



Hepatoprotective and Antioxidant Effect of Hibiscus Polyphenol Rich Extract (HPE) Against Carbon Tetrachloride (CCL₄) –Induced Damage in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author AOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AA managed the analyses of the study and the literature searches and wrote the final draf. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: *Hibiscus sabdariffa* is a medicinal plant that is consumed for its health benefits in Africa. The study was designed to investigate the hepatoprotective potentials of Hibiscus polyphenolic rich extract (HPE), (a group of phenolic compounds occurring in the dried calyx of *Hibiscus sabdariffa*) against CCl₄-induced damaged in rats.

Place and Duration of Study: Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomosho, Nigeria, between January 2011 and June 2012.

Methodology: Liver injury was measured in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and thiobarbituric acid reactive substances (TBARS) along with reduction of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH). The antioxidant activity of HPE was evaluated using DPPH and ABTH radical scavenging assay *in vitro*.

Results: The antioxidant investigation showed that HPE was able to scavenge the ABTS and DPPH radicals and these radicals scavenging abilities were found to be dose-dependent. Pretreatment of rats with different doses of HPE (50 and 100 mg/kg)

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significantly lowered serum ALT, AST, ALP, LDH and TBARS levels in CCl₄ treated rats. GSH, SOD and CAT were significantly increased by pre-treatment with the HPE, in CCl₄ treated rats. HPE was found to contain high level of total phenolic content (140.78mg/g in GAE/g dried weight). Hence, these data indicate that the dietary supplement of Hibiscus extract may inhibit liver damage in rats.

Conclusion: The hepatoprotective activities observed in this study could be due to the ability of phenolic compounds to neutralize the free radicals produced from the metabolism of CCl₄.

Keywords: *Hibiscus sabdariffa*; antioxidants; lipid peroxidation; carbon tetrachloride; hepatoprotective.

1. INTRODUCTION

Human exposure to xenobiotics on a daily basis is causing serious health problems in many countries of the world. Liver damage can be caused by certain xenobiotics and microbial infiltration from ingestion or infection [1,2]. Most current modern medical options for the treatment of liver disease are associated with serious complications. For instance, treatment with antiviral drugs, steroids and vaccines, has been reported to be with poor therapeutic success and may be associated with severe risks of toxicity [2].

In many parts of the world where the reliable liver protective drugs are scarce and expensive, a number of medicinal plants may be alternative options for the treatment of liver disorders [3,4,5]. HPE is one of such important herbal hepatoprotective drug. *H. sabdariffa* is a natural phenolic rich plant that has been reported to have a wide range of pharmacological properties, such as antioxidant activity and free radical scavenging capacity. HPE extracted from *H. sabdariffa* fruits is a mixture of phenolic compounds [6] and its antioxidative effects were determined in this study. HPE which is a group of phenolic compounds was isolated from the dried flowers of *H. sabdariffa*. Previous studies showed that the constituents of the extract of *H. sabdariffa* have strong antioxidant properties [6,7]. It has been postulated in several studies that effectiveness of plant extracts against many diseases are due to their antioxidant actions [8-10]. Oxidative stress and liver damage have been induced in rats in many studies with CCl₄. CCl₄ is a toxic substance that is used to induce liver damage in laboratory animals [11,12]. CCl₄ is biotransformed by the Cytochrome P₄₅₀ in the liver endoplasmic reticulum to the highly reactive trichloromethyl free radical [13]. CCl₄ can damage a number of tissues particularly the liver and kidney of many species [14]. It has been hypothesized that one of the principle causes of CCl₄ induced liver injury is lipid peroxidation induced by free radical derivative of CCl₄. Thus, antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl₄ induced liver injury [15]. Based on the reported strong antioxidant potentials of *H. sabdariffa*, the possibility of HPE in the prevention of CCl₄-induced toxicity is hereby investigated in this study.

