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Moringa oleifera leaf fractions attenuated *Naje haje* venom-induced cellular dysfunctions via modulation of Nrf2 and inflammatory signalling pathways in rats

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ABSTRACT

Naja haje envenoming could activate multiple pathways linked to haematotoxic, neurological, and antioxidant systems dysfunctions. *Moringa oleifera* has been used in the management of different snake venom-induced toxicities, but there is no scientific information on its antivenom effects against *Naja haje*. This study thus, investigated the antivenom activities of different extract partitions of *M. oleifera* leaves against *N. haje* envenoming. Forty five male rats were divided into nine groups (n = 5). Groups 2 to 9 were envenomed with 0.025 mg/kg (LD_{50}) of *N. haje* venom while group 1 was given saline. Group 2 was left untreated, while group 3 was treated with polyvalent antivenom, groups 4, 6 and 8 were treated with 300 mg/kg^{-1} of N-hexane, ethylacetate and ethanol partitions of *M. oleifera,* respectively. Groups 5, 7 and 9 were also treated with 600 $mgkg^{-1}$of the partitions, respectively. Ethanol extract and ethyl acetate partition of *M. oleifera* significantly improved haematological indices following acute anaemia induced by the venom. Likewise, haemorrhagic, haemolytic and anti-coagulant activities of *N. haje* venom were best inhibited by ethanol partition. Envenoming significantly down-regulated Nuclear factor erythroid 2-related factor 2 (Nrf2) with the consequent elevation of antioxidant enzymes activities in the serum and brain. Treatment with extract partitions however, elevated Nrf2 levels while normalising antioxidant enzyme activities. Furthermore, there were reduction in levels of pro-inflammatory cytokines (TNF-α and interleukin-1β) in tissues of treated envenomed rats. This study concludes that ethanol partition of *M. oleifera* was most effective against *N. haje* venom and could be considered as a potential source for antivenom metabolites.

1. Introduction

Elapids are venomous snakes which form a geographically widespread group that includes kraits, coral snakes, mambas, sea snakes and cobras [1]. Several species of cobras are natives to Africa, among them is *Naja haje* commonly called Egyptian cobra, a large and slender snake found in habitats of Nile valley, western and southern Egypt to northern South Africa and Northern Nigeria [1–3]. *N. haje* is one of the poisonous cobra responsible for quite a number of snakebite fatalities in Nigeria [3]. Some of the clinical manifestations of *N. haje* envenoming include local pain, severe swelling, blistering, necrosis and variable non-specific effects [4].

Venom components of *N. haje* are capable of altering a steady physiological state of the body system in several ways in envenomed victims which may lead to a diseased state. Some of these components include phospholipase A2 (PLA2) – an hydrolytic enzyme that destroys the cell membrane and snake venom metalloproteinases (SVMPs) which have been reported to promotes inflammatory response leading to tissue

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damage [5,6]. PLA2 is a major enzyme present in *N. haje* venom which causes cardiotoxicity, myotoxicity, neurotoxicity, blood lyses, hypotension, inhibition of platelet aggregation and coagulopathy [7,8]. PLA2s enzymes has Ca^{2+} as co-factors and hydrolyses 2-acyl ester bonds of membrane glycerol-phospholipids generating free fatty acids including lyso-phospholipids which are important precursors of inflammatory mediators [9]. Studies have reported that PLA2 may affect the mitochondrial respiratory functions, blood lytic effects or increase the generation of reactive oxygen species (ROS) leading to oxidative stress [10,11]. Undoubtedly, excessive generation of ROS can activate multiple signalling pathways of vascular disease, resulting in dysfunctions of antioxidant system such as nuclear factor erythroid 2-related factor 2 (Nrf2) which are associated with elimination of ROS [12]. In addition, snakes venom are capable of modulating the immune system as venom component can act directly on leukocytes, macrophages and endothelial cells to stimulate inflammatory cytokine proliferation [13].

Treatment of snake envenoming requires a standard antivenom, but snake venom antiserum development and standardization is expensive and not easily accessible due to storage requirements [14]. In most tropical countries, rural snake endemic areas are faced with storage difficulties because of epileptic or non-availability of power supply and most victims of snakebite have limited accessibility to medical treatments.

Consequently, most locals make use of medicinal plants as alternative therapy for treatment of snake envenoming. Medicinal plants have been used traditionally to effectively manage various cases of snake envenoming since ages [9,15]. Several studies have investigated the reputation of plants in neutralizing the toxic effects of snake venoms with positive outcomes [9,16,17]. Earlier studies have documented the effectiveness of *Moringa oleifera*, a medicinal plant in neutralizing several snake venom induced toxicities [18,19]. Despite the available information on the use of medicinal plant in the management of snake bite, no empirical data exist on the effect of *M. oleifera* extract on *N. haje* envenoming. Accordingly, this study evaluated the efficacy of *M. oleifera* leaf partitions on the antioxidant and inflammatory pathways against *N. haje* venom intoxication in albino rats.

2. Materials and methods

2.1. Experimental animals

Eighty Seven adult male Wistar rats weighing between 120 and 140 g was purchased from the animal house of the Department of Zoology, University of Ibadan, Nigeria. The animals were transported via well ventilated pathogen free cages to the Animal House of the Department of Zoology, Federal University of Agriculture Abeokuta, Nigeria, where the experiment was carried out. The rats were acclimatized for two weeks under standard conditions (12 h light and 12 h dark, cycle and ambient temperature) in the animal house. The rats were fed with standard chow and water *ad libitum*. All animal experiments complied with the National Research Council publication on guide for the care and use of laboratory animals [20].

2.2. Plant materials

M. oleifera leaves was obtained from premises of University of Ibadan campus, Nigeria and authenticated in the herbarium of Department of Botany, University of Ibadan, Nigeria with voucher no: UIH-224601.

2.2.1. Preparation and extraction of the M. oleifera leaves

Freshly collected leaves were air-dried at room temperature in the laboratory. Dried leaves were pulverized and cold maceration method was employed for the extraction [21]. Briefly, approximately 1200 g of the powdered leaf of *M. oleifera* was soaked in 3 L of absolute ethanol for 72 h thereafter the content was filtered into a conical flask. Filtrates was concentrated using rotatory evaporator at 40 °C.

2.2.2. Partitioning process of M. oleifera extract

M. oleifera extract was further partitioned using *n*-hexane and ethyl acetate [22]. Filtrates collected consist of N-hexane, ethyl acetate and ethanol partitions which were concentrated into a paste using rotatory evaporator at 40 °C.

2.3. Venom and anti-venom

Crude venom of *N. haje* was procured from the Department of Veterinary Physiology, Ahmadu Bello University Zaria, Nigeria. The lyophilized venom was kept as room temperature in the laboratory. Polyvalent anti-venom (EchiTAb-Plus ICP) was purchased from Instiudo Clodomiro Picado, University of Costa Rica, Costa Rica.

2.4. Anti-venom effects of M. oleifera extract partitions against N. haje venom in rats

2.4.1. Study design, envenoming and treatment procedures

Forty five male albino rats were randomly divided into nine groups (n = 5). Group 1 was injected with saline and served as normal control. Group 2 to 9 were injected intraperitoneally with 0.2 mL of 0.025 $mgkg^{-1}$ lethal dose concentration (LD_{50}) of *N. haje* venom [23] dissolved in 1 mL of normal saline. Treatment of envenomed rats commenced 1 h post envenomation. Group 2 was left untreated post envenomation (venom control) while group 3 was treated by intravenous injection of 0.2 mL of polyvalent anti-venom. However, group 4, 6 and 8 were treated with oral dose of 0.2 mL of 300 $mgkg^{-1}$ of N-hexane, ethyl acetate and ethanol extract partitions of *M. oleifera* in saline respectively. Additionally, group 5, 7 and 9 were treated orally with 0.2 mL of 600 $mgkg^{-1}$of *n*-hexane, ethyl acetate and ethanol extract partitions of *M. oleifera* in saline respectively. Treatment of envenomed rats was done for seven consecutive days thereafter blood was collected through the abdominal veins from the experimental rats for haematology and biochemical analyses. Rats were then sacrificed following guides [24]. Organs of experimental rats such as brain, kidney and liver were harvested and stored in 10% formalin for histopathological studies.

2.5. Haematological analysis

Hematological parameters such as Packed Cell Volume, White Blood Cell and differentials, Red Blood Cell, haemoglobin level and platelet were measured [25].

