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## Lethality of *Naja nigricollis* Reinhardt venom and antivenom activity of *Azadirachta indica* A. Juss. leaf extracts on albino rats

Ibrahim Sani<sup>1,\*</sup>, Umar Aliyu Rabi'u<sup>2</sup>, Sanusi Wara Hassan<sup>2</sup>, Umar Zaki Faruq<sup>3</sup> and Fatima Bello<sup>1</sup><sup>1</sup> Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.<sup>2</sup> Department of Biochemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria.<sup>3</sup> Department of Pure and Applied Chemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria.

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### Abstract

Snakebite remains an important cause of morbidity and mortality in Nigeria. Conventionally, the means of treatment is parenteral administration of serum-based antivenins. But due to the limitations of these antivenins, snakebite victims in many Nigerian communities are mostly treated with herbal antidotes. Hence, this research was designed to evaluate the lethality of *Naja nigricollis* Reinhardt venom and screen the antivenom activity of *Azadirachta indica* A. Juss. leaf extracts with a view to replacing the serum-based antivenin. The methanol extract of *A. indica* leaf was screened against venom-induced lethal effect in albino rats using standard methods. The crude methanol extract was subjected to solvent-fractionation, and the fractions were also screened against the venom-induced lethal effect in albino rats. The lethal doses, 50% (LD<sub>50</sub>) and 100% (LD<sub>100</sub>) of the venom were 0.389 and 3.891 mg/kg b. wt. respectively. The crude extract and its fractions exhibited antivenom activities with varying degrees of efficacy. The crude methanol extract and its hexane and ethyl acetate fractions presented animal mean survival time of 22.51 ± 2.38 h, 23.68 ± 0.89 h and 21.98 ± 1.36 h respectively against LD<sub>100</sub> of the venom. These are significantly different when compared with the mean survival time of the venom control groups. Hence, these findings suggest that *A. indica* leaf has potent antivenom activity and this can serve as leads for the development of safe, readily available and affordable antivenoms.

**Keywords:** *Naja nigricollis*; Venom, Lethality, *Azadirachta indica*; Antivenom

### 1. Introduction

Snakebite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria [1]. It is a neglected public health problem in most of the countries in Africa [2]. Rural populations are frequent victims as they go about their daily food production and animal rearing activities and as they reside in the comfort of their homes. Unfortunately, many of these snakebite cases go unreported and thus do not appear in official epidemiological statistics [3]. Health workers often have little or no formal training in the management of snakebite, and appropriate antivenom is rarely available [4].

According to WHO [4], about 5.4 million snakebites occur each year, resulting in 1.8 to 2.7 million cases of poisonous envenomation. There are between 81,410 and 137,880 snakebite related deaths and around three times as many amputations and other permanent disabilities each year. Most of these occur in Africa, Asia and Latin America. In Asia up to 2 million people are envenomed by snakebites each year, while in Africa an estimated 435,000 to 580,000 snakebites occurs annually that need treatment. Envenoming affects women, children and farmers in poor rural communities in low- and middle-income countries. The highest burden occurs in countries where health systems are weakest and medical resources sparse [5].

\* Corresponding author: Sani Ibrahim

The authorities should worry about the prevalence of fatal snakebites in Nigeria. According to a recent report by the News Agency of Nigeria (NAN), statistics of snakebites obtained from some medical centers across the country revealed a steady rise with the Kaltungo General Hospital in Gombe, Nigeria for instance, recording an average of 20 cases every day. Most of these victims visit herbalists because they could not afford the conventional antivenom.

Application of medicinal plants with antsnake venom activities might be useful in treating victims of snakebites [6], which is particularly important in rural areas where antivenins are not readily available [1,7,8]. However, antivenins have some disadvantages, thus limiting their efficient use. For example, they can induce adverse reactions ranging from mild symptoms to serious anaphylaxis and in addition, they do not neutralize local tissue damage [9,10]. Therefore, complementary therapeutics needs to be investigated, with plants being considered as a major source.

Plant extracts constitute an extremely rich source of pharmacologically active compounds [11,12] and a number of extracts (methanol, ethanolic, aqueous, etc.) for many plants such as, *Musa paradisiaca*, *Tamarindus indica*, *Mangifera indica*, *Andrographis paniculata*, *Aristolochia indica*, *Emblica officinalis*, *Mimosa pudica*, *Vitex negundo*, *Calotropis gigantean*, *Annona senegalensis*, *Parkia biglobosa*, *Vitis negundo*, etc., have been shown to act against snake venom [13-16]. The medicinal value associated with a plant can be confirmed by the successful use of its extract on snakebite wounds [17-19].

