Indian Journal of Novel Drug Delivery

IJNDD

An Official Publication of Karnataka Education and Scientific Society

Research Article

Hepatoprotective Effect of Aqueous and Ethanol Root Extracts of *Millettia Aboensis* on Carbon Tetrachloride-Induced Hepatotoxicity in Experimental Rats

ATTAMA AA, OFOKANSI KC, KENECHUKWU FC*, UGWUEZE ME

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria

ARTICLE DETAILS

Article history:
Received on 31 January 2014
Modified on 25 May 2014
Accepted on 03 June 2014

Keywords:
Millettia aboensis,
Hepatoprotective effect,
Carbon tetrachloride,
Liv-52®.

ABSTRACT

The entire plant including the root of *Millettia aboensis* Hook F. (Fabaceae) has been used in Nigerian folkloric medicine for treatment of veneral diseases, acute malaria, ulcer and liver disorder. The aim of this research was to evaluate the hepatoprotective effect of aqueous and ethanol root extracts of *Millettia aboensis* (AREMA and EREMA) on carbon tetrachloride-induced liver damage in rats. The extracts (AREMA and EREMA) were studied for their hepatoprotective effect on carbon tetrachloride-induced acute liver damage on Wistar albino rats. The degree of protection was measured using biochemical parameters such as serum enzymes and bilirubin levels. Further, the effects of both extracts on histopathological changes in liver were studied and compared with Liv-52®, a standard hepatoprotective agent. The extracts (AREMA and EREMA) at a dose level of 215 mg/kg and 431 mg/kg produced significant ($P<0.05$) hepatoprotection by dose-dependently decreasing the activity of serum enzymes and bilirubin. While hepatoprotective effect of AREMA was less than that of EREMA, at all dose levels, hepatoprotective effect of the latter (431 mg/kg) was comparable to that of Liv-52® (1 mL/kg). The hepatoprotective effect was confirmed by histopathological examination of the liver tissue of control and treated animals. This study has shown that *Millettia aboensis* root possesses hepatoprotective activity, which resides more in ethanol than aqueous extract. Further experiments are underway to identify the active constituent(s) responsible for the observed hepatoprotective effect and the mechanism(s) of action.

INTRODUCTION

The liver plays a crucial role in regulating various physiological processes in the body such as metabolism, secretion and storage. It generates reactive oxygen species that induce oxidative tissue damage. These radicals react with cell membranes and thus induce lipid peroxidation or cause inflammation, and are important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer. A major body protective or defence mechanism is the antioxidant enzymes, which convert active oxygen molecules into non-toxic compounds.[1] In addition to strengthening the in-built protective or defence mechanisms exogenous administration of antioxidant(s) may be useful in protecting various organs in the body.[2]

Author for Correspondence:
Email: chimafrankduff@yahoo.com

Liver diseases remain serious health problems and are caused among others by various chemicals, drugs, alcohol and toxins. Orthodox medicine does not have suitable answers for many disease conditions including liver disorders, asthma, cardiovascular disorders etc.[3] Presently only few allopathic hepatoprotective drugs with significant action like silymarin and those from natural sources are available for the treatment of serious liver disorders even after overwhelming advances have been made in medical science.[4-6] Herbal drugs play a role in the management of various liver disorders most of which speed up the natural healing processes of the liver.

Recently, a lot of work has been carried out with the intention of validating scientifically the efficacy of hepatoprotective herbal remedies used in folkloric medicine, particularly plant drugs and plant-derived polyherbal formulations with hepatoprotective activities.[7-

12] In order to investigate the efficiency of hepato-protective and antioxidant substances, various methods of free radical scavenging such as reduced glutathione, glutathione peroxidase and S-transferase, lipid peroxidation, catalase peroxidation, superoxide dismutase or *in vitro* techniques such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging models are employed. Hepatic damage is usually done using such drugs as paracetamol, isoniazid and rifampicin or chemicals such as carbon tetrachloride.[2] Carbon tetrachloride (CCl_4) is a well known hepatotoxin and exposure to this chemical is known to induce oxidative stress and cause liver injury by the formation of free radicals.[13] It has been established that metabolism of CCl_4 involves the production of free radicals through its activation by drug-metabolizing enzymes located in the endoplasmic reticulum.[14]

Millettia aboensis (Family Fabaceae), also called 'uturuekpa'in South Eastern Nigeria, is a small tree of about thirty feet high and up to two feet in girth but usually 12 metres high with reddish-brown pubescence on the petioles, branches, inflorescence and fruits. [15] The plant which is found commonly in low land rain forest, has conspicuously rusty-hairy leaves and handsome purple flowers in erect terminal racemes at branches.[16] Almost all parts of the plant possess medicinal properties. The leaf is used by traditional herbalists for general healing including ulcer healing and as laxatives while the root is used in treating gastro intestinal disturbances and liver diseases. Also the leaf, stem and roots mixed with other plant materials (herbs) is used in Nigerian folkloric medicine to cure veneral diseases such as gonorrhoea, syphilis etc. [15-18]

