Hindawi Publishing Corporation
Journal of Toxins
Volume 2015, Article ID 167492, 7 pages
http://dx.doi.org/10.1155/2015/167492

Research Article

Cytotoxiciy of *Naja nubiae* (Serpentes: Elapidae) and *Echis ocellatus* (Serpentes: Viperidae) Venoms from Sudan

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Received 25 December 2014; Revised 1 March 2015; Accepted 2 March 2015

Academic Editor: A. M. Soares

In Sudan, as in many African countries, no local specific antivenom is manufactured resulting in snake bite victims being treated by antivenoms imported from abroad. In the present work we measured the cytotoxic effect of the recently described spitting cobra (*Naja nubiae*) and the carpet viper (*Echis ocellatus*) snake venoms using a cell based assay. We also investigated the efficacy of four antivenoms CSL (Australia), SAIMR (South Africa), snake venom antiserum (India), and EchiTAb-Plus-ICP (Cost Rica) to neutralize the cytotoxic effect of the two venoms. The venoms resulted in a remarkable inhibition of cell viability with *N. nubiae* being more cytotoxic than *E. ocellatus*. The four antivenoms studied were effective in neutralizing *N. nubiae* cytotoxicity. However, only partial efficacy in neutralizing the cytotoxic effect of *E. ocellatus* was achieved using CSL (Australia) and SVA (India) antivenoms. Based on the cross neutralization by the four antivenoms, the Sudanese *N. nubiae* venom most likely has homologous epitopes with similar snakes from Australia, South Africa, India, and Cost Rica, while *E. ocellatus* venom from Sudan shares little homology with similar snakes from other countries.

1. Introduction

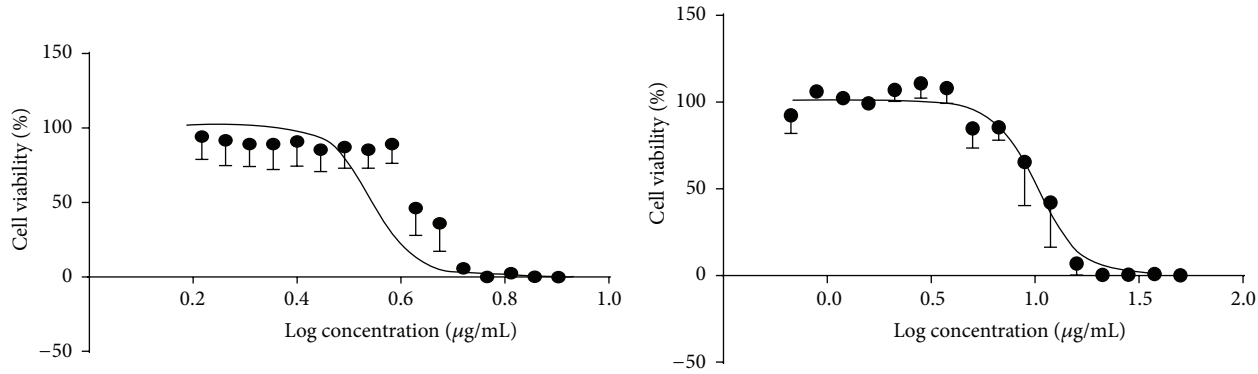
Snakes belonging to the genus *Echis* (saw-scaled viper) and *Naja* (cobras) are widely distributed in Africa and are of great medical importance [1–3]. Some species in the genus *Naja* (nonspitting cobras) are predominantly neurotoxic and produce progressive paralysis without necrosis [3]. The other species (spitting cobras) are characterized by cytotoxic pattern of envenomation which includes swelling at the bite site with blistering and bruising that may lead to necrosis [3, 4]. The abundance of cytotoxins and cytotoxic PLA_2s in the venom of spitting cobras is suggested to be the main factor responsible for these clinical features [5, 6]. The venom of the recently described spitting cobra, *Naja nubiae* [7], is unique as it displays both cytotoxic and neurotoxic properties [5]. The preclinical testing of cytotoxicity in the case of cytotoxic *Naja* deserves attention since local tissue damage is a major consequence [3, 8].

The saw-scaled or carpet viper (genus *Echis*) is the most important cause of snakebite mortality and morbidity in the sub-Saharan savannah region [3, 9]. Bites by these snakes produce moderate to severe local swelling, blistering, and necrosis with sever systematic haemostatic disorders [3, 10, 11].

