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

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**Received:** 05-10-2020, **Accepted:** 25-01-2021, **Published online:** 16-02-2021

**doi:** www.doi.org/10.14202/IJOH.2021.6-11

**How to cite this article:** Imam TS, Tukur Z, Bala AA, Ahmad NB, Ugya AY (2021) *In vitro* trichomonocidal potency of *Naja nigricollis* and *Bitis arietans* snake venom, *Int. J. One Health*, 7(1): 6-11.

**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 (LD<sub>50</sub>) value for both snake species was calculated by probit analysis using a statistical package for the sciences version 20.0 with an LD<sub>50</sub> 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 (IC<sub>50</sub>) of 0.805 µg/mL in *B. arietans* while 95% for *N. nigricollis* at an IC<sub>50</sub> 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 pie. 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...
challenge; therefore, there is a need for efficient and safer anti-protozoal agents [10-12]. The proteins con-
tain in snake venom are responsible for the biological
effect associated with it, these proteins which constitu-
tute about 95% of snake venom, are either toxins (neu-
rotoxins), non-toxins, or hydrolytic enzymes.

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

This study aimed to assess Naja nigricollis and
Bitis arietans snake venoms’ efficiency as an antibac-
terial 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 character-
ized by open grassland used by pastoralists for cat-
tle 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 herpeto-
fauna 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±
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
poisoning of the snake venom was determined by adopt-
ing 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 gra-
vidity 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, centrifuged
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 frac-
tion, 100 µL BSA standard and 100 µL distilled water
to the tubes labeled test, standard, and blank, respec-
tively, 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 tempera-
ture (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 coag-
ulation method [20]. Briefly, fresh egg yolk (lecithin)
was homogenized in distilled water to give a concen-
tration 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.

\[ N = \frac{Z_a^2 \cdot P \cdot Q}{d^2} \]

Where
- \( N \) = sample size
- \( Z_a \) = table value for given risk a
- \( Z_b \) = table value for given risk b
- \( \text{y} \) = population standard deviation
- \( d \) = physical difference in mean response

\[ N = \frac{Z_a^2 \cdot P \cdot Q}{d^2} \]

Therefore, \( N = 125.850816 \approx 126 \)

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 (IC_{50}) 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 (LD_{50}) presented result shows that the venom of both N. nigricollis and B. arietans is toxic to the experimental animal. The LD_{50} 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 LD_{50} of N. naja (Cobra) was approximate between 6 and 7 µg/dose. The LD_{50} of the genus Bitis has been reported contradicting varying value of this study such include: Study on the LD_{50} 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.

<table>
<thead>
<tr>
<th>Venom</th>
<th>Dose (µg/mL)</th>
<th>Animal injected</th>
<th>Weight (g)</th>
<th>Injection route (Intravenous in the tail)</th>
<th>Survival after 24 h</th>
<th>LD_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naja nigricollis</td>
<td>2</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>4</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bitis arietans</td>
<td>2</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>100</td>
<td>I.V</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Venoms</th>
<th>Total protein (µg/mL)</th>
<th>PLA2 (µmoles mg/h) <em>STD</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>N. nigricollis</td>
<td>212.33</td>
<td>490±45.5</td>
</tr>
<tr>
<td>B. arietans</td>
<td>210.0</td>
<td>456±40.5</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Venom</th>
<th>Concentrations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Naja</td>
<td>16.80±15.52 a</td>
<td>21.60±14.5 b</td>
</tr>
<tr>
<td>Bits</td>
<td>19.10±13.40 a</td>
<td>24.10±15.19 b</td>
</tr>
<tr>
<td>+ve control</td>
<td>46.00±1.00 b</td>
<td>46.00±1.00 b</td>
</tr>
<tr>
<td>-ve control</td>
<td>1.00±0.00 b</td>
<td>1.00±0.00 b</td>
</tr>
</tbody>
</table>

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

![Figure-1](Available at www.onehealthjournal.org/Vol.7/No.1/2.pdf) Mortality of Trichomonas vaginalis after exposure to various concentration of Bitis arietans snake venom in comparison to positive and negative control.

![Figure-2](Available at www.onehealthjournal.org/Vol.7/No.1/2.pdf) 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\textsubscript{50} of 3.2±2.0 mg/mL [29]. Recent literature described L-MAO isolated from Bothrops piirajai 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 µg/mL, which is less than the LD\textsubscript{90} of the venom. The LD\textsubscript{90} value of the N. nigricollis and B. arietans of the envenomed rat was evaluated in relevance to the previous literature with an LD\textsubscript{90} of 4.04 µg/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.

Acknowledgments

The authors appreciate the support rendered by B.G, Kurfi, Department of Biochemistry, Bayero University Kano. The authors are also thankful to Nigeria Snakebite Research and Intervention Center, Bayero University, Kano, for kind support. The authors did not receive any funds for this study.

Competing Interests

The authors declare that they have no competing interests.

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