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ORIGINAL ARTICLE

Antihaemolytic, Antihaemorrhagic and Antifibrinolytic Effects of Fractions of *Buchholzia coriacea* Seeds on *Naja nigricollis* Crude Venom

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SUMMARY

Bulchhozia coriacea (Capparaceae) seeds are used in managing snake bite in Western Nigeria were investigated against *Naja nigricollis* (Spitting cobra) venom-induced hemolytic, hemorrhagic and fibrinolytic effects. This study was aimed at determining the antihaemolytic, antihaemorrhagic as well as antifibrinolytic effects of *B. coriacea* on *N. nigricollis* crude venom. Microwave-assisted extraction with hexane, chloroform, ethyl acetate, and methanol was carried out. *Naja nigricollis* venom-induced erythrocyte lysis (100 %) was significantly reduced to 18% by the chloroform fraction at 0.625 mg/mL. At the concentration of 0.625 mg/mL, the hexane, chloroform, ethyl acetate and methanol fractions administered in combination with the venom reduced percentage hemorrhagic activity to 23%, 17%, 49%, and 87%, respectively. In conclusion, *Bulchhozia coriacea* seed fractions exhibited significant antihaemolytic, antihaemorrhagic and antifibrinolytic activities against *N. nigricollis* crude venom and may beneficial as a pre-treatment the while victim is transferred to a healthcare facility to receive the definite treatment to ensure speedy recovery.

Key words: Antihaemolytic, antihaemorrhagic, fibrinolytic, venom, *Bulchhozia coriacea*

INTRODUCTION

Snake envenomation is common in rural areas due to alarming death rates (Gutiérrez *et al.*, 2010). Snake envenomation is usually high during the rainy season and was recognise as Neglected Tropical Diseases (NTD) by the Who Health Organisation (WHO) in June 2017. This was due to the prevalence of snake bite in both developing and industrialised country . The snakes particularly responsible for this alarming death rates is the *Naja nigricollis*.

Naja nigricollis is a species of spitting cobra dominant mostly in sub-Saharan Africa. It is moderately sized snakes that can grow up to of 1.2 to 2.2 m in length. It is a medically important venomous snake with body coloration and

markings varying greatly with respect to environment of origin (Richard, 2012; Luca 2001). Envenomation by *Naja nigricollis* causes symptoms, like swelling, necrosis, blistering and neurotoxicity as a common symptom of systemic envenomation (Chidambaram *et al.,* 2011). Antivenom development and standardization are tasking financially and require a modern storage facility such as freezers, which is often lacking in remote snake endemic areas (Soares *et al.,* 2009). Ethnic groups in western Nigeria use of *Bulchhozia coriacea* seed paste in the treatment of snake bites due to *Naja nigricollis* (Anowi *et al.,* 2012: Nwachukwu *et al.,* 2012). Plants remain the only alternative antivenom that is handy in most rural areas, where there is poor or complete lack of hospitals and storage facilities for conventional synthetic antivenom (Alam and Gomes, 2003). The use of this plant is only a form of first aid treatment for snake envenomation. Efforts should be made to administer conventional antivenom on the victim as this is the only definite treatment for snake envenomation recognised by the World Health Organization. In Nigeria, treatment of snake envenomations is crippled by scarcity of specific antivenom and other supportive measures like clotting factors and cryoprecipitate (Fadare 2012). The use of specific anti-snake venoms is rare because of scarcity, availability of non-specific anti-venoms and unaffordability of specific anti-venoms, (Warrel, 2008). The most available anti-venom used in Nigerian is the polyvalent non-specific type produced by the Pasteur anti-venom. (Laing et al., 2003). Snake envenomation may lead to damage to blood capillaries by haemorrhaging and phospholipase A2 reactions (Furtado *et al.,* 2003; Koh *et al.,* 2006). This is manifested as haematuria, hemoptysis, gum bleeding, haematemesis, and skin bleeding (Warrell, 2004). However, plants used in the treatment of snake envenomation may contain a significant quantity of secondary metabolites flavonoids and alkaloids, which possess venom neutralizing property (Daduang *et al.,* 2005).

MATERIALS AND METHODS

Plant material

The seeds of *Bulchhozia coriacea* were collected from Kwara state Nigeria, during the dry season in March. The plants alongside flowers were identified in the Herbarium section of the Department of Botany, Faculty of Lifesciences, Ahmadu Bello University (ABU), Zaria, Nigeria. The plant was given a voucher no. 2349, deposited at the herbarium.

