

Antioxidant Status, Organ Integrity and Lipid Profile of Carbon Tetrachloride Intoxicated Rats Following Pre-treatment with Methanolic Extract of *Crossopteryx febrifuga* Benth Leaf

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Authors' contributions

This work was carried out in collaboration between all authors. Author SEOA designed the study, wrote the protocol, interpreted the data and wrote the manuscript, while Authors SY, EO, PI, MO, HY and SDM anchored the animal study and laboratory analyses, performed preliminary data analysis and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To evaluate the antioxidant and organ protective activities *Crossopteryx febrifuga* Benth, in male albino rats.

Methods: Animals were intraperitoneally pretreated with the methanolic extract of the leaf at 10mg/kg dose before intoxication with CCl₄ (0.6mL/Kg). The hepatoprotective activity was assessed by measuring levels of aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), packed cell volume (PCV) and hemoglobin (Hb), while the antioxidant status was monitored by levels of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase, and kidney function was evaluated by levels of urea and creatinine. Total cholesterol and HDL-cholesterol were determined spectrophotometrically at 540nm.

Results: Compared to the CCl₄ control group, pre-treatment with methanolic extract of *C. febrifuga*

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leaves or vitamin E caused a significant ($p<0.05$) decrease in the levels of AST, ALT, urea, creatinine and total cholesterol with a concomitant boosting in the levels of SOD, catalase, HDL-cholesterol, PCV and hemoglobin concentrations.

Conclusion: These results suggest that *C. febrifuga* leaf contain substances that possess significant antioxidant, organ protective and hypolipidemic effects to justify its use in traditional medicine and further evaluation of its pharmacological properties.

Keywords: Antioxidant effect; hepatoprotective effect; *Crossopteryx febrifuga*; disease chemoprevention; medicinal plant; oxidative stress; lipid profile; haematological effect.

1. INTRODUCTION

Liver damage is one of the most serious diseases that have accompanied the adoption of modern food styles, liberal intake medications and exposure to many agricultural and environmental pollutants whose number is increasing by the day. There has been a sharp upward trend in the use of phytomedicines over the last decades in Europe and USA [1,2]. Hence, oriental and African herbal medicines have recently attracted the interest of modern scientific communities as alternative therapy.

African indigenous herbal medicines are widely used throughout the continent, despite an apparent lack of scientific evidence for their quality, safety and efficacy [3]. However, recent reports of African medicinal plants from our laboratories and elsewhere have shown that many Nigerian medicinal plants [4-6] and plant foods [7-8] possess therapeutic attributes, including antioxidant [9-11], anticancer [12] and hepatoprotective effects [13-14]. But many other Nigerian medicinal plants including *Crossopteryx febrifuga* Benth, are yet to receive such attention.

C. febrifuga Benth, (Family *Rubiaceae*), also known as ordeal tree in English or "Ohiapele" among the Igala speaking ethnic group of Central Nigeria, is a twisted tree with conspicuous tubular flowers, which is widely distributed throughout the Savannah region of Central, East and West Africa. Preparations of the tree is used in traditional medicine for symptomatic relieve of dry cough and for treatment of septic wounds, respiratory infections, fever, dysentery and pain [15-17]. In northern Nigeria, the plant has been used for treatment of liver related diseases, pain and malaria for generations, and its efficacy is widely acclaimed among the Hausa [18], Igala and other ethnic groups in Northern Nigeria. Previous studies using crude methanolic extract of *C. febrifuga* revealed that it contains biologically active substances with potential values in the treatment of trypanosomiasis,

malaria and infection caused by *Staphylococcus aureus* [19,20]. Apart from the aforementioned, there are fewer other documented data to our knowledge, on the activity profile of this important medicinal plant, especially with respect to the scientific evaluation of the plant for its acclaimed efficacy in the management of liver-related and oxidative stress-mediated diseases. Hence, this study was undertaken to evaluate the protective effect of the methanolic extract of the leaf of the plant against carbon tetrachloride induced oxidative stress and injury and thus validate its medicinal use.

