Original Article

Evaluation of Mango Seed Kernel Methanolic Extract on Metalloproteases in Carpet Viper (*Echisocellatus*) Venom: An in Vitro Experiment

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ABSTRACT

Background: The global incidence of snakebite has become a major concern to the community. This study aimed to evaluate the effect of mango seed kernel methanol extract on metalloproteases in Carpet Viper (*Echis ocellatus*) venom.

Methods: Mango seed kernel methanolic extract was evaluated in vitro for its anti-venom activity and inhibition of metalloproteases of Carpet Viper's (*Echis ocellatus*) venom. Metalloprotease portion was partially purified from the venom of *E. ocellatus* with a yield of 71%, a purification fold of 2.63 and a specific activity of 19.00 µmol/min/mg protein.

Results: The enzyme appeared as a band on SDS-PAGE with a molecular weight of 23 kDa. The kinetic properties of the enzyme showed a K_m of 0.31 mg mL^{-1} and a V_{max} of 9.09 µmol min^{-1}. When the enzyme was incubated with the extract, kinetic studies revealed a mixed non-competitive pattern of inhibition with K_m values of 0.56 and 1.11 mg mL^{-1} and V_{max}values of 6.67 and 4.17-µmol min^{-1} for 5% and 20% inhibitor concentrations, respectively. An estimated K_i value of 0.168 mg mL^{-1} was obtained from a secondary plot demonstrating that the extract had a high affinity for the partially purified enzyme; thus, could serve as an effective inhibitor.

Conclusion: Methanol extract of mango seed kernel has a high affinity for the partially purified enzyme, and it might provide an inexpensive and readily available alternative to sheep serum in the management of snakebite envenomation. Therefore, further in vivo studies are necessary to assess its effectiveness and safety.

Keywords: Mangifera, Metalloproteases, Snake Bites, Viperidae, Viper Venoms.

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INTRODUCTION

Snakebite is a common, devastating environmental, and occupational hazard, especially in rural areas of tropical developing countries like Nigeria. One of the most common poisonous snakes in Nigeria is West African carpet viper (*Echis ocellatus*) from the Viperidae family. It accounts for 90% of bites and 60% of the fatalities in this country, which add up to 20% of all African cases [1-6]. In humans, envenomation by *E. ocellatus* causes severe blistering, edema, and necrosis at the bite site, and life-threatening systemic effects including hemorrhage, coagulopathy, hemotoxicity and occasionally hypovolemic shock [2, 3, 5]. Snake venom metalloproteases (MPs) are common components in many snake venoms, especially in Viperidae and play key roles in envenomation. Mango (*Mangifera indica Linn*.) is one of the most important tropical fruits in the world, including Nigeria. The kernel content of the seeds ranges from 45.7% to 72.8% and comprises about 20% of the whole fruit depending on the variety [7]. Mango seed kernel extract is very rich in polyphenols and has been pharmacologically documented to have antioxidant, anti-tyrosinase, anti–inflammatory, and hepatoprotective effects as well as anti-enzymatic activities against snakes' venom [8, 9].

World Health Organization has mentioned snake bite as an ignored disease occurring mostly in Asia and Africa, with an estimated 5.5 million

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bites each year, resulting in 2.5 million envenomations and 125000 deaths worldwide [5]. "Antivenom immunotherapy which has been the only specific treatment against snakebite envenomation is burdened with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness" [10].

In addition, production of antiserums in animals is time-consuming, expensive and requires ideal storage conditions. In Africa, especially in Nigeria, the availability of these antivenoms is restricted, thus, limiting the use of serum therapy [11-14]. The use of natural venom inhibitors could complement or substitute sheep serum anti-venoms and could minimize the socio-medical problems of snakebites in tropical countries. Mango seeds are readily available in Nigeria as agro-waste and might provide a cheaper and more accessible alternative for snake antivenoms.

