EFFECTS OF *ALAFIA MULTIFLORA* STAPF ON LIPID PEROXIDATION AND ANTIOXIDANT ENZYME STATUS IN CARBON TETRACHLORIDE-TREATED RATS.

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Abstract

Carbon tetrachloride (CCl_4) is a common model of heart, hepatic and kidney failure. Acute exposure to CCl_4 produces rapid cellular injury and lipid peroxidation of membranes. The present study was designed to evaluate the efficacy of *Alafia multiflora* on CCl_4-induced oxidative stress in rat tissues. The methylene chloride/methanol extract of *A. multiflora* (125 and 250 mg/kg) was given once daily, orally, to male wistar rats for eight days prior to subcutaneous injection of CCl_4 (2 ml/kg in olive oil) or vehicle. Rats were killed 48 h after CCl_4 administration. Oxidative stress in heart, liver and kidney tissues was estimated using malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). CCl_4 caused a marked rise in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, creatinine and lipid peroxidation markers in the control. The administration of *A. multiflora* (250 mg/kg) conferred significant protection against the elevation of creatinine levels by 80.55% but failed to provoke significant

reduction of ALT, AST and total bilirubin levels which increased after CCl_4 administration.
A. multiflora (250 mg/kg) administration ameliorated the decreased level of SOD by 41.14%, 65.05% and 72.72%, in the rat heart, liver and kidney respectively. MDA levels were significantly reduced by 23.58% in the heart and by 28.68% in the kidney respectively, at the dose of 250 mg/kg. The plant extract failed to improve GSH and catalase levels in the liver. Our study demonstrated that prior administration of *A. multiflora* extract has protective effect against CCl_4 intoxication in the rat. The increase of antioxidant defence system activity in experimentally CCl_4-treated animals induced by *A. multiflora* extract might lead to decreased oxidative status provoked by CCl_4 through the inhibition of the production of reactive oxygen species.

Key words: *A. multiflora*, CCl_4 intoxication, lipid peroxidation,
oxidative stress.

Alafia multiflora Staff in a large liana belonging to the family Apocynaceae. It is a multiple usage medicinal plant distributed in West and Central Africa. The latex mixed with bark scrapings is applied to wounds and leg ulcers, and to ulcers caused by syphilis. In Ghana, the latex diluted with water is taken orally to cure stubborn wounds. In Cameroon the fresh latex, either alone or mixed with *Oncinotis glabrata* (Baill.) Stapf ex Hiern, is also applied to treat yaws. The stem bark or fruits in decoction are taken to relieve abdominal pain. The seeds are an ingredient of arrow poison in DR Congo. (1). The pyrrolizidine alkaloid, alafine, has been extracted from *Alafia multiflora* seeds (2). Vanillic acid, isolated from an ether extract of the latex was found to be responsible for the antibacterial properties (3). Therefore, the popular and scientific interest to screen extracts of plants used in traditional medicine in all over the world took us in this study.
It is generally known that aerobic organisms consume molecular oxygen for normal respiration and obligatorily generate toxic by-products with double-edged beneficial and

deleterious consequences. Carbon tetrachloride (CCl_4)-intoxication in animals is an experimental model that mimics oxidative stress in many pathophysiological situations (4,5). Various studies have demonstrated that CCl_4 intoxication causes free radical generation in many tissues such as liver, kidney, heart, lung, testis, brain and blood (6,7,8). Oxidative stress parameters have been observed in these tissues including defects in enzymatic antioxidant mechanism and increased thiobarbituric reactive substances concentrations (9,10, 11,12). Currently used therapeutics strive to scavenge the reactive oxygen species (ROS), and a more therapeutically viable approach is to inhibit specifically this source of superoxide (13). Studies also show that certain natural extracts containing antioxidants protect against the CCl_4-induced increase in lipid peroxide levels and repair the oxidant status of various tissues (6,12,1). The present investigation was, conducted to evaluate the oxidant/antioxidant capacity of *A. multiflora* extract in the rat tissues treated with CCl_4.

