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Research Article
Antisnake Venom Effect of *Diospyros mespiliformis* Stem-bark Extract on *Naja nigricollis* Venom in Albino Rats

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Abstract

Background and Objective: Snakebite is one of the major health problems in Nigeria. It is frequently treated with parenteral administration of serum-based antivenins. Despite the success of the serum therapy, antivenins have some limitations particularly in relation to the neutralization of local tissue damage. Hence, this research was aimed at evaluating the antisnake venom potential of *Diospyros mespiliformis* stem-bark ethanol extract on *Naja nigricollis* (spitting cobra) venom in albino rats. **Materials and Methods:** The LD_{50} of the venom was determined using probit analysis and preliminary phytochemical screening was conducted using standard methods. For the antivenom activity evaluation of the plant extract, experimental animals were grouped into six; normal control, venom control, venom and extract (150 mg kg^{-1} b.wt.), venom and extract (300 mg kg^{-1} b.wt.), extract control, venom and conventional antivenin. **Results:** The LD_{50} of the venom was determined to be 0.380 mg kg^{-1} b.wt. The result of the phytochemical screening revealed the presence of saponins, tannins, glycosides, terpenoids and alkaloids. The antisnake venom activity of the extract presented significant increased ($p<0.05$) in the Mean Survival Time (MST) of 6.72 ± 0.64 and 16.99 ± 1.24 h at extract dose of 150 and 300 mg kg^{-1} b.wt., respectively when compared with the venom control group with MST of 0.82 ± 0.03 h. **Conclusion:** Based on these findings, it was established that *D. mespiliformis* stem-bark extract has antisnake venom activity and can serve as lead in isolating the active principle that can be affordable and readily available.

Key words: Snakebite, *Naja nigricollis*, *Diospyros mespiliformis*, venom, antivenom

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Snakes are ubiquitous creatures that vary in their morphologic characteristics and potential for envenomation[1]. The non-venomous snakes belong to the family Colubridae (e.g., grass snake) while others known to cause severe envenomation belong to Elapidae (such as; cobras, kraits, mambas and coral snakes), Viperidae (such as; American rattlesnake, russell's viper and adders) and Hydrophidae (such as; sea snake)[2]. Three species; black-necked spitting cobra (*Naja nigricollis*), carpet viper (*Echis ocellatus*) and puff adder (*Bitis arietans*) belonging to the first two families, are the most important snakes associated with snakebite injuries in northern part of Nigeria[3].

Naja nigricollis is considered one of the most poisonous among snake species capable of injecting or spitting its venom into/onto the victim[3]. It is documented to belong to the most diverse and widespread genus of cobras[4]. They are medium-sized, less than two meters and black in colouration. Other species of this genus such as; *Mozambique* spitting cobra is red in colouration. Spitting cobras are categorized as generalist predators. They are well equipped to prey on a wide variety of small vertebrates[5]. They also tend to feed on fairly larger species that are relative to their own body size. In all, they have an adaptive capability in which they could prey on several different species when they are exposed to different microhabitats. When the spitting cobra feels threatened it exhibits a defensive behaviour by spitting its venom in the face of its harassers and/or predators, thus given the common name "spitting cobra"[6]. The venom of the spitting cobra is non-toxic to the skin, but is harmful to the eyes of its aggressors. The patho-physiology of envenomation includes both local and systemic effects. The magnitude of toxicity depends on the dose and potency of the venom toxins and their diffusion rate into the general circulation from the site of bite. However, the extent of systemic toxicity greatly varies depending on the body mass for identical bites. Therefore, envenomation may include only local effects (haemorrhage, oedema, myonecrosis and extracellular matrix degradation) or may include systemic effects (neurotoxicity, myotoxicity, cardiotoxicity and alterations in haematological systems)[7].

Diospyros mespiliformis (Ebony tree) belongs to the family Ebenaceae. It is known as Kaiwa or Kanya in Hausa[8]. It grows mostly in savanna and woodland, often on termite hills. The tree is widely distributed throughout the eastern part of the African continent, from Ethiopia to the south of Swaziland. It grows well in areas with adequate water and little or no frost[9]. The plant is a tall, evergreen tree 15-50 m high, with dense, rounded and buttressed stem. Bark grey-black or black, smooth in young trees rough with small regular scales in older trees, pinkish when slashed. Young branchlets are green, tomentellous with pinkish white hairs, glabrescent later. Crown is very branchy with dense foliage. The leaves are simple, alternate, leathery and dark green with small hair on the underside of old leaves. The fruit is a fleshy berry, with an enlarged calyx yellow to orange when ripe[10].