This study therefore aimed to evaluate the effect of mango seed kernel methanolic extract on metalloproteases in Carpet Viper (*E. ocellatus*) venom

MATERIALS AND METHODS

Snake Venom

The study was conducted between March, 2014 and June, 2015 at the Department of Biochemistry, Ahmadu Bello University (ABU), Zaria-Nigeria. Freeze-dried *E. ocellatus* venom was obtained from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University (ABU), Zaria-Nigeria.

All procedures were in accordance with ethical guidelines for care and use of laboratory animals. The study was approved by the Experimental Animals Committee of Ahmadu Bello University, Zaria Nigeria.

Seed Kernel

Mangifera indica (mango) seeds were gathered from Zaria metropolis and identified in the Ahmadu Bello University herbarium Zaria – Nigeria.

Reagents

Sephadex G-75 and DEAE-cellulose were purchased from Sigma Chemical Co. St. Louis, MO, USA. All other chemicals were obtained from reputable chemical companies.

Preparation of Extract

The kernels were manually removed from the seeds' coat and then were sun-dried and grounded to powder. Then the extraction was carried out with methanol using soxhlet apparatus. Subsequently the solvent was removed by rotary evaporator under reduced pressure to obtain the crude extract.

Partial Purification of Metalloproteases

Ion Exchange Chromatography on DEAE Sephadex

DEAE-cellulose was prepared by dissolving 2 g of anion-exchanger in 20 ml of phosphate buffer, pH 7.4. The slurry was then poured into a 3.0 X 20 cm column. Crude *E. ocellatus* venom (100 mg) was dissolved in 10ml phosphate buffer, pH 7.4 in a beaker. This was transferred to a centrifuge tube and the insoluble components were removed by centrifugation. Then 2 ml of the recovered supernatant was loaded onto DEAE Sephadex column (3.0 x 20 cm) pre-equilibrated with 0.2 M phosphate buffer pH 7.4. The column was eluted stepwise with NaCl gradient (0.0 – 0.5 M) at a flow rate of 1ml per minute. Thirty fractions were collected and assayed for metalloprotease activity and total protein. The fractions showing highest specific activities were pooled.

Gel Filtration Chromatography on Sephadex G-75

The gel was prepared by dissolving 2 g of Sephadex G-75 in 20 ml phosphate buffer, pH 7.8 for 24 h at room temperature and then mixed with a glass rod to float the swollen particles from the slurry. The slurry was then poured into a 2 by 100 cm column packed with glass wool at the bottom. The column was first equilibrated with phosphate buffer, pH 7.8 before the sample was applied. The pooled metalloprotease active fractions from the ion exchange chromatography were loaded onto Sephadex G-75 column equilibrated with phosphate buffer (pH 7.4). The column was eluted with the same buffer, maintaining a flow rate of 1ml/min.

The most active fraction was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis according to the method of Laemmli, [15] and was prepared with silver staining.

Metalloprotease Assay

Metalloprotease assay was carried out according to an earlier study [16]. In brief, 0.5 ml of 20 mg/ml enzyme solution with 0.05 ml of 1 mg/ml casein solution in 0.2 M phosphate buffer pH 7.5 was incubated for 30 min at 50 °C. The reaction was stopped by adding 1.0 ml of 10% TCA and the absorbance of TCA-soluble peptide was measured at 280 nm [17]. A control assay, without the enzyme in the reaction mixture, was also carried out and used as the blank in all spectrophotometric measurements.

Effect of the Extract on the Partially Purified Metalloproteases

Varying concentrations of the extract preparations (0, 5, 10 and 20% w/v) were used as substrate to inhibit the partially purified metalloproteases. The initial velocity data obtained was used for double reciprocal plots and a secondary plot was obtained from the primary plot to determine the inhibition binding constant (K_i) of the extract.

RESULTS

Purification of Metalloprotease Enzymes from Echis Ocellatus Venom

The results of partial purification of metalloproteases from *E. ocellatus* venom are summarized in Table 1. The crude extract contained about 1.10 mg of protein with total and specific activities of 10.17-µmol/min and 9.25 µmol/min/mg of protein, respectively. Fractionation of crude venom on DEAE cellulose chromatography produced a specific activity of 11.71 µmol/min/mg. Subsequent gel filtration on Sephadex G-75 chromatography demonstrated an active peak (Figure1) with a specific activity of 19.00 µmol/min/mg of protein, 2.63 purification fold, and 71% recovery.