Liv-52®, a herbal medicine commonly used to treat liver ailments, was developed in the mid 1950s by the Himalaya Herbal Healthcare group of India and it contains the following: Capers (*Capparis spinosa*, 65 mg), a hepatic stimulant and protector which improves the functional efficiency of the liver; Wild Chicory (*Cichorium intybus*, 65 mg), a powerful hepatic stimulant which increases bile secretion and acts on liver glycogen to promote digestion; Black Nightshade (*Solanum nigrum*, 32 mg), which promotes liver and kidney health and has shown hepatoprotective activity in cases of toxicity induced by drugs and chemicals; Arjuna (*Terminalia arjua*, 32 mg), a tonic for heart and liver and regulates hepatic cholesterol biosynthesis; Negro Coffee (*Cassia occidentalis*, 16 mg), a digestive and hepatic tonic; Yarrow (*Achillea millefolium*, 16 mg), a stimulant tonic for liver; Tamarisk (*Tamarix gallica*, 16 mg) a hepatic stimulant which also provides digestive support.[19] It possesses both antioxidant and enzyme-regulating properties and also works as a cholesterol regulator. Liv-52® maintains the functional effectiveness of the liver by protecting the hepatic parenchyma. In many parts of the world, this wonder drug is used as a preventive measure for risks of contracting life-threatening liver diseases. Alcoholics take it as a vitamin to maintain a healthy liver amid load of toxins they take each day. Patients suffering from liver diseases also find Liv 52® useful in improving their health condition.[19]

To the best of our knowledge, the hepatoprotective activity of roots of *Millettia aboensis* has not been clinically evaluated so far. A potent and safe drug is needed for the treatment of hepatic diseases. Thus, the primary objective of this investigation was to examine the phytochemical constituents, acute toxicity and hepatoprotective activity of ethanolic and aqueous root extracts of *Millettia aboensis* (EREMA and AREMA) using carbon tetrachloride intoxicated rats as experimental model. Liv 52® was used as a standard drug for comparison in the study.

MATERIALS AND METHODS

Plant materials and preaparation of extracts

The roots of *Millettia aboensis* were harvested from Ehandiagu in June, 2010 in Nsukka Local Governement Area of Enugu State, Nigeria. Their botanical identities were determined and authenticated by Mr. A. Ozioko, a taxonomist with the International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka. The voucher specimen was deposited at the centre for future references. The root of *Millettia aboensis* was shade-dried and pulverized to coarse powder using an electrically operated mill. A 0.5 kg of shade-dried pulverized roots was extracted with 2 L of ethanol (90 % v/v) using a soxhlet apparatus and concentrated under vacuum. The aqueous extraction was done by placing 50 g of the powder in a 250 mL percolator which was initially plugged with a cotton wool at the base . A volume of 150 mL of distilled water was added and thoroughly mixed. The mixture was then allowed to macerate, filtered and concentrated under vacuum.

Determination of yield of the extracts

The percentage yield of the extracts (AREMA and EREMA) were determined by weighing the coarse plant root before extraction and after concentration and calculated using the formula

$$Yield\ (\%) = \frac{W_E}{W_P} \times 100 \qquad \ldots\ldots\ldots (1)$$

Where W_E = Weight of extract (g), W_P = Weight of drug powder (g).

Preliminary phytochemical analysis

Standard procedures [17, 20] were followed in the tests for the phytochemical constituents of AREMA and EREMA. The following constituents were tested for alkaloids, resins, reducing sugars, acidic compounds, fats and oil, steroids, terpenoids, glycosides, flavonoids, tannins, carbohydrates, saponins, proteins and amino acids.

Experimental animals

Wistar albino rats of either sex weighing between 66 g and 153 g and mice weighing between 25 g and 30 g procured from the animal house of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka were used. They were housed in well ventilated stainless-steel cages at room temperature (25 ^{0}C) in hygienic conditions under natural light and dark schedule and were fed on standard laboratory diet. Food and water were given ad libitium. Permission for the use of animal and animal protocol was obtained from the Ethics Committee of the University of Nigeria, Nsukka. The animal studies were carried out in accordance with Ethical Guidelines of Animal Care and Use Committee (Research Ethics Committee) of University of Nigeria, Nsukka, following the 18th WMA General Assembly Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Drugs and chemicals

Liv-52® was procured from the Himalaya Herbal Healthcare group of India. SGOT, SGPT, ALP, Total Bilirubin and Conjugated Bilirubin kits were procured from Span Diagnostics, Surat, India. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was obtained from Sisco research laboratories, Mumbai, India. All other reagents used for the experiments were of high analytical grade.

