Chronic Toxicological Evaluation and Reversibility Studies of Moringa Oleifera Ethanolic Seed Extract in Wistar Rats

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Abstract

This study evaluated the chronic toxicity and reversibility Potential of *Moringa oleifera* seed in Rats. Forty-four Wistar rats were randomized into four groups of 11 rats each, three groups were treated with doses of 50, 200, and 800 mg/kg of MOESE *p.o*, for 90 days while one group served as control and was administered distilled water *p.o*. After 90 days, half of the animals were sacrificed and blood samples collected for determination of biochemical and haematological parameters; antioxidants and *malondialdehyde (MDA*); sperm count, motility and morphology. Organs were harvested for weight determination and histopathological assessments the liver. The other half were left for another 30 days without treatment with the extract, after which they were sacrificed. MOESE produced significant reduction in food and water intake, relative weights of liver, heart, testes, lung and spleen at all treatment dose, these changes were overall small magnitude, within the range of historical control. There was significant increase in AST (200 and 800 mg/kg), LDH (200 mg/kg), ALP (800 mg/k), albumin (800 mg/kg) and K^+ (800 mg/kg), but decrease in serum Ca^{2+} (50 and 200 mg/kg). A significant increase in serum antioxidant was observed at all treatment doses, while semen pH were reduced. These effects on organ relative weights and all biochemical parameters except AST and ALP were reversed after 30 days of the reversibility study. Histopathological presentations showed necrosis in the liver (50 and 200 mg/kg). Our study demonstrated that ethanol extract of *Moringa oleifera* seeds could cause infertility in male rats due to decreased semen pH. Our results also showed that the extract may be hepatotoxic due to persistent high level of AST and ALP even after cessation of exposure to the extract, this is also consistent with the histopathological results that shows hepatic necrosis associated with chronic exposure to the extract.

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Introduction

Many plants have shown very effective medicinal values for various diseases in humans. As a result, pharmacological evaluation of medicinal plants has recently witnessed a growing interest amongst researchers worldwide. Studies have shown that several of the pharmaceuticals currently available have had a long history of use as herbal remedies.[1] Quinine, antimalarial drug from cinchona bark;[2] digoxin and digitoxin drugs for heart failure and atrial dysrhythmias from digitalis leaves[1,3] are examples of the contributions of traditional pharmacopoeia. In Nigeria, despite immense progress in the delivery of health care at the primary health care level, an estimated 75 percent of the population still prefers to solve their health problems by consulting the traditional healers.[4] In addition, herbal medicines are extensively used in the developing world where they offer a more affordable alternative to pharmaceutical drugs.

Moringa oleifera belongs to the family of moringaceae, a single genus with 14 known species.[5] It is a fast growing drought resistant tree native of sub-Hamalayan tracts of Northern India but now distributed worldwide in the tropics and sub tropics[6,7] including Nigeria. The seed extracts of *Moringa oleifera* in particular have been reported to possess anti-inflammatory, antioxidant, anticancer, antimicrobial, Hypolipidaemic, antiulcer, antidiabetic, diuretic and hepatoprotective activities.[8-13]

Various studies have been conducted to investigate biological activities and efficacy of *Moringa oleifera* seed extracts,[14,15] while little attention is being paid to its toxicity studies. Most of the studies on toxicity of *Moringa Oleifera* were focused on acute and sub-chronic toxicity[14] and none of the studies has a report on reversibility of any toxic effect observed, from MOESE. This study therefore aims to investigate the chronic toxicity of *MOESE* and possibility of reversal of any toxic effect produced after 30 days of none treatment

Materials and methods

2.1 Identification and Preparation of Seeds Extract:
Moringa oleifera seed was bought from Avuco farms company Ltd, in Kaduna State Northwest Nigeria in

February (2015) and authenticated by a plant taxonomist Professor F.A Oladele of the Department of Plant Biology, University of Ilorin. The plant material was stored in the herbarium of the same department with a voucher number UIH/011/1011.

The seed husk was removed to get the seeds that were then crushed with a clean mortar. One kilogram of the crushed seeds of *Moringa oleifera* was defatted by macerating with 5L of Petroleum ether (BDH) at room temperature and allowed to extract for 72h with intermittent shaking (model 3017 rotary shaker. GFL, Burgwedel, Germany). The solution was filtered and the defatted seed was then re-extracted with absolute ethanol (BDH) for another 72hrs.The resultant extract was then freeze-dried to yield 32.3 g of semi-solid residue. The residue was reconstituted in distilled water and appropriate concentrations were used in the experiments.

2.2 Preliminary Phytochemical Screening

The qualitative analyses of the plants constituents were carried out using the methods described by Harbone.[16,17] The presence of alkaloids, glycosides, tannins, saponins, terpenoids, ?avonoids, glycosides, anthraquinones, volatile oil, and steroids were tested.

Experimental Animals

Forty-four (32 males and 12 females) healthy and matured albino rats (*Rattus novergicus*) weighing 120-170g were obtained from Animal Housing facility of the Biological Garden of the University of Ilorin, Ilorin, Kwara state, Nigeria. The rats were housed in polycarbonate cages (TECHNIPLAST cages, Italy) in the Animal House facility of the Faculty of Basic Medical Sciences, University of Ilorin, and were maintained under standard conditions with a 12-hour light/dark cycle. A certified rodent chow (Oluwagbemisola feeds) and drinking quality water were available *ad libitum*. Animal use procedures were approved by the Animal Care and Use Committee of the University of Ilorin. Animal care and use was conducted in accordance with the principals stated in the Guide for the Care and Use of Laboratory Animals and in accordance with all applicable regulations. The study was performed in accordance with National Institute of Health (NIH) guidelines for the care and use of laboratory animals.[18]

Single Dose Toxicity Study

A single dose study of *Moringa oleifera* ethanolic seed extract (MOESE) was carried out using Lorke's method.[19] A total of 12 healthy and mature male mice, without food for 3-4 hours, were used for the study. In the first phase, 9 of the mice were equally distributed into 3 groups and administered 10mg/kg, 100mg/kg and 1000mg/kg of the MOESE intraperitoneally (*i.p.*), respectively. Mice were generally observed for toxic symptoms and behavioural changes (Sedation, hyperactivity, diarrhea, writhing, restlessness etc.) for 2h post-administration and mortality for 24h post-administration. In the second phase, 3 mice were given different doses of 1600, 2900, and 5400 mg/kg also intraperitoneally (*i.p.*). They were also observed for symptoms of toxicity and mortality within 24h post-administration. The median lethal dose was calculated using the formula: $LD_{50} = \sqrt{(D_0 \times D_{100})}$; where D_0 is the highest dose that did not cause mortality, and D_{100} is the lowest dose that caused mortality.

