



EFFECT OF *Allium sativum* ON PHOSPHOLIPASE FROM *Naja mossambica* (COBRA)

A. Abdullahi¹, A. B. Salau², E. C. Egwin¹, H. L. Muhammad¹, F. S. Adefolalu¹ and K. Ebisine¹

¹Department of Biochemistry, Federal University of Technology, Minna, Niger State

²Department of Biochemistry, Ahmadu Bello University, Zaria

Corresponding author: abkad2008@yahoo.com

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Abstract

Methanolic extract of *Allium sativum* (garlic) was investigated for its inhibitory action on both crude and partially purified phospholipase activity from *Naja mossambica* venom. The purification scheme employed both ammonium sulphate precipitation and size exclusion (gel filtration) chromatography in sephadex G-50. The enzyme was inhibited by different concentrations of the extract in a dose dependent pattern. Double reciprocal plot of the enzyme activity against both substrate and inhibitor concentration showed an uncompetitive pattern of inhibition. The V_{max} and K_m of both crude and partially purified phospholipase gave 67×10^5 M/min, 1.28 mg/ml and 98×10^5 M/min, 2.41 mg/ml respectively. Inhibition constant (K_i) to determine binding affinity of inhibitor to enzyme for both crude and partially purified gave 0.17 mg/ml and 0.04 mg/ml respectively suggesting a higher binding affinity and may be a good source of potential inhibitor of phospholipase.

Keywords: *Allium sativum*, methanolic extract, phospholipase, *Naja mossambica*,

INTRODUCTION

Snake bite is a major health hazard that leads to high mortality and great suffering in victims. Conservative sources estimate that the number of accidents involving snake globally reach one million, resulting in 600,000 envenomations and more than 20,000 deaths annually (Chippaux, 1998). Other sources place annual incidences globally at 5 million with about 40,000 or more deaths close to 10% mortality attributed to malaria (Warrell, 1996).

Although not all snakes are poisonous, or their bites deadly, there are however several varieties of snake whose bites may result in immediate casualties. However, notwithstanding the poison injected into a victim's body through a snake bite, it is possible to save the victims' lives by using the appropriate anti-venom. Cobras, vipers, and closely related species use venom to immobilize or kill their prey. The venom is modified saliva, delivered through fangs. The fangs of 'advanced' venomous snakes like viperids and elapids are hollow to inject venom more effectively, while the fangs of rear-fanged snakes such as the boomslang merely have a groove on the posterior edge to channel venom into the wound. Snake venoms are often prey specific, their role in self-defense is secondary. Snake venom is broadly divided into three categories based on toxicity from envenomation. These categories are (i) hemotoxins, which promote hemorrhaging primary to ex-extensive local swelling and necrosis, (ii) neurotoxins, which disable muscle contraction and paralyze the heart as well as hinder respiration, and (iii) cardiotoxins, which elicit specific toxicity to cardiac and muscle cells, causing irreversible depolarization of cell membranes (Hati *et al.*, 1999). Irrespective of internal consequences, it also causes extensive local tissue damage and inflammation by

the direct action of cytotoxic factors (Dong-Zhong *et al.*, 1997).

The Mozambique Spitting Cobra (*Naja mossambica*) is a type of cobra, native to Africa. It is considered one of the most dangerous snakes in Africa, second only to the Mamba. Like the Rinkhals, it can spit its venom. Its bite causes severe local tissue destruction (similar to that of the puff adder). Venom to the eyes can also cause impaired vision or blindness. Mozambique Spitting Cobra venom causes Local pain, swelling, blistering, necrosis ± flaccid paralysis. Snake venoms generally contain more than 90% protein and most of them are enzymes, which are proteinases, phospholipases, sphingomyelinases and hyaluronidases. Among these proteins, Phospholipase A2 (PLA2) are abundant (40%) in almost all snake venoms (Walter *et al.*, 1999; Murari *et al.*, 2005; Kini, 2005). PLA2 enzymes are one of the most toxic proteins present in snake venom; in addition to the digestion of prey, they exhibit many pharmacological effects by disturbing the normal physiological process of victims (Kini, 2003; Doley and Mukherjee, 2003; Doley *et al.*, 2004; Mukherjee, 2007). The most effective and accepted therapy for snakebite patients is immediate administration of specific or polyvalent antivenom following envenomation; however, this therapy carries an associated risk of anaphylaxis and serum reactions. Also, due to regional variation in venom composition of snakes, antivenom raised against the venom of a snake from a particular geographical origin may not be able to neutralize or prevent local effects of envenomation by snakes from other geographical location (Cannon *et al.*, 2008). Therefore in addition to administration of antivenom, there should be an alternative therapy for the snake bite patients. Numerous plant species are s

used as folk medicine to treat venomous snake bite in Nigeria, but without any scientific validation. Therefore, this type of treatment remains questionable and needs thorough scientific investigation.