Acute toxicity studies

The safety of the extracts (AREMA and EREMA) to the mice was evaluated by determining the oral acute toxicity (LD_{50}) using a modified Lorke's method. [21] The treatment and control animals were weighed and marked appropriately. The acute toxicity test of the plant extracts was tested on mice using six doses (10, 100, 1000, 1600, 2900 and 5000 mg/kg body weight) administered orally for each plant extract. The study was carried out in two phases. In the phase one of the study, nine mice were randomized into three groups of three mice each and were given 10, 100 and 1000 mg/kg b. wt. of each of the extract orally. The mice were observed for paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first 4 h and subsequently daily for 7 days. In the second phase of the study, another fresh set of nine mice were randomized into three groups of three mice each and were given 1600, 2900 and 5000 mg/kg b. wt. of each of the extract orally based on the result of the first phase. These were observed for signs of toxicity and mortality for the first critical 4 h and thereafter daily for 7 days. The LD_{50} was then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose, i.e. the geometric mean of the consecutive doses for which 0 and 100 % survival rates were recorded in the second phase. The oral median lethal dose was calculated using the formula:

LD_{50} = √Minimum toxic dose x Maximum tolerated dose ……. (2)

Experimental design for hepatoprotective activity (Acute model)

The hepatoprotective activity of the extracts (AREMA and EREMA) was tested using carbon tetrachloride (CCl_4) model. Liv-52® (1.0 mL/kg b.wt.) was used as a reference standard drug in this study.[12] Liv-52®, a herbal medicine commonly used to treat liver ailments, was developed in the mid 1950s by the Himalaya Herbal Healthcare group of India, and has been introduced fairly recently as a hepatoprotective agent. [19] The rats were divided randomly into seven groups of six animals per group according to the following protocol.

Treatment protocol

GROUP I: Normal control (n=6, the animals were given 1 mL/kg distilled water only).

GROUP II: Hepatotoxic control (n=6, the animals were given 0.7 mL/kg CCl_4).

GROUP III: Treatment control (n=6, the animals were given 0.7 mL/kg CCl_4 and 1 mL/kg Liv-52®)
GROUP IV: Treatment group (n=6, the animals were given 0.7 mL/kg CCl_4 and 215 mg/kg of aqueous root extract of *Millettia aboensis*, AREMA).
GROUP V: Treatment group (n=6, the animals were given 0.7 mL/kg CCl_4 and 215 mg/kg of ethanol root extract of *Millettia aboensis*, EREMA).
GROUP VI: Treatment group (n=6, the animals were given 0.7 mL/kg CCl_4 and 431 mg/kg of aqueous root extract of *Millettia aboensis*, AREMA).
GROUP VII: Treatment group (n=6, the animals were given 0.7 mL/kg CCl_4 and 431 mg/kg of ethanol root extract of *Millettia aboensis*, EREMA).

Rats were treated as per the treatment protocol for a period of nine days. While CCl_4 was administered intraperitoneally, the drugs were given orally. The carbon tetrachloride was diluted with liquid paraffin in the ratio of 1:1 before administration. Food was withdrawn 12 h before carbon tetrachloride administration to enhance the acute liver damage in the animals, which were sacrificed 24 h after the administration of CCl_4.

Assessment of hepatoprotective activity

The hepatoprotective activity of the extracts (AREMA and EREMA) was determined biochemically by using carbon tetrachloride-induced hepatotoxic rat model.[2] After nine days of drug treatment, the animals were dissected under ether anesthesia. Blood from each rat was withdrawn from carotid artery at the neck and collected in previously labeled falcon tubes and allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30 ^{o}C for 15 min and utilized for the estimation of some biochemical parameters namely alanine aminotransferase (ALT/ SGPT), aspartate aminotransferase (AST/SGOT), alkaline phosphatase (ALP), total bilirubin and conjugated bilirubin using standard kits. [1, 22-25]

Serum hepatospecific markers

Activities of serum glutamate oxaloacetate transaminase (SGOT/AST) and serum glutamate pyruvate transaminase (SGPT/ALT) were estimated by the method of Reitman and Frankel. [22] Briefly, 0.05 mL of serum with 0.25 mL of substrate (aspartate and α-ketoglutarate for SGOT; alanine and α - keto glutarate for SGPT, in phosphate buffer pH 7.4) was incubated for 1 h in case of SGOT and 30 min for SGPT. A 0.25 mL of DNPH solution was added to quench the reaction and kept for 20 min in room temperature. After incubation, 1 mL of 0.4 N NaOH was added and the absorbance was read at 505 nm in a UV-Vis spectrophotometer (UNICO 2102 PC UV-Vis Spectrophotometer, USA). Activities were expressed as IU/L. Based on the method of King and Armstrong,[23] alkaline phosphatase activity was assayed using disodium phenyl phosphate as substrate. The colour developed was read at 510 nm in a UV-Vis spectrophotometer (UNICO 2102 PC UV-Vis Spectrophotometer, USA) after 10 min. Activities of ALP was expressed as IU/L. Total bilirubin level was estimated based on the method of Malloy and Evelyn.[24] Diazotised sulphonilic acid (0.25 mL) reacts with bilirubin in diluted serum (0.1 mL serum + 0.9 mL distilled water) and forms purple coloured azobilirubin, which was measured at 540 nm in a UV-Vis spectrophotometer. Activities of total bilirubin were expressed as mg/dL. The level of conjugated bilirubin was estimated based on the method of Gornall et al.[25] Biuret reagent (1 mL) reacts with serum (10 µL) and the colour developed was read at 578 nm in UV-Vis spectrophotometer (UNICO 2102 PC UV-Vis Spectrophotometer, USA). Activities of conjugated bilirubin were expressed as mg/dL.