Recently, a MTS based cell cytotoxicity assay is being widely used as an alternative model for testing the cytotoxic effect of venoms as well as the efficacy of antivenoms with the advantage of avoiding the use of experimental animals [6, 12, 13]. In this study, a rat skeletal muscle cell line, L6, was used to examine the cytotoxic activity of *Naja nubiae* and *Echis ocellatus* venoms. Four antivenoms, CSL (Australia), SAIMR (South Africa), snake venom antiserum (India), and EchiTAb-Plus-ICP (Cost Rica), were also used to determine their ability to neutralize the cytotoxic effect of the two venoms.

2. Results and Discussion

2.1. Cytotoxicity of N. nubiae and E. ocellatus Venoms on L6 Cells. Incubation of L6 cells with serially diluted *N. nubiae*



(a) *N. nubiae* venom (b) *E. ocellatus* venom

FIGURE 1: Sigmoidal growth curves of (a) *N. nubiae* venom (1.647–8 μg/mL) and (b) *E. ocellatus* venom (0.668–50 μg/mL) displayed as percentage of maximum cell viability in L6 cells ($n = 4$).

venom (1.647–8 μg/mL) and *E. ocellatus* venom (0.668–50 μg/mL) resulted in a concentration-dependent inhibition of cell viability with an IC_{50} value of 4.27 μg/mL for *N. nubiae* and 10.33 μg/mL for *E. ocellatus* ($n = 4$; Figure 1). Previously the same cell-based assay examined the cytotoxic effect of the *Naja nigricollis* and *Naja mossambica* venoms on L6 skeletal muscle cell line [13] and found $IC_{50} = 7.2 \pm 0.6$ μg/mL for *N. nigricollis* and $IC_{50} = 3.1 \pm 0.4$ μg/mL for *N. mossambica*. This indicates that *N. nubiae* possesses venom comparable with the highly cytotoxic spitting cobras. It was observable that *N. nubiae* venom was markedly more cytotoxic than *E. ocellatus* since the concentration used for *N. nubiae* venom was sixfold less than *E. ocellatus* venom. The high cytotoxicity of *N. nubiae* venom is attributed to the presence of high concentrations of cytotoxic components such as cytotoxins and cytotoxic PLA_2. Cytotoxins account for 58%–73% of the venom proteins of spitting cobras [5, 6]. The results of this study agreed with Chiam-Matyas and Ovadia [14] who postulated that the cytotoxic activity of elapids is higher than vipers. It is also supported by a cell-based study which examined myonecrotic PLA_2 of *Naja nigricollis* and *Vipera russelli* snakes [15]. In agreement with the current finding, *Naja nigricollis PLA*$_2$ was more potent at eliciting the myotoxic effect than *Vipera russelli*. These findings are consistent with the general consideration that spitting cobras are mainly cytotoxic with little, if any, neurotoxicity [8, 16]. It also agrees with clinical features that characterize envenoming by spitting cobras [3, 4].

