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Inhibition of Snake Venom Enzymes and Antivenom Adjuvant Effects of *Azadirachta indica* A. Juss. (Meliaceae) Leaf Extracts

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Authors' contributions

This work was carried out in collaboration among all authors. Author IS designed the study and performed the statistical analysis. Authors RAU and SWH wrote the protocol. Author UZF managed the literature searches. Author IS wrote the first draft of the manuscript. Authors FB and AA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Snake venom enzymes are the key substances involved in snake venom toxicity. Thus, inactivating these enzymes is generally considered to be the fundamental step in the management of snakebite. Conventionally, snakebite envenomation is treated parenterally with serum-based antivenins, and adjuvants to these antivenins are required for maximum protection of victims. Hence, this research was aimed at evaluating the inhibitory effect of *Azadirachta indica* leaf extracts on *Naja nigricollis* Reinhardt venom enzymes and screens for their antivenom adjuvant

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effects. *A. indica* leaf was collected, authenticated and extracted using 95% methanol followed by fractionation using hexane and ethyl acetate. The venom enzymes inhibition assays was evaluated using *in vitro* methods, while, adjuvant effect was screened using Albino rats. The results revealed that both the hexane and the ethyl acetate fractions showed capability of inhibiting the venom enzymes significantly (P<0.05) when compared with the venom controls in varying degrees of efficacies. For the adjuvant effect, no significant effect (P>0.05) of the venom at the administered dose was observed on bleeding time, clotting time, defibrinogenating and haemorrhagic effects compared to the normal control. However, the size of necrotic lesion and the percentage haemolysis were significantly (P<0.05) higher in the venom control rats. Both the hexane and the ethyl acetate fractions significantly mitigated these effects in the treated animals. The degree of protection was about 3 folds more than when the antivenin was used alone. Finally, these findings would be of importance in the area of drug development with a view to actualizing the substitution or enhancing the effect of conventional snakebite therapeutic options.

Keywords: Azadirachta indica; snake venom; enzyme inhibition; antivenom; adjuvant effect.

1. INTRODUCTION

Currently, serum-based antivenin is the only medically approved antidote against snakebite envenomation [1]. It is usually produced by milking venom from the desired snake. The venom is then diluted and injected into an animal (mostly a horse). The animal will undergo an immune response to the venom, producing antibodies against the venom's toxins which can then be harvested from the animal's blood and used to treat envenomation [2-4].

Considering the limitations of antivenin, such as causing adverse reactions like anaphylactic shock, pyrogen reaction and serum sickness, as well as not providing enough protection against the venom induced toxicities, such as, necrosis, haemorrhage, cytotoxicity, nephrotoxicity, etc. [5], application of medicinal plants with antisnake venom activities might be useful in treating victims of snakebite envenomation [6,7].

Herbal antivenoms have been reported to neutralize toxic venom constituents through several mechanisms [4]. These include; inhibition of venom enzymes [8] and through antivenom adjuvant action [9] or combination of these activities [10].

Snake venom enzymes are the key substances involved in snake venom toxicities [11,12]. Thus, inactivation of these enzymes is generally considered to be the fundamental step in the management of snakebite.

The most common enzymes in snake venoms are phospholipase A_2s, serine proteinases, metalloproteinases, acetylcholinesterases, L-amino acid oxidases, phosphomono- and phosphodi-esterases, nucleotidases and hyaluronidases [12,13].

Digestive hydrolases, L-amino acid oxidases, phospholipases, serine proteinases and metallo-proteinases damage vascular endothelium [14]. Phosphodiesterases interfere with prey's cardiac system, mainly to lower blood pressure [11]. Phospholipase A2 causes haemolysis by lysing the phospholipid cell membranes of red blood cells. Amino acid oxidases and proteinases are used for digestion. Amino acid oxidase also triggers some other enzymes and is responsible for the yellow colour of the venom of some species. Hyaluronidase increases tissue permeability to accelerate absorption of other enzymes into tissues. Snake venoms typically contain neurotoxins, but they also often include enzymes that promote various hydrolysis reactions. The neurotoxins carry out the task of immobilizing victim by interrupting the ability of the nerve cells to stimulate muscle movement. Hydrolysis helps make the tissues of the victim easier for the snake to digest if it is eaten. These hydrolytic enzymes may include molecules capable of breaking down collagen and phospholipids as well as other enzymes [15].

