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IN VITRO EVALUATION OF ANTIBACTERIAL ACTIVITIES OF SEED AND SHELL EXTRACTS OF *Moringa oleifera* AGAINST SOME HUMAN PATHOGENIC BACTERIA

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Abstract

Phytochemical analyses of aqueous and methanolic extracts of Moringaoleiferaseed powder demonstrated only the presence of saponins, reduced sugar, and carbohydrates.The antibacterial properties of aqueous and methanolic extracts ofMoringaolifera seed powder were determined in vitro against Pseudomonas aeruginosa, Salmonnellatyphi, Staphylococcus aureus and Bacillus subtilis usingdisc diffusion and minimum inhibitory concentration method (MIC). The aqueous extract of the seed powder displayed a potential antibacterial activity against the two tested gram-negative bacteria: Pseudomonas aeruginosa and Salmonnellatyphi, and the two tested gram-positive bacteria: Staphylococcus aureus and Bacillus subtilis. The methanolic extract of the seed powder however displayed antibacterial activity for onlyPseudomonas aeruginosa and Bacillus subtilis. The zones of inhibition for seed powder of aqueous and methanolic extracts were 4.33 -12 mm and 0.83 - 5 mm respectively. The results suggest that the seed powder extracts of M. oleifera can be used as antibacterial agents that could be developed into chemotherapeutic products.

Keywords: *Moringaoleifera, gram-negative, gram-positive, minimum inhibitory concentration, extracts.*

INTRODUCTION

The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. The frequency of these life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immuno-compromised patients in developing countries (Al-Bari *et al.*, 2006). The increasing prevalence of multi-drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raised the spectre of 'untreatable' bacterial infections and adds urgency to the search for new infection-fighting strategies (Zy *et al.*, 2005; Rojas *et al.*, 2006). For a long time, plants have been important sources of natural products for human health. World Health Organization (WHO), 2002 noted that majority of the world's population depend on traditional medicine for primary healthcare services because they constitute a major source of natural organic compounds. Plants have their antimicrobial properties as secondary metabolites such as alkaloid and flavonoid compounds. The practice of complementary and alternative medicine is now on the increase in developing countries in response to World Health Organization directives culminating in several pre-clinical and clinical studies that have provided the scientific basis for the efficacy of many plants used in folk medicine to treat infections (Vijaya and Ananthan, 1997; Dilhuydy, 2003). Despite the existence of potent antibiotic and antifungal agents, resistant or multi-resistant strains are continuously appearing, imposing the need for a continuous search and development of new drugs. Some strains of *S. aureus* are capable of producing staphyloxanthin (a carotenoid pigment that acts as virulent factor). It has an antioxidantaction that helps the microbe evade death by reactive oxygen species used by the hostimmune system. It is therefore very necessary that the search for newer antibiotic sources be a continuous process. Plants are the cheapest and safer alternative sources of antimicrobials (Pretorius and Watt, 2001; Sharif and Banik, 2006; Doughari *et al.*, 2007). *M. oringaoleifera* is the most widely cultivated species of a monogeneric family, the Moringaceae that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Fahey, 2005). It is widely used for treating bacterial infection, fungal infection, anti-inflammation, sexually transmitted diseases, malnutrition and diarrhoea. *Moringa*species have long been recognized by folk medicine practitioners as having value in the treatment of tumors (Ramachandran *et al.*, 1980). Hence, the present study was undertaken specifically to investigate the role of aqueous and methanolic extracts of *M. oleifera* seed powder as potential antimicrobial agent against some human pathogenic bacteria.

MATERIALS AND METHODS

All practical analysis and evaluations were carried out in Biochemistry and Microbiology laboratories, Federal University of Technology, Bosso campus, Minna-Niger state, Nigeria.

Sample Collection

The pods of *M. oringaoleifera* Lam were collected from Bosso area in Minna, Niger State in the month of August, 2010 and identified at the herbarium unit of Biological Science Department, Federal University of Technology, Minna, Niger State. The pods were air dried, and cracked. The seed powder was further air dried, made into powder, and sieved through 2 mm brass.

Test Microorganisms

The four bacterial strains used in this study are two gram-negative: *Pseudomonas aeruginosa*and *Salmonella typhi*, and two gram-positve: *Bacillus subtilis and Staphylococcus aureus*. All the tested strains were collected from the Department of Microbiology, Federal University of Technology, Bosso Campus Minna, Niger State. The bacteria were grown at 37°C (in incubator) and maintained on nutrient agar slants at 4°C.

Plant Sample Extraction

Aqueous Extracts of Seed Powder

Fifty gramsseed powderof *M. oringaoleifera* was placed in 400 ml of distilled water in a round bottom flask and fixed to a reflux extractor via glass adaptor for four hours at 100°C. This was filtered off into a clean beaker using muslin cloth and subjected to steam bath evaporation at 40°C. The extract obtained was stored in the refrigerator at 4°C till ready for antibacterial activity test (Akueshi *et al.*, 2002).

