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Research Article
Protective Effect of *Azadirachta indica* Leaf Fractionated Extracts on Renal and Haematological Indices against Snake Venom Toxicity in Albino Rats

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Abstract

Background and Objective: Snake venoms constitute different toxins which display different modes of action. Likewise, venoms from different snake species vary significantly in their toxin composition. Snake venom can cause morbidity and even mortality through tissue destruction. Hence, this research was aimed at evaluating the protective effect of *Azadirachta indica* leaf extracts on renal and haematological indices in albino rats. **Materials and Methods:** *A. indica* leaf was collected, authenticated and extracted using methanol followed by fractionation using hexane and ethylacetate. Albino rats of both sexes were used. They were grouped into 5, normal control, venom control, venom and hexane fraction (100 mg kg^{-1} b.wt.), venom and ethylacetate fraction (100 mg kg^{-1} b.wt.), then venom and conventional antivenom. **Results:** The hexane and the ethylacetate fractions significantly rendered protection on the renal and haematological indices probably via inactivation of the venom cytotoxins. **Conclusion:** This research has provided a scientific proof on the antivenom properties of *A. indica* leaf *and* the plant extracts can be used for detoxification of the snake venom toxicity.

Key words: *Azadirachta indica*, fractionated extracts, renal, haematological, snake venom toxicity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biochemically, snake venoms are complex mixtures of active substances, mainly proteins and peptides which interfere with the course of several biological processes in humans[1]. A large number of protein toxins have been purified and characterized from snake venoms[2] and snake venoms typically contain from 30 to over 100 protein toxins. Some of these proteins exhibit enzymatic activities, whereas several others are non-enzymatic proteins and peptides.

Different toxins from snake venom exhibit different modes of action. Furthermore, venoms from different species vary greatly in their toxin composition[3].

Snake venom can cause death by causing tissue destruction and widespread haemorrhage (bleeding from some internal organ/part of the body associated with defective clotting mechanism in the body)[4].

Over the years, a number of toxins that affect blood circulation have been isolated and characterized from various snake venoms. Some of them affect platelet aggregation, whereas others affect blood coagulation[5]. Venom proteins affecting blood coagulation can functionally be classified as pro-coagulant or anticoagulant proteins on the basis of their ability to shorten or prolong the blood-clotting process. Pro-coagulant proteins are either serine proteinases or metalloproteinases. They induce blood coagulation either by specifically activating zymogen, one of the blood coagulation factors, or by directly converting soluble fibrinogen into an insoluble fibrin clot[6].

Azadirachta indica (Neem tree) belongs to the Meliaceae (mahogany) family. It is known as '*Dogon yaro* or *Darbejiya'* in Hausa Language. The neem tree has long been recognized for its unique properties in improving human health[7]. It is grown in most tropical and sub-tropical areas of the world for shade, reforestation and for the production of raw material for natural insecticides and medicines[8]. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity[9].

Different parts of neem plants such as leaf, bark, root, seed and flowers show role in disease management through modulation of various biological activities[10].

It has been demonstrated by earlier studies that crude extract of neem leaves possesses significant hypoglycemic as well as hypolipemic activities in addition to hepatoprotective and hypertensive activities[11,12]. Hence, this research was aimed at evaluating the protective effect of *A. indica* leaf extracts on renal and haematological indices in albino rats.

MATERIALS AND METHODS

Study area: The research was conducted between November, 2018 to March, 2019 in Aliero Local Government Area of Kebbi State, Nigeria. It was performed in Biochemistry Research Labouratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

Experimental animals: Adult Wister albino rats of both sexes aged 4-5 months and weighing between 150-280 g were used for the experiments. They were purchased from National Veterinary Research Institute, Vom, Nigeria and kept under standard laboratory conditions (22-24°C, 12:12 h dark/light cycle). The animals were allowed free access to both food (commercial rodents pellets) and water ad libitum[13], they were allowed to acclimatize for 2 weeks. Weight of each rat was taken before the commencement of the experiment. All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals[14].