Many plants with snake venom enzymes inhibitory effect, such as *Curcuma longa* L. (Zingiberaceae), *Cordia verbenacea* A.DC. (Boraginaceae), *Hemidesmus indicus* (L.) R.Brex Schult. (Apocynaceae), *Areca catechu* L. (Arecaceae), *Crinum jagus* (J. Thomps.) Dandy, (Amaryllidaceae), *Quercus infectoria* G. Olivier (Fagaceae) etc. were believed to contain high level of polyphenols [16]. Polyphenols such as tannins are specialized metabolites found in many plants species that have been shown to interact with enzymes from snake venom by non-

specific binding to the proteins [17,18]. In relation to the mode of action, several studies [19-22] have concluded that the inhibition of polyphenolic compounds on snake venom enzymes is due to the interactions between the enzyme and the hydroxyl groups present in this type of metabolites through hydrogen bonds resulting in the formation of a stable complex [23]. However, the activity of polyphenol compounds may involve varying degrees of interactions such as hydrophobic connections mediated by aromatic rings [11].

For the adjuvant action, a compound (2-hydroxy-4-methoxy benzoic acid) potentiated venom neutralizing action of commercial snake venom antiserum [9]. High degree of neutralizing both cobra and viper venoms was achieved by this compound. This effect was executed through increased antiserum retention and venom antigen presentation for better neutralization. Thus, the compound has a dual action in this phenomenon; (a) showing its adjuvant effect and (b) increasing antiserum efficacy.

2. MATERIALS AND METHODS

2.1 Study Area

The research was conducted in Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

2.2 Experimental Animals

Adult Wistar albino rats of both sexes aged 3 – 4 months and weighing between 150 – 200 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* [24]; 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 [25].

2.3 Standard Snake Venom Antiserum (Antivenin)

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

2.4 *Naja nigricollis* Reinhardt

The snake specie (*Naja nigricollis* Reinhardt) used was captured and housed in a wooden cage with the help of a snake-charmer. After collection, it was duly identified and authenticated by a zoologist at the Department of Animal and Environmental Biology, Kebbi State University of Science and Technology, Aliero, Nigeria. Its venom was milked and used for the experiments.

2.5 Milking of Venom

The venom was collected between 5.00 pm to 6.00 pm, in a low light condition at an ambient temperature according to the method of Goswami et al., [16] with modification 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 cleaned and sterilized container.

2.6 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) [26].

2.7 Dosing of the Venom

The dose of the venom used for the *in vivo* studies was according to the LD_{50} of the venom as reported by Sani et al., [27].

2.8 Collection and Authentication of the Plant Material

Azadirachta indica A.Juss. 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, Nigeria and voucher specimen; VN:083 was deposited there.

2.9 Preparation of Crude *A. indica* Leaf Methanol Extract

The extract was prepared according to the method of Dupont et al., [28]. The collected leaf was cleaned with 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.

2.10 Solvent-Fractionation of 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 ethyl acetate in increasing order of polarity. Two hundred grams (200 g) of the dried methanol extract were reconstituted in 400 ml of distilled water in a 1 liter separating funnel. This was then partitioned sequentially with equal volume of n-hexane, and ethyl acetate to yield the n-hexane and ethyl acetate 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).

2.11 Inhibition Assays of the Venom Enzymes

For the venom enzymes inhibition studies, venom was pre-incubated with extracts for 30 minutes at 37°C and then used.