2.2. The Effect of Antivenoms on N. nubiae Venom Cytotoxicity. L6 cells were incubated with serially diluted *N. nubiae* venom (1.647–8 μg/mL) in a media supplemented with CSL, SAIMR, SVA, or EchiTAb-Plus-ICP antivenoms. The effects of the four antivenoms on the venom concentrations that caused 100% (IC_{100}) and 50% (IC_{50}) cell death are shown in Figure 2. All the antivenoms significantly inhibited the cytotoxic effect of *N. nubiae* venom at a venom concentration that caused 100% and 50% cell death (one-way ANOVA, $P < 0.05$). The four antivenoms were effective in neutralizing the cytotoxic effect of *N. nubiae* venom despite the fact that none of the antivenoms contain *N. nubiae* in its immunization mixture. This cross neutralization indicates the presence of antibodies capable of neutralizing *N. nubiae* cytotoxins in the four tested antivenoms. SAIMR polyvalent antivenom was raised against the venom of 10 species of viperid and elapid snakes including *N. mossambica* venom whereas EchiTAb-Plus-ICP antivenom contains *N. nigricollis* venom in its immunization mixture. The venoms of both *N. mossambica* and *N. nigricollis* are highly cytotoxic [3]. Since all spitting cobras have similar cytotoxic PLA_2s [5], it is not surprising that antibodies derived from *N. nigricollis* and *N. mossambica* cytotoxins are capable of neutralizing *N. nubiae* cytotoxicity. This finding is supported by a recent cell-based assay which found SAIMR antivenom effective against the cytotoxic effect of both *N. nigricollis* and *N. mossambica* venoms [13]. Another cell-based assay by Méndez et al. reported effective neutralization of *N. nubiae* venom cytotoxicity by EchiTAb-Plus-ICP antivenom [6]. Interestingly, they found the antivenom efficiency higher in the case of *N. nubiae* venom than *N. nigricollis*, the venom used in the manufacture of EchiTAb-Plus-ICP which explains the complex factors participating in the neutralization of snake venom toxins. Moreover, recent investigation of the efficacy of EchiTAb-Plus-ICP antivenom against PLA_2 activity of *N. nubiae* venom revealed high antivenomic neutralization potency [5]. The CSL polyvalent antivenom which is produced against group of Australian elapids is also capable of neutralizing the cytotoxicity of *N. nubiae* venom. This indicates the presence of antibodies capable of neutralizing *N. nubiae* cytotoxins in the CSL antivenom. The polyvalent SVA antivenom contains the cytotoxic Indian cobra *Naja naja* venom in its immunization mixture which may contribute to its efficacy.

2.3. The Effect of Antivenoms on E. ocellatus Venom Cytotoxicity. L6 cells were incubated with serially diluted *E. ocellatus* venom (0.668–50 μg/mL) in a media supplemented with CSL, SAIMR-Echis, SVA, or EchiTAb-Plus-ICP antivenoms (5 units/mL). The effects of the four antivenoms on the venom concentrations that caused 100% (IC_{100}) and 50% (IC_{50}) cell death are shown in (Figure 3). All the antivenoms failed to

