SCREENING DEVELOPMENT FOR ANTI-HEMOTOXIC ACTIVITY OF THAI HERBS AGAINST EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM

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การพัฒนาวิธีการคัดกรองสมุนไพรไทยที่มีฤทธิ์ต้านความเป็นพิษต่อเลือดของพิษงูแมวเซา *Daboia siamensis* (Smith, 1917)

นายพชร สิทธิชีวภาค

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	SCREENING DEVELOPMENT FOR ANTI-HEMOTOXIC ACTIVITY OF THAI HERBS AGAINST EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM
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พชร สิทธิชีวภาค : การพัฒนาวิธีการคัดกรองสมุนไพรไทยที่มีฤทธิ์ต้านความเป็นพิษต่อเลือดของพิษงูแมวเซา *Daboia siamensis* (Smith, 1917). (SCREENING DEVELOPMENT FOR ANTI-HEMOTOXIC ACTIVITY OF THAI HERBS AGAINST EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM) อ.ที่ปรึกษาหลัก : ผศ. ดร.นพดล กิตนะ, อ.ที่ปรึกษาร่วม : รศ. ดร.สุชาดา สุขหร่อง,ผศ. ดร.จิรารัช กิตนะ

งูแมวเซา *Daboia siamensis* เป็นหนึ่งในงูพิษที่สำคัญในประเทศไทย พิษงูแมวเซามีผลต่อเลือดทำให้เกิดพยาธิสภาพในระบบไหลเวียนเลือดและส่งผลไปที่ไต ซึ่งวิธีมาตรฐานในการรักษา คือ การใช้เซรุ่ม แต่ด้วยราคาของเซรุ่ม อาการที่เซรุ่มไม่สามารถรักษาได้ และอาการแพ้ในผู้ที่ได้รับเซรุ่ม ทำให้มีความสนใจแสวงหาการรักษาทางเลือกรวมทั้งการใช้พืชสมุนไพร อย่างไรก็ตามการหาสมุนไพรที่มีฤทธิ์ยับยั้งพิษงู จำเป็นต้องมีวิธีการคัดกรองที่เหมาะสม ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อพัฒนาวิธีการคัดกรองในหลอดทดลองและในสิ่งมีชีวิตเพื่อใช้คัดกรองสมุนไพรที่มีฤทธิ์ยับยั้งความเป็นพิษต่อเลือดของพิษงูแมวเซา โดยในการทดสอบในหลอดทดลอง ได้พิจารณาใช้การทดสอบที่สัมพันธ์กับฤทธิ์ทางชีวภาพของของพิษงูแมวเซา 5 ด้าน และ พบว่าการทดสอบการทำงานของเอ็นไซม์ phospholipase การกระตุ้นการแข็งตัวของเลือด และการทำลาย fibrinogen สามารถแสดงความแตกต่างระหว่างกลุ่มควบคุมบวกและกลุ่มควบคุมลบได้ชัดเจน ส่วนการทดสอบการทำงานของเอนไซม์ย่อยโปรตีนและฤทธิ์กระตุ้นการเกาะกลุ่มของเซลล์เม็ดเลือดแดง ไม่สามารถใช้คัดกรองได้ เนื่องจากพบการออกฤทธิ์ในพิษงูแมวเซาน้อยมาก ในการทดสอบในสิ่งมีชีวิตสามารถใช้เอ็มบริโอไก่เป็นสัตว์ทดลองทางเลือกเพื่อศึกษาพิษงูแมวเซาต่อเลือด และพบว่าจุลกายวิภาคของเนื้อเยื่อหลอดเลือดในเอ็มบริโอไก่สามารถบ่งชี้ความเป็นพิษต่อเลือดได้ เมื่อนำสารสกัดสมุนไพร 10 ชนิด มาคัดกรองด้วยวิธีการในหลอดทดลองที่ได้พัฒนา พบว่าสารสกัดหมากสง *Areca catechu* L. สามารถยับยั้งการทำงานของพิษงูแมวเซาได้มากที่สุดถึงร้อยละ 45.93 จึงนำสารสกัดหมากสงความเข้มข้นต่าง ๆ มายับยั้งพิษงูในระดับความเข้มข้นที่สามารถฆ่าเอ็มบริโอไก่ได้ร้อยละ 50 (LD_{50} เท่ากับ 6.35 µg/µL) ก่อนนำไปทดสอบกับเอ็มบริโอ พบว่าสารสกัดหมากสงสามารถลดการตายของเอ็มบริโอไก่ได้ตามระดับความเข้มข้นของสารสกัด เมื่อคำนวณความเข้มข้นของสารสกัดหมากสงที่สามารถลดการตายของเอ็มบริโอไก่ที่ได้รับพิษงูแมวเซามา 4 ชั่วโมงได้ครึ่งหนึ่ง (ID_{50}) ได้ค่าเท่ากับ 4.42 µg/µL ผลการศึกษาแสดงให้เห็นว่าสารสกัดหมากสงมีประสิทธิภาพในการยับยั้งความเป็นพิษต่อเลือดของพิษงูแมวเซา ทั้งยังมีความเป็นพิษต่ำ (ค่า LD_{50} ของสารสกัดหมากสง เท่ากับ 445.16 µg/µL) จึงเป็นสมุนไพรที่มีศักยภาพนำไปพัฒนาเป็นตำรับยาเพื่อใช้ในการรักษาผู้ถูกงูพิษกัดต่อไป

สาขาวิชา สัตววิทยา ลายมือชื่อนิสิต .....

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Patchara Sittishevapark : SCREENING DEVELOPMENT FOR ANTI-HEMOTOXIC ACTIVITY OF THAI HERBS AGAINST EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM. Advisor: Asst. Prof. NOPPADON KITANA, Ph.D. Co-advisor: Assoc. Prof. Dr. SUCHADA SUKRONG,Asst. Prof. JIRARACH KITANA

The eastern Russell's viper, *Daboia siamensis*, is one of the important venomous snakes in Thailand. Its venom possesses hematotoxin causing pathological alterations to circulatory and renal systems. Although antivenom serum is used for standard medical treatment, its cost per dose, ineffectiveness for some symptoms, and potential to develop allergic reactions in patients has called attention to an alternative remedy including the medicinal herb. To find effective herbs, appropriate screening assays are needed. This study aims to develop *in vitro* and *in vivo* screening assays and use for screening Thai herbs with anti-hematotoxic activity against *D. siamensis* venom. For *in vitro* assay, five assays corresponding to 5 biological activities of venom were developed. Three assays for phospholipase, coagulation, and fibrinogenolytic activities have been successfully developed and effectively showed a marked difference between positive and negative controls. Two assays for proteolytic and hemagglutination activities were proven to be not suitable for the screening because of the low activity in *D. siamensis* venom. For *in vivo* assay, an alternative chick embryo assay for hematotoxic activities of *D. siamensis* venom was developed. The microanatomy of embryonic vasculature was verified as suitable markers for the hematotoxic effect of snake venom. Subsequently, 10 herbal extracts were screened with the validated *in vitro* assays. The results showed that *Areca catechu* L. is the most effective herb with 45.93% inhibition against the venom. Various concentration of *A. catechu* extract was used to neutralize *D. siamensis* venom at LD_{50} concentration (6.35 µg/µL), and the mixture applied to the chick embryo. It was found that *A. catechu* extract reduced the mortality of chick embryo in a dose dependent manner. The median inhibitory dose (ID_{50}) of *A. catechu* against *D. siamensis* venom in chick embryo during 4-hour exposure was calculated at 4.42 µg/µL. With the strong anti-hemotoxic effect and the low toxicity of *A. catechu* (LD_{50} of 445.16 µg/µL), this herb should be further developed into the herbal remedy for snakebit treatment.

Field of Study:	Zoology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I
INTRODUCTION

Venomous snakes can be found worldwide, especially in tropical regions such as Southeast Asia (Nijman, 2010; Somaweera and Somaweera, 2010). In Thailand, various venomous snake species are commonly found in a rural area or forest edges where they could pose serious public health problems to people. The annual report on snakebite incidents in Thailand between 2006-2015 showed that the average number of snakebite patients was 6,155 people (Muangdang, 2015). Although the number of snakebite patients was decreased every year, this number didn't display the actual number of snakebite patients. In most countries, their report about the snakebite mortality based on only hospital data in which the actual number was higher because some of the patients didn't inform the authority or went to the local doctor for treatment (Fox et al., 2006).

In Thailand, one of the important venomous snake species is the eastern Russell's viper, *Daboia siamensis* (Smith, 1917). The *D. siamensis* is known to contain a strong hemotoxicity causing pathologic alterations to the circulatory system of snakebite patients including blood coagulation, the changing of red blood cell's morphology, kidney failure and death (Mitrakul, 1979; Napathorn et al., 1998; Thamaree et al., 2000).

To treat snakebite patients, in general, antivenom serum is recommended by the World Health Organization (WHO) as the standard medical treatment (WHO, 1981). However, concerns have been raised over the following disadvantages of antivenom serum. First, it is ineffective to treat certain symptoms, such as local necrosis, nephrotoxicity, hemorrhage, etc. Also, antivenom serum cost per dose is expensive. As a result, only some hospitals, usually in the main city, have the antivenom serum. For people lived in a rural area or far away from the main hospital, it is hard to reach this antivenom serum in time (Bawaskar and Bawaskar, 2001). The most important adverse effect is an allergic reaction. According to some case reports that used antivenom serum for treating snakebite patients, it was shown that 5-80%

of patients who received antivenom serum can potentially develop allergic reactions which can cause redness, illness or even death (Williams et al., 2007; Cannon et al., 2008; Dhanya et al., 2009).

For these reasons, alternative treatments that are effective, safe, and easy to find are thus in need. One of the most popular treatments that have been used for a long time by local healers in Thailand is the use of medicinal herbs. Local healers who use medical herbs as a snakebite treatment have been found in every parts of Thailand such as Mr. Lae Sornkurd (Nakhon Sawan province), Mr. Som Khatchiangsaen (Lampang province), Mr. Khluean Thongrot (Trang province), etc. Each local healer has a different set of medical herbs and the treatment method, but one of the local healers that incorporated his traditional herb with modern medical practice is Mr. Aia Saikrasoon (Kap Choeng Hospital, Surin province). His treatment can heal the symptoms and also reduce other adverse effects from venom that might happen in the future. Currently, the traditional herb of Mr. Aia Saikrasoon has also been incorporated with the modern practice at Phanom Dong Rak Hospital (The King's 80th Birthday Celebration Phanom Dong Rak Hospital), Surin province, especially during the waiting time for confirmation of the snake species or the appearance of signs of systemic poisoning (Phongphladisai and Panyawatthananukun, 2011). Although there are many case reports that show the therapeutic effect of the herb as a treatment, there is still a limited number of scientific reports to prove the effectiveness and safety of traditional herbs (Coe and Anderson, 2005; Veronese et al., 2005). This causes uncertainty to the patients on the use of traditional herbs as alternative treatment

In general, an *in vivo* assay based on rodent models is needed for testing the efficacy of herbs. This assay is time-consuming and expensive, further limiting the validation of such remedies for snakebite patients. This study thus aims to develop screening assays for medicinal herbs with anti-hemotoxic activity against the eastern Russell's viper venom (Figure 1-1) using a battery of tests both *in vitro* (phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay: Chapter III) and *in vivo* (chick embryo as an alternative animal model: Chapter IV). After validation as the appropriated assay for screening

herbs with anti-hemotoxic activities against the eastern Russell's viper, efficacy of a selected group of Thai herbs against *D. siamensis* venom were screened and verified (Chapter V). The overall results were summarized into the schematic procedure of bioassay-guided isolation and the important issues related the current and future works were discussed (Chapter VI). In addition to a standard assay for screening herbs with anti-hemotoxic activities against the eastern Russell's viper venom, it is anticipated that a potential candidate Thai herb for treating snakebit patient effectively and safely can be obtained.

Objectives

1. To develop the *in vitro* and *in vivo* screening assays for Thai herbs with anti-hemotoxic activity against *Daboia siamensis* venom
2. To screen for Thai herbs with anti-hemotoxic activity against *Daboia siamensis* venom

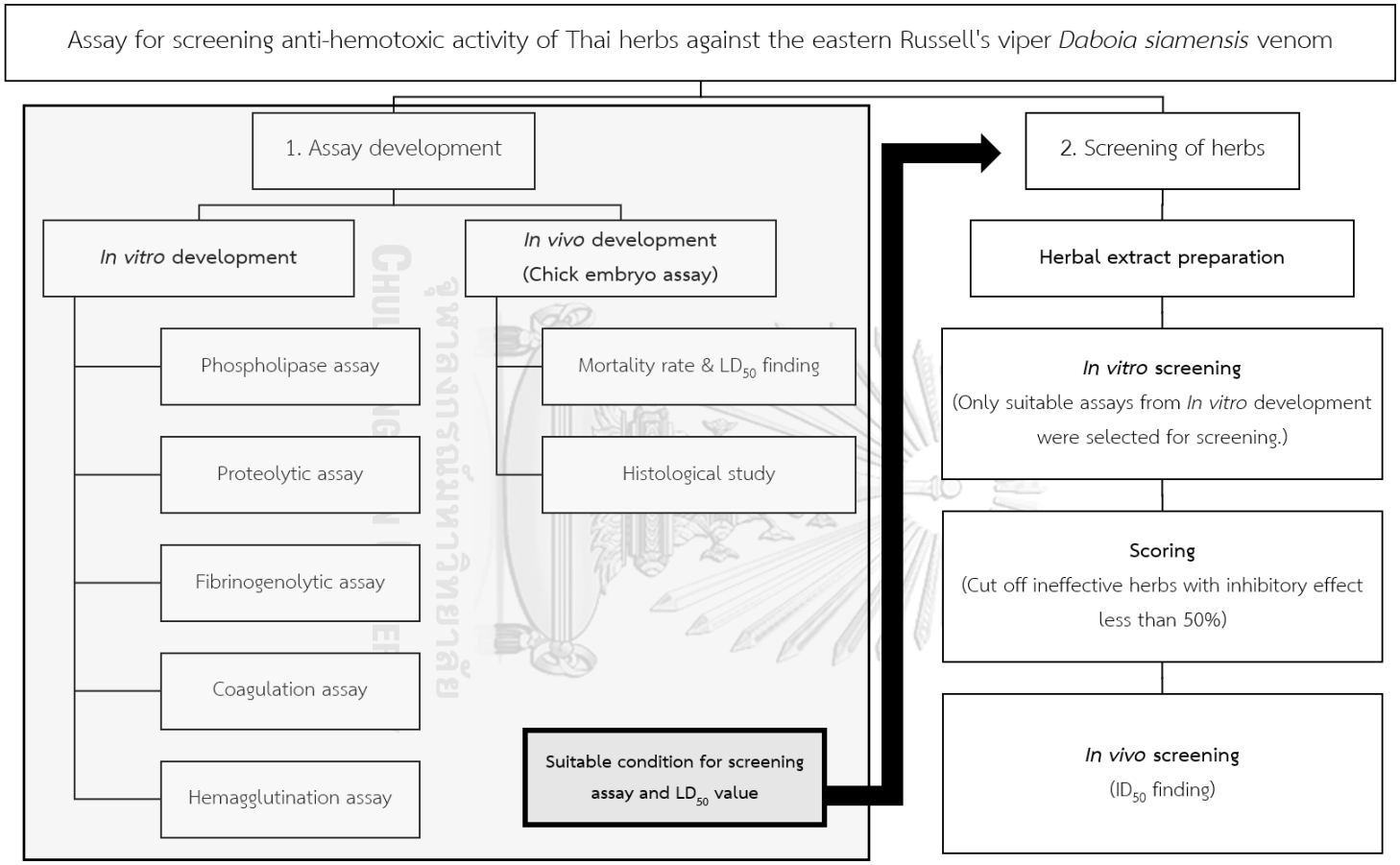

Figure 1-1 Research framework for the development of assays for screening anti-hemotoxic activity of Thai herbs against the eastern Russell's viper *Daboia siamensis* (Smith, 1917) venom

CHAPTER II

LITERATURE REVIEW

The Eastern Russell's Viper *Daboia siamensis*

Daboia siamensis, Smith, 1917 or the eastern Russell's viper is a venomous snake in the family Viperidae. Snake in genus *Daboia* was recently divided into 2 species based on geographical location including *D. russelii* and *D. siamensis* (Wüster et al., 1992; Wüster, 1998). *D. siamensis* distributes in southeast Asia, southern China, Taiwan, and Thailand, especially in the central and eastern parts of Thailand (McDiarmid et al., 1999).

D. siamensis has light-brown body color with dark brown dorsal spots that spread throughout the body. Its head shape is triangular or arrow-like. Once threatened, it would coil itself and make a warning hissing sound by inhaling the air to enlarge the body and then exhaling out (Figure 2-1). It is frequently found in grasslands near the plantation, the sandy ground, the rocky hills, and bushy areas, especially in the rural areas (Chanhome and Pauwels, 2007). When the ambient temperature is high, the snake would usually hide in the rodent hole, termite mold, or between the rocks to avoid sunlight (Cox, 1991).

Figure 2-1 External morphology of *Daboia siamensis* in curling position

The proteomic study showed that *D. siamensis* venom can be classified based on the sequence and structure into 6 protein families: phospholipase A_2, metalloproteinases, serine proteinases, L-amino acid oxidases, vascular endothelial growth factors (VEGFs) and C-type lectin-like proteins (Risch et al., 2009). Phospholipase A_2 has abilities to cause oedema, hemolysis, myolysis, and also the neurotoxic effect (Mizuno et al., 2001). Serine protease and metalloprotease play a major role in the coagulopathy which involves the prothrombin activation (Nicolaes and Dahlback, 2002) and leading to the formation of thrombin and fibrin clots (Mizuno et al., 2001), respectively. L-amino acid oxidase has strong effect against some type of tissue which leads to necrotic and apoptotic cell death (Lewis et al., 1990; Gopalakrishnakone et al., 1995; Du and Clemetson, 2002). C-type lectins show an anti-coagulant activity that contribute to the total coagulopathic activity of venom (Morita, 2005). VEGFs or vascular endothelial growth factors regulate the vasculogenesis and also enhance vascular permeability, further causing the leakage of the blood vessels and hypotension in several patients (Ferrara, 2004; Tokunaga et al., 2005).

In clinical and pathological aspects, *D. siamensis* venom mainly interferes with hemostatic alterations, especially coagulation disturbances. In human, this coagulation not only cause clot formation but also the imbalance of the coagulation enzyme that can cause various critical diseases (Palta et al., 2014). Coagulation cascade contains several enzymes that work together to maintain the normal state (Figure 2-2). In *D. siamensis* venom, several enzymes in family serine proteinases and metalloproteinases are found (Risch et al., 2009) and can interfere with the coagulation cascade. One of the identified enzymes in serine proteinases from Russell's viper venom is RVV-V or thrombin-like factor V-activating proteinase. Similar to thrombin, RVV-V has the ability to activate factor V to yield the final product of cross-linked fibrin (Tokunaga et al., 1988). Although this enzyme is the proteinase, it did not show proteolytic activity against other coagulation factors such as prothrombin, factor VIII, factor XIII, and fibrinogen (Segers et al., 2006). Metalloproteinase in Russell's viper or RVV-X can activate factor X in the similar way as factor IXa and VIIa during the coagulation cascade (Furie et al., 1974). Moreover, both alpha- and beta-fibrinogenase-like found in *D. siamensis* venom can degrade alpha and beta chain of fibrinogen, respectively (Sukkapan et al., 2011). The active site of these enzymes could be different from thrombin and hence leading to the different form of fibrin (Komori et al., 1985).

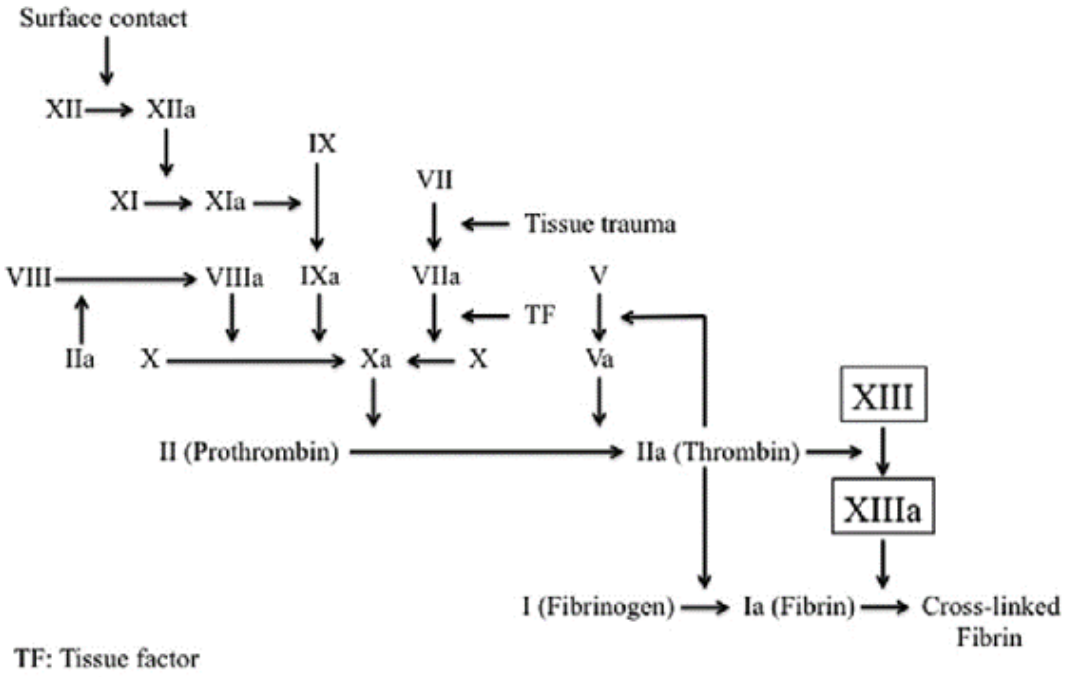

Figure 2-2 Factors and pathways in the coagulation cascade (Shimogawa et al., 2017)

As many pathological alterations can be developed to a more complicated symptoms, one of the most serious clinical symptoms is acute kidney injury which can be developed to acute renal failure (Kanjanabuch and Sitprija, 2008). Underlying process of acute renal failure are the disseminated intravascular coagulation (DIC), and ischemia by the fallen blood pressure and intravascular hemolysis (Sitprija et al., 1974). Although, most of the protein fraction in *D. siamensis* (phospholipase A_2, metalloproteinases, L-amino acid oxidases) including platelet-activating factor has a different action, they could synergistically affect the alteration of renal function (Chaiyabutr et al., 2020).

There are several factors that cause the snake in the same species and locality to have varied venom component. The younger snake tends to have a smaller volume of reserved venom, but greater defibrinogenation and edema-inducing activity than the adult. As a result, the venom from a younger snake tend to cause more serious renal failure in the patients. This age-dependent variation is not only found in *D. siamensis*, but also in *Bothrops moojeni* (Furtado and Kamiguti,

1985), *Crotulus horridus* (Bonilla et al., 1973) and *Crotulus utrox* (Reid and Theakston, 1978). Moreover, the length of the body of Russell's viper is the factor to increase the effect of the bite. Several studies showed that, with the longer body of the snake, higher amount of venom yield from the venom gland and greater defibrinogenation and swelling in the bite area can be found (Warrell and Phillips, 1985; Pe and Cho, 1986; Warrell, 1991). In addition, other aspects that also affect the variability of venom include geographic, ontogenic, taxonomic, and even sex-based variations (Serrano et al., 2005; Lomonte et al., 2014; Zelanis et al., 2016).

Snakebite and Antivenom Serum Production

Snake envenomation affects the health of Thai people in form of injuries and death every year. The recent annual report on the prevalence of snakebite patient in 2015 shows that 1.8 / 100,000 people were bitten by venomous snakes. With the lack of species identification, only 1 case (0.02%) was reported as *D. siamensis*. Majority of this prevalence (4,532 cases or 98.14%) fell into the unknown snake species category (Muangdang, 2015). Further, in a less serious case, the patient may choose not to go to the hospital. Therefore, it is likely that the prevalence of *D. siamensis* envenomation may be greater than it has been recorded.

Antivenom serum is regarded as the standard medical treatment for snakebite patients according to the WHO recommendation (WHO, 1981). With the risk in an allergic reaction or other side effects, the proper use of antivenom serum is very crucial for clinical manifestations. First and foremost, it is crucial to know the species of snake since the antivenom serum is species specific. In addition, the other symptoms of an allergic reaction are needed to be monitor after receiving antivenom serum for further treatment (Ramathibodi Poison Center, 2018).

In the antivenom serum production, it is purified from the plasma of animals that have been immunized against a snake venom or the mixture of venom. According to the WHO guideline for antivenom production (WHO, 2010), the process starts with venom collection from snakes that may originate from the wild or the snake farm. Since the intra-species variation of venom component may cause the inconsistent enzyme composition in a snake venom (Furtado and Kamiguti, 1985), the venom must be collected from snakes in the same locality where the anti-venom is intended to be used. The optimized dose of venom is used for immunization of healthy animals (usually horse). Blood or plasma sample of the animal subject was routinely collected for fractionation of plasma to extract the antivenom immunoglobulins.

For quality control of every batch of the obtained antivenom serum, a series of tests was carried out. One of the important analyses is the venom-neutralizing potency test (WHO, 2010) to check for the effectiveness of antivenom serum against the overall toxic activity of the snake venom. There are two continuous steps in this

analysis including an assay to find the LD_{50} of the venom and another assay to find the median effective dose (ED_{50}) of antivenom serum. For the first assay, various doses of venom are injected intravenously to animal subject (mice), and the mortality rates were observed at 24-hours after the injection. The median lethal dose (LD_{50}) or the dose of the venom that could kill half of the tested animal is subsequently used in the second assay. Antivenom serum at various doses are mixed with the venom at the LD_{50} concentration. The antivenom serum and venom are mixed in the test tube and incubated at 37°C for 30 minutes before injecting to the animal subject. The dose of antivenom serum that can reduce the death of mice that received the venom at LD_{50} dose by half is regarded as the median effective dose (ED_{50}). In general, the Probit analysis is used for LD_{50} and ED_{50} calculation.

Medicinal Herb for Snakebite Treatment

Several herbs with antivenom activity were recorded in traditional varieties in many countries including Thailand. One of the Thai Materia Medica named "Tamra Phra Osot Phra Naray" (Picheansoonthon et al., 1999) provided a list of 19 herbs with antivenom activity and the description of how to prepare and use of these herbs. Furthermore, uses of medicinal herbs by local healers have been found in every part of Thailand. The healers used the different herb depending on the available herbs in the area. Some of the healers mixed several type herbs into their own remedy or used different techniques to prepare the herbal remedy and to perform the treatment. Large number of patients showed sign of healing after treatment, raising interest in herbal remedy by researchers and physicians in the hospital (Phongphladisai and Panyawatthananukun, 2011).

One of the well-known local snakebite healers is Mr. Aia Saikrasun from Surin province (Phongphladisai and Panyawatthananukun, 2011). He has used the mixture between *Trigonostemon reidioides* (Kurz) Craib, *Araca catechu* L. and lime juice to make a herbal paste and applied onto the wound area of the snakebite patient. Not only his treatment can heal the symptoms, but also reduce other adverse effects from the venom. Nowadays, his treatment has been incorporated with modern medical practice. As the snakebite patients were commonly left untreated for a certain period of time to confirm the snake species or the signs of systemic poisoning in order to reduce the risk in allergic reaction from antivenom serum treatment, herbal remedy has been applied to the patient during this waiting time to alleviate the symptoms.

T. reidioides and *A. catechu* were subsequently tested against *Naja kaouthia* venom and found to show antivenom activity which can effectively neutralize venom and reduce the death of mice (Prapavicha, 1998; Srithamma et al., 2004). These studies confirmed the valid use of herbal remedy for snakebite treatment and the need for additional screening for other type of medicinal herbs.

Screening of herbs has been used as a convenient way to find the herb with antivenom activity. The experiment usually involves the animal model since it can represent the interaction between the tested substance and the effect in living

organisms. In the herb screening for antivenom activity, the measurement of a lethal dose of venom (LD_{50}) is the first step that required as a baseline for the finding of the effective dose of herb (ED_{50}) (WHO, 2010). In early toxinology research, many animal models were used including pigeon, dogs, cats, rabbits, mice, etc. (Geoffroy and Hunauld, 1737; Fontana, 1781; Sewall, 1887). Currently, most of the studies on venom toxinology was performed on mice (WHO, 1981; Theakston and Reid, 1983).

Chick Embryo as an Alternative Animal Model

Embryo of egg laying animal, such as chicken, has been used in various field of biological researches including developmental toxicology (Sells et al., 1997; Sells et al., 1998; Krishnan et al., 2014). The fact that the development of chick embryo occurred outside the mother allows for various observation *ex vivo*. The normal development of chick embryo can be completed within 21 days at the appropriate temperature (Guthery, 2005). Somatic development of chick embryo at each stage has been thoroughly investigated and recorded (Hamburger and Hamilton, 1951) (Figure 2-3).

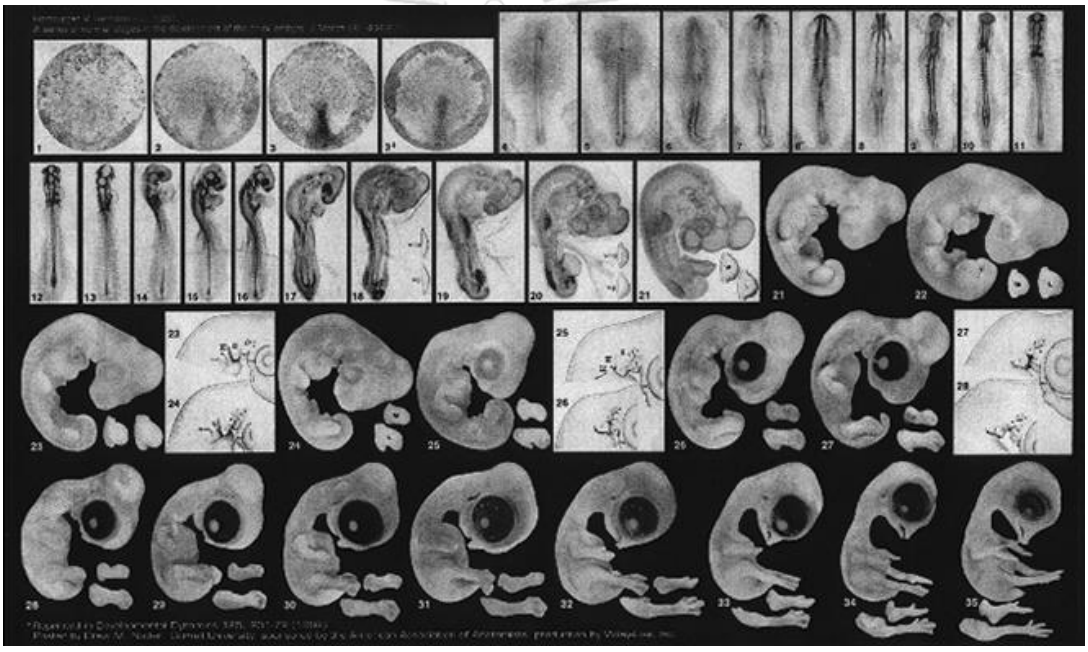

Figure 2-3 The normal development of chick embryo (*Gallus gallus domesticus*) from day 0 to day 21 (hatching) can be divided into 35 discrete stages (Hamburger and Hamilton, 1951).

The optimal temperature for chick embryo development is around 37.8°C (Barott, 1937). During the first 24 to 48 hours, the blood-island starts to form around the embryonic area, and the surrounding yolk content tends to change in appearance (Figure 2-4A). At the same time, heart formation starts, and some chick embryo may show sign of the heart activity. These two signs can be used as markers of normal development in the chick embryo. Although there are several somatic developments in this period, the transparent body of the embryo make the observation less obvious (Figure 2-4B).

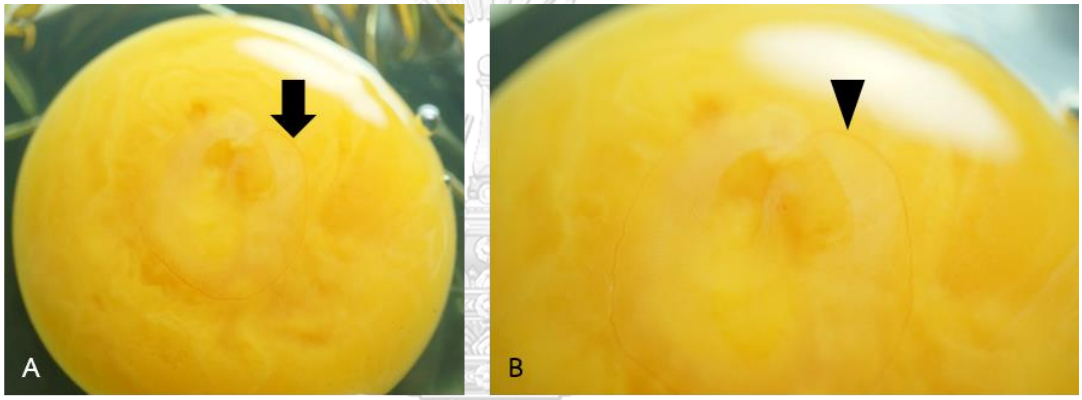

Figure 2-4 (A) The 2-day old chick embryo with a blood-island (tip of the black arrow), and the surrounding yolk content changes in appearance. (B) In some embryos, the heart activity can be seen in the middle of blood-island and inside the embryo (triangle).