In previous studies, *Hibiscus sabdariffa* extracts showed antioxidant, antigenotoxic and biomodulatory effects on various cells and animals exposed to different agents [6,8,9,10,16]. Literature reviews indicated that the hepatoprotective activity of *Hibiscus sabdariffa* has been sparingly evaluated so far. However, it is unclear if HPE also has protective effects against CCl₄ toxicity. Therefore, this study investigated the antioxidant effect of HPE *in-vitro* as well as examining its protective effect on the CCl₄-induced liver damage in rats.

2. MATERIALS AND METHODS

2.1 Reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sufonic acid) ABTS, gallic acid, thiobarbituric acid (TBA), nicotinamide adenine dinucleotide reduced (NADH) were obtained from Sigma–Aldrich Chemical Co. Ltd. (England). Nitrobluetetrazolium (NBT), 5,5-dithiobisnitro benzoic acid (DTNB) was obtained from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

2.2 Plant Material

The dried calyx of *Hibiscus sabdariffa* was purchased at Sabo market in June 2011, in Ogbomoso. The identification and authentication of the plant was done by Dr A.J. Ogunkunle at Department of Pure and Applied Biology, Ladoko Akintola University of Technology, Ogbomoso, where a specimen was deposited in the herbarium. The dried fruits were further dried at room temperature and blended to a coarse powder.

2.3 Preparation of Hibiscus Polyphenol Rich Extract (HPE)

HPE was prepared according to the method of Lin et al. [17]. Briefly 100 g of *Hibiscus sabdariffa* powder were extracted three times with 300 mL of methanol at 50°C for 3 h. The samples were filtered after each extraction and the solvent was removed from the combined extracts with a rotary evaporator at 37°C. The residue was then dissolved in 100 mL of water and extracted with 200 mL hexane to remove some of the pigments (i.e. chlorophyll, caratenoids). The aqueous phase was extracted three times with 180 mL ethyl acetate, and the ethyl acetate fraction was evaporated using rotary evaporator at 37°C. The residue was re-dissolved in 250 mL water and was lyophilized to obtain 1.5 g of HPE and stored at -20°C before use.

2.4 Determination of Total Phenolic Compounds in HPE

The content of total phenolic compounds in HPE was determined by Folin–Ciocalteu method as described by Miliuskas et al. [18]. Briefly, 1 mL aliquots of 0.03, 0.08, 0.10 and 0.30 mg/ml ethanolic gallic acid solutions were mixed with 5 mL Folin-ciocalteu reagent (diluted ten-fold) and 4 mL (75 g/L) sodium carbonate. The absorption was read after 30 min at 20°C at 765 nm to prepare the calibration curve. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated using the following formula:

$$C = c \cdot V/m'$$

Where: C-total content of phenolic compounds, mg/g plant extract, in GAE; c-the concentration of gallic acid established from the calibration curve, mg/ml; V- the volume of extract, ml; m' - the weight of pure plant methanolic extract, g.

2.5 Trolox Equivalent Antioxidant Capacities (TEAC) with Manganese Dioxide

The assay was performed as described by Schelesier et al. [19]. The ABTS radical cation was prepared by filtering a solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2 µm syringe filter. This solution was diluted in 5 mM PBS pH 7.4, adjusted to an absorbance of 0.700 ± 0.020 at 734 nm and preincubated at room temperature prior to use for 2 h. 1 mL of ABTS^{•+} solution and various concentrations of the extracts (diluted with water) were vortexed for 45 seconds in reaction tubes, and the absorbance (734 nm) was taken exactly 2 minutes after initiation of mixing. PBS blanks were run with each assay. The antioxidant activity of the extracts was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\% \text{ antioxidant activity} = ((A_{(ABTS^{\bullet+})} - A_{(Extracts)}) / (A_{(ABTS^{\bullet+})}) \times 100.$$

