



## Anti-snake Venom Activity of Aqueous and Ethanolic Extracts of *Crinum jagus* Bulb

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### Authors' contributions

This work was carried out in collaboration between all authors. Authors AUW, PKM and AHZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SS and PKM managed the analyses of the study. Authors PKM and AHZ managed the literature searches. All authors read and approved the final manuscript.

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### ABSTRACT

**Aim:** To determine the anti-snake venom effect of crude aqueous and ethanolic extracts and separated fractions of *Crinum jagus* bulb on rats injected with *Echis ocellatus* venom.

**Study Design:** Evaluation of LD<sub>50</sub> of venom, anti-lethal effects of different extract preparations and their effect on neutralising haemorrhage; the hallmark of *Echis ocellatus* venom toxicity.

**Place and Duration of Study:** Department of Biochemistry laboratory, Modibbo Adama University of Technology, Yola, Nigeria between March and October 2017.

**Methodology:** venom was collected by the milking method. Fractionation of crude extracts was done by column chromatography separately with benzene/methanol, acetic acid/methanol and ethyl acetate/methanol. Thirty rats were used to determine LD<sub>50</sub> of venom while twenty-four albino rats were used in the anti-venom study; divided into 6 groups of 4 rats each. Group one served as the normal control, group two served as venom untreated control while groups 3, 4, 5 and 6 were injected with 0.2 mg/mL *Echis ocellatus* venom and treated with 200 mg/kg body weight crude extracts, fractions I, II and III respectively then observed for 24 hours. Results: LD<sub>50</sub> of venom was found to be 5

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mg/mL; column chromatography of crude extracts gave three fractions each designated I, II and III respectively. Phytochemical analysis of both crude aqueous and ethanolic extract of *Crinum jagus* bulb revealed the presence of alkaloids, flavonoids, terpenoids, steroids, tannins and phenolics. Only fraction III of both aqueous and ethanolic extract maintained all phytochemicals from the crude. Both extracts and their fractions were all able to neutralise the venom. For the aqueous extract, the crude extract and fraction III gave the best result while for the ethanolic extract, fractions II and III caused the best neutralisation.

**Conclusion:** *Crinum jagus* bulb has demonstrated significant anti-venom activity, this can be exploited for the development of new anti-snake venom drugs.

**Keywords:** *Crinum jagus*; *Echis ocellatus*; venom; column chromatography.

## 1. INTRODUCTION

Snakes constitute a particularly alarming public health problem in most developing countries including Nigeria where the rural communities are the worst affected. More than five million snake bites occur worldwide every year, causing more than 100,000 deaths [1]. However, the unreported cases may be even more. When venomous snakes bite their prey, their fangs cause puncture wounds which result in envenomation; even though this method of killing prey is common, majority of snake species are non-venomous. The non-venomous types of snakes usually wrap themselves around a critical part of their prey and constrict to death. Several species of venomous snakes have been reported, and they can be found on every continent except Antarctica [2,3]. Many snake venoms, especially those from viperine and crotaline snakes cause local and systemic bleeding [4]. This bleeding is the consequence of the damage to blood vessel walls by venom components. *Echis ocellatus* venom consists primarily of proteins and peptides including snake venom metalloproteinases including zinc metalloproteinase which is involved in haemorrhagic activity, serine proteases, neurotoxins, nephro and cardiotoxins and procoagulants [5,6,7]. In Nigeria, State governments continue to expend appreciable part of the annual budgets on importation of polyvalent antivenins (PVA) used to treat venomous snake bites [8]. Unfortunately, PVA's are not only expensive; some cause anaphylaxis in patients [5,6,9]. Polyvalent antivenins are often unavailable in most health centers in Nigeria due to storage difficulties as a result of erratic electricity supply. The need for the development of alternative or similar antivenin compounds from naturally occurring substances thus becomes paramount. Medicinal plants have been used to treat infectious diseases for many years worldwide leading to a growing interest in the

development of drugs of plant origin [10]. Nigeria is one of the countries in the world with a unique wealth of medicinal plants and vast traditional knowledge of the use of herbal medicine for treatment of various diseases [11]. Furthermore, medicinal plants have been previously reported to effectively manage various cases of envenomation in different parts of Nigeria [12].

