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Preclinical efficacy evaluation of two commercially available anti-snake venom against *Naja nigricollis* induced envenomation

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ABSTRACT

Background: The biochemical and immunological variations of snake venom components lead to many challenges in manufacturing appropriate anti snake venom (ASV). These variations have negatively impacted clinical outcomes due to the availability of ineffective ASVs in countries where the manufacturing venom does not originate. There are reports of ineffective ASVs exported to some African countries with public health and economic consequences on the already debilitating crisis. Recently, there have been calls and publications to draw the attention of policymakers and regional regulators on the need for preclinical and clinical data related to ASV, especially when manufactured from other regions. We therefore, screened the two most commercially available antisnake venom in Northern Nigeria against the most medically important cobra venom (*N. nigricollis*).
Methods: *N. nigricollis* venom was manually milked from five *N. nigricollis* captured from the wild and the LD_{50} of venom was determined using probit analysis. The efficacy evaluation was conducted using the classical world health organization's preclinical mixing of venom/ASV methods on the lethal, hemorrhagic, hemolytic and necrotic effect of the venom in mice and rabbit blood.
Result: The median lethal dose (LD_{50}) of *Naja nigricollis* venom was estimated to be 1.0 mg/kg as calculated using Probit analysis. The two ASVs used for this study; EchiTab Plus-ICP and Premium Antivenom (PAV), provided protection (100%) against venom-induced lethality in mice except at the dose of 100 l/mouse, where the PAV provided only 33% protection. All the administered doses of both EchiTab-Plus-ICP and PAV showed statistically significant reduction ($p<0.001$) in the mean hemorrhagic diameter when compared with the control group (19.12±1.95 mm). There was also significant reduction ($p<0.001$) in the mean necrotic diameter in all the groups compared to the control group (8.58±1.33 ml). Two dilutions of EchiTab-Plus-ICP (100 and 200 l) were able to significantly reduce (>50%) venom-induced hemolysis by 58 and 62% respectively, compared with the venom control group. On the other hand, such reduction was not observed with PAV.
Conclusion: The two most commercially available ASV in Northern Nigeria, EchiTab Plus-ICP and Premium Antivenom, were significantly ($P > 0.01$) effective against lethality and venom-induced pathological parameters from *Naja nigricollis* envenoming, including; hemolysis, hemorrhagic and necrotic lesions, with EchiTab Plus-ICP showing better activity.

1. Background

Snakebite envenoming (SBE) is a neglected tropical disease that causes public health problems in Latin America, Asia and Africa. The problem is on the increase in Nigeria and other West African countries.[1] This crisis's medical and economic implications have not been given significant recognition in the scientific community until recently when the World Health Organization (WHO) enlisted SBE as a category A neglected tropical disease.[1,2] Affected societies in Asia and African countries face snakebite-related disadvantages with negative impacts on the economy and the healthcare sectors in these regions.[2] The burden of SBE has been estimated at 1.03 million disability-adjusted life years per annum in sub-Saharan African, out of which Nigeria has the highest burden with

43% of the total burden in West Africa.[3] There are four families of venomous snakes found in Nigeria, including Viperidae, Elapidae, Columbridae and Actraspididae.[4] Carpet viper (*Echis ocellatus*), black-necked spitting cobra (*Naja nigricollis*), belonging to the Viperidae and Elapidae families are the most medically important snakes associated with envenoming in northern Nigeria.[4,5] The cobras represent the most medically crucial elapid snake group throughout Africa. Generally, spitting cobras have locally active venom that causes ulceration and necrosis around the bite site, with systemic neurotoxic effects. The hospital record of snakebite victims' history has shown that *Naja nigricollis* is the most urbanized snake in northern Nigeria.[4]

Anti-snake venom (ASV) is currently the standard effective treatment of snakebite envenoming. Although effective and high-quality ASV against the medically important snakes exist, availability and affordability have become a massive hindrance for most snakebite victims in West African countries; these lead to importation and marketing of ASVs with doubtful efficacy in the sub-Sahara Africa region.[6-8] Some of the marketed ASVs for Africa are inappropriately manufactured with venoms from non-African snakes, causing uncertain efficacy of the available ASVs, which exacerbates the complexity of intervention measures to reduce the burden of snakebite in the sub-Saharan Africa.[7,8] There are reports of ineffective ASVs exported to some African countries with public health and economic consequences on the already debilitating crisis of ASV paucity.[6,9] This study selected the two most commercially available antisnake venom in Northern Nigeria[10] and screened them for activity against *Naja nigricollis* snake venom.

