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Alternative assay for the haemotoxic activities of the venom from the eastern Russell's viper *Daboia siamensis* (Smith, 1917)

Patchara Sittishevapark[a], **Jirarach Kitana**[a,b], **Suchada Sukrong**[c], **Noppadon Kitana**[a,b,*]

[a] Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330 Thailand
[b] BioSentinel Research Group (Special Task Force for Activating Research), Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330 Thailand
[c] Research Unit of DNA Barcoding of Thai Medicinal Plants, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330 Thailand

*Corresponding author, e-mail: noppadon.k@chula.ac.th

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ABSTRACT: Russell's viper is one of the common venomous snakes across Asia, known for inducing haemotoxic effects. While antivenom is the standard treatment for envenomation, its high cost and risk of severe allergic reactions have prompted interest in alternative therapeutic agents. To evaluate the efficacy and safety of these agents, suitable models and assays for assessing haemotoxic activities of snake venom are required. In this study, the chick embryo was developed as a screening model for venom-induced toxicity. Eggs were incubated at around 37 °C from day 0 after laying, the embryos were transferred to plastic wrap hammocks on day 2, and incubated until day 6. Venom solutions at various concentrations and normal saline (control) were applied to filter paper and placed on the lateral vitelline vein. Mortality was recorded at 4 h post-treatment, with an LD_{50} of 8.83 µg/µl. Microscopic examination revealed basement membrane disruption, clotted blood, and loss of membrane integrity in the venom-treated embryos. These results confirm the haemotoxic effect of *Daboia siamensis* venom on chick embryonic vasculature and provide a reference guide for testing the efficacy of therapeutic agents against snake toxins. In conclusion, the overall protocol that has been developed can be applied to validate the potential use of domestic chick embryos as an alternative assay in toxicological studies.

KEYWORDS: alternative animal model, blood vessel, coagulation, domestic chicken, haemorrhage, LD_{50}

INTRODUCTION

In a tropical region, such as Southeast Asia, venomous snakes are relatively common [1], and can cause serious public health problems to people, especially those who live in a rural area or forest edge in, or close to, suitable habitats of these snakes. The growth in the human population with expansion up to and into former suitable habitats for snakes increases human-snake encounters and conflicts, including from habitat fragmentation or deforestation, and these lead to increased incidents of snakebites [2, 3]. In Thailand, several venomous snake species pose risks to humans. However, one group that is particularly influenced by human population expansion, associated with a rise in snakebite incidents, is the viper group, especially the eastern Russell's viper (*Daboia siamensis*) [4]. Its venom possesses haemotoxin, which causes various pathological alterations to the circulatory system, including morphological changes of the erythrocytes and blood vessels, blood coagulation, kidney failure and death [5]. Although antivenom serum is generally used in snakebite patients for standard medical treatment [6], its cost per dose is expensive and it is ineffective at treating some symptoms [7]. Moreover, 5–80% of antivenom serum receivers may develop allergic reactions that can vary from redness through serious illness or even death [8, 9]. Alternative treatments that are more effective but safer are, therefore, needed.

Nowadays, several alternative treatments are being studied for snakebite patients, including therapeutic synthetic agents and modern drugs. However, obtaining such substances often requires complex synthesis and costly protocols. One group of treatments that Thai people have been used for a long time is herbal extracts. Due to their lower cost and widespread availability across the country, local healers are known to use medicinal herbs as an alternative treatment for snakebite patients. Nowadays, traditional herbs have been incorporated with modern medical treatment for treating snakebite patients in several hospitals in Thailand [10]. However, the number of scientific reports to support the effectiveness and safety of traditional herbs is still limited, making it less widely accepted [11]. In general, an *in vivo* assay based on rodent or more complex animal models is needed for testing the efficacy of herbs. However, screening herbs may require a large number of animals, which is both time-consuming and costly [12], further limiting the validation of such remedies for snakebite patients.

