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Investigating the Effect of Flavonoid, Saponin, Alkaloids and Tannins Extracted from *Combretum dolichopentalum* Diels in CCl_4 –Induced Hepatotoxicity

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ABSTRACT

Modification of biological molecules caused by oxidative stress is the most popular harmful b side reaction leading to pathological changes. This study investigated the impact of flavonoids, a saponins, and tannin extracted from *Combretum dolichopentalum* on CCl_4-induced hepatotoxicit five male rats were sorted into nine groups, and allowed access to water and food. Groups received distilled water only, groups III and IV pre-treated with 250 and 500 mg/kg body weig ethanol extract of *C. dolichopentalum* (EECD) respectively, group V pre-treated with 50 mg silymarin, group VI to IX pre-treated with 100 mg/kg bwt of flavonoids, saponins, alkaloids and t *C. dolichopentalum* respectively. After seven days, all animals (except normal control) were int with 0.4 mL/kg bwt CCl_4, and sacrificed 48 h after. Samples of liver and blood were used for h and biochemical studies respectively. Results showed that CCl_4 -induced massive fatty cha centrilobular necrosis in the liver, marked elevations in serum malondialdehyde (MDA), live enzymes and other biochemical changes were observed. However, animals administered alkaloids, flavonoids, tannins and saponins of *C. dolichopentalum* presented improved liver his feature. The biochemical parameters of treated rats approached that of normal control w administered alkaloid and flavonoid showed more significant decrease in MDA and total biliru results from the current study show that *C. dolichopentalum*, especially flavonoid and alkaloid clearly protects the liver from CCl_4-induced hepatotoxicity. Furthermore, the saponin fracti *dolichopentalum* appears to be hepatotoxic to the liver.

Keywords:*Combretum dolichopentalum*, Flavonoids, Saponins, Tannins, Hepatotoxicity.

Introduction

Oxidative stress occurs when there is an imbalance of free radicals and antioxidants in a biological system. This phenomenon is triggered by imbalance in generation and accumulation of reactive oxygen species (ROS) and the capacity to scavenge the reactive metabolites by biological systems.[1,2] These reactive metabolites when in excess can induce adverse metabolic changes in biological systems such as lipids, protein and DNA damage.[3,4] Under normal conditions, antioxidant mechanisms, including antioxidants molecules and enzymes scavenge reactive oxygen species and defend the organism from the harmful effects of oxidizing species. Cellular antioxidants help to control oxidative stress *in vivo*, but cellular antioxidant interception and scavenging of ROS is not 100 % efficient, and this calls for the introduction of plant-based antioxidants for the enhancement of endogenous antioxidants. *Combretum dolichopentalum* is one of such plants.[4,5] Africans and Indians are known for the traditional use of different species of Combretum. Locals in Ogwa Imo State Nigeria use *C. dolichopentalum* for the treatment and management of diseases of gastrointestinal disorders.[5,6] The warm aqueous extract of *C. dolichopentalum* is consumed to recondition the uterus after parturition by indigenous women of Mbaise, in Imo State Nigeria.[6]

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Plant based drugs used in traditional medicine practice have become the model of current research because they are affordable, effective, and with minimal side effects. Research shows that *C. dolichopentalum* possess a very high content of minerals such as sodium, potassium, and vitamins such as vitamins A, thiamine and niacin. It also contains essential amino acids like leucine and arginine and acidic amino acids.[4,6] *Combretum dolichopentalum* leaves contain in varying concentration, flavonoids such as epicatechin, anthocyanin, rutin, and kaempferol and other bioactive compounds.[7,8,9] Furthermore, *C. dolichopentalum* is bactericidal as expressed against *Salmonella typhi* and *Escherichia coli*,[10] *Staphylococcus aureaus*, *P. aeruginosa* and *C. albicans*.[11] The use of plant extracts have been reported to normalize adverse biochemical changes *in vivo*.[2,12,13] However, there are scarce materials on specific phytochemical fractions involved in ameliorating adverse biochemical and physiological changes. Therefore, this study investigated the effect of flavonoids, alkaloids, saponins, and tannins extracted from *Combretum dolichopentalum* on CCl_4-induced hepatotoxicity. The study desired to elucidate the effect of the crude extract vis-a vis the specific phytochemical fractions of *C. dolichopentalum* leaf responsible for the hepatoprotective potentials.

