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A Novel Phospholipase A_2 from *Najanigricollis* venom: Purification, Characterization and Inhibitory effect of Ursolic acid on the Isolated Phospholipase A_2

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Abstract
The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin which is limited by problems of hypersensitivity reaction in some individuals and its inability to resolve the local effects of the venom.The studyaimed to isolate, characterize Najanigricollis venom phospholipase A_2 and investigate the inhibitory effect of Isolated Ursolic acid from Psidiumguajava leafon its activity. Phospholipase A_2 (EC. 3.1.1.4) was isolated and partially purified from the venom of Najanigricollis. A two-step purification process on DEAE-Cellulose and Sephadex G-75 column chromatography gave a specific activity of 12.50µmol/min/cm^3/mg protein, 156.25 purification fold and 50% recovery. Initial velocity studies revealed a K_M and V_{max} of 1.40 x 10^{-1}mg/cm^3 and 7.70x10^{-1}µmol/min respectively. SDS-Page of the partially purified PLA_2 shows molecular weight of 45.55KDa.Studies on the inhibition kinetics revealed that the compound exhibit a mixed type of inhibition, the Ki and Kii values are 0.43 and 0.06mg/cm^3 for Ursolic acid. The relevance of these findings would be of importance in the area of drug development that could serve as an alternative to the conventional therapy.

Keywords: Inhibition, Phospholipase A_2, *Psidiumguajava*, Snake venom,Ursolic acid.

INTODUCTION

Snake venoms are complex mixture of compounds with a wide range of biological and pharmacological activities, which more than 90% of their dry weight is composed of proteins, comprising a variety of enzymes, such as proteases (Metalo and Serine), phospholipases A_2, L-amino acid oxidases, esterases and others(Kang *et al*., 2011).

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Snake venom is one of the most abundant sources of secretory PLA_2 ($sPLA_2$), which are one of the potent molecules in snake venoms (Maung- Maung *et al.,* 1995; Chakrabarty *et al.,* 2000).
Phospholipase A_2 (PLA_2) are enzymes found to catalyze the hydrolysis of fatty acyl ester bonds in the 2-position of 3- sn-phospholipid to release fatty acid and lysophospholipid; the fatty acid so formed may act as either a second messenger or a precursor of eicosanoids (Fuly*et al.,* 2002; Huang and Mackessy, 2004; Sallau*et al.,* 2008).

The enzyme from snake venoms is primarily used for tropic and defense 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 (Bharati *et al.,* 2003; Hasson *et al.*, 2003; Shashidharamurthy and Kemparaju, 2006; Higuchi *et al.,* 2007). The enzyme is therefore a highly interesting molecule to venom researchers because in addition to digesting the prey, it mimics the pathological action of the whole venom poisoning (Shashidharamurthy and Kemparaju, 2006).Ursolic acid (UA)(3β-hydroxyurs-12-en-28-oic) is a pentacyclictriterpene, occuring in many plants parts including fruits and seed. It was considered to be pharmacologically inactive for a long time. However, upon closer examination, UA was found to be medicinally active both topically and internally (Ibrahim and Francis, 2013). This ubiquitous pentacyclictriterpene has been studied for its anti-inflamatory, hepatoprotective, analgesic and antimicrobial activity (Ibrahim and Francis, 2013).

Therefore its isolation and characterization will not be over emphasized. This study is aimed to isolate, characterize and investigate the inhibitory potentials of Ursolic acid against phospholipase A_2 from *Najanigricollis* venom.

MATERIALS AND METHODS

Six (6) adult *Najanigricollis* snakes were obtained from Kaltungo, Gombe State, Nigeria.

Milking of Venom

The Venom was collected by milking method as described by Markfarlane (1967). Briefly silica gel was activated at 80°C for 2 hours in an oven placed indesiccators and kept in a refrigerator. The snakes were restrained at the position of the joint between the last cervical vertebrae and the skull, using the thumb and the fore-finger, care was taken to avoid strangulation. A cellophane piece was used to cover a beaker and held in position using rubber band. The second hand was used to hold the beaker and brought to make contact with the mouth of the snake, such that as the snake bites through the cellophane using its upper fangs, venom was ejected into the beaker. The venom samples from all the *N. nigricollis* snakes were pooled and immediately placed indesiccators with activated silica and allowed to crystallize at 4°C.The crystallized venom was then transferred into eppendorf tubes, labelled properly and kept in a deep freezer at – 18°C. These were referred to as crude venom.

Assay of Phospholipase A_2 Activity:

The activity of phospholipase A_2 was determined as described by Sallau*et al.* (2008) using egg yolk as a substrate: Exactly 25□l of 1mg/cm^3 of the substrate was incubated with10□l of the enzyme for 10 minute at 37°C. The reaction was terminated by immersing the tube in a boiling water bath for 2min and the amount of released free fatty acid was measured titrametrically at pH 8.0. The activity of PLA_2 was defined as amount of enzyme that hydrolyses 1 □mole of fatty acids from the substrate per minute under standard conditions.

