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Anti-Snake Venom Activity of *Indigofera Hirsuta* Extracts Against *Naja Nigricollis* Venom

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Abstract:

Indogofera hirsuta has reportedly been used in traditional medicine to treat snake envenomation. The study is aimed at exploring the potential of the plant for antivenom activity using standard methods. Preliminary phytochemical screening on the extracts revealed the presence of secondary metabolites such as alkaloids, flavonoids, tannins, steroids/terpenes, cardiac glycosides and saponins. Acute toxicity studies conducted on mice (ip) for different extract (Methanol, Butanol and Chloroform) using Lorkes method gave an LD_{50} of 1131.3mg/kg each, suggesting the plant to be less toxic. The antivenin effect of the extracts was tested against *Naja nigricollis* venom using mice. Maximum protection was observed in *ex vivo* model at an administered dose of (LD_{99} +260) with 100% survival for all mice used the extracts and a moderate survival rate (50%) at a dose of (LD_{99} + 80). Appreciable protection was observed *in-vivo* model at an administered dose of (LD_{99} + 500) with 83.3% survival for methanolic extract, moderate survival rate (50%) at a dose of (LD_{99} + 250) and relatively lower survival rate (33.3%) at a dose of (LD_{99} + 125) for all mice used the extracts. The results confirmed that extracts of *Indigofera hirsuta* contains bioactive constituents with significant antivenin activity ($P<0.05$) under both *ex vivo* and *in vivo* conditions and thus providing scientific evidence to support the traditional claims for use in the treatment of snake bite.

Keywords: *Indogofera hirsute, anti-snake venom, ex-vivo, in-vivo.*

Introduction

Snake venom is one of the leading causes of disability and death in many developing countries, especially in rural areas where healthcare facilities are severely limited (Aleku et al., 2014). Snake venom is composed of over 90% proteins of which most of them are enzymes. The composition of venom depends largely on the species of snake, geographical differences, seasonal changes and other factors. Reflecting the complications involved in snakebite management (Gomes et al., 2010). Species carpet viper (*Echis Ocellatus*) black – necked spitting cobra (*Naja nigricolis*) and puff adder (*Bitis arietans*) belonging to viperidae and Elapidae family. The most important venom-related snakes in Nigeria, is commonly found in the Northern part especially Sokoto, Kebbi, Zamfara, Katsina, Kaduna, Plateau, Gombe, Bauchi and Taraba states and some areas of northern Enugu (Ameen et al., 2015).

The current recognized and acceptable treatment for snakebite patients is immediate administration of serum antivenom, but the

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availability of the drugs were severely limited and largely unavailable due to cost and access (Paul et al., 2011 year). Refrigeration requirements for medicines limit local distribution due to limited electricity availability (Anas et al., 2010). Antivenoms often trigger allergic reactions in some patients, limiting their use (Shantosh & Shivaji, 2004), and high-dose antivenoms often lead to adverse effects (Ariaratnam et al., 2001). All of these challenges have forced us to find effective alternative antivenom antidotes that are cheaper, more readily available and available in our local area.

Indigofera hirsuta have several medicinal uses; in Nigeria, leaf juice is taken internally for liver diseases, leaf decoction is used for jaws, ulcers, gravel, epilepsy or convulsions in infants, it is also used for diabetes, leprosy, tuberculosis, infections, snake bites and for malaria and eyelids Inflammation (Burkill, 1995). Other literature has reported pharmacological applications of this plant, including analgesic and anti-inflammatory activities (Abbas et al., 2013).

Many *Indigofera* species have been reported as potential sources of antivenoms. As described in the literature on the medicinal uses of the plants under study, *Indigofera hirsuta* is also expected to have a similar potential like other species. The plant is also claimed to be used locally to treat snake venom in localized areas of northern Nigeria.

Therefore, this study tends to explore the antivenom potential of the *Indigofera hirsuta* extracts against *Naja nignicollis* venom.

Materials and Methods

Plant Collection and Identification

Fresh aerial part of *Indigofera hirsuta* was obtained from Zaria Local Government of Kaduna State, Nigeria. The sample was identified and authenticated by Mallam Namadi Sanusi of Herbarium Unit, Department of Botany, Faculty of Life Science, Ahmadu Bello University Zaria, where a voucher specimen number V/NABU0503 was obtained.

Plant Preparation

The fresh aerial part of *Indigofera hirsuta* were properly cleaned and washed carefully with distilled water (3 litres) but not excessive and shade dried, pulverized, labelled and stored at room temperature.

