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Research Article

Kolaviron Isolated from *Garcinia kola* Seed Inhibits Snake Venom Phosphodiesterase Activity

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ABSTRACT

Most of the snake venom toxic enzymes that induce severe pathological effects belong to the hydrolytic class, including phosphodiesterase (svPDE) and kolaviron (KV) from *Garcinia kola* seed (GKS) happens to be a potent inhibitor against this class of enzymes. This study demonstrated the inhibitory effect of KV against svPDE activity. KV, isolated through successive extraction methods from GKS, was validated via Liquid Chromatography-Mass Spectrometry (LC-MS). KV was assessed enzymatically for its ability to inhibit svPDE. About 1% KV was obtained from 50 g pulverized GKS following LC-MS spectra analysis. KV inhibited svPDE in a dose-dependent pattern with an IC_{50} value of 40 µg/mL. Lineweaver-Burk plot of initial velocity data of inhibition by KV revealed a non-competitive pattern with a K_i value of 22 µg/mL at 40 µg/mL of KV. The K_M remained constant at 2.4 µg/mL, while V_{max} changed from 10.6 µmol/min/mg to 3.7 µmol/min/mg in the presence of 40 µg/mL of KV. This study suggests that KV could act as a potential inhibitor capable of countering the toxic action of svPDE in a non-competitive manner and may be used in treating snakebite victims.

Keywords: *Snake venom, Toxin, Hydrolytic enzyme, Phosphodiesterase, Kolaviron, Inhibition pattern*

INTRODUCTION

Most severe clinical complications induced by snake venom are largely due to the presence of a hydrolytic class of enzymes namely; phospholipase A_2, protease, hyaluronidase, L-amino acid oxidase, and phosphodiesterase etc. (Bickler, 2020; Okafor and Onyike, 2021). Snake venom phosphodiesterases (svPDEs) are universally present in all snake venoms and considered responsible for the hydrolysis of polynucleotides via endonucleolytic cleavage (Ullah *et al.*, 2019; Shah *et al.*, 2020).

The svPDE (EC 3.1.4.1) performs a crucial role in the degradation of extracellular nucleotides through step-by-step removal of 5′ mononucleotides starting from 3′-end of polynucleotides (Uzair *et al.*, 2018). This enzyme regulates nucleotide-based cell-cell signaling processes leading to hypertension, heart attack, stroke, and even death in snakebite victims (Tehara and Keasling, 2003; Mitra and Bhattacharyya, 2014; Ibrahim *et al.*, 2016; Delhaye and Bardoni, 2021).

Most predominant inhibitors are specifically for intracellular PDE, for instance: resveratrol (naturally occurring polyphenol), genistein (isoflavone derivative), caffeine, and theophylline (xanthine derivative), among others (Nichols and Morimoto, 2000; Bender and Beavo, 2006; Salehi *et al.*, 2018; Baillie *et al.*, 2019; Bondarev *et al.*, 2022). According to Mamillapalli *et al.* (1998), extracellular snake venom PDE inhibitors are limited to only polyclonal antivenin antibodies and heparin. Hence, a systematic search for a more efficient, cost-effective, safer, and specific svPDE inhibitor is justified.

Okafor and Onyike (2021) reported that KV (a biflavonoid derivative) isolated from *G. kola* seed could inhibit certain hydrolytic enzymes including phospholipase A_2, protease, hyaluronidase, and L-amino acid oxidase. However, there is no evidence of KV inhibitory effect against svPDE, which also belongs to the hydrolytic class of enzymes. Hence, this study attempts to evaluate the inhibitory potential of KV against svPDE.

MATERIALS AND METHODS

Chemicals and reagents

Bis-p-nitrophenyl phosphate (Bis-pNPP), hydrochloric acid, barbitone sodium and snake venom PDE were products of Sigma Chemicals (St. Louis, MO).

Plant material

Garcinia kola seeds were procured in August 2023 from local farmers in Nkpukpuevula, Abia State, Nigeria. Botanical identification was carried out at the Department of Botany, University of Calabar, Nigeria, and the specimen voucher number was UC-162.

Ethical approval

The study protocol was approved by the Research and Ethics Committee of the Faculty of Basic Medical Sciences, University of Cross River State (UNICROSS), Okuku Campus, Nigeria (UNICROSS/FBMS/IREC/2022-A08), in line with the Helsinki Declaration (1964) guidelines, as revised in 2013.

Test sample

Kolaviron (KV) was extracted according to the method described by Cotterill *et al.* (1978). The powdered seeds (50 g) were extracted with light petroleum ether (b.pt. 40-60°C) in a "Soxhlet" extractor for 24 h. The defatted, dried marc was re-extracted with acetone. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate (6×250 ml). The concentrated ethyl acetate fraction gave a yellow solid sample (known as kolaviron) and was later authenticated by Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. KV was further dissolved and considered as the test sample.

