PROTECTIVE ACTIVITY OF THE STEM BARK METHANOL EXTRACT OF *ALAFIA MULTIFLORA* AGAINST CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present study was designed to evaluate the hepatoprotective activity of the stem bark methanol extract of *Alafia multiflora* against CCl_4 induced liver damage in rats. The extract (125 and 250 mg/kg) was administered orally to the animals with hepatotoxicity induced by CCl_4 (40%, 0.5 mL/kg). The plant extract was effective in protecting the liver against the injury induced by CCl4 in rats. This was evident from significant reduction in serum enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and creatinin. It was also observed that extract of *A. multiflora* conferred protection against CCl_4-induced superoxide dismutase depletion in liver and kidney (30 %); CCl_4-induced catalase depletion in the same organs (>100 %); CCl_4-induced reduced glutathione depletion (30 and 68 %) and CCl_4-induced lipid peroxidation in the liver (28 %). Histopathological studies showed marked reduction in fatty degeneration and centrizonal necrosis, in animals receiving different doses of *A. multiflora* along with CCl_4 as compared to the control group. The results suggest that the stem bark methanol extract of *A. multiflora* possesses hepatoprotective activity against CCl_4-induced hepatocellular damage in rat and this effect of *A. multiflora* may be due to its antioxidant content. However, further studies are needed to evaluate the role of this plant on liver regeneration.

Keywords: *Alafia multiflora,* methanol *extract,* liver function, hepatoprotection

INTRODUCTION

Liver is the chief site for intense metabolism and excretion. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. But it is continuously and variedly exposed to environmental toxins, and abused by poor drug habits, and alcohol and prescribed and over-the-counter drug which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease[1,2].

Treatment options for common liver diseases and the effectiveness of treatments such as interferon, colchicine, penicillamine, and corticosteroids are inconsistent at best and the incidence of side-effects profound. All too often the treatment is worse than the disease[3].

In spite of tremendous advances in modern medicine no effective drugs are available, which stimulate liver functions and offer protection to the liver from the damage or help to regenerate hepatic cells[4]. Therefore, many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective liver damage in experimental animal model.

Alafia multiflora (Apocynaceae) is a medicinal plant traditionally used in ulcerous wound and sometimes in abdominal pain. In a previous works, protective effect of the methanol/methylene chloride/ (1:1) extract of *A. multiflora* on CCl_4-induced oxidative stress in rats has been study[5]. Furthermore, antibacterial and antiradical activities of different extracts of this plant have been demonstrated, as well as the LD_{50} (>5g/kg) in rats[6]. Phytochemical screening of the extract showed the presence of phenols, tannins, flavonoids, anthraquinones and alkaloids[6].

In the present study, we aimed to examine whether the stem bark methanol extract of *A. multiflora* could exert *in vivo* cytoprotective effect on the CCl_4 model of liver toxicity. This plant effect was evaluated on biochemical markers and histological analysis.

MATERIALS AND METHODS

Animals

Experiments were performed using male albinos rats (Wistar rats, 150-200 g) bred in the animal house of Animal Physiology laboratory, Faculty of science, University of Yaounde I. The animals were maintained on a 12/12 h light/dark natural cycle with standard laboratory chow and water *ad libitum.* Rats were divided into five groups, each one consisting of five animals. Prior authorization for the Cameroon National Ethics Committee (Reg. N° FWA-IRB00001954) as early indicated[6].

Extract preparation

Fresh stem bark was collected in the Centre Region of Cameroon in May and authenticated at the National Herbarium-Yaounde, where the voucher specimen was conserved under the reference number 43196/HNC. A methanol/methylene chloride extract of the stem bark of *Alafia multiflora* was then prepared as previously described (Dimo et al., 2006). 36 g of this crude extract

was then partitioned with 500 mL of and evaporated in a rotary vacuum to obtain dried methanol extract. The yield of extraction was 37 %.

The carbon tetrachloride-induced hepatic damage

Hepatic injury was induced by treating rats intra peritoneally with CCl_4-olive oil (40%, 0.5 mL/kg) mixture 48 h before sacrifice on day 10. Rats received by gavage *A. multiflora* at doses of 125 or 250 mg/kg of body weight during 10 days, combined with CCl_4 on day 8. Control rats were treated with olive oil (0.5 mL/kg) 48 h before sacrifice.

