Evaluation Of The Protective Potential Of Methanol Leaf Extract Of *Pyrenacantha staudtii* Hutch And Dalz (Icacinaceae) And 3-Carbomethoxypyridine Isolated From It On Chronically-Induced Liver Damage In Rats

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ABSTRACT

Pyrenacantha staudtii Hutch and Dalz (Icacinaceae) is a woody climber used in folk medicine for the treatment of stomach colic, gastric ulcer, dysmenorrhea, threatened abortion and liver diseases. Earlier studies with 3-carbomethoxypyridine (3-CMP) isolated and characterized from *P. staudtii* had shown potential hepatoprotective effects on acute hepatocellular damage. The present study evaluates the hepatoprotective effects of the methanol leaf extract of *P. staudtii* (PS) and 3-CMP isolated from it on chronically-induced hepatocellular damage, using rodent models of hepatotoxicity and assesses the short-term toxicity profile of 3-CMP.Rats were administered *P. staudtii* (25- 100 mg/kg body weight/day) and 3-CMP (5-20 mg/kg body weight/day) orally for 56 days. Liver damage was induced by twice weekly intraperitoneal administrations of carbon tetrachloride (CCl_4, 1 ml/kg), or thioacetamide (TAA, 200 mg/kg) for eight weeks. Body weight changes, biochemical {alanine transaminases (ALT), aspartate transaminases (AST), alkaline phosphatase (ALP), albumin and bilirubin} and histopathologic parameters were evaluated. The urine ascorbic acid content of the 3-CMP treated rats was also determined. A 42-day short-term oral toxicity evaluation of 3-CMP was also carried out. Oral administration of the methanol extract, for 56 days, did not significantly decrease the serum enzyme levels or increase the total protein/albumin levels but reversed the decreased body weight induced by CC_{l4}. Treatment with 3-CMP significantly ($p<0.05$) and dose-dependently increased the total body weight and urine ascorbic acid content and reduced the ALT, AST and ALP levels without affecting bilirubin or total protein in CCl4-intoxicated rats. However, 3-CMP had no significant effects on the body weight changes, biochemical markers or urine ascorbic acid content in TAA-hepatotoxic rats. Liver sections from both CCl_4- and TAA-hepatotoxic rats showed improved histological appearances on treatment with all doses of 3-CMP and *P. staudtii*. 3-CMP possesses significant antihepatotoxic effect greater than methanol extract of PS, as seen by its ability to decrease liver enzymes increased by CCl_4, in a dose-dependent manner, similar to the standard drug sylimarin. This was further confirmed by its effect on urine ascorbic acid content as well as improved liver histology. The more pronounced effect of 3-CMP on CCl_4-induced than on TAA-induced liver damage suggests that it might have greater efficacy in liver fibrosis than cirrhosis. Short term toxicity studies indicated that 3-CMP has no immediate or delayed toxic effects on rats.

Key words: 3-carbomethoxypyridine; *Pyrenacantha staudtii*; CCl_4; Liver damage; Thioacetamide

INTRODUCTION

The liver has the tremendous capacity for detoxification and excretion of many toxic end products of metabolism as well as synthesis of useful ones. Therefore damage to the liver by hepatotoxic agents is of grave consequences.

Chronic hepatic diseases are a major global public health concern, especially in developing nations, such as Nigeria, because of their high morbidity and mortality rate. These may result from consumption of contaminated or over-processed foods, environmental chemicals, synthetic therapeutic agents and viruses or autoimmune diseases. The management of liver diseases is still a challenge to orthodox medicine. The conventional drugs have little to offer and some adversely affect the liver function (Meyer and Kulkarni, 2001 Mohan *et al*, 2007; Kamble *et al*., 2008), hence the tendency towards alternative herbal remedies.

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Herbal drugs have gained prominence and popularity in recent years because of their perceived safety, efficacy and cost effectiveness. One of the important and well documented uses of plant products is their use as hepatoprotective agents. Hence, liver disorders have benefited from therapeutic strategies employing compounds extracted from plants and herbs (Alshawsh *et al*., 2011).

Pyrenacantha staudtii (Icacinaceae) is a high climbing glabrous shrub that inhabits the tropical rainforest and farmlands. It has pure green or pink leaves in mostly fairly low corymbrose inflorescence, with fruiting follicles over 0.6 m long, and fruits mainly during dry season (Busari, 1976). *P. staudtii* finds ethnopharmacological usage in the treatment of various intestinal disorders/worm infections, stomach colic, dysmenorrhea and threatened abortion (Hutchinson and Dalziel, 1966). Scientific studies of *Pyrenacantha staudtii* have shown anti-abortifacient, anti-dysmenorrheal (Aguwa and Okunji, 1986) and in vitro antimalarial (Mesia *et al*., 2005) activities. Others include antiulcer (Aguwa and Mittal, 1981; Akubue *et al*., 1983; Aguwa and Okunji, 1986); tocolytic (Falodun *et al*., 2005) and hepatoprotective (Anosike *et al*., 2008) activities.

Phytochemical screening of the various morphological parts of *P. staudtii* had revealed the presence of alkaloids, saponins, tannins, fatty acids, flavonoids and sugars (Aguwa and Mittal, 1981, Falodun *et al*., 2009). A chemical compound, 3-carbomethoxypyridine (3-CMP), consisting of pale yellow to brown crystals with a melting point of 40-42°C has been isolated and characterized from the plant (Falodun *et al*., 2006). The compound is reported to have a vasodilating effect on the skin of humans (Leopold et al., 1995), a relaxant effect on uterine smooth muscle (Falodun *et al*., 2006) and anti-ulcer activity (Okpo *et al*., 2012).

The protective effect of 3-CMP isolated from Pyrenacantha staudtii on acute hepatocellular damage induced by carbon tetrachloride had earlier been demonstrated (Okpo *et al*., 2013).The present study evaluates the effects of 3-carbomethoxypyridine and methanol leaf extract of *Pyrenacantha staudtii* on chronic hepatotoxicity induced by carbon tetrachloride (CCl_4) and thioacetamide (TAA). The short time toxicity profile of 3-CMP has also been assessed.

