Trop J Nat Prod Res, November 2018; 2(11):482-488 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Musanga cecropioides (Cecropiaceae) Attenuates Carbon Tetrachloride-Induced Non-Alcoholic Fatty Liver Disease in Wistar Rats

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ARTICLE INFO

Article history:
Received 07 November 2018
Revised 21 November 2018
Accepted 24 November 2018
Published online 27 November 2018

ABSTRACT

Musanga cecropioides (M. cecropioides) is a medicinal plant used traditionally in Africa to induce labour, reduce elevated blood pressure, reduce high blood sugar as well as inhibit dysentery. In this study, the *in vivo* hepatoprotective activity of *M. cecropioides* was investigated in carbon tetrachloride (CCl_4)-treated wistar rats. Animals were randomized into 5 groups with group 1 serving as control while animals in groups 2 to 5 were administered CCl_4 (30% v/v with olive oil). Animals in groups 3 and 4 were pretreated with 250 and 500 mg/kg *M. cecropioides*, respectively, whereas group 5 animals (control group) were pretreated with 25 mg/kg silymarin (standard liver protective drug). Following treatment, alterations in biochemical parameters such as serum alanine amino-transferases (ALT), aspartate amino-transferases (AST), alkaline phosphatase (ALP), total protein (TP), Catalase (CAT), Superoxide Dismutase (SOD) and Malondialdehyde (MDA) as well as histopathological changes in the liver of experimental rats were investigated. Findings show increased serum biochemical and antioxidant profiles (AST, ALT, ALP and MDA), reduced TP, CAT and SOD as well as increased fat deposits and inflammatory infiltrates in the liver sections of animals treated with CCl_4. However, following pretreatment with *M. cecropioides* and silymarin, altered biochemical parameters were observed to be retrieving towards normal while the histo-architecture of the liver was markedly improved. These results suggest that *M. cecropioides* could be a potent hepatoprotective agent against CCl_4-induced liver injury and these activities might in part, be attributed to the high total phenolic contents earlier reported to be present in this plant.

Keywords: Carbon-tetrachloride, Hepatotoxicity, Antioxidants, *Musanga cecropioides*.

Introduction

The incidence of non-alcoholic fatty liver disease (NAFLD) is on the rise globally largely due to increased obesity and change in lifestyle among adults and children resulting to accumulation of fat in the liver.[1] NAFLD has also been implicated as a risk factor for cardiovascular diseases which is the most common mortality associated with patients and is increasingly the main reason for liver transplantation lately.[2, 3] The presence of fatty liver or hepatic steatosis marks the onset of the disease and this could advance to steatohepatitis with subsequent inflammation of hepatocytes. Although, it has been reported that about 5 to 20% of patients with fatty liver progresses to non-alcoholic steatohepatitis (NASH) during the disease, but over 10 to 20% may advance to higher-grade fibrosis while less than 5% degenerates to cirrhosis of the liver.[4] NAFLD-induced cirrhosis has also been reported to heighten the progression of liver disease to hepatocellular carcinoma.[5] The parthogenesis of NAFLD remains unclear, however, documented evidence show type 2 diabetes mellitus, insulin resistance accumulation of fatty acids which can alter reactive oxygen species (ROS) has been implicated as risk factors.[6-8]

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Citation: Omoruyi SI and Enogieru AB. *Musanga cecropioides* (Cecropiaceae) Attenuates Carbon Tetrachloride-Induced Non-Alcoholic Fatty Liver Disease in Wistar Rats. Trop J Nat Prod Res. 2018; 2(11):482-488. doi.org/10.26538/tjnpr/v2i11.4

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Also, the exposure of humans to certain toxic environmental chemicals such as the carcinogen, carbon tetrachloride (CCl_4), has been reported to play a role in the induction of NAFLD.[9, 10]

