European Journal of Medicinal Plants
9(4): 1-10, 2015, Article no.EJMP.18927
ISSN: 2231-0894

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Aqueous Extract of the Fruits of *Xylopia aethiopica* (Dunal) A. Rich. Protects against Carbon Tetrachloride - Induced Hepatotoxicity in Rats

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Authors' contributions

This work was carried out in collaboration between both authors. Author OBA contributed in running the laboratory work, analysis of the data and drafting of the manuscript. Author NEJO contributed in designing the study, supervised the laboratory work and in critical reading of the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2015/18927
Editor(s):
(1) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Anonymous, Guangzhou University, China.
(2) Anonymous, National Pingtung University of Science and Technology, Taiwan.
(3) Anonymous, Egypt.
Complete Peer review History: http://sciencedomain.org/review-history/10143

Original Research Article

Received 17th May 2015
Accepted 9th June 2015
Published 13th July 2015

ABSTRACT

Aim: This study was designed to examine the hepatoprotective effect of aqueous extract of the fruits of *Xylopia aethiopica* using the carbon tetrachloride (CCl_4) model.
Place and Duration of Study: Department of Chemical Sciences, Biochemistry unit, Afe Babalola University, Ado-Ekiti, Nigeria between November, 2013 and April, 2014.
Methodology: Thirty six rats divided into 6 groups of 6 animals each were used for the investigation. Group 1 served as control, while groups 2, 3 and 4 were pre-treated with aqueous extract of the fruit of *X. aethiopica* at the respective dose of 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight for 21 days prior to a single intraperitoneal administration of CCl_4. Animals in group 5 received only the fruit extract at a dose of 1000 mg/kg body weight, while those in group 6 were given only CCl_4. All animals were sacrificed 24 h after the administration of CCl_4. Liver functions

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was assessed by measuring the plasma levels of AST, ALT, ALP, total protein and albumin. Plasma lipid profile and the degree of lipid peroxidation in the liver were determined in addition to histopathological evaluations.
Results: Whereas CCl_4 administration resulted in significant elevations in plasma ALT, AST, and ALP, there was a significant reduction in both plasma total protein and albumin. In addition, histopathological changes were observed with CCl_4. Analysis of the data obtained for MDA, SOD and catalase suggest that the plant extract exerts its protective effect probably by inhibiting CCl_4-induced lipid peroxidation in liver tissue.
Conclusion: It can be suggested that *X. aethiopica* fruits has the ability to offer a significant degree of protection to liver cells against carbon tetrachloride-induced hepatotoxicity in Wistar albino rats by antioxidant mechanism of action.

Keywords: Carbon tetrachloride (CCl_4); hepatoprotective; hepatotoxicity; oxidative stress; Xylopia aethiopica.

1. INTRODUCTION

The liver is known to play a number of vital roles that contribute to overall well-being. These functions are often compromised in liver pathology. Diseases affecting the liver have fast become a major health challenge [1]. Despite tremendous advances in modern medicine, prevention and treatment options still remain limited. Till date, resection and transplantation remain the only hope of long term survival for patients. Unfortunately these are often outside the reach of many people especially in developing countries. Consequently, most of the patients die within three months of presentation.

The goal of this study therefore was to contribute to the search for a cheap, safe and readily available remedy against liver diseases. Understandably, plants and plant-derived products have taken centre stage in man's quest for therapeutic agents. In this particular study, the possible hepatoprotective activity of *Xylopia aethiopica* was investigated in rats using the CCl_4 model.

Xylopia aethiopica or African negro pepper enjoys a huge patronage both in nutrition and in ethnomedicine [2,3]. The plant is thought to be rich in a number of physiologically and pharmacologically active agents, including volatile oils [4,5]. The fruits of *Xylopia aethiopica* have been reported to act as antioxidant, hypolipidemic and hypoglycaemic agent [6]. Flavonoids from *X. aethiopica* have been shown to exhibit both antioxidant and anti-inflammatory properties [7]. Kaurene diterpenes isolated from *Xylopia aethiopica* include xylopic acid which has antiplasmodial [8], analgesic [9], cardiovascular and diuretic effects [10]. Others include kaurenoic acid which has antitrypanosomic, analgesic and anti-inflammatory effects [11,12], acetylgrandifloric acid was reported to have antibacterial effect [13] and ent-15-oxokaur-16 en-19-oic acid which is antiproliferative [14].