Garlic (*Allium sativum* Linn.) is one of those plants that have been seriously investigated over the years to counteract snake venom and other poisonous bites. It has been used for centuries to fight infections (Onyeagba *et al.*, 2006). The early Egyptians used it to treat diarrhoea the ancient Greeks used it to treat intestinal and extraintestinal diseases, while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections (Ankri and Mirelman, 1999; Jaber and Al-Mossawi, 2007). *Allium sativum* (garlic) is reported to exhibited little snake repellent properties and snake venom protection. Previous experiment shows that pretreatment with the therapeutic dose of garlic for ten days induced a prophylactic activity against the pathogenic effects of the venom in both tissues, which appeared more or less normal except for very minor abnormalities. However, application of double therapeutic dose of garlic for the same duration did not induce any prophylactic activity (Rahmy and hammaid, 2001). Therefore, this study is to investigate the inhibitory action of solvent action of *Allium sativum* on phospholipase activity from Snake Venom.

MATERIALS AND METHODS

Plant Collection and Extraction

Fresh bulb of *Allium sativum* was bought from Minna central market, Niger state and dried at room temperature for three weeks after which it was grounded in mortar and crushed to powder form using blender. 100g of the resulting garlic powder was weighed into a distillation flask and extracted with 300ml of methanol by reflux for two hours. The extract was obtained by filtering the mixture into conical flasks through a cheese cloth and evaporated to dryness at 50°C in a steam bath and collected into sterile sample bottle.

Source of Snake Venom

Freeze dried *Naja mossambica* venom (0.2g) was obtained from Dr. Balarabe, department of Biochemistry, A.B.U Zaria.

Protein Content of Crude Venom

This was carried out using biuret has described by (Albert, 1999) 0.2ml of each of the above solution was introduced into a test tube each containing 0.8ml of distilled water. 4.0ml 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 against the blank (containing 1.0ml of 0.15M NaCl solution and 4.0ml of biuret reagent).

Phospholipase Assay

This was carried out by modification of (Habermann and Neunmann, 1984).

The activity of phospholipase is defined as the amount of enzyme required to hydrolyze 1mg of free fatty acid from lecithin present in the egg yolk under standard assay conditions.

Determination of Km and Vmax for Crude Phospholipase

Egg yolk suspension (0.5ml, 2mg/ml) was introduced into a clean test tube containing 50µl of 1mM CaCl₂ and 100µl of distilled water. To this, 100µl of 20mg/ml venom solution was added. This was incubated at 37°C for 1 hour. After the incubation, the enzyme was inactivated by heating the tube at 100°C for 2 minutes. One drop of phenolphthaleine was added, this was titrated against 2mM 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 here was subtracted from the volume above in the presence of the enzyme. This gave 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 4mg/ml, 6mg/ml, 8mg/ml. activity obtained for the respective substrate concentration were used to plot Lineweaver Burk's plot to determine Vmax and Km for the crude phospholipase.

Effect of Methanol Extract of *Allium sativum* on Crude Phospholipase Activity

Egg yolk suspension (0.5ml, 2mg/ml) was introduced into a clean test tube containing 50µl of 1mM CaCl₂ and 100µl of 5% solution of methanol extract of *Allium sativum*. To this, 100µl of 20mg/ml venom solution was added. This was incubated at 37°C for 1 hour. After the incubation, the enzyme was inactivated by heating the tube at 100°C for 2 minutes. One drop of phenolphthaleine was added, this was titrated against 2mM NaOH. To determine volume of NaOH used by the free fatty acid content of the yolk, the titration was carried out on the yolk suspension without the enzyme. The volume obtained here was subtracted from the volume obtained above in the presence of the enzyme. This gave the volume of NaOH used up by the fatty acid released from lecithin content of the yolk by the phospholipase. Phospholipase activity was also

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determined using 4mg/ml, 6mg/ml, 8mg/ml egg yolk suspension as the substrate. This was repeated with 10% and 20% extract of *Allium sativum*.