Experimental design

Rats were divided into five groups consisting of five animals each.

- Group 1: control rats received the vehicle (olive oil, 0.5 mL/kg s.c.) at day 8.
- Group 2: received CCl_4 in olive oil (0.5 mL/kg, s.c) at day 8
- Group 3: received *A. multiflora* extract 250 mg/kg daily using an intragastric tube for 10 days
- Group 4: received *A. multiflora* extract 125 mg/kg (p.o) for 10 days and simultaneously administered CCl_4 (0.5 mL/kg, s.c) at day 8
- Group 5: received *A. multiflora* extract 250 mg/kg (p.o) for 10 days and simultaneously administered CCl_4 (0.5 mL/kg s.c) at day 8

All rats had free access to food and drinking water during the study. The animals were killed on day 10 by cervical decapitation. Blood samples were collected, from carotid arterery, allowed to clot at room temperature and serum separated by centrifuging at 3000 rpm for 10 min for various biochemical parameters. The heart, liver and kidney were quickly harvested and weight. Liver and kidney were homogenized in Tris-HCl buffer (0.01 M, pH 7.4) and heart in distilled water using a potter homogenizer to give a 10 % of homogenate. Such portion of the same organs was fixed in 10% formalin for histopathological observation.

Assessment of liver and renal function

Biochemical parameters parameters in serum i.e. aspartate aminotransferase (AST)[7], alanine aminotransferase (ALT)[7], total bilirubin[8], serum creatinin[9], total proteins[10] were analyzed according to the reported methods.

Assessment of oxidative stress in tissue

Superoxide dismutase (SOD) determination

Tissue superoxide dismutase was determined by the method of Misra and Fridovich[11]. SOD was monitored at 480 nm using an assay mixture containing 1666 mL carbonate buffer (0.2 M, pH 10.2) and 0.134 mL of 10% homogenate. The reaction was started by adding 0.2 mL of epinephrine (0.3 mM). Change in absorbance was recorded at 480 nm at 20 sec for 1 min interval. Suitable control lacking enzyme preparation was run simultaneously. One unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of auto oxidation of epinephrine.

Catalase assay

Catalase activity (CAT) was measured by the method of Sinha[12]. The reaction mixture consisted of 0.75 mL phosphate buffer (0.1 M, pH 7.5) and 0.2 mL 10% homogenate. The reaction was started by adding 0.2 mL H_2O_2 (50 mM) and stopped 1 min later by adding 2 mL of perchloric acid. The tubes were boiled in water bath for 10 min and cooled immediately under running tap water. Changes in absorbance were recorded at 570 nm. H_2O_2 was quantified using a calibration curve and the CAT activity was expressed as µmol of H_2O_2/min/mg proteins.

Lipid peroxidation assay

Free radical mediated damage was assessed by measuring the extend of lipid peroxidation in term of malondialdehyde (MDA). It was determined by thiobarbituric acid reaction. The reaction mixture consisted of 1 mL trichloroacetic acid 20%, 2 mL thiobarbituric acid 0.67%, 1 mL homogenate supernatant. Then, the tubes were boiled in water bath for 1 hour and cooled immediately under running tap water. Then the tubes were centrifuged at 4000 rpm for 30 min. the upper layer was aspired out and absorbance was measured at 530 nm. MDA was quantified using an extinction coefficient of 1.56×10^5 M^{-1}.cm and expressed as µmol of MDA/mg wet tissue[13].

Estimation of reduced glutathione (GSH)

Reduced glutathione in the rat tissues was assessed by the method previously described by Ellman[14]. The reaction mixture consisted of 3 mL of Ellman reagent and 0.02 mL of the homogenate supernatant. The tubes were mixed and kept at room temperature for 1 hour. Changes in absorbance were read at 412 nm on a spectrophotometer.

Histopathological studies

Pieces of heart (ventricle), liver and renal tissues were excised, washed with normal saline and processed separately for histopathological observation. Initially the material was fixed in 10% buffered neutral formalin, dehydrated in gradual ethanol (50-100%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin (H-E) dye. The sections were examined microscopically for histopathology changes, including cell necrosis, fatty change, and ballooning degeneration.

Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was evaluated by one way-ANOVA), followed by Tukey test using SPSS software. [The $p<0.05$ was considered statistically significant.

