FRACTIONS OF METHANOLIC EXTRACT OF *ANNONA SENEGALENSIS* PERS DETOXIFIES *BITIS ARIETANS* ENVENOMING IN MICE

Supported by tetfund TERTIARY EDUCATION TRUST FUND

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Received: April 16, 2024 Accepted: June 28, 2024

Abstract: Snakebite envenoming is classified as a neglected tropical disease by the World Health Organization due to its public health importance and the little attention it receives from the global community (WHO, 2021). Millions of people around the world get bitten by snakes yearly. *Bitis arietans* commonly known as the African Puff Adder is one of the most venomous snakes in the world. Many poor regions of the world including Africa, lack access to potent and affordable antidotes. To address this challenge, this research was designed to assess the potential of the methanolic extract of *Annona senegalensis* Pers leaves (an indigenous plant in African) in detoxifying *B. arietans* venom. The results obtained from this study revealed that the fractions (F-III and F-IV) exhibited anti-hemorrhagic properties which is evident on the dorsal part of the excised mice skin. Also, both fractions inhibited fibrinogen clotting activity (F-III: 35.71±0.1; F-IV: 32.30 ± 0.5) and detoxified the venom in experimental mice resulting in 100 % survival. The findings from this study presents *Annona senegalensis* Pers leaves as a source of bioactive compounds for the development of affordable antidotes against snakebites.

Keywords: Envenoming, *Bitis arietans*, *Annona senegalensis* Pers, Antidotes, Snakebite, Venom

Introduction

Snakebite induced envenoming is a life-threatening neglected tropical and sub-tropical disease affecting mostly rural areas of Africa, Asia and Latin America. Approximately 5.4 million people are bitten by snakes every year with about 50% being envenomed. Over 137, 000 people die yearly from snakebite envenoming with much more amputations or other disabilities (WHO, 2021). Since snake bite envenoming affects majorly the poorest of the world population, it attracts little or no attention (Basnyat and Shilpakar, 2022, WHO, 2021). One of the major challenges in treating envenoming is the unavailability and non-affordability of effective anti-venin as well as the complexity and variation in the venom of snakes belonging even to the same genus and in some cases, the same species (Calvete et al., 2007, Emmanuel et al., 2014).

The use of medicinal plants in the treatment of snake bite envenoming has been established in literature and dates back centuries (Giovannini and Howes, 2017). It has also been established through research that the usefulness of medicinal plants in the management of diseases is linked to the phytochemicals they possess (Panda and Kumari, 2019). *Annona senegalensis* Pers also known as African custard-apple, is a shrub of about 6-11 meters consumed in some part of Africa as fruit. It contains phytochemicals with medicinal properties. Different parts of the plant have been reportedly used in folk medicine to treat different diseases including snake bites (Emmanuel et al., 2014, Mogha et al., 2022).

Bitis arietans commonly known as the African Puff Adder is one of the most venomous species of snakes responsible for the highest deaths resulting from snakebites, especially in Africa (Megale et al., 2020). To address this public health challenge, we conducted research to establish the ability of methanolic extract of leaves of *Annona senegalensis* Pers, an indigenous African plant to detoxify *Bitis arietans* venom.

Materials and Methods

Annona senegalensis

Annona senegalensis Pers leaves were collected from the wild along Funtua road in Zaria, Kaduna State, Nigeria. The plant was authenticated by a Taxonomist at the Department of Botany, Ahmadu Bello University, Zaria-Nigeria. The plant has been registered with a voucher number (900167) and deposited at the Departmental Herbarium.

Crude extraction

Leaves of *A. senegalensis* Pers were collected, washed, air-dried, and then pulverized into fine powder. The fine powdered leaves of *A. senegalensis* Pers was extracted for 72 hours on an orbital shaker, using absolute methanol. The recovered methanolic extract was filtered, concentrated, and dried on a water bath preset at 40 °C. The extraction yield was 5.08 % w/w.

Partial purification of crude extracts

The partial purification of the crude extract was done following the method we previously reported (Emmanuel et al., 2014). The extract was mixed with activated aluminum oxide dissolved in methanol in the ratio of 4:1. The mixture was allowed to dry in an oven at 40 °C. The dried mixture was placed on a 50 × 1.5 cm pre-packed aluminum oxide column. The adsorbed components of the extract were eluted with 0.4 L of each of the solvent combinations sequentially; benzene: methanol (10:1), ethylacetate: methanol (19:1), acetone: methanol (5:3), acetic acid: methanol (1:1) and water: methanol (1:9). The fractions were evaporated to dryness and stored in snap cap tubes at 4 °C prior to use.

