Nig. J. Pure & Appl. Sci. Vol. 35 (Issue 2, 2022)
e-ISSN 2756-4045

www.njpas.com.ng

Ethyl Acetate Fraction of *Moringa oleifera* Leaves Mollified Toxicological Activities Actuated by *Bitis arietans* (Puff adder) Venom

[1*]Ajisebiola, B.S., [1]Oyinlola, R.R., [1]Akintunde, S.O., [2]Paul, O. and [3]Adeyi, A.O.

[1]Department of Zoology, Osun State University, Osogbo, Osun State, Nigeria.
[2] Department of Chemistry, Bells University of Technology, Ota, Nigeria
[3]Animal Physiology Unit, Department of Zoology, University of Ibadan, Ibadan, Nigeria.

Date Received: 05-03-2022
Date Accepted: 18-06-2022
DOI: https://doi.org/10.48198/NJPAS/22.A08

ABSTRACT

Bitis arietans (Puff adder) venom possess numerous biological active toxins exhibiting toxicological actions after envenoming. *Moringa oleifera* crude extract has been documented to extenuate toxicities induced by *B. arietans* venom. This study reported the efficacy of n-hexane, ethyl acetate and ethanol solvent fractions obtained from crude extract of *M. oleifera* against the lethal dose toxicity, biological activities and oxidative stress induced by *B. arietans* venom to ascertain the best fraction with active antivenom phytochemicals. Forty-five male rats were randomly selected into nine groups (n=5) for the anti-oxidative stress study. Groups 1 served as control; group 2 to 9 were envenomed by a single intraperitoneal injection of 1.5 mg/kg (LD_{50}) of the venom. Group 2 was not treated post envenomation while groups 3 was treated with polyvalent antivenom. Group 4, 6 and 8 were treated with 300 mg/kg while group 5, 7 and 9 were treated with 600 mg/kg of n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera* respectively. High dose of *M. oleifera* ethyl acetate solvent fraction best neutralized the lethal dose toxicity and strongly inhibited the heamorrhagic and anticoagulant activities of *B. arietans* venom. The venom induced oxidative stress with significant ($P<0.05$) enhancement of Superoxide Dismutase, Catalase and Malondialdehyde levels in serum and heart tissues of untreated envenomed rats. However, ethyl acetate solvent fraction was most effective in normalizing the antioxidant enzyme activities of envenomed treated rats. Results showed that ethyl acetate fraction of *M. oleifera* possesses active antivenom phytochemicals against *B. arietans* venom induced toxicities.

Keywords: *Bitis arietans,* fertile eggs, Snake venom, *Moringa oleifera,* Antioxidants, Envenomation

Introduction

Snake venoms are complex mixtures of diverse biological active compounds such as proteins, peptides and nucleotides that can illicit toxic responses in a biological system. Several of these venom constituents disrupt hemostatic functions in human inducing various harmful effects after envenomation (Yamazaki and Morita, 2007). Studies have identified numerous constituents of venom primarily responsible for these toxic activities including metalloproteinases, phospholipases A2, snake venom serine proteinases, acetylcholinesterase, L-amino acid oxidases,

Corresponding Author: **Babafemi Siji Ajisebiola**
Department of Zoology, Osun State University, Osogbo, Osun State, Nigeria.
Email: dbabslinks@yahoo.com **Phone No:** +2348066690747

nucleotidases, and snake venom hyaluronidases (Kang *et al.,* 2011).

Bitis arietans commonly called puff adder is a venomous specie of snakes which belongs to the family Viperidae and produces venom that can be deadly following envenomation in humans and animals (WHO, 2010). The venom of *B. arietans* is composed of numerous toxic enzymes with biological properties capable of causing severe clinical effects after envenomation. The instant toxic effects experienced by victims after envenoming are local tissue damage, coagulopathy, spontaneous systemic bleeding, hypotension and myocardial damage (Currier *et al.,* 2010). *B. arietans* has been reported to be of high medical importance in Nigeria as bites from this species have resulted in many deaths most especially in the Northern region (Yusuf *et al.*, 2015).

Studies have documented that the major enzymes present in *B. arietans* are metalloproteinase (SVMPs) and phospholipases A2 (PLA2) exhibiting toxic biological activities (Currier *et al.,* 2010; Megale *et al.,* 2018). The former is responsible for spontaneous heamorrhage, delays coagulation, extracellular matrix degradation, blistering and local myonecrosis while the later often causes heamolysis, cardiotoxicity, myotoxicity, neurotoxicity and hypotension after envenoming (Fox and Serrano, 2005; Gutierrez and Ownby, 2003). In addition, SVMPs and PLA2s promote inflammatory responses that may facilitate tissue alterations and other systemic damages (Laing *et al.,* 2003; Teixeira *et al.*, 2003). Reactive oxygen species (ROS) are major initiators of inflammatory responses, alterations in cellular physiology and contribute significantly to systemic pathologies as evident in victims of snake envenomation (Carroll *et al.,* 2007). Furthermore, free radicals are directly involved in damaging cellular components, induces oxidative stress and play a major role in venom induced toxicity (Al-Quraishy *et al.,* 2014).

