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EFFECT OF AQUEOUS EXTRACT OF HENSIA CRINITA ON CARBON TETRACHLORIDE– INDUCED TOXICITY DAMAGE IN WISTAR ALBINO RATS

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ABSTRACT

The effect of aqueous extract of H. crinita on carbon tetrachloride–induced liver damage in Wistar albino rats was investigated. Animals were injected intraperitoneally with a single dose of 0.3% CCl_4 (per body weight) in 1:1 olive oil and left for 24 hours to establish liver damage. Treatment of various groups of rats with 240, 480 and 720mg/kg of extract for 4 weeks on a daily basis after CCl_4 – induced liver damage was established significantly reduced ($p<0.05$) aspartate transaminase, alkaline phosphatase and white blood cell count with an increase in dosage. Alanine transaminase, liver malondialdehyde, serum albumin, total protein and haemoglobin concentration were restored to normal with an increase in dosage. The liver architecture was restored from a necrotic liver to a normal liver with focal areas of hepatocytes degeneration. The result of this study shows that the extract protected against after CCl_4 – induced liver damage in rats.

Keywords: Carbon tetrachloride, induced rat liver damage, *Hensia crinita* extract, protection.

INTRODUCTION

The liver is the largest organ in the body and performs a large number of functions that impact on all body systems. The liver along with the pancreas and intestines work together to digest, absorb and process food. Since the liver is the primary site for biotransformation, it is vulnerable to the toxic action of xenobiotics that are bioactivated to more toxic compounds. The fact that it receives blood directly from the gastrointestinal tract also renders it particularly susceptible to damage by ingested toxicants (chemicals, medications and microorganisms) (Gupta and Singhvi, 2011).

Liver toxicity refers to damage done to the liver by medications, chemicals and virus. Toxicity to liver manifests itself with the presence of liver diseases such as hepatitis (liver inflammation), cholestasis, steatosis (fat accumulation), cancer, cirrhosis and liver failure. It is difficult to detect early warning symptoms specific to liver metabolic imbalances and an individual may suffer for a long time from a liver disorder without knowing it. Thus, the administration of a known dose of hepatotoxin like carbon tetrachloride, paracetamol, D-galactosamine, thioacetamide, ethyl alcohol etc., may produce measurable effects, the magnitude of which can be measured by carrying out various liver function tests

(Kashaw et al., 2011). Carbon tetrachloride is one common hepatotoxin used in the experimental study of liver damage (Obi et al., 1998; Ulicna et al., 2003 and Yan et al., 2004).

Heinsia crinita (Afzel.) G. Taylor (Rubiceae) is a small tree 8-13m high with woody stems and branches in the under storey of high evergreen forest. The white, black and Ekoi varieties are the most common edible varieties. The black variety has dull leathery, dark green leaves with dense brown hairs along the nerves; 7.5 cm long and 3 cm wide on the average. It is because of the very dark green color of the leaves that it is called black, as compared with the white variety which has characteristic light green colored leaves. Phytochemical screening of the black variety of *H. crinita* shows that it contains steroids, anthranoids, anthraquinones, saponin, flavonoids, cardiac glycosides and alkaloids (Etuk et al., 1997 and Kola et al., 2003). The black variety is known to be the most aromatic of the three varieties, its lipid analysis indicates lecithins as the most prominent. It is also rich in the short-chained fatty acids and has been found to have the highest ash, crude fibre, hydrocyanic acid, tannin, and alkaloids content of all three varieties, giving it a distinct "pluses" for plants with high medicinal potential (Etuk et al., 1997).

