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Inhibition of *Naja nigricollis nigricollis* venom acetylcholinerase activity by kolaviron isolated from *Garcinia kola*

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ABSTRACT

Acetylcholinerase (AChE) enzyme often predominant in cobra venom causes paralysis, respiratory failure and other life-threatening complications, but kolaviron (KV) inhibits brain AChE, showing therapeutic potential. This study attempt to investigate the inhibitory effect of KV against *Naja nigricollis nigricollis* (NNN) venom AChE activity. The identity and purity of KV compound was authenticated using Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The activity of AChE and its inhibition studies were performed via standard protocol. The chromatographic analysis of KV gave six (6) peaks, which corresponds to its basic components. The activity of NNN AChE was inhibited in dose-dependent pattern, when pre-incubated with increasing doses of KV ranging from 0.2 to 1.0 µg/mL. A maximum inhibition of 90 % was observed at 1.0 µg/mL of KV and half maximal inhibitory concentration (IC_{50}) of KV was estimated at 0.59 µg/mL. The data suggest that kolaviron is a potent inhibitor of snake venom acetylcholinerase (AChE), and its inhibitory activity likely occurs through direct contact, a common mechanism characteristic of flavonoid compounds and may be employed in snakebite treatment.

Keywords: Cobra venom, *Naja nigricollis nigricollis*, neurotoxin, acetylcholinerase, acetylcholine, kolaviron, physical interaction

INTRODUCTION

Envenoming by the black-necked spitting cobra (*Naja nigricollis nigricollis*) is one of the most important species responsible for a significant portion of snakebite-related morbidity in Nigeria [1]; [2]; [3]. *Naja nigricollis nigricollis* (NNN) venom can cause a range of systemic complications, including neurotoxicity, which may result in respiratory paralysis, a life-threatening condition, as well as other complications such as cardiovascular instability, renal dysfunction, and local tissue damage [4]; [5]. Acetylcholinerase (AChE; EC 3.1.1.7) is a predominant enzyme in cobra snake venoms including NNN, and it plays a crucial role in the breakdown the neurotransmitter acetylcholine (ACh) into choline and acetate [6]. Acetylcholine is responsible for the transmission of nerve impulse across the synapse [7]; [8]. NNN venom AChE has a high affinity for ACh and rapidly hydrolyzes it, thereby preventing signal transmission at the neuromuscular junction and making it a potent neurotoxin (Adewunmi *et al.*, 2024). This can lead to muscle paralysis and potentially life-threatening respiratory failure, hallmarks of NNN envenomation [9]. The presence of AChE in NNN venom contributes significantly to the venoms neurotoxicity and makes it a key target for developing antidotes for snake bites. Kolaviron (KV), a complex biflavonoid from *Garcinia kola* seed, has been widely investigated [10];[11] for its potential to neutralize or mitigate the toxic effects of snake venom components. For instance, reports have shown that KV can mitigate snake venom-induced blood disorders, and vital organ damage [12]; [4]. Interestingly, the work of [13] clearly demonstrated that KV can inhibit acetylcholinestrase activities in the brain of wistar rats. Based on these reports, it was of interest to investigate the inhibitory effect of kolaviron (KV) against *Naja nigricollis nigricollis* venom acetylcholinerase activity. This is promising area of research, as it could lead to the development of new treatments or antidotes for snake bites

MATERIALS AND METHODS

Materials

Acetylthiocholine (ACh) and 5, 5 – dithiobisnitrobenzioc acid (DTNB) were products of Sigma Aldrich Chemical (St Louis, MO, USA). Sodium dihydrogen phosphate, Disodium hydrogen orthophosphate, Sodium chloride, Sodium hydroxide and Hydrochloric acid were obtained from May and Baker Ltd., England.

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Test sample

Kolaviron was provided by Prof. S.A Onasanwo, Department of Physiology, University of Ibadan, Nigeria and authenticated using Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. KV was further dissolved and considered as the test sample.