Standard snake venom antiserum (Antivenom): The lyophilized polyvalent snake venom antiserum (Batch No.: 01AS83659, Man. Date: March, 2018, Exp. Date: February, 2021) was used as standard to compare with the efficacy of the plant extract. It was produced by a standard pharmaceutical company (VINS Bioproducts Limited Andhra Pradesh, India).

***Naja nigricollis*:** The snake specie (*Naja nigricollis*) used was captured and housed in a wooden cage with the help of a snake charmer. After collection, it was duly identified by a zoologist. Its venom was milked and used for the experiments.

Milking of venom: The venom was collected at 5:30 pm, in a low light condition at ambient temperature according to the method of Goswami *et al.*[15] by using a short-acting general anesthesia, halothane (Piramal Healthcare Limited, U.K.). The glands below the eyes of the snake were compressed to release the stored venom into a clean and sterilized container.

Preparation of venom: After milking, the venom was lyophilized using a freeze-dryer (Millrock Technology, USA) and kept inside a refrigerator (HR135A, Haier-Thermocool, Lagos, Nigeria) in a light resistant and air-tight container. Before use, the lyophilized venom was reconstituted in 0.9% saline (regarded as the venom) and kept at 4°C. The venom concentration was expressed in terms of dry weight (mg mL^{-1})[16].

Collection and authentication of the plant material: *Azadirachta indica* leaf was collected within Aliero town, Kebbi State, Nigeria. It was authenticated at the herbarium of the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero and voucher specimen was deposited there; VN:083.

Preparation of the crude *A. indica* leaf methanol extract: The extract was prepared according to the method of Dupont *et al.*[17]. The collected leaves were washed with clean water and air-dried under shade, pulverized using pestle and mortar. One kilogram (1 kg) of the powdered leaf was measured and soaked in 2.5 L of 95% methanol. The mixture was then kept at room temperature for 24 h and filtered twice; initially with a muslin cloth and later with a Whatman filter paper No. 1. The filtrate was evaporated to dryness at 45°C using rotary evaporator. The residue was further fractionated.

Solvent-fractionation of the crude *A. indica* leaf methanol extract: The crude methanol extract of the *A. indica* leaf was fractionated by liquid-liquid extraction using n-hexane and ethylacetate in increasing order of polarity. Two hundred grammes (200 g) of the dried methanol extract were reconstituted in 400 mL of distilled water in a 1 L separating funnel. This was then partitioned sequentially with equal volume of n-hexane and ethylacetate to yield the n-hexane and ethylacetate fractions. The fractions were concentrated to dryness and the residues were kept in a refrigerator in an air-tight container for further use. Before use, each fraction was reconstituted in 1% aqueous solution of Tween-80 (polysorbate) and was expressed in terms of dry weight (mg mL^{-1}).

Evaluation of the renal and haematological indices protection effect: To evaluate the protection effect of the hexane and the ethylacetate fractions, biochemical parameters of renal functions and haematological indices were analyzed.

Thirty rats were used. They were distributed randomly into (5) groups of (6) rats each (3 males and 3 females) and were treated as follows:

Group 1 : Received 1% aqueous solution of Tween-80 orally (p.o) and served as normal control

Group 2 : Injected (i.p.) only with 0.2 mg kg^{-1} b.wt., of the snake venom and served as venom control

Group 3, 4 : Injected (i.p.) with 0.2 mg kg^{-1} b.wt., of the snake venom, then after 30 min they were administered orally (p.o.) with 100 mg kg^{-1} b.wt., of the n-hexane and ethylacetate fractions of the extract, respectively

Group 5 : Injected (i.p.) with 0.2 mg kg^{-1} b.wt., of the snake venom, then after 30 min., they were administered (i.v.) with the standard conventional serum antivenin at the dose of 1 mL/ 0.6 mg venom

All the groups received same volume of preparations and were monitored for 24 h.

