

Haematopoietic induction and hepatic protective roles of Hepacare® in CCl₄-induced hepatic damaged rats

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Received: 12 August 2016 / Accepted: 7 February 2017
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Abstract Herbal formulations are plant parts used as raw materials for self-administered pharmaceutical remedies, and many of them are being sold without any scientific validation for their potency and efficacy. This research work was aimed at evaluating the haematopoietic, biochemical, and histological effects of Hepacare®, a popularly sold herbal formulation in Nigeria against carbon tetrachloride (CCl₄)-mediated liver damage in rats. Haematological analysis showed significant reduction ($p < 0.05$) in haemoglobin, red blood cell, packed cell volume, and platelet counts in CCl₄-treated group when compared with the untreated group. These parameters were however reversed across the groups treated with the herbal formulation. Levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were significantly ($p < 0.05$) reduced after treatment of rats with the formulation which were previously elevated ($p < 0.05$) in the CCl₄-treated group when compared with the untreated group. The CCl₄-treated group exhibited significantly different activities in liver SOD and GSH enzymes. The level of MDA was lowered in the liver tissue samples of treated rats when compared with the CCl₄-exposed untreated rats. The groups treated with the formulation showed signs of protection against this toxicant as evidenced by the absence of necrosis. Hepacare® showed reversal effects on the previously increased haematological parameters and damaged liver tissues with a potential to ameliorate oxidative stress in hepatic dysfunction.

Keywords Hepacare® · Carbon tetrachloride · Haematologic · Liver injury · Hepatic marker enzymes · Antioxidant markers · Histological examination

Introduction

The use of herbal medicine has been in existence for a relatively long period of time. Herbal formulations are parts of plants commonly used as raw materials for self-administered pharmaceutical remedies and as supplementary products in the general population (Chizzola et al. 2003). Despite the development in contemporary medicine, liver diseases remain a universal health challenge; thus, the search for new drugs is still an ongoing process. Chemotherapeutic drugs used for treating liver diseases are insufficient and sometimes come with side effects. As a result of objectionable side effects of such drugs, there is a growing need to focus on systemic research procedures and assess scientific basis for the herbal formulations with potential hepatoprotective activity (Baranisrinivasan et al. 2009). There are diverse plants and herbal formulations that are readily obtainable for treating liver diseases (Schuppan et al. 1999). Hundreds of commercial herbal formulations with potential hepatoprotective ability are being sold all round the globe. Hepacare® is used to treat patients with sickle cell disease; it is also used as protection against liver damage which may be as a result of incessant alcohol consumption. According to the manufacturer, one capsule (250 mg) is to be taken with each meal three times daily for up to 30 days. Hepacare® is a blend of three plants which include: *Calliandra portoricensis*, *Uvaria chamae*, and *Canarium schweinfurthii*. *C. portoricensis* (Jacq.) is a flowering recurrent plant belonging to the family Mimosaceae (Amujoyegbe et al. 2014). It is often used in folklore medicine as an abortifacient in women (Orishadipe

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et al. 2010) and as a laxative/worm expeller (Agunu et al. 2005). The plant has been reported to have antidiarrhoeal, antiplasmodic and analgesic, and anticonvulsant activities (Aguwa and Lawal 1988; Oyebode et al. 2012). *U. chamae*, commonly known as finger root or bush banana, belongs to the family Annonaceae. It is a small tree native to the tropical rainforest of west and central Africa where it grows in wet and coastal shrub lands (Okon et al. 2013). It is used to treat fever and relieve pains in injuries (Obadoni and Ochuko 2001). *C. schweinfurthii* Engl. (Burceraceae) commonly known as 'African canarium' or 'African olive' is a large forest tree which grows to a height of 150 ft with straight cylindrical bole of 90 ft. It spreads across the west, east, and central Africa (Taniguchi et al. 2004). The fruits, stems, and bark are used for treating coughs, venereal diseases, and exudates. The leaves and rhizomes are used to increase nervous activity in the body and also to cure constipation, malaria, sexual infections, rheumatism, and diarrhoea (Koudou et al. 2005). The fruit pulp is made up of 50% oil and often used for the production of biofuel (Tchiegang 2004; Agbo et al. 1992).

Several herbal formulations have been examined for their potential hepatoprotective activity against liver damage in experimental animal models at the expense of other physiological activities of the body.

Carbon tetrachloride (CCl₄) is a toxic substance that exerts its toxic effect by altering the endoplasmic reticulum, resulting in the loss of metabolic enzymes located in the intracellular structures which has downstream effects on the haematology of mammals. Moreover, CCl₄ is metabolized by the cytochrome P₄₅₀ system to yield the trichloromethyl free radicals that disrupt the cell envelope and organelles, resulting in lipid peroxidation (Bussieres and Habra 1995). The study is therefore aimed at examining the effects of Hepacare[®] on the blood and verifying the liver protective potential of this drug in CCl₄-mediated hepatic cell damaged rats.

Materials and methods

Source and composition of herbal formulation

Hepacare[®] is a proprietary blend of the stem bark dried powder of *C. portoricensis*, *U. chamae*, and *C. schweinfurthii* and packaged into 250 mg gelatine capsules in ratio 1:3:1, respectively. The formulation was obtained from a pharmaceutical outlet at Ikeja, Lagos State, Nigeria in January 2014.