RESULTS

1. Effect of *A. multiflora* on liver enzymes, renal functions and relative organ weight

Serum levels of ALT, AST, total bilirubin, and creatinine of the control group were accounted respectively for 42.61 U/L, 54.46 U/L, 34.02 mg/L, and 6.60 mg/L. CCl_4 when

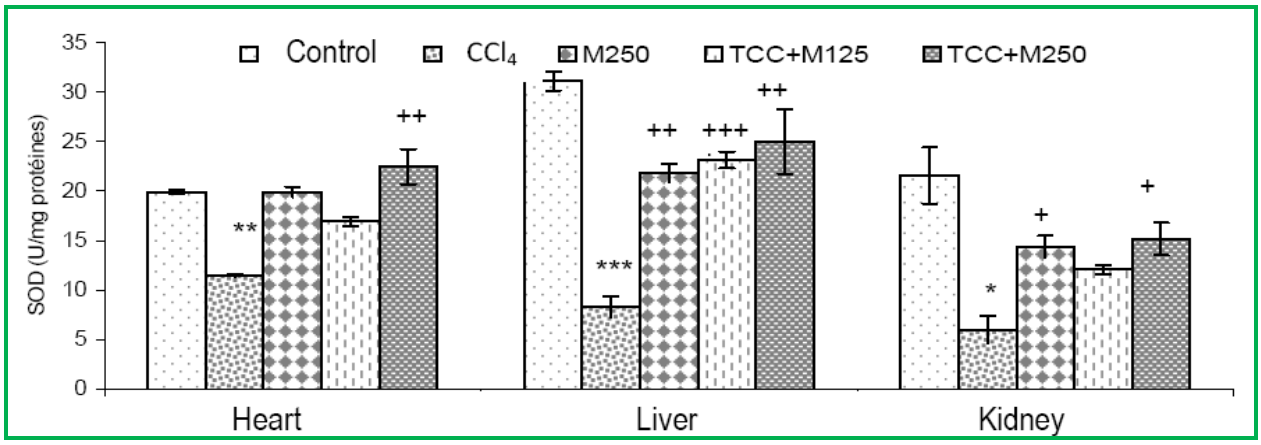
administered alone 48 h before sacrifice caused significant increased in serum levels of ALT (34.40%), AST (49.49%), total bilirubin (172.33%) and creatinine (200%), when compared to controls. The above changes were altered with the administration of the plant extract. Noticeable decrease of creatinin??? (about 40.52%) had been observed, as compared to control group (6.60 mg/L). *Alafia multiflora* at doses of 125 and 250 mg/kg significantly reduced CCl_4-induced increase in ALT, AST, total bilirubin and creatinin levels in the serum, when compared to CCl_4 group. Daily administration of a single dose of the stem back methanol extract of *A. multiflora* at 250 mg/kg during 10 days in normal rats did not significantly affect serum transaminases, total bilirubin and creatinin concentrations, when compared to the control group. The plant extract alone or combine with CCl_4 slightly increased liver and kidney relative weight when compared to the controls. CCl_4 did not cause significant changes in heart, liver and renal relative weight as compared to controls (Table 1).

Table 1: Serum concentration of transaminases, total bilirubin, creatinin, relative weight of heart, liver and kidney in CCl_4 and *A. multiflora* treated rats.

	Controls	CCl_4	M250	M125+ CCl_4	M250+ CCl_4
ALT (U/L)	42.61±2.91	57.27±0.51*	$40.40\pm2.43^{+}$	48.16 ± 1.62	$45.20\pm1.25^{+}$
AST (U/L)	54.46±1.05	81.41±0.77*	54.60±0.00	$70.20\pm2.55^{+}$	$61.08\pm2.82^{++}$
Bilirubin (mg/L)	34.02±14.45	92.63±4.15*	$33.04\pm5.53^{++}$	$44.20\pm5.59^{++}$	$42.14\pm4.25^{++}$
Creatinine(mg/L)	6.60 ± 2.71	$28.80\pm3.61^{*}$	$5.71\pm0.90^{+}$	$7.43\pm0.70^{+}$	$6.86\pm1.46^{+}$
Heart (mg/g bw)	3.33 ± 0.01	3.60 ± 0.01	4.36 ± 0.01	3.07 ± 0.02	3.31 ± 0.00
Liver (mg/g bw)	28.78± 0.03	38.72± 0.06	32.14 ± 0.04	38.06 ± 0.09	34.72 ± 0.06
Kidney(mg/g bw)	5.78 ± 0.02	7.25 ± 0.1	13.34 ± 0.08	8.38 ± 0.00	7.12 ± 0.01

Values represent means ± SEM; n=5. *p< 0.05 as compared to control, $^{+}$p<0.05 when compared to CCl_4; $^{++}$p<0.01 when compared to CCl_4. CCl_4= rats received CCl_4; M250 = rats received *A. multiflora* at 250 mg/kg; M125+ CCl_4 = rats received *A. multiflora* at 125 mg/kg and CCl_4; M250+CCl_4 = rats received *A. multiflora* at 250 mg/kg and CCl_4.