Repeated Dose Toxicity Study

A total of 44 rats were randomly allotted to 4 groups of 8 male (140–170g) and 3 female (120–140g) animals each housed separately in polycarbonate cages. The animals were daily treated p.o. with distilled water (control) and MOESE at doses of 50, 200, and 800 mg/kg (representing one-fourth of the pharmacologically active dose, the pharmacologically active dose, and four times the pharmacologically active dose respectively[20] for 90 days. All treatments were administered via oral gavage.

All animals were observed daily for clinical signs (hyperactivity, diarrhea, depression, behavioral changes, etc.). Food and water intake were measured daily while the animals' body weights were taken pre-exposure, and weekly during exposure. At the end of the 90 day treatment period, 5 rats from each group were comatosed by cervical dislocation. Blood samples were collected from the rats via the descending aorta and cardiac puncture, into non-heparinized tubes for determination of biochemical and antioxidant parameters, and into EDTA (ethylenediamine-tetra acetate) bottles for haematological parameters, respectively. Semen was obtained from the epididymis of the male animals for semen analysis. Vital organs including the heart, lungs, spleen, kidneys, lungs, testes and pancreas were excised, carefully observed for gross lesions, and weighed. The organs were then preserved in 10% formaline for histopathological assessment. Mortality in each treatment group was recorded during the course of the 90 day administration of MOESE.

Reversibility Study

The remainders of the animals were left without MOESE administration for thirty (30) days for the reversibility studies, with food and water *ad libitum*. At the end of the 30 Days, rats were comatosed by cervical dislocation, and assays conducted in the main study were carried out.

Biochemical Analysis

The blood samples collected were allowed to clot, and then centrifuged at 3000rpm for 5 min to

obtain the serum. The serum was analyzed for liver function parameters (AST, ALT, ALP, albumin, total bilirubin and conjugated bilirubin), kidney function parameters (creatinine, urea, glucose and uric acid) and serum lipid profiles (total cholesterol, HDL, LDL and triglycerides) using commercial kits (Randox Laboratories Ltd., United Kingdom). Total protein concentration was determined using biuret method.[21,22] Serum electrolytes concentrations were determined by established methods: sodium and potassium concentration by flame photometry, chloride and bicarbonate concentration by titrimetric method, and calcium concentration by cresol-phthalein complexone method.[23-25]

Determination of *in vivo* antioxidants and malondialdehyde (MDA) levels

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA) were determined according to established methods,[26] using standard kits (Biorex Diagnostics, United Kingdom).

Haematological Analysis

Blood samples were analyzed using established procedures by automated haematology analyzer. Parameters analyzed include packed cell volume (PCV), red blood cell (RBC) count, haemoglobin (Hb), platelet count, total and differential white blood cell (WBC) count, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV), and mean cell haemoglobin (MCH).

Sperm Analysis

Sperm analysis to assess seminal fluid for motility, count and morphology was carried out according to the methods of Cheesbrough, 2000[27] and Ogli et al. 2009.[28] Male rats were comatosed and strapped astride on their back on dissecting board. The testis was removed with its ipsilateral epididymis into a beaker after incision on the right scrotum. Subsequently, semen was expelled out of the epididymis into a beaker placed in water bath at 36°C.[28] (Ogli *et al*., 2009).

Sperm Motility

Semen (10–15 µl) was placed on a slide in a way that the spermatozoa were evenly distributed and covered with a glass. After appropriate focus, several fields of the specimen were assessed for motility using the 40 × objective of the microscope. The number of motile cells was noted out of a total of 100 spermatozoa.

Sperm Count

Using sodium bicarbonate-formalin diluting fluid, a 1 in 20 dilution of semen was carried out with thorough mixing. An improved Neubauer ruled chamber was filled with well-mixed diluted semen using a Pasteur pipette. The number of spermatozoa in an area of 2 sq mm was counted using the 10 × objective of the microscope after 3–5 min. Estimation of the number of spermatozoa in 1 ml of fluid was done by multiplication of the number counted by 100 000.

Sperm Morphology

A thin smear of the liquefied well-mixed semen, made on a slide, was fixed with 95% v/v ethanol while still wet for 5–10 min. This was allowed to air-dry after which it was washed with sodium bicarbonate-formalin solution. This was to remove any present mucus. Rinsing with water was carried out many times. Subsequently, the smear was covered with dilute carbon fuchsin (1 in 20). This was allowed to stain for 3 min and the stain was washed off with water. Dilute Loeffler's methylene blue (1 in 20) was used to cover the smear for 2 min to achieve counterstaining. This was washed off with water, drained and allowed to dry. Normal and abnormal spermatozoa were examined using the 40 × objective of the microscope. Estimation of percentage normal and abnormal morphology was done from the counting of hundred spermatozoa.

Histopathological Study

Liver obtained from experimental animals were fixed in 10% formol-saline were dehydrated in graded alcohol, embedded in paraffin, and cut into 6µm thick sections. Hematoxylin-eosin was used to stain the sections for photomicroscopic assessment.[29] Slides were examined using the × 40, × 100, and × 400 objectives.

Statistical Analysis

Data were expressed as the mean ±SEM and statistically analyzed by one-way analyses of variance (ANOVA). Comparisons between control and treatment groups were made by LSD and Dunnet's post-test, using SPSS (version 17) statistical software. Values of $p \leq 0.05$ were considered significant.[30]

Results

Acute Toxicity

No mortality and visible signs of toxicity were observed upon administration of MOESE *i.p* at doses of 10, 100 and 1000mg/kg using Lorke's method. Writhing, grooming, and decreased locomotor activity were the behavioral manifestations observed at 1600 and 2900 mg/kg Mortality was recorded at the highest dose of 5400mg/kg within an hour of administration, with the LD_{50} estimated to be 3957.27mg/kg.

Effect of MOESE on Body and Organ Weights

The change in body weight of the male and female rats treated with MOESE doses (50, 200 and 800 mg/kg b.w) for 90 days are presented in Figures 1 and 2 respectively. In respect to male rats, MOESE at the dose

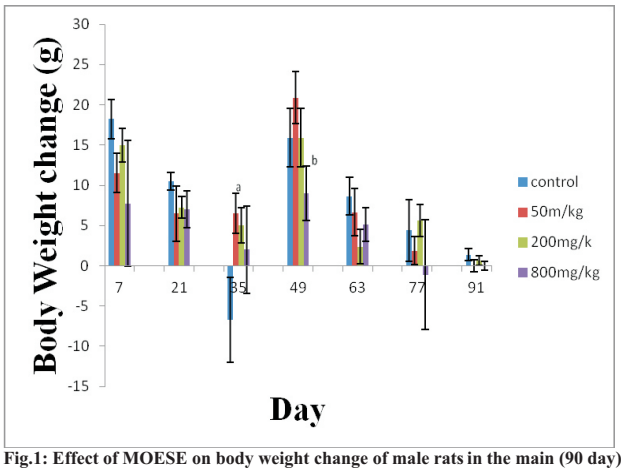

Fig.1: Effect of MOESE on body weight change of male rats in the main (90 day) study. Values are mean ± SEM. (n = 8).