Ammonium Sulphate Precipitation

The crude enzyme solution were precipitated using 30-85% ammonium sulphate and the solution was kept for 24 hours. The resulting precipitate was dialysed against 0.1M sodium phosphate buffer at pH 7.5. The dialysate was centrifuged to remove minor insoluble materials (Alan, 1994).

Partial Purification of Phospholipase from the Venom of *Naja mossambica* Using Sephadex

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.

RESULTS AND DISCUSSION

In the 20mg/ml *naja mossambica* venom used for the experiment, there was 2.0mg/ml of protein. Figure 1

shows the lineweaver-Burk plot for crude phospholipase using egg yolk as substrate. The slope of the graph is 0.032 while the intercept on the Y-axis is 0.025mM/hr. The V_{max} therefore is $67 \times 10^5 M/min$, and the K_m is 1.28mg/ml. *Allium sativum* shows uncompetitive inhibition with crude phospholipase activity as shown in figure 2. The inhibition observed was dose dependent with maximum inhibition at 20% of the extract and the least was observed at 5% of the extract. The K_i value is 0.17mM/hr.

Figure 3 shows crude phospholipase activity in the presence of plant extract. 20% of extract produced highest inhibition with 53.85%, followed by 10% with 68.30% while 5% gave the least inhibition with 90.34%. Table shows the elution profile of phospholipase from sephadex G-50 column eluted with phosphate buffer pH7.5 with the aim of partially purifying the enzyme. Test tubes 5, 6, 7 showed the highest activity with average flow rate for each testtube as 1.202 ± 0.027 .

Figure 4 shows the lineweaver Burk plot for the purified phospholipase using egg yolk as substrate. The slope of the graph is 0.041 while the intercept on the Y-axis is 0.017mM/hr. V_{max} obtained is $98 \times 10^5 M/min$ while the K_m is 2.41mg/ml.

Methanolic extract of *Allium sativum* showed uncompetitive inhibition. The inhibition pattern is represented by figure 10. The inhibition observed was dose dependent with the maximum inhibition at 20% of the extract concentration, and the least was observed in 5% extract concentration. The K_i value is 0.04mg/ml.

Figure 6 shows crude phospholipase activity in the presence of plant extract. 20% of extract produced highest inhibition with 25.46%, followed by 10% with 30.09% while 5% gave the least inhibition with 38.19%.

