In vitro Anti venom Effect of *Catunaregam nilotica*(STAPF) Root Extracts against *Echis ocellatus* Phospholipase A_2 Activity

ABSTRACT

Aims: This study evaluates the inhibitory effect of the crude extract of *Catunaregam nilotica* (STAPF) and its fraction against *Echis ocellatus* phospholipase A2 (PLA 2) activity using an in vitro approach.
Study design: In-vitro Antivenom Study.
Place and Duration of Study: Department of Science Laboratory Technology, Federal Polytechnic Kaura Namoda, Zamfara State, Nigeria, between September to December, 2024.

Methodology: Crude extraction was performed through cold maceration and fractionated via solvent-solvent partitioning using solvents that increased polarity. The phytochemical contents were screened using standard methods. The inhibitory effect of the crude extracts and their fraction against PLA2 activity was evaluated via the in-vitro acidimetry method.
Results: The phytochemical analysis result revealed alkaloids, anthraquinones, flavonoids, phenols, saponins, steroids, tannins, terpenoids, and steroids in the CMECNR and all the fractionated extracts. The in-vitro inhibition assay results revealed that both the hexane and the ethyl acetate fractions significantly inhibited the activity of PLA2 ($P<0.05$) compared with the venom controls in varying degrees of efficacy.
Conclusion: In conclusion, this study supports the potential of medicinal plants in developing effective antivenom therapies. Further studies are recommended to isolate the active compound and elucidate its mechanism of action

Venomous snakes are species that produce venom in their venom glands. Phospholipase A2, commonly found in snake venoms, plays a key role in breaking down phospholipids and inducing local and systematic biological effects. This study evaluates the inhibitory effect of the crude extract of *Catunaregam nilotica* (STAPF) and its fraction against *Echis ocellatus* phospholipase A2 (PLA 2) activity using an in vitro approach. The crude extract was fractionated via solvent-solvent partitioning using solvents that increased polarity. The phytochemical contents were screened using standard methods. The inhibitory effect of the crude extracts and their fraction against PLA2 activity was evaluated via the in-vitro acidimetry method. The phytochemical analysis result revealed alkaloids, anthraquinones, flavonoids, phenols, saponins, steroids, tannins, terpenoids, and steroids in the CMECNR and all the fractionated extracts. The in-vitro inhibition assay results revealed that both the hexane and the ethyl acetate fractions significantly inhibited the activity of PLA2 ($P<0.05$) compared with the venom controls in varying degrees of efficacy. In conclusion, this study supports the potential of medicinal plants in developing effective antivenom therapies. Further studies are recommended to isolate the active compound and elucidate its mechanism of action.

Keywords: **In vitro, Anti venom, PLA_2, *Catunaregam nilotica, Echis ocellatus***

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1.0 INTRODUCTION

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Snakes belong to the Phylum *Chordata*, Order *Squamata*, Sub-order *Serpentes* and Class *Reptilia.* Linnean taxonomy places all modern snakes within the Sub-order *Serpentes*, part of the Order *Squamata*(Alejandro, 2007;Database, 2023) and various families, including Colubridae, Boidae, Elapidae, Pythonidae, and Viperidae (Adrião, et al., 2022). Venomous snakes are species that produces venom in their venom glands and these group of snakes have been classified into four families: *Viperidae, Atractaspididae, Elapidae* and *Colubridae*(Gutiérrez, et al., 2017) of these, *Naja nagricollis* and kraits of *Elapidae* family cause maximum envenomation (WHO, 2016; Ajisebiola et al., 2022).Venomous snakes are prevalent on most of the continents except for Antarctica, Ireland, New Zealand, and many small Atlantic and Central Pacific islands (Nicola, et al., 2021). *Elapidae* families (African cobra, Asian kraits, African mambas, American coral snakes, Asian cobra, Australian and New Guinean venomous snakes, and sea snakes) and *Viperidae* (Asian pit vipers, American rattlesnakes and pit vipers, and old-world vipers) are medically important snakes (Guimarães, et al., 2014). These medically significant snakes can cause snakebite envenomation (SBEs), which can lead to symptoms such as pain, swelling, bleeding, severe tissue damage, local necrosis, hemorrhage and edema at the bite site, tissue death, and neurological issues(WHO, 2016). In severe cases, SBEs can cause loss of function in the affected limb, acute respiratory and renal failure which can lead to death (Nicola et al., 2021;Monteiro et al., 2020).
In Nigeria the majority of snake species that are of medical importance belong to three families' viz., Viperidae (Vipers and Adder), Elapidae (Cobras and Mambas) and Colubridae (Boomslag). The saw-scaled or carpet viper (*Echis ocellatus*), Cobras (Naja spp.) and puff adders (Bitisarietans) have proved to be the most important cause of mortality and morbidity. Specifically, the *Echisocellantus* is by far the most common cause of morbidity and mortality in North-Eastern Nigeria (Shah et al., 2022).