The use of phytotherapy as antivenom have gained much attention from researchers due to limited availability and accessibility of serum antivenom in several regions most especially in Asia, Latin America and Sub-Saharan Africa (Gutierrez *et al.,* 2015). *Moringa oleifera* Lam. is a common medicinal plant that has been explored for its usefulness in diverse treatment of diseases including snakebite envenoming (Adeyi *et al.,* 2020). Our previous studies have demonstrated that ethanol crude extract of *M. oleifera* leaves possesses neutralizing potency against pathophysiological effects actuated by *B. arietans* venom *in vivo* and *in vitro* models (Ajisebiola *et al.,* 2021). However, there is no available information on the efficacy of *M. oleifera* solvent fractions on toxicity induced by *B. arietans* venom. Hence, this current study focused to determine the best effective solvent fractions of *M. oleifera* crude extract against the toxic biological effects, venom lethal toxicity and oxidative stress induced by *B. arietans* venom using *in vivo* and *in vitro* methods.

Materials and Methods

Venom Collection

Lyophilized crude venom of *B. arietans* was procured from the Department of Veterinary Physiology and Pharmacology, Amadu Bello University, Zaria, Nigeria. It was stored at 2–4 °C in the Department of Zoology laboratory, University of Ibadan, Nigeria prior to the experiment.

Antivenom

A polyvalent EchiTAb-Plus ICP antivenom produced for treatment of *Echis ocellatus*, *Bitis arietans* and *Naja nigricollis* envenoming was used as standard drug for this study. The drug was produced at Instituto Clodomiro Picado, University of Costa Rica, Costa Rica.

Collection of plant material

Fresh leaves of *M. oleifera* were collected within the residential quarters of University of Ibadan, Nigeria. Samples of obtained leaves were deposited at the University of Ibadan Herbarium where it was identified and assigned specimen voucher no: UIH-22442.

Preparation and extraction of M. oleifera leaves

Fresh leaves of *M. oleifera* were rinsed in tap water, allowed to air-dry at room temperature, pulverized using electrical blender and stored in an amber bottle. Ethanol extraction of *M. oleifera* leaves was carried out using cold maceration method as previously described (WHO, 1998).

Solvent fractionation of ethanol crude extract of M. oleifera

Solvent fractionation was carried out using the protocol described by Wagenen *et al.* (1993). Exactly 194.22 g of ethanol crude extract of *M. oleifera* was fractionated using n-hexane and ethyl acetate solvents based on their polarity.

Phytochemical screening

Qualitative and quantitative analysis of n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera* extract were carried out for the detection of active secondary metabolite using standard methods (AOAC, 2005; Trease and Evans, 2002).

Experimental models

Collection of animals for in vivo studies

Eighty one adult male Wistar rats weighing between 120-150 g were obtained from the Animal House of the Department of Zoology, University of Ibadan, Nigeria. The animals were maintained in pathogen-free plastic cages under standard conditions (12h light and 12h dark, 27ºC cycle) in the animal house. They were fed with grower pelletized feed (Vital feeds, Nigeria) and allowed free access to tap water *ad labitum*. Thirty six rats were randomly selected into twelve groups of three rats (n=3) each for the antihaemorrhagic assay while fourty five rats were divided randomly into nine groups of five rats (n=5) each for the anti-oxidative stress study. Animal experimental procedure was approved by the University of Ibadan-Animal Care and Use Research Ethics Committee (UI-ACUREC) with assigned number: UI-ACUREC/19/0030.

Collection of fertile eggs for effective dose (ED_{50}) study

A total number of 100 fertile chick eggs were procured from hatcheries section of Obasanjo farms located in Ibadan, Oyo state for the determination of effective dose (ED_{50}) of *M. oleifera* solvent fractions. The chick eggs were incubated for 6 days to allow development of the eggs embryo with a vascularized yolk sac and primitive heart. The fertile eggs were transported via plastics containers to the Animal Physiology Laboratory, Department of Zoology, University of Ibadan, Nigeria where the experiment was carried out. The eggs were randomly divided into five groups (n=20 eggs) and eggs of each groups were used to determine venom neutralization assay by *M. oleifera* solvent fractions.

In vitro antivenom study of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera extract against B. arietans venom activities

The anti-haemorrhagic assay was determined according to Theakson and Reid (1983) while anti-heamolytic and coagulant activity were carried out as described by Gomes and Pallabi (1999) with modifications (Adeyi *et al.,* 2020).

Effective Dose (ED_{50}) assay for neutralization of venom lethal dose using M. oleifera solvent fractions

The effective dose was determined using chick embryo as previously developed by Sells *et al.* (1998). This was carried out to determine the best active solvent fraction obtained from *M. oleifera*

crude extract that can neutralized the lethal dose of the venom when injected into fertile eggs. Twenty fertile eggs were randomly selected into 5 groups (n=5 eggs) to determine the ED_{50} for n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera*. Exactly 0.1 ml of $3\times LD_{50}$ of venom in 1 ml of saline were pre-incubated with different amounts of solvent fractions of *M. oleifera* (0.1 ml of 200, 400, 600 mg) in respective separate tubes for 30 min at 37 °C. Modification to sells *et al.* (1998) method, contents of egg was not transferred out of the egg instead mixtures of venom/test samples were transferred into the egg opening at the apex without distorting the egg contents (Valk *et al.,* 2014). Exactly 0.1 ml of the venom and venom/test samples was applied to filter paper discs and placed over the vitelline vein on the yolk sac membrane of fertile egg in the respective venom group and groups treated with *M. oleifera* solvent fractions. After 6 h, the number of embryo deaths was counted in each group and recorded. The median effective dose (ED_{50}) as defined it protects 50% of the fertile eggs injected with $3LD_{50}$ of the venom. The ED_{50} was estimated using probit analysis.