*Crinum jagus* commonly called Harmattan lily belongs to the family *Amaryllidaceae*; a heterogenous family of 90 genera and about 1310 species [13] that are widely distributed throughout the world. Several species are cultivated in tropical and subtropical regions of the world as ornamentals and for medicinal purposes. They are bulbous plants with umbels of lily-like flowers. Its local name is "Ogede-Odo" in Yoruba and "Gadali" in Hausa [14]. The plant has previously been reported to have properties such as antibacterial, antifungal [15], anticonvulsant [16], anti-asthmatic [12], and antioxidant activities [8]. It was reported by [17] that methanolic extract of the bulb of *Crinum jagus* significantly protected mice from death, myonecrosis and haemorrhage induced by the effect of snake venoms. The people of Western Cameroon use the bulb of *Crinum jagus* in the management of diabetes [18]. It is commonly used in the management of cases of snake bite by the *Igede* speaking tribe of Oju local government area in Benue state and the Fulani nomads from Northern Nigeria living among them. They drink a decoction of the bulb either alone infused in water or with a dash of local gin. We therefore chose to work with aqueous and ethanolic extracts of this plant to reflect this traditional use. This study is therefore aimed at investigating the ethnobotanical claim of the anti-venom property of *Crinum jagus* as it is used traditionally using venom from carpet viper snake (*Echis ocellatus*).

## 2. METHODOLOGY

### 2.1 Collection of Plant Sample

Fresh *Crinum jagus* plant was harvested from Sattede in Dumne, Song local Government area of Adamawa state in March, 2017. It was authenticated in the Department of Plant Science Modibbo Adama University of Technology, Yola and the Department of Forestry, Ministry of Environment Jimeta-Yola. The bulb was cut into pieces using a knife and allowed to air dry at room temperature for two weeks. The dried bulb was crushed into powder using a blender and stored in an airtight container until needed for use.

### 2.2 Experimental Animals

Adult male and female rats of the *Rattus norvegicus* strain weighing between 100-150g were obtained from the animal breeding unit of the Department of Biochemistry, University of Jos, Plateau State. The rats were housed in plastic cages and maintained under standard laboratory conditions with free access to rat pellets and tap water *ad libitum*.

### 2.3 Collection of Snake Venom

A carpet viper snake was caught by a hunter in Malabu, Girei Local Government Area of Adamawa State. The venom was collected by the milking method of [7]. To collect the venom, a sterile glove was put over the mouth of a sterile glass jar and a rubber band was used to secure the glove in place. The snake handler then wore a latex glove, grabbed the snake by the neck and brought it towards the covered glass jar. The snake was then made to "bite" the sterile glove covering the jar by sinking its fangs into the glove. Its venom glands were then massaged manually and venom dripped from its fangs into the glass jar. The collected venom was reconstituted with normal saline to obtain various concentrations and used immediately.

### 2.4 Chemicals and Reagents

Sodium hydroxide (NaOH) concentrated sulphuric acid (H<sub>2</sub>SO<sub>4</sub>), ethanol, methanol, sodium sulphate, chloroform, benzene, ammonia solution, acetic anhydride, formaldehyde and methane were obtained from JHD Sci-Tech Company limited, Guandong Guanghua, South Road, Guangzhou City, China. All other reagents used were of analytical grade.

### 2.5 Extract Preparation

For the aqueous extract, five hundred grams (500 g) of the powdered bulb was macerated in 2000 mL of distilled water with intermittent shaking for 48 h and after that filtered using Whatman filter paper No 1. The filtrate was evaporated to dryness in a water bath at a temperature of 40°C.

While for the ethanolic extract, the same amount of powdered bulb was macerated in the same volume of 99.7% ethanol and subsequently treated similarly as the aqueous extract.

#### 2.5.1 Fractionation of the extracts by column chromatography

Slurry was prepared by dissolving 30g silica gel in 100mL methanol: water (1:1) and packed in a column (1.5x30 cm.) the column was loaded with 15 mL of the crude aqueous and ethanolic extracts of *Crinum jagus* bulb separately and sequentially eluted with benzene/methanol (9:1) and acetic acid/methanol (1:1), ethylacetate/methanol (19:1) [19]. There was a colour separation in the column which gave rise to three distinct bands: yellow, light green and dark green. Each of these bands was collected separately and designated as fractions I, II and III respectively. The collected fractions were then separately concentrated under pressure using a rotary evaporator. For each extract, three fractions were obtained and designated I, II and III.