MATERIALS AND METHODS

Collection and preparation of extract and compound (3-CMP)

The leaves of *Pyrenacantha staudtii* were collected around the premises of University of Benin, Ugbowo, Benin City, Nigeria and authenticated at the Forestry Research Institute of Nigeria (FRIN) Ibadan, where a herbarium specimen (No FHI 107624) was deposited. The leaves were dried under the shade, pulverized into coarse powder and extracted with absolute methanol using a soxhlet extractor. The resulting methanol extract was evaporated to dryness in a rotary evaporator set at 40^{o}C. A stock solution of the dried extract (200mg/ml) was prepared from which other concentrations were made as required. 3-carbomethoxypyridine, isolated and characterized from the methanolic leaf extract of *Pyrenacantha staudtii* using gradient chromatography was provided pure and in crystalline form (Falodun and Usifo, 2006).

Animals

Albino mice (28±3.5g) and Sprague-Dawley rats (230±60g) were in-bred or obtained from the Animal House of Ambrose Alli University, Ekpoma, Edo State, Nigeria. Animals were maintained under standard environmental conditions, with access to food and water ad libitum, and handled according to standard protocols for the use of laboratory animals (National Institute of Health USA; Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002).

Acute toxicity study

Rats and mice were randomly selected into different control and test groups of 5 animals each. Four doses of 3-CMP (0.25, 0.5, 1 and 2g/kg body weight) were given by oral intubation and the control Group received distilled water orally. General symptoms of toxicity and mortality were recorded for 48 hours and a further 2 weeks for any signs of delayed toxicity.

Short term toxicity study

Rats were randomly allotted to control and different test groups of 6 animals per group. Three doses of 3-CMP (50, 100 and 200 mg/kg body weight/day) were given by oral intubation for 42 days. Control group received water (5ml/kg) via the same route. Body weight of each rat was recorded at weekly intervals. Behavioral changes and any external general symptoms of toxicity were noted. On day 42, blood was collected, under light ether

anaesthesia, via the abdominal aorta for haematological and biochemical assays.

Carbon tetrachloride- and Thioacetamide-induced liver damage

Rats were randomly divided into 8 groups of at least 10 animals each and starved for 24 hours with free access to water. Group 1 (vehicle control) was given distilled water (2 ml/kg). Animals in groups 2, 3 and 4 received via the oral route 25, 50 and 100 mg/kg/day of methanolic extract of *Pyrenacantha staudtii* respectively, while groups 5, 6 and 7 were administered 3-carbomethoxypyridine at doses of 5, 10 and 20 mg/kg/day, respectively. Silymarin (50 mg/kg/day), used as the standard drug, was administered orally to animals in group 8. Liver damage was induced by intraperitoneal administration of carbon tetrachloride (CCl_4) in 30% v/v olive oil (1ml/kg, twice weekly) to rats in group 9 (Reddy *et al*., 1992). All administrations were for 56 days.

In thioacetamide-induced hepatotoxicity study, 3-CMP (5, 10 and 20 mg/kg/day) and the standard drug, silymarin (50mg/kg/day) were given for 56 days. Liver damage was induced in rats by oral administration of thioacetamide (200 mg/kg, twice weekly) for 56 days (Saraswat *et al*., 1996).

The body weight of each rat was recorded at weekly intervals and behavioural changes and any external general symptoms of toxicity were noted.

Animals in all the groups were placed in metabolic cages and urine collected over 24 hours (Ishak, 1982; Visweswarm *et al*., 1994). Ascorbic acid content of the urine was determined according to a standard method (Roe and Keuther, 1943) slightly modified by Niro and Shah (1986) using 2, 4-dinitrophenylhydrazine.

On the 57th day, blood was collected via the abdominal aorta under light ether anaesthesia, in all the groups. Assessment of liver function using serum marker enzymes such as aspartate aminotransferase, alanine aminotransferase (Reitman and Frankel, 1957), alkaline phosphatase (Young *et al*., 1975), bilirubin (Jendrassik *et al*, 1938); and total protein (Thomas, 1998) levels were done using standard techniques.

Haematological estimations of red blood cell (RBC), white blood cell (WBC), haemoglobin (Hb) and platelet count (PLT) were done using standard laboratory procedures (Dacie and Lewis, 1991; Ghai, 1995; John, 1972).

After collection of blood samples, the rats in different groups were sacrificed and their liver isolated. Sections of the liver were fixed in 10% buffered formalin for histopathological studies.

Data analysis

Results are presented as mean ± standard error of mean (SEM) and n represents the number of animals per group. Data comparisons were made using one-way ANOVA with Dunnett'sposthoc test (GraphPad Prism 5, GraphPad Software, Inc. San Diego, California, USA) and values were considered significant at $p<0.05$.

RESULTS

Acute toxicity studies

3-carbomethoxypyridine up to an oral dose of 2 g/kg caused neither death nor any observable symptoms of toxicity in the rats. In addition, no delayed toxic effects were observed in rats after two weeks.

Short-term toxicity studies

Body weight and organ weight indices

Rats in the control group (given water over a six week period) exhibited a slight increase (4.42%) in the total body weight. Administration of 3-CMP during the period showed significant ($p<0.05$) weight reductions for the 100 and 200 mg/kg groups compared to control. However the lowest dose (50 mg/kg) group showed an increase in the total body weight similar to the control. All the doses of 3-CMP administered did not cause any significant changes in heart, liver and kidney weight indices compared to control (Table 1).

Haematological parameters

All doses of 3-CMP administered for 42 days showed no significant alterations in the haematological parameters (namely: WBC count, RBC count, haemoglobin concentration, haematocrit and platelet count compared to control (Table 2).

Biochemical markers

Treatment of rats with 3-CMP did not cause any significant changes in ALT, total and conjugated bilirubin. The highest dose of the extract (200 mg/kg/day) caused appreciable reductions in ALP, total protein and albumin. However, these were not significantly different from the control (Table 3).

