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Some Biochemical Effects of Methanolic Extract of *Afzelia africana* Seed in Rats Following Single or Repeated Carbon Tetrachloride Intoxication

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Authors' contributions

This work was carried out in collaboration between both authors. Author SEA designed the study and wrote the protocol, while author UDI managed the analyses and the literature searches, performed the statistical analysis and wrote the first draft. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To establish the *in vivo* effects of the methanolic extract of *Afzelia africana* seed on CCl_4- induced organ damage on some biochemical parameters.
Methods: The rats were divided into 7 groups of 5 rats each. In the single administration experiment, rats were intraperitoneally pre-treated with the extract (10mg/kg) for two days before CCl_4 intoxication at 0.6ml/kg. In the repeated intoxication experiment, rats were administered the extract (5mg/kg) by intraperitoneal injection for 10 consecutive days, and

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CCl_4 (0.6ml/kg) 72 hourly intervals for 10 days. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin (conjugated and total) were analyzed for hepatoprotective effect, while urea and creatinine were used to evaluate kidney function. **Results:** Data obtained showed significant reduction (P=.05) in the activities of ALT and AST, as well as in levels of bilirubin, urea and creatinine in the *Afzelia africana* extract-treated rats compared to the CCl_4 control.
Conclusion: These results indicate that the seeds of *A. africana* contain constituents that could protect the kidney and liver and ameliorate them from both acute and chronic injuries.

Keywords: Afzelia africana; biochemical effects; antioxidant; oxidative stress; chronic effect; acute effect; in vivo effect.

1. INTRODUCTION

Occurrence of acute and chronic organ diseases, particularly, those of the liver and kidney has become issues of public health concern to health practitioners in many developing countries, including Nigeria. Because of the possible roles of orthodox medicines in the increasing rates of these diseases, there is growing interest towards phytochemicals from natural sources, particularly, those in foods, as possible agents in the management and prevention in these diseases [1,2].

Called "Ojawala" in the Igbo language of Nigeria, *Afzelia africana a member of the family Leguminosae* and sub-family *caesalpinaceae*, is largely found in the fringing forest and the drier parts of the forest regions of Africa. Like melon and *Irvingia gabonensis* seeds [3], the seeds are generally used in southern part of Nigeria as soup thickening ingredient. The seed is rich in crude proteins (27%) minerals and other nutritional constituents [4-6]. Besides, phytochemical evaluations has revealed the presence of flavonoids oil and other phytochemicals of medicinal importance [7,8].

The leaf of the plant is used in local medicine for the treatment of several diseases, including general pain relief, digestive problems, e.g. constipation and vomiting, internal bleedings or haemorrhage [4], diarrhoea, gastrointestinal disorders and gonorrhoea [7], but the bark is used as poison for fishing. These numerous nutritional and medicinal uses notwithstanding, information on its organ protective effects in acute and chronic disease conditions are rather rare. Hence, we evaluated its relevance in the prevention and management of potential chronic and acute diseases of vital organs.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Methanol, petroleum ether, vitamin E as tocopherol acetate, carbon tetrachloride, corn oil, were of analytical grades and were purchased from Sigma chemical Co, Ltd (USA) and assay kits were acquired from Randox Laboratories Ltd., Antrim, United Kingdom. 3

2.1.1 Plant collection and authentication

The vegetative part and seeds of *A. africana* were collected in the morning hours in May/June 2009 from Achalla in Awka North Local Government Area of Anambra State,

Nigeria. It was authenticated at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria Nigeria where voucher number 900245 was assigned.

2.1.2 Sample preparation and extraction

Previously sun-dried seeds of the plant were dried to constant weight in the oven at 50℃ for 24 hours and pulverized using laboratory mortar and pestle. It was then filtered through a mesh guaze of 2µm. Fifty grams (50g) of the filtered sample was weighed and extracted beginning with petroleum ether (for 9hrs) followed by methanol (5hrs × 3 times) using soxhlet extractor. The methanol extracts were combined and dried *in vacuo*, weighed and stored at 4℃ until required.

2.1.3 Experimental animals

Male albino rats (7-8weeks old and weighing about 120-150g) were purchased from the animal house of the National Research Institute for Chemical Technology (NARICT), Basawa-Zaria, Kaduna State, Nigeria. They were preconditioned for two weeks prior to experimentation. They were fed *ad libitum* on tap water and growers mash (Vital feeds, Bukuru-Jos, Plateau State, Nigeria) and weighed prior to commencement and at termination of the experiment. To use the animals for this study, permission was obtained from the University Committee on the use of laboratory animals, and experiments were performed in accordance with national and international standards regulating the handling and use of experimental animals.