Blood samples were then collected into tubes with and without EDTA for haematological and biochemical analyses respectively. The coagulated blood samples for biochemical analyses were centrifuged at 3,000 rpm for 10 min to obtain the sera.

Serum biochemical analysis: The following parameters were analyzed in the sera collected, urea[18], uric acid[19], electrolytes and creatinine[20].

Haematological analysis: These were performed using an automatic haematological analyzer (Coulter STKS, Beckman Coulter, California, USA) by determining the amount of the following blood parameters[21], 2001), Packed cell volume (PCV), haemoglobin (Hb), red blood cell (RBC), mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC), lymphocytes, neutrophils, monocytes, basophils and platelets.

Statistical analysis: The data generated from the study are presented as Mean±SEM and subjected to one way analysis of variance (ANOVA) and statistical difference between the means were separated using new Duncan's multiple range test at $p<0.05$ with the aid of a statistical package (IBM SPSS Statistics 20).

RESULTS

Injection of the *N. nigricollis* venom to the albino rats at 0.2 mg kg^{-1} b.wt., caused significant decrease ($p<0.05$) in the serum levels of uric acid and creatinine (Table 1). These decreases were 59 and 21% at $p<0.05$, respectively when compared to the normal control group. The hexane fraction significantly ($p<0.05$) increased the serum level of uric acid in the envenomed rats when compared to the venom control (Table 1). None of the administered fractions (but only the standard antivenin) had significant effect on the levels of

Table 1: Renal function indices of envenomed rats treated with hexane and ethylacetate leaf fractions of *A. indica*

	Treatment				
Test parameters	Normal control	Venom control	Venom+hexane fraction	Venom+ethylacetate fraction	Venom+standard antivenom
Urea (mmol L^{-1})	007.05±1.63[a]	006.00±2.52[a]	006.50±0.73[a]	006.83±1.90[a]	008.57±0.83[a]
Uric acid (mg dL^{-1})	004.60±0.42[b]	001.90±0.56[a]	003.50±0.19[b]	002.77±0.36[ab]	002.17±0.25[a]
Creatinine (mmol L^{-1})	119.00±1.41[b]	094.33±9.68[a]	098.00±2.30[a]	100.67±4.62[a]	127.67±5.35[b]
Na^+ (meq L^{-1})	144.50±0.71[a]	144.67±2.52[a]	144.00±1.08[a]	139.67±4.04[a]	140.67±4.93[a]
K^+ (meq L^{-1})	007.10±0.42[a]	036.73±0.40[b]	008.60±0.64[a]	007.13±1.10[a]	007.33±0.32[a]
Cl^- (meq L^{-1})	108.00±6.41[a]	109.00±4.73[a]	110.00±3.01[a]	108.00±5.57[a]	108.33±2.58[a]
HCO_3^- (meq L^{-1})	029.00±1.41[a]	031.67±4.04[a]	027.00±0.27[a]	022.33±2.50[a]	026.00±4.00[a]

Results are presented as Mean±SEM (n = 6), values carrying different superscripts from the normal control for each parameter are significantly ($p<0.05$) different using ANOVA and Duncan multiple range test

Table 2: Haematological indices of envenomed rats treated with hexane and ethylacetate leaf fractions of *A. indica*