2.6. Enzymatic antioxidant status

Serum and homogenates of brain tissues of the experimental rats were used in determining the superoxide dismutase activity [26], catalase [27] and levels of malondialdehyde [28].

2.7. Assay of cytokines in serum and brain tissues

2.7.1. Tissue preparation

Frozen samples were homogenized in 1.5 mL RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS pH = 7.6) supplemented with Protease inhibitors at 4 °C. The homogenate was incubated on ice for 30 min and then centrifuged at 10, 000 g for 30 min at 4 °C. Following centrifugation, the supernatants were transferred to labelled Eppendorf and stored at −80 °C for cytokine measurement.

2.7.2. Pro-inflammatory cytokines and NRF2 levels in the serum and brain

Quantitative measurement of the levels of cytokines was performed using Mini Enzyme-Linked Immunosorbent Assay (ELISA) Development Kits (Peprotech). A total of 96-well plates were set up according to the manufacturer's instructions and read using an ELISA plate reader at 405 nm with 650 nm as the correction wavelength. Concentrations of the

Table 1
Effects of different partitions of *M. oleifera* on haematological disorder caused by *N. haje* venom.

Groups	PCV (%)	Hb (g/dl)	RBC (cell/L)	Platelet (x10^5 cell/L)	MCV (fl)	MCH (pg/cell)	MCHC (%)
1 (Saline)	57.67 ± 0.06^{b}	18.27 ± 0.059^{b}	9.08 ± 0.056^{b}	156.33 ± 0.06^{abc}	$635.93 \pm .076^{b}$	201.46 ± 0.06^{b}	31.69 ± 0.00^{b}
2 (Venom only)	34.33 ± 0.06^{a}	11.23 ± 0.059^{a}	5.71 ± 0.056^{a}	847.67 ± 0.06^{ab}	$400.61 \pm .076^{a}$	131.07 ± 0.67^{a}	21.81 ± 0.00^{ab}
3 (Venom+0.2 mL AV)	50.33 ± 0.06^{b}	16.33 ± 0.059^{b}	8.53 ± 0.056^{b}	1027.67 ± 0.69^{abc}	$590.67 \pm .076^{b}$	192.15 ± 0.67^{b}	32.52 ± 0.06^{b}
4 (Venom+300 mg/kg NH)	43.67 ± 0.06^{ab}	12.63 ± 0.059^{a}	6.23 ± 0.056^{a}	663.33 ± 0.76^{ab}	$462.65 \pm .076^{ab}$	135.08 ± 0.67^{ab}	19.91 ± 0.06^{a}
5 (Venom+600 mg/kg NH)	34.67 ± 0.05^{a}	11.70 ± 0.059^{a}	5.67 ± 0.056^{a}	633.33 ± 0.06^{ab}	$406.76 \pm .076^{a}$	137.46 ± 0.07^{ab}	22.55 ± 0.00^{ab}
6 (Venom+300 mg/kg EA)	43.67 ± 0.06^{ab}	15.37 ± 0.059^{ab}	7.27 ± 0.056^{ab}	122.67 ± 0.09^{abc}	$603.64 \pm .076^{b}$	211.03 ± 0.67^{b}	34.98 ± 0.06^{b}
7 (Venom+600 mg/kg EA)	48.00 ± 0.05^{ab}	16.00 ± 0.059^{ab}	7.81 ± 0.056^{ab}	179.33 ± 0.09^{abc}	$614.03 \pm .076^{b}$	204.56 ± 0.07^{b}	33.31 ± 0.06^{b}
8 (Venom+300 mg/kg ET)	15.67 ± 0.05^{a}	5.27 ± 0.059^{a}	2.57 ± 0.056^{a}	443.33 ± 0.16^{a}	$203.20 \pm .076^{a}$	68.31 ± 0.07^{a}	11.20 ± 0.06^{a}
9 (Venom+600 mg/kg ET)	54.00 ± 0.06^{b}	17.80 ± 0.059^{b}	8.89 ± 0.056^{b}	102.00 ± 0.069^{abc}	$607.08 \pm .076^{b}$	200.07 ± 0.67^{b}	32.95 ± 0.010^{b}

Values are mean ± S.E. M (η = 5). Values in the same column with different superscripts are significantly different ($P < 0.05$).
Hb: Haemoglobin, RBC: Red Blood Cell, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration.
AV: Anti-venom, NH: N-hexane, EA: Ethyl Acetate, ET: Ethanol.

NRF2, TNF-α and IL-1β were estimated [13].

2.8. Biological studies

2.8.1. Anti -haemorrhagic assay

Forty two male albino Wistar rats weighing between 120 and 140 g was used for this study with modification to previous method [29]. Fifteen rats were randomly selected into five groups (n = 3) and used for the Minimum Haemorrhagic Dose (MHD) determination. These groups consist of a normal control and four envenomed groups. Twenty seven rats were divided randomly into nine groups (n = 3) for the anti-haemorrhagic study consisting of a normal control, venom control, antivenom group and the six extract treated groups. MHD was determined by injecting intradermally, 0.1 mL of varying concentrations of the venom from a serial dilution (5.0, 2.5, 0.25 and 0.625 mg) of 10 mg venom in normal saline into the rats. MHD was defined as the least amount of venom which when injected intradermaly, (i.d.) rats showed a hemorrhagic foci of 10 mm diameter in 3 h. Anti-hemorrhagic activity was carried out by mixing 0.2 mL of a fixed amount of venom (2 × MHD in saline) with 0.2 mL of different amount (300 and 600 mg/kg^{-1} in 1 mL of saline) of each extract partitions and antivenom (0.2 mL) separately. The mixtures of venom/partitions and venom/antivenom were incubated at 37 °C for 1 h and 0.1 mL of the mixture was injected intradermaly into rats. The hemorrhagic foci was measured after 3 h and expressed as percentage inhibition [30].

Table 2
White blood cell and differentials of *N. haje* envenomed rats treated with extract partition of *M. oleifera*.

GROUPS	WBC (x10^3 cell/L)	LYM (%)	NEUT (10^3cm^3)	MONO (10^3µl)	EO (10^3µl)
1 (Saline)	2966.67 ± 0.05^{ab}	81.67 ± 0.06^{ab}	15.00 ± 0.05^{ab}	1.33 ± 0.25^{ab}	2.00 ± 0.26^{ab}
2 (Venom only)	2000.00 ± 0.05^{b}	55.00 ± 0.06^{a}	10.33 ± 0.03^{a}	1.00 ± 0.20^{a}	0.33 ± 0.05^{a}
3 (Venom+0.2 mL AV)	3150.00 ± 0.05^{ab}	79.67 ± 0.06^{ab}	16.67 ± 0.02^{ab}	1.67 ± 0.50^{ab}	1.33 ± 0.16^{ab}
4 (Venom+300 mg/kg NH)	1833.33 ± 0.05^{b}	53.00 ± 0.03^{a}	9.33 ± 0.05^{a}	1.33 ± 0.50^{ab}	1.33 ± 0.21^{ab}
5 (Venom+600 mg/kg NH)	2150.00 ± 0.05^{ab}	52.67 ± 0.06^{a}	12.67 ± 0.06^{a}	1.00 ± 0.51^{a}	0.33 ± 0.02^{a}
6 (Venom+300 mg/kg EA)	4250.00 ± 0.05^{ab}	73.33 ± 0.05^{ab}	23.33 ± 0.05^{ab}	1.67 ± 0.21^{ab}	1.67 ± 0.22^{ab}
7 (Venom+600 mg/kg EA)	3583.33 ± 0.05^{ab}	76.67 ± 0.04^{ab}	21.33 ± 0.05^{ab}	1.67 ± 0.20^{a}	0.33 ± 0.02^{a}
8 (Venom+300 mg/kg ET)	800.00 ± 0.057^{a}	24.67 ± 0.07^{a}	7.33 ± 0.01^{a}	0.67 ± 0.22^{a}	0.67 ± 0.52^{a}
9 (Venom+600 mg/kg ET)	2833.33 ± 0.05^{ab}	66.45 ± 0.03^{ab}	17.22 ± 0.05^{ab}	1.33 ± 0.50^{ab}	1.33 ± 0.16^{ab}

Values are mean ± S.E (η = 3). Values in the same column with different superscripts are significantly different ($P < 0.05$). WBC: white blood cell, LYM: Lymphocytes, NEUT: Neutrophils, MONO: Monocytes, EO: Eosinophils. AV: Antivenom, NH: N-hexane, EA: Ethyl Acetate, ET: Ethanol.