Figure 1. Effect of the methanol extract of *A. multiflora* on SOD activity in heart, liver and kidney of CCl_4 treated rats.



Each bar represents mean ± SEM, n=5. TCC CCl_4= rats received CCl_4; M250 = rats received *A. multiflora* at 250 mg/kg; M125+ CCl_4 = rats received *A. multiflora* at 125 mg/kg and CCl_4; M250+CCl_4 = rats received *A. multiflora* at 250 mg/kg and CCl_4. * p<0.05 when compared to controls; **p<0.01 when compared to controls; ***p<0.001 when compared to controls; $^{+}$ p < 0.05 when compared to CCl_4; $^{++}$ p < 0.01 when compared to CCl_4; $^{+++}$ p < 0.001 when compared to CCl_4.

2. In vivo antioxidant activities of the stem bark methanol extract of *A. multiflora*

Superoxide dismutase activity

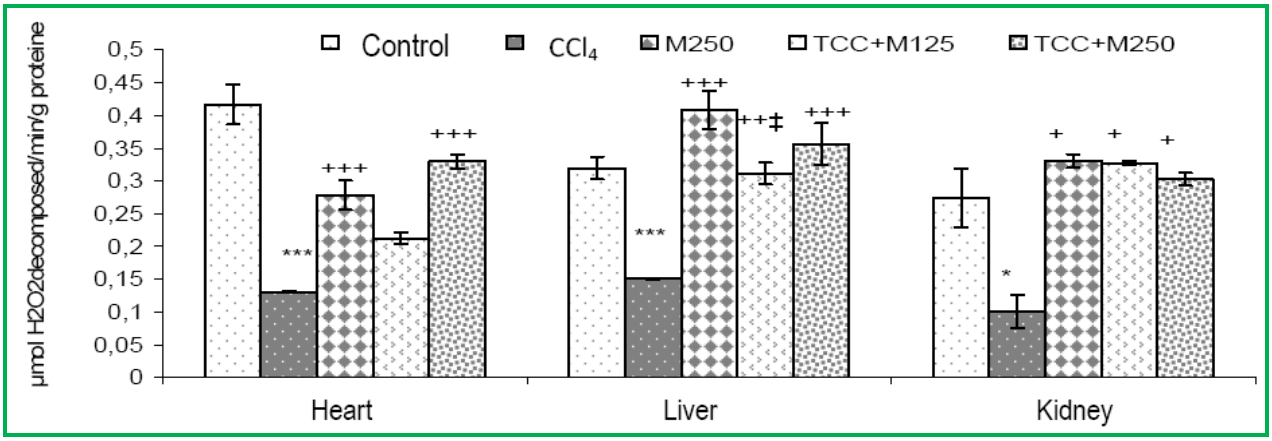
Heart, liver and renal levels of SOD in control rats were 19.5, 32.5, and 23.4 U/mg protein respectively. CCl_4 when administered alone 48 h before sacrifice significantly decreased these values for 42.44, 73.24 and 72.16 % in heart, liver and kidney, respectively. Treatment with *A. multiflora* extract significantly increased the level of SOD as compared to CCl_4 control group (Figure 1). The effect produced by *A. multiflora* extract in this context was even better, since the levels of this enzyme were brought (p > 0.05) almost to nearly that of control rats (group 1). Daily administration of a single dose of the methanol extract of *A. multiflora* at 250 mg/kg during 10 days did not significantly affect SOD levels in investigated tissues as compared to control normal rats.