From a standpoint of a clear understanding of the mechanistic basis of carbon tetrachloride-induced toxicity, plants such as *Xylopia aethiopica*, possessing significant antioxidant or free radical scavenging activities are considered plausible candidates for the prevention of cellular injury occasioned by CCl_4. Various studies have shown that carbon tetrachloride (CCl_4) intoxication causes free radical generation in many tissues including the liver, kidney, heart, lung, testis, brain and blood [15–18]. Authors are in agreement that processes involving reactive oxygen species contribute significantly to the aetiology of CCl_4-induced damage in a vast array of biological systems and tissues, including the liver [15]. It is now known that, CCl_4 is metabolically converted by cytochrome P_{450} enzymes, notably CYP2E to trichloromethyl (CCl_3) radicals, resulting in enhanced generation of trichloromethylperoxyl radicals (Cl_3COO^-) and hydrogen peroxides [19]. It is hoped that data or information from the present study would raise new possibilities in our quests for an effective plant based remedy against liver diseases.

2. MATERIALS AND METHODS

2.1 Chemicals

CCl_4 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and kits (from Randox laboratories, Crumlin, United Kingdom) used throughout this investigation were of the highest analytical grade commercially available while the water was glass distilled.

2.2 Collection and Identification of Plant Material

Fruits of *Xylopia aethiopica* were obtained in May, 2013 from a forest near Warri in Delta State, Nigeria, and authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria. The voucher specimen of the plant is available. A sample of the plant portion was deposited at the Departmental Herbarium for future reference.

2.3 Preparation and Administration of Plant Extract

The plant sample was air dried and blended. The blended plant material was then soaked in warm water (initial temperature, 45ºC) for 24 hours following which it was filtered using Whatman filter paper number 1. The filtrate was then concentrated at 45ºC using a rotary evaporator and dried by means of a freeze dryer. The extract was administered orally using gavage according to the required doses of 250, 500 and 1000 mg/kg body weight for 21 days.

2.4 Grouping of Animals

A total of 36 Wistar strain albino rats, weighing between 180 to 220 g was used for the experiment. The animals were well-monitored and of good health before the start of the experiment as they did not show any sign of adversity. The animals were then divided into 6 groups of 6 animals each. Group I animals served as normal control and received normal chow while animals in group II received normal feed and *X. aethiopica* extract at a daily dose of 250 mg/kg body weight. Group III animals received normal feed and plant extract at a daily dose of 500 mg/kg body weight while their group IV counterparts received normal feed and extract at a daily dose of 1000 mg/kg body weight. Animals in group V received normal feed and fruit extract at a daily dose of 1000 mg/kg body weight. Group VI animals received normal feed and a single intraperitoneal administration of CCl_4 only.

All treatments with extract lasted for 21 days and preceded CCl_4 administration. Only animals in group II, III, IV and VI were given the single intraperitoneal injection of CCl_4. All animal were handled in strict accordance with the NIH guide for the care and use of laboratory animals.

2.5 Administration of CCl_4

The CCl_4 model described by Huo et al. [20], with some modifications, was employed for inducing liver damage (3 ml/kg body weight) of a 1:1 preparation of CCl_4 and groundnut oil (in the ratio 1:1). Where necessary, each animal received this preparation. Administration of CCl_4 was by a single intraperitoneal injection. Administration was done after a twenty-one day treatment with *X. aethiopica,* and following an overnight fast.

2.6 Preparation of Plasma and Liver Homogenate

The rats were sacrificed 24 h after CCl_4 administration by cervical dislocation and then dissected. Blood samples were collected from the heart using the heart puncture technique. Blood was collected in heparinized bottles. The blood samples were subsequently centrifuged at 3000 rpm for 10 min using a bench centrifuge to obtain plasma. The plasma obtained was separated, and transferred into fresh plain sample bottles and used for the subsequent biochemical analyses. The liver of each animal was excised immediately after sacrifice. They were rinsed in ice-cold 1.15% potassium chloride, blotted with filter paper and weighed. Weighed portions were minced with scissors in 4ml of ice-cold 0.1M phosphate buffer, pH 7.4 and homogenized in a Potter–Elvehjem homogenizer. The homogenates were later centrifuged using refrigerated centrifuge at 12,000 x *g* for 15 min at 4ºC to obtain clear supernatant, which were used for subsequent biochemical analyses.

