Ameliorating Effects of Aqueous Extract of *Tetracarpidium conophorhum* against Iron-Overload Induced damage in Rats

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ABSTRACT: Iron, though an important nutrient but in excess can produce serious damaging effects on organs of the body. Intake of plants and or plant products with iron chelating ability, instead of synthetic iron chelators, can produce less adverse effects and may be more effective. The potential effect of *Tetracarpidium conophorhum (T. conophorhum)* as a chelating agent of plant origin has not been determined hence the present study. Iron overload condition was induced by oral administration of iron II chloride in the rats. Twenty five rats were randomly divided into five groups. Groups A and B were the normal and negative control (iron overload only) respectively. Groups C-E were the iron overload induced rats treated with 250, 500 and 1000mg/kg body weight of *T. conophorhum* extracts. Animals in groups C-E received daily oral administration of extracts for thirty days. Thereafter, we studied the effects of *T. conophorhum* on liver function enzymes, antioxidant enzymes, hematological parameters and lipid peroxidation. Iron overloaded rats showed significant increase $(p<0.05)$ in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total and direct bilirubin levels and a significant decrease $(p<0.05)$ in total protein, albumin, antioxidant enzymes. *T. conophorhum* extract at a high dose of 1000mg/kg body weight was able to restore the levels of these parameters to normal. These findings therefore suggest that high dose of *T. conophorhum* seed extract may be effective in reducing the resultant effects produced in the iron overload-induced liver damage.

Iron is an important trace element required by most biological systems because of its tendency to act as both an electron donor and acceptor (Halliwell and Gutteridge, 1990). It is also involved in red blood cell function, myoglobin activity, immune function, cognitive performance, brain function, as well as function and synthesis of neurotransmitters. Therefore, iron deficiency can cause multiple organ malfunctions. However, iron overload can be as destructive and dangerous as its deficiency (Barton, 2007). Liver is one of the largest organs in the human body and the main site for intense metabolism and excretion. Hepatotoxicity is the most common finding in patients with iron overloading as liver is mainly the active storage site of iron in our body. Iron must be bound to proteins such as ferritin, hemosiderin, and transferrin to prevent its destructive effects, but there is increase in the amount of iron, the liver fails to produce enough protein to store iron. Therefore, excessive free iron will be produced as a result. Since the body cannot deal with this large amount of excessive iron, it deposits iron into organs such as the heart, liver, and endocrine organs, which leads to dysfunction of these organs. The consequences of this disease represent as liver dysfunction, cardiomyopathy, diabetes, and even death. Excess iron causes a serious damage to the liver that is the main storage site of iron in our body, by forming hydroxyl radical mediated oxidative stress as these free radicals attack cellular molecules and develop carcinogenic molecules within cells (Hershko *et al.*, 2003; Shander *et al.*, 2009). Treatment of iron overload-induced diseases entails iron removal using iron chelation therapy. Some of the iron-chelating drugs used are deferoxamine, deferiprone, and deferasirox. The use of these agents has several limitations as the drugs produces severe adverse effects on the body (Al-Refaie *et al*., 1992; Richardson, 1999). It has been found that most plants used for treatment of liver diseases are effective antioxidants (Lopez *et al*., 2003; Rice-Evans *et al.,* 1996) and iron chelation is very important part of their antioxidant activity (Cook and Samman, 1996). Thus, search for crude drugs of the plant origin with antioxidant activity has become a central focus of study of hepatoprotection. *Tetracarpidium conophorum* (conophor nut) commonly called the African walnut is a perennial climbing shrub found in the forest zones of sub-

saharan Africa (Oluwole and Okusanya, 1993). The tropical African walnut, known as *Tetracarpidium conophorum* or *Plukenetia conophora* (Oyekale *et al*. 2015), belongs to the family Euphorbiaceae (Edem *et al*., 2009). In Nigeria, and it is known as *okhue* or *okwe* among the Bini tribe of Edo State (Chijoke *et al.,* 2015; Kanu *et al*., 2015). The anti-diarrhea (Nwachoko and Jack, 2015), fertility enhancing (Ikpeme *et al.* 2014), Obianime and Uche (2010)), antioxidant (Amaral *et al*. 2004), anti-chelating (Olabinrin *et al*., 2010), anti-inflammatory(Olaniyi *et al*. 2016) properties of this plant has been evaluated. The present study seeks to evaluate the ameliorating effects of aqueous extract of *tetracarpidium conophorum* on iron overload induced Wistar rats.