2.11.1 Phospholipase A2 inhibition assay

The venom phospholipase A2 activity was determined acidimetrically using the method of Tan and Tan, [29]. Constant volumes of substrates comprising Calcium chloride (18 mM), sodium deoxycholate (8.1 mM) and egg yolk were mixed and stirred for 10 min to get homogenous egg yolk suspension. The pH of the mixture was adjusted to 8.0 using 1 M of Sodium hydroxide. Snake venom (3 mg/ml) was added to the above mixture (15 ml) to initiate the process of hydrolysis and saline was used as control. A decrease in pH of the suspension was noted after two minutes with the help of a pH meter. A decrease of pH by one (1) unit corresponded to 133 µmole of fatty acid released. Enzyme activity was expressed as µmole of fatty acid released per minute [30]. Enzyme activity and percentage activity were obtained using the following formulae:

$$\text{Enzyme Activity (µmole FA/min.)} = \frac{\text{µmole of FA released}}{\text{Time taken in minutes}}$$

Where: FA = Fatty acid

$$\% \text{ Activity} = \frac{\text{Enzyme Activity of the test sample}}{\text{Enzyme Actviity of the venom control}} \times 100$$

2.11.2 Proteinase inhibition assay

The venom's proteinase activity assay was performed according to the method of Greenberg, [31]. The reaction mixture composed of 0.5% casein, 1.0 ml of Tris–HCl buffer (pH 8.0) and 0.5 ml of 0.25% crude venom was incubated for 4 h at 37°C. At the end of 4 h, the reaction was stopped by adding trichloroacetic acid (TCA) and the mixture was filtered. The filtrate (1.0 ml) was used for protein estimation using the method of Lowry et al., [32]. L-tyrosine was used as standard. In the above investigation, 1 unit of enzyme activity was defined as the amount that yielded 0.02 µmole of tyrosine/h under the experimental conditions described.

2.11.3 Phosphomonoesterase inhibition assay

The venom's phosphomonoesterase activity was determined using the method of Bessey *et al.,* [33]. The reaction mixture contained 1.0 ml of Tris–HCl buffer (pH 8.0), 1.0 ml of disodium-p-nitrophenol phosphate, 0.5 ml of 0.25% crude venom. The mixture was incubated at 37°C for 3 h, and the absorbance was then measured at 425 nm. p-Nitrophenol was used as the standard. One (1) unit of enzyme activity was defined as the amount that yielded 0.1 µmole of p-nitrophenol/h under the experimental conditions.

2.11.4 Phosphodiesterase inhibition assay

The venom's phosphodiesterase activity was determined using the method of Lo et al., [34]. The assay mixture contained 0.1 ml of venom solution, 0.5 ml of 2.5 mM Na-p-nitrophenyl phosphate, 0.3 ml of 10 mM $MgSO_4$ and 0.5 ml of 0.17 M Tris–HCl (pH 8.0). The absorbance was measured at 400 nm. Phosphodiesterase activity was expressed in nmole of phosphate released/minute. Molar extinction coefficient at 400 nm was 8100 Cm^{-1} M^{-1} [30].

2.11.5 L-Amino acid oxidase inhibition assay

The venom's L-amino acid oxidase activity assay was carried out according to the method of Li et al., [35]. The reaction mixture contained 1.0 ml of L-leucine, 2.0 ml of Tris–HCl buffer (pH 8.0), 0.25 ml of 0.1% dianisidine hydrochloride, 0.15 ml of 0.1% horseradish peroxidase and 0.04 ml of 0.5% crude venom solution. It was allowed to stand for 10 minutes at room temperature and the absorbance was measured at 415 nm. One (1) unit was defined as the amount of enzyme that catalyzed the formation of 1 µmol H_2O_2 per minute.

2.11.6 Acetylcholinesterase inhibition assay

The venom's acetyl-cholinesterase activity assay was conducted using the method of Ellman *et al.,* [36]. The reaction mixture contained 3.0 ml of phosphate buffer (pH 8.0), 10 µl of 10 mmol/l DTNB and 20 µl of 158.5 mmol/l acetylethiocholine iodide. A total of 50 µl of 0.1% crude venom and 3 mls of buffer solution were incubated at room temperature for 5 minutes. Then, 10 µl of 5,5'-Dithio-bis-(2-Nitrobenzoic Acid) (DTNB) (a strong oxidizing agent) and 20 µl of substrate acetylethiocholine iodide were added in order to reach a final concentration of 1 mmol/l. The increase in absorbance at 412 nm was measured on a double beam spectro-photometer against control mixture prepared at the same time. However, in the latter case, 50 µl of enzyme was replaced with 50 µl of buffer solution.

