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Effect of *Vernonia amygdalina* on phospholipase activity from *Naja Mossambica* (Cobra)

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Abstract

Background: *Phospholipases* are one of the numerous enzymes found in the *Naja mossambica* venom. They play a major role in snakebite envenomation, and also responsible for the hydrolysis of a phospholipid, disrupting the membrane integrity. In this study, we evaluated the effect of *Vernonia amygdalina* on *Phospholipase* activity from *Naja mossambica* (Cobra) Results: Partially purified *phospholipase* had maximal velocity (V_{max}) and Michaelis Menten constant (K_m) of 7.6 × 10^{-5} mol/min and 1.7mg/ml, while the crude *phospholipase* had V_{max} and K_m of 9.4 × 10^{-5}mol/min and 2.5mg/ml respectively. inhibition study of aqueous extracts of *Vernonia amygdalina* leaf shows that the extract is a potent inhibitor of crude *phospholipase* in a dose-dependent pattern. The different doses of extract 15 %, 10 % and 5% produced percentage inhibition of 74.04 %, 78.6 % and 86.63% respectively. The kinetic binding constant (K_i) values of crude phospholipase for different concentrations of extracts 5%, 10% and 15% were 0.21mg/ml, 0.29mg/ml and 0.39mg/ml, while the partially purified *phospholipase* for different concentrations of extracts 5%, 10% and 15% were were 0.48mg/ml, 0.42mg/ml and 0.41mg/ml respectively. It can be deduced from the results that the extract inhibits the *phospholipase* activity in an uncompetitive manner. Conclusion: Aqueous extract of *Vernonia amygdalina* leaves may contain some bioactive agents that could serve as potent inhibitors of phospholipase from *Naja mossambica* venom.

Keywords: Activity, inhibition, *Vernonia amygdalina, Naja mossambica,*

1. Introduction

Snakebite accidents are a socio-medical problem in tropical countries. It is a serious health threat that leads to high mortality and great distress in victims. The global accidents are estimated as five million, accounting for 600,000 envenomation and no less than 40,000 deaths yearly [1,2]. The major pathophysiological problems of envenomation usually start with the early development of systemic effects such as headache, abdominal pain, diarrhea, nausea, vomiting, low blood pressure followed by late systemic effects such as respiratory failure, neuromuscular blockage, hemorrhage, and renal failure. Sometimes, it is associated with local effects like pain, swelling, and necrosis of tissues which may lead to amputation [3]

Phospholipases (PL; EC 3.1.1.4) are enzymes that abundantly found in ophidian venoms with the high hydrolytic activity of phospholipids. This enzyme induces various pharmacological effects such as edema, neurotoxicity, hemorrhagic,

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anticoagulant, modulation of platelet aggregation, and myotoxic effects [4, 5]. They also act on components of the extracellular matrix and of the basement membrane, as well as several components of the coagulation cascade and receptors. These enzymes display up to 96% of similarity with their human homologues [6, 7, 8]. *Phospholipases* are considered as important regulators of the arachidonic acid pathway. They hydrolyze phospholipids thus generating bioactive lipid mediators or messengers that control multiple cell functions and regulate physiological and pathophysiological processes [9,10,11].

Vernonia amygdalina (V. amygdalina) is commonly known as bitter leaf [12], Previous work on the phytochemical analysis of this plant have revealed the presence of steroid glucosides (Vernoniosides A_1-A_4 and B_1-B_3), several sesquiterpene lactones (Vernodalin,vernolide, hydroxyvernolide and vernodalol), Terpenoids, Flavonoids, Saponins, Tannins, Alkaloids, Cardiac glycosides and also, more importantly, Vernolepin, a natural compound isolated from this plant. *Vernonia amygdalina* is a shrub that grows throughout tropical Africa and South-East Asia [13]. The plant is generally known as bitter leaf due to the bitterness of its leaves. Based on previous research [14, 15, 16, 17]. These acclaimed potentials necessitated the need to study this plant with a view to check its role in the treatment of snakebite.

2. Material and methods

2.1. Source of Venom and Vernonia Amygdalina Leaves

Freeze dried crystal powder of *Naja mossambica* venom was gift from Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. Leaves of *Vernonia amygdalina* were obtained from Bosso environs in Minna, Niger state and identified at the Department of Biological sciences, Federal University of Technology Minna.