Table 1**:** Effect of 42-day oral administration of 3-CMP on body weight and relative organ weights of rats

Treatment	Dose (mg/kg)	Body weight change (%)	Relative organ weight (g/100g)		
			Liver	Heart	Kidney
Control	2 ml/kg	4.42±1.28	2.80 ± 0.18	0.36 ± 0.03	0.28 ± 0.02
3-CMP	50	4.17±1.83	2.77 ± 0.14	0.33 ± 0.01	0.27 ± 0.01
	100	-0.44±1.28*	2.69 ± 0.07	0.37 ± 0.01	0.28 ± 0.01
	200	-0.34±0.66*	2.91 ± 0.04	0.35 ± 0.01	0.29 ± 0.01

Values are mean ± SEM.*p<0.05, significantly different from control; (n= 6 animals).

Table 2**:** Effects of 42-day oral administration of 3-CMP on haematological parameters in rats

Treatment	Dose (mg/kg)	WBC ($x10^3$/ µL)	RBC ($x10^6$/ µL)	Hb conc. (g/dL)	HCT (%)	PLT ($x10^3$/µL)
Control	2 ml/kg	6.50± 0.93	6.03± 0.29	11.08± 0.67	32.13± 1.71	625.0± 76.84
3-CMP	50	7.25± 1.96	6.68± 0.26	12.30± 0.84	34.12± 0.89	777.50± 131.22
	100	7.21± 1.19	6.52± 0.12	11.55± 0.17	33.50± 0.40	717.33± 102.58
	200	5.52± 0.28	6.03± 0.20	10.80± 0.38	32.41± 1.11	671.17± 75.68

Values are mean ± SEM

3-CMP = 3-Carbomethoxypyridine; **Hb conc** = Haemoglobin concentration; **HCT** = Haematocrit; **PLT** = Platelet; **RBC** = Red Blood Cell; **WBC** = White Blood Cell

Table 3**:** Effect of 42-day oral administration of 3-CMPon biochemical parameters

Treatment	Dose (mg/kg)	ALT (IU/L)	ALP (IU/L)	Total protein (g/dL)	Albumin (g/dL)	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)
Control	2 ml/kg	159.00± 13.20	60.50± 7.32	0.54± 0.18	0.30± 0.10	12.62± 0.24	5.68± 0.11
3-CMP	50	180.00± 21.11	53.33± 10.15	0.58± 0.16	0.34± 0.10	12.35± 0.26	5.57± 0.12
	100	143.17± 9.71	45.79± 9.33	0.36± 0.08	0.24± 0.06	11.77± 0.30	5.36± 0.13
	200	165.50± 9.09	36.68± 3.27	0.34± 0.08	0.14± 0.02	11.92± 0.21	5.37± 0.10

Values are mean ± SEM

3-CMP = 3-Carbomethoxypyridine; **ALP** = Alkaline phosphatase;ALT = Alanine transaminase

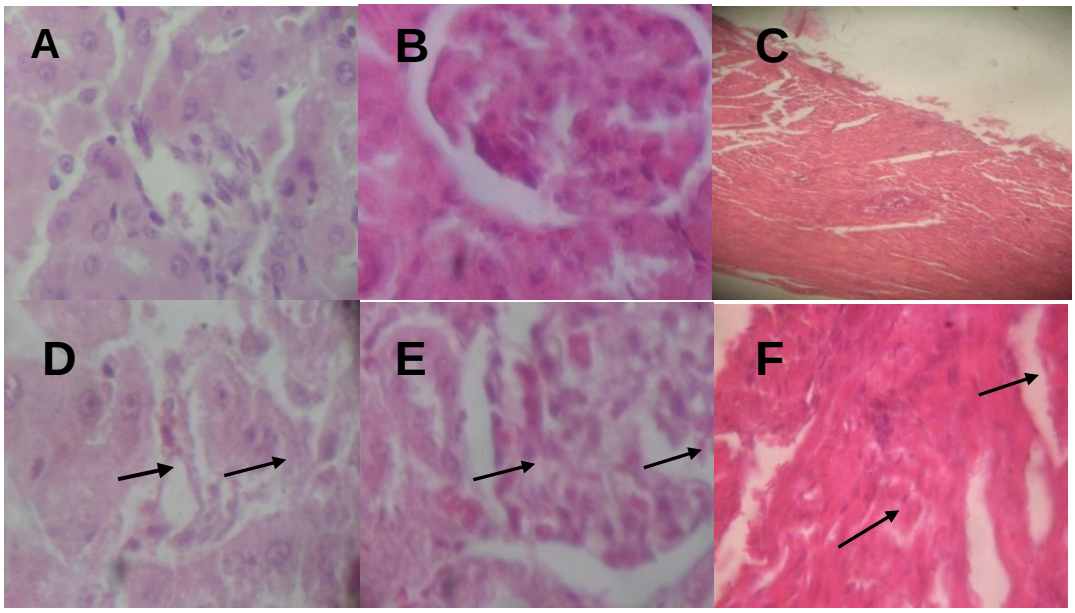

Figure 1: Sections of the liver (A), kidney (B) and heart (C) of control rats given water for 42 days showing normal structure and architecture. Treatment with 3-CMP (200 mg/kg/day) shows mild vascular congestion in the liver (D); mild interstitial congestion in the kidney (E) and moderate transmural oedema and mild infiltrates of chronic inflammatory cells in the heart (F) [H&E x40]. Arrows (↓) show points at which lesions occurred.

Table 4: Effects of *Pyrenacantha staudtii* and 3-CMP on body weight in CCl_4-induced hepatotoxicity.

Treatment	Dose (mg/kg/day)	Total change in body weight (%)	
		P. staudtii	3-CMP
CCl_4 alone	-	4.66 ± 2.72	4.66 ± 2.72
P staudtii	10	11.76 ± 3.78	-
	25	14.66 ± 4.86*	-
	50	14.61 ± 3.48*	-
3-CMP	5	-	13.42 ± 4.88*
	10	-	17.46 ± 4.45**
	20	-	21.96 ± 5.92**
Sylimarin	50	25.61 ± 5.01**	25.61 ± 5.01**
Distilled water	5ml	6.76 ± 3.30	6.76 ± 3.30

Values are mean ± SEM. *p<0.05, **p<0.001 significantly different from CCl_4 alone; (n= 8 animals).

