

**INTERNATIONAL JOURNAL OF SCIENCE FOR GLOBAL SUSTAINABILITY****In-Vivo Antivenom Effect of *Annona senegalensis* (Pers) Against Biological Activities of *Naja nigricollis* (Hallowell) Envenomation**Idris Aliyu Kankara^a, Aliyu Abdullahi Maru^a, Gayus Aminu Paulina^a and Binta G. Kurfi^b^a Department of Science Laboratory Technology, Federal Polytechnic Kaura Namoda, Zamfara State, Nigeria.^b Department of Biochemistry, Faculty of Biomedical Science Bayero University, Kano, NigeriaCorresponding Author's Email & Phone No.: aliyukankara@gmail.com, 07035228026

Received on: August, 2023

Revised and Accepted on: September, 2023

Published on: October, 2023

ABSTRACT

Snake bites are a common, serious medical issue often neglected in developing countries. This study aims to investigate the effectiveness of methanolic extract from the root bark of *Annona senegalensis* in neutralizing the biological activities of *Naja nigricollis* envenomation in vivo. The methanolic extract was fractionated using a liquid-liquid fractionating method. The inhibition of venom-induced edematogenic and hemorrhagic effects was evaluated in albino rats using a pre-incubation protocol. The aqueous-methanol fraction (AM) of *A. senegalensis* AM root methanolic extract significantly inhibited the hemorrhagic and edema-inducing activities of the venom. The methanolic extract derived from the root bark of *A. senegalensis* has exhibited promising potential as an effective anti-snake venom agent.

Keywords: *Annona senegalensis* (Pers), *Naja nigricollis* (Hallowell.), Envenomation and Biological activities.

1.0 INTRODUCTION**1.1 Snake Envenomation**

Snake bites are a common, serious medical issue often neglected, especially among the poorest populations living in areas where snakes are abundant. According to studies conducted by Pucca *et al.* (2019) and Abubakar *et al.* (2023), there are an estimated 5.4 million snake bites each year, leading to 1.8-2.7 million cases of envenoming. This results in approximately 410 - 137,880 deaths and three times as many amputations and other permanent disabilities every year, as per WHO's 2018 report. Typically, snake bites affect people who play a vital role in the socio-economic activities of the community, such as farmers, fishermen, and hunters.

1.2 Venomous Snakes

Venomous snakes belonging to the Viperidae, Atractaspididae, Elapidae, and Colubridae families can produce and store venom in the salivary gland located in the upper jaw ventral posterior of their eyes. Among them, *Naja nigricollis* and kraits from the Elapidae family are known to cause the most severe snake envenomation (Dias da Silva *et al.*, 2022; Mp *et al.*, 2019). The venom of these snakes is a complex mixture of inorganic ions, such as sodium, zinc, magnesium (Laustsen, *et al.*, 2018), calcium, cobalt, and copper, as well as organic

compounds like carbohydrates, serotonin, histamine, bradykinin potentiating peptide, disintegrins, proteins, toxins, and enzymes such as L-amino acid oxidases, hyaluronidases, serine proteases, metalloproteases, and PLA2 (Pucca *et al.*, 2019; Menaldo *et al.*, 2015). These protein toxins and other venom components can act independently or in combination to cause local or systemic tissue damage, neurotoxicity, and myotoxicity (Abubakar *et al.*, 2023; Dias da Silva *et al.*, 2022). In addition, they can cause cardiotoxicity, hemolytic, hemostatic, coagulant, hemorrhagic, and oedema effects (Bala *et al.*, 2018). Snake bites can be life-threatening and require immediate medical attention (Dias da Silva *et al.*, 2022). The only authorized and effective treatment for snake envenomation is anti-snake venom (Ahmad *et al.*, 2020). However, this treatment is expensive and requires ideal storage facilities (Abubakar *et al.*, 2023), which may not be available in rural communities of developing countries (Felix-Silva *et al.*, 2017).