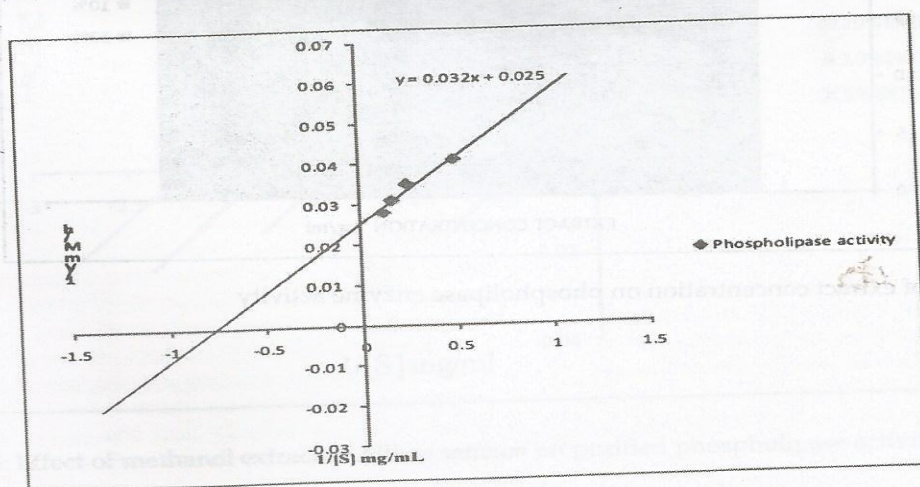


Fig 1: Lineweaver-burk plot for crude phospholipase

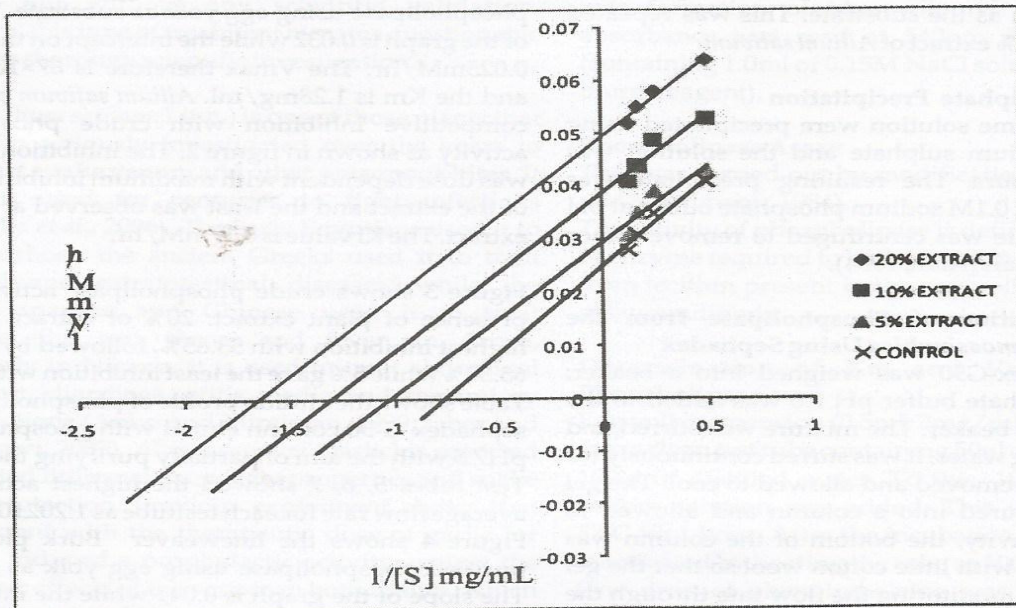


Fig 2: Effect of methanolic extract of *Allium sativum* on crude phospholipase activity

