



Research Article

Comparative Study of Phospholipase A₂ Activity in *Echis ocellatus* and *Bitis arietans* Venoms and Its Inhibition by Aqueous Bark Extract of *Boswellia dalzielii*

*Maina Isa Muhammad, Yahaya Tijani, Bulama Burah, Ali Mohammed Alkali, Abba Kura Shettima and Hassan Zanna

Department of Biochemistry, Faculty of Life Sciences, University of Maiduguri, Nigeria

*Corresponding author's Email: mainaisahmuhammad@gmail.com, doi.org/10.55639/607.020100107

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ABSTRACT

Phospholipase A₂ (PLA₂) enzymes are important toxic components of many snake venoms and play a significant role in venom-induced tissue damage and inflammation. Understanding the biochemical properties of these enzymes may contribute to the development of improved therapeutic strategies for snakebite envenomation. This study investigated the phospholipase A₂ activities of *Echis ocellatus* and *Bitis arietans* venoms and evaluated the inhibitory potential of aqueous bark extract of *Boswellia dalzielii*. Enzyme activity was determined using a spectrophotometric assay with phosphatidylcholine as substrate. The effects of pH and temperature on PLA₂ activity were also evaluated, and kinetic parameters were determined using Lineweaver–Burk analysis. The optimum pH for PLA₂ activity in both venoms was 7.0, while the optimum temperature was 45 °C for *Echis ocellatus* and 40 °C for *Bitis arietans*. The extract exhibited inhibitory activity against PLA₂ with IC₅₀ values of 1.58 µg/ml and 1.52 µg/ml for *Echis ocellatus* and *Bitis arietans*, respectively. Kinetic analysis suggested mixed inhibition for *Bitis arietans* venom and uncompetitive inhibition for *Echis ocellatus* venom. These findings indicate that *Boswellia dalzielii* extract contains bioactive compounds capable of inhibiting venom PLA₂ activity. However, further studies including phytochemical characterization and in vivo validation are required to confirm its therapeutic potential.

Corresponding author: Maina Isa Muhammad, **Email:** mainaisahmuhammad@gmail.com
Department of Biochemistry, Faculty of Life Sciences, University of Maiduguri, Nigeria

INTRODUCTION

Snake types such as *Echis ocellatus*, *Bitis arietans*, *Naja melanoleuca*, *Naja nigricolis* and *Naja mossambica* are responsible for poisonous snakebites in Nigeria (Juliana *et al.*, 2023).

Envenomation by snakes leads to various pathological conditions due to a complex mix of proteins present in snake venom, which is primarily composed of enzymatic and non-enzymatic proteins, making up approximately

90% of the venom's content (Juliana *et al.*, 2023). These proteins include, but not limited to, serine proteases, metalloproteinases, L-amino acid oxidases, and notably, phospholipase A₂ (PLA₂). PLA₂ enzymes, prevalent in various venomous snake families, are pivotal due to their primary function in the hydrolysis of biological membranes, which is crucial for their role in venom's pathogenic effects (Bashir *et al.*, 2020). Notably, some PLA₂ enzymes exhibit neurotoxic effects by targeting the presynaptic junctions, leading to severe neurological impairments (Resende *et al.*, 2017).

The use of plant products or herbs for medicinal purposes has been part of the practice of man since time immemorial for developing immunity or resistance against diseases. Most of the people in the rural areas rely on herbs for remedies or ailments such as stomatitis, diarrhoea, cancer, snakebite and other diseases (Sofowora, 1993). According to the WHO, around 80% of the population globally uses traditional medicine TM with Tanzania having a 60% usage estimates (WHO, 2022). Most governments in developing countries requested WHO's support in creating a body of reliable evidence and data on traditional medicine TM practises and products. In 2013, the WHO developed a strategy in response to the World Health Assembly resolution on the development and use of TM (Mujinja., 2022)

Snakebite envenomation remains a significant public health problem in many tropical and subtropical regions, particularly in sub-Saharan Africa where access to effective antivenom therapy is limited. Among the various toxic components of snake venom, phospholipase A₂ (PLA₂) enzymes play an important role in causing inflammation, haemolysis, neurotoxicity and tissue necrosis. Although conventional antivenoms are effective in neutralising systemic toxicity, they are often less effective against local tissue damage induced by venom enzymes. Consequently, there is growing interest in identifying natural inhibitors of venom enzymes from medicinal plants. *Boswellia dalzielii* is widely used in traditional medicine for the treatment of inflammatory disorders and other ailments; however, limited information exists regarding its inhibitory effects on snake venom PLA₂ enzymes. Therefore, this study aimed to

evaluate the phospholipase A₂ activity of *Echis ocellatus* and *Bitis arietans* venoms and investigate the inhibitory potential of aqueous bark extract of *Boswellia dalzielii*.

