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Snake-Antivenom Activities of Aqueous Extracts of *Amaranthus spinosus* L. Against *Naja subfulva* Venom

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Abstract:- The present management regime of snake bites requires the use of anti-venom immunoglobulins (Igs). However, these anti-venoms have the limitations of being expensive, requiring cold storage facilities, and having problems of hypersensitivity reactions in some individuals. *Amaranthus spinosus* plant medicine has traditionally been used in managing snake bites in Uasin-Gishu County, Kenya. However, its efficacy has not been scientifically validated. Therefore, this study aimed to determine *in vivo* and *in vitro* the efficacy of the medicinal plant against *Naja subfulva* venom using the mouse model, agarose-erythrocyte-egg yolk gel plate, and human-citrated plasma methods. The antivenom studies suggest that the aqueous plant extracts possess antivenom activity against *N. subfulva* venom both *in vivo* and *in vitro*. In conclusion, this study confirmed that aqueous extracts of *Amaranthus spinosus* were effective in neutralizing *in vivo* and *in vitro* snake venom activity of *Naja subfulva*.

Keywords:- *Median effective dose (ED_{50}), Median lethal dose (LD_{50}), Phospholipase A_2, Toxicity, Phytochemicals.*

I. INTRODUCTION

Snake envenomation is a significant medical concern in tropical countries and many other parts of the world. Globally, the incidence of clinically significant snakebite envenomation has been calculated at above 421000 and 20000 deaths annually [1]. The only validated and recommended therapy for treating snake bite envenomation effectively is through antivenoms; a serum separated from the blood of hyperimmunized horses [2]. Antivenom neutralizes the effects of snake venom envenomation stopping further damage to the body, but it does not reverse the damage already done. Other limitations of snake antivenom therapy include its unavailability, high cost, inadequate storage facilities in developing countries, and difficulty in identifying the snakes. Some individuals may also suffer from an immediate hypersensitivity reaction after antivenoms treatment [3].

The current crisis in the supply of antivenom to Sub-Saharan Africa has prompted a need to look for alternative antivenoms that are either synthetic or natural, which will substitute or complement the animal-derived anti-venoms [4]. Herbal remedies for snakebite treatment are readily available in rural areas, but most of these phytochemicals used in various parts of the world against snake envenomation are seldom studied [5]. This study, therefore, aims at determining *in vivo* and *in vitro,* using the mouse model, the efficacy and safety of *Amaranthus spinosus* medicinal plant used to treat snake bites in parts of Uasin-Gishu County, Kenya.

II. MATERIALS AND METHODS

A. Collection and Identification of Medicinal Plants

Whole plant parts were collected from their natural habitats using a local name (Chepkerta-Nandi) and plant identity authenticated as per the International Code of Botanical Nomenclature by an acknowledged authority in taxonomy from the East African herbarium at the National Museums of Kenya. Coordinates for the location of collection points for the plant were taken and recorded as E35.1381903N.5550996.

B. Preparation of Plant Extracts

One hundred grams (100 g) of shade-dried powdered plant materials were mixed with 2000 ml double distilled water in 2500 ml screw-caped conical flasks and kept in a water bath at 60ºC for 2h. The extracts were then filtered by using a muslin cloth and then re-filtered using Whatman filter paper No. 1. The filtrate was then stored in a refrigerator at 4^0C until they were freeze-dried with a lyophilizer. Freeze-dried materials were weighed and kept in a freezer at -20^0C until use.

C. Venom and Experimental Animals

Lyophilized snake venom powder of *Naja subfulva* was obtained from Bio-Ken snake farm, Watamu, Malindi, and stored at 4ºC. Swiss albino mice of either sex (18-20 g) were used for efficacy and toxicity studies. The mice were bred

under standard laboratory conditions of 12 hours exposure to light, 25 ± 2ºC temperature, and 35-60 % humidity in the animal houses of the Departments of Biochemistry, Microbiology and Biotechnology and Medical Laboratory Sciences (MLS) of Kenyatta University and Jomo Kenyatta University of Agriculture and Technology respectively. They were fed with standard mice pellets from Unga Feeds Limited and provided with water *ad libitum.*

D. In Vivo Venom-Neutralization Potency Tests of the Aqueous Plant Extracts

The only validated means of assessing venom toxicity and antivenom neutralizing efficacy by manufacturers and regulatory authorities is by determining venom lethality (LD_{50}) and antivenom neutralizing capacity (ED_{50}) [6].

➢ *Venom Median Lethal Dose (LD_{50}) Assay*

The median lethal dose (LD_{50}) of *Naja subfulva* venom was assayed as per the method developed by. Various doses of venom dissolved in 0.2 ml of physiological saline were injected into the tail vein of mice, using groups of 5 mice for each venom dose. The LD_{50} was estimated by probit analysis at a 50% probability of deaths occurring within 24h of venom injection.