Methanol (100%) Extracts of Seed Powder

Fifty grams seed powder of *M. oringaoleifera* was placed in 200 ml of methanol (100%) in a round bottom flask and fixed to a reflux extractor via glass adaptor for four hours at 60°C. This was filtered off using muslin cloth into a clean beaker and subjected to steam bath evaporation at 40°C. The extract obtained was stored in a refrigerator at 4°C for antibacterial activity test (Akueshi *et al.*, 2002).

Phytochemical Screening of Extracts

Phytochemical tests for various constituents of extracts were carried out by the methods of Trease and Evans (1983). The extracts were screened for the presence of alkaloids, flavonoids, saponins, glycosides, tannins, reduced sugar, carbohydrates, amino acids, steroids, phenols, volatile oils and proteins.

Experimental Design

The antibacterial assay involves the antibacterial activity of samples of *M. oleifera* extracts;

I. Aqueous extract of seed powder;
II. Methanolic extract of seed powder.

In vitro Antibacterial Test

The *In vitro* antibacterial test was carried out by disc diffusion method (Bauer *et al.*, 1996; Barry, 1980) using 25 ìl of standardized suspension of tested bacteria spread on nutrient agar plates. The discs (5 mm in diameter) were impregnated with 10 ìl of 20 mg ml^{-1} (200 ìg $disc^{-1}$), air-dried and placed on seeded agar plates. Negative controls were prepared using the same solvents to dissolve the plant extracts. Tetracycline (30 ìg disc-1) was used as positive control to determine the sensitivity of bacterial strain. The plates were incubated at 37°C for 24hrs. Antimicrobial activity was evaluated by measuring the zones of inhibition against the tested bacteria. Each assay was carried out in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of two samples of *M. oleifera* was determined by two-fold serial dilution method (Chandrasekaran and Venkatesalu, 2004). The dose levels of seed powder of 20 mg ml^{-1} was serially diluted in a nutrient broth of 5 ml with varying concentrations; 10, 5, 2.5, 1.25 and 0.625 mg ml^{-1}. The test tubes were incubated at 37°C for 24 hrs. Controls were used with the test organisms, but with distilled water instead of the plant extract. The least concentration of the samples with no visible growth was taken as the MIC (Adesokan, 2007).

RESULTS AND DISCUSSION

Table 1 shows the phytochemical properties of the extracts. The two extracts demonstrated trace presence of saponins, reduced sugar and carbohydrate.

Table 2 shows diameter of zones of inhibition of bacterial growth at varying concentrations of seed powder of *M. oringaoleifera*. The aqueous extract of seed powder showed stronger antibacterial activity against the studied gram-negative bacteria (*Psuedomonasaeruginosa* and *Salmonella typhi*) and gram-positive bacteria (*Bacillus subtilis*and *Staphylococcus aureus*) with the respective diameter zones of inhibition as: 11.33±1.52, 12.00±2.64 and 5.00±3.00, 4.33±2.08 mm. Methanolic extract of seed powder also exhibited a relatively potent inhibitory effect against all the tested gram-negative bacteria (*Psuedomonasaeruginosa* and *Salmonella typhi*) and gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) with their respective diameter zones of inhibition was 3.33±2.86, 5.00±1.0 and 1.5±1.32, 1.83±1.25 mm.

Table 1: Phytochemical Constituents of *Moringaolifera* Seed Powder

Phytochemical Components	Aqueous Extract	Methanolic Extract
Saponins	+	+
Tannins	-	-
Flavonoids	-	-
Alkaloids	-	-

Table 3: Minimum Inhibitory Concentration (MIC) of *M. oringaoleifera*seed powder and shell extracts against the tested human pathogenic bacteria.

Bacteria		Aqueuosextracts[a]		
		Seed powder	Seed powder	Distilled Water
Gram-Negtive	*Pseudomonas aeruginosa*	2.5	10	-
	Salmonella typhi	2.5	ND	-
Gram-positive	*Bacillus subtilis*	5	10	-
	Staphylococcus aureus	5	ND	-

KEY:

[a]Minimum inhibitory concentration (values in mg ml^{-1}).

ND: Not detected

Distilled water is the control.

Table 3: Minimum Inhibitory Concentration (MIC) of *M. oringaoleifera*seed powder and shell extracts against the tested human pathogenic bacteria.

Bacteria		Aqueousextracts*		
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Gram-Negtive	*Pseudomonas aeruginosa*	2.5	10	-
	Salmonella typhi	2.5	ND	-
Gram-positive	*Bacillus subtilis*	5	10	-
	Staphylococcus aureus	5	ND	-

KEY:

*Minimum inhibitory concentration (values in mg ml^{-1}).