Material and methods

Animals

Three-month old male Wistar rats (150-200 g) raised in the animal house of the Department of Animal Biology and Physiology, University of Yaounde I, Cameroon, were used. The animals were housed in plastic cages and maintained in ambient temperature and normal light/dark cycle. They were fed standard laboratory food and water *ad libitum*.

Plant material

Stem barks of the leafy plant of *A. multiflora* were collected in Nkolntara, village near Yaounde city (Central Province of Cameroon) and authenticated in the National Herbarium, Yaounde where voucher specimen **N° 43196/HNC** was deposited. The stem bark was sun-dried and powdered. 2400 g were macerated in 7 L of methylene chloride/methanol (1/1) at room temperature for 72 h with frequent shaking. The filtrate was concentrated under reduced pressure and evaporated in an oven at 35°C to obtain 23,8 g representing a yield of 0,9 %**.**
Preliminary phytochemical properties of this extract were tested using the following chemicals and reagents: alkaloids

(Mayer and Dragendoff's reagents), saponins (frothing test), tannins ($FeCl_3$), glycosides (Salkowski test), flavonoids (NaCl and HCl) and phenols ($FeCl_3$ and $K_3Fe(CN)_6$ (15).

Experimental design

Different groups of rats (n =5 per group) received the methylene chloride/methanol extract of *A. multiflora* once daily at doses of 125 or 250 mg/kg orally for 8 days. The animals were then challenged with a single dose of CCL_4 2 mL/kg s.c (40% v/v in olive oil) on the 8^{th} day. The CCL_4 control rats treated with vehicle (distilled water, 10 mL/kg) were given a single dose of olive oil (2 mL/kg s.c). In addition, another group received the highest dose of *A. multiflora* (250 mg/kg) once daily, but not CCL_4 in order to examine the effect of the plant extract alone on oxidative status and lipid peroxidation. All rats had free access to food and drinking water during the study.

After 48 h of CCL_4 treatment, the animals were sacrificed. Blood was withdrawn from the carotid artery, allowed to coagulate at room temperature for 30 min and serum separated by centrifugation at 3000 rpm for biochemical analysis. The heart, liver and kidney were quickly harvested, weight and homogenised. The homogenates were then centrifuged at 4800 rpm for 60 min at 4°C and the supernatant was stored at –20 °C till further biochemical estimations.

Biochemical assessment of serum

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were measured using the Reitman and Frankel method (16). Total serum bilirubin was estimated using the method described by Cheesbrough (17), with standard dirtrol as control. The determination of serum creatinine levels was done according to the method of Bartel et *al.* (18).

Assessment of oxidative stress in tissues

Estimation of lipid peroxidation

The extent of lipid peroxidation in tissues was assessed by measuring the level of malondialdehyde (MDA) according to the method of Wilbur (19). Briefly, 1 mL of trichloroacetic acid (TCA) 20% and 2 mL of thiobarbituric acid (TBA) 0.67%

were added to 2 mL of homogenate supernatant. The absorbance of the mixture was recorded at 530 nm. MDA was quantified using an extinction coefficient of 1.56 X 10^5 M^{-1}/cm^{-1}**)** and expressed as µmol of MDA per mg of wet tissue.

Estimation of reduced glutathione

Reduced glutathione (GSH) in the rat heart, liver and kidney was assayed by the method previously described by Ellman (20). Briefly, 0.02 mL of the homogenate supernatant was added to 3 mL of Ellman reagent. The samples were mixed and kept at room temperature for at least 1 hour. Changes in absorbance were read at 412 nm on a spectrophotometer.

Estimation of superoxide dismutase

The ability of superoxide dismutase (SOD) to inhibit the autoxidation of adrenaline at pH 10.2 makes this reaction a basis for a simple assay for dismutase. Superoxide anion (0_2^-) generated by the xanthine oxidase reaction causes the oxidation of adrenaline to adrenochrome and the yield of adrenochrome produced by 0_2 increases with pH and concentration of adrenaline. The superoxide dismutase activity in tissues was determined in our study by the method of Misra and Fridovich (21). Briefly, the assay mixture consisted of 134 µL tissue homogenate supernatant and 1666 µL carbonate buffer (50 mM, pH 10.2). In the cuvette, 0.2 mL of a freshly prepared adrenaline solution (0.3 mM) was added to the above mixture. The auto-oxidation of adrenaline was observed by measuring after one minute intervals, the absorbance at 480 nm. SOD was quantified as follows:

- % inhibition = $100 - [(\Delta DO_{sample})/\Delta DO_{blanck}] \text{ X } 100$
- 50 % inhibition correspond to one unit
- **X%** inhibition to N units

Specific SOD activity or SOD units per mg protein = [(SOD units /mL)/mg protein] X dilution factor.

