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DETERMINATION OF LETHAL DOSE (LD_{50}) CONCENTRATION OF *ECHIS OCELLATUS'* VENOM AND ETHANOL EXTRACT OF *Moringa oleifera* PLANTS ON THE PARAMETERS OF HAEMATOLOGY AND HISTOPATHOLOGY OF MALE ABINO RATS.

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ABSTRACT

Abino Rat is the most venomous viper species which constitutes a severe economic and public health problem all over the world, specifically, in African countries. It's responsible for 90% of the bites and over 60% of the several thousand annual deaths from snakebite in West Africa, particularly to farming communities and antivenom is the only available specific treatment but its use often triggers serious physiological responses. Moringa oleifera has been reported as a medicinal plant used traditionally in Nigeria for the treatment of many diseases. This study therefore investigated the LD_{50} of the E. ocellatus snake venom and the LD_{50} of M. oleifera extract. For the LD_{50} of E. ocellatus experiment, twenty (20) male albino rats weighing between 150-180g were randomly divided into four (4) groups of five rats each. Group A served as the normal control while group B, C, and D

Introduction:
Venoms are modified saliva containing a mixture of different bioactive proteins and polypeptides used by an animal for defense or to immobilize its prey (Gomes *et al.*, 2010). Snake venom is a cocktail of thousands of different peptides, enzymes, chemicals and majorly proteins which produced in the glands throughout the life of the snake and contained anti-clothing proteins which produces toxicity only when contacted with the blood but harmless if ingested in liquid or crystal form after passing through mouth and excreted unchanged.).The constituents of snake venom can be preserved for longer period if the dried solid crystal form is stored properly and the crystal mass can dissolve

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were envenomed by a single intraperitoneal injection of 0.56, 0.28 and 0.14mg/kg respectively, while sixteen (16) male albino rats weighing between 150-180g were randomly divided into four (4) groups of four (4) rats each. Mortality rate, weight change and behavioural response were observed during the experiment. More so, haematology and histopathology of heart were investigated in the LD_{50} of the E. ocellatus snake venom and the concentrations of the blood component of the rats were significantly decreased ($p<0.05$) in haematological study of the LD_{50} of the E. ocellatus snake venom. Heart lesion was observed in all the groups compared to the control group. The findings were determined using analysis of Probits in SPSS 2019 and *revealed that E. ocellatus has LD_{50} of 0.22mg/kg and M. oleifera has 800mg/kg as LD_{50}. Moreover, M. oleifera has very low toxicity profile in all the tested animals, it is relatively safe for herbal oral medication and this research might become a highly effective therapeutic agent for the treatment of diseases. Further study would be effect of M. oleifear as an antidote for Echis ocellatus envenomation.*

Keywords: *Echis ocellatus, Moringa oleifera, haematology and histopathology.*

Readily in blood and water (Jin *et al.,* 2004). *Echis ocellatus* (Africa saw-scaled viper) is a venomous viper species that ranges in its African savannah distribution from Mali in the west to Cameroon and Chad in the east, Stemmler, (1979). Despite its relatively small size (averaging 30–50 cm), it is considered the single most medically important snake species in West Africa and constitutes a severe economic and public health problem, particularly to farming communities, Laing, *et al.,* (1995). It is reported to be responsible for 90% of the bites and over 60% of the several thousand annual deaths from snakebite in West Africa, Chippaux, (2002) and Chippaux, *et. al.,* (2008). The incidence of snake bite in rural West Africa is estimated to be as high as 174 per 100,000 population, with 11–17% mortality rate caused by *E. ocellatus*, Visser, (2008). Its venom is extremely potent with an intravenous LD_{50} for mice of 20.1µg, Laing, *et al.,* (1995).

For the past century, polyvalent anti-venom (PVA) is the only available specific and conventional method of treatment of snake envenomation but this is not readily achievable in rural settings in Nigeria as a result of erratic power supply for the preservation of the PVA. However, PVA does not provide enough protection against venom-induced haemorrhage, necrosis, nephrotoxicity and often develops hypersensitivity reactions (Sutherland, 1977; Corrigan *et al*, 1978). The symptoms in humans envenoming by *E. ocellatus* prompts a quantitatively similar pathophysiological effects such as severe blistering, oedema and necrosis at the bite site, and life threatening systemic effects including haemorrhage, coagulopathy and occasionally hypovolaemic shock, Fox, (2005). The administration of anti-venom in post envenomation usually neutralized the system effects successfully but elimination of local tissues damage is a more problematic chore. In many of cases of snakebite, lack of neutralization of local effects results in permanent tissue damage and organ dysfunction, Onyeama, *et. al.,* (2012).