On day 3-4 of development, the embryo enlarges and the complexity of the blood vessel is increased. In this period, the wall of the yolk sac is thinner than the younger embryo to facilitate the expansion of the embryo. The change in morphology of embryo is more prominent such as the network of blood vessel, eyes (completely pigmented on day 4), limb bud, the rotation or bending of the embryo, and some internal organs (Figure 2-5).

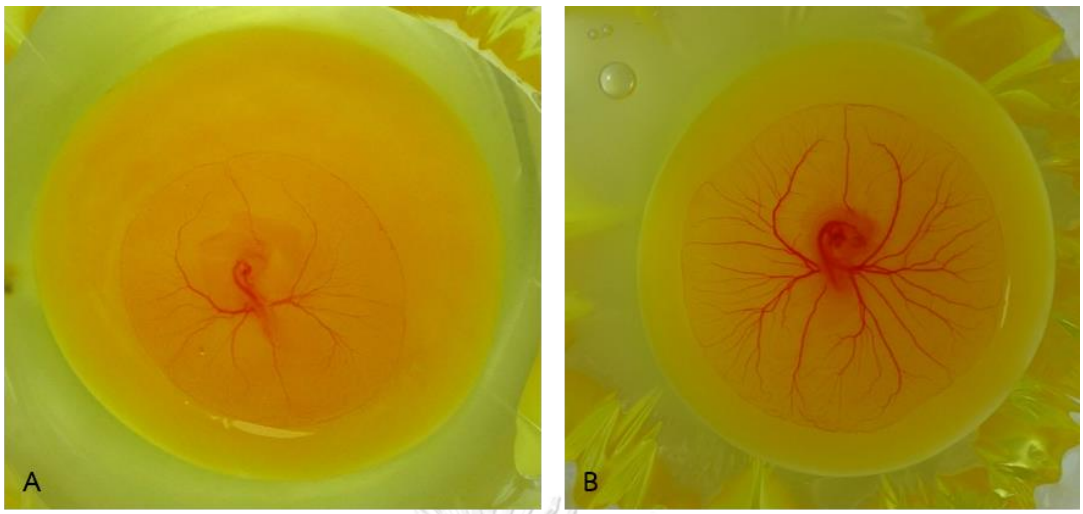

Figure 2-5 (A) The 3-day chick embryo shows a clear embryo with the blood-circulation. The area of embryonic development is separated from the yolk sac underneath. (B) The 4-day chick embryo shows a larger size of the embryo with more complex blood vessels.

At day 5-6 of development, the embryonic development area expands and the blood complexity is very obvious (Figure 2-6). The development of a blood vessel is completed on day 8 (Hamburger and Hamilton, 1951; Sissman, 1970). To avoid the difficulty of tissue collection from complex blood vessel, *in vivo* assay used in this study is performed up to 6 days of embryonic development.

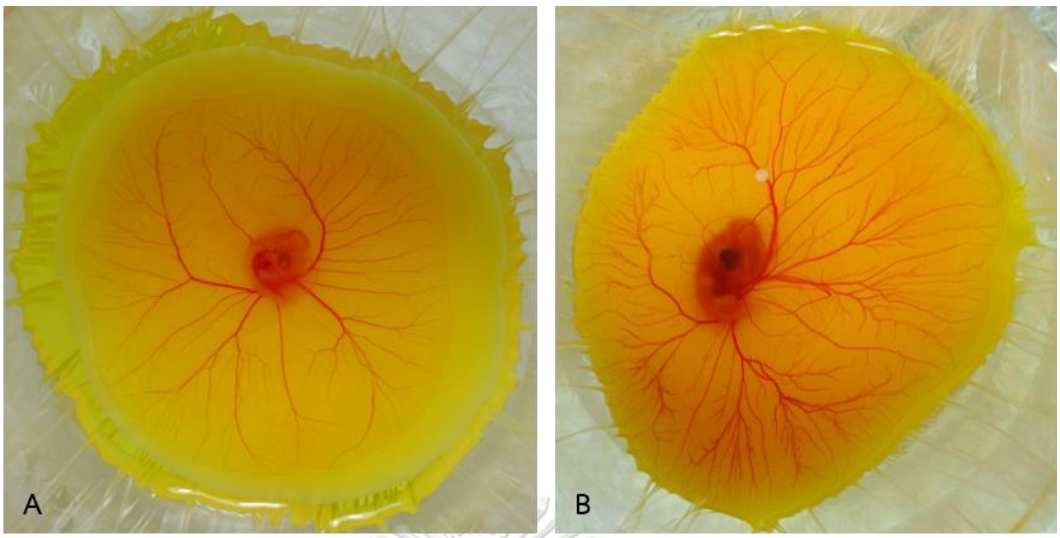

Figure 2-6 (A) The 5-day chick embryo shows the enlarged development area and the expansion of vasculature. (B) The 6-day embryo shows the most complex degree of blood vessel with the full expansion of the development area to the edge of the plastic hammock.

The Principles of the 3Rs

Major guiding principle of research on animal is the 3Rs (replacement, reduction, and refinement) that has been proposed by Russell and Burch in 1959 (Russell and Burch, 1959) for the humane treatment of animal in research. In this study, the developments of *in vitro* and *in vivo* screening assay have been based on the 3Rs principle as follows.

The *in vitro* screening was developed to remove the ineffective herbs from the screening before starting the *in vivo* screening, hence reducing the use of animals (replacement). The initial number of animals used in the *in vivo* screening was minimal and gradually increase until the statistical power is comparable to other studies, hence the small number of animal with the scientifically sound result was used (reduction). Chick embryo at day 6 was selected as a model for the screening. Since the complex neural development of chick would complete on day 10 of incubation (Rosenbruch, 1989) and the evidence of insensitivity of 7-day chick embryo to external stimuli (Rosenbruch, 1997), the 6-day chick embryo used in this study would not experience the pain (refinement).

CHAPTER III

DEVELOPMENT OF *IN VITRO* ASSAYS FOR HEMOTOXIC ACTIVITIES OF THE EASTERN RUSSELL'S VIPER VENOM

Introduction

Venomous snakes can be found worldwide, especially in the tropical regions such as Southeast Asia (Nijman, 2010; Somaweera and Somaweera, 2010). In Thailand, various venomous snake species are commonly found in rural area or forest edges where they could pose serious public health problems to people. One of the most important venomous snake species is the eastern Russell's viper or *Daboia siamensis* (Muangdang, 2015) which is known to contain hemotoxic venom causing pathologic alterations to circulatory system of snakebite patient including blood coagulation, red blood cell morphology change, kidney failure and death (Mitrakul, 1979; Napathorn et al., 1998; Thamaree et al., 2000). In general, antivenom serum is regarded as a standard medical treatment for snakebite. However, concerns have been raised over the use of antivenom serum since it is ineffective to treat some certain symptoms, the cost per dose is expensive (Cheng and Winkel, 2001) and 5-80% of patients who received serum can potentially develop allergic reactions which can cause redness, illness or even death (Williams et al., 2007; Cannon et al., 2008; Dhanya et al., 2009). Alternative treatments that are more effective and safer are thus needed.

In many parts of Southeast Asia, including Thailand, local healers have been known to use medicinal herbs as a treatment for snakebite patients. Recently, treatment of snakebite patients has incorporated traditional herbs with modern medical practice in several hospitals in Thailand (Phongphladisai and Panyawatthananukun, 2011). Unfortunately, there is still a limited number of scientific reports to prove the effectiveness and safety of traditional herbs (Coe and Anderson, 2005; Veronese et al., 2005). Several attempts have been made to test for efficacy of traditional herbs by the screening methods. However, the screening assays

for efficacy of herbs requires the time-consuming and expensive use of animals, thus further limited the availability of scientific discovery of remedy for snakebite patients.

In this study, appropriate *in vitro* assays for screening medicinal herbs that have anti-hemotoxic activity against *D. siamensis* venom were developed. There are 5 assays corresponded to important biological activities of the venom found in most case of patients bitten by *D. siamensis* or related species (Mukherjee et al., 2000; Kularatne, 2003; Antonypillai et al., 2011), namely phospholipase A_2 activity, proteolytic activity, coagulation activity, fibrinogenolytic activity, and hemagglutination activity. Although standard protocol for screening assays were established in most, if not all, of the activities (Mukherjee et al., 2000; James et al., 2013; Krishnan et al., 2014; Gopi et al., 2015; Oliveira et al., 2016), most of them based on *D. russelli* venom, requires large quantity of venom, or specific chemicals and conditions to work with (Sharma et al., 2015). Therefore, optimization of assay conditions is necessary for the development of the screening assay specific for *D. siamensis*. As a result, preliminary tests were carried out based on the standard protocol from Oliveira's work (Oliveira et al., 2016). Assay optimizations were detailed in Appendix C. In this chapter, only the final developed methods are presented.

The aims of this study was to develop screening assays for medicinal herb with anti-hemotoxic activity against eastern Russell's viper venom using a battery of *in vitro* tests including phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay, and hemagglutination assay. The suitable assays were further used as the standard assay for screening of anti-hemotoxic herbs.

Materials and Methods

Five *in vitro* assays modified from the Oliveira et al. (2016) covering major mode of hemotoxicity in snakebite patients were developed. Each assay was carried out using the same experiment groups including

- negative control (-ve ctrl): venom and normal saline
- positive control (+ve ctrl): venom and a dose of antivenom serum that completely inhibited the venom
- normal control (ctrl): (only normal saline or phosphate-buffered saline (PBS)).

Some assay might have additional groups that were added to fulfill the result as described in the materials and methods of each assay. The aim of the development was to find optimal conditions, volume, concentration, or the observation method of each assay. The optimal conditions of the assay should show a marked difference between the negative control group and the positive control group. Doses of venom used in this part were 0.1-10 µg/µL which is corresponded to LD_{50} value (dose of venom that resulted in 50% mortality of the 6-day old chick embryos) from the previous study (Sittishevapark, 2015).

1. Preparation of Venom and Serum Solution

Lyophilized snake venom of the eastern Russell's viper (purchased on July 2017; Figure 3-1A) and its antivenom serum (Lot No. WR00117; Figure 3-1B) were purchased from Snake Farm, the Queen Saovabha Memorial Institute, Bangkok, Thailand. To prepare the stock solution, venom powder was dissolved and adjusted the concentration to 10 µg/µL with normal saline. Antivenom serum was dissolved in distilled water at the recommended volume. One microliter of the stock antivenom serum could neutralize 0.6 microliter of *D. siamensis* venom at 10 µg/µL concentration). Both solutions were aliquoted to a small volume and stored in microtube at -20°C for further use (Figure 3-1C).

Figure 3-1 (A) Lyophilized venom of *D. siamensis* and (B) antivenom serum of *D. siamensis* venom were bought from Snake Farm, the Queen Saovabha Memorial Institute, Bangkok, Thailand. (C) They were prepared as the stock solution and stored for further use.

2. Development of Phospholipase Assay

Agar plates technique was used for assessing the phospholipase activity as described by Oliveira et al. (2016). The preparation started with making 1% bacteriological agar and adjusted the pH to 7.2. The mixture was heated on a hotplate (Figure 3-2A) until it reached 90°C. Then, the mixture was added with 0.005% of sodium azide solution and 0.01 M of $CaCl_2$ solution. After the temperature of the mixture reduced to 40-45°C, a 33% yolk solution in PBS at pH 7.2 (1:3, PBS: yolk) was added and stirred until it became the homogenous mixture. It was then poured into the Petri dish and left at room temperature (25-30°C) for solidification. After that, the gel was punctured to make 0.5 cm diameter holes by using a hollow steel tube (Figure 3-2B).

The test substances including the control group (normal saline), the negative control group (venom at 10 µg/µL), and the positive control group (venom and antivenom serum) were applied at 10 µL per well (Figure 3-2C). Then, all plates were incubated at 37°C for 18 hours (Figure 3-2D).

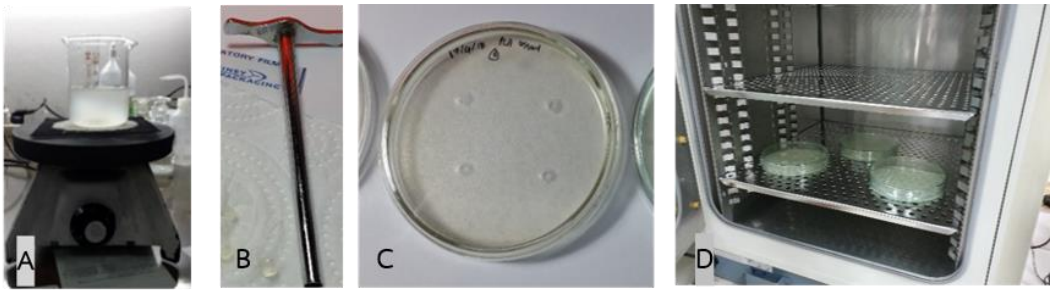

Figure 3-2 (A) The 1% bacteriological agar at pH 7.2 was mixed and heated on the hot plate. After adding all substances, the mixture was poured into the Petri dish for solidification. (B) A small hollow steel tube was used for puncturing the 0.5 cm diameter holes. (C) The test substances in each group were applied to the well and (D) incubated at 37°C for 18 hours.

After incubation, phospholipase activity can be observed from the clear zone around the hole. The diameter of the clear zone around each well was measured by a vernier caliper (Figure 3-3A) 3 times at 3 different axes (Figure 3-3B). The experiment was replicated 7 times per group. The data was checked for normality and homogeneity of variance. If the result complied with a parametric assumption, the data would be assessed by one-way ANOVA with Student-Newman-Keuls (SNK) post hoc test. If not, the Kruskal-Wallis one-way ANOVA on rank with SNK post hoc test would be used. The analysis was performed in the SigmaPlot version 11. Significant difference was determined at $p < 0.05$.

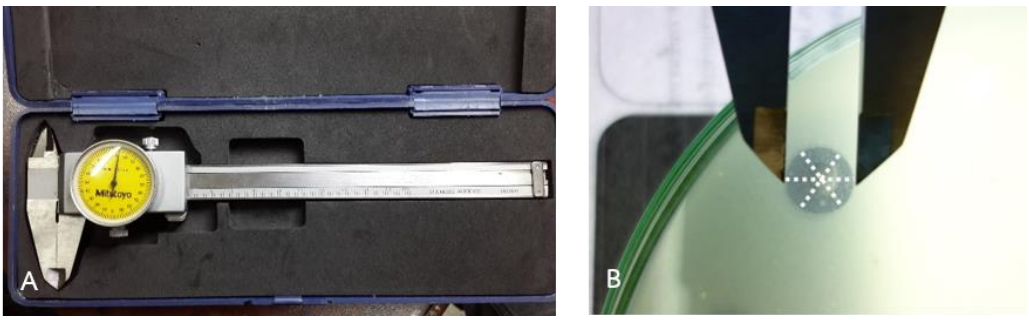

Figure 3-3 (A) The vernier caliper was used for measuring the clear zone diameter. (B) Three different lines of diameter were measured to average the value in case the clear zone was not circular.

In addition, clear zones were stained with 1% methyl red to verify the presence of fatty acid which is the product from phospholipase activity.

3. Development of Proteolytic Assay

This assay was modified from Wang and Huang (2002). Azocasein was used as a substrate of protease. The stock of azocasein solution was prepared at 5 µg/µL. Azocasein was mixed with test substances in each group including the control group (normal saline) and the negative control group (venom at 10 µg/µL). In addition, to enhance the effect of proteolytic enzyme in the venom (Wang et al., 2004), venom at 10 µg/µL with the solution of divalent metal ion including $CaCl_2$ group (venom and 5 mM of $CaCl_2$ solution) and $MgCl_2$ group (venom and 5 mM of $MgCl_2$ solution) were also added to the experiment. Lastly, the pancreatin group (pancreatin solution at 5 µg/µL) was used as a positive proteolytic activity

Substances in each group were mixed in a microtube and incubated at 37°C for 90 minutes. Then, the trichloroacetic acid (TCA) solution at 5% was added and the mixture was centrifuged at 1,000 xg for 5 minutes. After that, the supernatant was transferred to a 96-well plate and added with NaOH at 0.5 M before measuring the absorbance at 450 nm with a microplate reader (Multiskan EX). Azocasein is reddish orange in color, while the cleaved azo dye is yellowish in color. Higher absorbance at 450 nm represents a higher amount of azo dye indicating high proteolytic activity of

the venom. There were 3 replications in each experiment group. The average absorbance value of these experiment groups was assessed for normality of distribution and homogeneity of variance. If the result complied with the parametric assumption, the data would be assessed by one-way ANOVA with Student-Newman-Keuls (SNK) post hoc test. If not, the Kruskal-Wallis one-way ANOVA on rank with SNK post hoc test would be used. The analysis was performed in the SigmaPlot version 11. Significant difference was determined at $p < 0.05$.

4. Development of Coagulation Assay

The evaluation of coagulation activity was modified from the previous studies (Rodrigues et al., 2000; Oliveira et al., 2016). The discarded human whole blood (Figure 3-4A) was obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand. The use of human blood was carried out with prior permission from the National Blood Center's committees (COA No. NBC 12/2018). The whole blood was centrifuged at 700 xg at 4°C for 10 minutes for the blood fractionation. Plasma in the supernatant was separated and kept at -20°C until assay (Figure 3-4B).

Figure 3-4 (A) The discarded human whole blood bag obtained from the National Blood Center was centrifuged for (B) plasma collection.

Before the assay development, the obtained plasma contained the anticoagulant CPDA-1 (Beutler and West, 1979). This anticoagulant contains citrate which has the property of binding with free calcium ions in the solution to inhibit the coagulation cascade. To resume the cascade, the $CaCl_2$ solution was added as the source of free calcium ion. The range-finding method was used for finding the suitable concentration of $CaCl_2$ solution that neutralized the anticoagulant in the plasma stock.

Briefly, there were 3 main experiment groups of this part: the control group (plasma and PBS), the calcium group (plasma and $CaCl_2$ at x mol/L (M)) and the calcium and venom group (plasma, $CaCl_2$ at x M and venom at 5 µg/µL). Concentration "x" refers to various concentrations of $CaCl_2$ solution used in this study. The volume of plasma, $CaCl_2$ solution and venom solution in each well was 90, 5 and 5 µL, respectively. The group without $CaCl_2$ solution or venom solution was filled with PBS until the volume was 100 µL per well. After incubation at 37°C for an hour, optical density at 405 nm was measured with the microplate reader (Multiskan EX). The initial concentration of $CaCl_2$ was 0.46 M which came from the calculation between the amount of citrate molecule in the plasma stock and the reaction between citrate and calcium ion. Other concentration values were adjusted from the preliminary test for the best result. The suitable concentration of $CaCl_2$ solution is the concentration that yields the largest difference in optical density (OD) between the calcium group (low OD) and the calcium and venom group (high OD).

Once the CPDA-1 was neutralized, the neutralized plasma (95 µL) was mixed with test substances in each group including the control group (5 µL PBS), the negative control group (5 µL of venom at 5 µg/µL) and the positive control group (5 µL of the mixture of venom and antivenom serum) in a 96-well plate. After incubation at 37°C for an hour, OD_{405} was measured with the microplate reader (Multiskan EX). There were 3 replications in each experiment group. The result was tested for the normality and homogeneity of variance. To compare results between PBS and venom groups at the same $CaCl_2$ concentration, the data would be analyzed by the Student *t*-test if they fitted with the parametric assumption. Otherwise, the data would be compared by Mann-Whitney U-test. Significant

difference was determined at $p < 0.05$. From the preliminary test, 60 minutes is the shortest incubation time with the highest OD value, and the OD_{405} is the suitable wavelength for detecting the fibrin or the coagulation activity in each group. Higher OD_{405} represents a higher amount of fibrin indicating high coagulation activity of the venom.

5. Development of Fibrinogenolytic Assay

The evaluation of fibrinogenolytic activity was described by Oliveira et al. (2016). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for detecting the separation of alpha and beta-peptide from fibrinogen which is the sign of fibrinogenolytic activity. Fibrinogen (6 µL at 10 µg/µL) was mixed with test substance in each group including the control group (19 µL PBS), the negative control group (6 µL of venom at 10 µg/µL and 13 µL PBS), the positive control group (6 µL antivenom serum and 13 µL PBS), the 2x venom group (6 µL of venom at 20 µg/µL and 13 µL PBS), and fibrinogen with venom and antivenom serum group (13 µL fibrinogen solution, 6 µL of venom at 10 µg/µL and 6 µL antivenom serum). Other groups used in the experiment were antivenom serum only group (6 µL antivenom serum and 19 µL PBS), and venom only group (6 µL of venom at 10 µg/µL and 19 µL PBS). Amount of protein used in each experiment group was based on prior quantification of total protein content with Bradford's assay and adjusted to 3-5 µg.

The mixture were incubated at 37°C for 1 hour in a water bath, then the reaction was added with 5 µL of the mixture between beta-mercaptoethanol for stopping the reaction in the sample and bromophenol blue for staining the band (Figure 3-5A). Then, the mixtures were boiled for 5 minutes (Figure 3-5B). All samples and protein standards (Precision Plus Protein™ Dual Color Standards, Bio-Rad) were transferred to 12% polyacrylamide gel (12% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad) and run the current at 90 V for 2 hours (Figure 3-5C). Gels were stained with Coomassie Blue G-250 and destained with 10% acetic acid before visualization the position of protein band in each lane. The fibrinogenolytic activity of venom

represents by the separated band of alpha-fibrinopeptide. High intensity of the separated alpha-fibrinopeptide band means the high fibrinogenolytic activity.

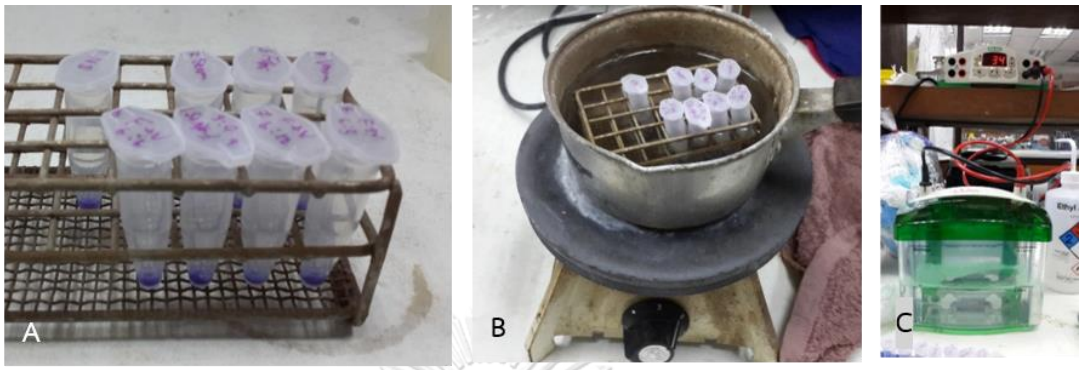

Figure 3-5 (A) The sample buffer was added to the mixture of each group before (B) boiling for 5 minutes. (C) After that, the SDS-PAGE technique was used for detecting the separated band of alpha-fibrinopeptide.

6. Development of Hemagglutination Assay

The hemagglutination activity was evaluated based on Oliveira et al. (2016) with some modifications. The whole blood obtained from the National Blood Center was centrifuged at 700 xg at 4°C for 10 minutes for the blood fractionation (Figure 3-6A). After removal of the plasma, erythrocytes were washed 3 times with normal saline and finally diluted to 2% suspension in normal saline. Erythrocyte suspension (90 µL) was mixed with substances in each group including the control group (10 µL PBS), the negative control group (5 µL of serial dilutions of venom and 5 µL PBS), and the positive control group (5 µL of mixture between venom and the serial dilution of antivenom serum and 5 µL PBS). The serial dilution of venom started with the original concentration of venom at 10 µg/µL. The venom was serially diluted at 2-fold until the final concentration reached 1/128 of the original stock. For the serial dilution of antivenom serum, original stock of antivenom was serially diluted at 2-fold until the final concentration reached 1/128 of the original stock. Each dilution of antivenom serum (5 µL) was mixed with 5 µL of the venom at the concentration of 10 µg/µL before mixing with the erythrocyte suspension.

In addition, positive hemagglutination activity was prepared by mixing the erythrocyte suspension with the serial dilution of an antibody against the specific blood type at 1/1 ratio to 1/128 ratio. The serial dilution of antibody was prepared in similar fashion to the venom dilutions (Figure 3-6B).

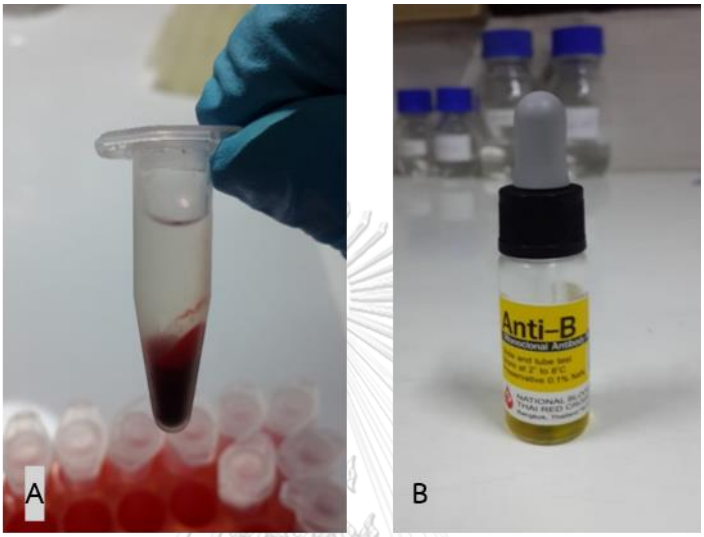

Figure 3-6 (A) The whole blood was centrifugated for separation of the erythrocytes. The 2% erythrocyte suspension was tested with various experiment group in the 96-well plate. In addition, erythrocyte suspension was tested against (B) the antibody of the same blood type to serve as positive hemagglutination of erythrocyte.

Mixtures in every experiment groups were transferred to the u-shape bottom 96-well plate for visualization. After incubation at 37°C for 2 hours, there was 2 types of result: hemagglutination or the spreading pattern of erythrocyte on the surface of the well (Figure 3-7A) and non-hemagglutination or the group of small sinking blood droplet at the bottom of the well (Figure 3-7B). Then, the minimum dilution of venom, antivenom serum, or antibody that represents the hemagglutination was transformed to titer value for the comparison between the experiment group.

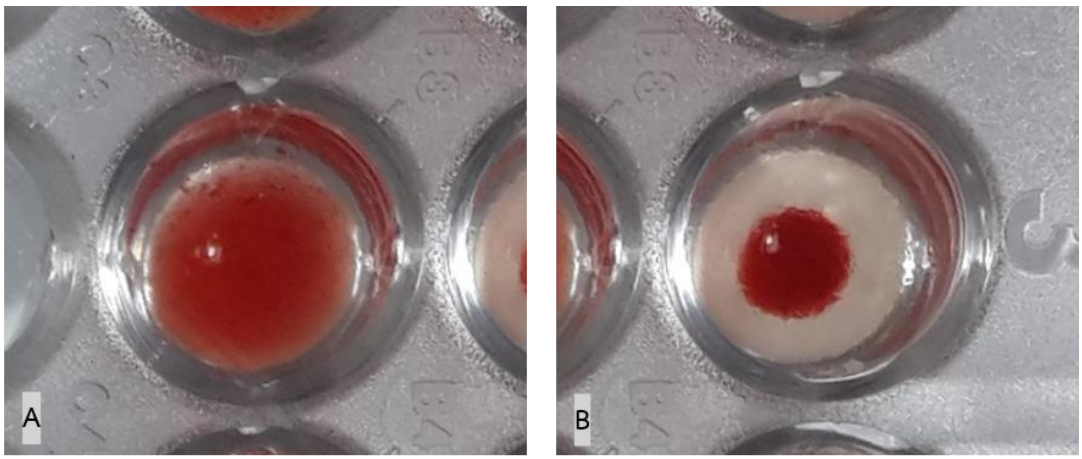

Figure 3-7 (A) Representative results of hemagglutination assay. The set with hemagglutination activity showed the spreading pattern of erythrocytes on the surface of the well while (B) the non-hemagglutination activity showed the group of small sinking blood droplets at the bottom of the well.

Results

1. Development of Phospholipase Assay

After incubation for 18 hours, the negative control group (venom) showed the clear zone as a turbid area around the well (tip of the arrow in Figure 3-8). The average diameter of the negative control group is 0.33 ± 0.03 cm. No clear zone was found in other experiment groups including the control group (normal saline) and the positive control group (the mixture of venom and antivenom serum) (Figure 3-9). The clear zone diameter of the negative control group was significantly higher than the control group and positive control group, while there was no significant difference between the control group and positive control group (Kruskal-Wallis one-way ANOVA on rank with SNK post hoc test; $H = 19.038$, $df = 2$, $p < 0.001$).

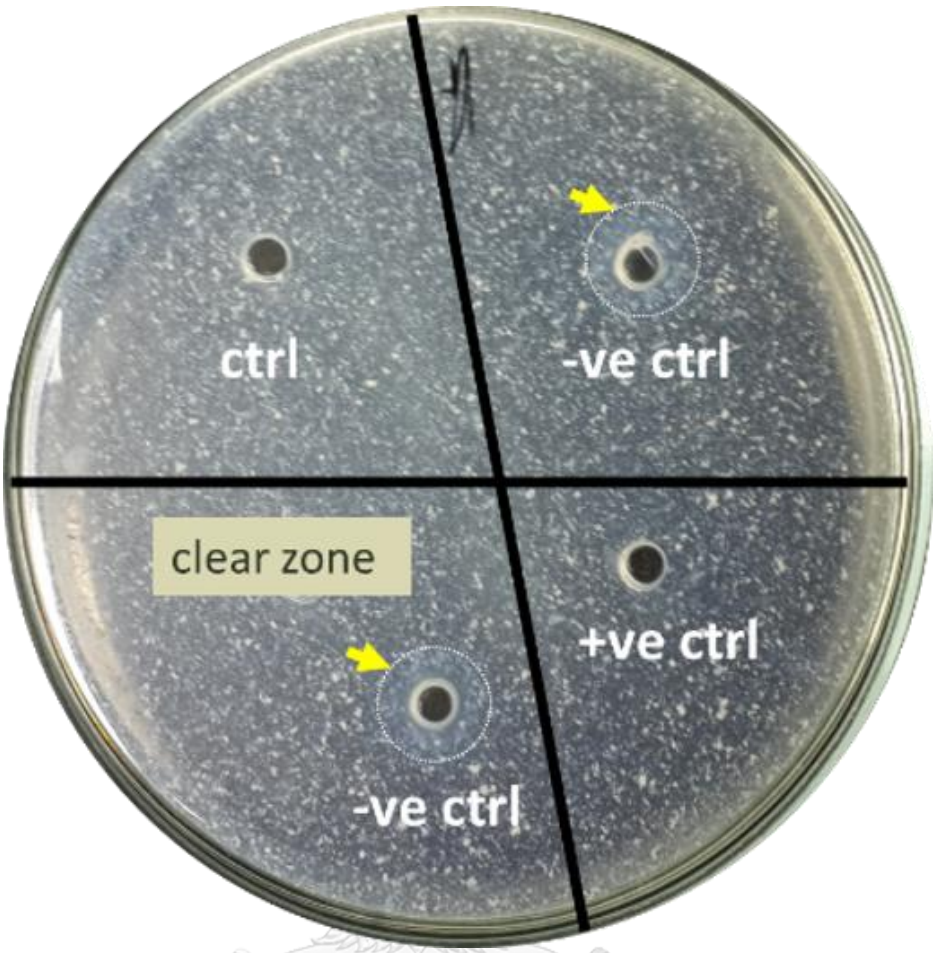

Figure 3-8 Representative agar plate of the phospholipase assay showed a large clear zone in the negative control group (-ve ctrl group; venom). Arrows point at the margin of the clear zone and the circle surrounding the area of the clear zone, while the control group (ctrl group; normal saline) and positive control group (+ve ctrl group; the mixture of venom and antivenom serum) show no clear zone.