Materials and Methods

Collection and authentication of plant material

Fresh plants of *C. dolichopentalum* were collected from a farm at Obinze in Owerri West Local Government Area of Imo State in March, 2015. Authentication of the plant sample was done by Mr. A. Ozioko, a staff of the Bioresource Development and Conservation Program (BDCP), at the University of Nigeria, Nsukka, Enugu State, Nigeria. The sample specimen was deposited with voucher number IMSUH12 at Imo State University Herbarium.

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Ethanol extraction of plant
Fresh leaves of *C. dolichopentalum* were plucked, washed, air dried for 4 weeks, and pulverized to powder with a grinding mill (Kenwood BL357). Ethanol extract was achieved with 300 g of the powder macerated in 1.75 L of 80% ethanol in 3 portions for 48 h, and pooled together. The mixture was subjected to coarse and fine filtration using a muslin cloth and Whatman No 1 filter paper respectively. Rotary evaporator was used to concentrate the filtrate which was named ethanol extract of *C. dolichopentalum* (EECD).

Extraction of alkaloid, saponin, flavonoid and tannin for in vivo studies
*Alkaloid extraction:*Alkaloid was extracted by adding 50g of the pulverised sample into a 1000 mL beaker. To this, 500 mL 29% acetic acid containing ethanol was added, allowed without agitation for 6 h and filtered subsequently. The filtrate was reduced to ¼ of the initial volume over laboratory water bath. Concentrated NH_4OH was used to precipitate alkaloid. The precipitate was recovered by filtration with Whatman No. 1 filter paper.[14]

Saponin extraction:
Saponin was extracted by adding 50 g of pulverised sample in 1000 ml beaker. To this, 500 mL 20 % ethanol was added and stirred. The mixture was placed on a water bath at 55°C for 4 h with continuous stirring. Following filtration, the residue was re-extracted using same solvent. At water bath temperature of 90°C, the combined filtrate was concentrated to 40 mL, delivered into a separating funnel, and 50 mL diethyl–ether was added. The setup was vigorously agitated and the aqueous–layer recovered. This procedure was carried out three consecutive times and 60 mL n–butanol was added. The aqueous–layer was washed with 10 ml 5 % NaCl twice and the recovered solution dried to constant weight over water bath.[14]

*Flavonoid extraction:*Flavonoid was repeatedly extracted by adding 50 g pulverised sample into a beaker containing 500 mL 80 % aqueous methanol at 25±3°C. With Whatman No. 45 filter paper the recovered solution was filtered, and the combined filtrate dried over water bath.[15]

*Tannin extraction:*Tannin was extracted by adding 50 g pulverised sample in a beaker containing 500 mL distilled H_2O. After filtration, to eliminate neutral substances, the filtrate was re-extracted thrice using ethyl acetate, reduced to pH 2 with concentrated HCl, re-extracted using ethyl acetate and thereafter, evaporated to dryness.[14]

Experimental Design
Male Wistar albino rats (65) were sourced from the Department of Veterinary Medicine, University of Nigeria Nsukka, Enugu State, Nigeria. The rats were acclimatized for 7 days at room temperature in metal cages under 12/12 h light and dark cycle. The rats were given unhindered access to clean water and rat pellets (Pfizer Feeds, Aba, Nigeria). The protocol of this study adhered to the laws and regulations for handling experimental animals[16] and was endorsed by the ethics committee of the Department of Biochemistry, FUTO with reference number FUTO/BCH/MAR/XXI/15/01
To arrive at 250 and 500 mg/kg EECD, the acute toxicity study of ethanol extract *of C. dolichopentalum,* presented no mortality or any observable adverse changes in animals' (mice) behaviour after a dose of 2500 mg/kg body weight. The acute toxicity study for the selection of 100 mg/kg for flavonoids, saponins, alkaloids and tannins, showed no mortality or observable adverse changes in the behaviour of animals (mice) after a dose 2000 mg/kg body weight. The animals were grouped and treated as shown in Table 1. Ethanol extract *of C. dolichopentalum* and the extracted flavonoids, saponins, alkaloids and tannins were administered daily by oral gavages, for 7 days. After 7 days, animals in all the groups (except normal control) were intoxicated with 0.4 mL/kg bwt $CC1_4$ in liquid paraffin (2:1), and the toxicant was allowed to act on the animals for 48 h. After nightlong fast the animals were anaesthetized in dimethyltetrachloride chamber, blood drawn by cardiac puncture and liver excised.

