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Anti-hemorrhagic Activity of Wild Custard Apple (Annona senegalensis) Ethanolic Leaf Extract on Spitting Cobra (Naja negricollis) Metalloprotease

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Abstract

The main feature of *Naja nigricollis* venom is its high proteolytic activity responsible for most of local and systemic effects observed during envenomation by this snake. In this present work we reported the purification and characteristics metalloprotease from *Naja nigricollis* venom by combination of two step chromatography; a gel filtration (sephadexG-75) and an ion – exchange(DEAE–sephadex –A-50). Ethanolic extraction of the leaf of *Annona senegalensis* was carried out. An hemorrhagic *Naja nigricollis* metalloprotease(NNMP) was partially purified from *Naja negricolis* venom using sephadex G-75 and DEAE-sephadex A-50 column chromatography. Purification of the hemmoraghic metalloprotease was about 11-fold with a total recovery of 5.8% from the crude *Naja negricollis* venom. Biochemical characterization of NNMP show a Km of 10.5M and V_{max} of 45.5 unitsmg-1. The hemorrhagin show optimal stability at P^H 5.5 acetate buffer and thermally stable at 40^0C. NNMP has activation energy as 0.25kjmol-1 and it caused hemorrhage when injected intra-dermally in albino mice suggesting hemorrghic metalloprotease. NNMP hemorrhagic activities were highly inhibited by ethylenediaminetetraacetic acid (EDTA) and the ethanolic leaf extract of *Annona senegalensis*.

Keywords: Hemorrhagic; ethanolic extraction; biochemical characterization; metalloprotease; NNMP; Annona senegalensis.

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1. Introduction

Annona senegalensis or Africa custard apple is a species of plant in the Annonaceae family. The genus name "Annona" is from the Latin word "anon" meaning "Yearly produce" referring to the specific name senegalensis means is "of Senegal" which is where the type of specimen was first collected. Wild fruit trees of this species are found in semi-arid to sub humid in all regions of Africa. The species occurs along river banks, fallow land, and swamp forests. Commonly grows as a single plant in the understory of savannah woodlands. *Annona senegalensis* is a shrub or small tree 2-6meters tall but may reach 11m under favorable condition, with leaf scars and roughly circular flakes exposing paler patches of under bark. The Leaves are alternate, simple, oblong, ovate or ecliptic, of size 6 - 18.5 x 2.5 - 11.5cm. Fruit formed from many fused carpels, with fleshy and lumpy egg shaped; 2.5 - 5 x 2.5 - 4cm.The unripe fruit is green, turning yellow to orange on ripening; stalk 1.5 - 5cm long; produce numerous seeds, which is cylindrical or oblong in shape and orange-brown in color. The leaves are sometimes used as vegetables, while the edible white pulp of the ripe fruit has a pleasant, pineapple-like taste; flowers serves as spice for various meals. Effective insecticide is obtained from the plant bark. The bark is also used for treating guinea worms, diarrhea, gastroentritis,lungs infections, snake bites, toothache and respiratory infections. Gum from the bark is used for sealing cuts and wounds [1;2]. The plant decoction has been reported to be used traditionally in the treatment of sleeping sickness in Northern Nigeria [3].Traditional folks has also reported the use of the leaf of this plant in the treatment of snake bite. It is also used in the folkloric medicine in the treatment of cancer [4]. *Naja negricollis* belongs to the cobra genus Naja under the family Elapidae. *Naja nigricollis* "the black spitting cobra" feeds on rodents. It bites quickly and then waits while its venom damages the nervous system of its prey, like all other snakes, *Naja nigricollis* swallows its prey whole. This species sometimes enters building in search of rodent prey [5]. The spitting cobra eats rats and mice that carry diseases and human food. Its venom is a potential source of medicine, including anti-cancer drugs and pain killer [6]. *Naja nigricollis* is highly venomous and its bite can be lethal, because it hurts rodents that live around people, it is often encountered by accident and many people die each year from *Naja nigricollis* venom poisoning. The spitting cobra (*Naja nigricollis*) is the cause of numerous sebous snake-bite incidents in Africa [7]. Snake venoms are highly modified saliva that contains many different powerful toxins. There are at least 2, 500 species of snakes living at the present time of which over 600 are known to produce venom. Unlike most other predators, all snakes swallow prey whole, so are especially vulnerable to injury if their prey animals are active. Most snake venoms contain specific proteins that paralyze the prey so that it no longer moves, or interfere with normal blood clotting mechanisms, so that the animals goes into shock and begin the process of digestion by breaking down the tissues of the prey animal. Venom also helps to deter predators, and is an important defense mechanism for the snake. The actions of different snake venom are broad and understanding of multiple poisoning processes is desirable in formulation of a satisfactory antidote. Snake venoms are complex mixtures of components with diverse array of actions both on prey and human victims, and they are generally rich sources of water soluble enzymes and polypeptides [8]. Hemorrhage as defined by the "Stedman's Pocket Medical Dictionary" is the bursting forth of blood or an escape of blood from the vessels and it describes hemorrhagic factors as related to or marked by hemorrhage. A definition by [9] describes hemorrhage as excessive bleeding. Most of the hemorrhagins found in snakes such as *Naja Nigricollis* are proteases, they act by hydrolyzing proteins into amino acids, and some are fibrinolytic enzymes causing fibrinogen depletion by direct