Determination of protein concentration:
The total protein concentration was determined by the Bradford (1976) method using bovine serum albumin (BSA) as standard.

Ion Exchange Chromatography:
The technique ion exchange chromatography was carried out as described by Sallau *et al.*, (2008); Briefly 2cm^3 of the crude enzyme (10mg/cm^3) was loaded onto DEAE cellulose column (1.5x 30 cm) pre-equilibrated with 50mM phosphate buffer pH 6.8, the column was eluted step wise with NaC1 gradient (0.01-0.1M) at a flow rate of l cm^3/min, and thirty fractions were collected (3cm^3 each). Both the enzyme activity and the total protein concentration were determined.

Gel filtration:
The active fractions from ion exchange chromatography step were pooled together and loaded onto Sephadex G-75 column pre equilibrated with 50mM phosphate buffer pH 6.8, the column was eluted with the same buffer, the flow rate was maintained at 1 cm^3/min. Thirty fractions were collected (5cm^3 each), the activity of the enzyme and the total protein concentration was determined.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)
Exactly 0.1cm^3 of the partially purified enzyme sample, the crude enzyme and molecular weight marker were treated with 0.5cm^3 of treatment buffer (1%SDS,1% mercaptoethanol and 0.1M phosphate buffer at pH 6.8) and incubated at 100°C for 5minutes. Bromophenol blue (15µl) was added to each sample to serve as the tracking dye together with 10µl of glycerol.

The prepared samples were then loaded onto the gel (12% running gel and 6% stacking gel prepared in slap) and the electrophoresis was performed concurrently at a current of 5mA for about four hours. Trisglycine buffer pH 6.8 was used as the running buffer. After the electrophoresis, the gel was removed and stained over night with coomasie brilliant blue. It was then rinsed and also distained many times in 7% acetic acid.

Molecular Weight Determination
At the end of electrophoresis, the relative mobilities of the proteins were calculated as the ratio of distance moved by protein to the distance moved by the tracker dye. The plot of the log of the MW Vs. RF was made from where the molecular of the partially purified enzyme was estimated.

Effect of pH and on partially purified PLA_2
The activity of partially purified PLA_2 was determined as a function of pH using the following buffers: 50mM acetate buffer for the pH range of 5.0-6.0, phosphate buffer for the pH range of 6.5-7.5 and Tris-HC1 buffer for the pH range of 8.0-9.0 .The plot of PLA_2 activity against pH was prepared to determine the optimum pH.

Effect of temperature on partially purified PLA_2
The activity of partially purified PLA_2 was determined as a function of temperature. The reaction mixture were incubated progressively at the intervals of 5°C starting from 25°C to 70°C maximum. The activity of the enzyme at different temperatures were plotted to determine the optimum temperature.

Initial Velocity Studies

The partially purified PLA_2 was assayed at various concentrations of the substrate (egg yolk) from 0.2-1.0mg/cm^3 at regular interval of 0.2mg/cm^3. A plot of reciprocal of the enzyme activity against reciprocal of substrate concentrations used was plotted (Lineweaver-Burk plot) and K_M and V_{Max} were determined from the plot.

Effect of Some Divalent Cations:

The enzyme activity was determined in the presence of divalent cations at final concentration of 10mM: Ca^{2+}, Mg^{2+}, Co^{2+} and Cu^{2+}.

Inhibition Studies:

The same procedure for PLA_2 assay as described above was used in the presence of Isolated Ursolic acid from *Psidiumguajava* leaf extract (UAPGL) pre-incubated with partially purified PLA_2,and tannic acid was used as a standard inhibitor. In another set of experiment, different concentrations of the substrate were incubated with different concentration of the isolated ursolic acid 0.1, 0.5 and 1mg/cm^3 to plot Lineweaver-Burk plot.

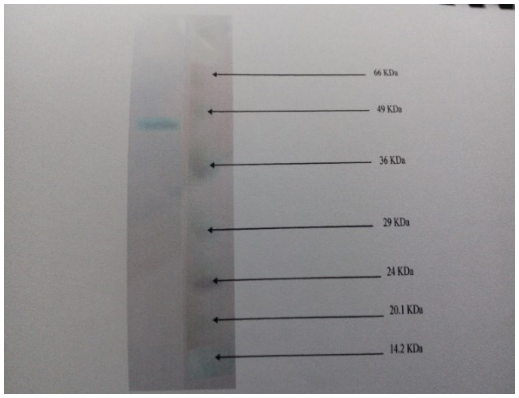
RESULTS AND DISCUSSION

Najanigricollis venom phopholipase A_2 was partially purified by a factor of 156.25 and a recovery of 50% (Table 1) by a combination of ion exchange chromatography using DEAE-cellulose and gel filtration using Sephadex G-75. It was indicated by a single peak on SDS-PAGE (Plate 1).