Preparation of herbal formulation

The encapsulated formulations were collected and opened. The contents of the capsule were further grinded and dissolved in distilled water and adjusted to 5 ml/kg bw of animals.

Experimental animals

Forty male albino rats purchased from the University of Agriculture, Abeokuta, Ogun State, Nigeria, ranging from 150 to 200 g were used for the experiment. The rats were kept under natural light-dark cycle at a controlled temperature and humidity and held in the animal house of the Biological Science Department, Covenant University, Ogun State, Nigeria. Feed and water were given to the animals ad libitum, and they were allowed to adjust for 2 weeks preceding the experiment. This research was approved by the Biological Sciences Research Ethics Committee, Covenant University. All animals were also treated in line with the National Institutes of Health (NIH) guidelines for the use and care of animals in the laboratory (National Institute of Health (NIH) 2011).

Experimental design

The model described by Adebayo and colleagues was employed (Adebayo et al. 2014). The choice of the dose was the unreported LD₅₀ in a pilot study conducted prior to this experiment. The animals were divided into five groups of eight rats each. Group A represents the normal control group and was given only vehicle (distilled water, 1 ml/kg bw) for 7 days. Animals in group B were treated with vehicle for 4 days and with the vehicle and CCl₄ (50% solution of CCl₄ in olive oil, 2 ml/kg bw) on the fifth, sixth, and seventh days. The animals in groups C, D, and E were administered with 500, 1000, and 1500 mg/kg bw of the herbal formula, respectively, and distilled water for the first 4 days and with distilled water, drug, and CCl₄ for the remaining 3 days. Prior to dissection, the animals were anaesthetized in diethyl ether and blood samples were obtained via cardiac puncture into lithium-heparinized containers using a syringe. The blood was centrifuged at 10,000×g min⁻¹ for 15 min and the plasma was stored at -20 °C until required for biochemical assays (Adebayo et al. 2011). The kidney and liver were immediately removed, washed with ice-cold 1.15% KCl buffer, and weighed. Liver homogenate was prepared by 0.1 M Tris HCl homogenizing buffer at pH 7.5. Samples from the liver and kidney were fixed with 10% formaldehyde and processed for histological examination while the liver homogenate was used for the antioxidant assay.

Haematology

The haematological indices: packed cell volume (PCV), haemoglobin (Hb), red blood cell (RBC) count, white blood cell (WBC) count, and platelet (PLT) count were analysed using an automated haematology system analyser (ADVIA 60 Open Tube, New York, USA).

Biochemical assay

The test kits for all biochemical indices measured were procured as obtained from Randox Laboratories, UK. Standard methods were used to assess the levels of alkaline phosphatase (ALP) (Tietz et al. 1983), alanine aminotransferase (ALT), aspartate amino transferase (AST) (Reitman and Frankel 1957), albumin (Doumas et al. 1971), total bilirubin (Doumas et al. 1973), total protein (Weichselbaum 1946), urea (Krieg et al. 1986), creatinine (Larsen 1971), and cholesterol (Zoppi and Fellini 1976).

Antioxidant assays

Proven analytical antioxidant assay techniques were employed to quantify GSH (reduced glutathione) (Moron et al. 1979), superoxide dismutase (SOD) (Misra and Fridovich 1972), and catalase (CAT) (Sinha 1972), whereas lipid peroxidation was analysed and expressed as the amount of MDA (malondialdehyde) formed (Niehaus and Samuelson 1968).

Histology

Small pieces of the liver tissues were fixed in 10% formalin and implanted in liquid paraffin (Aliyu et al. 2007). Specimens of 5–6 μm thickness stained with haematoxylin and eosin were examined for histological changes using a compound microscope.

Statistical analysis

Differences between the control and experimental groups were deduced using the statistical analysis software package SPSS (version 13.0) and GraphPad Prism (version 5). One-way analysis of variance (ANOVA) test was used to compare between groups. Significant differences were estimated from post hoc test by employing Tukey. All data were reported as mean \pm SEM; p values less than 0.05 were considered to be significant.

Results

Haematological studies

Haematological analysis (Fig. 1) showed significant reduction ($p < 0.05$) in haemoglobin, red blood cell, packed cell volume, and platelet counts in the CCl_4 -treated group when compared with the untreated control group. These parameters were significantly ($p < 0.05$) reversed across the groups treated with the herbal formulation. However, white blood cell count in the

animal's blood was not significantly ($p > 0.05$) different from the control groups.

Biochemical studies

ALP, AST, ALT, and total bilirubin levels were significantly ($p < 0.05$) elevated in the CCl_4 group as compared with the untreated group. There was however significant reductions ($p < 0.05$) across the treated groups in these parameters except in the levels of ALP and total bilirubin in which the group dosed with 1500 mg/kg bw of the drug exhibited significant ($p < 0.05$) decrease. Although there was a slight increase in the concentrations of total protein and albumin, it was not statistically different from the control groups (Fig. 2). There was no significant difference ($p > 0.05$) in the levels of urea, creatinine, and total cholesterol (Fig. 3).