ND: Not detected

Distilled water is the control.

Displays of strong inhibition of aqueous extract of seed powder against all the tested bacteria were notice within the concentrations of 2.5 mg ml^{-1} and 5.0 mg ml^{-1}. The methanolic extract of the seed powder however showed inhibition only for *Pseudomonas aeuginosa* and *Bacillus subtilis* at 10 mg ml^{-1}. The highest zones of inhibition against all the tested bacteria were found in the aqueous extract of the seed powder and this showed relativity with the positive control drug, tetracycline, (with respect to *Salmonella typhi* and *Bacillus subtilis)*. The methanolic extract of the seed powder also showed inhibitory potency but was less by half the activity of the aqueous extracts on all tested bacteria. The inability of the methanolic extract to effectively inhibit some of the tested bacteria could be due to incomplete extraction of its active components. The secondary metabolites like alkaloids and flavonoids may therefore be too low to demonstrate antibacterial activities against the tested bacteria. The alkaloids are nitrogenous heterocyclic organic compounds produced by plants to protect it self against predators. Saponins on the other hand have anti-inflammatory, anticholinergic, and hepatoprotective effects. The lowest MIC wasrecorded in respect of the two gram-negative bacteria (*P. aeruginosa* and *Salmonella typhi)*. These suggest that *M. oleifera* seed powder used contain bio-active components whose antibacterial activities against the tested gram-negative, and gram-positive bacteria are closely related to that of the antibiotic, tetracycline. The *in vivo* activity of the aqueous extract of the seed powder of *M. oringaoleifera* showed a better antibacterial activity over the methanolic extract. This is observed by the stronger inhibitory potentials exhibited by the aqueous extract. The claim by the traditional medical practitioners that *M. oleifera* seed powder is used to treat some infections has not been authenticated by our research. It may however have some potential applications in pharmaceutical industry for treating some pathogenic bacteria. Other solvents can be used for extraction (e.g. ethanol) to see whether it will show greater efficacy.

CONCLUSION

The inability of the two extracts to effectively inhibit the tested bacteria could be due to incomplete extraction of the active components especially alkaloids, tannins, and saponins. If seed extracts are to be used for medicinal purposes, issues of safety and toxicity should also be considered.

REFERENCES

Adesokan, A. A.; Akanji, M. A.; Yakubu, M. T. 2007. "Antibacterial potentials of aqueous extract of *Enantiachlorantha* stem bark". *African Journal ofBiotechnology*, 6: 2502- 2505.

Akueshi, C. O.; Kadiri, C. O; Akueshi, E. U.; Agina, S. E.; Ngurukwem, B. 2002. "Antimicrobial potentials of *Hyptissauvedens*". *Journal of Botany*, 15: 37 - 41.

Al-Bari, M. A.; Sayeed, M. A.; Rahman, M. S.; Mossadik, M. A. 2006. "Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis*", a novel species collected in Bangladesh. *Research Journal of Medicine and Medical Science*, 1: 22 - 34.

Barry, A. I. 1980. Procedure for testing antimicrobial agent in agar media. In: Antibiotica in laboratory medicines. Willims and Wilkins Co. Baltimore, pp. 1 - 23.

Bauer, A. W.; Kibry, W. M. M.; Sherris, J. C.; Turck, M. 1996. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, **45:** 493 - 496.

Chandrasekaran, M.; Venkatesalu, V. 2004. Antibacterial and antifungal activity of *Syzygiumjambolanum* seeds. *Journal of Ethnopharmarcology*, .**91:** 105 - 108.

Dilhuydy, J. M.; 2003. "Patients' attraction to complementary and alternative medicine (CAM): A reality which physicians can neither ignore nor deny. *Bulletin of Cancer.*, **90:** 623- 628.

Doughari, J. H.; El-mahmood, A. M.; Manzara, S. 2007. Studies on the antibacterial activity of root extracts of *Carica papaya* L. *African Journal of Microbiology Research*, **5:** 037 - 041.

Pretorius, C. J.; Watts, E. 2001. Purification and identification of active components of *Carpobrotusedulis*. *Journal of Ethnopharmacology*, **76:** 87 - 91.

Ramachandran, K.; Szkudelski, P.; Torben, Y. A. 1980. Effect of aqueous extracts of leaf of *Eucalyptus camaldulensis* on *Staphylococcus aerus*. *American journal of Medicine*. **4:** 45 - 47.

Rojas, R. F.; Hoff, W.; Boer, R. 2006. Antibacterial and Antifungal activity of *Syzygiumjambolanum* seeds. *Journal of Ethnopharmacology*, **19:** 105-108.