degradation of fibrin polymer [10]. Most of the hemorrhagins found in snakes such as *Naja nigricolis* are proteases which could be metalloproteases, serine proteases and others. The hemorrhagic activities of both the snake venom and metalloproteases are inhibited by natural plants, hence the study seek to isolate and characterized metalloprotease from venom of *Naja negricollis* and evaluate the inhibitory ability of ethanolic leaf extracts of Annona senegalensis. Snake bites has pose serious public health problem in many part of the world, especially in Africa, Asia, Latin America and Oceania [11]. Collected data has shown that there are about 1.2 to 5.5 million snakebites yearly, resulting to 25,000 to 125,000 deaths [12]. Despite this alarming death threat pose by snakebites it seem to be neglected by national and international health authorities and other relevant agencies. Thus, snake envenomation is listed by World Health Organization (W.H.O) in 2009 as a Neglected Tropical Diseases (NTDs) [13]. The available treatment for snake venomation is the anti-venom serum therapy, whose effectiveness has limitations. Thus, the search for complementary alternatives for the treatment of snakebites is important [14].

2. Materials and methods

Crude freeze dried venom from *Naja nigricollis* was gotten from Biological science department, of Ahmadu Bello University Zaria, Nigeria. The *Annona senegalensis* leaves were gotten from the wild at zaria in the Northern part of Nigeria. Albino mice were obtained from the Department of pharmacognosy and drug development,Ahmadu Bello University,Zaria,Nigeria.

2.1. Reagents

Sodium Chloride, Ethanol, Tris-HCL, Fibrin, Herpes buffer, sephadex-G-75, DEAE-sephadexA-50, DistilledWater, fibrinogen, Ethylenediaminetetraacetic acid (EDTA), Trichloroacetic acid (TCA), Phyenylmethyl sulfonyl fluoride (PMSF). All chemicals are of analytical grades.

2.2. Purification of the metalloprotease (NNMP)

100mg of freeze dried venom was dissolved in 5ml 0f Tris-Hcl buffer P^H 7.2 to make it up to 20mg/ml. The prepared snake venom was then eluted in a sepadex-G-75 column (1.5x47cm) and DEAE-sephadex-A-50 column (1.6x54cm) which was previously equilibrated with Tris-Hcl buffer, P^H 7.2.The elution was done with 50mM Tris-Hcl buffer,P^H 7.2 at a flow rate of 0.02ml/min as described by [15]. 30 fractions of 5ml/tube of eluent were collected after sepadex-G-75 chromatography, their absorbance at 280nm were read. The prominent peaks were used to check inhibitory study which the fraction with the positive inhibition to EDTA (F24), was eluted in DEAE-sephadex-A-50 column (1.6x54cm) that was previously equilibrated with the same buffer. Different concentration of Nacl (0.1M-0.5M) was used to elute the fraction (F24). The flow rate was 0.06ml/min and 5ml/tube was collected.

2.3. Protein concentration determination

The protein concentration was determined by method described by [16]. This was done by taking absorbance at 260nm and 280nm

Protein concentration = $1.55A_{280}$ - $0.76A_{260}$.

2.4. Metalloprotease activity

Proteolytic activity was tested using fibrin as substrate and following the method described by [17] modified by [18]. 50µl of the partially purified hemorrhagin were incubated with 100µl, 1% fibrin in 0.05M Tris-Hcl buffer, P^H 7.2, for 30mins at 37^0c .The blank was also prepared in a similar manner with the exception of the partially purified hemorrhagin. 200µl, 1M Trichloroacetic acid (TCA) was then added for protein precipitation. The samples were centrifuged for 20mins at 2800xg and the supernatant absorbance was recorded at 280nm.