*Azadirachta indica* A. Juss. (Neem tree) belongs to the Meliaceae (mahogany) family. It is known as 'Dogon yaro or Darbejiya' in Hausa language. The tree can grow up to 30 m tall with spreading branches covering some 10 m across [20]. The neem tree has long been recognized for its unique properties both against insects and in improving human health [21]. It is grown in most tropical and sub-tropical areas of the world for shade, reforestation and for the production of raw materials for natural insecticides and medicines [22]. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity [23].

Different parts of *Azadirachta indica* tree such as seeds, leaves, flowers, and the bark are widely used for different purposes [24]. Different phytochemicals such as quercetin and azadirachtin and liminoids such as nimbin, nimbinin, and nimbidin have been purified from the different parts of the plant. Moreover, the leaves also contain mixture of compounds such as nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol, nimbiol, various amino acids, and several other types of ingredients [25]. Neem tree extracts have been extensively used in health management since ancient times and have a variety of health-promoting properties [26].

In Northern Nigeria, plant extracts have been used traditionally in the treatment of snakebite envenomation without scientific validation. Thus, vegetal extracts have been found to constitute many compounds with a range of antsnake venom properties [6,27]. The present work explores the neutralization of *Naja nigricollis* venom by the extracts of *Azadirachta indica* leaf used in Kebbi State, Nigeria.

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## 2. Materials and methods

### 2.1. Study area

The research was conducted within Aliero town, Nigeria. It was performed 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 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* [28], 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 [29].

### 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 standard to compare with the efficacy of the plant extract. 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 by a zoologist in 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 the hour of 17.30-18.00, in a low light condition at ambient temperature according to the method of Goswami *et al.*, [16] 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.

#### **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) [2].

#### **2.7. Determination of venom lethal doses, 50 % (LD<sub>50</sub>) and 100 % (LD<sub>100</sub>)**

The lethal doses of the venom were determined using a modified method of Theakston and Reid, [31]. Twenty (20) rats were randomly distributed into five (5) groups of four (4) rats each.

Group 1: served as normal control and were administered with normal saline intraperitoneally (i.p.).

Groups 2 to 5: were injected (i.p.) with the venom at the dose of 1.0, 2.0, 3.0 and 4.0 mg/kg b. wt. respectively in normal saline.

Mortality was recorded within 24 h of venom administration and the lethal doses (LD<sub>50</sub> and LD<sub>100</sub>) were estimated using probit analysis [30-32].

#### **2.8. 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.

#### **2.9. Preparation of crude *A. indica* leaf methanol extract**

The extract was prepared according to a modified method of Dupont *et al.*, [33]. 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.

#### **2.10. 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 solvents 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, ethyl acetate, and n-butanol to yield the n-hexane, ethyl acetate, n-butanol (saturated with water) and aqueous fractions.

The fractions were concentrated to dryness and the residues were kept in a refrigerator in an air-tight container for further analyses. Before use, each fraction was reconstituted in either distilled water or 1 % aqueous solution of Tween-80 (polysorbate) and was expressed in terms of dry weight (mg/ml).

#### **2.11. Antivenom activity screening of the crude *A. indica* leaf methanol extract**

Thirty five (35) albino rats were randomly distributed into seven (7) groups of five (5) rats each.

Group 1: received orally with only distilled water and served as normal control.

Group 2: were injected intraperitoneally (i.p.) only with LD<sub>100</sub> of the snake venom and served as venom control.

Group 3 to 5: served as treatment groups and they were injected (i.p.) with the LD<sub>100</sub> of the snake venom, then after 30 min they were treated orally with the crude methanol extract at doses of 100, 200 and 400 mg/kg b. wt. respectively.

Group 6: received only the crude methanol extract at the dose of 400 mg/kg b. wt. orally and served as extract control.

Group 7: were injected (i.p.) with the LD<sub>100</sub> of the snake venom, then after 30 min, they were administered intravenously (i.v.) with the standard conventional serum antivenin at the dose of 1 ml/0.6 mg venom and served as standard control.