MATERIALS AND METHODS

Snake venoms were obtained in lyophilised form from the Department of Biochemistry, University of Maiduguri. The venoms were stored at -20 °C until use. Prior to analysis, venom samples were reconstituted in buffer solution and the protein concentration was determined using standard protein estimation methods. Aliquots were prepared to prevent repeated freeze thaw cycles and *Boswellia dalzielii* (stem bark) was collected at Auno village of Maiduguri, Borno State, Nigeria, identified by a botanist department of botany, faculty of Life Sciences University of Maiduguri. The substrate lecithin was extracted from chicken egg yolk. All chemicals used in this study were of analytical grades.

Extract Preparation

The aqueous extract of *Boswellia dalzielii* stem bark was prepared according to the method described by (Soforowa 2008). Six hundred grams of the fine powder of the sample was soaked in six (6) litres of distilled water for 24 hours. The sample was filtered using muslin cloth. The residue was discarded and the filtrate was subjected to evaporation using hot plate at the temperature ranging from 40-45°C. The weight of the extract obtained was 37.4 grams and the percentage yield was 6.23%. The dried extract obtained was used for the study (Soforowa, 2008).

Assay of Phospholipase A₂

Samples (venoms) were freshly diluted in cold saline containing 2 mM HEPES at pH 7.5. 20 µL of each sample mixture, or HCl at different concentrations, or buffer as the negative control in 96 well microplate. Exactly 180 µL of an assay mixture containing 5 mM TritonX-100, 5 mM phosphatidylcholine, 2 mM HEPES, 10 mM calcium chloride, and 0.04% bromothymol blue dye in water at pH 7.5 at 37 °C were added. The phosphatidylcholine was dissolved at 160 mg/mL in methanol and stored at -70 °C, and resuspended at 45°C immediately before use. The plate was immediately analyzed at 620 nm with a microplate reader. Rates of change in absorbance for triplet replicates was blank

adjusted and multiplied by 1000 and dilution factor for ease of reporting as PLA₂ activity (Reynolds *et al.*, 1992). All reagents were freshly prepared and calibrated prior to analysis. The enzyme activity was determined spectrophotometrically at 620 nm and the rate of change in absorbance was used to estimate PLA₂ activity.

Effect of pH on PLA₂ Activity

The effect of pH on PLA₂ activity was studied using a standard PLA₂ assay with modification (Price, 2007), by varying the pH of the medium. Phosphatidylcholine, CaCl₂, BSA, and BTB were taken at concentrations of 25 μM, 1 mM, 1 mg/mL, and 0.05 μM, respectively. The mixture volume was filled up to 995 μL with HBS with eight different pH levels ranges between (5 to 8.5). The change in PLA₂s activity were compared.

Effect of Temperature on PLA₂ Activity

The effect of temperature on PLA₂ activity was studied using a standard PLA₂ assay with modification (Price, 2007), varying the Temperature while keeping the rest of the assay component unchanged. Briefly, in a glass cuvette, Phosphatidylcholine, CaCl₂, BSA, and BTB were taken at concentrations of 25 μM, 1 mM, 1 mg/mL, and 0.05 μM, respectively at pH 7.5. The mixture volume was filled up to 995 μL with HBS with eight different Temperature levels ranging between (25°C to 60°C).

Assay Method for PLA₂ Kinetic Study Using L-α-Phosphatidylcholine.