CCl_4 was well known for its use as a dry-cleaning agent, vermicide (kills worms) and refrigerant until its toxicity was established and its use was thereafter limited.[11] CCl_4 is quite stable and is a well-known environmental pollutant which may pollute the air and also contaminate groundwater supplies.[9] CCl_4 is a widely established hepatotoxin and is routinely used in animal models for inducing liver damage.[12-14] CCl_4 induced liver damage is characterized by increased generation of free radicals, build-up of inflammatory cytokines, toxic lipid and protein peroxidation production leading to steatosis, fibrosis and necrosis of hepatocytes.[15-18] These adverse effects of CCl_4 have been attributed to cleavage of CCl_4 to its toxic reactive metabolites, trichloromethyl free radicals by cytochrome P450 enzyme.[19, 20]

Plant extracts and plant-derived natural products have been previously reported to mitigate increased ROS activities and liver toxicity induced by CCl_4.[21-24] A notable medicinal plant is *M. cecropioides*, also known as the umbrella tree and mostly found in the tropical forests of West Africa stretching from Guinea to Congo. Traditionally, the plant is used to induce labour, reduce elevated blood pressure, reduce high blood sugar, as well as treat cough, chest infection, malaria and jaundice.[25-28] Other validated pharmacological activities for the stem bark aqueous and ethanol extracts include its hypotensive and hypoglycemic effects in experimental animal models.[29-32] The leave extracts have also been reported to possess vasodilating, hypotensive, anti-inflammatory and anti-nociceptive activities in animal models.[33-36] Considering the biological activities reported for *M. cecropioides*, this study therefore investigates the potential hepatoprotective activity of *M. cecropioides* in CCl_4-induced liver injury as a model of NAFLD, with silymarin as a reference standard (pharmacological control).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Plant material

Fresh stem barks of *M. cecropioides* were collected from a forest around Oluku area within Benin City during the month of September 2011 and were identified by Mr. Sunny Nweke of the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin. Authentication of this plant had initially be done at the Forest Research Institute of Nigeria, Ibadan, Nigeria where a herbarium specimen number FHT106428 was deposited.[29] The sheaths were washed with normal saline, sorted, air-dried at room temperature and protected from direct sunlight and heat for two weeks until completely free from moisture. They were then pulverized using the laboratory hammer-mill and the powdered samples were stored in air and water-proof containers until required for extraction.

Preparation of aqueous extract

Approximately, 2.0 kg of the powdered stem bark of *M. cecropioides* was extracted over 24 h using 2 L of distilled water. The mixture was filtered using Whatman filtered paper, and the filtrate evaporated at 60°C using a vacuum rotary evaporator. The moist residue was freeze-dried using a vacuum freeze-drier and dried powder was preserved in a refrigerator at 4°C until needed. The crude extract was dissolved in distilled water to make a concentration of 100 mg/mL from doses of 250 and 500 mg/kg body weight were reconstituted and administered orally.

Experimental animals and their management

A total of 30 adult male wistar rats with average weight of 240 to 250 g were used for this study. The animals were inbred rats obtained from the rat colony of the Animal House of the Anatomy Department, University of Benin, Benin City. The animals were maintained in cages under natural conditions with temperature between 25 - 30ºC, and standard photoperiod of approximately 12 hours of light alternating with approximately 12 hours of darkness and were fed with grower's marsh (Bendel Feeds and Flour Mills Limited, Ewu, Edo State, Nigeria) and potable water *ad libitum*.

Experimental design and induction of CCl_4 hepatotoxicity

Animals were randomly divided into 5 groups of 6 animals per group (n = 6) such that the average weight differences between groups did not exceed ± 10 g. Animals in group 1 served as the normal control while animals in groups 2 to 5 were administered CCl_4 (Merck Chemicals, Germany) 30% v/v dissolved in olive oil intraperitoneally every 72 h. Animals in groups 3 to 5 were treated daily with 250 mg/kg, 500 mg/kg of *M. cecropiodes* and 25 mg/kg of silymarin. The silymarin (a standard hepatoprotective drug) treated group served as the pharmacological control. The chosen dose for CCl_4 and silymarin was based on previous research.[37] Extract and silymarin were administered daily for fourteen days and all animals were sacrificed on day fifteen of experiment. The treatment regimen is as shown in Table1 below.