2.2. Preparation and extraction of plant

The fresh leaves of *V. amygdalina* were air dried for at least 2 weeks then pounded to increase the surface area for extraction. A 50 g of dried homogenizes plant was weighed into a distillation flask and extracted with 200 ml of solvent by refluxing for 2 hours. The mixture was filtered using a muslin cloth. Evaporation of the filtrate was carried out by heating on a steam bath. The evaporated extract was then weighed and stored in a sterile sample bottle. The bottle was labeled and refrigerated

2.3. Determination of Protein Concentration Using Biuret Method

The standard protein solutions used were 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml. 0.2ml of each of the above solution was introduced into a test tube each containing 0.8 ml of distilled water 4.0 ml of freshly prepared biuret reagent was added to each tube. All the tubes were placed in a water bath at 37 °C for 10 minutes. There was formation of blue coloured complex whose absorbance was read at 540nm.

A 0.2 ml crude venom solution (20 mg/ml) was added to a test tube containing 0.8 ml of distilled water. 4.0 ml of freshly prepared biuret reagent was added. The blank was also prepared by adding 1.0ml of distilled to 4.0 ml of biuret reagent. The two tubes were placed in a water bath at 37 °C for 10minutes after which the absorbance of the blue coloured solution formed was read against the blank at 540 nm. All the absorbance values were converted to protein concentration by extrapolating from the standard curve.

2.4. Determination of Phospholipase Activity of Crude Venom

PLA2 activity was assayed according to the method established by Dole [18] with modification. Egg yolk suspension (0.5 ml, 2 mg/ml) was introduced into a clean test tube containing 50 µl of 1mM $CaCl_2$, pH 8.5 buffer and 100 µl of distilled water. To this, 100 µl of 20 mg/ml venom solution was added. This was incubated at 37 °C for 1 hour. After incubation, the enzyme was inactivated by heating the tube at 100 °C for 2 minutes. One drop of phenolphthalein was added, this was titrated against 20 mM NaOH. To determine volume of NaOH used by the free acid content of the yolk, the titration was carried out on the yolk suspension without the enzyme. The volume obtained was subtracted from the volume obtained above in the presence of the enzyme. This gives the volume of NaOH used up by the fatty acid released from lecithin content of the yolk by the phospholipase. Phospholipase activity was also determined using 4 mg/ml, 6 mg/ml, and 8 mg/ml egg yolk suspension. Activities obtained for the respective substrate concentration were used to plot the Lineweaver Burk's plot to determine the V_{max} and K_m for the crude phospholipase.

Effect of Aqueous Extract of V. Amygdalina Leaves on Crude: Phospholipase Activity; Egg yolk suspension (0.5 ml, 2 mg/ml) was introduced into a clean test tube containing 50 µl of 1mM CaCl2 and 100 µl of 5 % solution of aqueous extract of V. amygdalina. To this, 100 µl of 20mg/ml venom solution was added. This was incubated at 37 oC for 1 hour. After

incubation, the enzyme was inactivated by heating the tube at 100oC for 2 minutes. One drop of phenolphthalein was added, this was titrated against 20mM NaOH. To determine volume of NaOH used by the free acid content of the yolk, the titration was carried out on the yolk suspension without the enzyme. The volume obtained was subtracted from the volume obtained above in the presence of the enzyme. This gives the volume of NaOH used up by the fatty acid released from lecithin content of the yolk by the phospholipase. Phospholipase activity was also determined using 4 mg/ml, 6 mg/ml, and 8 mg/ml egg yolk suspension as the substrate. This was repeated with 10 % and 20 % aqueous extract of V. amygdalina.

2.5. Phospholipase Enzyme Purification

Ammonium sulphate and precipitation Sephadex-G50 were used in the purification. In the ammonium sulphate precipitation, 30-80 % ammonium sulphate solutions were prepared into different test tubes. The enzyme was added gradually and stirred until precipitates were formed. The suspensions formed were centrifuged at 3000 rpm for 10 min. The supernatant was decanted and the precipitate collected. The phospholipase activity and protein content were tested for. Further purification was then carried out.