MATERIALS AND METHODS

Plant material: Fresh Walnuts seeds were obtained from New Benin market in Edo State, Nigeria. It was identified and authenticated in of the Department of Plant and Biology and Biotechnology, University of Benin, Benin City. Voucher specimen was deposited in the herbarium with voucher number UBHe0153

Preparation of plant extract: The back was removed and the sample were chopped and then allowed to dry for few days after which it was pulverized with a mechanical grinder. 400grams of the pulverized sample was macerated in 900ml distilled water for 48 hours. The crude extracts obtained were filtered with Whatman filter paper and the filtrates concentrated using a rotary evaporator (BÜCHI Labortechnik AG, Switzerland). The freeze dried extract was kept in the freezer until use.

Animals: Twenty five (25) adult female Wistar rats weighing between 180-250g were bred in the animal house of the Department of Anatomy, University of Benin, Benin City. They were acclimatized under 12hours light/ dark cycle and room temperature was 22° C to 25° C and they were allowed free access to food and water.

Experimental design: Induction of iron overload was by single dose administration of 2mls iron II chloride orally to the rats at a dose of (0.01mg/kg body weight) The rats were divided into five groups (A, B, C, D, and E) of five rats each.
Group A served as the normal control rats
Group B were negative control (iron overload only)
Group C were Iron overload rats treated with low dose of walnut (250mg/kg body weight).
Group D were iron overload rats treated with medium dose of walnut (500mg/kg body weight)
Group E were iron overload rats treated high dose of the walnut extract (1000mg/kg body weight)

Extracts were administered daily for thirty (30) days. At the end of the experimental period, the animals were anaesthetized under chloroform, blood samples were collected through cardiac puncture into plain sample tubes for biochemical investigations (liver function test and antioxidant enzymes) and into EDTA sample bottles for hematological assay. The blood samples for serum biochemical assays were centrifuged at 3000 g for 10 min to obtain serum, which was later used for the estimation of biochemical parameters. The liver tissue was also collected for histopathological examinations.

Biochemical parameters: Liver function parameters: Alanine amino transferase (ALT) and aspartate amino transferase (AST) activities were measured by Reitman and Frankel (1957) method while serum ALP was assayed by the method of Englehardt *et al* (1970). Serum protein levels were assayed using Tietz *et al* (1991) method, serum albumin levels were measured by Doumas and Biggs (1972) method. Serum total and direct bilirubin levels were assayed by Jendrassik and Grof (1938) method. All parameters were assayed using commercially available kits.

Lipid peroxidation assay: The malondialdehyde (MDA) level was used to estimate the level of lipid peroxidation. MDA level was determined by the method of Varshney and Kale (1990).

Antioxidant enzymes: Catalase activity was determined in the serum by the method of Cohen (1970). Superoxide dismutase activity was determined using the method of Misra and Fridovich (1972). Gluthathione peroxidase was assayed using the method of Nyman (1959).

Hematological Parameters: RBC count, WBC count, platelets count, PCV, Hemoglobin, differential leukocyte count were assayed for using the automated hemoanalyzer.

Histopathological examinations: Excised liver samples were cleaned with normal saline and fixed for two days in 10 % buffered neutral formalin. Sections (5 μm thick) were paraffin-embedded and stained with hematoxylin and eosin. The sections of the liver were obtained and examined under Leica DM750 research microscrope with a digital camera (LeicaICC50) attached. Digital photomicrographs of the tissue sections were taken at x400 magnifications.

Statistical Analysis: All data collected was presented as graphs, tables and figures also as mean ± standard error of mean (SEM) of controls and experimental groups. The data was subjected to analysis of variance

(ANOVA) using Statistical Package for Social Sciences (SPSS), version 17. A value of p < 0.05 was taken as significant.

