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Specificity Studies on Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum

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Authors' contributions

This work was carried out in collaboration between all authors. Author FAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MB and POY managed the analyses of the study. Author AJN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin following envenomation which is limited by problems of hypersensitivity reactions in sensitive individuals and its inability to resolve the local effects of the venom. In this study, we report the specificity studies of Phospholipase A_2 (PLA_2) Inhibitor isolated from *Echis ocellatus* Serum (PIES) towards snake venom neurotoxic PLA_2 and non-toxic mammalian secretory PLA_2. Membrane stabilizing and protective ability of PIES was recorded by its potential to reduce hemolysis due to venom PLA_2 from 81.20% to 35.30% *in vitro.* Coagulant potentials of PIES were also seen in its ability to restore plasma coagulation time to less than a minute. Interestingly, PIES does not affect the enzymatic activity of mammalian secretory PLA_2 but strongly inhibits PLA_2 activity of *Echis ocellatus* (carpet viper) in this study. The present study shows that PIES holds a good promise for the development of novel antivenin drug in future.

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1. INTRODUCTION

The number of known venomous species of snakes does not exceed 650 [1]. About 100 different species have been reported in Africa. In Nigeria, 57 species have been documented of which only 10 are known to be fatally venomous [2,3]. In the recent past, the incidence of snakebite worldwide has been reported to be in excess of 300,000 per year with more than 150,000 deaths [4] mainly due to *Echis ocellatus* (carpet viper). Based on hospital records, the four most deadly Northern Nigerian snakes are *Echis ocellatus*, *Naja nigricollis*, *Bitis arietans* and *Naja katiensis* [5],

The carpet viper, *Echis ocellatus* has been reported to be the most medically important snake and among the commonest cause of envenomation in West Africa, being responsible for approximately 95% of the reported cases in northern Nigeria causing several hundreds of deaths annually [6]. *Echis ocellatus* is commonly found in the Benue- Niger valley axis and the hilly north-eastern part of Nigeria [7].

Snake venom, the most complex of all poisons is a mixture of enzymatic and non enzymatic toxic compounds as well as other nontoxic proteins, non proteins including carbohydrates and metals all stored in the poison gland [8].

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid, mainly arachidonic acid (AA) The enzyme from snake venoms is primarily used for trophic and defence functions in most species but show wide range of pharmacological activities such as neurotoxicity, myotoxicity, cardiotoxicity, but with a greater impact on platelet aggregation and blood coagulation [9].

The treatment for snake bite is as variable as the bite itself. The only available treatment is the use of antivenin against snake bite. Antivenin binds to and neutralises the venom, stopping further damage, but do not reverse the damage already done [10]. Some individuals may react to the antivenin with an immediate hypersensitivity reaction [11]. Other alternative treatment involves the use of folk and traditional medicines.

Venomous snakes are resistant to their own venom and several natural neutralizing proteins have been identified in their plasma [12]. In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined [13]. However, information as regards the nature and specificity of these inhibitors from *E. ocellatus* is still scanty and yet to be fully elucidated. Thus, this study aims at investigating the specificity of PLA_2 inhibitor towards snake venom neurotoxic PLA_2s (β-neurotoxins) and nontoxic mammalian PLA_2s and its potential as therapeutic drug for snakes envenoming.

2. MATERIALS AND METHODS

Reagents were purchased from Sigma Chemical Company, St. Louis, U. S. A. twelve (12) adult *Echis ocellatus* snakes were obtained from Kaltungo, Gombe State, Nigeria and identified at the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria. Blood samples of Bovine, Ovine and Caprine were collected from Zangon Shanu abattoir, Zaria, Kaduna State, Nigeria.

2.1 Collection and Preparation of Venom

Echis ocellatus identified were kept in wooden boxes. They were fed with raw meat fortnightly. Venom was collected by the modified milking method [14] from twelve (12) adult *Echis ocellatus* of both sexes, pooled and lyophilized using a dessicator with activated silica as the dessicant and stored at 4℃ until required. These was referred to as crude venom.