2.1.4 *In vivo* evaluation of plant extract effects

For the single carbon tetrachloride intoxication, rats were divided into 7 groups with each group containing 5 rats as follows; extract only, extract + CCl_4, solvent only, solvent + CCl_4, vitamin E only, vitamin E + CCl_4, and the untreated control. The same concentration of the extract (10mg/kg) was administered by intraperitoneal injection for two days before administration of CCl_4 (0.6ml/kg), which was done after 1 hour of administrating the plant extract on the third day. Rats were sacrificed 24 hours following CCl_4 intoxication.

For the repeated intoxication, rats were divided into 7 groups as listed above. The same dose of CCl_4 (0.6ml/kg body weight) was administered by intraperitoneal injection before the administration of the first extract or vitamin E one hour later, and subsequently 72 hourly intervals for 10 days before termination of the experiment. After the first day, extract and vitamin E were administered daily at a dose of 5mg/kg for 10 days. Seventy two (72) hours following the last intoxication and twenty four hours after the last extract treatment, rats were sacrificed under chloroform anaesthesia.

2.1.5 Blood collection

At the point of sacrifice, blood from each rat was withdrawn from carotid artery at the neck and collected in previously labeled test tubes and allowed to stand for 4 hours. Clear serum were collected from the blood into eppendorf tubes and stored at -20℃ for biochemical assays.

2.2 Liver Function Tests

2.2.1 Aspartate aminotransferase

Aspartate aminotransferase was determined as previously described [9], using Randox Assay kit (Randox Laboratories Ltd., Antrim, United Kingdom). This procedure involves 0.5µl

of reagent 1 (phosphate buffer, L-aspartate and α-oxoglutarate) in a clean test tube containing 0.1ml of serum, mixed and incubated for exactly 30 min at 37℃ and 0.5ml of reagent 2 (2,4-dinitrophenylhydrazine) was added, mixed and allowed to stand for exactly 20 min at 25℃. Sodium hydroxide (0.5ml) was added, mixed and absorbance was read against the reagent blank at 540nm after 5 minutes. The AST concentration was determined by extrapolation from a calibration curve.

2.2.2 Alanine aminotransferase

Using Randox Assay Kit, alanine aminotransferase was determined as previously described [9]. This involves adding 0.5µl of reagent 1 containing phosphate buffer, L-alanine and α-oxoglutarate to a clean test tube containing 0.1ml of serum, mixing and incubating for exactly 30 minutes at 37℃. Exactly 0.5ml of reagent 2 (containing 2,4-dinitrophenylhydrazine) was added, mixed and allowed to stand for another 20 minutes at 25℃. Reaction was stopped with 0.5ml of sodium hydroxide (0.4M) and absorbance was read against the reagent blank at 540nm after 5 minutes. The ALT concentration was determined by extrapolation from the standard calibration curve.

2.3 Determination of Bilirubin concentration

Conjugated, unconjugated and total bilirubin levels were estimated as described by [10] using Randox assay kit. For total bilirubin; one drop (0.05ml) of reagent 2 (sodium nitrite) was added into a clean test tube containing 0.20ml of reagent 1 (sulphanilic acid and hydrochloric acid) and 1.00ml of reagent 3 (caffeine and sodium benzoate) followed by 0.20ml of serum, mixed and allowed to stand for 10 minutes at room temperature. Then, 1.00ml of reagent 4 (tartarate and sodium hydroxide) was also added, mixed and allowed to stand for 10 minutes at room temperature. The absorbance of the sample against sample blank (A_{TB}) was read at 560 nm and the value of the total bilirubin (mg/dl) was obtained by $10.8 \times A_{TB}$.

For direct bilirubin, 1 drop (0.05ml) of reagent 2 was added into a clean test tube containing 0.20ml of reagent 1. Then, 2.00ml of sodium chloride (9 g/l) was added followed by 0.20 of serum, mixed and allowed to stand for exactly 5 minutes at room temperature. The absorbance was read against the sample blank (A_{DB}) at 530 nm and the value of direct bilirubin (mg/dl) was obtained by $14.4 \times A_{DB}$. Indirect bilirubin was obtained by difference.