2.11.7 Hyaluronidase inhibition assay

The venom's hyaluronidase activity was determined turbidometrically using the method of Pukrittayakamee et al., [37]. The assay mixture contained Tris–HCl buffer (pH 8.0), 50 µg of hyaluronic acid (0.5 mg/ml in buffer) and enzymes in a final volume of 1.0 ml. The mixture was incubated for 15 minutes at 37°C and the reaction was quenched by the addition of 2 ml of 2.5% (w/v) cetyltrimethylammonium bromide in 2% NaOH (w/v). The absorbance was read at 400 nm (within 10 minutes) against a control solution containing 1 ml of the same buffer and 2 ml of 2.5% (w/v) cetyltrimethylammonium bromide in 2% NaOH (w/v). Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube in which no enzyme was added as 100%. One (1) unit was defined as the amount of enzyme that triggered 50% turbidity reduction. Specific activity was defined as turbidity reducing units per milligramme of enzyme.

2.12 Antivenom Adjuvant Action Potentiation

Thirty five (35) rats were randomly distributed into seven (7) groups of five (5) rats each as follows:

Group 1: Injected intradermaly (*i.d.*) (through shaved dorsal skin of the rats) with normal saline, then 1% aqueous solution of Tween-80 was administered orally (*p.o*) and served as normal control.

Group 2: Injected intradermal (*i.d.*) (through shaved dorsal skin of the rats) only with 0.190 mg/kg body weight of the snake venom and served as venom (negative) control.

Group 3 and 4: Injected (*i.d.*) (also through shaved dorsal skin of the rats) with 0.190 mg/kg b. wt. of the snake venom, followed by immediate oral administration (*p.o*) of 100 mg/kg b. wt. each of the *n*-hexane and ethyl acetate extract respectively.

Group 5: Injected (*i.d.*) with 0.190 mg/kg 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/0.6mg venom. They served as positive control.

Group 6 and 7: Injected (*i.d.*) with 0.190 mg/kg 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/0.6mg venom, followed by immediate oral administration of 100 mg/kg b. wt. each of the *n*-hexane and ethyl acetate extract respectively.

All the groups received the same volume of preparations. Neutralization of the venom toxic effects by the extracts was evaluated as follows:

2.12.1 Determination of bleeding time

For the determination of the bleeding time, the method of Mohammed et al., [38] was used. Two (2) hours after the animal treatment, the tail of each rat was gently pierced with lancet. 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 was noted when the paper was no longer stained with blood.

2.12.2 Determination of clotting time

For the determination of clotting time, the method of Igboechi and Anuforo, [39] was used. Clotting time is the time required for a firm clot to be formed in fresh blood on glass slides. Three (3) hours after the animal treatment, blood samples were collected from the rats 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 then recorded.

2.12.3 Inhibition of venom defibrinogenating activity

The defibrinogenating activity (DFA) of venom is defined as the effect of venom which when injected (*i.d.*) into rat causes incoagulable blood 4 h later. Four (4) hours after the animal treatment, the coagulability of the blood was examined.

2.12.4 Inhibition of venom haemorrhagic activity

The Minimum Haemorrhagic Dose (MHD) of venom is defined as "the least amount of venom which when injected intradermaly (*i.d*) into rats, results in a haemorrhagic lesion of 10 mm diameter 6 h later" [40]. Six (6) hours after the animal treatment, the haemorrhagic lesions were measured and recorded.

2.12.5 Inhibition of venom necrotizing activity

The Minimum Necrotizing Dose (MND) of a venom is defined as "the amount of venom which when injected (*i.d*) in to rats, results in a necrotic lesion of 5 mm diameter three (3) days later" [40]. To assess the anti-necrotizing effect of the extract fractions, three (3) days after the animal treatment, the necrotic lesions were measured and recorded.