The elution profile of MPs from ion exchange chromatography on DEAE Cellulose showed four major peaks as shown in Figure 2. These fractions had the highest metalloprotease activity when the eluates were assayed for activity with casein and thus were pooled for the next purification step.

The elution profile of MPs on Sephadex G-75 pooled from DEAE Cellulose active fractions showed one prominent peak with the highest metalloprotease activity as shown in Figure 1. When the eluates were subjected to treatment with the active fraction of the mango seed kernel extract, metalloprotease activity was significantly reduced, suggesting that the active fraction of mango seed kernel extract had some anti-metalloprotease activity against *Echis ocellatus* venom.

The purification profile (Table 1) showed that the metalloprotease specific enzyme activity for crude venom was 9.25 µmol/min/mg proteins. However, when it was subjected to purification steps, the specific enzyme activity increased to 19.00 µmol/min/mg proteins with a total yield of 71%.

The double reciprocal plot of partially purified MPs obtained from *E. ocellatus* venom demonstrated that the enzyme had an estimated K_m of 0.31 mg/ml and V_{max} of 9.09-µmol/min (Figure 3).

Result of SDS-PAGE for Metalloprotease

The purity and the molecular weight of the partially purified inhibitory metalloproteases were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Figure 4 shows the electrophoretic pattern of the sample under denaturing conditions. A faint band of the protein sample was visualized against the standard marker proteins on the gel and the molecular weight of partially purified metalloproteases (lane 3 and 4) was estimated to be 23 kDa.

Lineweaver-Burk and the Secondary Plot of the Inhibition of Partially Purified Metalloprotease Activity by the Extract

The Lineweaver-Burk (double reciprocal) plot of the inhibition of metalloprotease activity by the most active fraction of the mango seed kernel extract in the presence of the substrate suggested the presence of non-competitive inhibitory effects with K_m values of 0.56 and 1.11 mg mL^{-1} and V_{max}values of 6.67 and 4.17 µmol min^{-1} for 5% and 20% inhibitor concentrations, respectively. Whereas, the solution with 0% of inhibitor showed a K_m of 0.31 mg mL^{-1} and a V_{max} of 9.09 µmol min^{-1}. The secondary plot of the Lineweaver-Burk plot showed an estimated K_i (inhibition binding constant) value of 0.168 mg mL^{-1}(Figure 5 and 6).

Table 1. Purification Table for metalloproteases.

Purification step	Protein (mg)	Total enzyme activity (µmol/min)	Specific enzyme activity (µmol/min/mg protein)	Purification fold	% yield
Crude venom	1.10	10.17	9.25	1	100
DEAE Cellulose	0.45	5.27	11.71	1.27	52
Sephadex G-75	0.38	7.22	19.00	2.63	71

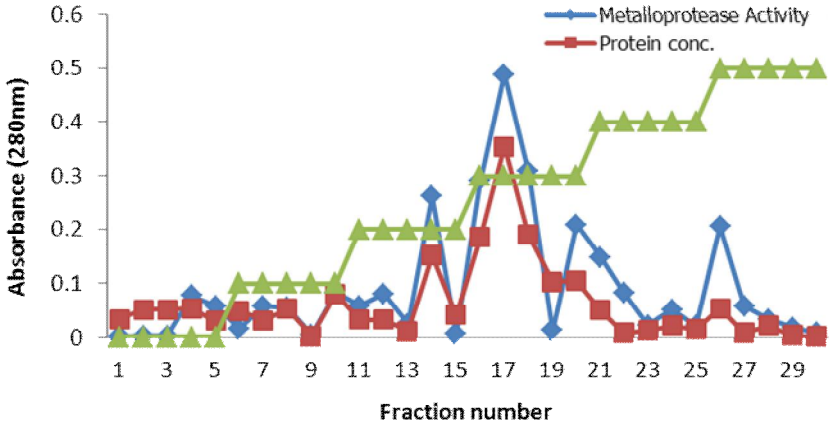

Figure 1. Elution profile of partially purified metalloproteases from *E. ocellatus* on Sephadex G-75 column chromatography.