RESULTS AND DISCUSSION

Iron plays a very important role in the human body and since the liver is the primary site for iron storage, extreme deposition of iron in the liver can increase oxidative stress in the liver and lead to further injuries, such as hepatocellular necrosis, inflammation, fibrosis, and cancer(Cannon, 2006).
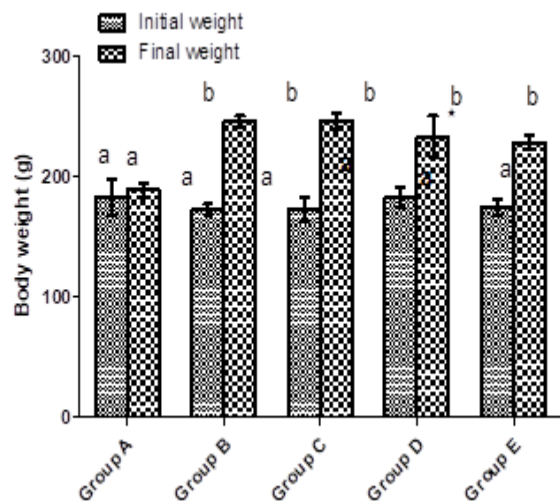

Fig 1: Graph showing initial and final body weight following the administration of *T. conophorum* Extract at different doses. Values are Mean ± SEM, *n* = 5 rats in each group. *p* < 0.05 as statistical significance, Different superscript indicates statistically significant difference in treated groups compared with the control
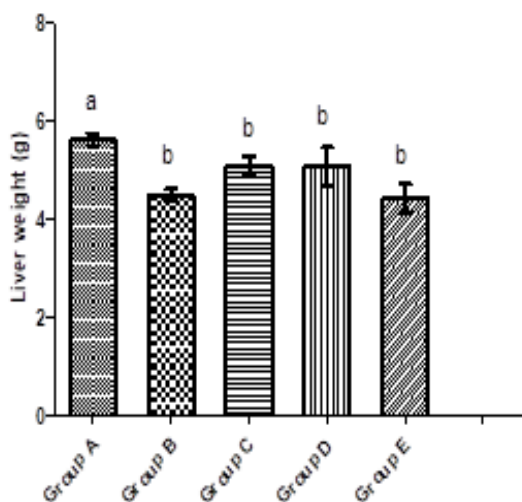
Fig 2: Graph showing initial and final body weight following the administration of *T. conophorum* extract at different doses. Values are Mean ± SEM, *n* = 5 rats in each group. *p* < 0.05 as statistical significance, Different superscript indicates statistically significant difference in treated groups compared with the control