Assay for snake venom phosphodiesterase inhibition studies and kinetic parameters

Phosphodiesterase activity was assayed as described by Mamillapalli *et al*. (1998). One (1) mL of 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM Bis-pNPP (substrate) and snake venom PDE (10 µg/mL) were incubated for 60 minutes at 37°C. The reaction was terminated by the addition of 50 µl of 50 mM NaOH. The absorbance was measured at 405 nm in a UV-VIS spectrophotometer and activity was expressed as µmol/min/mg (Bis-pNPP extinction coefficient = 8.10 x 10^3 $cm^{-1}M^{-1}$). For the inhibition study, svPDE was pre-incubated for 15 min at 370°C with a different concentration of inhibitor (kolaviron) ranging from ratio 1:0 to 1:5 (enzyme: inhibitor). A similar experiment was carried out at different substrate concentrations (0.313 – 5.000 mM) in the absence and presence of an inhibitor (i.e. KV). Initial velocity data obtained were then used to construct Lineweaver-Burk's plot and to determine kinetic parameters (K_M and V_{max}), inhibition pattern, and inhibitory constant (K_i).

Statistical analysis

Data obtained from the experiment were shown 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

Each fifty grams (50 g) of pulverized *G. kola* seed subjected to successive extraction methods gave a yellow solid sample often referred to as kolaviron (0.5 g) with a percentage yield of 1.0%. The isolate was further validated using Liquid Chromatography-Mass Spectrometry (LC-MS). KV chromatogram revealed six (6) different peaks in the positive mode direction (Figure 1). The molecular formulas of these peaks were further deduced from Electrospray Ionization Mass Spectrometer (ESI-MS) and values presented as (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 (see Table 1). Based on their respective base peak (m/z), the components of kolaviron were identified as: 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 = $C_{30}H_{22}O_{11}$).

A fixed concentration of svPDE (10 µg/mL) was pre-incubated with varying concentrations of KV ranging from 10 µg/mL to 50 µg/mL at 5 mM Bis-pNPP (substrate) and assayed using standard protocol previously described. The activity of the enzyme (svPDE) reduces as the concentration of kolaviron increases in a dose-dependent fashion with the half-maximal inhibitory concentration (IC_{50}) of 40 µg/mL (Figure 2). Similarly, the activity of svPDE (10 µg/mL) was further assessed at different substrate concentrations (0.313 – 5.000 mM) in the absence and presence of 40 µg/mL KV (inhibitor). Lineweaver-Burk plot of initial velocity data of inhibition by KV revealed a non-competitive pattern with K_i value of 22 µg/mL at 40 µg/mL of KV. The K_M remained constant at 2.4 µg/mL, while V_{max} changed from 10.6 µmol/min/mg to 3.7 µmol/min/mg in the presence of 40 µg/mL of KV (Figure 3).