2.12.6 Inhibition of venom haemolytic activity

The venom induced haemolysis and its neutralization by the extract fractions was determined using the method of Vijayabharathi et al., [41]. Blood samples were collected from healthy rats via tail vein using heparin as anticoagulant. The blood was then centrifuged at 3000 rpm for 10 min. and packed cells were separated. The cells were washed thrice with phosphate buffer (0.15 M, pH 7.4), and then re-centrifuged to collect the cells. Venom (0.1 mg/ml, 1 ml), phosphate buffer (0.15 M, pH 7.4, 1 ml) and RBCs (1% v/v, 1 ml) were mixed and incubated at 37°C for 30 minutes. Subsequently, the mixture was centrifuged at 1000 rpm for 3 minutes. Absorbance of the supernatant (due to the release of haemoglobin) was measured spectrophotometrically at 540 nm. For the anti-haemolytic activity, the snake venom was pre-incubated with 0.5 mg/ml of the extract at 37°C for 30 minutes. Solution of saline and the solution of the venom and extract served as control for the haemolytic and anti-haemolytic assays respectively. All assays were conducted in triplicates. Finally, percentage haemolysis and protection were calculated by using the following formulae:

$$\%\ \text{Hemolysis}\ ;\ \mathrm{Y} = \frac{\mathrm{A_T}}{\mathrm{A_C}} \times 100$$

$$\%\ \text{Protection} = 100 - \mathrm{Y}$$

Where,

A_T = Absorbance of treated sample, A_C = Absorbance of control

2.13 Data 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).

3. RESULTS

3.1 Inhibition of Venom Enzymes

Table 1 presents the effects of the hexane and ethyl acetate fractions on the activities of the venom enzymes. Acetylcholinesterase, hyaluronidase, phospholipase A2 and L-amino acid oxidase had significantly ($P<0.05$) higher enzymatic activity compared to phosphomono-esterase, while proteinases and phosphodies-terase have significantly ($P>0.05$) lower enzymatic activity.

Both the hexane and the ethyl acetate fractions showed capability of inhibiting the venom enzymes significantly (P<0.05) when compared with the venom controls. The hexane fraction

significantly (P<0.05) inhibited the activities of hyaluronidase, phosphodiesterase and proteinases more than the inhibition by the standard antivenin. However, there was no significant (P>0.05) difference between the inhibitory effect of the hexane fraction and that of the standard antivenin on the activities of phospholipase A2, L-amino acid oxidase and acetylcholinesterase.

The inhibitory effect of ethyl acetate fraction was significantly (P<0.05) higher than that of the standard antivenin on hyaluronidase. Statistically similar inhibitory effect was observed against phospholipase A2, proteinases and phosphomonoesterase. But, on phosphodiesterase, L-amino acid oxidase and acetylcholinesterase, the inhibitory effects of the standard antivenin are significantly higher (P<0.05) than that of the ethyl acetate fraction.

3.2 Antivenom Adjuvant Effect

Table 2 presents the neutralization of venom toxic effects and antivenom adjuvant action potentiation by the hexane and ethyl acetate fractions. No significant effect (P>0.05) of the venom at the dose of 0.190 mg/kg b. wt. was observed on the bleeding and clotting times compared to the normal control group (Table 2). Similarly, no defibrinogenating effects (non coagulable blood) as well as significant change in the size of haemorrhagic lesions were observed in all the animal groups.

However, the size of necrotic lesion and the percentage haemolysis were significantly (P<0.05) higher in the venom control rats. Both the hexane and the ethyl acetate fractions significantly mitigated these effects compared to the venom control (Table 2). Interestingly, the envenomed rats administered with both the standard antivenin and the hexane or the ethyl acetate fractions presented necrotic lesion and percentage haemolysis that were not significantly different (P>0.05) from the normal control. The degree of protection was about 3 folds more than when each of the fractions or the antivenin was used alone (Table 2).

4. DISCUSSION

4.1 Inhibition of Venom Enzymes

The hexane and the ethyl acetate fractions showed capability of inhibiting the venom enzymes when compared with the venom controls. The hexane fraction significantly inhibited the activities of hyaluronidase, phosphodiesterase and proteinases more than the inhibitory effect of the standard antivenin. However, the inhibitory effect of the hexane fraction was not significantly different from that of the standard antivenin on the activities of phospholipase A2, L-amino acid oxidase and acetylcholinesterase.