In all iron overload-induced diseases, iron removal by iron chelation therapy is an effective life-saving strategy. The limitations such as lower specificity, limited capacity to enter cells and higher side effects especially hepatic failure (Gagliardo *et al*., 2008; Cappellini *et al*., 2006) has led to the search of alternative source of therapy.
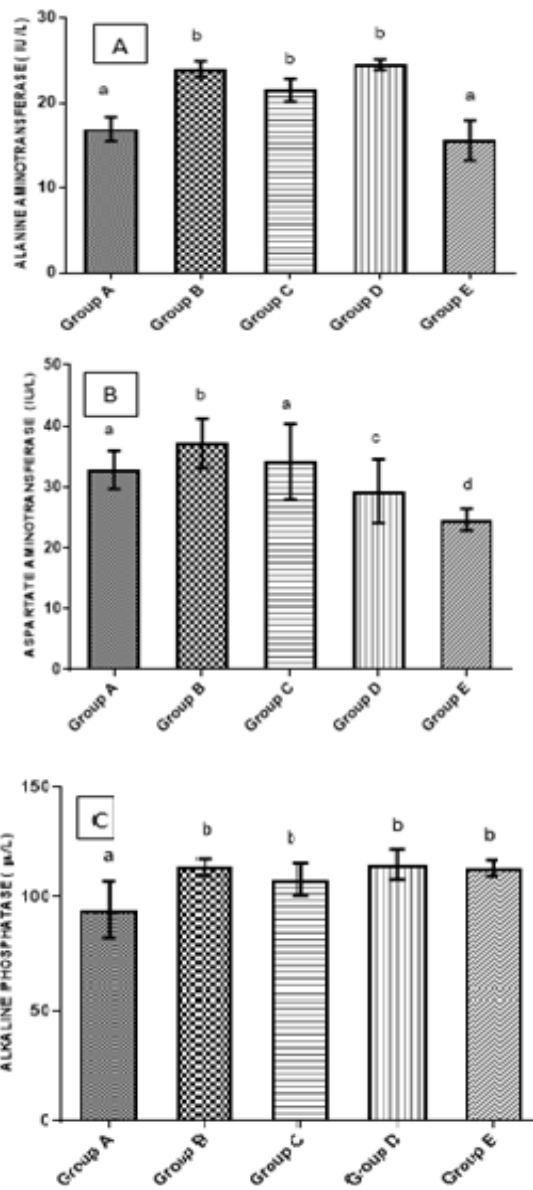
Fig 3 Effects of varying doses of *T. conophorhum* extract on serum liver biomarkers on iron overload induced rats. A)Alanine amino transferase (ALT) activity B) Aspartate amino transferase (AST) activity C) Alkaline phosphatase (ALP) activity. Values are Mean ± SEM, *n* = 5 rats in each group. *p* < 0.05, a as compared with the normal control group
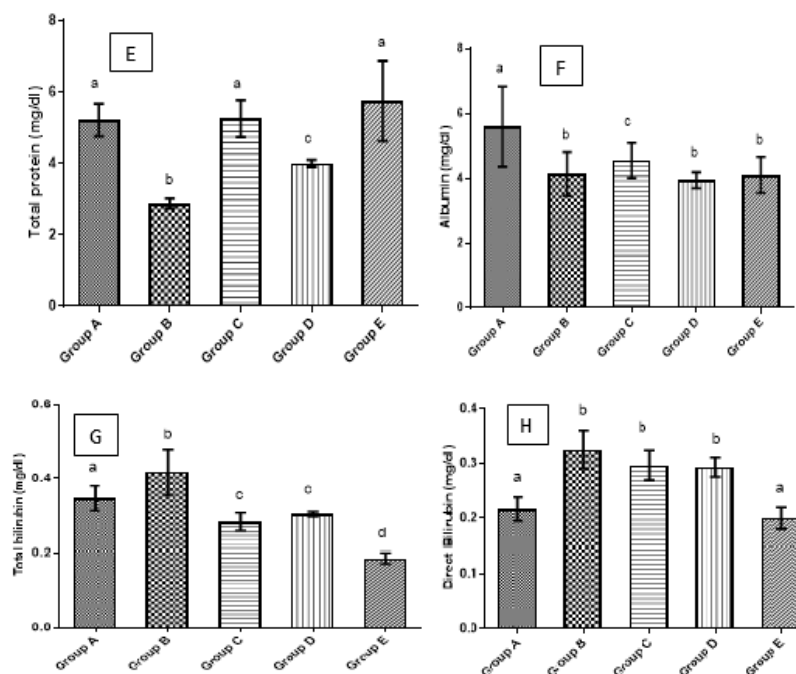
Fig 4: Effects of varying doses of *T. conophorhum* extract on serum liver biomarkers on iron overload induced rats. E) Total protein value F) serum albumin concentration G) direct bilirubin value H) total bilirubin concentration

Hence, herbal therapy may be evaluated as alternative therapeutic strategies against iron overload. Several bio resources form plants have already been reported as natural antioxidants with iron chelating potentials (Sarkar *et al*., 2012; Hazra *et al.,* 2012). We recorded a 42.3 % increase in the weight of the negative control rats, 42.3 % for the low dose treated rats, a 27.4% for the middle dose treated rats, and 30.9% in the high dose treated rats (fig 1). This clearly shows administration of high dose *T. conophorhum* extract led to a significant decrease in the weight of the iron overload rats that were significantly increased due to iron overload. A significant decrease ($p<0.05$) in the weight of the liver was observed in the negative control and high dose treated *T. conophorum* iron overload group when compared with the normal control. This shows that administration of high dose (1000mg/kg body weight of *T. conophorhum* extract) did not normalize the increase weight in the liver resulting from iron overload (fig 2).In this study, injury to the liver were assessed by measuring AST, ALT, ALP, total protein, and albumin level. As these enzymes are predominantly found in the hepatic cell, and liver damage due to excess iron leads to the release of these intracellular enzymes into the blood (Pulla and Lokesh, 1996). This was evident by the increased AST, ALT, ALP activity observed in this study (fig 3). Increase in serum alkaline phosphatase activities is the indicative of cellular damage due to loss functional integrity of cell membranes. Albumin concentrations increase in inflammation, and the antioxidant activities