Echis ocellatus venom comprises a diverse array of enzymatic families, including snake venom metalloproteinases (SVMPs), snake venom serine proteases (SVSPs), and snake venom phospholipase A2, which collectively constitute approximately 70% of the complete venom proteome (Oliveira*et al.,* 2022). These enzymes possess the capability to function either synergistically or independently, thus inducing local tissue injury, myotoxic effects and hemorrhagic manifestations through the degradation of the basement membrane of capillary vessels (Muhammad *et al.,* 2022; Ajisebiola et al., 2022). The hemorrhagic consequences associated with *Echis ocellatus* venom can lead to mortality as they cause bleeding from crucial organs by inflicting damage upon the vascular endothelium (Shah et al., 2022).

Phospholipases A2 (PLA2; EC 3.1.1.4), commonly found in snake venoms, play a key role in breaking down phospholipids and can induce biological effects such as edema, platelet aggregation modulation, and neurotoxic, anticoagulant, and myotoxic activities. Myotoxic PLA2s bind to lipids or proteins on the plasma membrane, compromising its integrity either through enzymatic hydrolysis or direct interaction with the membrane. This disruption leads to uncontrolled ion permeability, primarily Ca^{2+} influx which triggers destructive processes like muscle hypercontraction, membrane damageand mitochondrial Ca^{2+} overload which ultimately result in muscle cell necrosis (Pereañez*et al.,* 2011)

PLA_2, abundant in viperid and crotalid venomsplays a key role in the development of myotoxicity following snakebites, which can manifest as local or systemic muscle damage. Their action can cause irreversible injuries, potentially leading to limb amputation. Antivenoms, while effective against systemic effects, have limited efficacy in preventing local tissue damage. This highlights the need for alternative inhibitors and complementary therapies to enhance conventional antivenom treatment.

Antivenom (ASV) represents the onlyapproved pharmacological intervention employed to mitigate the effects of snake envenomation, thereby enhancing the immune response following a snake bite (Mukherjee, 2020). Nonetheless, ASV is associated with numerous limitations. It can precipitate acute anaphylaxis or anaphylactic reactions attributed to its heterologous nature, which may vary from mild to severe and manifest within one-hour post-administration of the antivenom (Bhaumik *et al.,* 2020). Sometimes, it fails to confer adequate protection against local toxicities induced by snake venom, such as hemorrhage, necrosis, and nephrotoxicity (Roy, 2021). The procurement of ASVs is economically burdensome and necessitates optimal storage conditions (Liaqat et al., 2022), which may be inadequate in rural regions endemic to snake populations.

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Plants are reputed to neutralise the toxic effects of snake venom, with many plants claimed to be antidotes for snake bites in traditional folk medicine (Liaqat et al., 2022). These plants are used, single or in combination as antidotes for snake envenomation by rural populations in Nigeria and many parts of the world (Omara et al., 2020). There are several reports of the widespread use of medicinal plants against snake bites around the world, especially in tropical and subtropical regions such as Asia, Africa and South America (Sani *et al.,* 2020). However, literatures have shown that very few plants undergo scientific validation.

Catunaregam nilotica is a medicinal plant commonly found in Northern Nigeria, widely used in the treatment of bites from two of the most venomous snakes in the region *Naja nigricollis* (black-necked spitting cobra) and *Echis ocellatus* (saw-scaled viper) as well as scorpion stings. Communities in Isa (Sokoto state),Zuru(Kebbi state) and Gumel(Jigawa state) have long utilized this plant for its broad range of traditional applications. The root and stem bark are especially used to treat various poisons, particularly snakebites. Additionally, the plant holds significant value in managing gonorrhea and other sexually transmitted diseases (using the leaves), as a genital stimulant (using the root), and for its use as anti-dysenteric, anti-inflammatory, and anti-fertility properties (root and stem bark) (Hassan et al., 2023).