Determination of anti-oxidative stress effects of solvent fractions of M. oleifera against B. arietans venom in rats

Animal groupings

Fourty five male albino Wistar rats were randomly divided into nine groups of five rats each. Group 1 was injected with saline (Normal control) while group 2 to 9 were envenomed. Group 2 was not treated post envenomation (Venom control) while group 3 was treated with polyvalent antivenom (antivenom control). Groups 4, 6 and 8 were treated with 300 mg/kg^{-1} of n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera* respectively while groups 5, 7 and 9 were treated with 600 mg/kg^{-1} of n-hexane, ethyl acetate, and ethanol solvent fractions of *M. oleifera* respectively.

Venom injection and treatment procedures

Envenomed rats were administered a single intraperitoneal injection of 1.5 mg/kg^{-1} (LD_{50}) of *B. arietans* venom (Ajisebiola *et al.,* 2015) dissolved in 2 ml of normal saline. After 1 hour, the envenomed rats were treated orally with 0.2 ml of varying concentrations (300 and 600 mg/kg) of the respective *M. oleifera* solvent fractions (n-hexane, ethyl acetate and ethanol fractions), while 0.2 ml of the antivenom was injected intramuscularly. Envenomed rats were treated for seven consecutive days.

Blood sample and organs collection

After seven days of treatment, the experimental rats were sacrificed using the method of Rowett, (1977). Blood samples from rats were collected into pre-labelled EDTA bottles by cardiac puncture method into EDTA bottles while the hearts and kidneys were removed for biochemical analysis.

Antioxidant profiles in serum and tissues of experimental rats

Lipid peroxidation were determined using the method of Ohkawa *et al.* (1979) while catalase activity was carried out as described by Aebi, (1984). SOD activity was assessed using the method as described by Marklund and Marklund, (1974).

Statistical analysis

Results were expressed as means ± standard error (S.E) and statistical significance of differences among experimental groups were evaluated using one way analysis of variance (ANOVA) and Duncan Multiple Test. SPSS computer software (version 25) was used to analyse the data and a value of ($P<0.05$) was considered statistically significant.

Results

Qualitative and quantitative phytochemical analysis of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera

The results of the qualitative and quantitative phytochemical screening of *M. oleifera* solvent fractions (n- hexane, ethyl acetate and ethanol fractions) were shown in Table 1 and 2 respectively.

Table 1: Qualitative phytochemical constituents of n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera.*

Test	N-hexane fraction	Ethyl acetate fraction	Ethanol fraction
Alkaloids	+	++	+
Flavonoids	+	++	+
Saponins	-	+	+
Tannis	-	++	++
Anthraquinones	+	+	+
Terpenoids	+	++	++
Cardiac Glycosides	+	+	-
Steroids	+	++	++
Phenol	+	++	+

+ : Present, **++ :** Abundant, **-:** Absent

Table 2: Quantitative phytochemical constituents of n-hexane, ethyl acetate, and ethanol solvent fractions of *M. oleifera.*

Test	n-hexane fraction	Ethyl acetate fraction	Ethanol Fraction
	Yield (%)	**Yield (%)**	**Yield (%)**
Alkaloids	2.33	42.50	14.75
Flavonoids	6.23	22.30	12.30
Saponins	-	4.60	15.10
Tanins	-	0.26	0.77

- : Absent

Antivenom activities of M. oleifera solvent fractions

Anti-haemorrhagic effects of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera against B. arietans venom activity

There was no lesion observed in group 1 which served as the normal control whereas, 100% heamorrhage was recorded in group 2 (venom control), a value significantly ($P<0.05$) higher when compared to other groups treated with *M. oliefera* fractions and antivenom. *M. oleifera* fractions and antivenom displayed a strong inhibitory activities in the treated groups. However, heamorrhage induced by the venom was best inhibited in group treated with 600 mg of *M. oleifera* ethyl acetate solvent fraction with 68% inhibition (Table 3).

Table 3: Anti-heamorrhagic effects of *M. oleifera* solvent fractions against *B. arietans* venom

Groups	Test samples	% Inhibition
1	Normal control	No lesion
2	Venom control	No inhibition (100% haemorrhage)
3	Venom/antivenom (ml)	64.00 ± 1.00^{fg}
4	Venom/n-hexane 200 mg	29.00 ± 1.00^{a}
5	Venom/n-hexane 400 mg	38.00 ± 1.73^{c}
6	Venom/n-hexane 600 mg	37.00 ± 1.00^{bc}
7	Venom/ethyl acetate 200 mg	31.33 ± 0.67^{ab}
8	Venom/ethyl acetate 400 mg	55.67 ± 1.67^{e}
9	Venom/ethyl acetate 600 mg	68.00 ± 2.31^{g}
10	Venom/ethanol 200 mg	42.33 ± 2.33^{cd}
11	Venom/ethanol 400 mg	55.00 ± 1.00^{e}
12	Venom/ethanol 600 mg	62.00 ± 1.15^{fg}

Values are expressed as mean ± S.E (n=3). Values in the same column with different superscripts are significantly different ($P<0.05$).

Anti-heamolytic effects of M. oleifera n-hexane, ethyl acetate and ethanol solvent fractions against B. arietans venom activity

A complete (100%) heamolysis was recorded in group 1 (normal control) and the value obtained was significantly (P<0.05) higher when compared to venom control and other treated groups. The antivenom, 600 mg of n-hexane and ethyl acetate fractions of *M. oleifera* showed the highest antiheamolytic effects with 82, 83 and 82% inhibition respectively, although the values were statistically (P<0.05) not different. However, n-hexane fraction exhibited the highest antiheamolytic effect with 83% inhibition (Table 4).