Table 1: Experimental treatment

Groups	Group Identity	Treatment
I	Normal control (NC)	feed and water
II	Positive control (PC)	feed, water and CCl_4
III	Treated group (T_{250})	250 mg/kg bwt of EECD and CCl_4
IV	Treated group (T_{500})	500 mg/kg bwt of EECD and CCl_4
V	Sylimarin group	50 mg/kg bwt of silymarin and CCl_4
VI	Flavonoid group	100 mg/kg bwt of flavonoid and CCl_4
VII	Saponin group	100 mg/kg bwt of saponin and CCl_4
VIII	Alkaloid group	100 mg/kg bwt of alkaloid and CCl_4
IX	Tannin group	100 mg/kg bwt of tannin and CCl_4

Preparation of liver samples for histopathology and SOD activities
Some portions of the liver samples were washed in a buffer of 1.15 % KCl, dabbed with paper and weighed. The liver samples of rats in Groups I, II, III, IV, V and VII were subjected to histological investigation by the method of Okoro.[17] The other portions of the liver samples were homogenized in 10 mM of KCl phosphate buffer containing EDTA at pH 7.4. The supernatant obtained by centrifuging the homogenate at 12000 x g for 10 min, was used to assay the activities of superoxide dismutase (SOD).[18]

Biochemical Assays
To obtain serum used to assay various biochemical parameters, blood samples collected from the rats were centrifuged at 600 x g for 15 min after clotting. The method of Reitman and Frankel[19] was used to assay serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. The concentration of malondialdehyde (MDA) was determined by the method of Wallin et al.[20], protein by the method described by Tietz,[21] and albumin was carried out according to the method of Doumas.[22] Total globulin fraction was determined by subtracting the concentration of albumin from total protein concentration. Albumin/globulin ratio was calculated by dividing the albumin by the globulin. The concentration of serum total bilirubin was determined by the method of Zoppi*et al*.[23]

Statistical analysis
Experimental data were analysed using the statistical program SPSS (version 18, USA). Data were analysed by one-way analysis of variance (ANOVA). Values were considered significant at $p<0.05$. Values of (n = 5) determinations are represented as mean ± SD.

Results and Discussion

At the end of pre-treatment with varying fractions of *C. dolichopentalum,* serum MDA concentration varied non−significantly ($p<0.05$) in the entire groups (except Alkaloid group ($p= 0.006$)) when compared to the Normal control (Figure 1). However, when compared to the Positive control serum MDA concentration reduced nonsignificantly ($p<0.05$) in the groups except in Alkaloid ($p = 0.001$) and Flavonoid ($p = 0.023$) groups which presented significantly reduced MDA concentration of 0.65± 0.16 mg/dl and 0.97± 0.17 mg/dL respectively. This agrees with the observation of other studies that induced hepatoxicity with CCl_4.[24,25] Malondialdehyde is a toxic aldehydes of lipid peroxidation, capable of spread by chain reaction and causing damage across biomembranes.[26,27] Animal groups pre-treated with EECD, flavonoid, saponin, alkaloid and tannin extracts of *C. dolichopentalum* showed decreased MDA with the alkaloid treated rats showing the least MDA concentration.This shows that the bioactive compounds in EECD and the flavonoids, saponins, alkaloids and tannins fractions used in pre-treatment inhibited lipid peroxidation in the liver cells. This is in line with the works that ameliorated lipid peroxidation by administration of plant extracts.[12,13,25,28] When compared to the Positive control the activity of superoxide dismutase

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in CCl_4−induced oxidative stress (Figure 2) varied nonsignificantly (p = 0.96) in the groups except tannin pre−treated rats that presented significantly reduced (p = 0.000) SOD activity (9.65 0.415 U/g liver). The removal of superoxide by SOD is indeed a detoxication reaction, although basically, the cytosolic form (Cu, Zn - SOD) is more susceptible to the pre-oxidants, while the mitochondrial form of SOD can be induced under oxidative stress.[29] It was also observed that tannin control groups was significantly reduced compared to the flavonoid, saponin alkaloid groups.