Table 1: Treatment regimen

Group	Treatment
Group 1	Normal saline (10 mL/kg of body weight)
Group 2	Carbon Tetrachloride control CCl_4 30% v/v with olive oil (1 mL/kg of body weight) (every 72 h)
Group 3	*M. cecropiodes* (250 mg/kg of body weight) daily + CCl_4 30% v/v with olive oil (every 72 h)
Group 4	*M. cecropiodes* (500 mg/kg of body weight) daily + CCl_4 30% v/v with olive oil (every 72 h)
Group 5	Silymarin (25 mg/kg of body weight) daily + CCl_4 30% v/v with olive oil (1 mL/kg of body weight) (every 72 h)

Assay for serum hepatic enzymes

Blood samples (average of 4 mL per animal) were collected via cardiac puncture and stored in plain bottles. The blood samples were centrifuged at 3000 revolutions/min (rpm) using a bench-top centrifuge (Shanghai Surgical Instrument Factory, Shanghai, China) at 37°C for 15 min to separate the sera. Serum alanine aminotransferases (ALT), aspartate aminotransferases (AST), alkaline phosphatase (ALP) as well as total protein (TP) were assayed spectrophotometrically, using Randox colorimetric assay diagnostic kits (Randox, Northern Ireland).

Estimation of antioxidant Activity

After collection of blood samples, the rats in different groups were sacrificed by cervical dislocation and after that liver tissues excised immediately and washed in ice cold normal saline. Part of the livers were chopped off, homogenized, centrifuged at 3500 rpm for 5 min and the clear supernatants were collected into an empty specimen container using a micropipette. Assays for catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) as a marker for lipid peroxidation were done as previously described.[38-40]

Histopathological studies

Liver tissues were fixed in 10% buffered formalin for routine histological tissue processing. Briefly, tissues were dehydrated in ascending grades of ethanol, embedded in paraffin wax and sections of 5 µm thickness were cut, mounted on glass slides and stained with haematoxylin and eosin (H&E) for histopathological examination.

Data Analysis

Data generated from this study were analysed using GraphPad Prism Version 6 and expressed as mean ± S.D. The significance of difference in the means of all parameters was determined using one-way analysis of variance with 95% confidence interval and statistical significance was set at $P < 0.05$.

Results and Discussion

Impact of M. cecropiodes on serum liver enzymes and protein in CCl_4 treated animals

In this study, the impact of *M. cecropiodes* on hepatic enzymes as well as protein was evaluated in wistar rats exposed to CCl_4. Figure 1 shows that CCl_4 administration significantly led to a marked increase in serum liver function enzymes (AST, ALT and ALP) as well as a reduction in total protein. However, in animals pretreated with 250 and 500 mg/kg of body weight of *M. cecropiodes* as well as those treated with the reference drug silymarin (25 mg/kg of body weight), there was a reversal of serum markers towards normal. Together, these results suggest that *M. cecropiodes* protects the liver from CCl_4-induced toxicity in adult wistar rats by modulating the activities of serum liver enzymes.

Effect of M. cecropiodes on liver antioxidant enzymes in CCl_4 treated animals

To further evaluate the hepatoprotective potentials of *M. cecropiodes* in CCl_4-induced liver toxicity, the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) as well as the concentration of malondialdehyde (MDA) were measured in liver homogenates. Results show that CCl_4 administration led to a decrease in liver SOD and CAT as well as an increase in MDA levels. Contrary to the results obtained from the CCl_4 treated animals, a remarkable improvement on the levels of antioxidant enzymes in animals pretreated with *M. cecropiodes* was observed (Figure 2). Taken together, these results suggest that *M. cecropiodes* protects the liver from CCL4-induced toxicity by regulating changes in liver antioxidant enzymes.