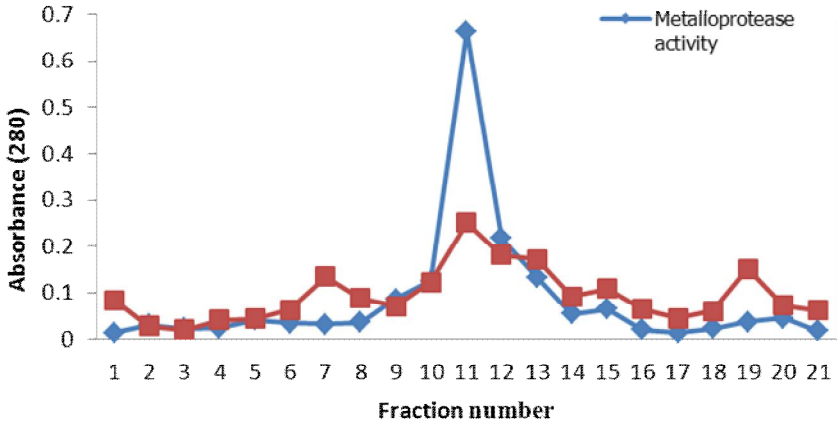

Figure 2. Elution profile of ion exchange chromatography of metalloproteases on DEAE Sephadex.

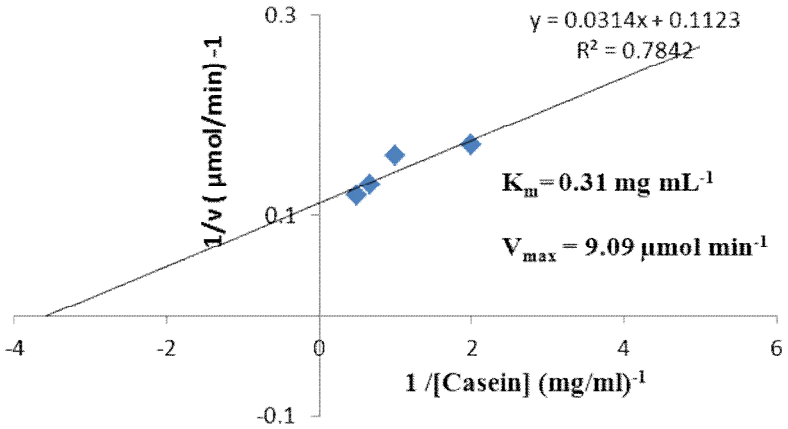

Figure 3. Double reciprocal plot of partially purified metalloprotease from *Echisocellatus* venom, showing K_m and V_{max}.

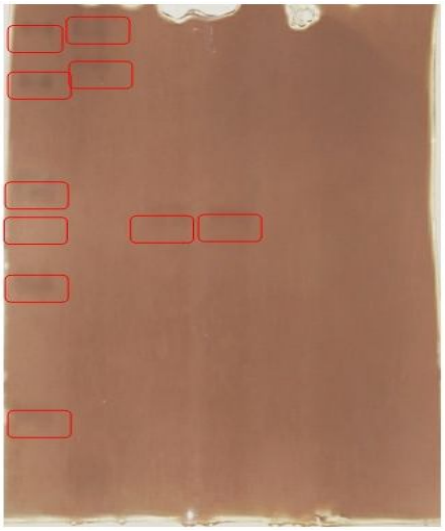

Figure. 4. Electrophoretic pattern of partially purified metalloprotease of *Echisocellatus* on SDS-PAGE.