For the sephadex-G50 Gel Purification, 10g of Sephadex-G50 was weighed into a beaker; 100ml of phosphate buffer pH 7.0 was added to the sephadex in the beaker. The mixture was stirred and placed in boiling water. It was stirred continuously for about an hour, removed and allowed to cool. The gel formed was poured into a column and allowed to settle under gravity, the bottom of the column was initially packed with little cotton wool so that the gel can settle while monitoring the flow rate through the column. Phosphate buffer pH 7.5 was added to the column then 5ml of the crude venom solution was also introduced into the column. From time to time, phosphate buffer pH 7.5 is introduced into the column to elute the purified venom solution. The time taken to obtain 5 ml effluents is noted and recorded. This was done for 10 consecutive test tubes. The value obtained was used to calculate the flow rate of each tube. The protein content of each tube was determined using biuret reagent.

2.6. Statistical analysis

The analysis was performed using SPSS statistical package for WINDOWS (version 21.0; SPSS Inc, Chicago). Data were expressed as the Mean ± SEM of three determinations. Results were subjected to ANOVA followed by DMRT.

3. Results

3.1 Determination of K_m and V_{max} for Crude *Phospholipase*

In the 20mg/ml *Naja mossambica* (Spitting cobra) venom used for the experiment, there was 2.0mg/ml of protein**.** The **c**rude *phospholipase* activity obtained was 38.52 mM/hr. Figure 1 shows the Lineweaver-Burk plot for crude phospholipase using egg as substrate. The slope of the plot was 0.038 while the intercept on the Y-axis was 0.022 $mM^{-1}hr$. V_{max} was 76×10^{-5} mol/min and K_m was 1.7mg/ml.

3.2 Effect of Aqueous Extract of *V. Amygdalina* Leaves on Crude *Phospholipase* Activity.

As shown in Figure 2, the inhibition of the crude *phospholipase* activity by the extract was dose- dependent. Highest inhibition was observed with 15 % extract. The pattern of inhibition was observed to be uncompetitive from the plot. Fig 5 represents the crude phospholipase activity in the presence of plant extract. The percentage inhibition produced by 15 % extract was highest with 74.04 %, 10 % extract produced 78.6 % while 5% had the lowest inhibition of 86.63%. The K_i values obtained for different concentrations of extract were: 5 % 0.21mg/ml, 10% 0.29mg/ml and 15% 0.39mg/ml.

3.3 Determination of K_m and V_{max} for Partially Purified *Phospholipase*.

Partially purified phospholipase activity obtained was 45.48mM/hr. Figure 3 shows the Lineweaver-Burk plot for crude phospholipase using egg as substrate. The slope of the plot was 0.046 while the intercept on the Y-axis was 0.0178$mM^{-1}hr$. The K_m obtained was 2.5mg/ml while the V_{max} was 94×10^{-5}mol/min.

3.4 Effect of Aqueous Extract of *V. amygdalina* Leaves on Partially Purified *Phospholipase* Activity.

As shown in Figure 4, the inhibition of the partially purified phospholipase activity by the extract was dose- dependent. Highest inhibition was observed with 15% extract. The pattern of inhibition was observed to be uncompetitive from the plot. Figure 6 6 represents the partially purified phospholipase activity in the presence of plant extract. The percentage

inhibition produced by 15% extract was highest with 73.08%, 10% extract produced 81.70% while 5% had the lowest inhibition of 88%. The K_i values obtained were 5% 0.48mg/ml, 10% 0.42mg/ml and 15% 0.41mg/ml.

3.5 Elution of Phospholipase from Sephadex-G50 Column

Figure 7 shows elution profile of phospholipase from sephadex G-50 column eluted with phosphate buffer with the aim of partially purifying the enzyme. The elution had an average flow rate of 1.202±0.027 with test tubes 5 and 6 showing highest activity.

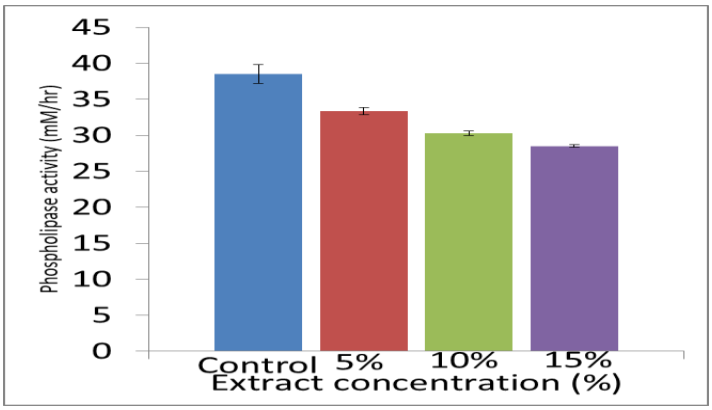

Figure 1 Effect of different concentration of extract on the activity of crude phospholipase**.** The results are expressed as mean values of three experiments (n=3).

