

Original Research Article

The inhibitory potentials of seed kernel methanol extract of *Mangifera indica* L.

anacardiaceae and fractions on metalloprotease-induced toxic effect from

***Echis ocellatus* venom.**

ABSTRACT

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 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.

Key words: *Mangifera indica*, Snake venom, bleeding time, clotting time, haematological parameters.

25 INTRODUCTION

26 Snakebite poisoning is an important neglected disease in most of the developing countries.
 27 Viper snakes like *Echis ocellatus* are among the most common types of venomous snakes
 28 which are responsible for many envenoming and deaths in most tropical areas. In humans,
 29 envenomation by *Echis ocellatus* causes severe blistering, oedema and necrosis at the bite
 30 site, and life threatening systemic effects including haemorrhage, coagulopathy,
 31 haemotoxicity and occasionally hypovolaemic shock (WHO, 2010). Snake venom
 32 metalloproteases (SVMPs) are one of the abundant components in many snake venoms,
 33 especially in viper snakes and they play key roles in envenomation. SVMPs possess diverse
 34 functions such as the disruption of homeostasis mediated by procoagulant or anticoagulant
 35 effects, platelet aggregation, and apoptotic or pro-inflammatory activities. Antivenom
 36 immunotherapy which has been the only specific treatment against snake bite envenomation
 37 is burdened with various side effects such as anaphylactic shock, pyrogen reaction and serum
 38 sickness (Abdulrazaq, 2011). Because of these problems in antiserum therapy, numerous
 39 medicinal plants have been proposed for the management of snake bites (Selim *et al.*, 2013).

40 Medicinal plants have been used for many years to treat a great variety of diseases including
 41 envenomations by animal bites. These plants play important roles in human health, as they
 42 are a good source of inhibitors and pharmacologically active compounds. Plant extracts that
 43 have been shown to antagonize the activity of various kinds of venoms and toxins are known
 44 to contain arrays of active principles such as aristolochic acid, alkaloids, steroidal, flavanoids,
 45 phenols, pterocarpanes, quinonoid xanthenes, resveratrol, glycoside and tannins. *Mangifera*
 46 *indica* Linn. seed kernel extract are very rich in polyphenols and have been
 47 pharmacologically documented to have antioxidant, anti-tyrosinase, anti-inflammatory, and

48 hepatoprotective activities as well as anti-enzymatic activities against snake venom
49 (Pithayanukul, 2009; Humphery *et al.*, 2014).

50 MATERIALS AND METHOD

51 **Snake venom:** Freeze dried *Echis ocellatus* venom was obtained from the Department of
52 Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria-Nigeria.

53 **Seed kernel:** *Mangifera indica* seed were collected from Zaria metropolis and identified in
54 the Ahmadu Bello University herbarium Zaria – Nigeria.

55 Preparation of extract and its fractionation

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

63

64 Animal Grouping

65 Wistar albino mice were randomly divided into ten groups of three mice:

66 **Group 1:** Control group that received normal saline

67 **Group 2:** Eenvenomed mice that did not receive any extract treatment

68 **Group 3:** Mice that received the extract alone

69 **Group 4:** Envenomed mice treated with the extract

70 **Envenomation of Mice**

71 The *in vivo* study was carried out according to the method of Omale *et al.* (2013). The
72 venom was administered intradermally at a dose of 0.1 mg/kg (0.01mg/ml) body weight of
73 mice. After which, the extract was also administered intradermally at a dose of 40mg/kg
74 (4mg/ml) body weight of mice. After envenomation, the mice were left for four hours then
75 bleeding time and clotting time were measured. The animals were sacrificed and a 2ml blood
76 was collected for the measurement of haematological properties.

77 **Measurement of Bleeding and Clotting time in envenomed Mice.**

78 **Bleeding time:**

79 For the determination of the bleeding time, modified procedure of Mohamed *et al.* (1969)
80 was used. Four hours after the treatment of the animals, the tail of each rat was gently pierced
81 with lancet and a piece of white filter paper was used to blot the blood gently from the
82 punctured surface of the body. The readings were taken every 15 sec. The end result occurred
83 when the paper was no longer stained with blood.

84 **Clotting time:**

85 For the determination of the clotting time, the modified method of Igboechi and Anuforo
86 (1986) was used. Clotting time is the time required for a firm clot to be formed in fresh blood
87 on glass slides. The blood sample was collected from the mice via tail bleeding and a drop
88 was placed on a clean plain slide and every 15 sec, a tip of office pin was passed through the
89 blood until a thread-like structure was observed between the drop of blood and tip of the pin.
90 The thread-like structure was an indication of a fibrin clot. The time was recorded.

91 **Measurement of Haematological Parameters**

92 **Packed Cell Volume (PCV) of Mice**

93 Anti-coagulated blood were placed in plain capillary tubes and one end of the tubes sealed
94 with clay. The filled tubes were placed in a micro-haematocrit centrifuge and centrifuged for
95 5 minutes at 1000 RPMs. The centrifugal force layered the blood components according to
96 weight. The heaviest components are the red blood cells and are pushed to the bottom of the
97 tube. The total height of the column (cells + plasma) and the height of the packed red cell
98 column were measured.