2.7 Biochemical Analyses

Biochemical analyses carried out included measurement of the activities of plasma alanine aminotransferase (ALT), aspartate amino-transferase (AST), gamma-glutamyl transferase (γ-GT), and alkaline phosphatase (ALP); plasma total protein and albumin concentrations. In addition, triglyceride and total cholesterol concentrations were also measured in both plasma and liver homogenate. The determination of the concentrations of these biochemical parameters was done using commercially available test kits, products of Randox Laboratories (Crumlin, United Kingdom).

2.8 Antioxidant Assays

Lipid peroxidation was assessed by measuring the formation of thiobarbituric (TBA) reactive substances according to the method described by Varshney and Kale [21]. Catalase (CAT) activity was determined according to the method of Sinha [22]. The level of superoxide dismutase (SOD) activity was measured as described by Misra and Fridovich [23].

2.9 Histopathological Assessment

Liver sections from rats of different groups were fixed in 10% neutral formalin solution, dehydrated in graded alcohol, and embedded in paraffin. Fine sections obtained were mounted on glass slides and counter-stained with hematoxylin–eosin for light microscopic analyses. The slides were which were coded, were examined and photographed by a qualified histopathologist. The histopathologist had no knowledge of the treatment groups.

2.10 Statistical Analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) using windows SPSS software package. Post hoc testing was performed for inter-group comparisons using the Least Significant Difference (LSD) test according to the method described by Zar [24]. In all instances *P* values <.05 were considered statistically significant.

3. RESULTS

The results obtained from the study are presented in Tables 1-5.

3.1 Plasma Marker Enzymes

Table 1 shows the effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in some serum enzymes. Intraperitoneal administration of CCl_4 resulted in marked liver damage as shown by significant elevations in the activities of plasma alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) when compared with normal control group (*P*<.05). Pretreatment with *X. aethiopica* (fruits) at 250 mg/kg, 500 mg/kg and 1000 mg/kg resulted in a significant decrease in the activities of these enzymes when compared with CCl_4 group in a dose dependent manner. However, there was no significant different among the three doses of the extract in the activities of ALT, AST, GGT and LDH. The higher doses of 500 and 1000 mg/kg body weight appear to have been more effective.

3.2 Plasma Total Protein and Albumin

Table 2 showed the effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in plasma total protein and albumin levels. A significant decrease in the levels of total protein and albumin was observed following a single intraperitoneal administration of CCl_4 when compared with normal control group (*P*<.05). Pretreatment with aqueous extract of the fruits of *X. aethiopica* at the three dose levels tested resulted in a significant increase both plasma total protein and albumin when compared with CCl_4 group.

3.3 Plasma Triglyceride and Cholesterol

Table 3 showed the effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in plasma triglyceride and cholesterol. Intraperitoneal administration of CCl_4 resulted in marked increase in the levels of plasma triglyceride and total cholesterol when compared with normal control group (*P*<.05). Pretreatment with aqueous extract of the fruits of *X. aethiopica* at all three doses tested produced a significant decrease in triglyceride and total cholesterol when compared with CCl_4 group.

3.4 Liver Total Protein, Triglyceride and Cholesterol Levels

The effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in liver total protein, triglyceride and cholesterol is shown in table 4. Administration of rats with CCl_4 produced a significant decrease (*P*<.05) in total protein level, and a significant increase (*P*<.05) in triglyceride and total cholesterol level when compared with normal control group. A significant increase was observed in total protein level, and a significant decrease in triglyceride and total cholesterol levels was observed when animals were pretreated with aqueous extract of the fruits of *X. aethiopica* at the three doses tested.

Table 1. Effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in ALT, AST, ALP, GGT and LDH

Groups	Treatment	Plasma ALT (U/L)	Plasma AST (U/L)	Plasma ALP (U/L)	Plasma GGT (U/L)	Plasma LDH (U/L)
I	Normal control	40.24^{a}±3.91	88.36^{a}±6.71	42.35^{a}±3.45	1.54^{a}±0.22	95.96^{a}±9.28
II	Fruit extract (250 mg/kg) + CCl_4	108.04^{b}±4.86	171.76^{b}±4.53	80.03^{b}±7.18	3.38^{b}±0.53	112.36^{ab}±8.74
III	Fruit extract (500 mg/kg) + CCl_4	107.87^{b}±1.29	170.70^{b}±1.92	108.90^{c}±10.61	2.83^{ab}±0.13	111.00^{ab}±3.83
VI	Fruit extract (1000 mg/kg) + CCl_4	99.36^{b}±2.95	166.62^{b}±2.13	126.50^{c}±11.48	2.57^{ab}±1.12	96.23^{ab}±2.19
V	Fruit extract (1000 mg/kg) only	39.85^{a}±2.50	84.81^{a}±5.99	55.44^{ab}±6.21	1.24^{a}±0.23	93.17^{a}±7.01
VI	CCl_4 only	133.58^{c}±4.02	220.67^{c}±11.74	204.60^{d}±18.17	5.28^{c}±0.84	152.00^{c} ±9.58