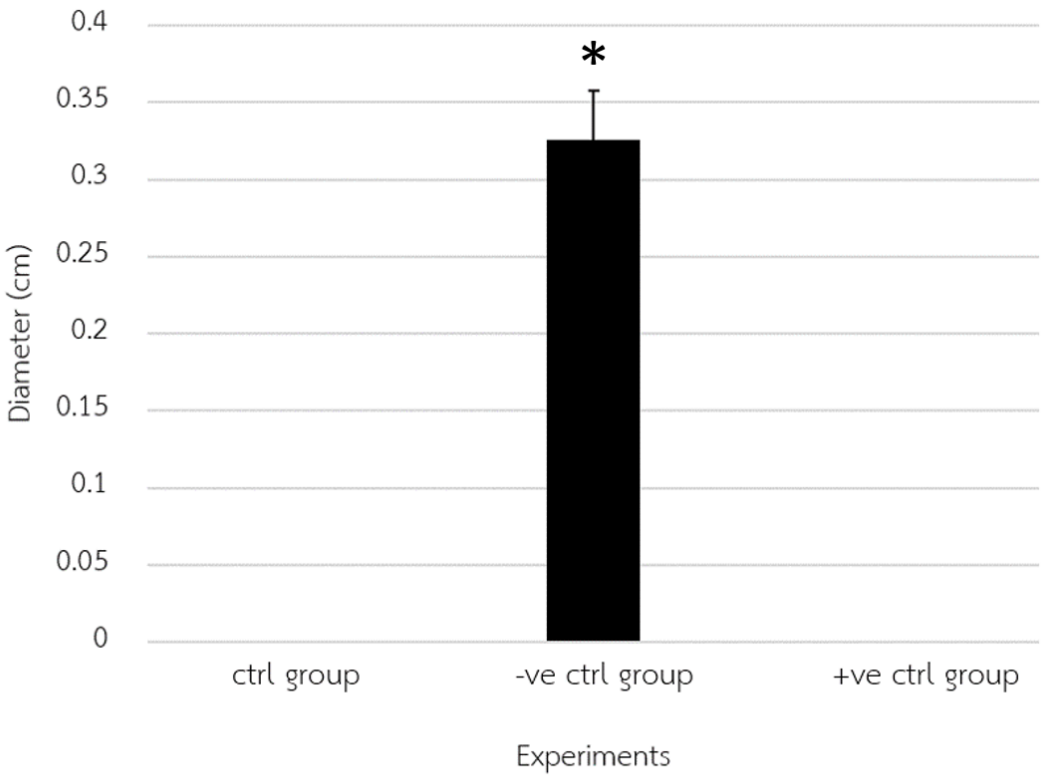

Figure 3-9 Bar graph (mean ± S.D.) of the diameter of a clear zone in the agar plate of the phospholipase assay. The negative control group (-ve ctrl group; venom) shows the clear zone of 0.33 ± 0.03 cm, while the control group (ctrl group; normal saline) and the positive control group (+ve ctrl group; the mixture of venom and antivenom serum) show no clear zone formation. An asterisk (*) indicates significant difference (Kruskal-Wallis one-way ANOVA on rank with SNK post hoc test; $H = 19.038$, $df = 2$, $p < 0.001$) from the other groups.

Methyl red staining was used to verify that the clear zone area in the agar plate occurred as a result of the phospholipase activity. The agar plate mainly consists of agar and yolk solution with pH of 6.0 – 7.0. The reaction between phospholipase in *D. siamensis* venom and phospholipid in agar produces fatty acid as the product, resulting in a decrease in pH. Therefore, the color of the agar would appear yellowish, while the area with fatty acid would turn to orange or red (Vallee and Gibson, 1948).

The result from methyl red staining showed a clear zone in orange compared to the nearby agar in yellow. This result indicated that the clear zone has low pH and confirmed the presence of fatty acid in the clear zone as well as the phospholipase activity in the venom (Figure 3-10B).

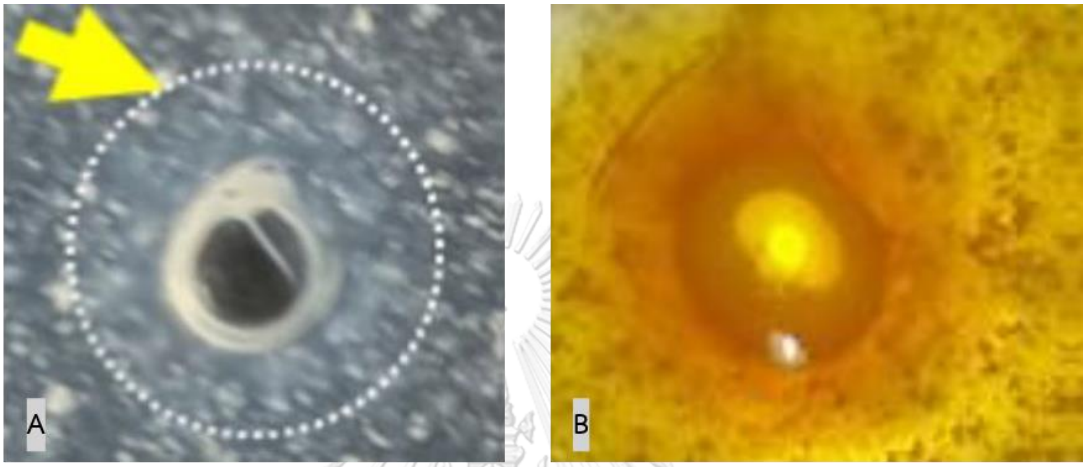

Figure 3-10 (A) The negative control group (venom) showed a clear zone around the well. (B) After staining with 1% methyl red, the clear zone was orange in color while the nearby area was yellow in color.

2. Development of Proteolytic Assay

After the addition of a TCA solution and centrifugation, the color of supernatant in the pancreatin group (pancreatin solution) changed to yellow indicating presence of an azo dye as a result of proteolytic activity. Meanwhile, the control group (normal saline), the negative control group (venom), the $CaCl_2$ group (venom at 10 µg/µL and $CaCl_2$ at 5 mM) and the $MgCl_2$ group (venom at 10 µg/µL and $MgCl_2$ at 5 mM) show the clear solution with yellow sediment of azocasein at the bottom of microtube, indicating minimal proteolytic activity (Figure 3-11).

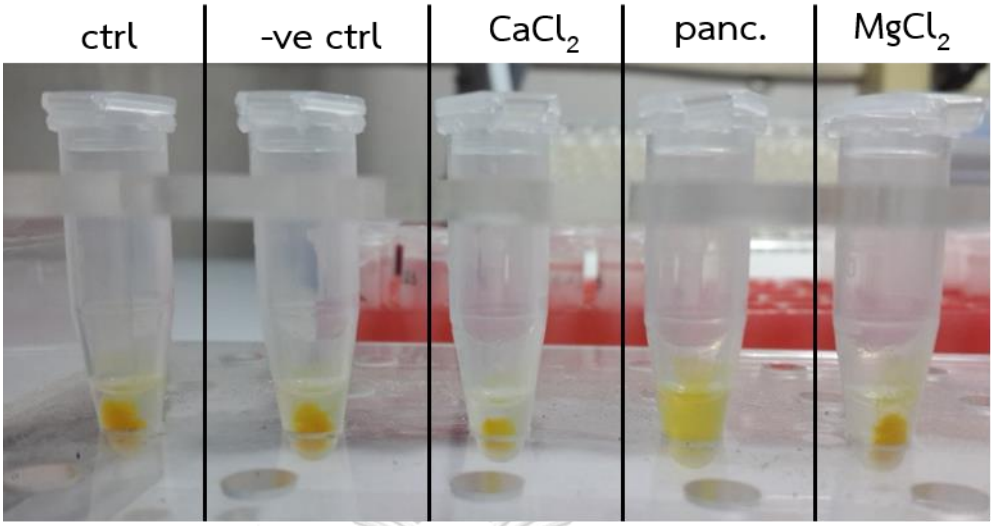

Figure 3-11 The result of the proteolytic assay showed that the positive proteolytic activity (yellow color solution) was found only in the pancreatin group (panc. group; pancreatin solution), while other groups including the control group (ctrl group; normal saline), the negative control group (-ve ctrl group; venom at 10 µg/µL), the $CaCl_2$ group (venom with calcium ion), and the $MgCl_2$ group (venom with magnesium ion) show clear solution with yellow sediment.

Measuring for absorbance at 450 nm of the supernatant also confirmed that the high A_{450} value was found in only the pancreatin group. The statistical analysis showed that the A_{450} of the pancreatin group was significantly higher than other groups (one-way ANOVA and SNK post hoc, $p < 0.05$), while other pair-wise comparisons showed no significant difference (Figure 3-12). Although calcium and magnesium are known to enhance the proteolytic activity of snake (*Agkistrodon acutus*) venom (Wang et al., 2004), addition of these divalent metal ions (venom with Ca^{2+} and Mg^{2+}) did not yield a marked difference from the control group (normal saline) nor the negative control group (venom). As a result, the assay was not further tested with the positive control group (the mixture of venom and antivenom serum) in the experiment.

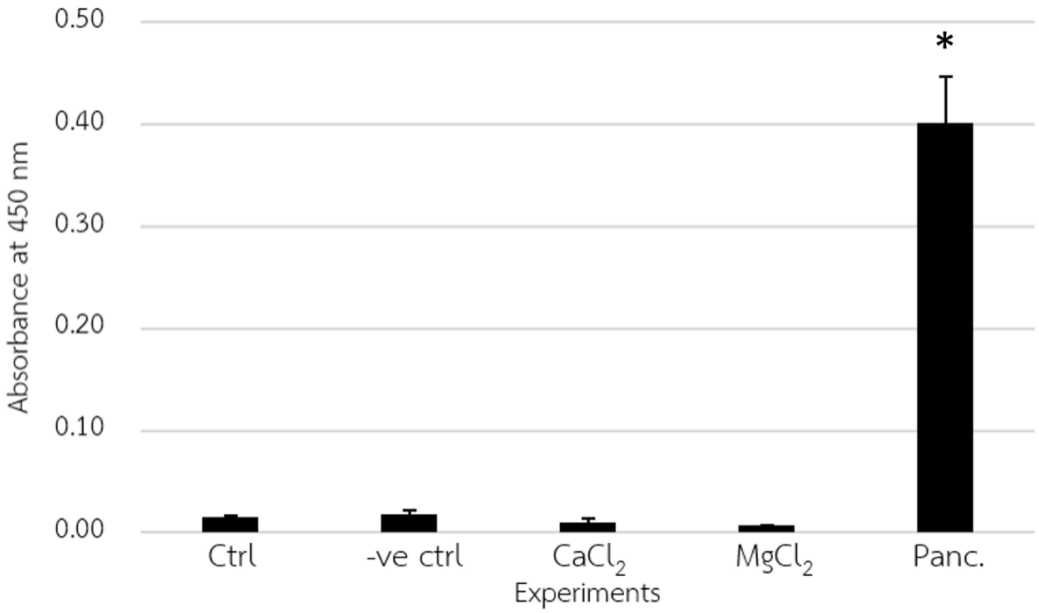

Figure 3-12 Bar graph (mean ± S.D.) showing absorbance at 450 nm of the supernatant from the proteolytic assay. The high A_{450} value was found only in the pancreatin group (panc. group) at 0.401 ±0.046, while the control group (ctrl group), negative control group (-ve ctrl group), $CaCl_2$ group and $MgCl_2$ group show very low A_{450} value. An asterisk (*) indicates a significant difference (one-way ANOVA and SNK post hoc, $p < 0.05$) from the other groups.

3. Development of Coagulation Assay

Since the plasma used in this study contained CPDA-1 anticoagulant, it is thus necessary to find the optimal concentration of $CaCl_2$ required for deactivating (or neutralizing) the anticoagulant. The optimal concentration of $CaCl_2$ solution was calculated from the range-finding method. From Figure 3-13, the concentration that showed the largest difference in OD_{405} value between venom added (high OD) and non-venom added (low OD) is "CaCl2_0.2875" or the optimal concentration of 0.2875 M. Comparison between the PBS group and the venom group at the same concentration of $CaCl_2$ revealed that significant differences were found at 0.2875 M (Student *t*-test; t = -3.458, df = 4, p = 0.026) and 0.23 M (Student *t*-test; t = -3.900, df = 4, p = 0.018), while no significant difference was found at other $CaCl_2$

concentration (Student *t*-test; $p > 0.05$). The result of incubating the neutralized plasma (95 µL) with test substances showed that turbid plasma was found in the negative control group (5 µL of venom at 5 µg/µL) or venom-added group (middle well, Figure 3-14) while the control group (5 µL PBS) and the positive control group (5 µL of mixture between venom and antivenom serum) showed clear plasma (upper and lower well, Figure 3-14).

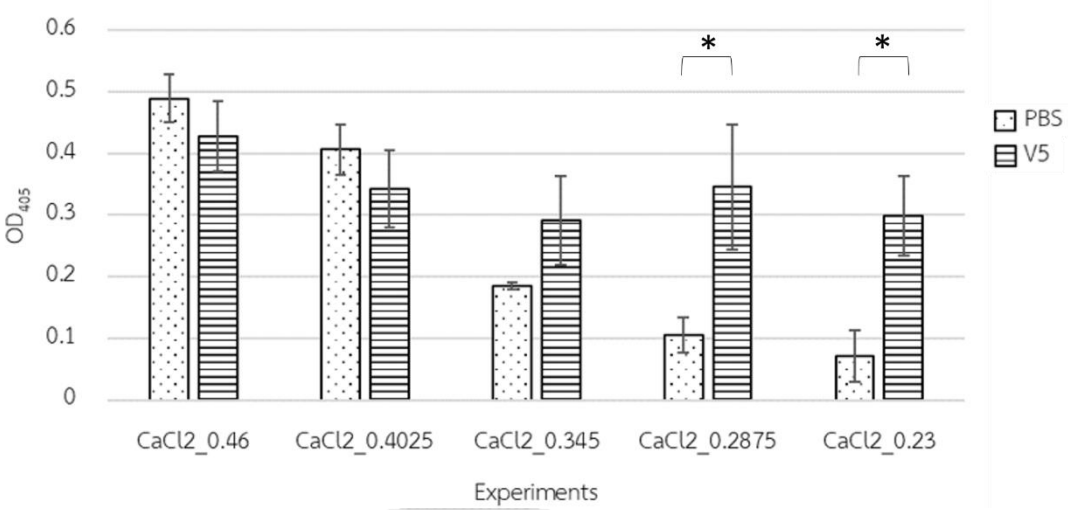

Figure 3-13 Bar graph (mean ± S.D.) representing optical density at 405 nm of plasma in the range-finding assay for optimal concentration of $CaCl_2$ that neutralizes the anticoagulation activity. The graph with the dot pattern shows the control group (plasma with PBS) and the graph with the cross pattern shows the negative control group (plasma with venom). The optimal concentration is 0.2875 M of $CaCl_2$ solution as shows in the "CaCl2_0.2875" column. The legend "PBS" was the result on PBS-added group while "V5" is the group with venom at 5 µg/µL. ("CaCl2_x" when "x" means the concentration of $CaCl_2$ solution) A significant difference (Student *t*-test; $n = 3$, $p < 0.05$) between the group is indicated by an asterisk (*).

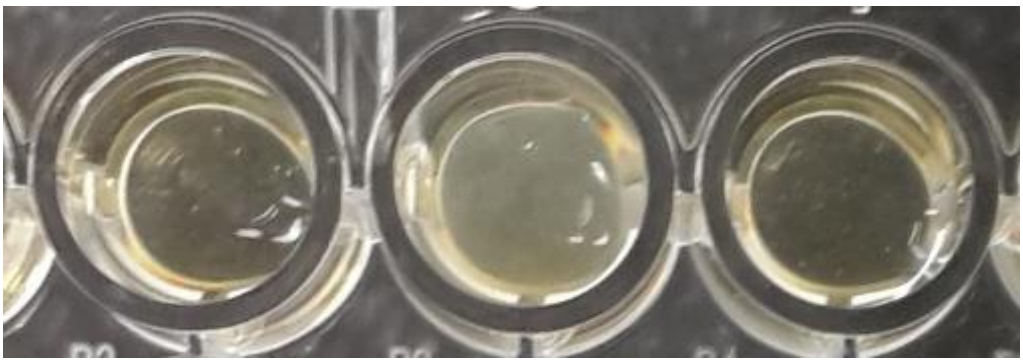

Figure 3-14 The result of the control group or PBS-added group (left well), the negative control group or venom-added group (middle well) and the positive control group or the mixture between venom and antivenom serum (right well) showed that the turbid solution, i.e. coagulating plasma, was found only in the negative control group.

4. Development of Fibrinogenolytic Assay

Fibrinogen composes of 3 subunits with different molecular weights including alpha, beta, and gamma subunits. Alpha-fibrinopeptide is the heaviest, followed by beta and gamma-fibrinopeptide, respectively (McKee et al., 1970). In Figure 3-15, the major difference between lane 4 (fibrinogen) and lane 6 (fibrinogen and venom) is the absence of the uppermost band or alpha-fibrinopeptide in the venom treated fibrinogen (arrows on Figure 3-15), while beta and gamma-fibrinopeptide are intact. The rectangle box in lane 6 shows the putative fragment of alpha-fibrinopeptide. In addition, a comparison between lane 4 (fibrinogen) and lane 7 (fibrinogen and the mixture of venom and antivenom serum) showed the neutralizing effect of antivenom serum against venom that can be verified by the existence of the alpha-fibrinogen band.

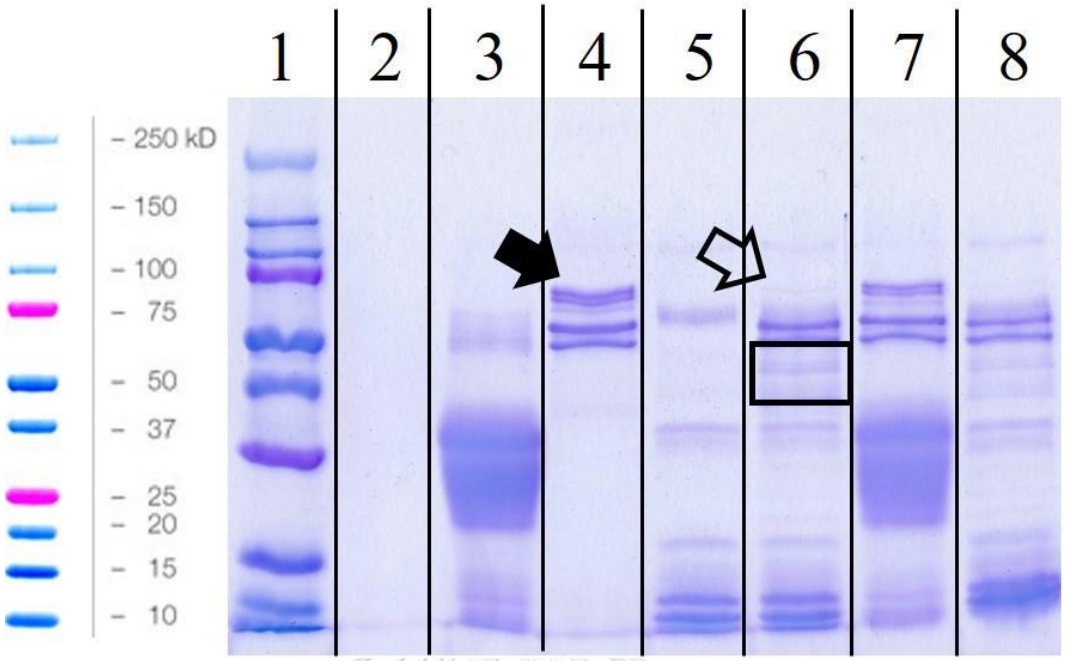

Figure 3-15 SDS-PAGE of the fibrinogen and the different test substances. A solid arrow points at the alpha-fibrinopeptide band while the transparent arrow points at the disappearance of alpha-fibrinopeptide caused by *D. siamensis* venom. The rectangle box in Lane 6 indicates the fragment of alpha-fibrinopeptide. Lane 1 is protein standard with the corresponding molecular weight shown in the adjacent chart (on the left); Lane 2 is PBS; Lane 3 is antivenom serum; Lane 4 is fibrinogen; Lane 5 is *D. siamensis* venom; Lane 6 is the mixture of fibrinogen and venom; Lane 7 is fibrinogen and the mixture of venom and serum; Lane 8 is the mixture of fibrinogen and 2x venom.

5. Development of Hemagglutination Assay

To test for erythrocyte hemagglutination activity, the antibody of the same blood type was used as the positive hemagglutinant. Hemagglutination was evident as a spreading pattern of erythrocyte on the well surface as seen in wells with a high concentration of antibody (1/8 to 1; Figure 3-16). On the contrary, incubating erythrocyte with *D. siamensis* venom shows very little (negligible) hemagglutination even with the high concentration of venom.

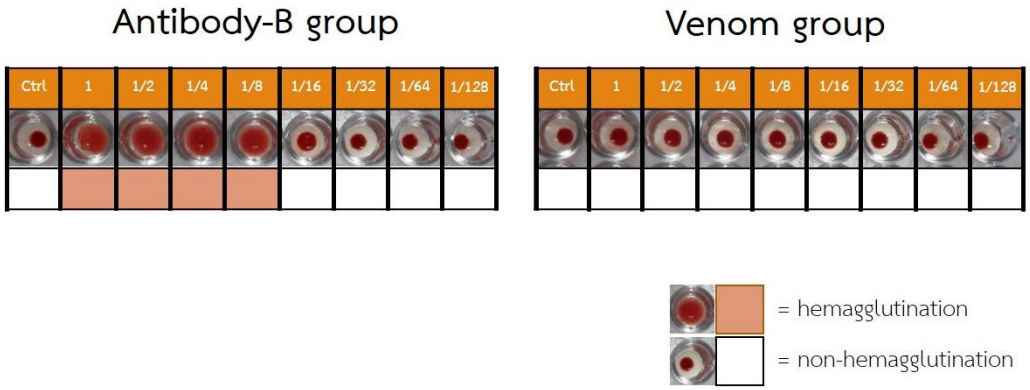

Figure 3-16 The reaction between 2% erythrocyte suspension and test substance (antibody on the left and venom on the right). The antibody group shows the hemagglutination, while the venom group shows non-hemagglutination at any dilution. The letter above the photograph refers to the experiment groups (ctrl = control group, 1 = the concentration of substance at 10 µg/µL, 1/2 = the concentration of substance at 5 µg/µL, and so on) The table below the photograph shows the record of hemagglutination (filled cells represent hemagglutination; blank cells represent non-hemagglutination).

Discussion

To develop screening assays for medicinal herb with anti-hemotoxic activity against eastern Russell's viper venom, *in vitro* assay based on biological activity of *D. siamensis* venom is needed. In this study, a set of *in vitro* tests including phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay were developed.

Phospholipase A_2s (PLA_2) includes many protein families with a common enzymatic activity (Burke and Dennis, 2009). In *D. siamensis* venom, a secretory phospholipase A_2 ($sPLA_2$) is a major enzyme in the venom (Risch et al., 2009). In the phospholipase activity assay, the yolk solution is the main source of phospholipid which contains 31% of total lipid in the yolk (Aro et al., 2009). PLA_2 can hydrolyze phospholipid into lysophospholipid and fatty acid (Kini and Evans, 1989). The presence of acidic clear zone from fatty acid in the phospholipase assay confirms the presence of PLA_2 in *D. siamensis* and validate that this assay is suitable to be used as one of the tests for the hemotoxic activity of the snake venom.

In the coagulation assay, the whole blood obtained from the National Blood Center, the Thai Red Cross Society was used. This blood has CPDA-1 as an anticoagulant allowing a long term storage for up to 28 days (Fernandes da Cunha et al., 2005). Citrate found in CPDA-1 solution can bind to calcium in the plasma, thus preventing the calcium to initiate a coagulation cascade (Davie et al., 1991). In the coagulation assay, $CaCl_2$ solution was added to increase calcium ion for reacting with free citrate molecules to neutralize the anti-coagulation activity. As a result, the presence of any active coagulant in snake venom may initiate the coagulation cascade right away. In *D. siamensis* venom, the enzyme in family metalloproteinase, as well as other groups, can inhibit or enhance the coagulation activity (Chen et al., 2008; Risch et al., 2009).

In this study, optical density at 405 nm was used for turbid measurement based on the preliminary study which screened 6 wavelengths (405, 450, 540, 595, 620 and 690 nm) and found that the wavelength that yielded the largest difference in OD value between the control group and the negative ctrl group (venom group) was 405 nm. The use of this wavelength was also reported in other studies

(Kraus, 1996; Nakamura et al., 1999; Suwanchaikasem et al., 2013). The incubation time is also the key factor in this assay. In the preliminary test, the 96-well plate was observed every 10 minutes during the incubation period of upto 120 minutes. Sixty minutes was selected for the proper incubation time with the highest difference in OD value between each experiment group.

It is known that *D. siamensis* venom contains a lot of enzymes in the family metalloproteinase and serine protease that can cause hematotoxic symptoms (Castro et al., 2004). The fibrinogenolytic activity is one of the effects of the enzyme in this family. The current result showed that *D. siamensis* venom selectively cleaved the alpha chain but not beta and gamma chain, inferring that *D. siamensis* venom has alpha-fibrinogenolytic activity. This activity also found in the venom of other snake species (Lee et al., 2014; Coimbra et al., 2018). Activity of the alpha-fibrinogenase found in *D. siamensis* venom could cause the degradation of alpha-fibrinopeptide of fibrinogen as seen in this study (Sukkapan et al., 2011).

For the proteolytic assay, azocasein is the non-specific protease substrate used for the detection of the protease activity (Tomarelli, 1949). High OD_{405} value means higher amount of protease in the solution. In this study, pancreatin has been successfully used as a positive proteolytic agent in the assay. However, the negative ctrl group (venom at 10 µg/µL) and the 2 groups of venom with divalent metal ion (venom + $CaCl_2$ solution and venom + $MgCl_2$ solution) did not show any increase in OD_{405} compared to the control group (normal saline). Although calcium and magnesium ions were known to enhance the proteolytic activity of *Agkistrodon acutus* venom, a hemotoxic venom similar to *D. siamensis* venom (Wang et al., 2004), addition of these divalent ions did yield any change in OD_{405}. It could be concluded that *D. siamensis* venom has very little (negligible) proteolytic activity.

The important protein involved in the agglutination activity in other snake species is C-type lectins (Zelensky and Gready, 2005). This protein can bind with oligosaccharide in the surface of the cell to induce the agglutination of erythrocyte (Carvalho et al., 1998) and human platelet (Ogilvie et al., 1989). However, result of this study showed that *D. siamensis* venom did not cause hemagglutination even

with the high concentration of venom, suggesting that the venom contains very little (negligible) hemagglutination activity.

Although previous case reports (Phillips et al., 1988; Ratcliffe et al., 1989; Kularatne, 2003) showed that the venom from *Daboia* species contains 5 major biological activities tested in this study, proteolytic and hemagglutination activities were reported to be minor symptoms with the lowest effect to snakebite patients. This indicates that proteins involved with these 2 activities might be present in minute quantity, and thus unlikely to be tested with the further screening assays.

Conclusion

In this study, three *in vitro* assays including phospholipase assay, coagulation assay, and fibrinogenolytic assay have been successfully developed and effectively showed a marked difference between positive and negative controls. Two *in vitro* assays including proteolytic assay and hemagglutination assay were proven to be not suitable for the screening assay because of the low to lack of activity in *D. siamensis* venom. Proteolytic and hemagglutination activities were reported to be minor symptoms of *Daboia* bite with the lowest effect to snakebite patients (Phillips et al., 1988; Ratcliffe et al., 1989; Kularatne, 2003). The proteins involved with these two activities might be present in minute quantity, and thus unlikely to be tested with the current assays. As a result, three successful *in vitro* assays including phospholipase assay, coagulation assay, and fibrinogenolytic assay could be further used, potentially with an *in vivo* assay, to screen for medicinal herbs with anti-hemotoxic activity against *D. siamensis* venom.

CHAPTER IV
DEVELOPMENT OF AN *IN VIVO* ASSAY FOR HEMOTOXIC ACTIVITIES OF THE EASTERN RUSSELL'S VIPER VENOM

Introduction

In a tropical region, such as Southeast Asia, venomous snakes are relatively common (Nijman, 2010; Somaweera and Somaweera, 2010) 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 (Yue et al., 2019). The eastern Russell's viper (*Daboia siamensis)* is one of the most important venomous snakes in Thailand with a high incidence of snakebites (Muangdang, 2015). Its venom possesses hematotoxin, which causes various pathological alterations to the circulatory system, including morphological changes of the erythrocytes and blood vessels, blood coagulation, kidney failure, and death (Mitrakul, 1979; Napathorn et al., 1998; Thamaree et al., 2000). Although antivenom serum is generally used in snakebite patients for standard medical treatment, its cost per dose is expensive and it is ineffective at treating some symptoms (Bawaskar and Bawaskar, 2001). Moreover, 5–80% of antivenom serum receivers may develop allergic reactions that can vary from redness through serious illness or even death (Williams et al., 2007; Cannon et al., 2008; Dhanya et al., 2009). Alternative treatments that are more effective but safer are, therefore, needed.

Local healers from many parts of Southeast Asia, especially Thailand, are known to use medical 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 (Phongphladisai and Panyawatthananukun, 2011). However, the number of scientific reports to support

the effectiveness and safety of traditional herbs is still limited, making it less widely accepted (Coe and Anderson, 2005; Veronese et al., 2005)

In order to test the therapeutic effect of herbs, the reliable and scientifically proven screening method is needed. However, the test of the interaction between the test substances and the biological mechanisms in living organisms or *in vivo* study cannot be performed directly in humans but must be first validated their safety and efficiency in animal models. Most of the studies in venom lethality and neutralization used mice as an animal model (WHO, 1981; Theakston and Reid, 1983). Also, an *in vivo* assay based on rodent models is normally used for testing the efficacy of herbs. This assay is time-consuming and expensive, 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 (Sells et al., 1998). One can handle many 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 (Rosenbruch, 1989), less than 10-day-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 (Rosenbruch, 1997). In fact, both the National Institute of Health, USA (National Institute of Health, 1991) and Thailand's Animals for Scientific Purposes Act B.E. 2558 (A.D. 2015) (Institutional Animal Care and Use Committee, 2015) 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.

This study aimed to develop an *in vivo* assay for the hemotoxic activities of the eastern Russell's viper's venom using 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 *in vivo* screening assay for medicinal herbs with anti-hematotoxic activity against the snake venom.

Materials and Methods

1. Animals

According to Thailand's Animals for Scientific Purposes Act B.E. 2558 (A.D. 2015) (Institutional Animal Care and Use Committee, 2015), 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. 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.

2. 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.

3. Chick Embryo Preparation

Fertilized chicken eggs on the day of laying (day 0) were transferred from Luangsuwanvajokkasikit farm, Kasetsart University to the laboratory at CU (Figure 4-1A). Eggs were sequentially cleaned with distilled water, 70% (v/v) ethanol solution, and povidone-iodine, and then dried with sterile gauze (Figure 4-1B). Eggs were labeled and incubated in the incubator. The incubating temperature used in this study was 36.9 ± 0.9 °C (mean ± S.D.) with relative humidity in excess of 80%, which was close to 37.8 °C or the suitable temperature for the incubation of chick embryos (Barott, 1937). During the incubation, eggs were laid on the plastic-coated wire mesh to prevent them from rolling. A tray with distilled water was placed inside the incubator to increase humidity in the incubator (Figure 4-1C). The eggs were

turned 4–6 times/day at 180 degrees which could facilitate the embryos to increase their blood oxygen concentration, hence allowing them to develop normally (Tazawa, 1980).

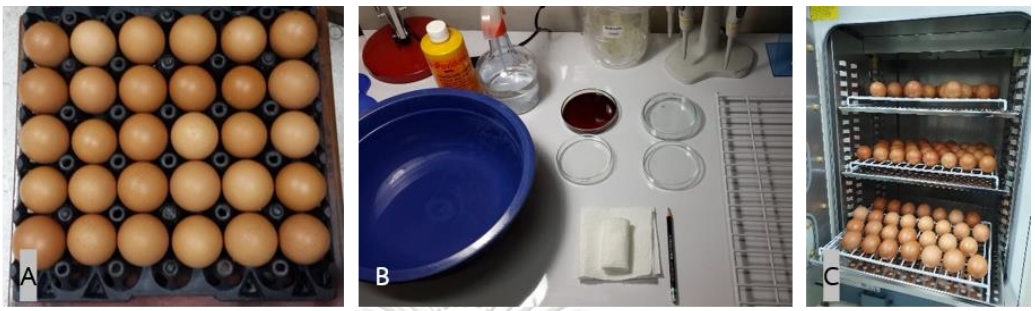

Figure 4-1 (A) Eggs on the day of laying were transferred to CU, (B) then cleaned by distilled water, 70% (v/v) ethanol, povidone-iodine solution, and then dried with sterile gauze, respectively. (C) All eggs were labeled and incubated in the incubator at 36.9 ± 0.9 °C with relative humidity in excess of 80%.

For the actual assay, a shell-less culture ("hammock technique") which was modified from Tahara and Obara (Tahara and Obara, 2014) was used to facilitate the treating protocol and to enable one to observe the chick embryo while under treatment. On the day 2 of incubation, eggs were cracked open (Figure 4-2A) and the egg content, including embryos, yolk, and albumen, was weighed (Figure 4-2B) and transferred onto the culture vessel (Figure 4-2C). The preparation of culture vessel started with 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.

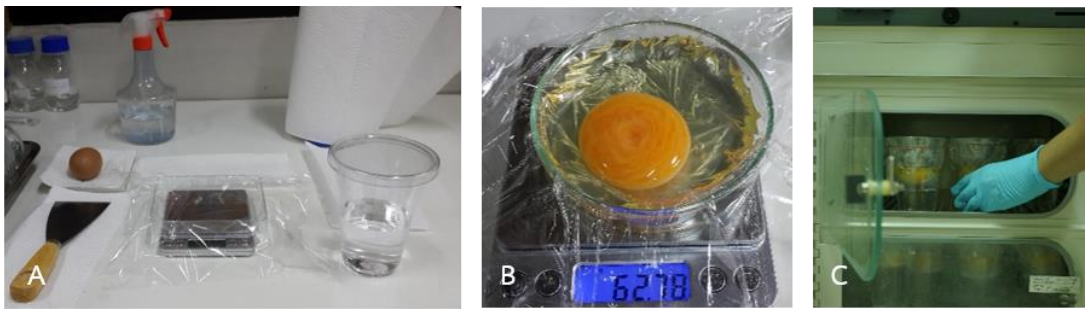

Figure 4-2 (A) The utensils used for cracking open the egg. (B) The content inside the egg was weighted before (C) being placed to culture vessel by hammock technique for incubation until day 6.