2. MATERIALS AND METHODS

2.1. STUDY AREA

The study was conducted in the Kaura Namoda, Nigeria, within the Biochemistry Research Laboratory situated in the Department of Science Laboratory, under the School of Science, Federal Polytechnic Kaura Namoda, Zamfara State, Nigeria.

2.2. STANDARD SNAKE VENOM ANTISERUM (ANTIVENIN)

The polyvalent snake venom antiserum known as African Snake Venom Antiserum (Asna Anti-venom C), designated with Batch Number AF10/10 and manufactured in October 2021 with an expiration date set for December 2025, was obtained from Bharat Serums and Vaccines Ltd, India. This antiserum served as a benchmark for evaluating the comparative efficacy of the botanical extract.

2.3. COLLECTION of SNAKE VENOM

Lyophilized venom of *Echis ocellatus* was procured from the pharmacology department at Ahmadu Bello University, Zaria, Nigeria.

2.4. Preparation of venom

The lyophilised venom was reconstituted in 0.9 % saline and kept at 4°C. The venom concentration was expressed in terms of dry weight (mg/ml) (Razi et al., 2011)

2.5. Collection and authentication of the plant material

The fresh root barks of *Catunaregam nilotica* were collected from Gabake village situated within the Birnin Magaji Local Government Area of Zamfara State, Nigeria. The collected specimens were subsequently transported in polyethylene bags to the biochemistry laboratory, Kebbi State University of Science and Technology, Aliero. The sample was authenticated by Professor Dramendra Singh at the herbarium unit of the university and assigned a voucher number Ksusta/PSB/H/Voucher No. 234A. The roots were meticulously washed and air-dried under shade until it reached a constant weight.

2.6. Preparation of crude methanol extract of *Catunaregam nilotica* root bark (CMECNR)

The extract was prepared in accordance with the methodology established by Jimoh et al. (2021). The harvested root was cleaned using distilled water, air-dried in a shaded environment and ground into a fine powder using pestle and mortar. The root powder of *Catunaregam nilotica* (1kg) was immersed in 2.5 L of methanol (95%). The resultant mixture was maintained at ambient temperature for 72 hours and filtered twice, first employing a muslin cloth followed by a Whatman filter paper No.1. The resulting filtrate wasevaporated to dryness at a controlled temperature of 45°C using a rotary evaporator.

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2.7 Solvent-Fractionation of crude methanol extract*Catunaregam nilotica* root bark

The crude methanol extract derived from the root bark of *Catunaregam nilotica* was subjected to fractionation through liquid-liquid extraction using n-hexane, ethyl acetate, and n-butanol, arranged sequentially according to their increasing polarity. The crude extract, amounting to 100 grams, was reconstituted in 200 milliliters of distilled water within a 500-milliliter separating funnel and subjected to vigorous shaking. Subsequently, the resulting solution was partitioned with an equivalent volume of n-

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hexane and agitated intensely. After allowing the components to settle, the lower section of the separating funnel was opened to facilitate the removal of the aqueous layer. The residual contents within the separating funnel were transferred into a clean vessel designated for the n-hexane fraction. An additional equal volume of n-hexane was introduced, followed by further shaking and separation. This process was repeated continuously until the extraction yielded negligible amounts of extract in the n-hexane layer. An analogous procedure was executed for both ethyl acetate and n-butanol. The resulting fractions underwent concentration to dryness via a rotary evaporator, culminating in the acquisition of n-hexane (n-HF), ethyl acetate (EAF), n-butanol (n-BF), and aqueous fractions (AF) (Ahmed *et al.,* 2022; Dawurung*et al.,* 2021; Abubakar and Haque, 2020). The fractions were concentrated to dryness, and the resultant residues were maintained in a refrigerator within an airtight container for subsequent analysis. Prior to utilization, each fraction was reconstituted in either distilled water or a 1% solution of Tween80 (polysorbate) and quantified in terms of dry weight (mg/ml).

2.8. Qualitative phytochemical screening

5g of CMECNR was dissolved in 40 ml of distilled water and thereafter subjected to phytochemical screening through established methodologies (Harborne, 1973; Trease and Evans, 1989; Sofowora, 1993).