2.6 Trolox Equivalent Antioxidant Capacities with Potassium Persulfate

The assay was performed essentially as described by Re et al. [21]. ABTS radical cation was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–24 h before use. The ABTS^{•+} solution was diluted with water for the hydrophilic assay and with ethanol for the lipophilic assay and adjusted to an absorbance of 0.700 ± 0.020 at 734 nm. For the photometric assay, 1ml of the ABTS^{•+} solution and various concentrations of the extracts were mixed for 45 seconds and measured immediately after 1 min at 734 nm. The antioxidant activity of the extracts was calculated by determining the decrease in absorbance at different concentrations by using the following equation.

$$\% \text{ antioxidant activity} = ((A_{(ABTS^{\bullet+})} - A_{(Extracts)}) / (A_{(ABTS^{\bullet+})}) \times 100.$$

2.7 DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

Briefly, 1.95 ml DPPH[•] solution (2.4 mg/ 100 mL methanol) and 50 µL antioxidant solution were mixed. At first, the absorbance of DPPH[•] was measured as blank, then the antioxidant solution was added and mixed. The reaction was measured at 5 min interval at 515 nm until $\Delta A = 0.003 \text{ min}^{-1}$ [21]. The anti-oxidative activity was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\% \text{Inhibition activity} = ((A_{(DPPH^{\bullet})} - A_{(Extracts)}) / (A_{(DPPH^{\bullet})}) \times 100$$

2.8 Animal model and Experimental Design

Twenty four male Wistar albino rats (180-220 g) were obtained from the animal house at LAUTECH Agricultural Department, Ogbomoso, Oyo state and were maintained under standard environmental conditions and had free access to feed and water. Animal studies were approved by the Committee for Ethical Animal Care and Alternatives to Animal Use in Research, Testing, and Education of 1986. Seven days after acclimatization, the rats were divided into four groups with six rats each. Group 1 served as control, group 2 received single dose of equal mixture of carbon tetrachloride (CCl₄) and olive oil (50%, v/v, 1.25 ml/kg i.p.) on the 7th day. Group 3 and 4 animals were treated with Hibiscus polyphenol rich extract (HPE) at dose level of 50 and 100 mg/kg per day p.o., respectively for 7 days and on

the 7th day; a single dose of an equal mixture of carbon tetrachloride and olive oil (50% v/v 1.25 ml/kg i.p.) was administered.

2.9 Preparation of Serum and Liver Homogenates

Twenty-four hours after the animals were administered with a single dose of CCl₄, they were sacrificed by cervical dislocation. Blood samples of each animal were collected by heart puncture and were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 4000Xg for 5 min and analyzed for various biochemical parameters (serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), and serum lactate dehydrogenase (LDH)). The liver samples were cut into small pieces and homogenized in phosphate buffer saline (PBS) to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 12,000 rpm for 15 min. The supernatant obtained was used for assay of superoxide dismutase, catalase, reduced glutathione, thiobarbituric acid reactive substances (TBARS) content, and protein estimation.

2.10 Biochemical Analysis

Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in serum were determined using enzymatic kits (Labkit, Spain) according to the manufacturer's instructions.

2.11 Measurement of Hepatic Lipid Peroxidation

MDA levels were measured by the double heating method Draper and Hadley, [22]. The method is based on spectrophotometric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 0.5 mL of liver homogenate was mixed with 2.5 mL of trichloroacetic acid (TCA, 10%, w/v) solution followed by boiling in a water bath for 15 min. After cooling to room temperature, the samples were centrifuged at 3000 rpm for 10 min and 2 mL of each sample supernatant was transferred to a test tube containing 1mL of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm with respect to the blank solution. The concentration of MDA was calculated based on the absorbance coefficient of the TBA-MDA complex ($\epsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and it was expressed as nmol/mg protein.