Histopathology findings

Histopathology of processed liver tissues stained with H and E provides supportive evidence for the biochemical analysis. Control group showed a normal liver architecture with well-delineated hepatocytes, liver sinusoids and portal triad (Figure 3A). Liver section of group 2 animals showed characteristic liver histopathological lesions following administration of CCl_4 such as portal vein congestion, severe inflammatory infiltrates, large vacuoles indicative of fat deposits and necrosis of hepatocyte. Compared to the group 2 animals, micrographs obtained from liver sections of animals treated with *M. cecropiodes* displayed a dose-dependent reduction of severity of damage inflicted by CCl_4. Importantly, the standard hepatoprotective agent silymarin at 25 mg/kg daily also showed improved liver histology which was similar to the control animals (Figure 3B to E). Together, these results suggest that *M. cecropiodes* may protect liver histology following damage from toxins.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

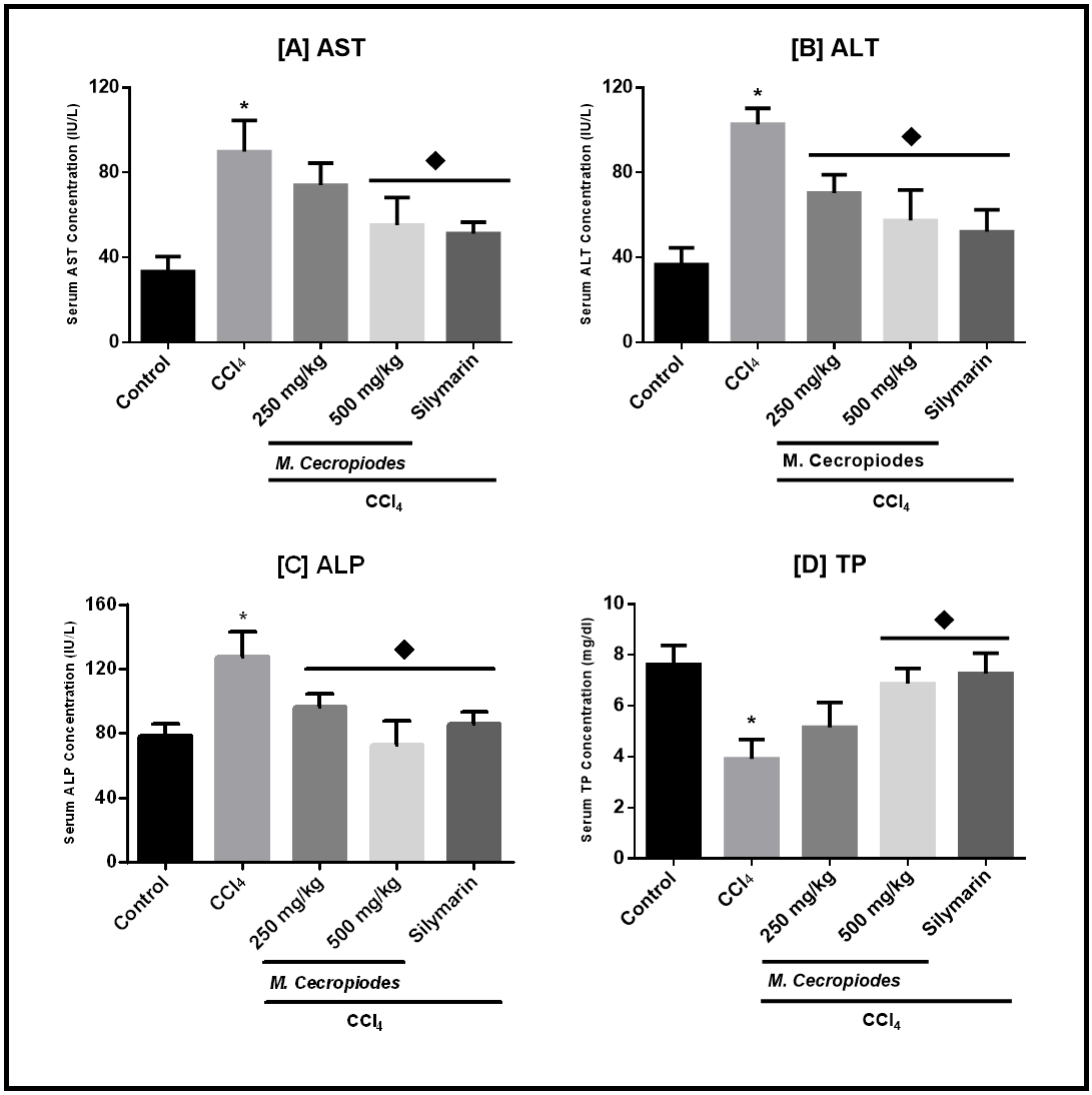

Figure 1: *Musanga cecropiodes* modulates altered serum hepatic enzymes and protein of experimental animals exposed to CCl_4. ALT - Alanine amino-transferases, AST - Aspartate amino-transferases, ALP - Alkaline phosphatase, TP - Total protein. Results show mean ± S.D of n = 4. * and ◆ shows significance at $P < 0.05$ when compared to control and CCl_4 treated groups, respectively.

