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The Inhibitory Potentials of Seed Kernel Methanol Crude Extract and Fractions of *Mangifera indica* L. Anacardiaceace on Metalloprotease-induced Toxic Effect of *Echis ocellatus* Venom

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Authors' contributions

This work was carried out in collaboration between all authors. Author SI designed and edited the work. Author MM edited and approved the final manuscript. Authors OOO and EAA managed the literature searches. Author OOI managed the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The study was carried out to determine the effect of methanol extract of *Mangifera indica* seed kernel and its solvent fractions on metalloprotease induced toxic effect on mice. Total of twelve albino mice were used for different treatment group which were replicated in triplicates. This research was carried out in the laboratory of the department of pharmacognosy Ahmadu Bello University, Zaria, August, 2015. *Mangifera indica* seed kernel methanol extract was partitioned using solvent with increasing polarity (ethyl acetate, n-butanol and aqueous). The most active ethyl acetate fraction was used to determine the *in vivo* anti venom metalloprotease induced toxic effect

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on blood coagulation system (bleeding and clotting time) and some haematological parameters (packed cell volume, haemoglobin, white blood cell and total protein count) in mice. The results obtained from the *in vivo* studies showed a significant increase in the bleeding and clotting time of the mice treated with the venom alone when compared with that of the control. In the mice treated with venom and the extract, the increase in bleeding and clotting time was observed to be significantly (p=0.05) reduced. Also, the results obtained for haematological parameters showed a significant reduction, except for the WBC count which was slightly elevated in the mice treated with venom alone when compared to the control. Normalcy was significantly restored in the groups treated with the venom and the extract. This suggests that the extract has some inhibitory effect *in vivo* against the action of *Echis ocellatus* venom. The relevance of these findings would be of importance in the management of snake bite envenomation and the development of anti venom.

Keywords: Mangifera indica; snake venom; bleeding time; clotting time; haematological parameters.

1. INTRODUCTION

Snakebite poisoning is an important neglected disease in most of the developing countries. Viper snakes like *Echis ocellatus* are among the most common types of venomous snakes which are responsible for many envenoming and deaths in most tropical areas. In humans, envenomation by *Echis ocellatus* causes severe blistering, oedema and necrosis at the bite site, and life threatening systemic effects including haemorrhage, coagulopathy, haemotoxicity and occasionally hypovolaemic shock [1]. According to data presented by Nasidi [2], globally over 3 million humans are bitten annually by venomous snakes, resulting in more than 150,000 deaths. Nigeria is reported to have one fifth of all West African region cases occurring in the country. This number does not account for unreported incidences, and those who survive envenomation will often deal with secondary effects such as necrosis or limb amputation. Snake venom metalloproteases (SVMPs) are one of the abundant components in many snake venoms, especially in viper snakes and they play key roles in envenomation. SVMPs possess diverse functions such as the disruption of homeostasis mediated by procoagulant or anticoagulant effects, platelet aggregation, and apoptotic or pro-inflammatory activities. Antivenom immunotherapy which has been the only specific treatment against snake bite envenomation is burdened with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness [3]. Because of these problems in antiserum therapy, numerous medicinal plants have been proposed for the management of snake bites [4].

Medicinal plants have been used for many years to treat a great variety of diseases including envenomations by animal bites. These plants play important roles in human health, as they are a good source of inhibitors and pharmacologically active compounds. Plant extracts that have been shown to antagonize the activity of various kinds of venoms and toxins are known to contain arrays of active principles such as aristolochic acid, alkaloids, steroidal, flavonoids, phenols, pterocarpanes, quinonoid xanthenes, resveratrol, glycoside and tannins. *Mangifera indica Linn*. seed kernel extract are very rich in polyphenols and have been pharmacologically documented to have antioxidant, anti-tyrosinase, anti–inflammatory, and hepatoprotective activities as well as anti-enzymatic activities against snake venom [5,6]. The use of natural venom inhibitors from *Mangifera indica* seed extracts, which are widely known by many could complement or substitute for the action of sheep serum anti-venoms. It would be an alternative way to minimize the socio-medical problem of snake bite in tropical countries, especially in Nigeria. *Mangifera indica* seed kernel extract may provide this cheap and readily accessible alternative.

2. MATERIALS AND METHODS

2.1 Snake Venom

Freeze dried *Echis ocellatus* venom was obtained from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria-Nigeria. This was partiarlly purified on a two-step purification process using ion exchange chromatography on DEAE sephadex and gel filtration on sephadex G-75.