2.12 Superoxide Dismutase Activity Assay

SOD activity was measured according to method of Kakkar et al. [23]. Assay mixture contained 0.1 mL of supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 mL of phenazine methosulphate (186 μM), 0.3 mL of nitrobluetetrazolium (300 μM) and 0.2 mL of NADH (750 μM). NADH was added to the mixture and after incubation at 30°C for 90 s, the reaction was stopped by addition of 0.1 mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 mL of *n*-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as that amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

2.13 Determination of Reduced Glutathione

GSH was assayed by the method of Aebi [24], with slight modification. An aliquot of 0.5 mL of each tissue homogenate was precipitated with 0.5 mL of trichloroacetic acid (10% w/v). The precipitate was removed by centrifugation. 0.8 mL of the filtered sample was mixed with 0.3 DTNB (4 mg/mL) and 0.9mL phosphate buffer (0.1 M, pH 7.4). The yellow colour developed was read at 412 nm. Reduced glutathione was expressed as µg/mg of protein.

2.14 Catalase Assay Activity

Catalase activity was measured by the method of Aebi [24]. An aliquot (10µl) of each tissue supernatant was added to cuvette containing 1.99 µl of 50mM phosphate buffer (pH 7.0). Reaction was started by addition of 1000µl of freshly prepared 30mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometric ally at 240 nm. Activity of catalase was expressed as U/mg of protein.

2.15 Statistical Analysis

The results were recorded as means ± SEM from five experiments and analyzed with the use of student's t-test. Differences were considered to be statistically significant at p<0.05.

3. RESULTS AND DISCUSSION

3.1 Trolox Equivalent Antioxidant Capacity (TEAC) Assays of Trolox, Gallic Acid and Hibiscus Polyphenol Rich Extract (HPE)

In the three versions of the TEAC assay; the TEAC value of Trolox is 1.00. Gallic acid responded in all the assays as the strongest antioxidant, but the TEAC value analyzed in TEAC III (hydrophilic version) was higher when compared with the value obtained under TEAC II and TEAC III (lipophilic version), in addition, the two versions also showed comparable antioxidant activity both in gallic and HPE (Table 1).

Table 1. Trolox Equivalent Antioxidant Capacity (TEAC) assay of Trolox, Gallic acid and Hibiscus polyphenol rich extract (HPE)

Assay/Antioxidant	Trolox	Gallic	HPE
TEAC II	1.00	4.40	1.50
TEAC III(hydrophilic)	1.00	5.78	1.63
TEAC III (lipophilic)	1.00	4.25	1.42

3.2 Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity and Total Phenolic Content of HPE Extracts

The Hibiscus polyphenol rich extract (HPE) demonstrated a concentration dependent scavenging activity by quenching DPPH radicals (data not shown) and was compared with gallic acid, as a positive control. The IC₅₀ values for DPPH scavenging by HPE and gallic acid were 140.86 µg/mL and 16.33 µg/mL respectively (Table 2). The total amount of phenolic compounds present in HPE was found to be 104.78 mg/g in gallic acid equivalent (Table 2).

Table 2. Total phenolic content and DPPH radical scavenging value of HPE

Sample	Total phenol ^a	DPPH scavenging activity (IC 50) ^b
HPE	104.8 ± 3.2	140.9 ± 8.2
Gallic	ND	16.3 ± 1.5

Each value represents the mean ± SEM. (n=3).

^aTotal phenolic content was expressed as mg gallic acid equivalents/g dried extract.

^bExpressed as µg/mL; ND, not detected

3.3 Effect of Treatment with Carbon Tetrachloride on the Levels of AST, ALT and ALP Activities

CCl₄ treatment resulted in significant ($p < 0.05$) rise in the levels of AST, ALT and ALP when compared to the control group (Fig. 1). Oral administrations of HPE at two different doses (50mg/kg and 100mg/kg) lower the levels of these marker enzymes, namely, AST, ALT and ALP significantly ($p < 0.05$).