1.3 Antivenom Ethnomedicinal Plants

Various ethnomedicinal plants have been said to possess anti-snake venom properties, as Sani *et al.* (2020) stated. However, only a select few have undergone scientific testing in vivo and in vitro. Shivashankar *et al.* (2019) have identified numerous ethnomedicinal plants that have proven

to be potent inhibitors against Hyaluronidase, phospholipase A2, and proteases of *N. nigricollis* venom. Felix-Silva *et al.* (2017) demonstrated that the methanol extract of *A. senegalensis* Pers. roots reduced the lethality caused by *N. nigricollis* Reinhardt venom. Further research by Muthusamy *et al.* (2017) found that Ikshusterol3-O-glucoside isolated from *Clematis gouriana* Roxb. ex DC. root has a potent snake-venom neutralizing capacity.

Guiera senegalensis leaf extract was found to detoxify both *Echis carinatus* and *N. nigricollis* venom. Ibrahim *et al.* (2011) concluded that *L. egyptiaca* and *N. rustica* leaf extracts inhibit the proteolytic activity of *N. nigricollis* venom. Lastly, Abubakar *et al.* (2016) observed the efficacy of ethanol stems bark extract of *Commiphora africana* (a. Rich.) Engl. (burseraceae) against pharmacological actions induced by *N. nigricollis* venom through non-competitive inhibition of PLA2 activity and significant protection of albino rats against venom-induced mortality.

1.3.1 *Annona Senegalensis*

Annona senegalensis is a plant species that belongs to the *Magnoliophyta* division, *Magnoliopsida* class, *Magnoliidae* subclass, *Magnoliales* order, *Annonaceae* family, *Annona* genus, and the *senegalensis*. It is commonly known as the African custard apple or wild custard apple and is referred to as Gwándàn dààjìì (in Hausa) or dukun-hi (in Fulani). *Annona senegalensis* is a tropical perennial and has various ethnomedicinal uses. Its root helps treat male sexual impotence, gastritis, snakebites, erectile dysfunction, and tuberculosis. The stem bark has been used to treat snakebites and hernias. The leaves have been utilized for the treatment of yellow fever and smallpox. The plant has many beneficial effects, including anticonvulsant, anti-trypanosomal, anti-oxidant, antimalarial, antimicrobial, antidiarrheal, anti-inflammatory, anti-parasitic, and antinociceptive properties. These properties make it a valuable plant for medicinal purposes (Mustapha, 2013). Traditional herbalists claim to have effective cures and are immune to snakebites through ethnomedicinal plants. They are the first people to be called upon when snakebites occur in their communities. There are many instances where their medication has cured what could have been fatal envenomation, and the source of this 'power' is ethnomedicinal plants. Therefore, this study

aims to evaluate the in-vivo antivenom effect of *Annona senegalensis* (pers) against the biological activities of *Naja nigricollis* (hallowell) envenomation.

2.0 MATERIAL AND METHODS

2.1 Collection of Snake Venom

Lyophilized *Naja nigricollis* venom was bought from the pharmacology department at Ahmadu Bello University Zaria. The venom was extracted manually from the adult specimen, lyophilized, and stored at room temperature in a specimen container until use. The Venom solutions were made using phosphate-buffered saline (PBS) at pH 7.4.

2.2 Experimental Animals

Twenty-one (21) albino rats of both sexes weighing about 18-40g were used for this study. The rats were kept at the animal's house under normal environmental conditions and maintained with free access to pelletized growers' feed and access to water ad libitum. The animal procedures in this study adhered to the guidelines set forth by the National Institute of Health's Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. Before conducting the experiments, we obtained permission from the institutional animal ethical committee at Federal Polytechnic Kaura Namoda in Zamfara State, Nigeria.

2.3 Collection and identification of *A. senegalensis*

The fresh root bark of *A. senegalensis* was collected randomly from Dan Nakwabo village in Kankara Local Government Area of Katsina State, Nigeria. The sample was then transported in a specimen container to the Department of Biological Science laboratory at Federal Polytechnic Kaura Namoda, Zamfara State. It was authenticated at the Herbarium section of the Botany Unit, Bayero University Kano, Nigeria, and given voucher number BUKHAN 3. The root bark of *A. senegalensis* was thoroughly washed to remove sand and then air-dried.

2.4 Preparation of plant extract

To ensure homogeneity, the root bark of *A. senegalensis* was ground into a fine powder using a wooden mortar and pestle. Then, 500 grams of the shade-dried powdered root bark of *A. senegalensis* was added to methanol in a 1:10 (w/v) ratio and immersed for 24 hours, with

continuous stirring at room temperature (maceration). The extraction process was carried out over three consecutive days, with the solvent (methanol) being changed every 24 hours. The resulting filtrate was concentrated using a water bath at 40°C to produce an extract. This extract was then dried and stored in a labelled plastic container until needed.