➢ *Aqueous Extracts Median Effective Dose (ED_{50}) Assay*

The venom-neutralizing potential of aqueous extracts of the five selected plants was determined against $2LD_{50}$ ("challenge dose") of *Naja subfulva* venom. Various doses of plant extracts (100, 200, 300, 400, and 500 mg/kg body weight) were mixed with $2LD_{50}$ of *Naja subfulva* venom sample in a total of 0.2 ml and incubated at 37ºC for 30 minutes and injected intravenously into mice. Control mice received a mixture of venom "challenge dose" with physiological saline solution alone to confirm that the venom "challenge dose" induces 100 % lethality. Five mice were used for each antivenom dose. The median effective dose (ED_{50}) was calculated from the number of deaths occurring within 24h of venom/antivenom mixture injection by probit analysis. The lower the ED_{50} value, the higher the neutralizing ability of the antivenom [8].

E. In Vitro Venom-Neutralization Potency Tests of the Aqueous Plant Extracts

➢ *Phospholipase A_2 Activity*

Phospholipase A_2 activity was determined using an indirect hemolytic assay on an agarose-erythrocyte-egg yolk gel plate as per the methods described by Gutiérrez *et al.*[9]. Increasing doses of *N. subfulva* venom were added into 3mm wells in agarose gels (0.8 % in PBS, pH 8.1) containing 1.2 % sheep erythrocytes, 1.2 % egg yolk as a source of lecithin and 10 mM $CaCl_2$. Slides were then incubated at 37ºC overnight and the diameters of the hemolytic halos were measured. Control wells contained 15 µl of physiological saline. The minimum indirect hemolytic dose (MIHD) corresponds to a venom dosage, producing a hemolytic halo of 11 mm diameter. The efficacy of plant extracts in neutralizing the Phospholipase A_2 activity was determined by mixing a constant dose of venom (MIHD) with various doses of plant extracts and incubating for 30 minutes at 37ºC. Aliquots of 10 µl of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contained venom without plant extracts. Plates were then incubated at 37ºC for 20 h. Neutralization was expressed as the plant extracts/venom's MIHD mixture that reduced the diameter of the hemolytic halo by 50 % when compared to the effect induced by venom's MIHD alone.

➢ *Procoagulant Activity*

The procoagulant activity was assayed according to the method described by Theakston & Reid [7] and as modified by Laing *et al.* [8]. Various amounts of venom dissolved in 100 µL PBS (pH 7.2) were added to human citrated plasma at 37ºC. Coagulation time was recorded and the minimum coagulation dose (MCD) was determined as the venom dose which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as a control. In neutralization assays, a constant dose of venom was mixed with various dilutions of plant extracts. The mixtures were then incubated for 30 minutes at 37ºC. 0.1 mL of the mixture was then added to 0.3 mL of citrated plasma and the clotting times were recorded. In control tubes, plasma was incubated with venom or plant extracts. Neutralization was expressed as an effective dose (ED), defined as the plant extracts/venom dose (MCD) at which the clotting time increased three times when compared with the clotting time of plasma incubated with two times MCD of venom alone.

F. Qualitative Phytochemical Screening of the Aqueous Plant Extracts

The presence or absence of phytochemicals in the aqueous plant extracts was carried out using standard methods [10, 11].

G. Data Management and Statistical Analysis

Data were recorded in the Laboratory notebook and then entered into Excel Spreadsheet for cleaning after which it was exported to MINITAB 18 for statistical analysis LD_{50} and ED_{50} were determined by probit analysis.

H. Ethical Approval

This study was approved by Kenyatta University Ethics Review Committee and licensed by the National Commission for Science, Technology, and Innovation (NACOSTI) of Kenya (license number NACOSTI/P/22/15100).

III. RESULTS

A. Lethality of Naja Subfulva Venom and in Vivo Neutralization Potency of the Five Aqueous Plants Extracts in Mice

The lethality of *Naja subfulva* venom after its intravenous injection in mice through the tail vein is reported in Table 1. Results indicate that after intravenous injection of various doses of *Naja subfulva* venom in mice via the tail, the LD_{50} of the venom was found to be 0.99 µg/g body weight (Figure 1). The neutralization potency of the aqueous extract of *A. spinosus* was determined by mixing various doses of the plant extract with $2LD_{50}$ (2 µg/g body weight) of venom sample and incubating the mixture at 37ºC for 30 minutes before intravenous injection into mice. Results indicate that

the ED_{50} obtained for the aqueous extracts was 87.53mg/kg body weight (Table 2; Figure 2).