2.2 Collection and Preparation of Serum

The blood was obtained by bleeding the snakes through the caudal vein. After blood clotting at room temperature, the serum was separated by centrifugation at 3000xg for 5mins and kept in the refrigerator until further use [15].

Blood samples were collected each from five (5) Bovine, Ovine and Caprine from Zangon Shanu abattoir in Zaria, Kaduna State, Nigeria. The blood were allowed to stand for 30 minutes at room temperature and thereafter centrifuged at 3000xg for 5 mins to collect the serum. The serum obtained from these animals were regarded as the non toxic secretory PLA_2.

2.3 Partial Purification of Phospholipase A_2 from Crude Venom *Echis ocellatus*

2 ml of 10 mg/ml of crude *E. ocellatus* venom was loaded on sephadex G-75 column equilibrated with 50 mM phosphate buffer (pH 6.5). The column was eluted with the same buffer, maintaining a flow rate of 1 ml/min. 2 ml each of 50 fractions were collected and assayed for PLA_2 [16] and total protein concentration by taking absorbance at 280 nm [17]. The PLA_2 active fractions were used for the study.

2.4 Purification of PLA_2 Inhibitor from *Echis ocellatus* Serum

5 ml of serum collected from the *E. ocellatus* snakes was directly fractionated at 4℃ on a sephadex G-200 column, pre equilibrated with 0.05 M Tris HCl buffer, pH 8.1 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 2 ml/hr. 2 ml of twenty fractions were collected and inhibition studies (assay for residual PLA_2 activity) was carried out and fractions exhibiting inhibitory activity against the partially purified PLA_2 from *Echis ocellatus* venom were pooled together and directly loaded on a DEAE cellulose column pre equilibrated with 0.05 M phosphate buffer, pH 6.8. The column was eluted with a stepwise linear gradient of sodium chloride (0.01- 0.1 M NaCl) at a flow rate of 5 min/ml. 2 ml of fifty Fractions collected were assayed for residual PLA_2 activity and total protein and fractions exhibiting inhibitory activity against *E. ocellatus* PLA_2 were pooled together, dialysed and stored at -4℃ until further use [18].

2.5 *In vitro* Studies of Effect of PIES on *Echis ocellatus* Venom

2.5.1 Red blood cell fragility test

Membrane stabilizing activity of the partially purified PIES was assessed using hypotonic solution-induced rat erythrocyte haemolysis. The tail tips of 3 clean healthy Albino rats were clipped and 200 µL of blood was collected each in heparinised capillary tubes and transferred to 5 ml of phosphate buffered saline pH 7.4 and centrifuged. The washing was repeated until a clear supernatant was seen. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 10 µl of partially purified PLA_2 from *Echis* venom, 4 ml of 10 mM sodium phosphate buffered saline (pH 7.4) and 25 µl of the partially purified PIES (5, 10 and 20%). The control sample consisted of 0.5 ml of RBC mixed with 4 ml hypotonic -buffered saline solution alone. The mixtures were incubated for 2 hours at room temperature and centrifuged at 3000xg for 10 min and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated [19].

% Inhibition of heamolysis = 100 x {OD1-OD2/OD1}

Where:

OD1 = Optical density of hypotonic saline solution alone
OD2 = Optical density of test sample.

2.6 Determination of Anticoagulant Activity

A method with modification was adopted. Citrated bovine plasma was prepared by adding 1% w/v of sodium citrate to 2 ml of blood. The blood was then centrifuged at 3000xg for 5 mins to obtain the plasma. The test sample consisted of 200 µl of plasma, 100 µl of crude *E. ocellatus* venom and 25 µl of 5%, 10% and 20% of PIES each. While the control samples consisted of 200 µl of plasma for the normal and 200 µl of plasma and 100 µl of crude venom for the positive controls respectively. The samples were incubated in a water bath at 37℃ for 60 seconds and a final 100 µl of 25 mM $CaCl_2$ was added, and the coagulation time (Recalcification time) recorded [20].