2. MATERIALS AND METHODS

2.1 Chemicals

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, total cholesterol and high density lipoprotein cholesterol "HDL-cholesterol" kits were obtained from Randox Laboratory Ltd. U.K. Methanol, ethanol, alpha-tocopherol, carbon tetrachloride, thiobarbituric acid, trichloroacetic acid and other chemicals were obtained from Sigma-Aldrich Germany.

2.2 Sample Collection and Identification

The leaf of *C febrifuga* Benth were collected from Emere village in Ankpa Local Government Area of Kogi State, Nigeria. It was identified at the Herbarium Section of the National Institute for Pharmaceutical Research and Development (NIPRD), Idu-Abuja Nigeria, where a Voucher specimen with number 5878 was deposited.

2.3 Experimental Animals

Wistar strain of male Albino rats, body weight ranging from 160-200g bred in the Animal House of Department of Biochemistry, Ahmadu Bello University, Zaria were used. The rats were fed on pelleted commercial growers' mash (Vital feed, Jos, Nigeria). They were kept at room

temperature and were maintained *ad libitum* on tap water and growers mash (ECWA Feed, Bukuru-Jos, Plateau State, Nigeria) except in the last 15 hours before termination of the experiment. The animals were housed in plastic cages under conditions of 12h light/12h dark cycle and at 25°C. The rats were weighed prior to commencement and termination of the experiment. Experiments were performed in accordance with national and international standards regulating the handling and use of experimental animals.

2.4 Extraction of Plant Sample

The leaves of *C. febrifuga* Benth were collected and air-dried at room temperature and made into fine powder using mortar and pestle. Pulverized material (35g) was placed in the thimble of soxhlex extractor and extracted first, using petroleum ether (300mL) for 8 hours each and then methanol (300mL), three times for 5 hours each. The methanol extracts were combined and dried *in vacuo* at 45°C using a rotary evaporator, and were thereafter stored at -20°C until required.

2.5 Experimental Grouping and Treatment

The capacity of the extract to protect against hepatic injury and oxidative stress was investigated by randomly dividing the experimental rats into the following groups with six rats each: controls: solvent only (corn oil) and CCl₄; Reference Standards: vitamin E only (50 mg/kg), Vitamin E pre-treatment + CCl₄; *C. febrifuga* only (10 mg/kg) and *C. febrifuga* pre-treatment + CCl₄. All carbon tetrachloride treatments were performed at a dose of 0.6mL/kg from a 33.3% solution in corn oil. Animals were pre-treated with the extract (10 mg/Kg) for three days before intoxication with carbon tetrachloride (0.6mL/kg) that was administered one hour after the extract treatment on the third day [21,12-14].

2.6 Animal Sacrifice

All animals were sacrificed 24 hours following last administration of drug or *C. febrifuga* Benth extract. Animals were sacrificed under chloroform anesthesia and whole blood was collected and allowed to stand for two hours for collection of serum. The samples were kept in Eppendorf tubes and stored at -20°C until required for assay of biochemical parameters

[12-14,21]. The organs were immediately harvested, rapidly rinsed in ice-cold normal saline and immediately homogenized or stored at -20°C for analysis of malondialdehyde as indicator of lipid peroxidation.

2.7 Tissue Homogenization

The whole liver, kidneys and heart from each animal was removed after sacrificing the animal and were rinsed in normal saline and immediately stored in deep freezer. Tissues were homogenized in 10 parts in ice-cold potassium phosphate buffer (pH 7.4) using mortar and pestle [12-14,21]. The homogenate was centrifuged at 3000 rpm for 15 minutes and the supernatant collected, and the protein concentration of the sample determined by the Biuret method, using bovine serum albumin as standard.

2.8 Determination of Liver Function Parameters

Aspartate aminotransferase and alanine aminotransferase were determined colorimetrically at 546nm using Randox assay kits based on the principle described by Reitman and Frankel [22]. Also, using the Randox kit, the colorimetric assay method for conjugated bilirubin employed, involves reaction with diazotized sulphanilic acid in alkaline medium to form a blue complex, while total bilirubin was determined in the presence of caffeine, which releases albumin-bound bilirubin that then reacts with diazotized sulphanilic acid [23].