Bitis arietans venom

Bitis arietans venom was obtained from the Bioresource Development Centre of the National Biotechnology Development Agency, Bayelsa State, Nigeria.

Experimental animals

Male mice with average weight of 25 g were obtained from the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria-Nigeria and were maintained on food and water *ad libitum* at the animal house situated at the Department of Biochemistry, Ahmadu Bello

University, Zaria-Nigeria. The guide for the care and use of laboratory animals, 1996 of the Institute for Laboratory Animal Research (ILAR) Commission on Life Science, National Research Council was duly followed.

Lethal dose determination

The minimum dose of *Bitis arietans* venom that is lethal to mice was ascertained by intraperitoneal injection of the reconstituted venom into the mice at 5 mg/kg and 20 mg/kg body weight respectively, while the normal control mice were administered normal saline alone. The mortality rate was determined 24 hours post injection.

Fibrinogen-clotting assay

The clotting time was determined as reported previously (Cavinato et al., 1998). 20 µL of *Bitis arietans* venom (7.5 mg/kg) was formulated with 100 mg/kg of the fractions (I-V) in the ratio of 1:30 w/w in normal saline, These were pre-incubated at 37 °C for 30 min and 900 µL of fibrinogen solution (2 mg/mL of fibrinogen in 10 mM Tris buffer pH 7.4, 10 mM $CaCl_2$ and 100 mM NaCl) was added and further incubated at 37 °C. Coagulation was expressed using the coagulation index (CI) obtained from the equation:

$CI = t^{-1} \times 1000$

where t^{-1} is the inverse of time.

Neutralization of hemorrhagic activity of Bitis arietans venom

To determine the effectiveness of the fractions (I-V) in neutralizing the hemorrhagic activity of *Bitis arietans* venom, 50 % of the minimum lethal dose of *Bitis arietans* venom (3.75 mg/kg) was pre-incubated at 37 °C with the fractions (I–V) and the crude extract respectively in the ratio 1:30 w/w.

Five groups of albino mice with average weight of 23 g (2 mice per group) were injected with the respective mixtures intradermal. The mice were anesthetized and sacrificed 3 hours post-injection. The dorsal part of the skin was then removed and the diameter of the hemorrhagic lesion on the skin was used as an index of the anti-hemorrhagic activity of the fraction(s).

Bitis arietans venom-induced death neutralization

Bitis arietans venom (7.5 mg/kg); the venom and the 100 mg/kg of various fraction formulated in 1:30 w/w in normal saline and the fractions alone were pre-incubated respectively at 37 °C for 30 min. Seven groups (4 mice per group) were then injected respectively with a mixture of *Bitis arietans* venom and the fractions; the fractions alone and venom alone.

Result and Discussion

We have reported the presence of two fractions (F-III and F-IV) from the methanolic extracts of the leaves of *A. senegalensis* Pers that detoxifies *Echis ocellatus* venom (Emmanuel et al., 2014). To validate the effectiveness of these fractions in neutralizing the venom of *B. arietans* which is one of the most deadly snake known to man, we employed the standard approach of venom and antidote pre-incubation; a method we and other researchers have previously used and reported (Silva et al., 2017, Vieira et al., 2021).

We demonstrated in this study that fraction III and IV were able to detoxify *B. arietans* venom resulting in 100 % survival of envenomed mice. These fractions (F-III and FIV) also had significant anti-hemorrhagic activity, which could be the mechanism by which they exert their detoxifying effects on the highly hemorrhagic, *B. arietans* venom. The potent fractions (F-III and F-IV) also inhibited the fibrinogen clotting activity of *B. arietans* venom.

Table 1: Inhibition of fibrinogen clotting activity of *Bitis arietans* venom by fractions of the crude methanolic extracts of the leaves of *Annona senegalensis* Pers.

S/N	Incubates	Coagulation Index (CI) ± SE
1	20 µl of fraction I (100 mg/kg) + 20 µl of venom + 30 min. Incubation + 900 µl Fibrinogen solution	17.24 ± 0.4
2	20 µl of fraction II (100 mg/kg) + 20 µl of venom + 30 min. Incubation + 900 µl Fibrinogen solution	67.24 ± 0.8
3	20 µl of fraction III (100 mg/kg) + 20 µl of venom + 30 min. Incubation + 900 µl Fibrinogen solution	35.71 ± 0.1
4	20 µl of fraction IV (100 mg/kg) + 20 µl of venom + 30 min. Incubation + 900 µl Fibrinogen solution	32.30 ± 0.5
5	20 µl of fraction V (100 mg/kg) + 20 µl of venom + 30 min. Incubation + 900 µl Fibrinogen solution	22.72 ± 0.7
6	20 µl of venom + 30 min. incubation + 900 µl Fibrinogen solution.	50.20 ± 0.5
7	Fractions (I-V) + 30 min. Incubation + 900 µl Fibrinogen solution	-