2.4 Kidney Function Test

2.4.1 Urea

Urea was analyzed based on the principle that urea in serum was hydrolysed to ammonia in the presence of urease, and this ammonia was then measured colorimetrically by Berthelot's reaction using Randox analytical kit [11]. The procedure involves adding 100µl of reagent 1 (containing EDTA, sodium nitroprusside and urease) to clean test tubes containing 10µl of serum, mixing and incubating at 37℃ for 10 minutes and 2.50ml of reagent 2 (diluted phenol) and 2.50ml of reagent 3 (containing diluted sodium hypochlorite and sodium hydroxide) which were mixed immediately and incubated at 37℃ for 15 minutes before reading the Absorbance at 540nm. Urea concentration was then calculated by simple proportion using the absorbance of a known concentration of the standard.

2.4.2 Statistical analysis

Utilizing SPSS Version 20, the results obtained were statistically analyzed using one way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The significance level was set at P=.05.

2.4.3 Creatinine

Creatinine level was assayed using Randox reagent kit, based on the principle that creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration [11]. In the method, 0.2ml of serum was added into a clean test tube containing 2.0ml of creatinine working reagent (picric acid and sodium hydroxide). They were mixed and absorbance A_1 was read after 30 sec and at exactly 2 minutes later, absorbance A_2 was read at 510nm. Creatinine level was calculated as change in the sample absorbance divided by the change in the standard absorbance multiply by the concentration of the standard.

3. RESULTS

3.1 Effect of Daily Extract Administration on Rats Repeatedly Intoxicated with CCl_4

3.1.1 Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) Activities

The group of rat administered CCl_4 alone was observed to have significantly (P=.05) elevated levels of ALT and AST compared to the normal control and CCl_4 intoxicated rats treated with extract. The group treated with both extract and CCl_4 was observed to have ALT activity significantly higher than the normal control group, but not significantly different from the AST activity of the normal control group. There was no significant difference between the ALT and AST activities of the normal control, vitamin E and extract treated groups (Fig. 1).

3.1.2 Serum total, conjugated and unconjugated bilirubin concentration

There was a significant (P=.05) increase in the levels of conjugated, total and unconjugated bilirubin in the CCl_4 control group. However, in the CCl_4 groups treated with vitamin E or extract, there were no significant difference in the conjugated bilirubin concentration. But the CCl_4 group treated with vitamin E was observed to have significantly (P=.05) lower concentration of total and unconjugated bilirubin than the CCl_4 group treated with the extract (Fig. 2). There was no significant difference in the concentration of conjugated bilirubin of the extract, vitamin E only treated groups and the normal control. Also, there was no significant difference in the concentration of total and unconjugated bilirubin of the groups treated with extract and vitamin E only (Fig. 2).

3.1.3 Serum urea and creatinine concentration

The levels of urea and creatinine in the CCl_4 group were significantly (P=.05) higher than in every other group (Table 1), but treatment with extract or vitamin E caused a significant (P=.05) reduction. However, as with the groups treated with extract and vitamin E only, there was no significant difference in the levels of urea and creatinine in the CCl_4 groups treated with either extract or vitamin E.

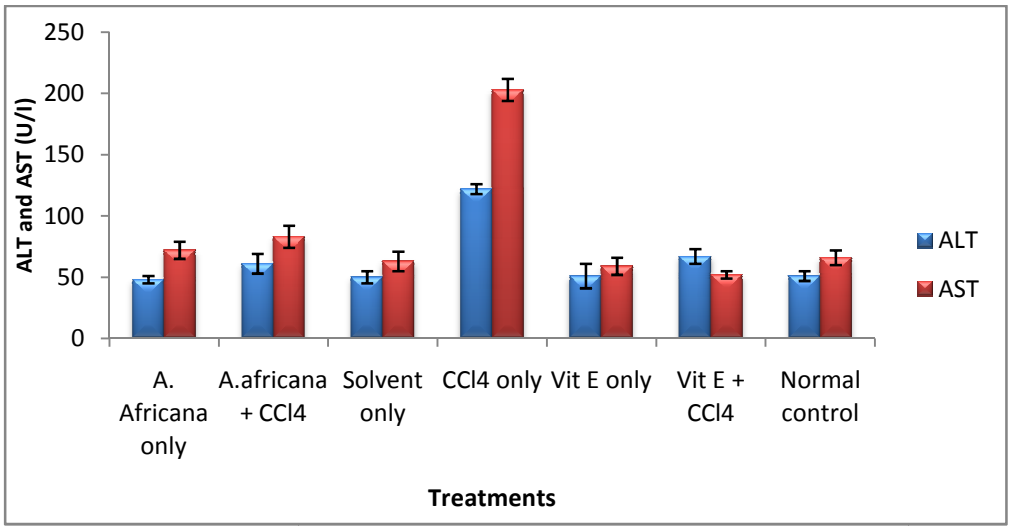

Fig. 1. Levels of rat serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in serum after 10 days following daily intraperitoneal injection of *A. africana* extract 5mg/kg with 72 hourly carbon tetrachloride intoxication