Histopathologic evaluation

Histological examination of the various organs (Figures 1A, 1C and 1E), showed that control rats given distilled water during the 42 days study period maintained normal structure and architecture of the liver, heart and kidney. The group treated with 3-CMP (200 mg/kg/day) showed mild vascular congestion of the liver (Figure 1B), mild interstitial congestion of the kidney (Figure 1D) and moderate transmural oedema of the heart (Figure 1F).

Table 5: Effects of *Pyrenacantha staudtii* and 3-CMP on biochemical parameters in CCl_4-induced hepatotoxicity

Treatment	Dose (mg/kg)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Albumin (g/dL)	Total protein (g/dL)	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)
Control		104.80±7.25	39.50±10.75[b]	35.55±8.12[b]	3.42±0.29	7.64±0.67	0.58±0.16	0.18±0.06
CCl_4 alone	-	158.00±12.31	82.86±13.45	101.03±10.95	3.16±0.32	7.07±0.79	0.71±0.15	0.43±0.08
CCl_4 +								
P. staudtii	10	156.29±17.75	97.21±5.61	92.85±11.11	2.97±0.26	8.12±0.74	1.02±0.28	0.51±0.07
	25	136.63±8.69 [a]	76.69±12.49	57.48±8.96	3.07± 0.71	7.41±0.49	0.41±0.07	0.30±0.06
	50	172.90±22.43	87.57±8.68	85.70±10.59	2.58±0.25	7.96±0.42	0.35±0.06	0.21±0.06
CCl_4 +								
3-CMP	5	86.86± 9.88[b]	26.04± 1.78[c]	50.98± 12.41[a]	3.46± 0.47	8.76± 0.32	0.49± 0.09	0.32± 0.08
	10	69.93± 7.08[c]	23.87± 2.29[c]	65.59± 17.80	2.52± 0.17	8.32± 1.39	0.50± 0.13	0.39± 0.12
	20	46.40± 4.09 [c]	24.10± 3.18[c]	47.17± 11.78[b]	2.42± 0.18	6.29± 0.39	0.53± 0.16	0.36± 0.04
Sylimarin	50	90.23±10.21[a]	21.45±3.68[c]	37.65±12.48[b]	3.74±0.88	7.77±1.10	0.56±0.13	0.32±0.05

Values are mean ± SEM. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ compared to CCl_4 alone

Effect of P. staudtii and 3-CMP on CCl_4-induced hepatotoxicity

Body weight changes

The higher doses of the extract (25 and 50 mg/kg) caused significant increases in the body weight of the treated rats compared to control. Administration of 3-CMP significantly increased, in a dose-dependent manner, the total body weight of rats compared to the CCl_4 –alone group (Table 4).

Biochemical markers

In the CCl_4 alone-treated control group, serum enzymes and bilirubin increased appreciably with decreased levels of total protein and albumin when compared to normal control group (Table 5). No significant changes in these biochemical parameters were observed with all doses of extract compared to control (Table 5). In contrast, serum enzymes (AST, ALT and ALP) decreased in the groups treated with 5, 10 and 20 mg/kg of 3-CMP in a dose-dependent manner but these tended towards normal values, especially at the highest dose of the compound, comparable to the effects produced by sylimarin. The bilirubin and protein levels were not significantly altered.

Haematological parameters

The study showed significant reduction in WBC count with 10 and 20 mg/kg of 3-CMP compared to control. Sylimarin also showed a similar effect. Further analysis revealed no significant difference in RBC count, haemoglobin concentration and platelets count between control and treatment groups (Table 6).

Table 6: Effects of 3-CMP on haematological parameters in CCl_4-inducedhepatotoxicity

Treatment	Dose (mg/kg)	WBC ($x10^3$/µL)	RBC ($x10^6$/µL)	Hgb Conc. (g/dL)	PLT ($x10^3$/µL)
Control	-	13.37± 2.07	8.59± 1.92	13.45± 0.34	783.17± 136.54
CCl_4 alone	-	28.03± 4.10	7.83± 0.46	11.77± 0.60	516.25± 74.92
CCl_4 +					
3-CMP	5	25.69± 3.18	7.12± 0.24	12.77± 0.41	481.43±51.84
	10	17.47± 1.19*	7.24± 0.11	12.59± 0.22	503.78± 28.31
	20	17.43± 2.10*	7.03± 0.21	12.17± 0.61	519.29± 33.36
Sylimarin	50	14.68± 1.86**	7.19± 0.26	12.73± 0.37	557.33± 64.29

Values are mean ± SEM. *p<0.05, **p<0.01 significantly different from CCl_4 alone group; n=8 animals.

3-CMP = 3-Carbomethoxypyridine; **WBC** = White Blood Cell;**RBC** = Red Blood Cell; **Hb conc** = Haemoglobin concentration; **PLT** = Platelet

Table 7: Effect of 3-CMP on body weight in thioacetamide-induced hepatotoxicity.

Treatment	Dose (mg/kg)	Total body weight change (%)
Thioacetamide alone	-	8.00 ± 5.53
3-CMP	5	-1.72 ± 2.92
	10	-2.26 ± 4.34
	20	-7.07 ± 2.78
Sylimarin	50	15.07 ± 5.37*
Distilled water	5ml	6.76 ± 3.30

Values are mean ± SEM. *p<0.05, significantly different from thioacetamidealone;(n= 8 animals).