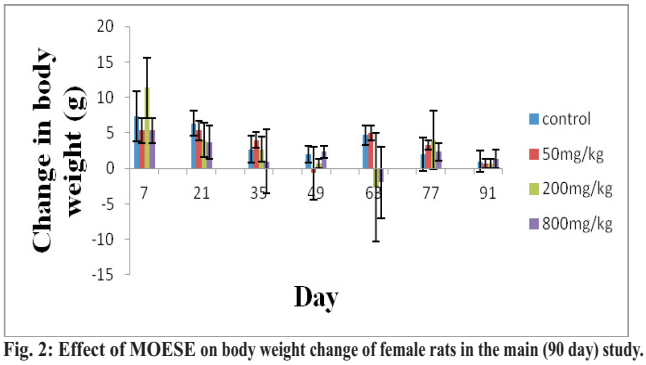

Fig. 2: Effect of MOESE on body weight change of female rats in the main (90 day) study. Values are mean ± SEM. (n = 3).

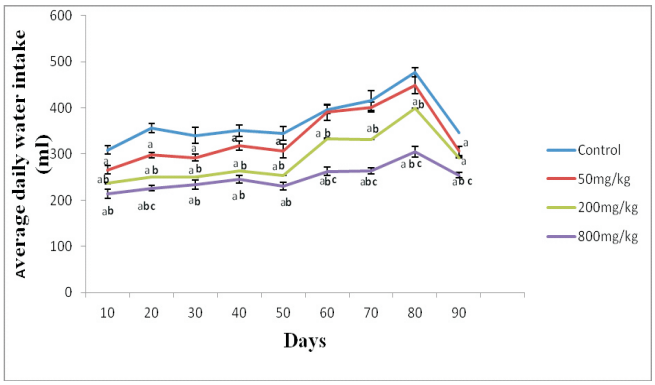

Fig. 4: Effect of MOESE on daily water intake of rats in the main (90 day) study. Values are mean ± SEM. (n = 11).

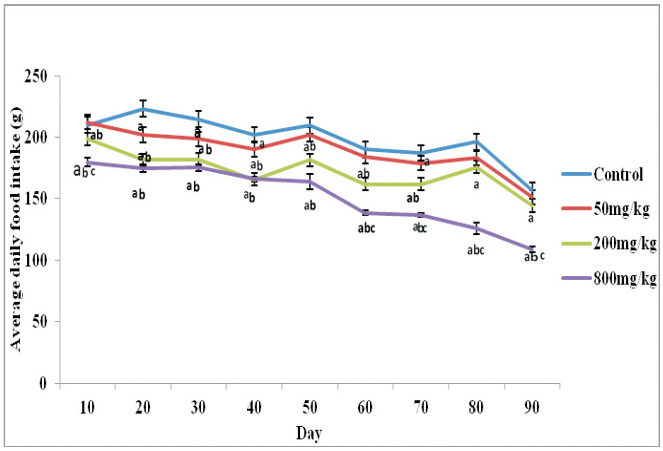

Fig. 3: Effect of MOESE on daily food intake of rats in the main (90 day) study. Values are mean ± SEM. (n = 11).

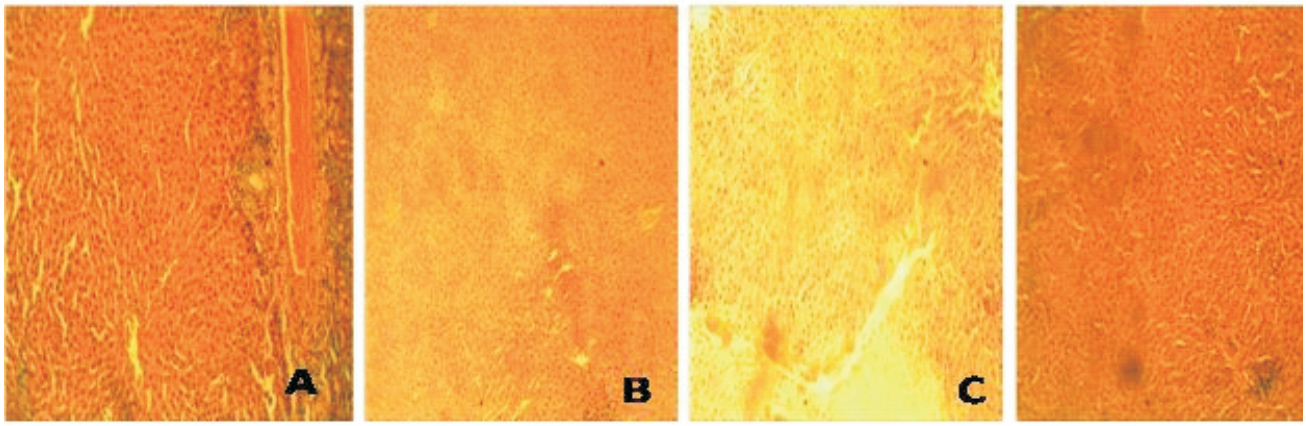

Figure 5. Photomicrograph of transverse section of the liver of rats administered v distilled water and ethanol extracts of *M. oleifera* seed for 90 days. A: Distilled w treated liver shows normal hepatic plates and hepatocyes with normal histolog features and intact stroma; B: 50 mg/kg body weight ethanol extract of *M. oleifera* tre liver shows dilated blood vessels and areas of marked hepatic necrosis; C: 200 mg/kg b weight ethanol extract of *M. oleifera* treated liver shows dilated blood vessels, sinuso haemorrhage and extensive areas of necrosis; D: 800 mg/kg body weight ethanol extrac *M. oleifera* treated liver shows dilated blood vessels and extensive areas of necrosis. H magnification 400×.