Catalase activity

Catalase (CAT) activity registered in control rats in tem of µmol of H_2O_2 decomposed was about 0.43, 0.32 and 0.28. CCl_4 given 48 h before sacrifice significantly decreased this activity by 68.59%, 52.89% and 63.26%, respectively in the heart, liver and kidney. However, Rats receiving plant extract at doses of 125 and 250 mg/kg, concomitantly with CCl_4 showed increased CAT activity when compared to CCl_4 group. At 125 mg/kg for example, this activity was 62.49%, 106.81% and 224.76% in heart, hepatic and renal tissue respectively lesser than CCl_4

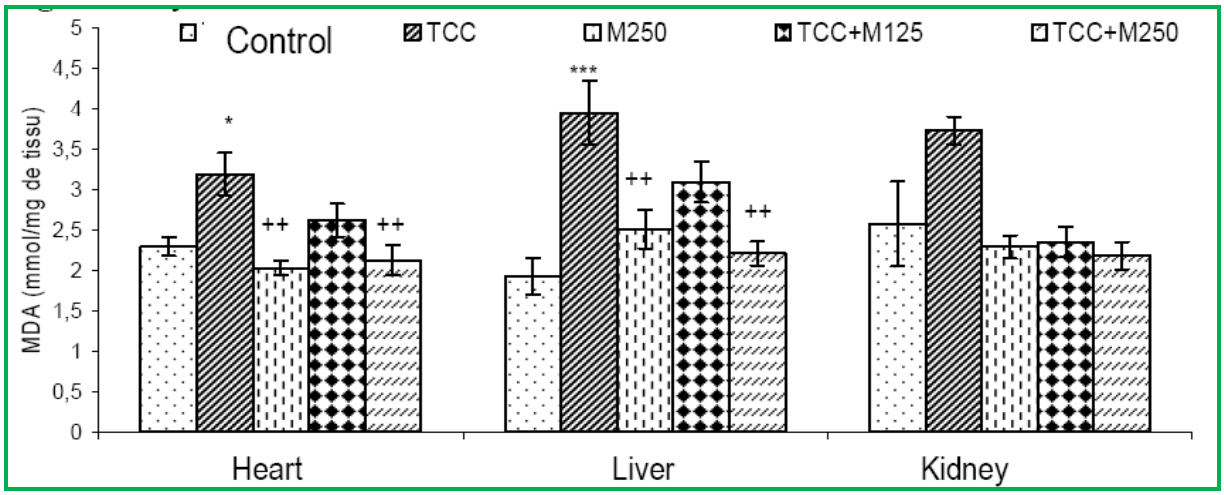
group. At 250 mg/kg, these values were 152.17 %, 136.65 % *and* 200.47 %, respectively in the same organs (Figure 2). The methanol extract of *A. multiflora* when administered alone at the dose of 250 mg/kg during 10 days did not cause any significant variation in CAT activity in the same organs, when compared to controls.

Figure 2. Effect of the methanol extract of *A. multiflora* on CAT activity in heart, liver and kidney of CCl_4 treated rats.



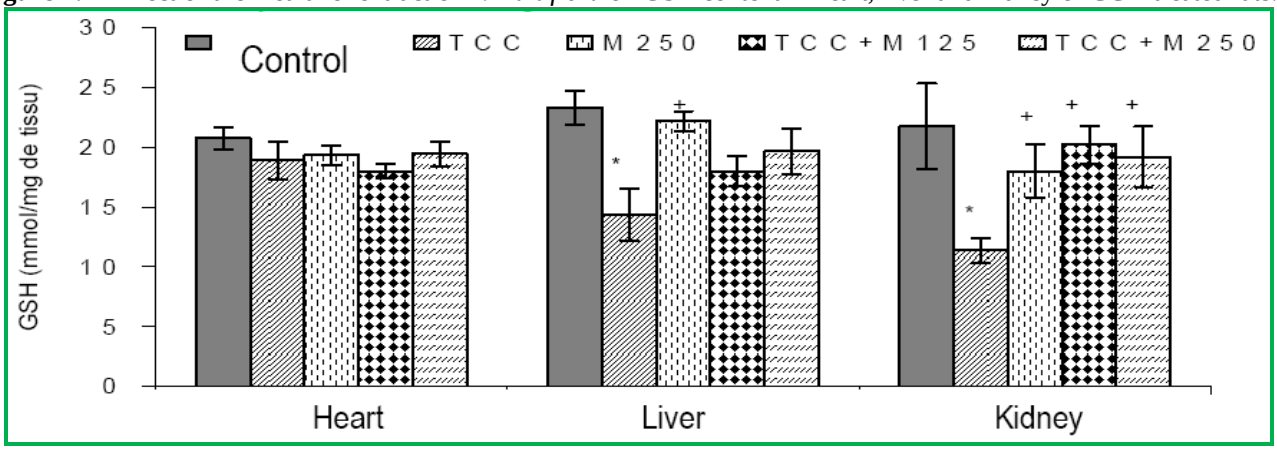
Each bar represents mean ± SEM, n=5. TCC CCl_4= rats received CCl_4; M250 = rats received *A. multiflora* at 250 mg/kg; CM125+ CCl_4 = rats received *A. multiflora* at 125 mg/kg and CCl_4 ; M250+ CCl_4 = rats received *A. multiflora* at 250 mg/kg and CCl_4.. * p<0.05 when compared to controls ; ***p<0.001 when compared to controls; $^{+}$ p < 0.05 when compared to CCl_4; $^{++}$ p < 0.01 when compared to CCl_4; $^{+++}$ p < 0,001 when compared to CCl_4.