	Treatment				
Test parameters	Normal control	Venom control	Venom+hexane fraction	Venom+ethylacetate fraction	Venom+standard antivenom
WBC ($\times10^3$ μL^{-1})	007.50±00.66[a]	007.50±02.97[a]	003.60±00.76[a]	004.33±01.12[a]	008.03±00.19[a]
LYM (%)	074.40±01.86[a]	078.60±01.41[a]	069.50±03.02[a]	069.63±02.25[a]	055.73±05.80[a]
MON (%)	008.73±00.69[b]	002.95±00.21[a]	009.90±01.36[b]	006.47±01.63[ab]	010.17±00.49[b]
NEU (%)	016.07±01.41[a]	014.50±02.55[a]	019.90±00.97[a]	016.23±01.86[a]	024.37±01.96[a]
EOS (%)	001.10±00.17[a]	000.55±00.07[a]	000.70±00.02[a]	001.10±00.20[a]	001.30±00.21[a]
BAS (%)	009.70±00.78[b]	003.40±00.99[a]	010.00±01.05[b]	006.57±01.10[ab]	008.43±00.27[b]
RBC ($\times10^6$ μL^{-1})	005.98±01.41[b]	001.55±00.28[a]	004.68±00.16[b]	006.08±00.44[b]	006.04±00.55[b]
HGB (g dL^{-1})	011.73±02.83[b]	007.90±00.14[a]	012.60±00.94[b]	011.97±00.84[b]	012.03±00.87[b]
HCT (%)	033.10±02.07[b]	010.90±00.99[a]	027.70±03.20[b]	032.27±01.66[b]	033.03±01.46[b]
MCV (μm^3)	055.53±01.36[a]	051.80±00.70[a]	059.20±04.39[a]	052.67±02.95[a]	054.90±03.93[a]
MCH (pg)	019.57±00.47[a]	018.20±00.57[a]	018.40±00.62[a]	019.50±00.44[a]	019.93±00.45[a]
MCHC (g dL^{-1})	035.30±01.22[a]	035.10±00.57[a]	031.00±02.01[a]	037.10±01.25[a]	036.43±02.38[a]
PLT ($\times10^3$ μL^{-1})	424.67±49.13[a]	468.50±14.66[a]	480.00±15.11[a]	458.67±84.44[a]	387.33±43.32[a]
MPV (μm^3)	006.80±00.70[a]	006.65±00.35[a]	006.80±00.20[a]	006.30±00.20[a]	007.33±00.92[a]
PCT (%)	000.28±00.08[a]	000.31±00.10[a]	000.47±00.02[a]	000.29±00.03[a]	000.29±00.06[a]

Results are presented as Mean±SEM (n = 6), values carrying different superscripts from the normal control for each parameter are significantly ($p<0.05$) different using ANOVA and Duncan multiple range test, LYM: Lymphocytes, MON: Monocytes, NEU: Neutrophils, EOS: Eosinophils, BAS: Basophils, RBC: Red blood cells, HGB: Haemoglobin, HCT: Haematocrit, MCV: Mean cell volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, PLT: Platelets, MPV: Mean platelets volume, PCT: Plateletcrit

creatinine. K^+ level was significantly ($p<0.05$) higher in the serum of venom control rats compared to the normal control. This was not observed in the envenomed rats administered with hexane fraction, ethylacetate fraction or the standard antivenin. The serum levels of urea, Na^+, Cl^- and HCO_3^- were not significantly ($p>0.05$) affected by the administration of 0.2 mg kg^{-1} b.wt., venom when compared to the normal control group.

The *N. nigricollis* venom at the dose of 0.2 mg kg^{-1} b.wt., did not significantly ($p>0.05$) affect the levels of some haematological parameters in the envenomed albino rats with the exception of monocytes (MON), basophils (BAS), red blood cells (RBCs), haemoglobin and haematocrit when compared to the normal control (Table 2). The levels of MON and BAS decreased significantly ($p<0.05$) following the administration of the venom. The envenomed rats administered with the hexane fraction indicated significant increase in the levels of the MON and the BAS returning to the normal levels compared to the venom control group (Table 2). The levels of RBCs and haematocrit significantly ($p<0.05$) decreased in the venom control compared to the normal control. These decreases were not observed in the envenomed rats administered with the hexane fraction, ethylacetate fraction or the standard antivenin. Thus, the levels of RBCs and haematocrit in these treatment groups were not significantly ($p>0.05$) different compared to the normal control group.