With the references to the incidence of snakebites in West Africa and specifically, the reports of the crisis of E. *ocellatus* in Nigeria, It has therefore, become necessary to look for new methods to neutralize these toxins through phytotherapy so as to turn out to be accessible and affordable choice for the treatment of snakebite victims since the PVA with its known anaphylactic reactions in some people is expensive and unaffordable to the rural people who constitute the majority of snakebite victims. In the ethnobotanical study of snakebites specifically, the viperian species, traditional herbalists in southern parts of Nigeria have found the leaves of *Moringa oleifera* valuable in neutralizing viperian venom. The phytochemical analysis of the plants revealed its various glycosides of thiocarbamate and isocyanide class. Pterygospermin, moringyne, Niaziridin, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate, etc., are few of them which are isolated and therapeutically proved by scientific studies as antihypertensive, antiasthmatic, antidiuretic, anticancer, antibiotic, antiulcer, analgesic, CNS- depressant, antiepileptic, anti-inflammatory, anthelmintic, antiurolithiatic, and also contain a mixture of several

hydrolytic enzymes, in which proteases are the key enzymes responsible for the observed pharmacological actions and many more. Therefore, this study was aimed to investigate the lethal dose concentration (LD_{50}) test of ethanol leaf extract of *M. oleifera* and *E. collatus* in male wistar rats.

MATERIALS AND METHOD

Collection of Venom

The venom was collected by the usual milking method of Markfarlane (1967) from locally caught. Lyophilized venom pooled from adult species of *E. ocellatus* was collected in Zaria town, Kaduna State and obtained from the Department of Pharmacology, Amadu Bello University, Zaria, Nigeria. It was stored at 2–4°C prior to the experiments and dilutions to desired concentrations were done when needed.

Collection of the Plant material

Fresh leaves of *M. oleifera* were collected from campus premises of the University of Ibadan and the leave were identified and authenticated by trained Taxonomist with voucher no: UIH-22442 in the herbarium section of the Department of Botany, University of Ibadan, Ibadan. The leaves were subsequently air-dried at room temperature between (25 to 27^{0}C) in the laboratory and grinded to powdery form using a Qlink electric blender and sieved.

Extraction of the Ethanol Extract of the Plant Leaf

Cold maceration method was employed in the extraction of the plant material. Five hundred grammes (500g) of the powdered leaf of *M. oleifera* were soaked in 1liters of 100% ethanol for 72 hours to obtain the ethanol extract. The content was filtered through a Whatman filter paper lined funnel into a conical flask. The filtrates was concentrated using rotator evaporator at 40^{o}C before being gently evaporated to dryness at room temperature (25 to 27^{0}C) and then stored in separate clean dry bottles and kept in the refrigerator at 40^{o}C. Dilutions to desired concentrations were made when required.

Experimental Animals

Adult male albino rats weighing 150-180g were purchased from the Animal House of Department of Physiology, University of Ibadan, Nigeria. The rats were acclimatized for two weeks under standard conditions (12h light and 12h dark cycle), well ventilated, pathogen free cages at room temperature (27^0C) in the animal house of the Department of Zoology, University of Ibadan. They were fed with standard mouse pellets and water was supplied *ad libitum.*

Lethal concentration dose test (LD_{50}) for the venom

After the two weeks of acclimatization, the LD_{50} of the venom was determined. The lethal dose (LD_{50}), values of the E. *ocellatus* crude venom was conducted according to the Spearman-Kärber method (1978). Twenty (20) animals weighing 150-180g were divided into four (4) groups containing five rats in each group.

Group 1 (Control group): This group consisted of five male albino rats and they were intraperitoneal injected with 0.2ml of normal saline.