Plant kingdom provides an inexhaustible source of various herbal compounds with pharmacological potential[11]. A plethora of medicinal plants, available locally are used widely by traditional healers in the form of plant sap, pastes, decoctions, powders and pills in the treatment of snakebite envenomation[12].

Currently, many researchers focus on the investigation of plants used in different parts of the world for treatment of venomous snake bites with the view of finding the active compounds or developing an active formula. There are several reports of such efforts with some recorded successes and failures particularly in tropical countries of Africa, Asia and South America[13].

However, there are claims of very potent plant-based antivenoms with snake charmers. Hence, it is worthy to study these plants with the view of scientifically authenticating their potency claims and this may benefit rural dwellers and similar communities around the world. Therefore, this research was aimed at investigating the antisnake venom potential of *Diospyros mespiliformis* stem-bark ethanol extract on *Naja nigricollis* (spitting cobra) venom in albino rats.

MATERIALS AND METHODS

Study area: The research was conducted between August, 2018-February, 2019 in Aliero Local Government Area of Kebbi state, Nigeria. It was performed in Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

Standard snake venom antiserum (antivenin): 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).

Experimental animals: Thirty adult Wister albino rats of both sexes aged 2-3 months and weighing between 250-310 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*[14], 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[15].

***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.

Collection and authentication of the plant material: The *Diospyros mespiliformis* stem-bark for the antivenom screening in this study was collected on Saturday, 8th August, 2018 from Aliero town, Kebbi state, Nigeria. It was authenticated at the herbarium in the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero and voucher specimen was deposited there; VN:231.

Preparation of *D. mespiliformis* stem-bark ethanol extract: The extract was prepared according to the method of Dupont *et al*.[16]. The collected plant parts were washed with clean water and air-dried under shade, pulverized using pestle and mortar. About 500 g of the powdered plant material was weighed and soaked in 2.5 L of 95% ethanol. 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 used for the antisnake venom activity screening.

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*.[17] by using a short-acting general anesthesia; halothane (Piramal Healthcare Limited, UK). 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[18] (mg mL^{-1}).

Determination of venom median lethal dose (LD_{50}): The LD_{50} of the collected venom was determined using the method of Theakston and Reid[19]. Twenty rats were randomly distributed into five groups of four rats each:

- **Group 1:** The group served as control and received (intraperitonially; i.p.) 0.2 mL of normal saline
- **Groups 2-5:** Animals were injected (i.p.) with 0.2 mL of different concentrations of the venom (1.0, 2.0, 3.0 and 4.0 mg kg^{-1} b.wt., respectively) in normal saline

Mortality was recorded within 24 h of venom administration and the LD_{50} was estimated using probit analysis[19-21].

Qualitative phytochemical screening on the *D. mespiliformis* ethanol stem-bark extract: Five grams of the *D. mespiliformis* stem-bark extract was dissolved in 40 mL of distilled water and then subjected to phytochemical screening using the methods of Mbatchou and Kosoono[22]. The presence of flavonoids, phenols, tannins, saponins, phlobatannins, alkaloids, terpenoids, steroids, anthraquinones and glycosides were determined.

Screening of the *D. mespiliformis* ethanol stem-bark extract for antivenom activity: Thirty rats were randomly distributed into six groups of five rats each. The test was conducted as follows:

- **Group 1:** Rats received only distilled water and served as normal control
- **Group 2:** Rats were injected intraperitoneally (i.p.) only with 2 LD_{50} dose of the snake venom and served as venom control
- **Group 3-4:** They were served as treatment groups and injected (i.p.) with the 2 LD_{50} dose of the snake venom, then after 30 min they were treated orally with the plant extract at doses of 150 and 300 mg kg^{-1} b.wt., respectively
- **Group 5:** It received only plant extract at the dose of 300 mg kg^{-1} b.wt., orally and served as extract control

- **Group 6:** Rats were injected (i.p.) with the 2 LD_{50} dose 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, number of rats survived and signs of toxicity[21] were recorded for 24 h.

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).

RESULTS

Venom median lethal dose (LD_{50}): The lethality data of the *N. nigricollis* venom is presented in Table 1. Using the probit curve (Fig. 1), the LD_{50} of the venom was determined to be 0.380 mg kg^{-1} b.wt.