Key: Lane 1= molecular weight marker, Lane 3 and 4 = Partially purified metalloprotease (23kDa)

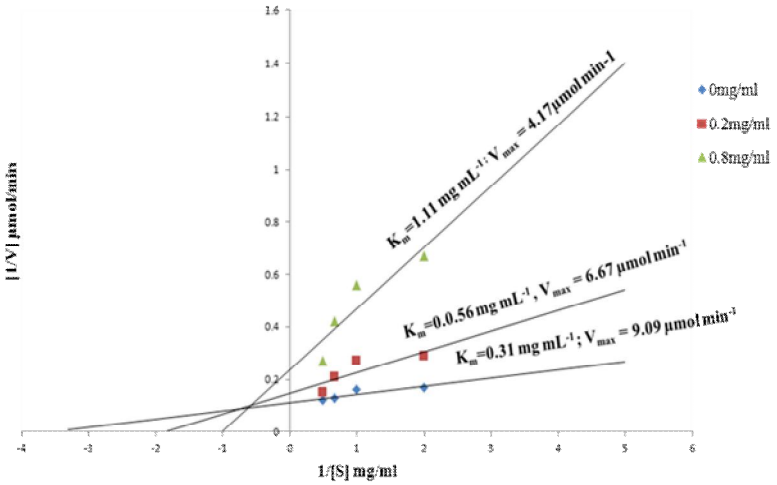

Figure 5. Double reciprocal plot showing the effects of different concentrations of the extract with most inhibitory effect on partially purified metalloproteases activity.

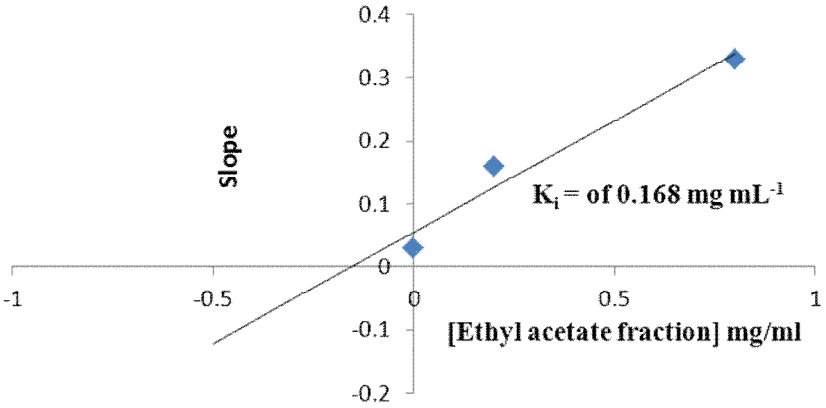

Figure 6. Secondary plot of slope against inhibitor concentration showing the inhibition binding constant (K_i).

DISCUSSION

The global incidence of snakebites and their associated mortality are overwhelming and the majority occurs in rural areas of resource-poor countries; therefore, many victims do not seek hospital treatment but prefer traditional remedies [18, 19]. Plant extracts have been traditionally used as folk medicine in the treatment of snakebites all over the world [8, 9, 12, 20], which is of particular importance especially in resource-poor countries where antivenin is not readily available. As a result, efforts are being made by research groups to identify more traditionally available plant extracts that could curb the menace of snakebite envenomation [18, 20].

Thus, this study has been carried out to evaluate the potency of mango (*M. indica*) seed kernel methanolic extract and its different solvent fractions in ameliorating snake envenomation. Crude methanolic extract of mango seed kernel has significant inhibitory activity against metalloproteases present in the venom of *E.ocellatus,* believed to be the major component of venom from the Viperidae family, and is responsible for the anticoagulant effect, which is the primary cause of mortality in snakebite victims. The methanolic extract of *Giurera senegalensis* inhibited metalloprotease and phospholipase A_2 present in *E. ocellatus*'s venom [21]. Mango's methanolic extract has inhibitory effect on enzymatic activities of *Naja nigricollis* venom [9]. In addition, Kapok tree (*Ceiba pentandra*) leaves extract neutralizes *E.ocellatus* venom [22].