Table 1: Purification Table for *N. nigricollis*Phospholipase A_2

Faction	Total Volume (cm^3)	Protein (mg/cm^3)	Enzyme Activity (□mol/min/cm^3)	Specific Activity (□mol/min/cm^3/mg protein)	Yield (%)	Purification Fold
Crude Enzyme	5	1.303	0.10	0.08	100	1.00
DEAE-Cellulose Fraction	3	0.057	0.06	1.68	60	21.00
Sephadex G-75 Fraction	5	0.004	0.05	12.50	50	156.25

The increase in specific activity of partially purified PLA_2 after two purification steps could be due to the removal of some other interacting compounds present in the venom, since it is a complex mixture of protein and non-protein substances. The very high percentage yield obtained was higher than those obtained from ostrich pancreatic PLA_2 and dromedary PLA_2 (Bacha*et al.,* 2007), which could mean that the purification steps used here were more appropriate for this enzyme.



A B

Plate 1: SDS-PAGE of partially purified *N. nigricollis* PLA_2 and Protein Markers
Lane A: Partially Purified PLA_2 Obtained fromSaphadex G-75 Column, Lane B: Protein Markers,

New advances in materials and or choice of more appropriate materials have contributed with proteins purification processes, which gives enzyme with high degree of purity and quantity. Other reports show the materials used (Stationary phase) in their respective purifications are different from the ones used in this study. Quintero and Soares (2010) reported 14 KDa Phospholipase A_2 in which CM-Sepharose and Phenyl-Sepharose were used in the two step purification. Higher yield and relative purity of this enzyme obtained could be attributed to the nature of the material used (Stationary phase) and the higher number of different fractions collected. In another work by Numes*et al.* (2011) described the isolation of an acid phospholipase named BL-PLA_2, obtained from *Bothropsleuculus* through two sequential chromatographic steps using CM-sepharose and Phenyl-Sepharose CL-4B column which gave 15KDa and good percentage yield.

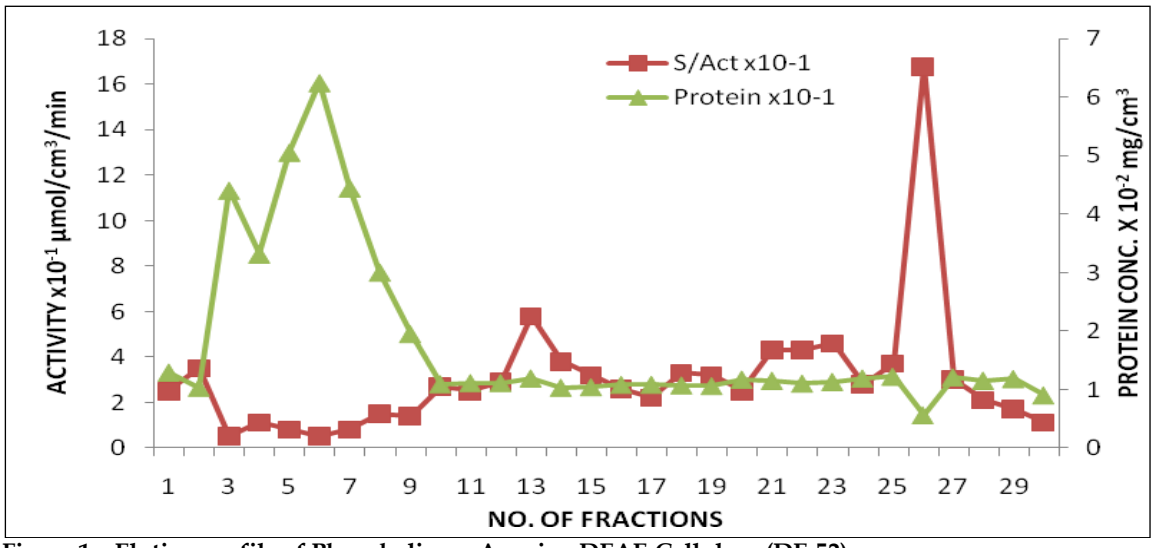


Figure 1a: Elution profile of Phospholipase A_2 using DEAE-Cellulose (DE-52)