Figure 3. Effect of the methanol extract of *A. multiflora* on MDA content in heart, liver and kidney of CCl_4 treated rats.



Each bar represents mean ± SEM, n=5. TCC CCl_4= rats received CCl_4; M250 = rats received *A. multiflora* at 250 mg/kg; CM125+ CCl_4 = rats received *A. multiflora* at 125 mg/kg and CCl4 ; M250+CCl_4 = rats received *A. multiflora* at 250 mg/kg and CCl_4. * p<0.05 when compared to controls; ++ p < 0.01 when compared to CCl_4.

Figure 4. Effect of the methanol extract of *A. multiflora* on GSH content in heart, liver and kidney of CCl4 treated rats.



Each bar represents mean ± SEM, n=5. TCC CCl4= rats received CCl_4; M250 = rats received *A. multiflora* at 250 mg/kg ; CM125+ CCl_4 = rats received *A. multiflora* at 125 mg/kg and CCl_4 ; M250+CCl_4 = rats received *A. multiflora* at 250 mg/kg and CCl4.. * p<0.05 when compared to controls; $^{+}$p < 0.05 when compared to CCl_4.

Figure 5. Light micrographs of heart section taken from rats (HEX400). A, Normal control group; B, CCl_4 control group (0.5 mL/kg); C, methanolic extract (250 mg/kg) +CCl_4

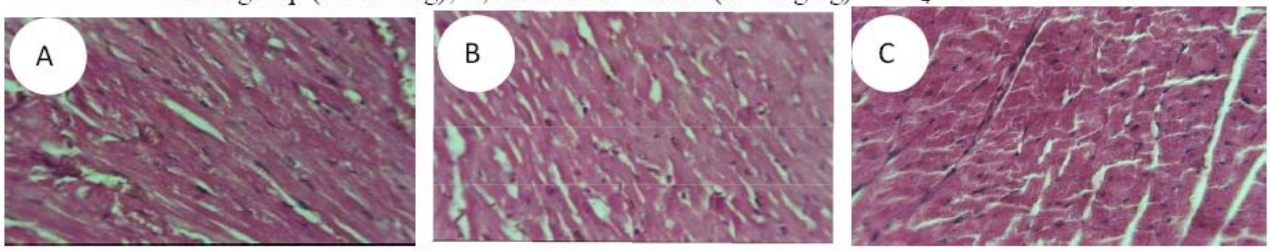
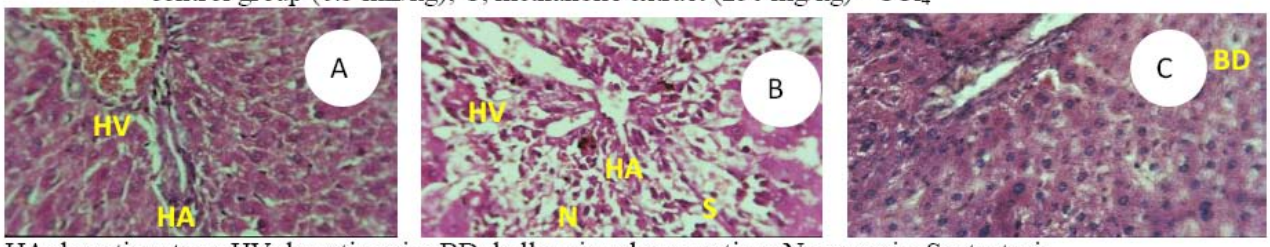
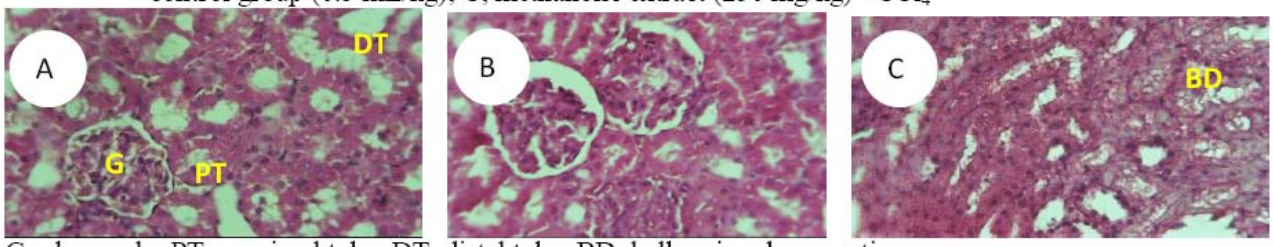