2.5 Fractionating of the Crude Extract

The methanolic root bark extract of *A. senegalensis* was dissolved in 500 ml of distilled water in a separating funnel. The mixture was then extracted with hexane, dichloromethane, and ethyl acetate, using liquid-liquid fractionating to produce fractions with varying polarities. The fractions were concentrated on a rotary evaporator and air-dried at room temperature. The fractions were reconstituted into a solution with PBS pH 7.4 when required for the study.

2.6 Inhibition of edema inducing activity of the venom

Briefly, 5 µg (half of LD50) of the *N. nigricollis* venom was pre-incubated with dichloromethane (DCM), ethyl acetate (EA), and aqueous-methanol (AM) fractions of the extract, respectively, for 1 hour at 37 °C in the ratio 1:5, 1:10 and 1:20 venom: extract (w/w). The pre-incubated venom fractions were injected in the right footpads of albino rats (n=3), while the left footpads were injected with PBS pH 7.4 only. Albino rats injected with the venom alone served as positive controls. After 45 minutes, the two legs were removed at the ankle joint. The weight (oedema ratio) increase was calculated using the formula below for the (Costa et al., 2018; Escalante et al., 2009).

$$\frac{\text{Weight of edematous leg}}{\text{Weight of control leg}} \times 100$$

2.7 Inhibition of Hemorrhagic inducing activity of the venom

The hemorrhagic activity of *N. nigricollis* venom was determined using a modified method of Kondo et al. (1960) and Liu et al. (2021). Haemorrhage was induced by intradermal injection of 5 µg (half of LD50) of *N. nigricollis* venom in the dorsal region of the Albino rat. The extent to which DCM, EA and

AM fractions of methanolic extract root bark of *A. senegalensis* could inhibit the hemorrhagic activity of the venom was determined using pre-incubation protocol. 5 µg of the *N. nigricollis* venom pre-incubated with DCM, EA and AM fractions of the extract for 1hr at 37 °C in the ratio 1:5, 1:10 and 1:20 venom: extract (w/w) were injected intradermally in the dorsal region of the Albino rats, while the control group received venom only. One hour after venom injection or pre-incubated mixture, the animals were sacrificed by cervical dislocation. The diameter of the hemorrhagic halo of the treated groups was measured in mm with the aid of a ruler and compared with the control group.

2.8 Data Analysis

The data generated from the study are presented as mean ± SEM and subjected to one way analysis of variance (ANOVA) and statistical difference between the means were evaluated using New Duncan's Multiple Range Test at P<0.05

3.0 RESULT AND DISCUSSION

3.1 Determination of the activity of fractions

The n-hexane fraction was negligible and not considered for further study. The fractions of dichloromethane (DCM), ethyl acetate (EA), and aqueous-methanol (AM) were evaluated for possible anti-venom activity.

3.2 Inhibition of Edematogenic Activity

The injection of 5 µg of *N. nigricollis* venom caused significant (p<0.05) swelling of the left footpad in albino rats, as shown in Figure 1. The venom's oedema-inducing pathophysiology depends on the pharmacological activities of various enzymes and protein toxins, such as PLA2, prostaglandins, leukotrienes, bradykinin, histamine thromboxanes, and complement factors (Zamune and Teixeira, 2002), present in the venom. However, when the AM, EA, and DCM fractions of the extract were administered at a ratio of 1:20w/w, they significantly reduced the oedema ratio from 155±3 (Figure 1) to 105.33±2.08 (Figure 4c), 112±1.73 (Figure 3c), and 117.67± 2.0 (Figure 2c), respectively. The AM fraction (Figure 4c) had the most significant (p<0.05) inhibitory effect, followed by the EA fraction (Figure 3c) and the DCM fraction (Figure 2c).