Table 1: Median lethal dose (LD_{50}) of *Naja subfulva* venom in mice

Group	Dose (µg/g body weight)	Mortality (24hr)	% Death	Log dose
1(Control)	0.2 ml saline	0/5	0	-
2	2.5	5/5	100	0.40
3	2.0	5/5	100	0.30
4	1.5	3/5	60	0.18
5	1.0	2/5	40	0.00
6	0.5	1/5	20	-0.30

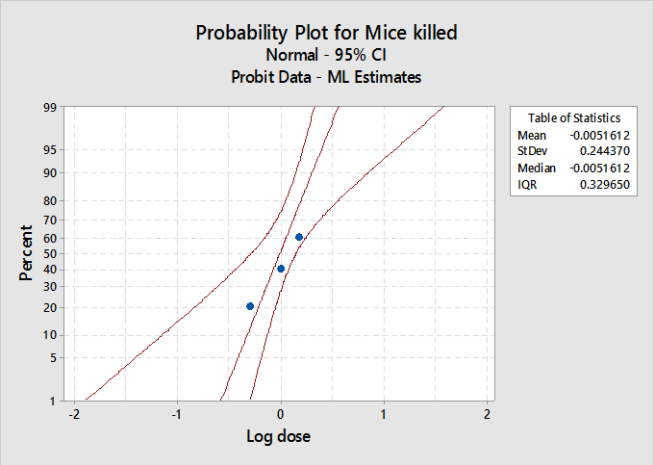

Fig 1: SNAKE VENOM LD_{50}

Mean of log dose (Log_{10}) = -0.0051612

LD_{50} = $10^{-0.0051612}$ = 0.99µg/9g body weight

Table 2: Effect of intravenous administration of a preincubated mixture of $2LD_{50}$ (2 µg/g body weight) *Naja subfulva* venom and the aqueous plants extract to mice

Group	Dose (mg/kg body weight) extract	Mortality (24 hr)	% Survival	Log dose
	Amaranthus spinosus			
1 Control	0 (venom only)	5/5	0	-
2	100	2/5	60	2.00
3	200	1/5	80	2.30
4	300	1/5	80	2.48
5	400	0/5	100	2.60
6	500	0/5	100	2.70

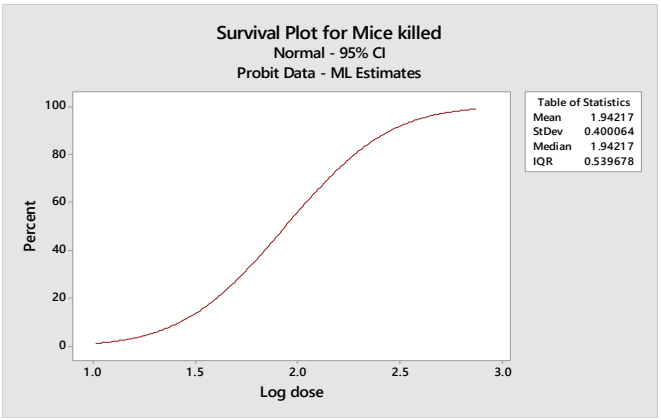

Fig 2: The median effective dose (ED_{50}) for *Amaranthus spinosus*

Mean of log dose (Log_{10}) = 1.94217

ED_{50} = $10^{1.94217}$ =87.53mg/kg body weight

B. In vitro venom neutralization potency of the five aqueous plants extracts

➢ *Phospholipase A_2 Activity*

1.5µg of *N. subfulva* venom produced 11mm diameter hemolytic halos, which is considered 1U (U/10µg) as well as the Minimum Haemolytic Dose (MHD). This indicates that *N. subfulva* venoms possess the enzymes (phospholipase A_2) that can lyse sheep RBCs. Incubating different concentrations of aqueous extracts of *Amaranthus spinosus* from 100-500mg with 1.5µg of *N. subfulva* venom, it was found that the extract prevented hemolysis by a minimum of 57.3% at 100mg/kg to a maximum of 66.4% at 500mg/kg ranging from 4.7±0.08 to 3.7±0.10mm diameter (Tables 3 and 4)

Table 3: The effect of *N. subfulva* venom (PLA_2) induced hemolysis on sheep RBCs plate (n=3)

Venom(µg)	Haloes(mm)
0 (control)	3.00±0.00
2.5	13.00±0.20
2.0	12.10±0.20
1.5	11.03±0.25
1.0	7.03±0.12
0.5	6.03±0.06

Table 4: The effect of aqueous extract of *Amaranthus spinosus* on *N. subfulva* venom (PLA 2) induced hemolysis on sheep RBCs plate

Treatment		
Venom (1.5µg) + extract	**Hallos in mm**	**% inhibition**
100mg/kg	4.7±0.08	57.30
200mg/kg	4.6±0.06	58.20
300mg/kg	4.4±0.10	60.00
400mg/kg	3.9±0.13	64.50
500mg/kg	3.7±0.10	66.40

➢ *Procoagulant Activity*

No activity was observed when *Naja subfulva* venom was tested for procoagulant activity using the protocols described by Theakston and Reid [7].