Antioxidant studies

SOD activity was significantly ($p < 0.05$) depleted in the CCl_4 -exposed untreated rats, but the animals treated with the herbal formulation showed significantly ($p < 0.05$) increased activity across the treatment groups. GSH activity was significantly ($p < 0.05$) elevated in the group treated with 1500 mg/kg bw. The concentrations of MDA were significantly ($p < 0.05$) lowered in the liver tissue samples of all treated rats when compared with the untreated CCl_4 -exposed rats (Fig. 4).

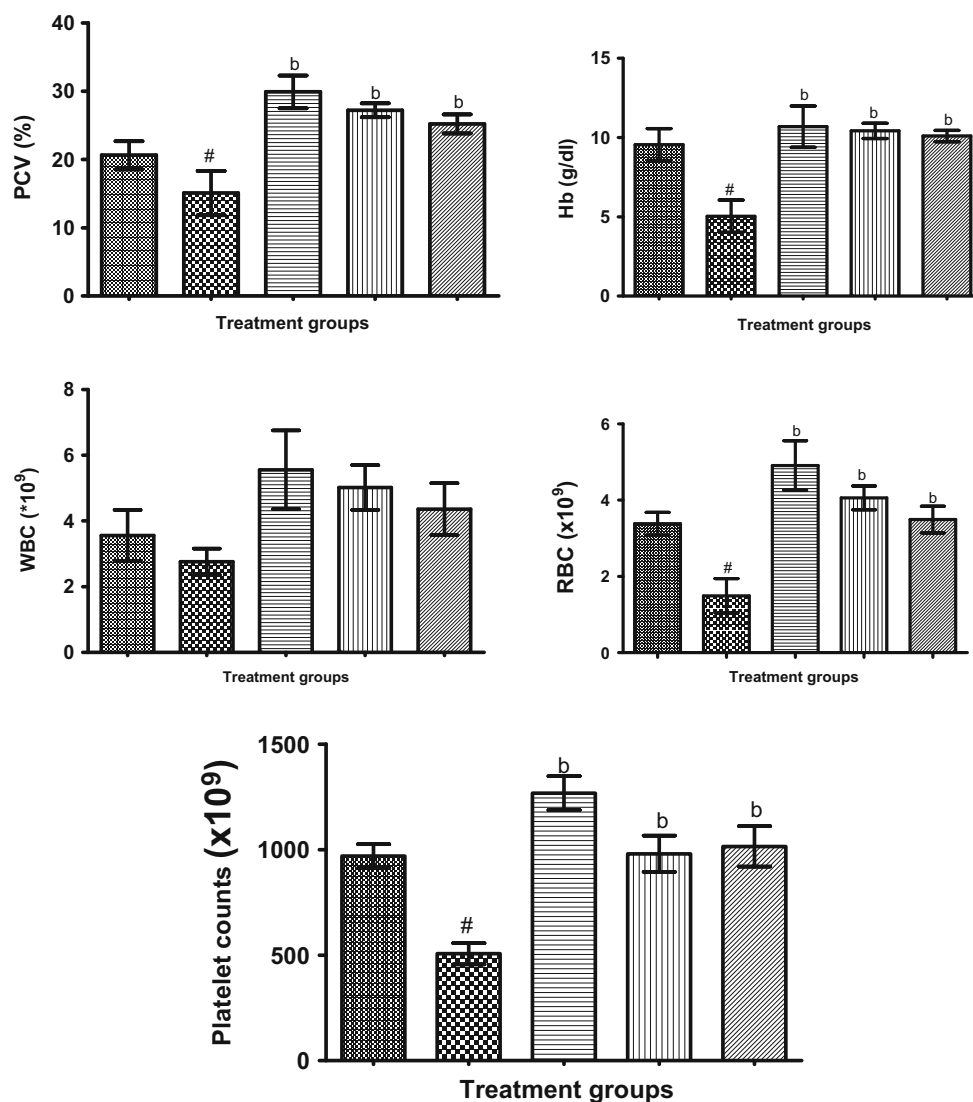
Histological studies

Histopathological assessment of the liver tissues showed centrilobular necrosis with rats treated with CCl_4 alone. As they were treated with Hepacare[®], there was a dose-dependent reversal effect, leading to reduced sinusoidal dilation and centrilobular fatty degeneration (Fig. 5). Similarly, the kidney tissues of rats in CCl_4 -treated group showed acute tubular necrosis and glomerular widening. But the group treated with Hepacare[®] showed a dose-dependent reversal effect on the kidney tissues, leading to reduced sinusoidal dilation and centrilobular fatty degeneration across the treatment group (Fig. 6).

Discussion

The liver is rich in protein which partly consists of antioxidants because of the numerous metabolic processes it oversees (Valencia et al. 2001). These antioxidants scavenge the liver cells of superoxides, hydroxyl radicals, nitric oxides, peroxides, and a host of others (Farombi and Owwoeye 2011). Since free radicals are excited by CCl_4 , radicals formed tend to overwhelm the radical scavenging activity of certain antioxidants