Figure 3 showed ALT activity significantly increased in the positive control (p= 0.00) and Saponin group (p = 0.000) compared to the normal, and other treated groups. Significant capacity in the reduction of ALT activities were presented by flavonoid (p = 0.000) and alkaloid (p = 0.000) fractions of *C. dolichopentalum* pre-treated rats compared to Positive Control. Figure 4 shows AST activities reduced significantly in Tannin (p = 0.000), Normal control (p = 0.000) and Silymarin (p = 0.029) groups with activities of 50.20± 13.31 IU/L, 67.20± 14.13 IU/L and 82.47± 6.90 IU/L respectively when compared to the Positive control (106.6 IU/L). EECD treated rats showed dose dependent reduction of ALT and AST activities. Both AST and ALT are biomarker enzymes of hepatic parenchyma injury after a toxic insult.[30,31] Decrease in activities of transaminases in EECD and flavonoids, alkaloids and tannins pre-treated rats indicates stabilization of plasma membrane as well as protection of hepatic tissues from damage caused by CCl_4.[32]

Results (Figure 5) also showed nonsignificant (p = 0.193, 65.46±11.24 g/L) decrease in total protein in positive control rats when compared to Normal control (p = 77.30±1.45) and other groups. Albumin (Figure 6) decreased nonsignificantly (p = 0.779, 34.13±10.21 g/L) in positive control compared to Normal control, 250 mg/kg, 500 mg/kg and Silymarin groups. When compared to the Normal control, globulin concentrations (Figure 7) reduced nonsignificantly in the positive control (p = 0.995), 250 mg/kg EECD (p = 0.998, 27.08±6,86 g/L) and 500 mg/kg EECD (p = 0.971, 24.86±8.22 g/L) pre-treated groups. Whereas, globulin increased nonsignificantly in flavonoids (p =1.000, 34.26±1.96 g/L), Saponin (p = 0.627, 47.33±2.47 g/L), alkaloid (p = 1.000, 33.95±5.57 g/L) and Tannin (p = 0.992, 10.72±4.79 g/L) groups when compared to Positive control (p = 0.995, 31.34±6.49 g/L). The albumin/globulin ratio (Figure 8) showed nonsignificantly elevated values in rats pre-treated with 250 mg/kg bwt, 500 mg/kg bwt and silymarin. A drop in the albumin/globulin ratio reveals hypoproteinemia as observed in the positive control, and groups pre-treated with flavonoid, saponin, alkaloid and tannin extracts of *C. dolichopentalum*. This could be attributed to decreased synthesis of albumin due to liver cirrhosis and/or non availability of the precursors for albumin synthesis. The pure/single phytochemical extracts of *C. dolichopentalum* seem to lack important bioactive ingredients necessary for synergy in such complex metabolic process. However, rats pre-treated with EECD and silymarin presented higher albumin/globulin ratio, indicating protection of the liver from toxic assault by the full compliments of *C. dolichopentalum* phytochemical constituents. The higher albumin/globulin ratio of the EECD pre-treated groups compared to the flavonoid, saponin, alkaloid, and tannin groups confirms the fact that protein haemostasis can be maintained better with the crude extract than with the individual phytochemicals. Furthermore, elevated concentrations of total bilirubin (Figure 9) were observed in Positive control compared to Normal and other treated groups. These results are in line with other studies that reported decrease in protein profile and increase in total bilirubin.[28,31] A fall in protein and albumin concentrations have been reported in severe parenchymal liver damage, especially from poisoning with CCl_4,[23,34] reflecting synthetic incapability of the liver intoxicated with CCl_4. The incapacitation of the liver may alter the liver's ability to actively participate in buffering action and enzymatic activities, fluid exchange, binding and transport functions. The increased concentration of total bilirubin in positive control shows increase in the breakdown of haemoglobin or other heme containing proteins such as myoglobin, catalase and cytochrome. Also, it indicates the liver's inability to remove bilirubin by glucoronidation.[35] However, pretreatment with the extract protected heme containing proteins from oxidative damage.

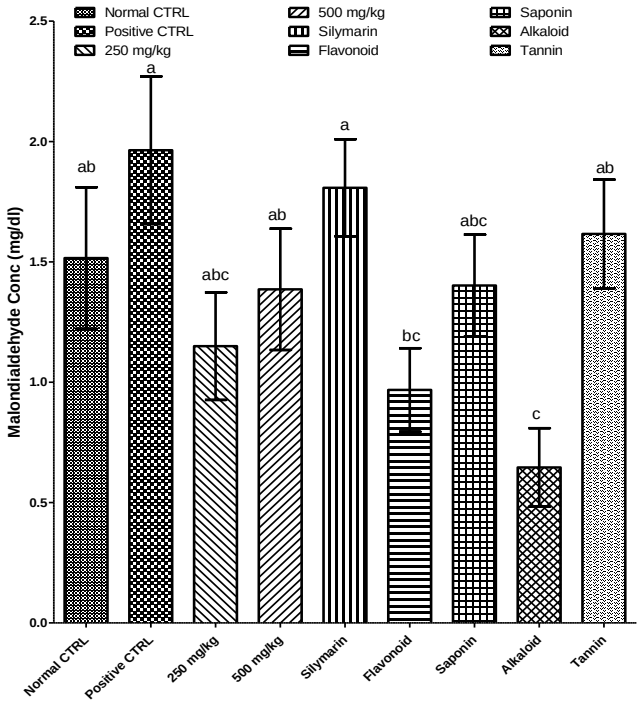

Figure 1: Effect of EECD in malondialdehyde (MDA) concentration in CCl_4-induced oxidative stress. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