2.2 Seed Kernel

Mangifera indica seed were collected from Zaria metropolis and identified in the Ahmadu Bello

University herbarium Zaria – Nigeria with the voucher number: 1312 *Mangifera indica* Anacardiaceae.

2.3 Preparation of Extract and Its Fractionation

The kernel was removed from the seed coat, sun-dried and pulverized to powder. Extraction was carried out with methanol using soxhlet apparatus for six (6) hours at 70℃ and the solvent was removed by rotary evaporator under reduced pressure to obtain the crude extract. The crude methanol extract was suspended in aqueous: methanol (4:1) followed by an assay guided fractionation with ethyl acetate and n-butanol (saturated with water). Each solvent fraction was tested for activity by inhibiting the action of metalloprotease present in the snake venom and the most active fraction was recorded.

2.4 Animal Grouping

Albino mice were randomly divided into ten groups of three mice:

Group 1: Control group that received normal saline
Group 2: Envenomed mice that did not receive any extract treatment
Group 3: Mice that received the extract alone
Group 4: Envenomed mice treated with the extract

2.5 Envenomation of Mice

The *in vivo* study was carried out according to the method of Omale et al. [7]. The venom was administered intra-dermally at a dose of 0.1 mg/kg (0.01 mg/ml) body weight of mice. After which, the extract was also administered intra-dermally at a dose of 40 mg/kg (4 mg/ml) body weight of mice. After envenomation, the mice were left for four hours then bleeding time and clotting time were measured. The animals were sacrificed and a 2 ml blood was collected for the measurement of haematological properties.

2.6 Measurement of Bleeding and Clotting Time in Envenomed Mice

2.6.1 Bleeding time

For the determination of the bleeding time, modified procedure of Mohamed et al. [8] was used. Four hours after the treatment of the animals, the tail of each mouse was gently pierced with lancet and a piece of white filter paper was used to blot the blood gently from the punctured surface of the body. The readings were taken every 15 sec. The end result occurred when the paper was no longer stained with blood.

2.6.2 Clotting time

For the determination of the clotting time, the modified method of Igboechi and Anuforo [9] was used. Clotting time is the time required for a firm clot to be formed in fresh blood on glass slides. The blood sample was collected from the mice via tail bleeding and a drop was placed on a clean plain slide and every 15 sec, a tip of office pin was passed through the blood until a thread-like structure was observed between the drop of blood and tip of the pin. The thread-like structure was an indication of a fibrin clot. The time was recorded [10].

2.7 Measurement of Haematological Parameters

2.7.1 Packed cell volume (PCV) of mice

Anti-coagulated blood were placed in plain capillary tubes and one end of the tubes sealed with clay. The filled tubes were placed in a micro-haematocrit centrifuge and centrifuged for 5 minutes at 10000 RPMs. The centrifugal force layered the blood components according to weight. The heaviest components are the red blood cells and are pushed to the bottom of the tube. The total height of the column (cells + plasma) and the height of the packed red cell column were measured using a micro-haematocrit reader [11].

2.8 Haemoglobin Concentration

Twenty micro litres (0.02 ml) of blood were diluted with 5 ml of cyanmethaemoglobin (Drabkin's) reagent. After 10 minutes, the absorbance was measured at 540 nm with water as blank. The haemoglobin concentration was obtained from calibration curve using cyanmethaemoglobin standard [12].

2.9 White Blood Cell Count

Using Thoma white cell pipette, blood was drawn up to 0.5 mark, followed by the diluting fluid to

the 11 marks. This gives 1 to 20 dilutions. The haemocytometer chamber was then filled and the white cells are allowed to settle for 1-3 minutes. The counting chamber was then placed on the microscope stage with a lower power objective ×10 to ascertain the cells are evenly distributed and then ×40 objectives was used for clearer viewing and counted.

2.10 Determination of Total Protein

The refractometer is a device used to measure the refractive index of a solution. Refractive index is defined as the degree that the light is bent when it is passed through a liquid, and is a function of the amount and type of solid material dissolved in the liquid. In clinical practice, this instrument is calibrated to give direct readings of total protein in serum samples.

Refractometer model 7416 USA was used for protein determination. The refractometer was set to zero reading using distilled water and the protein concentration was taken.