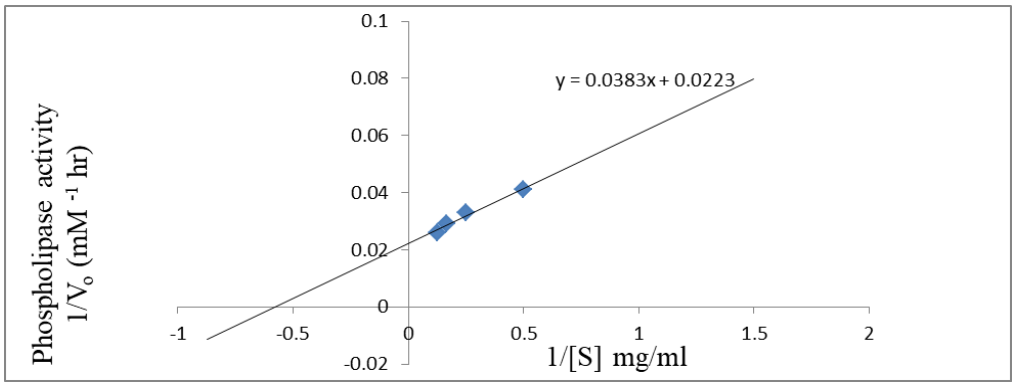

Figure 2 Lineweaver-Burk plot to determine Km and Vmax of crude phospholipase.

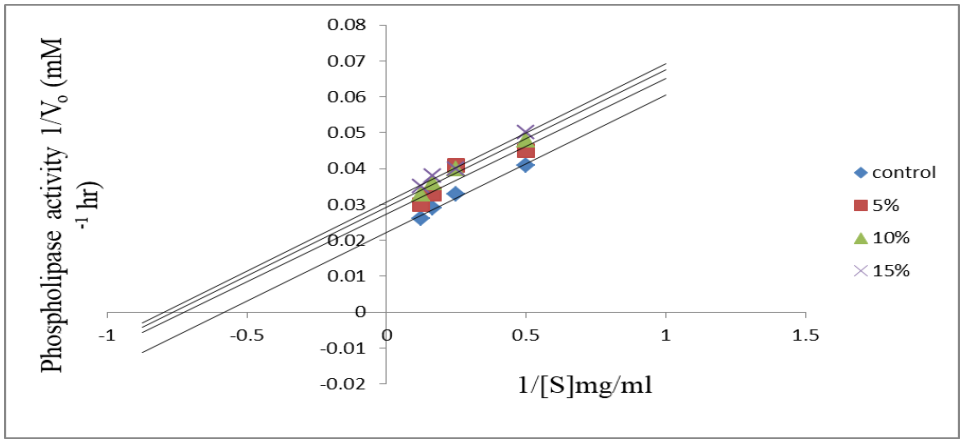

Figure 3 Double reciprocal plot to determine inhibition pattern of the extract on crude phospholipase.

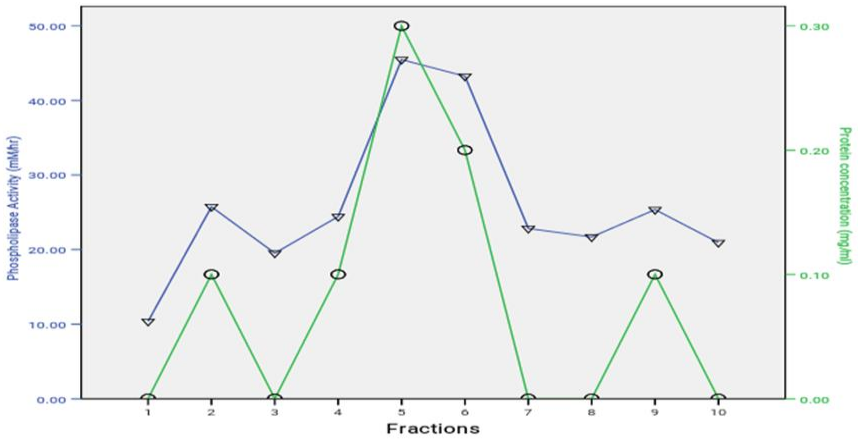

Figure 4 Elution profile of phospholipase from Sephadex G-50 column.