Histopathological studies

One animal from each group was utilized for this purpose. Small pieces of liver tissues in each group were collected in 10 % neutral buffered formalin for proper fixation for 24 h. These tissues were processed and embedded in paraffin wax. Sections of 5- 6 µm in thickness were cut and stained with hematoxylin and eosin (H & E). These sections were examined photomicroscopically for necrosis, steatosis and fatty changes of hepatic cell. [1, 12]

Statistical analysis

The experimental results were expressed as the Mean ± SEM for six animals in each group. Difference between means were determined statistically using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (DMCT). P value of < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

The present study was undertaken to evaluate the hepatoprotective effect of *Milletia aboensis*, a commonly used plant in Nigerian traditional medicine for treatment of liver diseases. [15-18] Ethnopharmacological data has been one of the useful ways for the discovery of biologically active compounds from plants.[26] Ethnopharmacological use of plants could form the basis for phytochemical and phytopharmacological investigation. Extraction yields (w/w) were 49 % and 46 % for AREMA and EREMA respectively in terms of dry weight. Thus AREMA had higher yield than EREMA. Preliminary phytochemical tests of the extracts of *Millettia aboensis* revealed the presence of phytoconsituents as presented in Table 1. It could be seen from Table 1 that AREMA contains high amounts of saponins, resins, proteins, amino acids and carbohydrate while EREMA had high amounts of flavonoids, alkaloids, steroids and terpenoids. The two extracts had equal amounts of glycosides and reducing sugar, but tannins, acidic compounds and fats and oil were absent in the extracts.

Table 1: Phytochemical constituents of EREMA and AREMA.

Constituent	Observation	
	EREMA	*AREMA*
Alkaloids	*+++*	*+*
Resins	*-*	*+++*
Reducing sugars	*+*	*+*
Acidic compounds	*-*	*-*
Fats and Oil	*-*	*-*
Steroids	*++*	*+*
Terpenoids	*++*	*+*
Glycosides	*+++*	*+++*
Flavonoids	*+++*	*+*
Tannins	*-*	*-*
Carbohydrates	*++*	*+++*
Saponins	*+*	*++++*
Proteins and amino acids	*+*	*+++*

KEYS: +++ = relatively high abundance of compound,++ = moderate abundance of compound,+ = relative low presence of compound, ND = not detected, EREMA = ethanol extract of Millettia aboensis, AREMA = aqueous extract of Millettia aboensis.

In other words, phytochemistry of the extracts showed that AREMA contains high amounts of saponins, resins, proteins, amino acid and carbohydrates while EREMA had high amounts of flavonoids, alkaloids, steroids and terpenoids; both extracts had equal amounts of glycosides and reducing sugars. Phytoconstituents like flavonoids, triterpenoids, saponins and alkaloids are known to possess hepatoprotective activity. [27-29] A large number of studies have suggested that flavonoids commonly function as antioxidants and may protect plants against oxidative stress caused by suboptimal environmental conditions.[30, 31] Perhaps the hepatoprotective effect of AREMA and EREMA could be attributed to the presence of these phytoconstituents present in the plant extracts. In addition, liver protective herbal drugs have been shown to contain a variety of chemical constituents like phenols, coumarins, ligands, essential oil, monoterpenes, carotenoids, glycosides, organic acids, lipids, xanthenes, etc.[32-48] Thus it is possible that other secondary metabolites of the plant as observed in the present study may be responsible for the hepatoprotective activity of the plant.

The LD_{50} of AREMA and EREMA was calculated to be 2154 mg/kg. The extracts were found to be safe in the dose used and there was no mortality up to a dose of 2154 mg/kg, b.w. for both extracts. Hence, 1/5th and 1/10th of the lethal dose i.e. 215 and 431 mg/kg b.w. p.o. of both extracts were selected for further evaluation. Behavioural signs of toxicity observed in mice given 5000 mg/kg and above include drowsiness, weakness, reduced activity in treated mice and paw licking. The necropsy and autopsy were performed and signs of toxicity included diarrhea and congested lungs with focal areas of necrosis. The results of acute toxicity test indicated that the LD_{50} of AREMA and EREMA was 2154 mg/kg. This implies that doses below this could be safe while doses above it could be detrimental to the human system.[21] Hence, 1/5th and 1/10th of the lethal dose i.e. 215 and 431 mg/kg b.w. p.o. of both extracts were selected for the hepatotoxicity test (acute model). The acute toxicity results indicated that the extracts were practically non-toxic acutely[21] and thus provided a guide in the choice of doses for further *in vivo* studies. This high safety profile might have contributed to the widespread use of *Millettia aboensis* in different ethno-therapeutic interventions, particularly as a hepotoprotective.

The effects of AREMA and EREMA on ALT, AST, ALP, total bilirubin and conjugated bilirubin in CCl_4–induced liver damage in rats are summarized in Table 2.

Table 2: Effect of EREMA, AREMA and Liv-52® on serum enzymes, total bilirubin and conjugated bilirubin on carbon tetrachloride-induced liver damage in rats.