Eggs were transferred to the culture vessel on day 2 because of the appropriate thickness of yolk sac membrane. Prior study showed that the 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 (Sittishevapark, 2015). 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 at the same condition until ready for the assay on day 6. Dead embryo was routinely checked and quickly removed from the incubator. 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 (Hamburger and Hamilton, 1951; Sissman, 1970).

Number of chicken eggs used and number of chick embryos available for the *in vivo* assay after incubation are shown in Table 4-1. With the starting number of 300 eggs, 95 embryos were available for the assay, resulting in a 31.7% yield of chick embryos.

Table 4-1 Number of chicken eggs used and number of chick embryos available for the *in vivo* assay after incubation 36.9 ± 0.9 ℃. The 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	31.7

4. Mortality Rate and LD_{50} in Chick Embryo

Chick embryos on day 6 were used as the model for finding the LD_{50} value of the venom. Based on the egg content weight on 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 ℃ for 30 minutes before treating the embryo. The volume of venom in each group and the control group (normal saline) were normalized to 2 μL and dropped onto a 0.2 cm diameter circle filter paper (Whatman No. 1; Figure 4-3A, B) that was then placed over the lateral vitelline vein of the chick embryo (Figure 4-3C). The targeted blood vessel must be closed to the embryo and the tissue must float to the surface for increasing the success in tissue collection. Observation of the embryo's health was performed every hour and the number of dead embryos (no sign of heartbeat) was recorded at 4 hours after venom application. The number of deaths in the control group and at each venom concentration was calculated into the mortality rate and used in LD_{50} calculation by Probit Analysis in the SPSS statistics version 22 software.

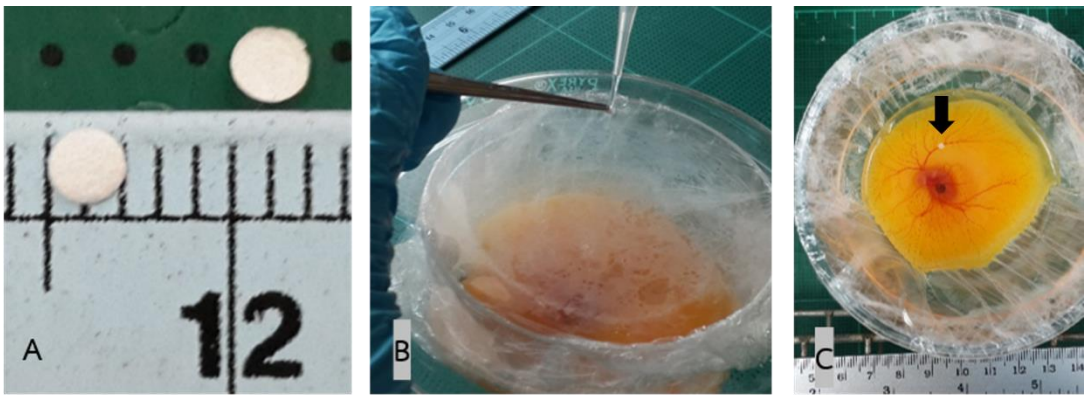

Figure 4-3 (A) The 0.2 cm diameter filter paper (Whatman No. 1) was used as an applicator onto the chick embryo blood vessel. (B) Aliquot (2 µL) of *D. siamensis* venom or normal saline was applied onto a 0.2 cm diameter filter paper. (C) The treated filter (arrow) paper was then placed over the lateral vitelline vein of the chick embryo.

5. Histological Study

5.1 Tissue Collection and Storage

Blood vessels at the lateral vitelline vein and surrounding membrane were collected with the aid of filter paper. A square filter paper (2 x 2 cm) with a diamond-shaped hole was placed onto the embryonic blood vessel, with the treated area located in the middle (Figure 4-4A). The membrane was cut along the border of the filter paper and carefully lifted together with the filter paper (Figure 4-4B, C). The membrane was cleaned with normal saline (Figure 4-4D) and immersed in 10% neutral buffered formalin for 24 h for fixation (Figure 4-4E). 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. The dead embryo was autoclaved before discarding as the biohazard waste.

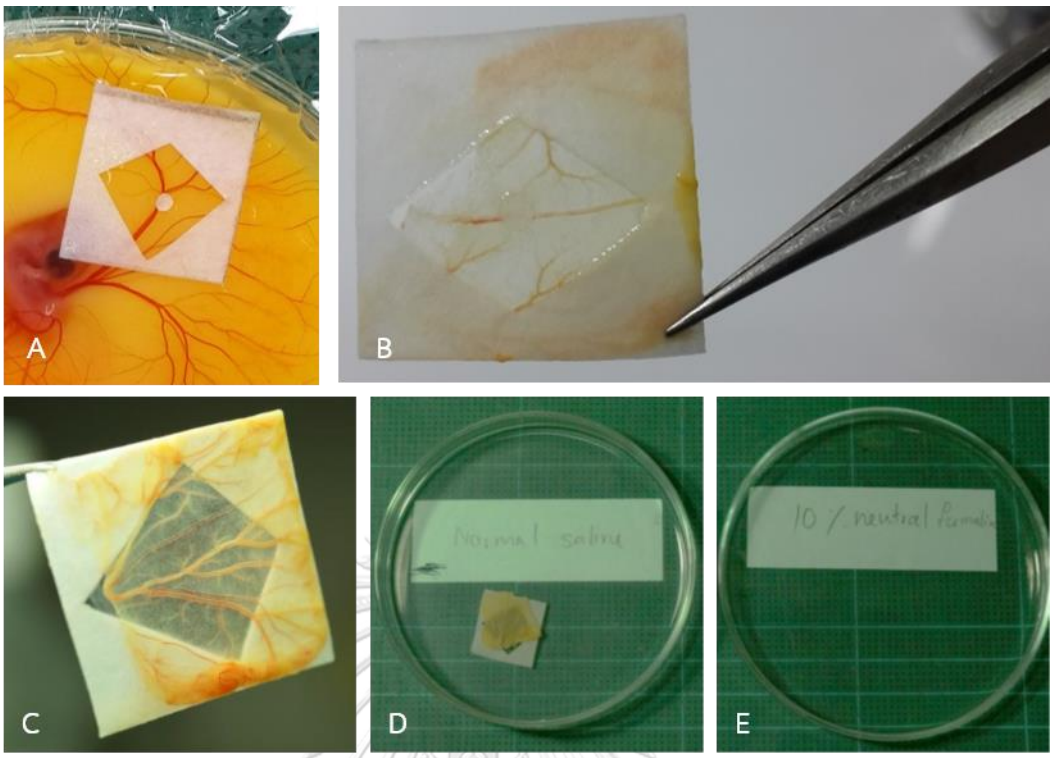

Figure 4-4 (A) Square filter paper with a diamond-shaped hole was placed over the treated area of the blood vessel of the chick embryo. (B, C) The filter paper with tissue was cut and lifted. (D) The tissue was washed with normal saline and (E) fixed in 10% neutral buffered formalin before storing it in the 70% ethanol until it was processed by the standard whole mount or paraffin method.

5.2 Tissue Processing and Staining

Two types of tissue processing and staining were used in this study: the whole-mount staining with hematoxylin and eosin (H&E) and cross-section staining with Periodic Acid–Schiff (PAS). PAS staining is used for staining the basement membrane (McManus, 1946) which is one of the targets of *D. siamensis* venom. Basement membrane has carbohydrates and carbohydrate-containing molecule (Ireland, 1978) which can be potentially interacted by PAS staining (Mcmanus, 1948). For the whole mount staining, formalin-fixed and alcohol preserved tissues were trimmed for the designate embryonic blood vessel with a minimum area of the surrounding membrane. These tissues were processed through the standard protocol for H&E staining (Suvarna and Layton, 2012). Tissues were attached to the slide by Permount™ for further microscopic observation. 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 the 5-μm thickness, and attached to the slides. Tissue slides were processed through the standard PAS procedure (Suvarna and Layton, 2012). Pathological alterations of the endothelial tissue and surrounding tissue were observed under a light microscope and recorded.

Results

1. Mortality Rate and LD_{50} of *D. siamensis* Venom in Chick Embryo

After the method for preparing embryos had been optimized (Sittishevapark, 2015), the mortality rate of each group (the control group and venom-treated groups) was calculated for the lethal dose of *D. siamensis* venom (Table 4-2). The LD_{50} of this batch of *D. siamensis* venom at 4-hour post-treatment was 16.54 µg/embryo or 8.27 µg/µL.

Table 4-2 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 doses of *D. siamensis* venom for 4 hours.

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

2. Histological Study

To examine the hematotoxic effects of venom, various histopathological markers were used, including the presence of clotted blood in the vessel (Homsi-Brandeburgo et al., 1988), changes in the basement membrane, loss of membrane integrity (Gutierrez et al., 2016), and ruptures in the blood vessel or signs of leaking of erythrocytes and other components to the outside (Ownby et al., 1974; Gutierrez et al., 2016).

Results from the whole mount staining of the venom-treated group showed an overview of the blood vessel and surrounding cells (Figure 4-5A). The blood

vessel was stained as a purple tube with a spongy-liked layer around the tube (Figure 4-5B). In some areas, the rupture of the endothelial cell and leaked erythrocytes and other components from the vessel were found (Figure 4-5C). In addition, densely packed erythrocytes, and other components in the blood vessel (blood clots) were found in the vessels of the venom-treated group (Figure 4-5D).

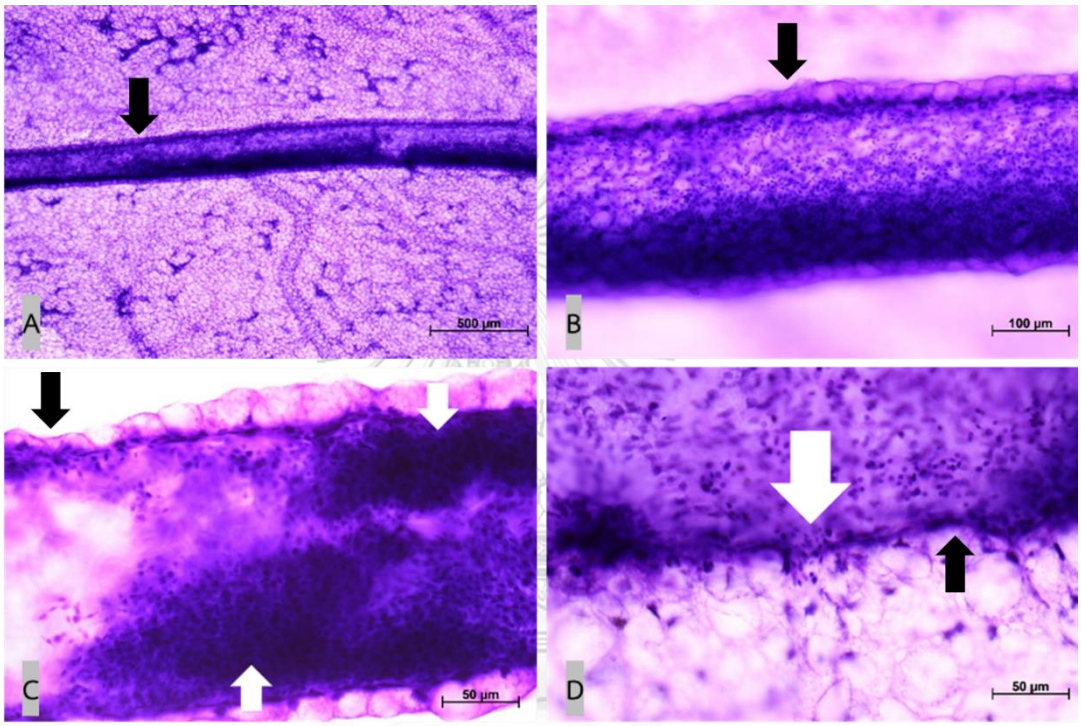

Figure 4-5 Representative micrographs of a blood vessel (black arrows) of the chick embryo in the *Daboia siamensis* venom-treated group (H&E stained). (A) Overview of the blood vessel at low (50 x) and (B) high (100 x) magnification. (C) Clotted blood (white arrow) can be found in some areas. (D) Endothelial wall showing signs of rupture and leakage of erythrocytes and other components (white arrow).

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 (McManus, 1946; Mcmanus, 1948; Ireland, 1978). Compared to the control group (Figure 4-6A), it was found that the venom treated group showed various changes in the blood vessel, including blood clots, which showed as a dense pack of

erythrocytes and other components (Figure 4-6B). Of importance to this assay, a high number of thrombocytes was found in the venom-treated group (Figure 4-7), inferring that the blood was clotting (Grant and Zucker, 1973). The basement membrane showed membrane shrinkage or loss of membrane integrity (Figure 4-6C). In addition, ruptures of the blood vessel and leakage of erythrocytes or other components were found (Figure 4-6D).

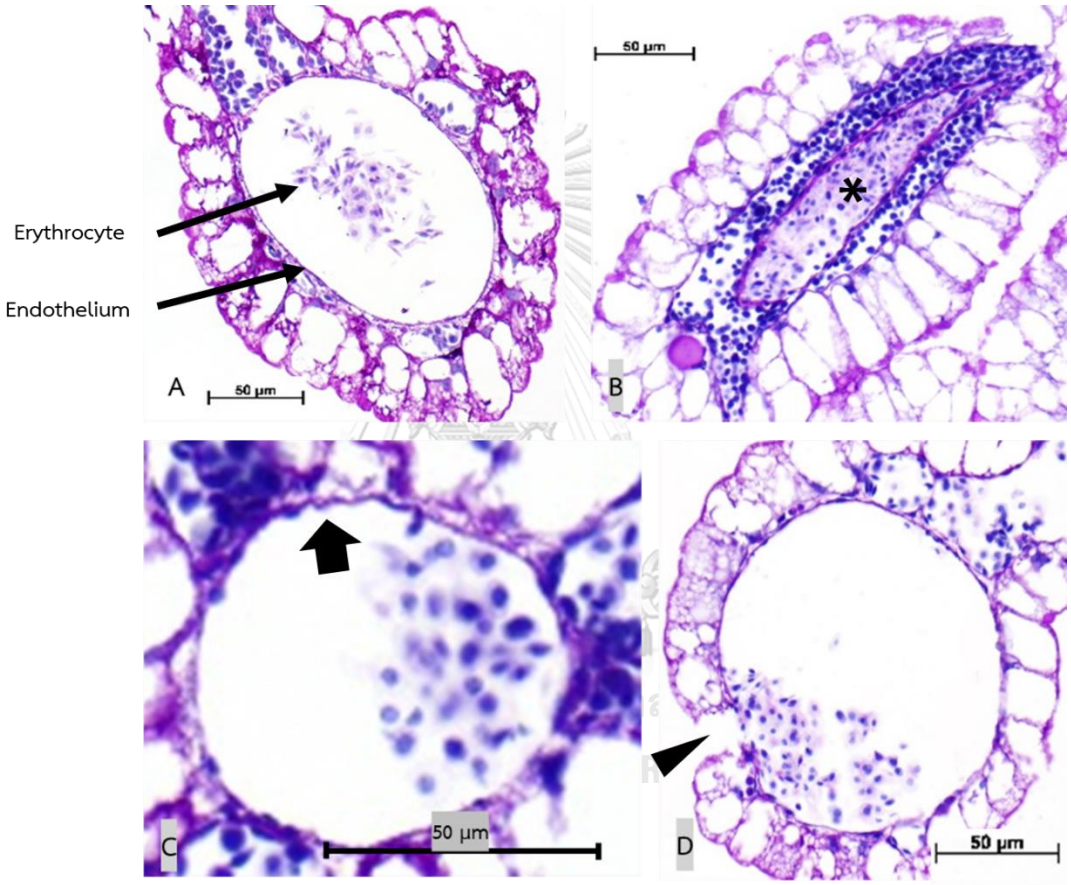

Figure 4-6 Representative micrographs of a blood vessel of the chick embryo in the *D. siamensis* venom-treated group (PAS stain). (A) Comparison was made between the control group (x 200), and (B-D) the venom-treated group. The pathological changes potentially relate to the hematotoxic effect of venom included (asterisk; B) blood clotting, (arrow; C) membrane shrinkage or loss of membrane integrity, and (arrowhead; D) the rupture of the endothelial wall and leaking of erythrocytes and other components.

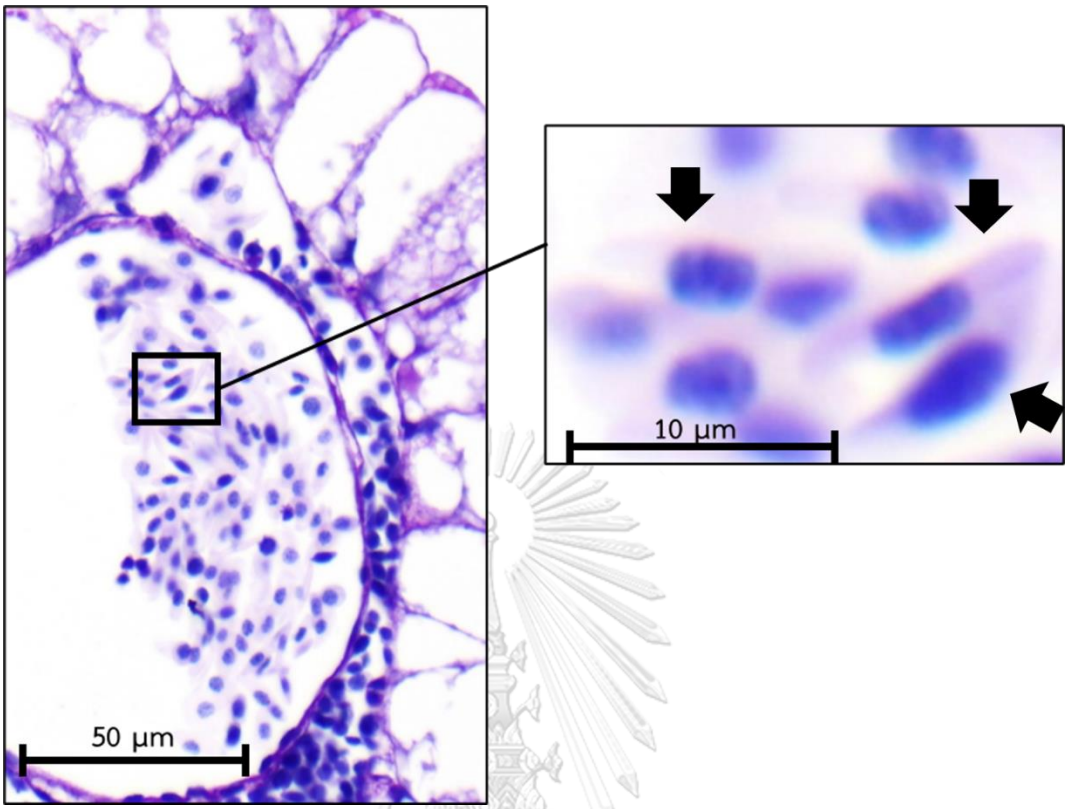

Figure 4-7 Representative micrographs of a blood vessel of the chick embryo in the *D. siamensis* venom-treated group showing the blood clotting and presence of thrombocytes (arrows), as ovoid or discoid shaped cells (PAS stain).

Discussion

The LD_{50} of this batch of *D. siamensis* venom, based on the day 6 chick embryo assay, was 16.54 µg/embryo, which was much higher than that was previously reported (6.12 µg/embryo) in a study using the same chick embryo assay and exposure method to test for the lethal dose of *D. siamensis* venom (Sittishevapark, 2015). 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 (Currier et al., 2010), sexual dimorphism (Furtado et al., 2006), 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 (Chippaux et al., 1991). 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.

The venom of *D. siamensis* is well known to show hematotoxic effects on blood circulation (Mukherjee et al., 2000; Risch et al., 2009). 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. 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 (Mitrakul, 1979). For the blood clotting reaction, RVV-V and RVV-X from venom stimulate factors V and X (Figure 2-2), respectively, then both factors accelerate the coagulation and the formation of blood clots (Mitrakul, 1979). 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 (Figure 4-7) and so could also be inferred that the blood was clotting (Grant and Zucker, 1973).

Secondly, changes in the membrane integrity and rupture of the blood vessel wall were also linked with snake hemotoxin. In Viperidae snakes, including *D. siamensis*, the enzyme frequently found in the venom is a group II secretory

phospholipase A_2 ($sPLA_2$) (Dennis, 2000; Chakraborty et al., 2002), which is a multifunction enzyme that is involved in the generation of oxidative stress (Tietge et al., 2005). The process starts with the $sPLA_2$-catalyzed hydrolyzation of phospholipids in the cell membrane to produce lysophospholipid and free fatty acids (Kini and Evans, 1989; Burke and Dennis, 2009; Risch et al., 2009), including arachidonic acid. This oxidative metabolite can further produce reactive oxygen species (ROS) (Muralikrishna Adibhatla and Hatcher, 2006) that then further cause oxidative damage at the membrane by the apoptosis pathway. Amongst the many alterations caused by apoptosis, one is the shrinkage of membranes (Clarke et al., 2007), 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 and hemorrhage (Bieth, 2001; Baldo et al., 2010).

Overall, the results confirm the hematotoxic effect of *D. siamensis* venom on the chick embryonic vasculature and provided a reference guide for testing the efficacy of herbal extracts against snake hematotoxin.

Conclusion

In this study, an alternative *in vivo* assay for hematotoxic activities of the eastern Russell's viper, *D. siamensis*, venom was developed. The LD_{50} concentration of the snake venom based on this assay was 16.54 µg/embryo. This value could be used as the standard concentration of venom in further screening for herbs with an anti-hematotoxic activity. The microanatomy of embryonic vasculature was examined and verified as suitable markers for the hematotoxic 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.

CHAPTER V
SCREENING THAI HERBS WITH ANTI-HEMOTOXIC ACTIVITIES AGAINST THE EASTERN RUSSELL'S VIPER VENOM

Introduction

The eastern Russell's viper, *Daboia siamensis* (Smith, 1917), is one of the important venomous snakes causing serious public health problems in Thailand. In general, antivenom serum has been used as the standard treatment for snakebite patients as recommended by the WHO (WHO, 1981). However, since antivenom serum commonly used for treating snakebite patients may lead to lethal allergic reactions (Dhanya et al., 2009) as well as not readily available in some rural area, the medicinal herb has become an important alternative remedy.

Several medicinal herbs were reported to have active compounds with antivenom property (Mors et al., 2000; Gomes et al., 2010; Gupta and Peshin, 2012). Of importance to note, the extract of *Trigonostemon reidioides* (Kurz) Craib and *Araca catechu* L. have been used for snakebite treatment by the well-known local healer in Surin province. Based on scientific experiments, *T. reidioides* was found to have antivenom activity against *Naja kaouthia* venom (Srithamma et al., 2004). This led to the development of herbal remedy for the actual treatment in the hospital. Currently, there are several hospitals in Thailand that apply the herbal treatment as a first step for treating snakebite patients before using the antivenom serum (Phongphladisai and Panyawatthananukun, 2011).

On the other hand, concerns have been raised against the used of herbal medicine to treat snakebite patients over its actual antivenom activity. In the snakebite incident, 20-60% of the incidents could be a dry bite without envenomation when the patients have the fang sign but without the local or systemic symptoms (Naik, 2017). This dry bite can be found in most venomous snake species (Russell et al., 1975). For this reason, it was believed that patients treated by the local healers and showed sign of healing might be due to the dry bite and a placebo effect rather than the true healing. In addition, there is a limited number of

scientific reports to confirm the efficacy and safety of medicinal herb, making it less widely accepted as the treatment for snakebite patients (Coe and Anderson, 2005; Veronese et al., 2005).

With the diverse and large number of medicinal herbs in Thai traditional medicine, it is possible that the potentially effective herbs against the snake venom are available. However, to screen a large number of unknown herbs, the time-consuming and expensive use of animals in the *in vivo* screening could become an obstacle for the discovery of the new herbal remedy. In this study, the *in vitro* assays (Chapter III) have been developed as primary screenings in order to screen out an ineffective herb and select a potential candidate prior to testing in the animal model. In addition, *in vivo* assay based on chick embryo (Chapter IV) has been developed with histological markers for hematotoxic effects of the venom to confirm the biological activities found in the *in vitro* assays.

The aims of this chapter was to find potential candidate Thai herb(s) that can be used to treat snakebite patient effectively and safely. Briefly, ten Thai herbs were selected based on the potential constituents and screened for anti-hemotoxic activity against snake venom using the validated *in vitro* assays. The inhibitory effect of the herb against the venom was used as a criterion for herb selection. Herb with more than 50% inhibition were subsequently tested in the *in vivo* assay.

Materials and Methods

1. Herb Preparation

1.1 Selection of Herbs

Based on the 49 Thai herb extracts available at Associate Professor Dr. Suchada Sukrong's laboratory, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Table 5-1) and two herb samples of *Trigonostemon reidioides* (Kurz) Craib (Figure 5-1A) and *Areca catechu* L. (Figure 5-1B), 10 herbs with potential inhibitory effect against snake venom were selected. The selection criteria included: prior use in scientific study, actual use in the snake bite treatment at the hospital or rural area, and record in Thai Materia Medica.

- Herbs with antivenom activity based on the scientific studies included 1) *Thunbergia laurifolia* Lindl. (Keeratichandacha et al., 2015), and 2) *Ageratum conyzoides* L. (Jain and Srivastava, 2005).
- Herbs used by local healers or doctors in the snakebite treatment included 3) root of *Trigonostemon reidioides* (Kurz) Craib, 4) dry seed of *Areca catechu* L. In addition, herbs in the same family as *T. reidioides* (Euphorbiaceae) were also selected. This included 5) whole plant of *Phyllanthus polyphyllus* Willd. 6) leaves of *Antidesma acidum* Retz., 7) whole plant of *Phyllanthus taxodiifolius* Beille, and 8) leaves of *Mallotus repandus* (Rottler) Müll.Arg..
- Herbs that has been recorded in the Thai Materia Medica included 9) vines of *Tinospora cordifolia* Miers, and 10) fruits of *Myristica fragrans* Houtt. (Figure 5-2).

All plant material of the herb extract at Associate Professor Dr. Suchada Sukrong's laboratory were collected in Thailand, and identified by Associate Professor Dr. Thatree Phadungcharoen, Department of Pharmacognosy and pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn

University. The voucher specimen was deposited at the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

T. reidioides and *A. catechu* were collected and identified by the local healer (Mr. Aia Saikrasun). Both herbs were bought at the 16th National Herbs Expo, IMPACT Muang Thong Thani, Nonthaburi province. The voucher specimen of *T. reidioides* (SS-AS-001) and *A. catechu* (SS-AS-002) were deposited at the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Table 5-1 Lists of available herb extracts in the stock of Assoc.Prof.Dr.Suchada Sukrong at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Yellow-highlighted names are the selected herb. (Scientific name and family are cited from the International Plant Names Index (IPNI) (http://www. ipni.org) and Thai common name are cited from the "Thai plant names" by Tem Smitinand (Smitinand, 2001))

No.	Scientific name	Thai common name	Family	Plant part	Reference	Voucher Number
1	*Afgekia mahidoliae* B.L.Burtt & Chermsir.	กันภัยมหิดล	Leguminosae	Leaves		SS-SA-2555-001
2	*Ageratum conyzoides* L.	สาบแร้งสาบกา	Asteraceae	Leaves	Scientific study	SS-SA-2555-002
3	*Aglaia odorata* Lour.	ประยงค์	Meliaceae	Leaves		SS-SA-2555-003
4	*Ampelocissus martini* Planch.	ส้มกุ้ง	Vitaceae	Leaves		SS-SA-2555-004
5	*Antidesma acidum* Retz.	เม่าสร้อย	Euphorbiaceae	Leaves	Same family as *T. reidioides*	SS-SA-2555-005
6	*Aquilaria crassna* Pierre ex Lecomte	กฤษณา	Thymelaeaceae	Leaves		SS-SA-2555-006
7	*Asparagus racemosus* Willd.	จั่นดิน	Asparagaceae	Whole plant		SS-KI-080753
8	*Asparagus setaceus* (Kunth) Jessop	โปร่งฟ้า	Asparagaceae	Leaves		SS-TB-080753-3
9	*Bacopa monnieri* (L.) Wettst.	พรมมิ	Scrophulariaceae	Whole plant		CU-MN 20170126
10	*Barleria lupulina* Lindl.	เสลดพังพอน	Acanthaceae	Whole plant		SS-SA-2555-007
11	*Bauhinia aureifolia* K.Larsen & S.S.Larsen	ใบสีทอง	Leguminosae	Leaves		SS-SA-2555-008
12	*Chaetocarpus castanocarpus* (Roxb.) Thwaites	สำเภา	Euphorbiaceae	Leaves		SS-SA-2555-009
13	*Chaetocarpus castanocarpus* (Roxb.) Thwaites	สำเภา	Euphorbiaceae	Branches		SS-SA-2555-009

No.	Scientific name	Thai common name	Family	Plant part	Reference	Voucher Number
14	*Chassalia curviflora* (Wall.) Thwaites	เข็มพระราม	Rubiaceae	Leaves		SS-SA-2555-010
15	*Chassalia curviflora* (Wall.) Thwaites	เข็มพระราม	Rubiaceae	Branches		SS-SA-2555-010
16	*Ixora lucida* R.Br. ex Wall.	เข็มขาว	Rubiaceae	Leaves		SS-SA-2555-011
17	*Cinnamomum camphora* (L.) J.Presl	การบูร	Lauraceae	Leaves		SS-SA-2555-012
18	*Clausena wallichii* Oliv.	เพี้ยฟาน	Rutaceae	Branches		SS-SA-2555-013
19	*Clitoria hanceana* Hemsl.	หนอนตายอยาก	Leguminosae	Root		SS-SA-2555-014
20	*Colubrina asiatica* (L.) Brongn.	คันทรง	Rhamnaceae	Leaves		SS-SA-2555-015
21	*Dregea volubilis* Benth. ex Hook.f.	กระทงหมาบ้า	Asclepiadaceae	Vines		SS-SA-2555-016
22	*Harrisonia perforata* Merr.	คนทา	Simaroubaceae	Branches		SS-SA-2555-017
23	*Holarrhena pubescens* Wall.	โมกใหญ่	Apocynaceae	Leaves		SS-SA-2555-018
24	*Hydnophytum formicarum* Jack	หัวร้อยรู	Rubiaceae	Stems		SS-SA-2555-019
25	*Lagerstroemia speciosa* Pers.	อินทนิลน้ำ	Lythraceae	Leaves		SS-TVT-2011-01
26	*Mallotus repandus* (Rottler) Müll.Arg.	มะกายเครือ	Euphorbiaceae	Leaves	Same family as *T. reidioides*	SS-SA-2555-020
27	*Microcos tomentosa* Sm.	พลับพลา	Tiliaceae	Branches		SS-SA-2555-021
28	*Micromelum minutum* (G.Forst.) Wight & Arn.	หัสคุณ	Rutaceae	Stems		SS-SA-2555-022
29	*Mitragyna diversifolia* Havil.	กระทุ่มน้ำ	Rubiaceae	Leaves		TTP-050901
30	*Mitragyna diversifolia* Havil.	กระทุ่มน้ำ	Rubiaceae	Branches		TTP-050901
31	*Mitrephora vandaeflora* Kurz	นางแดง	Annonaceae	Leaves		SS-SA-2555-023
32	*Moringa oleifera* Lam.	มะรุม	Moringaceae	Seeds		SS-SA-2555-024

No.	Scientific name	Thai common name	Family	Plant part	Reference	Voucher Number
33	*Murraya siamensis* Craib	โปร่งฟ้า	Rutaceae	Roots		SS-SA-2555-025
34	*Murraya siamensis* Craib	โปร่งฟ้า	Rutaceae	Arial part		SS-SA-2555-025
35	*Myristica fragrans* Houtt.	จันทน์เทศ	Myristicaceae	Fruits	Record in Thai Materia Medica	SS-SA-2555-026
36	*Pandanus odoratissimus* L.f.	เตยทะเล	Pandanaceae	Roots		SS-SA-2555-027
37	*Phyllanthus polyphyllus* Willd.	เสียวใหญ่	Euphorbiaceae	Whole plant	Same family as *T. reidioides*	SS-SA-2555-028
38	*Phyllanthus taxodiifolius* Beille	ไคร้หางนาค	Euphorbiaceae	Whole plant	Same family as *T. reidioides*	SS-SA-2555-029
39	*Pueraria candollei* Wall. ex Benth.	กวาวเครือ	Leguminosae	Rhizomes		TH090505
40	*Saccharum officinarum* L.	อ้อย	Poaceae	Stems		SS-SA-2555-030
41	*Salacia chinensis* L.	กำแพงเจ็ดชั้น	Celastraceae	Stems		SS-SA-2555-031
42	*Sambucus simpsonii* Rehder	พวงไข่มุก	Caprifoliaceae	Leaves		SS-SA-2555-032
43	*Scaphium macropodum* (Miq.) Beumée ex K.Heyne	สำรอง	Sterculiaceae	Leaves		SS-SA-2555-033
44	*Siphonodon celastrineus* Griff.	มะดูก	Celastraceae	Leaves		SS-SA-2555-034
45	*Smilax corbularia* Kunth	ข้าวเย็นเหนือ	Smilacaceae	Stems		SS-SA-2555-035
46	*Smilax glabra* Roxb.	ยาหัว	Smilacaceae	Stems		SS-SA-2555-036
47	*Thunbergia laurifolia* Lindl.	รางจืด	Acanthaceae	Leaves	Scientific study	SS-1109201
48	*Tinospora crispa* Miers	บอระเพ็ด	Menispermaceae	Vines	Record in Thai Materia Medica	SS-SA-2555-037
49	*Vitex trifolia* L.	คนทีสอทะเล	Lamiaceae	Whole plant		SS-WP-160311-12
50*	*Trigonostemon reidioides* (Kurz) Craib	โลดทะนง	Euphorbiaceae	Roots	Obtained from Mr. Aia Saikrasoon	SS-AS-001
51*	*Areca catechu* L.	หมากสง	Arecaceae	Seeds	Obtained from Mr. Aia Saikrasoon	SS-AS-002

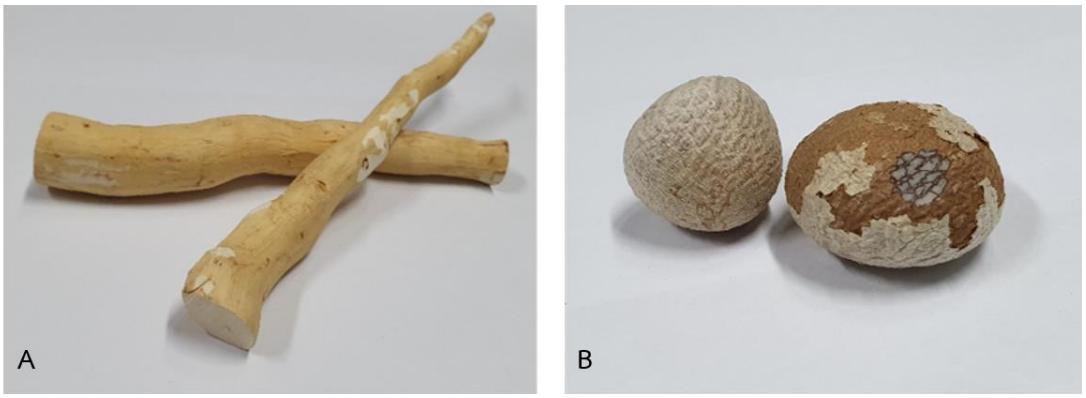

Figure 5-1 (A) Samples of *T. reidioides* (1-2 cm diameter and 15-20 cm length; Left) and (B) *A. catechu* (1.5-2.5 cm diameter and 1-1.5 cm height; Right) were purchased from the well-known local healer in 2019.