2.0 Materials and Method

2.1 Animals

One hundred and fifty swiss albino mice (18 to 20 g) and three rabbits (2 to 2.5 kg) were obtained and maintained according to Bayero University guidelines for the use and care of laboratory animals. The animals were housed and fed at the Animal House Facility, Department of Pharmacology and Therapeutics, Bayero University Kano. The study was approved by the Ethics Committee of the College of Health Sciences, Bayero University Kano (BUK/CHS/HREC/VII/66). All animals were treated according to Bayero University ethical code for animal experimentation

2.2 Materials

EchiTab Plus-ICP produced by Instituto Clodomiro Picado, Costa Rica (Exp. date 09/2021), Premium antivenom produced by Premium Serums, India (Exp. date 06/2021), Crude venom, Vanier caliper, spectrophotometer (PerkinElmer, USA), Centrifuge (PerkinElmer, USA).

2.3 Antivenom selection and venom collection

Two most commercially available ASV in Northern Nigeria[10] were selected for the study and the ASV samples were purchased from a licensed distributor. Doses of the ASVs were calculated based on the manufacturers' recommendations. *N. nigricollis* snakes were captured from Duguri area of Bauchi State-Nigeria.They were identified and kept at the serpentarium of Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. Venoms from the captured snakes were manually pooled at Veterinary Medicine serpentarium, Ahmadu Bello University Zaria, using the method described by Markflane.[11] Briefly, the snake was held captive by the handler, mouth opened and fangs placed on the edge of a glass container covered with polythene. The milking was enhanced by pressing on and off of the snake tail while the venom gradually drops into the container.[11]

2.4 Determination of LD_{50} and anti-lethality assay

Thirty Swiss albino mice weighing 18 to 20 g were divided into five groups (n = 6). Various doses of venom were administered *intraperitoneally (i.p)*. Deaths occurring within 48hrs were recorded, and the Median Lethal Dose (LD_{50}) was estimated by probit analysis.[12]

Fifty-four mice were divided into nine groups (n=6). Group I - received a fixed amount of venom alone dissolved in normal saline, corresponding to $2LD_{50}$, *intraperitoneally (IP.)*. To group II–V; 4 increasing doses of the premixed EchiTab Plus-ICP with $2LD_{50}$ of the venom incubated for 30minutes at 37°C were administered *intraperitoneally (i.p.)*. To group VI–X; 4 increasing doses of the premixed Premium Antivenom (PAV) with $2LD_{50}$ of the venom incubated for 30 minutes at 37°C were administered *intraperitoneally (i.p.)*. 48 hours after administrations, mortality was recorded, and the results were analyzed.[1]

2.5 Determination of Minimum Hemorrhagic Dose (MHD)

The minimum hemorrhagic Dose (MHD) was determined as described by Theakston and Reid[12]. Eight groups of four mice each (18–20 g) were injected intradermally *(i.d.)* with various doses of venom dissolved in distilled water. Three

hours after injection, mice were sacrificed, the skins were removed, and the hemorrhagic area in the inner side of the skin was measured using a Vanier caliper.[12]

2.5.1 Venom-Induced Hemorrhage Neutralization Assay

Fifty-four mice were divided into nine groups of 6 mice each. Group I - received a fixed amount of venom alone dissolved in distilled water, corresponding to 2MHD intradermally (i.d). To group II–V, 4 increasing doses of the premixed EchiTab Plus-ICP with 2MHD of the venom incubated for 30 minutes at 37°C were administered *intradermally (i.d)*. To group VI–X -4, increasing doses of the premixed PAV with 2MHD of the venom incubated for 30 minutes at 37°C were administered intradermally (i.d). Three hours after, the mice were sacrificed, and the injected skins were removed; the size of the hemorrhagic lesion in the inner side of the skin was measured as described by Theakston and Reid.[12]

2.6 Determination of minimum necrotizing dose (MND)

The Minimum Necrotizing Dose (MND) was determined as described by Theakston and Reid[12]. Eight groups of four mice each (18–20 g) were injected *intradermally (i.d.)* with various doses of venom in distilled water. Three days post-injection, the skins were removed, and the necrotic area in the inner side of the skin was measured using a vernier caliper.[1]