All the groups received same volume of preparations. In all the groups, the duration of survival and number of rats survived were recorded for 24 h [32].

### 2.12. Antivenom activity screening on the fractionated extracts

Thirty five (34) rats were used. They were randomly distributed into seven (7) groups of five (5) rats each as follows:

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

Group 2: injected intraperitoneally (*i.p.*) only with LD<sub>100</sub> of the snake venom and served as venom control.

Group 3 to 6: served as treatment groups and they were injected (*i.p.*) with LD<sub>100</sub> of the snake venom, then after 30 min, they were treated orally with 200 mg/kg b. wt. of the *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of the extract respectively.

Group 7: injected (*i.p.*) with the LD<sub>100</sub> of the snake venom, then after 30 min, they were administered intravenously (*i.v.*) with the standard conventional serum antivenin at the dose of 1 ml/0.6 mg venom.

In all the groups, the duration of survival and number of animals survived were recorded for 24h.

### 2.13. Qualitative phytochemical screening

Five gram (5 g) each from the methanol residue of the *Azadirachta indica* leaf and its fractions were each dissolved in 40 ml of distilled water and then subjected to phytochemical screening using the methods of Mbatchou and Kossono, [34]. The presence of flavonoids, phenols, tannins, saponins, phlobatannins, alkaloids, terpenoids, steroids, anthraquinones, cardiac glycosides, saponins glycosides and anthraquinone glycosides were determined.

### 2.14. Quantitative phytochemical screening

#### 2.14.1. Determination of total flavonoids content

The method of Harborne, [35] was used. To about 2.5 g of the extract, 25 ml of 80 % aqueous methanol were added. The solution was then filtered through Whatman filter paper No. 1. The filtrate was transferred to a crucible and evaporated to dryness over a water bath and weighed. The total flavonoids content was determined using the following formula:

$$\text{Flavonoids (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}}$$

#### 2.14.2. Determination of total tannins content

The method of Amorim *et al.*, [36] was used. A 0.5 g extract was weighed into 100 ml plastic bottle, then, 50 ml of distilled water was added and covered. It was allowed to stand for 5 min, and thereafter; shaken for 1 h in a mechanical shaker. This was then filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a tube and mixed with 3 ml of 0.1 M FeCl<sub>3</sub> in 0.1N HCl and 8 mM Potassium ferrocyanide. The absorbance was measured spectrophotometrically at 520 nm within 10 min. A blank sample was prepared using tannin acid to get 100 ppm and measured. The following was used to calculate the total tannins content.

$$\text{Tannins} = \frac{\text{Absorbance of Test Sample}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard (mg/ml)}$$

### 2.14.3. Determination of total saponins content

The method of Obadoni and Ochuko, [37] was used. To 5 g extract in a conical flask, 25 ml of 20 % aqueous ethanol were added. The sample was heated over a water bath for 1 h with continuous stirring at about 55 °C. The concentrate was transferred into a 250 ml separating funnel and 5 ml of diethyl ether were added and shaken vigorously. The aqueous layer was recovered and the ether layer discarded. About 15 ml of n-butanol were then added followed by addition of 2.5 ml of 5 % aqueous NaCl. The remaining solution was heated over a water bath. After evaporation, the sample was dried in the oven to a constant weight.

$$\text{Saponins (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}}$$

### 2.14.4. Determination of total phenols content

The total phenol content was determined by Folin-Ciocalteu Spectrophotometric method [38]. Folin-Ciocalteu reagent (0.1 ml) was added to 2 ml of the extract. The mixture was allowed to stand for 15 min. Then 5 ml of saturated sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) were added. The mixture was allowed to stand for 30 min at room temperature and the total phenol content was determined by measuring the absorbance at 765 nm. Gallic acid was used as standard. Total phenol value was expressed in terms of mg/g Gallic acid equivalent per gram of the sample using linear regression equation obtained from a standard gallic acid calibration curve.