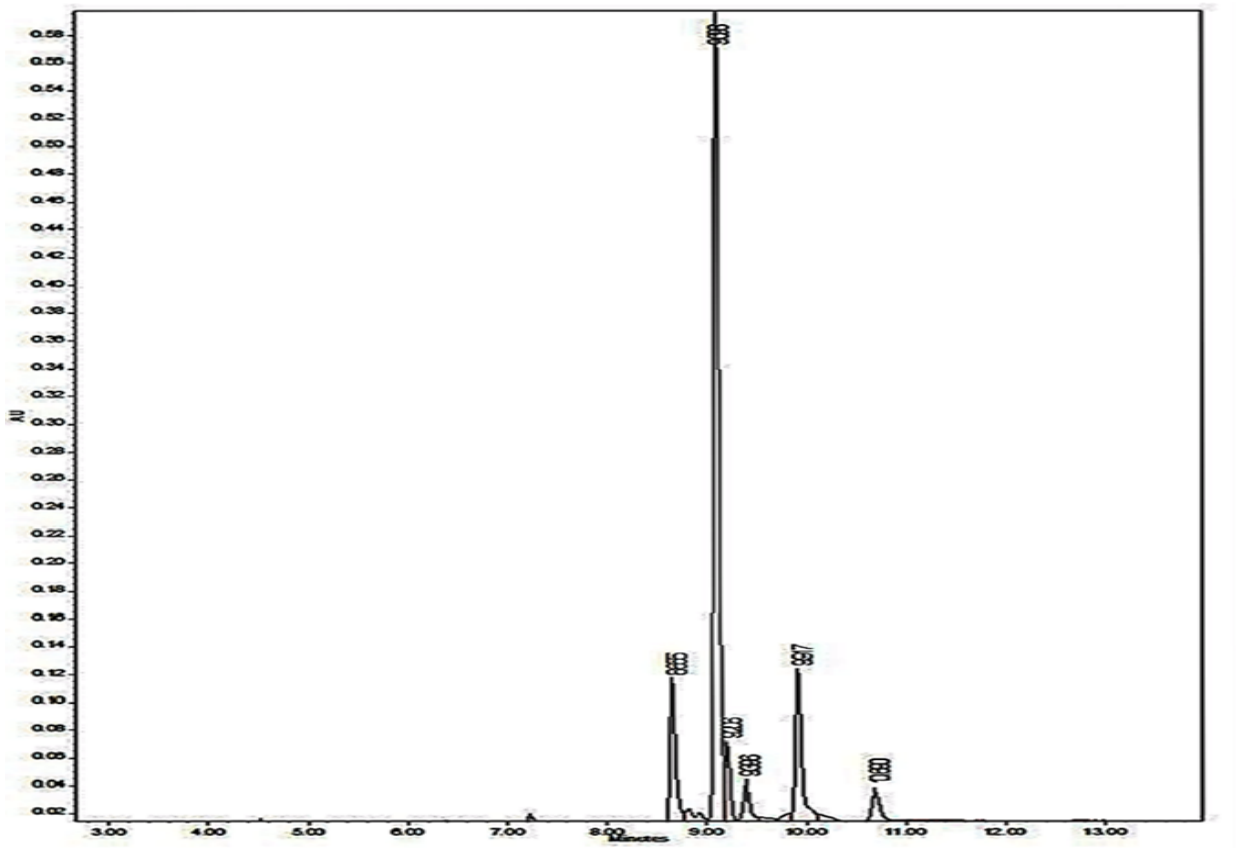

Figure 1. Chromatographic Analysis of Kolaviron Compound using LC-MS

Table 1. Components of Kolaviron Compound Revealed 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

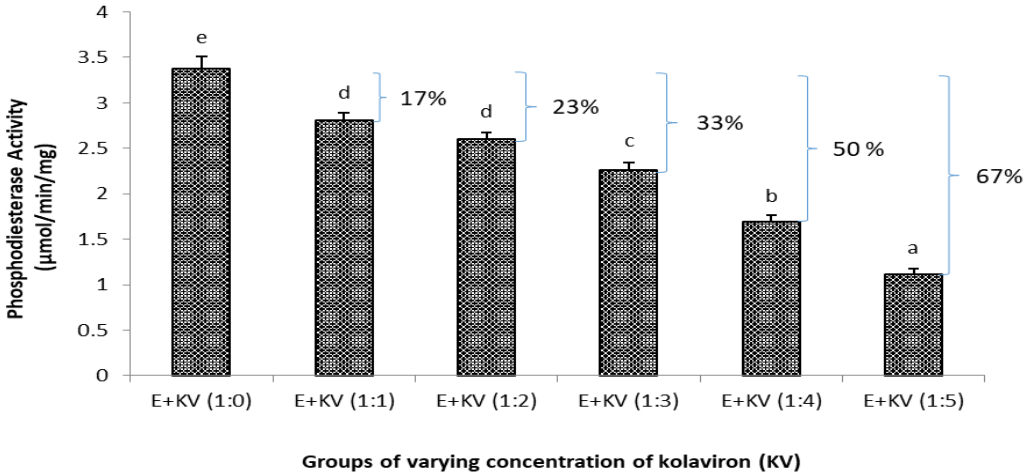

Figure 2. Inhibition of svPDE by Increasing Concentration of KV.

The results are presented as mean ± SD (n=3). E+KV signifies enzyme (svPDE) plus KV meaning Kolaviron