*Corrected formula[21]:

$$0\%\ \text{dead} = 100\left(\frac{0.25}{n}\right) = 100\left(\frac{0.25}{4}\right) = 6.25$$

$$100\%\ \text{dead} = 100\left(\frac{n-0.25}{n}\right) = 100\left(\frac{4-0.25}{4}\right) = 93.75$$

where, n is the number of rats in a group.

The corresponding log dose at 50% probit of mortality is 2.58.

Hence, the venom LD_{50} = Antilog of 2.58 = $10^{2.58}$ = 380.2 µg kg^{-1} = 0.380 mg kg^{-1} b.wt.

Phytochemical composition of the *D. mespiliformis* stem-bark extract: The qualitative phytochemical composition of the stem-bark ethanol extract of *D. mespiliformis* is presented in Table 2. Tannins, saponins, alkaloids, terpenoids and glycosides were detected in the ethanol extract, while, flavonoids, phenols, phlobatannins, steroids and anthraquinones were not detected.

Antisnake venom activity of the *D. mespiliformis* stem-bark extract: The *N. nigricollis* venom at LD_{100} produced 100% mortality in the envenomed rats. The Mean Survival Times (MST) of the animals administered with the snake venom without any treatment was less than 1 h (Table 3).

The ethanol extract of *D. mespiliformis* stem-bark significantly increased MST at the dose of 150 mg kg^{-1} b.wt., but could not protect the animals from death at that dose

Table 1: Probit values for determination of the venom LD_{50}

Groups	Average animal weight (g)	Venom dose (mg kg^{-1})	Average dose of venom administered (µg kg^{-1})	Log dose	Dead total	Death (%)	*Corrected (%)	Probit
1	160.60	-	-	-	0/4	0	0.00	0.00
2	198.40	1	186	2.27	0/4	0	6.25	3.45
3	173.70	2	348	2.54	1/4	25	25.00	4.33
4	182.20	3	546	2.74	3/4	75	75.00	5.67
5	139.50	4	558	2.75	4/4	100	93.75	6.55

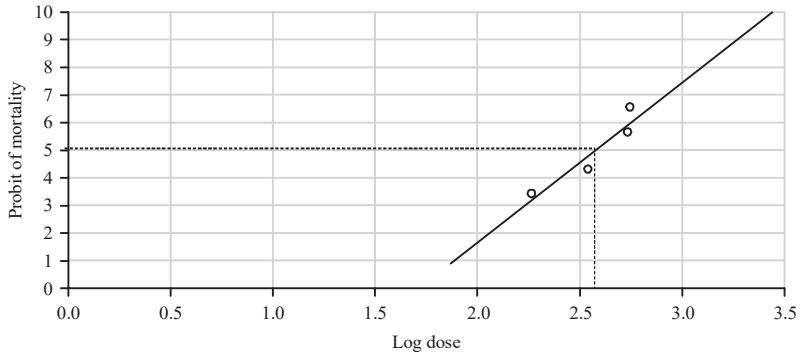

Fig. 1: Probit curve for the determination of venom LD_{50}

Table 2: Qualitative phytochemical composition of *D. mespiliformis* stem-bark extract

Phytochemicals	Observation
Flavonoids	-
Phenols	-
Tannins	+
Saponins	+
Phlobatannins	-
Alkaloids	+
Terpenoids	+
Steroids	-
Anthraquinones	-
Glycosides	+

+: Present, -: Not detected

Table 3: Antisnake venom activity of the *D. mespiliformis* stem-bark ethanol extract

Groups	Treatment	Extract (mg kg^{-1})	Venom (mg kg^{-1})	Standard antivenin (1 mL/0.6 mg venom)	Survival total	Survival (%)	Mean survival time (h)
1	Control	-	-	-	5/5	100	24.00 ± 0.00^{c}
2	Venom only	-	0.71	-	0/5	0	0.83 ± 0.10^{a}
3	Venom+extract	150	0.71	-	0/5	20	6.72 ± 0.64^{b}
4	Venom+extract	300	0.71	-	0/5	60	16.99 ± 1.24^{bc}
5	Extract only	300	-	-	5/5	100	24.00 ± 0.00^{c}
6	Venom+antivenin	-	0.71	1.30	5/5	100	24.00 ± 0.00^{c}

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). The dose of the plant extract when doubled to 300 mg kg^{-1} b.wt., was found to be more effective against the venom, presenting MST of 16.99±1.24 h.