#### 2.5.2 Phytochemical screening

Preliminary screening of the secondary metabolites was carried out on the aqueous and ethanolic extracts of the bulb and also on the fractions as described by [20,21,22].

#### 2.5.3 Determination of median lethal dose (LD<sub>50</sub>)

The venom was reconstituted with normal saline to obtain concentrations ranging between 4 mg/mL and 10mg/mL according to methods of [23] and [24]. Thirty rats were divided into five groups of six rats each. They were then injected with 0.2mL of 4, 6, 8 and 10 mg/mL of the reconstituted venom through the intraperitoneal route respectively. The control group received 0.2 mL of normal saline. The times of death was recorded within twenty four (24) hours after administration of the venom. The assay was

carried out for the venom of carpet viper and the LD<sub>50</sub> was determined by probit analysis ( $t, \lim_{n \rightarrow \infty} n/n = ct > 0$ ) [25].

#### 2.5.4 Determination of neutralization effect of the lethal venom effect

For each extract, twenty-four rats were randomly divided into six groups of four rats each. Equal doses of the reconstituted venom (5 mg/mL) were then injected intraperitoneally into animals in groups 2, 3, 4, 5 and 6. Fifteen minutes after the injections, animals in groups, 3, 4, 5 and 6 were orally administered with 200 mg/kg body weight of each crude extract and fractions I, II, and III obtained from both aqueous and ethanolic extracts of *Crinum jagus* bulb respectively. Group 1 served as the normal control and was injected with 0.2 mL normal saline. Group 2, the untreated venom control was injected with 0.2 mL of the reconstituted venom. The animals were observed for twenty-four hours. The surviving rats were then sacrificed, the skin was removed to expose the area at the point of injection and the presence/absence and degree of haemorrhage were observed [25].

### 2.6 Statistical Analysis

All values are expressed as Mean  $\pm$  SEM. Comparisons between the groups were conducted using student *t*-test; *p*- values <0.05 were considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Phytochemical Analysis

Phytochemical analysis of both the crude aqueous and crude ethanolic extract of *Crinum jagus* bulb revealed the presence of alkaloids, flavonoids, terpenoids, steroids, tannins and an appreciable quantity of phenolics. Fraction I of aqueous extract also contained all six except flavonoids; fraction II contained only flavonoids, tannins, and phenolics while fraction III contained all six. However, fraction I of the ethanolic extract

revealed the presence of tannins, terpenoids, flavonoids and steroids; fraction II contained all but steroids and fraction III contained all six.

### 3.2 Percentage Yield after Fractionation

After fractionation of the crude aqueous extract of *Crinum jagus* bulb, the percentage yield was 71.20, 70.00 and 71.10% respectively. While the ethanolic extract yielded 70.90, 82.90 and 80.20% respectively.

### 3.3 LD<sub>50</sub> of the Carpet Viper (*E. ocellatus*) Venom

At the highest concentration of 10 mg/mL reconstituted venom, all the rats in the group died (0% survival) while at lowest concentration of 4mg/mL only one out of six rats died producing (83.3% survival), the mean time of death was calculated and the median lethal dose was determine to be 5 mg/mL (Table 1).

### 3.4 Neutralization of Lethal Activity of Carpet Viper (*Echis ocellatus*) Venom by *Crinum jagus* Bulb

#### 3.4.1 Aqueous extract

Table 2 shows the anti-lethal activity of the aqueous extract and separated fractions of *Crinum jagus* bulb in carpet viper snake venom injected rats. A total of 9 rats out of 16 rats (56.25%) survived within the 24 h period of treatment. The crude aqueous extract and fraction III showed the highest activity.

#### 3.4.2 Ethanolic extract

Table 3 shows the anti-lethal activity of the ethanolic extract and separated fractions of *Crinum jagus* bulb in carpet viper snake venom injected rats. The crude ethanolic extract gave 50% neutralization effect, fractions II and III produced 75% neutralization, while fraction I produced 0% neutralization.