Values are expressed as Mean±SEM (n=6)
Values with different letter along the same column are significantly different

Table 2. Effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in plasma total protein and albumin levels

Groups	Treatment	Plasma total protein (g/dl)	Plasma albumin (g/dl)
I	Normal control	4.64^{a}±0.11	3.08^{a}±0.23
II	Fruit extract (250 mg/kg) + CCl_4	3.62^{b}±0.09	1.56^{b}±0.17
III	Fruit extract (500 mg/kg) + CCl_4	4.08^{ab}±0.14	1.97^{bc}±0.17
IV	Fruit extract (1000 mg/kg) + CCl_4	4.45^{a}±0.39	2.43^{c}±0.32
V	Fruit extract (1000 mg/kg) only	4.70^{a}±0.34	3.55^{a}±0.26
VI	CCl_4 only	1.14^{c}±0.24	0.57^{d}±0.12

Values are expressed as Mean±SEM (n=6)
Values with different letter along the same column are significantly different

Table 3. Effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in plasma triglyceride and cholesterol

Groups	Treatment	Plasma triglyceride (mg/dl)	Plasma cholesterol (mg/dl)
I	Normal control	101.97^{a}±8.71	22.38^{a}±2.30
II	Fruit extract (250 mg/kg) + CCl_4	124.22^{bc}±8.17	32.41^{b}±0.33
III	Fruit extract (500 mg/kg) + CCl_4	137.91^{b}±2.45	29.51^{b}±0.24
IV	Fruit extract (1000 mg/kg) + CCl_4	129.61^{b}±12.63	26.35^{ab}±2.67
V	Fruit extract (1000 mg/kg) only	104.78^{ac}±7.49	22.64^{a}±1.39
VI	CCl_4 only	173.22^{d}±7.84	52.69^{c}±2.14

Values are expressed as Mean±SEM (n=6)
Values with different letter along the same column are significantly different

3.5 Antioxidant and Lipid Peroxidation Biomarkers

Table 5 showed the effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in liver lipid peroxidation (LPO), Superoxide dismutase (SOD) and Catalase (CAT). Malondialdehyde (MDA), an index of lipid peroxidation was significantly increased with the administration of CCl_4 when compared with the

normal control group (P<.05). The induced peroxidation was significantly prevented by pretreatment with aqueous extract of *X. aethiopica*.

3.6 Histological Assessment of the Liver

Photomicrographs revealing the hepatic morphology of all the treatment groups are shown in plate 1(a) to (f). Histological examination of liver sections of normal control rats showed no visible pathological alterations and no significant lesions (plate 1 (a)). Rats pre-treated with aqueous extract of the fruit of *X. aethiopica* (250 mg/kg, orally) plus CCl_4 showed mild lymphocytic parenchyma infiltration; fragmentation of the hepatocytes with progressive occlusion of the sinusoid (plate 1 (b)); Rats pre-treated with aqueous extract of the fruit of *X. aethiopica* (500 mg/kg, orally) plus CCl_4 showed mild lymphocytic and parenchyma infiltration (plate 1 (c)); Rats pre-treated with aqueous extract of the fruit of *X. aethiopica* (1000 mg/kg, orally) plus CCl_4 showed mild lymphocytic infiltration with the degeneration of the hepatocytes (plate 1 (d)). Rats treated with 1000 mg/kg dose of the aqueous extract of fruit of *X. aethiopica* are intact with preserved histological profile (plate 1 (e)). However, evidence of cellular degeneration in the hepatocytes; multiple foci of hepatocellular necrosis with cellular infiltration by lymphocytes and macrophages were seen on sections of rats treated with CCl_4 only (plate 1 (f)).