2.5. Biochemical characterization

2.5.1. Inhibitory studies

The inhibitory studies were done as described by [19]. 50µl of the hemorrhagin, with 50µl of the different inhibitors (EDTA, PMSF, IAA, TRYPSIN) were incubated with 200µl of fibrin in Tris-Hcl buffer P^H 7.2 for 30mins and activity was checked.

2.5.2. Optimum temperature determination

The effect of temperature on the rate of hydrolysis of fibrin by the enzyme was studied at 100c, 25Oc, 400c, 50^Oc, 60^Oc, 70^Oc, 80^Oc, 90^Oc and 100^Oc as described by[20]. It was incubated for one hour and absorbance was taken at 280nm.

2.5.3. Optimum P^H determination

The enzyme optimum P^H was determined as described by [21] with slight modifications. It was studied using various buffers such as acetate buffer at P^H 4.0-5.5, Phosphate buffer at P^H 6.0-7.5 and Herpes buffer at P^H 8.0. The enzyme was incubated with the fibrin in this various buffer P^H for 30mins and absorbance at 280nm was taken.

2.5.4.Thermostability of the enzyme

The residual enzyme activity was studied by incubating the enzyme with herpes buffer P^H 8.0 at temperatures of 10^0c, 25^Oc, 40^0c, 50^Oc and 60^Oc for 15mins. The substrate was added and absorbance at 280mn was taken to get the residual enzyme activity.

2.5.5. Incubation of metaloprotease with substrate

100µ of the enzyme solution was incubated into several concentrations of the substrate, (20mgml-1, 17mgml-1, 15mgml-1, 10mgml-1 and 5mgml-1) at 37Oc for 1hr and absorbance at 280nm was taken to determine activity.

2.6. Ethanolic extraction

110g of the leaves of Annona senegalensis was homogenized and soaked in 600mls of ethanol. The ethanolic extract was evaporated to dryness in a pre-set water bath at 60oc. The green paste of the recovered extract was weighed and used for anti-hemorrhagic studies.

2.7. Hemorrhagic studies

The hemorrhagic studies of the venom and partially purified metalloprotease were tested using the method described by [22]. One group of the mice were intra-dermally injected 2ml doses of 10µg/50µ/20g body weight of the crude venom, some received 46µg/ml/20g body weight of the metalloprotease, other group where given 1.48g/10ml/20g body weight of the extract and the last group received Tris-Hcl buffer P^H 7.2, these were to serve as controls. The test mice received the metalloprotease and the extract in ratio of 1:20, 1:30, and 1:40 respectively. After 3hrs the mice were humanely euthanized and the back skin was removed to check hemorrhagic activity.

3. Results and discussion

3.1. Purification of protein

Crude venom (100mg) was purified by a two-step chromatography. The prepared crude venom was first eluted in a sepadex-G-75 column and 30 fractions were collected. Fractions (F20, F21, F24 and F28) with higher yield ranging from 10.5% to 13.3%. A significant high purification level of the four fractions was also observed, that is between 7 to 9-fold. The total protein observed in the four fractions range from 0.415mg to 0.525mg. Relatively high enzymatic activities were observed among the four fractions (0.167unit-0.183unit). Therefore, fraction (F24) that show the highest yield of 13.3% and highest total protein of 0.525mg as showned on table 1, was selected for further purification.

Table 1: Purification of crude venom using sephadex-75.

Steps	Protein(mg)	Total Protein(mg)	Activity(unit)	Specific Activity(unit/mg)	Yeild(%)	Purification level
SephadexG-75						
Crude venom	0.790	3.950	0.188	0.238	100	1
F20	0.100	0.500	0.167	1.817	12.7	8
F21	0.098	0.490	0.183	1.867	12.4	8
F24	0.105	0.525	0.178	1.695	13.3	7
F28	0.083	0.415	0.173	2.084	10.5	9
TOTAL	0.386		0.701			

The crude venom from *Naja nigricollis* (100mg) was applied into a sephadex G -75 columns, resulting in four peaks (fig 1). Proteolytic activity upon fibrin was investigated for peaks 1, 2, 3 and 4 [23]. Furthermore, peak 3 show positive inhibitions by EDTA