The liver is a highly complex organ and plays central role in regulating metabolic functions such as nutrient storage, maintenance of homeostasis, synthesis of proteins, lipid metabolism as well as secretory and excretory functions.[41, 42] Considering the critical physiological functions of the liver, it is usually prone to injuries from drugs and chemical toxins, which sometimes results in NAFLD. Furthermore, the incidence of NAFLD is gradually on the rise and it has been adjudged as the most common cause of liver dysfunctions and possibly the major reason for liver transplantation.[43, 44] Lesion to liver hepatocytes could directly be as result of changes in membrane integrity and intracellular function or indirectly as a result of immune-mediated membrane damage. Also noticeable in cases of liver toxicity are elevated serum liver enzymes, alterations in liver antioxidant enzymes as well as changes in the liver histo-architecture.[45] In the present study, the potential protective effect of the crude aqueous of *M. cecropiodes* was investigated in CCl_4-induced liver injury as a model for NAFLD in experimental animals.

It is widely known that CCl_4 is one of the strongest chemical toxins capable of inducing severe liver injury and its toxicity has been linked to NAFLD.[14] CCl_4 is rapidly absorbed by the liver due to its lipophilicity and easy permeability through cell membranes.[46] The mechanism of CCl_4 toxicity has been attributed to its intermediate reactive metabolite trichloromethyl free radical which interacts with cellular components, resulting to lipid peroxidation, damage to plasma membranes and finally leakage of hepatic markers.[47, 48]

In cases of liver damage following chemical toxins, the serum hepatic enzymes, AST, ALT and ALP are used as markers due to their unusually elevated levels.[49] Similarly, an early marker in cases of hepatic steatosis leading to NAFLD is a rise in the level of these liver enzymes, with ALT levels appearing higher than AST levels. This differential is used as a yardstick to differentiate hepatic steatosis from NAFLD and alcoholic fatty liver, with the latter being demonstrated by an increase in the AST:ALT ratio.[50, 51] Findings from this study show that CCl_4 administration to experimental animals led to an increase in hepatic serum markers and the pretreatment of animals with *M. cecropiodes* and silymarin attenuated CCl4-induced elevation of hepatic serum enzymes. These findings are consistent with our earlier study demonstrating that *M. cecropiodes* protected the liver from paracetamol toxicity.[52]

Furthermore, It is expected that an effective liver protective agent should be able to restore altered physiological processes or reduce any deleterious effects from liver toxins.[53] A reduction in TP levels can be used as an indication to determine the severity of liver damage and CCl_4 is widely known to trigger this reduction.[54-56] In this study, administration of CCl_4 led to a reduction in TP levels in experimental animals indicating liver injury. Conversely, TP levels were observed to be significantly higher following pretreatment with *M. cecropiodes* and silymarin, thus indicating promotion of protein synthesis. These findings are consistent with previous studies which reported that treatment with plant extracts stimulated an increase in TP levels in CCl_4-induced liver injury. [56, 57]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