Chick embryos have been proposed as an alternative animal model since they can be used as a surrogate for higher vertebrate animal models for studying changes in several organ systems [13]. One can handle

a large number of eggs in a limited time or space in each experiment, making it a simpler and cheaper method. In addition, with the complex neural development completed by day 10 of incubation [14], less than 10-d-old chick embryos can be regarded as a model with minimal pain and suffering of experiment procedure. Moreover, there is evidence that chick embryos on day 7 are insensitive to any external stimuli [15]. In fact, both the National Institute of Health, USA [16] and Thailand's Animals for Scientific Purposes Act B.E. 2558 (A.D. 2015) [17] mandated that a chick embryo that had not reached half of its incubation time would not experience pain and can, therefore, be used for experimentation without any ethical restrictions or prior protocol approval, simplifying the planning process. According to USDA Animal Welfare regulations, this model is categorized under Category C, and all experimental procedures can be conducted in accordance with the established guidelines [18].

This study aimed to develop an *ex-ovo* assay for the haemotoxic activities of the eastern Russell's viper's venom using the domestic chick embryos. It is believed that, ultimately, the LD_{50} value based on the mortality rate of the embryo and histopathological alterations of embryonic vasculature observed in this study can be used in the screening assay for medicinal herbs or therapeutic agents with anti-haemotoxic activity against the snake venom.

MATERIALS AND METHODS

Ethical use of animals

In this study, 300 fertilized brown eggs of *Gallus gallus domesticus* (white leghorn) were bought at day 0 (day of laying) from Luangsuwanvajokkasikit Farm, Kasetsart University, Bangkok, Thailand and then transferred to Chulalongkorn University (CU) for the experiment. At the farm, eggs laid on the day prior to transfer to CU were kept below 15 °C to stop embryonic development, which was subsequently resumed synchronously after transfer to CU by incubating the eggs at 37 °C. According to Thailand's Animals for Scientific Purposes Act B.E. 2558 (A.D. 2015) [17], a chick embryo that had not reached half of its incubation time can be used for experimentation without any ethical restrictions or prior protocol approval.

Preparation of venom solution

Lyophilized snake venom of the eastern Russell's viper was purchased from the Snake Farm, Queen Saovabha Memorial Institute, Bangkok, Thailand (purchased on August 1, 2018). Venom was dissolved in normal saline and adjusted to a concentration of 10 µg/µl for use as a stock solution. Venom solution was aliquoted into 1.5-ml microtubes and stored at −20 °C for further use.

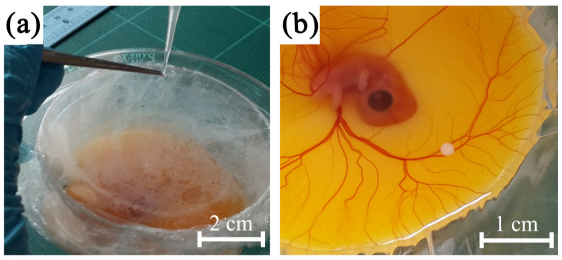

Fig. 1 (a) Aliquot (2 µl) of *D. siamensis* venom or normal saline was applied onto a 0.2 cm diameter filter paper. (b) The treated filter paper was then placed over the lateral vitelline vein of the chick embryo.

Chick embryo preparation

Fertilized chicken eggs at the day of laying (day 0) were transferred to the laboratory at CU and sequentially cleaned with distilled water, 70% (v/v) ethanol solution, and povidone-iodine, and then dried with sterile gauze. All eggs were labeled and incubated at 36.9 ± 0.9 °C (mean ± S.D.) with a relative humidity in excess of 80%. The eggs were turned 4–6 times/d until day 2 of incubation, when they were cracked open and the egg content, including embryos, yolk and albumen, was weighed and transferred onto the culture vessel. The hammock technique used for chick embryo culture was modified from Tahara and Obara [19]. Briefly, 100–120 ml of distilled water was added into a 470-ml plastic cup. A plastic film or food wrap was placed over the cup to form a concave hammock and the egg content was transferred into the hammock. A sterile glass Petri dish was used as a cover. Each chick embryo was incubated in the same condition as the egg until ready for the assay on day 6. Number of chicken eggs used and number of chick embryos available for the *ex-ovo* assay after incubation are shown in (Table 1). With the starting number of 300 eggs, 95 embryos were available for the assay, resulting in 31.7% yield of chick embryos.