2.7 Effect of PIES on the Enzymatic Activity of *E. ocellatus* PLA_2 and Non Toxic Mammalian Secretory PLA_2s

2.7.1 Effect of PIES on bovine, ovine and caprine serum

The effect of partially purified PIES on Bovine, Ovine and Caprine serum was determined after measuring activities of the following groups:

Group 1: Bovine, Ovine and Caprine Serum PLA_2 + Substrate
Group 2: Tannic acid + Bovine, Ovine and Caprine Serum PLA_2 + Substrate (standard control)
Group 3: Bovine, Ovine and Caprine Serum PLA_2 + partially purified PIES (10%) + Substrate.
Group 4: Bovine, Ovine and Caprine Serum PLA_2 + partially purified PIES (10%) + *Echis* PLA_2 + Substrate.

2.7.2 Effect of PIES on partially purified *E. ocellatus* PLA_2

Group 1: Partially purified *Echis* PLA_2 + Substrate
Group 2: Tannic acid + partially purified *Echis* PLA_2 + Substrate (standard control)
Group 3: Partially purified *Echis* PLA_2 + partially purified PIES (10%) + Subtrate
Group 4: Partially purified *Echis* PLA_2 + partially purified PIES (30%) + Subtrate
Group 5: Partially purified *Echis* PLA_2 + partially purified PIES (40%) + Subtrate
Group 6: Partially purified *Echis* PLA_2 + partially purified PIES (50%) + Subtrate

3. RESULTS

3.1 *In vitro* Studies of Effect of Partially Purified PIES on Partially Purified *Echis ocellatus* Venom

The *in vitro* hemolytic effect of partially purified *Echis* PLA_2 significantly ($p<0.001$) reduced from 81.2±0.010 in blood samples without PIES, to 64.3±0.019% and 35.3±0.017% in blood samples treated with 10% and 20% PIES respectively. However, the percentage observed in untreated blood samples (81.2±0.010) was not significantly different from samples treated with 5% PIES (83.6±0.024), showing a dose dependent effect (Table 1).

The anticoagulant activity of *E. ocellatus* venom in the presence and absence of partially purified PIES was given as the average recalcification time (in minutes) of citrated bovine plasma; and from the results (Table 2), it decreased significantly from 4.32 minutes in plasma without the inhibitor to 0.54 minutes on addition of 20% PIES. The recalcification time was found to be dose-dependent as it decreased with increasing concentration of PIES at 5% (3.56 minutes), and 10% (1.23 minutes).

3.2 Effect of Partially Purified Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES) on Non-toxic Secretory Phospholipase A_2

Table 3 shows the effect of partially purified PIES on non-toxic secretory PLA_2 from Bovine, Ovine and Caprine. Tannic acid completely inhibited non-toxic PLA_2 (0.200±0.000 µmol/min) and was significantly lower ($p< 0.05$) than values obtained in Bovine, Ovine and Caprine samples.

Table 1. Inhibition of hemolysis due to phospholipase A_2 from *Echis* venom by partially purified phospholipase A_2 inhibitor from *Echis ocellatus* serum (PIES)

Parameter	Haemolysis (%)
Hypotonic solution + blood only	100
Venom PLA_2 + blood only	81.2±0.010[a]
Venom PLA_2 + 5%PIES + blood	83.6±0.024[a]
Venom PLA_2 + 10%PIES + blood	64.3±0.019[b]
Venom PLA_2 + 20%PIES + blood	35.3±0.017[c]

Mean ±SD for three determinations; values with different superscripts are significantly different at $p<0.001$ one way ANOVA

Table 2. Effects of partially purified PIES on anticoagulant activity of *E. ocellatus* venom

Sample	Recalcification time (min)
Plasma only	4.23
Crude venom	***
Venom + plasma + 5% PIES	3.56
Venom + plasma + 10% PIES	1.23
Venom + plasma + 20% PIES	0.54

*Average recalcification time for three determinations; ***No clotting after 30 mins*

In Bovine, samples without treatment (6.880±0.759 µmol/min) were not significantly different ($p< 0.05$) when compared with those treated with 10% PIES (5.720±0.668 µmol/min). However, samples treated with 10% and partially purified PLA_2 from *E. ocellatus* (4.965±0.487 µmol/min) had values lower than the treated and untreated with partially purified PIES.