2.8.2. Anti-haemolytic assay

Anti-haemolytic study was carried out using 20 mL of citrated bovine erythrocytes. Centrifugation of blood was done 10 times at 2400 rpm for 10 min using 5 mL of saline (0.9%) to obtain free packed cells. Thereafter, a 10% cell suspension was prepared. Exactly, 0.2 mL of 2 × LD_{50} of the venom (0.05 mg/kg dissolved in 2 mL of normal saline) was mixed with 10 mL of 10% cell suspension. Thereafter, 0.2 mL of the mixtures were added to 0.2 mL of varying dose of the extract partitions of *M. oleifera* (300 and 600 mg/kg^{-1} of 1 mL in saline) and polyvalent antivenom in separate tubes. The mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 3 mL of chilled phosphate buffer saline (PBS pH 7.2) to the tubes. The tubes were centrifuged at 2400 rpm for 10 min and absorbance of the supernatant was measured at 540 nm. Supernatant treated with 3 mL distilled water was taken as 100% lyses and serves as control [30]. Percentage inhibition was calculated using the formula.

$$\% \text{ Inhibition} = \frac{\text{Control} - \text{Test Sample}}{\text{Control}} \times 100$$

2.8.3. Anti-coagulating activity

The method of Gomes and Pallabi [29] was adopted using citrated bovine plasma. Nine tubes were arranged in a test tube rack, test tube 1 contain 0.2 mL of normal saline (normal control). 0.2 mL of 2 × LD_{50} of the venom (0.05 mg/kg/mL dissolved in 2 mL of normal saline) was put in test tubes 2–9.0.2 mL of bovine citrated plasma was added to all the test tubes. Test tube 2 serves as the venom control. 0.2 mL of each *M. oleifera* extract partitions (300 and 600 mg/mL^{-1}dissolved in 1 mL of normal saline) and 0.2 mL of polyvalent anti-venom was added to test tubes 3–9 respectively. Then 0.2 mL of $CaCl_2$ was added to all the mixtures and incubated for 30 min at 37 °C and clotting time recorded.

2.9. Histopathological studies

The histopathological examination of the kidney, brain and liver were carried out using conventional techniques of paraffin-wax sectioning and hematoxylin-eosin staining [31].

2.10. Statistical analysis

The Statistical Package for Social Sciences (SPSS version 16) software produced by IBM Corp. Ltd. was used for data analysis. Data were expressed as Mean ± Standard Error of mean (SEM). Data were analysed by one way analysis of variance (ANOVA) and Duncan multiple test. The

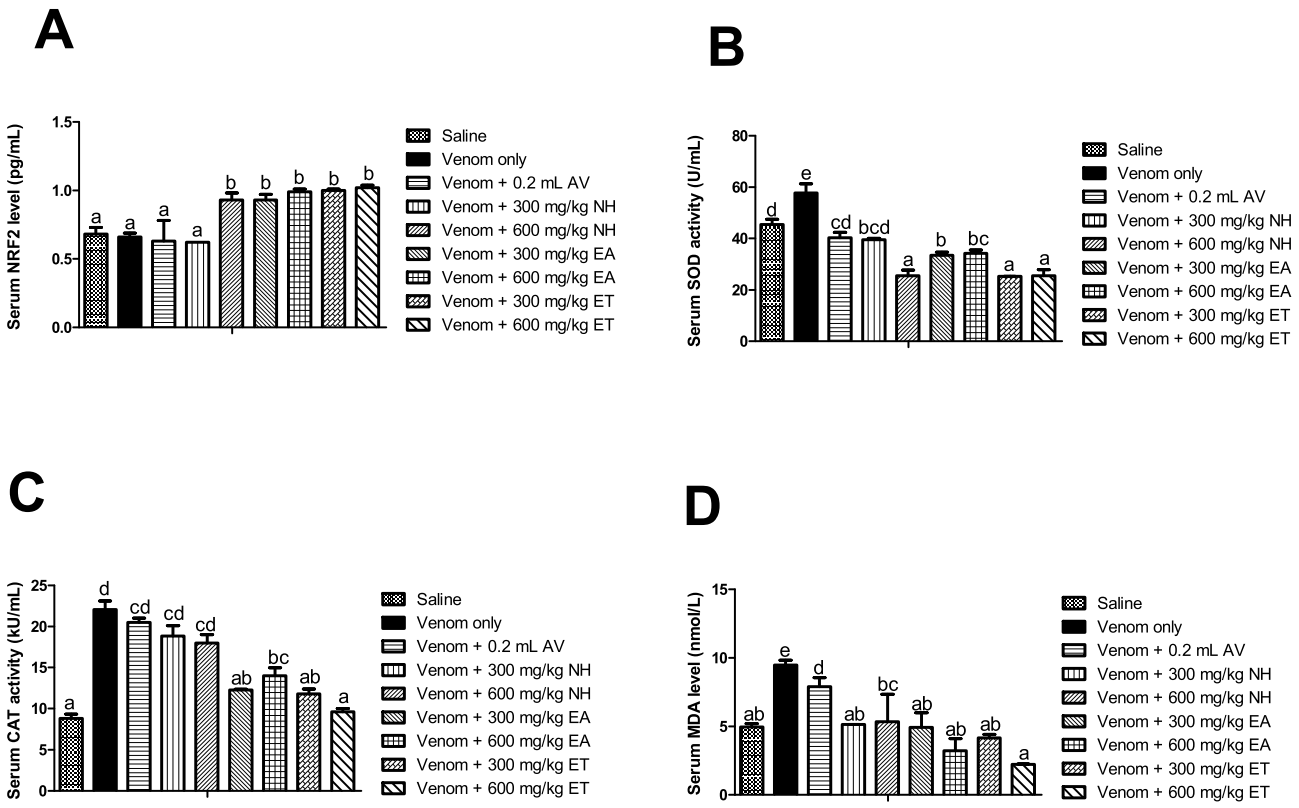

Fig. 1. Effects of *M. oleifera* extract partitions on the serum antioxidant indices and oxidative damage marker in rats exposed to *N. haje* venom. Data are expressed as mean ± Standard error of mean (S.E.M). Bars with different letters are statistically distinct at $p < 0.05$ (n = 5). (A) Serum NRF2 level (B) Serum superoxide dismutase (SOD) activity (C) Serum catalase (CAT) activity (D) Serum malondialdehyde (MDA) level. AV: Anti-venom, NH: N- hexane partition, EA: Ethyl acetate partition, ET: ethanol partition.

differences between mean values were considered significant at $p < 0.05$.

4.0. Results

4.1. Mortality of the envenomed rats within the experimental groups

No mortality was recorded in normal control, polyvalent antivenom treated group and groups treated with 300 mg/kg *n*-hexane and 600 mg/kg ethyl acetate extract partition of *M. oleifera*. However 40% mortality was also observed in envenomed groups treated with 300 mg/kg ethyl acetate and ethanol extract partitions. Groups treated with 600 mg/kg *n*-hexane and ethanol extract partitions also recorded 20% mortality each.

4.2. Haematology of envenomed rats after treatment with extract partitions of M. oleifera

The PCV, Hb and RBC counts of untreated group were significantly ($P<0.05$) lower compared to normal group (Table 1). The PCV, Hb and RBC counts of groups treated with antivenom and 600 mg/kg of ethyl acetate and ethanol extract partitions of *M. oleifera* were significantly higher compared to untreated group and groups treated with 300 mg/kg of the extract partitions. The platelets counts of normal control group were significantly ($P<0.05$) higher compared to untreated group but not significantly different from groups treated with antivenom and 600 mg/kg of ethyl acetate and ethanol extract partitions.

4.2.1. White blood cell and differentials

There was a significant decrease in WBC counts of venom control group compared with normal control rats (Table 2). The WBCs of groups treated with antivenom and 600 mg/kg of ethanol, ethyl acetate and *n*-hexane extract partitions were significantly higher compared to venom control rats. The lymphocytes, neutrophils and monocytes counts of venom control rat group were significantly ($P<0.05$) lower compared to normal rats.