Sharif, J. P.; Banik, Y. K. 1997. Effect of ethanolic extract of *Salvadoraoleoides* in infected mice. *Indian Journal of Pharmacology*, **40:** 23 - 27.

Trease, G. E, Evans, M. C. 1983. Textbook of pharmacology. 12th edition BaillereTindall London, pp. 343 - 383.

Vijaya, K.; Ananthan, S. 1997. "Microbiological screening of Indian medicinal plants with special reference to enteropathogens". *Journal of Alternative and Complement Medicine*, 3: 13-20.

World Health Organisation (WHO) 2002. Department communicable disease surveillance. Definition, Diagnosis, and Classification of Microbial Diseases pp 20

Zy, S. G.; Kuhnt, N.; Rimpler, R. 2007. Biological and pharmacological activities of constituents of *Hyptisverticillata*. *Planta Medica*, **61:** 227 - 232.

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

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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 filteration) 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, 1.28mg/ml and 98×10^{-5}M/min, 2.41mg/ml respectively. Inhibition constant(Ki) to determine binding affinity of inhibitor to enzyme for both crude and partially purified gave 0.17mg/ml and 0.04mg/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 mortal 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 $CaCl_2$ 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 phenolpthaleine 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 $CaCl_2$ 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 phenolpthaleine 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

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 Vmax therefore is 67×10^{-5}M/min, and the Km 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 Ki 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. Vmax obtained is 98×10^{-5}M/min while the Km 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 Ki 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%.