In Ovine, there was no significant difference ($p=0.105$) between samples without treatment (7.260±0.263 µmol/min), samples treated with partially purified 10% PIES (6.480±0.580 µmol/min) and samples treated with 10% PIES and partially purified PLA_2 from *E. ocellatus.* (6.800±0370 µmol/min).

In Caprine, samples without treatment (6.580±0.944 µmol/min) were not significantly different ($p< 0.05$) when compared to those treated with 10% partially purified PIES (6.425±0.286 µmol/min), whereas, samples treated with partially purified 10% PIES and partially purified *Echis* PLA_2 (5.342±0.628 µmol/min) were lower than values obtained for the untreated samples and samples treated with 10% PIES.

3.3 Effect of Partially Purified PIES on *E. ocellatus* PLA_2

The partially purified PIES significantly ($p< 0.001$) reduced the activity of partially purified PLA_2 from *E. ocellatus* venom. Treatment of *E. ocellatus* PLA_2 with tannic acid (0.200±0.000 µmol/min) significantly ($p< 0.001$) reduced the *Echis* PLA_2 activity when compared with untreated samples in group 1 (5.531±0.11 µmol/min) and samples treated with 10% (3.281±0.28 µmol/min), 30% (2.277±0.87 µmol/min), 40% (2.114±0.71 µmol/min) and 50% (1.587±0.09 µmol/min) PIES. Also the PLA_2 activity of samples treated with 40% and 50% PIES were significantly ($p< 0.001$) lower than the untreated samples in group 1 (5.531±0.1 µmol/min), but were not significantly ($p< 0.001$) different from samples treated with 10% (Table 4).

4. DISCUSSION

The partially purified *E. ocellatus* PLA_2 catalysed the extensive haemolysis of RBCs (Table 1). About 80% haemolysis was achieved after 2 hours post incubation. However, in the presence of 20% (v/v) PIES, there was a significant decrease ($p<0.001$) in the haemolysis of RBCs to about 35.3±0.017%. It can be construed that the haemolytic activity may be linked to hydrolysis of membrane lecithin since the hydrolysis was followed as a function of released free fatty acids [21]. More so, the hydrolysis of lecithin generates free fatty acid and lysolecithin, while the former leads to acidosis, the later is a powerful hemolyzing agent with detergent-like effects [22]. In a related finding, *Andrographis paniculata* and *Aristolochia indica* extracts were capable of inhibiting PLA_2 dependent hemolysis of Ovine RBCs induced by *Echis* venom in a dose dependent manner [23]. PLA_2 causes a complete degradation of glycerol phospholipids in the cell membrane as they attack lecithin-converting 2/3 of the phospholipids into lysoderivative [24]. The destruction of cell membrane integrity may in turn lead to phospholipase activation and release of arachidonate followed by the biosynthesis and release of eicosanoids to surrounding tissue, which, by themselves cause considerable membrane damage [25]. In this context, an inhibition of PLA_2 enzyme plays a significant role in preventing membrane damage [26]. The suppression of haemolysis by PIES suggests that PIES- PLA_2 interaction may protect the cell from haemolytic anaemia.

The anticoagulant action of venoms due to *Viperidae, Crotalidae* and *Elapidae* bites is mainly due metalo and serino proteinases but PLA_2 enzymes have a great importance too, though, there are quite a number of non PLA_2 anticoagulant from the same source [27,28]. It has also been reported that crude venom from *Bothrops jararaca* snakes has anticoagulant and PLA_2 activities [29]. Table 2 shows that *E. ocellatus* venom is capable of preventing coagulation of citrated bovine plasma. Treatment with partially purified PIES reduced the recalcification time from over 30 minutes to less than 60 seconds. These shows that PIES is also likely to be a proteinase inhibitor because our results conforms with the findings who reported that both *Indigofera pulchra* nad *Aristolochia albida* were found to neutralise the anticoagulant,