2.6.1 Venom-Induced necrosis neutralization assay

Fifty-four mice were divided into nine groups of 6 mice each. Group I - received a fixed amount of venom alone dissolved in distilled water, corresponding to 2MND *intradermally (i.d)*. To group II–V, 4 increasing doses of the premixed EchiTab Plus-ICP with 2MND of the venom incubated for 30 minutes at 37°C were administered *intradermally (i.d)*. To group VI–X. 4, increasing doses of the premixed PAV with 2MND of the venom incubated for 30 minutes at 37°C were administered *intradermally (i.d)*. Three days post-injection, the skins were removed, and the necrotic area in the inner side of the skin was measured using a vernier caliper.[12]

2.7 Anti-hemolytic activity of the antisnake venom

Pilot study was conducted to determine the amount of venom that induced 100% hemolysis in 1ml of 2% rabbit erythrocytes. The amount was premixed with various dilutions of the two antivenoms and incubated at 37 °C for 30 minutes while the control contained venom solution alone. Each mixed solution was added to 500µl of 2% erythrocytes and incubated at room temperature; this was followed by measuring the absorbance of the liberated hemoglobin at 540 nm. The percentage hemolysis was calculated by dividing the sample's absorbance on control absorbance multiplied by 100.[13]

2.8 Statistical Analysis

Data were analyzed using a statistical package for social science students (SPSS) version 20 and presented as mean ± standard error of the mean (SEM).[14] Statistical differences between treatments and controls were conducted using a one-way Analysis of Variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

3.0 RESULTS

3.2 Snake venom

2g of venom was pooled from five *Naja nigricollis* at the serpentarium of the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria.

3.3 Median lethal dose (LD_{50}) of *Naja nigricollis* venom

The LD_{50} of *Naja nigricollis* was determined using probit analysis, and was found to be 1.0 mg/kg in mice, as shown in figure 2

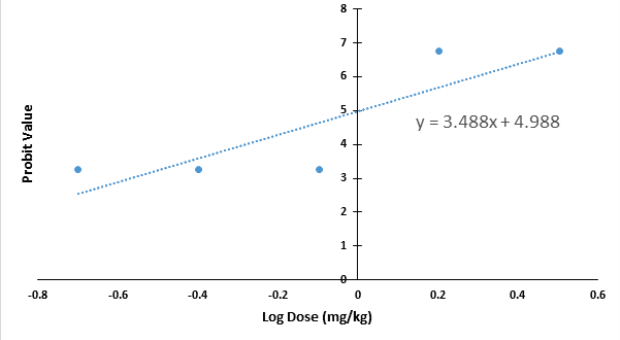

Figure 2. Linear graphical representation of *Naja nigricollis* venom LD_{50} determination

LD_{50} = 1.0mg/kg in mice, Probit value = corresponding value on probit table, Log Conc = log 10 of the corresponding concentration, n = 6,

3.4 Effect of EchiTab-Plus-ICP and Premium Antivenom on *Naja nigricollis* venom-induced Lethality in Mice

All the doses of EchiTab-Plus-ICP tested provided 100% protection against the $2LD_{50}$ venom induced lethality compared to the control group. Similar result was obtained with PAV except the dose of 100 ul/mouse which provided only 33% protection against lethality as shown in figure 3.