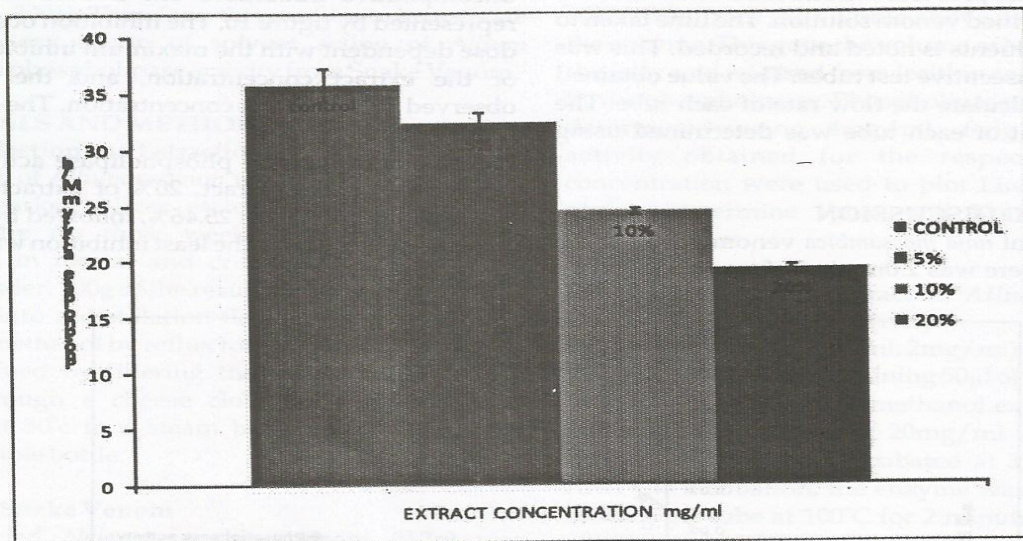


Fig 3: Effect of extract concentration on phospholipase enzyme activity

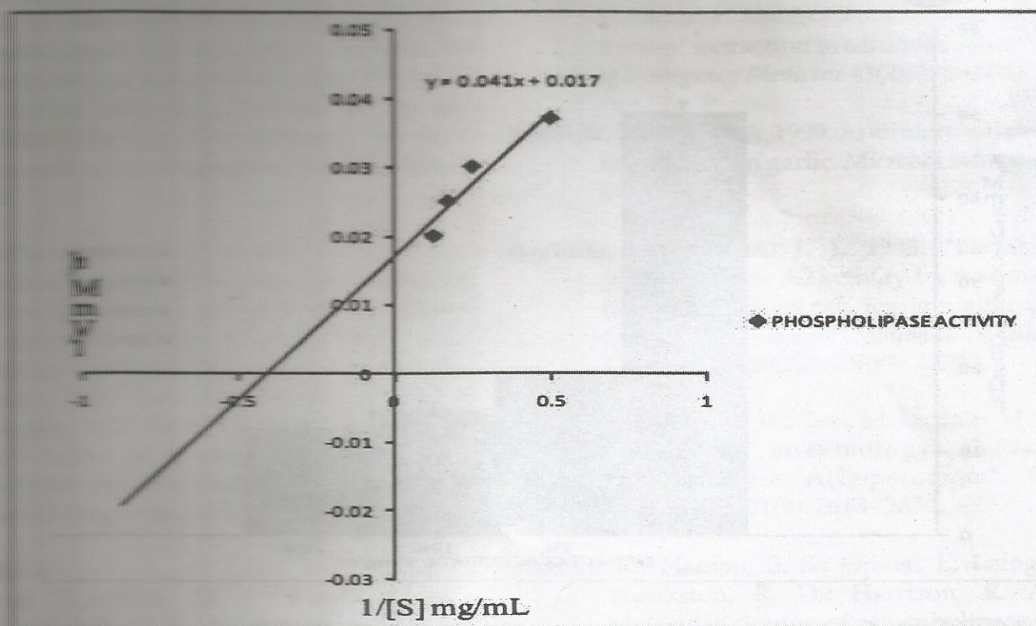


Fig 4: Lineweaver-burk plot for purified phospholipase

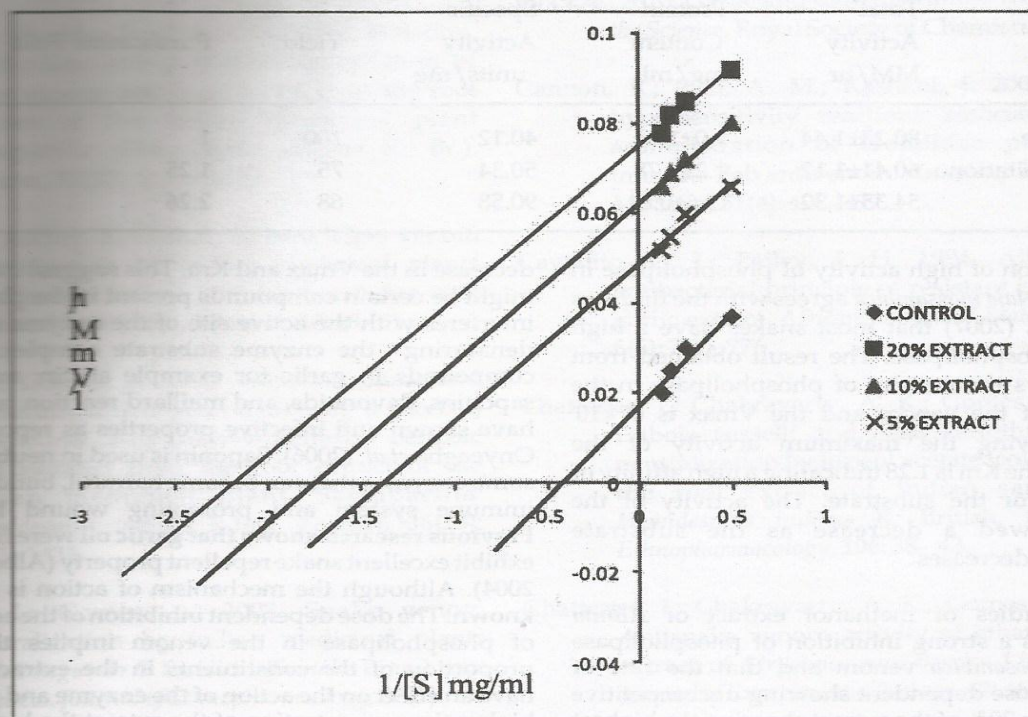


Fig 5: Effect of methanol extract of *Allium sativum* on purified phospholipase activity