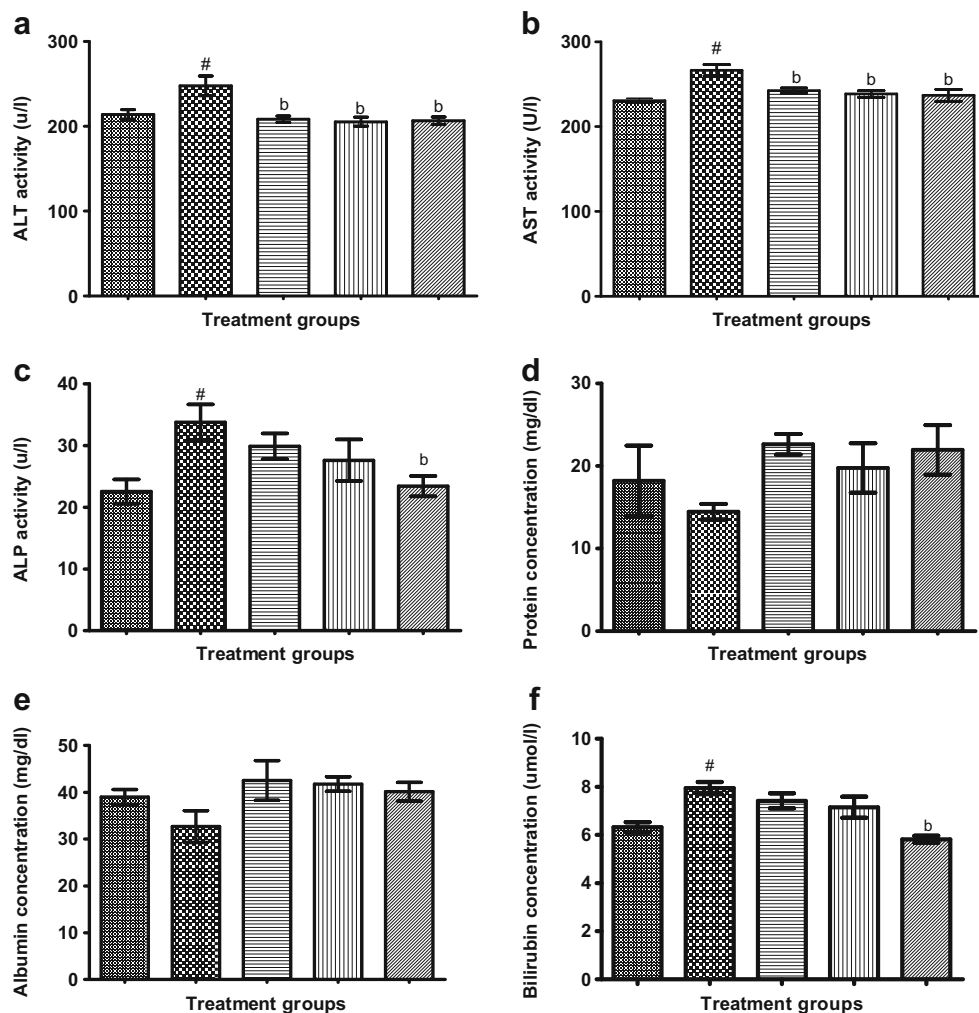
Fig. 1 Effects of Hepacare® on selected haematological parameters of albino Wistar rats. *WBC* white blood cell, *Hb* haemoglobin, *RBC* red blood cell, *PCV* packed cell volume. Values represent mean \pm SEM of eight replicates. ^a $p < 0.05$, significantly different from the negative control group. ^b $p < 0.05$, significantly different from the CCl₄ control group. [#] $p < 0.05$, the CCl₄ control group significantly different from the untreated control group



found in hepatic cells, thus igniting a cascade of other radicals that eventually damage the liver (Iwalewa et al. 2005). We were able to substantiate hepatic damage by the characteristic increased activity levels of liver function enzymes such as ALT, AST, and ALP, which were significantly higher ($p < 0.05$) in the CCl₄ group when compared with the untreated group (Fig. 2). We observed in our results that Hepacare® was able to significantly increase the activity levels of SOD and GSH ($p < 0.05$), although GSH was only significantly increased in the group treated with 1500 mg/kg bw while the increase observed for CAT was not statistically significant ($p < 0.05$) in all treatment groups when compared alongside with the untreated and CCl₄-treated groups (Fig. 4). The increase in the level of these antioxidants in Hepacare®-treated rats suggests that increased antioxidant activities of SOD, CAT, and GSH induced by the herbal formulation were able to ameliorate the hepatotoxic effect of CCl₄ which follows the same pattern in the observed activity levels of ALT, AST, and ALP in these

groups. These parameters were reduced in Hepacare®-treated groups; however, ALT level was more significantly lower ($p < 0.05$) when compared alongside with the untreated control group. Our findings are in consistent with the histological results of the liver tissues where CCl₄-treated animals showed marked centrilobular necrosis but became reduced following treatment with Hepacare® (Fig. 5). This result is quite similar with the results obtained by Rhunzi and colleagues using oroxylin as a hepatoprotective agent (Runzhi et al. 2013). Certain phytochemicals in the drug formulation are likely to repress hepatic cell necrosis that could arise from inflammatory response caused by CCl₄ (Taniguchi et al. 2004). Since antioxidant enzymes observed are inversely proportional to liver function enzymes, the reduction in plasma liver function enzymes mediated by Hepacare® herbal formulation may be attributed to the increased protein concentration of antioxidant enzymes mediated by the drug formulation and the individual free radical scavenging activity of drug component mediated by