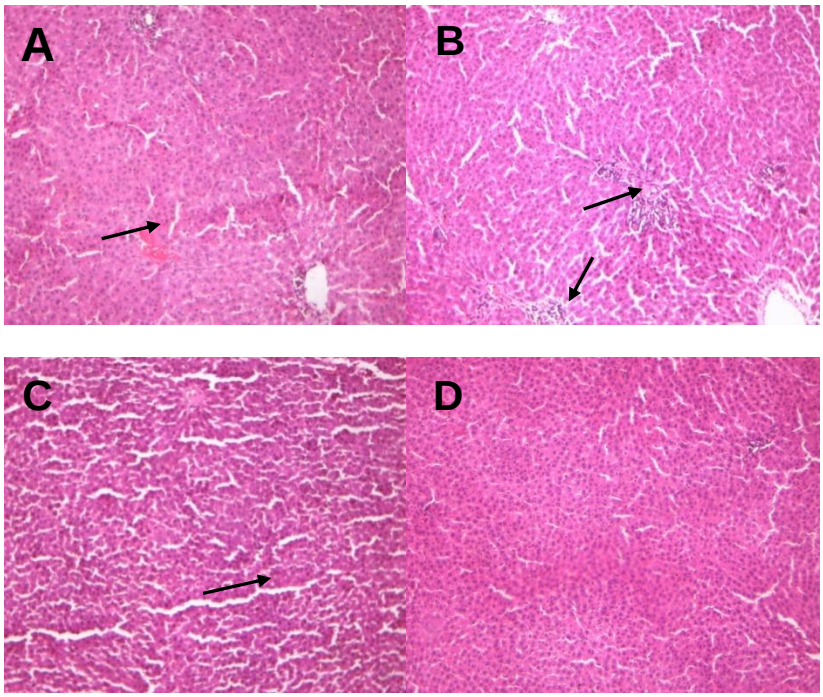

Figure. 2.Histological sections of the liver; (A): normal rats (given water) showing normal hepatocytes, (B) CCl_4 (1 ml/kg, twice weekly) showing focal inflammatory infiltrates withlymphocytes and plasma cells, (C) *Pyrenacantha staudtii* (50 mg/kg/day) + CCl_4 and (D) Sylimarin (50 mg/kg/day) + CCl_4 treatedrats showing normal hepatocytes with round to ovoid nuclei and abundant eosinophilic cytoplasm (H&E x 400). Arrows (↓) show points at which lesions occurred.

Liver histopathology

The extract (50 mg/kg) and sylimarin partially restored the structure and architecture of the liver by reducing the focal inflammatory infiltrates consisting of lymphocyte and plasma cells present in the control (Figure 2).

There was a significant change in the structure of the liver between control and the treatment group given 3-CMP (20 mg/kg) and sylimarin both showing mild vascular congestion (Figures 3C and 3D) and mild microvesicular steatosis, in sylimarin (Figure 3D). Liver from rats given CCl4 alone showed focal inflammatory infiltrates with lymphocytes and plasma cells (Figure 3B).

Effect of 3-CMP in thioacetamide (TAA)-induced hepatotoxicity

Body weight changes

There was a slight increase in body weight in the group given thioacetamide alone for 8 weeks. Treatment of the rats with different doses of 3-CMP caused marked decreases in body weight which were not significantly different from the thioacetamide alone group. However it was observed that there was a proportional increase in weight reduction as the dose of 3-CMP increased (Table 7). The Sylimarin group showed a significant ($p<0.01$) gain in total body weight when compared to the group given thioacetamide alone.

Biochemical markers

A significant elevation of the AST level was observed with the two higher doses of 3-CMP (10 and 20 mg/kg) (Table 8). No significant differences were observed between control and treatment groups in the other biochemical parameters (Table 8).

Effect of 3-CMP on liver histology of thioacetamide hepatotoxic rats

Liver sections from the thioacetamide-intoxicated rats showed moderate bridging fibrosis (cirrhosis) manifesting as radiating arms (Fig. 4B). In the 3-CMP-treated group, there was mild vascular congestion and attenuation of the bridging fibrosis (Fig. 4C). These effects were similar to those observed with the group treated with sylimarin (Fig. 4D).

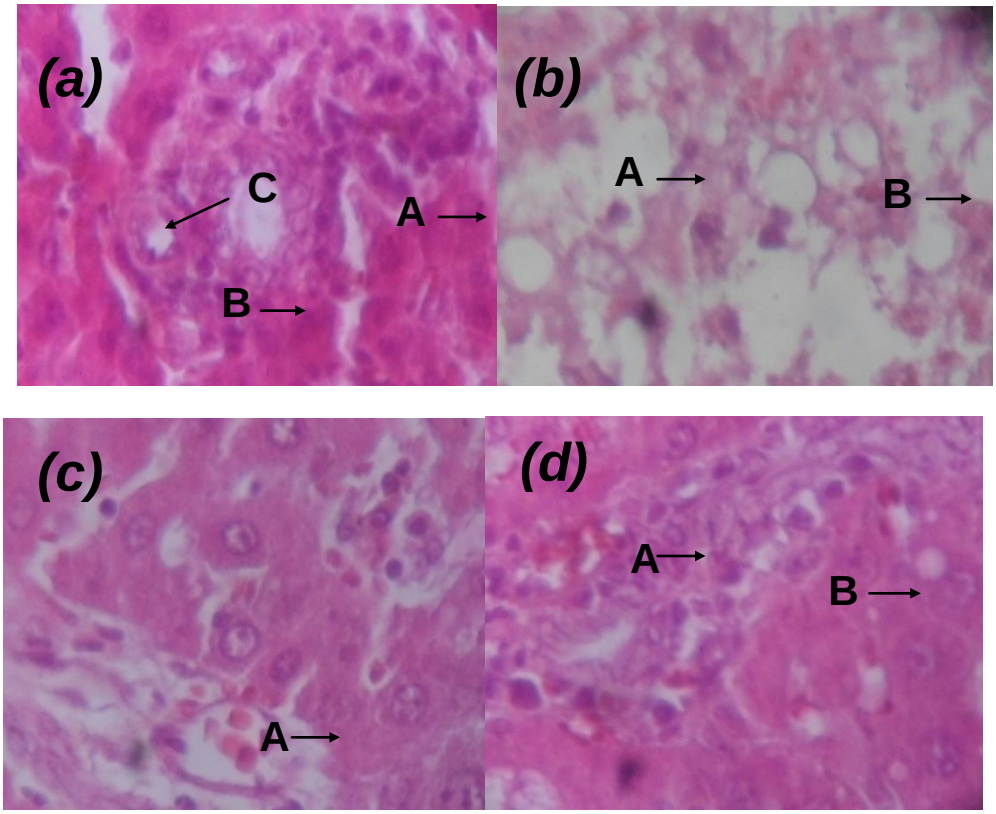

Figure. 3.Histopathological sections of the liver showing ***(a)*** normal healthy rats given water alone showing hepatocytes (A), portal triad (B) and sinusoids (C); ***(b)*** rats given CCl_4 (1 ml/kg, twice weekly) for 8 weeks showing severe macrovesicular steatosis (A) and mild vascular congestion (B); ***(c)***rats treated with 3-CMP (20 mg/kg) + CCl_4 showing mild vascular congestion A; ***(d)*** Sylimarin (50 mg/kg) + CCl_4 showing mild vascular congestion (A) and mild microvesicular steatosis (B) (H&E x 400). Arrows (↓) show points at which lesions occurred.