DISCUSSION

The fractionated extracts (hexane and ethylacetate) used in this research have significantly ($p<0.05$) rendered protection on the renal and haematological indices probably via inactivation of the venom cytotoxins[5,22]. This research revealed that the injection of crude venom of *Naja nigricollis* caused a significant increase in the serum level of K^+ in the envenomed rats compared to the normal control group (Table 1). There are few investigations regarding the effect of snake venoms on serum electrolytes. Mohamed *et al.*[23]

reported a decrease in Na^+ and an increase in K^+ serum levels following envenomation with *N. nigricollis* venom in albino rats. In the current study, the significant increase in the serum K^+ level might be due to haemolysis of the RBCs by the venom toxic proteins which leads to the release of the intracellular K^+, thereby increasing its serum level in the envenomed rats[24]. The hexane and the ethylacetate fractions were capable of reducing the serum level of the K^+ possibly by inactivation of the venom toxic proteins compared to the venom control group.

The reduction in serum uric acid and creatinine levels in the venom control rats compared to the normal control group might be due to impaired renal function (Table 1). Renal damages with different types of lesions (glomerular, tubular, interstitial or vascular) due to the action of cytotoxic proteins manifested by a decrease in the serum urea level were also reported by Schneemann *et al.*[25].

The mechanisms of venom cytotoxin-mediated toxicity include haemolysis of RBCs due to their ability to bind to cell membranes leading to alterations in the organization and function of the lipid bilayers[22]. Similarly, the venom phospholipase A2 is a well-known agent of haemolysis[26] through its hydrolytic activity on the RBCs membrane phospholipids[27]. The haemolysis of RBCs observed in the present study consequently leads to the decrease in their concentration which eventually results in the decrease in the percentage of HCT. The hexane and the ethylacetate fractions used were able to prevent the haemolysis as confirmed by an increase in the level of the RBCs together with increase in the percentage of HCT in the hexane and the ethylacetate treatment groups.

Significant reduction in the levels of monocytes and basophils in the venom control rats compared to the normal control group in this research agreed with the findings of James *et al.*[24] who reported a reduction in the levels of monocytes and basophils in the envenomed rats compared to the normal control rats. Interestingly, it was observed that, the hexane and ethylacetate fractions used were capable of protecting the effect of the venom on monocytes and basophils (Table 2). This might be due to the inactivation of the toxic proteins in the *N. nigricollis* venom[5].

Many researches dealing with effects of snake venom on blood cells, marrow cells and in cells from other organs of animals, like muscle, liver, kidney and skin, showed varying results depending on the experimental concentrations of the venoms, exposure time, site of administration and type of the venom toxin[28]. Toxic effects which manifest as tissue necrosis, inflammation, cytotoxicity, myotoxicity and haemolysis produced by cytotoxic proteins and complement depletion are associated with *N. nigricollis* venom[5,26,29,30].

Snake venom cytotoxins are highly basic amphipathic proteins and they constitute as much as 40-70% of cobra venom. They include lytic factors, cobramins, cytolysins and membranotoxins[22]. However, inactivation of these cytotoxic proteins can neutralize the toxicity and lethality of the cobra venom[5].

CONCLUSION

The advantages of herbal remedies are that, they are cheap, readly available, stable at room temperature and could neutralize a wide range of venom toxins. The fractionated extracts of the *A. indica* leaf showed an effective protection on the renal and haematological indices against the *Naja nigricollis* venom with varying degrees of efficacy. Therefore, this research has provided a scientific proof on the antivenom properties of *A. indica* leaf and the plant extracts can be used for detoxification of the snake venom toxicity.

SIGNIFICANCE STATEMENT

This study discovers the antivenom properties of *A. indica* leaf and this can be used for the detoxification of the snake venom toxicity thereby preventing tissue/organ distraction. It is obvious that snake venoms have physiological effect on human body, because they target almost every tissue or organ of the body. Organ failure as a result of snake venom toxicity can lead to death. Hence, this study will help researchers to develop cheap, readily available and easily storable plant-based antivenom for the benefit of rural communities around the world.

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