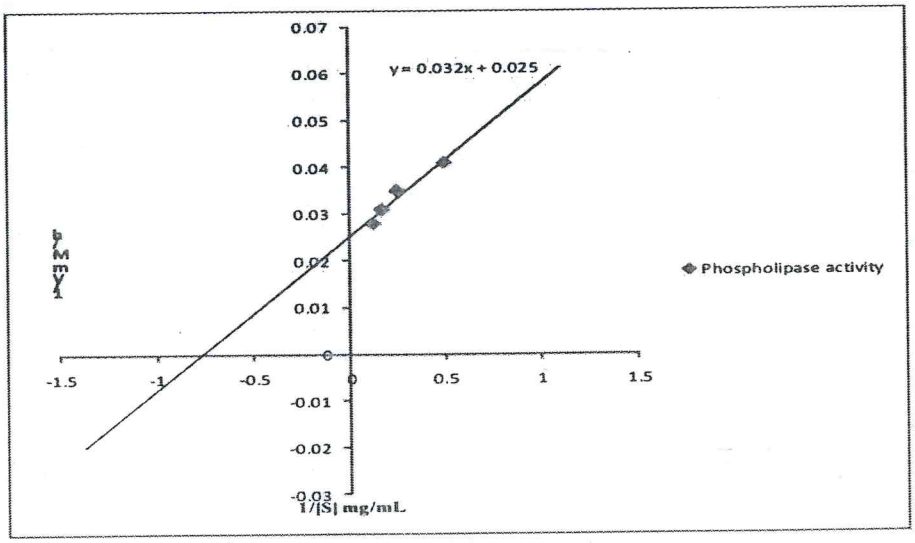

Fig 1: Lineweaver-burk plot for crude phospholipase

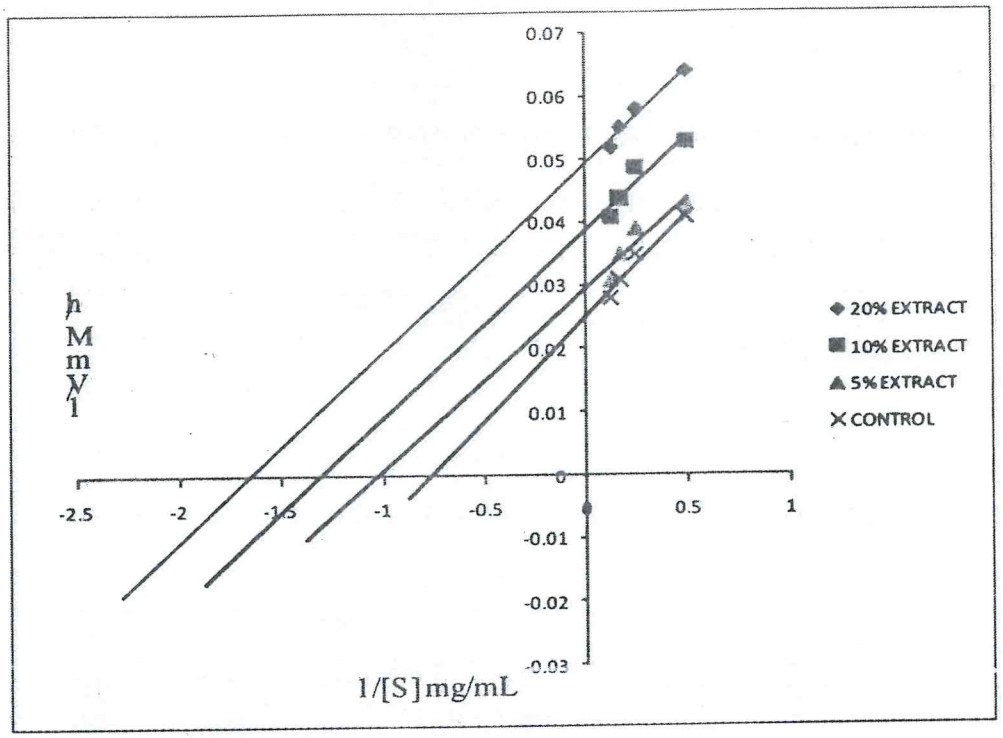

Fig 2: Effect of methanolic extract of *Allium sativum* on crude phospholipase activity

