

***In vitro* trichomonocidal potency of *Naja nigricollis* and *Bitis arietans* snake venom**

Tijjani Sabiu Imam¹, Zainab Tukur¹, Aisha Abdullahi Bala¹, Nuraddeen Bello Ahmad² and Adamu Yunusa Ugya^{3,4}

1. Department of Biological Sciences, Bayero University Kano, Kano, Nigeria; 2. Department of Biological Sciences, Kaduna State University, Kaduna State, Nigeria; 3. Key Lab of Groundwater Resources and Environment of Ministry of Education, Key Lab of Water Resources and Aquatic Environment of Jilin Province, College of New Energy and Environment, Jilin University, Changchun, China; 4. Department of Environmental Management, Kaduna State University, Kaduna, Nigeria.

Corresponding author: Adamu Yunusa Ugya, e-mail: ugya88@kasu.edu.ng

Co-authors: TSI: tsimam.bio@buk.edu.ng,

ZT: zaintuk@gmail.com, AAB: balaaisa04@gmail.com, NBA: nbeeahmerd@gmail.com

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Abstract

Background and Aim: *Trichomonas vaginalis* drug's limited efficacy and high toxicity, justify the need to explore other therapeutic agents, including animal toxins. In this study, the *Naja nigricollis* and *Bitis arietans* snake venoms were used to assess such trichomonocidal effect.

Materials and Methods: The median lethal dose (LD₅₀) value for both snake species was calculated by probit analysis using a statistical package for the sciences version 20.0 with an LD₅₀ of 4.04 µg/mL for the *N. nigricollis*, and no mortality was observed in the *B. arietans* envenomed rats.

Results: The trichomonocidal potency of the snake venom on *T. vaginalis* was evident with a growth inhibitory concentration of 89% with a half-maximal inhibitory concentration (IC₅₀) of 0.805 µg/mL in *B. arietans* while 95% for *N. nigricollis* at an IC₅₀ of 0.411 µg/mL.

Conclusion: The statistical analysis of one-way analysis of variance shows a significant difference (p<0.05) between the venoms and positive control group (p<0.001), and there is no significant difference between each venom and its varying concentration (p>0.05). As the least concentration can be useful, interestingly, there is no significant difference in the efficacy of *N. nigricollis* and *B. arietans* to *T. vaginalis* (p>0.05); as such, either of the venom can be used for the treatment of trichomoniasis.

Keywords: *Bitis arietans*, *Naja nigricollis*, *Trichomonas vaginalis*, trichomonocidal activities.

Introduction

Trichomonas vaginalis is the most popular flagellated protozoan parasite that causes the disease trichomoniasis, which is the most endemic non-viral sexual transmitted disease worldwide [1]. This parasite is morphologically different from other protozoan parasites such as *Giardia lamblia* and *Entamoeba histolytica*. It only exists in only one morphological state of trophozoite and cannot also form cyst as such can survive up to 24 h in urine, semen, and water samples. One of the unique features of *T. vaginalis* is the adherence factor, which favors the organisms in colonizing the vaginal epithelium cells [2]. Trichomoniasis is asymptomatic in men, while symptoms in women include foul-smelling vaginal discharge, genital itching, swelling of the vagina, severe pain during urination, and continuous urge to pee. Infection with

T. vaginalis may increase the risk of human immunodeficiency virus type 1 (HIV-1) transmission, especially in developing countries. It is widespread in areas with the highest prevalence of HIV-1 infections [3,4]. An estimate of 220 million cases of trichomoniasis has been reported worldwide, the prevalence of Trichomoniasis in Europe and the United States is minimal with about 2.3 million cases, about 8 million cases have been reported in North America, and the highest prevalence of 25% reported from Africa with 32 million these cases endemic in sub-Saharan Africa [5,6]. Several factors are responsible for the prevalence of Trichomoniasis in Africa, including poor personal hygiene, low socio-economic status, low level of education, and poverty [7,8].

Meanwhile, many epidemiological studies have been made about the magnitude of trichomoniasis; such studies revealed diverse prevalence rates across the globe [1]. Furthermore, *T. vaginalis* infections are an intriguing problem affecting the northern part of Nigeria, especially in enhancing HIV transmission, cervical cancer, and adverse complications during pregnancy, such as abortion, premature, and low-weight birth babies [9]. Similarly, metronidazole resistance and its associated cytotoxic effects are a severe

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challenge; therefore, there is a need for efficient and safer anti-protozoal agents [10-12]. The proteins contain in snake venom are responsible for the biological effect associated with it, these proteins which constitute about 95% of snake venom, are either toxins (neurotoxins), non-toxins, or hydrolytic enzymes.