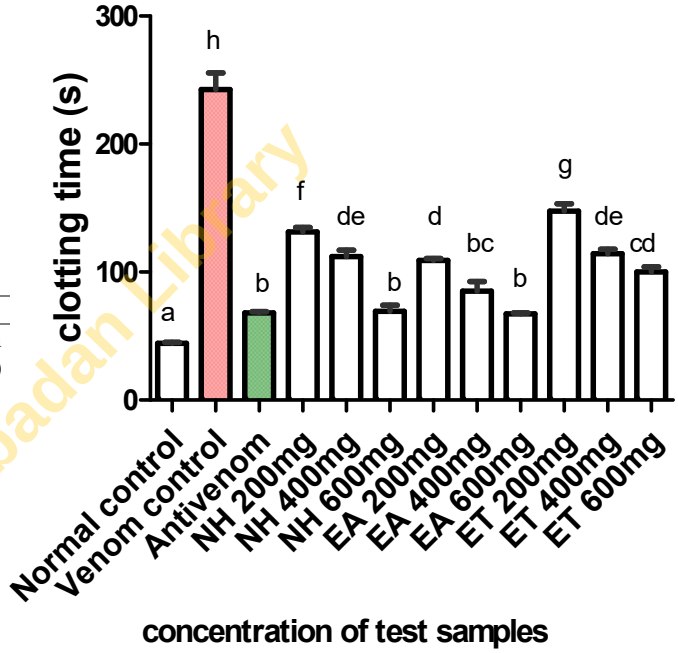
Table 4: Anti-heamolytic effects of *M. oleifera* solvent fractions against *B. arietans* venom

Groups	Test Samples (Ml)	Inhibition (%)
1	Normal control	No inhibition (100% heamolysis)
2	Venom control	16.00 ± 1.06^{g}
3	Venom/antivenom (ml)	82.33 ± 1.45^{fg}
4	Venom/n-hexane 200 mg	68.33 ± 0.88^{e}
5	Venom/n-hexane 400 mg	75.00 ± 0.58^{ef}
6	Venom/n-hexane 600 mg	83.00 ± 1.53^{fg}
7	Venom/ethyl acetate 200 mg	45.67 ± 2.84^{c}
8	Venom/ethyl acetate 400 mg	73.67 ± 1.86^{e}
9	Venom/ethyl acetate 600 mg	82.33 ± 2.19^{fg}
10	Venom/ethanol 200 mg	27.67 ± 0.67^{a}
11	Venom/ethanol 400 mg	37.00 ± 5.13^{b}
12	Venom/ethanol 600 mg	43.00 ± 2.31^{bc}

Values are expressed as mean ± S.E (η =3). Values in the same column with different superscripts are significantly different (P<0.05).

Coagulant effects of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera against B. arietans venom activity

Group 1 which served as the normal control clotted at 44 s with value significantly (P<0.05) lower when compared to the venom control which clotted at 242 s. The anticoagulant activity of the venom was strongly inhibited in group treated with antivenom (68 s), n-hexane (69 s) and ethyl acetate (67 s) solvent fractions of M. oleifera. However, the best coagulating effects was noticed in group treated with 600 mg of ethyl acetate fraction (Fig 1).



Values are expressed as mean ± S.E (n =3). Values with different superscripts are significantly different (P<0.05).

Figure 1: Coagulant effects of *M. oleifera* solvent fractions against *B. arietans* venom

Neutralization of the lethal toxicity of B. arietans venom by M. oleifera solvent fractions in fertile eggs model

The mortalities of embryo observed and results of the effective dose (ED_{50}) as determined using fertile eggs embryo having a vascularized yolk sac and primitive heart were presented in table 5 and 6. The least ED_{50} was recorded in ethyl acetate solvent fraction of *M. oleifera* with estimated

value at 120.316 mg, thus the most active fraction of *M. oleifera*.

Table 5: Mortalities of egg embryos after injection with mixtures of venom and *M. oleifera* solvent fractions

Groups	N-hexane fraction	Ethanol fraction	Ethyl acetate fraction
1 (Normal saline)	0	0	0
2 (Venom only)	5	4	4
3 (Venom/200mg test sample)	3	3	2
4 (Venom/400mg test sample)	2	2	1
5 (Venom/600mg test sample)	0	2	0

Table 6: Effective dose (ED_{50}) values using probit estimation

M. oleifera solvent fractions	Effective Dose (ED_{50}) values (mg)
N-hexane fraction	258.66
Ethanol fraction	305.757
Ethyl acetate fraction	120.316

Anti-oxidative stress activities of M. oleifera solvent fractions in rats

Mortalities observed post envenoming in rats treated with M. oleifera solvent fractions

During the exposure and treatment period, no mortality was recorded in the normal control group, however, 40% mortality were recorded in all the envenomed treated groups except in group 7 envenomed and treated with 600 mg/kg of ethyl acetate fraction of *M. oleifera* where 20% mortality was recorded.

Serum antioxidants profiles of envenomed rats treated with n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera

The values obtained for serum enzymes activities of Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT) of group 1 (normal control) was significantly ($P<0.05$) lower compared to group 2 (venom control). The MDA, SOD and CAT of envenomed rats treated with solvent fractions of *M. oleifera* and antivenom were significantly ($P<0.05$) lower compared to the venom control. Group 7 envenomed and treated with 600 mg/kg of ethyl acetate fraction recorded the lowest SOD and MDA activity while group 3 and 9 envenomed and treated with antivenom and 600 mg/kg of ethanol fraction respectively, recorded the least CAT activity (Table 7).