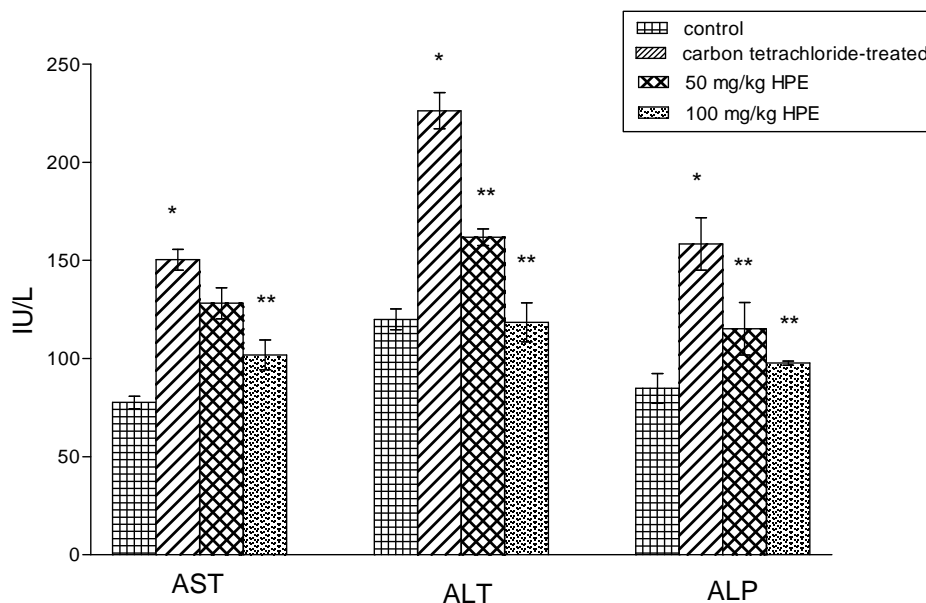


Fig.1. Effect of HPE on serum levels of AST (IU/L), ALT (IU/L) and ALP (IU/L) during CCl₄ treated oxidative stress in rats. Values are mean ± SEM. *Group 2 (CCl₄ treated rats) compared with Group 1 (control rats). **Groups 3 and 4 (HPE treated rats) compared with Group 2 (CCl₄ treated rats)

3.4 Effect of Treatment with CCl₄ on the Level of Lactate Dehydrogenase Activity

CCl₄ treatment resulted in significant ($P < 0.05$) rise in the level LDH when compared to the control group (Fig. 2). Oral administrations of HPE at two different doses (50mg/kg and 100mg/kg) lower the level of this marker enzyme, LDH significantly ($p < 0.05$).