The non-toxins in snake venom have some pharmacological properties, including anticancer properties, antimicrobial properties, and analgesic properties [13,14]. Although it is a fact that the non-toxic protein in snake venom possesses antimicrobial potentials, no or scanty literature exists showing the efficacy of this venom as an alternative to metronidazole in the eradication of *T. vaginalis* [15].

This study aimed to assess *Naja nigricollis* and *Bitis arietans* snake venoms' efficiency as an antibacterial against *T. vaginalis*.

Materials and Methods

Ethical approval

This study was approved by the Ethical Committee of the Department of Biological Sciences, Bayero University Kano, Nigeria.

Study period and location

The study was conducted in three villages, namely Kaltungo LGA (Gombe State), Alkaleri LGA (Bauchi State) and Karim Lamido LGA (Taraba State) in Nigeria, from July to November 2018. Agriculture is the main economic activity practiced by community members in these villages. The villages are characterized by open grassland used by pastoralists for cattle grazing. They are also situated in a semi-arid area characterized by a prolonged dry season lasting up to 7 months. The selection of the study sites was based on the fact that the areas present microhabitats whose ecological features are associated with rich herpetofauna and thus highly prone to reports of snakebite cases.

Acute toxicity assay

Experimental animal and design

Adult male Wistar albino rats of equal weight were obtained from the Department of Biological Science, Bayero University Kano. The animals were kept in wire-bottom cages at a temperature of 25+ or -1°C and under a standard condition of illumination with 12 h light – darkness cycle. They were provided with constant water and a balanced diet. In general, the animals' care was following the WHO guidelines for the care and use of laboratory animals [16]. The acute toxicity of the snake venom was determined by adopting the method described by Reed and Muench [17]. Thirty adult male albino rats with an average weight of 100 g±5 g were randomly selected to avoid gravidity that may affect the research findings if female albino rats were used. The selected male albino rats were placed into seven different cages, including the negative control with five animals per group. The rats were injected intravenously with 0.2 mL of 2

µg, 4 µg, and 6 µg, respectively, of *N. nigricollis* and *B. arietans* snake venoms. The animals were observed and compared with control for toxic symptoms such as weakness, loss of appetite, difficulty in movement, nose bleeding, mouse bleeding, and mortality for the first 2 h and 24 h post-envenoming.

Venom collection and preparation

Venom was collected from the wild and extraction was made by the milking method of Macfarlane [18]. The crude venom was collected by holding the head between the index finger and thumb, whereby the body was held between the truck and the personal arm. The jaws were forced open to expose the fang. The fangs were then pushed through the plastic/paraffin membrane hooked over the lip of a glass vessel, and gentle pressure was applied to the gland below the eye area in dim light to squeeze out the venom. Collected venom was diluted in de-ionized water, centrifuge at 10,000 gravity for 15 min. It was then vacuum dried and stored at -20°C.

Determination of total protein concentration

This was determined by adopting the protocol of Bradford [19] whereby a calibration curve covers the range 0-100 µg/mL standards with bovine serum albumin (BSA) serving as standard. Dilutions were made in duplicates using water as diluents to a total volume of 800 µL. The fractions were also analyzed by arranging the test tubes labeled as a test, standard, and blank. Distilled water (700 µL) was dispensed into all tubes followed by 100 µL of sample or fraction, 100 µL BSA standard and 100 µL distilled water to the tubes labeled test, standard, and blank, respectively, making a total volume of 800 µL. Bradford reagent (200 µL) was added to all tubes. The tube was mixed and incubated for 2 min at room temperature (24°C). The absorbance's of the blue-colored solutions were measured against the reagent blank at 595 nm.

Phospholipase A₂ (PLA₂) activity of the crude venom

The PLA₂ activity of *N. nigricollis* and *B. arietans* snake venoms was determined by the modified coagulation method [20]. Briefly, fresh egg yolk (lecithin) was homogenized in distilled water to give a concentration of 100 mg/mL, and then 10 µL of the venom and 10 µL of 50 Mm Tris/HCl buffer pH (pH=8) were incubated in 100 µL of the substrate at 37°C for 10 min, the mixture was then immersed in boiling water for 2 min to stop the reaction. The liberated fatty acid was titrated against 20 mM of sodium hydroxide (NaOH) using phenolphthalein as an indicator.