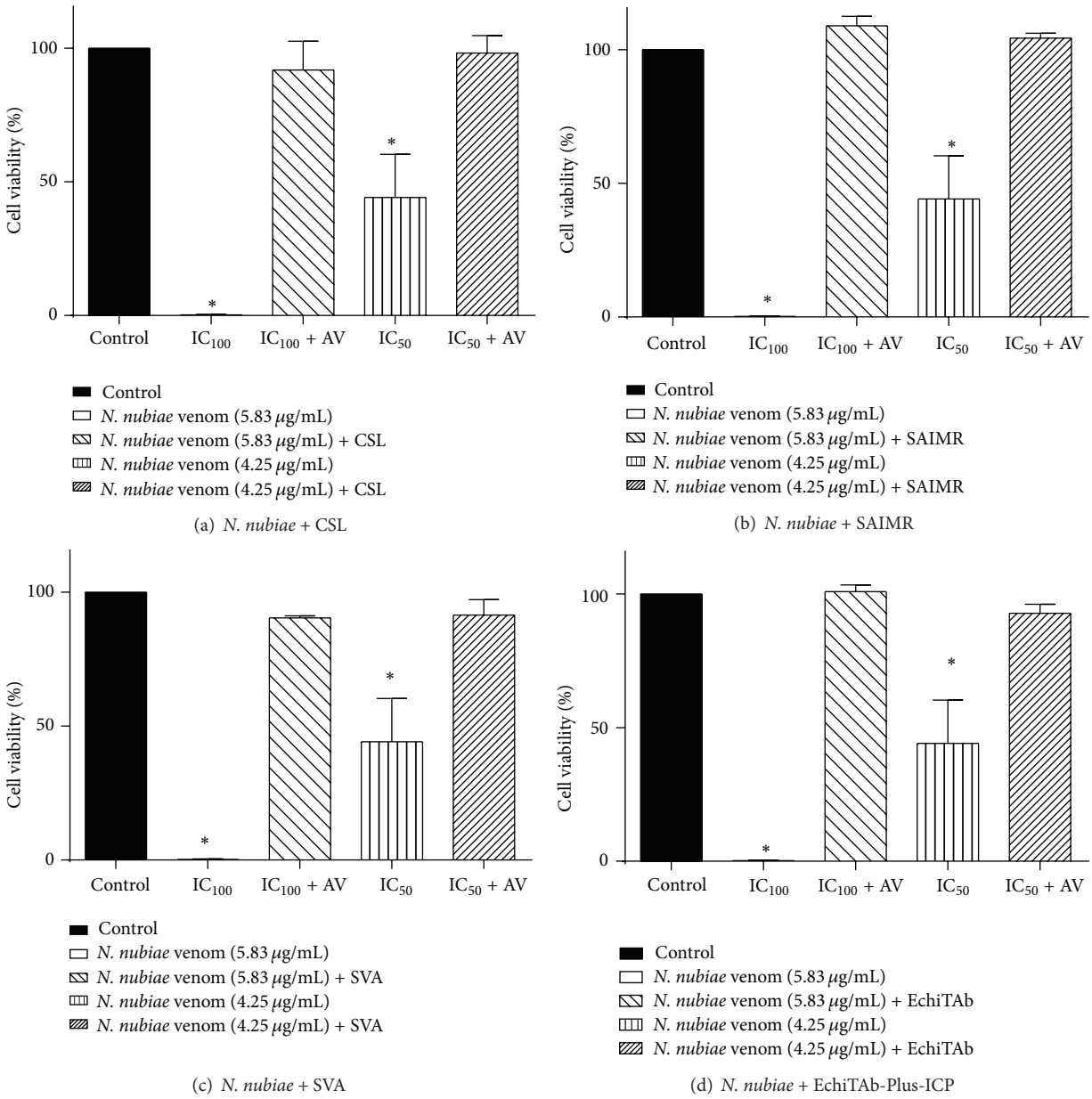


Figure 2: L6 cells incubated with *N. nubiae* venom and (a) CSL antivenom, (b) SAIMR antivenom, (c) SVA antivenom, or (d) EchiTAb-Plus-ICP antivenom using two venom concentrations: the first concentration caused 100% cell death (IC_{100}) and the second caused 50% cell death (IC_{50}) ($n = 4$). Cell viability is expressed as a percentage of the control. Statistical analysis was made by comparing venom with venom + antivenom using a one-way ANOVA, $P < 0.05$ followed by Bonferroni multiple comparison test.

significantly inhibit the cytotoxic effect of *E. ocellatus* at IC_{100}. However, CSL and SVA were able to significantly inhibit the effect of *E. ocellatus* venom at IC_{50} (one-way ANOVA, $P < 0.05$). It is surprising that SAIMR-Echis and EchiTAb-Plus-ICP antivenoms were unable to inhibit the cytotoxic effect of *E. ocellatus* venom at IC_{100} and IC_{50}. The former is monospecific antivenom against *E. ocellatus* while the latter is polyspecific antivenom against *E. ocellatus, B. arietans,* and *N. nigricollis*. This deficit is more likely to be due to lack of neutralizing antibodies against the cytotoxic PLA_2 and cytotoxins in the two antivenoms in spite of the fact that *E. ocellatus* has been used in the immunization mixture for the production of the two antivenoms. Furthermore it is surprising that the effect in the case of EchiTAb-Plus-ICP antivenom led to more cell death than the venom alone. A possible explanation for this might be the large volume of antivenom used coupled with its low efficacy which results in media dilution and more cell death. Our results are constant with previous studies which observed impairment in the neutralization of some snake cytotoxins. A cell-based assay found death adder antivenom (CSL Ltd.) unable to prevent the cell death caused by the death adder (*Acanthophis* spp.) even