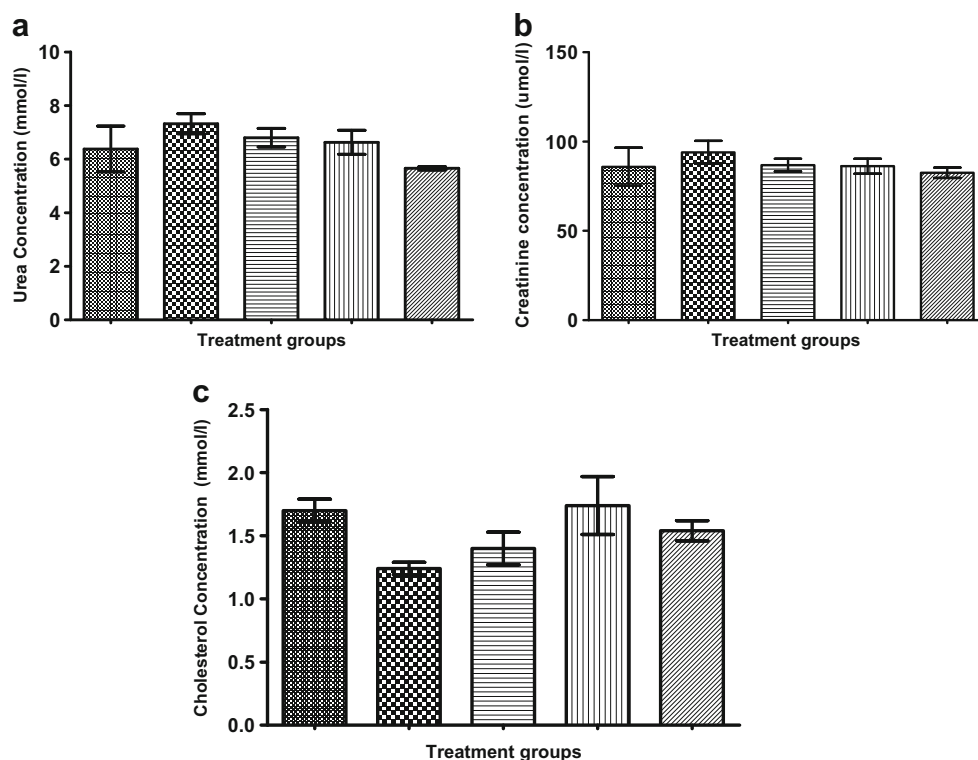
Fig. 2 Effects of Hepacare® on liver functions of albino Wistar rats. **a** *ALT* alanine aminotransferase. **b** *AST* aspartate aminotransferase. **c** *ALP* alkaline phosphatase. **d** Total protein. **e** Albumin. **f** Bilirubin. Values represent mean \pm SEM of eight replicates. ^a $p < 0.05$, significantly different from the negative control group. ^b $p < 0.05$, significantly different from the CCl₄ control group. [#] $p < 0.05$, the CCl₄ control group significantly different from the negative untreated control group



flavonoids and phenolic compounds present. Radicals that oxidize membrane lipids have the potential to release membrane-bound alkaline phosphatase from the endoplasmic reticulum and plasma membrane of hepatic cells (Ojiako and Nwajo 2005). This was observed in the amount of lipid peroxidation that was found in the CCl₄ group. Lipid peroxidation is characterized by the amount of MDA formed when polyunsaturated fatty acids of biological membranes are oxidized and broken down (Gawel et al. 2004). Treatment with Hepacare® reduced the concentration of MDA in a dose-dependent pattern. Chemoprevention against chemical carcinogens and oxidative stress breakdown and chemical carcinogens by natural products may be linked to their antioxidant status (Farombi and Owoye 2011). We can also suggest the presence of certain bioactive components in this herbal formulation that have the ability to induce the expression and synthesis of protein that includes various antioxidant enzymes. Since plasma total protein points to the viability of hepatic tissues in mammals, the rise in plasma total protein and albumin in the entire treated groups, although not significantly different from

the untreated and CCl₄-treated groups, could show that the formulation increased the protein synthesis activity of the liver. This is probably the underlying mechanism for the elevation of antioxidants observed in the liver cells. This result is in line with the findings of Sanjay and colleagues on the hepatoprotective activities of *Amorphophallus companullatus* (Sanjay et al. 2009). Serum total protein basically contains globulins and albumin which are globular proteins that act as transporters of xenobiotics. It is therefore important for the synthesis of these proteins for effective elimination of the ingested foreign compounds that makes the herbal formulation. Dixon and Paterson reported an association between body posture and plasma total protein and cholesterol (Dixon and Paterson 1978). Because the liver of animals in the CCl₄ group was severely damaged, animals in the group had lower levels and always had a lying posture; these parameters were consequently reduced in the group. CCl₄-administered animals suffer from distress and therefore are unable to take in fat and protein from dietary sources, thus crumbling the level of cholesterol in the circulatory system. Hepatic

Fig. 3 Effects of Hepacare® on plasma concentration of urea, creatinine, and cholesterol in albino Wistar rats. **a** Urea. **b** Creatinine. **c** Cholesterol. Values represent mean ± SEM of eight replicates. ^a*p* < 0.05, significantly different from the negative control group. ^b*p* < 0.05, significantly different from the CCl₄ control group. [#]*p* < 0.05, the CCl₄ control group significantly different from the untreated control group



damage compromises this physical activity (Adedapo et al. 2005). Since the inability to ingest cholesterol triggers its de novo synthesis which takes place in the liver of mammals to complement the dietary source, the animals suffer from low blood levels of cholesterol as a result of damage to the liver.