Table 1: Effect of MOESE on relative organ weight of rats in the main (90 day) study

Dose (mg/kg)	Liver	Kidney	Lungs	Heart	Testes	Spleen	Pancreas	Brain
Control	3.01±0.02	0.29±0.01	0.79±0.02	0.32±0.00	0.74±0.08	0.39±0.01	0.25±0.02	0.68±0.03
50	2.66±0.06[a]	0.28±0.01	0.71±0.02[a]	0.29±0.01	0.82±0.01	0.29±0.01[a]	0.28±0.02	0.70±0.01
200	2.53 ±0.02[ab]	0.27±0.00	0.57±0.01[ab]	0.27±0.01[a]	0.48±0.01[ab]	0.31±0.01[a]	0.36±0.01[ab]	0.77±0.04[a]
800	2.79±0.01[abc]	0.29±0.01	0.59±0.02[ab]	0.31±0.01[c]	0.49±0.02[ab]	0.27±0.02[a]	0.29±0.01[c]	0.65±0.01[c]

Values are mean ± SEM. (n = 5).

Table 2: Effect of MOESE on organ weight of rats in the reversibility study

Dose (mg/kg)	Liver	Kidney	Lungs	Heart	Testes	Spleen	Pancreas	Brain
Control	2.85±0.03	0.24±0.01	0.84±0.05	0.31±0.00	0.98±0.05	0.33±0.02	0.37±0.06	0.74±0.03
50	2.86±0.06	0.28±0.01[a]	0.76±0.02	0.30±0.00	0.94±0.04	0.33±0.00	0.44±0.03	0.88±0.08
200	2.76±0.04	0.26±0.00[a]	0.79±0.02	0.33±0.01	0.99±0.04	0.20±0.10	0.32±0.03	0.96±0.02[a]
800	2.77±0.11	0.29±0.00[a]	1.20±0.31	0.30±0.01	1.04±0.03	0.31±0.01	0.29±0.01	0.86±0.02

Values are mean ± SEM. (n = 5).

Table 3: Effect of MOESE on liver function parameters in rats in the main (90 day) study

Dose (mg/kg)	AST (u/L)	ALT (u/L)	ALP (u/L)	Protein (g/L)	Albumin (g/L)	TB (µmol/l)	CB (µmol/L)
0	234.33 ±1.20	47.33 ±1.20	84.67 ±1.76	47.33 ±0.67	18.33 ±0.33	8.33 ±0.33	0.23 ±0.03
50	223.00 ±21.00	46.00 ±2.65	84.33 ±0.88	52.00 ±1.53	20.67 ±1.20	8.33 ±0.67	0.367 ±0.03
200	356.33 ±22.66[ab]	45.00 ±2.65	81.33 ±1.33	48.00 ±0.58	20.33 ±0.33	9.00 ±0.58	0.33 ±0.03
800	330.00 ±2.89[ab]	47.33 ±0.67	91.00 ±0.58[abc]	52.33 ±3.18	21.67 ±1.45[a]	8.33 ±0.33	0.23 ±0.07

Values are mean ± SEM. (n = 5).

Table 4: Effect of MOESE on liver function parameters in rats in the reversibility study

Dose (mg/kg)	AST (u/L)	ALT (u/L)	ALP (u/L)	Protein (g/L)	Albumin (g/L)	TB (µmol/l)	CB (µmol/L)
0	139.67 ±0.88	42.33 ±2.19	90.33 ±1.33	66.67 ±1.45	23.00 ±0.00	0.57 ±0.03	0.20 ±0.05
50	137.33 ±0.33	37.00 ±0.00	92.00 ±1.00	69.00 ±2.08	25.67 ±0.88[a]	0.53 ±0.08	0.23 ±0.03
200	268.67 ±0.67[ab]	41.00 ±0.58	90.33 ±1.45	65.33 ±2.85	24.00 ±1.00	0.57 ±0.03	0.23 ±0.03
800	282.67 ±2.03[abc]	39.67 ±2.40	99.00 ±0.58[abc]	61.33 ±1.76[b]	25.00 ±0.00	0.73 ±0.12	0.23 ±0.03

Values are mean ± SEM. (n = 5).

Table 5: Effect of MOESE on serum kidney function parameters in rats in the main (90 day) study

Dose (mg/kg)	Creatinine (µmol/L)	Urea (mmol/L)	Glucose (mmol/L)	Uric acid (mmol/L)
0	46.33 ± 1.45	8.30 ± 0.15	4.20 ± 0.15	0.38 ± 0.03
50	49.33 ± 2.03	8.23 ± 0.12	4.13 ± 0.15	0.44 ± 0.04
200	48.33 ± 2.40	8.47 ± 0.09	4.30 ± 0.15	0.51 ± 0.04
800	48.33 ± 1.67	8.47 ± 0.07	4.67 ± 0.39	0.43 ± 0.06

Values are mean ± SEM. (n = 5).

Table 6: Effect of MOESE on serum electrolytes in rats in the main study

Dose (mg/kg)	Na (mmol/L)	K (mmol/L)	Cl^- (mmol/L)	HCO_3^- (mmol/L)	Ca (mmol/L)
0	133.67± 1.20	6.60± 0.15	88.67± 1.45	21.00± 0.58	3.14± 0.05
50	134.00± 2.31	6.97± 0.12	89.67± 2.60	19.00± 1.52	2.72± 0.19[a]
200	133.67± 0.33	6.77 ± 0.30	95.00± 1.00	20.33± 0.33	2.67± 0.10[a]
800	135.00± 1.15	7.30 ± 0.23[a]	95.00± 3.21	19.33± 0.88	2.82± 0.08

Values are mean ± SEM. (n = 5).

Table 7: Effect of MOESE on serum electrolytes in rats in the reversibility study

Dose (mg/kg)	Na (mmol/L)	K (mmol/L)	Cl^- (mmol/L)	HCO_3^- (mmol/L)	Ca (mmol/L)
0	135.00± 0.58	6.03± 0.83	98.67± 1.86	17.33± 0.33	2.68± 0.04
50	135.33± 1.20	6.97± 0.57	97.33± 0.88	18.67± 0.67	2.59± 0.09
200	135.00± 1.53	7.47± 0.30	89.67± 1.45[ab]	22.00± 0.58[ab]	2.74± 0.05
800	136.00± 1.73	7.10± 0.84	95.33± 1.76[c]	20.00± 0.58[ac]	2.67± 0.06

Values are mean ± SEM. (n = 5).

Table 8: Effect of MOESE serum lipid profiles of rats in the main study

Dose (mg/kg)	Trig (mmol/L)	Total chol (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
0	1.13 ± 0.07	3.97 ± 0.03	1.01 ± 0.07	2.60 ± 0.15
50	1.17 ± 0.03	4.00 ± 0.10	0.96 ± 0.05	2.73 ± 0.15
200	1.20 ± 0.11	4.23 ± 0.09[ab]	0.94 ± 0.12	3.17 ± 0.09[a]
800	1.17 ± 0.09	3.73 ± 0.03[abc]	1.03 ± 0.04	2.60 ± 0.09[c]

Values are mean ± SEM. (n = 5).