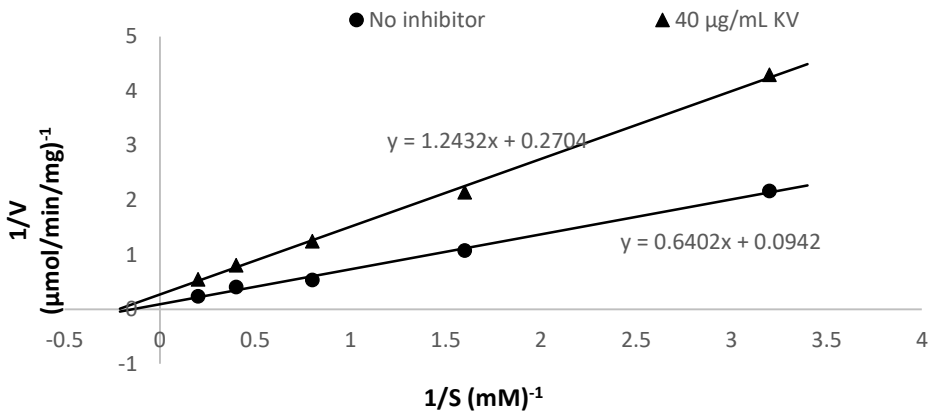

Figure 3. Lineweaver-Burk plot (inverse of initial velocity V vs. inverse of substrate concentration S) of svPDE activity in the absence (circle) and presence (triangle) of 40 µg/mL KV showing non-competitive pattern of inhibition

DISCUSSION

The evidence that KV (a biflavonoid derivative) isolated from *G. kola* seed (Onasanwo *et al.*, 2011) could inhibit certain hydrolytic enzymes (namely; phospholipase A_2, protease, hyaluronidase, and L-amino acid oxidase) has previously been investigated and documented in the literature (Okafor and Onyike, 2021). However, there is no available information about its inhibitory action on another important hydrolytic enzyme such as snake venom phosphodiesterase (svPDE). Hence, this study is justified. Before inhibition studies, the chromatogram of the isolated test sample (KV) was performed using Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, to validate the compound. Kolaviron chromatogram revealed six (6) peaks (Figure 1) and when analysed through an Electrospray Ionization Mass Spectrometer (ESI-MS) (Table 1) correspond to *Garcinia* biflavonoid 1 A, *Garcinia* biflavonoid 1 B, *Garcinia* biflavonoid 2, kolaflavanone, kolaflavones and binaringenin. These corroborate the findings of Okafor *et al.* (2022).

Snake venom phosphodiesterase (svPDE) is one among many hydrolytic enzymes, present in snake venom, responsible for the degradation of extracellular nucleotide (Mamillapalli *et al.*, 1998; Uzair *et al.*, 2018). The svPDE performed a major pathophysiological role such as regulating nucleotide-based cell-cell signalling processes leading to life-threatening conditions and in some cases even death in snakebite victims (Mitra and Bhattacharyya, 2014; Ibrahim *et al.*, 2016). Snake venom PDE can also cause deleterious DNA and RNA degradation (Mamillapalli *et al.*, 1998). The inhibition of svPDE can therefore be significant in countering the toxic effect of svPDE-induced toxicities by snake venom.

In the present study, the enzymatic assay performed in the *in vitro* model demonstrated that KV interaction with svPDE could lead to a significant inhibition (Figure 2). KV appeared to inhibit svPDE activity in a dose-dependent pattern with a half-maximal inhibitory concentration (IC_{50}) value of 40 µg/mL. The IC_{50} value connotes the dose of a compound required to elicit 50% inhibition in an *in vitro* model. The value of IC_{50} is inversely proportional to inhibitory activity, and thus smaller value observed in this study connotes more potency (Abubakar *et al.*, 2020). Interestingly, there are shreds of evidence documented in the literature that depicts herbal-based polyphenol and isoflavone derivatives as potential inhibitors of intracellular PDE (Salehi *et al.*, 2018; Baillie *et al.*, 2019; Bondarev *et al.*, 2022), but no information yet about extracellular PDE, which exists in snake venoms (Mamillapalli *et al.*, 1998).

The inhibitory potential of KV was further demonstrated to inhibit the activity of svPDE in a unique non-competitive pattern (Figure 3). In this context, it might be proposed that the inhibition of svPDE by KV might have involved sites other than the active site and could be one of the mechanisms responsible for mitigating venom-induced toxicities (Mamillapalli *et al.*, 1998; Nok *et al.*, 2002). The observed lower value of K_i (22 µg/mL) in the presence of KV inhibitor (40 µg/mL) further justifies the possibility of the inhibitor having a high affinity for the enzyme, independent of substrate availability. It is often said that the lower the value of K_i, the higher the affinity and the more the enzyme: inhibitor interaction (Nok *et al.*, 2002). Thus, it is likely that svPDE: KV interacted through direct physical interaction mechanism which is usually peculiar and unique to many naturally occurring polyphenol compounds (Abubakar *et al.*, 2020) like kolaviron.

CONCLUSION

This study suggests that kolaviron could act as a potential inhibitor capable of countering the toxic action of snake venom phosphodiesterase in a non-competitive manner and may be used in treating snakebite victims.

LIMITATION AND STRENGTH

This study focuses on the interaction between kolaviron and snake venom phosphodiesterase and it's inhibitory effects

FUNDING STATEMENT

This research work did not receive sponsorship from any funding agencies.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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