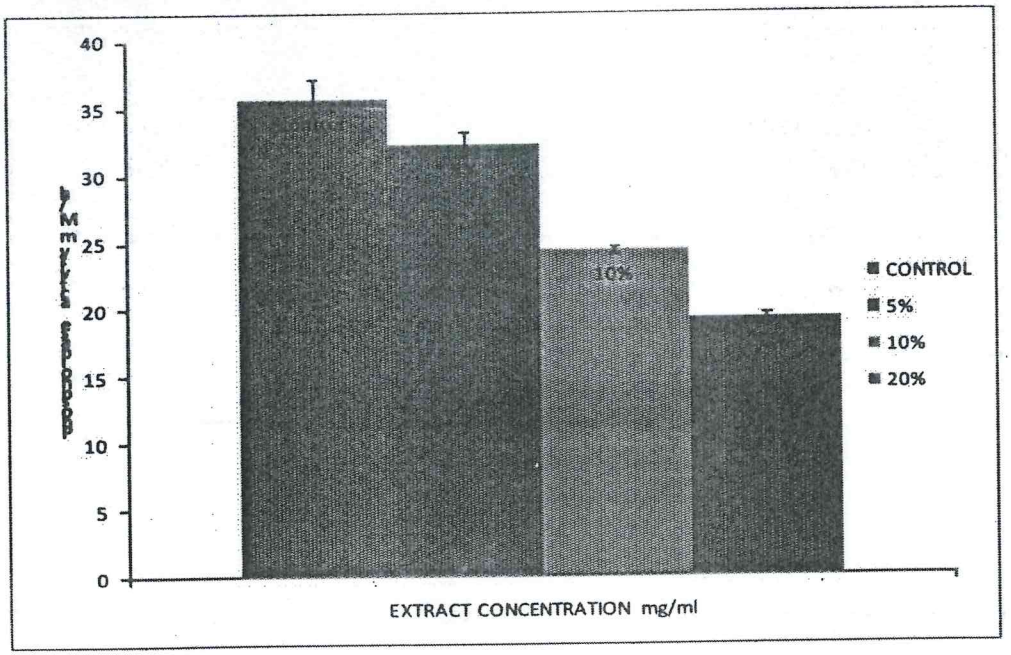

Fig 3: Effect of extract concentration on phospholipase enzyme activity

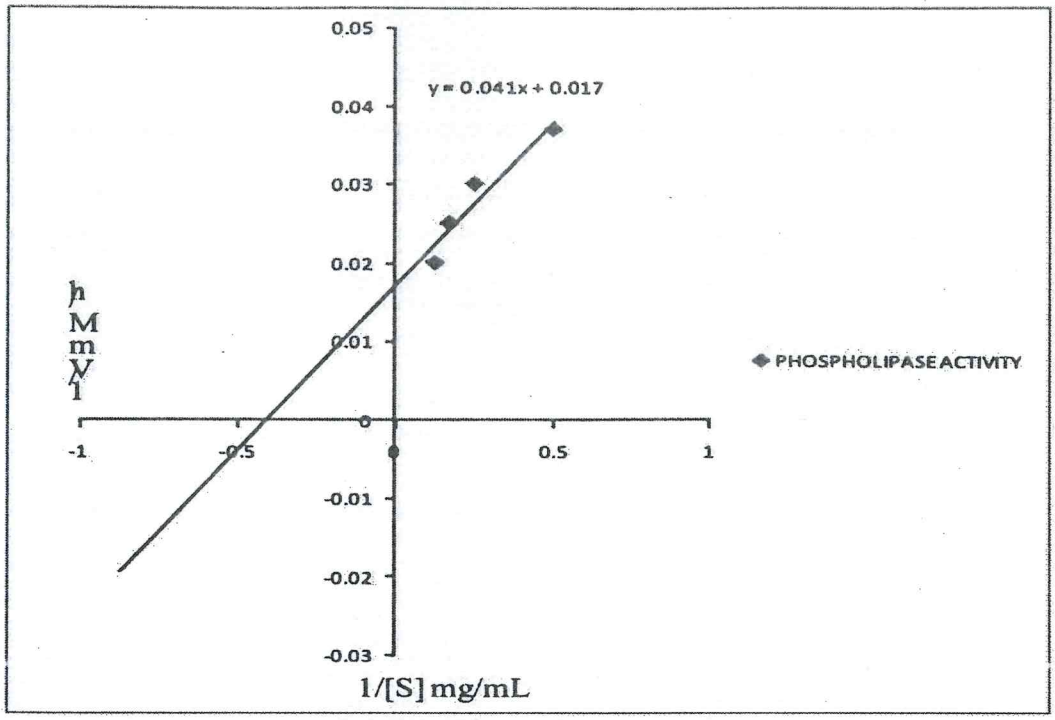

Fig 4: Lineweaver-burk plot for purified phospholipase

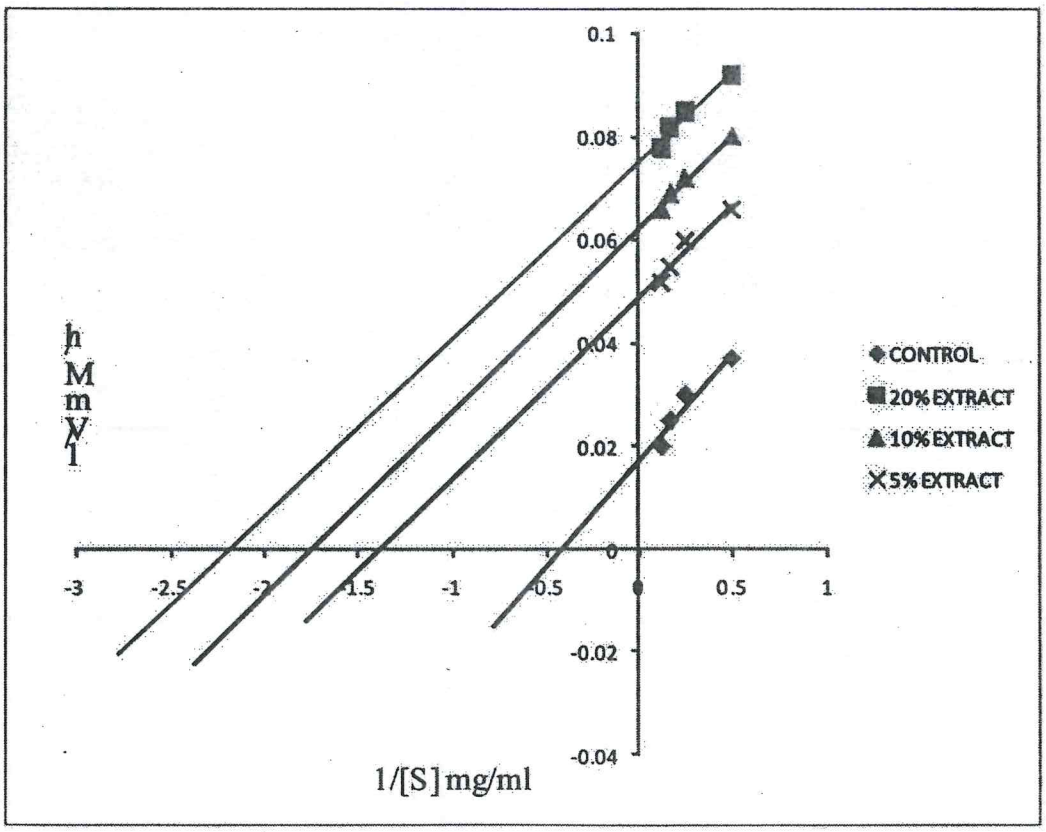

Fig 5: Effect of methanol extract of *Allium sativum* on purified phospholipase activity