C. Phytochemical Screening

Phytochemical analysis of aqueous plant extracts revealed the presence of alkaloids, tannins, terpenoids, and saponins in aqueous *Amaranthus spinosus*. The results are replicated in table 4

Table 4: Phytochemical analysis of aqueous extracts of *Amaranthus spinosus*

Phytochemicals	***Amaranthus spinosus***
Alkaloids	+++
Tannins	++
Cardiac glycosides	++
Steroids	-
Triterpenoids	-
Flavonoids	+++
Terpenoids	+
Phenols	++
Flavones	-
Saponins	++

KEY: +++ High presence; ++ Moderate presence; + Trace; - Negative results

IV. DISCUSSION

Snakebite is a major public health hazard that leads to a high mortality rate worldwide. World Health Organization (WHO) estimates that 2.5 million snakebites occur worldwide each year and out of these, 125,000 are fatal [12]. Snake antivenoms are currently the only validated antidotes for snake envenomation, but they have several limitations which include hypersensitivity reactions among some patients and inefficient supply systems leading to their unavailability in rural areas. Although the use of plants against the effects of snake bites has long been recognized, more scientific attention has only been given to this area of research in the last 20 years [13]. The present study examined antivenom potential, phytochemical composition, mineral elements composition, and acute and sub-acute toxicity effects of aqueous extracts of *Amaranthus spinosus*. The most important step in determining the antivenom potential of plant extracts is the pre-clinical testing using *in vivo* and *in vitro* methods to assess their neutralizing potential against a wide range of venom effects [14].

The only validated protocol for assessing venom toxicity and antivenom-neutralizing potency by both manufacturers and regulatory authorities is the test for determining venom lethality (LD_{50}) and antivenom-neutralizing capacity (ED_{50}) [15]. In this study, therefore, *in vivo* and *in vitro* pharmacological tests for venom lethality, procoagulant activity, and phospholipase activity (PLA_2) caused by *Naja subfulva* venom were investigated. The design for the conventional antivenoms rodent ED_{50} tests requires premixing of the venom with the test antivenom before intravenous injection into the animal [16]. This, however, does not replicate the clinical situation where the events occur at different times, that is, envenomation followed by antivenom therapy. Following this protocol, neutralization studies on the plant extracts showed that the aqueous extracts of *Amaranthus spinosus* exhibited varying abilities to neutralize the lethality induced by *N. subfulva* venom in a dose-dependent manner. Since snake venom is mainly made up of proteinaceous compounds (approximately 90-95%) mainly enzymes, the most likely mechanism for the plant extracts' ability to neutralize *N. subfulva* venom could be due to the ability of the extract's triterpenoids, terpenoids, polyphenolic, tannins and tannin like substances to bind venom proteins resulting in inhibition and precipitation of the venom proteins [17].

Naja subfulva venom showed the presence of phospholipase A_2 enzymes by producing hemolytic haloes in indirect hemolytic assays due to the formation of phospholipid hydrolysis products such as lysophospholipids and free fatty acids which are lytic. Different doses of the aqueous extracts inhibited PLA_2 in a dose-dependent manner. This inhibition may be due to the extracts' phytochemicals such as flavonoids, phenols, terpenoids, and quinonoid activity which have been documented to deactivate snake venom constituents [18]. Plant secondary metabolites react and sequester phospholipases and pose a hindrance in the binding of the latter to their target site (s) hence reversing the anticoagulation action of phospholipase A_2 [19].

The procoagulant activity was studied using human-citrated plasma but no coagulation of the plasma was observed. This was in agreement with studies done by Suntravat [20], which showed that *N. subfulva* venom had no significant activity on both procoagulant and anticoagulant activities. *Naja subfulva* toxic phospholipase A_2 probably affects coagulation due to its interaction with plasma phospholipids.

V. CONCLUSIONS

i) The aqueous plant extracts of *Amaranthus spinosus* demonstrated significant antivenom activity both in *in vivo* and *in vitro* assays.
ii) Qualitative phytochemical analysis of the aqueous plant extracts showed the presence of tannins, cardiac glycosides, phenols, triterpenoids, terpenoids, saponins, alkaloids, and flavonoids that could have contributed to the plant's ability to neutralize snake venom.

DATA AVAILABILITY

The quantitative and qualitative data used to support the findings of this study are included in the article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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