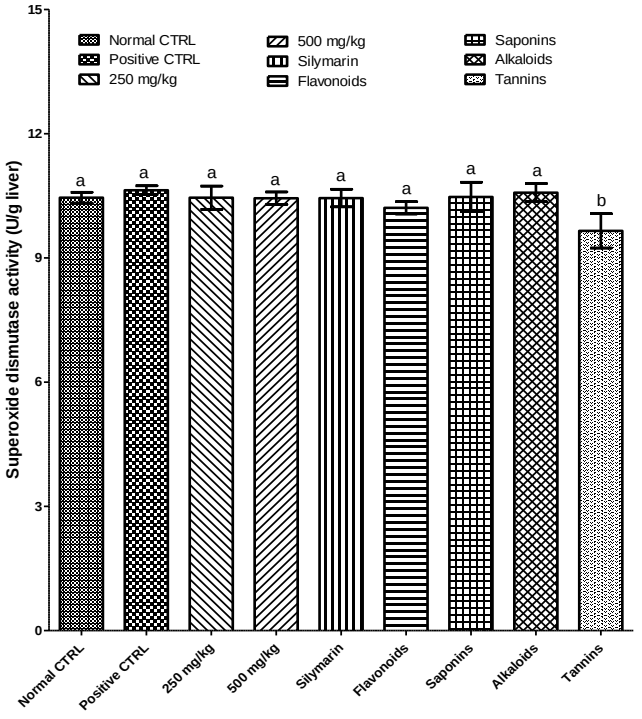

Figure 2: Effect of EECD and the extracted phytochemicals on superoxide dismutase (SOD) activity in CCl_4-induced oxidative stress. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

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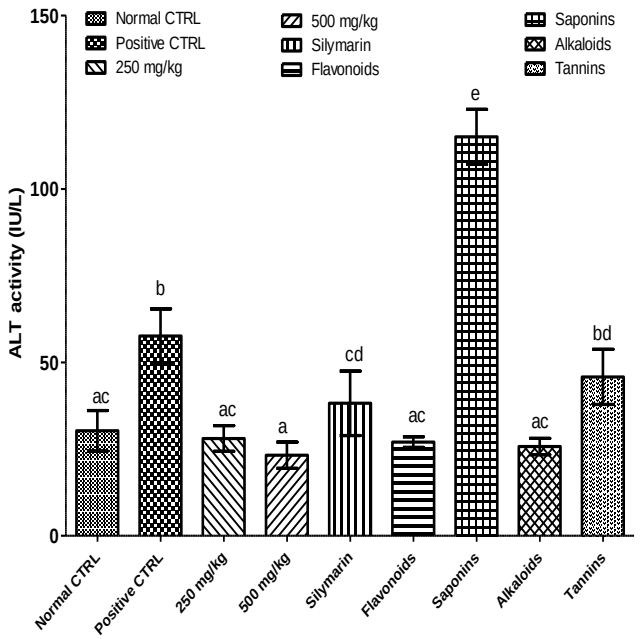

Figure 3: Effect of EECD and the extracted phytochemicals on alanine aminotransferase (ALT) activity in CCl_4-induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