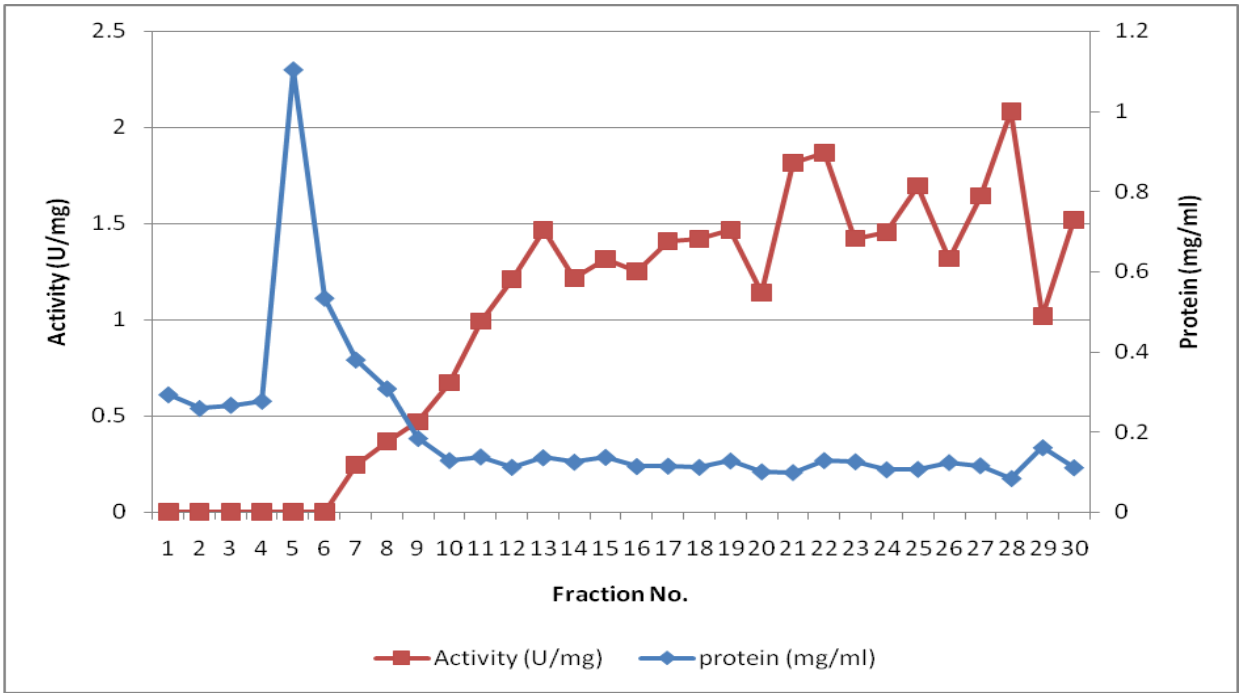

Figure 1: Elution profile for the Gel chromatography of *Naja nigricollis* protease on Sephadex-G-75 eluted with 50mM Tris-Hcl buffer,P^H 7.2 at a flow rate of 0.02ml/min.

Fraction (F24) was further eluted by DEAE Sephadex-A50 and 30 fractions was collected. Four peaks was observed(F7, F15, F18,and F25) with high purification level ranging from 5 to 11-fold. A significant decrease in enzymatic activity(0.018unit-0.136unit),total protein(0.230mg-0.370mg) and yield (5.8%-9.37%) was observed as shown on table 2. Fraction (F18) with the highest purification fold(11-fold) was selected for biochemical characterization.

Table 2: further purification of fraction 24 (F24) isolated, this time using DEAE Sephadex-A50.

Steps	Protein(mg)	Total Protein(mg)	Activity(unit)	Specific Activity(unit/mg)	Yeild(%)	Purification level
DEAE Sephadex-A50						
F24**						
F7	0.049	0.245	0.063	1.286	6.2	5
F15	0.064	0.320	0.136	2.125	8.1	9
F18	0.046	0.230	0.018	2.526	5.8	11
F25	0.074	0.370	0.031	2.395	9.37	10
TOTAL	0.233		0.248			

Since our aim was to partially purify metalloprotease, peak 3 was then re-chromatographed in DEAE–sephadex–A-50 (fig 2). Four prominent peaks were selected for proteolytic activity test.