Table 7: Antioxidant enzymes activity in serum of envenomed rats after treatment with solvent fractions of *M. oleifera*

Treatment Groups	Serum SOD (U/mL)	Serum CAT (kU/L)	Serum MDA (nmole/L)
1 (Control)	18.75 ± 0.00^{b}	2484.20±65.48	3341.90 ± 21.04^{ab}
2 (Venom only)	32.64 ± 2.00^{f}	3158.00 ± 4.03^{d}	3872.00 ± 6.49^{e}
3 (Venom+0.2 ml AV)	17.82 ± 0.46^{ab}	1768.00 ± 2.28^{a}	3587.60 ± 4.27^{cd}
4 (Venom+300 mg/kg NH)	29.20 ± 2.90^{ef}	2710.00 ± 1.67^{bcd}	3753.40 ± 24.29^{de}
5 (Venom+600 mg/kg NH)	21.39 ± 0.93^{bc}	2419.60 ± 2.12^{bc}	3784.20 ± 55.68^{de}
6 (Venom+300 mg/kg EA)	18.05 ± 2.12^{ab}	2387.40 ± 2.88^{bc}	3653.80 ± 9.79^{cde}
7 (Venom+600 mg/kg EA)	13.89 ± 0.40^{a}	2477.70 ± 1.07^{bc}	3257.30 ± 53.16^{a}
8 (Venom+300 mg/kg ET)	26.62 ± 1.01^{de}	2322.90 ± 69.79^{abc}	3630.30 ± 14.96^{cd}
9 (Venom+600 mg/kg ET)	32.07 ± 0.81^{f}	2068.10 ± 12.90^{ab}	3504.30 ± 73.03^{bc}

Values are mean ± S.E (η =3). Values in the same column with different superscripts are significantly different ($P<0.05$).

CE: Crude Extract, AV: Antivenom, NH: N-hexane, EA: Ethyl Acetate, ET: Ethanol, MDA: Malondialdehyde, SOD: Superoxide Dismutase, CAT: Catalase

Effects of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera on antioxidant enzymes activity in kidney tissues of envenomed treated rats

The activities of Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT) on kidney of venom control rats did not present any significant ($P<0.05$) increase when compared to normal control. Also, envenomed groups treated with respective solvent fractions of *M. oleifera* were statistically ($P<0.05$) not different from the normal and venom control in the MDA measurement (Table 8).

Table 8: Antioxidant enzymes activity in kidney of envenomed rats after treatment with solvent fractions of *M. oleifera*

Treatment Groups	Kidney SOD (U/mL)	Kidney CAT (kU/L)	Kidney MDA (nmole/g)
1 (Control)	25.84±1.33^{abc}	7680.00±62.87^{b}	33.97±5.85^{a}
2 (Venom only)	26.69±2.40^{abc}	7787.20±21.50^{cd}	34.19±1.06^{a}
3 (Venom+0.2 ml AV)	25.63±3.89^{abc}	7825.10±33.88^{d}	30.38±1.78^{a}
4 (Venom+300 mg/kg NH)	26.51±1.61^{abc}	7780.00±40.71^{cd}	32.41±0.06^{a}
5 (Venom+600 mg/kg NH)	25.51±0.82^{abc}	7621.90±19.55^{b}	34.36±0.26^{a}
6 (Venom+300 mg/kg EA)	26.78±1.61^{abc}	7610.60±29.88^{b}	35.02±0.13^{a}
7 (Venom+600 mg/kg EA)	23.61±0.80^{ab}	7576.70±28.87^{b}	34.68±0.20^{a}
8 (Venom+300 mg/kg ET)	24.76±1.67^{a}	7633.20±11.29^{b}	34.13±1.04^{a}
9 (Venom+600 mg/kg ET)	24.14±3.61^{a}	7452.50±19.55^{a}	33.61±0.53^{a}

Values are mean ± S.E (η =3). Values in the same column with different superscripts are significantly different ($P<0.05$). CE: Crude Extract, AV: Antivenom, NH: N-hexane, EA: Ethyl Acetate, ET: Ethanol, MDA: Malondialdehyde, SOD: Superoxide Dismutase, CAT: Catalase

Effects of n-hexane, ethyl acetate and ethanol solvent fractions of M. oleifera on antioxidant enzymes activity in heart tissues of envenomed treated rats

The tissue enzymes activities of Superoxide Dismutase (SOD), Malondialdehyde (MDA) and Catalase (CAT) of group 2 (venom control) was significant ($P<0.05$) higher compared to normal control. Also, the MDA, CAT and SOD levels in heart tissues of the venom control rats significant ($P<0.05$) increased compared to envenomed groups treated with solvent fractions of *M. oleifera.* Also, there was significant ($P<0.05$) reduction in the SOD, CAT and MDA levels of rats treated with antivenom when compared to venom control group. However, Group 7 envenomed and treated with 600 mg/kg of ethyl acetate faction of *M. oleifera* best normalized the SOD, CAT and MDA activities across the treated groups (Table 9).