### 2.14.5. Determination of total alkaloids content

Alkaloids were determined by gravimetric method [35]. Five grams (5 g) of the extract were weighed into a conical flask containing 50 ml of 10 % ammonium hydroxide. The mixture was stirred and allowed to stand for 4 h before filtering. The filtrate was evaporated to one quarter of its original volume on a hot plate and concentrated ammonium hydroxide solution was added, drop-wise, to the mixture in order to precipitate the alkaloids. The precipitate was filtered using a weighed filter paper and washed with 10 % ammonium hydroxide solution. The precipitate was dried with the filter paper in an oven at 60 °C for 30 min and then reweighed. The total alkaloids were obtained by using the formula:

$$\text{Alkaloids (mg/g)} = \frac{\text{Weight of residue}}{\text{Weight of sample}}$$

## 2.15. Data analysis

The data generated from the study are presented as mean  $\pm$  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

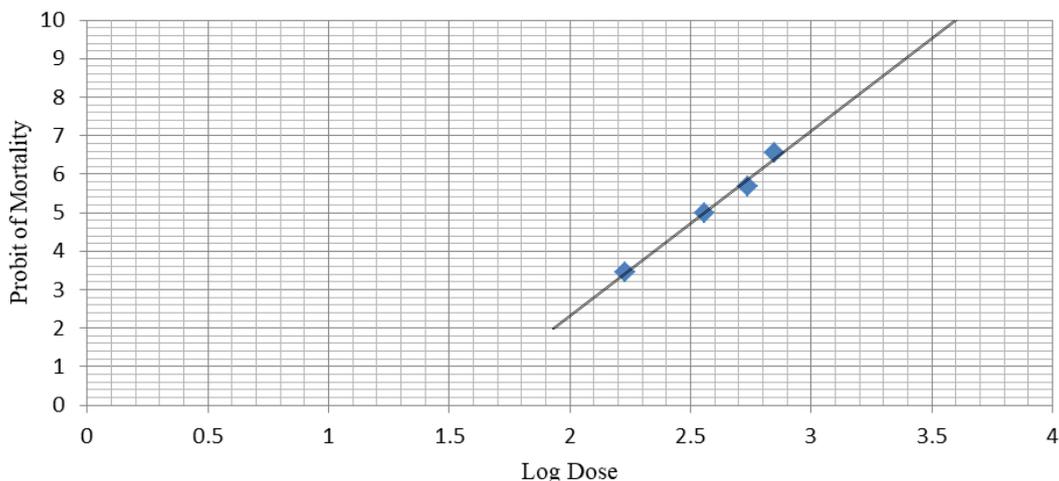
### 3.1. Venom lethal doses ( $\text{LD}_{50}$ and $\text{LD}_{100}$ )

The lethality data of the *Naja nigricollis* venom is presented in table 1. Using probit curve (Figure 1), the  $\text{LD}_{50}$  (median lethal dose) of the venom was determined to be 0.389 mg/kg body weight, while, its  $\text{LD}_{100}$  was 3.981 mg/kg b. wt.

**Table 1** Probit analysis for determination of venom  $\text{LD}_{50}$  and  $\text{LD}_{100}$

Group	Average animal weight (g)	Venom dose (mg/kg b. wt.)	Average dose of venom administered ( $\mu\text{g}/\text{kg}$ b. wt.)	Log dose	$\frac{\text{Dead}}{\text{Total}}$	% Death	% Corrected*	Probit
1	172.10	-	-	-	0/4	0.00	0.00	0.00
2	168.70	1.00	168.70	2.23	0/4	0.00	6.25	3.45
3	183.30	2.00	366.60	2.56	2/4	50.00	50.00	5.00
4	182.90	3.00	548.70	2.74	3/4	75.00	75.00	5.67
5	176.60	4.00	706.40	2.85	4/4	100.00	93.75	6.55

\*Corrected formulae:  $0\% \text{ dead} = 100 \left( \frac{0.25}{n} \right) = 100 \left( \frac{0.25}{4} \right) = 6.25$  [32].  $100\% \text{ dead} = 100 \left( \frac{n-0.25}{n} \right) = 100 \left( \frac{4-0.25}{4} \right) = 93.75$ . Where n is the number of rats in a group.



**Figure 1** Probit curve for determination of venom LD<sub>50</sub> and LD<sub>100</sub>

From figure 1, the corresponding Log dose at 50% probit of mortality is 2.59.

Hence, the venom LD<sub>50</sub> = Antilog of 2.59 =  $10^{2.59} = 389.05 \mu\text{g/kg} = 0.389 \text{ mg/kg}$

While, the corresponding Log dose at 100 % probit of mortality is 3.6.