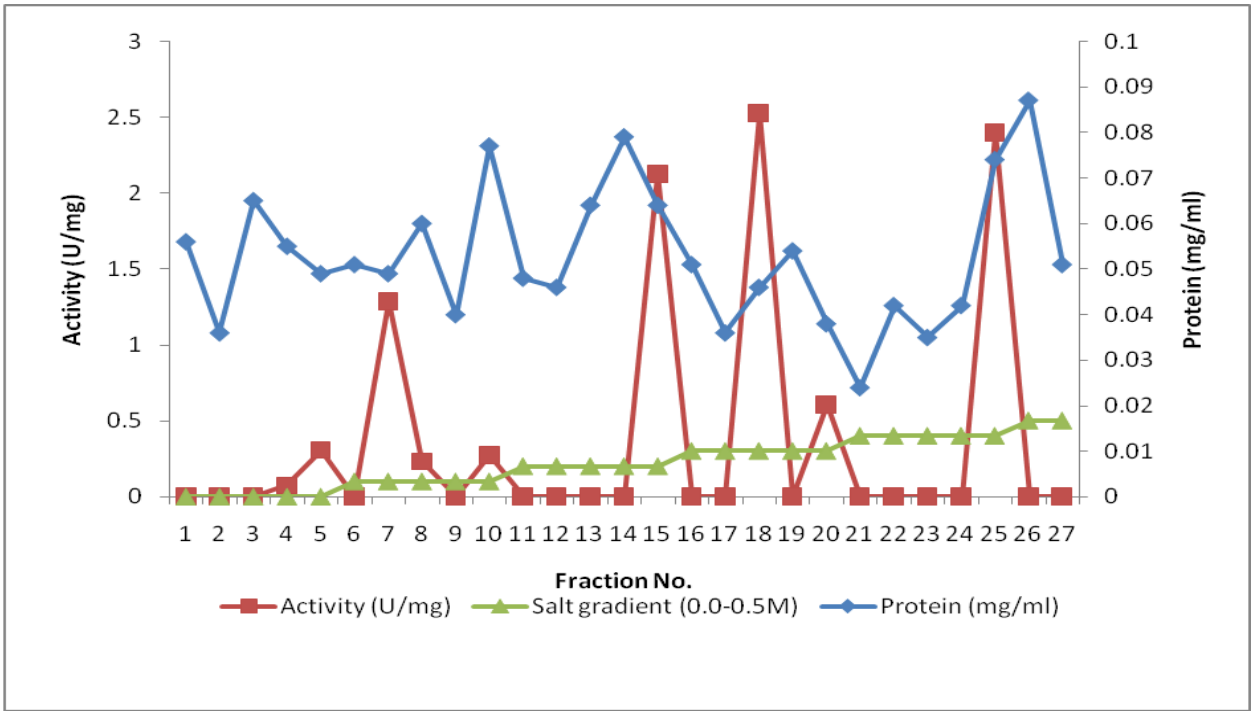

Figure 2: Elution profile for ion- exchange chromatography of *Naja negricollis* metalloprotease on DEAE-sephadex-A-50 equilibrated with 50mM Tris- HCl buffer pH 7.2 at a flow rate of 0.06ml/min and step gradient elution with NaCL of 0.1-0.5M.

3.2. Biochemical characterization

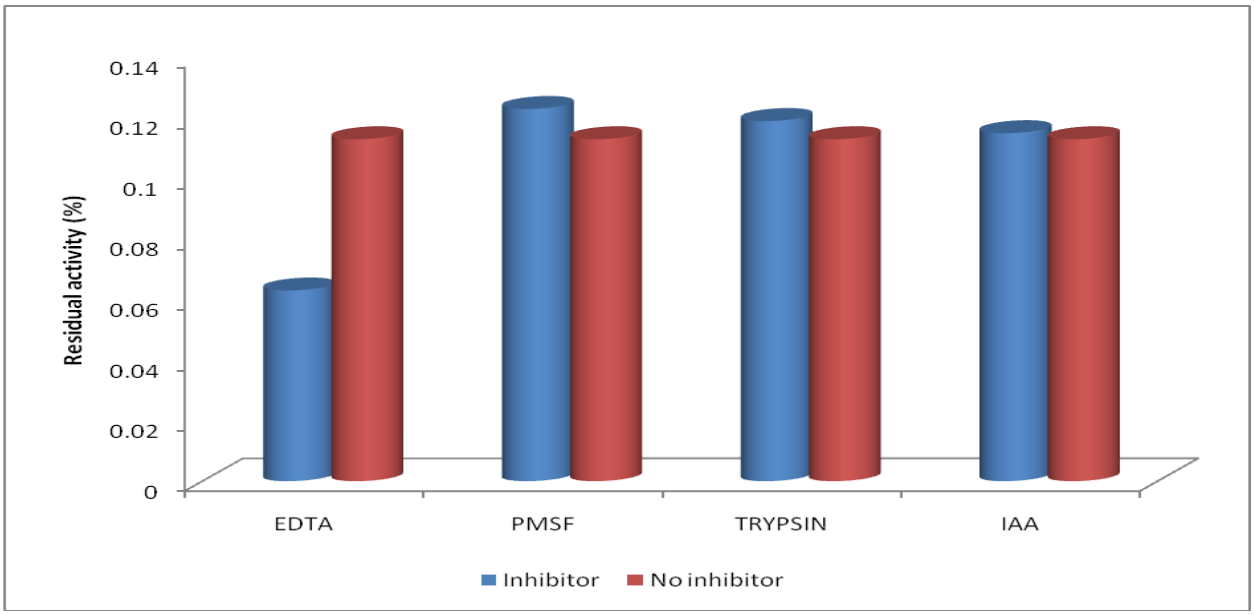

Figure 3: Inhibition of metalloprotease activity by various inhibitors.