2.9 Determination of Packed Cell Volume (PCV) and Hemoglobin Concentration

Whole blood samples were collected into heparinized capillary tubes, filled up to about 2/3 the length, sealed with plasticine and centrifuged at 3000rpm for 10 minutes. Packed cell volume was determined using hematocrit reader, and the hemoglobin concentration was calculated from the PCV values.

2.10 Assay for Lipid Peroxidation

Malondialdehyde level as indicator of lipid peroxidation was determined as Thiobarbituric Acid Reactive Substances (TBARS) as described by earlier workers [12-14]. In this reaction, lipid peroxidation induced by the administered CCl₄ generates peroxide intermediates which upon

cleavage release malondialdehyde, a product that reacts with thiobarbituric acid to form a colored complex which is measured at 535nm. In summary, the method is as follows; one milliliter of 14% trichloroacetic acid was measured into a test tube, 1mL thiobarbituric acid (0.6%) and 50 μ L of the tissue homogenate were then added. The mixture was incubated at 80°C for 30 minutes in a water bath, allowed to cool rapidly in ice for 5 minutes followed by centrifugation at 3000 rpm for 10 minutes. Malondialdehyde was measured colorimetrically at 535nm and the level of the level of lipid peroxidation was calculated using the molar extinction coefficient of malondialdehyde (1.56×10^5 mol/L/cm) using the formula, $A = \Sigma CL$ where A = absorbance, Σ = molar coefficient, C = concentration and L = path length. All MDA concentrations were expressed in nmol/mg tissue protein.

2.11 Determination of the Activity of Endogenous Antioxidant Enzymes

The ability of the extracts to boost the capacity of antioxidant enzymes was evaluated by determining the activity of two endogenous antioxidant enzymes, namely catalase (CAT) and superoxide dismutase (SOD) as follows:

2.11.1 Catalase (CAT)

Catalase (CAT) activity was measured using Abei's method [24]. Briefly, the method is as follows: 10 μ L of serum was added to test tube containing 2.80mL of 50mM phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1mL of fresh 30mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240nm for 5 min on a spectrophotometer (Jenway 640 UV/Vis). A molar extinction coefficient of $0.0411 \text{mM}^{-1} \text{cm}^{-1}$ was used to calculate catalase activity.

2.11.2 Superoxide dismutase (SOD)

Superoxide dismutase activity was evaluated based on the principle that auto-oxidation of hematoxylin is inhibited by SOD at the assay pH; The percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. The amount of SOD in the sample is determined in the "standard cytochrome C" SOD unit by measuring the ratios of auto-oxidation rates in the presence and absence of the sample [25]. The method can be summarized thus; exactly 920 μ L of assay buffer was added into clean test tube containing 40 μ L

of sample, mixed and incubated for 2 min at 25°C, following which 40 μ L of hematoxylin solution was added, mixed quickly and the absorbance was measured immediately at 560nm.

2.12 Determination of Total and HDL-Cholesterol

The method of Roeschlau and Gruber [26] was applied using assay kits (Randox Laboratories Ltd, UK) to determine the total serum cholesterol spectrophotometrically at 546nm after enzymatic hydrolysis and oxidation. On the other hand, the HDL cholesterol was determined using assay kits (Randox Laboratories Ltd, UK) after low density lipoprotein (LDL and VLDL) and chylomicron fractions were precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction, in the supernatant was assayed colorimetrically 540nm.

2.13 Determination of Urea and Creatinine

Urea was analysed based on the principle that in the presence of urease, urea in the serum is hydrolysed to ammonia which is trapped by Berthelot's reaction using analytical kits (Randox Laboratories) and measured spectrophotometrically as described by Stephen et al. [27]. Urea concentration was then calculated by simple proportion using the absorbance of a known concentration of the standard.