Hepacare® was able to restore the levels of cholesterol in the treated groups; however, there was no observed significance (*p* > 0.05) in all the experimental groups, but there was a correlation with protection against liver injury when compared with observed activity of liver function parameters (Fig. 2).

Fig. 4 Effects of Hepacare® on hepatic antioxidant of albino Wistar rats. **a** SOD superoxide dismutase. **b** GSH reduced glutathione. **c** CAT catalase. **d** MDA malondialdehyde. Values represent mean ± SEM of eight replicates. ^a*p* < 0.05, significantly different from the negative control group. ^b*p* < 0.05, significantly different from the CCl₄ control group. [#]*p* < 0.05, the CCl₄ control group significantly different from the untreated control group

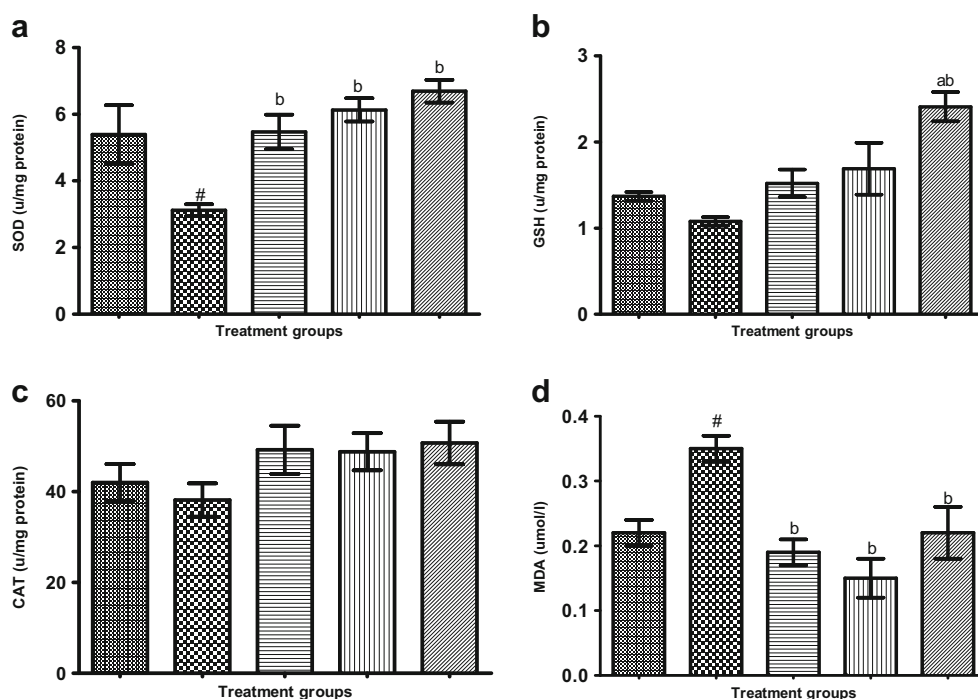
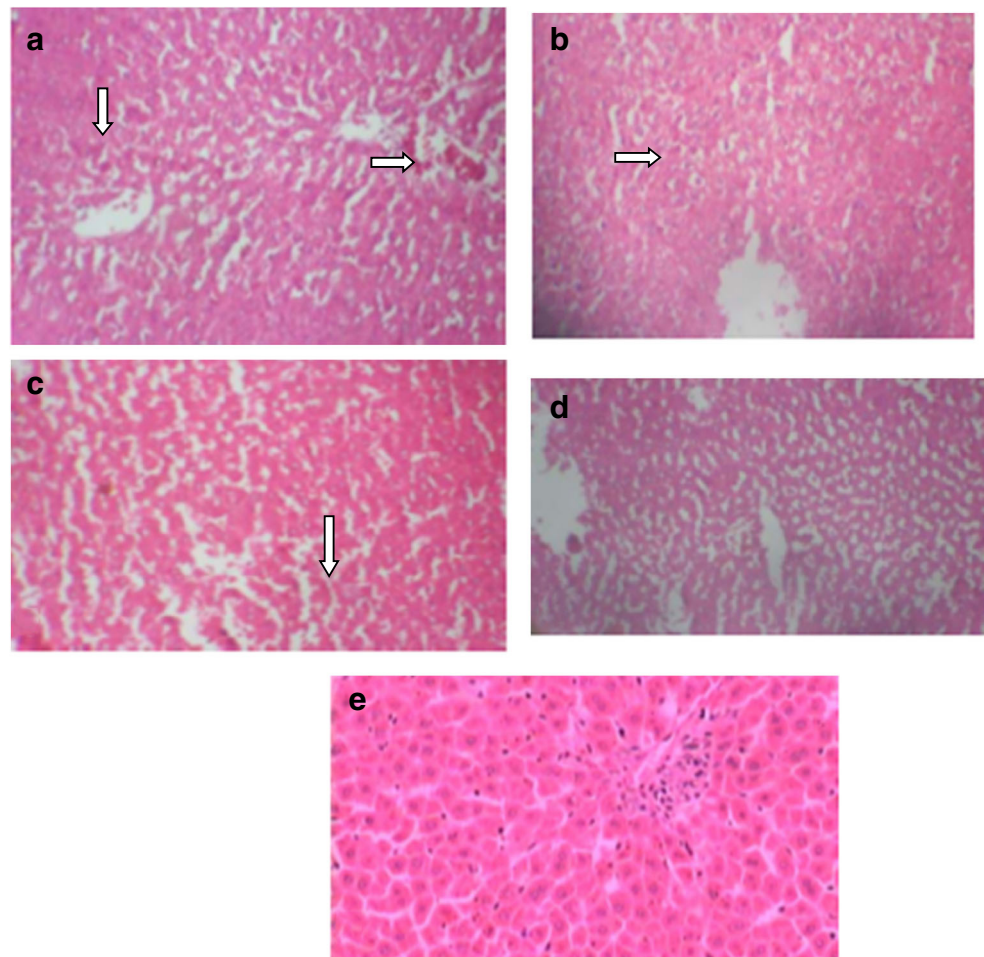