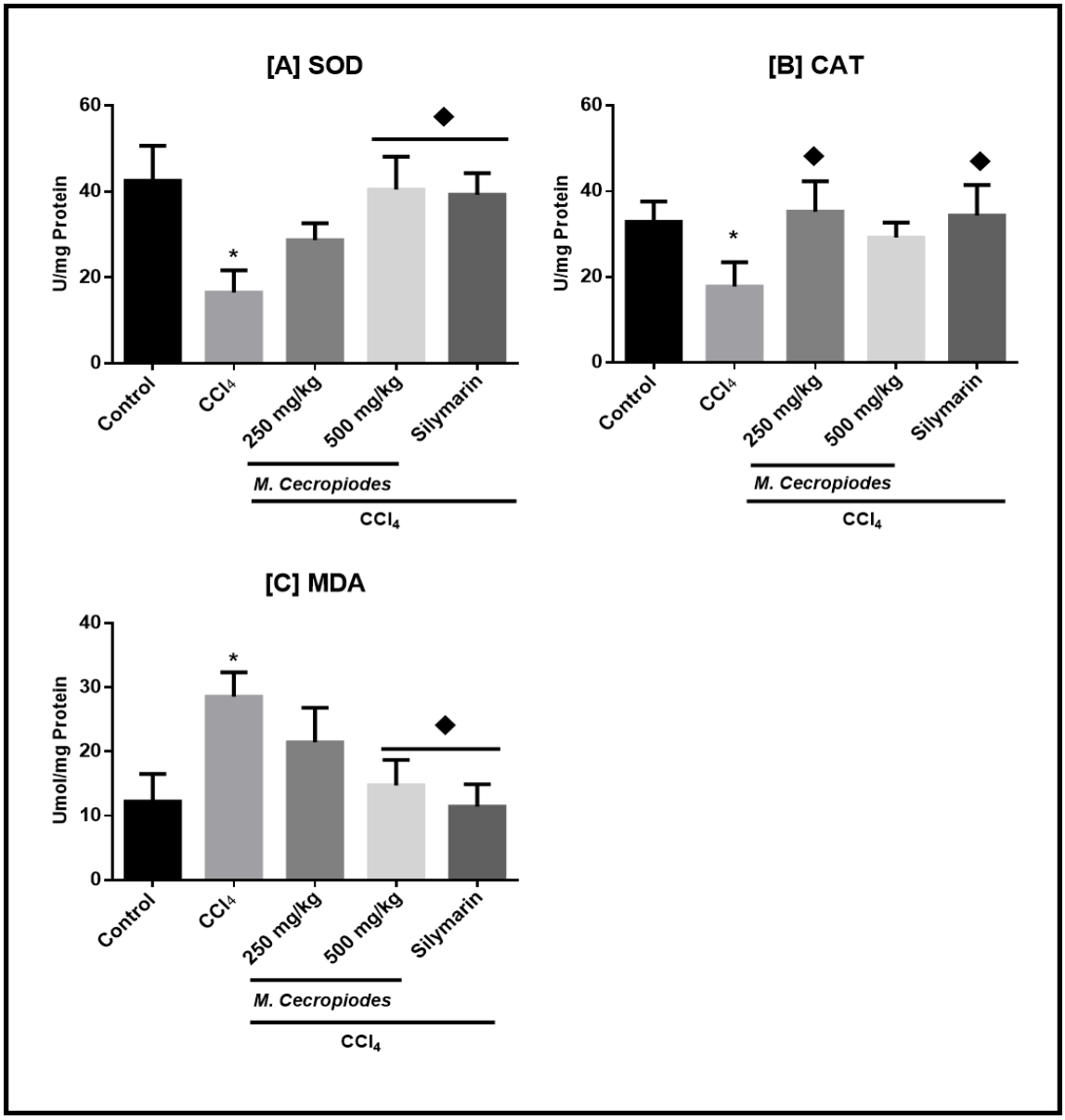

Figure 2: *Musanga cecropiodes* restores depleted antioxidant enzymes of experimental animals exposed to CCl_4. CAT - Catalase, SOD - Superoxide Dismutase, MDA - Malondialdehyde. Results show mean ± S.D of n = 4. * and ◆ shows significance at $P < 0.05$ when compared to control and CCl_4 treated groups, respectively.

Additionally, hepatocyte toxicity is indicated by an alteration in antioxidant enzymes which could stimulate the generation of free radicals leading to oxidative stress. Antioxidant enzymes present a front line of defense against free radicals in living organisms.[58] It is well documented that uninhibited generation of ROS promotes several human disease conditions including diabetes, cancer and hepatotoxicity due to alteration in physiological processes.[59, 60] In this study, CCl_4 treatment led to alterations in SOD, CAT and MDA activities in the liver of experimental animals.