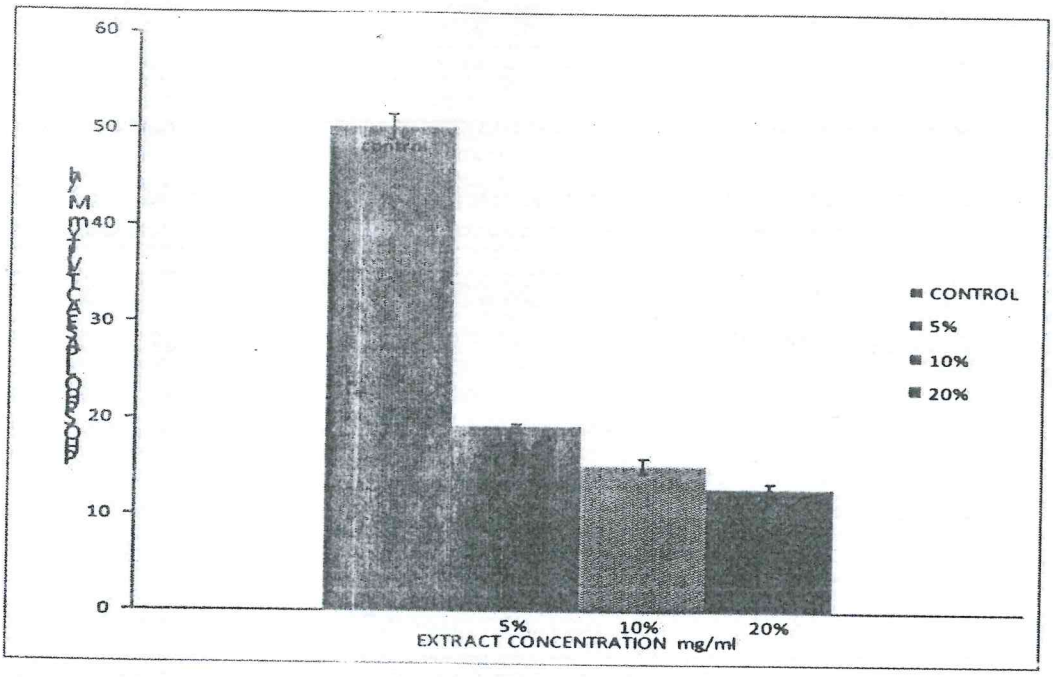

Fig 6: Effect of extract concentration on purified enzyme activity

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_4SO_4 Precipitation	60.41±1.12	1.2±0.78	50.34	75	1.25
Gel Filtration	54.35±1.32	O.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 yet 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.

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, its 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.

REFERENCES

Abubakar, M. S.; Sule, M. I.; Pateh, U. U.; Abdulrahman, E. N.; Haruna, A. K.; Jahum, M. B 2000. Invitro snake detoxifying action of leaf extract of *Guiena senegalensis*. *Journal of enthnopharmacology*, **69:** 253 - 257.

Alam, M. I.; Auddy, B.; Gomes, A. 1994. Isolation, purification and partial characterization of viper venom inhibiting factor from the root extract of the Indian medicinal plant sarsaparilla (*Hemidesmus indicus* R. Br.). *Toxicon.*, 32(12): 1551 -1557.

Alam, M. I.; Auddy, B.; Gomes, A. 1996. Viper venom neutralization by Indian medicinal plants *Hemidesmus indicus* and *Pluchea indica* root extract. *Phytotherapy Research*, **10:** 58 - 61.

Alam, M. I.; Gomes, A. 1998. Adjuvant effect and antiserum action potentiation by a (herbal) compound 2-hydroxy-4-methoxy benzoic acid isolated from the root extract of the Indian medicinal plant 'Sarsaparilla' (*hemidesmus indicus* R.Br.). *Toxicon*, **36(10):** 1423 - 1431.

Alam, M. I.; Gomes, A. 2003. Snake venom neutralization by Indian medicinal plants (*Vitex negundo & Emblica officinalis* Linn.) root extracts. *Journal of Ethnopharmcology*, **86(1):** 75 -80.

Alberts, M.; Shalit, M.; LoGalbo, F. 2004. "Suction for venomous snakebite: a study of "mock venom" extraction in a human model". *Annals of Emergency Medicine* **43(2):** 181 - 186.

Ankri, S., Mirelman, A. 1999. Antimicrobial properties of allicin from garlic. *Microbes Infection*, **2:** 125-129.

Argiolas, A.; Pisano, J. J. 1983. "Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom". ***Journal of Biological Chemistry*, 258(22): 13697- 13702.**

Berg, O. G.; Gelb, M. H.; Tsai, M. D.; Jain, M. K. 2001. "Interfacial enzymology: the secreted phospholipase A(2)-paradigm". *Chemical Revolution*, 101(9): 2613 - 2654.

Bharati, K.; Hasson, S. S.; Oliver, J.; Laing, G. D.; Theakston, R. D.; Harrison, R. A. 2003. Molecular cloning of phospholipase A2 from venom gland of *Echis* carpet vipers. *Toxicon.*, **41:** 941 - 947.

Block, E. 2010. Garlic and Other Alliums: *The Lore and the Science*. Royal Society of Chemistry.

Cannon, R.; Ruh, A. M.; Kashani, J. 2008. Acute hypersensitivity reactions associated with administration of crotalidae polyvalent immune Fab antivenom. *Annals of Emergency Medicine*, **51(4):** 407 - 411.

Cavallito, C. J.; Bailey, J. H. 1994. Allicin, the antibacterial principle of resistant bacteria to garlic extract. *African Journal of Biotechnology*, **6(6):** 771- 776.