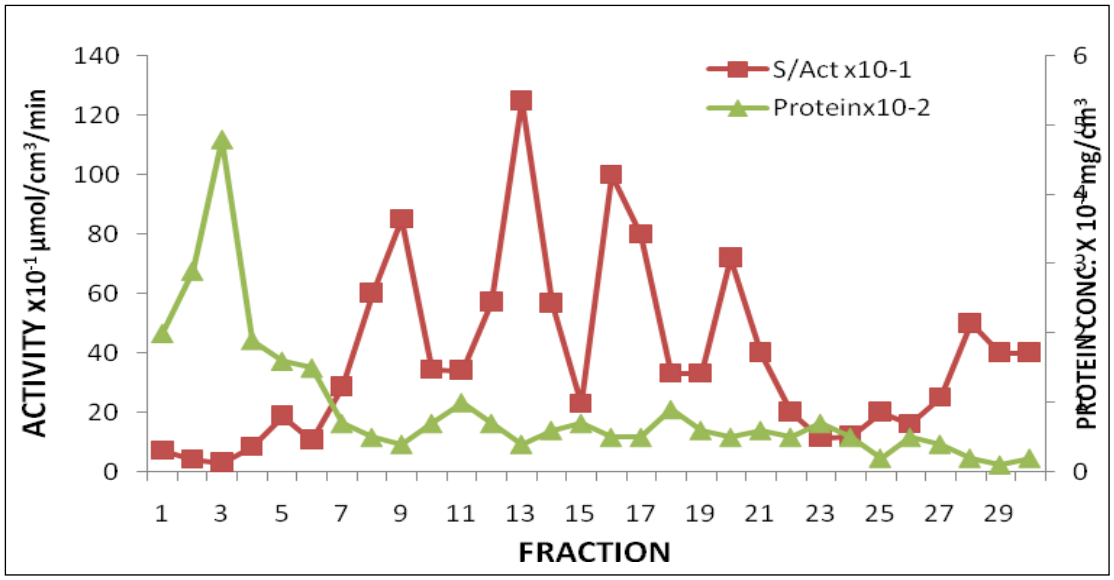


Figure 1B: Elution profile of Phospholipase A_2 using Sephadex G-75

The *Najanigricollis* venom PLA_2 optimal activity at pH 7.5 was found to be similar to *L. muta* and *N. naja* venoms PLA_2 as reported by Fuly*et al.* (2002); Shashidaramurthy and Kemparaju, (2006), *E. Ocellatus* by Sallau*et al.* (2008) and could be attributed to the pathological role of the enzyme in envenomations as basic pH contributes to the enhancement of the enzyme action,due to the general base catalytic mechanism. General base mediated attack on productively bound substrates has been described by Scott and Sigler (1994) to be the first out of the three essential steps in catalysis of PLA_2 enzyme, as deduced from crystallographic studies on the enzyme. The PLA_2 optimum temperature agrees with other findings by Shashidarammurthy and Kemparaju (2006); andBacha*et al.*(2007). The increases in body temperature of snake bitten victims above the physiological temperature could also make the condition favorable for PLA_2 to exert its hydrolytic function effectively (Sallau*et al.*, 2008).