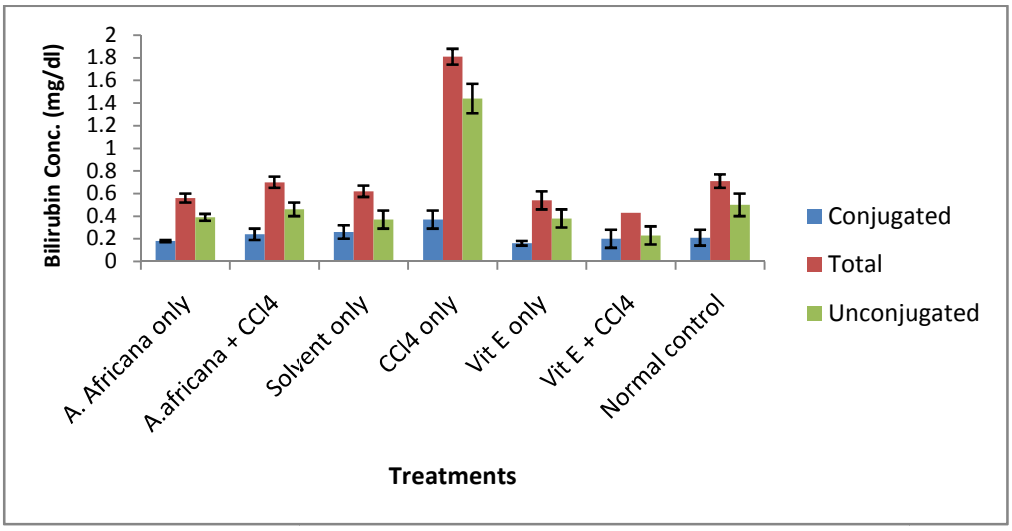

Fig. 2. Bilirubin concentrations in serum of rats after 10 days following daily intraperitoneal injection of *A. africana* methanolic extract 5mg/kg with 72 hourly carbon tetrachloride intoxication

3.2 Results of the Acute Liver Injury Model

3.2.1 Serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) Activities

The group of rats administered CCl_4 were observed to have significantly (P=.05) higher levels of ALT compared to the untreated control group. However, treatment with the extract was observed to significantly lower these levels, although not as much as the group treated

with the vitamin E, the reference antioxidant controls (Fig. 3). But, there was no significant difference between ALT activities of the groups treated with extract and CCl_4 control. Similarly, there was no significant difference in the AST activities of the groups administered extract only, vitamin E only and the normal control (Fig. 3).

Table 1. Mean creatinine and urea concentration in serum of rats after 10 days following daily intraperitoneal injection of *A. africana* extract 5mg/kg with72 hourly carbon tetrachloride intoxication

Urea and creatinine conc mg/dL			
Group	**Treatment**	**Urea**	**Creatinine**
1	*A. Africana* only	68 ± 3^a	1.2 ± 0.2^a
2	*A. africana* + CCl_4	71 ± 5^a	1.4 ± 0.3^a
3	Solvent only	87 ± 7^b	1.2 ± 0.1^a
4	CCl_4 only	130 ± 9^c	2.4 ± 0.3^b
5	Vit E only	70 ± 6^a	1.2 ± 0.1^a
6	Vit E + CCl_4	82 ± 7^b	1.2 ± 0.2^a
7	Normal control	85 ± 4^b	1.0 ± 0.0^a

Values are Mean±SD; Values having different letters across the column are significantly different (p<<0.05)