Preparation of Fractions

The seeds of *Bulchhozia coriacea* were air-dried and pulverized. The powdered seeds were extracted by the microwave-assisted extraction method (Daily and Vuong, 2016). A household microwave oven was used for the extraction, in order to gain maximum yield of extracts. 300 g of the pulverized seeds were soaked with 1 litre of hexane. The suspensions were irradiated with microwaves set at of 30 ^{0}C for 3 minutes. After cooling, it was then irradiated again with microwave for 3 minutes. Super boiling of the solution did not occur. The suspension was filtered and concentrate using a rotary evaporator with a water bath set at 30 ^{0}C. This extraction process was exhaustively and successively repeated using chloroform, ethyl acetate, and methanol.

Source of Venoms

Venom extraction was by milking locally caught *Naja nigricollis* (Markfarlane, 1967). The extracted venom was lyophilized using a freeze dryer (Bionics Scientific Technologies (P) LTD BST-LY101) and stored at about 2 ^{0}C before the experiments.

Animals

Ethical approval was given for the use of the life models used. 25 Swiss albino mice, weighing 20-25 kg and of either sex, were used for the study. They were kept at the Animal Experimental Laboratory at 23 °C and about a 12-hour light-dark-light circle. They were fed with animal pellets (Vital Feeds, Kaduna, Nigeria) and water. Each experimental group for the hemorrhagic activity consisted of five animals, housed in separate cages.

***Buchholzia coriacea* seeds extracts on Venom-induced hemolysis**

The antihaemolytic activities of fractions of the plant were evaluated *in vitro* by the method described by Pipelzadeh and Pipelzadeh (2012). Briefly, a total of 20 mL of the bovine blood sample was collected from an abattoir in Zaria using a 3.8 % sodium citrate. The bovine blood was centrifuged at 1548 ×*g* for 10 minutes and the plasma decanted. Exactly 5 mL of normal saline was mixed with the packed cell layer and centrifuged again at 1548 × g for 10 minutes, and the supernatant discarded. This procedure was repeated 10 times to obtain plasma-free packed cells. A total of 2 mL of prepared *B. coriacea* seed fractions of dilutions equivalent to 10, 5, 2.5, 1.25 and 0.625 mg/mL were immediately transferred into five different test tubes, each containing 10 mL of 1% cell suspension in saline mixed with 2 mL of *N. nigricollis* venom (10 mg/mL). The control group was a mixture of 1% cell suspension in saline mixed with venom alone. The mixture was incubated for 1 hour at 37 ^{o}C. The reaction was stopped by adding 3 of chilled phosphate-buffered saline. Thereafter, the tubes were centrifuged at 1075 x *g* for 10 minutes and absorbance of the supernatant measured spectrophotometrically (Agilent 2344 spectrophotometer) at 540 nm. The supernatant of the experimental tube treated with 3 mL chilled water was taken as 100 % lysis; Antihaemolytic activity was determined as follows (Gomes *et al.*, 2010)

$$\text{Antihaemolytic activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where

A_0 = Absorbance of RBC + Venom

A_1 = Absorbance of RBC + Venom + Extracts

***Buchholzia coriacea* seeds extracts on Antifibrinolytic activity**

The bovine blood sample was collected and 3.8 % sodium citrate (9:1v/v) to prevent coagulation. The blood was centrifuged at 1680 x g and stored at 4 ^{o}C for 15 minutes to obtain platelet-free plasma. Fibrinolytic activity was determined by the method of Theakston and Reid (1983) and by using bovine citrated plasma (2.0 mg/mL). The *B. coriacea* seed fractions of dilutions equivalent to 10, 5, 2.5, 1.25 and 0.625 mg/mL were transferred into five different test tubes. The tubes, already containing exactly 50 µL of venom (10 mg/mL) were mixed with 0.2 mL of plasma solution, and clotting time was determined at 37 ^{o}C (the control group was a mixture of 0.2 mL of plasma solution with the venom). All fibrinolytic activities were expressed as the reciprocal of the clotting times, recorded in minutes (Theakston and Reid, 1983).

***Buchholzia coriacea* seeds extracts on Haemorrhagic Effect**

The method used was a modification of that of Omori-Satoh *et al.* (1972). Briefly, a stock solution (10 mg/mL) of crude venom samples of *Naja nigricollis* was prepared. Five test tubes each containing 1 mL of normal saline were arranged in a test tube rack. Two-fold serial dilution was made to obtain concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/mL of *B. coriacea* seed fractions. Exactly 0.2 mL of each dilution was injected intradermaliy into mice 2 minutes after the venom was injected (the control group **received normal saline injection). The** mice were sacrificed by cervical dislocation 60 minutes post-injection, skinned and the hemorrhagic foci, induced were measured in cm using a transparent ruler (Omori-Satoh *et al.*, 1972; Furtado *et al.*, 2003). For each of the fraction concentration, the area of the hemorrhagic foci (AHF) was determined using the formula: $\pi r2$.

AHF = $\pi r2$ .Where AHF = Area of the haemorrhagic foci, π = 3.14, r = radius of haemorrhagic foci. The extent of damage in the venom group of mice was taken as 100%.