1.2 Crude Extract Preparation

Plant materials of the available herb extract were kept at Associate Professor Dr. Suchada Sukrong's laboratory, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. *T. reidioides* and *A. catechu* extracts were prepared by the senior lab member in Associate Professor Dr. Suchada Sukrong's laboratory. The preparation of crude extract started with cutting and grinding of the herb sample into small pieces. The sample was freeze dried and ground into powder by mortar and pestle. The herb powder was extracted by soaking in methanol overnight. The extracted solvent was poured out and the powder re-extracted with the new methanol. The process was repeated 3 times to increase the yield. The solvent from every extraction steps was combined and evaporated with the rotary evaporator. The crude extract of each herb was kept at -20°C for further use (Figure 5-2).

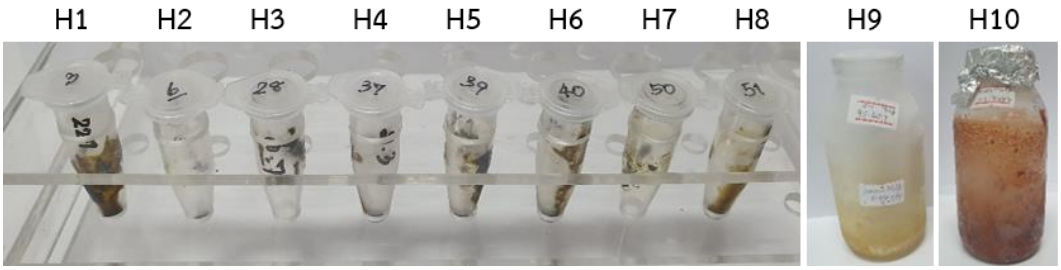

Figure 5-2 Crude extract of ten herbs used in the screening process were labeled with the following code number: *Ageratum conyzoides* L. (H1), *Antidesma acidum* Retz. (H2), *Mallotus repandus* (Rottler) Müll.Arg. (H3), *Myristica fragrans* Houtt. (H4), *Phyllanthus polyphyllus* Willd. (H5), *Phyllanthus taxodiifolius* Beille (H6), *Thunbergia laurifolia* Lindl. (H7), *Tinospora cordifolia* Miers (H8), *Trigonostemon reidioides* (Kurz) Craib (H9), and *Areca catechu* L. (H10).

1.3 Herb Solution Preparation

The crude extract was dissolved in dimethyl sulfoxide (DMSO) which was considered as a low toxic solvent for most organism (Kloverpris et al., 2010). To avoid the toxicity of DMSO, several studies suggested that DMSO at 0.5% (v/v) was the most suitable for keeping high cell viability in various types of cells (Chen and Thibeault, 2013; Leon-Garcia et al., 2017). In this study, the DMSO control group was included in every assay to check for the effect of DMSO.

In the dilution process, the DMSO was added to the crude extract in a small increment until the crude extract completely dissolved with the minimum amount of DMSO. Then, PBS was used for diluting the concentration of DMSO in the solution to below 0.5% (range of the actual concentration at 0.2-0.35%). Each herb solution was diluted to the stock concentration of 8 µg/µL, aliquoted to 20 µL and kept at -20°C for further use (Figure 5-3).

Code numbers were assigned to each herb and used in the assay. These included *Ageratum conyzoides* L. (H1), *Antidesma acidum* Retz. (H2), *Mallotus repandus* (Rottler) Müll.Arg. (H3), *Myristica fragrans* Houtt. (H4), *Phyllanthus polyphyllus* Willd. (H5), *Phyllanthus taxodiifolius* Beille (H6), *Thunbergia laurifolia*

Lindl. (H7), *Tinospora cordifolia* Miers (H8), *Trigonostemon reidioides* (Kurz) Craib (H9), and *Areca catechu* L. (H10).

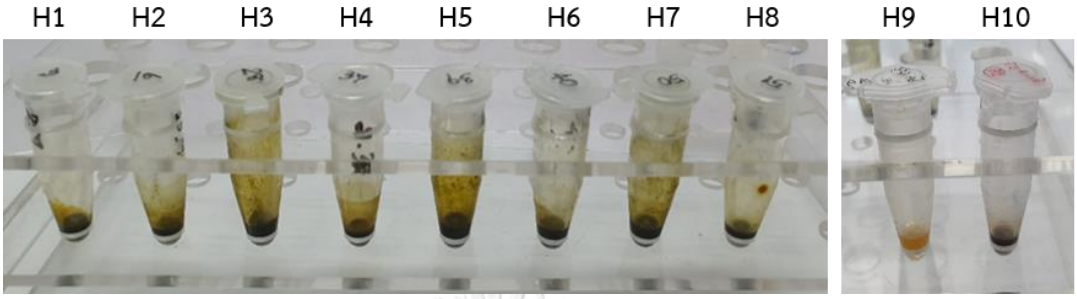

Figure 5-3 Crude extracts of ten herbs used in the screening process were dissolved in DMSO and PBS. The stock concentration of each herb was kept at 8 µg/µL.

2. *In vitro* Screening Assays

Three *in vitro* assays (Chapter III) were used as screening assays. The aim of this part is to find an effective herb against the hematotoxic activity of *D. siamensis* venom. All herbs and venom were prepared at 8 µg/µL by using PBS as a solvent. This concentration was equivalent to the LD_{50} value of *D. siamensis* on chick embryo after 4-hour incubation (Chapter IV). Each assay was carried out using the same experiment groups including 1) negative control (with venom), 2) positive control (with venom and antivenom serum), 3) blank PBS (only PBS), 4) blank DMSO (only DMSO), 5) herb toxicity test (with herb), and 6) screening (with venom and herb at various ratio). Components of each groups are shown in the following list.

- **negative control group** (-ve ctrl): venom at 8 µg/µL and PBS
- **positive control group** (+ve ctrl): venom and a dose of antivenom serum that completely inhibited the venom
- **control group** (ctrl): PBS
- **blank DMSO group** (DMSO): only DMSO

- **herb toxicity group at the various concentration** (HX; “X” refers the code number of herbs as shown in Figure 5-2). Range of herb concentration including 1) “HX_(1)” (herb solution at the same concentration as the venom), 2) “HX_(0.5)” (herb concentration at one half of the venom concentration), and 3) “HX_(0.1)” (herb concentration at one tenth of the venom concentration)
- **screening group** (V+HX_1:Y; “X” refers the code number of herb and “Y” refers to the concentration of herb) including 1) “V+HX_1:1” (herb concentration at the same concentration as the venom), 2) “V+HX_1:0.5” (herb concentration at one half of the venom concentration), and 3) “V+HX_1:0.1” (herb concentration at one tenth of the venom concentration)

2.1 Venom and Serum Preparation

Lyophilized snake venom of the eastern Russell’s viper and its antivenom serum were purchased from Snake Farm, the Queen Saovabha Memorial Institute, Bangkok, Thailand. Snake venom (purchased on August 2018) was dissolved with dH_2O to make 8 µg/µL solution (the previously reported LD_{50} value), then aliquoted and stored at -20 ℃. Antivenom serum (Lot No. WR00117) was dissolved with 1,000 mL distilled water. After that, the prepared antivenom serum was aliquoted to a small portion and stored at -20℃.

2.2 Phospholipase Assay

Based on the validated phospholipase assay, every controls (control group, positive control group, and negative control group) must be tested in every agar plate. As a result, Petri dishes with a diameter of 150 mm was used in order to increase number of experimental wells per plate.

The phospholipase assay was carried out on an agar plate consisted of 1% agar and yolk solution as described in Chapter III. Briefly, the agar mixture was heated on the hotplate until it reached 90℃, then added with 0.005% of sodium azide

solution and 0.01 M of $CaCl_2$ solution. The mixture was poured into the 150 mm Petri dish and left at room temperature for solidification. After that, the gel was punctured to make 0.5 cm diameter holes by using a hollow steel tube (Figure 5-4A). The mixture of each experiment group was dropped into the wells at the total volume 10 µL per well. After 1-hour incubation (Figure 5-4B), phospholipase activity can be observed from the clear zone around the hole. The diameter of the clear zone around each well was measured by a vernier caliper at 3 different diameters. The experiment was replicated 5 times per group. Average diameter of the clear zone in each group was calculated into percent inhibition using the following formula:

$$\%\text{inhibition} = \frac{\text{Avg. diameter of the -ve ctrl group - Avg. diameter of the experiment group}}{\text{Avg. diameter of the -ve ctrl group - Avg. diameter of the +ve ctrl group}} \times 100$$

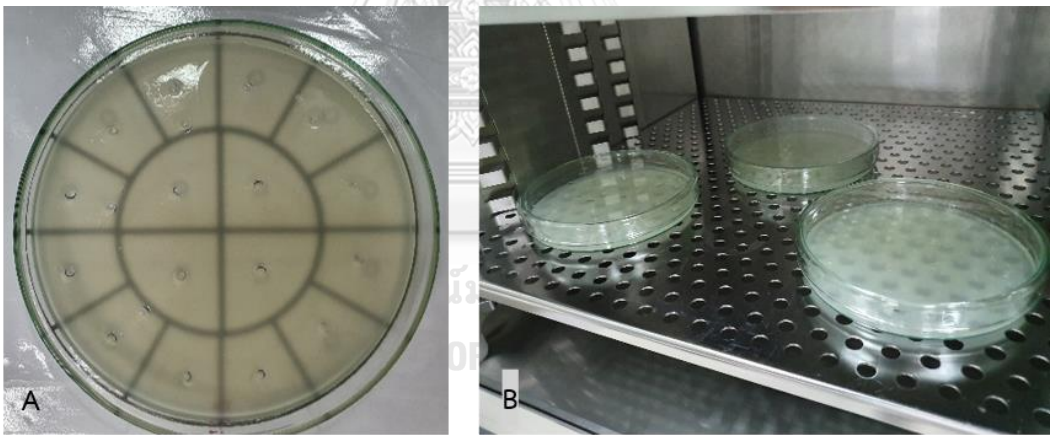

Figure 5-4 After the gel solidification, (A) the Petri dish was divided and punctured into 16 wells with an aid of the outline on the tracing paper. (B) After adding a sample to each plate, the plates were incubated at 37°C for 1 hour.

2.3 Coagulation Assay

The coagulation assay modified from Rodrigues et al. (2000) and Oliveira et al. (2016) was carried as described in Chapter III. The discarded human blood was

obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand with prior permission from the National Blood Center's committees (COA No. NBC 12/2018). The whole blood was centrifuged at 500 xg at 4°C for 5 minutes for the blood fractionation. Plasma in the supernatant was separated and kept at -20°C until assay.

Since the obtained plasma contained the anticoagulant CPDA-1 (Beutler and West, 1979) that can inhibit the coagulation cascade, 0.2875 M of $CaCl_2$ solution was added as the source of free calcium ion in order to neutralize the anticoagulant in the plasma stock.

The coagulation assay was based on the spectrophotometry technique on a microplate. The blank control group (plasma and PBS) was used for checking the OD_{405} without $CaCl_2$ solution. The neutralized plasma was mixed with test substances in each group including $CaCl_2$ group (plasma with $CaCl_2$ solution), the control group (PBS), the negative control group (venom), and the positive control (the mixture of venom and antivenom serum), the herb toxicity group (plasma + $CaCl_2$ solution + herb solution at LD_{50} concentration), and the screening groups (plasma + $CaCl_2$ solution + the mixture of venom and various concentration of herb solution). The label on screening group was "V+HX (1:Y)" when X refers to herb code number, and Y was the ratio of herb concentration (0.1 and 1).

Firstly, the mixture of venom and antivenom serum and the mixture of venom and herb were incubated at 37°C for 30 minutes in the water bath (Figure 5-5A). After that, the mixture were mixed and transferred to the 96-well plate. Lastly, the neutralized plasma was added to each well by a multichannel pipette. The plate was covered and shook for 1 minute. After incubation at 37°C for an hour (Figure 5-5B), the optical density at 405 nm was measured with the microplate reader (Multiskan EX). There were 3 replication per experiment and each plate was measured 3 times with 5-minutes interval between reading. Higher OD represents a higher amount of fibrin indicating high coagulation activity of the venom. The average OD_{405} of each experiment were compared and turned into percent inhibition by the following formula:

$$\%\text{inhibition} = \frac{\text{Avg. OD405 of the -ve ctrl group - Avg. OD405 of the experiment group}}{\text{Avg. OD405 of the -ve ctrl group - Avg. OD405 of the +ve ctrl group}} \times 100$$

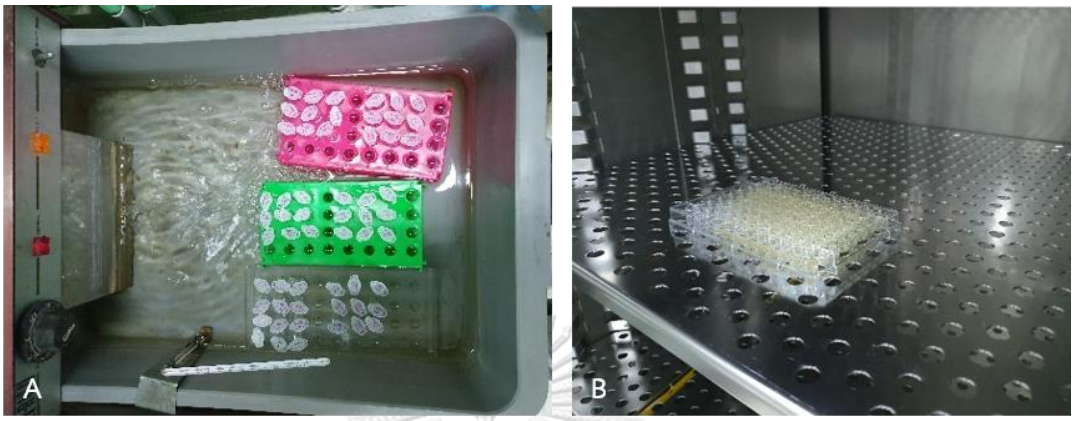

Figure 5-5 (A) The mixture of venom and antivenom serum and the mixture of venom and herb were incubated at 37°C for 30 minutes in the water bath. (B) After mixing all ingredients and transferring to a 96-wells plate, the plate was added with the neutralized plasma and incubated at 37°C for 1 hour.

2.4 Fibrinogenolytic Assay

The fibrinogenolytic assay modified from Oliveira et al. (2016) was carried out as described in Chapter III. SDS-PAGE was used for detecting the separation of alpha and beta-peptide from fibrinogen which is the sign of fibrinogenolytic activity. The amount of protein added in each well of SDS-PAGE was optimized with the same sample dilution process as done in Chapter III except the fact that protein concentration in the herb was not determined. Fibrinogen solution at 8 µg/µL was used as a substrate. The experiment groups in this part included: 1) the blank group (PBS), 2) the control group (fibrinogen + PBS), 3) the negative control group (fibrinogen + venom), 4) the positive control group (fibrinogen + mixture of venom and antivenom serum), 5) the DMSO group (fibrinogen + DMSO), 6) herb toxicity group (fibrinogen + herb solution at LD_{50} concentration), and 7) the screening group (fibrinogen + mixture of venom and herb solution at "Y" ratio or "F+VHX(1:Y)"]. "X"

refers to the code number of herb from 1-10 and “Y” refers to the ratio of herb concentration (0.1, 0.5, and 1).

The mixture of venom and antivenom serum and the mixture of venom and herb were incubated at 37°C for 30 minutes. After that, the fibrinogen was added to the mixture, then incubated at 37°C for 30 minutes. PBS was added to the mixture for optimized the concentration of protein. The reaction was stopped with beta-mercaptoethanol and bromophenol blue was added before boiling all tubes for 5 minutes (Figure 5-6A). Samples and protein standards (Precision Plus Protein™ Dual Color Standards, Bio-Rad) were transferred to 12% polyacrylamide gel (12% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad) and run the current at 200 V for 40 minutes (Figure 5-6B). Gels were stained with Coomassie Blue G-250 and destained with 10% acetic acid before visualization for protein bands in each lane. (Figure 5-6C). The picture of gels was captured by using the scanner (Figure 5-6D).

The fibrinogenolytic activity of the venom represents by the cleavage of the alpha-fibrinopeptide band. Addition of antivenom serum or herb that can neutralize the venom would result in inhibitory effect that can be verified by the existence of the alpha-fibrinogen band. Compared to the negative control (fibrinogen + venom), herb extract that showed less or no fragment of alpha-fibrinopeptide was considered as having the inhibitory effect against the venom. While herb extract that show the same pattern of alpha-fibrinopeptide as the negative group was considered as having no inhibitory effect against the venom.

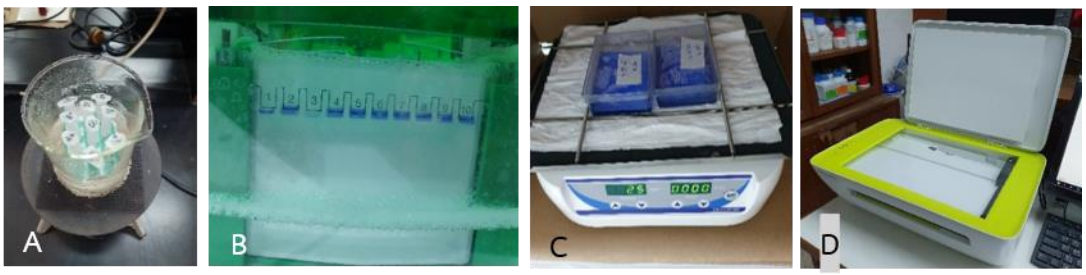

Figure 5-6 (A) Sample in each group was boiled for 5 minutes and (B) transferred to the gel. After running the current at 200V for 40 minutes, (C) gels were stained and destained with the aid of an orbital shaker before (D) scanning the whole gel.

3. Scoring

In the actual snakebite cases, the patient who was envenomated by *D. siamensis* had many possible symptoms. However, not all of these symptoms were fatal. Some of them can be relieved without any treatment. The effective treatment is thus important to focus on the fatal symptoms rather than the mild symptoms. In order to rank the fatality of symptoms, the information was gathered from the actual case report by *D. siamensis* bite (Phillips et al., 1988; Warrell, 1989; Kularatne, 2003). It was found that the most frequent and fatal symptoms was coagulation activity and fibrinogenolytic activity. The second, third, and fourth were phospholipase, hemagglutination, and proteolytic activity, respectively. However, according to the *in vitro* development assay, hemagglutination and proteolytic activity were proven to show very little (negligible) activity in *D. siamensis* venom, hence excluding from the *in vitro* screenings.

To select the effective herb for further assay *in vivo*, percent inhibitions of coagulation and phospholipase assays and the result of fibrinogenolytic assay were used. For each herb, the percent inhibition was multiplied by degree of the fatality listed below. Afterward, the calculated value was termed “weighted inhibition”.

- Primary effect: Coagulation activity x 4
- Primary effect: Fibrinogenolytic activity yes/no
- Secondary effect: Phospholipase activity x 3

Since the result of fibrinogenolytic assay was on a nominal scale, the result was used solely for verifying the coagulation activity. Since these 2 activities are on the same cascade, if the herb showed no inhibitory effect against the fibrinogenolytic activity, the percent inhibition in the coagulation activity was also regarded as 0.

Afterward, percent inhibition of the negative control group (venom) was also calculated. Then, the total percent inhibition was calculated from the following formula. The herb with more than 50% of the inhibitory effect was selected for further *in vivo* screening.

$$\text{Total \%inhibition} = \frac{\text{Weighted inhibition of phospholipase assay + Weighted inhibition of coagulation assay}}{\text{Degree of fatality for phopholipase assay + Degree of fatality for coagulation assay}}$$

4. *In vivo* Screening

4.1 Animals

According to Thailand's Animals for Scientific Purposes Act B.E. 2558 (A.D. 2015) (Institutional Animal Care and Use Committee, 2015), 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. In this study, 941 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.

4.2 Chick Embryo Preparation

Fertilized chicken eggs on the day of laying (day 0) were transferred from Luangsuwanvajokkasikit farm, Kasetsart University to the laboratory at CU. Eggs were 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 37.5 ± 0.5 °C (mean ± S.D.) with relative humidity in excess of 80%. The eggs were turned 4–6 times/day until day 2 of incubation, when they were cracked open. The egg content including embryo, yolk, and albumin was weighed and transferred to culture vessel. The hammock technique used for chick embryo culture was modified from Tahara and Obara (Tahara and Obara, 2014). Briefly, 200 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 all three *in vivo* screening steps (LD_{50} of venom, LD_{50} of *A. catechu*, and ID_{50} of *A. catechu* against *D. siamensis* venom) after incubation are shown in Table 5-2. With the starting number of 941 eggs, 354 embryos were available for the assay, resulting in a 37.6% yield of chick embryos.

Table 5-2 Number of chicken eggs used and number of chick embryos available for all three *in vivo* screening steps after incubation 37.5 ± 0.5 °C. The 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	941	765	581	349
Success rate (%) compared to the preceding step	N/A	81.3	75.9	60.1
Overall success rate (%)	N/A	81.3	61.7	37.1

4.3 Mortality Rate and LD_{50} of *D. siamensis* venom

D. siamensis was obtained from the Snake Farm, the Queen Saovabha Memorial Institute, Bangkok, Thailand. Since the protein composition in the venom can be varied between batch and affected the result on the screening assay. In this part of the study, the new batch of venom was tested on the chick embryo assay for finding a new LD_{50} value.

Chick embryos on day 6 were used as the model for finding the LD_{50} value of the venom. Based on the egg content weight on day 2, embryos were assigned into five groups so that every group had a similar mean weight. Snake venom (0, 1.25, 2.5, 5, and 10 µg/µL) were incubated at 37.5 ± 0.5 °C for 30 minutes before treating to the embryo. The volume of venom in each group were normalized to 2 µL with PBS and added to a circle filter paper (Whatman No. 1, 0.2 cm diameter). The filter paper was then placed over the lateral vitelline vein of the chick embryo. Observation of the embryo's health was performed every hour and the number of dead embryos (no sign of heartbeat) was recorded at 4 hours after venom application. Minimum

number of chick embryos used in each experiment was 15. Numbers of death in the control group and at each venom concentration was calculated into the mortality rate and used in LD_{50} calculation by Probit Analysis in the SPSS statistics version 22 software.

4.4 Mortality Rate and LD_{50} of Herb Extract

After *in vitro* screenings, the selected herbs were tested in the chick embryo assay in order to obtain the LD_{50} value of the herb. Based on the egg content weight on day 2, chick embryos were divided into seven groups so that every group had a similar mean weight. Various concentrations of herb solution (0, 0.1, 1, 10, 100, 200, and 500 µg/µL) were treated to the embryo by the same method as the previous part (4.3). Minimum number of chick embryos used in each experiment was 15. Numbers of death in the control group and at each herb concentration were calculated into the mortality rate and used in LD_{50} calculation by Probit Analysis in the SPSS statistics version 22 software.

4.5 Herb Screening

Assay based on day 6 chick embryos was used as the *in vivo* screening for the herb with anti-hematotoxic effect against *D. siamensis* venom. Based on the egg content weight on day 2, chick embryos were divided into seven groups so that every group had a similar mean weight. The experiment groups included 1) the control group (PBS), 2) the negative control group (venom at LD_{50} concentration), 3) the positive control group (the mixture of venom and antivenom serum), 4) herb toxicity group (herb solution at the same concentration as the LD_{50} of venom), 5) venom and herb (V+H) at 1:1 ratio, 6) venom and herb (V+H) at 1:0.5 ratio, and 7) venom and herb (V+H) at 1:0.1 ratio. PBS pH 7.2 was used as a diluent for every solution preparation. Samples were mixed and incubated at 37°C for 30 minutes in the water bath before treating the embryo. Minimum number of chick embryos used in each group was 15.

The mortality rate was recorded and calculated into the percent inhibition by the following formula. Subsequently, percent inhibition of every herb concentration was plotted in Microsoft Excel. Linear regression analysis was performed on SigmaPlot version 11 program to obtain the regression equation for determining the median inhibitory dose (ID_{50}) of the herb.

$$\%\text{inhibition} = \frac{\%\text{mortality of the -ve ctrl group - \%mortality of the experiment group}}{\%\text{mortality of the -ve ctrl group - \%mortality of the +ve ctrl group}} \times 100$$

Results

1. *In vitro* Screening

1.1 Phospholipase Assay

After incubation and clear zone measurement (Figure 5-7A), total diameter of a sample group (venom and herb) was compared with the positive control group (venom and antivenom serum: 100% inhibition) and the negative control group (venom: 0% inhibition). Three different line of diameters were measured to get the average value in case that a clear zone was not circular (Figure 5-7B). The plate was observed and measured over the black background for better visualization.

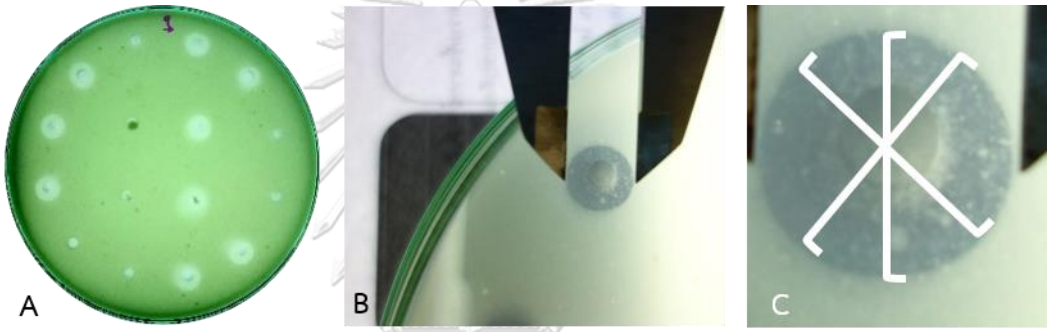

Figure 5-7 A sample agar plate for phospholipase assay. (A) The clear zone indicated venom activity. (B) Clear zone was measured with a vernier caliper (C) at 3 diameter lines at a different axes to compensate for uncircular pattern.

After percent inhibition calculated, these values were presented in Figure 5-9. The result showed that H10 at ratio 1:1 seem to have the highest inhibitory effect (50.25%) against the venom, while the second and third was H3 at ratio 1:1 (39.35%) and H2 at ratio 1:1 (36.46%), respectively.

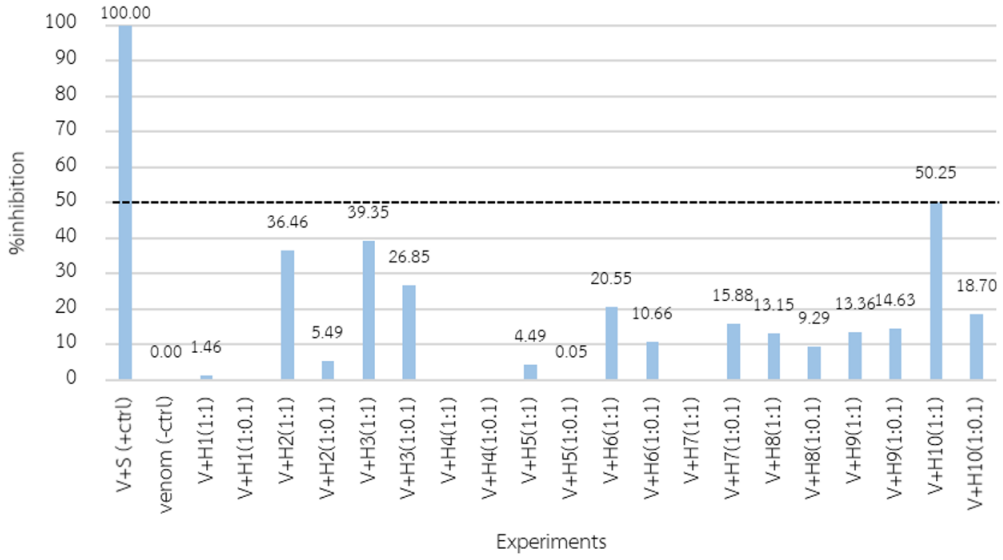

Figure 5-8 The inhibitory effect of herbs (H1-H10) against phospholipase activity of *D. siamensis* venom at LD_{50} (8 µg/µl). The horizontal dash line indicates the 50% inhibition. (V+S = the mixture of venom and antivenom serum, V+HX (1:Y) = the mixture of venom and herb solution No. "X" when "X" refers to the code number of herb and "1:Y" refers to the ratio concentration of herb compared to venom)

1.2 Coagulation Assay

Percent inhibition was calculated for every experiment group and shown in Figure 5-10. The %inhibition of each group showed that the H10 at ratio 1:1 has the highest inhibitory effect against venom (42.69%) while the second and third were H4 at ratio 1:1 and 1:0.1, respectively.

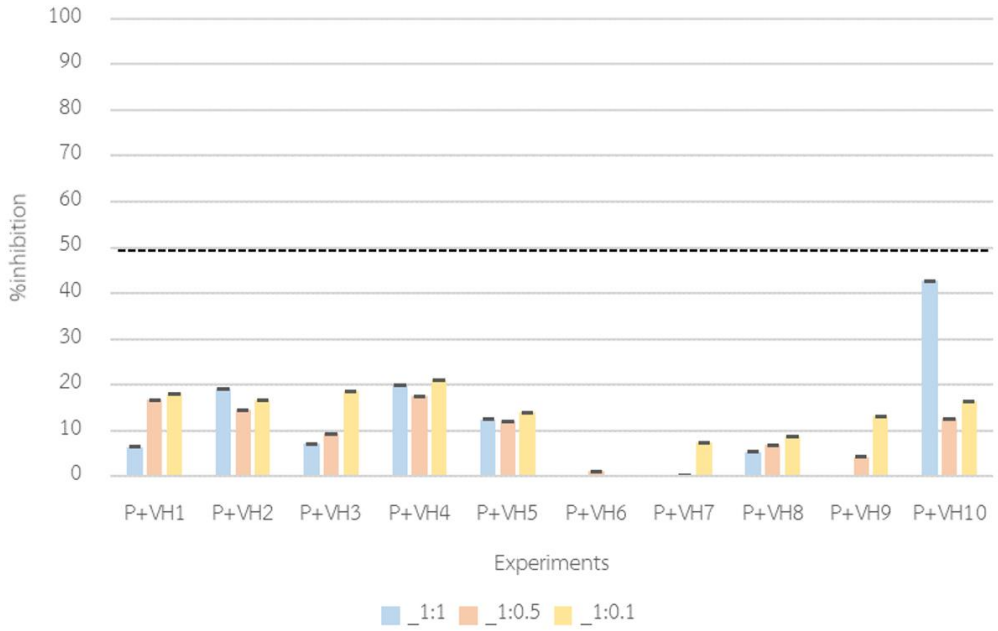

Figure 5-9 The inhibitory effect of herbs (H1-H10) against coagulation activity of *D. siamensis* venom at LD_{50} (8 µg/µL). The horizontal dash line indicates the 50% inhibition. (P +VHX = plasma + the mixture of venom and herb solution No. "X" when "X" refers to the code number of herbs. The legend of "1:Y" refers to the ratio concentration of herb compared to venom.)