In this study, metalloproteases were partially purified from *E. ocellatus* venom with an increase in purification from 1.27 to 2.63 folds.The specific activity increased from 11.71 µmol/min/mg proteins to 19.00 µmol/min/mg proteins. These findings are similar to another study where an increase in purification fold and specific activity of the crude venom metalloprotease after the two purification steps could be attributed to the removal of other synergistically interacting components of the venom [23]. The partially purified metalloproteases showed a distinct band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with an estimated molecular weight of 23 kDa. This result is also in line with the work of Gomes et al [24] that a metalloprotease (BthMP) from Brazilian lance head (*Bothrops moojeni*) venom with a molecular weight of 23.5 kDa. Furthermore, a metalloprotease with a molecular mass of approximately 23 kDa was purified from the venom of fer-de-lance (*Bothropsasper*) snake [25], which is similar to other previously identified metalloproteases such as *B. asper* hemorrhaging BH2 and BaP1 with molecular masses of 26 and 24 kDa, respectively [26]. The low K_m value of 0.31 mg/ml and the high Vmax value of 9.09 µmol/min obtained is an indication of high affinity of the partially purified enzyme for its substrate, which further substantiates the observed toxicities in Viperidae snakes as a result of the activities of metalloproteases [22, 23].

There are several compounds with diverse chemical structures known in plants which have been accounted for with the ability to interact with peptides and proteins (enzymes) of snake venom. The mechanism of action of these compounds are still not clear and might be attributable to inactivation of molecular structures prone to chemical attacks that may block the active sites of the snake venom components. Another mechanism of action of the plant compounds is inhibition of metalloproteases present in the snake venom. This is due to the metal chelator substances in the plant extracts [27]. The results in Table 2 reveal a reduction in the activity of the enzyme by 58% when treated with the crude extract while treatment with the active fraction showed a further inhibition of 63%. This suggests that the ethyl acetate fraction contained more active constituents than the methanolic extract, and thus is more effective in the inhibition of metalloproteases' activity of *E. ocellatus*' venom. In another study by WHO, the methanolic extract of the seed kernel of *M. indica* was reported to have inhibitory effect on the phospholipase A_2 activity of black-necked spitting cobra's (*Najanigricolis*) venom [5]. The Lineweaver-Burke plot of varying concentrations of the extract on the substrate indicated that the extract inhibited the enzyme in a non-competitive manner with increasing K_m and decreasing V_{max}. In a related study[11], a non-competitive pattern of inhibition of metalloprotease, activity of saw-scaled, viper's (*E.carinatus*) venomwas reported by aqueous extract of *Guiera senegalensis* leaves that is also in consonance with Ibrahim et al.[12] findings.

In this study, the low K_i (inhibition binding constant) value of the extract for the metalloproteases is an indication of a high affinity of the extract for these enzymes. Therefore, mango seed kernel could be used in designing novel *E. ocellatus* antivenins.

Table 2. The inhibitory effects of the methanol extract of *Mangiferaindica* seed kernel and its solvent fractions on the metalloproteases activity of *Echisocellatus*venom.

Group (0.1mg/ml)	Metalloproteases Activity (µmol/min)	Relative Enzyme activity (%)	% Inhibition
Blank (Buffer only)	2.76 ± 0.02	0	0
Venom+ Buffer	10.17 ± 0.01	100	0
Venom + Methanol Extract	4.31 ± 0.01	42	58
Venom + Ethyl acetate fraction	3.74 ± 0.02	37	63
Venom + *n*-Butanol fraction	8.64 ± 0.01	85	15
Aqueous fraction	8.71 ± 0.02	86	14

CONCLUSION

The results revealed a mixed non-competitive pattern of inhibition of metalloproteases activity with an estimated K_i value of 0.168 mg mL^{-1}, demonstrating that methanol extract of mango seed kernel has a high affinity for the partially purified enzyme. Therefore, it might provide an inexpensive and readily available alternative to sheep serum in the management of snakebite envenomation. Further in vivo studies are necessary to assess its effectiveness and safety.

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