The assay protocol was developed by optimizing the assay parameters following guide lines from the published literature (Price, 2007). All components were mixed in the following order in HBS 1Xbuffer: 10 μL of 100 mg/mL BSA, 10 μL of 100 mM CaCl₂, and 10 μL of 5mM BTB, making a final concentration of 1 mg/mL BSA, 1 mM Ca₂⁺, and 0.05 mM BTB, respectively. The L-α-lecithin was added separately in 8 different concentrations (0.78–100 μM) into 8 sets of experiments. The reaction mixture was mixed for 20s, and readings were taken every minute for 10 min. Absorbance was recorded at 620 nm with a SpectraMax M2. The kinetic parameters Km and Vmax were estimated using Lineweaver–Burk plots derived from reciprocal plots of substrate concentration versus reaction

velocity. Linear regression analysis was used to obtain correlation coefficients (R²) and estimate kinetic constants.

PLA₂ Inhibition Study with the Developed Assay Using a *Boswellia dalzielli*

Ten (10) mM *Boswellia dalzielli* stock solution was prepared by dissolving 1.0 mg of *Boswellia dalzielli* dry powder in 1ml of distilled water. The stock solution was diluted 1000-fold using HBS to a working inhibitor concentration of 10 μM. Exactly 10 μL of 10 mM *Boswellia dalzielli* stock in distilled water was diluted with 990 μL of HBS to yield a 100 μM stock. Inhibition kinetics of *Boswellia dalzielli* bark extract were determined using 100 nM of extract with L-α-lecithin at varying concentrations (0.78 μM, 1.56 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM). The inhibitory concentration (IC₅₀) was determined by plotting percentage inhibition against extract concentration and calculating the concentration required to reduce enzyme activity by 50%. A typical Michaelis–Menten enzyme kinetics graph was produced when the initial velocity is plotted as a function of substrate concentration. The curve was linearized using the Lineweaver–Burke double reciprocal plots (Price, 2007).

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using appropriate statistical tests and differences were considered significant at p < 0.05.

RESULTS

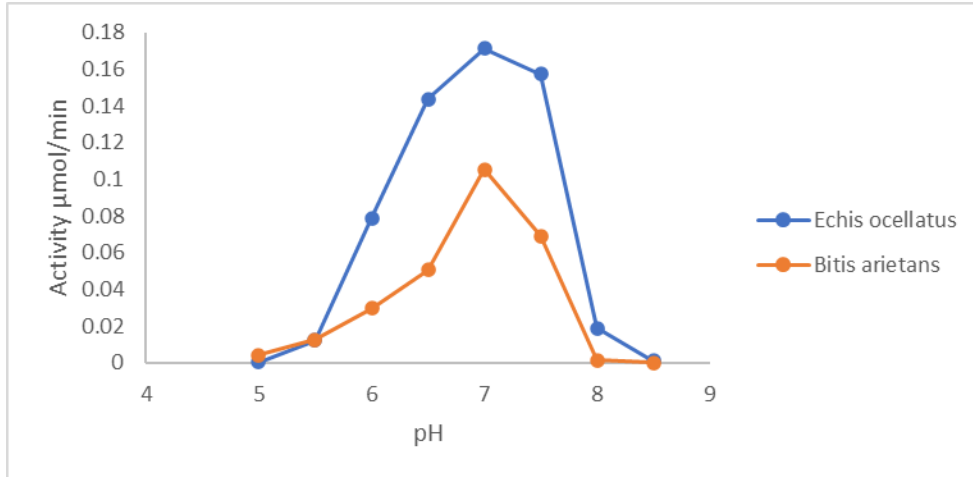


Figure 1: Effect of pH on PLA₂ activity of *Echis ocellatus* and *Bitis arietans* venoms on L- α -Phosphatidylcholine.