4.3. Effects of extract partitions of M. oleifera on serum and brain tissue antioxidant enzymes and oxidative stress index of N. haje envenomed rats

The activities of SOD and CAT as well as MDA levels in serum (Fig. 1) and brain (Fig. 2) of untreated envenomed group were significantly higher compared to normal control. The level of Nrf2 recorded in the sera and brain tissues of normal control group were significantly ($P<0.05$) higher compared to untreated and anti-venom treated groups whereas Nrf2 expression recorded in sera and tissues of untreated and anti-venom groups were significantly ($P<0.05$) lower compared to groups treated with extract partitions of *M. oleifera*. However, among groups treated with extract partition of *M. oleifera,* groups treated with 300 mg/kg of N-hexane partition and 600 mg/kg of ethanol partition recorded the least and highest Nrf2 expression on sera and tissues respectively (Fig. 3A and B). Groups treated with antivenom and various extract partitions of *M. oleifera* had significantly lower SOD, CAT and MDA levels compared to untreated group. Across groups treated with extract partitions of *M. oleifera,* groups treated with 300 and 600 mg/kg of ethanol partition recorded the lowest enzymes activities in sera and

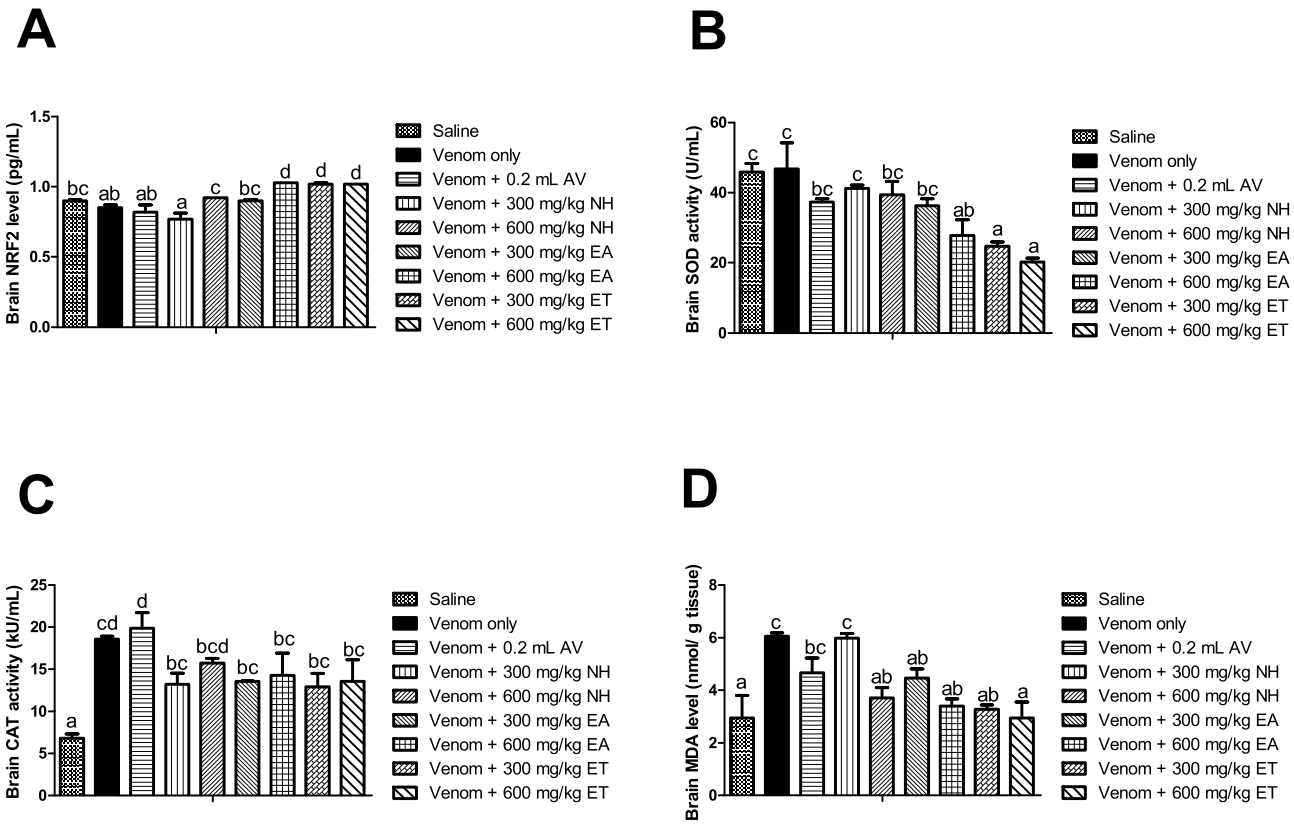

Fig. 2. Effects of *M. oleifera* extract partitions on the brain antioxidant indices and oxidative damage marker in rats exposed to *N. haje* venom. Data are expressed as mean ± Standard error of mean (S.E.M). Bars with different letters are statistically distinct at $p < 0.05$ ($n = 5$). (A) Brain NRF2 level (B) Brain superoxide dismutase (SOD) activity (C) Brain catalase (CAT) activity (D) Brain malondialdehyde (MDA) level. AV: Anti-venom, NH: N- hexane partition, EA: Ethyl acetate partition, ET: ethanol partition.

brain.

4.4. Effects of extract partitions of M. oleifera on serum and brain levels of Nrf2, interleukin1 –beta (IL1-β) and tumor necrosis factor–alpha (TNF-α) expression in N. haje envenomed rats

The IL1-βand TNF-α levels in the sera and brain tissues of untreated group were significantly ($p < 0.05$) higher compared to normal control including groups treated with anti-venom and respective extract partitions of *M. oleifera*. In envenomed but treated groups, group treated with 300 mg/kg of N-hexane partition of *M. oleifera* recorded the lowest TNF-α and IL1-β in sera while the lowest TNF-α and IL1-β in brain tissues was recorded in group treated with 300 mg/kg N-hexane and ethyl acetate partitions of *M. oleifera* respectively (Fig. 3C–F).

4.5. Anti-haemorrhagic activity of partitions of M. oleifera leaf extract on N. haje envenomed rat

The minimum dose of the venom was 2.5 mg/mL with the hemorrhagic foci of 10 mm diameter. Polyclonal anti-venom showed 54% while high dose (600 mg/kg) of *n*-hexane and ethanol extract partitions of *M. oleifera* had 55% and 76% inhibition, respectively (Table 3).

4.6. Anti-haemolytic activity of M. oleifera extract partitions on N. haje envenomed rats

Total haemolysis was observed in the normal control samples. *N. haje* venom had 88% haemolysis of citrated bovine erythrocytes however, extract partitions of *M. oleifera* inhibited haemolysis induced by the venom at different concentrations. The highest inhibitory activity was observed in sample mixed with 600 mg/kg of ethyl acetate with 72% inhibition while antivenom recorded 48% inhibition (Table 4).

4.7. Anti-coagulant activity of M. oleifera extracts partitions on N. haje venom

Normal control samples clotted in 44 s but samples of *N. haje* venom which served as venom control clotted in 720 s. However, various extract partitions of *M. oleifera* exhibited anticoagulant activities, as samples treated with high dose (600 mg/kg) and low dose (300 mg/kg) of ethyl acetate and ethanol partition of *M. oleifera* extract inhibited the anticoagulant activity of the venom which was dose dependent. Samples treated with 600 mg/kg of ethyl acetate partition recorded the strongest inhibitory effects at 74 s. Anti-venom samples clotted at 60 s (Fig. 4).

4.8. Histopathology

4.8.1. Brain tissues of the envenomed treated rats

Tissues of the normal control showed no observable lesion but a fused neuronal degeneration and necrosis was revealed in brain tissue of untreated group. Also, envenomed group treated with 600 mg/kg of ethanol partition and group treated with 300 mg/kg of ethyl acetate showed a severe congestion of the meninges (the membranes that enveloped the brain) while other envenomed treated groups showed no visible lesion (Fig. 5).