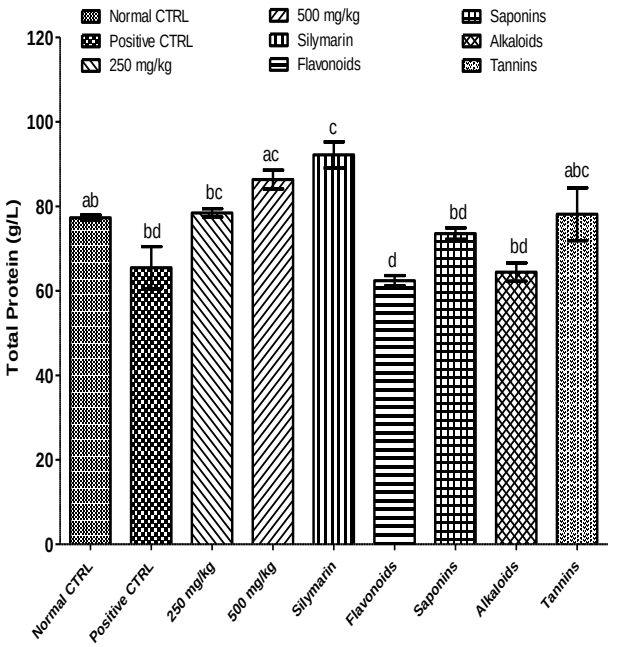

Figure 5: Effect of EECD and the extracted phytochemicals on serum total protein in CCl_4-induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

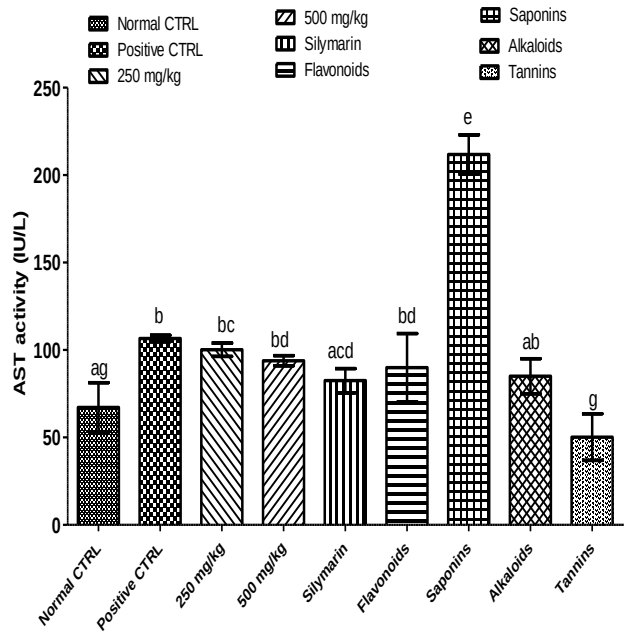

Figure 4: Effect of EECD and the extracted phytochemicals on activity of serum aspartate amino transferase (AST) in CCl_4-induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

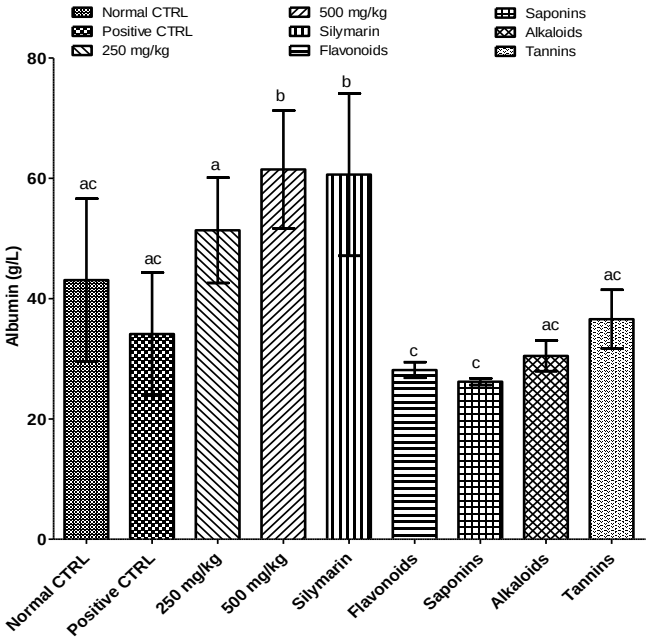

Figure 6: Effect of EECD and the extracted phytochemicals on serum albumin concentration in CCl_4–induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

The reduction suggests that EECD possesses liver protective activities. This reduction is in line with the work which showed that rutin a component of *C. dolichopentalum* can cause a reduction in total bilirubin by protecting heme containing proteins.[7,31] The EECD perhaps prevented the destruction of haemoglobin or aided the liver by enhancing its ability to remove bilirubin by glucoronidation.

Also, the alkaloids precipitated from *C. dolichopentalum* better prevented the destruction of heme containing proteins as well as enhanced bilirubin uptake, conjugation and subsequent excretion by the liver compared to the flavonoid, saponin and tannin groups. The result of histological study showed massive fatty change and centrilobular necrosis in most areas of the liver in CCl_4-intoxicated and untreated group and the saponin pre-treated.