Creatinine level was assayed using assay kits as described by Stephen et al. [27]. The analysis is based on the principle that creatinine in alkaline solution reacts with picric acid to form a coloured complex whose intensity is directly proportional to the creatinine concentration. The creatinine concentration (mg/dL) was calculated by simple proportion from the standard using the difference between A_1 and A_2 in both cases.

2.14 Statistical Analysis

The results on AST, ALT, SOD, catalase, TBARS, bilirubin, total and HDL-cholesterols are presented as mean $\pm$ standard deviation and statistical evaluation was performed using Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The significance level was set at $P=0.05$.

3. RESULTS AND ANALYSIS

Compared to the CCl₄ control group, pre-treatment with methanol extract of *C. febrifuga* or vitamin E control caused a significant ($P=0.05$) decrease in the levels of aspartate aminotransferase and alanine aminotransferase (Fig. 1). Similarly, the statistically significant elevation in bilirubin level (both total and direct

bilirubin) owing to CCl₄ intoxication was significantly prevented by pretreatment with *C. febrifuga* leaf extract. Similar trend was observed for the packed cell volume, haemoglobin concentration (Fig. 2) and bilirubin levels (Fig. 3), as no significant difference was found between the group pre-treated with Vitamin E control and that pre-treated with the *C. febrifuga* leaf extract for all the parameters.

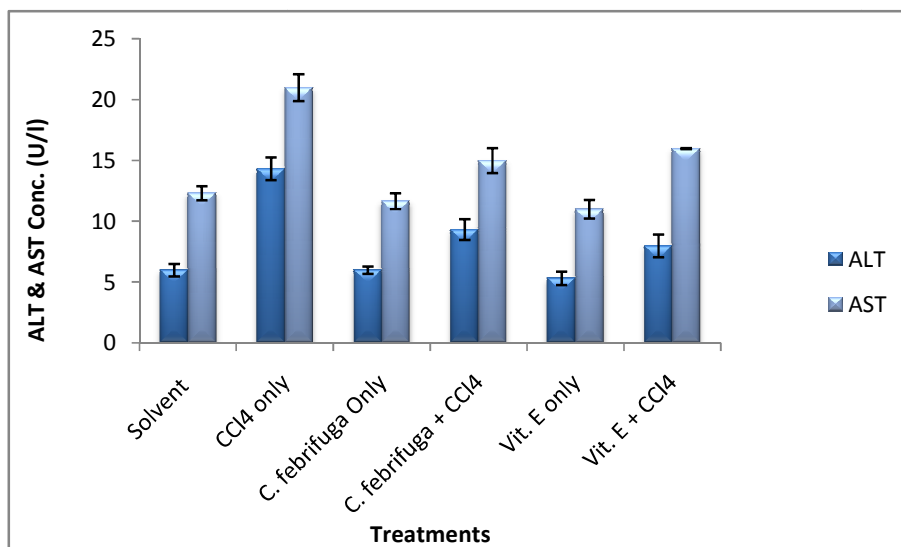


Fig. 1. Alanine aminotransferase and aspartate aminotransferase concentrations of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