**Table 1. Median lethal dose (LD<sub>50</sub>) determination of carpet viper snake venom**

Groups	Conc. of venom (mg/mL)	No. of dead rats/total rats in the group	% of rats alive	Mean death time (Min)
1	Control	0/6	100	-
2	4	1/6	83.3	845 $\pm$ 5.0
3	6	5/6	16.6	647.40 $\pm$ 1.12
4	8	5/6	16.6	643.40 $\pm$ 0.67
5	10	6/6	0	637.33 $\pm$ 0.21

Values means of 6 replicates  $\pm$  SEM

**Table 2. Anti-lethal effect of *Crinum jagus* bulb crude aqueous and separated fractions on carpet viper venom injected albino rats**

Groups	Treatment	No. of dead rats/no of rats in group	Mean time of death (Min)	percentage of surviving rats
1	Normal control	0/4	-	100
2	Negative control (0.2 mg/mL venom)	4/4	1095±139.96	-
3	Crude aqueousextract (200mg/kg)	1/4	1336±49.50	75
4	Fraction I (200 mg/kg)	2/4	1410±108.67	50
5	Fraction II (200 mg/kg)	3/4	896±79.00	25
6	Fraction III (200 mg/kg)	1/4	1337±62.00	75
Total		7/16		56.25

Values are means 4 replicates ±SEM

**Table 3. Anti-lethal effect of *Crinum jagus* bulb crude ethanolic and separated fractions on carpet viper venom injected albino rats**

Groups	Treatment	No. of Dead rats out of the total in the group	Percentage of Surviving rats	Mean Death Time (Min)
<b>Crude extract</b>	200 mg/kg	2/4	50	175.2 ± 31.00
	Fraction I			
	200 mg/kg	4/4	0	11.8 ± 7.00
Fraction II	200 mg/kg	1/4	75	1387 ± 33.00
Fraction III	200 mg/kg	1/4	75	6.2 ± 31.00
Negative control	(0.2 mg/mL venom)	0/4	0	183 ± 139.96
Normal control	200 mg/kg	0/4	100	-

Values are means of 4 replicates ± SEM

### 3.5 Neutralization of Haemorrhagic Activity

#### 3.5.1 Aqueous extract

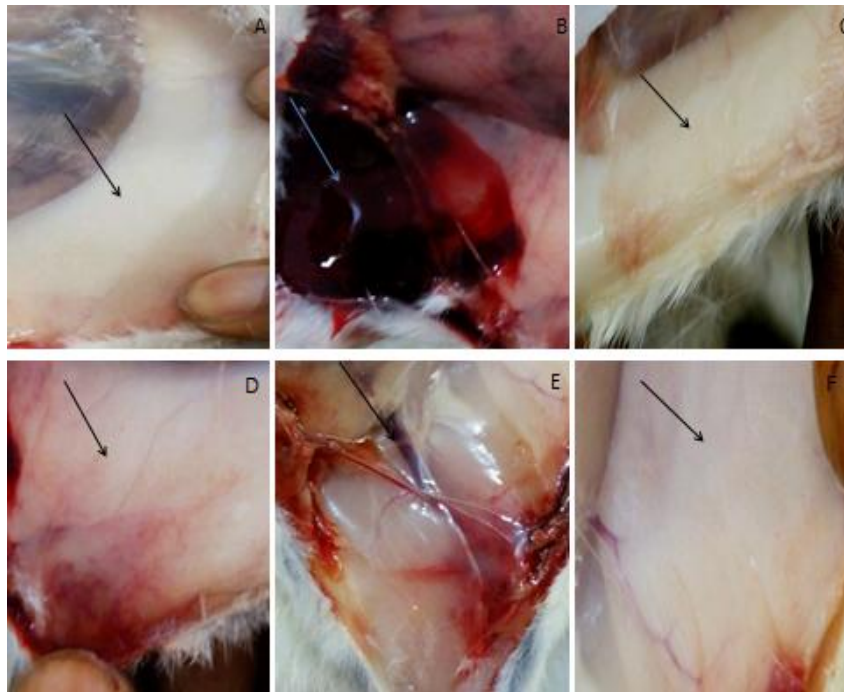
The effects of crude aqueous extract and its three fractions in neutralizing the haemorrhagic activity of the carpet viper venom are shown on Plate 1. The crude aqueous extract and fraction III achieved complete neutralization of hemorrhagic activity (coagulopathy) after a 24 h treatment period compared to the untreated animals which had severe hemorrhagic activity. Fraction I caused moderate neutralization of the hemorrhagic activity while fraction II caused partial neutralization after the 24 h treatment period compared to control (arrows show injection site).