Tissue protein was evaluated using theBiuret method of protein assay.

Estimation of catalase

Catalase (CAT) activity was determined in tissues according to the method of Sinha (22). In this method dichromate in acetic acid is reduced to chromic acetate when heated in the presence

of hydrogen peroxide (H_2O_2), perchromic acid is formed which is an H_2O_2 unstable intermediate. The chromic acid produced is measured calorimetrically at 570 nm. Briefly, 50 µL of homogenate supernatant was added to 750 µL of phosphate buffer (0.1 M, pH 7.5). Then, 200 µL of hydrogen peroxide substrate (50 mM) was added. Exactly 1 min later, the reaction was stopped by adding 2 mL of dichromate (5%) in acetic acid (300 mL). After that, the tubes were kept at 100 °C for 10 min. After cooling with tap water, changes in absorbance were recorded at 570 nm. Hydrogen peroxide was quantified using a calibration curve and the CAT activity was expressed as µmol of H_2O_2 per minute per mg of protein.

Statistical analysis

All experimental data were expressed as mean ± SEM and statistically assessed by one-way analysis of variance (ANOVA). The differences between test animals and controls were analysed using scheffe test. $P< 0.05$ was considered to be significant.

Results

Phytochemistry

Polyphenol, flavonoids, saponins, alkaloids and glycosides were identified.

Effect of *A. multiflora* on liver enzymes and renal functions

The activities of AST, ALT, total bilirubin and creatinine were significantly increased in the sera of CCl_4-induced animals when compared with control rats. *A. multiflora* at the dose of 250 mg/kg alone didn't significantly affect the levels of these enzymes as compared to normal control. Treatment of rats with the plant extract (250 mg/kg) slightly, but not significantly, prevented the increase of AST, ALT and total bilirubin induced by CCl_4. The level of serum creatinine was significantly reduced by 80.55% at the dose of 250 mg/kg as compared to the CCl_4-control group. CCl_4 intoxication increased urine excretion which was significantly attenuated by *A. multiflora* extract at the dose of 250 mg/kg (data not shown).

Table 1: Effect of the methylene chloride/methanol extract of *Alafia multiflora* on body weight, relative heart, liver and kidney weighst, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and creatinine levels in CCl_4-intoxicated rats.

	Control	**CCl_4**	**AM(250 g/kg)**	**AM(125 g/kg) + CCl_4**	**AM(250 g/kg) + CCl_4**
Body weight (g)	170.00 ± 2.88	172.40 ±7.62	164.40 ± 7.81	169.60± 9.00	177.20 ± 9.55
Heart (mg/g bw)	3.33 ± 0.01	3.60 ± 0.01	3.79 ± 0.00	3.53 ± 0.00	3.43 ± 0.01
Liver mg/g bw)	28.78 ± 0.04	38.72 ± 0.07	31.10 ± 0.05	39.38 ± 0.08	28.73 ± 0.03
Kidney (mg/g bw)	5.78 ± 0.02	7.25 ± 0.01	5.82 ± 0.01	6.98 ± 0.01	5.86 ± 0.03
ALT (U/L)	42.61 ± 2.91	57.27 ± 0.51†	48.92 ± 0.26*	62.97 ± 3.49†‡	51.44 ± 0.75
AST (U/L)	54.46 ± 1.05	81.41 ± 0.77†	59.19 ± 0.98*	94.46 ± 5.24†*	77.16 ± 1.12
Total Bilirubin(mg/L)	34.01 ± 4.44	92.62 ± 4.15†	55.25 ± 9.76*	122.30 ± 8.25†	84.82 ± 6.01†
Creatinine (mg/L)	9.60 ± 0.71	28.80 ± 3.61†	8.00 ± 0.21*	21.60 ± 2.99†	5.60 ± 0.08*