The effect of inhibitors on proteolytic activity was tested upon fibrin with EDTA, PMSF, IAA and TRYPSIN (fig 3).These tests reveal that the *Naja nigricollis* metalloprotease is a protease ion–depedent since its

proteolytic activity upon fibrin was inhibited by EDTA. The fibrinolytic activity was not inhibited by PMSF, IAA, and TRYPSIN inhibitors. With respect to the results of the inhibitors we can include *Naja nigricollis* metalloprotease in the large class of venom (SVMPs) [24]. Temperature studies of the enzyme show that it has optimal activity and thermally stable at 40^{O}c. Subsequent temperature increase leads to denaturation of the secondary, tertiary and quartnary structures of the enzyme. The great increase in proteolytic activity of the enzyme at temperature between 20^{0}c to 40^{0}c was in accordance to *Bothrops moojeni* metalloprotease obtained by[25]. The metalloprotease is thermally stable at temperature 40^{0}c where the enzyme show high activity and low activity was observed at temperature between 60^{0}c to 100^{0}c as shown in figure 4.

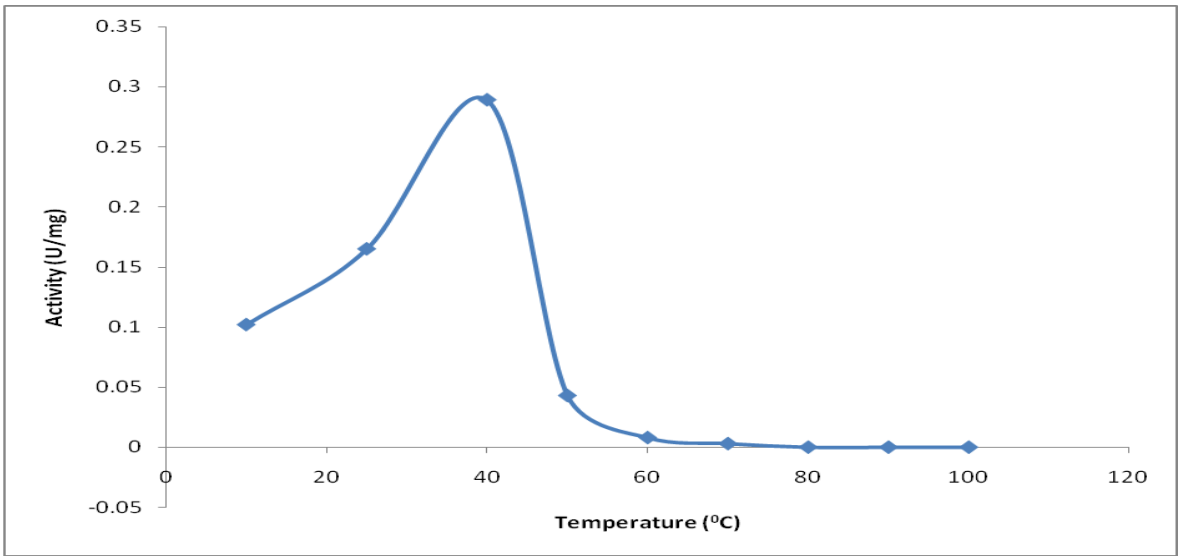

Figure 4: Temperature optimum of *Naja nigricollis* metalloprotease.