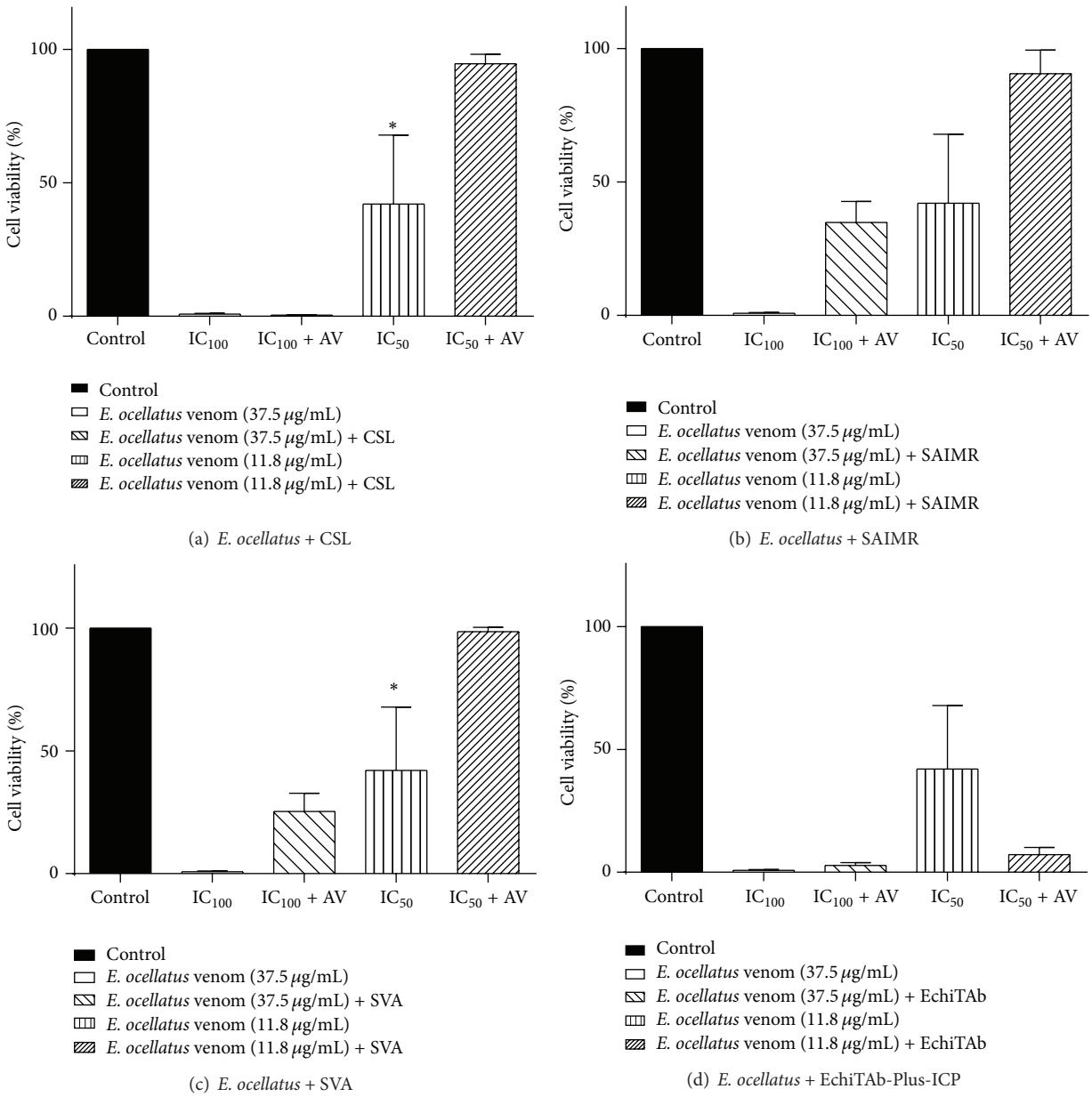


Figure 3: L6 cells incubated with *E. ocellatus* venom and (a) CSL antivenom, (b) SAIMR-Echisantivenom, (c) SVA antivenom, or (d) EchiTAb-Plus-ICP antivenom using two venom concentrations. The first concentration caused 100% cell death (IC_{100}) and the second concentration caused 50% cell death (IC_{50}) ($n = 4$). Cell viability is expressed as a percentage of the control. Statistical analysis was made by comparing venom with venom + antivenom using a one-way ANOVA, $P < 0.05$ followed by Bonferroni multiple comparison test.

at high antivenom concentration [13], although death adder antivenom was able to inhibit the myotoxic and the neurotoxic effects of *Acanthophis* spp. [17]. Moreover Gowda and Middlebrook reported impaired neutralization of *N. nigricollis* PLA_2 by rabbit antiserum produced against the same toxin [15]. However, rabbit antiserum prepared the same way easily neutralized the lethal effects of the snake venom PLA_2 neurotoxins [18]. On the other hand, the findings of the current study do not support previous reports which showed that EchiTAb-Plus-ICP antivenom was effective in eliminating the myotoxic effect of mice injected intramuscularly with *E. ocellatus* venom [19]. This difference in the antivenom potency may be explained by the difference in the assay used for assessment (*in vivo* and *in vitro*) or it may be due to the geographical difference between the two venoms examined (*E. ocellatus* from East and West Africa). CSL, which is polyspecific antivenom produced against group of Australian snakes, was found effective in neutralizing the cytotoxic effect of *E. ocellatus* venom at IC_{50} while not effective at IC_{100}. Since all medically important Australian snakes are elapid, this partial neutralization may be attributed to the presence of neutralizing antibodies against elapid cytotoxins and cytotoxic PLA_2 in CSL polyvalent antivenom. Partial neutralization was also reported with the Indian SVA which is