Haematological indices

Treatment of rats with 3-CMP did not significantly alter the haematological profile compared to thioacetamide alone-treated animals (Table 9). However, there was an increase in WBC with decreased RBC, haemoglobin and platelet counts in thioacetamide-hepatotoxic rats compared to the normal control.

Effect of 3-CMP on urine ascorbic acid content of CCl_4 and thioacetamide treated rats

The effect of 3-CMP on urine ascorbic acid content in CCl4 and thioacetamide intoxicated rats is shown in Table 10. There was a significant decrease in ascorbic acid content in the CCl4 and thioacetamide groups compared to the normal control (distilled water) group. Treatment with different doses of 3-CMP resulted in dose-dependent elevations in urine ascorbic acid content compared to CCl4 alone group, with a significant increase at the highest dose (20 mg/kg/day), an effect comparable to that of sylimarin. Conversely, in the thioacetamide-induced hepatotoxic rats, 3-CMP caused a non-significant dose-dependent reduction in the ascorbic acid concentration of the urine compared to the thioacetamide alone group (Table 10). Sylimarin elicited a significant elevation of the ascorbic acid content relative to the control. This effect was comparable to the sham-treated (distilled water) group.

Table 8: Effects of 3-CMP on biochemical parameters in TAA-induced hepatotoxicity

Treatment	Dose (mg/kg)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total protein (g/dL)	Albumin (g/dL)	Total bilirubin (mg/dL)	Conjugated bilirubin (mg/dL)
Control		104.80±7.25	39.50± 10.75	35.55± 8.12[b]	7.64± 0.67[b]	3.42± 0.29	0.58± 0.16	0.18± 0.06
TAA alone	-	50.12± 5.32	28.10± 2.05	91.56± 17.81	5.92± 0.35	2.24± 0.24	0.53± 0.11	0.50± 0.11
TAA +								
3-CMP	5	83.18± 11.97[a]	28.18± 0.69	70.52± 15.56	7.29± 0.28	2.67± 0.18	0.75± 0.14	0.26± 0.07
	10	108.53± 5.49[c]	32.92± 2.19	61.21± 7.93	8.49± 0.75[a]	3.14± 0.24	0.87± 0.15	0.35± 0.11
	20	102.47± 10.79[c]	25.07± 0.88	129.63± 19.45	6.47± 0.28	2.88± 0.23	0.71± 0.19	0.58± 0.11
Sylimarin	50	77.63± 8.02	24.22± 2.08	86.21± 9.64	7.11± 0.31	2.57± 0.18	0.80± 0.13	0.31± 0.07

Values are mean ± SEM. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$

ALP = Alkaline phosphatase; ALT = Alkaline transaminase; AST = Aspartate transaminase;

3CMP = 3 Carbomethoxypyridine; TAA = Thioacetamide

Table 9: Effect of 3-CMP on haematological parameters in thioacetamide-induced hepatotoxicity

Treatment	Dose (mg/kg)	WBC ($x10^3$/µL)	RBC ($x10^6$/µL)	HgbConc. (g/dL)	PLT ($x10^3$/µL)
Control	2	13.37± 2.07	8.59± 1.92	12.17± 0.61	783.17± 136.54
TAA alone	-	19.63± 1.97	7.58± 0.15	11.77± 0.60	543.50± 85.36
TAA +					
3-CMP	5	16.10± 3.07	7.35± 0.18	13.28± 0.53	432.25±50.41
	10	17.58± 1.62	7.53± 0.18	13.60± 0.32	470.43± 56.42
	20	17.43± 2.10	7.03± 0.21	13.62± 0.30	519.29± 33.36
Sylimarin	50	13.80± 1.16	7.63± 0.26	14.20± 0.50	616.00± 83.08

Values are mean ± SEM; n = 8

3-CMP = 3-Carbomethoxypyridine; **WBC** = White Blood Cell; **RBC** = Red Blood Cell; **Hb conc** = Haemoglobin concentration; **PLT** = Platelet.

Table 10: Effects of 3-CMP on urine ascorbic acid content in CCl_4-and thioacetamide - induced hepatotoxicity in rats

Treatment	Dose (mg/kg/day)	Ascorbic acid content (mg/dL)	
		+ CCl_4	+Thioacetamide
Alone	-	1.63± 0.49	2.19± 0.55
3-CMP	5	1.14± 0.31	0.99± 0.44
	10	3.30± 0.67	0.60± 0.29
	20	4.02± 0.29*	0.54± 0.16
Sylimarin	50	4.42± 1.11*	4.14± 0.53*
Distilled water	5ml	4.23± 0.51*	4.23± 0.51*

Values are mean ± SEM. *p<0.05, significantly different from CCl_4 or thioacetamide alone; (n= 8 animals)