Table 3. Effect of partially purified PIES on non toxic secretory PLA_2 activity

Group	Bovine	Ovine	Caprine
1	6.880±0.759[a]	7.260±0.263[a]	6.580±0.944[a]
2	0.200±0.000[b]	0.200±0.000[b]	0.200±0.000[b]
3	5.720±0.668[a]	6.800±0.370[a]	6.425±0.286[a]
4	4.965±0.487[ab]	6.480±0.580[a]	5.342±0.628[ab]

F=0.239; p=0.791; F=2.741; p=0.105; F=2.132; p=0.365

Statistically significant difference exists at p<0.05. Mean values were separated by Duncan's Multiple Range test

All values are expressed as phospholipase A_2 activity in µmol/min

Group 1: L-α-lecithin + Bovine, Ovine and Caprine serum

Group 2: L-α-lecithin + tannic acid + Bovine, Ovine and Caprine serum

Group 3: 10% PIES + L-α-lecithin + Bovine, Ovine and Caprine serum

Group 4: 10% PIES + Echis PLA_2 + L-α-lecithin + Bovine, Ovine and Caprine serum

haemolytic and PLA_2 activities of crude venom from viperidae snakes [30]. Similarly, it has also been reported that PLA_2 purified from *Daboia russeli siamensis* venom significantly ($p<0.05$) shortened the rate of coagulation activity of human citrated plasma [31] and this may be due to the binding of clotting factor Xa (FXa) to inhibit prothrombinase complex, which is the target protein for anticoagulant PLA_2s as previously reported [32].

Table 4. Effect of partially purified PIES on E. ocellatus PLA_2

Group	*E. ocellatus* PLA_2
1	5.531 ± 0.11^{a}
2	0.200 ± 0.00^{c}
3	3.281 ± 0.28^{ab}
4	2.277 ± 0.87^{ab}
5	2.114 ± 0.71^{b}
6	1.587 ± 0.09^{b}

F=9.476; p< 0.001; a, b, c = statistically significant difference exists at p<0.01. Mean ±SD for three determinations separated by ANOVA*
All values are expressed as phospholipase A_2 activity in µmol/min
Group 1: partially purified Echis PLA_2 + Substrate
Group 2: Tannic acid + partially purified Echis PLA_2 + Substrate (standard control)
Group 3: Partially purified Echis PLA_2 + partially purified PIES (10 %) + Substrate
Group 4: Partially purified Echis PLA_2 + partially purified PIES (30 %) + Substrate
Group 5: Partially purified Echis PLA_2 + partially purified PIES (40 %) + Substrate
Group 6: Partially purified Echis PLA_2 + partially purified PIES (50 %) + Substrate

From our results, treatment of Bovine, Ovine and Caprine serum with partially purified PIES did not affect the PLA_2 in the serum samples tested (Table 3); but significantly ($p<0.001$) decreased PLA_2 activity in partially purified *Echis* PLA_2 (Table 4). Our findings are similar to the reports that a PLA_2 inhibitor isolated from *Crotalus durissus terrificus* snake serum; Crotoxin Inhibitor from *Crotalus* Serum (CICS) inhibits the PLA_2 activity and neutralizes the pharmacological action of crotoxin, the main neurotoxin from *Crotalus durissus terrificus* venom. It also inhibits and neutralizes other PLA_2 β-neurotoxins from *Viperidae* snake venom, but does not act on PLA_2 β-neurotoxins from *Elapidae* venom or the toxic or nontoxic PLA_2s from other sources [33]. In a related study, extract of *Guiera senegalense* and *Sterculia setigera* gave 33.4% and 44.3% protection to mice treated with a minimal lethal dose of *Viperidae* venom but offered no protection to mice treated with minimal lethal dose of *Elapidae* venom [34]. Thus, our findings suggest that the carpet viper contain in its blood a PLA_2 inhibitor that inhibits the snake venom PLA_2 but does not act on the non-toxic secretory PLA_2 from mammals, indicating its specificity.

