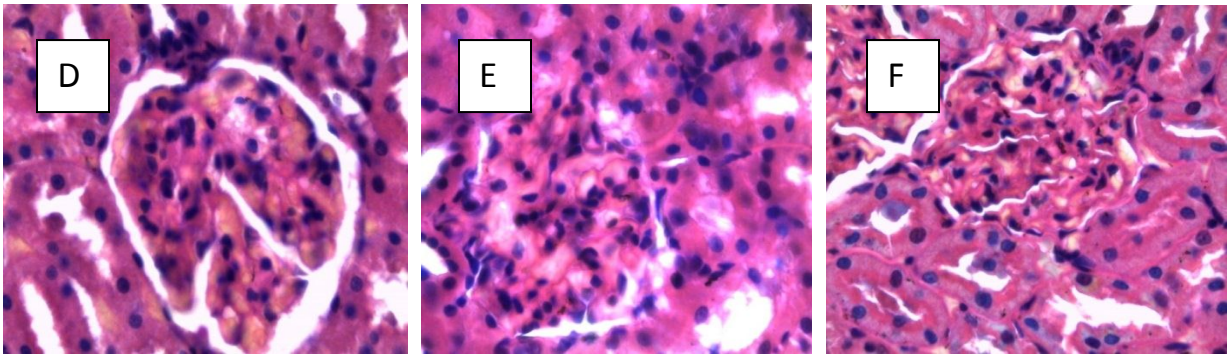


Plate 1: The Histological morphology of the cross section of rat kidney tissues (x 150).

A: Control (salt-free diet/water); B: (salt-free-diet/water + 250 mg/kg bwt flavonoid fraction); C: (salt-free diet/water + 500 mg/kg bwt flavonoid fraction); D:(4% salt-diet/1 % salt-water + 250 mg/kg bwt flavonoid fraction); E:(4 % salt-diet/1 % salt-water + 500 mg/kg bwt flavonoid fraction); F: (4 % salt-diet/1 % salt-water only).

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