Phospholipase A_2 represents a superfamily of lipolytic enzymes which specifically catalyze the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids resulting in the generation of fatty acid (arachidonate) and lysophospholipids [42].

Snake venom PLA2s are considered to be target specific and mimic the whole venom poisoning by exhibiting a wide variety of toxic effects. Type II are the chief toxins responsible for the expression of inflammatory, vasodilating and vasoconstriction mediators including prostaglandins, histamine, kinins, eicosanoids, platelet activating factor, catecholamines, dopamine, nitric oxide and endothelins during envenomation [43] leading to systemic toxicity of the venom. In addition, type I PLA2s are also involved in exerting several additional effects including cardiotoxicity, myotoxicity, pre- or post-synaptic neurotoxicity, oedema, haemolysis, hypotension, convulsion, and can also lead to platelet aggregation inhibition and anticoagulation [18,44,45].

In order to understand the interaction between tannins and other polyphenols (from plants) with PLA2 from *Naja nigricollis*, a previously reported research on molecular docking indicated that these molecules selectively bind to the active sites or modify conserved residues that are important to the catalytic activity of PLA2 enzymes [46]. Therefore, this might be the mechanism of the inhibitory effect of the hexane and ethyl acetate fractions on the activity of PLA2 in this research due to their high content of tannins and other polyphenols (data not included).

There have been numerous reports on natural compounds from plant resources that are capable of inhibiting PLA2 [1,6]. These compounds include aristolochic acid, vitamin E and atropine [47]. The hydroxyl groups of both aristolochic acid and vitamin E form two hydrogen bonds with the side chains of His48 and Asp49. The conserved water molecule in

both cases has been replaced by the hydroxyl moieties of these compounds and generates direct hydrogen bonding interactions [48,49].

Serine proteinases catalyze the cleavage of covalent peptide bonds in proteins and thereby affecting many biological processes such as; digestion, blood coagulation, the immune system and inflammation [50]. Venom serine proteinases (VSPs) interfere with the regulation and control of key biological reactions in the blood coagulation cascade, fibrinolysis and blood platelet activation [12,18,51-55].

Chandrashekara et al., [56] demonstrated that extracts from *Morus alba* (Moraceae) are active against *Daboia russelli* venom, inhibiting the caseinolytic and procoagulant activities. This inhibition was attributed to the presence of tannins which are capable of forming complexes with proteins [57,58]. Hence, it can be inferred that the fractions used in this research were able to inhibit the activity of the venom serine proteinases from 0.79 ± 0.02 U/L (as observed in the venom control) to 0.37 ± 0.09 and 0.46 ± 0.11 U/L for the hexane and ethyl acetate fraction respectively.

L-amino acid oxidase is a flavoenzyme found in such diverse organisms as bacteria, fungi, algae, fish, snails as well as venoms of snakes from the families *Viperidae*, *Crotalidae* and *Elapidae* [12,59].

Flavin present in LAAO is responsible for the characteristic yellow colour of many snake venoms and contribute to their toxicity because of the oxidative stress that results from the production of H_2O_2 [60-62]. This feature allows the classification of LAAOs as FAD-dependent oxidoreductases. They are capable of catalyzing the stereospecific oxidative deamination of L-amino acid substrates to α-keto acids [63].

Faust et al., [64] reported that LAAO contributes a role to the toxicity of the venom and suggested that the enzyme causes induction of apoptosis, cytotoxicity, inhibition or induction of platelet aggregation, haemorrhage and haemolysis.

Interestingly, the fractions of the *A. indica* leaf used in this study were able to significantly inhibit the activity of the venom LAAO from 12.60 ± 0.83 U/l (as observed in the venom control) to 1.75 ± 0.14 and 6.00 ± 1.20 U/l for the hexane and ethyl acetate fraction respectively. This could be attributed to their high content of phenolic compounds (data not included).