Plant Extraction

The powdered plant material (1000g) was extracted in 5 litres gallon containing 3 litres of methanol for seven (7) days, the extract was filtered using Whatman No.1 filter paper and solvent was evaporated using rotary evaporator. The resulting crude extract was dried and subsequently weighed to determine the percentage yield. The crude methanolic extract (80g) was successively subjected to liquid-liquid extraction with chloroform (500cm^3) and butanol (700cm^3) in sequential order of polarity, afforded chloroform and butanol extracts respectively.

Qualitative Phytochemical Screening

Each extract (2 g) was subjected to a preliminary phytochemical screen for the presence of secondary metabolites according to the procedures described by Trease and Evans (2008) and Silva et al. (1998).

Venom Sample

Venom of adult *Naja nigricollis* was collected by the milking method of Markfalane (1967). Lyophilized and stored venom at 4 ^{o}C until needed (Yusuf et al., 2019).

Experimental Animals

Swiss albino mice (16-30 g) of both sexes were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Nigeria. They were fed with a laboratory diet and water, then placed in propylene cages at room temperature under standard conditions (12-h light and 12-h dark cycle).

Median Lethal Dose (*LD50*) Determination of the Extracts

The route of administration was intraperitoneal using the method described by Lorke (1983). In the first stage, exactly nine mice of different

sexes were divided into three groups of three mice each. Groups 1, 2 and 3 received 10, 100 and 1000 mg/kg, respectively. In the second phase, four mice were used based on the results of the first phase. Each of the four mice received different doses of the extract, 200, 400, 800 and 1600 mg/kg. The experiment was repeated for butanol and chloroform extracts using the same graded doses. The median lethal dose was calculated as follows:

$$LD_{50} = \sqrt{MLDxMSD} \quad (1)$$

Where:

MLD – Minimum lethal Dose

MSD – Maximum survival Dose

Lethality Assay of the Venom

The venom was reconstituted with normal saline to obtain concentrations ranging from 1 to 5 mg/ml. The method of Theakston and Reid (1983) was used in which three groups of mice, each containing five (5) mice, were injected intraperitoneally with 0.2 ml of various concentrations of recombinant venom. The control group (5 mice) received normal saline only (0.2 ml each, i.p.). Time of death was recorded within 24 h after venom injection. LD99 values (minimum lethal dose, MLD) were determined by probit analysis.

Ex vivo Snake Venom Detoxifying Effect by the Extracts

The method described by Abubakar et al. (2000) was used. Four groups of mice, each containing six (6) mice. The first group (control group) received only 0.2 ml of LD99 (4.6 mg/kg) of black-necked cobra venom. Groups 2, 3 and 4 (as treatment groups) were given LD99-equivalent venom of the black-necked cobra containing 80, 170 and 260 mg/kg of methanolic extract, respectively. The venom and extract were incubated at 37°C for 10 min, and then 0.2 mL of the incubated mixture was injected into each animal in the treatment group. The route of administration is intraperitoneal. The number of deaths and survival times were recorded within 24 hours. The experiment was repeated for butanol and chloroform extracts using the same graded doses.

In vivo Snake Venom Detoxifying Effects by the Extracts

The method described by Theakston and Reid (1983) was used. Twenty-four (4) mice were divided into four groups, each group containing six (6) mice. Group 1 (control group) received 0.2 ml normal saline, and groups 2, 3 and 4 (treatment groups) received 125, 250 and 500 mg/kg methanol extract, respectively. The route of administration was intraperitoneal. All the animals in the various groups were then injected with LD_{99} (4.6 mg/kg) of the *Naja nigricollis* venom for one (1) hour after injecting the extract and then observed for mortality and survival within 24 hours. The experiment was repeated for Butanol and chloroform extracts using the same graded doses.

Statistical Studies

Statistical analysis of differences between means was performed by one-way analysis of variance (ANOVA), followed by multiple comparisons of values in control versus treatment groups using post hoc Dunnett's test. The results were considered significant at $p \leq 0.05$. All the analysis was done using statistical package for the social sciences (SPSS) version 20.0.

Results and Discussion

The percentage yield of methanolic crude extract obtained from 1000g of pulverized aerial part of *Indigofera hirsuta* was (86.76g) which represents (8.68%w/w). Subjecting the crude extract to liquid-liquid extraction, the percentage yield recorded are 8.56g and 12.86g equivalent to 0.86% 1.29% for chloroform and Butanol respectively.

The qualitative phytochemical screening of *Indigofera hirsuta* on the extracts revealed the presence of secondary metabolites such as alkaloids, flavonoids, tannins, steriods/terpenes, cardiac glycosides and saponins as shown in Table 2. These components have been reported to be associated with different pharmacological activities of the plant (Mors et al., 2000).