1.3 Fibrinogenolytic Assay

The fibrinogenolytic activity of *D. siamensis* venom was evidented by the cleavage of alpha-fibrinopeptide band (lane 2 of Figure 5-11). Addition of antivenom serum or herb that can neutralize the venom would result in inhibitory effect that can be verified by the existence of the alpha-fibrinogen band (black rectangle area of lanes 3, 5, 6, 7 in Figure 5-11). Using the negative control (fibrinogen + venom) as a reference, herb extract that showed less or no fragment of alpha-fibrinopeptide was considered as having the inhibitory effect against the venom, while herb extract that show the same pattern of alpha-fibrinopeptide as the negative group was considered as having no inhibitory effect against the venom.

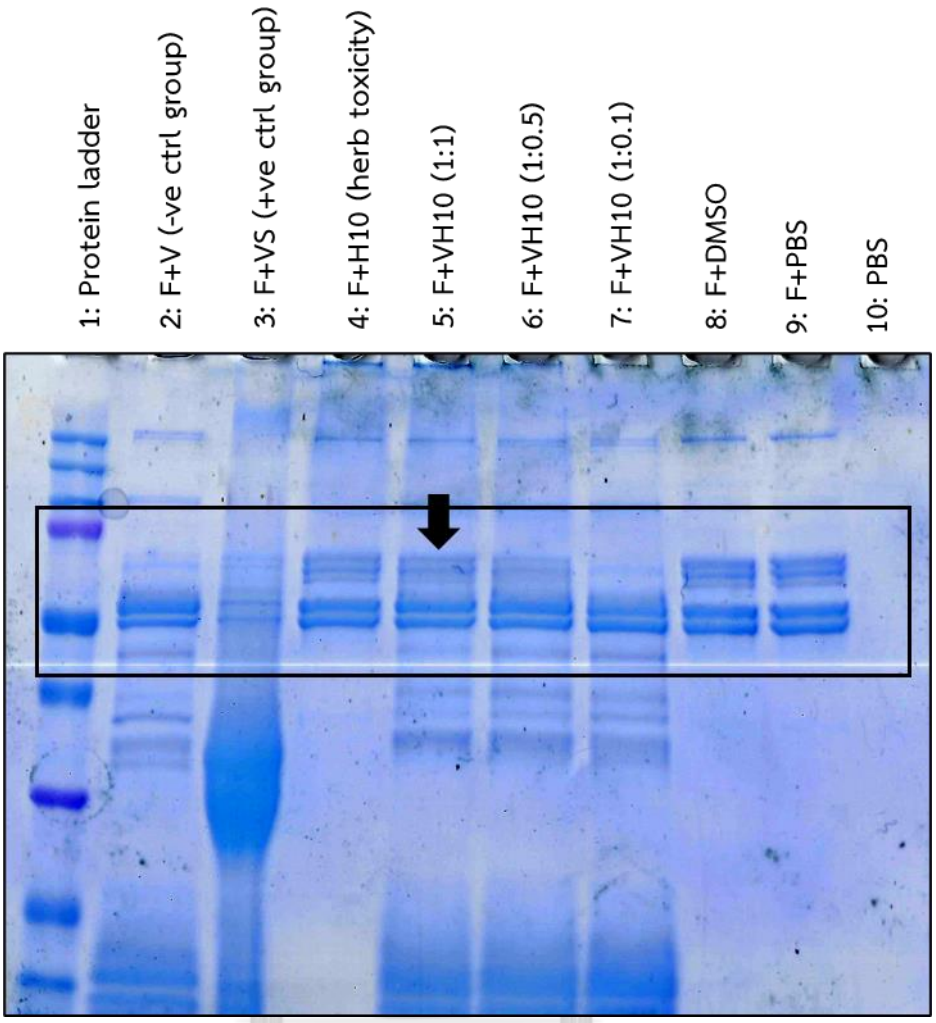

Figure 5-10 A sample of SDS-PAGE result of H10. An undigested band of alpha-fibrinogen (arrow) in the mixture between venom and herb at 1:1 ratio inferred that there was an inhibitory effect against *D. siamensis* venom. The rectangle shows the area used for the analysis. (F+V = fibrinogen + venom, F+VS = fibrinogen + the mixture of venom and antivenom serum, F+HX = fibrinogen + herb solution No. X, F+VHX (1:Y) = fibrinogen + the mixture of venom and herb solution when "X" refers to the herb code number of herb and "Y" refers to the ratio concentration of herb compared to venom, F+DMSO = fibrinogen + DMSO, and F+PBS = fibrinogen + PBS)

The results SDS-PAGE of the selected 10 herbs are shown in the Figure 5-12. Each gel is cropped to show only in the result of the negative control group (venom, lane 2), the herb toxicity group (lane 4), and herb screening groups (lanes 5-7). The results showed that H4, H5, H6, H7, H9, and H10 show the inhibitory effect (Table 5-3).

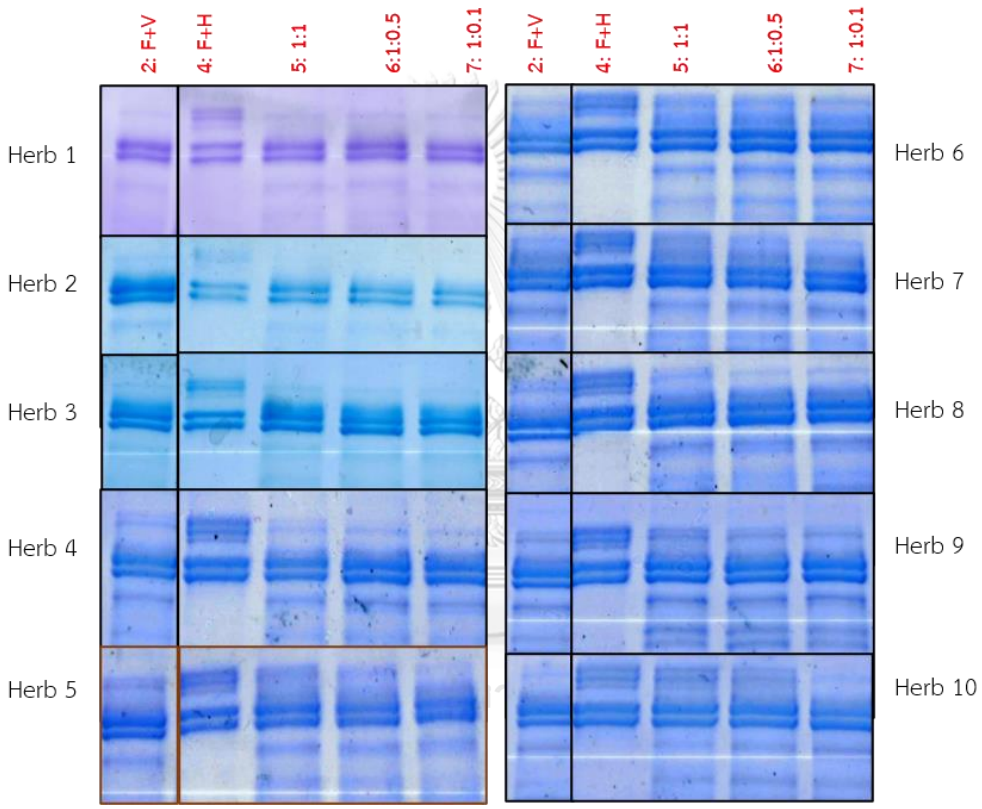

Figure 5-11 Representative results of SDS-PAGE from the screening for herbs against fibrinogenolytic activity of *D. siamensis* venom. The patterns on alpha-fibrinopeptide bands of the screening group (venom and herb) on lane 5, 6, and 7 were compared with the negative control group (venom) on lane 2. (F+V = fibrinogen + venom, F+H = fibrinogen + herb solution, 1:Y = fibrinogen + the mixture of venom and herb solution when “Y” refers to the ratio concentration of herb compared to venom.)

Table 5-3 Tabulated results of the screening for herbs against fibrinogenolytic activity of *D. siamensis* venom. The letter "+" refers to inhibitory effect, while the letter "-" refers to no inhibitory effect.

Herb	Ratio of venom : herb	Activity	Herb	Ratio of venom : herb	Activity
H1	VH1 (1:1)	-	H6	VH6 (1:1)	+
	VH1 (1:0.5)	-		VH6 (1:0.5)	+
	VH1 (1:0.1)	-		VH6 (1:0.1)	+
H2	VH2 (1:1)	-	H7	VH7 (1:1)	+
	VH2 (1:0.5)	-		VH7 (1:0.5)	+
	VH2 (1:0.1)	-		VH7 (1:0.1)	-
H3	VH3 (1:1)	-	H8	VH8 (1:1)	-
	VH3 (1:0.5)	-		VH8 (1:0.5)	-
	VH3 (1:0.1)	-		VH8 (1:0.1)	-
H4	VH4 (1:1)	-	H9	VH9 (1:1)	+
	VH4 (1:0.5)	-		VH9 (1:0.5)	-
	VH4 (1:0.1)	-		VH9 (1:0.1)	-
H5	VH5 (1:1)	+	H10	VH10 (1:1)	+
	VH5 (1:0.5)	+		VH10 (1:0.5)	+
	VH5 (1:0.1)	-		VH10 (1:0.1)	-

2. Scoring

Percent inhibition in each assay and the degree of fatality were used to calculate the total percent inhibition by the scoring method as previously described. Overall results in Table 5-4 shows that H10 at 1:1 ratio has the highest percent inhibition against *D. siamensis* venom (45.93%). As a result, H10 (*Areca catechu* L.) was selected as a candidate herb for further *in vivo* screening, albeit the number is lower than the expected 50% inhibition.

Table 5-4 Summary result of the *in vitro* screenings for Thai herbs with anti-hemotoxic activity against *D. siamensis* venom. Total percent inhibition is shown in the last column ("normalize to 100%"). The green highlight indicates the herb with the most inhibitory effect against *D. siamensis* venom. The letter "+" refers to inhibitory effect, while the letter "-" refers to no inhibitory effect.

Code number	Experiment	Phospholipase assay	Fibrinogenolytic assay	Coagulation assay	Phospholipase x 3	Coagulation x 4	Sum of % inhibiton	Normalize to 100% max
H1	VH1 (1:1)	1.457	-	6.520	4.372	0	30.451	4.350
	VH1 (1:0.5)	N/A	-	16.795	0.000	0	67.181	9.597
	VH1 (1:0.1)	-3.279	-	18.235	0.000	0	72.942	10.420
H2	VH2 (1:1)	36.463	-	19.274	109.389	0	186.484	26.641
	VH2 (1:0.5)	N/A	-	14.480	0.000	0	57.918	8.274
	VH2 (1:0.1)	5.493	-	16.833	16.480	0	83.810	11.973
H3	VH3 (1:1)	39.350	-	7.116	118.049	0	146.512	20.930
	VH3 (1:0.5)	N/A	-	9.522	0.000	0	38.087	5.441
	VH3 (1:0.1)	26.850	-	18.780	80.549	0	155.671	22.239
H4	VH4 (1:1)	-0.865	-	20.045	0.000	0	80.181	11.454
	VH4 (1:0.5)	N/A	-	17.680	0.000	0	70.721	10.103
	VH4 (1:0.1)	-0.818	-	21.183	0.000	0	84.730	12.104
H5	VH5 (1:1)	4.488	+	12.544	13.464	50.176	63.641	9.092
	VH5 (1:0.5)	N/A	+	12.074	0.000	48.295	48.295	6.899
	VH5 (1:0.1)	0.047	-	13.916	0.140	0	55.805	7.972
H6	VH6 (1:1)	20.547	+	-0.972	61.641	0	61.641	8.806
	VH6 (1:0.5)	N/A	+	1.261	0.000	5.043	5.043	0.720
	VH6 (1:0.1)	10.659	+	-0.359	31.978	0	31.978	4.568
H7	VH7 (1:1)	-1.295	+	-6.222	0.000	0	0.000	0.000
	VH7 (1:0.5)	N/A	+	0.367	0.000	1.469	1.469	0.210
	VH7 (1:0.1)	15.879	-	7.432	47.637	0	77.364	11.052
H8	VH8 (1:1)	13.153	-	5.428	39.459	0	61.173	8.739
	VH8 (1:0.5)	N/A	-	6.905	0.000	0	27.620	3.946
	VH8 (1:0.1)	9.291	-	8.736	27.874	0	62.817	8.974
H9	VH9 (1:1)	13.358	+	-2.848	40.073	0	40.073	5.725
	VH9 (1:0.5)	N/A	-	4.331	0.000	0	17.324	2.475
	VH9 (1:0.1)	14.630	-	13.171	43.889	0	96.572	13.796
H10	VH10 (1:1)	50.250	+	42.689	150.750	170.756	321.506	45.929
	VH10 (1:0.5)	N/A	+	12.660	0.000	50.642	50.642	7.235
	VH10 (1:0.1)	18.696	-	16.577	56.088	0	122.394	17.485

3. *In vivo* Screening

3.1 Mortality Rate and LD_{50} of *D. siamensis* venom

Mortality rate of each group (control group and venom-treated groups) was calculated for the lethal dose of *D. siamensis* venom (Table 5-5). It was found that the LD_{50} after 4-hour incubation was 12.69 µg/embryo (6.35 µg/µL). This value was relatively smaller than the batch used in Chapter IV (16.54 µg/embryo or 8.27 µg/µL) indicating higher toxicity of the new batch of snake venom.

Table 5-5 Total number of chick embryos (6 day after incubation at 37.5 ± 0.5°C) used in each treatment, number of dead/alive embryos, and mortality rate after exposure to various dose of *D. siamensis* venom for 4 hours.

Venom concentration (µg/µL)	Start embryo	Death hour (hr after treating)				Total death	Alive	Mortality rate
		1	2	3	4			
0	19	0	0	1	0	1	18	5.26
1.25	18	0	0	0	0	0	18	0.00
2.5	17	1	1	0	0	2	15	11.76
5	15	6	1	0	1	8	7	53.33
10	16	8	1	1	0	10	6	62.50

3.2 Mortality Rate and LD_{50} of Herb Extract

After the *in vitro* screening, only the selected herb (H10, *A. catechu*) was tested for LD_{50} in the chick embryo. After treating chick embryo with the herb solution for 4 hours, the mortality rate of chick embryo was recorded (Table 5-6). The LD_{50} of H10 was 445.16 µg/µL which is approximately 70 times higher than the maximum concentration used in the *in vivo* herb screening (6.35 µg/µL). This indicates that *A. catechu* is quite safe to the chick embryo.

Table 5-6 Total number of chick embryos (6 day after incubation at 37.5 ± 0.5°C) used in each treatment, number of dead/alive embryos, and mortality rate after exposure to various dose of *A. catechu* solution (0.1, 1, 10, 100, 200, and 500 µg/µL) for 4 hours.

Herb concentration (µg/µL)	Start embryo	Death hour (hr after treating)				Total death	Alive	Mortality rate
		1	2	3	4			
ctrl	20	0	0	0	0	0	20	0.00
0.1	12	0	0	1	0	1	11	8.33
1	16	0	0	0	0	0	16	0.00
10	16	0	0	1	1	2	14	12.50
100	15	0	0	1	1	2	13	13.33
200	15	0	0	0	3	3	12	20.00
500	16	0	1	2	6	9	7	56.25

3.3 Herb Screening

The result shows that the control group (PBS) and negative control group had 1 death (a 5 % and 4.76% mortality rate, respectively). The negative control group or venom treating at LD_{50} concentration showed the mortality rate of 47.62% which is closed to the expected 50% mortality. The *A. catechu* treated group showed 0% mortality inferring the herb do not adversely affect the chick embryo. Mixing the herb with the venom at LD_{50} at 1:1 and 1:0.5 ratio could reduce the mortality rate of chick embryo to 5 % and 38.10%, respectively. However, mixing the herb with the venom at LD_{50} at 1:0.1 ratio could not inhibit the embryo death and resulted in 71.43% mortality (Table 5-7).

Table 5-7 Total number of chick embryos (6 day after incubation at 37.5 ± 0.5°C) used in each treatment, number of dead/alive embryos, and mortality rate after exposure to various doses of *A. catechu* solution with *D. siamensis* venom at LD_{50} for 4 hours.

Groups	Start embryo	Death hour (hr after treating)				Total death	Alive	Mortality rate
		1	2	3	4			
ctrl (PBS)	20	0	1	0	0	1	19	5.00
-ve ctrl (venom at LD_{50})	21	6	1	2	1	10	18	47.62
+ve ctrl (V+S)	21	0	0	0	1	1	20	4.76
herb toxicity (herb solution at LD_{50} of venom)	20	0	0	0	0	0	20	0.00
V+H at 1:1	20	0	1	0	0	1	19	5.00
V+H at1:0.5	21	2	4	2	0	8	13	38.10
V+H at1:0.1	21	6	6	2	1	15	6	71.43

The mortality rate of the screening group (V+H) at various ratios was used to calculate to %inhibition as previously described. The 100% inhibition was assigned to the positive control group (mixture of venom and antivenom serum), while 0% inhibition was assigned to the negative control group (venom at LD_{50}). The percent inhibition and *A. catechu* concentration were plotted in Microsoft Excel. Dose-dependent inhibitory effect of *A. catechu* against the *D. siamensis* venom was found (Figure 5-12). After the linear regression analysis, the median inhibitory dose (ID_{50}) of *A. catechu* against *D. siamensis* venom in chick embryo during 4-hour exposure was calculated at 4.42 µg/µL. The result was assessed by linear regression analysis and the p-value was 0.042 indicating that the relation between concentration of extract and %inhibition was dose-dependent.

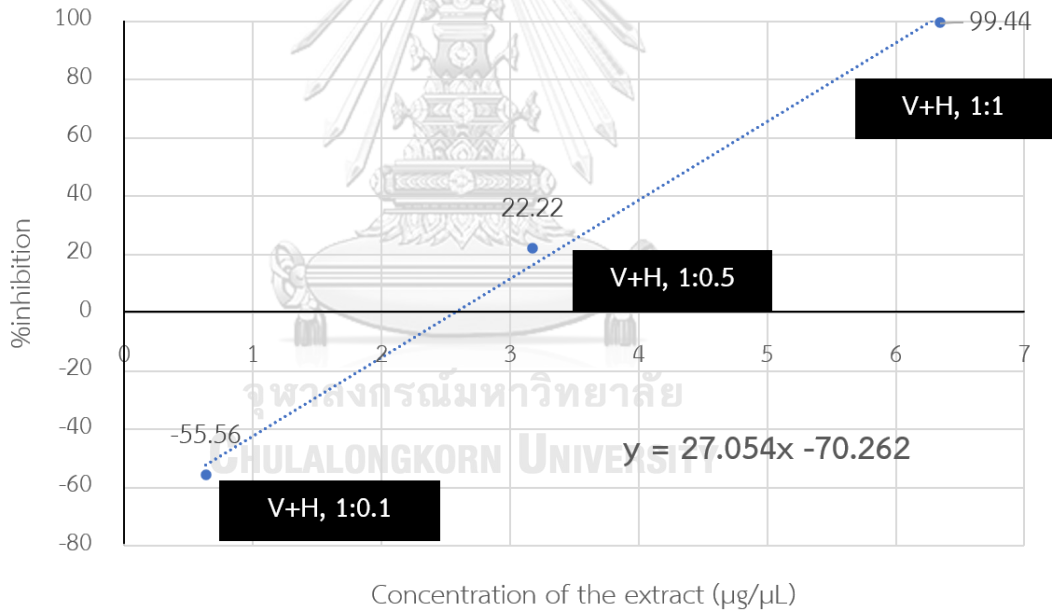

Figure 5-12 Linear regression line showing relationship between concentrations of *A. catechu* extract vs. percent inhibition against the *D. siamensis* venom at LD_{50} value (6.35 µg/µL). The relationship between concentration of the extract and %inhibition was dose-dependent (linear regression; $F(1,1) = 228.454$, $p = 0.042$).

Discussion

Potential candidate Thai herb(s) that can be used to treat *D. siamensis* envenomated patient were screened in this study. Ten Thai herbs were selected and screened for anti-hemotoxic activity against snake venom using the validated *in vitro* assays (phospholipase assay, fibrinogenolytic assay, and coagulation assay). The inhibitory effect of the herb against the venom was used as a selection criterion. It was found that *A. catechu* was the selected herb with more than 45.93 % inhibition against the snake venom, and subsequently tested in the *in vivo* assay. After an *in vivo* screening, the median inhibitory dose (ID_{50}) of *A. catechu* against *D. siamensis* venom in chick embryo during 4-hour exposure was calculated at 4.42 µg/µL.

A. catechu was previously found to have antivenom activity (Jaiswal et al., 2011) as well as other therapeutic effects including anti-bacteria (Faden, 2018), anti-microbe (Cyriac et al., 2012), and anti-inflammatory (Pithayanukul et al., 2009). An aqueous extract of *A. catechu* was also found to show the inhibitory effect against *Naja kaouthia* in the *in vitro* neutralizing method (Pithayanukul et al., 2005). One potential active constituent in *A. catechu* extract that was reported to show antivenom activity is tannin (Okuda, 1991) Tannin has a role in the *in vivo* screening by inhibiting the lethality of venom by 2 means including the specific inhibitory effect at the nicotinic acetylcholine receptor and the non-specific inhibitory effect by precipitation the protein in venom (Jaiswal et al., 2011). In addition to tannin, other constituents in *A. catechu* could also play role in these inhibitory effects and should be further studies.

Although *A. catechu* was found to poses an inhibitory effect against the hematotoxic activity of *D. siamensis* venom, it is uncertain whether the herb may inhibit other toxic effect of the *Daboia* venom. In *D. russelii*'s case reports (Phillips et al., 1988; Kularatne, 2003; Antonypillai et al., 2011), symptoms found in the snakebite patients included coagulopathy, hemorrhage, myotoxicity, necrosis and neurotoxicity. Although neurotoxicity and myotoxicity is not common in *D. siamensis* (Warrell, 1989), these effects should be targeted for further assay development.

Meanwhile, the potential adverse effects of *A. catechu* should also be considered before further use in the remedy. It has been widely report that chewing *A. catechu* may lead to various disease including leukoplakia, oral submucous fibrosis, carcinoma of oral squamous cell, and pharyngeal carcinoma (Jeng et al., 1999; Trivedy et al., 2002; Lee et al., 2005). In addition, toxicity of *A. catechu* is quite high to the point that it can be used as a potential molluscicide (Jaiswal and Singh, 2008). Physicians who routinely ground the *A. catechu* also reported that particulate matter of *A. catechu* could also cause the irritation to the airway.

One of the well-known herb for snakebite treatment is *Trigonostemon reidioides* (Kurz) Craib (H9). According to the actual treatment, most of the snakebite patient bitten by *N. kaouthia* was successfully treated with the mixture of *T. reidioides* and *A. catechu* (Srithamma et al., 2004). However, the result on the screening showed that *T. reidioides* had a very little inhibitory effect against *D. siamensis* venom (5.72% to 6.26% total inhibition). In the previous study from Srithamma et al. (2004), the pre-incubated of filtered extract of *T. reidioides* and *N. kaouthia* venom was intra-muscularly treated to the mice also failed to exert an inhibitory effect against the venom. In another study that orally treated the mice with the unfiltered *T. reidioides* extract after *N. kaouthia* venom injection showed that the extract could prolong the mortality rate for 48 hours (Prapavicha, 1998). It is of interest to note that *N. kaouthia* venom was the target in most of the previous studies. This is possibly due to the effect of Rediocides A and G, major components in *T. reidioides*, on neutralizing against *N. kaouthia* venom especially on the alpha-cobratoxin. The Rediocides A and G was found to share the same binding sites with the cobratoxin, hence the competition between these components may result in the inhibitory effect of *T. reidioides* to this toxin (Utsintong et al., 2009). The minimum inhibitory effect of *T. reidioides* against *D. siamensis* suggested that components in this herb are specific to certain components in the snake venom.

For the herb with lesser inhibitory effect against *D. siamensis* venom, *A. acidum* and *M. repandus* are also interesting for the further screening study. These

herbs were selected by the criteria that they were in the same family as *T. reidioides*. The leaves of *A. acidum* showed the therapeutic effect against stomachache and dysentery (Khan and Yadava, 2010). In the phytochemical test of the ethanol extract of *A. acidum*, tannin and alkaloid were found in the moderate amount (Patil and Jadhav, 2014). A closely-related species in genus *Antidesma, A. ghaesembilla*, was found to have cytotoxic, antioxidant, and antibacterial activities in the phytochemical screening (Habib et al., 2011). The leaves of *M. repandus* has a strong therapeutic effect against inflammation (Saijo et al., 1989; Hasan et al., 2014), moderate in relieving muscle pain (Chuakul, 2000), inhibitory effect against snakebite (Lin and Kan, 1990). The phytochemical screening of methanolic extract showed various active compounds such as carbohydrates, glycosides, flavonoids, saponins including tannin and alkaloid (Hasan et al., 2014). These herb extracts contain tannin and alkaloid as active compounds which are the potential active compounds against the snake venom (Soares et al., 2005) and might be the good candidate for further screening of herb with antivenom activity.

Conclusion

Based on three *in vitro* screenings, *Areca catechu* (H10) showed the most promising result as candidates for an *in vivo* screening. In the *in vivo* screening, *A. catechu* at the ratio 1:1 and 1:0.5 showed the inhibitory effect against the venom with a very high LD_{50} concentration. The median inhibitory dose (ID_{50}) of *A. catechu* against *D. siamensis* venom in chick embryo during 4-hour exposure was calculated at 4.42 µg/µL.

With the strong anti-hemotoxic effect of *A. catechu* found in both *in vitro* and *in vivo* assays, this herb should be further developed into the herbal remedy for snakebite treatment. In addition, this newly modified *in vitro* and *in vivo* screening assays showed the potential for screening for the herb with anti-hematotoxic activity.

CHAPTER VI
GENERAL DISCUSSION & CONCLUSION

Conclusion

Venomous snake could pose serious public health problems to people. One of the important venomous snake species with the high incident of snakebite is the eastern Russell's viper, *Daboia siamensis* (Smith, 1917). Its venom possesses hematotoxin, which causes various pathological alterations to the circulatory system, including morphological changes of the erythrocytes and blood vessels, blood coagulation, kidney failure and death (Mitrakul, 1979; Napathorn et al., 1998; Thamaree et al., 2000). Although antivenom serum is generally used as the standard medical treatment, its lethal allergic reactions (Dhanya et al., 2009), its cost per dose as well as its scarcity in some rural area created a need for an alternative remedy such as the medicinal herb.

To find effective herbs, appropriate screening assays specific to snake venom are needed. This study aims to develop screening assays for the medicinal herb with anti-hemotoxic activity against the eastern Russell's viper venom using a battery of tests both *in vitro* (phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay: Chapter III) and *in vivo* (chick embryo as an alternative animal model: Chapter IV). After validation as the appropriate assay for screening herbs, the efficacy of a selected group of Thai herbs against *D. siamensis* venom was screened and verified (Chapter V).

For *in vitro* assay development, three *in vitro* assays including phospholipase assay, coagulation assay, and fibrinogenolytic assay have been successfully developed and effectively showed a marked difference between the negative control group (venom) and the positive control group (venom and antivenom serum). Two *in vitro* assays including proteolytic assay and hemagglutination assay were proven to be not suitable for the screening assay because of the limited activity in *D. siamensis* venom. This indicates that proteins involved with these two activities might be present in minute quantity or the assay doesn't have enough sensitivity for

detecting the presence of both activities in venom, thus unlikely to be tested with the current assays.

For an *in* vivo assay development, an alternative chick embryo assay for hematotoxic activities of *D. siamensis* venom has been developed. The LD_{50} concentration of the snake venom based on this assay was 16.54 μg/embryo (or 8.27 μg/μL). This value could be used as the standard concentration of venom in further screening for herbs with an anti-hematotoxic activity. The microanatomy of embryonic vasculature was examined and verified as suitable markers for the hematotoxic effect of snake venom, including blood clotting, loss of membrane integrity, 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. According to the experiment, the cost of chick embryo (15 baths per egg) and materials used (less than 10 baths per chick embryo) for screening assay is quite reasonable. Further, methods or techniques used in this study are relatively simple with the potency for screening the therapeutic effect of herbs against *D. siamensis* venom. Therefore, chick embryo is a suitable alternative model for further screening in toxicology and pharmacology.

After the development of *in vitro* and *in vivo* assays, the selected group of Thai herbs were tested for their anti-hematotoxic activity against *D. siamensis* venom using both screening assays. Based on three *in vitro* screenings, *Areca catechu* (H10) showed the most promising result as candidates for an *in vivo* screening. In the *in vivo* screening, *A. catechu* at the ratio 1:1 and 1:0.5 showed the inhibitory effect against the venom at the LD_{50} concentration (12.694 μg/embryo or 6.347 μg/μL). The median inhibitory dose (ID_{50}) of *A. catechu* against *D. siamensis* venom in chick embryo during 4-hour exposure was calculated at 4.42 μg/μL. With the strong anti-hemotoxic effect of *A. catechu* found in both *in vitro* and *in vivo* assays, this herb should be further developed into the herbal remedy for snakebite treatment.

Overall, this study successfully developed the suitable and cost-effective *in vitro* and *in vivo* screening assays for medicinal herb with anti-hematotoxic effect against *D. siamensis* venom. *A. catechu* was initially found to be a potential candidate Thai herb for treating snakebite patient. With the high diversity of Thai herb

and the readily available set of assays to be used in the screenings, it is expected that growing number of Thai herb with potential anti-hematotoxic effect against the snake venom will be found in the future.

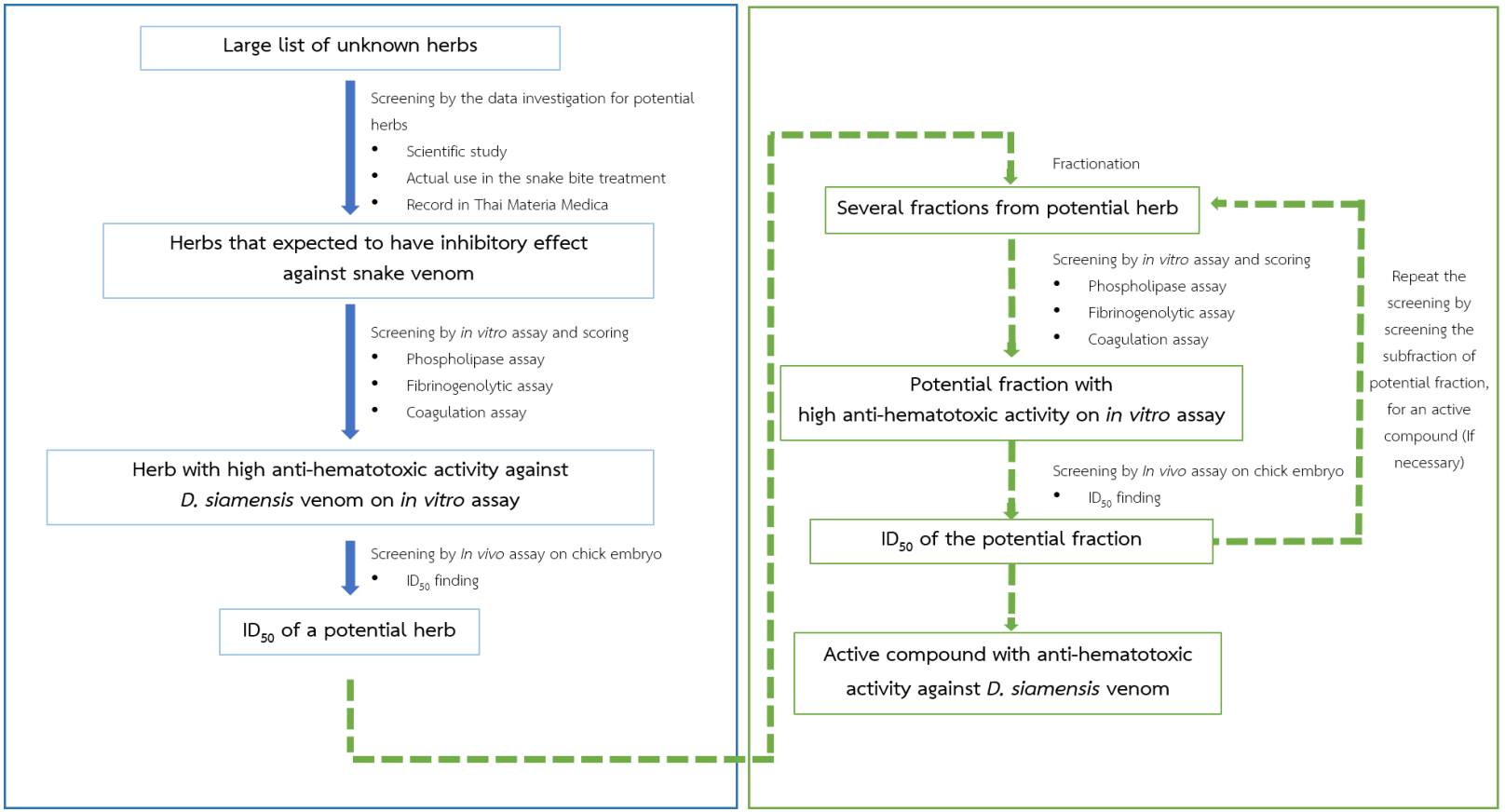

Figure 6-1 The bioassay guided isolation of herb with anti-hematotoxic activity against *D. siamensis* venom. The blue rectangle / line represents the screening process of the current work, while the green rectangle / dash line represents the potential future work on the focused herb or fraction.