Venom sample

The venom was collected by the usual milking method from locally caught spitting cobra (*Naja nigricollis nigricollis*), kept at the herpetarium, Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. The pooled venoms were lyophilized and stored prior to the experiments

In vitro assay for inhibition of snake venom acetylcholinesterase activity by kolaviron

Acetylcholinesterase (AChE) activity in NNN venom was measured by the method of Ellman *et al.* (1961). Exactly, 0.4 mL of venom sample (10 µg/mL) was added to 2.6 mL of phosphate buffer (0.1 M, pH 8.0) containing 100 µl of DTNB (5, 5'-dithiobis-2-nitrobenzoic acid). This was pre-incubated for 2 min at room temperature and reaction commenced immediately upon the addition of 20 µl of substrate acetylcholine (30 mM). The product of thiocholine reaction with DTNB was determined at 412 nm continuously for a period of 3 min at 30 seconds intervals. The activity was expressed as micromole per minute per milligram protein, using the extinction coefficient of 1.36×10^4 $cm^{-1}M^{-1}$. For the inhibition study, venom sample was pre-incubated for 15 min at 37^0C with a different concentration of inhibitor (kolaviron).

Statistical analysis

The experiment was performed in triplicate for each sample and results obtained were presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA), followed by Duncan's multiple range test was carried out using Graph Pad Prism software. Data were considered statistically significant at $p < 0.05$.

RESULTS

The chromatographic profile of kolaviron (KV) was authenticated using Liquid Chromatography-Mass Spectrometry (LC-MS) as shown in Figure 1. In the positive mode direction, KV chromatogram revealed six (6) different peaks. The molecular formulas of each peak were deduced from mass-to-charge ratio (m/z) and values obtained through Electrospray Ionization Mass Spectrometer (ESI-MS) analysis. The results are presented as follows: (1) 573.19 *m/z*, (2) 557.28 *m/z*, (3) 587.38 *m/z*, (4) 557.26 *m/z*, (5) 541.30 *m/z* and (6) 555.30 *m/z* (Table 1). It was based on these results that the following components of kolaviron were identified as listed: *kolaflavones* (555.30 = $C_{30}H_{21}O_{11}$), *Garcinia biflavonoid* 2 (573.19 = $C_{30}H_{22}O_{12}$), *binaringenin* (541.30 = $C_{30}H_{22}O_{10}$), *kolaflavanone* (587.38 = $C_{31}H_{24}O_{12}$), and *Garcinia biflavonoid* 1 A & B (557.28, 557.26 = $C_{30}H_{22}O_{11}$). Figure 2 depicts the effect of kolaviron on NNN acetylcholinesterase (AChE) activity. The concentration of AChE activity in 0.4 mL of NNN venom sample (10 µg/mL) was found to be 1.47 ± 0.06 (µmol/min/mg). AChE activity was further assessed after pre-incubation with increasing concentrations of KV (ranging from 0.2 µg/mL to 1.0 µg/mL) using standard protocol previously described. The inhibitory effect of KV on AChE activity showed a dose-dependent pattern as the concentration increases. A maximum inhibition of 90 % was observed at 1.0 µg/mL of KV and half maximal inhibitory concentration (IC_{50}) of KV was estimated at 0.59 µg/mL (Figure 3).

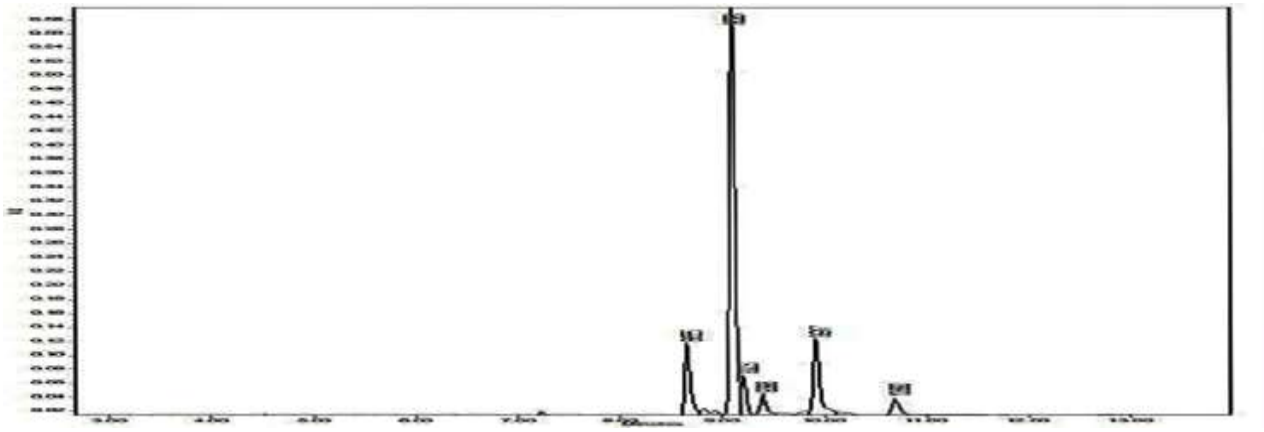

Figure 1. The chromatographic profile of kolaviron compound using LC-MS

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Table 1. Components of kolaviron compound identified via mass spectra

