Assessment of L-Amino Acid Oxidase Activity in Snake Venoms by Prussian Blue Agar Assay: Purification and Characterization of the King Cobra Enzyme

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Abstract

L-amino acid oxidase (LAAO) is a flavo-dependent enzyme found in many snake venoms, noted for its cytotoxic and antimicrobial activities through the production of hydrogen peroxide (H_2O_2). This study aimed to develop and evaluate a Prussian blue agar (PBA) assay for LAAO activity in snake venoms, and to purify the enzyme from *Ophiophagus hannah*, confirming its identity and biochemical characteristics. Venoms from five snake species were screened, and *Ophiophagus hannah* (King Cobra) venom displayed the highest LAAO activity, resulting in the most prominent blue halo on PBA. This blue coloration resulted from the reaction between H_2O_2 and ferricyanide, leading to the formation of Prussian blue. The halo diameter was quantitatively correlated with known H_2O_2 concentrations. Therefore, it can be used for quantitative assessment. The inclusion of reduced glutathione (GSH), an antioxidant, significantly decreased halo size, illustrating its inhibitory effect on LAAO activity. LAAO was further purified from *O. hannah* venom through gel filtration, ion-exchange, and affinity chromatography. The identity and purity of the enzyme were verified via SDS-PAGE and protein sequencing. Reverse-phase high-performance liquid chromatography (RP-HPLC) revealed a distinct LAAO peak at approximately 80 minutes, coinciding with the corresponding peak in crude venom. These findings confirmed the presence and retention behavior of LAAO. The PBA method proved to be a rapid, visual, and cost-effective tool for assessing LAAO activity and potential modulators. The PBA method proved to be a rapid, visual, and cost-effective tool for assessing LAAO activity and potential modulators. It holds significant value for applications in toxicology research, enzyme characterization, and the investigation of agents that inhibit its activity.

Keywords: L-amino acid oxidase, King cobra venom, Hydrogen peroxide, Prussian blue, Glutathione

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Received date: June 19, 2025 **Revised date:** August 29, 2025 **Accepted date:** April 3, 2026

การประเมินกิจกรรมของเอนไซม์แอล-อะมิโนแอซิดออกซิเดสในพิษงู ด้วยวิธีพรัสเซียนบลูอะการ์: การทำให้บริสุทธิ์และการวิเคราะห์เอนไซม์จากพิษงูจงอาง

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บทคัดย่อ

แอล-อะมิโนแอซิดออกซิเดส (L-amino acid oxidase; LAAO) เป็นเอนไซม์ในกลุ่มฟลาวินโปรตีนสามารถพบได้ในพิษงูหลายชนิด มีคุณสมบัติเป็นพิษต่อเซลล์และสามารถต้านจุลชีพได้ จากการสร้างไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาและประเมินวิธีพรัสเซียนบลูอะการ์ (Prussian blue agar; PBA) สำหรับการเกิดกิจกรรมของ LAAO ในพิษงูต่าง ๆ และทำให้เอนไซม์ดังกล่าวจากพิษงูจงอาง (*Ophiophagus hannah*) มีความบริสุทธิ์ ตลอดจนยืนยันเอกลักษณ์และลักษณะทางชีวเคมีของเอนไซม์ ผลจากการทดสอบพิษงู 5 ชนิด พบว่าพิษงูจงอางแสดงกิจกรรมของ LAAO สูงที่สุด โดยเกิดวงสีน้ำเงินเด่นชัดบนเพลต PBA ซึ่งเกิดจากปฏิกิริยาระหว่าง H_2O_2 และเฟอริไซยาไนด์จนเกิดผลึกพรัสเซียนบลู ขนาดของวงสีน้ำเงินมีความสัมพันธ์เชิงปริมาณกับความเข้มข้นมาตรฐานของ H_2O_2 จึงสามารถใช้ประเมินเชิงปริมาณได้ การเติมกลูตาไธโอนชนิดรีดิวซ์ (GSH) ส่งผลให้ขนาดวงสีน้ำเงินลดลงอย่างชัดเจน แสดงถึงฤทธิ์ต้านของ GSH ต่อกิจกรรมของ LAAO เอนไซม์ LAAO จากพิษงูจงอางผ่านการทำให้บริสุทธิ์ด้วยวิธีโครมาโทกราฟี และยืนยันโดย SDS-PAGE พร้อมหาลำดับกรดอะมิโนและการวิเคราะห์ด้วยโครมาโทกราฟีของเหลวสมรรถนะสูงแบบ reverse phase (RP-HPLC) เพื่อแสดงตำแหน่งของ LAAO ที่เวลาประมาณ 80 นาที (retention time) วิธีการ PBA แสดงให้เห็นว่าเป็นวิธีที่รวดเร็ว ต้นทุนต่ำ เหมาะสำหรับการประเมินกิจกรรมของ LAAO มีความสำคัญสำหรับการวิจัยทางพิษวิทยา การวิเคราะห์คุณสมบัติเอนไซม์ และการศึกษาสารต่าง ๆ ในการยับยั้งกิจกรรมของเอนไซม์นี้

คำสำคัญ: แอล-อะมิโนแอซิดออกซิเดส พิษงูจงอาง ไฮโดรเจนเปอร์ออกไซด์ พรัสเซียนบลู กลูตาไธโอน

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1. Introduction

LAAO is an FAD-dependent flavoenzyme commonly present in the venoms of snakes from the Elapidae and Viperidae families, where it contributes significantly to their toxic effects through the production of H_2O_2, a reactive oxygen species.[1] The enzyme catalyzes the oxidative deamination of L-amino acids, producing α-keto acids and ammonia along with H_2O_2, which is responsible for many of LAAO's cytotoxic, apoptotic, and antimicrobial actions.[2,3] In *Ophiophagus hannah*, LAAO is among the prominent enzymatic constituents of the venom, although its abundance was generally lower than that found in viper venoms.[4] Despite this, King Cobra LAAO displayed potent bioactivity, including antibacterial effects and the ability to induce apoptosis in mammalian cells, thus contributing to prey immobilization and tissue damage at the bite site.[5] Proteomic studies revealed that LAAO isoforms in King Cobra venom vary by geographical location, suggesting adaptive functional diversification.[6] The enzyme's structure typically consisted of two identical subunits and a tightly bound flavin adenine dinucleotide (FAD) cofactor, which is essential for catalysis.[7] LAAO was glycosylated, which is thought to enhance its stability and bioactivity.[8]