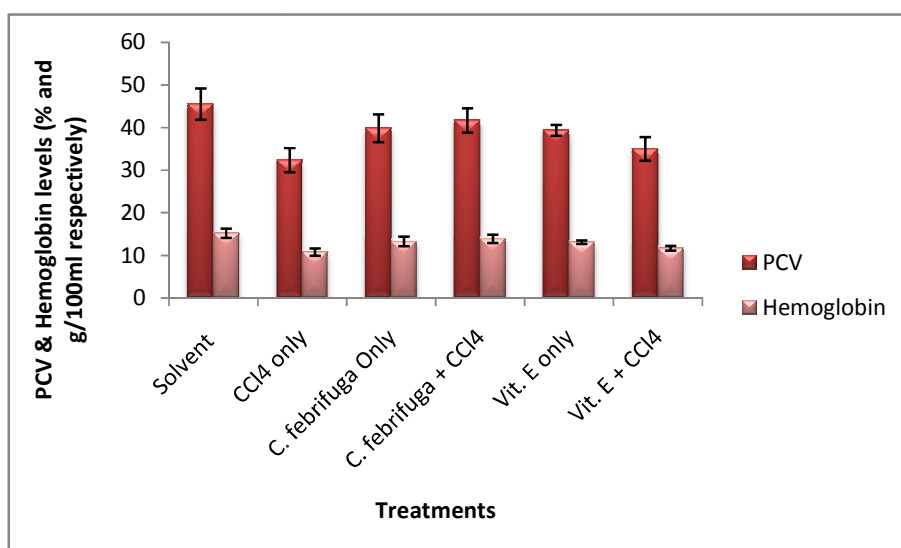


Fig. 2. Packed cell volume and hemoglobin levels of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

From Fig. 4, it can be observed that compared with the CCl₄ control group, the level of malondialdehyde in the group pre-treated with *C. febrifuga* leaf extract or vitamin E were restored to within the normal range for all organs, especially in the liver and the kidney. However, for most of the organs, no significant difference existed between the malondialdehyde level in the vitamin E and *C. febrifuga* leaf extract pre-groups

(Fig. 4). Similarly, compared to the CCl₄ control group that experienced statistically significant depletion in the levels of endogenous antioxidant enzymes, catalase and superoxide dismutase, the levels of these enzymes in vitamin E control or *C. febrifuga* leaf extract pre-treated groups were significantly ($P=0.05$) boosted (Figs. 5 and 6).

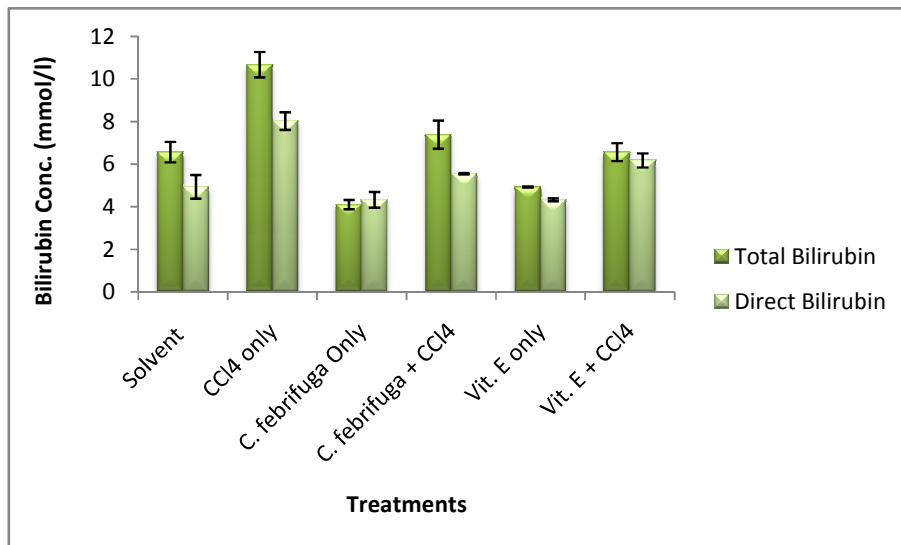


Fig. 3. Bilirubin concentrations of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanol extract of *C. febrifuga* leaf (10mg/Kg)