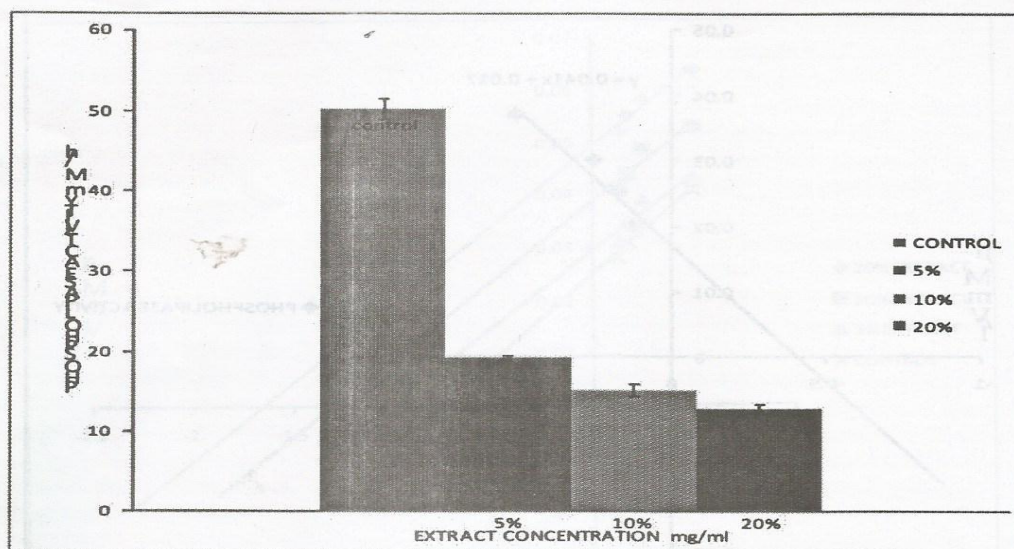


Fig 6: Effect of extract concentration on purified enzyme activity

Table 1: Purification profile of *Naja mossambica* phospholipase

Purification	Total Activity MM/hr	Protein Content mg/ml	Specific Activity units/mg	Yield	Purification Fold
Crude Enzyme	80.23±1.44	2.0±1.34	40.12	100	1
NH ₄ SO ₄ Precipitation	60.41±1.12	1.2±0.78	50.34	75	1.25
Gel Filtration	54.35±1.32	0.6±0.81	90.58	68	2.26

The observation of high activity of phospholipase in the venom of *Naja mossambica* agrees with the findings of Mukherjee, (2007) that most snakes have a high content of phospholipase. The result obtained from figure 6 shows the activity of phospholipase in the crude form of the venom and the Vmax is 67×10^{-5} M/min showing the maximum activity of the enzyme, and the Km is 1.28 indicating a high affinity of the enzyme for the substrate. The activity of the enzyme showed a decrease as the substrate concentration decreases.

Inhibition studies of methanol extract of *Allium sativum* shows a strong inhibition of phospholipase from *Naja mossambica* venom and that the rate of inhibition is dose dependent showing uncompetitive inhibition with 20% of the extract showing the highest inhibition suggesting that the inhibitor binds directly to the enzyme-substrate complex and not the free enzyme thereby distorting the active site of the enzyme. This type of inhibition shows considerable

decrease in the Vmax and Km. This suggest that there might be certain compounds present in the plant that interferes with the active site of the enzyme thereby denaturing the enzyme substrate complex. Many compounds in garlic for example allicin, minerals, saponins, flavonoids, and maillard reaction products have shown anti infective properties as reported by Onyeagba *et al.* (2006). Saponin is used in neutralizing some enzymes that can become harmful, building the immune system and promoting wound healing. Previous research shows that garlic oil were found to exhibit excellent snake repellent property (Albert *et al.*, 2004). Although the mechanism of action is not well known. The dose dependent inhibition of the activities of phospholipase in the venom implies that the proportion of the constituents in the extract could have an effect on the action of the enzyme and that the higher the concentration of the extract the higher the venom enzyme activity and the lower the toxicity ultimately imposed by the venom.

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After purification, the activity of the phospholipase increased due to removal of impurities and other compound in the venom that might interfere with the substrate. The Vmax and Km also increased. There is a reduction in the Ki value from 0.17mM/hr for the crude to 0.04mM/hr for the partially purified phospholipase indicating a high affinity of the extract to the enzyme.

CONCLUSION

From the result obtained, it is clear that methanol extract of *Allium sativum* is a potent inhibitor of both crude and purified phospholipase from *Naja mossambica* venom and that a high concentration of the extract preferably 20% of the extract will inhibit *Naja mossambica* venom. This therefore shows that *Allium sativum* could serve as a good source of *Naja mossambica* venom antidote and could be used in designing a novel drug to be used as an antivenin.

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