Table 1. Percentage Yield of *Indigofera hirsuta* Different Extracts

Extracts	Colour	Weight (g)	Yield (w/w%)
Chloroform	Light green	8.56	0.86
Butanol	Light brown	12.86	1.29
Methanol (Crude)	Greenish brown	86.76	8.68

Table 2. Results of Qualitative Phytochemical Screening of *Indigofera hirsuta* Different Extracts

Constituents	Test	Methanol	Butanol	Chloroform
Alkaloids	Mayer's Waganer's Dragendorff's	++ ++ ++	++ ++ ++	++ ++ ++
Flavonoids	Ferric Chloride	+++	+++	+
Steroids/Terpenes	Salkowski	++	-	++
Tannins	Ferric Chloride	++	++	-
Cardiac Glycosides	Killer Killani	++	++	-
Saponins	Frothing	++	++	-
Anthraquinones	Borntrager	+	+	-

++ = Significantly present + = Slightly present - = Absent

Table 3a. Determination of Median Lethal Dose (LD_{50} (*ip*)) for *Indigofera hirsuta* Extracts (First Phase)

Dose (mg/kg)	Number of Mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	2/3

Table 3b. Determination of Median Lethal Dose (LD_{50} (*ip*)) for *Indigofera hirsuta* Extracts (Second Phase)

Dose (mg/kg)	Number of Mice used	Mortality
200	1	0/1
400	1	0/1
800	1	0/1
1600	1	1/1

Note: LD_{50} = 1131.3 mg/kg

Table 4. Determination of Minimal Lethal Dose (LD_{99}) for *Naja Nigricolis* Venom

Groups	Dose (mg/ml)	Dead/Total	% of Survival	Toxic Symptoms
I	0	0/5	100	No toxic Symptoms
II	1	0/5	100	Weak and not active
III	3	2/5	60	Heavy Breathing & Eye closure
IV	5	5/5	0	Limp Paralysis

Note: LD_{99} was found to be 4.6mg/kg by probit analysis as shown below

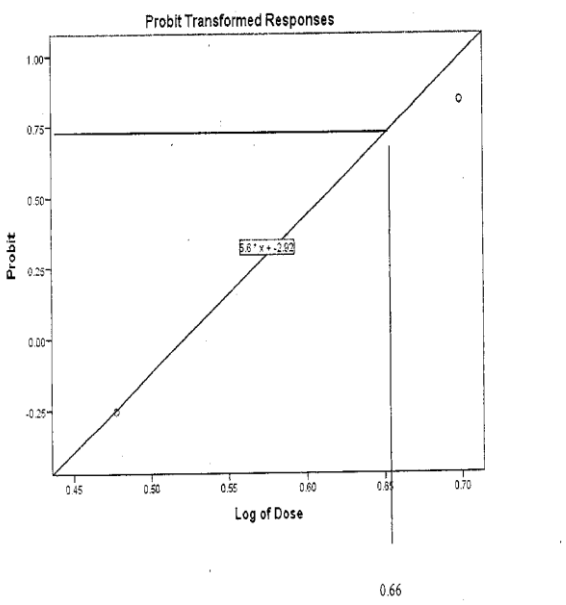


Figure1. LD99 Calculation of Naja nigricollis Venom in Mice Receiving Various Doses of Venom

LD_{99} = anti log of 0.66 = 4.6 m/kg

The acute toxicity profile of the extracts (methanol, butanol and chloroform) of *Indigofera hirsuta* was studied. The median lethal dose (LD_{50}) was found to be 1131.3 mg/kg each. This suggests that the extract is less toxic (Lorke, 1983) and relatively safe when administered intraperitoneally in antivenom doses. As shown in Figure 1, the lowest lethal dose (LD_{99}) of *Naja nigricollis* estimated by probit analysis was 4.6 mg/kg. Although Yusuf et al. (2019) reported a value (5.57 mg/kg) for the same species of snake. However, Abubakar et al. (2000) and Isah et al. (2015) reported higher values (9.55 mg/kg) and (9.7 mg/kg), respectively. This variability observed in the minimal lethal dose of venom may be due to differences in venom composition due to geographic location, subspecies, season, age, and diet of snakes (Chippaux et al., 1991; Daltry et al., 1996).