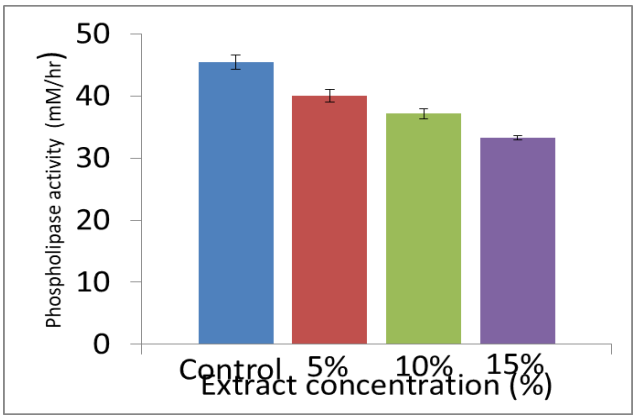

Figure 5 Effect of different concentration of extract on the activity of partially purified phospholipase. The results are expressed as mean values of three experiments (n=3).

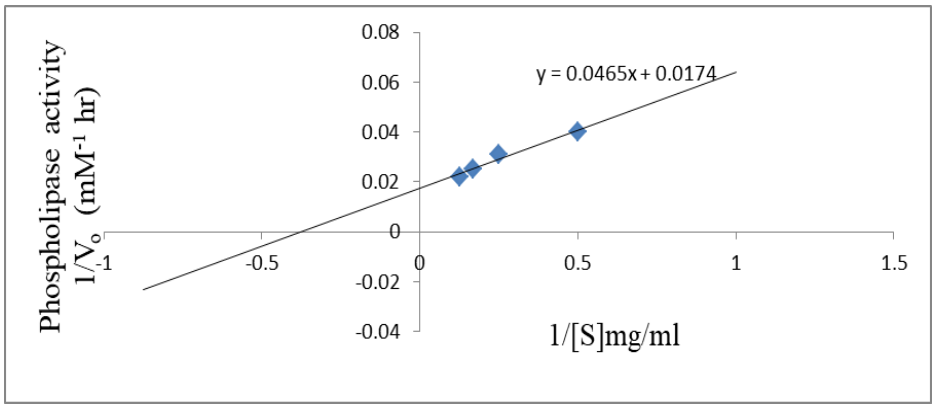

Figure 6 Lineweaver-Burk plot to determine K_m and V_{max} of partially purified phospholipase.

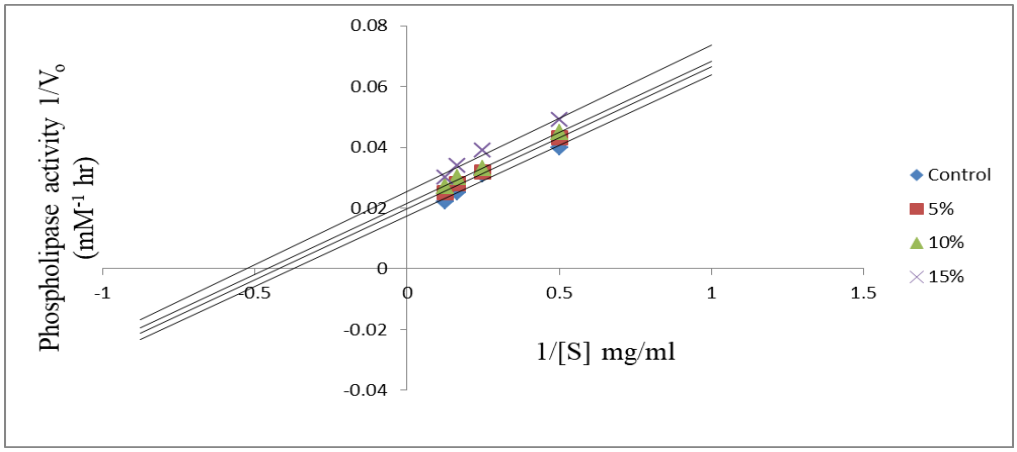

Figure 7 Double reciprocal plot to determine inhibition pattern of the extract on partially purified phospholipase**.**

Table 1 Purification table of *naja mossambica* venom phospholipase.