Table 9: Antioxidant enzymes activity in heart tissue of envenomed rats after treatment with solvent fractions of *M. oleifera*

Treatment Groups	Heart SOD (U/mL)	Heart CAT (kU/L)	Heart MDA (nmole/g)
1 (Control)	23.61±1.29^{a}	4058.20±16.13^{a}	35.33±2.58^{ab}
2 (Venom only)	31.89±1.95^{b}	6736.00±8.07^{c}	39.94±0.00^{c}
3 (Venom+0.2 ml AV)	25.69±1.39^{ab}	4258.20±89.93^{ab}	34.55±0.41^{ab}
4 (Venom+300 mg/kg NH)	27.09±0.70^{ab}	6033.00±23.26^{ab}	36.84±0.18^{b}
5 (Venom+600 mg/kg NH)	27.59±2.09^{ab}	6516.90±6.45^{ab}	34.63±0.77^{ab}
6 (Venom+300 mg/kg EA)	25.93±0.93^{ab}	6355.90±75.45^{ab}	36.77±0.44^{b}
7 (Venom+600 mg/kg EA)	21.55±3.72^{a}	3517.00±1.88^{a}	33.75±0.37^{a}
8 (Venom+300 mg/kg ET)	32.87±3.81^{b}	4968.20±53.69^{ab}	35.32±0.47^{ab}
9 (Venom+600 mg/kg ET)	23.61±1.06^{a}	4382.30±6.20^{ab}	34.94±0.49^{ab}

Values are mean ± S.E (η =3). Values in the same column with different superscripts are significantly different ($P<0.05$). CE: Crude Extract, AV: Antivenom, NH: N-hexane, EA: Ethyl Acetate, ET: Ethanol, MDA: Malondialdehyde, SOD: Superoxide Dismutase, CAT: Catalase

Discussion

Bitis arietans venom contains cytotoxic and hemotoxic enzymes which affects cells of the body, resulting in blood lyses and systemic heamorrhage after envenoming due to the presence of PLA_2 and majorly SVMPs (Currier *et al.,* 2010). Alteration in physiological steady state of the body due to snake venom toxic enzymes may results in death if proper treatment is not administered in time. In the *in vivo* study using rats, mortality recorded across the groups post-envenoming may be attributed to the toxic effects of the venom toxins. However, mortalities reduced drastically in envenomed rats treated with solvent fractions of *M. oleifera* most especially in group treated with ethyl acetate solvent fraction of *M. oleifera* which recorded the least death. This finding is an indication that the plant's phytoconstituents may have halted further actions of the venom toxins to increase their chances of survival. Interestingly, this result further strengthened findings from the neutralization of venom lethal dose toxicities study using fertile chick eggs as ethyl acetate solvent fraction of *M. oleifera* recorded the least ED_{50} thus, found to be best effective to neutralize the LD_{50} of the venom. According to Laing *et al.* (1992), the lower the ED_{50} value, the higher the neutralizing ability of the antivenom.

In this present study, the venom exhibited a significant heamorrhagic action in the venom control which can be attributed to the action of SVMPs enzymes present in *B. arietans* venom as they are known to cause degradation of the extracellular membrane components of the capillary vessels resulting in a break and loss of blood from the blood vessels (Currier *et al.,* 2010) and such observation has been earlier reported (Baggai *et al.,* 2020). Furthermore, antivenom and varying concentrations of *M. oleifera* solvent fractions displayed antiheamorrhagic activities, however, high dose of ethyl acetate fraction of *M. oleifera* was most effective against the heamorrhagic action of the venom. This observation aligned with earlier report on the antiheamorrhagic activity of crude extract of *M. oleifera* on *B. arietans* venom (Ajisebiola *et al.,* 2021). The antiheamorrhagic effects of ethyl acetate fraction of *M. oleifera* may be due to the presence of bioactive compounds that can directly inhibits SVMPs enzymes by interacting with divalent ion such as Zn2+ which is an essential cofactor of SVMP thereby leading to the inhibition of their enzymatic activity (Mors *et al.,* 2000).

B. arietans venom are known to disrupt blood coagulation in envenomed victims. As observed in this study, the venom caused a significant delay in plasma clotting time in the venom control, however, coagulating time was shortened in the treated groups. It should be noted that coagulation was best restored in group treated with antivenom and high dose of *M. oleifera* ethyl acetate fraction. The venom of *B. arietans* contains haemolysins such as phospholipase A2 (PLA2) which are responsible for blood lyses by interacting with phospholipids on cell membrane that results to hydrolysis of intact membrane releasing free fatty acids and lysopholipids (Choklingam *et al.,* 2012). The venom induced heamolysis conforms to findings of Baggai *et al.* (2020) however, heamolysis exhibited by the venom was best inhibited in group treated with high dose of *M. oleifera* n-hexane fraction and these findings corroborated previous report of anti-blood lyses effect of *M. oleifera* crude extract against *B. arietans* venom (Ajisebiola *et al.,* 2021).

The present study showed that *B. arietans* venom induced oxidative stress in serum and caused malfunctioning of vital organs through production of MDA contents and enzyme release. The venom induced oxidative stress as revealed by enhanced superoxide dismutase (SOD) and catalase (CAT) enzymes activities in serum and heart tissues of venom control rats which may be attributed to the generation of reactive oxygen species (ROS) by

the venom toxins. This observation was in tandem with our previous studies on oxidative stress induced by *Echis ocellatus* venom which is a viper (Adeyi *et al.,* 2021). ROS do play a major part in venom-induced toxicity by altering cellular physiology (Santhosh *et al.,* 2013). Also, the enzymatic/non-enzymatic toxins including membrane-perturbing peptides can damage the cell membrane as well as cause disruption of the mitochondrial membrane, respiratory burst and activities of various oxidases resulting in a surge of ROS into the system (Santhosh *et al.,* 2013). Superoxide dismutase (SOD) and catalase (CAT) are known to directly eliminate ROS and higher activities as observed in the venom control indicated oxidative stress. However, antivenom and *M. oleifera* solvent fractions were able to normalize the activity of SOD and CAT but the effects were significantly evident in group 7 envenomed and treated with 600 mg/kg of *M. oleifera* ethyl acetate fraction when compared to other envenomed treated groups. The results aligned with earlier studies on the effectiveness of *M. oleifera* solvent fractions against oxidative stress induced by *E. ocellatus* venom (Adeyi *et al.,* 2021).