Sample collection

The procedure, justification of the research, and informed consent of participant females seeking Antenatal and Gynecology care were obtained before sample collection. Similarly, the isolate was obtained from the parasitological laboratory of Sir Muhammad Sunusi Specialist Hospital Kano. Two sterile high vaginal swabs of susceptible females were collected.

Immediately after collection, wet mount preparations were made with the first swap followed by microscopic examinations under low power (10×) and high power (40×) magnification for the presence of motile *T. vaginalis*. In comparison, the second swap was inoculated into the cultured medium at 32°C and observed at different time intervals of 24, 48, and 72 h.

Sample size

The sample size was calculated using the prevalence rate of *T. vaginalis* in Kano State is 9%. A standard epidemiological formula (Fisher's formula for cross-sectional descriptive study) was then used to calculate the sample size.

$$N = [(z_a + z_b)^2][y^2/d^2]$$

Where

N = sample size

Z_a = table value for given risk a

Z_b = table value for given risk b

y² = population standard deviation

d² = physical difference in mean response

$$N = \frac{Z^2 PQ}{D^2}$$

Therefore, N = 125.850816 ≈ 126

126 is the minimum sample size for the study.

Inclusion criteria

- All women presenting with the symptoms of *T. vaginalis* within the reproductive ages of 15-55 years were included in the study.
- All pregnant women seeking antenatal care were also enrolled.
- Women undergoing metronidazole treatment with re-infection or persistent infection were included in the study.

Preparation of the culture medium

The media were prepared by dissolving the following, 1.3 g of nutrient broth, 1.0 glucose, and 0.2 g L cysteine hydrochloride in 90 mL of boiled distilled water to the obtained homogenized solution. It was sterilized by autoclaving at 121°C for 15 min and allowed to cool to 50°C. 80 mL of inactivated human serum was added, and chloramphenicol (10 g/L of medium) was added aseptically to the sterile medium. The pH was adjusted to 6.4 with 1 mol of NaOH and 2 mL of the medium were dispensed in sterile Bijou bottles.

Parasite cytotoxicity assay (susceptibility test)

The assay of *N. nigricollis* and *B. arietans* snake venom on *T. vaginalis* was prepared by dissolving the

venom in 1 mL of 0.5 % dimethyl sulfoxide (DMSO), which is not toxic for parasites. The trophozoite was treated with doubling the concentration of 1.2 µg, 2.4 µg, 3.8 µg, and 4.8 µg. These concentrations were tested against 2 mL of the test organism, while 100 µg of metronidazole was used as a positive control. About 0.5% DMSO was used as the negative control, respectively. All test tubes were incubated at 37°C, and observations were made at 24, 48, and 72 h' time interval. A drop of sample suspension prepared was placed on the hemocytometer, which was then covered with a coverslip; it was pressed gently to form a rainbow ring at the edges, and finally viewed under a microscope. Complete flagella active parasites were considered as viable [21]. The half-maximal inhibitory concentration (IC₅₀) was calculated based on direct counting of the formalin-fixed parasite using a hemocytometer.

Results and Discussion

The result presented in Table-1 shows the acute toxicity of the venom of *N. nigricollis* and *B. arietans* to the experimental animal. The median lethal dose (LD₅₀) presented result shows that the venom of both *N. nigricollis* and *B. arietans* is toxic to the experimental animal. The LD₅₀ of *N. nigricollis* obtained in the study was 4.08 µg/g. However, no mortality was observed from the *B. arietans* envenomed rat but has shown toxic symptoms such as weakness, loss of appetite, difficulty in movement, nose bleeding, mouse bleeding, and hair [22]. However, this could be due to several factors such as the route of injection, toxins isoforms, geographic variation of species with wide distributions, and the weight of the animal, and all these are factors that influence the venom toxicity [23]. The variation in venom composition is observed across all taxonomic levels of snakes, between families, genera, and species [24]. Furthermore, some variation was also reported from *Naja naja*, which belongs to the same genus as *N. nigricollis*. The LD₅₀ of *N. naja* (Cobra) was approximate between 6 and 7 µg/dose and *N. naja* 5.656 µg/dose. The LD₅₀ of the genus *Bitis* that has reported contradicting varying value of this study such include: Study on the LD₅₀ of various bitis species that showed, *B. arietans*, 0.96 µg/mouse; *Bitis nasicornis*, 123.67 µg/mouse; *Bitis rhinoceros*, 95.28 µg/mouse; *Naja melanoleuca*, 13.41 µg/mouse; *Naja mossambica*, 22.40 µg/mouse; *Bothrops atrox*, 76 µg/mouse, *Lachesis muta*, 123.4 µg/mouse,

Table-1: Acute toxicity assay of Wistar albino rat exposed to snake venoms.