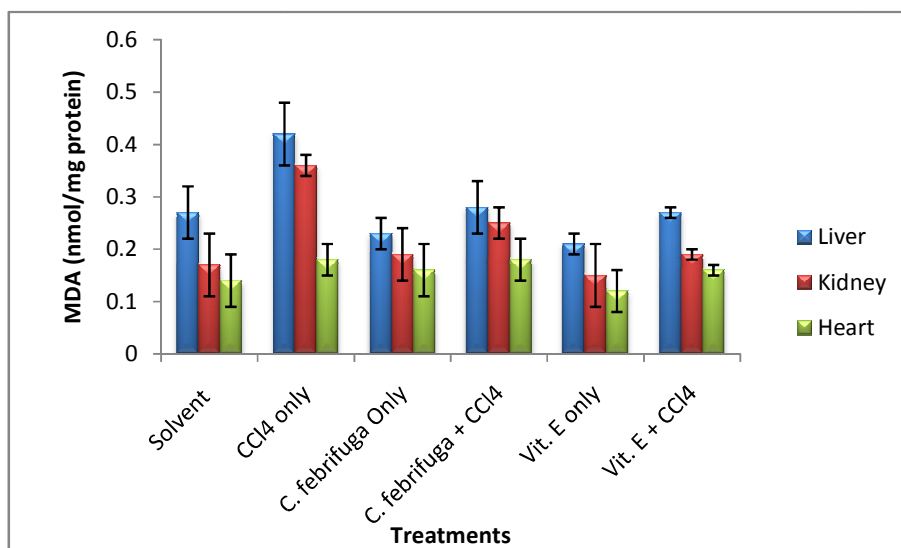


Fig. 4. Levels of malondialdehyde in the liver, kidney and heart of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

Figs. 7 and 8 present some lipids and kidney function parameters. Total cholesterol, urea and creatinine levels were significantly ($P=0.05$) elevated with a simultaneous depletion in the CCl_4 control group, but pre-treatment with the methanol extract of *C. febrifuga* leaf significantly

($P=0.05$) reversed this trend. For these parameters, no significant difference ($P=0.05$) existed between the vitamin E and extract treated groups, nor between the vitamin E and extract pre-treated groups).

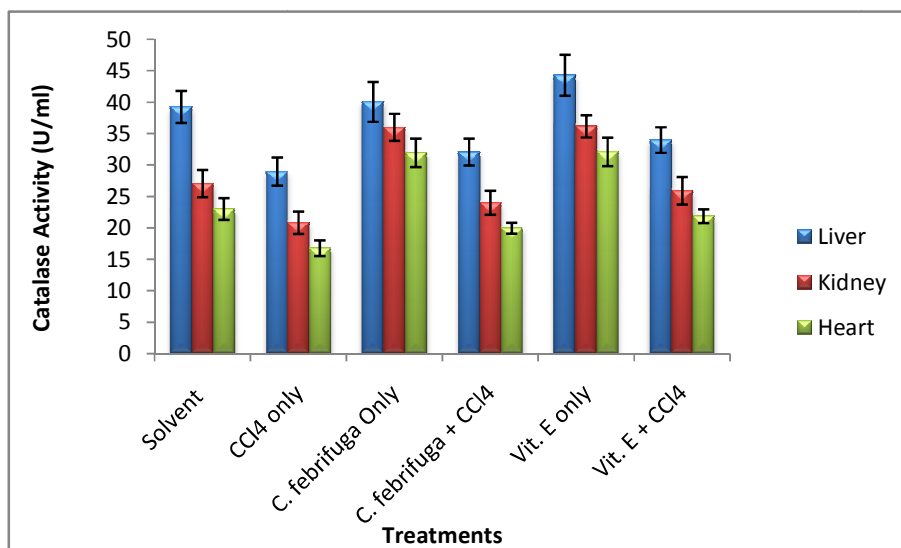


Fig. 5. Catalase activity in the some organs of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