Mortality rate and LD_{50} in chick embryo

Chick embryos at day 6 were used as the model for finding the LD_{50} value of the venom. Based on the egg content weight at day 2, embryos were assigned into five groups so that every group had a similar mean weight. Various concentrations of the same batch of venom (0, 1.25, 2.5, 5, and 10 µg/µl) were incubated at 36.9 ± 0.9 °C for 30 min before treating the embryo. The volume of venom in each group and normal saline (control group) was normalized to 2 µl and dropped onto a 0.2 cm diameter circle filter paper (Whatman No. 1; Fig. 1a) that was then placed over the lateral vitelline vein of the chick embryo (Fig. 1b). Observation of the embryo's health was performed every hour and the number of dead embryos (no sign of

Table 1 Number of chicken eggs used and number of chick embryos available for the *ex-ovo* assay after incubation 36.9 ± 0.9 °C. Success rate for each embryo preparation steps are shown as a percentage compared to the preceding step as well as a percentage compared to the original number of eggs.

	Procured eggs	Fertile eggs	Survived embryo at day 2	Survived embryo at day 6
Number	300	247	174	95
Success rate (%) compared to the preceding step	N/A	82.3	70.4	54.6
Overall success rate (%)	N/A	82.3	58.0	31.7

N/A = Not applicable; no preceding step for comparison.

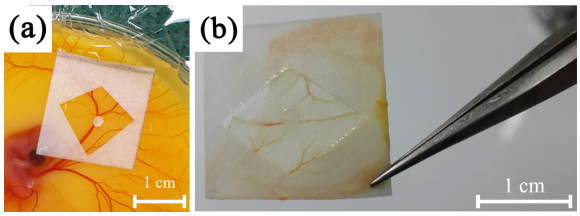

Fig. 2 (a) Square filter paper with a diamond-shaped hole was placed over the treated area of the blood vessel of the chick embryo. (b) The filter paper with tissue was cut and lifted up to be processed by standard whole mount or paraffin method.

heartbeat) was recorded at 1, 2, 3, and 4 h after venom application. The number of deaths in the control group and at each venom concentration at 4 h was calculated into the mortality rate, and used in a dose-response curve and LD_{50} calculation in RStudio 2024.12.0.

Histological study

Tissue collection and storage

Chick embryos were observed their mortality every hour for a duration of 4 h. Embryos that died were removed from the incubator, and their tissues were collected immediately upon confirming death. Blood vessels at the lateral vitelline vein and surrounding membrane were collected with the aid of filter paper. A square filter paper (2 × 2 cm) with a diamond-shaped hole was placed onto the embryonic blood vessel, with the treated area located in the middle (Fig. 2a). The membrane was cut along the border of the filter paper, and carefully lifted up together with the filter paper (Fig. 2b). The membrane was cleaned with normal saline and immersed in 10% neutral buffered formalin for 24 h for fixation. Tissues were washed and stored in 70% (v/v) ethanol for further histological analysis. Chick embryos were detached from the yolk and euthanized by prolonged freezing at −20 °C.