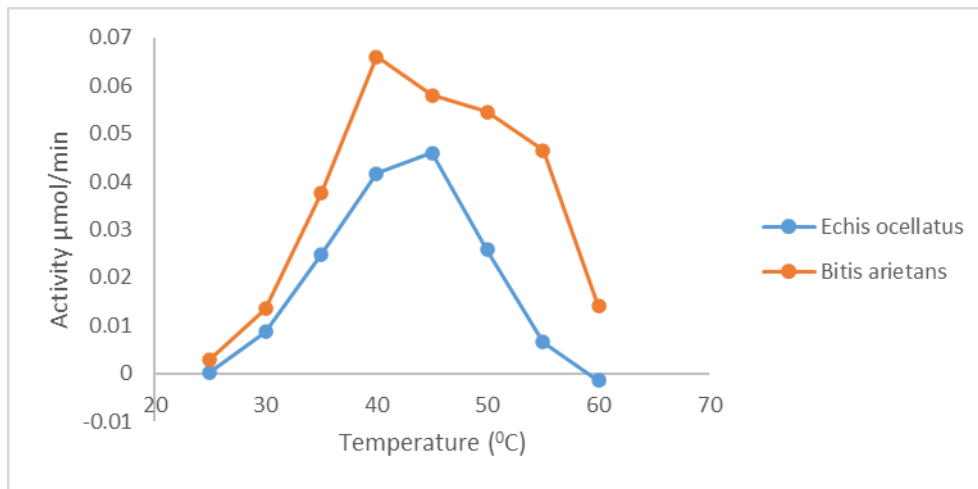


Figure 2: Effect of temperature on PLA₂ activity of *Echis ocellatus* and *Bitis arietans* on L- α -Phosphatidylcholine.

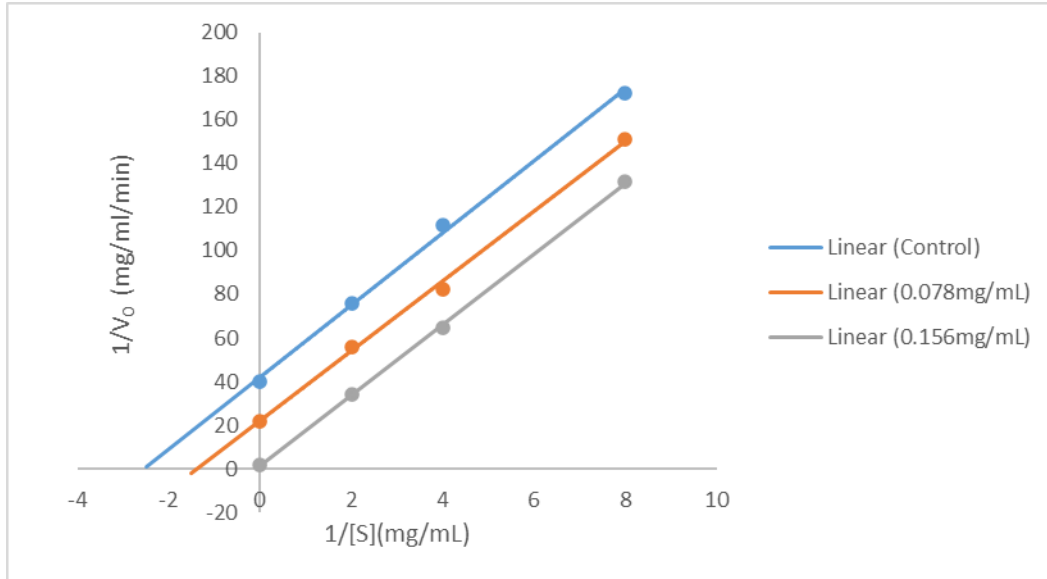


Figure 3a: Lineweaver-Burk Plot showing uncompetitive inhibition mechanisms of *Boswellia dalzielii* extract on L- α - Phosphatidylcholine against Phospholipase A₂ of *Echis ocellatus*.

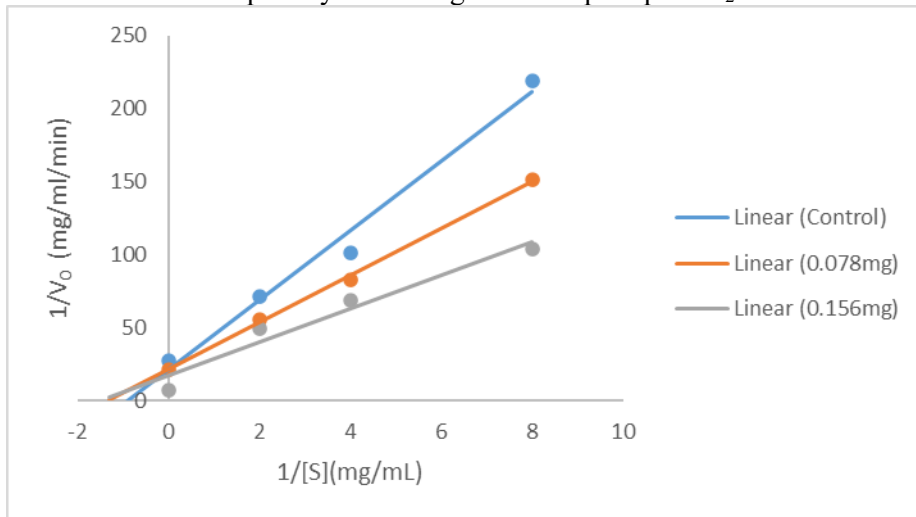


Figure 3b: Lineweaver-Burk Plot showing mixed inhibition mechanisms of *Boswellia dalzielii* extract on L- α - Phosphatidylcholine against Phospholipase A₂ of *Bitis arietans*