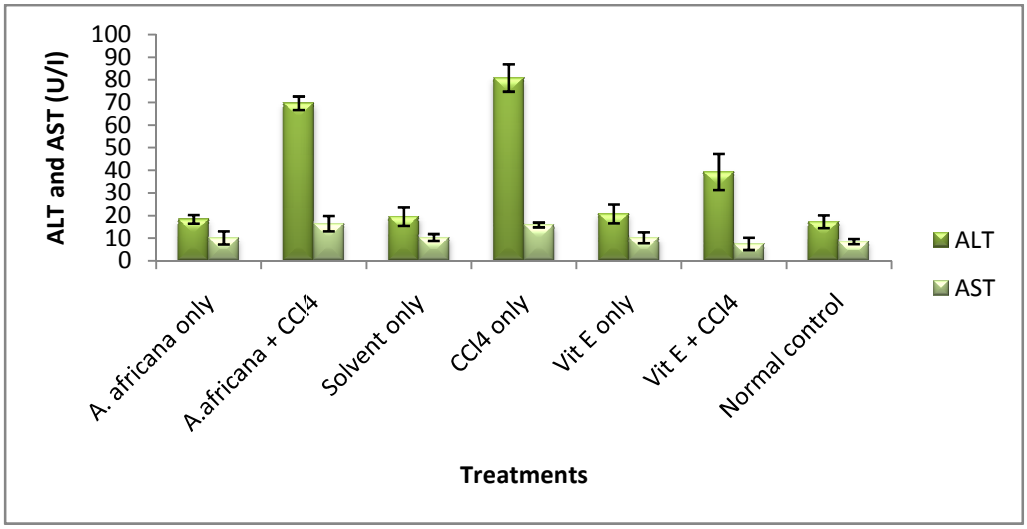

Fig. 3. Mean ALT and AST concentration in serum of rats on CCl_4 intraperitoneal intoxication following two day methanolic extract of *A. africana* seed pre-treatment (10mg/kg)

3.2.2 Serum total, conjugated and unconjugated bilirubin concentration

The levels of conjugated, total and unconjugated bilirubin in the CCl_4 only group were significantly (*P*=.05) higher than the groups treated with either vitamin E or extract, but there were no significant difference in the levels of conjugated, unconjugated and total bilirubin between the groups intoxicated that were either treated with vitamin E or the extract. However, the group administered extract alone had significantly lower concentration of total and conjugated bilirubin than the normal control group (Fig. 4).

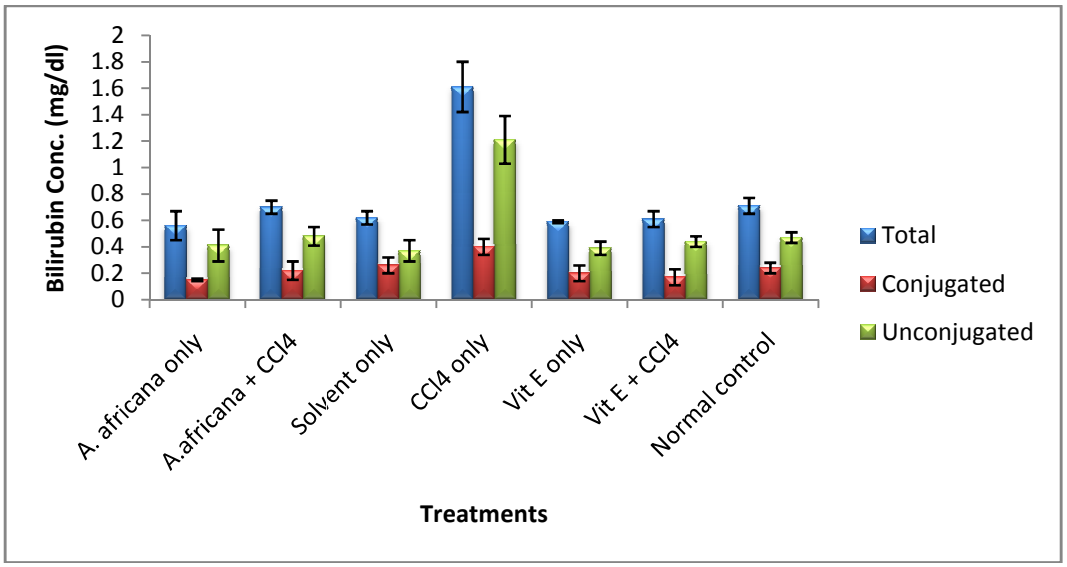

Fig. 4. Mean bilirubin concentration (total, conjugated and unconjugated) in rat serum on CCl_4 intraperitoneal injection following two day pre-treatment with methanolic extract of *A. africana* seed (10mg/kg)

3.2.3 Serum urea and creatinine concentration

Table 2 shows that there were significantly (*P*=.05) elevated levels of urea and creatinine in the CCl_4 control compared to the untreated control, but treatment with extract and CCl_4 was observed to have significantly high concentration of urea than the normal control group. However, there was no significant difference between the creatinine levels in the CCl_4 groups treated with either extract or vitamin E, and the normal control. Similarly, there was no significant difference between the urea and creatinine levels in the groups treated with extract and vitamin E only.