Group 2 (High dose): This group consisted of five male albino rats and they were intraperitoneal injected with 0.56mg/kg of the diluted crude venom of *E. ocellatus.*

Group 3 (Medium dose): This group consisted of five male albino rats and they were intraperitoneal injected with 0.28mg/kg of the diluted crude venom of *E. ocellatus.*

Group 4 (Low dose): This group consisted of five male albino rats and they were intraperitoneal injected with 0.14mg/kg of the diluted crude venom of *E. ocellatus.*

Lethal dose concentration (LD_{50}) test of the M. *oleifera*

The method of Turner (1965) was adopted. Sixteen (16) adult male were rats divided randomly into four (4) groups of four (4) rats per each were used. At the end of the two weeks of acclimatization, the extracts were diluted as required and administered once orally to the rats at 10.00am for the test. The groups were constituted as follows:

Group 1 (Control group): They were given only normal saline.

Group 2: They were administered with 150mg/kg of the dissolved ethanol leaf of M. *oleifera* in 1ml of normal saline.
Group 3: They were administered with 300mg/kg of the dissolved ethanol leaf of M. *oleifera* in 1ml of normal saline.
Group 4: They were administered with 800mg/kg of the dissolved ethanol leaf of M. *oleifera* in 1ml of normal saline.
Mortality as well as the behavioural responses of the rats was recorded during the 24 hour period to study of the lethality of the each dose and lethality of the mortality of rats were determined using analysis of Probits in SPSS 2019. After 12hours of envenomation with the E. *ocellatus*, the blood samples were collected into heparinised tubes from each surviving rat of the study by ocular puncture method and the sample bottles were gently rocked to allow a proper mixing of the blood with the anticoagulants in the sample bottles which was properly labeled for histological and the rats were sacrificed according to guides by Rowett, (1977), and the heart samples were harvested from the various groups. These samples were blotted dry and fixed in 10% formaldehyde (pH 7.2 to 7.4) and sections of tissues were cut for histological analysis.

RESULTS

The LD_{50} test of the venom and Extract

Table 1, showed the mortality of the envenomed rats during the toxicity test. One hour after a single intra-peritoneal injection of the venom, the envenomed rats showed signs of dizziness and reduced activity and restriction of movement within the cages. Within 24 hours of envenomation, 20%, 60% and 100% mortalities were recorded in Group D, C and B respectively. More so, Table 2, showed the weight changes of the experimental animals during the 24 hours test (venom LD_{50}). Furthermore, the lethal dose concentration (LD_{50}) for the venom was estimated to be 0.22mg/kg using Probit analysis.
Table 3, showed the weight changes of the experimental animals during the 7days test of Extract LD_{50}. In the extract $LD_{50,}$ weight loss was recorded in Group B, C and D administered with extracts compared to the group A

(normal control) which recorded weight gain. Furthermore, the LD_{50} of the extract was established to be 800mg/kg.

Table 1: Mortality of the Rats for LD_{50} Assay

Group	4hours	8hours	12hours	16hours	20hours	24hour	Percentage (%) () Mortality at 24hrs
A	-	-	-	-	-	-	00
B	3	2	-	-	-	-	100
C	-	-	1	1	1	-	60
D	-	-	-	-	1	-	20

Table 2: Weight Changes of the Rats during LD_{50} Assay of the venom

	Mean Body Weight (g)		
Groups	Initial	Final	Weight changes (%)
A	150.50	151.08	0.58††
B	152.20	NS	-
C	162.00	153.68	8.52†
D	155.10	151.57	3.53†

Weight gain = †† Weight loss = †

NS = No survival

Legend for the Table 1-2

Group A – Control group

Group B – envenomed with 0.56mg/kg of crude venom of *E. ocellatus*

Group C – envenomed with 0.28mg/kg of crude venom of *E. ocellatus*

Group D – envenomed with 0.14mg/kg of crude venom of *E. ocellatus*

Table 3: Weight Changes of the Rats during LD_{50} Assay of the Leaf Extract.

	Mean Body Weight (g)		
Groups	Initial	Final	Weight changes (%)
A	104.40	105.80	1.34††
B	109.30	109.20	0.09†
C	113.70	111.10	2.28†
D	126.70	123.90	2.21†

Weight gain = †† **Weight loss = †**

Legend for Table 3.

Group A – Control group
Group B – administered with 150mg/kg of the extract.
Group C – administered with 300mg/kg of the extract.
Group D – administered with 800mg/kg of the extract.