Figure 6. Light micrographs of liver section taken from rats (HEX400). A, Normal control group; B, CCl_4 control group (0.5 mL/kg); C, methanolic extract (250 mg/kg) +CCl_4



HA: hepatic artery; HV: hepatic vein; BD: ballooning degeneration; N: necrosis; S: steatosis

Figure 7. Light micrographs of kidney section taken from rats (HEX400). A, Normal control group; B, CCl_4 control group (0.5 mL/kg); C, methanolic extract (250 mg/kg) +CCl_4



G: glomerule; PT: proximal tube; DT: distal tube; BD: ballooning degeneration

Estimation of lipid peroxidation

MDA concentration was about 2.34, 1.95 and 2.52 mmol/mg of tissue in heart, liver and kidney of in control rats. Lipid peroxide formation induced by CCl_4 was significantly higher in these organs. Administrations of the plant extract at the dose of 250 mg/kg significantly reduced the lipid peroxidation levels as compared to CCl_4 control group in the heart and liver (19.20% and 28.5%)(Figure 3). The methanol extract of *A. multiflora* (250 mg/kg) alone did not significantly affect MDA content of tissues examined.

3. Histopathological observations

Histology of the liver sections of control animals showed normal hepatic cells with well preserved cytoplasm, prominent nucleus, nucleolus and visible central veins (Figure 6 A). The liver sections of CCl_4-intoxicated rats exhibited macro vesicular fatty changes, intense periportal necrosis, ballooning degeneration and the loss of cellular boundaries (Figure 6 B). The histological architecture of liver sections of the rats treated with the methanol extract of *A. multiflora* showed significant liver protection against the toxicant as evident by normal hepatic cord, well preserved cytoplasm, prominent nucleus and nucleolus, absence of necrosis and lesser fatty infiltration (Figure 6 C).

Histological profile of the CCl_4-treated animals did not showed typical sign of heart (Figure 5 B) and nephro-toxicity (Figure 7 B), when compared to control group (Figures 5A, 7A). Rats treated with *A. multiflora alone* or in combination with CCl_4 showed no change in kidney morphology, but typical sign of ballooning degeneration of the epithelial cells (Figure 7 C)

DISCUSSION

In the present study, the stem bark methanol extract of *A. multiflora* was evaluated for the protective activity using tissue injury induced by CCl_4 in rat model. This study therefore gives some scientific evidences on the effect of the plant extract on enzyme status and histological observation. Administration of CCl_4, 48h before the pharmacological evaluation produced oxidative stress in rats as revealed by the increase levels of MDA. The increase in the levels of serum bilirubin reflected the failed of binding, conjugation and excretory capacity of hepatocytes[15] and the increase in transaminases was the clear indication of cellular leakage and loss of functional integrity of the cell membrane[16]. It is well documented that CCl_4 is biotransformed under the action of cytochrome P450 in the microsomal compartment of liver to trichloromethyl radical which readily reacts with molecular oxygen to form trichloromethyl peroxyl