This black variety is harvested wild from fast disappearing forests for food, but is more popular for its medicinal uses. In Southeastern Nigeria, it is used in oral hygiene as well as in the prevention and cure of decaying gums. Matured leaf extracts are used to cure many stomach disorders including peptic ulcer (Etuk et al., 1997 and Kola et al., 2003). According to the work of Kola et al. (2003), the butane fraction of the black variety of *H. crinita* has both antibacterial and antifungal activity against *S. aureus*, *P. aeruginosa* and *E. gametophyte* respectively. This study was designed to evaluate the ability of aqueous extracts of *H. crinita* to protect against liver damage due to CCl_4-induced toxicity

MATERIALS AND METHODS

(i) Chemicals

All chemicals used in the study were of analytical reagent grade.

(ii) Plant material

Fresh leaves of *H. crinita* were collected from Sapele in Delta State of Nigeria. The leaves were authenticated by Mr Edwin Nwosu of the herbarium section of the Department of Plant Science, University of Port Harcourt, River State, Nigeria and assigned voucher number UPH.NO.V.1139.

(iii)Preparation of aqueous extract

Fresh leaves were rinsed in distilled water, air dried at room temperature, shredded and ground into powder form with a Kenwood electric blender. Then, 300 g of the dried and grounded sample of the vegetable was soaked in distilled water (1:5 weight per volume) in conical flasks, agitated vigorously for 5 minutes and left for 18 hours on the bench before filtering with a Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator at 50^oC and evaporated to dryness using a water bath at 50^oC. The concentrated extract was removed from the evaporating dish with a spatula. Then, 8, 16 and 24 g respectively was weighed out and each was dissolved in 100 ml of distilled water. The aqueous extract was poured into sample bottles and stored at 4^oC in a refrigerator prior to use.

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(iv) Experimental Animals

Twenty four male and female Wistar albino rats weighing between 100-200 g of about three months old bred in the Animal House of the Department of Biochemistry, University of Port Harcourt were used for this study. The animals were housed in metabolic cages with commercial rat feed and water given *ad libitum.* The cages were cleaned daily and food and water changed daily.

(v) Experimental design

Animals were randomly assigned into six groups of four rats each and given food and water *ad libitum* throughout the experimental period (28 days). Liver damage was induced in all animals (except control) by injection with 0.3% CCl_4 (per body weight) in a ratio of 1:1 (olive oil and CCl_4) intraperitoneally for 24 hour. Treatment with different doses of the extract followed afterwards.

Group 1 - (Control)	Animals were fed with normal diet for 28 days
Group 2- (CCl_4 control)	Animals injected with a single dose of 0.3% CCl_4 (per body weight) in a 1:1 ratio with olive oil intraperitoneally on day 1 of the experiment and sacrificed 24 hrs later
Group 3- (CCl_4 group)	Animals injected with a single dose of 0.3% CCl_4 (per body weight) in a 1:1 ratio with olive oil intraperitoneally on day 1 of the experiment and left for 4 weeks without treatment
Group 4	Animals injected with a single dose of 0.3% CCl_4 (per body weight) in a 1:1 ratio with olive oil intraperitoneally on day 1 of the experiment and treated with 240 mg/kg of *H. crinita* for 4 weeks
Group 5	Animals injected with a single dose of 0.3% CCl_4 (per body weight) in a 1:1 ratio with olive oil intraperitoneally on day 1 of the experiment and treated with 480 mg/kg of *H. crinita* for 4 weeks
Group 6	Animals injected with a single dose of 0.3% CCl_4 (per body weight) in a 1:1 ratio with olive oil (per body weight) intraperitoneally on day 1 of the experiment and treated with 720 mg/kg of *H. crinita* for 4 weeks

(vi) Collection of blood sample

Whole blood was collected from each rat by direct cardiac puncture into ice-cold lithium heparinized and ethylenediaminetetraacetic acid (EDTA) bottles for biochemical assay and haematological studies respectively.

(vii) Collection of liver sample

The animals were dissected and the liver was quickly removed, the largest lobe was divided into two parts which were then used as follows: 1) submerged in 10% formal saline for preparation of histopathological sections and 2) put in ice-cold normal saline for malondialdehyde (MDA) assay.