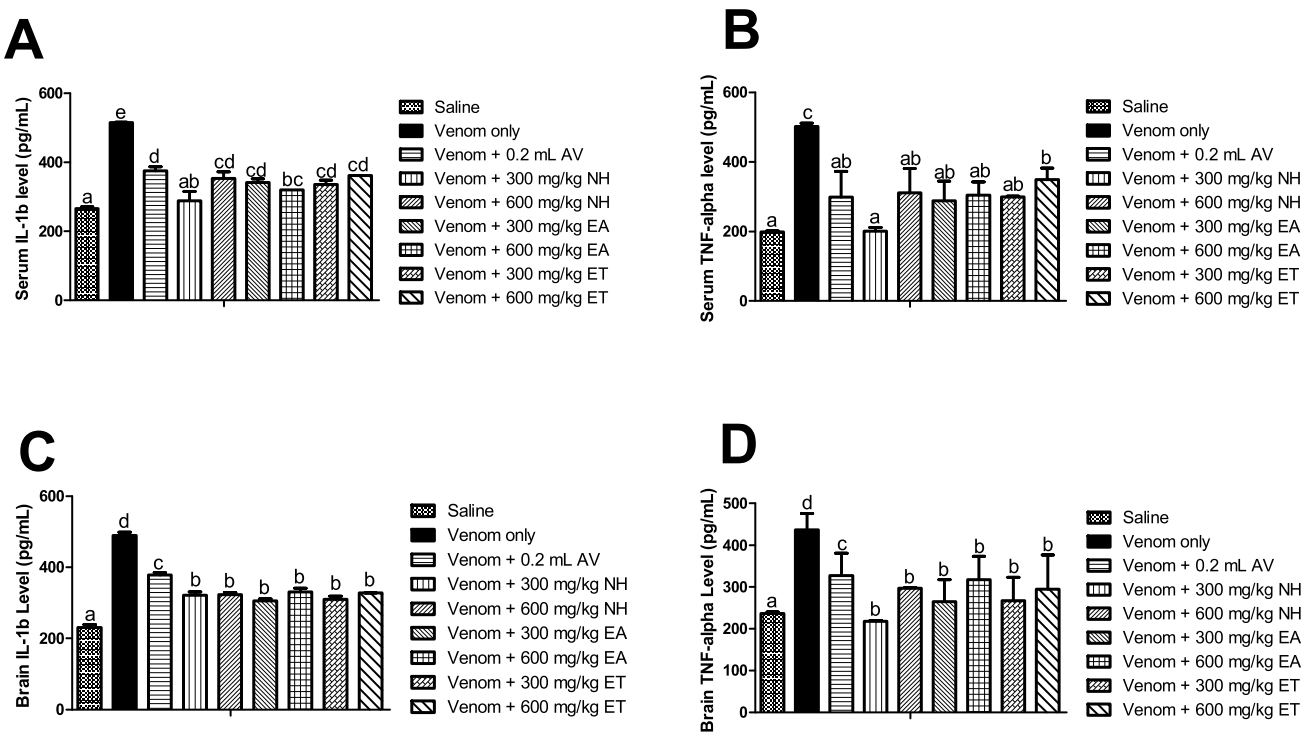

Fig. 3. Effects of *Moringa oleifera* extract partitions on the IL-1β, and TNF- α expression in rat exposed to *Naja haje* venom. Data are expressed as mean ± Standard error of mean (S.E.M). Bars with different letters are statistically distinct at $p < 0.05$ (n = 5). (A) Serum interleukin −1 beta (IL-1β) level (B)Serum Tumor necrosis factor-alpha (TNF- α) level (C)Brain interleukin −1 beta (IL-1β) level(D) Brain Tumor necrosis factor-alpha (TNF- α) level AV: Anti-venom, NH: N- hexane partition, EA: Ethyl acetate partition, ET: ethanol partition.

Table 3
Anti-hemorrhagic activity of *M. oleifera* extract partitions against N. *haje*-envenomed rats.

Treatment groups	Inhibition (%)
Group 1	–
Group 2	0 (No Inhibition)
Group 3	54.00 ± 1.53
Group 4	33.00 ± 2.52
Group 5	55.00 ± 3.61
Group 6	26.00 ± 3.46
Group 7	34.00 ± 3.71
Group 8	42.00 ± 1.15
Group 9	76.00 ± 1.15

Data are expressed as means ± S.E.M (n = 3). $P < 0.05$.
Legend-: No haemorrhagic foci.
Group 1: injected with saline (Normal control). **Group 2:** injected with venom not treated (Venom control). **Group 3:** injected with venom and treated with 0.2 mL of antivenom. **Group 4:** injected with venom and treated with 300 mg/kg of *n*-hexane partition. **Group 5:** injected with venom and treated with 600 mg/kg of *n*-hexane partition. **Group 6:** injected with venom and treated with 300 mg/kg of ethyl acetate partition. **Group 7:** injected with venom and treated with 600 mg/kg of ethyl acetate partition. **Group 8:** injected with venom and treated with 300 mg/kg of Ethanol partition. **Group 9:** injected with venom and treated with 600 mg/kg of Ethanol partition.

Table 4
Anti-haemolytic Activity of *M. oleifera* Extract partition against *N. haje*-Envenomed Rats.

Treatment groups	Inhibition (%)
Group 1	0
Group 2	12.00 ± 2.40
Group 3	48.00 ± 4.16
Group 4	49.00 ± 1.45
Group 5	62.00 ± 1.15
Group 6	51.00 ± 1.15
Group 7	72.00 ± 1.15
Group 8	52.00 ± 1.54
Group 9	58.00 ± 2.08

Data are expressed as means ± S.E. of three samples. $P < 0.05$.
Legend: **Group 1**: Mixture of distilled water/citrated RBCs (normal control). **Group 2**: Mixture of venom/citrated RBCs (venom control). **Group 3**:Mixtures of venom/citrated RBCs/0.2 mL of polyvalent antivenom. **Group 4**: Mixture of venom/citrated RBCs/300 mg/kg of *n*-hexane partition. **Group 5**: Mixture of venom/citrated RBCs/600 mg/kg of *n*-hexane partition. **Group 6**: Mixture of venom/citrated RBCs/300 mg/kg of ethyl acetate partition. **Group 7**: Mixture of venom/citrated RBCs/600 mg/kg of ethyl acetate partition. **Group 8**: Mixture of venom/citrated RBCs/300 mg/kg of Ethanol partition. **Group 9**: Mixture of venom/citrated RBCs/600 mg/kg of Ethanol partition.

4.8.2. Kidney tissues of the envenomed treated rats

Kidney of normal control showed unaffected tissue whereas untreated rats showed foci of tubular degeneration caused by interstitial haemorrhage. Envenomed group treated with 300 mg/kg of ethanol partition revealed degeneration of few tubules as well as envenomed rats treated with polyvalent anti-venom. Moderate congestion of renal cortex was seen in envenomed rats treated with 300 mg/kg of ethyl acetate partition. Nephrotoxic effect was fully ameliorated in envenomed

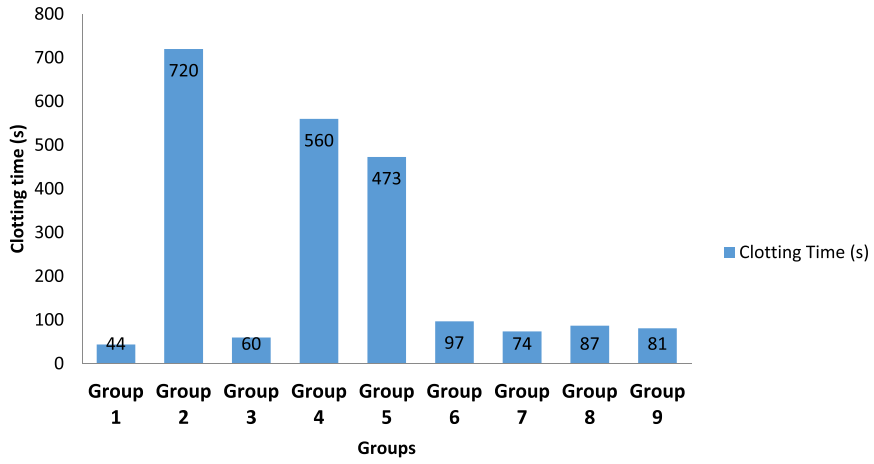

Fig. 4. Anti-Coagulant activity of *M. oleifera* extract partitions against *N. haje* venomData
Legend: **Group 1**: Mixture of distilled water/citrated plasma (normal control). **Group 2**: Mixture of venom/citrated plasma (venom control).**Group 3**: Mixtures of venom/citrated plasma/0.2 mL of polyvalent anti-venom. **Group 4**: Mixture of venom/citrated plasma/300 mg/kg of *n*-hexane partition. **Group 5**: Mixture of venom/citrated plasma/600 mg/kg of *n*-hexane partition. **Group 6**: Mixture of venom/citrated plasma/300 mg/kg of ethyl acetate partition. **Group 7**: Mixture of venom/citrated plasma/600 mg/kg of ethyl acetate partition. **Group 8**: Mixture of venom/citrated plasma/300 mg/kg of Ethanol partition. **Group 9**: Mixture of venom/citrated plasma/600 mg/kg of Ethanol partition.
are expressed as means ± S.E. of three samples. $P < 0.05$.