The H_2O_2 played a central role in oxidative stress-mediated damage, affecting both host immune cells and invading pathogens.[3,9] The biological potential of LAAO had sparked growing interest in its use as an antimicrobial or anticancer agent. However, practical applications remain limited by concerns over enzyme stability, immunogenicity, and off-target toxicity.[10-11] Nevertheless, it served as a functional biomolecule for studying redox biology and the evolution of venom. To measure LAAO activity, standard assays typically involve spectrophotometric detection of H_2O_2 or downstream chromogenic reactions.[12] The spectrophotometric technique often requires expensive equipment and complex sample handling. A more accessible alternative was the use of Prussian blue agar (PBA), which detected H_2O_2 visually through a colorimetric reaction between ferric ions and ferrocyanide, forming a blue-colored complex known as Prussian blue.[13] The mechanism of Prussian blue formation for H_2O_2 detection is

1) LAAO catalyzes the oxidative deamination of L-amino acids, producing hydrogen peroxide (H_2O_2):

$$\text{L-amino acid} + O_2 \longrightarrow \text{alpha-keto acid} + NH_3 + H_2O_2.$$ [1-2]

2) H_2O_2 acts as an oxidizing agent, converting ferrocyanide $[Fe(CN)_6]^{4-}$ into ferricyanide $[Fe(CN)_6]^{3-}$:

$$H_2O_2 + 2[Fe(CN)_6]^{4-} \longrightarrow 2[Fe(CN)_6]^{3-} + 2OH^-.$$ [9,13]

3) Ferricyanide then reacts with ferrous ions (Fe^{2+}) to form insoluble Prussian blue (ferric ferrocyanide):

$$4Fe^{3+} + 3[Fe(CN)_6]^{4-} \longrightarrow Fe_4[Fe(CN)_6]_3 \text{ (Prussian blue).}$$ [14-15]

The intensity of the blue color correlates with the concentration of H_2O_2. Therefore, measuring the production of H_2O_2 can indicate the presence and activity of LAAO.

Traditional methods for measuring LAAO activity rely on spectrophotometric or peroxidase-coupled systems to detect H_2O_2, which can be costly and require specialized equipment. The PBA method has been recognized as a reliable and straightforward colorimetric technique for detecting H_2O_2 production, which serves as a key indicator of LAAO catalysis. Its core principle involves the reaction between ferric ions and ferrocyanide/ferricyanide complexes, resulting in the formation of an insoluble Prussian blue precipitate within the agar medium. This precipitate manifests as visible halos surrounding wells or plugs containing enzymes or venom samples. Originally utilized in microbiology for the identification of oxidase-producing bacteria, the PBA method can be extended to venomous snake research. This method is cost-effective and

circumvents the need for spectrophotometric analysis. It enables straightforward observation and measurement of halos, providing a rapid screening tool for inhibitors such as antioxidants. Notably, PBA enables the direct visual quantification of LAAO activity zones on agar plates, thereby supporting the analysis of crude venoms, the identification of active venom fractions, and facilitating comparative assessments of enzymatic activity across different species populations.[16]

This investigation employed the PBA method to: (i) establish a quantitative standard curve correlating halo size with H_2O_2 concentration; (ii) assess LAAO activity across five snake venoms (*C. rhodostoma, D. siamensis*, *N. kaouthia*, *O. hannah*, and *T. albolabris*). Subsequently, LAAO was purified and authenticated from *O. hannah* venom utilizing chromatographic techniques, SDS-PAGE, and LC-MS/MS. The retention time was subsequently ascertained through RP-HPLC, and the inhibitory effect of reduced glutathione (GSH), a reactive oxygen species scavenger, was examined.

LAAO is a critical enzymatic component of venomous snakes, contributing to their toxic, antimicrobial, and apoptotic functions. PBA offers a practical and cost-effective method for detecting LAAO activity, thereby enhancing our ability to study venom components and supporting future applications in biotechnology and venom-based drug discovery.

2. Materials and Methods

2.1 Snake venom

Five freeze-dried snake venoms, including *Calloselasma rhodostoma*, *Daboia siamensis, Naja kaouthia*, *Ophiophagus hannah*, and *Trimeresurus albolabris*, were obtained from the snake farm at the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand, and were stored at 4°C before use.

2.2 Prussian blue agar (PBA) preparation

To evaluate LAAO activity, a PBA plate assay was developed using a modified chromogenic detection method.[17] The PBA was prepared by separately dissolving 0.1 g each of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and potassium hexacyanoferrate (III), $K3[Fe(CN)_6]$, in 5 mL of distilled water (DW), and then mixing them (solution A). 1.0 g of tryptone and 0.5 g of yeast extract were dissolved in 90 mL of DW (solution B). Solution A (10 mL) and 50 mM L-leucine (final concentration) were added to solution B (90 mL). The mixture was adjusted to a pH of approximately 7.4-7.5 with sodium hydroxide (NaOH), and 1.5% agar was added. It was autoclaved at 121°C for 15 minutes. 50 mL of Prussian blue solution was poured into a 145/20 mm Petri dish (Diameter: 145 mm; height: 20 mm) (Greiner Bio-One, Austria) to create an agar plate. The plate was then left to stand for 20 minutes, and circular wells with a diameter of 2 mm were made on the plate.