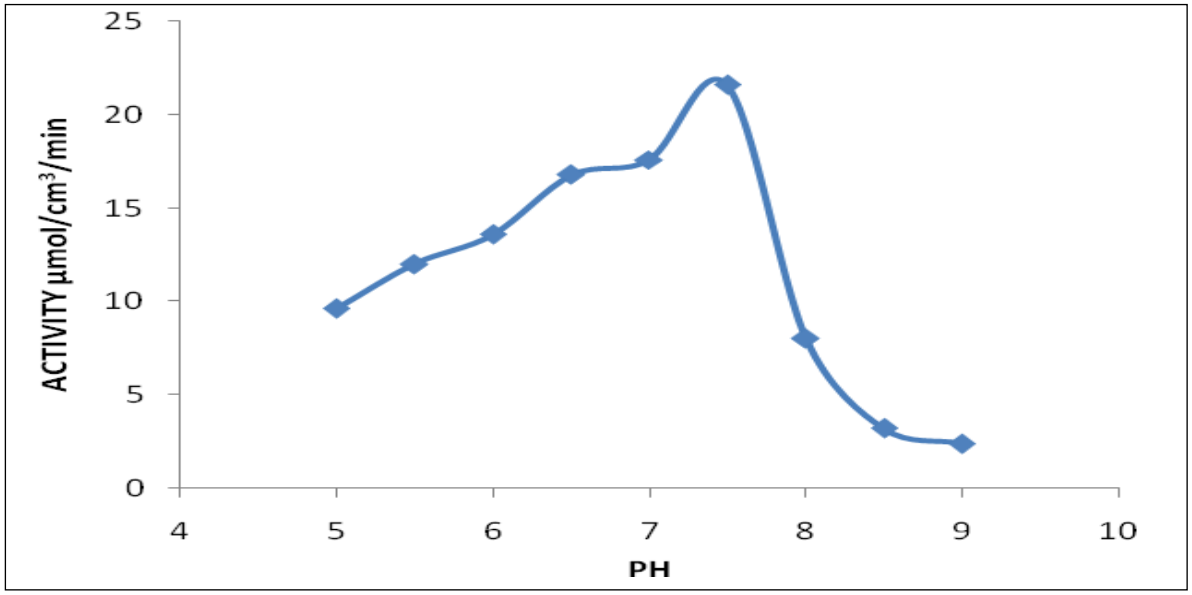


Figure1c: Effect of pH on *N. nigricollis* PLA_2 Activity

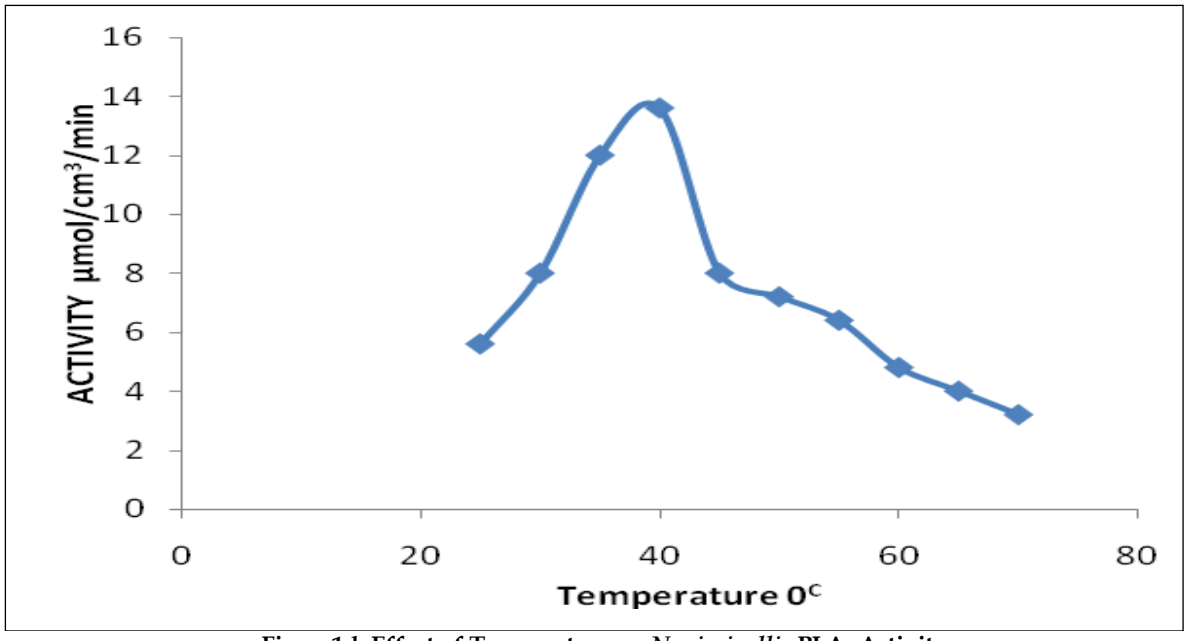


Figure1d: Effect of Temperature on *N. nigricollis* PLA_2 Activity

The K_m and V_{max} of this enzyme (*Najanigricolis* PLA_2) were found to be 1.4x 10^{-1}mg/cm^3 and 7.70 x10^{-1} µmol/min/cm^3 respectively. Both the values obtained are relatively similar to that obtained from the venom of *Echisocellatus* (Sallau*et al*., 2008). The fairly low K_m value is an indication of moderately high affinity of the enzyme for phospholipids, which further substantiate the observed toxicities, in snakes as a result of PLA_2. Furthermore, the V_{max} obtained implies that at the end of 1minute post *N. nigricollis* envenomation at least 7.70 x 10^{-1} µmoles of free fatty acids would have been excised from the victim's red blood cells leading to haemolysis of red blood cells observed after envenomation (Rosenberg, 1979). It could also lead to liberation of free fatty acids from other membrane phospholipids making the medium highly acidic, with the lysophospholipids generated exhibiting a detergent-like action that leads to degeneration of muscle fibres (myotoxicity) and/or inhibiting the release of neurotransmitter (neuroxicity) (Carasch*et al,* 1985).