polyvalent antivenom raised against two elapids (*Naja naja* and *Bungarus caeruleus*) and two vipers (*Echis carinatus* and *Daboia russelii*). Although *E. carinatus* is included in the immunization mixture, it seems that the presence of the elapid cytotoxins has a crucial role in the antivenom potency.

The results of this study indicate a low immunogenicity of *E. ocellatus* cytotoxins and suggest that their neutralization depends on the presence of antibodies against cytotoxic components belonging to other venoms such as elapids. However, previous reports showed that elapids and vipers appeared to have different mode of cytotoxicity. Elapids venom causes irreversible depolarization of the cell membrane which results in cell death within the first hour of incubation, displaying a cellular necrosis effect. While in the case of vipers, the cells become rounded, lose the attachment with the substrate, and finally die, displaying an apoptotic effect [14].

3. Experimental Section

3.1. Reagents. Bovine Serum Albumen (BSA), Dulbecco's Modified Engle's Medium (DMEM) with high glucose, and Dulbecco's Phosphate Buffer Saline (PBS) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The pierce BCA protein assay kit was purchased from Pierce Biotechnology (Illinois, USA). 1% penicillin/streptomycin and trypsin were purchased from Trace Scientific (Melbourne, Australia). The cell titer 96 aqueous one solution cell proliferation assay (MTS assay) was purchased from Promega (Melbourne, Australia). The Fetal Calf Serum (FCS) was purchased from CSL Ltd. (Melbourne, Australia).

3.2. Antivenoms. CSL polyvalent snake antivenom was purchased from CSL Ltd. (Melbourne, Australia). SAIMR (South African Institute of Medical Research) polyvalent and anti-*Echis* antivenoms were purchased from South African venom producers Ltd. (Johannesburg, South Africa). The snake venom antiserum was purchased from VINS bioproducts Ltd. (Andhra Pradesh, India). The EchiTab-Plus-ICP antivenom was a gift to MVG (Monash Venom Group) from Jose Gutierrez (Institute Clodomiro Picado, San Jose, Costa Rica).

3.3. Cells. The rat skeletal muscles myoblast cells line, L6, was purchased from the American Tissue Type Collection (ATTC Virginia, USA).

3.4. Preparation of the Venoms. The freeze-dried venoms were suspended in milliQ water and filtered through 0.22 μm millipore membrane (Millipore; Bedford, MA, USA). The protein concentration was determined using the bicinchoninic acid reagent (BCA) protein assay kit (Pierce; Illinois, USA). BSA was used as standard solution at dilutions (1–0.025 mg/mL) and milliQ water as blank. The venom samples were diluted to 1 : 2 and 1 : 4. Twenty-five microliters (25 μL) of the standard solutions and the venom samples were added to 96-well microtiter plate and the absorbance was measured at 562 nm in a fusion α microplate reader according to manufacturer's instructions (Packard bioscience; Connecticut, USA). Venom samples were divided into aliquots and stored at −20°C till used.

3.5. Cell Culture. L6 cells were cultured in 175 cm^2 flask (Greiner Bio-One; Frickenhausen, Germany) using DMEM culture media. Media were supplemented with 5% FCS and 1% penicillin/streptomycin (5% DMEM). Cells were incubated at 37°C and media was changed every second day in an atmosphere of 5% CO_2 until 50% confluence assessed under light microscope. Cells were lifted using trypsin and pelleted. The cell pellet was resuspended in 30 mL culture media and seeded at 100 μL/well in 96-well cell culture plates. Plates were incubated at 37°C in an atmosphere of 5% CO_2 and the media were changed every second day until cells were 90% confluent (assessed by eye using light microscope). Media were discarded from wells and replaced with DMEM media supplemented with 2% FCS and 1% penicillin/streptomycin 2% DMEM enabled the differentiation of myoblasts into myocytes. Plates were incubated at 37°C at atmosphere of 5% CO_2 and the media changed every second day until the differentiation was observed under light microscope.