In a research reported by Pithayanukul et al., [65], ethanolic extract from seed kernels of *Mangifera indica* (Anacardiaceae) and its pentagalloyl glucopyranose exhibited anti-haemorrhagic and anti-dermonecrotic characteristics and inhibited LAAO activity of *Calloselasma rhodostoma* and *Naja kaouthia* venoms. Molecular docking studies indicated that the phenolic compounds of *M. indica* could selectively bind to the LAAO hydrophobic binding pocket in the catalytic site, thus inhibiting the enzyme activity [17].

Acetylcholinesterase is a member of the cholinesterase family [66] and plays a vital role in acetylcholine (ACh) transmission in the nervous system by ensuring the hydrolysis of ACh to choline and an acetate group, thereby terminating the chemical impulse [44,67].

Table 1. Inhibition profile of venom enzymes treated with hexane and ethyl acetate fractions of *A. indica* leaf

Enzyme	Enzyme activity (U/l)			
	Venom control	Venom + hexane fraction	Venom + ethyl acetate fraction	Venom + standard antivenin
Phospholipase A2	14.08 ± 0.30^{b}	00.59 ± 0.03^{a}	00.94 ± 1.40^{a}	00.42 ± 0.08^{a}
Proteinases	00.79 ± 0.02^{c}	00.37 ± 0.09^{a}	00.46 ± 0.11^{b}	00.42 ± 0.10^{b}
Phosphomonoesterase	03.20 ± 0.18^{c}	01.17 ± 0.30^{b}	00.82 ± 0.22^{ab}	00.61 ± 0.13^{a}
Phosphodiesterase	00.70 ± 0.09^{c}	00.36 ± 0.03^{a}	00.64 ± 0.23^{c}	00.40 ± 0.13^{b}
L-Amino Acid Oxidase	12.60 ± 0.83^{c}	01.75 ± 0.14^{a}	06.00 ± 1.20^{b}	02.09 ± 0.40^{a}
Acetylcholinesterase	30.21 ± 5.30^{c}	02.31 ± 1.93^{a}	06.04 ± 1.53^{b}	03.00 ± 0.19^{a}
Hyaluronidase	16.50 ± 2.91^{c}	03.93 ± 0.13^{a}	04.05 ± 1.03^{a}	08.05 ± 2.07^{b}

Values are presented as mean ± SEM of triplicates. Values carrying different superscripts (along the row) from the venom control are significantly (P<0.05) different

Table 2. Antivenom adjuvant properties of hexane and ethyl acetate leaf fractions of *A. indica* in rats

Test parameters	Treatment						
	Normal control	Venom control	Venom + hexane fraction	Venom + ethyl acetate fraction	Venom + standard antivenin	Venom + antivenin + hexane fraction	Venom + antivenin + ethyl acetate fraction
Bleeding Time (s)	92.05 ± 3.63[a]	95.50 ± 2.52[a]	93.50 ± 2.73[a]	92.83 ± 1.90[a]	94.57 ± 0.63[a]	94.03 ± 3.20[a]	93.81 ± 2.96[a]
Clotting Time (s)	39.60 ± 0.42[a]	54.22 ± 3.16[a]	50.21 ± 1.19[a]	46.00 ± 0.36[a]	52.17 ± 2.25[a]	36.37 ± 5.15[a]	39.17 ± 1.22[a]
Haemorrhagic Lesion (mm)	02.75 ± 0.41[a]	03.80 ± 0.68[a]	03.50 ± 1.30[a]	02.80 ± 0.62[a]	03.17 ± 0.35[a]	02.93 ± 1.21[a]	03.22 ± 1.01[a]
Defibrinogenating Effect	-	-	-	-	-	-	-
Necrotic Lesion (mm)	01.00 ± 0.02[a]	33.16 ± 3.40[c]	11.75 ± 0.64[b]	12.00 ± 0.20[b]	16.33 ± 0.32[b]	02.75 ± 1.62[a]	05.15 ± 0.44[a]
Haemolysis (%)	03.06 ± 1.20[a]	69.93 ± 4.27[c]	13.06 ± 2.11[b]	15.30 ± 2.51[b]	13.33 ± 0.58[b]	05.12 ± 0.38[a]	06.01 ± 1.19[a]

Values are presented as mean ± SEM (n = 5). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test. - = No defibrinogenating effect (non-coagulable blood) was observed

Significant amounts of AChE are found in the venom of snakes, particularly in species belonging to the family *Elapidae*, with the exception of Dendroaspis species [12]. In contrast, AChE is not found in venoms of snakes belonging to the Viperidae and Crotalidae families [68].