Furthermore, oxidative stress resulting from venom induced toxicity was assessed by measuring lipid peroxidation. Lipid peroxidation is a baseline for manifestations of oxidative damage initiated by ROS (Santhosh *et al.,* 2013). Malondialdehyde (MDA) is a known end product of lipid peroxidation and considered as bio-indicator of lipid peroxidation (Othman *et al.,* 2014). *B. arietans* venom elevated levels of MDA in serum and heart of envenomed untreated rats as earlier reported by Adeyi *et al.* (2021) using a viper venom. This effect was ameliorated by solvent fractions of *M. oleifera* however, levels of MDA was most reduced in group 7 treated with high dose (600 mg/kg) of *M. oliefera* ethyl acetate fraction.

Gomes *et al.* (2110) have reported that plants constituents are active against snake venom toxins. On the other hand, phytochemical analysis of n-hexane, ethyl acetate and ethanol solvent fractions of *M. oleifera* extract revealed various metabolites which has been reported to be active against snake venom toxins. Inhibition of snake venom enzyme and protein binding properties have been associated with bioactive compounds of flavonoids, polyphenols, terpenoids, alkaloids, xanthenes etc (Selvanayagam *et al.,* 1996). Report abound that phenolics, especially polyphenols such as tannins, bind proteins acting upon the component of venom directly and disabling them to act upon the receptors (Selvanayagam *et al.,* 1996) and they could also act by competitive blocking of the receptors (Lans *et al.,* 2001). In other studies, polyphenols and tannins are attributable to the reduction in enzyme activities (Abubakar *et al.,* 2000). In this present study, it is pertinent to note that these major phytochemicals earlier mentioned are present abundantly in ethyl acetate fraction of *M. oleifera*. Furthermore, previous studies have attributed the inhibition of toxic enzymes of snake venom to the presence of these phytochemicals in *M. oleifera* crude extract (Adeyi *et al.,* 2020) which may also be applicable in this study.

Conclusion

In this study, *B. arietans* venom initiated some pathophysiological alterations and induced oxidative stress *in vitro* and *in vivo* but fractions of *M. oleifera* extract mollified these toxic actions actuated by the venom toxins. Ethyl acetate fraction of *M. oleifera* was best effective in attenuating the venom induced toxicities. However, further studies is important and hereby suggested to isolate and characterize bioactive compounds present in ethyl acetate fraction of *M. oleifera* which are responsible for the antivenom activity.

Declaration: Authors declare no conflict of interest

References

Abubakar, M.S., Sule, M.I., Pateh, U.U., Abdulrahaman, E.M., Haruna, A.K. and Jahun, B.M. (2000). *In vitro* snake venom detoxifying action of leaf extract of *Guierra senegalensis*. *Journal of Ethnopharm-acology*, 69:253–257

Adeyi, A.O., Adeyemi, S.O., Effiong, E.O.P., Ajisebiola, B.S., Adeyi, O.E. and James, A.S. (2021). *Moringa oleifera* extract extenuates *Echis ocellatus* venom-induced toxicities, histopathological impairments and Inflammation via enhancement of Nrf2 expression in rats. *Pathophysiology*, 28:98–115.

Adeyi, A.O., Ajisebiola, B.S., Adeyi, O.E., Alimba, G.C. and Okorie, U.G. (2020). Antivenom activity of *Moringa oleifera* leave against pathophysiological alterations, somatic mutation and biological activities of *Naja nigricollis* venom. *Scientific African,* 8, e00356.

Aebi, H. (1984). Catalase. *Methods in Enzymology*, 1059:121–5

Ajisebiola, B.S., Solomon, R., Anwar, U. and Adeyi, A. O. (2021). Neutralization of *Bitis arietans* venom-induced pathophysiological disorder, biological activities and genetic alterations by *Moringa oleifera* leaves. *Toxin Reviews*, 40:847-858.

Al-Quraishy, S., Mahamed, A., Dkhil, M.A. and Moneim, E.A. (2014). Hepatotoxicity and oxidative stress induced by *Naja haje* crude venom. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 20:42.

AOAC (Association of Official Analytical Chemists) (2005). Method of analysis. Washington, DC: AOAC.

Baggai, B.I., Yusuf, P.O., and Alloh, F.T. (2020). Inhibitory Studies of *Tamarindus indica* Seed Extract and Fractions on Hematological Activities of *Bitis arietans* Venom. *Journal of Advances in Biology and Biotechnology,* 23:48-57

Carroll, I.M., Andrus, J.M., Bruno-Barcena, J.M., Klaenhammer, T.R., Hassan, H.M. and Threadgill, D.S. (2007). Anti-inflammatory properties of Lactobacillus gasseri expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis. *American Journal of Physiology- Gastrointestinal and Liver Physiology,* 293:729–738.

Choklingam, S., Ghosh, S.S. and Mukherjee, A.K. (2012). Differential mode of attack on membrane phospholipids by an acidic phospholipase A2 (RVVA-PLA 2-I) from Daboia russelli venom. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1818:3149-3157

Currier, R.B., Harrison, R.A., Rowley, P.D., Laing, G.D. and Wagstaff, S.C. (2010). Intra-specific variation in venom of the African Puff Adder (*Bitis arietans*): Differential expression and activity of snake venom metalloproteinases (SVMPs). *Toxicon*, 55:864-873

Fox, J.W. and Serrano, S.M. (2005). Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon*. 45:969–985

Gomes, A., Bhattacharjee, P., Mishra, R., Biswas, A. K., Dasgupta, S. C. and Giri, B. (2010). Anticancer potencial of animals venom and

toxins. *Indian Journal of Experimental Biology*, 48:93–103.