Figure 1, Presented the food and water consumption of the experimental animals during LD_{50} assay of the leaf extract. The food and water consumption of control group A (normal control) was consistence throughout the 7days of the treatment compared to all administered groups. The food consumption in treatment groups was inconsistent at day 1 and 2 but gradually increases in day 3, and however, a marked increase in the food and water consumption was observed in day 4, 5, 6 and 7. More so, behavioural responses such as restriction of movement in their micro-environment (their cages), dizziness and low water consumption were first observed in the administered groups.

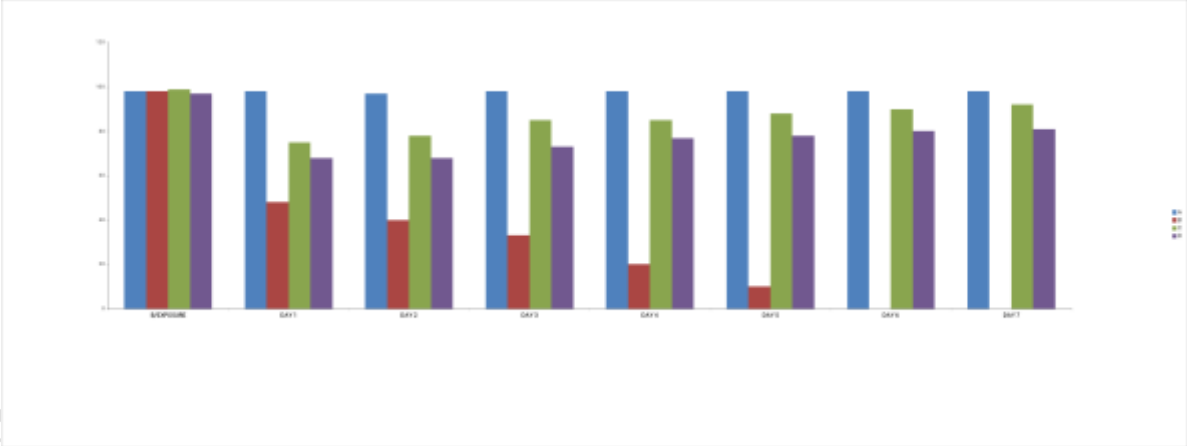


Figure 1: Percentage Food Consumption of LD_{50} of the Extract.

Haematology of LD_{50} in Envenomed rats.

Table 4, showed the haematological parameters of each experimental group. Group A (normal control) showed a significantly ($P<0.05$) higher

values in the Packed Cell Volume (PCV), Red Blood Cell (RBC), Heamoglobin (Hb) compared to the group B, C and D and this showed that the dose dependent on decrease in the PCV, RBC, Hb across the envenomed groups. However, the group B showed a significantly ($P<0.05$) higher values in the PCV, RBC, Hb compared to group C and D. The white blood cell (WBC) of the group A (normal control) showed significantly ($P<0.05$) higher values compared to group B, C and D. However, the (WBC) of group B showed no significantly ($P<0.05$) value compare to other envenomed groups. Also the platelet values were significantly ($P<0.05$) not different across all the experimental groups. Moreover, the result of Mean Corpuscular Volume (MCV) showed higher values in the all envenomed rats groups which were significantly ($P<0.05$) compared to group A (normal control). There were no significant ($P<0.05$) differences in the MCV (*fl*) values of Groups B compared to group C and Group D. And Group B and C showed no significant ($P<0.05$) differences in the MCV, MCHC and MCH values.

Table 4: Haematological parameters of LD_{50} in Envenomed rats.