(viii) Biochemical analysis

Serum aspartate transaminase (AST) and Alanine transaminase (ALP) activities were analysed according to the method of Reitman and Frankel (1957). The colorimetric end point method using the substrate developed by Roy (1970) was used for the assay of Serum Alkaline phosphatase (ALP). Total protein (TP) was determined by the RANDOX-Biuret method. The method of Grant and Kachmar (1987) was used to determine serum albumin concentration. The method of Hunter et al. (1963) modified by Gulteridge and Wilkins (1980) was adopted for the determination of liver malondialdehyde (MDA) concentration.

(ix) Haematological analysis

Packed cell volume (PCV), haemoglobin concenteration (Hb) and white blood cell count (WBC) were determined according to the method of Baker and Silverton (1985). Mean corpuscular volume (MCV) was derived from the ratio of the volume of packed red cells (haematocrit) to the total number of red blood cells (RBC).

(x) Histopathogical studies

The method of Baker and Silverton (1985) was adopted in the preparation of slides for histological examinations. Mayer's acid alum haematoxylin stain was prepared by dissolving 50g of Aluminum potassium sulphate (alum) and 1g haematoxylin dye in 100ml of distilled water. 0.2g of sodium iodate and 20ml of glacial acetic acid was then added to the mixture, it was then boiled, cooled and filtered. Following the decalcification, dehydration, impregnation, embedding and section cutting, the tissues were stained using Mayer's acid alum haematoxylin and eosin and mounted in natural balsam. The slides were then examined microscopically for histological changes. The slides were read at x 100 magnification.

(xi) Statistical analysis

All data are presented as mean ± standard deviation (n = 4). The one way analysis of variance (ANOVA) was used to analyse the data. The results were considered significant at P values of less than 0.05 ($P<0.05$).

RESULT

(i) Serum ALT, AST and ALP

As observed in Table 1, the injection of rats with a single dose of 0.3% per body weight of CCl_4 for 24 hours before sacrifice (G2) significantly ($p<0.05$) increased serum ALP, ALT and AST as compared with control (G1). On leaving the animals without treatment after CCl_4 injection for 4 week (G3), there was a significant decrease in ALP but no significant ($p<0.05$) change was observed in ALT and AST. A significant ($p<0.05$) decrease in ALT and ALP was observed with an increase in the dose of *H. crinita*. At 720 mg/kg (G6) ALT was not significantly ($p<0.05$) different from control. The extracts had no significant effect on serum AST level at 240 mg/kg (G4) when compared with G3. The decrease in AST produced at 480 (G5) and 720 mg/kg (G6) was significantly ($p<0.05$) lower when compared with that obtained in G2 and G3.

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Table 1. Liver enzymes of rats administered *H. crinita* extracts

Liver enzymes (IU/L)	Control (G1)	CCl_4 control (G2)	CCl_4 group (G3)	*H. crinita* for 4 week after CCl_4 injection		
				240 mg/kg (G4)	480 mg/kg (G5)	720 mg/kg (G6)
ALP	208.93±7.11[a]	345.50±4.58[b]	281.15±6.61[c]	266.05±8.27[d]	226.07±5.20[e]	192.33±7.84[f]
ALT	49.0±2.16[a]	82.5±3.77[b]	81.5±6.03[b]	72.0±2.94[c]	63.5±1.73[d]	42.5±6.02[a]
AST	121.08±5.78[a]	204.57±3.90[b]	200.37±9.68[bc]	187.82±7.82[c]	172.34±8.93[e]	159.28±8.15[e]

Groups with different subscript letters across a row are significantly ($p<0.05$) different from each other. Groups with the same subscript letters are not significantly different at the 5% level.