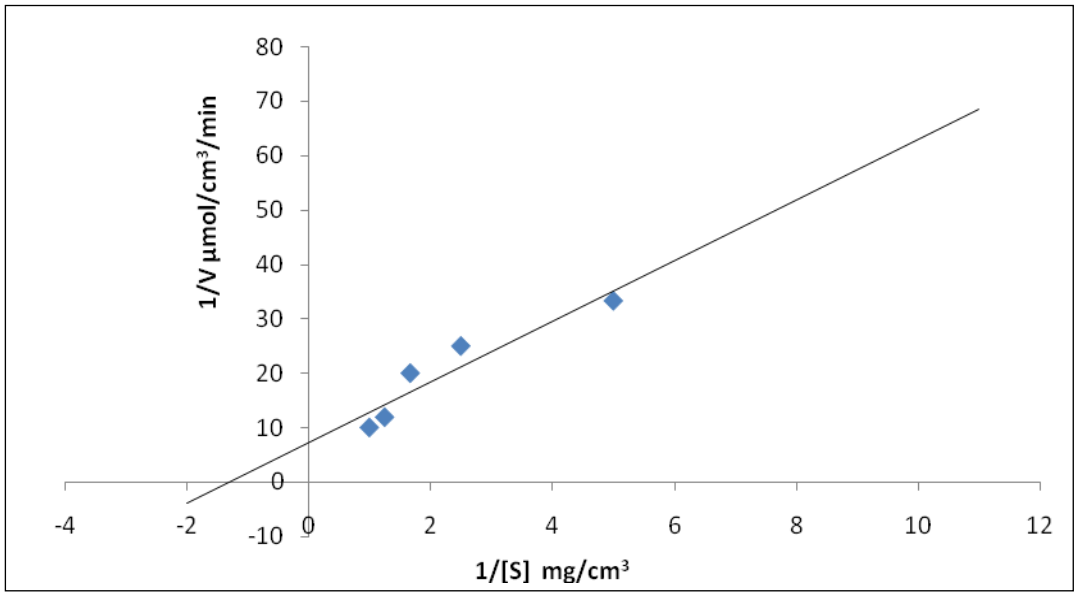


Figure 1e: Lineweaver-Burk plot of *N. nigricollis* PLA_2 activity against egg yolk as substrate.

The molecular weight of partially purified *N. nigricollis* PLA_2 was estimated to be 45.55KDa using protein markers as standard (Plate 4.1). Most PLA_2 from *Naja* species exists in multimeric forms and this molecular association is known to be essential for activity (Rosenberg, 1979); for example most neurotoxins isolated from *Naja* species are multimeric (Rosenberg, 1979; Condrea *et al*., 1980; Dufton and Hider, 1983;). The result shows that the partially purified *N. nigricollis* PLA_2 obtained is a high molecular weight enzyme compared to the low molecular weight PLA_2as reported by Salvador *et al*. (2011) (14KDa), *Daboiarusseliisiamensis* PLA_2 (13.67KDa) was also reported by Khunsap *et al*. (2011), and others from different snake species. This could be attributed to the different geographical areas where the snakes are found and species difference. However Abubakar *et al*. (2003) reported the presence of two high molecular weight PLA_2 (NNL and NN2) for the first time from *N. nigricollis* venom with 65KDa and 71KDa respectively. Therefore this should be the second time to obtain a high molecular weight PLA_2 from *N. nigricollis* venom.

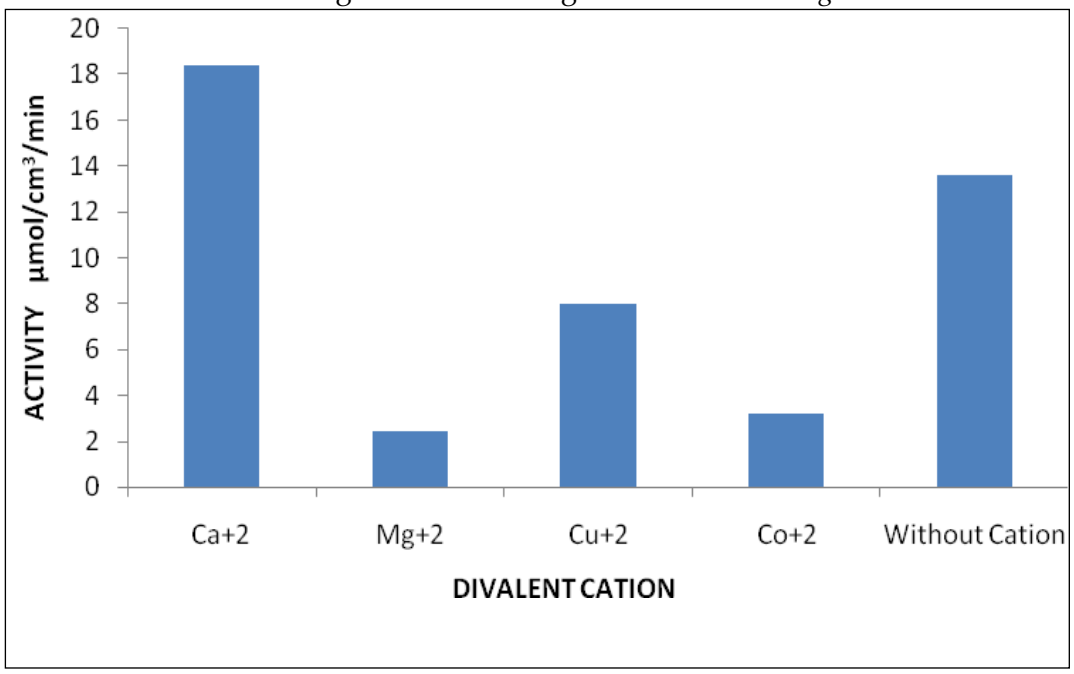


Figure 2a: Effect of divalent cations (10mM) on the activity of *N. nigricollis* PLA_2