3.6. MTS Assay

3.6.1. The Effect of the Venom on Cell Proliferation. Media were removed from wells and the venom stock solutions were diluted in culture media (2% DMEM) to a final concentration of 8 μg/mL for *N. nubiae* venom and subsequently serially diluted 1.1-fold 15 times (1.647–8 μg/mL). For *E. ocellatus* media were diluted to a final concentration of 50 μg/mL and then serially diluted 1.3-fold 15 times (0.668–50 μg/mL). Samples (100 μL/well) were added to the 96-well cell culture plate in quadruplicate. Control samples (cells + media, without venom) and media blanks (no cells) were also run in parallel and the plates were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. The cell culture plates were then removed from the incubator and washed three times with prewarmed PBS. DMEM (50 μL) and MTS solution (10 μL) were added to each well and the plates were incubated for 3 h at 37°C at atmosphere of 5% CO_2. Absorbance was measured at 492 nm utilizing a fusion α plate reader (Packared Bioscience; Connecticut, USA).

3.6.2. The Effect of Antivenoms on Cell Proliferation. The protein concentrations of the antivenoms were as follows: CSL polyvalent snake antivenom 814.2 mg/mL, SAIMR 481.6 mg/mL, SAIMR-Echis 548.5 mg/mL, EchiTAb-Plus-ICP 230 mg/mL, and snake venom antiserum (SVA) 173.9 mg/mL. The cell culture was carried out as described above; antivenoms (5 U/mL) were added to the cell media before serially diluting the venom 1.1-fold for *N. nubiae* and 1.3-fold for *E. ocellatus*. Control samples (cells + media + antivenoms) and media blanks (media + antivenom) were also run in parallel. When the number of units is not indicated in the vial, the antivenom was used in a mass to mass ratio as antivenom with units/mL indicated. The plates were incubated 24 h at 37°C with 5% CO_2. The plates were washed,

MTS was added, and the absorbance was measured as described above.

3.6.3. Data Analysis. Data were analyzed using the Graphpad Prism 5 software (Graphpad software Inc., California, USA, 2007). The sigmoidal growth curve displayed as a percentage of cell viability versus log venom concentration and the IC_{50} (the half maximal inhibitory concentration) was calculated for each venom. The results were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with statistical significance indicated when $P < 0.05$.

4. Conclusions

Using the cell-based assay, higher cytotoxicity was reported to *N. nubiae* venom compared to *E. ocellatus* venom.

The antivenoms CSL (Australia), SAIMR (South Africa), SVA (India), and EchiTAb-Plus-ICP (Cost Rica) showed high potency against *N. nubiae* cytotoxicity suggesting presence of homologous epitopes. *E. ocellatus* venom mostly likely has diverse immunological epitopes and was only partially neutralized with CSL (Australia) and SVA (India).

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Dr. Huda Khalid and Dr. Nicki Konstantakopoulos have done the practical work and wrote the paper. Dr. Nicki Konstantakopoulos and Professor Maowia Mukhtar contributed to the design of the study, obtaining the venoms, and revised the paper.

Acknowledgments

The authors are grateful to Professor Wayne Hodgson for his valuable suggestions and for Mr. Mohammed Missawi for his effort to provide the venoms. This work was partially funded by DAAD (German Academic Exchange Service) and the Ministry of Higher Education, Sudan.

References

[1] S. Spawls and B. Branch, *The Dangerous Snakes of Africa*, Southern Book, London, UK, 1995.

[2] R. D. G. Theakston, D. A. Warrell, and E. Griffiths, "Report of a WHO workshop on the standardization and control of antivenoms," *Toxicon*, vol. 41, no. 5, pp. 541–557, 2003.

[3] World Health Organization, *Guidelines for the Prevention and Clinical Management of Snakebite in Africa*, WHO Regional Office for Africa, Brazzaville, Congo, 2010, http://www.afro.who.int.

[4] D. A. Warrell, B. M. Greenwood, N. M. Davidson, L. D. Ormerod, and C. R. Prentice, "Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (*Naja nigricollis*)," *Quarterly Journal of Medicine*, vol. 45, no. 177, pp. 1–22, 1976.