2.9. Phospholipase A2 Assay

The acidimetric assay for PLA_2 enzymes, described by Tan and Tan (1988) and reported by Sani *et al.* (2020b) was adopted in this study. Constant volumes of substrates comprising Calcium chloride (18 mM), Tween eighty (1%) and egg yolk (2 mg/ml) were mixed and stirred for 10 min to get homogenous egg yolk suspension. The pH of the mixture was adjusted to 8.0 using NaOH (1 M) and HCL (1N). Snake venom (0.1mg/ml) was added to the above mixture (15ml) to initiate the process of hydrolysis, and saline was used as a control. For venom enzyme inhibition assay, *Echis ocellatus* venom (0.1mg/ml) was pre-incubated with CMECNR and its Fractions (0.1mg/ml) for 30 minutes at 37ºC to neutralize the hydrolytic action of PLA_2. A decrease in the pH of the suspension was noted after two minutes with the help of a pH meter. A pH decreases by one (1) unit corresponded to 133 µmole of fatty acid released. Enzyme activity was expressed as µmole of fatty acid released per minute (Yap *et al.,* 2011).

The inhibitory activity by the CPFs of the root of *C. nilotica* against the PLA_2 was calculated and expressed in terms of percentage activity using the following relationships.

Enzyme activity = $\frac{\mu mol\ of\ FA\ released}{Time\ taken\ in\ minutes}$

% Activity = $\frac{Enzyme\ activity\ of\ the\ test}{Enzyme\ activity\ of\ the\ control} \times 100$

Where: FA = Fatty acid

% Inhibition = $\frac{Enzyme\ activity\ of\ the\ control - Enzyme\ activity\ of\ the\ test}{Enzyme\ activity\ of\ the\ control} \times 100$

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3.0 Results and Discussion

50.6g of black crude extract was obtained after 72h of extraction with methanol. This is 5.1% yield of the extract. It was then labeled as crude methanol extract of *Catunaregam nilotica*root bark (CMECNR).

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3.1 Phytochemical composition of the crude extract and its fractions

Table 1 present the qualitative phytochemical compositions of the CMECNR and its fractions (n-hexane, ethyl acetate, n-butanol, and aqueous). Alkaloids, anthraquinones, Flavonoids, Phenols, Saponins, Steroids, Tanninsand Terpenoids were detected in the CMECNR and all the fractionated extracts. Phenols were detected in CMECNR, n-butanol and aqueous fraction. Terpenoids were detected in the CMECNR, n-hexane and ethyl acetate fractions. Saponins and Anthraquinones were not detected in n-hexane and aqueous fractions.

Table 1: Phytochemical Compositions of C. nilotica and Its Fractions

Test	CMECNR	n-HF	EAF	n-BF	AF
Alkaloids	+	+	+	+	+
Anthraquinones	+	+	+	-	-
Flavonoids	+	+	+	+	+
Phenols	+	-	+	+	+
Saponins	+	-	+	-	+
Steroids	+	+	+	+	+
Tannins	+	-	+	+	-
Anthracyanins	-	-	-	-	-
Glycosides	+	-	+	-	+

Key: CMECNR =Crude Methanol Extract of *Catunaregam nilotica* root bark; n-HF= n Hexane Fraction; EAF= Ethyl acetate Fraction; n-BF= n Butanol Fraction; AF=Aqueous Fraction, + = Present, - = Not detected

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3.2 In vitro antivenom Effects

Table 2 presents the in vitro detoxifying effects of different fractions of *Catunaregam nilotica* root extracts on the PLA_2 enzyme activity of *Echis ocellatus* venom. The n-hexane fraction exhibited the most significant inhibitory effect among the extracts, with a 75.04% reduction in PLA2 activity. The ethyl acetate fraction also showed a notable reduction inhibiting the enzyme by 56.49%, indicating moderate detoxifying potential. The n-butanol fraction showed 52.06% inhibition of PLA2 activity while the aqueous fraction had the lowest effect, with only 29.56% inhibition. This makes it the least effective of the tested extracts. In comparison, ASV was highly effective, nearly completely neutralizing the enzyme's activity with 97.92% inhibition.