Chatterjee, I.; Chakravarty, A. K.; Gomes, A. 2006. Daboia russelli and Naja kauuthia venom neutralization by lupeol acetate isolated from the root extract of Indian sarsaparilla *Hemidesmus indicus* R. British. *Journal of Ethnopharmacology*, **106:** 38- 43.

Chatterjee, I.; Chakravarty, A. K.; Gomes, A. 2004. Antisnake venom activity of ethanolic seed extract of *Strychnos nux vomica* Linn. *Indian Journal of Experimental Biology*, **42:** 468 - 475.

Chippaux, J. P. 1998. Snake-bites: Appraisal of the global situation. *Buletin WHO*, **76:** 515- 524.

Coborn, John 1991. The Atlas of Snakes of the World. New Jersey: TFH Publications.

Dennis, E. A. 1994. "Diversity of group types, regulation, and function of phospholipase A2". *Journal of Biological Chemistry*, 269(18): 13057-1360.

Fry, B. G.; Vidal, N.; Norman, J. A.; Vonk, F. J.; Scheib, H.; Ramjan, R. and Kuruppu, S. 2006. "Early evolution of the venom system in lizards and snakes". *Nature (Letters)*, **439**: 584-588.

Gernot, K. 2005. "Spice Pages: Garlic (*Allium sativum*, Garlick)". http://www.uni-graz.at/~katzer/engl/Alli_sat.html. Retrieved 2007-08-28.

Gomes, A.; Saha, A.; Chatterjee, I.; Chakravarty, A. K. 2007. Viper and cobra venom neutralization by beta sitosterol and stigmasterol isolated from the root extract of *Pluchea indica Less.* (Asteraceae). *Phytomedicine*, **14(9)**: 637-643.

Iwanaga, S.; Suzuki, T. 1979. Enzymes in snake venom. In: Handbook of Experimental Pharmacology.

Jaber, M. A.; Al-Mossawi, A. 2007. Susceptibility of some multiple resistant bacteria to garlic extract. *African Journal of Biotechnology*, 6(6): 771-776.

Leslie, C. C. 1997. "Properties and regulation of cytosolic phospholipase A2". ***Journal of Biological Chemistry*, 272(27): 16709- 16712.**

Mackessy, S. P. 2002. "Biochemistry and pharmacology of colubrid snake venoms". *Journal of Toxicology: Toxin Reviews*, 21(12): 43-83.

Mattison, C. 2007. The New Encyclopedia of Snakes. New Jersey: Princeton University Press, 272. ISBN 978-0691132952.

Mehrtens, J. M. 1987. Living Snakes of the World in Color. New York: Sterling Publishers, 480 p.

Nicolas, J. P.; Lin, Y.; Lambeau, G.; Ghomashchi, F.; Lazdunski, M.; Gelb, M. H. 1997. "Localization of structural elements of bee venom phospholipase A2 involved in N-type receptor binding and neurotoxicity". *Journal of Biological Chemistry*, 272(11): 7173-7181.

Onyeagba, R. A.; Ugbogu, O. C.; Okeke, C. U.; Iroakasi, O. 2006. Studies on the antimicrobial effects of garlic (*Allium sativum* Linn), ginger (*Zingiber officinale* Roscoe) and lime (*Citrus aurantifolia* Linn). *African Journal of Biotechnology*, 3(10): 552-554.

Pan, Y. H.; Epstein, T. M.; Jain, M. K.; Bahnson, B. J. 2001. "Five coplanar anion binding sites on one face of phospholipase A2: relationship to interface binding". *Biochemistry*, 40(3): 609-617.

Rahmy, T. R.; Hammaid, K. Z. 2001. Prophylactic action of garlic on the histological and histochemical patterns of hepatic and gastric tissues in rats injected with a snake venom. *journal of natural toxins*, 10(2): 137- 65.

Sakai, A. 2007. (Clinical feature of envenomation by the snake, Yamakagashi Rhabdophis tigrinus). ***Chudoku Kenkyu*, 20(3): 235-243.**

Sinha, K. 2006. "No more the land of snake charmers...". *The Times of India*. sunflower seeds based on focused microwave-assisted soxhlet textbook of biochemistry by DM Vasudevan and S Sreekumari 5th edition.

Warrell, D. A. 1996. Clinical features of envenoming of snake bites. In: Bon C, Goyffon M, editor. *Envenoming and their treatments*. Lyon, Fond. Marcel Merieux; 63-76.

Yamasaki, T.; Teel, R. W.; Lau, B. H. 1991. "Effect of allixin, a phytoalexin produced by garlic, on mutagenesis, DNA-binding and metabolism of aflatoxin B1". *Nutrition*, 16: 787-788.