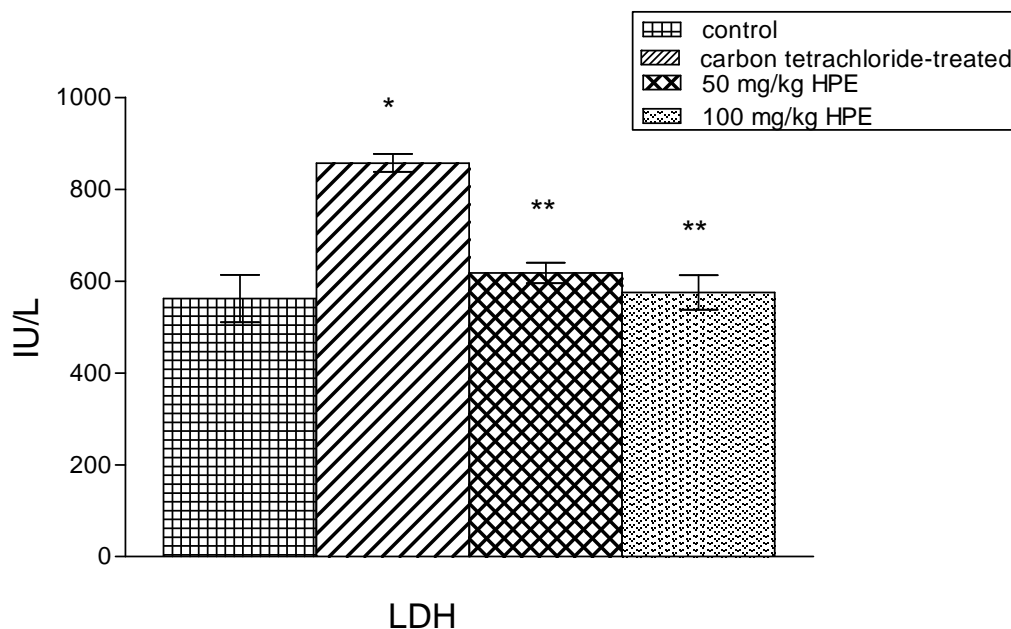


Fig. 2. Effect of HPE on serum levels of LDH (IU/L), during CCl₄ induced oxidative stress in rats. Values are mean ± SEM. *Group 2 (CCl₄ treated rats) compared with Group 1 (control rats). **Groups 3 and 4 (HPE treated rats) compared with Group 2 (CCl₄ treated rats)

3.5 Effect of Treatment with Carbon Tetrachloride on the Levels of Superoxide Dismutase and Reduced Glutathione

Administration of CCl₄ caused a significant ($P < 0.05$) decrease in SOD and GSH levels in rats when compared with normal animal. The HPE at 50 and 100 mg/kg showed significant ($P < 0.05$) increase in SOD and GSH levels when compared to CCl₄ treated rats (Fig. 3).

3.6 Effect of Treatment with CCl₄ on the Level of Catalase Activity and Thiobarbituric Acid Reactive Substance

The CCl₄-treatment caused significant ($P < 0.05$) decrease in the level of CAT in liver homogenate tissue, when compared with control group (Fig. 4). The pretreatment of plant extract at the dose of 50 and 100 mg/kg resulted in significant ($P < 0.05$) increase of CAT level when compared to CCl₄ treated rats. Rats treated with CCl₄ showed significant increase in lipid peroxidation level (LPO) of liver homogenates when compared to the rats in control group as shown in Fig. 5. Pretreatment with HPE at two different doses (50mg/kg and 100mg/kg) significantly reduced the LPO level when compared with CCl₄ treated rats.

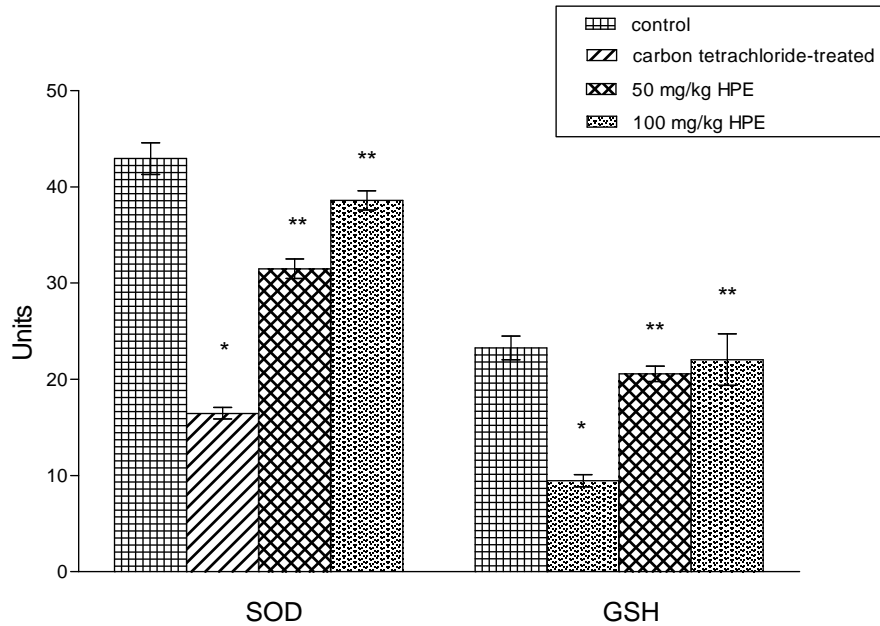


Fig. 3. Effect of Hibiscus polyphenol rich extract on hepatic level of SOD (U/mg protein) and GSH μ g/mg protein) during CCl₄ induced oxidative stress in rats. Values are mean \pm SEM. *Group 2 (CCl₄ treated rats) compared with Group 1 (control rats). **Groups 3 and 4 (HPE treated rats) compared with Group 2 (CCl₄ treated rats)