Tissue processing and staining

Two types of tissue processing and staining were used in this study: whole mount staining with haematoxylin and eosin (H&E: haematoxylin (Sigma-Aldrich, USA) and eosin Y (Merck, Germany)) and cross-section staining with Periodic Acid–Schiff (PAS: Periodic acid (Merck, Germany), Basic fuchsin (Sigma-Aldrich), and Sodium metabisulfite (Ajax, Australia)). For the whole mount staining, formalin-fixed and alcohol preserved tissues were trimmed for the designate embryonic blood vessel with a minimum area of surrounding membrane. These tissues were processed through the standard protocol for H&E staining [20]. Tissues were attached to the slide by Permount™ for further microscopic observation (Carl Zeiss Axio Scope A1 microscope equipped with a Canon EOS 7D camera.). In the cross-section staining, the standard paraffin method was used. Briefly, fixed tissues were trimmed and immersed into a series of increasing ethanol concentrations [50–100% (v/v)] for dehydration, a series of xylene for clearing and a series of paraffin wax for infiltration. Tissues were embedded into the paraffin block, sectioned at 5-µm thickness and attached to the slides. Tissue slides were processed through the standard PAS procedure [20]. Pathological alterations of the endothelial tissue and surrounding tissue were observed under a light microscope and recorded.

RESULTS

Mortality rate and LD_{50} of *D. siamensis* venom in chick embryo

After the method for preparing embryos had been optimized [21], the mortality rate of each group (control group and venom-treated groups) was calculated for the lethal dose of *D. siamensis* venom (Table 2). A dose-response curve and LD_{50} calculation were performed on these data using RStudio 2024.12.0 (Fig. 3). The LD_{50} of this batch of *D. siamensis* venom, 4 h post-treatment, was estimated using probit analysis with a 95% confidence interval to be 17.66 µg per embryo or 8.83 µg/µl.

Histological study

To examine the haemotoxic effects of venom, various histopathological markers were used, including the presence of clotted blood in the vessel [22], changes in the basement membrane, loss of membrane integrity and ruptures in the blood vessel or signs of leaking of erythrocytes and other components to the outside [23].

Table 2 Total number of chick embryos (6 day after incubation at 36.9 ± 0.9 °C) used in each treatment, number of dead/alive embryos, and mortality rate after exposure to various dose of *D. siamensis* venom for 4 h.

Treatment	Number of day 6 chick embryos			Mortality (%)
	Total	Dead	Alive	
Venom 0 µg/µl (control)	18	1	17	5.5
Venom 1.25 µg/µl	19	0	19	0
Venom 2.5 µg/µl	20	2	18	10
Venom 5 µg/µl	18	6	12	33.3
Venom 10 µg/µl	20	11	9	55

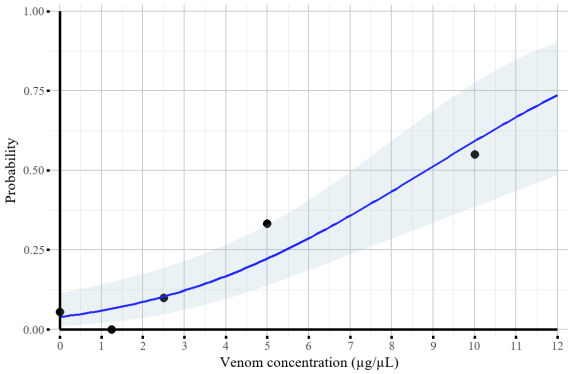

Fig. 3 A dose-response curve of chick embryo mortality following treatment with *D. siamensis* venom at various concentrations over a 4 h period was generated using RStudio 2024.12.0. Mortality rates were calculated based on the number of observed deaths at each dose. Probit analysis including a 95% confidence interval was used to estimate the LD_{50} value, which was approximately 8.83 µg/µl.