Table 2. Mean creatinine and urea concentration in serum of rats on intraperitoneal CCl_4 injection following two day pre-treatment with methanolic extract of *A. africana* seed (10mg/kg)

	Urea and creatinine concentration mg/dL		
Group	**Treatment**	**Urea**	**Creatinine**
1	*A. africana* only	43 ± 5^{abc}	1.2 ± 0.1^{a}
2	*A. africana* + CCl_4	59 ± 6^{c}	1.6 ± 0.2^{a}
3	Solvent only	32 ± 3^{a}	1.2 ± 0.2^{a}
4	CCl_4 only	92 ± 7^{d}	2.2 ± 0.3^{b}
5	Vit E only	46 ± 6^{abc}	1.2 ± 0.1^{a}
6	Vit E + CCl_4	54 ± 7^{bc}	1.2 ± 0.2^{a}
7	Normal control	37 ± 4^{ab}	1.0 ± 0.0^{a}

Values are Mean±SD; Values having different letters across the column are significantly different (P=.05)

4. DISCUSSION

In this investigation, carbon tetrachloride (CCl_4) was used as a model toxicant to induce organ injuries [12], because, upon metabolic activation, the reactive metabolite trichloromethyl radicals (.CCl_3) formed by cytochrome $P_{-450.}$ reacts rapidly with oxygen,, under high oxygen tension, generating many orders of magnitude of more reactive free radicals ($CCl_3OO.$). These free radicals initiate peroxidation of membrane polyunsaturated fatty acids and covalently bind to microsomal lipids and proteins [13] of important tissues and organs like the liver and kidney, thus damaging them. Like the reference antioxidant, Vitamin E used as the positive control, the capacities of the extract or any of its constitutents to prevent or ameliorate these CCl_4-induced injuries form the basis of this experimental model.

High level of bilirubin in the serum (Fig. 2) suggests haem degradation resulting from destruction of red blood cells [14] resulting from CCl_4 toxicity. Therefore, the significant (P=.05) decrease in the levels of bilirubin in *A. africana* extract-treated group suggests the potency of the extract to alleviate or protect damage to red blood cell membrane.

The extent of hepatic damage is assessed by the levels of serum marker enzymes like aspartate aminotransferase (AST) and alanine aminotransferase (ALT) which were significantly lowered by administration of *A. africana* methanolic extract (Figs. 1 and 3). This suggests the hepatoprotective potential of *A. africana* seed, since the serum level of AST and ALT are increased in good correlation with the severity of hepatic injury [14]. Similarly, bilirubin is excreted by the liver; hence its level in the blood is an index of liver function [14]. Thus, serum bilirubin (total, direct and indirect) levels which were also significantly (P=.05) elevated by CCl_4 treatment, but remarkably reduced by pretreatment or treatment with *A. africana* methanolic extract or vitamin E (Figs. 2 and 4) strongly suggest that the seed extract protected and ameliorated the rat liver against CCl_4-induced damage as much as vitamin E did.

Direct or indirect exposure of kidney to nephrotoxic agents may result in ultra-structural damage to any of the principal components of the nephron [15]. That the CCl_4 control group showed significantly (P=.05) higher levels of urea and creatinine (Tables 1 and 2) was an indication of kidney damage. Blood urea, which is derived from normal metabolism of protein and excreted in the urine is usually elevated in blood when there is glomerular damage. Similarly, creatinine, a metabolite of creatine, which under normal condition is excreted completely in the urine via glomerular filtration, accumulates in the blood, as observed in CCl_4 treated rats, only when there is impaired kidney function [16]. Thus, that the *A. africana* extract-treated group showed significantly (P=.05) lower concentration of creatinine and urea suggest the potency of the seed in protecting the kidney against or restoring it from oxidative damage.