2.3 Purification and confirmation of LAAO from *O. hannah* venom

The crude *O. hannah* venom was purified using a three-step procedure of column chromatography on an AKTA purification system (GE Healthcare Life Sciences, Germany). First, 20 mg/mL of crude *O. hannah* venom in 10 mM PBS (pH 7.3) was added to the Sephadex G- 75 column (3.2 cm x 150 cm) (GE Healthcare Life Sciences) and eluted with 10 mM PBS (pH 7.3). After elution, the first peak demonstrated the highest specific LAAO activity among the five peaks, as previously reported in 2023.[18] This fraction peak was collected, concentrated, and dialyzed against 50 mM Tris- HCl, pH 8.0. Second, it was purified using the Resource Q column (1 mL column size) (GE Healthcare Life Science), which was equilibrated with 50 mM Tris- HCl, pH 8.0, and eluted with a linear gradient of 0-1 M sodium chloride (NaCl) and 50 mM Tris- HCl, pH 8.0. The peak that gave LAAO activity was eluted from the Resource Q column, collected,

concentrated, and dialyzed with 50 mM Tris-HCl, pH 8.0. Third, the concentrated protein from Resource Q column was loaded onto the equilibrated HiTrap™ Heparin affinity chromatography column (1.6 cm x 2.5 cm, 5 mL size) (GE Healthcare Life Science, Germany) with 50 mM Tris- HCl (pH 8.0), and eluted with a linear gradient using 0-1 M NaCl and 50 mM Tris- HCl, pH 8.0. Peak one (purified LAAO) was collected and analyzed on 12% SDS-PAGE. The purified LAAO protein was identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A protein sample was subjected to in-solution tryptic digestion. Briefly, the purified protein was reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and digested overnight with sequencing-grade trypsin at 37°C. The resulting peptides were desalted using C18 ZipTips and dried under vacuum. Peptide separation was performed using a nano-liquid chromatography system coupled to a tandem mass spectrometer. The peptides were loaded onto a C18 reverse-phase column and eluted with a linear gradient of acetonitrile in 0.1% formic acid. MS data were acquired in positive ion mode using data-dependent acquisition. The MS/MS spectra were searched against the *Ophiophagus hannah* protein database (UniProt ID: P81383) using Mascot (or other relevant software).

2.4 Preparing a standard concentration of H_2O_2

H_2O_2 stock solution (30% w/v; ~9.8 M, Sigma-Aldrich) was stored at -20°C in amber vials to minimize photodegradation. Working solutions at concentrations of 100, 25, 6.25, 1.56, and 0.39 mM were freshly prepared in DW immediately before each experiment. All working standards were used within 2-3 h of preparation to avoid loss of activity due to spontaneous decomposition. The stability of H_2O_2 was periodically confirmed spectrophotometrically at 240 nm with the equation (according to the Beer-Lambert law):

$$c\ (\mathrm{M}) = \frac{A_{240}}{\varepsilon \cdot l} \quad \text{with } \varepsilon = 43.6\ \mathrm{M^{-1}\,cm^{-1}},\ l = 1.0\ \mathrm{cm}$$

A = absorbance at 240 nm, **ε** = molar extinction coefficient for H_2O_2 (43.6 M^{-1}·cm^{-1}), c = molar concentration (M), **l** = optical pathlength (cm; 1 cm cuvette). If the measured value c exceeds a deviation of plus or minus 5% from the target, it must be discarded, and a new sample prepared.[19]

2.5 LAAO activity assessment of snake venoms on the PBA assay

A standard curve of H_2O_2 was created by preparing a series of known concentrations of H_2O_2 at 100, 25, 6.25, 1.56, and 0.39 mM and spotting equal volumes (20 µL) of each concentration into the well of PBA. After incubating the plate at 37°C for 60 minutes, a blue halo appeared at each spot, and the diameter of these blue halos was measured in centimeters (cm). The results of the Prussian blue halos in zone diameter (cm) and H_2O_2 concentrations (mM) were plotted in a graph to establish a linear regression model and determined the linear equation and R-squared value. To evaluate LAAO in snake venom samples. 20 µL of crude venoms from *C. rhodostoma*, *D. siamensis*, *N. kaouthia*, *O. hannah*, *T. albolabris* venom, and purified LAAO of *O. hannah* (1 mg/mL each in 10 mM PBS, pH 7.3) were applied to the wells of PBA and incubated at 37°C for 60 minutes. The diameters of the blue halos were measured, as PBS served as a negative control. This experiment was conducted in triplicate.

2.6 Halo diameter measurement (PBA assay)

After observing the samples and incubating the PBA plates at 37°C for 60 minutes, halo diameters were measured from the outer edge to the outer edge of the uniform, continuous blue zone. Any faint, diffuse peripheral staining was excluded from the boundary. Each spot was measured along two perpendicular axes, and the mean was calculated as the diameter of the halo. Measurements were obtained using a digital caliper (INSIZE, Code No. 1108-150, 0-150 mm [0-6 inches] range, resolution 0.01 mm). The caliper was zeroed prior to each measurement.

2.7 Reduction of LAAO activity by GSH (a scavenger of reactive oxygen species (ROS))

10 µL of 1 mg/mL crude and purified LAAO from *O. hannah* venom in 10 mM PBS at pH 7.3 were mixed with 10 µL of 20 mM GSH in PBS at pH 7.3 and then applied to the wells of PBA and incubated at 37°C for 60 minutes. The diameters of the blue halos were measured, with PBS serving as a negative control. This experiment was conducted in triplicate.

2.8 Determining the LAAO position from *O. hannah* venom using reverse-phase high-performance liquid chromatography (RP-HPLC)

Materials and reagents: A C18 column (250 mm x 4.6 mm), manufactured by Agilent, USA, with a particle size of 5 µm, was used in this experiment. The mobile phase consisted of solvent A, which was water containing 0.1% trifluoroacetic acid (TFA) for ionization, and solvent B, which was acetonitrile with 0.1% TFA. Samples for injection consisted of 1 mg/ml (in 10 mM PBS, pH 7.3) of crude and purified LAAO from *O. hannah* venom. The HPLC model 1,100 series (Agilent, USA) was set as follows: the flow rate was set to 1.0 mL/min, the column temperature was maintained at room temperature, and the injection volume was 30 µL. Mobile phase composition: isocratic elution, with a constant ratio of solvents A and B, was applied throughout the entire run. Gradient elution with the ratio of solvents A and B was gradually varied during the run as follows: 0-5 minutes: 90% A, 10% B; 5-15 minutes: 50% A, 50% B; 15-25 minutes: 10% A, 90% B; and 25-30 minutes: returning to initial conditions (90% A, 10% B). Data analysis was performed to examine the chromatogram and identify peaks corresponding to various compounds by comparing the retention times of crude and LAAO from *O. hannah* venom that overlapped. This method was applied based on Bruno Lomonte's 2017.[20]

2.9 Measuring protein concentration

The Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) was used to determine protein concentration.