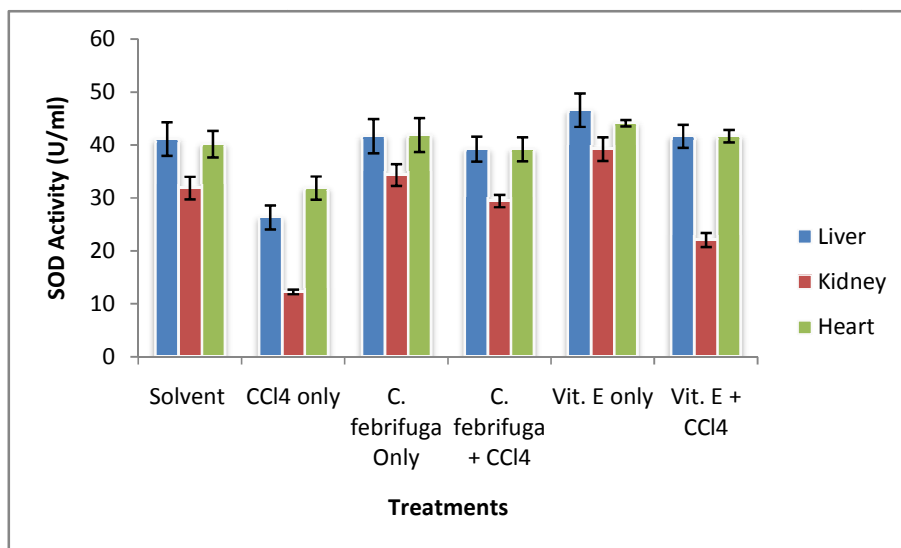


Fig. 6. Superoxide dismutase (SOD) activity in some organs of rats (n=6) intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

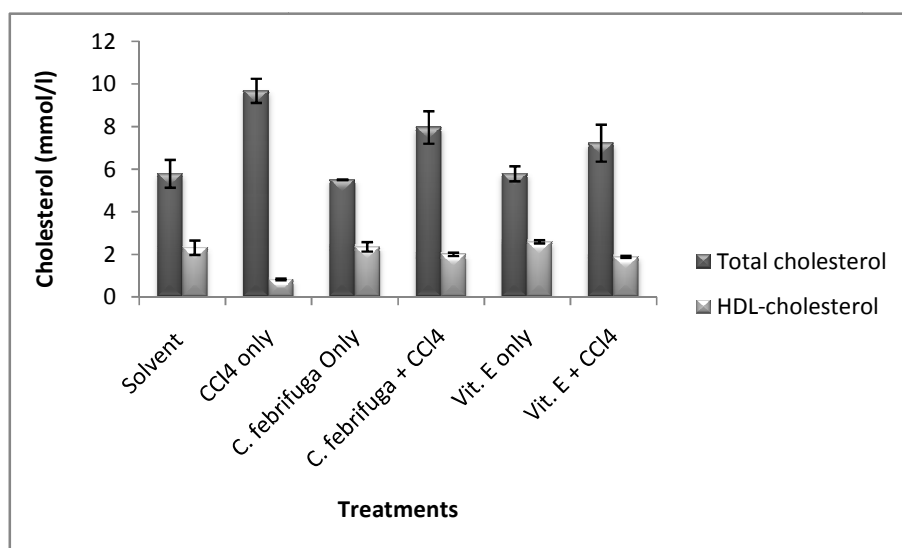


Fig. 7. Serum cholesterol levels of rats intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

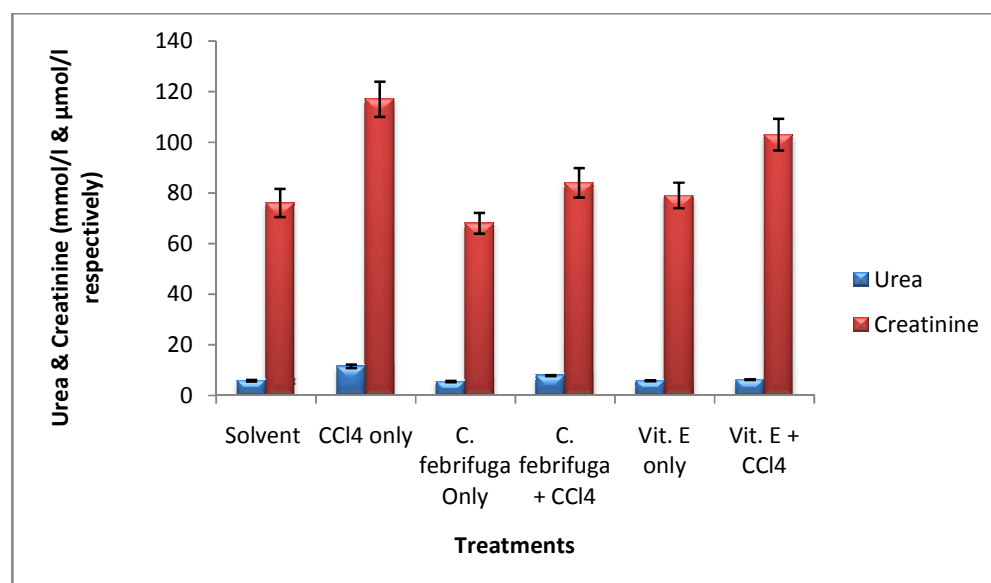


Fig. 8. Serum urea and creatinine levels of rats intoxicated with carbon tetrachloride (0.6mL/Kg) following three days pre-treatment with methanolic extract of *C. febrifuga* leaf (10mg/Kg)