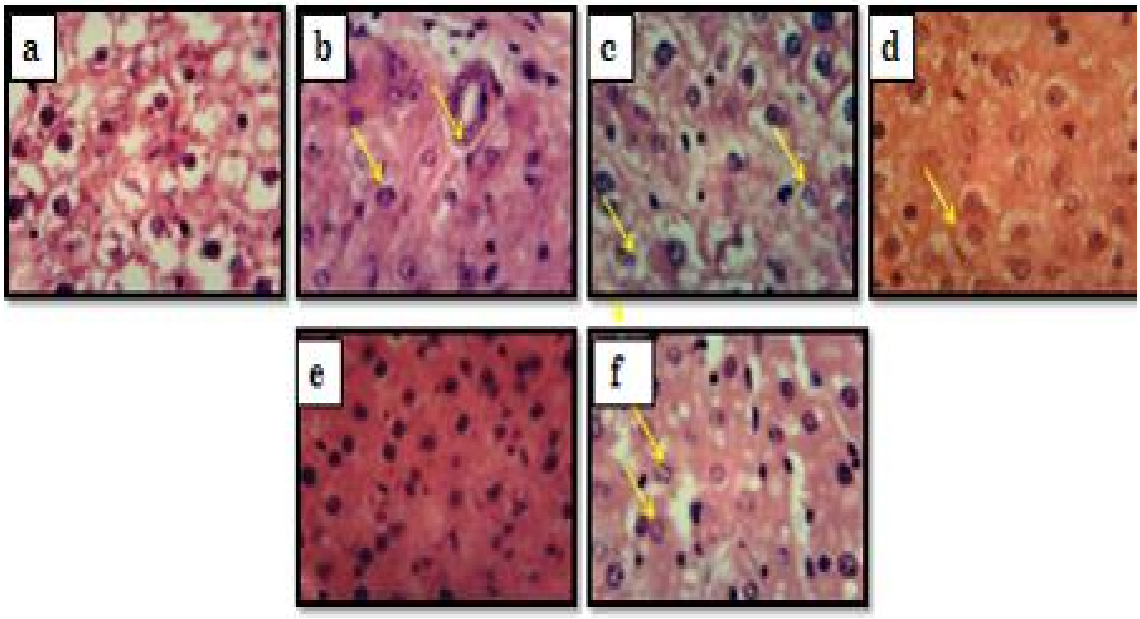

Plate 1. Photomicrograph of rat liver sections (Initial magnification 400 x)

Table 4. Effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 induced changes in liver total protein, triglyceride and cholesterol

Groups	Treatment	Liver total protein (g/g liver weight)	Liver triglyceride (g TG /g liver weight)	Liver cholesterol (mg/g liver weight)
I	Normal control	3.03^{a}±0.24	3.03^{a}±0.24	0.77^{a}±0.09
II	Fruit extract (250 mg/kg) + CCl_4	4.07^{b}±0.59	4.07^{b}±0.59	1.25^{b}±0.15
III	Fruit extract (500 mg/kg) + CCl_4	3.41^{b}±0.54	3.41^{bc}±0.54	1.24^{b}±0.20
IV	Fruit extract (1000 mg/kg) + CCl_4	3.30^{ab}±0.22	3.30^{ac}±0.22	1.00^{ab}±0.02
V	Fruit extract (1000 mg/kg) only	3.04^{a}±0.18	3.04^{a}±0.18	0.78^{a}±0.05
VI	CCl_4 only	0.78^{c}±0.36	5.17^{d}±0.43	2.30^{c}±0.17

Values are expressed as Mean±SEM (n=6)
Values with different letter along the same column are significantly different

Table 5. Effect of aqueous extract of the fruits of *Xylopia aethiopica* on CCl_4 - induced changes in liver LPO, SOD and CAT

Groups	Treatment	Liver LPO (µM MDA activity/g liver wt)	Liver SOD (Units SOD/g liver wt)	Liver CAT activity (mmole H_2O_2 consumed/g liver wt)
I	Normal control	8.02^{a}±0.74	2.69^{a}±0.21	0.31^{a}±0.03
II	Fruit extract (250 mg/kg) + CCl_4	14.51^{b}±1.42	2.02^{b}±0.41	0.23^{bc}±0.01
III	Fruit extract (500 mg/kg) + CCl_4	13.48^{b}±2.27	2.02^{ab}±0.00	0.24^{bc}±0.03
IV	Fruit extract (1000 mg/kg) + CCl_4	12.91^{b}±0.95	2.18^{ab}±0.17	0.28^{bc}±0.10
V	Fruit extract (1000 mg/kg) only	8.98^{a}±0.81	3.27^{ac}±0.20	0.31^{ac}±0.06
VI	CCl_4 only	19.45^{c}±1.54	0.67^{d}±0.07	0.10^{d}±0.01

Values are expressed as Mean±SEM (n=6)
Values with different letter along the same column are significantly different