2.10 Statistical Analysis

All conditions were performed in triplicate technical replicates on the PBA plate, a replicate number that enables the reliable estimation of within-assay variance for parametric statistics (e.g., ANOVA and linear regression) and aligns with guidance on assay development and precision assessment. Halo diameters were summarized as mean ± SD (n = 3 spots). One-way ANOVA with Bonferroni's multiple comparisons (α = 0.05) was used for group comparisons; the calibration was obtained by simple linear regression of halo diameter versus $\log_{10}[H_2O_2]$, reporting slope, intercept, 95% CI, and R^2. Analyses were performed with PRIMER of Biostatistics v6.0.

3. Results

Venom from *O. hannah* has been previously reported to contain high LAAO activity; however, further investigation into the properties and sequence of the purified enzyme was necessary for a better understanding of its biochemical characteristics. Therefore, this study focused on purifying LAAO from *O. hannah* venom and characterizing its enzymatic activity using the PBA method. The following section outlines the purification steps and amino acid sequence analysis of the isolated enzyme.

3.1 The purification of LAAO and the amino acid sequencing of the purified LAAO from the venom of *O. hannah*

To investigate the properties of LAAO from the venom of *O. hannah*, a multi-step purification process was employed. The demonstration showed the purification of LAAO using three different chromatographic techniques. The process was monitored to isolate the LAAO enzyme from the crude venom, allowing for

further characterization and amino acid sequencing. The image presented both the chromatographic profiles and SDS-PAGE analysis, confirming the successful purification of the enzyme (Fig. 1).

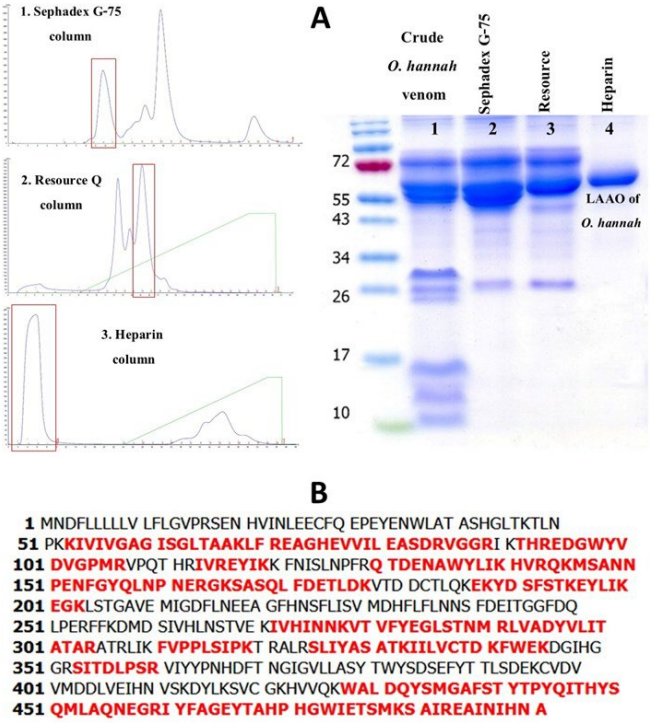


Fig. 1 Crude *O. hannah* venom was purified through three chromatography steps: 1. Sephadex G-75, 2. Resource Q and 3. Heparin column. The red box that framed the protein chromatogram peak in Fig. 1A (left) displayed the presence of LAAO at each step of purification. On the right-hand side, the protein bands of LAAO-purified steps of *O. hannah* venom were displayed at 12% SDS-PAGE: Lane 1: Crude *O. hannah* venom before purification; Lane 2: The crude *O. hannah* venom was purified via Superdex G-75 column; Lane 3: The pooled LAAO fraction of *O. hannah* venom from the Superdex G-75 column was purified using Resource Q column; Lane 4: The purified LAAO following the final Heparin column, the molecular weight of LAAO was around 60 kDa (Fig. 1A). The peptides detected during LC-MS/MS were the sequences highlighted in bold red in the image (Fig. 1B), which represented the regions of the purified LAAO protein that LC-MS/MS successfully identified. These peptides matched 52% of the reference sequence of *O. hannah* L-amino acid oxidase; its molecular weight was 55.941 kDa, and its pI was 5.99 (UniProt ID: P81383), confirming the identity of the purified enzyme.

A PBA-based method was used to visually and quantitatively assess LAAO activity by detecting H_2O_2, which reacted with potassium ferrocyanide to form a blue-colored complex. The diameter of the blue halo indicated the amount of H_2O_2 produced, reflecting the relative LAAO activity. This description detailed the development of standard H_2O_2 concentrations, which were subsequently used to compare the LAAO activity of various crude snake venoms based on this assay.

3.2 The presentation of various standard H_2O_2 concentrations (blue color halo) on PBA

A PBA was conducted by applying different concentrations of H_2O_2 in wells, allowing for observation of the resulting blue halos, which indicate the presence of H_2O_2. The diameters of the blue halos were measured for each H_2O_2 concentration, ranging from 0.39 mM to 100 mM, along with a control using PBS (Fig. 2).

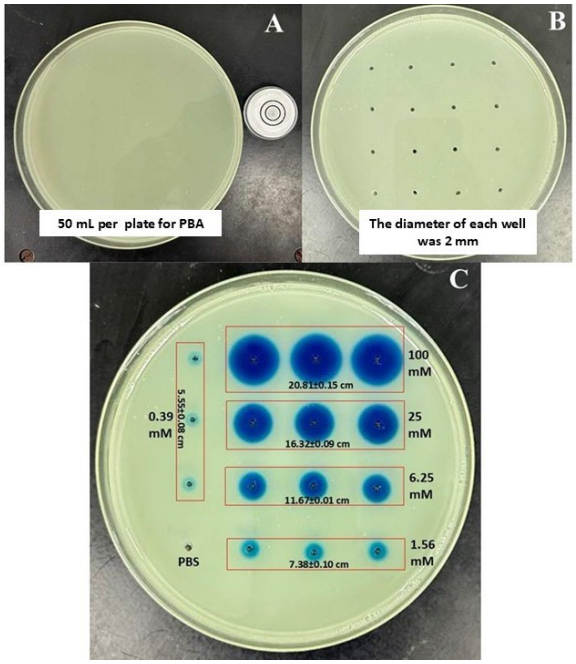


Fig. 2 PBA appeared light yellow or pale green due to its chemical composition, which includes ferric salts. It was poured onto a flat area or horizontal surface to allow the agar to solidify evenly, as checked by a circular bubble level (bubble vial) (2A). Each well on PBA was created with a hole measuring 2 mm (2B). The standard concentrations of H_2O_2 at 100, 25, 6.25, 1.56, and 0.39 mM were spotted by adding equal volumes (20 µL) of each concentration to the wells of PBA. After 1 hour of incubation at 37°C, blue spots appeared, the intensity of which correlated with the concentration of H_2O_2 (2C).