So, the venom LD<sub>100</sub> = Antilog of 3.6 =  $10^{3.6} = 3981.1 \mu\text{g/kg b. wt.} = 3.981 \text{ mg/kg b. wt.}$

### 3.2. Antivenom activity of the crude *A. indica* methanol extract

The *N. nigricollis* venom at the lethal dose, 100 % (LD<sub>100</sub>) produced 100 % mortality on the albino rats. The mean survival time (MST) of the animals administered with the venom without any treatment was less than 1 h (Tables 2 and 3).

The methanol extract of *A. indica* leaf significantly increased the mean survival time (MST) at a dose of 100 mg/kg b. wt., but could not protect the animals from death at that dose (Table 2). The dose of the plant extract when doubled to 200 mg/kg b. wt. was found to be more effective against the venom, presenting MST of  $22.46 \pm 1.85$  h. At this dosage, the mortality of animals was significantly reduced to 40 % (Table 2). At 400 mg/kg b. wt. of the extract, the percentage of animal survival was significantly increased to 80 % compared to the animal group treated with the standard antivenin with 100 % survival rate (Table 2).

**Table 2** Antivenom activity of crude methanol leaf extract of *Azadirachta indica*

Group	Treatment	Extract (mg/kg b. wt.)	Venom (mg/kg b. wt.)	Standard antivenin (1ml/0.6mg venom)	Survival Total	% Survival	Mean survival time (h)
1	Control	-	-	-	5/5	100	24.00 ± 0.00 <sup>c</sup>
2	Venom only	-	3.891	-	0/5	0	00.83 ± 0.10 <sup>a</sup>
3	Venom + Extract	100	3.891	-	1/5	20	15.33 ± 5.67 <sup>b</sup>
4	Venom + Extract	200	3.891	-	3/5	60	22.46 ± 1.85 <sup>c</sup>
5	Venom + Extract	400	3.891	-	4/5	80	22.51 ± 2.38 <sup>c</sup>
6	Extract only	400	-	-	5/5	100	24.00 ± 0.00 <sup>c</sup>
7	Venom + Antivenin	-	3.891	1.3	5/5	100	24.00 ± 0.00 <sup>c</sup>

Mean survival times are presented as Mean ± SEM (n = 5). Mean survival times carrying different superscripts from the venom control (group 2) are significantly (P<0.05) different using ANOVA and Duncan multiple range test.

**Table 3** Antivenom activity of leaf fractions of *A. indica*

Group	Treatment	Extract (mg/kg b. wt.)	Venom (mg/kg b. wt.)	Standard antivenin (1ml/0.6mg venom)	Survival Total	% Survival	Mean survival time (h)
1	1% Tween-80 (Control)	-	-	-	5/5	100	24.00 ± 0.00 <sup>c</sup>
2	Venom only	-	3.891	-	0/5	0	00.66 ± 0.13 <sup>a</sup>
3	Venom + <i>n</i> -Hexane fraction	200	3.891	-	4/5	80	23.68 ± 0.89 <sup>c</sup>
4	Venom + Ethyl acetate fraction	200	3.891	-	3/5	60	21.98 ± 1.36 <sup>c</sup>
5	Venom + <i>n</i> -Butanol fraction	200	3.891	-	0/5	0	00.96 ± 0.21 <sup>a</sup>
6	Venom + Aqueous fraction	200	3.891	-	0/5	0	06.45 ± 2.04 <sup>b</sup>
7	Venom + Antivenin	-	3.891	1	5/5	100	24.00 ± 0.00 <sup>c</sup>

Mean survival times are presented as Mean ± SEM (n = 5). Mean survival times carrying different superscripts from the venom control (group 2) are significantly (P<0.05) different using ANOVA and Duncan multiple range test.

It was observed that, there was no significant difference between the MST of animals treated with 200 mg/kg b. wt. of the *A. indica* leaf extract ( $22.46 \pm 1.85$  h) and that of the animals treated with 400 mg/kg b. wt. of the extract ( $22.51 \pm 2.38$  h). When compared, the MSTs of animals administered with 200 and 400 mg/kg b. wt. of the *A. indica* leaf extract, were not significantly different ( $P > 0.05$ ) from the MST of the animals administered with standard antivenin (Table 2).