(ii) Liver MDA, Serum Albumin and Total protein

Injection of rats with a single dose of 0.3% body weight of CCl_4 for 24 hours before sacrifice (G2) significantly ($p<0.05$) increased liver MDA, decreased serum albumin and total protein as compared with control (G1) (Table 2). On leaving the animals for 4 weeks after CCl_4 injection without treatment (G3), liver MDA level was significantly ($p<0.05$) decreased but no significant difference was observed in serum albumin and total protein. Administration of 240 mg/kg of *H. crinita* (G4) significantly ($p<0.05$) reduced liver MDA when compared with G2 and G3. At 480 and 720 mg/kg of *H. crinita* liver MDA level was significantly ($p<0.05$) lower than that observed in G4, G3 and G2 but was not significantly ($p>0.05$) different from control (G1). At 240 mg/kg of *H. crinita* (G4), serum albumin concentration was not significantly different from G2 and G3 but at 480 and 720 mg/kg respectively, serum albumin value was not significantly different from control. Total protein value at all doses of *H. crinita* respectively, was significantly higher than that observed in G2 and G3 but was not significantly different from control.

Table 2. Effect of *H. crinita* extracts on liver MDA, serum albumin and total protein

Parameter	Control (G1)	CCl_4 control (G2)	CCl_4 group (G3)	*H. crinita* for 4 week after CCl_4 injection		
				240 mg/kg (G4)	480 mg/kg (G5)	720 mg/kg (G6)
Liver MDA (nmole/mg protein)	0.556±0.01[a]	0.983±0.04[b]	0.716±0.06[c]	0.585±0.04[d]	0.571±0.01[a]	0.564±0.03[a]
Serum albumin (g/dl)	4.61±0.48[a]	3.85±0.30[b]	4.02±0.06[b]	4.09±0.58[b]	4.53±0.32[a]	4.61±0.40[a]
Total protein (g/dl)	7.14±0.57[a]	6.20±0.84[b]	6.15±0.59[b]	7.03±0.40[a]	7.38±0.47[a]	7.48±0.53[a]

Groups with different subscripts across a row are significantly ($p<0.05$) different from each other. Groups with the same subscript are not significantly ($p>0.05$) different from each other.

(iii)PCV, Hb, WBC and MCV

As observed in Table 3, the injection of rats with a single dose of 0.3% b.d. wt of CCl_4 for 24 hours before sacrifice (G2) significantly ($p<0.05$) increased WBC and MCV while a significant decrease was observed in Hb and PCV. On leaving the animals for 4 weeks after CCl_4 injection without treatment (G3) a significant ($p<0.05$) decrease was observed in WBC but no significant difference was observed in Hb, PCV and MCV when compared with G2. Administration of *H. crinita* after CCl_4 injection had no significant effect on WBC at 240 mg/kg (G4) but at 480 (G5) and 720 mg/kg (G6) respectively WBC was significantly increased when compared with G2 and G3. Hb value observed at 240 (G4) and 480 mg/kg (G5) was not significantly different from that observed in G2 and G3. At 720 mg/kg of *H. crinita* (G6), Hb level was significantly different from G1 (control). At all doses of *H. crinita* respectively PCV and MCV values respectively were not significantly different from G2 and G3.

Table 3. Effect of *H. crinita* extract on PCV, Hb, WBC and MCV of Wistar albino rats.

Parameter	Control (G1)	CCl_4 control (G2)	CCl_4 group (G3)	*H. crinita* for 4 week after CCl_4 injection		
				240 mg/kg (G7)	480 mg/kg (G8)	720 mg/kg (G9)
PCV (%)	42.9 ± 1.71^a	38.0 ± 2.12^b	39.0 ± 2.34^b	39.3 ± 4.21^b	39.7 ± 0.22^b	41.1 ± 1.97^b
Hb (g/dl)	14.2 ± 0.75^a	11.9 ± 0.62^b	12.3 ± 0.65^b	12.3 ± 0.79^b	12.6 ± 0.26^b	13.5 ± 0.48^a
WBC x 10^9 (g/dl)	8.2 ± 0.43^a	9.0 ± 0.44^b	4.2 ± 0.26^c	4.4 ± 0.26^c	5.9 ± 0.17^e	6.5 ± 0.48^f
MCV (Fl)	53.2 ± 1.70^a	59.6 ± 2.12^b	58.3 ± 1.53^b	58.2 ± 1.98^b	57.2 ± 1.92^b	56.3 ± 2.05^b

Groups with different subscripts across a row are significantly ($p<0.05$) different from each other. Groups with the same subscript are not significantly ($p>0.05$) different from each other.