The rationale for selecting the specified concentrations of H_2O_2 was grounded in standard concentrations of 0.39-100 mM because these concentrations were deliberately chosen to encompass the range of H_2O_2 outputs observed in both crude venoms and purified enzymes, while ensuring that the halos remained within a quantifiable and approximately linear response range on PBA.

After measuring the diameters (in cm) of the blue halos with standard concentrations of H_2O_2, the values of the Prussian blue halos were converted to logarithmic form to create a linear regression model (Table 1). This model was used to determine the linear equation and the R-squared value.

Table 1 The measurement data on the relationship between H_2O_2 standard concentrations and the diameter of the blue halo on PBA.

H_2O_2 concentration (mM)	Log [H_2O_2 concentration]	Diameter of the Prussian blue halo on PBA (cm), n = 3
100	2.00	20.81 ± 0.15
25	1.40	16.32 ± 0.09
6.25	0.80	11.67 ± 0.01
1.56	0.19	7.38 ± 0.10
0.39	-0.41*	5.55 ± 0.08

The correlation between the standard curve of H_2O_2 concentration and the diameter of Prussian blue halos showed the relationship between the amount of H_2O_2 produced and the size of the halo formed around the test area. The diameter of the Prussian blue halo (cm) was converted to a log to enable linear regression for quantification and to estimate unknown sample concentrations. Negative log values (-0.41) at 0.39 mM represented concentration near or below detection limits, which may introduce variability or nonlinear behaviour. For more straightforward presentation, better linearity, and easier interpretation, it was generally recommended to avoid negative log values and instead use concentrations within the assay's reliable dynamic range.[21] Consequently, this value was excluded in the construction of the standard curve to maintain its linearity, which is essential for accurate quantification.

3.3 The relationship between H_2O_2 concentration and the diameter of Prussian blue halos on PBA

A regression model was developed to estimate H_2O_2 concentrations produced by LAAO from various snake venoms, creating a helpful tool for assessing the enzymatic activity of LAAO in venom samples, as displayed in a graph (Fig. 3).

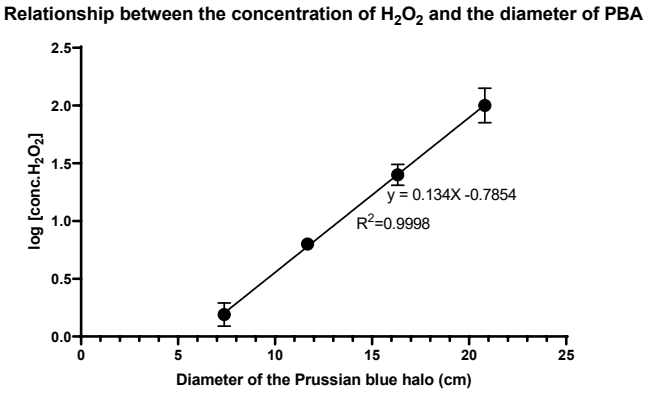


Fig. 3 The relationship between the concentration of H_2O_2 and the diameter of the PBA produced in the experiment. Y-axis (vertical): STD of H_2O_2 concentration was converted to logarithm (base 10). X-axis (horizontal): Diameter of the Prussian blue halo (cm) was the size of the blue color zone formed in the assay, indicating the presence and strength of H_2O_2. Regression equation on the graph: y = 0.134X - 0.7854 and R^2 = 0.998, indicating a robust positive correlation. This equation was used to estimate H_2O_2 concentrations generated by LAAO activity from various snake venoms based on measured halo diameters. Error bars represented the standard deviation (n = 3).

3.4 LAAO activities derived from five snake venoms and purified LAAO from *O. hannah* resulted in the formation of a Prussian blue color on PBA

This experiment was conducted to assess and compare the LAAO activity of various crude snake venoms and the purified LAAO enzyme from *O. hannah* venom by observing the formation of Prussian blue halos on PBA (Fig. 4).

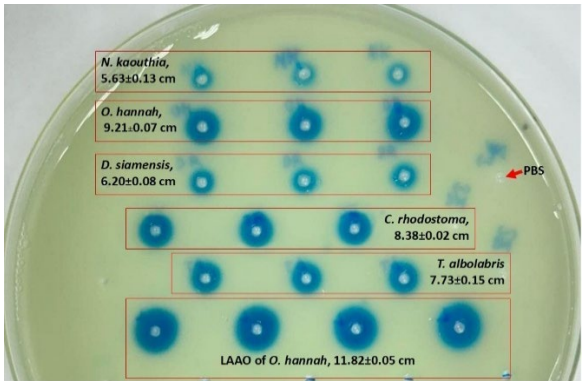


Fig. 4 PBA assay results used to evaluate LAAO activity from different crude snake venoms and purified LAAO from *O. hannah* venom. Each venom sample was applied at a concentration of 1 mg/mL (20 µL) and incubated at 37°C for 1 hour. The formation of blue halos indicated the production of H_2O_2, a byproduct of LAAO activity. The diameter of each blue halo was measured in centimeters and used to estimate the enzymatic activity. Among the crude venoms, *O. hannah* exhibited the largest halo (9.21 ± 0.07 cm), indicating vigorous LAAO activity, followed by *C. rhodostoma* and *T. albolabris* with moderate activity. The purified LAAO from *O. hannah* venom produced the largest halo (11.82 ± 0.05 cm), confirming its highest enzymatic potency.