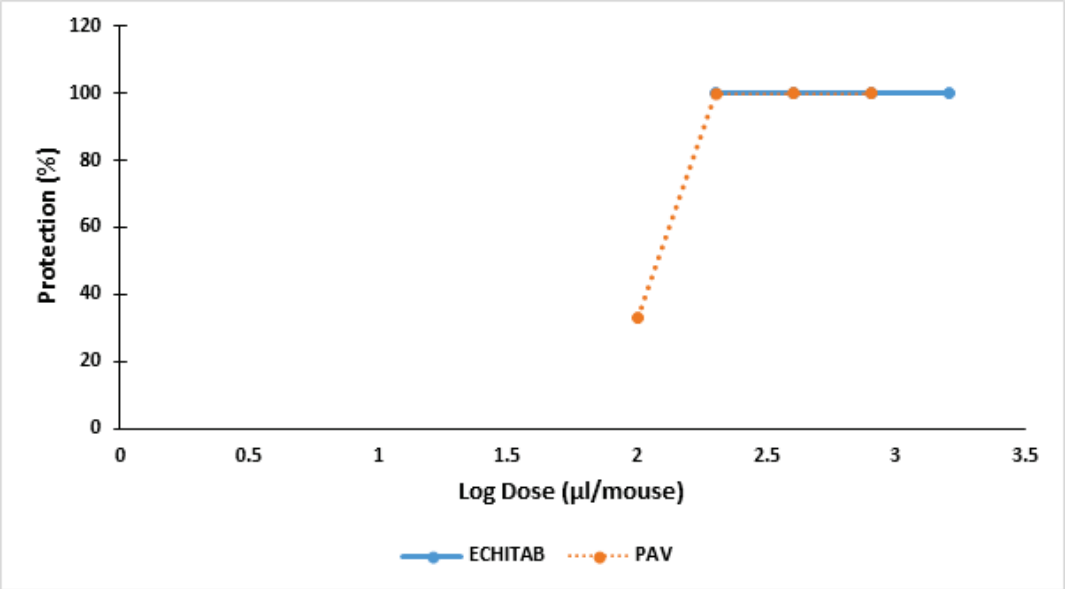

Figure 3: Effect of EchiTab-Plus-ICP and Premium Antivenom on (Naja nigricollis) venom-induced Lethality in Mice.

ECHITAB = EchiTab Plus-ICP, PAV = Premium Antivenom, % protection = protection from death, Dose = volume of ASV administered per mouse, Venom group = 0% protection from death, n = 6

3.5 Determination of Minimum Hemorrhagic Dose (MHD)

The minimum hemorrhagic dose was determined using probit analysis, and it was found to be 251 µg/kg in mice as shown in figure 4.

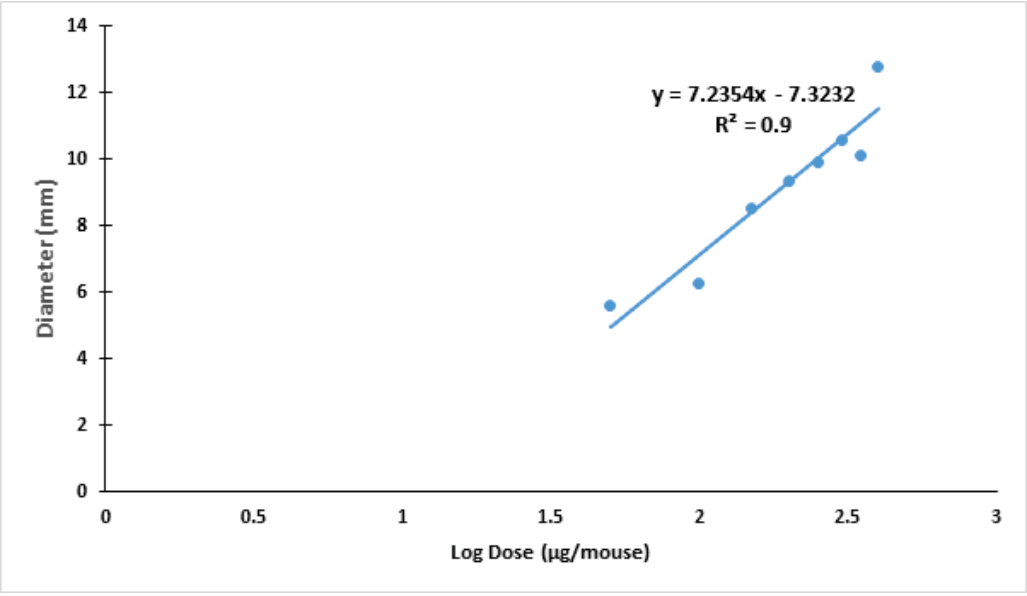

Figure 4. Linear graphical representation of *Naja nigricollis* venom MHD determination

Minimum Hemorrhagic Dose (MHD) = 1.0mg/kg in mice, Log Dose = Volume of antivenom administered per mouse, n= 6, mm = millimeter

3.6 Effect of EchiTab-Plus-ICP and Premium Antivenom on *Naja nigricollis* venom-induced Haemorrhage in Mice

All the administered doses of both EchiTab-Plus-ICP and PAV showed statistically significant reduction ($p<0.001$) in the mean hemorrhagic diameter when compared with the control group (19.12±1.95). The highest dose EchiTab-Plus-ICP reduced the hemorrhagic diameter to as low as 0.33±0.1 while that of PAV reduced the hemorrhagic diameter to 1.65±0.52 as shown in figure 5.