(vi) Histopathology of the liver

Intreperitoneal injection of a single dose of CCl_4 (0.3% body wt) for 24 hours prior to sacrifice showed a liver with extensive necrosis (G2) (Figure 1). On leaving the animals for 4 weeks without treatment (G3), microscopic examination of the liver showed a liver architecture altered by numerous fatty changes and balloon degeneration (apoptosis due to steatohepatitis). The cytoplasm of the hepatocytes had a lot of vacuolated fatty substances with the normal round to ovoid nuclei and a lot of congested blood vessels. However a few areas of normal hepatocytes were observed in the periphery (G3). Oral administration of 240 mg/kg of *H. crinita* for 4 weeks after CCl_4 induced liver damage (G4) revealed hepatocytes with balloon degeneration and active inflammation characterized with the presence of neutrophils. At a higher dose of 480 mg/kg (G5), hepatocytes with moderate fatty change and areas of balloon degeneration and focal areas of inflammation were observed. A further increase in dose to 720 mg/kg (G6), a normal liver architecture with focal areas of hepatocytes degeneration and congested blood vessels was observed.

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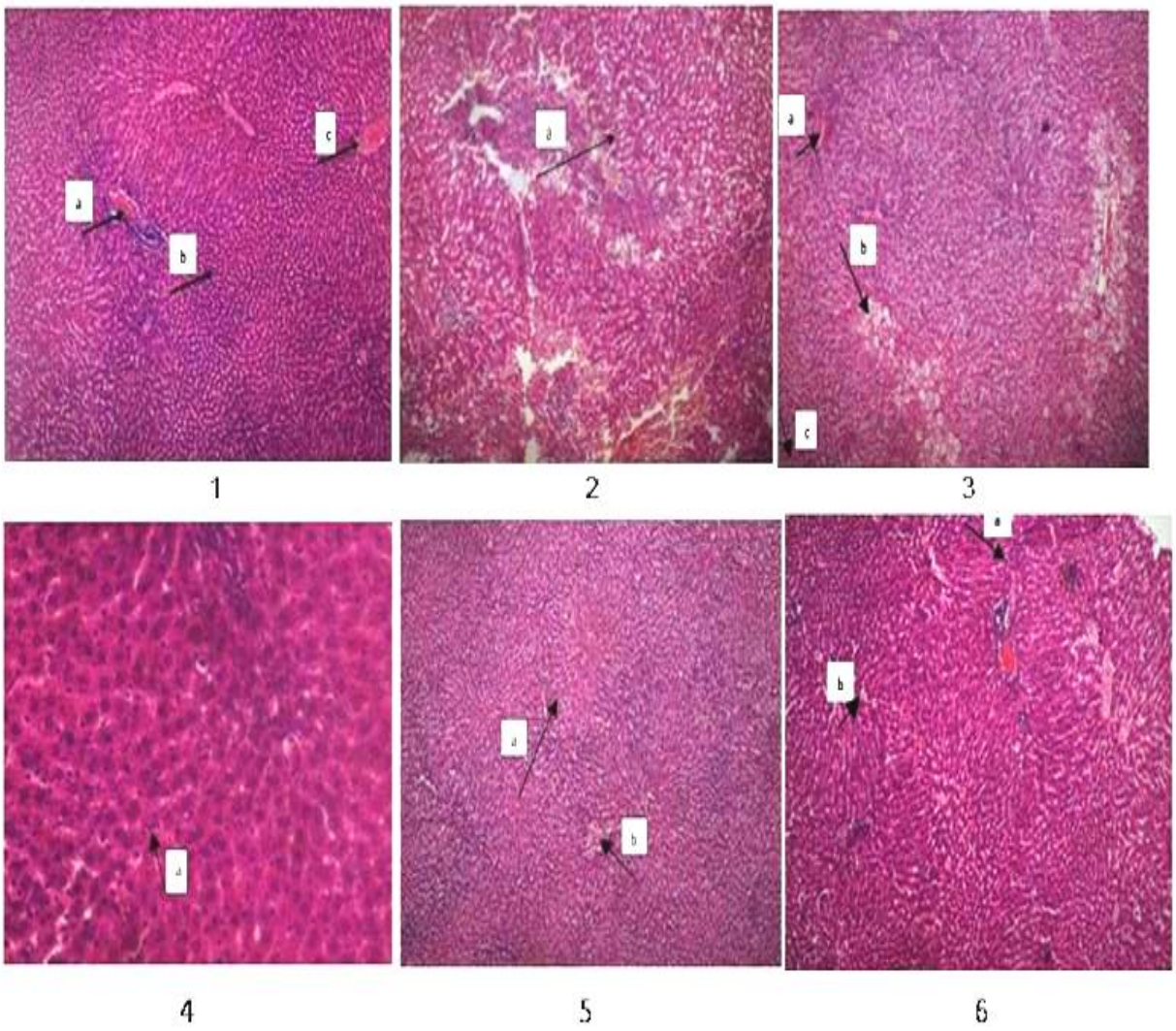