SOD is a detoxification enzyme and a major antioxidant in cellular processes which acts as an important first line defense against ROS in cells. It catalyses the dismutation of the highly reactive superoxide anion to O_2^- to a less reactive hydrogen peroxide (H_2O_2) or ordinary oxygen molecule (O_2).[61] Furthermore, formed H_2O_2 from dismutation of O_2^- is detoxified by CAT which protects cells from H_2O_2 generated within them. CAT converts H_2O_2 to water and oxygen and plays a critical role in promoting tolerance to oxidative stress when cells are faced with stress conditions.[62] In this study, while CCl_4 treatment inhibited SOD and CAT activities in liver tissues, pretreatment with *M. cecropiodes* and silymarin attenuated the activity of CCl_4. This indicates that the antioxidant enzymes activities were properly regulated following pretreatment with extracts and silymarin. The ability of plant extracts to boost antioxidant activities in liver tissues is widely known and this is attributed to their phenolic contents. In support of this, Ayinde et al., (2007) had earlier isolated two phenolic compounds from the stem bark of *M. cecropiodes* which showed antioxidant activities.[63] Also, a different study demonstrated that stem bark and leaf extracts of *M. cecropioides* contain many phytochemicals, with an important amount of phenolic compounds, and possess antioxidant activities.[64]

More so, an important marker for increased generation of free radicals is malondialdehyde which arises from lipid peroxidation of polyunsaturated fatty acids.[65] It is well established that an increase in lipid peroxidation is associated with liver damage and further suggests that endogenous antioxidants failed to inhibit the formation of free radicals.[66, 67] A mechanism of CCl_4 induced toxicity is by its induction of lipid peroxidation and findings from the present study show that CCl_4 led to an increase in MDA levels in experimental animals, but pretreatment with *M. cecropioides* and silymarin rescued MDA levels toward normal. Together, our findings suggest that *M. cecropioides* protected the liver of experimental animals exposed to CCl_4 by regulating altered activities of antioxidant enzymes.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