Groups and treatment	Dose	SGOT (IU/L)	SGTP (IU/L)	SALP (IU/L)	Total Bilirubin (mg/dL)	Conjugated Bilirubin (mg/dL)
Group I-Normal (Distilled water)	1 mL/kg	50.00±2.03	25.17±0.99	110.78±4.65	17.92 ±0.87	7.24±0.31
Group II-CCl_4 control	0.7 mL/kg	110.52±2.99	71.04±1.63	340.98±7.54	45.90±1.42	15.87± 0.88
Group III-LIV-52® + CCl_4	1 mL/kg + 0.7 mL/kg	84.76±3.09	58.11±2.44	220.75±7.16	23.84±1.99	9.01±0.22
Group IV-AREMA + CCl_4	215 mg/kg + 0.7 mL/kg	105.33±4.88	65.00±2.15	240.81±8.73	25.64±1.00	13.07±0.99
Group V-EREMA + CCl_4	215 mg/kg + 0.7 mL/kg	100.76±2.48	59.18±1.97	200.65±8.91	20.97 ±1.44	11.53±0.78
Group VI-AREMA + CCl_4	431 mg/kg + 0.7 mL/kg	95.61±2.07	55.04±1.98	200.53±4.75	18.26 ± 1.90	8.47 ±0.13
Group VII-EREMA + CCl_4	431 mg/kg + 0.7 mL/kg	90.56±2.31	50.10±1.16	190.68±3.97	17.55 ± 1.00	6.92 ±0.44

Values are Mean ± SEM; n = 6 animals in each group; EREMA = ethanol extract of Millettia aboensis, AREMA = aqueous extract of Millettia aboensis.

Administration of CCl_4 (0.7 mL/kg i.p., body weight) resulted in a significant ($P<0.05$) elevation of hepatospecific serum markers SGOT, SGPT, SALP, total bilirubin and conjugated bilirubin in CCl_4–treated group (Group II), in comparison with the normal control (Group I). On administration of the test samples (AREMA and EREMA i.e. Groups IV to VI, Table 2) and Liv-52® at the dose of 1 mL/kg (Group III, Table 2) the level of these enzymes were found to decrease towards normalcy. The extracts showed dose-dependent liver protective activity. Comparative analysis of the effect of various extracts on ALT, AST and ALP levels revealed that EREMA and AREMA (431 mg/kg body weight) showed protection against the hepatoxins. In other words, EREMA and AREMA at a dose level of 431 mg/kg and LIV-52® (1 mL/kg) produced a significant reduction ($p<0.05$) in the marker enzymes (AST, ALT, ALP) as well as total and conjugated bilirubin. Lower dose level of both extracts (215 mg/kg) also showed significant reduction in marker enzyme levels but the effect was less compared to that produced by 431 mg/kg. Similarly, the results showed a dose-dependent decrease in total bilirubin and conjugated bilirubin levels in all treated rats. At all dose levels EREMA caused greater decrease in total bilirubin when compared to AREMA. The level of conjugated bilirubin in EREMA-treated rats at a dose level of 431 mg/kg was comparable with that of the control (LIV-52®). Furthermore, administration of CCl_4 produced moderately significant increase in the liver weights. Higher doses of EREMA and AREMA, LIV-52®, and lower dose of EREMA produced significant decrease in the liver weights.

In this study, the administration of CCl_4 increases the level of AST, ALT, ALP, total bilirubin and conjugated bilirubin. Carbon tetrachloride-induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effects of medicinal plants and drugs.[38, 39] The hepatotoxic effects of CCl_4 are largely due to generation of free radicals.[49] Hepatic function could be evaluated by estimating the activities of serum biomarker enzymes like ALT, AST, ALP as well as total and conjugated bilirubin. When there is hepatopathy, these enzymes leak into the blood stream at a level commensurate with the extent of liver damage. [50, 51] Treatment of experimental animals with carbon tetrachloride caused induction of hepatotoxicity by metabolic activation. Carbon tetrachloride is metabolically activated by the cytochrome P_{450} enzyme system in the endoplasmic reticulum to form trichloromethyl free radical ($.CCl_3$) which combines with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation. [52, 53] These result in changes in structures of the endoplasmic reticulum and other membranes, loss of metabolic enzyme activation, reduction

of protein synthesis and loss of glucose-6-phosphatase activation, leading to liver injury. These may explain what happened in the CCl_4-treated groups (Groups II-VII) in the present study, consistent with a lot of previous investigations.[2, 38-44]