Gomes, A., Das, R., Sarkhel, S., Mishara, R., Mukherjee, S., Bhattacharya, S. (2010). Herbs and herbal constituents active against snake bite. *Indian Journal of Experimental Biology*, 48:865–878.

Gutiérrez, J.M. and Ownby, C.L. (2003). Skeletal muscle degeneration induced by venom phospholipases A2: insights into the mechanisms of local and systemic myotoxicity. *Toxicon,* 42:915–931

Gutierrez, J.M.T., Burnouf, R.A., Harrison et al. (2015). A call for incorporating social research in the global struggle against snakebite. *PLoS Neglected Tropical Diseases*. 9, e0003960.

Kang, T.S., Georgieva, D., Genov, N., Murakami, M.T., Sinha, M., Kumar, R.P., Kaur, P., Kumar, S., Dey, S., Sharma, S., Vrielink, A., Betzel, C., Takeda, S., Arni, R.K., Singh, T.P. and Kini, RM. (2011). Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. *The Federation European Biochemical Society (FEBS) Journal*. 278: 4544–4576.

Laing, G.D., Clissa, P.B., Theakston, R.D., Moura-da-Silva, A.M. and Taylor, M.J. (2003). Inflammatory pathogenesis of snake venom metalloproteinase-induced skin necrosis. *European Journal of Immunology*, 33:3458–3463

Laing, G.D., Theakston, R.D.G., Leite, R.P., Dias da Silva, W., Warrell, D.A., BIASG (Butantan Institute Antivenom Study Group) (1992). Comparison of the potency of three Brazilian Bothrops antivenoms using in vivo rodent and in vitro assays. *Toxicon*, 30:1219–1225.

Lans, C., Harper, T., Georges, K. and Bridgewater, E. (2001). Medicinal and ethnovetrinary remedies of hunters in Trinidad. BMC *Compliment and Alternative Medicine*, 1:10.

Marklund, S. and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, 47: 469-474

Megale, A.A.A., Magnoli, F.C., Kuniyoshi, A.K., Iwai, L.K., Tambourgi, D.V., Portaro, F.C.V., and da Silva, W.D. (2018). Kn-Ba: a novel serine protease isolated from Bitis arietans snake venom with fibrinogenolytic and kinin-releasing activities. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 24:38

Mors, W.B., Nascimento, M.C., Pereira, B.M., and Pereira, N.A. (2000). Plant natural products active against snake bite— the molecular approach. *Phytochemistry*, 55:627–642.

Ohkawa, H., Ohnishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95:351–8.

Othman, M.S., Safwat, G., Aboulkhair, M. and Abdel Moneim, A.E. (2014). The potential effect of berberine in mercury-induced hepatorenal toxicity in albino rats. *Food and Chemical Toxicology*, 69:175–181.

Rowett, H.G.O. (1977). Dissecting guides of rats with notes on mouse. Bulter and tanner LTD, London, UK. 111:5-23.

Santhosh, S.M., Hemshekhar, M., Thushara, R.M., Devaraja, S., Kemparaju, K., and Girish, K.S. (2013). Viper russelli venom-induced oxidative stress and hematological

alterations: Amelioration by crocin a dietary colorant. *Cell Biochemistry and Function*, 31:41-50.

Sells, P.G., Loannou, P and Theakston, R.D.G. (1998). A hummane, alternative to the measurement of the lethal effects (LD_{50}) of non-neurotoxic venoms using hens' eggs. *Toxicon*. 36:985.

Selvanayagam, Z.E., Gnanavendhan, S.G., Balakrisha, K., Rao, B.B., Sivaraman, J. and Subramanian, K. (1996). Ethretianone, a novel quinonoid xanthene from *Ehretia buxifolia* folia with anti-snake venom activity. *Journal of natural products*, 59:664–667

Teixeira, C.F., Landucci, E.C., Antunes, E., Chacur, M. and Cury, Y. (2003). Inflammatory effects of snake venom myotoxic phospholipases A2. *Toxicon*, 42:947–962

Theakston, R.D.G. and Reid, H.A. (1983). The development of simple standard assay procedures for characterization of snake venoms. *Bulletin of the World Health Organization*, 61:949–956

Trease, G.E. and Evans, W.C. (2002). Pharmacognosy, 15th edition, India, Reed Elsevier publication.

Valk, T.V., Arie, V. and Meijden, A. (2014). Toxicity of scorpion venom in chick embryo and meal worm assay depending on the use of the soluble fraction versus the whole venom. *Toxicon*, 88:38

Wagenen, V.B.C., Larsen, R., Cardellina, J.H., Randazzo, D., Lidert, Z.C. and Swithenbank, C. (1993). Ulosantoin, a potent insecticide from the sponge Ulosaruetzleri. *The Journal of Organic Chemistry*, 58:335-337

World Health Organization. (1998). WHO monographs on the selected medicinal plants. WHO, Geneva. Accessed July 20, 2000.

World Health Organization. (2010). Guidelines for the prevention and clinical Management of snakebite in Africa: accessed June 29, 2012.

Yamazaki, Y. and Morita, T. (2007). Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. *Current Pharmaceutical Design,* 13:2872–2886.

Yusuf, P.O., Mamman, M., Ajagun, E., Suleiman, M.M., Kawu, M.U., Shittu, M., Isa, H.I., Tauheed, M. and Yusuf, A. (2015). Snakes Responsible for Bites in North-Eastern Nigeria – A Hospital Based Survey. *Journal of Environmental Science, Toxicology and Food Technology*. 9:118-121.