Peak Number	Retention Time (Min)	Base peak (*m/z*)	Molecular formulae	Compound name
1	8.655	573.19	$C_{30}H_{22}O_{12}$	*Garcinia* biflavonoid 2
2	9.098	557.28	$C_{30}H_{22}O_{11}$	- *Garcinia* biflavonoid 1A
3	9.206	587.38	$C_{31}H_{24}O_{12}$	Kolaflavanone
4	9.396	557.26	$C_{30}H_{22}O_{11}$	*Garcinia* biflavonoid 1B
5	9.917	541.30	$C_{30}H_{22}O_{10}$	Binaringenin
6	10.690	555.30	$C_{30}H_{21}O_{11}$	Kolaflavones

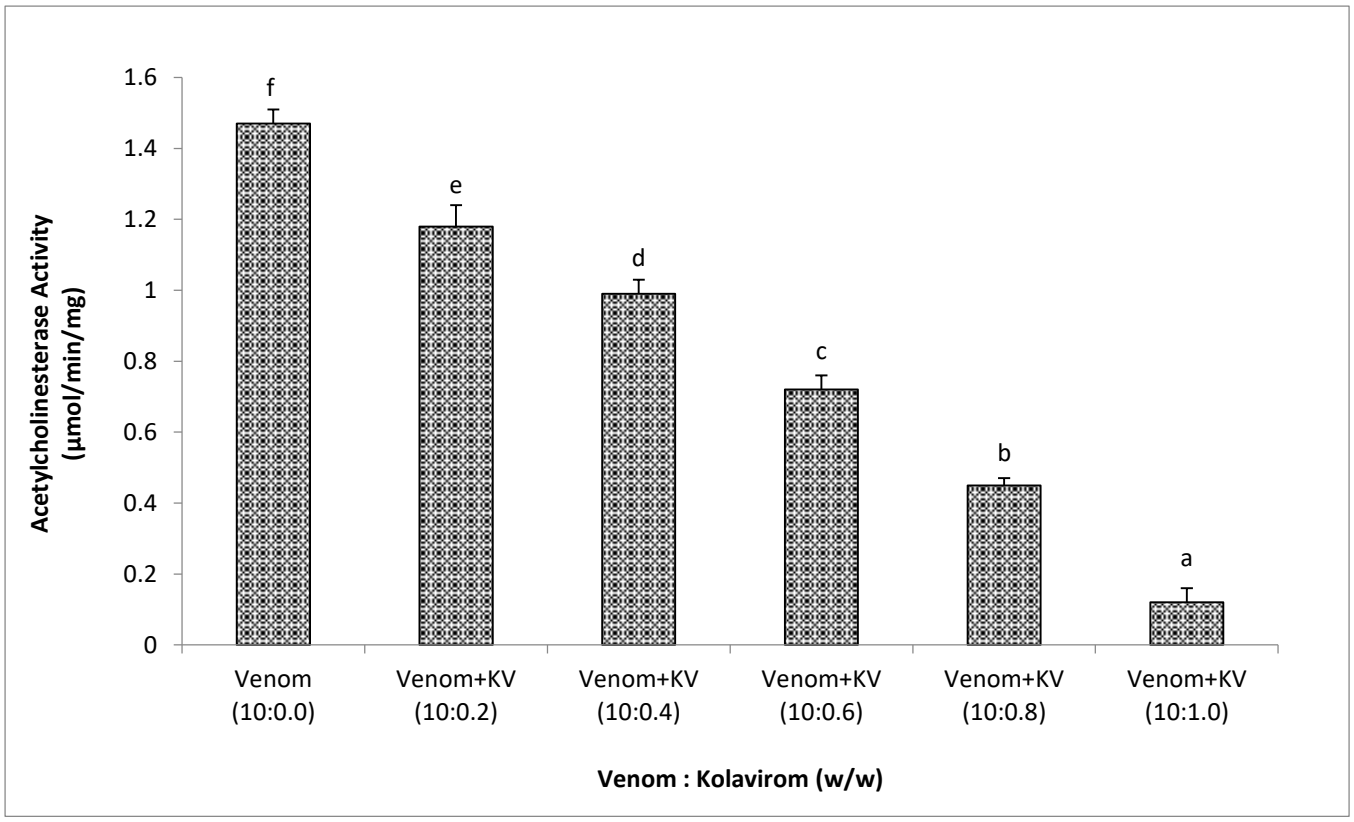

Figure 2. Inhibition of acetylcholinesterase activity in *Naja nigricollis nigricollis* venom by increasing concentration of kolaviron. Data are mean ± SD of three independent experiments and significant at $p<0.05$.