Millettia aboensis treated animals groups (Groups IV to VII) showed hepatoprotection against the injurious effects of CCl_4 resulting in the hindrance of formation of hepatotoxic free radicals. The reduction of the elevated serum biomarker enzymes by AREMA and EREMA may be due to the prevention of the leakage of intracellular enzymes by their membrane stabilizing activity, consistent with previous reports.[32-34] Overall, the results of this study showed that EREMA and AREMA at 215 and 431 mg/kg dose levels offered significant dose-dependent protection against CCl_4-induced hepatic damage in rats, but the effect produced by EREMA (421 mg/kg) was almost similar to that produced by Liv-52® (1 mL/kg p.o.), a standard hepatoprotective agent.[12, 19] Although the mechanism behind the hepatoprotection by EREMA and AREMA is still unknown, it may be related to the stimulation of antioxidant defence mechanism (due to the presence of flavonoids) against the free radicals generated by CCl_4 or by modulating the effects of the cytochrome P_{450} enzyme which is responsible for the production of the toxic free radicals from CCl_4.[52, 53] In addition, accumulation of bilirubin (one of the most useful clinical clues to the severity of necrosis) gives a measure of binding, conjugation and excretory capacity of hepatocytes.[49] Attenuation of total and conjugated bilirubin after treatment with AREMA and EREMA in liver damage induced by CCl_4 indicated the effectiveness of the extracts in maintaining normal functional hepatic status. Thus, reduction of total and conjugated bilirubin levels by AREMA and EREMA is suggestive of an early improvement in the secretory mechanism of the hepatic cells, in agreement with previous studies.[50, 51] The efficacy of any hepatoprotective drug is dependent on its capacity in either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by a hepatotoxin.[2] Both Liv-52®, AREMA and EREMA decreased CCl_4-induced elevated serum biomarker enzyme and bilirubin levels indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells. In other words, the stabilization of total and conjugated bilirubin, of SGPT, SGOT, and ALP levels by AREMA and EREMA is a clear indication of the improvement of the functional status of the liver cells.

Histopathological results indicate that the rats treated with vehicle control (Group I) showed normal structure and visible portal tract. Histopathological examination of the liver tissues from CCl_4-intoxicated animals (Group II) showed profound inflammation and congestion especially in the sinusoids. Hydropic degeneration and steatosis in the periportal region was also observed. Pretreatment of animals with Liv-52® (Group III) and 431 mg/kg each of EREMA and AREMA (Groups VI and VII) showed reduction in inflammation of the skin and significantly prevented degeneration of hepatocytes.

The hepatoprotective findings were further corroborated with histopathological studies. The histopathological examination clearly reveals that the hepatic cells, central vein, and portal triad are almost normal in AREMA and EREMA – treated groups (Groups VI and VII) as well as in the reference (Liv-52®) group (Group III) in contrast to group which received CCl_4 (Group II). Thus, AREMA and EREMA possessed effective hepatoprotective effect, having ameliorated almost to normalcy the damage caused by CCl_4 to hepatic function.

CONCLUSIONS

In conclusion, the present investigation has shown that *Millettia aboensis* ethanolic and aqueous root extracts (EREMA and AREMA) exerted significant dose-dependent protection against CCl_4-induced hepatotoxicity by the potential of the extracts to ameliorate the lipid peroxidation through the free radicals scavenging activity, which enhanced the levels of antioxidant defense system. This justifies the continuous folkloric use of *Millettia aboensis* root as a remedy for liver disorder in Nigeria. Higher dose of EREMA (431 mg/kg) has shown liver protective activity that is comparable to the reference drug Liv-52®. The hepatoprotection may be attributed to the antioxidant principles present in the plant, particularly flavonoids. However, further identification and elucidation of the structures of the actual constituents responsible for this activity is underway.

ACKNOWLEDGEMENT

The authors wish to thank Professor Ihedioha of the Department of Pathology, Faculty of

Veterinary Medicine, University of Nigeria, Nsukka for assisting in the histopathological studies.

REFERENCES

[1] Dash DK, Yerigar VC, Nayak SS, Ghosh T, Rajalingam D, Sengupta P. Evaluation of hepatoprotective and antioxidant activity of *Ichnocarpus frutescens* (Linn.) R. Br. on paracetamol induced hepatotoxicity in rats. Trop J Pharm Res, 2007; 6: 755-765.

[2] Osadebe PO, Okoye FBC, Uzor PF, Nnamani NR, Adiele IE, Obiano NC. Phytochemical analysis, hepatoprotective and antioxidant activity of *Alchornea cordifolia* methanol leaf extract on carbon tetrachloride-induced hepatic damage in rats. Asian Pac J Trop Med, 2012; 5:289-293.

[3] Nanjaian M. Herbal drug development for liver disorders and hyperlipidemia. 2012. Available online at: http://www.pharmainfo.net/reviews/herbal-drug-development-liver-disorders-and-hyperlipidemia. Assessed on 10/10/12.

[4] Khatri A, Garg A, Agrawal S. Evaluation of hepatoprotective activity of aerial parts of *Tephrosia purpurea* L. and stem bark of *Tecomella undulata.* J Ethnopharmacol, 2009; 122:1-5.

[5] Pramyothin P, Chirdchupunsare H, Rungsipipat A, Chaichantipyuth C. Hepatoprotective activity of *Thunbergia laurifolia* Linn extract in rats treated with ethanol: *In vitro* and *in vivo* studies. J Ethnopharmacol, 2005; 102:408–411.

[6] Kumar P, Rao D, Setty R. Antioxidant and hepatoprotective activity of tubers of *Momordica tuberose* Cogn. against CCL_4 induced liner injury in rats. Indian J Exp Biol, 2008; 46:510-513.