DISCUSSION

Herbal anti-venoms have been reported to neutralize toxic venom constituents through several mechanisms. These include; inhibition of venom enzymes, inactivation of venom toxic proteins, antioxidant activity, adjuvant action, chelation activity and combination of these activities[23].

Various phytochemicals with protein binding properties, active against snake envenomation include flavonoids, polyphenols, saponins, tannins, terpenoids, xanthenes, quinonoids, steroids and alkaloids. These bind to toxic venom proteins thereby inactivating them[24]. They could also competitively block the target receptors[11]. Hence, the presence of some of these phytochemicals in the ethanol extract of the *D. mespiliformis* used in this study may be responsible for its anti-venom activity.

Snake venom enzymes are the key substances involved in snake venom toxicity[24]. Thus, inactivation of these enzymes is generally considered to be the fundamental step in the management of snakebite[25].

The anti-snake venom efficacy of *D. mespiliformis* in this research may be attributed to its tannins content. 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[26]. In relation to the mode of action, Makhija and Khamar[27] have concluded that the inhibition of tannins 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 results in the formation of a stable complex[28]. However, the activity of tannins may involve varying degrees of interactions such as hydrophobic connections mediated by aromatic rings[24].

The lethality of snake venom is mainly attributed to its highly active enzymatic component, Phospholipase A2 (PLA2) that hydrolyzes cellular phospholipids, resulting in the release of arachidonic acid[29]. Oxidative metabolism of arachidonic acid generates potentially toxic Reactive Oxygen Species (ROS) including superoxide and hydroxyl free radicals[30]. An imbalance between the excessive generation and poor removal of ROS causes lipid peroxidation leading to cellular damage[31].

Plants secondary metabolites such as; flavonoids, terpenoids, tannins, other polyphenols and some minerals (selenium) have the capability of neutralizing free radicals; hence, they are valuable natural antioxidants that scavenge and remove oxygen free radicals, stabilize cell membranes and act as immunomodulators[32]. These classes of compounds are known to be powerful antioxidants both in hydrophilic and lypophilic environments. They can prevent, stop or reduce oxidative damage as a result of PLA2 activity by selectively binding to the active sites or modify conserved residues

that are critical for the catalysis[26] of the PLA2. Hence, presence of tannins and terpenoids in the ethanol extract of the *D. mespiliformis* stem-bark used in this research can be attributed to the antivenom activity of the plant.

Soares *et al*.[33] suggested that plant extracts have compounds that bind to divalent metal ions, which are required for some enzymatic activities. Presence of proper metal ion coordination is a pre-requisite for the hydrolytic activities of PLA2 and metalloproteinases[34]; hence, any metabolite that can weaken the enzyme-metal ion interaction would result in the inactivation of the hydrolytic activity[24].

The mechanisms by which plants can act against snake venom enzymes have been extensively studied. Among them, the phytochemicals as catequines, flavones, anthocyanines and tannins were related in their abilities in the chelation of Ca^{2+} required for catalytic activity of snake venom[24] PLA2. Thus, presence of tannins in the extract used in this study can be a reason for the plant antivenom activity provably involving chelation of the metal ion required by the venom enzymes.

CONCLUSION

The natural herbal remedies are showing promising expectations in the treatment of snakebite. Based on the findings, it was established that *D. mespiliformis* stem-bark extract has anti *Naja nigricollis* venom activity. It can serve as lead for the development of safe, readily available and affordable antivenoms.

SIGNIFICANCE STATEMENT

This study discovered that, snakebite is a neglected public health problem in most of the countries in Africa. 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. Health workers often have little or no formal training in the management of snakebite and appropriate anti-venom is rarely available. However, antivenins also have some disadvantages, thus limiting their efficient use. For example, production of antivenins is a painstaking, resource-intensive and time-consuming process. Another important issue with the conventional antivenins is the knowledge of which species of snakes inflicted the bite. If antivenin for a wrong species is administered it may not be effective. The general problem with these conventional antivenins is the allergic effect of animal serum to a number of victims. The allergy can be so severe as to cause death, otherwise must be treated separately. Additionally, antivenins do not neutralize the local tissue damage. There are claims of very potent broad-spectrum plant-based antivenoms with snake-charmers in the villages which are not properly authenticated. Therefore, it is pertinent to study these plants with the view to scientifically authenticate the potency claims so as to develop cheap, readily available and easily storable plant-based antivenom.

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