Venom	Dose (µg/mL)	Animal injected	Weight (g)	Injection route (Intravenous in the tail)	Survival after 24 h	LD ₅₀
<i>Naja nigricollis</i>	2	5	100	I.V	4	4.04
	4	5	100	I.V	2	
	6	5	100	I.V	0	
<i>Bitis arietans</i>	2	5	100	I.V	5	
	4	5	100	I.V	5	
	6	5	100	I.V	5	

Crotalus durissus terrificus, 4.32 µg/mouse, *Bothrops jararaca*, and 32.6 µg/mouse [25]. The snake venom contains diverse compounds, but about 90% of those compounds are protein and enzyme. Phospholipases are enzymes that are involved in the metabolism of lipids by stereospecific hydrolysis of 3-sn- phosphoglycerides. The total protein content and PLA₂ of the crude venoms are shown in Table-2. The *N. nigricollis* has higher protein content as well as PLA₂. The higher protein content of *N. nigricollis* venom over *B. arietans* venom could be due to local adaptation for feeding on different prey. The previous literature has documented that the Viperidae family's venoms had long been recognized for their complexity of molecular composition [26]. The result presented in Figures-1 and 2 showed *Naja nigricollis* susceptibility and *B. arietans* snake venom on *T. vaginalis*. In the study, three concentrations of 1.2, 2.4, and 3.6 µg/mL less than the established LD₅₀ of venoms were used. The mortality of such concentration on *T. vaginalis* was observed at a different time interval of 24, 48-, and 72-h incubation. Interestingly, this is the first study that showed anti-parasitic activity of snake venom on *T. vaginalis*. Three concentrations were used that were less than the established LD₅₀ of both venoms. Both *N. nigricollis* and *B. arietans* snake venom have shown to be very effective against *T. vaginalis* with an IC₅₀ of 0.411 µg/mL and mortality of 95% for *Naja* IC₅₀ 0.805 µg/mL and 89% mortality for *Bitis* at 72 h, respectively. In which 100 g of metronidazole was used, the control group yielded mortality of 99.8%. The effect of both venoms on *T. vaginalis* was evident as early as 24 h after the start of treatment (Table-3). The venom anti-parasitic activity could be due to PLA₂, L-amino acid oxidase (L-MAO), and cysteine-rich secretory proteins (CRISPs), which both *N. nigricollis* and *B. arietans* are known to possess. Based on statistical analysis of one-way analysis of variance, the result

shows a significant difference (p<0.05) between the venoms and positive control group (p<0.001), and there is no significant difference between each venom and its varying concentration (p>0.05) meaning that the treatment is not concentration-dependent. The least concentration can be effective as such a high concentration can be regarded as a waste of resources. Interestingly, there is no significant difference in the efficacy of *N. nigricollis*, and *B. arietans* to *T. vaginalis* (p>0.05); as such, either of the venom can be used for the treatment of trichomoniasis [27]. This correlates with several studies that reported anti-protozoan activity of snake venom, and such include: A study of Castillo *et al.* [28] that assessed the antiplasmodial effect of *Bothrops* as per whole and a purified fraction of Phospholipases A2 enzyme has on *Plasmodium falciparum*; it proves that both purified fraction and the whole snake venom have the antiplasmodial activity of an IC₅₀ values 1.42±0.56 µg/mL and 22.89±1.22 µg/mL, respectively. Interestingly, PLA₂ of the eastern diamondback rattlesnake (*Crotalus adamanteus*) has been shown to blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface when PLA₂ was added to infected chicken blood and fed to mosquitoes [28]. Thus, PLA2 is an excellent candidate for expression in transgenic mosquitoes as

Table-2: Total protein content and PLA₂ of the *N. nigricollis* and *B. arietans* snake venoms.

Venoms	Total protein (µg/mL)	PLA ₂ (µmoles mg/h) ^(STD)
<i>N. nigricollis</i>	212.33	490±45.5
<i>B. arietans</i>	210.0	456±40.5

PLA₂=Phospholipase A₂, *N. nigricollis*=*Naja nigricollis*, *B. arietans*=*Bitis arietans*

Table-3: The effect of *Naja nigricollis* and *Bitis arietans* on *Trichomonas vaginalis* at different concentration.