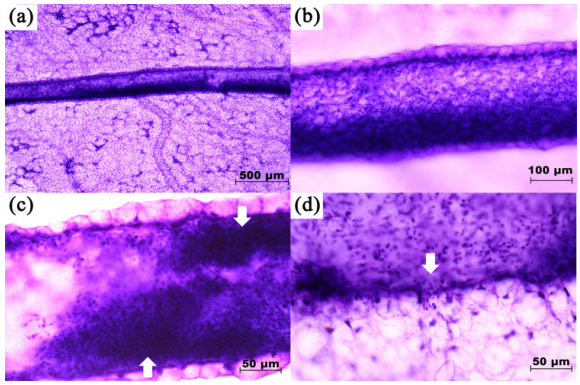

Fig. 4 Representative micrographs of a blood vessel of the chick embryo in the *D. siamensis* venom-treated group (H&E stained). (a, b) Overview of the blood vessel at (a) low (50×) and (b) high (100×) magnification. (c) Clotted blood (white arrow) can be found in some areas. (d) Endothelial wall showing signs of rupture and leakage of erythrocytes and other components (white arrow).

Results from the whole mount staining of the venom-treated group showed an overview of the blood vessel and surrounding cells (Fig. 4a). The blood vessel was stained as a purple tube with a spongy-liked layer around the tube (Fig. 4b). In some areas, rupture of the endothelial cells and leaked erythrocytes and other components from the vessel were found (Fig. 4c). In addition, densely packed erythrocytes and other components in the blood vessel (blood clots) were found in the vessels of the venom-treated group (Fig. 4d).

In the cross-section, PAS was used for staining the basement membrane so that the loss of membrane integrity and the rupture of the blood vessel could be observed [24]. Compared to the control group (Fig. 5a), it was found that the venom-treated group showed various changes in the endothelial tissue, including blood clots, which showed as a dense pack of erythrocytes and other components in the blood vessel (Fig. 5b). Of importance to this assay, a high number of thrombocytes was found in the venom-treated group (Fig. 6), inferring that the blood was clotting [25]. The basement membrane showed membrane shrinkage or loss of membrane integrity (Fig. 5c), and, in addition, ruptures of blood vessel and leakage of erythrocytes or other components were found (Fig. 5d).

DISCUSSION

Although the use of chick embryos in this study falls below the regulatory threshold for formal ethical approval, the model was employed under scientific necessity and in alignment with the principles of humane research. Chick embryos at this developmental stage are not considered capable of experiencing pain [14], and are thus regarded as a less sentient model. In other words, using chick embryos as a model appropriately aligns with the 3Rs principle. They serve as an alternative model for sentient or more complex organisms (Replacement). The protocol minimized the number of embryos used (Reduction) while ensuring the reliability and robustness of the results (Refinement).

Optimization of the chick embryo assay involved adjusting several factors that play role in normal development of the chick embryo. Firstly, the incubating temperature used in this study was 36.9 ± 0.9 °C, which was close to 37.8 °C or the suitable temperature for

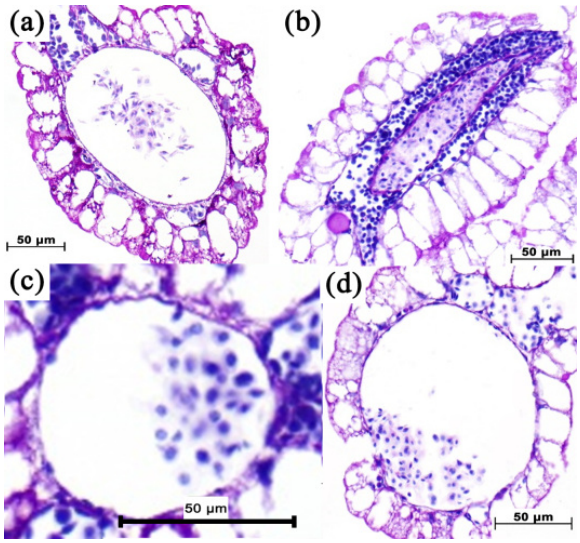

Fig. 5 Representative micrographs of a blood vessel of the chick embryo in the *D. siamensis* venom-treated group (PAS stain). Comparison was made between (a) the control group (×200), and the venom-treated group (b). The pathological changes potentially related to haemotoxic effect of venom included (b) blood clotting, (c) membrane shrinkage or loss of membrane integrity, and (d) the rupture of the endothelial wall and leaking of erythrocytes and other components.