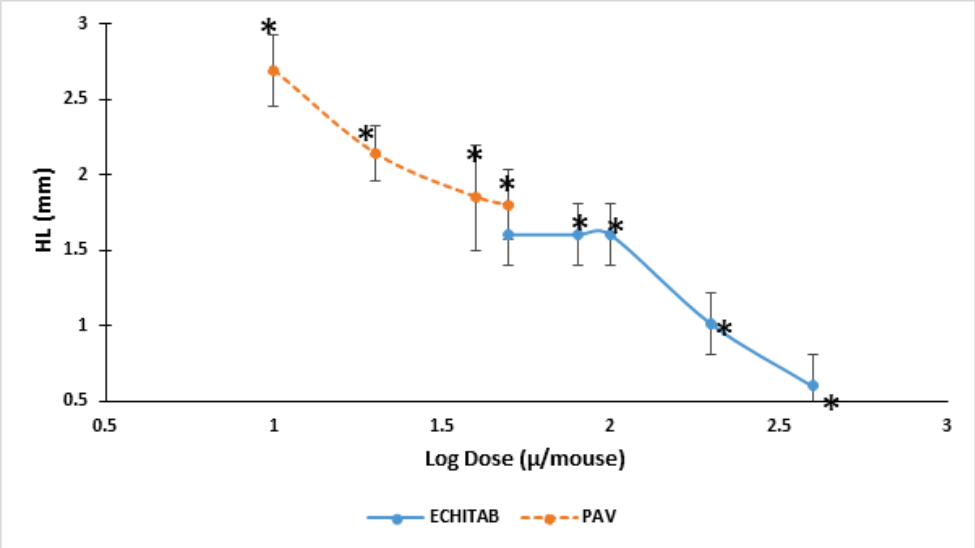

Figure 5: Effect of EchiTab-Plus-ICP and Premium Antivenom on *Naja nigricollis* venom-induced Haemorrhagic Injury in Mice.
ECHITAB = EchiTab Plus-ICP, PAV = Premium Antivenom, HL =Haemorrhagic lesion, Dose = Volume of ASV Administered per mouse. * = $p<0.01$ compared to Venom group (19.12 + 1.95 mm), n = 6

3.7 Determination of Minimum Necrotizing Dose (MND)

The minimum necrotizing dose was determined using probit analysis, and it was found to be 63 µg/kg in mice, as shown in figure 6.

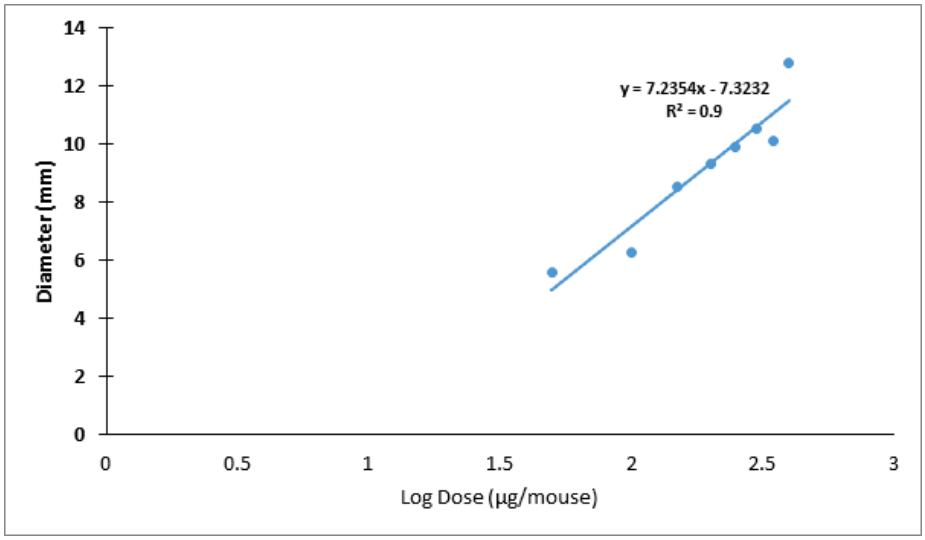

Figure 6. Linear graphical representation of *Naja nigricollis* venom MND determination
Minimum Necrotic Dose (MND) = 1.0 mg/kg in mice, Log Dose = Volume of antivenom administered per mouse, n= 6, mm = millimetre