radical[17]. Both the radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids[18]. In response to hepatocellular injury initiated by the biotransformation of CCl_4 to reactive radicals, "activated" Kupffer cells in liver respond by releasing increased amounts of active oxygen species and other bioactive agents. Various Studies demonstrated that liver is not the only target organ of CCl_4 and it causes free radical generation in other tissues also such as kidneys, heart, lung, testis, brain and blood[19-21]. In our study, CCl_4 selectively causes toxicity in liver, kidney and heart. In the present study, CCl_4 induced a severe damage in tissues investigated as represented by markedly elevated levels of ALT, AST and bilirubin coupled with a marked oxidative stress. In our investigation, CCl_4 increased lipid peroxidation, leads to inactivation of catalase, GSH and SOD as previously demonstrated in CCl4 intoxicated rats by Tirkey et al. [21]. Oxidative stress causes depletion of intracellular GSH, leading to serious consequences. *A. multiflora* administration ameliorated the increased level of lipid peroxidation after CCl_4 treatment. Improvement of hepatic GSH levels in *A. multiflora* -treated rats in comparison to CCl_4 intoxicated rats demonstrates the antioxidant effect of the stem bark extract of *A. multiflora.* Treatment of rats with 125 or 250 mg/kg doses of the methanol extract of *A. multiflora* (oral route) markedly prevented CCl4-induced elevation of serum ALT, AST, bilirubin and creatinin. The attributively of the observed alterations of serum transaminases and bilirubin levels to hepatic damage and dysfunction on healthy rats was confirmed by histopathological studies of liver which have shown that liver necrosis, which is a more severe form of injury, was markedly prevented by the methanol extract of *A. multiflora.* Cardiac and renal tissues were also injured at a lesser extend because SOD, CAT activities and GSH levels were depressed in CCl_4 group, instead histomorphology of these organs was normal. Improvements of these parameters by the plant extract point towards tissue protective activity of in the experimental model.

Therefore, it is possible that the *A. multiflora* extract at concentrations used have imposed ballooning to the liver and renal cells, but this toxic effect did not overcame the protective effect of the plant against CCl_4. Additionally, this observation which could be related to a potential retention of water in these organs, particularly the kidney, as noticed in a previous work[5] might explain the relative overweight of the liver and the kidney as described in table 1. Renal and cardiac structure were not alter after 48 hrs of CCl_4 administration in our study but even this short period of exposure led to a significant oxidative stress in kidneys. Fadhel and Amran[22] had also reported increased levels of renal lipid peroxidation in rats after CCl_4 exposure which could be improved by black tea extract. Similar observations were also reported with certain Indian ayurvedic Indian preparations[23].

In the present study it was noticed that the results are in accordance with the earlier reports on the hepatoprotective activity of the methanol/methylene chloride (1:1) extract of *A. multiflora* against CCl_4-induced lipid peroxidation in rats[5]. However, the effect was more pronounced with the methanol extract which succeeded to protect against GSH depletion and CAT inhibition than did the other one in the same model of rats. This observation hence rationalizes the use of this medicinal plant as a constituent of various herbal cytoprotective formulations.

Earlier phytochemical reports revealed that the methanol extract of *A. multiflora* was found to contain higher concentration of phenolics (69.83 gallic acid equivalent), and expressed antiradical activity against DPPH in solution (80.74 % at steady state) [6]. Taking together, the results obtained at this stage indicate that the *A. multiflora* stem bark exhibited hepatoprotective effect against CCL_4-induced liver damage. This may be due to the presence of phenolic compounds which have hepatoprotective and inhibition of lipid peroxidation properties. Further studies are needed to assess the effect of this plant on liver regeneration.

CONCLUSION

The methanolic extract of *A. multiflora*, claimed to be a hepatoprotective agent as evidenced by both biochemical and histopathological (normal livers) studies. This extract has shown the ability to maintain the normal functional statues of the liver. Possible mechanism that may be responsible for the protection of CCl4 induced tissue damage by *A multiflora* may be it action as radical scavenger intercepting those radicals involved in CCl4 metabolism by microsomal enzymes. Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance in oxidant status. Thus, tissue protective activity of *A. multiflora* may be due to its antioxidant content. Work is in progress to assess liver regeneration activity of the plant and to identify the antioxidant agents of this plant extract.

Acknowledgement: This work was carried at the Laboratory of Animal Physiology, University of Yaounde I and Laboratory of Anathomopathology, UHC Yaounde. Authors are highly thankful to Heads of Departments and Mr. NOMO (UHC) for providing the facilities.

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