5. CONCLUSION

From this study, it is concluded that phospholipase A_2 (PLA_2) inhibitor from *Echis ocellatus* serum (PIES) have membrane stabilising potentials by its protective ability against venom induced RBCs hemolysis. Because of its specificity, PIES might be therefore useful in the treatment of envenomations by the *Viperidae* snakes. In this context, it is important to note that PIES has no effect on the mammalian secretory PLA_2s, indicating that it should not interfere with the normal biological function of these enzymes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hile Jennifer. Snake venom may slow cancer growth. National Geographic News; 2004.
   Available:www.nationalgeographicnews.com
2. Abubakar MS. The biological activity, components and interaction studies of venom of *Naja nigricollis* reinhardt. A Ph.D, Ahmadu Bello University, Zaria, Nigeria; 2003.
3. Theakston RDG, Laing GD, Freire-Lascano A, Touzet JM, Vallejo F, Guderian RH, et al. Treatment of snake bites by *bothrops* species and *Lachesis mula* in Ecuador: Laboratory screening of candidates antivenoms. TRSTMH. 2003; 89:550-554.
4. EchiTAb. Annual medical records of antivenom treatment center, EchiTAb anti-snake study group UK/Nigeria, General Hospital, Kaltungo. Gombe State, Nigeria; 2008.
5. Bharati K, Hasson SS, Oliver J, Laing GD, Theakston RDG, Harrison RA. Molecular cloning of phospholipase A_2 from venom gland of *Echis* (carpet viper). Toxicon. 2003;41:941-947.

6. Hasson SS, Theakston RDG, Harrison RA. Cloning of a prothrombin activator-like metalloproteinase from the West African saw-scaled viper, *Echis ocellatus.* Toxicon. 2003;42:629-634.
7. Warrell DA, Arnett C. The importance of bites by the saw-scaled or carpet viper (*Echis carinatus*): Epidemiological studies in Nigeria and a review of the world literature. Acta Tropica. 1976;33(4):307-341.
8. Antony G, Rinku D, Sumana S, Roshnara M, Sangahamitra M, Shamik P, et al. Laboratory of toxicology and experimental pharmaco-thermodynamics. Ind. J. Biol. 2010a;48:865-879.
9. Shashidharamurthy R, Kemparaju K. A neurotoxic phospholipase A2 variant: Isolation and characterization from Eastern Regional Indian cobra (*Naja naja*). Toxicon. 2006;47:727-733.
10. Anthony G, Rinku D, Sumana S, Roshnara M, Murkharji S, Bhattacharya S, et al. Herbs and herbal constituents active against snakebite. IJEB. 2010b;48:865-878.
11. Canon R, Ruha AM, Kashani J. Acute hypersensitivity reaction associated with *Crotalidae* polyvalent immune fab antivenom. Annals Emerg. Med. 2008;51: 407.
12. Shao J, Shen H, Havsteen B. Purification, characterization and binding interactions of the Chinese cobra (*Naja naja atra*) serum antitoxic protein CSAP. J. Biochem. 1993; 293:559-566.
13. Biondo R, Soares AM, Bertoni WB, Franca SC, Periera AM. Direct organogenesis of *Mandevilla illustris* (Vell) woodson and effect of its aqueous extracts on the enzymatic and toxic activities of *Crotalus durissus terrificus* snake venom. Plant Cell Rep. 2005;22:549.
14. Markfarlane RG. Russel's viper venoms. Brit J Haem. 1967;13:437-451.
15. Perales J, Villela C, Domont GB, Choumet V, Saliou B, Moussatche ÂH, et al. Molecular structure and mechanism of action of the crotoxin inhibitor from *Crotalus durissus terrificus* serum. Eur. J. Biochem. 1995;227:19-26.
16. Sallau AB, Ibrahim MA, Salihu A, Patrick FU. Characterization of phospholipase A_2 (PLA_2) from *Echis ocellatus* venom. Afri J. Biochem Res. 2008;2(4):098-101.
17. Bhat MK, Gowda TV. Purification and characterization of myotoxic phospholipase A_2 from Indian cobra (*Naja naja)* venom. Biochem Int. 1989;25:1023-1034.
18. Higuchi DA, Barbosa CMV, Bincoletto C, Chagas JR, Magalhaes A, Richardson M. et al. Purification and partial characterization of two phospholipases A_2 from *Bothrops leucurus* (white tailed-jararaca) snake venom. Biochemie. 2007; 89:319-328.
19. Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf VO. Membrane stabilizing activity–a possible mechanism of action for the anti inflammatory activity of *Cedrus deodara* wood oil. Fitoterapia. 1999;70:251-257.
20. Theakston RDG, Reid HA. Development of simple standard assay procedures for the characterization of snake venoms. WHO Bulletin. 1983;61:949-956.
21. Nok AJ, Balogun E, Lori, JA, Abubakar M.S Inhibition of Naja nigricollis venom acidic Phospholipase A_2 catalysed hydrolysis of ghost red blood cells by columbin. IJMC. 2002;17(1):55-59.
22. Nok, AJ, Esievo KAN, Ibrahim S, Ukoha AI, Ikediobi ICO. Phospholipase A_2 from *Trypanosoma congolense*: characterization and haematological properties. Cell Biochem. Func. 1993;11: 125-130.
23. Meenatchisundaram SP, Parameswari G, Subbraj T, Michael A. Studies on antivenom activity of *Andrographis paniculata* and *Aristolochia indica* plant extracts against *Echis carinatus* venom. Int. J. Toxic. 2009;6:1.
24. Condrea E, Tang CC, Rosenberg P. Comparison of a relatively toxic phospholipase A_2 from *Naja nigricollis* snake venom with that of a relatively non-toxic phospholipase A_2 from *Hemachatus haemachatus* snake venom. Enzymatic activity on free and membrane bound substrates. Biochem Pharm. 1980;29: 1555-1563.
25. Kannagi R, Koizum IK, Masuda T. Limited hydrolysis of platelet membrane phospholipids. On the proposed phospholipase-susceptible domain in platelet membranes. J. Biol. Chem. 1981; 256:1177-1184.
26. Mukherjee AK. Correlation between the phospholipids domains of the target cell membrane and the extent of *Naja kaouthia* PLA_2-induced membrane damage: Evidence of distinct catalytic and cytotoxic