3.7 Effect of EchiTab-Plus-ICP and PANAF-Premium Antivenom on *Naja nigricollis* venom-induced Necrosis in Mice

There was significant reduction ($p<0.001$) in the mean necrotic diameter in all the mice groups that received EchiTab-Plus-ICP and PAV as compared with the control group (8.58±1.33). The EchiTab-Plus-ICP antivenom at the doses of 20, 40, 80, and 160 l/mouse reduced the necrotic length to 1.2±0.31, 0.7±0.23, 0.6±0.12, 0.3±0.12 in necrotic diameter respectively. On the other hand, the PAV at the doses of 5, 10, 20, 40 l/mouse reduced the necrotic length to 1.7±0.53, 1.1±0.39, 0.23±1.8, 0.2±0.36 in necrotic diameter respectively. The highest doses of EchiTab-Plus-ICP and PAV reduced the necrotic diameter to 1.2±0.31 and 1.7±0.53 respectively as shown in figure 7.

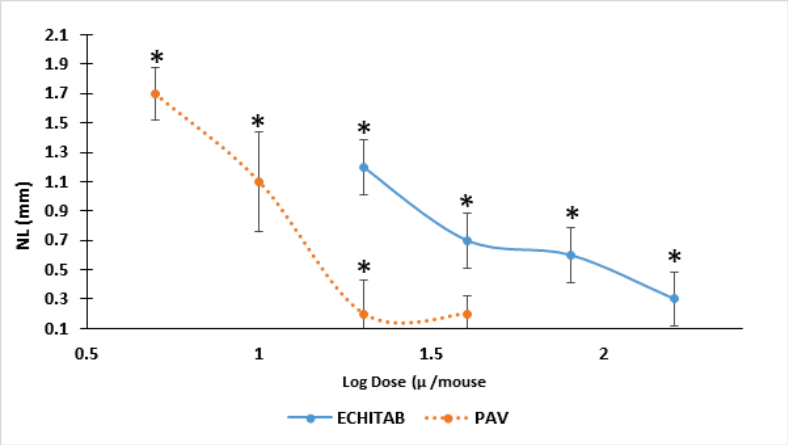

Figure 7: Effect of EchiTab-Plus-ICP and PANAF-Premium Antivenom on Naja nigricollis venom-induced Necrotic Injury in Mice
ECHITAB = EchiTab Plus-ICP, PAV = Premium Antivenom, NL =Necrotizing lesion, Dose =Volume of ASV administered per mouse, * = $p<0.01$ compared control group (8.58 + 1.33 mm), n = 6

3.8 Effects of Echitab-Plus-ICP and Premium Antivenom on *Naja nigricollis* venom-induced hemolysis in rabbit erythrocytes

Two dilutions of EchiTab-Plus-ICP (100 and 200 µl) were able to reduce venom-induced hemolysis to 58 and 62%, respectively, compared with the control group. On the other hand, PAV did provide more than 50% reduction in hemolysis as shown in figure 8.

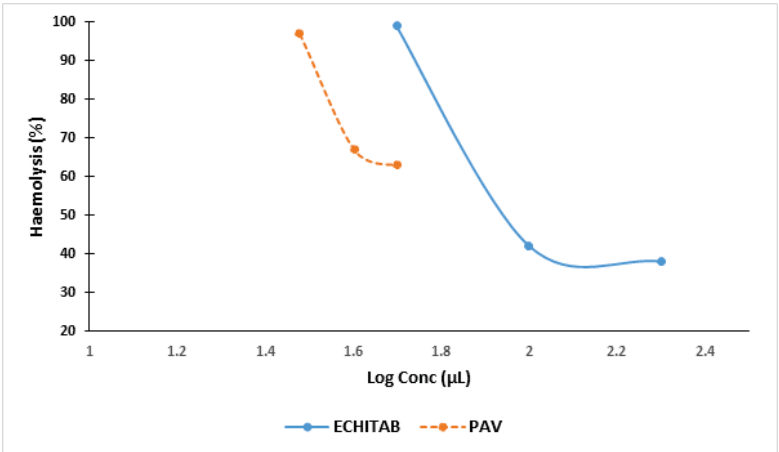

Figure 8: Effects of EchiTab-Plus-ICP and Premium Antivenom on *Naja nigricollis* venom-induced hemolysis in rabbit erythrocytes
ECHITAB = EchiTab Plus-ICP, PAV = Premium Antivenom, % = percentage hemolysis of red blood cells (RBC), Conc =Volume of ASV, Venom group = 100% hemolysis