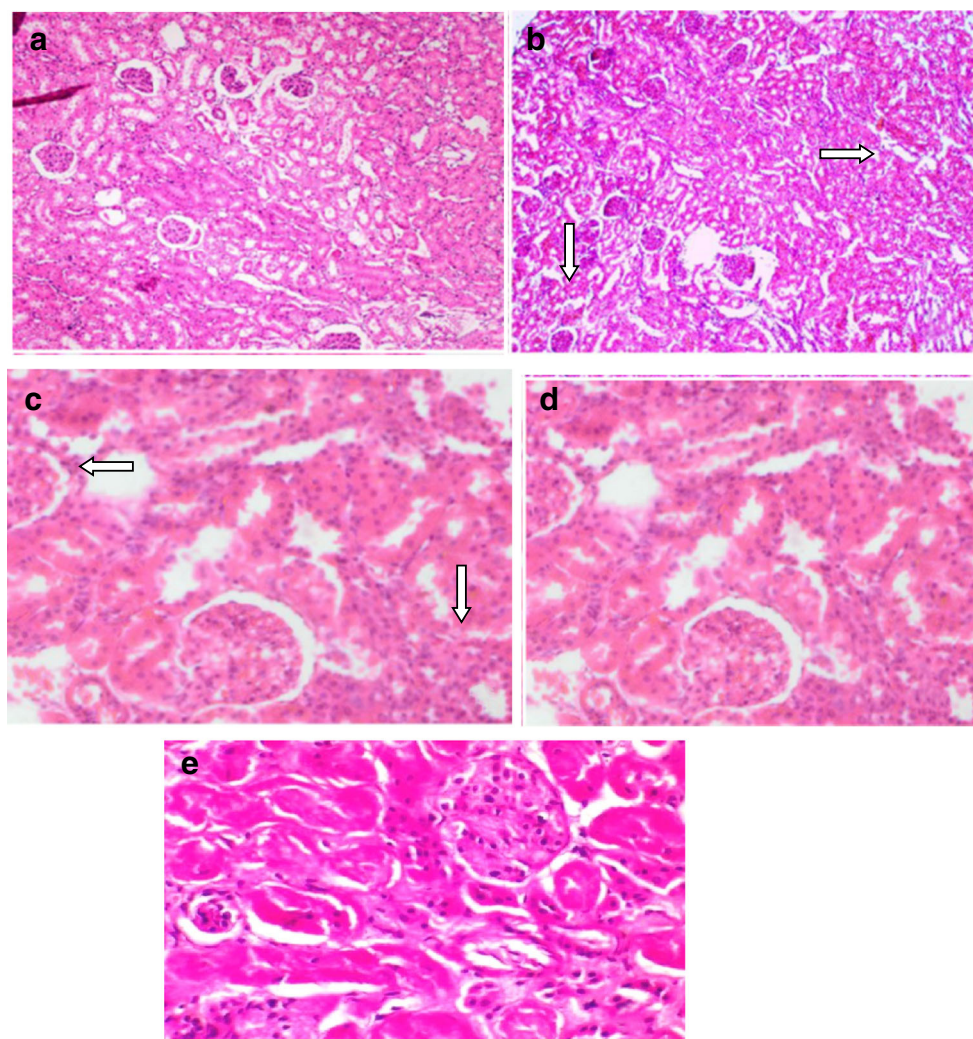
Fig. 5 Photomicrograph of **a** the liver of rat in the CCl₄-only group showing centrilobular necrosis (see the arrow), H&E, ×160; **b** the liver of rat treated with CCl₄ and 500 mg/kg bw of Hepacare[®] showing mild centrilobular fatty degeneration (see the arrow), H&E, ×160; **c** the liver of rat treated with CCl₄ and 1000 mg/kg bw of Hepacare[®] showing fatty degeneration and moderate sinusoidal dilation (see the arrow), H&E, ×160; **d** the liver of rat treated with CCl₄ and 1500 mg/kg bw of Hepacare[®] showing reduced sinusoidal dilation and centrilobular fatty degeneration, H&E, ×640; and **e** the liver of rat in the untreated control showing normal features, H&E, ×400