Table 9: Effect of MOESE on serum lipid profiles of rats in the reversibility study

Dose (mg/kg)	Trig (mmol/L)	Total chol (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
0	0.61 ± 0.03	4.63 ± 0.32	2.50 ± 0.25	2.50 ± 0.25
50	0.68 ± 0.12	4.43 ± 0.07	2.87 ± 0.03	2.87 ± 0.03
200	0.80 ± 0.02	4.43 ± 0.15	2.77 ± 0.15	2.77 ± 0.15
800	0.71 ± 0.10	4.40 ± 0.10	2.17 ± 0.27	2.17 ± 0.27

Values are mean ± SEM. (n = 5).

Table 10: Effect of MOESE on antioxidants and MDA levels in serum (90 day study)

Dose (mg/kg)	SOD (µl/ml)	GPX (µl/L)	MDA (µmol/L)
0	208.33±0.29	2399.70±45.11	3.07±0.07
50	277.67±5.55[a]	3025.30±43.56[a]	2.43±0.15[a]
200	229.33±4.84[b]	2561.00±21.03[ab]	2.13±0.15[a]
800	210.00±7.64[b]	3248.00±44.00[abc]	2.30±0.30[a]

Values are mean ± SEM (n=5).

Table 11: Effect of MOESE on antioxidants and MDA level in serum (30 day reversibility study)

Dose (mg/kg)	SOD (ul/ml)	GPX (ul/L)	MDA (umol/L)
0	204.33±5.81	2699.30±186.2	3.40±0.23
50	277.33±4.40[a]	3083.00±19.76	1.97±0.03[a]
200	309.00±3.06[ab]	3407.30±196.36[a]	2.03±0.03[a]
800	256.00±8.00[abc]	3186.00± 282.00	2.20±0.50[a]

Table 12: Effect of MOESE on hematological parameters in blood (90 day study)

Dose (mg/kg)	PCV (%)	WBC (10^9/L)	N (%)	L (%)	RBC (10^{12}/L)	Hb (g/dl)	PLT (10^9/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
0	36.67 ±0.33	5.51 ±0.47	69.00 ±0.57	30.33 ±0.33	6.26 ±0.06	15.77 ±0.22	255.00 ±24.70	59.00 ±0.00	26.43 ±0.13	43.93 ±0.68
50	36.67 ±0.33	6.87 ±0.79	69.67 ±0.33	30.00 ±0.33	6.27 ±0.11	15.83 ±0.37	359.67 ±25.34	56.33 ±0.88	25.53 ±0.18	44.73 ±1.14
200	35.00 ±1.15	4.95 ±0.38	69.67 ±0.33	30.00 ±0.00	6.24 ±0.08	17.20 ±0.17	312.00 ±50.14	55.33 ±0.33*	27.40 ±0.35	45.53 ±1.59
800	33.00 ±3.00	7.28 ±1.13	68.33 ±1.86	33.50 ±0.50	6.55 ±0.18	16.20 ±1.10	312.50 ±30.50	55.00 ±1.00*	27.00 ±0.20	48.95 ±0.65

Values are expressed as mean ± S.E.M. (n = 5).

Table 13: Effect of MOESE on some hematological parameters in blood (30 day reversibility study)

Dose (mg/kg)	PCV (%)	WBC (10^9/L)	N (%)	L (%)	RBC (10^{12}/L)	Hb (g/dl)	PLT (10^9/L)	MCV (fl)	MCH(pg)	MCHC (g/dl)
0	45.00 ±0.58	10.90 ±1.39	68.66 ±0.67	31.33 ±0.67	8.59 ±0.06	15.77 ±0.33	412.67 ±35.41	63.33 ±1.45	22.37 ±0.34	36.27 ±0.74
50	43.67 ±1.33	10.63 ±1.78	68.33 ±1.20	30.67 ±0.33	6.86 ±0.18	15.80 ±0.71	385.67 ±36.73	63.67 ±0.66	22.97 ±0.48	36.07 ±0.64
200	45.00 ±1.53	6.10 ±1.15	85.33 ±0.33*	35.33 ±0.33*	4.97 ±0.09*	15.33 ±0.79	404.33 ±31.15	64.67 ±1.33	21.20 ±0.49	30.33 ±0.23
800	38.50 ±0.50	5.99 ±0.29	65.50 ±0.50	34.50 ±0.50*	5.99 ±0.41	11.55 ±0.45	377.50 ±20.50	64.50 ±3.50	23.10 ±1.50	38.75 ±3.35

Values are expressed as mean ± S.E.M. (n = 5).

Table 14: Effect of MOESE on sperm motility, count, morphology and pH (90 day study)

Dose (mg/kg)	Sperm motility (%)	Sperm count (millions/ml)	Abnormality (%)	pH
0	75.33±7.75	39.67±0.67	7.20±1.72	4.70±0.20
50	66.67±6.67	42.33±0.88	8.33±2.01	4.20±0.06*
200	75.00±0.00	36.00±1.15	7.40±1.50	4.17±0.03*
800	57.50±2.50	41.00±6.00	5.50±1.71	4.25±0.05*

Values are expressed as mean ± S.E.M. (n = 5). $p \leq 0.05$ vs control
(One-way ANOVA followed by Dunnet posttest).

Table 15: Effect of MOESE on sperm motility, count, morphology and pH (30 day reversibility study)

Dose (mg/kg)	Sperm motility (%)	Sperm count (millions/ml)	Abnormality (%)	pH
0	80.00±7.63	53.33±4.67	2.67±1.67	4.23±0.12
50	80.00±2.89	63.33±6.57	3.00±1.67	4.53±0.29
200	75.00±5.00	51.33±6.22	2.90±1.67	4.50±0.25
800	70.00±10.0	48.50±0.50	2.80±2.50	4.30±0.20

Values are expressed as mean ± S.E.M. (n = 5). $p \leq 0.05$ vs control
(One-way ANOVA followed by Dunnet posttest).

of 50mg/kg caused statistically significantly increase ($p < 0.05$) in body weight on day 35, compared to control while a statistically significant reduction ($p < 0.05$) in body weight at dose of 800mg/kg on day 49 were observed, compared to 50mg/kg. However, in the female rats, no statistically significant differences in body weight were observed at all doses.