Based on this experiment, the constituents of *A. africana* seeds responsible for the observed organ protective effects cannot be listed with degree of certainty. However, considered against the backdrop of our earlier report that methanolic extracts of the seed possessed significant in vivo antioxidant effect [17], and it also produced result similar to that of vitamin E, a compound known for its potent antioxidant activity, it can reasonably speculated that polyphenols, widely reported in some other Nigerian plants [18,19] may, at least, in part, be responsible for the observed effects.

5. CONCLUSION

Put together, these results strongly suggest that regular consumption of *A. africana* seeds, as done in many parts of southern and central Nigeria may contributes significantly to the capacity of the populace to protect their vital organs, especially the liver and the kidney against acute and chronic injuries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Souri E, Amin G, Farsam H. Barazandeh TM. Screening of antioxidant activity and phenolic content of 24 medicinal plant extracts. DARU. 2008;16(2):83-87.
2. Atawodi SE. Antioxidant potential of African medicinal plants. Afri J Biotechnol. 2005;4(2):128-133.
3. Ejikeme PM, Obasi LN, Egbuonu ACC. Physico-chemical and toxicological studies on *Afzelia africana* seed and oil. Afri J Biotechnol. 2010;9(13):1959-1963.
4. Moctar S. *Afzelia africana* Seed leaflet. 2007;118. Available: www.kew.org/msbp.
5. Eddy NO, Udoh CL. Proximate Evaluation of Nutritional value of some soup thickners. Chem Class J. 2005;2:12-14.
6. Ojiako OA, Igwe CU, Agha NC, Ogbuji CA, Onwuliri VA. protein and Amino acid Compositions of *Sphenostyles stenocarpa, Sesamum indicum, Monodora myrstica* and *Afzelia africana* seeds from Nigeria. Pak J Nutr. 2010;9(4):368-372.
7. Akinpelu DA, Aiyegoro OA, Okoh AI. *In vitro* antimicrobial and phytochemical properties of crude extract of stem bark of *Afzelia africana*. Afri J Biotechnol. 2008;7(20):3665-3670.
8. Diedrichs A. Schmittmann B. The Seeds of *Afzelia africana*. Zeitsch. Unters. Nahr. Genussm. 1922;44:215-216.
9. Akram J, Mohammad JK, Zahra D, Hossein N. Hepatoprotective Activity of *Cichorium intybus* L. Leaves Extract Against Carbon Tetrachloride Induced Toxicity. Iran J Pharmaceut Res. 2006;1:41-46.
10. Sherine MR, Safinaz SI. Attenuation of N-nitrosodiethylamine-induced liver carcinogenesis in rats by naturally occurring diallyl sulfide. Afri J Biochem Res. 2008;2(10):197-205.
11. Stephen OA, Abdulkadir AS, Oladepo WD, Thajasvarie H. Effect of Melantonin on carbon tetrachloride induced kidney injury in wistar rats. Afri J Biomed Res. 2007;10:153-164.
12. Milena Nikolova M, Evstatieva L, Th uan Duy Nguyen TD. Screening of plant extracts for antioxidant properties. Botan Serb. 2011;35(1):43-48.
13. Catalá G. Lipid peroxidation modifies the picture of membranes from the “Fluid Mosaic Model” to the “Lipid Whisker Model” Biochim. 2012;94:101-109.
14. Colleen S. Allan MD. Micheal L. Marks Basic Medical Biochemistry: A Clinical Approach. Second Edition. 2003;453.
15. Barile FA. Clinical Toxicology: Principles and Mechanisms. CRC Press Boca Raton London New York Washington, D.C. 2004;233-243.
16. Frank CLu. Lu’s Basic Toxicology. Taylor & Francis, 11 New Fetter lane, London. Fourth Edition. 2002;38-97.

17. Atawodi SE, Iliemene UD, Onyike E. Effect of Methanol Extract of *Afzelia africana* Sm Seed Treatment on Acute and Chronic Liver Injury. Intern J Agric Biol 2014;16(3):597–602.
18. Atawodi SE, Atawodi JC, Idakwo GA, Pfundstein B, Haubner R, Wurtele G, Spiegelhalder B, Bartsch H, Owen RW. Polyphenol Composition and Antioxidant Potential of *Hibiscus esculentus* L.Fruit Cultivated in Nigeria. J Med Fd. 2009;12(6):1316-1320.
19. Atawodi SE. Polyphenol Composition and in vitro Antioxidant Potential of Nigerian *Canarium schweinfurthii* Engl. Oil. Adv Biol Res. 2010;4(6):314-322.

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