Parameters	GROUP A	GROUP B	GROUP C	GROUP D
PCV (%)	46.00 ± 2.00^{e}	42.01 ± 0.58^{d}	41.33 ± 0.58^{d}	40.34 ± 0.58^{d}
Hb (g/dl)	15.23 ± 0.82^{d}	14.88 ± 0.06^{c}	14.43 ± 0.06^{c}	13.44 ± 0.06^{c}
RBC (cell/L)	7.92 ± 0.35^{d}	7.99 ± 0.08^{d}	7.77 ± 0.08^{d}	6.78 ± 0.08^{d}
WBC ($x10^3$ cell/L)	6.5 ± 0.46^{b}	5.33 ± 0.30^{ab}	5.33 ± 0.20^{ab}	5.33 ± 0.10^{ab}
PLATELET ($x10^5$ cell/L)	1.31 ± 0.20^{a}	1.48 ± 0.40^{a}	1.47 ± 0.40^{a}	1.46 ± 0.40^{a}
LYM (%)	64.0 ± 3.0^{c}	67.0 ± 1.0^{b}	66.0 ± 1.0^{b}	65.2 ± 1.0^{b}
NEUT (%)	32.3 ± 3.5^{c}	20.0 ± 1.0^{b}	21.0 ± 1.0^{b}	23.2 ± 1.0^{b}
MONO (%)	1.7 ± 0.6^{bc}	1.7 ± 0.1^{c}	1.5 ± 0.3^{c}	1.3 ± 0.0^{c}
EO (%)	2.0 ± 1.0^{b}	1.6 ± 0.3^{ab}	1.5 ± 0.0^{ab}	1.3 ± 0.0^{ab}
MCV (*fl*)	58.1 ± 1.2^{c}	59.9 ± 0.3^{a}	59.8 ± 0.2^{a}	59.6 ± 0.2^{a}
MCH(*pg*/cell)	19.2 ± 0.2^{d}	19.8 ± 0.2^{a}	19.7 ± 0.3^{a}	19.6 ± 0.1^{a}

Values are Means $\pm$ S.D, $n \leq 3$

Mean with similar superscript on the same column are not significantly difference (P<0.05).

Legend for Table 4.

Group A (Control group): injected intraperitoneal injected with 0.2ml of normal saline.

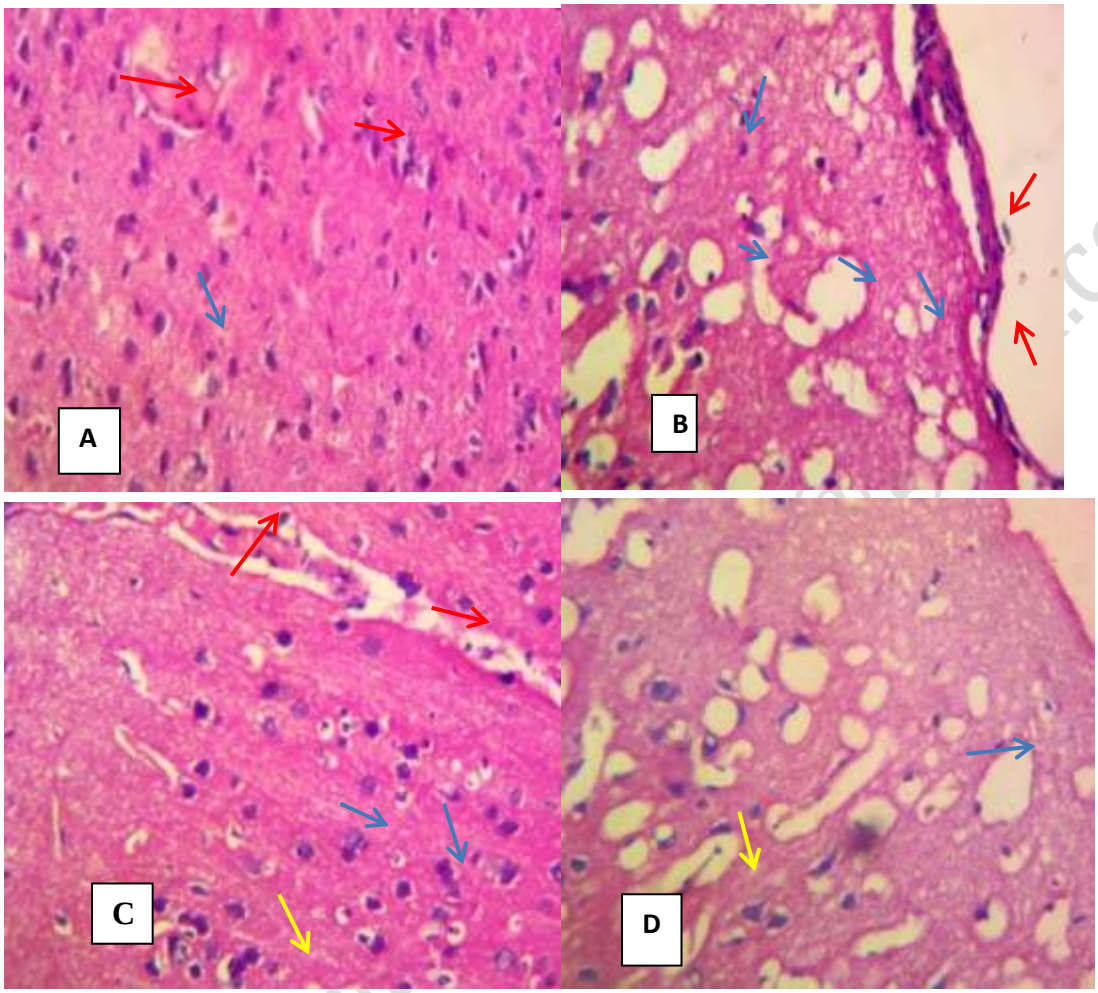
Group B (High dose): injected intraperitoneally with 0.56mg/kg of the diluted crude venom of *E. ocellatus.*