Data are expressed as means ± S.E. of three samples. $P< 0.05$

groups treated with 600 mg/kg of N-hexane, ethyl acetate and ethanol partitions of *M. oleifera* respectively (Fig. 6).

4.8.3. Liver tissues of the envenomed treated rats

Normal control showed unaffected hepatocytes but a moderate portal congestion, with very mild diffuse hydropic degeneration of hepatocytes was noticed in liver tissue of untreated rats. Group treated with anti-venom showed severe diffuse vacuolar degeneration and necrosis of hepatocytes. Further, a mild portal congestion with a moderate diffuse vacuolar degeneration of hepatocytes was noticed in group treated with 300 mg/kg, and 600 mg/kg of *n*-hexane. Group treated with 300 and 600 mg/kg of ethyl acetate showed a severe diffuse vacuolar degeneration and necrosis of the hepatocytes. In addition, a severe portal congestion was noticed in liver tissues of group treated with 300 mg/kg of ethanol partition. Envenomed group treated with 600 mg/kg of ethanol partition showed a severe portal and central venous congestion with severe diffuse vacuolar degeneration and necrosis of hepatocytes (Fig. 7).

5. Discussion

Envenoming by poisonous snake is a serious medical problem, especially in rural areas of tropical region. Thus, efficacious treatment should be available [32]. Screening of medicinal plants antidotes to ameliorate snake venom induced toxicity is very key in snakebite management [10] due to limitations of anti-venom. In this study, high mortality was observed in untreated envenomed rats as death was observed 2 h post-envenomation indicating *N. haje* venom was highly toxic and fatal [33]. However, extract partitions of *M. oleifera* reduced mortality in envenomed treated groups suggesting the protective effects of the plant against *N. haje* venom toxicities.

Reduction in blood indices of the untreated rats is an indication *N. haje* venom-induced haematotoxicity, because of the presence of phospholipase A2 enzymes. Phospholipase in venom are toxic to cells as they disrupt the structures of RBC membranes [34]. There was significant improvement in blood profiles of envenomed rats treated with high dose of extract partitions of *M. oleifera*. This result corroborated earlier studies that reported haematinic effects of *M. oleifera* against snake venom induced anaemia [19].

Higher activities of the antioxidant enzymes obtained in serum and brain tissue of untreated rats might indicate that *N. haje* venom induce oxidative stress and the effect may be attributed to the formation of reactive oxygen species (ROS). ROS are known do play a significant role in venom-induced toxicity by actively involved in inflammatory responses altering cellular physiology and playing a major role in pathological conditions [35,36]. Malondialdehyde (MDA) is a known biomarker of oxidative stress and cellular damage [37]. Significant increase in the MDA of serum and brain tissue of untreated rats further confirmed the ability of *N. haje* venom to induced oxidative damage as MDA is generally considered as a strong indicator of lipid peroxidation [38]. In addition, superoxide dismutase (SOD) and catalase (CAT) are key antioxidant enzymes because of their role in eliminating ROS directly [39]. Indeed, the RBC is the major source of ROS due to auto-oxidation of oxy-haemoglobin following envenomation. Superoxide radicals generated due to the snake venom toxicity are taken up by SOD and converted to hydrogen peroxide and oxygen. The hydrogen peroxide, if not taken care of, can be converted to hydroxyl radicals by interacting with metals such as iron leading to exacerbation of the oxidative stress. The dangerous hydrogen peroxide (H_2O_2) is later broken down to oxygen and water in a concerted effort to detoxify the ROS and prevent oxidative damage to biomolecules [40]. The activities of catalase and SOD was significantly high in serum and brain tissue of untreated rats confirming increased free radicals production and suggesting possible oxidative damage cause by the venom in the erythrocyte and brain tissue of rats. Studies have reported oxidant damage due to *N. haje* venom toxicities [23,41,42]. However, polyvalent antivenom and various partitions of *M. oleifera* extract showed significant normalised the antioxidant enzyme activities with ethanol extract partition of *M. oleifera* as the most effective. Studies have shown that *M. oleifera* is capable of ameliorating oxidative stress induced by snake venom in rats [19]. The ability of the extracts to normalize the activity of SOD and CAT as well as reducing the level of MDA may be attributed to its antioxidant properties due to the presence of electron–donating phytochemicals which has anti-ROS effects.

Cytokines are soluble protein mediators important for the orchestration of inflammatory responses of the human body [43]. However, excessive production of these mediators may significantly contribute to shock, multiple organ failure, and death [44,45]. Studies have shown snake venom components can produce large quantities of pro-inflammatory cytokines and mediate tissue damage because of their synergetic and important actions in initiating and promoting inflammatory responses [13]. Inflammatory cytokines are also implicated in the development of hepatic necrosis and activation of Kupffer cells leading to increased vascular permeability and apoptosis [46]. We observed in this study that *N. haje* display a significant ability to up-regulate TNF-α and IL-1β levels in serum and brain tissue of untreated rats confirming the venom activating specific immune responses most especially pro-inflammatory cytokines [47]. This is consistent with