The relative organ weights of the rats are shown in Table 1. At doses 50, 200, and 800 mg/kg, there were statistically significant reduction ($p < 0.05$) in the relative organ weights of the liver, lungs and spleen, compared to control. There was also statistically significant reduction ($p < 0.05$) in weight of testes at doses of 200 and 800 mg/kg, compared to control and 50mg/kg groups, while the relative weight of the heart, at the dose of 200 mg/kg, was statistically significantly reduced ($p<0.05$), compared to control. There was a statistically significant increase ($p<0.05$) in the relative weights of pancreas and brain at the dose of 200 mg/kg compared to control respectively. When compared to 50 mg/kg, there was a statistically significant reduction ($p<0.05$) in the relative weight of liver (at dose of 200 mg/kg), lungs and testes (at the doses of 200 and 800 mg/kg), while a statistically significant increase ($p < 0.05$) in the weights of liver (at the dose of 800 mg/kg) and pancreas (at the dose of 200 mg/kg) were also observed when compared to 50mg/kg.

When compared with 200 mg/kg, there was a statistically significant increase ($p<0.05$) in weights of liver and heart, and significant reduction ($p<0.05$) in the weights of pancreas and brain, at the dose of 800 mg/kg respectively. The significant differences observed were reversed after 30 days of cessation of treatment (Table 2).

Effect of MOESE on Food and Water Intake

Food consumption and water intake of rats treated with MOESE doses (50, 200 and 800) for 90 days are presented in Figures 3 and 4 respectively. In respect of food intake, compared to control, MOESE produced significant reduction ($p < 0.05$) in average

daily food intake at dose of 50 mg/kg on days 20, 30, 40, and 70. The same trend of effect (significant reduction) was obtained ($p < 0.05$) at doses of 200 mg/kg and 800 mg/kg but throughout the treatment period. Compared to 50 mg/kg treatment group, there was significant reduction ($p < 0.05$) in average daily food intake at doses of 200 mg/kg and 800 mg/kg all through the treatment period except on days 80 and 90 when there was no significant difference ($p > 0.05$) when 200 mg/kg was compared to 50 m/kg. Compared to 200 mg/kg, dose of 800 mg/kg produced significant decrease ($p < 0.05$) in average daily food intake on days 10, 60 and 80.

Concerning water intake, compared to control, MOESE at the dose of 50 mg/kg produced significant reduction ($p < 0.05$) in average daily water intake on days 10, 20, 30, 40, 50, and 90. Also, there was significant reduction at doses of 200 mg/kg and 800 mg/kg throughout the treatment period (days 10, 20, 30, 40, 50, 60, 70, 80 and 90).

EFFECT OF MOESE ON BIOCHEMICAL PARAMETERS

Effect of MOESE on Liver Function Parameters

There was significant increase ($p < 0.05$) in the activity of aspartate aminotransferase (AST) in the groups treated with 200 mg/kg (356.33 ± 22.66 U/L) and 800 mg/kg (330 ± 2.89 U/L) when compared to control (234 ± 1.20 U/L) (Table 3). When compared to 50 mg/kg treatment group, there was significant increase ($p < 0.05$) in AST activity in the groups treated with 200 mg/kg and 800 mg/kg. There was also a significant increase ($p < 0.05$) in the activity of alkaline phosphatase (ALP) in the groups treated with 800 mg/kg (91.00 ± 0.58) when compared to control (84.67 ± 1.76). A similar trend of increase occurred when compared 800 mg/kg to 50 mg/kg (84.33 ±0.88) and to 200 mg/kg (81.33 ± 1.33) treatment groups. The concentration of albumin was significantly increased ($p < 0.05$) in the group treated with *M. oleifera* seed at the dose of 800 mg/kg (21.67 ± 1.45) when compared to control (18.33 ± 0.33). The effects on AST and ALP were not reversed after 30 days of cessation of administration of the ethanol extract of *M. oleifera* seed because the significant increase observed after 90 days administration still persisted (Table 4). The effect on albumin was, however, reversed after 30 days of cessation of administration of the extract (Table 4).

Effect of MOESE on Kidney Function Parameters

There was a no significant difference ($p > 0.05$) in serum levels of these parameters at all treatment doses when compared to the control group (Table 5).

Effect of MOESE on Serum Electrolytes Concentrations

There was a significant increase ($p < 0.05$) in concentration of potassium (K) in the group treated with 800mg/kg of the extract (7.30 ± 0.23 mmol/L) when compared to the control group (6.60 ± 0.15 mmol/L) (Table 6). There was significant reduction ($p < 0.05$) in calcium concentrations in the groups treated with 50 mg/kg and 200 mg/kg of the extract when compared to the control. The effects on potassium and calcium were reversed after 30 days of cessation of the extract administration, while chloride concentration was significantly reduced in 200 mg/kg treatment group and bicarbonate was significantly increased in 200 mg/kg and 800 mg/kg groups (Table 7).

Effect of MOESE on Lipid Profiles

There was a significant increase ($p < 0.05$) in the concentration of total cholesterol in the group treated with the extract at the dose of 200 mg/kg (4.23 ± 0.09 mmol/L) when compared to the control group (3.97 ± 0.03 mmol/L); the increment ($p < 0.05$) is also significant when compared to 50 m/kg treatment group (4.00 ± 0.10 mmol/L) (Table 8). A significant reduction ($p < 0.05$) in concentration of total cholesterol was obtained in the group treated with 800 mg/kg (3.73 ± 0.03mmol/L) when compared to the control group (3.97 ± 0.03 mmol/L) and to the 50 mg/kg (4.00 ± 0.10mmol/L) and 200 mg/kg (4.23 ± 0.09 mmol/L) treatment groups (Table 8) . There was also a significant increase ($p < 0.05$) in the concentration of HDL in the groups treated with 200 mg/kg (3.17 ± 0.09 mmol/L) of the extract when compared to the control group (2.60 ± 0.15 mmol/L). At the dose of 800 m/kg, there was a significant decrease ($p < 0.05$) in HDL concentration when compared to 200 mg/kg but not significantly different from the control group (Table 8). The effects on total cholesterol and HDL were reversed after 30 days of cessation of administration of the extract (Table 9).

Effect of MOESE on Serum Antioxidants and Malondialdehyde Levels

At doses of 200mg/kg and 800mg/kg, there was no significant difference in the level of superoxide dismutase (SOD) in serum. However, a significant increase ($p < 0.05$) in the level of glutathione peroxidase (GPX) was observed with 200mg/kg dose of MOESE (2561.00 ±21.03ul/L vs control value of 2399.70± 45.11ul/L) (Table 10). Also, there was a significant increase ($p < 0.05$) in GPX with the 800mg/kg dose of MOESE (3248.00± 44.00 vs control value of 2399.70± 45.71) (Table 10). Also, there was a significant decrease ($p < 0.05$) in the level of SOD in the group that received 200mg/kg dose of MOESE (229.33 ± 4.84 ul/ml) compared to the 50mg/kg dose of MOESE (277.67±5.55 ul/ml) (Table 10). A significant decrease ($p < 0.05$) in the level of SOD in the group that received 800mg/kg dose of MOESE (210.00 ± 7.64 ul/ml) when compared to the group that received

50mg/kg dose of MOESE (277.67±5.55 ul/ml) was also recorded (Table 10).