3. RESULTS

3.1 Physical Signs of Envenomation Observed in the Mice after Envenomation

There were physical signs of envenomation as shown in Table 2 showing the effect of the venom alone and the effect of the venom in combination with extract.

3.2 The Effect of the Ethyl Acetate Fraction on the Blood Coagulation System (Bleeding Time and Clotting Time) in Mice

The effect of envenomation on the bleeding time and clotting time in mice as presented in Table 3 and 4 respectively, showed a significant increase in the bleeding time and clotting time of the group (2) that were treated with venom alone. This indicates the venom from *E. ocellatus* has deleterious effect on the blood coagulation system which is one of the indications of envenomation from viperidae snake. Meanwhile, in the group treated with venom and extract together (group 4), there were significant reductions in the bleeding time and clotting time suggesting that the extract has an inhibitory effect on the action of the snake venom component responsible for the deleterious effect of *E. ocellatus* on blood coagulation system.

3.3 The Effect of the Ethyl Acetate Fraction on Haematological Parameters

Hematological parameters were significantly ($p<0.05$) reduced in group 2 (envenomed mice) when compared with the venom and extract treated group (4) (Table 5). The WBC was slightly elevated in the group that received venom alone when compared with other hematological parameters.

Table 1. The yield of methanol extract of mango seed kernel and its solvent fractions with their effects on *Echis ocellatus*

Extracting solvent	Amount recovered (g)	% yield	Metalloprotease activity (µmol/min)	%inhibition
Methanol extract	11.10	10.10	4.31±0.01	58
Ethyl acetate fraction	4.45	44.5	3.74±0.02	63
n-Butanol fraction	0.93	9.30	8.64±0.01	15
Aqueous residue	2.97	29.7	8.71±0.02	14

*(Enzyme activity was determined from the slope of the line from the graph of absorbance vs. time and correlating the result with standard curve of tyrosin. Percentage inhibition was calculated using the formular; % inhibition = [(normal activity - inhibited activity) / (normal activity)] * 100%) with normal activity as the control without inhibition. The ethyl acetate gave the more inhibition than methanol probably because during the fractionation of the methanol extract, ethyl acetate was able to isolate or expose a component which activity was previously subdued in the methanol extract*

Table 2. Physical signs of envenomation observed within four hours after envenomation in mice

Groups	Treatment	Swelling	Echymosis	Palpitation
1	Normal saline	_	_	_
2	Venom alone	+ + +	+ +	+ + +
3	Extract alone	_	_	_
4	Venom + Extract	+	+	+

Table 3. Effect of the ethyl acetate fraction on bleeding time after envenomation

Groups	Treatment groups	Bleeding time (sec)
1	Normal saline	85±8.66[a]
2	Venom (0.1 mg/kg body weight)	275±8.66[b]
3	Extract (40 mg/kg body weight)	85±8.66[a]
4	Venom + extract	105±15.00[c]

Values are mean ± S.E.M (n = 3)
Values with the same superscript are considered statistically significant (p<0.05) when compared with the control

Table 4. Effect of the ethyl acetate fractions on clotting time after envenomation

Groups	Treatment administered	Clotting time (sec)
1	Normal saline	260±8.66[a]
2	Venom (0.1 mg/kg body weight)	125±31.22[b]
3	Extract (40 mg/kg body weight)	255±15.00[a]
4	Venom + extract	215±8.66[c]

Values are mean ± S.E.M (n = 3)
Values with the same superscript are considered statistically significant (p<0.05) when compared with the control

Groups 1 to 4 represent treatments with normal saline, venom alone, extract alone and venom + extract respectively.

4. DISCUSSION

Many compounds identified from plants having different chemical structure were reported to be capable of interacting with peptides and proteins (enzyme) of snake venom. The mechanism of action of the plant extracts/plant compounds are still not clear but may be attributed to the blocking of receptors-structure prone to chemical attack, and may block the active site of the snake venom. Other mechanism of action of the plant compounds are inhibition of enzymes present in the snake venom. This is due to the metal chelator substances present in the plant extracts/plant compounds [4]. It was observed from this study that the ethyl acetate fraction from the crude methanol extract has the highest inhibitory activity against the action of metalloprotease present in the venom of *Echis ocellatus* (Has been reviewed above in line 62 from the reviewers numbering). This enzyme is believed to be the major component of snake venom from the viperidae family responsible for the haemorrhagic effect which is the primary cause of envenomation and mortality observed in snake bite victims.