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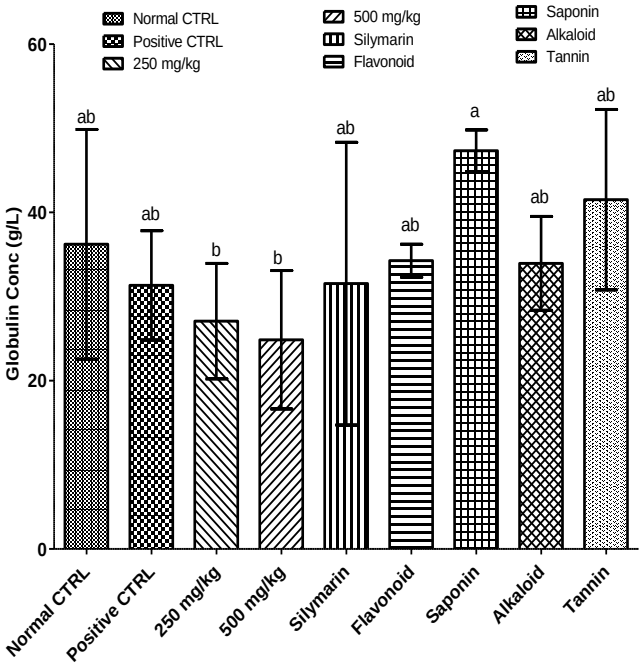

Figure 7: Effect of EECD and the extracted phytochemicals on serum globulin concentration in CCl_4–induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

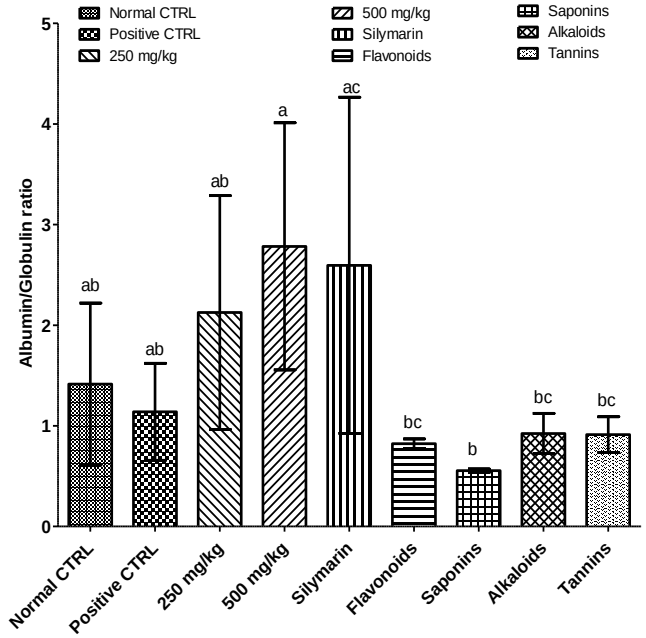

Figure 8: Effect of EECD and the extracted phytochemicals on albumin globulin ratio in carbon tetrachloride- induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

This is probably the reason for the increase in ALT and AST activities in these groups. Sumaiya *et al.*[36] reported CCl_4-induced fatty degeneration and vacuole formation in liver cells. The CCl_4 intoxicated rat groups presented also, mononuclear cell infiltration mostly macrophages and lymphocytes around the central vein and in the portal areas of the liver.

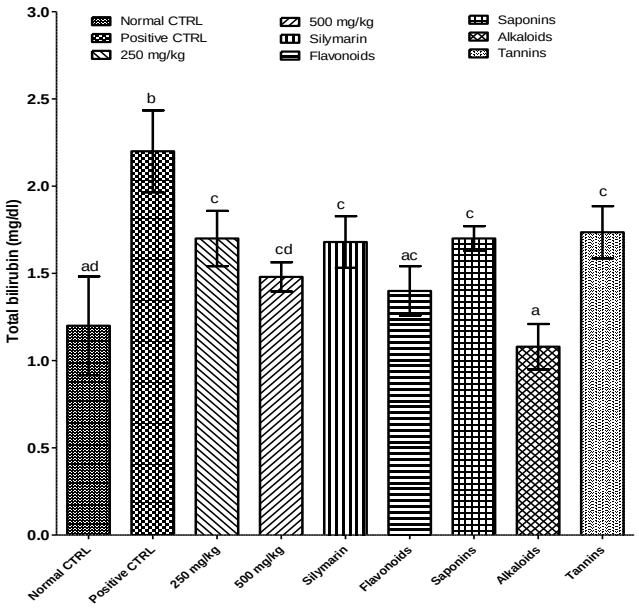

Figure 9: Effect of EECD and the extracted phytochemicals on serum total bilirubin in CCl_4 -induced hepatotoxicity. Significance ($p<0.05$) is indicated on bars with different alphabets. Values of (n = 5) determinations are represented as mean ± SD.