4. DISCUSSION

Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion of reduced liver glutathione as well as elevation in the serum levels of hepatic enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) which are indicative of cellular leakage [27,28]. Among all

xenobiotics, CCl₄ represents the most commonly used experimental model for induction of acute liver injury through its bioactivation to trichloromethyl free radicals that cause lipid peroxidation and consequently, hepatocellular damage [29,30]. In this study, CCl₄ induced severe liver damage as evidenced by the significant elevation ($P < 0.05$) of serum levels of ALT and AST as well as total and conjugated

bilirubin with a concomitant decrease in the levels of PCV and hemoglobin concentration. That this trend was reversed by pre-treatment with the methanolic extract of *C. febrifuga* leaf strongly suggests that the plant possess significant protective and possibly, ameliorative effect against acute liver injury.

The pathways of heam breakdown that produces bilirubin play significant roles in protecting cells from oxidative damage and in regulating certain cellular functions, especially when its products, particularly CO are in low concentration. Bilirubin is the most abundant antioxidant in mammalian tissues and is responsible for most of the antioxidant activity in serum. Its protective effects appear to be especially important in the developing brain of newborn infants, although cell toxicity may be associated with jaundice when bilirubin level is in excess of the serum albumin needed to solubilize it [31]. Thus, impaired liver function, especially lack of adequate glucuronyl bilirubin transferase or blocked bile secretion causes bilirubin to leak from the liver into the blood, resulting in a yellowing of the skin and eyeballs, a condition called jaundice. Hence, the estimation of the concentration of bilirubin in the blood may be useful in the diagnosis of the underlying liver disease. Therefore, improvement in liver function parameters upon pre-treatment with methanolic extract of *C. febrifuga* leaf suggests that the extract constituents may, at least in part, play some functional roles in the enzyme or process related to glucuronidation of bilirubin.

Oxidative stress generated by administration of CCl_4 was significantly prevented through pre-treatment with the methanol extract of *C. febrifuga* leaf as evidenced by decreased levels of malondialdehyde with concomitant boosting of the activities of catalase and superoxide dismutase in all organs evaluated. The roles of catalase and superoxide dismutase as endogenous antioxidant enzymes are well documented; SOD catalyses the dismutation of superoxide anion to H_2O_2 and O_2 , and because H_2O_2 is also harmful to cells, it is further decomposed by catalase to water [32]. Similarly, that pre-treatment with the methanol extract of *C. febrifuga* leaf prevented lipid peroxidation in the same way as vitamin E strongly suggests that the plant had good capacity to prevent the subsequent tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals [33] like

Vitamin E, a known potent antioxidant vitamin [34–36].

An abnormality in lipid profile is one of the most common complications in CCl_4 intoxication [37] and lipid peroxidation is associated with hypercholesterolemia and hypertriglyceridemia [38,39]. This is because acute CCl_4 intoxication initially causes a decrease in free fatty acid mobilization from adipose tissue resulting in increased production of cholesterol-LDL particles [39,40]. Significant lowering of total cholesterol and rise in HDL-cholesterol as mediated by the methanol extract of *C. febrifuga* leaf in this study, is a desirable biochemical event, especially with respect to prevention of atherosclerosis and ischemic conditions [41-43].

5. CONCLUSION

Put together, the results of this investigation proves that *C. febrifuga* leaf contain substances with significant antioxidant, hepatoprotective, nephroprotective and hypolipidemic effects to warrant further study to identify the exact active ingredients and their pharmaco-toxicological profiles.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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