Figure 1 . Histopathology slides of the liver showing the ameliorating effect of *H. crinita*
1 (G1): a – Central vein, b – Aggregation of hepatocytes, c – Central vein
2 (G2): a - Extensive necrosis
3 (G3): a –Congested blood vessel, b – Fatty change with feathery degeneration, c – Normal area
4 (G4): a – Inflammatory infiltrates
5 (G5): a – Mild fatty change, b – Perivascular inflammation
6 (G6): a – Congested blood vessel, b – Degenerated hepatocytes
Magnification histological examinations x 100

DISCUSSION

The bioactivation of CCl_4 by cytochrome (CYP) 2E1, CYP2B1 or CYP2B2 produces CCl_3* which combines with oxygen to produce trichloromethyl peroxy free radical (CCl_3OO^-) whichinitiates the chain reaction of lipid peroxidation, which attacks the polyunsaturated fatty acids in the liver membrane. Membrane lipid peroxidation by CCl_4 metabolites lead to impairment of functions and integrity. The distorted hepatic architecture impairs bile flow and

adequate blood flow to hepatocytes resulting in cholestatic induction of enzyme (ALP) synthesis (Obi et al., 1998; Ulicna et al., 2003). This mechanism of action was confirmed in this study with an increase in liver MDA (a biomarker for oxidative stress), serum ALT, ALP and AST due to CCl_4 induced liver toxicity.

Aldehydes formed through peroxidation of poly unsaturated fatty acids (PUFAs) are detrimental to cellular homeostasis. They deplete the natural antioxidant glutathione, increase production of the proinflammatory cytokine TNF-α, promote influx of inflammatory cells into the liver and activate stellate cells. These effects have the potential to directly induce inflammation, liver fibrosis, hepatocyte death and necrosis (Solanki and Jain, 2011). Histopathological slides of the present study showed areas with extensive necrosis in the liver sections of CCl_4 intoxicated rats. The findings of the histopathological examination is confirmed by the significant decrease in serum albumin and total protein level as the liver not only synthesize the protein it needs, but also produces numerous export proteins. Albumin is synthesized by the liver and it is a clinically useful marker of hepatic synthetic function. A decrease in serum albumin has been observed in liver diseases such as hepatitis, cirrhosis or hepatocellular necrosis (Berk and Korenblat, 2007).