The activity of *N. nigricollis* PLA_2 was determined in the presence of some divalent cations at concentration of 10mM. The increase in the activity of *N. nigricollis* PLA_2 in the presence of Ca^{2+} shows that this enzyme is Ca^{2+} dependent, and is similar to that of *Echisocellatus* PLA_2 reported by Sallau *et al*. (2008). Effects of isolated Ursolic acid on the activity of *N. nigricollis*PLA_2 was investigated. It was observed that the activity of the enzyme reduced in the presence of the isolated ursolic acid suggesting that this compound can be considered in antivenom design for *N. nigricollis* venom.

Studies of the inhibition kinetics of isolated Ursolic Acid revealed a mixed type of inhibition. The Ki and Kii values were 0.43 and 0.06 mg/cm^3 respectively. The findings are similar to the report of Borges *et al*. (2005) that said triterpenes like Ursolic acid are found to be present in varying proportions in plants and they have been previously reported for anti-snake venom activity.

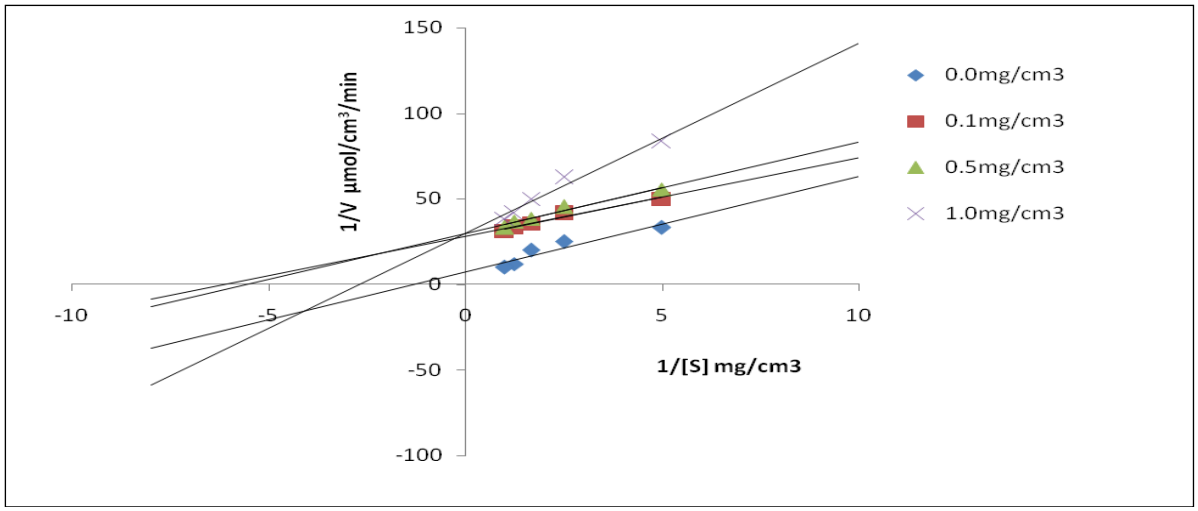


Figure 2b:Lineweaver-Burk plot of *N. nigricollis* PLA_2 activity against egg yolk as substrate and different concentration of isolated compound B3 (from *P.guajava*)