Statistical analysis:

One-Way ANOVA statistical tool was used. Values of $p < 0.05$ were considered significant.

RESULTS

The inhibitions induced by hexane, chloroform, ethyl acetate and methanol fractions of *in vitro* bovine hemolysis are shown in Figure 1. The hexane, chloroform, ethyl acetate, and methanol fractions inhibited venom-induced bovine erythrocyte lysis (haemolysis) by 26 %, 18 %, 50%, and 39 % respectively at 0.625. At a concentration of 1.25 mg/ mL, the percentage hemolytic activities of the hexane, chloroform, ethyl acetate and methanol extract of *B. coriacea* seed were 22%, 20%, 37% and 11%, respectively. Inhibition activities against venom-induced haemolysis at a concentration of 2.5 mg/mL of the fraction were 28 %, 22 %, 28 % and 28 %. The hexane, chloroform, ethyl acetate and methanol fractions of *B. coriacea* seeds inhibited venom-induced bovine hemolysis by 20%, 15%, 40% and 28% at a concentration of 5.0 mg/mL. At an increased concentration of 10 mg/mL of each fraction, the corresponding percentage hemolytic activities for the fractions were 28%, 36%, 67%, and 10%, respectively.

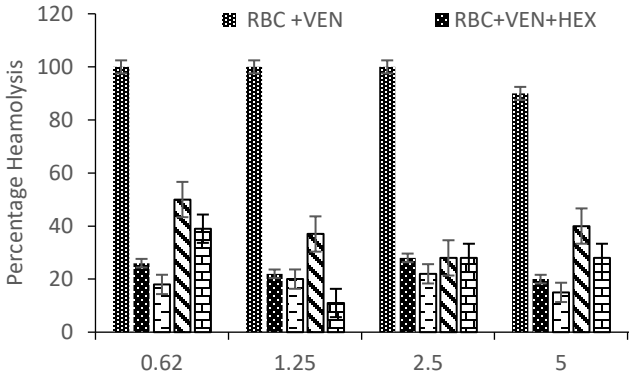

Figure 1. The antihaemolytic activity of fractions of the seeds of *B. coriacea*

KEY: RBC = Red Blood Cell, VEN = Venom, HEX = Hexane fraction, CHL = Chloroform fraction, ETH = Ethyl acetate fraction, METH = Methanol fraction,

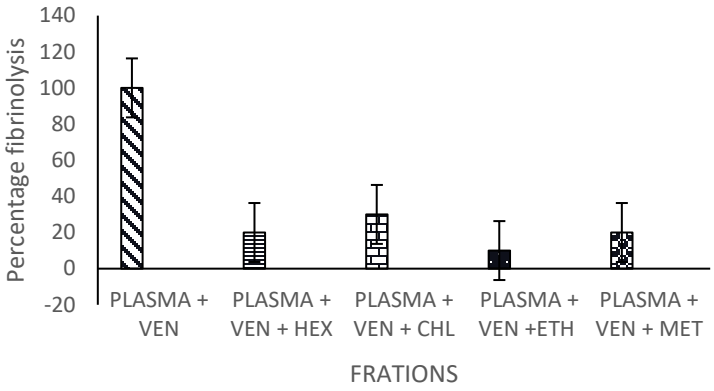

Figure 2: Antifibrinolytic activity of fractions of the seeds of *B. coriacea*

KEY: VEN = Venom, HEX = Hexane fraction, CHL= Chloroform fraction, ETH= Ethyl acetate fraction, MET= Methanol fraction.

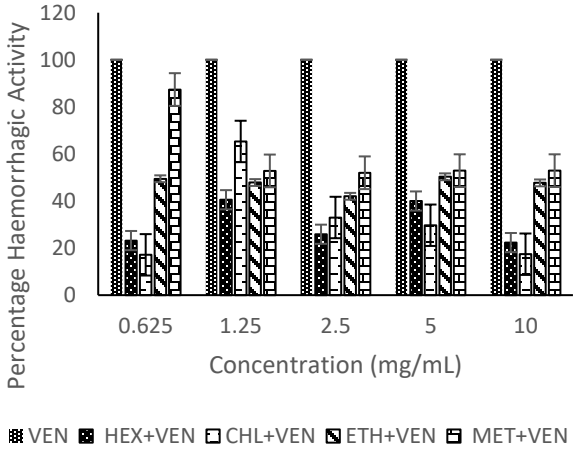

Figure 3: Haemorrhagic activity of fractions of the seeds of *B. coriacea*

KEY: VEN = Venom, HEX = Hexane fraction, CHL= Chloroform fraction, ETH = Ethyl acetate fraction + Venom, MET= Methanol fraction

Among the four fractions, the chloroform fraction at a concentration of 2.5 mg/mL showed the maximum antihaemolytic activity against venom-induced bovine hemolysis (Figure 1).