A decline in serum liver enzyme concentrations and liver MDA accompanied with an increase in serum albumin and total protein usually indicate recovery. The liver on its own makes an effort to battle toxic effects and regenerate itself when the source of damage is removed. This effort was evidenced in the decrease in liver MDA and serum ALP accompanied with the presence of balloon degeneration of hepatocytes observed in animals which were left for four weeks without treatment after CCl_4 – induced liver damage. Balloon degeneration is a form of apoptosis which may occur as the liver tries to remove hepatocytes which have been badly damaged and create new liver cell (regenerates itself) from healthy liver cells that still exist.

Treatment with different doses of aqueous extracts of *H. crinita* after liver toxicity clearly reduced ALP and AST with an increase in dose. ALT, liver MDA, serum albumin and total protein were restored to normal with an increase in dose. The liver architecture also improved from a necrotic liver to a normal liver with focal areas of hepatocytes degeneration and congested blood vessels.

In this study, CCl_4 toxicity resulted in the significant decrease of PCV and Hb with a significant increase in MCV and WBC. This was in accordance with the findings of Abd Elaziz et al. (2010). This may be due to deficiency of vitamin B_{12} as the liver is the organ for the storage of vitamin B_{12} which is essential for the production of red blood cell. In certain liver diseases, the liver becomes unable to store the normal amount of vitamin B_{12} leading to vitamin B_{12} deficiency and pernicious anaemia characterized by increased MCV and recticuloctyes (immature red blood cell). Liver disorders like fatty liver, liver cancer, hepatitis and bile duct obstruction all correlate with less vitamin B_{12} storage in the liver (Joske, 1963). The increase in WBC observed may be as a result of immune response. Aldehyde produced during CCl_4 induced lipid peroxidation may bind covalently to proteins forming adducts which may serve as neoantigens. This

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binding initiates harmful humoral and/or cellular responses (Kenna et al., 1988).

A drastic fall in WBC was also observed after 4 weeks without any form of treatment. This may be due to the immunosuppressive effect of CCl_4. As observed by Kaminski et al. (1989), Chakraborty et al. (2009) and SenGupta et al. (2011), apart from damaging the liver system, CCl_4 also suppresses the immune system by reducing nonspecific host responses parameters like morphological alteration, phagocytosis, nitric oxide release, myeloperoxidase release, cell adhesiveness and intracellular killing capacity of rats peritoneal macrophages after seven days of CCl_4 toxicity.

Administration of different doses of aqueous extracts of *H. crinita* had no significant effect on PCV and MCV but significantly reduced WBC and Hb was restored to normal with an increase in dose. More studies are needed to understand the influence of aqueous extract of *H. crinita* against the immunosuppressive effect of CCl_4.

The protecting effect of *H. crinita* may be due to the presence of anthraquinones and flavonoids. Anthraquinones derivative that have hydroxyl groups arranged at either the meta or ortho positions have been shown to inhibit lipid peroxidation. Flavonoids have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities (Kaur et al., 2008).

The fact that it is also rich in zinc, magnesium and lecithins may also have contributed as magnesium has membrane stabilizing properties and protects the membranes from lipid peroxidation by maintaining calcium homeostasis (Gueux et al., 1995). At high concentrations, magnesium inhibits lipid peroxidation directly, perhaps by competing with iron for phospholipid binding sites (Regan et al., 1998).

CONCLUSION

In conclusion, the oral administration of aqueous extract of *H. crinita* is effective in decreasing the effect of chronic liver injury via the restoration of membrane integrity, hepatic synthetic function, impeding lipid peroxidation, reducing cholestasis and the immunosuppressive effect of CCl_4 because of its antioxidant, anti-inflammatory and membrane stabilizing properties.

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