Table 5. *Ex vivo* Detoxifying Effect of *Indigofera hirsuta* Extracts on *Naja nigricollis* Venom

Treatment components	Control group (LD_{99} +NS)	% Survival of Mice within 24 hours on different treatments			Means survival time (hours)		
		(LD_{99} + 80) =T1	(LD_{99} + 170) =T2	(LD_{99} + 260) =T3	T1	T2	T3
Methanol	0 0	50.0	83.3	100.0	12.38±5.05*	17.25±4.36*	24.00±0.00*
Butanol	0	50.0	66.7	100.0	13.65±4.63*	14.72±4.15*	24.00±0.00*
Chloroform	0	50.0	66.7	100.0	14.08±4.44*	14.79±4.12*	24.00±0.00*
					Mean + SEM (Control) = 0.43±0.15		

Keys: NS = Normal Saline, T1 = (LD_{99} + 80) Treatment dose, T2 = (LD_{99} + 170) treatment dose and T3 = (LD_{99} + 260) treatment dose.

Note: Result were expressed as Mean ± SEM, represent $P \leq 0.05$ compared with control (one-way ANOVA) and Dunett Post hoc test.

In *ex vivo* study of *Indigofera hirsuta* extracts (methanol, Butanol, Chloroform) significantly ($P<0.05$) reduced the *Naja nigncollis* venom toxicity in mice injected (*ip*) with venom pre-incubated with extract in a dose dependent manner and thereby increased the survival time of mice. Mice injected with treatment (LD_{99} + 260) mg/kg was found to be alive even after 24hours of observation period with 100% survival. This could be due to complete neutralisation of the venom toxins. Methanolic extract with percentage survival recorded as 83.33% of (LD_{99} + 170) mg/kg treatment significantly ($P < 0.05$) reduced the venom toxicity more than Butanol and chloroform extracts. In general, *ex vivo* models reduced

venom toxicity in mice more significantly than *in vivo* models, where complete venom neutralization occurred at (LD99 260) mg/kg treatment for all mice as shown in Table 5.

Table 6. *In vivo* Detoxifying Effect of *Indigofera hirsuta* Extracts on *Naja nigricollis* Venom

Treatment components	Control group (LD_{99} +NS)	% Survival of Mice within 24hours on different treatments			Means survival time (hours)		
		(LD_{99} + 125) =T1	(LD_{99} + 250) =T2	(LD_{99} + 500) =T3	T1	T2	T3
Methanol	0 0	33.3	50.0	83.3	13.73±4.59*	13.86±4.54*	23.24±0.77*
Butanol	0	33.3	50.0	66.7	10.72±4.37*	14.23±4.37*	17.80±0.92*
Chloroform	0	33.3	50.0	66.7	9.76±4.51*	17.08±4.38*	18.27±3.62*
					Mean + SEM (Control) = 0.37±0.01		

Keys: NS = Normal Saline, T1 = (LD_{99} + 125) Treatment dose, T2 = (LD_{99} + 250) treatment dose and T3 = (LD_{99} + 500) treatment dose.

Note: Result were expressed as Mean ± SEM, represent $P \leq 0.05$ compared with control (one-way ANOVA and Dunett Post hoc test.

The results of the *in vivo* studies also revealed significant activity ($P<0.05$) against *Naja nigricollis* venom in mice with 83.3% survival at (LD_{99} + 500) mg/kg treatment for methanolic extract and 66.7% survival at the same treatment for Butanol and chloroform extracts, moderate survival rate (50%) at a dose of (LD_{99} + 250) and relatively lower survival rate (33.3%) at a dose of (LD_{99} + 125) for all mice used the extracts. The in vivo results obtained in a dose-dependent manner are consistent with the literature results reported by Maryam et al. (2022) for *Indigofera conferta*. These activities of the extracts observed from both models (ex vivo and in vivo) can be attributed to the individual or combined effects of the secondary metabolites present.

Flavonoids, Tannins and Terpenoids are reported to possess protein binding and enzyme inhibiting properties (Rajendran, et al., 2010). Steriods plays a vital role in the treatment of snake bites. (Carvalho et al., 2009 and Yusuf et al., 2015) reported that stagmasterol isolated from *Licania Arianeae* and *Neocarya macrophylla* showed neutralizing effect on snake venon with nearly 70% to 80% protection respectively. Therefore, the observed activity may be the result of the presence of steroids in the extract of *Indigofera hirsuta*. Qualitative results also showed that *Indigofera hirsuta* contains high amounts of tannins, in addition to other constituents which are known to non-specifically inactivate proteins. This may be a plausible mechanism for the detoxifying effects of the plant extracts and their claimed success against different snake species by traditional healers.

Conclusion

The study demonstrated that the *Indigofera hirsuta* is less toxic, relatively safe intraperitoneally and showed have significant activity ($P<0.05$) against *Naja nigricollic* venom both *ex vivo* and *in vivo* conditions and thus confirmed its ethonmedicinal claim of the plant in the treatment of snake bites which might be attributed to the secondary metabolites present in the plant.

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