Values are expressed as mean ± SEM, n = 5. †$p < 0.05$ compared to controls; *$p < 0.001$ compared to CCl_4; ‡$p < 0.001$ compared to AM (250).
AM = *Alafia multiflora*

Effect of *A. multiflora* on malondialdehyde levels

The malondialdehyde (MDA) levels in the heart, liver and kidney were significantly higher in the CCl_4 control group as compared to the normal control rats (Fig. 1). Administration of *A. multiflora* extract (250 mg/kg) once daily for 8 days prior to CCl_4, caused at a significant reduction in the levels of MDA in the heart(23.58%) and in the kidney (28.68%). When given alone for 10 days, the methylene chloride/methanol extract of *A. multiflora* did not significantly affect lipid peroxidation of tissues as compared to normal controls.

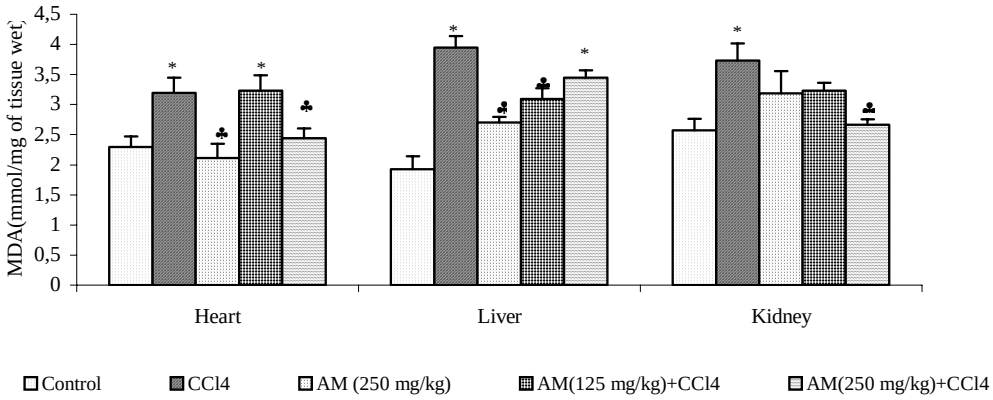

Figure 1 : Effect of the methylene chloride/methanol extract of *Alafia multiflora* (AM) on CCl_4-induced lipid peroxidation in rat heart, liver and kidney. Values are expressed as mean ± SEM, n=5. *p < 0.01 compared to controls, ♣p < 0.05 compared to CCl_4-treated rats.

Effect of *A. multiflora* on the reduced glutathione levels

CCl_4 challenge caused a significant depletion in reduced glutathione (GSH) levels, in the rat liver (40%) and kidney (50%) when compared to control group. *A. multiflora* extract (250 mg/kg) alone did not produce any significant change in the level of reduced glutathione either in heart, liver or kidneys. The plant extract failed to improve GSH levels in the liver but seemed to attenuate the reduction of GSH in the rat heart and kidneys (Fig. 2).

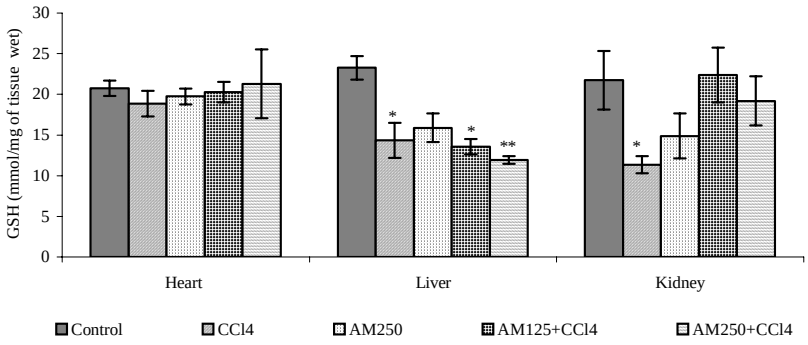

Figure 2 : Effect of the methylene chloride/methanol extract of *Alafia multiflora* on CCl_4-induced glutathione (GSH) depletion in rat heart, liver and kidney. Values are expressed as mean ± SEM, n=5. *p < 0.05 compared to controsl; **p < 0.01 compared to controls.