Figure 1: Albino rat paw showing the of edematogenic activity of *N. nigricollis* venom (5µg)



Figure 2a: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by DCM fraction (1:5 w/w) using pre- incubation protocol



Figure 2b: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by DCM fraction (1:10 w/w) using pre- incubation protocol

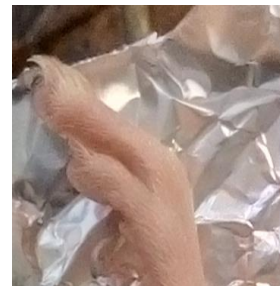


Figure 2c: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by DCM fraction (1:20 w/w) using pre- incubation protocol



Figure 3a: Albino rat paw showing the edematogenic activity of *N. nigricollis* venom (5µg)



Figure 3b: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by EA fraction (1:5 w/w) using pre- incubation protocol



Figure 3c: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by EA fraction (1:10 w/w) using pre- incubation protocol



Figure 3d: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by EA fraction (1:20 w/w) using pre- incubation protocol



Figure 4a: Albino rat paw showing the edematogenic activity of *N. nigricollis* venom (5µg)



Figure 4b: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by AM fraction (1:5 w/w) using pre- incubation protocol



Figure 4c: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by AM fraction (1:10 w/w) using pre- incubation protocol



Figure 4d: Albino rat paw showing the inhibition edematogenic activity of *N. nigricollis* venom by AM fraction (1:20 w/w) using pre- incubation protocol



Figure 5: Albino rat skin showing the of hemorrhagic activity of *N. nigricollis* venom (5µg)



Figure 6a: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by DCM fraction (1:5 w/w) using pre-incubation protocol



Figure 6b: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by DCM fraction (1:10 w/w) using pre-incubation



Figure 6c: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by DCM fraction (1:20 w/w) using pre-incubation protocol



Figure 5: Albino rat skin showing the of hemorrhagic activity of *N. nigricollis* venom (5µg)

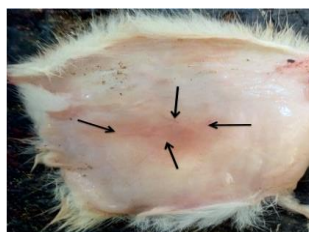


Figure 7a: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by EA fraction (1:5 w/w) using pre-incubation protocol



Figure 7b: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by EA fraction (1:10 w/w) using pre-incubation protocol



Figure 7c: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by EA fraction (1:20 w/w) using pre-incubation

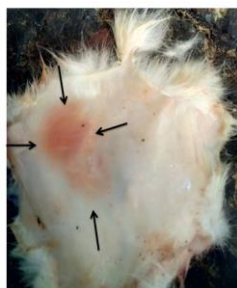


Figure 5: Albino rat skin showing the of hemorrhagic activity of *N. nigricollis* venom (5µg)

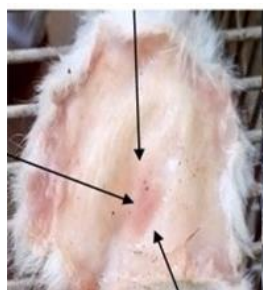


Figure 8a: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by AM fraction (1:5 w/w) using pre-incubation protocol

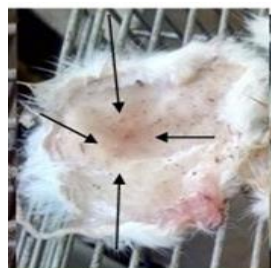


Figure 8b: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by AM fraction (1:10 w/w) using pre-incubation protocol



Figure 8c: Albino rat skin showing the inhibition hemorrhagic activity of *N. nigricollis* venom by AM fraction (1:20 w/w) using pre-incubation protocol