Crude venoms from five snake species and purified LAAO from *O. hannah* were tested for H_2O_2 production using a PBA, in which the diameter of the Prussian blue halo reflected the amount of H_2O_2 generated (Table 2).

Table 2 The ability of different crude snake venoms and the purified LAAO enzyme to generate LAAO activity on PBA (n = 3).

Crude snake venom and purified LAAO	Diameter of the Prussian blue halo (cm), n = 3	Concentration of H_2O_2 (mM), calculated from the regression equation*	Concentration of H_2O_2, resulting from LAAO activity (mM/mg)	Interpretation
O. hannah	9.21 ± 0.07	2.81	140.66	Strong activity
C. rhodostoma	8.38 ± 0.02	2.18	108.88	Moderate activity
T. albolabris	7.73 ± 0.15	1.78	88.91	Moderate activity
D. siamensis	6.20 ± 0.08	1.11	55.57	Low activity
N. kaouthia	5.63 ± 0.13	0.93	46.51	Very low activity
LAAO of *O. hannah*	11.82 ± 0.05	6.29	314.38	Highest H_2O_2 production due to purified enzyme

*The diameter of the blue halo (X value) was substituted into the regression equation, y = 0.134X - 0.7854, to find the y value (Log10[H_2O_2]), then it was used to convert the concentration of H_2O_2 to mM using the antilogarithm

Table 2 displayed a comparative analysis of LAAO activity from various crude snake venoms and purified LAAO from *O. hannah*, based on the production of H_2O_2 on PBA. The diameter of the blue halo produced by H_2O_2 was measured and used in a regression equation to estimate the concentration of H_2O_2. Among the crude venoms, *O. hannah* exhibited the most vigorous LAAO activity, while the purified LAAO from *O. hannah* venom produced the highest concentration of H_2O_2, confirming its high enzymatic potency. Activity levels were classified as very low, low, moderate, or vigorous based on the calculated H_2O_2 concentration in millimoles per milligram (mM/mg). This suggested species-specific differences in LAAO content.

3.5 LAAO activity in crude snake venoms measured by the PBA assay

The LAAO activity was measured and expressed as micromoles per milligram (mM/mg) in each venom sample, with statistical analysis performed to compare the activity levels across different species. Mean LAAO activity (mM/mg) in crude venoms from five snake species (n = 3 each) was statistically analyzed, as shown in the graph, Fig. 5.

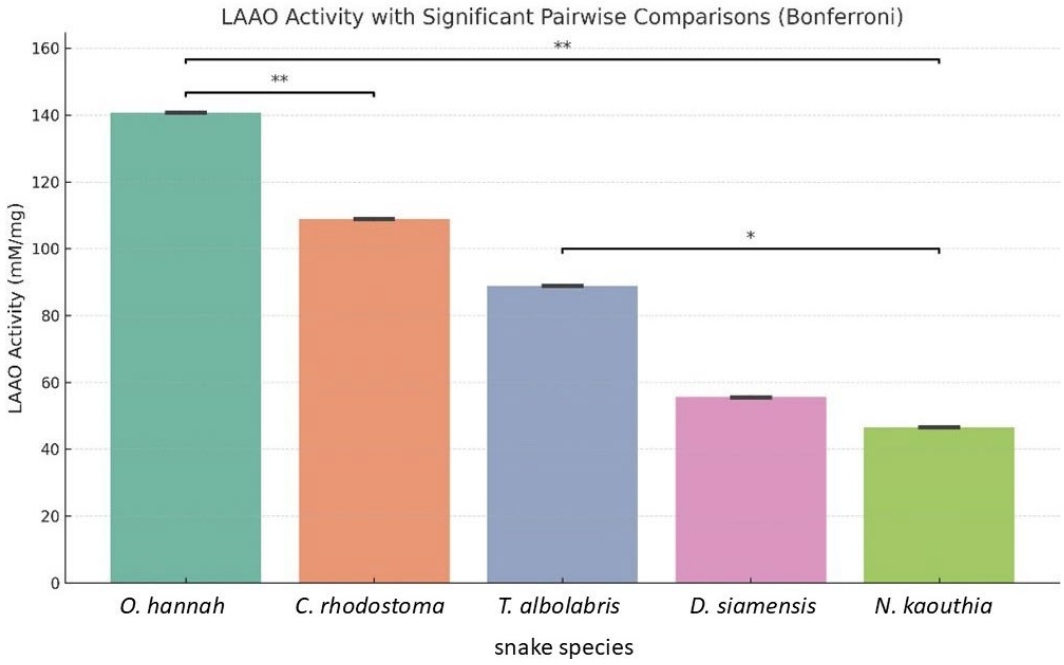


Fig. 5 LAAO activity (mM/mg) in the crude venoms of five snake species was measured using the PBA assay. Bars represent mean ± SD (n = 3). Bonferroni's multiple comparisons test was conducted to determine statistical significance. Significant differences between species were indicated: * $p < 0.05$, ** $p < 0.01$.

The statistical test revealed significant differences in LAAO activity among all five snake species ($p < 0.05$). *O. hannah* exhibited the highest enzymatic activity, followed by *C. rhodostoma*, *T. albolabris*, *D. siamensis*, and *N. kaouthia*. Pairwise comparisons, such as *O. hannah* vs. *C. rhodostoma*, *O. hannah* vs. *N. kaouthia*, and *T. albolabris* vs. *N. kaouthia*, were marked with $p < 0.01$ and $p < 0.05$, respectively, indicating strong significance. These findings support the robustness of the PBA assay in discriminating LAAO activity levels across different venoms.

3.6 Demonstration of LAAO reduction by GSH (Glutathione)

The observation of a decrease in the blue halo zone on PBA, when treated with either crude *O. hannah* venom or purified LAAO, due to GSH's inhibitory effect on LAAO activity, was shown in Fig. 6.