99 **Haemoglobin Concentration**

100 Twenty micro litres (0.02ml) of blood were diluted with 5ml of the reagent. After 10 minutes,
101 the absorbance was measured at 540nm with water as blank. The haemoglobin concentration
102 was obtained from calibration curve using standards

103 **White Blood Cell Count**

104 Using Thoma white cell pipette, blood was drawn up to 0.5 mark, followed by the diluting
105 fluid to the 11 marks. This gives 1 to 20 dilutions. The haemocytometer chamber was then
106 filled and the white cells are allowed to settle for 1-3 minutes. The counting chamber was
107 then placed on the microscope stage with a lower power objective $\times 10$ to ascertain the cells
108 are evenly distributed and then $\times 40$ objectives was used for clearer viewing and counted

109 **Determination of Total Protein**

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

112 **RESULT**

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

Extracting	Amount	% yield	Metalloprotease	%inhibition
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solvent	recovered (g)		Activity ($\mu\text{mol}/\text{min}$)	
Methanol extract	11.10	10.10	4.31 $\pm$ 0.01	58
Ethyl acetate fraction	4.45	44.5	3.74 $\pm$ 0.02	63
n-Butanol fraction	0.93	9.30	8.64 $\pm$ 0.01	15
Aqueous residue	2.97	29.7	8.71 $\pm$ 0.02	14

115

116 **Physical signs envenomation observed in the mice after envenomation.**

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

119 **Table 2:** Physical signs of envenomation observed within four hours after envenomation in
120 Mice.

GROUPS	TREATMENT	OBSERVATIONS
1	Normal saline	No signs of envenomation
2	Venom alone	Severe swelling and echymosis with palpitation and weakness
3	Extract alone	No signs of envenomation
4	Venom + Extract	Significantly reduced swelling, echymosis and palpitation

121 **The effect of the ethyl acetate fraction on the blood coagulation system (Bleeding time**
122 **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.

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

Groups	Treatment groups	Bleeding time (sec)
2	Normal saline	85±8.66 ^a
4	High dose venom	275±8.66 ^b
6	High dose extract	85±8.66 ^a
7	Low dose venom + low dose 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	High dose venom	125±31.22 ^b
3	High dose extract	255±15.00 ^a
4	High dose venom + low dose extract	215±8.66 ^c

137 Values are mean $\pm$ S.E.M (n = 3)
 138 Values with the same superscript are considered statistically significant (p<0.05)
 139 when compared with the control.

140 **The effect of the ethyl acetate fraction on haematological parameters:**

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

145 **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 $\pm$ 3.51 ^a	15.87 $\pm$ 0.81 ^a	6.73 $\pm$ 0.25 ^a	6.50 $\pm$ 0.46 ^a
2	22.33 $\pm$ 2.52 ^b	5.13 $\pm$ 0.42 ^b	9.77 $\pm$ 1.88 ^b	3.53 $\pm$ 0.31 ^b
3	43.00 $\pm$ 2.65 ^c	14.53 $\pm$ 0.50 ^a	7.33 $\pm$ 0.58 ^a	6.33 $\pm$ 0.58 ^a
4	41.33 $\pm$ 0.58 ^c	13.83 $\pm$ 0.40 ^a	7.43 $\pm$ 0.72 ^a	5.47 $\pm$ 0.42 ^a

146 Values are mean $\pm$ S.E.M (n = 3)
 147 Values in the same column with the same superscript are considered statistically
 148 significant (p<0.05) when compared with the control.

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

151

152

153 **DISCUSSION**

154 Many compounds identified from plants having different chemical structure were reported to
155 be capable of interacting with peptides and proteins (enzyme) of snake venom. The
156 mechanism of action of the plant extracts/plant compounds are still not clear but may be
157 attributed to the blocking of receptors-structure prone to chemical attack, and may block the
158 active site of the snake venom. Other mechanism of action of the plant compounds are
159 inhibition of enzymes present in the snake venom. This is due to the metal chelator
160 substances present in the plant extracts/plant compounds (Alam, 2014). It was observed from
161 this study that the ethyl acetate fraction from the crude methanol extract has the highest
162 inhibitory activity against the action of metalloprotease present in the venom of *Echis*
163 *ocellatus*. This enzyme is believed to be the major component of snake venom from the
164 viperidae family responsible for the haemorrhagic effect which is the primary cause of
165 envenomation and mortality observed in snake bite victims.

166 The 58% reduction in the activity of the enzyme when treated with the crude extract and
167 subsequent 63% reduction by the ethyl acetate fraction active fraction suggests that the ethyl
168 acetate fraction has extracted more active constituent from the methanol extract which is
169 more effective in the inhibition of metalloprotease activities of snake venom of *E. ocellatus*.
170 In another study, the methanol extract of the seed kernel of *M. indica* was reported to have an
171 inhibitory effect on the phospholipase A₂ activity of *Naja nigricolis* (Humphery *et al.*, 2014),
172 showing the potency of both the crude methanol extract and its ethyl acetate fraction in the
173 inhibition of snake bite envenomation.

174 *Mangifera indica* seed kernel extract appears to be a promising chemical agent for use as first
175 hand treatment, or in combination with antiserum in the management of snake bite victims.
176 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 (Warrell *et al.*, 1977). 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 Joseph *et al.*, (2012) who reported the neutralization of the 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.* (1995) 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 (Karthikeyan *et al.*,

2007). 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.*, (2008) who reported that Bangladesh snake venom caused leucocytosis after a period of injection. Lifshitz *et al.*, (2003) 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.

211 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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