[7] Handa SS, Sharma A, Chakraborty KK. Natural products and plants as liver protecting drugs. Fitoter, 1989; 57:307-51.

[8] Hikino H, Kiso Y. Natural products for liver diseases in economic and medicinal plant research. Academic press: London, 1988, 23-31.

[9] Sharma A, Shing RT, Sehgal V, Handa SS. Antihepatotoxic activity of some plants used in herbal formulations. Fitoter 1991; 62:131-8.

[10] Evans WC. An overview of drugs with anithepatotoxic and oral hypoglycaemic activities. In: Trease and Evans Pharmacognosy. Sanders Company Ltd: WD, 1996.

[11] Jamal AK, Yaacob WA, Laily BD. A chemical study on *Phyllanthus columnaris*. Eur J Sci Res, 2009; 28:76-81.

[12] Pramod JH, Pournima AS, Siddhalingesh GP, Yuvaraj DM, Ajay SK. Hepatoprotective activity of *Amorphophallus paeonoiifolius* tubers against paracetamol-induced liver damage in rats. Asian Pac J Trop Biomed, 2012; 2:S238-S242.

[13] Rubinstein D. Epinephrine release and liver glycogen levels after carbon tetrachloride administration. Am J Physiol, 1962; 203:1033-1037.

[14] Suja SR, Latha PG, Pushpangadan P, Rajasekharan S. Aphrodisiac property of *Helminthostachys zeylanica* in mice. J Trop Med Plants, 2002; 3:191-195.

[15] Adonu CC, Ugwueze ME, Ozioko CA. Phytochemical analyses of ethanol and water extracts of *Millettia aboensis*, *Cuscuta reflexa, Daniella oliveri* and *Synclisia scabrida*. Int J Pharm Biol Sci, 2013; 3:64-69.

[16] Uba BC. "Millettia". In Flora of Parkistan, page 51. Published by Science Press (Beijing) and Missouri Botanical Garden Press, 2010. Available online at //http//zzz.efloras.org. Assessed on 10/06/2010.

[17] Trease GE, Evans WC. Pharmacognosy, 13th Edition. ELBBS Oxford University Press, London, UK,1989, 245-263.

[18] World Health Organization (WHO). Guidelines for the assessment of herbal medicines. Programme on traditional Medicines, Geneva, 1991, pp.34-36.

[19] Arts ICW, Hollman PCH. Polyphenols and disease risk in epidemiologic studies. J Clin Nutr, 2005; 81:317-325.

[20] Harbone JB. Phytochemical methods, Third Edition, Thompson Science, 2-6 Boundary Row, London,UK, 1998, pp.107-150.

[21] Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol, 1983; 54:275-287.

[22] Retimen S, Frankel SA. Colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvate transaminases. Am J Clin Pathol, 1957; 28:56-63.

[23] King EJ, Armstrong AR. A convenient method for determining of serum and bile phosphatase activity. J Canad Med Assoc, 1934; 31:376-381.

[24] Malloy HT, Evelyn KA. The determination of bilirubin with the photometric colorimeter. J Biol Chem, 1937; 119: 481-490.

[25] Gornall AG, Bardwill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem, 1949; 177: 751-756.

[26] Kuria KAM, De Coster S, Muriuki G, Masengo W, Kibwage I, Hoogmartens J, et al. Antimalarial activity of *Ajuga remota* Benth (Labiatae) and *Caesalpinia volkensii* Harms (Caesalpiniaceae): *in vitro* confirmation of ethnopharmacological use. J Ethnopharmacol, 2001; 74:141-148.

[27] Pattanayak S, Nayak SS, Panda DP, Dinda SC, Shende V, Jadav A. Hepatoprotective activity of crude flavonoids extract of *Cajanus scarabaeoides* (L) in paracetamol intoxicated albino rats. Asian J Pharm Biol Res, 2011; 1(1): 22-27.

[28] Maheswari C, Maryammal R, Venkatanarayanan R. Hepatoprotective activity of *Orthosiphon stamineus* on liver damage caused by paracetamol in rats. Jordan J Bio Sci, 2008; 1(3): 105-108.

[29] Singh D, Gupta RS. (2011). Hepatoprotective activity of methanol extract of *Tecomella undulate* against alcohol and paracetamol-induced hepatotoxicity in rats. Life Sci Med Res, 2011; 3:87-95.

[30] Salah N, Miller NJ, Pagange G, Tburg L, Bolwell GP, Rice E, Evans C. Polyphenolic flavonoids as scavengers of aqueous phase radical as chain breaking antioxidant. Arch Biochem Bioph, 1995; 2:339-346.

[31] Okwu DE. Phytochemicals and vitamin content of indigenous species of South East Nigeria. J Sustain Agric Environ, 2004; 6:30-37.

[32] Ahsan MR, Islam KM, Bulbul IJ, Musaddik MA, Haque E. Hepatoprotective activity of methanol extract of some medicinal plants against carbon tetrachloride-induced hepatotoxicity in rats. Euro J Sci Res, 2009; 37: 302-310.