The 58% reduction in the activity of the enzyme when treated with the crude extract and subsequent 63% reduction by the ethyl acetate fraction active fraction suggests that the ethyl acetate fraction has extracted more active constituent from the methanol extract which is more effective in the inhibition of metalloprotease activities of snake venom of *E. ocellatus*. In another study, the methanol extract of the seed kernel of *M. indica* was reported to have an inhibitory effect on the phospholipase A_2 activity of *Naja nigricolis* [6], showing the potency of both the crude methanol extract and its ethyl acetate fraction in the inhibition of snake bite envenomation.

Mangifera indica seed kernel extract appears to be a promising chemical agent for use as first hand treatment, or in combination with antiserum in the management of snake bite victims. This was evident in the protective effect it conferred on mice after envenomation as observed in this study. A noteworthy observation was that a rapidly progressive swelling (edema which exceeded the point of venom introduction), echymosis, palpitation and weakness which has been consistently associated with systemic envenomation from Viperidae were observed in the mice that received venom alone within four hours following envenomation, while in the mice that received venom and extract together, there were mild swelling and echymosis which were confined only at the site of venom introduction. This indicates that the extract was efficient to subdue the effect of the venom thereby preventing further spreading of the earlier observed signs of envenomation. Meanwhile, the mice that received water, normal saline and the extract alone did not show any sign of envenomation in mice.

Spontaneous bleeding and coagulation disturbances are some of the haematological effects of *Echis ocellatus* in patients [13]. The increase in clotting time and bleeding time level observed in the group that received venom alone (Table 3 and Table 4) showed the anticoagulant effect of venom from *E. ocellatus* which was significantly abridged in the group that received venom and extract together showing the ability of the extract to neutralize the effect of the venom. This agrees with the work of Omale et al. [7] who reported the neutralization of the

Table 5. Effect of the ethyl acetate fractions some hematological parameters on mice

Groups	PCV (%)	Hgb (g/dl)	WBC ($\times 10^9$/L)	TP (g/dl)
1	46.33±3.51[a]	15.87±0.81[a]	6.73±0.25[a]	6.50±0.46[a]
2	22.33±2.52[b]	5.13±0.42[b]	9.77±1.88[b]	3.53±0.31[b]
3	43.00±2.65[c]	14.53±0.50[a]	7.33±0.58[a]	6.33±0.58[a]
4	41.33±0.58[c]	13.83±0.40[a]	7.43±0.72[a]	5.47±0.42[a]

Values are mean ± S.E.M (n = 3)
Values in the same column with the same superscript are considered statistically significant (p<0.05) when compared with the control

anticoagulant effect of venom of *Naja nigricollis.* The increase in bleeding time and clotting time observed could be as a result of the inhibition of platelet aggregation or the activation of prothrombin.

The Packed Cell Volume (PCV) of the envenomed mice were reduced significantly (p<0.05), when compare with non-envenomed Mice. This is in consonance with the report of Mwangi et al. [14] and could be as a result of systemic bleeding which characterizes envenomation by *E. ocellatus*. Also, the decrease in haemoglobin and total protein observed in the group that received venom alone as compared to the group that received the venom and extract together may be due to hemolysis which causes dilution of the blood [15]. White blood cells (WBC) are effectors of the immune system. The count of WBC increased after four hours in the group that received venom alone and this observation is consistent with observations of Amin et al. [16] who reported that Bangladesh snake venom caused leucocytosis after a period of injection. Lifshitz et al. [17] hypothesized that a sympathetic effect, as a result of the stress experienced by the victims could release temporarily WBCs from the marginal pools. Whereas, the group that received the venom and extract together did not show any increase in the WBC. This suggests that the plant extract must have antagonized the venom directly without cells of the immune system producing effectors cells.

5. CONCLUSION

The ethyl acetate fraction showed a significant Inhibitory activity on the metalloprotease induced toxic effect on blood coagulation system (bleeding and clotting time) and some hematological parameters (PCV, Hgb, WBC and TP) when evaluated *in vivo* and also reduced the signs of envenomation in Mice. *Mangifera indica* L might provide a less sensitive, inexpensive and readily available alternative to sheep serum in the management of snake bite envenomation. This study shows beneficial *in vivo* anti-hemorrhagic properties of MSKE and its ethyl acetate fraction against *E*. *ocellatus* venom.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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