Effect of *A. multiflora* on catalase levels

CCl_4 challenge caused a significant decrease in catalase (CAT) levels in the heart, liver and kidneys compared to control group. *A. multiflora* (250 mg/kg), when given alone had no significant effect on this enzyme. The extract failed to improve the level of CAT in tissues of CCl_4 treated rats (Fig.3).

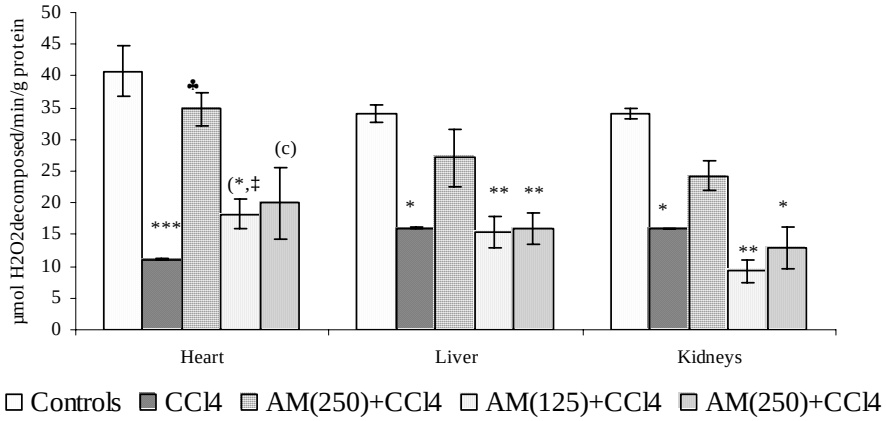

Figure 3 : Effect of different doses of methanol/methylene chloride extract of *Alafia multiflora* on CCl_4-induced depletion in catalase levels in rat heart, liver and kidney. Values are expressed as mean ± SEM, n = 5. *** $p < 0{,}001$ compared to controls; ** $p < 0.01$ as compared to controls; * $p < 0.05$ compared to controls; ♣$p<0{,}01$ compared to CCl_4; ‡$p < 0{,}05$ compared to AM
(250).

Effect of A. multiflora on superoxide dismutase levels

CCl4 administration significantly decreased, by 42.44%, 73.26% and 72.19%, the levels of superoxide dismutase (SOD), respectively, in the rat heart, liver and kidneys compare with the control group (Fig. 4). Administration of the methylene chloride/methanol extract of *A. multiflora* (250 mg/kg) alone did not significantly affect the tissue content of SOD as compared to normal control. Both doses of *A. multiflora* reduced the depletion of SOD levels as compared to CCl4 control rats. At the higher dose of 250 mg/kg, the depletion of the levels of SOD was reduced by 41.14%, 65.05% and 72.72%, respectively, in the rat heart, liver and kidnes.