[33] Nkosi CZ, Opoku AR, Terblanche SE. Effect of pumpkin seed (*Cucurbita pepo*) protein isolate on the activity levels of certain plasma enzymes in CCl_4-induced liver injury in low protein fed rats. Phyther Res, 2005; 19: 341-345.

[34] Palanivel MG, Rajkapoor B, Senthil KR, Einstein JW, Kumar EP, Rupesh KM. Hepatoprotective and antioxidant effect of *Pisonia aculeata* L. against CCl_4-induced hepatic damage in rats. Sci Pharm, 2008; 76: 203-215.

[35] Singh K, Singh N, Chandy A, Manigauha A. *In vivo* antioxidant and hepatoprotective activity of methanolic extracts of *Daucus carota* seeds in experimental animals. Asian Pac J Trop Biomed, 2012; 2: 385-388.

[36] Jain M, Kapadia R, Jadeja RN, Thounaojam MC, Devkar RV, Mishra SH. Cytotoxicity evaluation and hepatoprotective potential of bioassay guided fractions from *Feronia limmonia* Linn leaf. Asian Pac J Trop Biomed, 2011; 1:443-447.

[37] Ravikumar S, Gnanadesigan M. Hepatoprotective and antioxidant activity of a mangrove plant *Lumnitzera racemosa*. Asian Pac J Trop Biomed, 2011; 1:348-352.

[38] Sengupta M, Dutta Sharma G, Chakraborty B. Hepatoprotective and immunomodulatory properties of aqueous extract of *Curcuma longa* in carbon tetrachloride intoxicated Swiss albino mice. Asian Pac J Trop Biomed, 2011; 1: 193-199.

[39] Shi J, Aisaka K, Ikawa Y, Wake K. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. Am J Pathol, 1998; 153:515-25.

[40] Sun F, Hamagawa E, Tsutsui C, Ono Y, Ogiri Y, Kojo S. Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver. Biochim Biophys Acta, 2001; 1535:186-91.

[41] Zalatnai A, Sarosi I, Rot A, Lapis K. Inhibitory and promoting effects of carbon tetrachloride- induced liver cirrhosis on the diethylnitrosamine hepatocarcinogenesis in rats. Cancer Lett, 1991; 57:67-73.

[42] Mackinnon M, Clayton C, Plummer J, Ahern M, Cmielewski P, Isley A. Iron overload facilitates hepatic fibrosis in the rat alcohol/low-dose carbon tetrachloride model. Hepatology, 1995; 21:1083-1088.

[43] Natsume M, Tsuji H, Harada A, Akiyama M, Yano T, Ishikura H. Attenuated liver fibrosis and depressed serum albumin levels in carbon tetrachloride-treated IL-6-deficient mice. J Leukoc Biol, 1999; 66:601-8.

[44] Teocharis SE, Margeli AP, Skaltsas SD, Spiliopoulou CA, Koutselinis AS. Induction of metallothionein in the liver of carbon tetrachloride intoxicated rats: an

immunohistochemical study. Toxicology, 2001; 161:129-38.

[45] Tran QI, Adnyana IK, Tezuka Y, Nagaoka T, Tran QK, Kadota S. Triterpenesaponins from *Vietnamese ginseng* (*Panax vietnamensis*) and their hepatocyte protective activity. J Nat Prod, 2011; 64:456-61.

[46] Vijayan P, Prashanth H C, Dhanraj S A, Badami S, Suresh B. Hepatoprotective effect of total alkaloid fraction of *Solanum pseudocapsicum* leaves. Pharm Biol, 2003; 41:443-8.

[47] Tattini M, Galardi C, Pinelli P, Massai R, Remorini D, Agati G. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol, 2004; 163:547-61.

[48] Gould KS, Lister C. Flavonoid functions in plants. In: Andersen OM, Markham KR, eds. *Flavonoids: chemistry, biochemistry, and applications*. CRC Press: 2006, Boca Raton, 397-441.

[49] Srivastara SP, Chen NO, Holtzman JL. The *in vitro* NADPH-dependent inhibition by CCl_4 of ATP-dependent calcium uptake of hepatic microsomes from male rats in the status on the mechanism of inactivation of the hepatic microsomal calcium pump of the CCl_3 radical. J Biol Chem, 1990; 265:8392-8399.

[50] Lim JR, Faught PR, Chalasani NP, Molleston JP. Severe liver injury after initiating therapy with atomoxetine in two children. J Pediatric, 2006; 148:831-834.

[51] Kaplowitz N, Shinohara MJC. Endoplasmic reticulum stress and liver injury. J Semin Liver Dis, 2007; 27:367-372.

[52] Mujunmaddar AK, Upadhyne AS, Pradhan AM. Effects of *Azadirachta indica* leaf extract in CCl_4 induced hepatic damage in albino rats. Ind J Pharm, 1998; 24:571-578.

[53] Okpoku AR, Ndovel IM, Terbalanche SE, Hutching AH. *In vitro* hepatoprotective effects of *Rhoicissus tridendate* sub species Cuneifolia, a traditional Zulu medical plant, against CCl_4 induced acute liver injury in rats. South Africa J Bot, 2007; 73:372-377.