	Total activity	**Total Protein (mg)**	**Specific Activity Units/mg**	**Yield %**	**Purification fold**
Crude enzyme	127.87	2.0	64	100	1
NH_4SO_4 precipitation	98.04	1.2	82	77	1.3
Gel filtration on sephadex G-50 column	88.73	0.50	177	69	2.8

4. Discussion

The result obtained in figure 1 shows the activity of the *phospholipase* enzyme in crude form. The activity showed a decrease as the substrate concentration decreases since the activity of phospholipase is defined as the amount of

enzyme required to hydrolyze 1mg of free fatty acid from the lecithin present in the egg yolk under standard assay conditions.

The inhibition study of aqueous extract of *Vernonia amygdalina* leaves shows that the extract is a good inhibitor of phospholipase from *Naja mossambica* venom and that the rate of inhibition is dose-dependent. As the concentration of extract increases, the rate of inhibition increases (Figure 5 and 6). The dose-dependent inhibition of the activity of phospholipase in the venom implies that the proportion of the constituents in the extract indicated a significant effect on the action of the enzyme and that, the higher the concentration of the extract, the higher the phospholipase activity inhibition, as such they lower the toxicity ultimately posed by the venom. It showed an uncompetitive inhibition pattern known as a ping-pong reaction [19]. Uncompetitive inhibition implies that there might be in the extract, a compound interacting with the enzyme-substrate complex. The compound might have a destructive effect on the enzyme-substrate complex. The uncompetitive inhibitor which need not resemble the substrate presumably distorts the active site, thereby rendering the enzyme catalytically inactive. Adding substrate does not reverse the effect of an uncompetitive inhibitor because the binding of the substrate does not interfere with the binding site. Uncompetitive inhibition requires that the inhibitor affect the catalytic function of the enzyme but not its substrate binding. This is supported by the reports of [20, 21] who found out that the aqueous extract of the plant leaves they used contained tannins which is very much present in *Vernonia amygdalina* leaves [22] which have been reported to unspecifically inactivate proteins and enzymes. Previous studies confirmed that certain naturally occurring substances modify the actions of proteins and enzymes [23], especially the plant polyphenols which are the precursors of tannin. This can therefore be suggested to be the mechanism of the detoxifying action of aqueous extract of *V. amygdalina* leaves and why it is used in the treatment of snakebite as reported [24].

After purification, the activity of the *phospholipase* increased because the concentration of free fatty acid released from the lecithin content of egg yolk which is proportional to the activity of the enzyme increased though slightly. The slight increase in activity could be attributed to the assumption that the venom of *Naja mossambica* contains more *phospholipase* than other enzymes so the purification step was able to remove the little amount of other enzymes present which gave rise to the slight increase in activity [25, 26].

The proportional rate of inhibition of both crude and partially purified *phospholipase* by the plant extract indicates that the component in the leaves that inhibits the phospholipase is specific so that whether in the presence of impurities or pure, it targets the phospholipase directly and inhibits its action.

The K_i was lower than K_m for both purified and crude enzymes indicating that the extract/inhibitor binding affinity to the enzyme is higher than the substrate binding affinity to the enzyme. The K_i for crude was lower than that of the partially purified enzyme which means that the extract binds more to the crude venom than the partially purified venom and so it is a more effective inhibitor of the venom in general than the phospholipase enzyme alone, that is, the extract has an inhibitory effect on other components of the venom as well. Polyphenolic compounds, such as flavonoids, have already been reported to be PLA2 inhibitors [27].

5. Conclusion

The result of this work has shown that aqueous extract of *Vernonia amygdalina* leaves is a potent inhibitor of phospholipase from *Naja mossambica* venom and that the rate of inhibition increases as the extract concentration increases. It also shows that the extract has inhibitory effect on other components of the venom.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors affirm no conflict of interests in conducting this study.

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