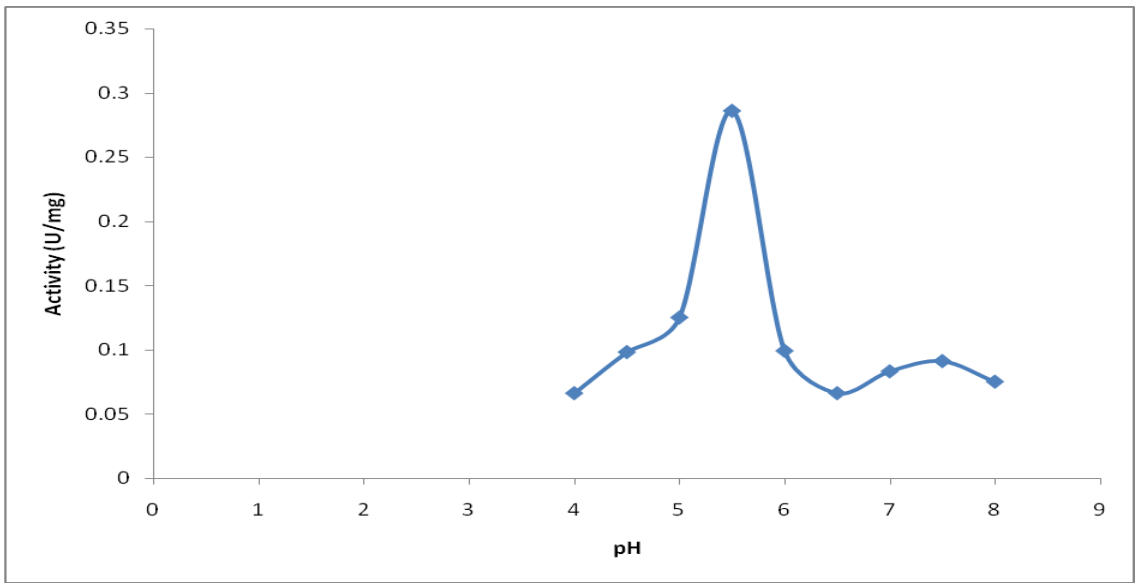

Figure 5: p^{H} optimum of *Naja nigricollis* metalloprotease .The reaction mixture contained in 100µl of fibrin, suitable amount of enzyme and 50mM acetate buffer (p^{H} 4.0-5.5), phosphate buffer (6.0-7.5), and herpes buffer 8.0.

At P^H 4 to 5.9 the enzyme activity show great increase with its peak at P^H 5.5 and activity decreases from P^H 6 t0 9 as seen in figure 5. The enzyme optimal P^H 5.5 was slightly different from that of *Bothrops moojeni* metalloprotease which was optimal between P^H 6 to 9. The slight P^H different might be due to differences of source, the *Naja nigricolis* metaloprotease might be from an acidic source. Activation energy of 0.25kjmol^{-1} indicates that the enzyme require little energy to carry out proteolytic activity. These results are found in the literature for other proteases from this class [26].

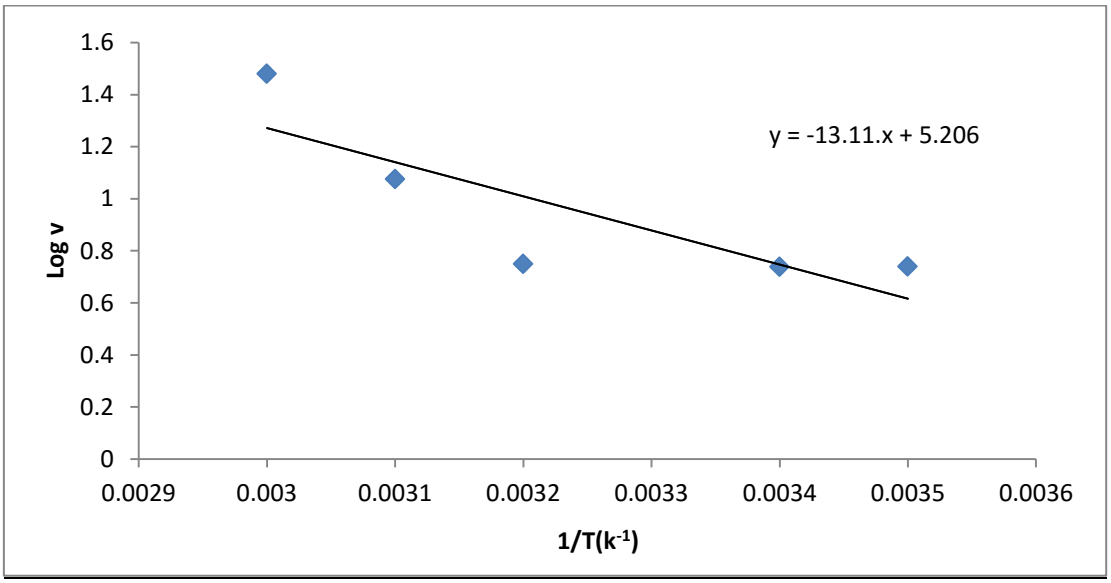

Figure 6: A graph showing the Arrhenius plot for the determination of Activation Energy of the *Naja nigricollis* metalloprotease. The activation energy were estimated to be 0.25Kjmol^{-1}.