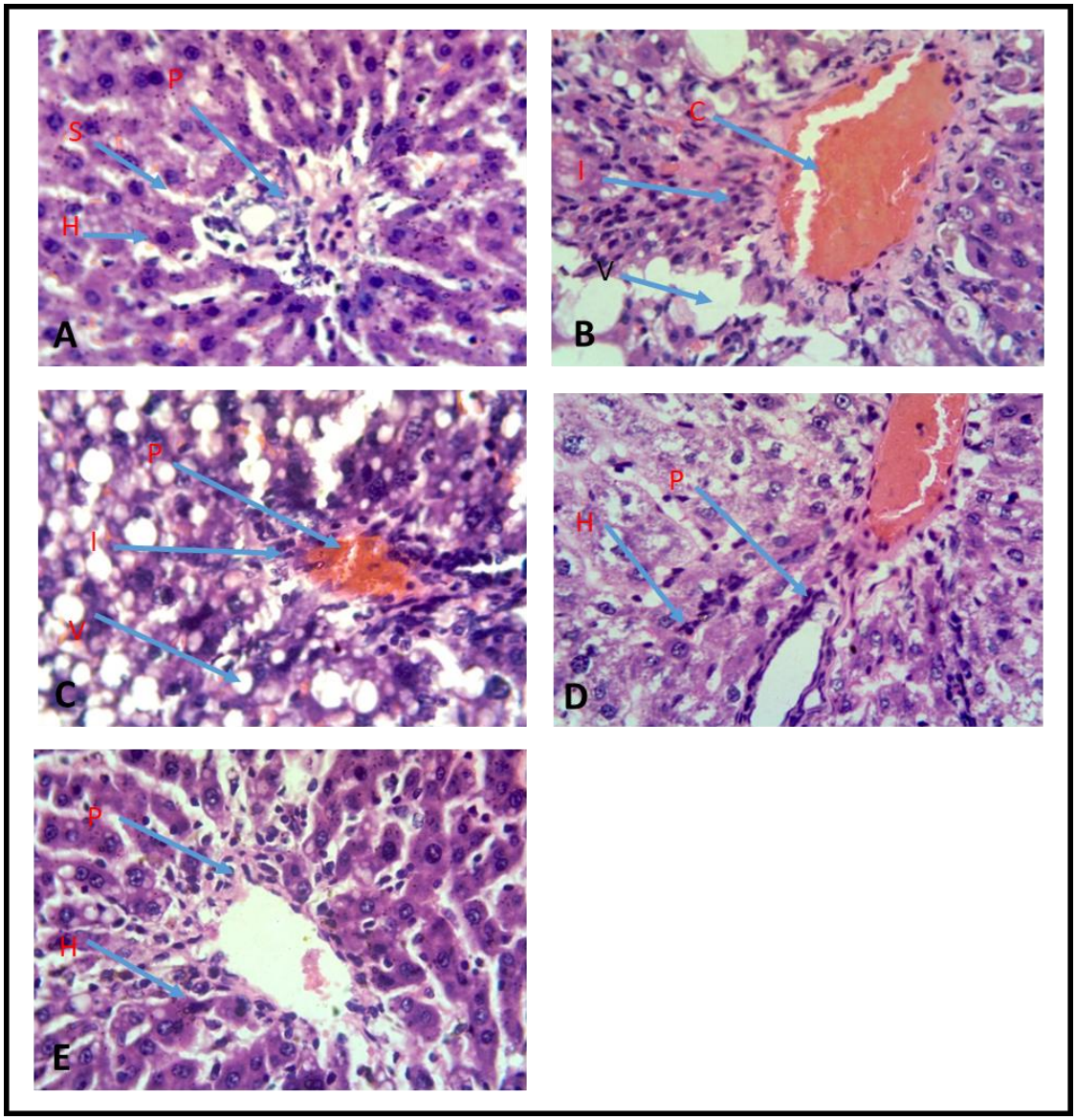

Figure 3: Liver sections from experimental animals stained with H and E. (A) Control animals showing normal hepatocytes and nuclei [H], portal area [P] and liver sinusoids [S]. (B) Liver section treated with CCl_4 showing portal congestion [C], Inflammatory infiltrates in the portal area [I], and vacuolations [V]. (C) Liver section treated with 250 mg/kg *M. cecropioides* showing portal congestion [P], reduction in severity of inflammatory infiltrates [I], reduction in vacuolar size [V]. (D) Liver section treated with 500 mg/kg *M. cecropioides* showing improvement in liver architecture with portal area [P], hepatocytes and nuclei [H], (E) Liver section treated with 25 mg/kg of silymarin showing hepatocytes and nuclei [H] and portal area with congestion [P].

In this study, the marked increase in serum hepatic enzymes, alteration in antioxidant enzymes following exposure of experimental animals to CCl_4 led to an aggregation of fat as well as inflammatory infiltrates in the parenchyma of the liver. This observation is consistent with what has been reported in previous studies.[68-70] This condition is known clinically as steatosis and marks the onset of the liver disease which could advance to steatohepatitis with inflammation of hepatocytes.[4] Our findings from this study show that *M. cecropioides* may have inhibited the accumulation of fat in the parenchyma of the liver as animals in groups 3 and 4 showed a lesser severity in vacuolations, thus suggesting a halt in the progression of lipid peroxidation.

Conclusion

This study evaluated the potential hepatoprotective activity of *M. cecropioides* following CCl_4-induced liver injury in experimental animals and findings from both biochemical and histopathological analysis showed that *M. cecropioides* was able to reduce elevated serum liver enzymes, modulate alterations in antioxidant enzymes as well as inhibit accumulation of fat in the liver parenchyma of rats treated with CCl_4. This study demonstrated that *M. cecropioides* might serve as a potent hepatoprotective agent and thus provides a strong rationale for further experiments to identify and characterize the bioactive compounds mediating this activity.

Conflict of interest

The authors declare no conflict of interest.

Author's Declaration

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

Acknowledgements

The authors wish to thank the technical staff of the Department of Anatomy, University of Benin led by Late Mrs. Comfort Braimah for their assistance during the tissue processing. The authors are also grateful to the technical staff of the Department of Medical Biochemistry, University of Benin for their support during the biochemical analyses.

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