Recommendations

During the course of assay development and herb screening, several modifications were made to the original protocol and some observations were noted in order to improve the outcome of this research. Specific remarks on this observation / modification are compiled in this list. In addition, the list also includes the possibilities to explore further questions as suggested by the current study.

1. **Quality of whole blood:** The coagulation and hemagglutination assays requires the use the whole blood. To avoid the ethical misconduct of obtaining blood from human, this study used the discarded blood from the National Blood Center. The blood was added with the CPDA-1 anticoagulant to prolong the storage of blood to a maximum of 35 days with the remaining 71% cell viable (Walker, 1993). As a result, the optimization of $CaCl_2$ concentration and volume to neutralize the CPDA-1 is needed for every batch of blood. In addition, the possibility to obtain the same quality of blood is uncertain. Some batch of old blood may be partially hemolyzed and cannot be used for the experiment. Therefore, the use of new whole blood, if available, is recommended. Also, centrifugation and fractionation for plasma should be done on the first day of arrival in order to prolong the plasma quality.

2. **The proteolytic and hemagglutination assays:** In this study, the proteolytic and hemagglutination assays were excluded from the screening (Chapter V) because of their very low (negligible) activity in *D. siamensis* venom (Chapter III). It is of important to note that the lack of these two activities cannot be universally applicable to every hematotoxic venom. Prior studies on the viper venom showed that high inter- and intra-species variability in venom protein was found (Gutierrez et al., 2011). Physical and biological factors such as age, body length, habitat may influence the composition of the enzyme in venom (Furtado and Kamiguti, 1985; Warrell and Phillips, 1985). For future study on hematotoxic venom of any snake species, the proteolytic and hemagglutination assays should still be included in the early step of assay development and validation.

3. **The active ingredients of *Areca catechu*:** Based on the *in vitro* and *in vivo* screenings, *A. catechu* extract has a prominent inhibitory effect against *D. siamensis* venom. However, the exact mechanism of the inhibition is still not known. The possible inhibitory reaction included the binding between tannin in *A. catechu* crude extract and the enzyme in the venom (Jaiswal et al., 2011). In addition, other constituents in the crude extract may also play roles in this inhibitory effects. It is recommended to undergo fractional extraction and characterization for active constituent(s) in the *A. catechu* crude extract. These candidate constituents could then be screened for anti-hematotoxic effect against the snake venom with the battery of *in vitro* and *in vivo* assays developed from this study.

4. **Note on the difference in the herb preparation:** In this study, methanolic extract of *Trigonostemon reidioides* (Kurz) Craib and *Areca catechu* L. were screened for anti-hematotoxic activity. It was found that only *A. catechu* showed a prominent inhibitory effect against *D. siamensis* venom. This has come as a surprise since these two herbs were widely used for treating snakebite patients by Mr. Aia Saikrasun, a well-known local healer in Surin province. In addition to the specific mechanism of *T. reidioides* against other snake species such as *Naja kaouthia* (Utsintong et al., 2009), it is also important to note the difference between the herbal remedy preparation and the extraction process used in this study. In traditional remedy, herbs are ground with the wooden mortar and pestle, and small amount of lime juice added to the paste. The herbal paste was then applied onto the envenomated area of the patient. It is possible that the acidic condition of the solvent and the synergism between these herbs may enhance the anti-venom effect of this herbal remedy. Therefore, the traditional extraction conditions as well as the combination of these two herbs should be further tested for the anti-hematotoxic activity against *D. siamensis* venom.

APPENDICES

APPENDIX A: HISTOLOGICAL PROCESS AND STAINING

1. Tissue Process

After the *in vivo* assay, the blood vessel of chick embryo was fixed in 10% neutral-buffered formalin solution for 24 hours and stored in 70% ethanol. The histological process was carried out as follows.

1.1 Trimming

The blood vessel tissue in 70% ethanol (Figure A-1A) was trimmed into a small piece so that only the treated area was selected. The exact location of the treated area was verified with the actual photograph taken on the day of tissue collection. After that, the tissue was washed by 70% ethanol (Figure A-1B).

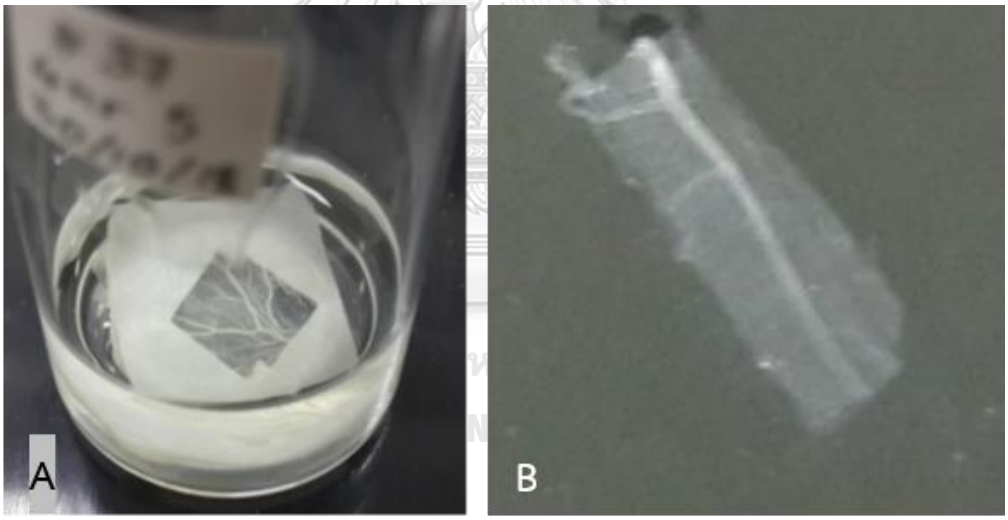

Figure A-1 The blood vessel tissue was stored in 70% ethanol (A). The trimmed tissue with small membrane area around the blood vessel (B).

1.2 Dehydration

The series of alcohol was used to gradually dehydrate the tissue. The chemicals used in this step are listed in Table A-1. The volume of chemicals used in each step was at least 10 times the volume of the tissue. During the immersion, the tissue was slowly shaken by the rotator for increasing the penetration of chemicals into the tissue. Eosin droplet was added to the 95% ethanol step for increasing the visibility of the tissue during the subsequent process, especially in the sectioning step. Tissue was subsequently immersed in xylene to clear the opacity of the tissue.

Table A-1 Series of chemicals and time used in each dehydration step

Chemicals	Time (minutes)
70% ethanol	15
90% ethanol	15
95% ethanol with 5 droplets of eosin	15
Butanol #1	15
Butanol #2	15
Xylene #1	15
Xylene #2	15

1.3 Infiltration and embedding

After dehydration, the paraffin wax was used to infiltrate the tissue for the ease of sectioning. The tissue was immersed in wax at 3 steps as shown in Table A-2.

Table A-2 Series of chemicals and time used in each infiltration step

Chemicals	Time (minutes)
Xylene and Wax I (1:1)	15
Wax I	15
Wax II	15

After these steps, a tissue was embedded in the molten wax in the embedding ring and left until setting at room temperature. This tissue block can be kept at 2°C to prevent from melting until further process (Figure A-2).

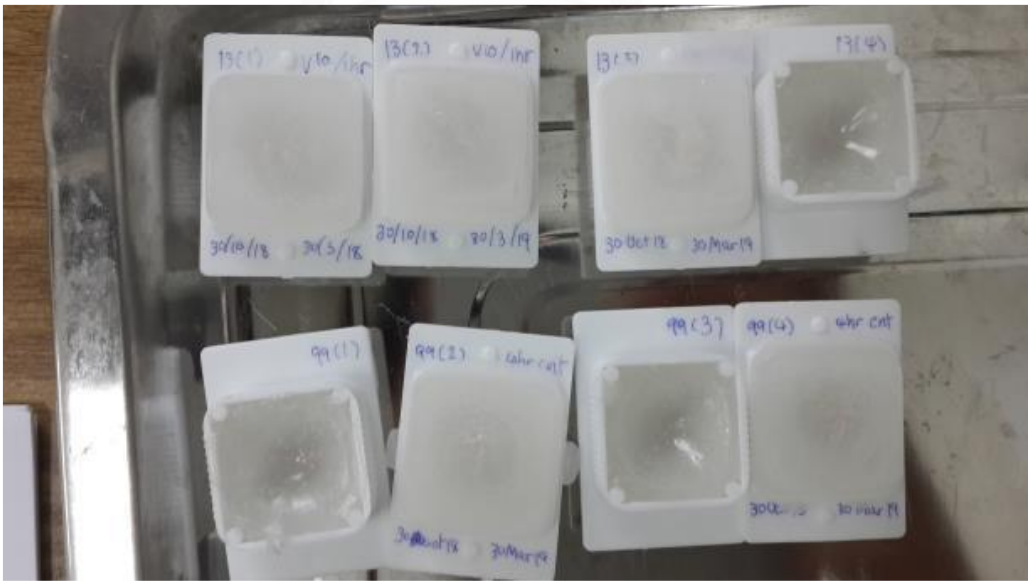

Figure A-2 The tissue block in paraffin wax to be sectioned in the next process

1.4 Sectioning and Attaching to the Slide

The wax around the tissue was trimmed by a razor blade or a knife to reduce the surface area of the wax before sectioning. Once trimmed, the tissue block was attached to a rotary microtome (Figure A-3A) and gradually sectioned into a ribbon with 0.6 µm thickness. The ribbon was transferred to a box with a black background for better visualization.

Before attaching the ribbon to the microscope slide, every slides was immersed in acidic alcohol overnight to clean the slide. Acidic alcohol solution was prepared by adding 10 mL of glacial acetic acid into 1,000 mL of 70% ethanol. After that, each slide was labeled and added with albumin solution (7-8 drops of albumin in 100 mL of distilled water). Tissue sections were placed onto the albumin solution on the slide, then the slide was place onto a hotplate at 40-45°C. The excess amount of albumin solution was wiped out by tissue paper. The dry slides were kept in the slide box until the staining process (Figure A-3B).

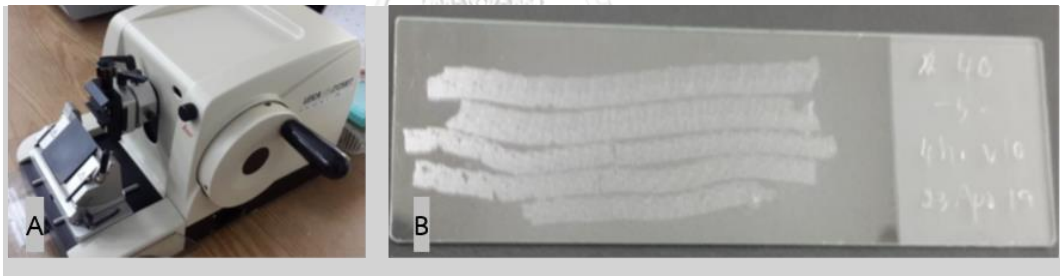

Figure A-3 A rotary microtome was used for sectioning the tissue into a ribbon with 0.6 µm thickness (A). The ribbon was attached to the microscope slide before the staining process (B).

2. Hematoxylin and Eosin Staining (H&E staining)

2.1 The Preparation of Chemical in H&E Staining

2.1.1 Delafield Hematoxylin Solution

- 4 g of hematoxylin
- 125 mL of absolute ethanol
- 6 g of ammonium alum
- 400 mL of distilled water
- 100 mL of glycerin

Four grams of hematoxylin were added into 25 mL of absolute ethanol, followed by 400 mL of saturated ammonium alum solution (6 g of ammonium alum in 400 mL distilled water, stirred under hear). The mixture was stored in the glass bottle with the cotton on the lid for the ventilation. The bottle was placed near the sunlight for 3-5 days for ripening the content. After that, the solution was filtered by the Whatman No. 4 filter paper. The solution was added with 100 mL of glycerin and 100 mL of absolute ethanol and left it at room temperature. The final solution was dark purple in color. It was kept at room temperature and needed to be filtered before use

2.1.2 Differentiator

One hundred mL of 70% ethanol was added with the 4 drops of 37% hydrochloric acid. The solution was kept in the amber glass bottle and away from the sunlight. The final solution must be a colorless solution.

2.1.3 Eosin Solution

One gram of eosin Y was dissolved in 100 mL of distilled water.

2.2 H&E Staining Process

To stain with hematoxylin and eosin, the tissue slide was immersed in a series of chemicals as shown in Table A-3.

Table A-3 Series of chemicals and time used for the hematoxylin and eosin (H & E) staining procedure

Chemicals	Time
Xylene I	5 minutes
Xylene II	3 minutes
n-Butanol	3 minutes
95% EtOH	3 minutes
90% EtOH	3 minutes
70% EtOH	3 minutes
Distilled water	3 minutes
Hematoxylin	3 minutes
Tap water	3 minutes
Differentiator (until background clear)	Dip 30 times
Tap water (until section blue)	3 minutes
70% EtOH	3 minutes
90% EtOH	3 minutes
Eosin	3 minutes
95% EtOH	3 minutes
n-Butanol I	3 minutes
n-Butanol II	3 minutes
Xylene I	3 minutes
Xylene II	5 minutes

2.3 Slide Mounting

After staining procedure, the slide was mounted with Permount and close with the coverslip. The slide was left at room temperature for an hour or until the Permount was completely dried before keeping in the slide box.

3. Periodic Acid Schiff Staining (PAS staining)

3.1 The Preparation of Chemical in PAS Staining

3.1.1 Periodic Acid Solution

- 1 g of periodic acid (H_5JO_6)
- 100 mL of distilled water

One gram of periodic acid was added into 100 mL of distilled water.

3.1.2 Schiff Reagent

- 1 g of basic fuchsin
- 1.9 g of sodium metabisulfite ($Na_2S_2O_5$)
- 100 mL of 0.15 N hydrochloric acid (HCl)

One hundred mL of 0.15 N hydrochloric acid was added with 1 g of basic fuchsin and 1.9 g of sodium metabisulfite, then stirred with a magnetic stirrer for 30 minutes or until no sediment was found in the solution. While stirring, 500 mg of activated charcoal was added to the solution and continue stirring for 1-2 minutes. The filter paper (Whatman No. 1) was used for filtering the charcoal from the solution. This step can be repeated until the solution was clear. The amber bottle was used for storing the Schiff Reagent at 2 – 4 °C. The solution was stable for several months.

3.2 PAS Staining Process

Tissue slide was immersed in a series of chemicals as shown in Table A-4 for PAS staining procedure.

Table A-4 Series of chemicals and time used for the PAS staining procedure

Chemicals	Time	Chemicals	Time
Xylene I	5 minutes	Distilled water	3 minutes
Xylene II	3 minutes	Differentiator	Dip 20 times
n-Butanol	3 minutes	Tap water	3 minutes
95% EtOH	3 minutes	70% EtOH	3 minutes
90% EtOH	3 minutes	90% EtOH	3 minutes
70% EtOH	3 minutes	95% EtOH	3 minutes
Tap water	2 minutes	95% EtOH	3 minutes
Periodic acid solution	15 minutes	n-Butanol I	3 minutes
Tap water	3 minutes	n-Butanol II	3 minutes
Schiff reagent	15 minutes	Xylene I	3 minutes
Tap water	10 minutes	Xylene II	5 minutes
Hematoxylin	4 minutes		

APPENDIX B: STATISTICAL DATA

1. The LD_{50} Calculation of *D. siamensis* Venom

1.1 The LD_{50} Calculation in the *in vivo* Development

Table B-1 The LD_{50} calculation of chick embryo in the *in vivo* development after treating with various concentrations of *D. siamensis* venom. The yellow highlight is on the LD_{50} of venom (8.270 µg/µL).

	Probability	95% Confidence Limits for Venom_conc		
		Estimate	Lower Bound	Upper Bound
PROBIT	.010	1.054	.206	1.906
	.020	1.341	.328	2.262
	.030	1.563	.441	2.527
	.040	1.754	.549	2.749
	.050	1.927	.657	2.947
	.060	2.087	.763	3.130
	.070	2.238	.871	3.302
	.080	2.383	.979	3.466
	.090	2.522	1.088	3.626
	.100	2.658	1.198	3.782
	.150	3.303	1.768	4.547
	.200	3.925	2.370	5.355
	.250	4.551	2.990	6.278
	.300	5.198	3.612	7.384
	.350	5.879	4.225	8.743
	.400	6.608	4.826	10.425
	.450	7.399	5.422	12.514
	.500	8.270	6.023	15.119
	.550	9.244	6.645	18.392
	.600	10.350	7.304	22.564
	.650	11.634	8.021	27.987
	.700	13.159	8.823	35.235
	.750	15.029	9.753	45.301
	.800	17.427	10.876	60.079
	.850	20.709	12.320	83.692
	.900	25.730	14.376	127.333
	.910	27.115	14.917	140.962
	.920	28.705	15.526	157.446
	.930	30.560	16.222	177.827
	.940	32.774	17.034	203.753
	.950	35.496	18.007	238.007
	.960	38.984	19.217	285.737
	.970	43.745	20.811	357.819
	.980	50.987	23.128	482.726
	.990	64.910	27.295	774.401

a. Logarithm base = 10.

1.2 The LD_{50} Calculation in the *in vivo* Screening

Table B-2 The LD_{50} calculation of chick embryo in the *in vivo* screening after treating with various concentrations of *D. siamensis* venom. The yellow highlight is on the LD_{50} of venom (6.347 µg/µL).

		Confidence Limits				Confidence Limits			
		95% Confidence Limits for Venom_conc				95% Confidence Limits for Venom_conc			
	Probability	Estimate	Lower Bound	Upper Bound	Probability	Estimate	Lower Bound	Upper Bound
PROBIT	.010	.953	.214	1.698	.550	7.032	5.179	11.776
	.020	1.190	.322	1.992	.600	7.803	5.702	13.966
	.030	1.370	.417	2.208	.650	8.689	6.264	16.749
	.040	1.523	.506	2.388	.700	9.732	6.886	20.376
	.050	1.660	.592	2.548	.750	10.999	7.596	25.273
	.060	1.787	.676	2.693	.800	12.605	8.445	32.235
	.070	1.906	.759	2.829	.850	14.774	9.522	42.948
	.080	2.019	.842	2.958	.900	18.042	11.037	61.839
	.090	2.128	.924	3.082	.910	18.934	11.432	67.561
	.100	2.233	1.007	3.203	.920	19.953	11.876	74.391
	.150	2.727	1.424	3.777	.930	21.137	12.382	82.715
	.200	3.196	1.858	4.350	.940	22.543	12.969	93.135
	.250	3.662	2.308	4.966	.950	24.261	13.671	106.655
	.300	4.139	2.771	5.659	.960	26.447	14.539	125.102
	.350	4.636	3.242	6.468	.970	29.406	15.678	152.260
	.400	5.163	3.717	7.433	.980	33.859	17.322	197.798
	.450	5.729	4.195	8.599	.990	42.285	20.251	299.042
	.500	6.347	4.680	10.022	a. Logarithm base = 10.			

2. The LD_{50} Calculation of *Areca catechu* in the *in vivo* Screening

Table B-3 The LD_{50} calculation of chick embryo in the *in vivo* screening after treating with various concentrations of *A. catechu* solution. The yellow highlight is on the LD_{50} of *A. catechu* (445.16 µg/µL).

		Confidence Limits		
		95% Confidence Limits for หมาก_conc		
	Probability	Estimate	Lower Bound	Upper Bound
PROBIT	.010	-173.370	-501.776	-32.991
	.020	-100.892	-376.046	21.813
	.030	-54.907	-297.295	57.604
	.040	-20.314	-238.807	85.283
	.050	7.825	-191.864	108.430
	.060	31.775	-152.473	128.695
	.070	52.775	-118.455	146.986
	.080	71.578	-88.488	163.854
	.090	88.678	-61.704	179.665
	.100	104.419	-37.502	194.671
	.150	169.591	56.890	262.613
	.200	221.388	123.607	324.914
	.250	265.825	174.472	384.735
	.300	305.730	215.686	442.921
	.350	342.709	250.866	499.850
	.400	377.798	282.208	555.910
	.450	411.747	311.108	611.572
	.500	445.158	338.518	667.383
	.550	478.568	365.149	723.974
	.600	512.517	391.595	782.090
	.650	547.606	418.425	842.662
	.700	584.585	446.269	906.927
	.750	624.490	475.929	976.666
	.800	668.927	508.590	1054.691
	.850	720.724	546.285	1146.014
	.900	785.896	593.284	1261.349
	.910	801.637	604.578	1289.264
	.920	818.737	616.827	1319.610
	.930	837.540	630.270	1353.002
	.940	858.540	645.257	1390.322
	.950	882.490	662.318	1432.919
	.960	910.629	682.323	1483.003
	.970	945.222	706.865	1544.628
	.980	991.207	739.413	1626.623
	.990	1063.685	790.564	1756.006

a. Logarithm base = 10.

APPENDIX C: RESEARCH DISSEMINATION

Proceedings

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom. *AIP Conference Proceedings*, *2120*(1), 070012. [SCOPUS]

Abstract (International Conference)

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2018) Development of screening assays for Thai herb with anti-hemotoxic activities against the eastern Russell's viper *Daboia siamensis* (Smith, 1917) venom. *Young Scientist Award 2018 (YSA)*, March 29-30, 2018, Pathum Thani, Thailand. [poster presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom. *AIP Conference Proceedings, 2120*(1), 070012. *International Conference on Biology and Applied Science 2019 (ICOBAS)*, March 20-21, 2019, Malang, Indonesia. [oral presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom. *18th Biological Sciences Graduate Congress (BSGC)*, December 18-20, 2018, Bangkok, Thailand. [poster presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2020). Vasculature anatomy as a marker for hematotoxic effect of the Russell's viper *Daboia siamensis* venom on chick embryo *Gallus gallus domesticus*. *Journal of Anatomy* 236 (Supplement I): 288-289. [Scimago Journal Rank 2019: Q1]

Meeting Abstract P2-V22, the *19th International Federation of Associations of Anatomists Congress (IFAA)*, August 9-11, 2019, London, UK. [poster presentation]

Proceedings

“AIP Conference Proceedings”

AIP Conference Proceedings

Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom

Cite as: AIP Conference Proceedings **2120**, 070012 (2019); https://doi.org/10.1063/1.5115729
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Development of *In Vitro* Assays for Hematotoxic Activity of the Russell's Viper *Daboia siamensis* venom

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Abstract The eastern Russell's viper, *Daboia siamensis*, is one of the important venomous snakes causing serious public health problems in Southeast Asia. Its venom contains a variety of enzymes and peptides that cause hemotoxic effects. Since antivenin commonly used for treating snakebite patients may lead to lethal allergic reaction, medicinal herbs have become an important alternative remedy. To find effective herbs, appropriate screening assays specific to snake venom are needed. This study aims to develop appropriate *in vitro* assays for screening medicinal herbs that have anti-hemotoxic activity against *D. siamensis* venom. Five assays corresponded to important biological activities of the venom were tested. Results revealed that 3 out of 5 assays were effective in showing markedly difference between positive and negative controls including 1) phospholipase A_2 assay examining diameter of clear zone around the well in agar plate with yolk solution, 2) coagulation assay measuring increase in optical density of the plasma after venom addition, and 3) fibrinogenolytic assay examining cleavage of alpha-fibrinopeptide in the SDS-PAGE. No significant difference between positive and negative controls was found in proteolytic and hemagglutination assays. These selected essays can be further incorporated with *in vivo* assay(s) to screen for effective herbs against *D. siamensis* venom.

INTRODUCTION

Venomous snakes can be found worldwide, especially in tropical regions such as Southeast Asia [1-2]. In Thailand, various venomous snake species are commonly found in a rural area or forest edges where they could pose serious public health problems to people. One of the most important venomous snake species is the eastern Russell's viper or *Daboia siamensis* [3] which is known to contain hemotoxic venom causing pathologic alterations to the circulatory system of snakebite patient including blood coagulation, red blood cell morphology change, kidney failure and death [4-6]. In general, anti-venin is regarded as a standard medical treatment for snakebite. However, concerns have been raised over the use of anti-venin since it is ineffective to treat some certain symptoms, the cost per dose is expensive [7], and 5-80% of patients who received serum can potentially develop allergic reactions which can cause redness, illness or even death [8-10]. Alternative treatments that are more effective and safer are thus needed.

In many parts of Southeast Asia, local healers have been known to use medicinal herbs as a treatment for snakebite patients. Recently, the treatment of snakebite patients has incorporated traditional herbs with modern medical practice in several hospitals in Thailand [11]. Unfortunately, there is still a limited number of scientific reports to prove the effectiveness and safety of traditional herbs [12-13]. In addition, assays for the efficacy of herbs require the time-consuming and expensive use of animals, thus further limited the availability of scientific discovery of remedy for snakebite patients.

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This study aims to develop screening assay for the medicinal herb with anti-hemotoxic activity against eastern Russell's viper venom using a battery of *in vitro* tests including phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay. The suitable assay will be used as the standard assay for further screening of anti-hemotoxic herbs.

EXPERIMENTAL DETAILS

Five *in vitro* assays covering major mode of hemotoxicity in snakebite patient were developed. Each assay was carried out using the same experiment groups including 1) negative control (venom and normal saline) 2) positive control (venom and a dose of anti-venin that completely inhibited the venom) and 3) normal control (only normal saline or phosphate-buffered saline (PBS)). The aim is to find optimal conditions, volume, or concentration of each substance. The optimal conditions of the assay should show a marked difference between negative control and positive control groups.

Preparation of Venom and Serum Solution

Lyophilized snake venom of the eastern Russell's viper and its anti-venin serum were purchased from Snake Farm, the Queen Saovabha Memorial Institute, Bangkok, Thailand. Venom was dissolved and adjusted the concentration to 10 µg/µL with normal saline. Serum was dissolved in distilled water at the recommended concentration. Both solutions were aliquoted to a small volume and stored in microtubes at -20 °C for further use.

Development of Phospholipase Assay

Agar plates were prepared by mixing the following chemicals: 1% bacteriological agar, yolk solution in normal saline, sodium azide and $CaCl_2$. After solidified in a petri dish, the gel was punctured to make 0.5 cm diameter holes. The test substances including normal saline (ctrl), venom (-ve ctrl) and the mixture between venom and anti-venin serum (+ve ctrl) were applied in each well. Then, all plates were incubated at 37 °C for 18 h. After incubation, phospholipase activity can be observed from the clear zone around the hole. The diameter of the clear zone around each well was measured by a vernier caliper. In addition, all clear zones were stained with 1% methyl red to verify the presence of fatty acid which is the product from phospholipase activity.

Development of Proteolytic Assay

In this assay, azocasein was used as a substrate of protease. Azocasein was mixed with the test substances including normal saline, venom and pancreatin solution (a positive proteolytic enzyme). In addition, venom with the solution of divalent metal ion, including $CaCl_2$ and $MgCl_2$ solution was added to the experiment to enhance the effect of the proteolytic enzyme in venom [14]. All substances were mixed and incubated at 37 °C for 90 min. Then, trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 1,000 xg for 5 min After that, the supernatant was transferred to a 96-well plate and added with NaOH before the absorbance measured at 450 nm with a microplate reader (Multiskan EX). Higher absorbance represents a higher amount of azo dye, indicating proteolytic activity of the venom.

Development of Coagulation Assay

Whole blood obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand, was centrifuged at 700 xg at 4 °C for 10 minutes for the blood fractionation. Plasma in the supernatant were separated and kept at -20°C until assay. Before the assay development, the range-finding method was used to find the suitable concentration of $CaCl_2$ solution that neutralized the anticoagulant in the plasma stock. Then, the neutralized plasma was mixed with test substances in each group including PBS, venom and the mixture of venom and anti-venin serum in a 96-well plate. After incubation at 37 °C for an hour, optical density (OD) at 405 nm was measured with the microplate reader (Multiskan EX). Higher OD represents a higher amount of fibrin indicating coagulation activity of the venom.

Development of Fibrinogenolytic Assay

Polyacrylamide gel electrophoresis was used for detecting the separation of alpha and beta peptide from fibrinogen, which is the sign of fibrinogenolytic activity. Fibrinogen and test substance in each group, including the mixture between PBS, venom and anti-venin serum were mixed and incubated at 37 ºC for 1 h, then the reaction was stopped with β-mercaptoethanol. All samples were run in 12% polyacrylamide gel at 90 V for 2 h. Gels were stained with Coomassie Blue G-250 and destained with 10% acetic acid before visualization the position of protein band in each lane.

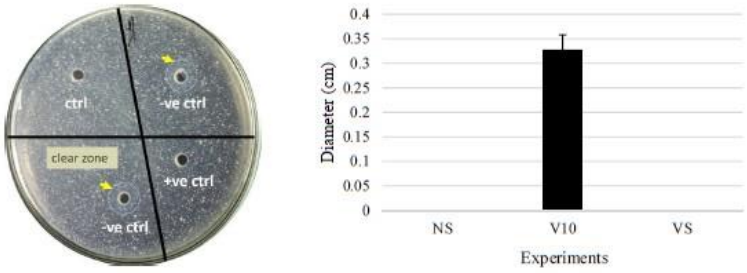
Development of Hemagglutination Assay

Whole blood obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand was centrifuged at 700 xg for 10 min at 4ºC for 10 min for the blood fractionation. After removal of the plasma, erythrocyte was washed 3 times with normal saline. Erythrocyte suspension (2%) was prepared and mixed with the mixture of venom and the serial dilution of anti-venin serum. In addition, erythrocyte suspension was tested against antibody of the same blood type to serve as positive hemagglutination of the erythrocyte. All experiment groups were transferred to the U-shape bottom 96-well plate for better visualization. After incubation at 37ºC for 2 h, there was 2 types of result: hemagglutination or the spreading pattern of erythrocyte on the surface of the well and non-hemagglutination or the group of small sinking blood droplet at the bottom of the well. Then, the minimum dilution of anti-venin serum or antibody that represent the hemagglutination was transformed to titer value for the comparison between experimental groups.

RESULT AND DISCUSSION

Development of Phospholipase Assay

After incubation for 18 h, the negative control group (-ve ctrl) showed a clear zone as the turbid area around the well (Fig. 1a) with a diameter of 0.3262 ± 0.0317 cm. In other experiment groups including ctrl (only normal saline) and +ve ctrl (the mixture of venom and serum), no clear zone was found. (Fig. 1b)

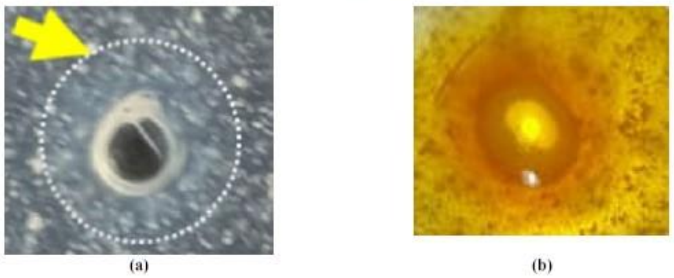


(a) (b)

FIGURE 1. (a) Representative agar plate showing the large clear zone in the negative control group (-ve ctrl). Arrows point at the margin of the clear zone. (b) Bar graph (mean ± S.D.) shows that diameter of clear zone in the negative control or venom at 10 µg/µL (V10) group is 0.3262 ± 0.0317 cm, while control or normal saline added group (NS) and positive control or the mixture of venom and anti-venin serum group (VS) show no clear zone formation.

To verify the phospholipase activity in the clear zone, 1% methyl red was used as a pH indicator. The reaction between phospholipase in *D. siamensis* venom and phospholipid in agar produces fatty acid as the product. This leads to pH decrease of the agar. The result from methyl red staining showed the clear zone in orange compared to the

nearby agar in yellow. This result indicated that the clear zone has low pH and confirmed the presence of fatty acid in the clear zone as well as the phospholipase activity in the venom (Fig. 2).



(a) (b)

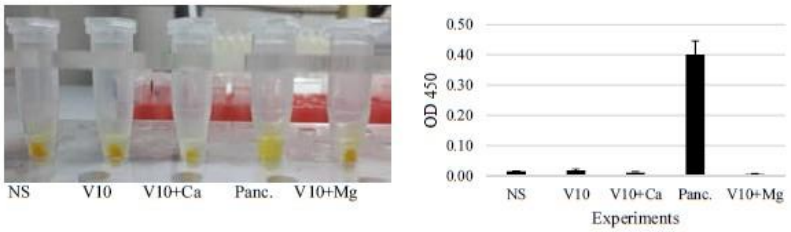
FIGURE 2. (a) The negative control group shows a clear zone around the well. (b) After staining with 1% methyl red, the clear zone was orange in colour while the nearby area was yellow.

Phospholipase A_2 (PLA_2) is known as a major enzyme in *D. siamensis* venom [15]. PLA_2 can hydrolyze phospholipid into lysophospholipid and fatty acid [16]. The presence of clear acidic zone in our phospholipase assay confirms the presence of PLA_2 in *D. siamensis* and validate that this assay is suitable to be used as one of the tests for the hemotoxic activity of the snake venom.

Development of Proteolytic Assay

After addition of a TCA solution and centrifugation, the colour of supernatant in the pancreatin group (Panc.) changed to yellow, which is the colour of azo dye as seen in Fig. 3a, while other groups (NS, V10) show the clear solution with yellow sediment of azocasein. OD measurement at 450 nm also confirmed that the highest OD value was the pancreatin added group (Fig. 3b). Although calcium and magnesium are known to enhance proteolytic activity of snake (*Agkistrodon acutus*) venom [14], addition of these divalent metal ions (V10+Ca and V10+Mg) did not yield a marked difference from the normal saline control (NS) nor the negative control (venom at 10 µg/µL: V10). As a result, the assay was not further tested with positive control (venom and anti-venin serum) in the experiment.