The area of the hemorrhagic foci for the *N. nigricollis* venom was taken as 100 per cent. The hexane, chloroform ethyl acetate, and methanol

fractions, when administered in combination with the venom, reduced the percentage hemorrhagic activity to 23%, 17%, 49% and 87% at concentrations of 0.625 mg/mL of each fraction, respectively. At a concentration of 1.25 mg/ mL of each fraction, corresponding reduced hemorrhagic activities of 40%, 65%, 47%, and 52% were observed. At a higher concentration of 10 mg/ mL, the hemorrhagic activities recorded for the hexane, chloroform, ethyl acetate, and methanol fractions were 22% 17.4 %, 47 %, and 52.9 %, respectively (Figure 2). The animal group treated with fractions plus venom had decreased hemorrhagic foci area to 17.4%, compared to the venom group (Figure 2).

The *N. nigricollis*-induced fibrinolysis was effectively antagonized by the fractions of *B. coriacea* seeds. All the seed hexane, chloroform, ethyl acetate, and methanol fractions inhibited fibrinolytic activity induced by *Naja nigricollis* venom. At a concentration of 2.25 mg/mL, the hexane, chloroform, ethyl acetate, and methanol fractions had a fibrinolytic activity of 20%, 30%, 10%, and 20% respectively while that of the venom was 100% (Figure III).

DISCUSSION

The result showed that venom of *N. nigricollis* induced bovine hemolysis at a 10 mg/mL. This was not unexpected as hemolysis by venom is due to phospholipase enzyme activity (Liu *et al.,* 2010). The inhibitory effect against hemolysis induced by the venom of *N. nigricollis* was highest (18 %) for chloroform fraction of *B. coriacea* at 0.625 mg/mL. Therefore, the chloroform fraction may be used to potentially reduce the effect of snake-bite; principally because of the large diversity of pharmacological compounds it contains (Kumarapppan *et al.,* 2011). The methanol fraction showed the reduced percentage hemolytic activity of 11% at a concentration of 1.25 mg/mL. The ability of the methanol fraction to neutralize the venom of *N. nigricollis* could also be attributed to the ability of bioactive secondary metabolites such as tannins and flavonids present in *B. coriacea* seeds, which bound venom proteins PLA2 and inhibited enzyme activity (Kalidhar, 2006; Kini, 2006). This mechanism may be involved in the action of many phytochemicals in plants and underlies the protective effects of plant fractions when preincubated with venom (Joshi *et al.*, 2010).

The *N. nigricollis*-induced fibrinolytic effect may be due to the finding that the venom contains a pro-coagulant enzyme, ecarin, which, activates prothrombin to produce thrombin, resistant to the anticoagulant, heparin (Theakston and Laing, 2014). The activity is responsible for lack of blood coagulability in of the victim and is due to the rapid depletion of coagulatory factors, such as fibrinogen, prothrombin, Factors V and VIII, and platelets (Theakston and Laing, 2014). The decreased platelets in the blood also bruising and bleeding after an injury. The pro-coagulatory action (direct activation of prothrombin) is the principal effect of the *N. nigricollis* venom on blood coagulation in humans (Manning, 1995). This effect was inhibited significantly by the fractions of *B. coriacea* seeds, with the ethyl acetate fraction showing the greatest inhibition at 2.25 mg/mL. The venom also contains haemorrhage, which causes direct endothelial damage to the blood vessel wall that leads to spontaneous bleeding (Reid and Theakston, 1983). Consequently, there is spontaneous oozing out of the blood into vital organs, especially the brain, causing lethality through cerebral haemorrhage (Warrell, 2004). The result showed that animal group treated with fractions plus venom had decreased percentage hemorrhagic activity of 22% 17.4%, 47% and 52.9% at 10 mg/ mL of the fractions respectively, compared to the venom group only. The haemorrhage was, apparently, due to dysfunction of blood coagulation and platelets, damage to the vascular endothelial cells and degradation of the basement membrane. Some of the fractions showed reduced inhibitions at high concentrations, possible due to the inability of plant fraction to diffuse through a medium at high concentration (Achika *et al.,* 2017). This study was carried out because we heard of the traditional medicine use of these seeds in areas where snakebite occurs and in areas where modern medicine is not available, we have sought to determine scientifically whether any components of these seeds have properties that

may make them useful in the treatment of snakebite. From our results it could be inferred that the use of these seed paste in treating snake envenomation can reduce the detrimental effect on snake venom temporarily before victim is treated with modern antivenom.

CONCLUSION

From the results above, we have shown that some components extracted from these seeds have therapeutic properties potentially useful in the treatment of snakebite. These properties may explain why these seeds have a place in traditional medicine in the treatment of snake bite, in instances where immunotherapy is not available

The Authors declare no conflict of interest.

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