4. DISCUSSION

The present report is centered on the hepatoprotective activities of aqueous extract of *Xylopia aethiopica* in carbon tetrachloride-induced liver damage. Oxidative stress-induce peroxidation is a prominent feature of CCl_4-induced hepatic injury. The mechanistic basis of CCl_4-induced cellular injury is now fairly well understood. Carbon tetrachloride is believed to be metabolized via a Cyt P_{450}-dependent pathway to the very reactive trichloromethyl intermediate [12,25]. The subsequent generation of trichloromethyl peroxyl radical is thought to promote the further generation of reactive oxygen species [26,27]. Free radical attack on biological molecules including proteins, lipids and DNA further increases the cell's susceptibility to damage. Consistent with this view, CCl_4-mediated cellular injury proceeds with massive elevations in both Plasma and target tissue levels of the product of lipid peroxidation, namely Malondialdehyde.

As always, the usual antioxidant defense system of the body attempts to rise up to the occasion in a bid to quench the highly reactive and offensive free radicals. In many instances, this effort ends up being futile as the natural antioxidant defense apparatus is soon depleted and overwhelmed. The end result is uncontrolled cell destruction with attendant organ dysfunction. Polyunsaturated fatty acids, especially those found in association with membrane phospholipids are particularly susceptible to free radical-mediated attacks following CCl_4 intoxication. This results in a loss in both the structural and functional integrity of organelle and cell membrane. With the cell membrane now compromised in both structure and function, critical ion gradients across the membrane are disrupted as ion flow across the membrane become increasingly haphazard. Massive increase in the intracellular (cytosolic) calcium ion concentration is particularly implicated in the cascade of events ultimately leading to cell death or necrosis.

As a secondary event to the loss o membrane integrity, intracellular enzymes and other proteins find their way into plasma, thus raising their plasma concentrations. Though not restricted to this organ, ALT and AST are to be localized within hepatocytes and are massively released into plasma following membrane damage. This explains the increased activities of these enzymes in plasma recorded in this study. The relative increase in plasma levels of ALT and AST is a pointer to the extent of hepatocyte damage. Where damage extends significantly to the mitochondria, the relative increase in plasma AST usually outweighs that in plasma ALT. This is because, whereas ALT has more of a cytosolic distribution, AST is localized principally in the mitochondrion [28].

Elevated plasma level of ALP is suggestive of biliary obstruction, such as occur in cholestatic disease of the liver. Differential diagnosis to ascertain the source of plasma ALP is provided by assay of gamma glutamyl transferase (GGT). GGT is a membrane bound enzyme whose activity in plasma increases significantly alongside that of plasma ALP following biliary obstruction [28,29]. Biliary obstruction, as suggested by the data obtained for both ALP and GGT in this study implies a failure of biliary secretion. This may account, at least in part for the observed increased intrahepatic cholesterol

concentrations since cholesterol is usually secreted along with bile acids. Another possible explanation is the inability of the liver to use up cholesterol and triglycerides in the synthesis and export of lipoproteins. Elevated levels of both triglycerides and cholesterol may be due to a failure in hepatic uptake secondary to hepatocyte damage.

The ability of *X. aethiopica* fruit extract to effectively protect the liver against carbon tetrachloride toxicity may be related to the remarkable antioxidant property of the plant previously reported by other workers [7,30,31]. Since carbon tetrachloride toxicity is mediated via a free radical dependent mechanism [32,33], agents with significant antioxidant activity are considered reasonable candidates for anti carbon tetrachloride therapies. Whether the plant extract possesses multiple bases for its anti carbon tetrachloride action remains to be verified. If indeed a multiple mechanistic basis exists, this would most likely revolve around inhibition of carbon tetrachloride uptake, the inhibition of its bioactivation to trichloromethyl and trichloromethylperoxyl radicals, possibly by inhibition of Cytochrome p 450 system or outright repression of that gene. While these other aspects of the study will continue to engage our attention and that of other workers, the outstanding hepatoprotective property of *X. aethiopica* demonstrated in this study, with glaring biochemical and histological evidences deserve special mention.

5. CONCLUSION

It can be concluded based on these findings that administration of aqueous extract of the fruits of *X. aethiopica* at doses up to 1000 mg/kg body weight has potential hepatoprotective activity against carbon tetrachloride-induced hepato-toxicity in Wistar albino rats. The mechanism of the plant extract is believed to be related to its antioxidant properties as demonstrated by its ability to boost antioxidant status in animals challenged with carbon tetrachloride.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by Afe Babalola University animal ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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