Group C (Medium dose): injected intraperitoneally with 0.28mg/kg of the diluted crude venom of *E. ocellatus.*

Group D (Low dose): injected intraperitoneally with 0.14mg/kg of the diluted crude venom of *E. ocellatus.*

Histopathology of Hearts of Envenomed Rats.

The results of the histopathological responses of the rats challenged with *Echis ocellatus* venom presented in Plate 5A-F, using light microscopy (x400 magnifications). The histopathological findings of the heart of normal control group A, showed a normal appearance of cardiomyocytes and absence of foci of haemorrhages between the cardiac muscle bundles compared to envenomed groups. The slide prepared from the heart of group B (high dose), revealed multiple foci of thinning and attrition of cardiomyocyte fibres, moderate congestion of coronary blood vessels and numbers of inflammatory cells and presence of foci of epicardial haemorrhages. The slide prepared from the heart of group C rats treated with (mid dose) showed minimal foci of thinning and attrition of cardiomyocyte fibres, moderate congestion of coronary blood vessels and numbers of inflammatory cells and presence of foci of epicardial haemorrhages. Moreover, the slide prepared from group D rats treated with (low dose) showed that revealed very small foci of thinning and attrition of cardiomyocyte fibres, moderate congestion of coronary blood vessels and numbers of inflammatory cells and presence of minimal foci of epicardial haemorrhages.



Light microscopy (x400 magnifications)

Plate 5A-D: Histological sections of the heart (A) cardiomyocytes appeared normal (B) multiple foci of thinning and attrition of cardiomyocyte and epicardial haemorrhages (C) extensive foci of mild pallor of the cardiomyocytes (D) focus of the cardiomyocytes

CONCLUSION AND RECOMMENDATION

The envenomination of wistar albino rats to *Echis ocellatus* venom brings on biological alterations in the haematological, histopathological activies of the experimental animals in this study. The venom was therefore, cytotoxic and haematoxic which was a frightening to human if not treated immediately and confirmed the veperian venom's expectation to body physiology. The alteration of haemoglobin when envenomized with

significant elevation of the PCV, RBC and platelets counts along with increase in the blood haemoglobin LD_{50} of the venom and the extract confirm that *M. oleifera* extracts can reverse the negative alteration in the haematological and histopathological studies in LD_{50} of the venom which are anaemia features such as haemolysis and bleeding encountered in *E. ocellatus* envenomination if subjected to experiment in the further studies. Also, the damage observed on the histopathological studies such as the marked reduction in the pathophysical damage in the LD_{50} of the envenomized rats such as injuries in the brain and heart of the experimental animals of the LD_{50} of the envenomized rats group can be ameliorated if subjected to further experiment. These results were correlated to the works of the earlier researchers like Sarkiyayi, (2011), Onyeama, *et. al.,* (2012), de Oliveira, *et. al.,* (2016).

RECOMMENDATION

The obtained results from this study have provided first hand information on the antivenomic properties of ethanol leaf extract of *Moringa oleifera* for the treatment of *Echis ocellatus* envenomination. However, with the growing knowledge on the biochemistry and pharmacology, efforts should be geared towards identification of specific fractions of the active components of the M. *oleifera* which is the keys inhibitors of venom toxins in *Echis ocellatus.* More so, efforts should be geared towards identification of natural inhibitors of *Echis ocellatus* venom toxins in varieties of plants and the combined natural inhibitors of varieties should be researched compare to single plant and this should be properly study alongside of its mechanisms of action.

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