3.3 Anti-Hemorrhagic activity

In albino rats, the injection of *N. nigricollis* venom resulted in severe haemorrhage damage around the injected area ($37 \pm 0.2 \text{ mm}$), as shown in Figure 5. However, various fractions of the extract exhibited a significant inhibitory effect against *N. nigricollis* venom-induced haemorrhage in rat skin, as demonstrated in Figures 5-8. The DCM fraction of the extract showed a significant ($p < 0.05$) reduction in the hemorrhagic lesion from ($37 \pm 0.2 \text{ mm}$) to ($20 \pm 0.5 \text{ mm}$) at a ratio of 1:5 w/w (venom: fraction), as shown in Figure 6a. At the ratio of 1:10 and 1:20 (w/w), the hemorrhagic lesion was significantly ($p < 0.05$) reduced to $17.0 \pm 0.2 \text{ mm}$ (Figure 6b) and ($7.4 \pm 0.6 \text{ mm}$) (Figure 6c), respectively. The AE and AM fractions at the ratio (1:10 and 1:20 w/w) showed a higher significant ($p < 0.05$) inhibitory effect against venom-induced haemorrhage (Figures 7b and 8c) compared to the inhibitory activity of DCM fraction at all concentrations (Figures 6a-6c) tested in this study. The AM fraction at the ratio of 1:20 venom: fraction (w/w) exerted the most potent anti-hemorrhagic effect (Figure 8c) compared to the anti-hemorrhagic effect of DCM (Figures 6a-6c) and EA fractions (Figures 7a-7c) of the extract. The study's result was similar to Emmanuel *et al.*'s (2014) finding on the anti-snake venom activity of fraction III of *A. senegalensis*, which was clearly shown by the complete neutralization of venom-induced haemorrhage.

4.0 DISCUSSION

The current findings show that the root bark of *Annona senegalensis* contains phytochemical compounds that could be used as a potential source for anti-snake venom drugs. The present findings are consistent with previous research conducted by Okoye *et al.* (2012) and Yande *et al.* (2017), which reported on the various phytochemical contents found in *Annona senegalensis*. Carvalho *et al.* (2013) have identified flavonoids, steroids, coumestan, alkaloids, terpenoids (mono-, di-, and triterpenes), and polyphenols as the bioactive components of ethnomedicinal plants, which have been found to inhibit classes of venom PLA2s. These plant metabolites possess protein-binding and enzyme-inhibiting properties and can neutralize snake venom (Raghavamma *et al.*,

2016). Mourão de Moura *et al.* (2017) have shown the ability of Tannins to precipitate various protein toxins, chelate metal ions and form complexes with metals such as Zn^{2+} and Ca^{2+} ions present in snake venom, thereby neutralizing various lethality induced by snake venom, particularly oedema, haemorrhage, hemolytic and coagulant activities. Snake venom enzymes and toxins responsible for inducing various lethality depend on metal ions for their three-dimensional structure or as cofactors to exert their biological activities (Nanjaraj *et al.*, 2015).

The phytochemical compounds in *A. senegalensis* might have contributed to the plant's anti-edematogenic activity against *N. nigricollis* venom. These findings are in line with Shivashankar *et al.* (2019) research, which established that PLA2 inhibitors significantly reduced oedema caused by Russell's viper, and Souza *et al.* (2015) research, which indicated a significant positive correlation between the edematogenic activity of Bothrops venoms and PLA2 activity in vivo. The inhibition of haemorrhage may occur due to the interaction between fatty acids or phytochemicals of the extract with venom enzymes or its components, leading to either blocking the active site of the metalloproteinase enzymes or forming a complex with the ions that are essential for the three-dimensional structure and the activity of the venom proteinase enzymes, thus inhibiting the activity of the venom metalloprotease enzyme mostly Zn^{2+} -dependent (Adrião, *et al.*, 2022). Strauch *et al.* (2013) concluded that the inhibitory effects of *Humirianthera ampula* extract on Bothrop's venom's hemorrhagic activity observed were due to the inhibitory effect of phytochemical compounds of the plant against the catalytic activity of snake venom metalloproteinase enzymes. Additionally, Alam (2014) stated that some phytochemical components of the plants could neutralize the venom's protein toxins due to their protein-binding ability and metal-chelating effects. Omara *et al.* (2020) attributed the hemorrhagic inhibitory effect of *Eclipta alba* to three of its constituents: wedelolactone, stigmasterol, and sitosterol. The present result was consistent with Emmanuel *et al.* (2014) finding on the anti-snake venom activity of the DCM fraction of *A.*

senegalensis, which was clearly shown by the complete neutralization of venom-induced haemorrhage. The anti-hemorrhagic activity of *A. senegalensis* might be attributed to its phytochemical compounds' inhibitory effect against the snake's venom-induced hemorrhagic ability (Kulatunga and Arawwawala, 2019)

5.0 CONCLUSION AND RECOMMENDATION

This study shows that the methanolic root bark extract of *A. senegalensis* has potential anti-venom effects in vivo against *N. nigricollis*-induced toxicity. This study suggests that *A. senegalensis* could be used to develop new anti-snake venom therapeutics.

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