The clotting time was determined as reported previously (Cavinato et al., 1998). 20 µL of *Bitis arietans* venom (7.5 mg/kg) was formulated with 20 µL 100 mg/kg of the fractions (I-V) in the ratio of 1:30 w/w in normal saline, These were pre-incubated at 37 °C for 30 min and 900 µL of fibrinogen solution (2 mg/mL of fibrinogen in 10 mM Tris buffer pH 7.4, 10 mM $CaCl_2$ and 100 mM NaCl) was added and further incubated at 37 °C. Coagulation was expressed using the coagulation index (CI) obtained from the equation: $CI = t^{-1} \times 1000$, were t^{-1} is the inverse of time.

Table 2: *Bitis arietans* venom detoxification by fractions of the crude methanolic extracts of the leaves of *Annona senegalensis* Pers.

Groups	Fraction and venom mixtures (1:3 w/w)	No of dead mice per group	Percentage survival of Mice	Death time (min±SE)
1	Venom alone	4/4	0	75.2 ± 16
2	Venom + 100 mg/kg Fraction I	2/4	50	1021 ± 45
3	Venom + 100 mg/kg Fraction II	3/4	25	840.1 ± 59
4	Venom + 100 mg/kg Fraction III	0/4	100	-
5	Venom + 100 mg/kg Fraction IV	0/4	100	-
6	Venom + 100 mg/kg Fraction V	4/4	0	117 ± 38
7	Fractions (I-V) alone	-	100	-

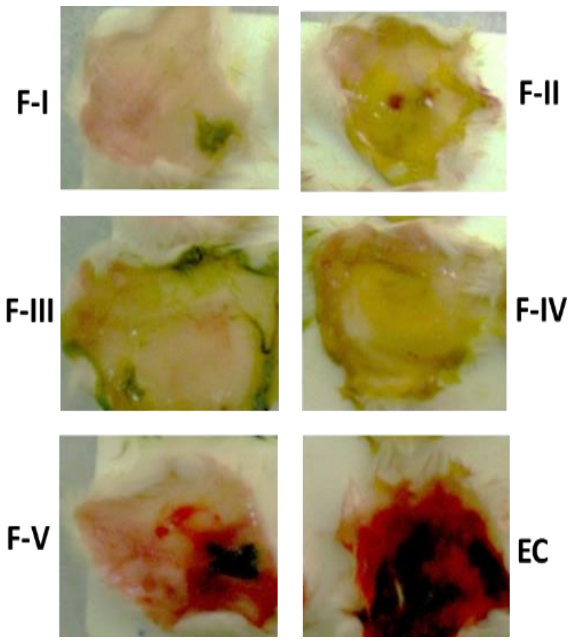

Figure 1: Anti-hemorrhagic activity of the various fractions (F-I to F-V) of the methanolic extracts of the leaves of *A. senegalensis* leaves Pers on *B. arietans* venom in comparison with the experimental control (EC).

We observed (Table 1) that another fraction (F-I) which had the strongest inhibitory action on the fibrinogen clotting activity of *B. arietans* venom was not able to improve the survival rate of the envenomed animals (Table 2). It can be deduced from this observation that the detoxifying effect of the methanolic extract of *A. senegalensis* Pers leaves does not rely only on its inhibitory action on fibrinogen clotting activity of *B. arietans* venom but rather uses other mechanism (which is presently unclear) to detoxify venom. Research has shown that *B. arietans* venom is a complex mixture with components belonging to various family of toxins (Calvete et al., 2007, Fasoli et al., 2010, Wang et al., 2020), these components are responsible for its toxicity and the potency of the venom rely on their activity. Hence, a potent antidote must be able to significantly inhibit the main components and/or other constituents of the venom to be able to ameliorate its detrimental effect on affected individuals.

Conclusion

We have previously reported the detoxification of *Echis ocellatus* venom-induced toxicity by fractions of *A. senegalensis* Pers leaves and in the present study, we also demonstrated that these fractions (F-III and F-IV) also detoxifies *Bitis arietans* venom. Therefore, it is conceivable that *A. senegalensis* contains bioactive principles that may possess broad snake venom detoxifying potential across species.

Declaration of Competing Interest

The authors declare that they have no-known competing interest that could have influenced the outcome of the research reported in this article.

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