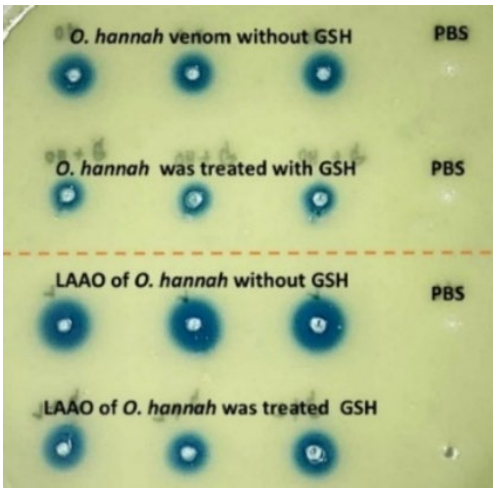


Fig. 6 Crude *O. hannah* venom without GSH (top row) produced intense blue colors, confirming active LAAO in the crude venom, which generates H_2O_2. Crude *O. hannah* venom with GSH resulted in reduced blue colors, indicating that GSH inhibited the oxidative activity of LAAO. Purified LAAO from *O. hannah* venom without GSH displayed intense blue colors, reaffirming the presence of an active enzyme that produces H_2O_2.

The results demonstrated that GSH effectively inhibited the oxidative activity of LAAO in *O. hannah* venom. Crude venom from *O. hannah* without GSH produced intense blue coloration, indicating the active presence of LAAO, generating H_2O_2. However, when GSH was added, the blue coloration significantly diminished, suggesting that GSH inhibited the enzyme's oxidative activity. A similar trend was observed with purified LAAO from the venom: an intense blue color in the absence of GSH confirmed the enzyme's activity, while a marked reduction in blue color upon treatment with GSH indicated that GSH directly inhibited LAAO. This highlighted the regulatory effect of GSH on LAAO activity, demonstrating its potential to modulate oxidative processes in the venom (Fig. 6).

3.7 Determining the LAAO position from *O. hannah* venom using reverse-phase RP-HPLC

Identifying the LAAO peak via RP-HPLC was a crucial step for in-depth biochemical characterization and analysis. This technique provided a consistent chromatographic profile with a distinct peak at a precise retention time, which was invaluable for confirming the presence and position of LAAO (Fig. 7).

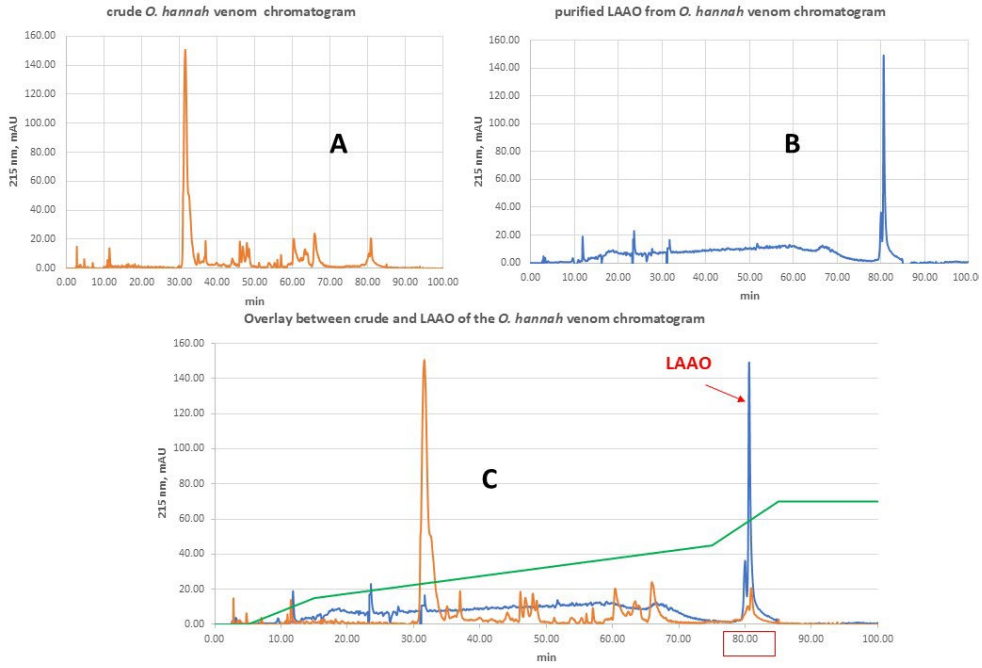


Fig. 7 The chromatogram of crude and purified LAAO from *O. hannah* venom obtained by HPLC was produced. The X-axis represented retention time (in minutes), which is the duration it took for a component to elute from the column and reach the detector. The Y-axis indicated the detector response (Absorbance Units, mAU), reflecting the amount of UV light absorbed by the eluting compounds. A peak represented one or more components that have been separated and were eluted from the column, with the area under the peak being proportional to the concentration of the element. The chromatograms of crude and purified LAAO from *O. hannah* venom were shown in 7A and 7B, respectively. The overlay chromatogram of purified LAAO and crude *O. hannah* venom was displayed in 7C.

RP-HPLC chromatographic profiles of crude *O. hannah* venom and purified LAAO were recorded at 215 nm. The chromatogram of crude *O. hannah* venom displayed multiple peaks (between 10 and 100 minutes), reflecting the complex mixture of proteins and peptides, with prominent peaks eluting between 30 and 35 minutes (Fig. 7A). The chromatogram of purified LAAO exhibited a dominant, symmetrical peak at approximately 80 minutes, indicating successful isolation and purity. Minor early peaks suggested the presence of residual impurities or minor co-eluting proteins (Fig. 7B). An overlay of the crude venom (orange) and purified LAAO (blue) chromatograms demonstrated that the LAAO peak at approximately 80 minutes was present in the crude *O. hannah* venom profile. The green line indicated the increasing gradient of the organic mobile phase (acetonitrile) used during the separation (Fig. 7C).