### 3.3. Antivenom activity of the fractionated leaf extracts

Table 3 presents the antisnake venom activity of the fractionated extracts. Animals administered with the LD<sub>100</sub> of the venom without any treatment served as venom control (negative control) and none of them survived. It was shown that, the MST of animals in the venom control was  $0.66 \pm 0.13$  h. But, there was significant increase ( $P < 0.05$ ) in the MST of animals treated with n-hexane fraction ( $23.68 \pm 0.89$  h) and ethyl acetate fraction ( $21.98 \pm 1.36$  h) when compared with that of the venom control group. High percentage survival of 80 % and 60 % were recorded for the animals treated with the n-hexane and ethyl acetate fraction respectively. These are significantly different from the percentage survival of animals treated with n-butanol and aqueous fractions where 0 % animal survival was recorded for each. The MSTs of animals treated with n-hexane and ethyl acetate fractions were not significantly different when compared with that of the animals treated with the standard antivenin ( $24.00 \pm 0.00$  h).

At the end of the screening, it was observed that, the antivenom activities of each of the fractionated extracts were: n-hexane (99 %), ethyl acetate (92 %), aqueous (27 %) and n-butanol (4 %) compared to standard antivenin.

### 3.4. Phytochemical composition of the crude extract and its fractions

The qualitative and quantitative phytochemical compositions of the leaf methanol extract of *A. indica* and its fractions (n-hexane, ethyl acetate, n-butanol and aqueous) are presented in tables 4 and 5 respectively. Phenols, tannins, terpenoids, steroids, cardiac glycosides, saponin glycosides, and anthraquinone glycosides were detected in the methanol extract and in all the fractionated extracts. Flavonoids were only detected in the crude methanol extract and aqueous fraction. Saponins were detected in the extract, and in the n-butanol and aqueous fractions. Terpenoids were detected in the extract, and in the n-hexane and ethyl acetate fractions. Phlobatannins, alkaloids and anthraquinones were not detected in all the extracts. From table 5, the concentration of flavonoid, phenols, tannins and saponins in the crude methanol extract are  $66.67 \pm 3.59$ ,  $403.00 \pm 72.20$ ,  $219.00 \pm 9.00$ ,  $179.07 \pm 9.04$  mg/g respectively. N-hexane and ethyl acetate fractions have higher phenols and tannins content than the n-butanol and aqueous fractions. The concentration of phenols in the n-hexane fraction was  $77.00 \pm 8.00$  mg/g, while tannins concentration in ethyl acetate fraction was  $94.08 \pm 3.26$  mg/g.

**Table 4** Qualitative phytochemical composition of *A. indica* leaf

Phytochemicals	Crude methanol extract	n-Hexane fraction	Ethyl acetate fraction	n-Butanol fraction	Aqueous fraction
Flavonoids	+	-	-	+	-
Phenols	+	+	+	+	+
Tannins	+	+	+	+	+
Saponins	+	-	-	+	+
Phlobatannins	-	-	-	-	-
Alkaloids	-	-	-	-	-
Terpenoids	+	+	+	-	-
Steroids	+	+	+	+	+
Anthraquinones	-	-	-	-	-
Cardiac Glycosides	+	+	+	+	+
Saponin Glycosides	+	+	+	+	+
Anthraquinone Glycosides	+	+	+	+	+

+ = Present, - = Not detected

**Table 5** Quantitative phytochemical composition of *A. indica* leaf

Phytochemicals	Crude methanol extract	n-Hexane fraction	Ethyl acetate fraction	n-Butanol fraction	Aqueous fraction
Total flavonoids (mg/g)	66.67 ± 03.59	-	-	63.08 ± 05.11	-
Total phenols (mg/g)	403.00 ± 72.20	77.00 ± 08.00	71.02 ± 13.71	60.50 ± 13.50	59.00 ± 05.50
Total tannins (mg/g)	219.00 ± 09.00	86.50 ± 02.50	94.08 ± 03.26	50.50 ± 05.52	63.50 ± 00.50
Total saponins (mg/g)	179.07 ± 09.04	-	-	31.67 ± 08.33	135.40 ± 21.25

Values are presented as mean ± SEM. Tests were conducted in triplicates. - = Not quantified