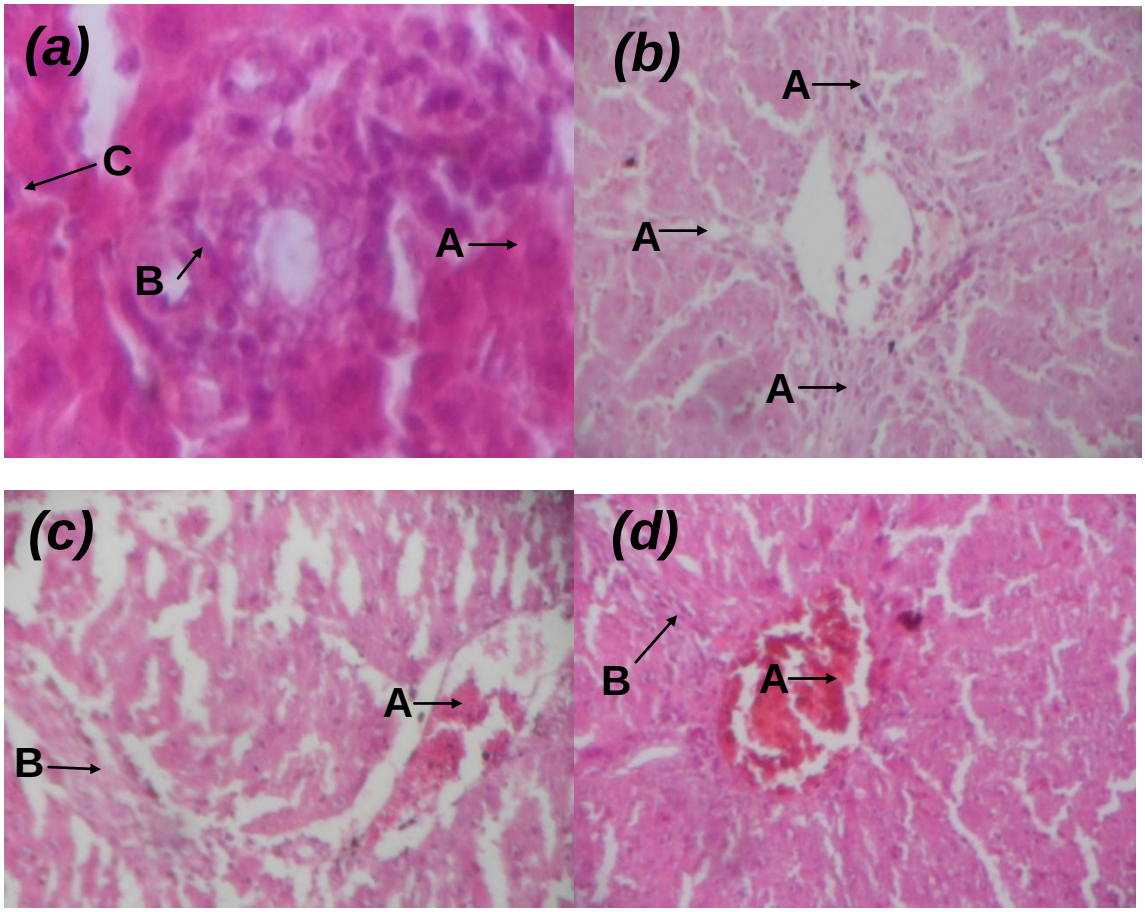

Figure. 4**.** Histopathological sections of the liver of rats given***(a)***: water for 56 days showing hepatocytes **(A)**, portal triad **(B)** and sinusoids **(C)**; ***(b)*** Thioacetamide alone (200 mg/kg, twice weekly) for 8 weeks showing moderate bridging fibrosis (cirrhosis) A. ***(c)*** 3-CMP (20mg/kg) + Thioacetamide showing mild vascular congestion **(A)** and mild bridging fibrosis **(B)** and ***(d)***Sylimarin (50mg/kg) + Thioacetamide showing severe vascular congestion(A) and mild bridging fibrosis (B) (H&E x 100**).** Arrows (↓) show points at which lesions occurred.

DISCUSSION

This study evaluated the hepatoprotective effect of methanol leaf extract of *Pyrenacantha staudtii* (PS) and its isolated compound, 3-Carbomethoxypyridine (3-CMP) in hepatocellular damage induced by carbon tetrachloride (CCl4) and thioacetamide (TAA). Previous studies had reported the hepatoprotective effects of P.S and 3-CMP (Anosike *et al*., 2008; Okpo *et al*., 2013). While these earlier studies investigated the effects of either the extract or the isolated compound on short-term hepatocellular damage and using high doses, the present study investigated their effects on chronic hepatocellular damage induced by two different hepatotoxins, namely carbon tetrachloride and thioacetamide.

3-CMP is an isolated pure compound from the leaves of *Pyrenacantha staudtii*. Acute and short-term toxicity studies revealed that it has no toxic effect on rats and mice. No mortality was observed up to a maximum oral dose of 2 g/kg body weight, neither were there any signs of delayed toxicity, so the oral LD50 could not be determined. According to OECD-423 guidelines for acute oral toxicity, an LD50 dose of 2,000 mg/kg is categorized as unclassified (OECD, 2000).

The reduction in body weight with the highest dose of 3-CMP in the short term toxicity studies may indicate an adverse reaction to the compound. Change in body weight of animals is useful in assessing response to therapy with drugs (Winder *et al*, 1969) and is indicative of adverse effects (Teo *et al*, 2002).

The relative organ (liver, kidney, heart) weights remained normal in the treated groups suggesting that 3-CMP may not be toxic to these vital organs. Lower doses of 3-CMP did not reveal any significant toxic effects on the organs. There were no visible colour changes or irregularities in the architecture of the various organs examined (liver, kidney and heart). However, the highest dose (200mg/kg) caused mild/serious lesions in all the organs studied. Since management of liver diseases requires long term treatment, it is necessary to exercise caution in the use of the compound at such high doses.

Hepatotoxicity caused by CCl_4 results from its activation, by CYP P450¬, to trichloromethyl radical (CCl_3.) which binds macromolecules, proteins, and lipids and with oxygen to yield other reactive oxygen species (ROS). This consequently leads to hepatic damage via alkylation of cellular proteins, lipid peroxidation and disruption of cell membrane structure and function (Brattin *et al*., 1985; Rikans *et al*., 1994).Thioacetamide, on the other hand, also covalently binds cell macromolecules and is metabolized by CYPE21 to toxic products that cause hepatic damage (Kim *et al*., 2000; Sadasivan et al., 2006). Hepatotoxicity of TAA progresses from fibrosis to cirrhosis.