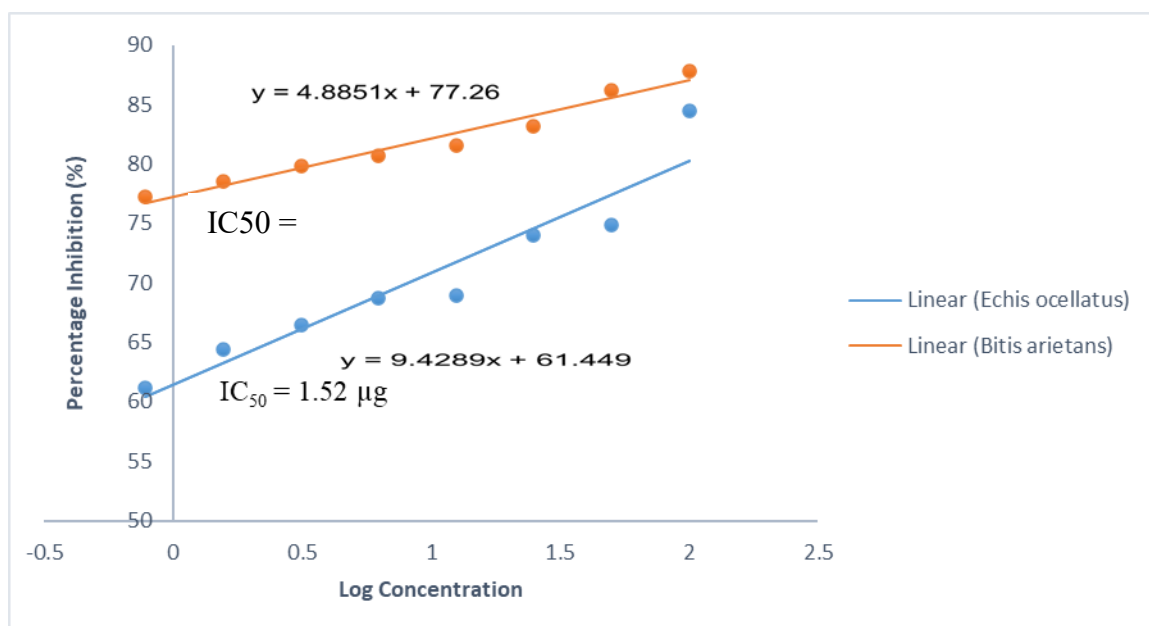


Figure 4: IC₅₀ of *Boswellia dalzielii* extract against the phospholipase A₂ of *Echis ocellatus* and *Bitis arietans* venoms.

DISCUSSION

Figure 1 revealed that *Echis ocellatus* venom had broad range of pH (from 6.5 to 7.5) with optimum pH at 7.0, increasing significantly when the pH raised and decreasing when the pH is at 7.5, 8.0 and 8.5 respectively while *Bitis arietans* venom revealed that the peak of PLA₂ activity is at 7.0, lowest enzymes activities were observed in alkaline pH of 8.5. These findings are in line with an earlier research by Resende *et al.*, (2017), which pinpointed the PLA₂ enzyme's optimal operational pH and temperature at 8.0 and 50°C, respectively. Furthermore, Bashir *et al.*, (2020) highlighted an optimal pH of 7.5 and temperature of 40°C for PLA₂. pH is an important factor for PLA₂ activity. PLA₂ works optimally around the physiologic pH of 7.4. However, in different disease conditions, the local pH may vary, affecting PLA₂ activity (Khan *et al.*, 2023).