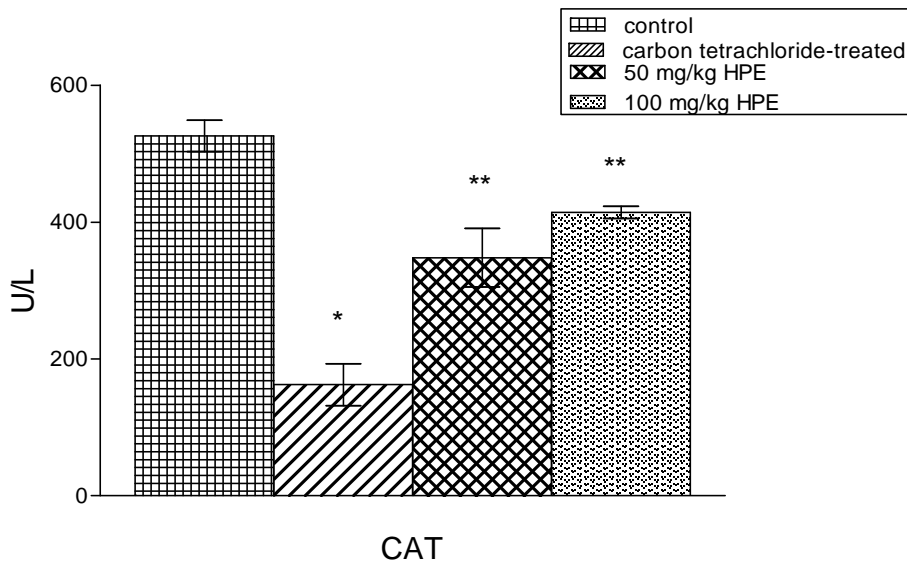


Fig. 4. Effect of Hibiscus polyphenol rich extract on hepatic level of catalase (U/mg protein) during CCl₄ induced oxidative stress in rats. Values are mean \pm SEM. *Group 2 (CCl₄ treated rats) compared with Group 1 (control rats). **Groups 3 and 4 (HPE treated rats) compared with Group 2 (CCl₄ treated rats)