#### 3.5.2 Ethanolic extract

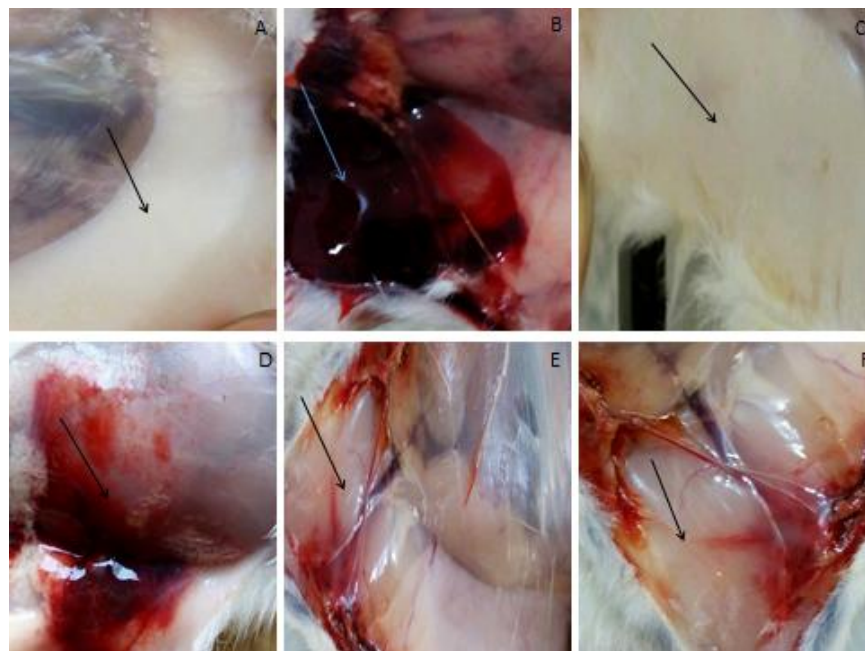
The effects of crude ethanolic extract and its three separated fractions in neutralizing the hemorrhagic activity of the carpet viper venom are shown on Plate 2. The crude ethanolic extract also achieved complete neutralization of haemorrhagic activity (Plate 2); fraction I gave poor neutralization while fractions II and III showed moderate neutralization of the haemorrhagic activity compared to the normal and untreated control (arrows show injection site).

### 3.6 DISCUSSION

Although snake bites are frequently treated using herbs, few of these claims have scientific justifications [26,27]. The phytochemical



**Plate 1. Pictures of the intraperitoneal injection sites of rats treated with aqueous extract of *Crinum jagus* bulb and fractions. The alphabets A-F correspond to the experimental groups: Normal control, untreated control, crude extract, fractions I, II and fraction III treated animals respectively. All animals in each group reacted similarly**



**Plate 2. Pictures of the intraperitoneal injection sites of rats treated with ethanolic extract of *Crinum jagus* bulb and fractions. The alphabets A-F correspond to the experimental groups: Normal control, untreated control, crude extract, fractions I, II and fraction III treated animals respectively. All animals in each group reacted similarly**

analysis of crude aqueous and ethanolic extracts of *Crinum jagus* bulb both revealed the presence of alkaloids, tannins, phenolics, terpenoids, steroids and flavonoids only. This finding is not in agreement with that of [17] who reported more bioactive components present in the plant. The observed differences may be due to environmental changes where the plant was collected or seasonal changes that could have altered the presence of the plant components. It could have also been as a result of changes during extraction and/or storage or as a result of uniqueness of the individual species of the plant as reported by [28] and [29] who reported that *Crinum jagus* has more than 3000 known species most of which are used to cure several ailments while some are flowers whereas others are poisons. The different fractions from the two crude extracts revealed that these phytochemicals were distributed differently in the various fractions; this could be attributed to the different solvent system used in the separation process.