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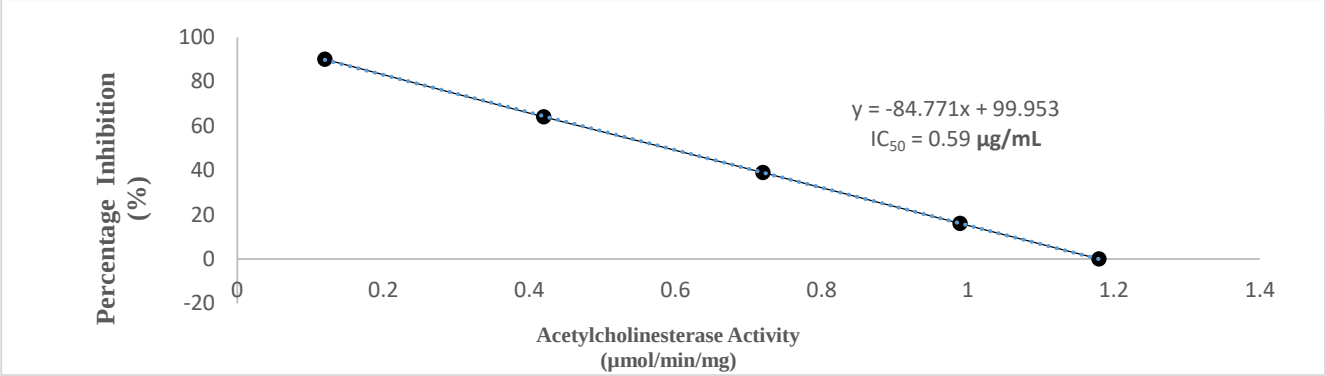

Figure 3. Half maximal inhibitory concentration (IC_{50}) of kolaviron against acetylcholinesterase activity in *Naja nigricollis nigricollis* venom

DISCUSSION

Previous studies have shown that kolaviron have the ability to neutralize or mitigate numerous snake venom toxicities [1]; [4]. This study reports the first evidence of kolaviron's inhibitory effect on *Naja nigricollis nigricollis* venom acetylcholinesterase activity, a finding not previously reported in the literature, whereas its effect on brain acetylcholinesterase activity has been established [13]. It is based on this background that this study was conducted. To verify the identity and purity of the test compound, kolaviron was authenticated using Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The chromatographic analysis revealed six peaks and further examination of their mass-to-charge (m/z) spectra identified components corresponding to *Garcinia biflavonoid* 1 A, *Garcinia biflavonoid* 1 B, *Garcinia biflavonoid* 2, *kolaflavanone*, k*olaflavones* and *binaringenin.* These results are in agreement with the findings of [12], providing further corroboration of their research. Acetylcholinerase (AChE) enzyme is a predominant neurotoxin component of cobra snake venom, which play vital role in the rapid breakdown of acetylcholine, thereby preventing the transmission of nerve impulses across the synapse leading to paralysis and respiratory failure [7]; [9]. The concentration of acetylcholinerase activity detected in *Naja nigricollis nigricollis* venom in the study supports its presence and significant role in the cobra venom's neurotoxicity. Therefore, the inhibition of AChE can be significant in attenuating its neurotoxic effect and other related complications. This study reveals that kolaviron exhibits potent inhibitory aactivity against acetylcholinerase (AChE), demonstrating significant potential as an AChE inhibitor. This is similar to the finding of [13]. The inhibition involves physical interaction in dose-dependent manner with a half-maximal inhibitory concentration (IC_{50}) value of 0.59 µg/mL. The IC_{50} value represents the concentration of a compound needed to achieve 50 % inhibition of enzyme activity. The smaller IC_{50} value observed in this study indicates higher potency, as it requires a lower dose of KV compound to achieve the same level of inhibition, suggesting that the value is inversely proportional to inhibitory activity [13].

CONCLUSION

The data suggest that kolaviron is a potent inhibitor of snake venom acetylcholinerase (AChE), and its inhibitory activity likely occurs through direct contact, a common mechanism characteristic of flavonoid compounds and may be employed in snakebite treatment.

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Conflicts of Interest

The author declares no conflict of interest.

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