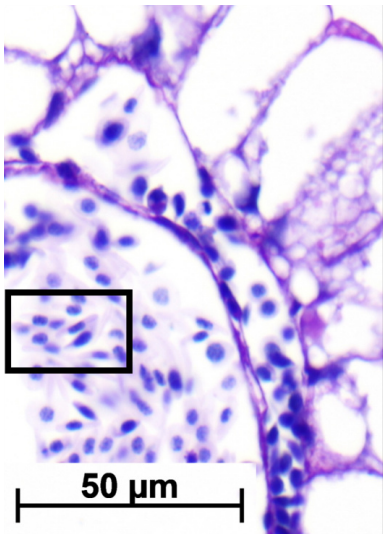

Fig. 6 Representative micrographs of a blood vessel of the chick embryo in the *D. siamensis* venom-treated group (PAS stain) showing the blood clotting and presence of thrombocytes, as ovoid or discoid shaped cells (within the rectangle).

incubation of chick embryos [26]. A previous study of Nakage and others in *Rhynchotus rufescens*, a medium-sized ground-living bird, showed that a change in the incubating temperature may decrease hatching and survival rates of the embryo [27]. In addition, the routine turning of eggs at 180 degree could facilitate embryos to increase their blood oxygen concentration, hence allowing them to develop normally [28].

For the actual assay, a shell-less culture ("hammock technique") was chosen to facilitate the treating protocol and to enable one to observe the chick embryo while under treatment. In this study, cracking eggs to the hammock culture was performed on day 2 of incubation because of the thickness of yolk sac membrane, where older eggs have a thinner yolk sac membrane and so tended to be easily torn by the sharp edge of the eggshell while cracking, whereas the day 2 egg had a thicker membrane and showed less incidents of tearing. In addition, using day 2 embryos in the hammock technique had the advantage of allowing researchers to verify the embryo development stage as well as the normal formation of the heart and blood vessels. Chick embryos were further incubated until ready for the assay on day 6. Using day-6 embryos for the exposure experiment is suitable for histological examination because the blood vessels are well formed and yet simple enough for tissue collection without harming the surrounding tissues [29].

The LD_{50} of this batch of *D. siamensis* venom, based on the day 6 chick embryo assay, was 17.66 µg/embryo, which was much higher than that (6.12 µg/embryo) previously reported using the same chick embryo assay and exposure method to test for the lethal dose of *D. siamensis* venom [21]. The different effect of venom (i.e. different proportion of enzyme or protein in venom) of the same species could be due to the intra-specific variation, since these studies each used a different batch of venom that may have come from different snakes with a different life history [30], sexual dimorphism [31], seasonal variation, diet, and age-dependent changes. Venom variability is known to have an impact on the use of antivenom serum, and must be considered beforehand [32]. It is, therefore, highly recommended that venom in the same batch of snake venom milking and lyophilizing is used throughout a trial. Otherwise, a new assay to verify the LD_{50} value must be performed for each and every batch of snake venom prior to further use. In this section, we have successfully optimized the conditions for venom injection and LD_{50} calculation using the *ex-ovo* assay. This protocol is sufficient to serve as a standard method for determining the LD_{50} value of other haemotoxic snake venom batches, as well as for applying venom to chick embryos.