#### 4. Discussion

In this research, the intraperitoneal LD<sub>50</sub> for *Naja nigricollis* venom was determined in rats to be 0.389 mg/kg body weight, while the LD<sub>100</sub> was 3.891 mg/kg b. wt. using probit analysis. Other researchers reported that, the LD<sub>50</sub> of the same venom is 0.35mg/kg body weight [39] and 0.40 mg/kg body weight [40]. These differences in the LD<sub>50</sub> could be attributed to differences in geographical location of the snake, seasonal variations in composition and potency of venoms [41]. As well, the route of venom injection and time for milking of the venom may also contribute to these differences in the LD<sub>50</sub>. According to Botham, [42], five distinct routes can be used for venom injection, i.e., intracerebroventricular (ICV), intramuscular (IM), intravenous (IV), intraperitoneal (IP) and subcutaneous (SC). The lowest LD<sub>50</sub> value is always obtained by the ICV route, whereas the SC route gives the highest LD<sub>50</sub> value. The smaller the LD<sub>50</sub> value, the more toxic is the venom. The opposite is also true; the larger the LD<sub>50</sub> value, the lower the toxicity [43].

*A. indica* leaf methanol extract was observed to be effective against the *N. nigricollis* venom as low mortality was recorded for each of the administered doses (Table 2). The mean survival time (MST) of the treatment groups was significantly increased, and the group which received the highest dose of the plant extract (400 mg/kg b. wt.) had lower mortality rate than those that received lower doses, which may be due to a number of factors such as dose of administration as reported by Abubakar *et al.*, [13] and Sani *et al.*, [3] who observed prolonged survival time with administered plant extract in a dose-dependent manner.

The antivenom activity observed in the rats administered with extract may be attributed to the presence of any of phenols, steroids, glycosides, tannins, saponins or terpenoids in the *A. indica* leaf extract [44]. The phytochemical components of *A. indica* leaf have been established in some reported researches and these include tannins, saponins, alkaloids, phenols, flavonoids, anthraquinones, cardiac glycosides and sterols [21,22,45]. Several studies have linked presence of these bioactive compounds in plant materials to antisnake venom activity [46].

The hexane and ethyl acetate fractions have higher phenolic contents. The high activity of the crude extract and its hexane and ethyl acetate fractions in neutralizing the snake venom lethal effects may be attributed to the presence of phenols; as plant phenols from the aqueous extracts of *Pentace burmanica*, *Pithecellebium dulce*, *Areca catechu* and *Quercus infectoria* have been shown to inhibit venom lethal effect by precipitation of *Naja kaouthia* venom [6,47-49]. Phenols have also been shown to possess dose-dependent inhibitory effects on phospholipase A<sub>2</sub>, hyaluronidase, and L-amino acid oxidase of *Calloselasma rhodostoma* and *Naja naja kaouthia* venoms in *in vitro* studies [50].

Tannins from persimmon, a fruit from *Diospyrus kaki* inhibits oedema in mice induced by sea snake and also improved the survival time in mice injected with *Laticauda semifasciata* and *Trimeresurus flavoviridis* venom [51]. Therefore, prolonged mean survival time observed in the treated groups of this research (Table 2) and the groups treated with hexane and ethyl acetate fractions (Table 3) may be attributed to the high amount of tannins in the crude methanol extract and the hexane and ethyl acetate fractions of the *A. indica* leaf (Table 5).

Terpenoids, flavonoids and polyphenols possess protein binding and enzyme inhibiting properties and also inhibit snake venom phospholipase A<sub>2</sub> activities of both viper and cobra venom [52,53]. A natural triterpenoid saponin extracted from the root of *Glycyrrhiza glabra* (licorice), has been characterized as a thrombin inhibitor [54] and this compound is known for its anti-inflammatory activity and it also exhibits antithrombotic properties against snake

venom *in vivo* [55,56]. The presence of terpenoids, flavonoids and saponins in the leaf extract of *A. indica* may also have contributed to its high antsnake venom activity in this research.

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## 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, stable at room temperature and could neutralize a wide range of venom toxins. The crude methanol extract of the leaf of *A. indica* showed antivenom activity against the *N. nigricollis* venom. Hence, this research has provided scientific proof on the antivenom properties of *A. indica* leaf. The extract fractions have also effectively neutralized the snake venom. This lends support to the traditional use of *A. indica* leaf in the treatment of snakebites envenomation. 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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## Compliance with ethical standards

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### *Disclosure of conflict of interest*

No existing conflict of interest.

### *Statement of 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.

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