The important functions of the liver are achieved through the involvement of various hepatic enzymes. Most hepatic damage disrupts cell membrane integrity and function causing leakage of hepatic enzymes into the systemic circulation, hence their increased serum concentrations (Ray *et al*, 2008).

Methanol extract of *Pyrenacantha staudtii* at all dose levels used neither attenuated the effect of CCl4 on body weight nor reduced the level of serum biochemical markers. This is in converse to the study by Anosike *et al*. (2008), which reported significant attenuation of elevated biochemical markers associated with CCl_4-induced hepatotoxicity. This may be as a result of the much lower dose of extract (10, 25 and 50 mg/kg/day) used in this study compared to the very high doses (750 mg/kg and 1500 mg/kg) employed by Anosike *et al*. (2008).

Administration of 3-CMP significantly elevated the body weights of CCl4-intoxicated rats while decreasing those of TAA-intoxicated rats. This implies that 3-CMP attenuated the possible weight loss associated with hepatic disease only in CCl4-induced hepatotoxicity.

In addition to the effect on weight loss, 3-CMP significantly decreased the levels of the transaminases, AST and ALT, in a dose-dependent manner.

Elevated serum levels of AST is a non-specific event as it can be from the myocardium, skeletal muscle or erythrocyte, but increased serum concentrations of ALT is most likely to reflect hepatic cell damage (Ozer *et al*., 2008).

The reduced levels of these transaminases observed with CCl4 -intoxicated rats treated with the 3-CMP suggests a possible preservation of the structural integrity of the hepatocellular membrane (by 3-CMP) to protect it against peroxidation by the reactive metabolites produced from exposure to CCl_4. This possibly prevented further damage to more hepatocytes and hence reduced further leakage of AST and ALT due to cell destruction.

On the other hand, the significantly increased serum AST levels (but not ALT) observed in TAA-intoxicated rats treated with 3-CMP points to a possible interaction between 3-CMP and TAA,

which causes the release of AST from other sources leading to a worsened hepatic state.

Total protein and serum albumin concentrations were not significantly elevated by 3-CMP in CCl4-treated rats. However, in TAA-treated rats, it significantly improved total protein without any significant increase in serum albumin. This observation suggests an increase in the protein synthetic function of the liver, which could be as a result of possible restorative effect on the damaged hepatocytes induced by TAA.

At all the dose levels used, 3-CMP decreased both conjugated and total bilirubin concentrations (though not significantly) without any significant changes in the same parameters on TAA intoxicated rats. Modulation of CCl4-induced increases in total and conjugated bilirubin by 3-CMP further shows its protective effect against CCl4-induced liver toxicity; however, it failed to show similar effect in TAA-induced hepatotoxicity.

Haematological evaluation revealed that 3-CMP and sylimarin significantly reduced WBC counts in CCl4-induced hepatotoxicity. This implies that 3-CMP may possibly possess immunomodulatory effects against inflammation associated with CCl4 type of hepatic damage. 3-CMP however, failed to significantly alter RBC count, haemoglobin concentration and platelets count in CCl4-induced hepatotoxicity as well as WBC count, RBC count and haemoglobin concentration in TAA treated rats.

A non-invasive method employed in studying the hepatoprotective potential of 3-CMP was the estimation of urine ascorbic acid content. Ascorbic acid, a water soluble vitamin with antioxidant property, is not metabolized by the liver and thus is excreted unchanged. Hepatic damage due to lipid peroxidation and alteration of cellular proteins reduce the membrane permeability to ascorbic acid leading to increased urine content. Hence, reduction in urinary excretion of ascorbic acid can be used as an index for CCl4 (and other hepatotoxins) - induced liver damage and to demonstrate hepatoprotective activity (Visweswaram *et al*., 1994.)

3-CMP significantly increased the urine ascorbic acid content in CCl4-treated rats but further potentiated the reduced urinary excretion initiated by TAA in rats. This further lends credence to the protective effect of 3-CMP on liver damage induced by CCl_4 and a possible hepatotoxic interaction between 3-CMP and TAA.

The histologic analysis of liver samples from animals given CCl_4 or TAA alone revealed structural distortions with enlarged portal spaces and fine brush-like conjuctive connective tissue projections joining together to form bridges and ultimately cirrhotic nodules of different sizes, establishing liver cirrhosis. This was more discernible in TAA treated rats. Methanol leaf extract of *Pyrenacantha staudtii* showed recovery from CCl_4-induced hepatic damage as demonstrated by the histopathology. This is contrary to the result of biochemical evaluation and may point to a possible hepatoprotective property of the plant.

The histopathological changes of liver sections obtained from rats fed with 3-CMP showed improved histological appearances in both CCl4- and TAA-induced hepatic damage.

3-CMP (like sylimarin) caused mild vascular congestion in CCl_4-induced hepatic damage (as against the severe macrovesicular steatosis observed in rats given CCl_4 alone) and in addition exhibited mild bridging fibrosis in TAA-induced hepatic damage. This further suggests that 3-CMP may possess hepatoprotective effects in CCl4-induced hepatic damage which, however, may be subdued in TAA-induced damage.

CONCLUSIONS

The study has revealed that although, the methanol leaf extract of *Pyrenacantha staudtii* did not significantly attenuate the effect of CCl_4 on the serum biochemical and haematologic indices, its effects on the liver histology points to a possible hepatoprotective activity. The lack of effect by the extract on the liver enzymes and other parameters evaluated may be a function of the doses used. Despite the seeming elevation of the liver enzymes and other biochemical indices caused by 3-CMP on thioacetamide-induced liver damage, attenuation of the nodularity of the liver architecture which presented as bridging fibrosis (cirrhosis) suggests a hepatoprotective effect.

This study has revealed that 3-CMP causes no immediate or delayed toxic effects on rodents and has greater hepatoprotective efficacy on CCl_4-induced liver damage linked with liver fibrosis than liver cirrhosis caused by thioacetamide. However, the exact mechanism of hepatoprotective activity of Pyrenecantha staudtii methanol extract and 3-CMP in CCl_4-induced liver damage and possible mechanisms of interaction between 3-CMP and TAA need further elucidation.

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