Sunitha et al., [69] investigated the molecular mechanism by which polyphenols from plant extracts interact with AChE from the venom of certain *Naja* snakes, which causes paralysis by hydrolyzing acetylcholine and prevent its binding to the muscle-type nicotinic acetylcholine receptor (nAChR). They evaluated the interaction between the AChE and the following phenolic compounds: Tannic acid, digallic acid, procyanidin dimer and procyanidin trimer, present in the extracts of *Quercus infectoria* (Fagaceae), *Pithecellobium dulce* (Leguminosae) and *Areca catechu* (Arecaceae). Molecular modeling showed the formation of hydrogen bonds between hydroxyl groups of tannins and amino acid residues surrounding the active site of the enzyme [69].

These findings by Sunitha et al., [69] can be used to explain why the hexane and the ethyl acetate fractions of the *A. indica* leaf tested in this research significantly inhibited the activity of the venom AChE due to their high content of tannins (data not included).

Venom's hyaluronidase (VHY) is rightly referred to as a 'Spreading factor' as it facilitates easy diffusion of venom toxins from the site of bite into general circulation [12,17,20]. Rapid hydrolysis of mega structure; hyaluronic acid (HA) into fragments of varied molecular size resulting in the decreased viscosity of the envenomed milieu aiding rapid diffusion of the toxins into circulation, which would otherwise, diffuse much slowly [70]. Further, the degraded end products of HA with high molecular mass are reported to be anti-angiogenic, anti-inflammatory and immunosuppressive [12], while low molecular mass fragments are pro-inflammatory, immune-stimulatory and angiogenic leading to complications [71].

The significant decrease in the activity of the VHY in this study could be attributed to a high content of phenolic compounds in the *A. indica* leaf fractions (data not included) as corroborated by other reported researches [17,20,65,70,72].

4.2 Antivenom Adjuvant Effect

In this research, it was shown that, the *Naja nigricollis* venom has no effect on bleeding and clotting times. Similarly, this venom has no haemorrhagic and defibrinogenating effects due to the absence of metalloproteinases (MPs) that are found only in viperid venoms [12,73]. MPs are primarily responsible for the haemorrhagic activity and the induction of local and systemic bleeding [4,12].

The significant reduction in the size of necrotic lesion and the percentage haemolysis in the envenomed rats co-administered with either hexane or the ethyl acetate fraction of the *A. indica* leaf and the standard antivenin signified potentiation of the effect of the standard antivenin through adjuvant action (Table 2).

Studies on the supplementation of conventional antivenins against snake venom or isolated venom enzymes with extracts from *Quercus infectoria* (Fagaceae) and *Areca catechu* (Arecaceae) and rosmarinic acid isolated from *Cordia verbenacea* (Boraginaceae) showed that, they enhanced the neutralization effect produced by the antivenin in mice [74-78].

5. CONCLUSION

The limitations of serum antivenin are well known and the world is searching for an alternative for snakebite treatment. Till date no suitable alternative measures are available, except the natural herbal remedies, which are showing promising expectations. The advantages of herbal compounds are that, they are cheap, easily available and stable at room temperature and could neutralize a wide range of venom toxins. Therefore, this research has provided scientific proof on the antivenom properties of *A. indica* leaf. The plant fractions tested have effectively neutralized the snake venom in the presence of serum antivenin, which is another advantage. It may be opined that the fractions having serum antivenin potentiating action as seen in this study might be selected for further trials. It is now obvious that, combination of antivenin and herbal compounds may provide a suitable alternative to the snakebite treatment in the near future. This lends support to the traditional use of *A. indica* leaf in the treatment of snakebites. Finally, these findings would be of importance in the area of drug development with a view to actualizing the substitution of conventional snakebite therapeutic options with

effective, cheap, accessible and less allergic plant-based compounds.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals, National Veterinary Research Institute, Vom, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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