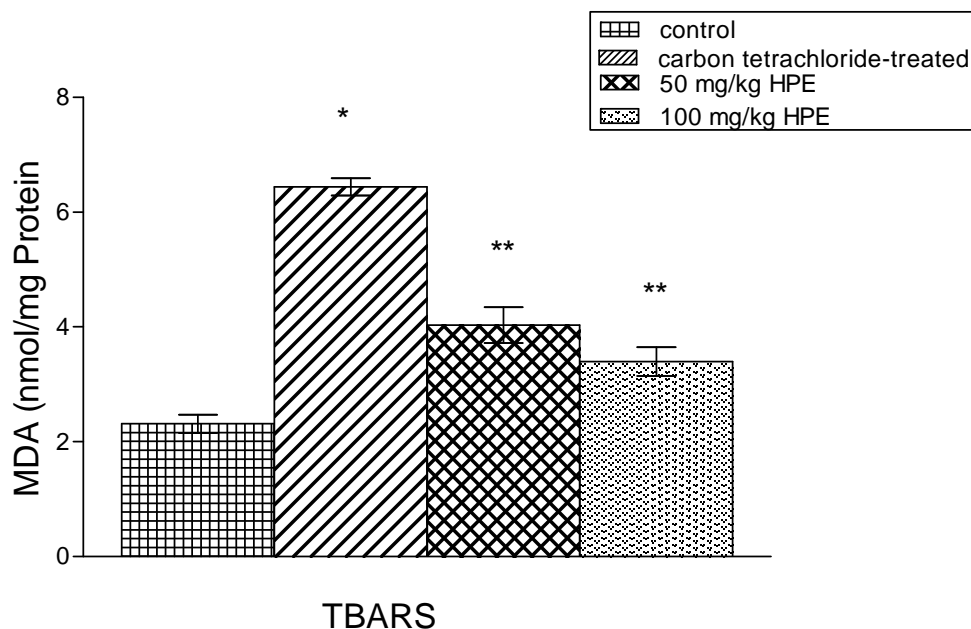


Fig. 5. Effect of Hibiscus polyphenol rich extract on hepatic level of thiobarbituric acid reactive substance (TBARS) (nM/mg protein) during CCl₄ induced oxidative stress in rats. Values are mean \pm SEM. * Group 2 (CCl₄ treated rats) compared with Group 1 (control rats). ** Groups 3 and 4 (HPE treated rats) compared with Group 2 (CCl₄ treated rats)

Phenolic compounds play an important role in plant resistance and defense against microbial infections which are intimately connected with reactive oxygen species (ROS) [25-26]. Polyphenolic compounds in plants also have the potential to prevent oxidative-damage related diseases including cancer and atherosclerosis [8,26].

PHE contains certain phytochemicals that are known to prevent ROS related diseases and thus, is examined for hepatoprotective potentials in rats *in vivo*. In this study, rat treated with a single dose of CCl₄ developed a significant hepatic damage and oxidative stress, which was observed from a substantial increase in the activities of serum AST, ALT, ALP and LDH. These are indicators of hepatocyte damage and loss of functional integrity. These results are in conformity with the reports of Yin et al. [28] and Ito et al. [29]. Any alteration in the activity of these enzymes causes tissue lesion and cellular impairment and dysfunction [2]. Decrease in the serum levels of these enzymes with HPE is an indication of the stabilization of plasma membrane as well as repair of liver damage caused by CCl₄. This observation is in agreement with the view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes [2,30].

In this study, there was a decreased in the activity of SOD and CAT due to hepatic injury caused by CCl₄. This could be linked to exhaustion of the enzyme as a result of oxidative stress caused by CCl₄ [2]. SOD and catalase activities were however, brought to near normal in rats pretreated with the extract at 50 mg/ml and 100 mg/ml prior to CCl₄-treatment. In addition, there was a decrease in liver reduced glutathione (GSH) level in rats treated with CCl₄ alone, but a dramatic rise in the level of liver GSH was observed in rats pre-treated with

HPE. The stabilization of these enzymes by the HPE is an indication of the improvement of the functional status of the liver. This might be related to the chemopreventive effects of HPE.

Lipid peroxidation has been postulated to be the destructive process in liver injury due to toxicant (CCl₄) [8]. In the present study, an elevation in the level of MDA in liver of animals treated with CCl₄ was observed. Enhanced lipid peroxidation expressed in terms of MDA (malonaldehyde) contents in CCl₄ treated rats confirmed hepatic damage by the earlier reports [6,9]. However, pre-treatment with HPE substantially reduce the level of lipid peroxidation. HPE is known to contain a considerable amount of phenolic content and exhibited strong free radical scavenging property in this study. Phenolics may efficiently reduce the excessive formation of free radical and maintain hepatic membrane integrity [26,27]. Consequently, the ability of the HPE extract to protect the rats against CCl₄ induced liver damaged may be attributed to the high antioxidant potentials exhibited in this study. This finding justifies the use of this HPE in traditional medicine in Africa for the treatment of liver diseases and supports the use of HPE in the prevention of chemically-induced toxicity.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Faculty of Basical Sciences, LAUTECH, Ogbomoso ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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