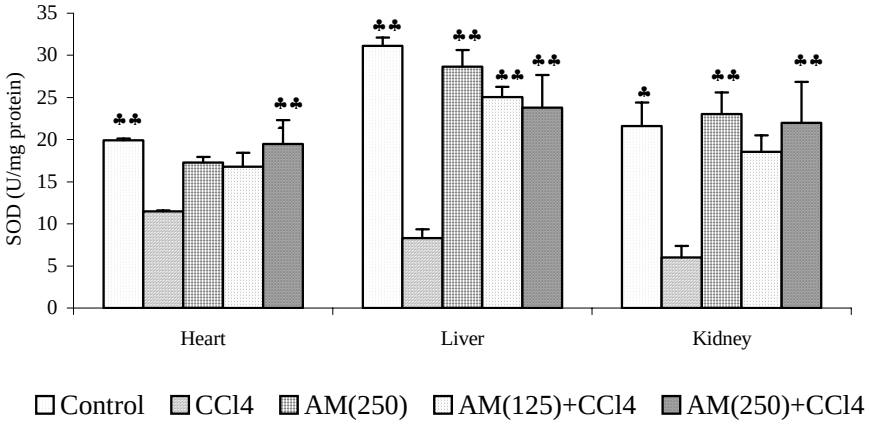

Figure 4: Effect of methylene chloride/methanol extract of *Alafia multiflora* (AM) on CCl_4-induced depletion in superoxide dismutase (SOD) in rat heart, liver and kidneyr. Values are expressed as mean ± SEM, n=5. a= statistically different at $p<0.001$ compared to CCl_4; b= statistical different at $p<0.01$ compared to CCl_4; c=
statistical different at $p<0.05$ compared to CCl_4.

Discussion

CCl4-intoxication elicited a significant decline in the antioxidant enzymes (SOD and CAT) and reduced glutathione concomitant with a significant elevation in MDA and serum ALT and AST levels. It is generally admitted that CCl4 is metabolised through the mitochondrial monooxigenase system (P450 2E1). Acute exposure to CCl_4 then produces rapid cellular injury due to reactive oxygen species (ROS), which generate lipid peroxidation of membranes (23). During metabolism, an unstable free radical, trichloromethyl, exits and is rapidly converted to trichloromethyl peroxide (5,24). The antioxidant systems such as antioxidant vitamins (A, C and E), superoxide dismutase, catalase, ceruloplasm and glutathione peroxidase protect the cells against lipid peroxidation, which is the basis of many pathologic processes (25). Protective effects of various natural products in CCl_4-intoxication have been reported (12,14).

Studies done with pentoxifylline and Ginseng showed that the antioxidant properties of these drugs contribute to protection against CCl_4-intoxication in rats (6,14).

In the present study, CCl_4 induced a significant increase in serum levels of ALT, AST, total bilirubin and creatinine, demonstrating a marked liver and kidney damage coupled with a marked hepatic and kidney oxidative stress.

We also observed significant elevation of urine excretion after CCl_4 administration (data not shown) indicating nephrotoxicity following CCl_4 administration in rats. CCl_4-intoxication in this study induced the generation of peroxyl radicals and O_2^- leading to the inactivation of catalase and SOD. Results of the present study also indicate that CCl_4-intoxication significantly decreased the levels of SOD and catalase in the rat heart, liver and kidney. *A. multiflora* treatment for 10 days significantly decreased the elevated MDA and seemed to improve liver enzyme levels at the higher dose of 250 mg/kg (p.o, once daily). The plant extract also increased, significantly, the reduced SOD and improved the level of GSH, and antioxidant enzyme levels in CCl_4-treated rats. Therefore, the methylene chloride/methanol extract of *A. multiflora* increased the activity of the antioxidant defence system in experimental CCl_4-treated animals.

Effective antioxidant defence requires concerted actions of both superoxide dismutase (which produces hydrogen peroxide from superoxide) and glutathione peroxidase (which removes the hydrogen peroxide) (26,27). In our study we observed the decrease of the ratio of superoxide dismutase/reduced glutathione in CCl4-treated rats (0.6) as compared to control group (0.9). This ratio was reversed in liver and kidney of rats given the plant extract at the dose of 250 mg/kg (>1.5). The most significant improvement of the reduced SOD levels in our finding suggests that *A. multiflora* exerts its protective effect by inhibiting superoxide radical production.

Many studies carried out over the past few years have shown that polyphenolic fractions from planst inhibit oxidative stress (28,29). Flavonoids have also been suggested to act as antioxidant through free radical scavenging (6). Preliminary phytochemical analysis of our plant extract revealed the presence of phenols and flavonoids. Thus, the protective effect of *A. multiflora* against CCl4-intoxication may be attributed to the presence of phenolic or flavonoids compounds, though this remaines to be confirmed.

Conclusion

In conclusion, our study demonstrated that CCl4 induces marked oxidative stress in rat heart, liver and kidney, which is attenuated by the methylene chloride/methanol extract of *A. multiflora*. The protective effects of A. multiflora against CCl4-intoxication might at least in part, be related to its superoxide anion inhibition or scavenging effect.

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