The K_m of the enzyme was seen as 10.5M and a V_{max} of 2.8unitmg^{-1}.

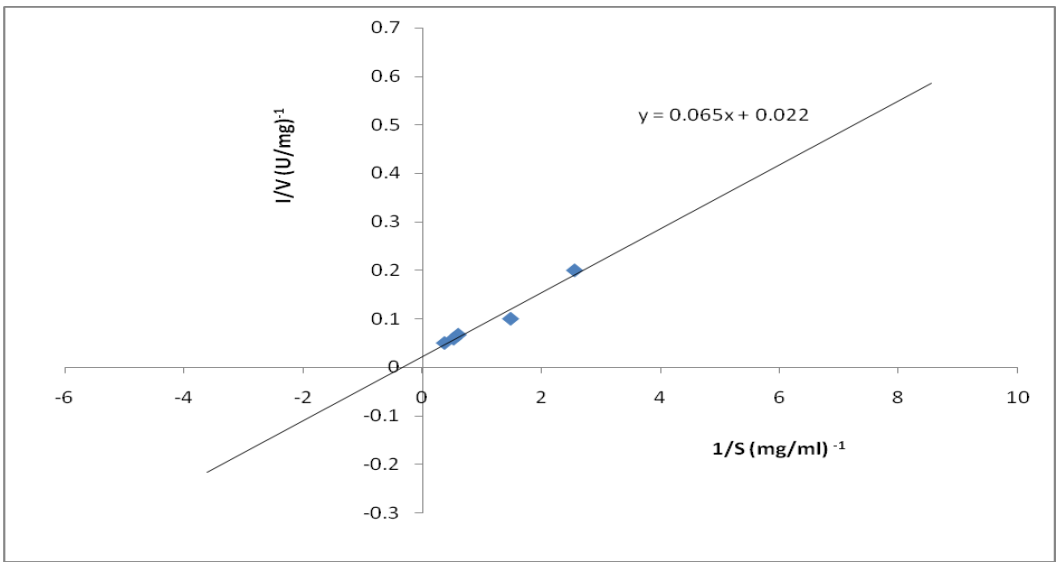

Figure 7: Line-Weaver Burk Plots of Initial Velocity data for the determination of K_M and V_{max} for *Naja nigricollis* metalloprotease.

3.3. Hemorrhagic studies

The dosage of 46µgml^{-1}/20g body weight of mice in 3hrs revealed hemorrhage and ethanolic extract of *Annona senegalensis* act as anti-hemorrhagic substance. The ethanolic extract drastically inhibits the development of hemorrhage as its concentration increases. It was deductively seen that administering of 1:40 of hemorrhagin to ethanolic extract, a clear skin was obtained (no hemorrhage). Also the same proportion of the ethanolic extract with crude venom inhibits hemorrhage. This indicates that the extract does not only stop hemorrhagic activity of metaloprotease but also other hemorrhagic enzymes or substances. These results suggest a better research on structure and function of metaloprotease and a probable therapeutic approach.

Figure 8: The hemorrhagic studies and the anti-hemorrhagic activity of ethanolic leaf extract of *Annona senegalensis* on the hemorrhagic metalloprotease in mice.

Plate numbering is from left top to bottom and so on. Plate 1= C, Plate 2= HG, Plate 3= E

Plate 4= HG : E (1:20), Plate 5= HG : E (1:30), Plate 6=HG : E (1:40), Plate 7= C:E (1:40), Plate 8= Control.

Key: C= Crude venom, E= Extract, HG= Hemorrhagin.

4. Conclusion

This research shows the presence of a metalloprotease from *Naja nigricollis* Venom. metalloprotease was also inhibited by ethylenediamine tetraacetate (EDTA) with Iodoacetate (IAA), phenylmethylsulfunylflouride (PMSF) and Trypsin showing no significant effect. The metalloprotease was optimally stable at 40^{o}c and has an optimal pH of 5.5 acetate buffer. The purification of the metalloprotease was about 11-fold with a total recovery of 5.8%. The V_{max} of the metalloprotease was seen as 2.8unitmg^{-1} and has K_M 10. 5M. The metalloprotease causes hemorrhage when intra-dermally injected into mice. Hemorrhagic activity of the metalloprotease was highly inhibited by the ethanolic extract of the leaf of *Annona Senegalensis.*

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