The effect of temperature on the crude *Echis ocellatus* PLA₂ activity showed a range of activity with an optimum value of 0.046 µmol/min (Figure 2) with the rise of temperature above 45°C the enzymes activity drastically decline indicating a drop in enzyme function. While crude *Bitis arietans* PLA₂ activities showed a broad range of activity with an

optimum value of 0.066 µmol/min, the enzyme continued to show some activity even at temperature above 40°C but in a declined manner representing a drop of more than 40%. The optimal operational temperature for the crude PLA₂ enzyme was established at 50°C. This temperature is marginally elevated compared to the PLA₂ extracted from the same *Echis ocellatus* venom, as reported by Sallau *et al.*, (2008). However, it closely aligns with findings from Egbe *et al.*, (2023) regarding PLA₂ from *Naja nigricollis* species, which exhibited an optimal temperature of 55°C, a value corroborated by the work of Alonazi *et al.*, (2023).

A comparison of the inhibition mechanism of PLA₂ enzyme of *Echis ocellatus* and *Bitis arietans* venoms using aqueous extract of *Boswellia dalzielii* is shown in Figure 3(a) and (b) respectively. Interestingly, *Boswellia dalzielii* had different mechanisms of inhibition, revealing the importance of the interfacial interactions in PLA₂ inhibition. In Figure 3(a and b), *Boswellia dalzielii* was used at a concentration of 100 nM to measure PLA₂ activity at different substrate concentrations. The Lineweaver–Burke plot was constructed in all cases and compared with similar plots obtained

without an inhibitor. The intersection of the two data sets indicated that *Boswellia dalzielii* acted in a mixed inhibition fashion in the case of *Bitis arietans* venom, figure 3(b). While in the case of *Echis ocellatus* venom, *Boswellia dalzielii* acted as an uncompetitive inhibitor against PLA₂ (Figure 3(a)). Khan *et al.*, (2024) also reported that the inhibition of PLA₂ using the newly developed assay to further evaluate its usefulness and sensitivity. The inhibitory activity observed in this study suggests that *Boswellia dalzielii* may contain bioactive compounds capable of interacting with venom PLA₂ enzymes. However, further studies including phytochemical characterisation and in vivo evaluation are required to confirm its therapeutic relevance.

The IC₅₀ data revealed an IC₅₀ values for *Echis ocellatus* and *Bitis arietans* venoms at 1.52 µg/ml and 1.58µg/ml respectively. Importantly, about 80% of the PLA₂ activities of *Bitis arietans* venom was inhibit by the aqueous extract of *Boswellia dalzielii*. However, the inhibition of PLA₂ activity of *Echis ocellatus* venom was not higher than 85% of the total enzyme activity. Mention must be made that the IC₅₀ value also varies within the traditional thio-PC assays because of the differences in the free fatty acid leaving the site after hydrolysis of the thio ester by the enzyme (Khan *et al.*, 2024)

A PLA₂ assay kinetic study was conducted between *Bitis arietans* and *Echis ocellatus* venoms using L- α -Phosphatidylcholine. The data after subjecting to Michaelis–Menten equation provided a V_{max} of 0.0129 nmols/min/mg and 0.01627 nmols/min/mg for PLA₂ in *Bitis arietans* and *Echis ocellatus* venoms, respectively. The K_M values for the substrate were 0.0632 µM and 0.1534 µM, respectively. The observed low K_M value is a clear indication of the high kinetic efficiency of the enzyme. Previous studies by Torres *et al.* (2012) and Chernyshenko *et al.* (2010) reported K_M values of 14.59 mg/ml and 8.5 µM from *Bothrops moojeni* and *Echis multisquamatis* venoms respectively. Laila *et al.*, (2019) reported a low K_M value, though a disadvantage in terms of envenomation but could be considered a great advantage in terms of clinical usefulness of these enzymes in the treatment of human diseases and as diagnostic reagents.

CONCLUSION

This study demonstrated that the aqueous bark extract of *Boswellia dalzielii* exhibits inhibitory activity against phospholipase A₂ enzymes from *Echis ocellatus* and *Bitis arietans* venoms. The kinetic analysis suggests possible enzyme–inhibitor interactions that reduce PLA₂ activity. Nevertheless, the study is limited to in vitro analysis and further investigations involving phytochemical identification, nonlinear kinetic modelling and in vivo validation are required to fully establish the potential of this plant extract as a complementary approach in snakebite management.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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