However, we can also suggest that the increased total protein and albumin though not significant in the groups administered with the herbal drug elevated these parameters and other parameters that readily bind these protein products such as cholesterol (Fig. 3). Total bilirubin is a marker for both hepatic injury and haemolysis. The increased levels of this parameter in CCl₄-treated group also corroborate the observed liver injury and haematological compromise. Considering this result, we can infer that the drug formulation attenuated CCl₄-induced damage on the hepatobiliary system of mammals in a concentration-dependent pattern with significant difference observed in the highest dose of the herbal formulation. Direct bilirubin leaks out of hepatic tissue into the circulatory system, thereby increasing the plasma concentration of total bilirubin, resulting in bilirubin deposits in the urine, skin, and mucous membrane of the eyes, leading to jaundice (Nyblom et al. 2004). Results obtained from haematological examination corroborate the protective effects of Hepacare[®] against any form of toxicity by CCl₄ (Fig. 1). The observed CCl₄-induced haemolysis was indicated by a reduction in RBC, Hb, and PCV. The haemolytic condition induced by CCl₄ was also proportional to the level of bilirubin observed in this

group which is a product of haemoglobin degradation (Tiribelli and Ostrow 2005). This haemolysis was then treated with Hepacare[®] as shown by a significant ($p < 0.05$) increase in PCV, Hb, and RBC in drug-treated groups in these blood parameters. CCl₄ was also observed to impair the formation of WBC, though not statistically significant ($p > 0.05$). Low WBC increases susceptibility to infection from the microenvironment and carcinogenesis by CCl₄. The ability of WBCs to carry out a 'search and arrest rogue cells' has not in any way compromised the system (Standish et al. 2008). The liver and kidney are thus liable to infection and haemorrhage within days. Hepacare[®] did not significantly ($p > 0.05$) alter the level of the WBC across the treated groups. The platelet count was significantly ($p < 0.05$) reduced in the CCl₄-treated group, leading to thrombocytopenia which may probably be due to decreased production of thrombopoietin. Thrombopoietin is a glycoprotein produced by the liver and kidney which controls the production of platelets. It stimulates the differentiation and production of the bone marrow cells that develop large numbers of platelets (Kaushansky 2006). Hepacare[®] significantly normalized the platelet counts and thus may act on its mechanism of action by stimulating the bone marrow cells for the

Fig. 6 Photomicrograph of **a** the kidney of rats in the untreated group showing normal glomerular and tubular histology; **b** the kidney of rats in the CCl₄-only-treated group showing acute tubular necrosis and glomerular widening (see the arrow); **c** the kidney of rat treated with CCl₄ and 500 mg/kg bw of Hepacare[®] showing focal necrosis of the proximal convoluted tubular lining epithelial cells (see the arrow); **d** the kidney of rat treated with CCl₄ and 1000 mg/kg bw of Hepacare[®] showing that focal areas of necrosis of the proximal tubular lining epithelial cells were still seen along with cellular swelling, desquamation, and loss of brush border; and **e** the kidney of rat treated with CCl₄ and 1500 mg/kg bw of Hepacare[®] showing reduced tubular necrosis and glomerular widening



production of this hormone. We also observed that Hepacare[®] was able to protect our experimental subjects from nephritic damage apart from the wide protection it has conferred on the liver at higher concentrations of the herbal formulation (Fig. 6). High plasma levels of creatinine and urea could result in the malfunctioning of cells of the kidney. The kidney is involved in the excretion of waste via ordered steps of ultrafiltration, selective reabsorption, and tubular secretion. A compromise of these activities leads to its damage which is characterized by the leakage of creatinine and urea out of the nephron. Thus, the kidney function test parameters such as urea and creatinine levels were low (but not statistically different) in Hepacare[®]-treated groups when compared with the control groups (Fig. 3). Creatinine resulting from muscle metabolism is usually filtered out of the plasma by the kidney. Increased plasma level of this parameter suggests an impairment of the glomerulus. However, this parameter cannot be measured in isolation because increased creatinine could result from increased muscle mass; therefore, blood urea nitrogen was measured alongside

creatinine. This was also insignificantly ($p > 0.05$) low in drug-treated groups. When the rats were treated with CCl₄, there were acute tubular necrosis and glomerular widening but subsequent treatment of the animals with Hepacare[®] especially at higher doses reduced these necrotic cells of the kidney (Fig. 6). Thus, Hepacare[®] was able to protect the animals from nephritic impairment.

Conclusion

In conclusion, we found Hepacare[®] to possess strong favourable effects in rat model against liver impairment and blood disorders caused by CCl₄ and potential toxicants similar to its mode of action through its high antioxidant-generating activities. The protective effect of Hepacare[®] therefore presents a clinical prospect in the development of novel curative or corrective agents for acute liver and kidney damage.

Acknowledgements This research was supported by Covenant University, Cnaan Land, Ota, Nigeria, through a seed grant (CUCERD-2014) given to AHA. The support from the technical staff of the Biochemistry Unit, Department of Biological Sciences, Covenant University, is greatly appreciated.

Compliance with ethical standards The research was approved by the Department of Biological Sciences Research Ethics Committee, Covenant University. All animals were also treated in line with the National Institutes of Health (NIH) guidelines for the use and care of animals in the laboratory (National Institute of Health (NIH) 2011).

Conflict of interest The authors declare that they have no conflict of interest.

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