At the dose of 50mg/kg, there was a significant increase ($p < 0.05$) in the level of SOD (277.67±5.55 vs control value of 208.33 ± 9.28 ul/ml), as well as a significant increase ($p < 0.05$) in the level of glutathione peroxidase (GPX), (3025.30 ± 43.56 ul/L vs control value of 2399.70± 45.71ul/L) (Table 10). Also, there was a significant decrease ($p < 0.05$) in the level of GPX in the group that received 200mg/kg dose of MOESE (2561.00 ±21.03 ul/L) when compared to the group that received 50mg/kg dose of MOESE (3025.30± 43.56 ul/L) (Table 10). There was a significant increase ($p < 0.05$) in the level of GPX in the group that received 800mg/kg dose of MOESE (3248.00± 44.00 ul/L) when compared to the group that received 50mg/kg dose of MOESE (3025.30± 43.56 ul/L) (Table 10). There was also a significant increase ($p < 0.05$) in the level of GPX in the group that received 800mg/kg dose of MOESE (3248.00± 44.00 ul/L) when compared to the group that received 200mg/kg dose of MOESE (2561.00 ±21.03 ul/L) (Table 10).

There were significant decreases in the level of MDA across the treatment group after the 90 day period (Table 10). At the dose of 50mg/kg, MOESE caused a significant decrease ($p < 0.05$) in the level of MDA (2.43 ± 0.15 vs control value of 3.07± 0.07 umol/L) (Table 10). There was a significant decrease ($p< 0.05$) in the level of MDA in the group treated with MOESE at the dose of 200mg/kg (2.13 ±0.15 vs 3.07±0.07umol/L) compared with the distilled water treated group (Table 10). A significant decrease ($p< 0.05$) in the level of MDA was also observed in the group treated with MOESE at the dose of 800mg/kg (2.30 ± 0.30 vs contol value of 3.07± 0.07umol/L) (Table 10).

After a 30 day cessation of treatment, there were significant increases in the level of superoxide dismutase (SOD) enzyme in the serum across the treatment groups (Table 11); this suggests that the increase in free radical generation mediated by MOESE were retained even with cessation of therapy. At the dose of 50mg/kg, there was a significant increase ($p < 0.05$) in the level of SOD (277.33±4.40 vs 204.33±5.81ul/ml in control) (Table 11), at 200mg/kg, there was also a significant increase ($p < 0.05$) in the level of SOD (309.00±3.06 vs 204.33±5.81ul/ml in control) (Table 11), and at 800mg/kg, a significant increase ($p < 0.05$) in the level of SOD (256.00 ±8.00 vs 204.33±5.81ul/ml in control group) was also recorded (Table 11). There was a significant increase ($p < 0.05$) in the level of SOD in the group that earlier received 200mg/kg dose of MOESE (309.00±3.06 ul/ml) when compared with the group that was previously treated with 50mg/kg dose of MOESE (277.33±4.40 ul/ml) (Table 11). There was a significant decrease ($p < 0.05$) in the level of SOD in the group that previously received 800mg/kg dose of MOESE (256.00±8.00 ul/ml) when compared with the 50mg/kg dose of MOESE previously treated animals (277.33±4.40 ul/ml) (Table 11). A significant decrease ($p < 0.05$) in the level of SOD was also recorded in the group that received 800mg/kg dose of MOESE (256.00±8.00 ul/ml) when compared with the group that received 200mg/kg dose of MOESE (309.00±3.06 ul/ml) (Table 11). The group treated with 200mg/kg dose of MOESE showed a significant increase ($p< 0.05$) in the level of GPX (3407.30±196.36 vs 2699.30±186.2 ul/L) when compared with the group treated with distilled water (Table 11). There were significant decreases in the level of MDA across the treated group after the 30 day cessation of therapy (Table 11). The group initially treated with MOESE at the dose of 50mg/kg showed a significant decrease ($p< 0.05$) in the level of MDA (1.97±0.03 vs 3.40±0.23 umol/L) when compared with the control group (Table 11). There was also a significant decrease ($p< 0.05$) in MDA level in the group that initially received 200mg/kg dose of MOESE (2.03±0.03 vs control value of (3.40±0.23 umol/L) (Table 11). A significant decrease ($p< 0.05$) in the level of MDA was also observed in the group that previously received 800mg/kg dose of MOESE (2.20±0.50 vs control value of 3.40±0.23 umol/L) after the 30 day cessation of therapy (Table 11).

Effect of *MOESE* on Haematologial Parameters in Blood

MOESE did not produce any significant effect ($P > 0.05$) on haematological parameters after the 90 day administration except for MCV in which there was a significant decrease ($p < 0.05$) in the group treated with MOESE at the dose of 200mg/kg (55.33±0.33 10^9/L) when compared with the group treated with distilled water (59.00±0.00 10^9/L) (Table 12), and also a significant decrease ($p < 0.05$) in the group treated with 800mg/kg *M. oleifera* (55.00±1.00 vs control value of 59.00±0.00 10^9/L) (Table 12).

After the 30 day cessation of treatment, a significant increase ($p < 0.05$) was recorded in the level of neutrophils in the group earlier treated with 200mg/kg dose of MOESE (85.33±0.33%) when compared with the distilled water treated group (68.66±0.67) (Table 13). Also, a significant increase ($p < 0.05$) in the level of lymphocytes in the group earlier treated with 200mg/kg dose of MOESE (35.33±0.33 vs 31. 31.33±0.67% in control group) was recorded (Table 13). A significant increase ($p < 0.05$) in the level of lymphocytes was also recorded in the group that earlier received 800mg/kg dose of MOESE (34.50 ± 0.50 vs control value of 31.33±0.67%) (Table 13). For RBC, a significant decrease ($p < 0.05$) in the group earlier treated with 200mg/kg dose of MOESE (4.97±0.0910^{12}/L) compared with control value of (8.59±0.06 10^{12}/L), was also observed (Table 13).

EFFECT OF MOESE ON SPERM MOTILITY, COUNT, MORPHOLOGY AND pH

MOESE did not produce any significant effect on sperm motility, count and abnormality after the 90 day administration period. However, there was significant decrease in pH across all the treated groups compared to control animals (Table 14). At the dose of 50mg/kg of MOESE, there was a significant decrease ($p < 0.05$) in pH (4.20±0.06 vs control value of 4.70±0.20) (Table 14). A significant decrease ($p< 0.05$) in pH was also observed in the group treated with 200mg/kg dose of MOESE (4.17±0.03) when compared with the distilled water treated group (4.7±0.20) (Table 14). It was also observed that the 800mg/kg dose of MOESE treated group showed a significant decrease ($p < 0.05$) in pH (4.25±0.05) when compared with the distilled water treated group (Table 14).