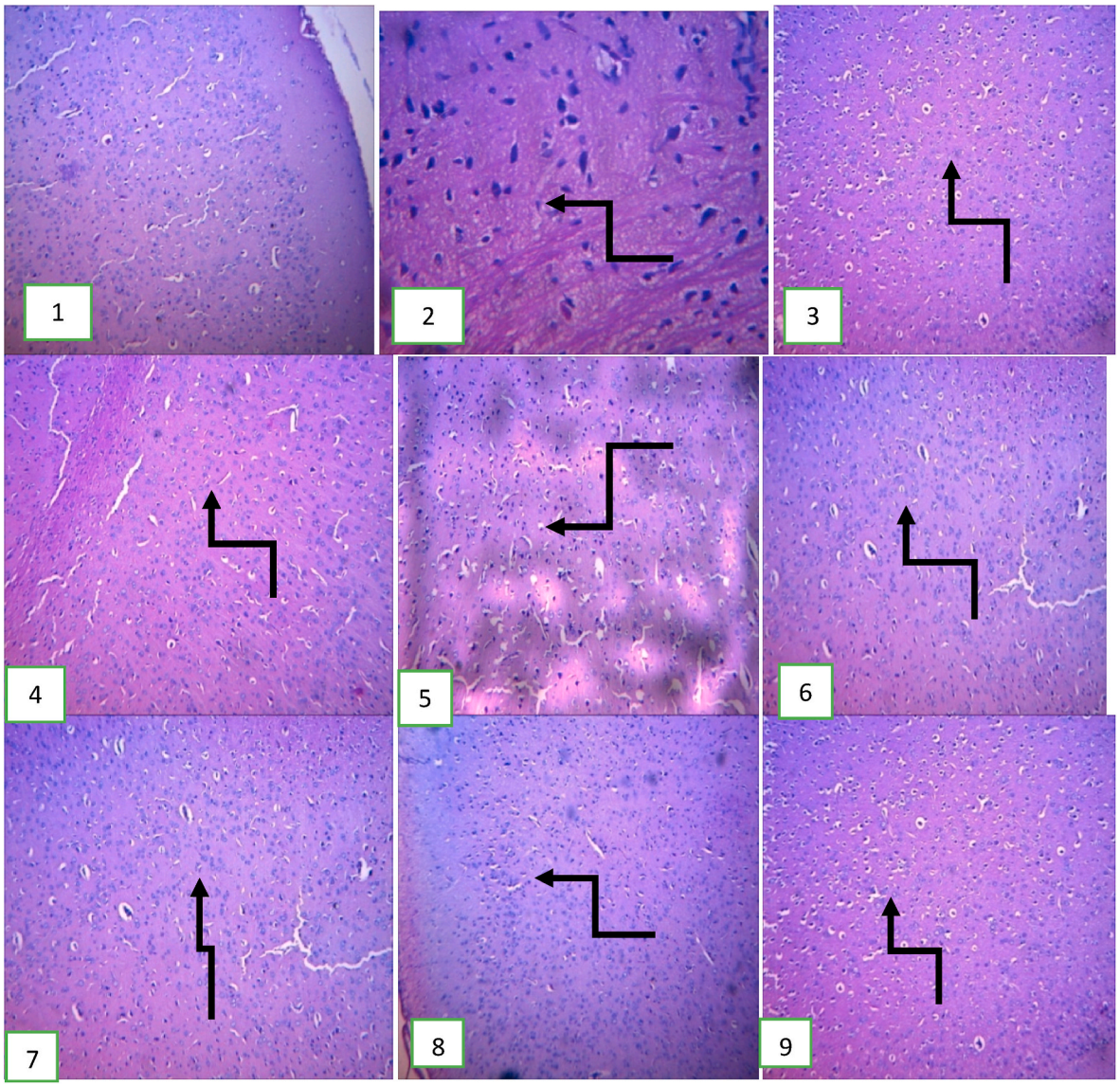

Fig. 5. Histopathological examination of brain tissues of the experimental rats.
Legend: **Group 1** (normal control): Neurons appeared normal, **Group 2** (venom control): neuronal degeneration and necrosis, **Group 3** (antivenom control): no visible lesion, **Group 4**(venom/300 mg/kg *n*-hexane): No visible lesion, **Group 5** (venom/600 mg/kg *n*-hexane): No visible lesion, **Group 6**(venom/300 mg/kg ethylacetate): severe congestion of the meninges, **Group 7**(venom/600 mg/kg ethylacetate): No visible lesion, **Group 8**(venom/300 mg/kg ethanol): No visible lesion, **Group 9**(venom/600 mg/kg ethanol):severe congestion of the meninges. H - hematoxylin, E – eosin.

previous studies using *Viper russelli* [46], *Montiviper abornmuelleri* [13], *Bothrops asper,* and *Bothrops jararaca* venoms [48]. The hydrolytic action of phospholipase A2 (PLA2) present in the venom on the poly-unsaturated fatty acid of the cell membranes produces arachidonic acid (a precursor to inflammatory mediators) resulting in the exacerbation of the inflammatory cascades [49]**.** However, antivenom and partitions of *M. oleifera* significantly reduced the pro-inflammatory cytokines levels. The anti-inflammatory effect of extract partitions of *M. oleifera* was more evident in groups treated with low dose (300 mg/kg) of the extract partition with *n*-hexane showing the least values and this may suggest that the partitions are most effective at low concentration against pro-inflammatory cytokine production.

Nuclear factor erythroid 2-related factor 2 (Nrf2), often expressed in the liver, lung, heart, and brain is known as the most significant endogenous factor related with cellular response to oxidative stress [50]. Nrf2 translocates into the nucleus and binds with an antioxidant response element (ARE) to upregulate a battery of antioxidative gene expressions, including SOD, catalase, and Peroxidases in response to antioxidants and oxidative stress [51]. Although results of this study showed that Nrf2 expression was downregulated in serum and brain tissue of untreated envenomed group and but upregulated catalase and SOD activities as observed in results of antioxidant profiles. The ability of the venom to downregulate the expression of Nrf2 confirms that *N. haje* venom is capable of inducing a dysfunction of the antioxidant system at a molecular level which may leads to substantial enrichment of ROS resulting in oxidative stress. Cells are capable of regulating the enzymatic system by a ‘‘coarse’’ mechanism whereby the rate of transcription of the enzyme is altered or by ‘‘fine’’ method whereby the activity of the available enzyme is regulated [52]. The increased activity of SOD and catalase may then be due to the anti- ROS mechanism by the cell (via regulation of the available enzymes). Nevertheless, antivenom and extract partitions of *M. oleifera* upregulated Nrf2 expression mostly in group treated with ethanol partition of *M. oleifera*. Monte et al. [53] reported that elevated Nrf2 expression is part of protective mechanism against induced toxicity. This result may indicate that *M. oleifera* can protect against *N. haje*-induced damage through the activation of the

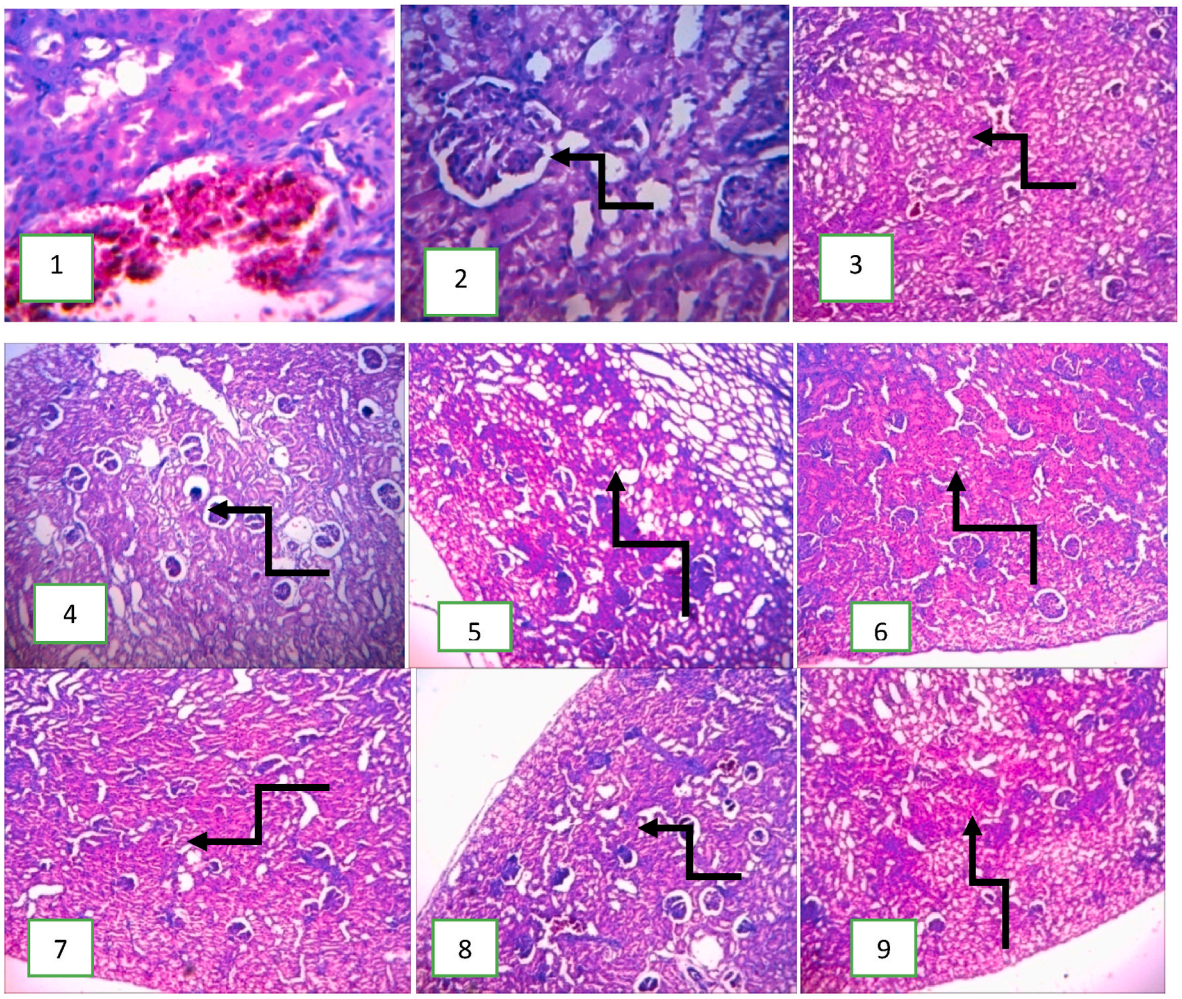

Fig. 6. Histopathological examination of Kidney tissues of the experimental rats.
Legend: **Group 1** (Normal control): No visible lesion, **Group 2** (Venom control): Few foci of tubular degeneration, **Group 3**(Antivenom control): Mild congestion of the renal cortex, **Group 4**(venom/300 mg/kg *n*-hexane): Few tubules and glomeruli appear degenerated (necrosis), **Group 5**(venom/600 mg/kg *n*-hexane): No visible lesion, **Group 6**(venom/300 mg/kg ethylacetate):Moderate congestion of renal cortex, **Group 7**(venom/600 mg/kg ethyl acetate): No visible lesion, **Group 8** (venom/300 mg/kg ethanol): Morderate congestion of the renal cortex, **Group 9**(venom/600 mg/kg ethanol): No visible lesion.