4. Discussion

The PBA assay was effectively established as a rapid, visual, and quantitative method for measuring LAAO activity in snake venom samples. The technique exploited the formation of Prussian blue through the reaction between H_2O_2 and ferricyanide, offering a safer and more accessible alternative to traditional chromogenic or spectrophotometric assays. Prussian blue detected H_2O_2 directly through a chemical redox reaction, unlike many conventional colorimetric assays that require enzymatic mediation. In this system,

H_2O_2 acts as an oxidizing agent that converts ferrocyanide $[Fe(CN)_6]^{4-}$ to ferricyanide $[Fe(CN)_6]^{3-}$, which then reacts with ferric ions (Fe^{3+}) to form the intensely blue pigment Prussian blue ($Fe_4[Fe(CN)_6]_3$). This reaction occurs spontaneously and does not require enzymes or cofactors, making it a direct and specific method for visualizing H_2O_2 production. In contrast, conventional assays—such as those using o-phenylenediamine (OPD), 3,3'-diaminobenzidine (DAB), or Amplex Red—depend on horseradish peroxidase (HRP) to catalyze the oxidation of colorless substrates into colored or fluorescent products. These enzyme-based assays, while sensitive, are indirect, often unstable, and involve reagents that may be toxic or light-sensitive. The Prussian blue method provided a safer, more accessible, and robust alternative, particularly in resource-limited or field-based settings where direct colorimetric visualization was advantageous.[13] This makes it highly suitable for applications such as screening LAAO, where H_2O_2 is a direct enzymatic product, or for venom research and enzymology in resource-limited contexts.

PBA was effective at approximately pH 7.5 because this pH provided optimal conditions for both the enzymatic activity of LAAO and the chemical formation of the Prussian blue complex. LAAO from snake venom displayed maximum activity in a near-neutral to slightly basic environment, and pH 7.5 preserved the structural integrity and catalytic efficiency of the enzyme, allowing for consistent production of H_2O_2. Meanwhile, the formation of the blue-colored ferric ferrocyanide complex (Prussian blue) required the presence of stable iron species and H_2O_2, both of which were favored under slightly alkaline conditions. At pH values lower than 7.0, iron ions might have precipitated or dissociated, while at higher pH, H_2O_2 could have degraded more quickly, reducing assay sensitivity. Therefore, maintaining the assay at pH 7.5 ensured a balance between enzymatic activity and chromogenic reaction efficiency, resulting in clear and quantifiable blue halos suitable for evaluating LAAO activity.[1,8] Prussian blue was dispersed more effectively in amino acid or peptide solutions than in water because these biomolecules contain functional groups that interact with the pigment's surface, creating a stabilizing layer around its particles. These interactions prevented aggregation and sedimentation by introducing steric and electrostatic stabilization, resulting in a more uniform colloidal suspension. In contrast, pure water lacks these stabilizing components, leading to poor dispersion and rapid particle settling. Thus, while Prussian blue remains chemically insoluble, amino acid- or peptide-rich solutions enhance its visual and functional dispersion through surface-binding and colloidal stabilization mechanisms.[22-23]

The establishment of a linear standard curve correlating halo diameter with H_2O_2 concentration demonstrated the assay's reliability and quantitative capacity. Among the five snake venoms tested, *O. hannah* venom produced the largest blue halo, correlating with the highest LAAO activity. This finding aligned with earlier studies reporting functionally potent LAAO in *O. hannah*, despite lower enzyme abundance compared to viperid venoms.[4]

Reduced GSH inhibited LAAO activity by H_2O_2, a key product of the enzymatic reaction. LAAO catalyzed the oxidative deamination of L-amino acids, producing H_2O_2, which reacted with ferrocyanide in PBA to form a visible blue halo. GSH, a potent intracellular antioxidant, reacted directly with H_2O_2 in a non-enzymatic redox reaction, converting it to water and oxidized glutathione (GSSG). This depletion of H_2O_2 prevented the formation of the Prussian blue complex, significantly reducing or eliminating halo formation on PBA, indicating effective inhibition of apparent LAAO activity.[2,8] These findings underscore the potential of GSH as a modulator in therapeutic or toxin-neutralization studies.

Purification of LAAO through gel filtration (Sephadex G-75), ion-exchange (Resource Q), and Heparin affinity chromatography yielded a protein of ~60 kDa, consistent with the known LAAO. It was confirmed via SDS-PAGE and LC-MS/MS. RP-HPLC analysis further validated the LAAO identity, with a significant peak

at ~80 minutes in both crude and purified venom, indicating the enzyme's hydrophobicity and chromatographic behavior. Earlier-eluting peaks at 30-35 min likely represented phospholipases A2 and three-finger toxins (3FTx), which correlated with the established elution profiles of these proteins in elapid venoms.[24] The combined use of colorimetric PBA screening and RP-HPLC profiling provided both functional and structural characterization. The method's strengths include fidelity, cost-effectiveness, and versatility for inhibitor screening and comparative venomics. However, limitations were also noted: potential interference from other H_2O_2-producing components and the assay's in vitro nature, which may not fully replicate in vivo conditions. Taken together, these findings affirmed that the PBA assay, supported by RP-HPLC validation, can significantly streamline LAAO activity profiling. This integrated approach provides a valuable platform for future studies on venom-derived enzymes, the development of therapeutic agents, and broader venom characterization projects.

5. Conclusion

In this study, a PBA method was successfully developed and applied to evaluate LAAO activity in various snake venoms. Among the tested species, *O. hannah* venom exhibited the most prominent LAAO activity, as evidenced by the largest Prussian blue halo zone on PBA, which correlated strongly with H_2O_2 generation. The inclusion of reduced GSH significantly decreased the intensity of the blue halos, demonstrating its inhibitory effect on LAAO activity, likely through the scavenging of hydrogen peroxide. Purification of LAAO from *O. hannah* venom through a three-step chromatographic procedure yielded a purified protein, which SDS-PAGE and amino acid sequencing confirmed. RP-HPLC analysis further confirmed the presence of LAAO in crude venom, eluting as a distinct peak at approximately 80 minutes. This peak aligned precisely with the major peak observed in the purified sample, validating the identity and retention position of LAAO in the venom chromatographic profile. Overall, the PBA assay proved to be a rapid, reliable, and visually interpretable platform for assessing LAAO activity. Combined with RP-HPLC profiling, this approach facilitated the identification, purification, and functional characterization of LAAO. These findings contribute valuable insight into venom enzymology and support the application of PBA as a practical tool for enzymatic screening, inhibitor testing, and venom-based therapeutic research.

6. Acknowledgements

The Scientific Committee of the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

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