Table 2: In vitro Detoxifying Effects of *Catunaregam nilotica* (STAPF) Root Extracts against *Echis ocellatus* Phospholipase A2 (PLA2) Enzyme

Treatment	Venom phospholipaseA2 Activity (μmol/l)	% (Inhibition)
Venom control	134.99 ± 0.01^{f}	0
Venom +ASV	2.81 ± 0.21^{a}	97.92
Ethyl acetate fraction	58.74 ± 0.44^{c}	56.49
n-Hexane fraction	33.69 ± 2.52^{b}	75.04
n-Butanol fraction	64.73 ± 0.09^{d}	52.06
Aqueous fraction	95.06 ± 0.04^{e}	29.56

PLA2 activity are presented as Mean ± SEM (n = 4). Mean activity carrying different superscripts are significantly (P=.05) different

3.3Discussion

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The potential of phytochemical compounds in the development of new drugs against deadly toxins is a significant area of research. Phytochemicals are known to counteract snake venom toxins through multiple mechanisms, including enzyme inhibition, deactivation of venom proteins, adjuvant effects, and chelation activities (Saravia-Otten *et al.,* 2022; Salihu *et al.,* 2024; Sani *et al.,* 2020). The phytochemical screening of the crude extract and its fractions revealed a promising array of bioactive compounds, including alkaloids, tannins, saponins, flavonoids, steroids, and terpenoids, each known for their antioxidant properties (Shivashankar *et al.,* 2019). These findings align with research byAbdlmageed et al. (2020) andHassan et al. (2023), highlighting the antivenom potential of *Catunaregamnolitca*.

Phytochemical compounds neutralize snake venom toxicity by inhibiting critical enzymes such as phospholipase A2 (PLA2), proteases, and hyaluronidase. For instance, alkaloids modulate neurotoxins through interactions with voltage-gated ion channels, potentially reducing pain and muscle spasms (Bhambhani *et al.,* 2021). Tannins and polyphenolic compounds, such as epigallocatechin gallate, exhibit strong inhibitory effects on PLA2 activity, binding venom proteins and mitigating enzymatic toxicity (Haifeng *et al.,* 2008; Pereañez*et al.,* 2011).

PLA_2 enzymes in *Echis ocellatus* venom are particularly harmful, causing blood vessel damage, haemorrhage, hypovolemic shock, and organ failure (Ajisebiola*et al.,* 2024). By degrading membrane phospholipids, these enzymes trigger inflammation, oxidative stress, and necrosis (Williams *et al.,* 2018; Tasoulis*et al.,* 2022; Ledsgaard *et al.,* 2023). The potent inhibition of PLA2 activity observed in this study suggests that the bioactive compounds in n-HF target these enzymes, potentially preventing severe envenomation symptoms.

The results demonstrated significant differences in inhibitory effects among the fractions. The n-HF fraction exhibited the highest inhibitory activity, followed by EAF and n-BF, while AF showed the least efficacy when compared to antiserum (ASV). This hierarchy highlights n-HF as the most promising fraction, containing potent bioactive compounds capable of neutralizing PLA_2 and mitigating inflammatory and hemorrhagic effects (Elda *et al.,* 2008).

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These findings are supported by previous studies showing that plant-based compounds such as rutin, quercetin, and naringenin effectively inhibit venom enzymes, improve survival, and restore hemostatic balance in envenomation cases (Sachetto*et al.,* 2022; Srimathi and Gurunathan, 2021). Similarly, saponins and steroids from *Andrographis paniculata* have shown robust anti-snake venom activity by reducing oxidative stress and inhibiting enzymatic functions (Khan, 2012; Adrião *et al.,* 2022). Tannins are particularly notable for their antioxidant, anti-inflammatory, and wound-healing properties (Sia *et al.,* 2011). Studies have demonstrated their ability to inhibit venom enzymes, neutralize free radicals, and promote tissue repair (Kuppusamy and Das, 1993; Lindahl and Tagesson, 1997). Alkaloids, on the other hand, provide neuroprotective effects by modulating ion channel activity, reducing the impact of neurotoxins, and alleviating symptoms such as pain and muscle spasms (Kaur and Arora, 2015).

3.4 Conclusion

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The potent PLA_2 inhibition observed with n-HF highlights its promise as a candidate for antivenom therapy. Future studies should focus on in vivo validation to confirm the therapeutic efficacy and safety of these phytochemicals in neutralizing snake venom toxicity. Further studies are recommended to isolate the active compound and elucidate its mechanism of action.

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