of albumin result from its ligand-binding capacities of ROS-producing metal such as copper and iron (Roche *et al.,* 2008). Administration of high dose (1000mg/kg body weight of extract) can be said to be effective in reducing the abnormal level in serum ALT and AST activity as observed in this study. Also the reduction in total protein and total bilirubin caused by the iron overload were all restored by administration of *T. conophorhum* extracts. Also high dose of *T. conophorhum* extract restored the direct bilirubin levels (fig 4).Adequate iron supply is essential for production of RBCs by erythropoiesis process (Ganong, 2005). RBC parameters are used most commonly to monitor erythropoiesis, these parameters include RBCs count, Hb, PCV, MCHC, MCH and MCV (Miyata *et al*., 1994). We recorded a significant decrease in the lymphocyte, monocytes and granulocyte count in the iron overloaded rats (table 1). Although administration of extracts of *T. conophorhum* did not restore these white cell markers to the normal values. The significant decrease in RBCs count, hematocrit, Hb observed in this study may result from decrease in serum total protein level resulting from iron overload as an increase in hemoglobin and red blood cells results from sufficiency of protein synthesis that mainly induces increase of an essential amino acids and long age of energy source of protein synthesis incorporated in hemoglobin production (Bersenyi *et al.,* 2004; Lavicoli, *et al*., 2003).
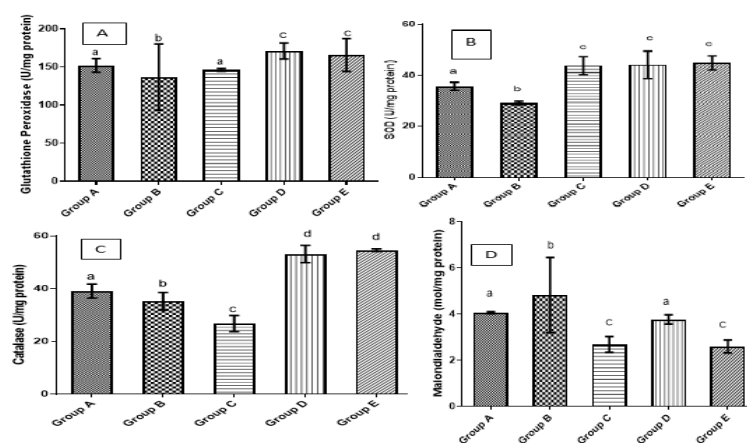
Table 1: Effects of varying doses of *T. conophorhum* extract on hematological indices on iron overload rats.

| Parameters	Group A (normal control)	Group B (negative control)	Group C (iron overload + 250mg/kg body weight of extract)	Group D (iron overload +500mg/kg body weight of extract)	Group E (iron overload +1000mg/kg body weight of extract)
WBC count (x10³/μl)	3.33 ± 0.12ᵃ	3.560 ± 0.27ᵃ	3.20 ± 0.45ᵃ	4.167 ± 0.79ᵃ	4.00 ± 0.40ᵇ
Lymphocytes (×10³/ul)	67.26 ± 15.80ᵃ	86.62 ± 1.56ᵇ	84.45 ± 3.65ᵇ	87.37 ± 2.03ᵇ	89.36 ± 0.83ᵇ
Monocytes (×10³/ul)	7.15 ± 3.06ᵃ	8.625 ± 0.82ᵇ	7.933 ± 1.35ᵃ	6.525 ± 1.00ᵇ	6.35 ± 0.50ᵇ
Granulocytes (×10³/ul)	4.050 ± 2.11ᵃ	6.90 ± 1.29ᵇ	5.633 ± 1.55ᵇ	2.70 ± 1.62ᵇ	2.740 ± 0.17ᵇ
RBC count (x10⁶/μl)	7.488 ± 0.09ᵃ	6.243 ± 0.25ᵇ	6.68 ± 0.26ᵃ	7.140 ± 0.26ᵃ	7.873 ± 0.25ᵃ
Haemoglobin (g/dl	14.76 ± 0.19ᵃ	11.40 ± 1.00ᵃ	12.60 ± 0.52ᵃ	14.23 ± 0.82ᵃ	15.80 ± 0.54ᵃ
Haematocrit (%)	45.56 ± 0.91ᵃ	36.13 ± 1.23ᵇ	38.95 ± 1.25ᵇ	41.55 ± 2.19ᵇ	47.36 ± 1.47ᵃ
MCV (fl)	60.90 ± 0.57ᵃ	58.92 ± 1.27ᵃ	59.47 ± 1.30ᵃ	62.40 ± 1.78ᵃ	60.06 ± 0.25ᵃ
MCH (pg)	19.68 ± 0.16ᵃ	18.86 ± 0.56ᵃ	19.53 ± 0.27ᵃ	20.33 ± 0.50ᵃ	20.38 ± 0.11ᵃ
MCHC (g/dl)	32.36 ± 0.44ᵃ	32.14 ± 0.84ᵃ	32.97 ± 0.48ᵃ	32.65 ± 0.25ᵃ	34.02 ± 0.27ᵃ
Platelet count (×10³ ul)	420.5 ± 45.51ᵃ	624.3 ± 58.11ᵇ	386.0 ± 163.7ᵇ	740.0 ± 27.59ᵇ	657.3 ± 72.52ᵇ