Carpet viper venom is haemotoxic; its LD<sub>50</sub> was determined to adjust the toxic dose of the venom to suit the individual experiment. The purpose was to obtain a dose of the venom which will not be too toxic to the rats (70-80% lethality) and allow the extract sufficient time to manifest its curative effects. In this study, 5mg/ml is regarded as the lethal dose which is in contrast with that of [17] who stated that 8.5mg/mL is the lethal dose. The observed difference might be due to the geographical location and environmental difference where the snakes are caught; milking procedure and storage and also may be due to the diet and/or the age of the snake. The *in vivo* analysis of the anti-lethal effect of the *Crinum jagus* bulb plant revealed that the oral administration of its aqueous and ethanolic crude extracts and most of their fractions (200 mg/Kg) given 15min after the intraperitoneal injection was able to cause some level of inhibition of snake venom toxin throughout the 24 h period of the study. This is also in contrast with the report of [17] who stated that the minimum inhibition dose is 500 mg/Kg body weight using the methanolic bulb extract of *Crinum jagus* plant. For the aqueous extract, the crude extract and fraction III which protected 75% of the animals from death were most active *in vivo*; this results also corresponds to that of the neutralization of hemorrhagic activity (Plate 1). Neutralization of haemorrhagic activity was determined by gross observation of the site of injection with venom of each experimental animal. For the treatments

that were effective, no haemorrhaging was observed while the reverse was true for failed or inadequate anti-venom activity. However, for the ethanolic extract; fractions II and III were most active *in vivo* but the crude ethanolic extract better neutralization of hemorrhagic activity compared to fractions II and III (Plate 2). The observed difference could be as a result of different solvent systems used in the fractionation process and the difference in the phytochemical components present in the individual fractions which implies that during the fractionation process, the active ingredient(s) were able to dissolve more in these fractions thus reducing antagonistic effects from other extract components that may otherwise hinder neutralization of lethal activity; this also suggests that the bioactive ingredients which acted against the venom resides in these fractions. Additionally, *in vivo* biotransformation effects on the extract could account for some of the observed differences. The results for fraction I of the ethanolic extract were consistent *in vivo* where all the rats died (Table 3) and in neutralization of hemorrhagic activity which was poor (Plate 2). This may be due to the absence of active ingredient(s) against the snake venom or the solvent system used in the fractionation process may have destroyed or reduced the effectiveness of the fraction against the venom. Certain naturally occurring substances are known to modify the action of proteins and enzymes responsible for haemorrhage in snake venom toxins, especially plant polyphenols, flavonoids, alkaloids etc. [25]. Phenolics are reported to bind to enzyme active sites rendering them inactive, while flavonoids and alkaloids are known to block sites that are targets to the toxins. The anti-venom activity of *Crinum jagus* bulb may be due to the presence of the bioactive components in the plant. The results of this study are consistent with the report of [30] who reported that the extract of *Crinum jagus* bulb contain high amount of flavonoids, alkaloids phenolics etc. in addition to other constituents that are known to specifically inactivate proteins. This might be the possible mechanism of the detoxifying action of the plant extract and its success against the venom of the carpet viper snake seen in this study.

#### 4. CONCLUSION

The results of this study have shown that both the crude aqueous and ethanolic extracts of *Crinum jagus* bulb and their separated fractions have anti snake venom activity against the

venom of the carpet viper snake at 200 mg/kg thus proving the traditional claim of using *Crinum jagus* bulb extract in snake bite treatment by traditional healers in North-Eastern Nigeria. Additionally, this study was able to establish that the active compound(s) responsible for this activity may reside in certain fractions of the crude extracts which may be further purified and explored for development of new anti-snake venom drugs.

## CONSENT

It is not applicable.

## COMPLIANCE WITH ETHICAL STANDARDS

All the animal experiments done in this study were approved by the appropriate Ethics Committee and have been performed in accordance with laid down standards for working with snake venoms.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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