Venom	Concentrations		
	1.2 µg/mL	2.4 µg/mL	3.6 µg/mL
<i>Naja</i>	16.80±15.52 ^a	21.60±14.5 ^a	29.90±17.9
<i>Bitis</i>	19.10±13.40 ^a	24.10±15.19 ^a	28.90±22.16
+ ^{ve} control	46.00±1.00 ^b	46.00±1.00 ^b	46.00±1.00
- ^{ve} control	1.00±0.00	1.00±0.00 ^c	1.00±0.00

Value are mean±standard deviation value with different superscript with the same column are considered significantly different (p<0.05)

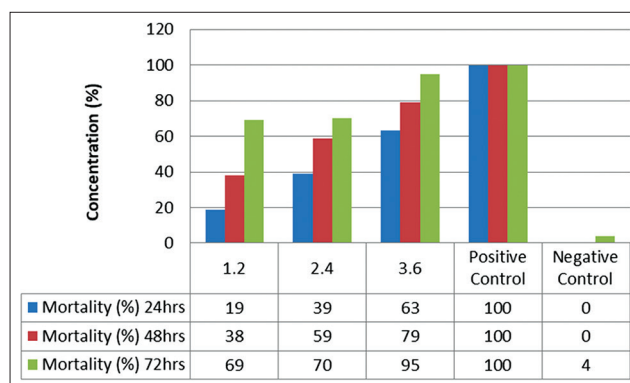


Figure-1: Mortality of *Trichomonas vaginalis* after exposure to various concentration of *Bitis arietans* snake venom in comparison to positive and negative control.

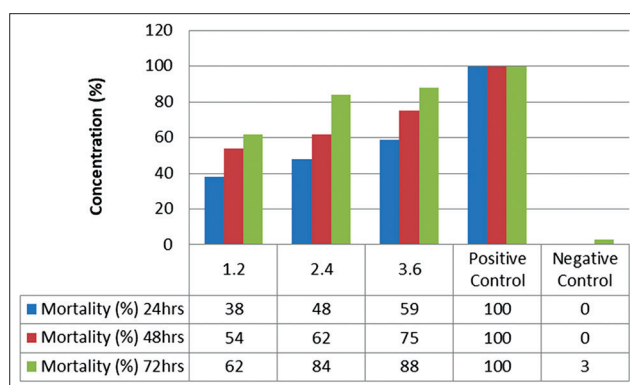


Figure-2: Mortality of *Trichomonas vaginalis* after exposure to various concentration of *Naja nigricollis* snake venom in comparison to positive and negative control.

a means of inhibiting the transmission of malaria. A similar study on BMP-1, a new metalloproteinase isolated from Bothrops Brazili snake venom has an *in vitro* anti-plasmodial property against *P. falciparum* with an IC_{50} of 3.2 ± 2.0 mg/mL [29]. Recent literature described L-MAO isolated from *Bothrops pirajai* and *Bothrops alternatus* venoms had inhibited *Escherichia coli* growth. Interestingly purified L-MAO of Australian elapid, *Pseudechis australis* (Australian king brown or mulga snake) also had antibacterial property [29]. Studies in this context include the effect of *Crotalus viridis viridis* snake venom on *Trypanosoma cruzi*, which shows a promising result of 76-93% reduction in the number of parasites per infected cell and a 94-97.4% reduction in the number of parasites per 100 cells after 96 h of infection [30].

Conclusion

In this study, the anti-parasitic activity of snake venoms on *T. vaginalis* was evident with a growth inhibitory concentration of 89% in *B. arietans* and 95% in *N. nigricollis*, a concentration of $3.6 \mu\text{g/mL}$, which is less than the LD_{50} of the venom. The LD_{50} value of the *N. nigricollis* and *B. arietans* of the envenomed rat was evaluated in relevance to the previous literature with an LD_{50} of $4.04 \mu\text{g/mL}$ for the *N. nigricollis*, and no mortality was observed in the *B. arietans*. More work is needed to isolate bioactive trichomonocidal compounds so as to improve the efficacy and safety of snake venom extract for human use.

Authors' Contributions

TSI: Investigation and data collection, data analysis and interpretation, writing and original drafting. ZT: Research conceptualization, supervision, review, and editing. AAB: Sample design and methodology. NBA: Review and editing. AYU: Review and editing. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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