[5] D. Petras, L. Sanz, Á. Segura et al., "Snake venomics of African spitting cobras: toxin composition and assessment of congeneric cross-reactivity of the Pan-African EchiTAb-Plus-ICP antivenom by antivenomics and neutralization approaches," *Journal of Proteome Research*, vol. 10, no. 3, pp. 1266–1280, 2011.

[6] I. Méndez, J. M. Gutiérrez, Y. Angulo, J. J. Calvete, and B. Lomonte, "Comparative study of the cytolytic activity of snake venoms from African spitting cobras (*Naja* spp., Elapidae) and its neutralization by a polyspecific antivenom," *Toxicon*, vol. 58, no. 6-7, pp. 558–564, 2011.

[7] W. Wüster and D. G. Broadley, "A new species of spitting cobra (Naja) from north-eastern Africa (Serpentes: Elapidae)," *Journal of Zoology*, vol. 259, no. 4, pp. 345–359, 2003.

[8] D. A. Warrell, "Clinical toxicology of snakebite in Africa and the Middle East/Arabian peninsula," in *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, J. Meier and J. White, Eds., pp. 433–492, CRC Press, Boca Raton, Fla, USA, 1995.

[9] D. A. Warrell, "Unscrupulous marketing of snake bite antivenoms in Africa and Papua New Guinea: choosing the right product-'What's in a name?'" *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 102, no. 5, pp. 397–399, 2008.

[10] D. A. Warrell, N. D. Davidson, B. M. Greenwood et al., "Poisoning by bites of the saw-scaled or carpet viper (*Echis carinatus*) in Nigeria," *Quarterly Journal of Medicine*, vol. 46, no. 181, pp. 33–62, 1977.

[11] W. P. Meyer, A. G. Habib, A. A. Onayade et al., "First clinical experiences with a new ovine Fab *Echis ocellatus* snake bite antivenom in Nigeria: randomized comparative trial with Institute Pasteur Serum (Ipser) Africa antivenom," *The American Journal of Tropical Medicine and Hygiene*, vol. 56, no. 3, pp. 291–300, 1997.

[12] N. Konstantakopoulos, G. K. Isbister, J. E. Seymour, and W. C. Hodgson, "A cell-based assay for screening of antidotes to, and antivenom against *Chironex fleckeri* (box jellyfish) venom," *Journal of Pharmacological and Toxicological Methods*, vol. 59, no. 3, pp. 166–170, 2009.

[13] Y. Kalam, G. K. Isbister, P. Mirtschin, W. C. Hodgson, and N. Konstantakopoulos, "Validation of a cell-based assay to differentiate between the cytotoxic effects of elapid snake venoms," *Journal of Pharmacological and Toxicological Methods*, vol. 63, no. 2, pp. 137–142, 2011.

[14] A. Chiam-Matyas and M. Ovadia, "Cytotoxic activity of various snake venoms on melanoma, B16F10 and chondrosarcoma," *Life Sciences*, vol. 40, no. 16, pp. 1601–1607, 1987.

[15] T. V. Gowda and J. L. Middlebrook, "Effects of myonecrotic snake venom phospholipase A2 toxins on cultured muscle cells," *Toxicon*, vol. 31, no. 10, pp. 1267–1278, 1993.

[16] J.-P. Chippaux, *Les serpents d'Afrique occidentale et centrale*, IRD Éditions, Paris, France, 2006.

[17] B. G. Fry, J. C. Wickramaratna, A. Jones, P. F. Alewood, and W. C. Hodgson, "Species and regional variations in the effectiveness of antivenom against the *in vitro* neurotoxicity of death adder (*Acanthophis*) venoms," *Toxicology and Applied Pharmacology*, vol. 175, no. 2, pp. 140–148, 2001.

[18] J. L. Middlebrook, "Cross-neutralizations of phospholipase A_2 neurotoxins from snake venoms," *Toxicon*, vol. 29, no. 12, pp. 1481–1487, 1991.

[19] J. M. Gutiérrez, E. Rojas, L. Quesada et al., "Pan-African polyspecific antivenom produced by caprylic acid purification of horse IgG: an alternative to the antivenom crisis in Africa," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 99, no. 6, pp. 468–475, 2005.