After the 30 day cessation of therapy, no significant difference in sperm parameters was observed (Table 15).

Effect of *M. oleifera* seed ethanolic extract on histological presentations

Concerning the liver, there was no adverse histolopathological presentations observed in the control group (Fig. 5A). The liver appeared normal with preserved hepatic architecture, hepatocyes with normal histological features and intact stroma. The liver of rats that received 50 mg/kg presented with dilated blood vessels, areas of marked necrosis and hyperchromatic foci (Fig. 5B), while that of 200 mg/kg showed dilated blood vessels, sinusoidal haemorrhage and extensive areas of necrosis (Fig. 5C). Dilated blood vessels and extensive areas of necrosis were also observed in the liver at the doses of 800 mg/kg (Fig. 5D).

Discussion

The reticence to the widespread use of medicinal plants is gradually being eroded by the inability of orthodox drugs to cure long standing and common ailments such as diabetes and hypertension. Additionally, anecdotal evidence of efficacy of medicinal plants has boosted confidence in the use of such products. In the present study, the 96h LD_{50} value of *M. oleifera* seed extract for the rats was found to be 2900mg/kg which indicates that *M. oleifera* seed extract could be relatively not toxic to rats at this concentration based on chemical labeling and clarification of acute systemic toxicity on oral LD50 values recommended by the Organization for Economic Cooperation and Development (OECD).[31] However, it has been reported that the LD50 is not an absolute value but an inherently variable biologic parameter that cannot be compared to constants sure as molecular weight or melting point.[32] Organ weight changes can be sensitive indicators of target organ toxicity, and significant changes in organ weights may occur in the absence changes in other pathology parameters.[33] In this study, there were no body weight, body weight gain, food consumption, absolute or relative organ weight, or water consumption or clinical changes associated with the administration of MOSES. Any body weight and food and water consumption changes were overall small magnitude, within the range of historical control (Table 1&2, Fig 1, 2&3). Liver function tests help in the diagnosis of any abnormal/normal condition of liver. Leakage of cellular enzymes into plasma indicates the sign of hepatic tissue damage.[34,35] Generally measurement of alanine aminotransferase (ALT) and AST are used as important diagnostic marker to indicate liver injury due to hepatotoxins. In this study, administration of MOSES extract significantly ($p<0.05$) increase serum AST, ALP and albumin levels but did not changed ALT values in treated animals relative to control (Table 3 & 4). The histology results also shows the damage of parenchymal and mitochondrial sites of the liver where ALT is located. Our results is dissimilar to that of Ferreira et al (2009)[36] who reported no alterations in hepatic enzymes in rat treated with 1300-1670mg/kg/day *Moringa Olefera* seed for 30days, but agrees with Bharani et al. (2003)[37] who reported that administration of the hydroalcoholic extract of *M. oleifera* drumsticks by oral route elevated levels of some hepatic enzymes involved in detoxification of xenobiotic substances, such as carcinogens and plant venomous compounds. The increased serum albumin level suggests a potential for the seed to cause imbalance fluid distribution. Albumin plays a major role in stabilizing extracellular fluid volume by contributing to oncotic pressure of plasma. Because smaller animals (for example rats) function at a lower blood pressure, they need less oncotic pressure to balance this, and thus need fewer albumins to maintain proper fluid distribution.[38] The increment in AST and ALT persisted while that of albumin was reversed after 30 days of cessation of the extract.

Reactive oxygen species (ROS) are related to oxidative stress and many scientific reports have shown that excessive production of ROS can further aggravate oxidative stress and have implicated ROS in a number of disorder/disease processes, including heart disease, diabetes, liver injury, cancer, and aging.[39-41] Equilibrium between ROS and enzymatic antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx) are crucial and could be an important mechanism for preventing damage by oxidative stress. The liver plays a central role in the maintenance of systemic lipid homeostasis and is especially susceptible to ROS damage, mechanism has been suggested to play role in preventing APAP toxicity.[42] In

this study, MOSES has shown a significant increase of these enzymes (Tables 10 & 11) suggesting the presence of antioxidants compounds. These results is consistent with previous studies described by Gupta et al. (2005)[43] and his colleagues that shows the capacity of *moringa* seeds to protect animals against oxidant stress caused by arsenic exposure. Several researches attributed this oxidative protection of the seeds to the presence of compounds with antioxidant activity against free radicals.[44,45]

No significant effect was produced by MOESE on hematologic parameters after the 90 day administration except a slight decrease in MCV in the group treated with MOESE at the dose of 200mg/kg and 800mg/kg when compared with the control group, the decrease in MCV was however reversed after the 30 day cessation of treatment. (Tables 12 & 13)

Regarding the semen analysis, significant differences were not observed in sperm count, motility and abnormality across the three treatment doses of MOESE used during the 90 day treatment period when compared with control animals. However, there was a significant decrease in the semen pH in all treated group when compared to control group (Table 14). The microenvironment of sperm, seminal plasma, is of great significance. Seminal plasma is a mixture of secretions from the testes, epididymides and accessory sex glands. Seminal plasma contains HCO_3^-/CO_2, inorganic ions, organic acids, sugars, lipids, steroids, amino acids, polyamines, nitrogenous bases and proteins.[46] As a result, semen has a very high buffering capacity, higher than that of most other body fluids. Therefore, the pH of the seminal fluid may play a significant role not only in maintaining the viability and quality of the sperm, but also in ensuring fertilization. Declined Na^+/K^+-ATPase activity at acidic pHs has been reported to result in decreased sperm movement and capacitation, which could be one of the mechanisms of male infertility,[47] this study shows marked decrease in the pH of sperm in animals treated with MOSES when compared with control, suggesting that the extract at given doses may cause infertility in male rats. The decrease in pH was reversed after the 30 day cessation of treatment in all three treated groups.
Histopathology of the liver from treated rats showed degenerative lesions on hepatic tissue. Some hepatotoxic drugs produce their effects through toxic metabolites and immunological reaction,[48] This result is buttressed by increased level of AST and ALP.

Our study clearly demonstrated that ethanol extract of *Moringa oleifera* seeds could cause infertility in male rats due to decreased semen pH. Our results also showed that the extract may be hepatotoxic due to persistent high level of AST and ALP even after cessation of exposure to the extract, this is also consistent with the histopathological results that shows hepatic necrosis associated with chronic exposure to the extract.

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