sites in PLA_2 molecules. Biochimica et Biophysica Acta. 2007;1770:187-195.
27. Pereanez JA, Nunez V, Huancahuire-Vega S. Biochemical and biological characterization of a PLA_2 from crotoxin complex of *Crotalus durissus cumanensis*. Toxicon. 2009;53:534-542.
28. Garcia D, Denegri ME, Acosta OC, Huancahuire-Vega S. Isolation and functional characterization of a new acidic PLA_2 Ba SpII RP4 of the *Bothrops alternatus* snake venom from Argentina. Toxicon. 2010;56:64-74.
29. Zingali RB, Carlinis CR, Francischett IM, Gunmeranes JA. *Bothrops Jararaca* snake venom: Effects on platelet aggregation. J. Thromb. Res. 1990;58(3):303-316.
30. Abubakar MS, Balogun E, Abdurahman EM, Nok AJ, Shok M, Mohammed A, et al. Ethnomedical treatment of poisonous snakebites: Plant extract neutralized *Naja nigricollis. Venom*. 2006;44(5):343-348.
31. Khunsap S, Pakmanee N, Khow O, Chanhome L, Sitprija V, Suntravat M, et al. Purification of a phospholipase A_2 from *Daboia russelii siamensis* venom with anticancer effects. Venom. 2011;(2):42-45.
32. Kerns RT, Kini RM, Stefansson S. Targeting of venom phospholipases: The strongly anticoagulant phospholipase A_2 from *Naja nigricollis* venom binds to coagulation factor Xa to inhibit the prothrombinase complex. Arch. Biochem. Biophys. 1999;369:107-113.
33. Grazyna F, Villela C, Perales J, Bon C, Interaction of the neurotoxic and nontoxic secretory phospholipases A_2 with the crotoxin inhibitor from *Crotalus* serum. Eur. J. Biochem. 2000;267:4799-4808.
34. Abubakar MS, Sule MI, Pateh UU, Abdurahman EM, Haruna AK, Jahun BM. *In vitro* snake venom detoxifying action of the leaf extract of *Guiera senegalensis*. Ethnopharm. J. 2000;69(3):253-257.

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