The venom of *D. siamensis* is well known to show haemotoxic effects on blood circulation [33]. The histopathological changes of the endothelial tissue of chick embryo was evidenced in the venom-treated group and corroborated with previous reports on *D. siamensis* venom components [21]. Firstly, the blood clotting could be caused by members of the serine proteinases (RVV-V) and metalloproteinases (RVV-

X) families, which are abundantly found in *D. siamensis* venom. For the blood clotting reaction, RVV-V and RVV-X from venom stimulate factor V and X, respectively, then both factors accelerate the coagulation and the formation of blood clots [34]. In addition, aggregation of thrombocytes, an avian blood cell that mainly serves a similar function to mammalian platelets in the blood clotting cascade, was found in this study (Fig. 5) and so could also be inferred that the blood was clotting [25].

Secondly, changes in the membrane integrity and rupture of the blood vessel wall were also linked with snake haemotoxin. In Viperidae snakes, including *D. siamensis*, the enzyme frequently found in the venom is a group II secretory phospholipase A_2 ($sPLA_2$) [35], which is a multifunction enzyme that is involved in the generation of oxidative stress [36]. The process starts with the $sPLA_2$-catalyzed hydrolyzation of phospholipids in the cell membrane to produce lysophospholipid and free fatty acids [37], including arachidonic acid. This oxidative metabolite can further produce reactive oxygen species (ROS) [38] that then further cause oxidative damage at the membrane by the apoptosis pathway [39]. Amongst the many alterations caused by apoptosis, one is the shrinkage of membranes [40], as found in this study. In addition, $sPLA_2$ can destroy the connective tissue, such as collagen and elastin, at the basal lamina and so consequently cause the rupture of the blood vessel walls or haemorrhage [41, 42]. These results are sufficient to describe the primary effects of *D. siamensis* venom on blood vessel tissue.

Compared to the *in-ovo* chick embryo model, the *ex-ovo* model enables direct visualization and rapid assessment of vascular changes following venom exposure, as previously described. In contrast, the chorioallantoic membrane (CAM) and other *in-ovo* models offer limited visibility and restricted space for treatment and manipulation. Initially, the *in-ovo* method was considered for administering treatments; however, it proved difficult to maintain the position of the filter paper on the targeted blood vessels. The curving of the eggshell allowed the embryo to rotate, causing the filter paper to detach and disperse the venom to unintended areas. This issue was lessened in the *ex-ovo* model, where the embryo is placed on a flat surface, preventing movement and improving filter paper stability. Contained in a transparent container (e.g., petri dish), the *ex-ovo* model facilitates high-resolution observation of venom responses at specific structural sites, generating dynamic data that are not easily captured in other animal models such as murine or higher vertebrates. Furthermore, this model allows researchers to administer substances precisely to targeted regions, expanding testing possibilities. This is particularly beneficial for screening herbal extracts or therapeutic agents that are not yet well characterized. In addition to assessing therapeutic efficacy, the model's overall visibility supports the detection of potential side effects in affected organ systems. These findings highlight the value of the *ex-ovo* model in venom bioassay development and preliminary therapeutic screening.

Overall, the results confirm the haemotoxic effect of *D. siamensis* venom on the chick embryonic vasculature, and provided a reference guide for testing the efficacy of herbal extracts and therapeutic agents against snake haemotoxins. However, further modifications are needed to adapt this model for broader screening applications. For other venomous species or venoms with differing modes of action, additional optimization is required to effectively capture the specific biological activity of each venom.

CONCLUSION

In this study, an alternative *ex-ovo* assay for haemotoxic activities of the eastern Russell's viper's (*D. siamensis*) venom was developed for studying the haemotoxic effects on blood vessels and for screening therapeutic agents that inhibit venom activity. The LD_{50} concentration of the snake venom based on this assay was 17.66 µg/embryo. This specific LD_{50} value could be used as the standard concentration of venom in further screening for therapeutic agents with an anti-haemotoxic activity. The microanatomy of embryonic vasculature was examined and verified as suitable markers for the haemotoxic effect of snake venom, including blood clotting, loss of membrane integrity or membrane shrinkage and rupture of the blood vessel wall. The results of this study could be applied to validate the potential use of chick embryos as an alternative assay in toxicological studies.

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