Nrf2 antioxidative pathways. The consequent decrease in antioxidant enzymes activities is the testament to the ability of treatment to mitigate the generated ROS.

Cobra venom contains cytotoxic lytic activity in synergy with phospholipase enzymes which may alter cell membranes of erythrocytes thereby resulting to haemolysis [54,55]. In this study, *N. haje* venom showed direct haemolytic activity on citrated blood but antivenom and extract partitions of *M. oleifera* protected erythrocytes from hemolytic effect of the venom in a dose dependent manner. High dose of ethyl acetate partition of *M. oleifera* showed the strongest anti-haemolytic activity however, the observed effect of *M. oleifera* is in tandem with previous study against cobra venom [18]. In addition, *N. haje* venom exhibited haemorrhagic effects as it contains snake venom metalloproteinases (SVMPs), and haemorrhagic enzymes which damage blood vessels wall resulting in systemic bleeding [56]. Although, extract partitions of *M. oleifera* countered the haemorrhagic activity of the venom as high dose (600 mg/kg) of ethanol partition recorded above 70% inhibition but total inhibition was not achieved. This result supported earlier report on anti-haemorrhagic activity of *M. oleifera* extract [19]. *N. haje* venom showed anti-coagulant activity as venom control clotted at 720 s, however, antivenom and high dose (600 mg/kg) of ethyl acetate and ethanol partitions of *M. oleifera* extract showed a strong pro-coagulant activity to counter this effect.

Snake envenoming are most times resulted into inflammation and tissue damage [57]. It was observed in this study that *N haje* venom causes pathological changes on vital organs of untreated rats as tissue degeneration were noticed on the brain, liver and kidney which supports earlier observations that *N. haje* venom is capable of affecting histology of vital organs after envenomation in rats [23]. However, antivenom and various extract partitions of *M. oleifera* showed ameliorative effects as no remarkable lesion was observed in groups treated with high dose (600 mg/kg) of the extract partitions. Studies have reported the ameliorative effects of *M. oleifera* extract on histological defects noticed on vital organs following envenoming by cobra and viper venom in rats [18,19]. The anti-venom ability of *M. oleifera* and ability to neutralize various toxicities induced by *N. haje* venom could be due to various compounds present in extract partitions. Although this study did not focus on profiling of the phytochemicals in *M. oleifera,* the presence of flavonoids, tannins and alkaloids has been confirmed by Fahal et al. [56]. Studies have suggested that compounds present in plant extracts could bind to divalent metal ions in the venom which are required for the hydrolytic activities of PLA2 thereby rendering it inactive, as metal ions are co-factors which are very crucial for enzymes stability and functionality [58]. Furthermore, *M. oleifera* has been shown to possess immunomodulatory, antioxidant and anti-inflammatory effects due to the aforementioned phytochemicals [59]. Thus, we pose that; the

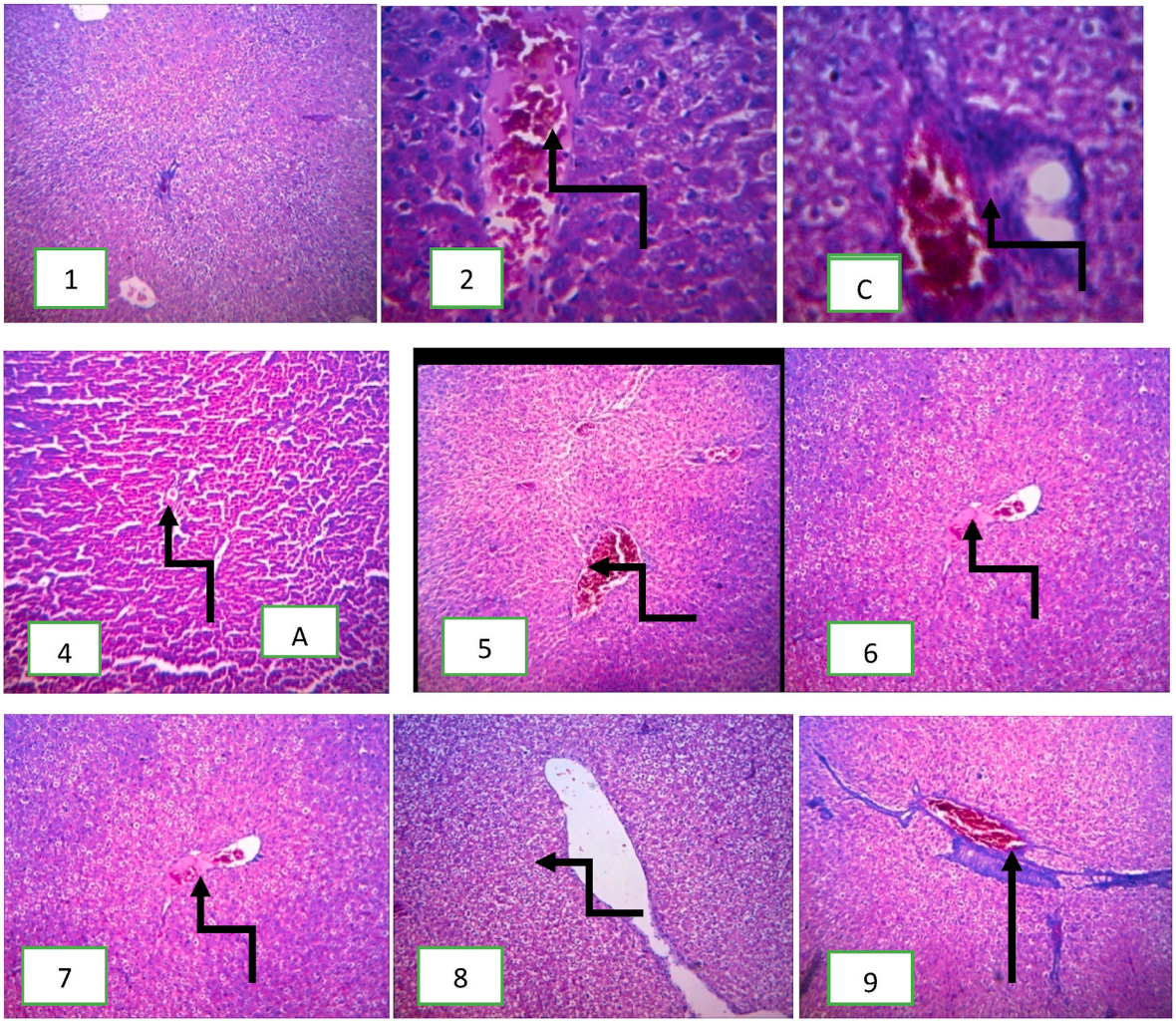

Fig. 7. Histopathological examination of liver tissues of the experimental rats.
Legend: **Group 1** (normal control): No visible lesion, **Group 2** (venom control): moderate portal congestion, with very mild diffuse hydropic degeneration of hepatocytes, **Group 3** (antivenom control): severe diffuse vacuolar degeneration and necrosis of hepatocytes, **Group 4** (venom/300 mg/kg *n*-hexane):severe diffuse vacuolar degeneration of hepatocytes, **Group 5** (venom/600 mg/kg *n*-hexane): severe diffuse vacuolar degeneration and necrosis of hepatocytes, **Group 6** (venom/300 mg/kg ethylacetate): mild portal congestion, with a moderate to severe diffuse vacuolar degeneration of hepatocytes, **Group 7** (venom/600 mg/kg ethylacetate): mild portal congestion, with a moderate to severe diffuse vacuolar degeneration of hepatocytes, **Group 8** (venom/300 mg/kg ethanol): severe diffuse vacuolar degeneration of hepatocytes, **Group 9** (venom/600 mg/kg ethanol):severe diffuse vacuolar degeneration and necrosis of the hepatocytes.

ameliorative effects of *M. oleifera* on the various pathologies induced by the venom may also be due to its ability to inhibit the activity of PLA2 which in turn renders the venom inactive.

6. Conclusion

Result obtained in this study established for the first, detailed report on *N. haje* venom-induced toxicities in *in vivo* and *in vitro* studies and possible inhibition using natural products from plants. The N-hexane, ethyl acetate and ethanol extract partitions of *M. oleifera* demonstrated their abilities in ameliorating *N. haje* toxic effects, however it should be noted that ethanol extract partition was most effective an indication of a potential anti-snake agent if properly explored.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial

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