Values are Mean ± SEM, n = 5 rats in each group. p < 0.05 as statistical significance, Different superscript indicates statistically significant difference in treated groups compared with the control

Iron overload causes the generation of various free radicals which is not always effectively normalized by the body's antioxidant defense machinery leading to oxidative stress related complications (Bonkovsky, 1991). These oxidative stress leads to a reduction in the levels of antioxidant enzymes hence, the decrease in the activities of these enzymes could be attributed to the excessive utilization of these enzymes in inactivating the free radicals caused by iron induced overload. The wide array of enzymatic and non-enzymatic antioxidant defenses of the body includes SOD, GPx, CAT, ascorbic acid,α-tocopherol, reduced glutathione,β-carotene, and vitamin A (Fu *et al*.,1999). gluthathione peroxidase (GPx) plays a role in the metabolism of glutathione by detoxifying harmful hydroperoxides in plasma, SOD helps convert superoxide radical to the less harmful hydrogen peroxide and oxygen and catalase (CAT) converts hydrogen peroxide to water and oxygen (Devasagayam *et al*., 2004). Treatment with *T. conophorhum* extract significantly restored the antioxidant enzymes. Our study recorded a 50.41%, 51.41%, and 53.94 increase in SOD activity in the 250,

500, and 1000mg/kg body weight of *T. conophorhum* treated rats when compared with the negative control (iron overload only)(fig 5). Also for catalase, we recorded 51% and 55.14% increase for the 500 and 1000mg/kg body weight of *T. conophorhum* treated rats (fig 5). For GPX activity, we recorded 6.95%, 25.07%, 21.21% increase in 250, 500 and 1000mg/kg body weight of *T. conophorhum* treated rats (fig 5). This could imply that *T. conophorhum* extract can act as a potent antioxidant agent in iron overload conditions. The significant increase in the antioxidant enzymes level may be compensated by the release and activation of stock enzymatic antioxidants from stores like liver and adipose tissue (Ranjbar *et al*., 2005). Our results is in line with the work of Chaudhuri *et al* (2016) who reported that Glycoside rich fraction from *Spondias pinnata* bark restored the antioxidant enzymes to normal levels in iron induced overload in mice. Also Khadiga *et al*., (2014) and Badria *et al* (2015) also reported a restoration of antioxidant enzymes to normal levels in iron induced overload conditions.

Fig 5: Effects of varying doses of *T. conophorhum* extract on serum antioxidant enzymes and lipid peroxidation biomarker on iron overload induced rats