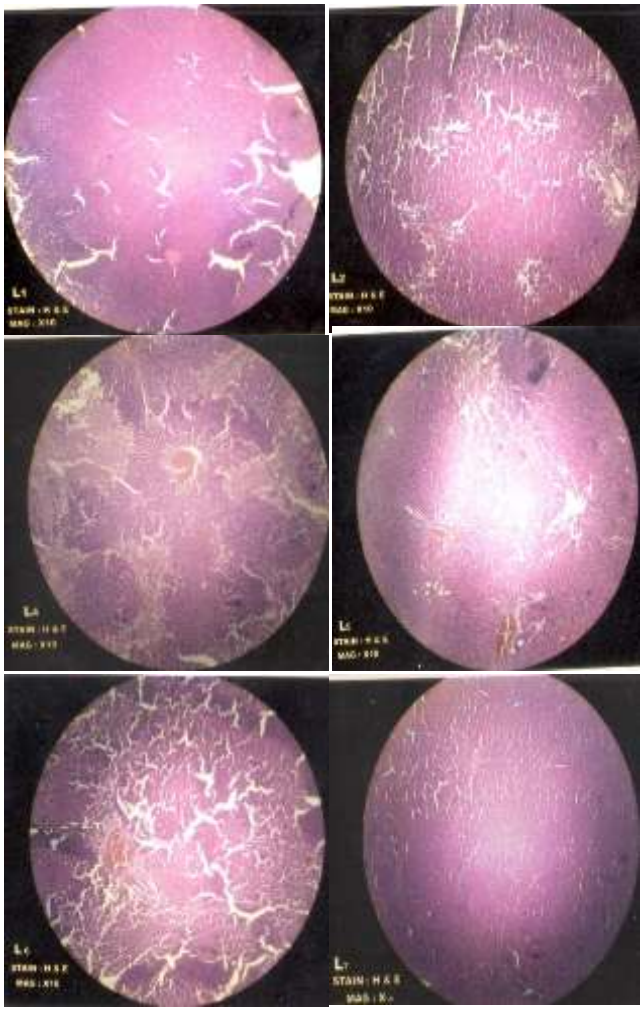

Figure 10: Light microphotographs of HE-stained sections of rat livers. L1=normal, L2= positive control, L4= 250 mg/kg, L5= 500 mg/kg, L6=saponin L7=Silymarin

The damage to liver tissues is reported to start after formation of trichloromethyl radical (•CCl_3) and trichloromethylperoxyl radical (•CCl_3O_2) formed from CCl_4 metabolism through cytochrome P450 enzyme.[13] These free radicals can bind to a liver cell component inducing inhibition of lipoprotein secretion. This causes fatty tissue to accumulate in the liver leading to fatty liver or steatosis. Fatty liver is also caused by factors interfering with secretory mechanisms. Studies have also suggested that flavonoid and alkaloid extract of *C. dolichopentalum* protects the kidney from CCl_4-induced nephrotoxicity.[37] However, the histopathological results showed that pretreatment with ethanol extract of *C. dolichopentalum* and silymarin were hepatoprotective in a dose dependent manner. It also confirms the hepatoprotective property of silymarin, a synthetic drug. Sumaiya et al.[36] reported reduced occurrence of lesions in the liver, on treatment with ethanol and aqueous extracts of saffron. The hepatoprotective capacity of ethanol extract of *C. dolichopentalum* can be attributed to attenuation of radical formation from CCl_4 by inhibition of activities of cytochrome P450 and enhanced radical scavenging by the plants bioactive compounds.

Conclusion

The phytochemical fractions derived from *C. dolichopentalum* revealed that; tannin fraction significantly reduced elevated concentration of MDA and enhanced the synthetic ability of the liver to form protein. While the alkaloid and flavonoid fractions significantly reduced activities of ALT and AST as well as concentrations of serum bilirubin thus, preventing hepatotoxicity. However, the saponin fraction introduced some levels of toxicity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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