4. Discussion

Many ASVs have no clinical data on efficacy, making healthcare professionals and regulatory agencies rely on preclinical testing reports for registration and ASV therapy in most sub-Saharan African countries.[6,15] The efficacy of ASV to neutralize the toxicity of medically relevant snake venoms has to be demonstrated through meticulous preclinical testing before their introduction into the clinical setting.[16,17] This is because many healthcare professionals and regulatory agencies rely on preclinical testing reports for registration and ASV therapy in most sub-Saharan African countries. [1,6] In this study, the LD_{50} of *Naja nigricollis* venom was estimated to be 1.0 mg/kg using Probit analysis. Similar value was reported by another study[18] and slightly higher by other researchers.[19] Interestingly other studies used the venom minimum lethal dose (LD_{99}) to induce lethality in mice; the LD_{99} doses range between 4.8 - 9.70 mg/kg.[20-22]. The variations in the LD_{50} from different studies could be due to differences in geographical locations of the snakes, sex, diet, seasonal variation, composition and relative abundance of toxins of the venom.[15] The most commercially available ASVs in northern Nigeria (EchiTab Plus and PAV) were able to protect (100%) against venom-induced lethality in mice. However, PAV could only offer 33% protection at the lowest dose used (100 µl/mouse) which could be as a result of regional venom variation as reported in Kenya.[6]

Elapid snakes have been reported to induce a range of pathological envenoming, including necrosis, hemorrhage, hemolysis and neurotoxicity.[22,23] We have tested the efficacy of EchiTab-Plus-ICP and Premium Antivenom on these pathologies and found a statistically significant difference ($p<0.001$) in the mean hemorrhagic diameter, mean necrotic diameter and percentage hemolysis when compared with the control group. These data provide more information on the preclinical efficacy of these ASVs against venom-induced pathologies, especially necrosis and hemolysis. EchiTab-Plus-ICP is manufactured by snakes' venom originated from Nigeria, including *Naja nigricollis Bitis arietans and Echis ocellatus*; unlike other ASVs, there are available preclinical and clinical data related to the efficacy of EchiTab-Plus-ICP in some treatment centers.[24-26]

The results showed that both ASVs might be effective in treating snakebite envenoming because the gold standard in the preclinical assessment and quality control of ASV is the neutralization of venom-induced lethality.[16] We have demonstrated the efficacy of EchiTab-Plus-ICP and PAV on some *N. nigricollis* venom toxicity profiles including; lethality, hemorrhage, necrosis and hemolysis compared to control group, thereby providing more information on the preclinical efficacy of these ASVs on some venom-induced pathologies. However, clinical data is currently lacking from many ASVs available in northern Nigeria, which could be the reason for the suspected inefficacy by some brands imported into sub-Saharan African countries.[27,28] The originality of venom used for immunization and proving clinical efficacy could be the reason EchiTab-Plus-ICP is more effective in our study than the other brand; this trend is also observed in other unpublished studies on snakes from northern Nigeria. Recently, there have been calls to draw the attention of policymakers and regional regulators on the need for preclinical and clinical data related to ASV, especially when manufactured from other regions.[1,17]

Limitation

Neurotoxicity is one of the major systemic toxicity effect of *N. nigricollis* envenoming and our study could not evaluate the efficacy of the two ASVs on neurotoxicity.

5. Conclusions

The two most commercially available ASV in Northern Nigeria, EchiTab Plus and Premium Antivenom, were significantly ($P > 0.01$) effective against lethality and other venom-induced pathological parameters from *Naja nigricollis* envenoming, including; hemorrhage, Necrosis and hemolysis. Even though both ASVs were effective against *Naja nigricollis* envenoming, EchiTab Plus provided 100% protection against lethality and significantly reduced hemolysis in all the doses used compared to Premium Antivenom that gave less than 40% protection in one of the doses used.

Competing interests

The authors declare no competing interest.

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No external funding was used for this study.

Statement of Authorship

We declared that this work was conducted by the authors named in this article. **AAB and BAZC** conceived the original idea, developed the methods, produced the theory.. **SM, MJ and BK** developed the techniques and co-

supervised the work. **AAB and YA** performed laboratory work and co-wrote the manuscript**. BAZC** gave the supervisory approval and finally revised the manuscript for intellectual content.

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