Lipid peroxidation is the process of oxidative degradation of poly unsaturated fatty acid and the products of lipid peroxidation inactivate cell constituents by oxidation or casing oxidative stress by undergoing radical chain reaction ultimately leading to the cell damage (Tribble *et al*., 1987). Iron is the most common cofactor within the oxygen handling biological machinery and, specifically, lipid peroxidation of biological membranes is the main pathogenic mechanism of iron overload induced tissue damage (Hershko *et al*., 1998). The lipid bilayer is destroyed by hydroxyl radical (lipid peroxidation) and results to end products such as malondialdehyde (MDA) which in turn activate the stellate cells to initiate liver fibrosis (Lee *et al*., 1995; Farinati *et al*., 1995). Enhanced lipid peroxidation has been proposed as an initial step by which iron causes structural and functional alterations in cell integrity (Bonkowsky *et al*., 1981). In this study we recorded a 44.3%. 21.93% and 46.20% decrease in MDA level for the 250, 500 and 1000mg/kg body weight of *T. conophorhum* treated rats respectively in comparison with the negative control only (fig 5). The significant decrease in malondialdehyde levels observed in the *T. conophorhum* treated rats respectively is due to the anti-oxidative nature of the extracts.
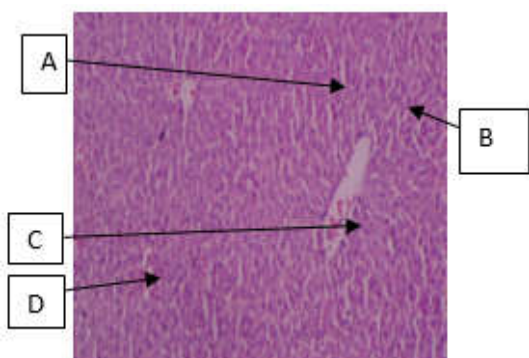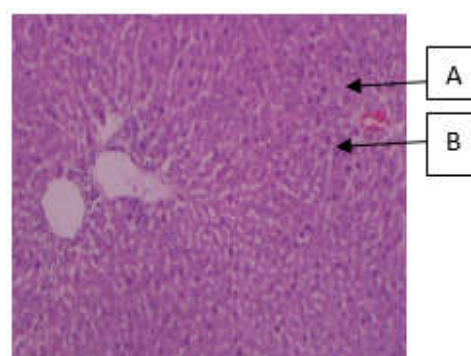

Plate 1 depicts (Group A) Control. A, hepatocytes, B, sinusoids, C, central vein and D, Portal vein (H&E x 100)
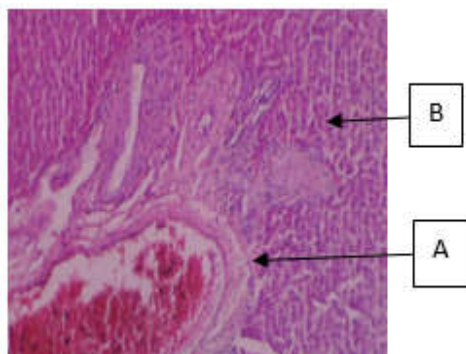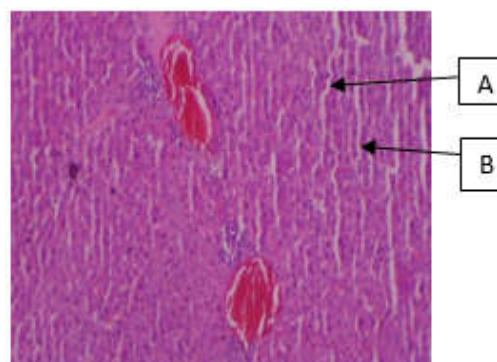

Plate 2 depicts (Group B) Rat given iron only: A, vascular congestion and B, periportal inflammatory infiltrates (H&E x 100)
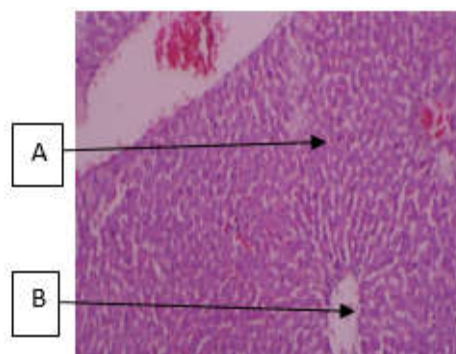
Plate 3 depicts (Group C) Rat given Iron plus low dose Extract: A, normal hepatocytes and B, vasculature (H&E x 100)

Plate 4 depicts (Group D) Rat given Iron plus moderate dose Extract: A, normal hepatocytes and B, kupffer cell activation (H&E x 100)

Plate 5 depicts (Group E) Rat given Iron plus high dose of Extract: A, normal hepatocytes and B, kupffer cell activation (H&E x 100)

Histological investigation of the liver tissues revealed that the iron overload group showed severe vascular congestion and periportal inflammatory infiltrate while those of the *T. conophorhum* treated groups showed normal hepatocyte with Kupffer cell activation (Plates 1-5). This therefore shows that the biochemical changes observed where corroborated by the histological findings.

Conclusion: Our findings therefore confirm that *T. conophorhum* extract at high doses can be effective in ameliorating the destructive effects of iron overload.

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