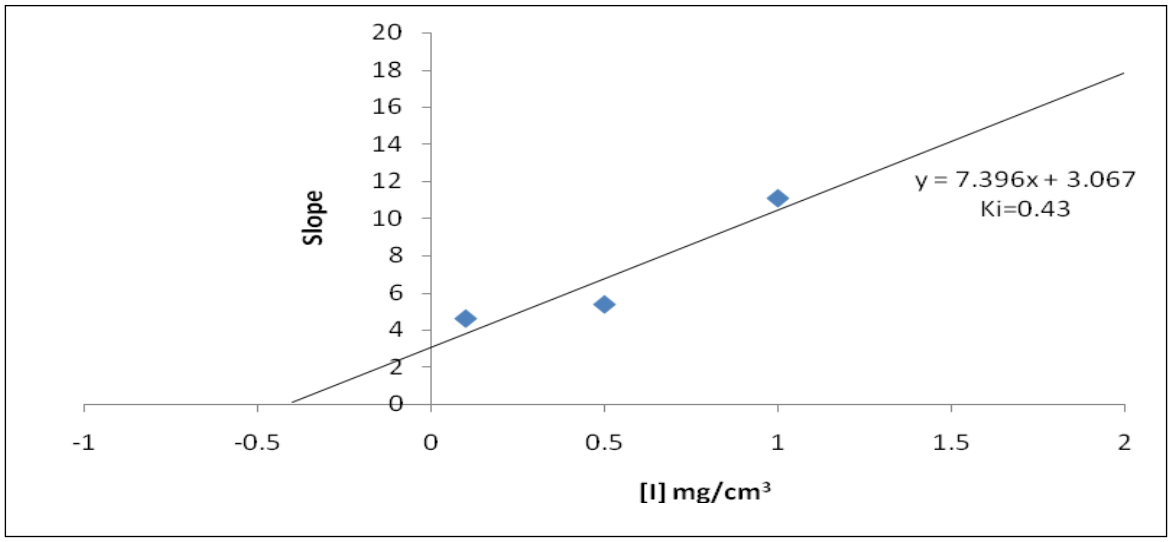


Figure 2c: Secondary Plot of slope against Inhibitor (B3) concentration showing Ki

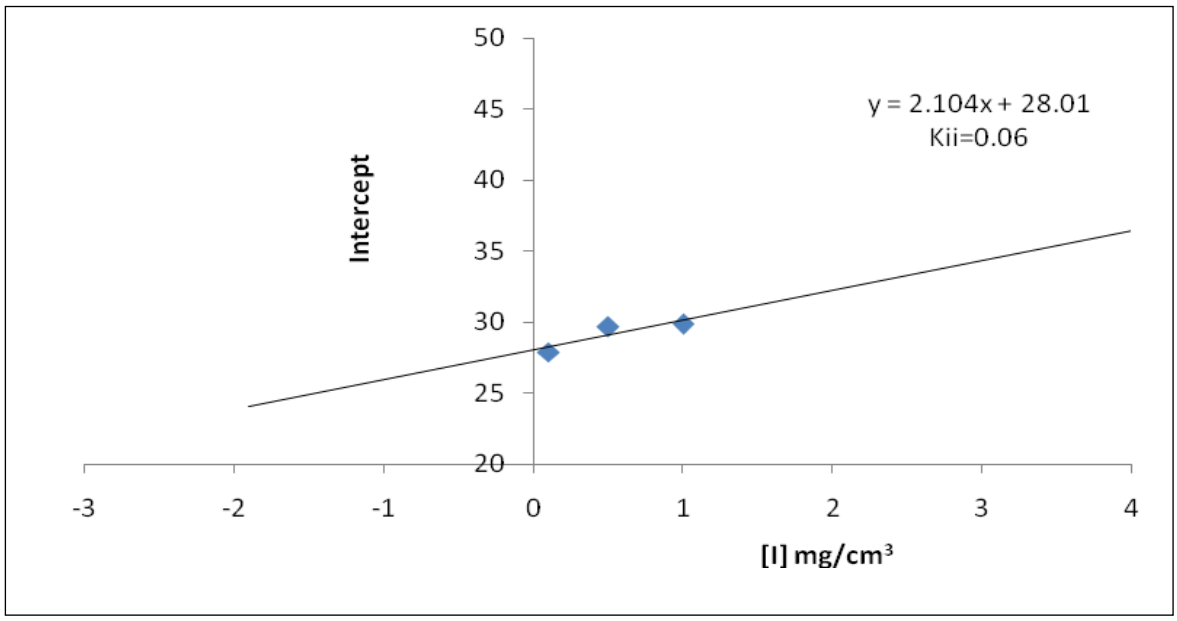


Figure 2d: Secondary Plot of Intercept against Inhibitor (B3) concentration showing Kii

CONCLUSION

Phospholipase A_2 was isolated, partially purified and characterized from *N. nigricollis* Venom. It shows specific activity of 12.50 µmol/min/cm3/mg protein, with purification fold of 156.25 and percentage recovery of 50%. The molecular weight of PLA_2 was found to be 45.55KDa, kinetic studies of the partially purified enzyme revealed a K_m and V_{max} of 1.40 x 10^{-1} mg/cm^3 and 7.70 x 10^{-1}µmol/min respectively. The isolated ursolic acid from *Psidiumguajava* leaf was found to be active against the activity of *N. nigricollis*PLA_2. The kinetic studies of the enzyme (PLA_2) with isolated compound revealed mixed type of inhibition. Therefore the relevance of these findings would be of importance in the development of drugs that could serve as an alternative to the conventional antivenin therapy.

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