Azocasein is the non-specific protease substrate used for detection of the protease activity by OD measurement at 450 nm [17]. Higher OD value means higher amount of protease in the solution. In this assay, pancreatin has been successfully used as a positive proteolytic agent in the assay. However, negative control (venom at 10 µg/µL: V10; V10+Ca or V10+Mg) did not show any increase in OD compared to the normal saline control (NS). It can be concluded that *D. siamensis* venom has very little (negligible) proteolytic activity.

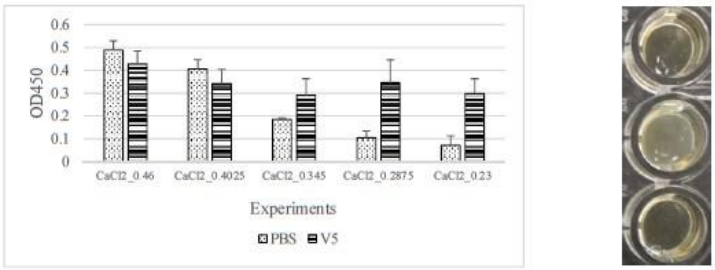


(a) (b)

FIGURE 3. (a) The result of proteolytic assay showed that the positive proteolytic activity (yellow color solution) was found only in pancreatin-added group (Panc.), while other groups including control group or normal saline (NS), negative control group or venom at 10 µg/µL (V10), venom with calcium ion (V10+Ca) and venom with magnesium ion (V10+Mg) show clear solution with yellow sediment. (b) Bar graph (mean ± S.D.) of supernatant OD450 shows the high OD value in the pancreatin added group (0.401 ±0.046).

Development of Coagulation Assay

Since the plasma used in this study contained CPDA-1 anticoagulant, it is thus necessary to find the optimal concentration of $CaCl_2$ required for deactivating (or neutralizing) the anticoagulant. The optimal concentration of $CaCl_2$ solution was calculated from the range-finding method. From Fig. 4a, the concentration that showed the largest difference in OD450 value between venom added (higher) and non-venom added (lower) is "CaCl2_0.2875" or the optimal concentration of 0.2875 µg/µL. The result of incubating the neutralized plasma with test substances showed that turbid plasma was found in the venom added group (middle well, Fig. 4b) while the control group (PBS) and the positive control group (the mixture between venom and anti-venin serum) show clear plasma (upper and lower well, Fig. 4b).



(a) (b)

FIGURE 4. (a) The result from range-finding assay for optimal concentration of $CaCl_2$ that neutralize the anticoagulation activity. The graph with the dot pattern shows a control group (plasma with PBS) and the graph with the cross pattern shows a negative control group (plasma with venom). The optimal concentration is 0.2875 µg/µL of $CaCl_2$ solution as shown in "CaCl2_0.2875" column. (b) The result of PBS group (upper well), venom (middle well) and the mixture between venom and anti-venin serum (lower well) show that the turbid solution, i.e. coagulating plasma, was found only in the negative group.

The whole blood obtained from the National Blood Center, the Thai Red Cross Society has CPDA-1 as an anticoagulant allowing long term storage for up to 28 days [18]. Citrate found in CPDA-1 solution can bind to calcium in the plasma, thus preventing the calcium from initiating a coagulation cascade [19]. In this assay, $CaCl_2$ solution

was added to increase calcium ion for reacting with free citrate molecules to neutralize the anti-coagulation activity. As a result, the presence of any active coagulant in snake venom may initiate the coagulation cascade. In *D. siamensis* venom, an enzyme in family metalloproteinase, as well as other groups, can inhibit or enhance the coagulation activity [20,15].

In this study, OD at 405 nm was selected for turbid measurement based on our preliminary study which screened 6 wavelengths (405, 450, 540, 595, 620 and 690 nm) and found that the wavelength that yielded the largest difference in OD value between control group and negative control group (venom group) was 405 nm. The use of this wavelength was also reported in similar studies [21-23].

Development of Fibrinogenolytic Assay

Fibrinogen composes of 3 subunits with different molecular weight including alpha, beta and gamma subunits. Alpha-fibrinopeptide is the heaviest, followed by beta and gamma-fibrinopeptide, respectively [24]. In Fig. 5, the major difference between lane 4 (fibrinogen) and lane 6 (fibrinogen and venom) is the absence of the uppermost band or alpha-fibrinopeptide (arrows on Fig. 5), while beta and gamma-fibrinopeptide are intact. The rectangle box in lane 6 shows the putative fragment of alpha-fibrinopeptide. In addition, comparison between lane 4 (fibrinogen) and lane 7 (fibrinogen and the mixture of venom and anti-venin serum) shows the neutralizing effect of anti-venin serum against venom that can be verified by the existence of alpha-fibrinogen's band.

It is known that *D. siamensis* venom contains a lot of enzymes in the family metalloproteinase and serine protease, which can selectively cleave the alpha chain but not beta and gamma chain [25]. An example of these enzymes includes the alpha-fibrinogenase-like enzyme which has an ability to degrade alpha-fibrinopeptide of fibrinogen as seen in this study [26].

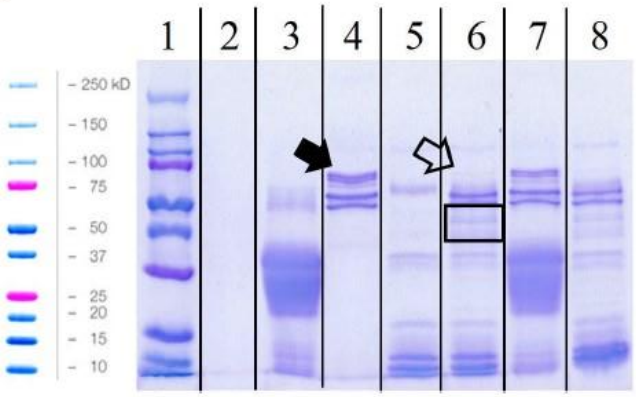


FIGURE 5. SDS-PAGE of the fibrinogen and the different test substances. Lane 1 is protein standard with corresponding molecular weight shown in the adjacent chart; Lane 2 is PBS; Lane 3 is anti-venin serum; Lane 4 is fibrinogen; Lane 5 is *D. siamensis* venom; Lane 6 is a mixture of fibrinogen and venom; Lane 7 is fibrinogen and the mixture of venom and anti-venin serum; Lane 8 is the mixture of fibrinogen and 2x venom. The solid arrow points at the alpha-fibrinopeptide band while the transparent arrow points at the disappearance of alpha-fibrinopeptide caused by *D. siamensis* venom. The rectangle box in Lane 6 indicates the fragment of alpha-fibrinopeptide.

Development of Hemagglutination Assay

To test for erythrocyte hemagglutination activity, the antibody of the same blood type was used as the positive hemagglutinin. Hemagglutination was evident as a spreading pattern of erythrocyte on the well surface as seen in

wells with a high concentration of antibody (1/8 to 1; Fig. 6). On the contrary, incubating erythrocyte with *D. siamensis* venom shows very little (negligible) hemagglutination even with the high concentration of venom.

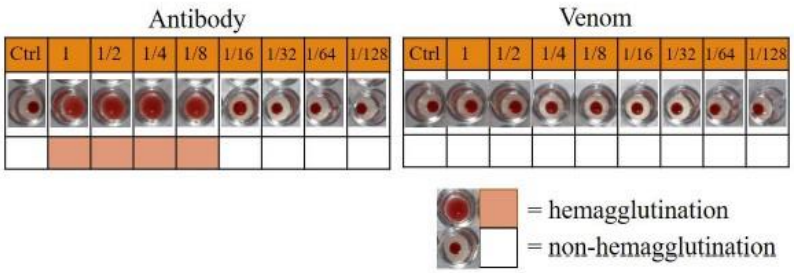


FIGURE 6. The reaction between 2% erythrocyte suspension and test substance (antibody on the left and venom on the right). Antibody group shows the hemagglutination, while venom group show non-hemagglutination at any dilution. The table below the photograph shows the record of hemagglutination (filled cells represent hemagglutination; blank cells represents non-hemagglutination)

SUMMARY

In this study, 3 *in vitro* assays, including phospholipase assay, coagulation assay and fibrinogenolytic assay have been successfully developed and effectively showed a marked difference between positive and negative controls. Two *in vitro* assays, including proteolytic assay and hemagglutination assay were proven to be not suitable for the screening method because of the lack of activity in *D. siamensis* venom. Although previous case reports [27-29] showed that the venom from *Daboia* species contains 5 major biological activities tested in this study, proteolytic and hemagglutination activities were reported to be minor symptoms with the lowest effect to snakebite patients. This indicates that proteins involved with these 2 activities might be present in minute quantity, and thus unlikely to be tested with the current assays. As a result, three *in vitro* assays including phospholipase assay, coagulation assay and the fibrinogenolytic assay will be further used, potentially with an *in vivo* assay, to screen for medicinal herbs with anti-hemotoxic activity against *D. siamensis* venom.

ACKNOWLEDGEMENT

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Poster Presentation

“Young Scientist Award 2018 (YSA)”

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DEVELOPMENT OF ASSAYS FOR SCREENING ANTI-HEMOTOXIC ACTIVITY OF THAI HERBS AGAINST EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM

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Introduction

The eastern Russell's viper (*Daboia siamensis*) is an important venomous snake causing serious public health problems in many parts of Thailand. Its venom shows hemotoxic property causing pathologic alterations to circulatory system. Anti-venin is commonly used for treating snakebite patients. However, it is ineffective to treat some certain symptoms and cause allergic reaction which may lead to death. Recently, treatment of snakebite patients has incorporated traditional herbs with modern medical practice in several hospitals in Thailand. Unfortunately, there is still a limited number of scientific reports to prove the effectiveness and safety. This study aims to develop screening assay for Thai herb with anti-hemotoxic activity against the eastern Russell's viper venom both *in vitro* and *in vivo*.

Experimental groups (5 *in vitro* assays)

negative control:	venom + normal saline
positive control:	venom + serum
control:	normal saline

Materials and Methods

1. Assay development

The aim of this topic is to find optimal conditions, volume, or concentration of substances.

In vitro

(modified from Oliveira et al., 2016)

phospholipase assay

- Agar plates: 1% bacteriological agar, 33% yolk solution, 0.005% sodium azide and 0.01M $CaCl_2$
- Applies the solution in each experiment groups into holes, then incubates at 37°C for 18 hours.
- Diameter of the clear zone around each well will be measured and recorded.

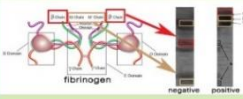
proteolytic assay

- Agar plates: 1% bacteriological agar, azocasein 5 mg/mL and sodium azide 0.005% (w/v)
- Applies the solution in each experiment groups into holes, then incubates at 37°C for 18 hours.
- Diameter of the yellow zone around each well will be measured and recorded.

coagulation assay

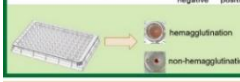
- Plasma will be mixed with 26 mM $CaCl_2$ and substance in each experiment group in a 96-wells plate.
- After incubation at 37°C for 1 hour, optical density (OD) at 405 nm will be measured.

fibrinogenolytic assay



- Polyacrylamide gel electrophoresis will be used for detecting the sign of fibrinogenolytic activity.
- Fibrinogen and substance in each experiment group will be run in 12% polyacrylamide gel at 90 V for 2 hours, followed by staining and visualizing under visible light.

hemagglutination assay



- Erythrocyte suspension will be mixed with substance in each group in 96-wells plate.
- After incubation at 37°C for 2 hours, hemagglutination activity will be observed from agglutination of erythrocyte.

In vivo

chick embryo preparation

incubator set at 37 °C and 80% humidity — hammock technique (Tahara and Obara, 2014) — ready for the assay

mortality rate & LD_{50}finding

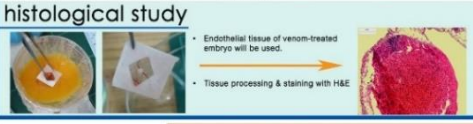
Experimental groups

control:	normal saline
Range finding:	various concentration of venom

- marker of death: no sign of heartbeat
- LD_{50} will be analyzed from mortality rate and concentration of venom.

0.2 cm diameter circle filter paper — drop substance in each group to paper — place on anterior vitelline vein — observe at 4 hours

histological study

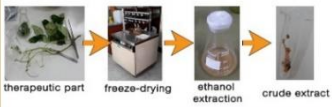


- Endothelial tissue of venom-treated embryo will be used.
- Tissue processing & staining with H&E

2. Screening of herbs

Trigonostemon reidioides (โลดทะนงแดง)

- Other herbs in Family Euphorbiaceae that have been used in snakebite treatment by local healers will also be selected for this study.
- Herb lists:
  - *Euphorbia heterophylla* L. (Desert poinsettia)
  - *Phyllanthus amarus* Schumach. & Thonn. (Carry me seed)
  - *Euphorbia neriifolia* L. (Indian spurge tree)
  - *Croton tiglium* L. (Croton oil plant)
  - *Excoecaria indica* (Mock willow)
  - *Jatropha curcas* L. (Barbados nut)
  - *Jatropha gossypifolia* L. (Belly-ache bush)
  - *Euphorbia tithymaloides* L. (Christmas candle)
  - *Ricinus communis* L. (Castor bean)



therapeutic part — freeze-drying — ethanol extraction — crude extract

Herb extract preparation

In vitro

Herb extract, venom and serum will be tested in 5 *in vitro* assays.

In vivo

- Embryos will be prepared according to the previous protocol.
- death count at 4 hours
- Mortality rate and concentration of herb will be used for calculating ED_{50} (effective dose that can lower the mortality rate to 50% of the negative control group).

Experimental groups

negative control:	venom (LD_{50}) + normal saline
positive control:	venom (LD_{50}) + serum
control:	normal saline
treatment:	herb (various conc.) + venom (LD_{50})
toxicity test:	herb (toxicity test)

Assay	Result
phospholipase assay	diameter
proteolytic assay	diameter
coagulation assay	optical density
fibrinogenolytic assay	separate or not
hemagglutination assay	titer

$$\% \text{ Inhibition} = \frac{\text{Different between ctrl (−) and treatment group}}{\text{Different between ctrl (−) and (+)}} \times 100$$

multiply by degree of fatality

Degree of fatality

Assay	Score
1st coagulation assay	x 4
1st fibrinogenolytic assay	Yes / No
2nd phospholipase assay	x 3
3rd hemagglutination assay	x 2
4th proteolytic assay	x 1

normalize total % inhibition of positive control to 100%, then use the same factor to normalize other groups

50 % cut off

- Only herbal extract with more than 50 % inhibition will be further used in the *in vivo* screening.

Expected outcomes

1) Standard assay for screening herbs with anti-hemotoxic activities against the eastern Russell's viper venom
2) Potential candidate Thai herb that can be used to treat snakebite patient effectively and safely

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DEVELOPMENT OF SCREENING ASSAYS FOR THAI HERB WITH ANTI-HEMOTOXIC ACTIVITIES AGAINST THE EASTERN RUSSELL'S VIPER *Daboia siamensis* (Smith, 1917) VENOM

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Keywords:

snake venom, herbal extract, *in vitro* assay, *in vivo* assay, alternative animal model

Background and Rationale

Venomous snakes can be found worldwide, especially in the tropical regions such as Southeast Asia (Nijman, 2010; Somaweera and Somaweera, 2010). In Thailand, various venomous snake species are commonly found in rural area or forest edges where they could pose serious public health problems to people. One of the most important venomous snake species is the eastern Russell's viper or *Daboia siamensis* (Muangdang, 2015) which is known to contain hemotoxic venom causing pathologic alterations to circulatory system of snakebite patient including blood coagulation, red blood cell morphology change, kidney failure and death (Mitrakul, 1979; Napathorn et al., 1998; Thamaree et al., 2001). In general, anti-venin is regarded as a standard medical treatment for snakebite. However, concerns has been raised over the use of ani-venin since it is ineffective to treat some certain symptoms, the cost per dose is expensive (Bawaskar and Bawaskar, 2001) and 5-80% of patients who received serum can potentially develop allergic reactions which can cause redness, illness or even death (Williams et al., 2006; Cannon et al., 2008; Dhanya et al., 2009). Alternative treatments that are more effective and safe are thus in needed.

In many parts of Thailand, local healers have been known to use medicinal herbs as a treatment for snakebite patients. Recently, treatment of snakebite patients has incorporated traditional herbs with modern medical practice in several hospitals in Thailand (Phongphladisai and Panyawatthananukun, 2011). Unfortunately, there is still a limited number of scientific reports to prove the effectiveness and safety of traditional herbs (Coe and Anderson, 2005; Veronese et al., 2005). In addition, assays for efficacy of herbs requires the time-consuming and expensive use of animals, thus further limited the availability of scientific discovery of remedy for snakebite patients. This study aims to develop screening assay for Thai herb with anti-hemotoxic activity against eastern Russell's viper venom using a battery of tests both *in vitro* (phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay) and *in vivo* (chick embryo as an alternative animal model). After validation as a assay for screening herbs with anti-hemotoxic activities against the eastern Russell's viper, efficacy of a selected group of Thai herbs against *D. siamensis* venom will be screened and verified. In addition to a standard assay for screening herbs with anti-hemotoxic activities against the eastern Russell's viper venom, it is anticipated that a potential candidate Thai herb for treating snakebite patient effectively and safely will be obtained.

Objectives

- To develop and verify *in vitro* and *in vivo* screening assays for Thai herbs with anti-hemotoxic activity against the eastern Russell's viper venom

Research Methodology

1. Development of *in vitro* assays for Thai herbs with anti-hemotoxic activities against the eastern Russell's viper venom (modified from Oliveira et al., 2016)

Five *in vitro* assays covering major mode of hemotoxicity in snakebite patient will be developed. Each assay will be carried out using the same experimental groups; 1) negative control (venom and normal saline) 2) positive control (venom and a dose of anti-venin that completely inhibited the venom) and 3) normal control (only normal saline). The aim of this topic is to find optimal conditions, volume, or concentration of substances. The optimal conditions of the assay should show a marked difference between negative control and positive control groups. Doses of venom used in this part will be 1-10 μg/μL which is corresponded to LD_{50} value (dose of venom that resulted in 50% mortality of the 6-day old chick embryos) from previous study. (Sittishevapark et al., 2016)

1.1 Preparation of venom and serum solution

Lyophilized snake venom of the eastern Russell's viper and its anti-venin serum will be purchased from Snake Farm, Queen Saovabha Memorial Institute, Bangkok, Thailand. Venom will be dissolved and adjusted the concentration to 10 μg/μL with normal saline. Serum will be dissolved in distilled water at recommended concentration. Both solutions will be store in microtube at -20°C for future use.

1.2 Development of phospholipase assay

Agar plates will be prepared by mixing the following chemicals: 1% bacteriological agar, 33% yolk solution in normal saline, 0.005% sodium azide and 0.01M $CaCl_2$. After solidified in a petri dish, gel will be punctured to make 0.5-cm diameter holes. The solution in each experiment groups will be applied in each well and incubated at 37°C for 18 hours. Phospholipase activity can be observed from the clear zone around the hole as phospholipid (from yolk solution) is cleaved to fatty acid and lysophospholipid by phospholipase enzyme in snake venom. Diameter of the clear zone around each well will be measured and recorded.

1.3 Development of proteolytic assay

Agar plates will be prepared by mixing the following chemicals: 1% bacteriological agar, 0.005% sodium azide and 5 mg/ml azocasein solution in Tris-HCl buffer. After solidified in a petri dish, gel will be punctured to make 0.5-cm diameter holes. The solution in each experiment groups will be applied in each well and incubated at 37°C for 18 hours. Proteolytic activity can be observed from the yellow zone around the hole as azocasein is cleaved to casein and azo dye (yellow color) by the proteolytic enzyme in snake venom. Diameter of the yellow zone around each well will be measured and recorded.

1.4 Development of coagulation assay

Whole blood will be obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand, and centrifuged at 700 xg for 10 minutes at 4°C. Plasma in the supernatant will be kept at -20°C until assay. Upon assay, plasma will be mixed with 26 mM $CaCl_2$ and substance in each experimental group in a 96-wells plate. After incubation at 37°C for 1 hour, optical density (OD) at 405 nm will be measured. Higher OD represents higher amount of fibrin indicating coagulation activity catalyzed by various enzymes in the venom.

1.5 Development of fibrinogenolytic assay

Polyacrylamide gel electrophoresis will be used for detecting the separation of alpha and beta peptide from fibrinogen which was the sign of fibrinogenolytic activity. Fibrinogen and substance in each experimental group will be incubated at 37°C for 1 hour, then stopped the reaction with β-mercaptoethanol. Sample will be run in 12% polyacrylamide gel at 90 V for 2 hours. Gel will be stained with Coomassie Blue G-250 and destained with 10% acetic acid before visualization.

1.6 Development of hemagglutination assay

Whole blood obtained from the National Blood Center, the Thai Red Cross Society, Bangkok, Thailand will be centrifuged at 700 xg for 10 minutes at 4°C. After removal of the plasma, erythrocyte will be washed and re-centrifuged with same condition for 3 times to

obtained erythrocyte suspension. The substance in each group will be incubated at 37°C for 5 minutes, then mixed with erythrocyte suspension in 96-wells plate. After incubation at 37°C for 2 hours, hemagglutination activity will be observed from agglutination of erythrocyte at different concentration of substance. Hemagglutination titer of each group will be recorded.

2. Development of *in vivo* assay for Thai herbs with anti-hemotoxic activities against the eastern Russell's viper venom

2.1 Chick embryo preparation

Fertilized chicken eggs will be obtained from Luangsuwanvajokkasikit farm, Kasetsart University at the day of laying (day 0). Eggs will be incubated at 38 °C with the relative humidity in excess of 80% and egg turning 4-6 times per day. Afterward, eggs will be cracked open to transfer embryos and yolk to culture vessel at day 2. Hammock techniques for chick embryo culture will be used according to Tahara and Obara (2014). Briefly, 100-120 ml of distilled water and 0.01 % benzalkonium chloride solution will be added into 240 ml plastic cup. A ventilation hole of 1-1.5 cm diameter and a straw will be made at the side of the cup. A polymethylpentene film or food wrap will be placed over the cup to form a concave hammock. Solution of 1% calcium chloride and 2.5-3 ml of distilled water will be added to the hammock before placing egg content into the hammock, followed by a sterile Petri dish as a cover. Chick embryo will be incubated at 38 °C with the relative humidity in excess of 80% until ready for the assay at day 6.

2.2 Mortality rate and LD_{50} in chick embryo

Chick embryos at day 6 will be treated with various concentration of venom in the range-finding group and normal saline for the control group. The volume of substance in each group will be normalized to 2 µl and dropped into a 0.2 cm diameter circle filter paper (Whatman No. 1) that placed over the anterior vitelline vein. Observation for embryonic health, especially the heart beat, will be carried out every hour and number of dead embryo will be recorded at 4 hours after venom application. LD_{50} will be analyzed from mortality rate and concentration of venom by Probit analysis in the SPSS statistics 22 software.

2.3 Histological study

Endothelial tissue of venom-treated embryo will be immersed in 10 % neutral buffered formalin for 4 hours. After that, tissue will be washed and stored in 70 % ethanol. Standard paraffin method will be used for dehydrating, clearing and embedding. Slide of tissue sectioned at 4-8 µm will be stained with hematoxylin and eosin (H&E). Pathological alteration of the endothelial tissue will be recorded under the light microscope.

3. Preparation of Thai herb extract

After the *in vitro* and *in vivo* assays are validated, efficacy of a selected group of Thai herbs that show potential activity against *D. siamensis* venom will be screened and verified. One of the most common and effective herb used for treating snakebite patient in Thailand is *Trigonostemon reidioides* (Family Euphorbiaceae) or "Lod-Tha-Nong-Daeng" in Thai (Phongphladisai and Panyawatthananukun, 2011). Previous report indicated that root extract of *T. reidioides* can completely inhibited activity of *Naja kaouthia* venom in mice (Praphawicha, 1998; Utsintong et al., 2009). Therefore, *T. reidioides* will be tested with the developed assays. In addition, other herbs in Family Euphorbiaceae that have been used in snakebite treatment by local healers will also be selected for this study. This includes *Euphorbia heterophylla* L. (Desert poinsettia), *Phyllanthus amarus* Schumach. & Thonn. (Carry me seed), *Euphorbia neriifolia* L. (Indian spurge tree), *Croton tiglium* L. (Croton oil plant), *Excoecaria indica* (Mock willow), *Jatropha curcas* L. (Barbados nut), *Jatropha gossypifolia* L. (Belly-ache bush), *Euphorbia tithymaloides* L. (Christmas candle) and *Ricinus communis* L. (Castor bean). Therapeutic part of each herb will be used to prepare a crude extract as follows. First, plant will be cut into small pieces, freeze-dried and ground into powder. Herb powder will be soaked in 70% ethanol overnight before collection of the ethanolic extract afterward. Extraction will be repeated 2 more times and these 3 ethanolic extract with be pooled. Rotary evaporator will be used for ethanol evaporation and the remaining crude extract will be collected and kept in microtube at -20 °C for future use.

4. Screening for Thai herbs with anti-hemotoxic activities against the eastern Russell's viper

Experimental groups will be divided into 5 groups including 1) negative control (venom at LD_{50} and normal saline), 2) positive control (venom at LD_{50} and a dose of anti-venin that completely inhibited the venom) 3) normal control (only normal saline) 4) treatment group (at least 3 concentration of each type of herb and venom at LD_{50}) and 5) toxicity test (only herb extract with the same concentration as the treatment group)

4.1 *In vitro* Screening

Herb extract, venom and serum will be tested in 5 *in vitro* assays (topics 1.2 to 1.6) including phospholipase assay, proteolytic assay, fibrinogenolytic assay, coagulation assay and hemagglutination assay. Data from each assay (except fibrinogenolytic assay) will be used to calculate % inhibition by the following equation:

$$\% \text{ inhibition} = \frac{\text{Difference between negative control group and treatment group}}{\text{Difference between negative and positive control group}} \times 100$$

Result from fibrinogenolytic assay (separation or no separation of alpha and beta peptide from fibrinogen molecule) will be used to verify the result of coagulation assay. If the separation is found, % inhibition of the coagulation will be further used. However, if there was no separation, % inhibition of coagulation assay will be changed to 0. .

Percent inhibition will be weighed with the degree of fatality listed according to symptoms of snakebite patient (Phillips et al., 1988; Warrell, 1989; Kularatne, 2003):

- Coagulation assay : × 4
- Phospholipase assay : × 3
- Hemagglutination assay : × 2
- Proteolytic assay : × 1

The adjusted % inhibition of every assay will be combined for each experimental group. After that, % inhibition of the positive control group will be normalized to 100%. Using the same normalization factor as the positive control group, % inhibition of every experimental groups will be adjusted accordingly. Only herbal extract with more than 50 % inhibition will be further used in the *In vivo* screening.

4.2 *In vivo* Screening

Embryos will be prepared according to the protocol in topic 2.1. The volume of substance in each group will be normalized to 2 µl and dropped into a 0.2 cm diameter circle filter paper (Whatman No. 1) that placed over the anterior vitelline vein. Observation for embryonic health, especially the heart beat, will be carried out every hour and number of dead embryo will be recorded at 4 hours after venom application. Mortality rate and concentration of herb will be used for calculating effective dose that can lower the mortality rate to 50% of the negative control group (ED_{50}) by Probit analysis in the SPSS statistics 22 software.

Expected Outcome

1) Standard assay for screening herbs with anti-hemotoxic activities against the eastern Russell's viper venom
2) Potential candidate Thai herb that can be used to treat snakebite patient effectively and safely

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Oral Presentation

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Sahid Montana 2 Hotel

Malang, Indonesia

Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom

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Abstract. The eastern Russell's viper, *Daboia siamensis*, is one of the important venomous snakes causing serious public health problems in Southeast Asia. Its venom contains a variety of enzymes and peptides that cause hemotoxic effects. Since antivenin commonly used for treating snakebite patients may lead to lethal allergic reaction, medicinal herbs have become an important alternative remedy. To find effective herbs, appropriate screening assays specific to snake venom are needed. This study aims to develop appropriate *in vitro* assays for screening medicinal herbs that have anti-hemotoxic activity against *D. siamensis* venom. Five assays corresponded to important biological activities of the venom were tested. Results revealed that 3 out of 5 assays were effective in showing markedly difference between positive and negative controls including 1) phospholipase A_2 assay examining diameter of clear zone around the well in agar plate with yolk solution, 2) fibrinogenolytic assay examining cleavage of alpha-fibrinopeptide in the SDS-PAGE, and 3) coagulation assay measuring increase in optical density of the plasma after venom addition. No significant difference between positive and negative controls was found in proteolytic and hemagglutination assays. These selected assays can be further incorporated with *in vivo* assay(s) to screen for effective herbs against *D. siamensis* venom.
Keywords: coagulation assay, fibrinogenolytic assay, hemotoxic venom, phospholipase assay

Poster Presentation

"18th Biological Sciences Graduate Congress (BSGC)"

December 18-20, 2018

Chulalongkorn University

Bangkok, Thailand

Development of *in vitro* assays for hematotoxic activity of the Russell's viper *Daboia siamensis* venom

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Introduction

The eastern Russell's viper, *Daboia siamensis* (Smith, 1917), is one of the important venomous snakes causing serious public health problems in Thailand. Its venom contains a variety of enzymes and peptides that cause hemotoxic effects. Since antivenin commonly used for treating snakebite patients may lead to lethal allergic reaction, medicinal herbs have become an important alternative remedy. To find effective herbs, appropriate screening assays specific to snake venom are needed. This study aims to develop *in vitro* screening assays for Thai herbs with anti-hemotoxic activity against the eastern Russell's viper venom.

Materials & Methods

***In vitro* assay development:**
The aim is to find optimal conditions that show a marked difference between negative (with venom activity) and positive control groups (without venom activity).

1. **Phospholipase assay**
The assay was carried out on an agar plate which mainly consisted of agar and yolk solution. Agar was punctured to make a holes and the test substances including venom, normal saline and serum was dropped into the wells.

2. **Proteolytic assay**
Azocasein (substrate of protease) and test substances including venom, normal saline and pancreatin solution were mixed and incubated. Then, TCA solution was added and centrifuged. After that, the supernatant was measured for absorbance at 450 nm.

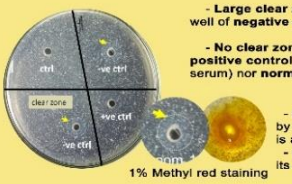
3. **Fibrinogenolytic assay**
The optimal amount of protein in the mixture of fibrinogen and test substances were adjusted for SDS-PAGE. Test substances were mixed and incubated. The sample was run in 12% polyacrylamide gel at 200 V for 40 minutes. The gel was stained and destained before visualization.

4. **Coagulation assay**
Since the whole blood from the National Blood Center was used, the minimum concentration of $CaCl_2$ solution that can neutralize anticoagulant in NBC plasma are needed. Upon assay, plasma was mixed with $CaCl_2$ solution and venom. After incubation, optical density (OD) at 405 nm was measured.

5. **Hemagglutination assay**
Whole blood from the National Blood Center was centrifuged for erythrocyte. Erythrocyte was washed and diluted to 2% erythrocyte suspension, then mixed with test substances. After incubation for 2 hours, hemagglutination activity was observed.

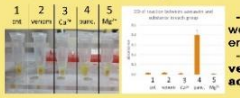
Results

1. Phospholipase assay



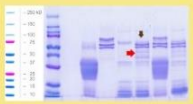
- **Large clear zone** was found around the well of **negative control**
- **No clear zone** was found in neither **positive control** (the mixture of venom and serum) nor **normal control** (normal saline)
- Phospholipid is hydrolyzed by PLA_2 **into fatty acid** which is **acidic.**
- Methyl red stain change its color to orange.

2. Proteolytic assay



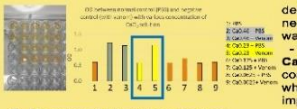
- **Calcium and magnesium ion** were used to **enhance** proteolytic enzyme in venom.
- *Daboia siamensis* venom has **very little (negligible) proteolytic activity.**

3. Fibrinogenolytic assay



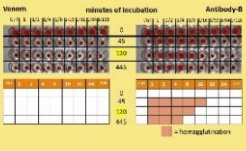
- **Brown arrow** point at the **disappear of alpha-fibrinopeptide** band.
- **Red arrow** point at the **fragment of alpha-fibrinopeptide.**
- **Snake venom metalloproteinases** (SVMPs) selectively cleave the alpha chain but not cleave beta and gamma chain. (Fox and Gutierrez, 2017)

4. Coagulation assay



- Marked difference in optical density of plasma between negative and positive control was found after $CaCl_2$ addition.
- **The minimum conc. of $CaCl_2$** that can induce coagulationis **is 0.2875 M** which will be further used for improving the assay.

5. Hemagglutination assay



- No change in any venom dilution.
- *Daboia siamensis* venom has **very little (negligible) hemagglutination activity.**

Conclusion

- Three *in vitro* assays has been developed for screening anti-hemotoxic activity of Thai herbs against *D. siamensis*.

Reference

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Development of *In vitro* assay for hematotoxic activity of the Russell's viper *Daboia siamensis* venom

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Venomous snakes cause a pose serious public health problem in Thailand. One of the important species is the eastern Russell's viper or *Daboia siamensis* (Smith, 1917). Its venom contains a variety of enzymes and peptides which is hemotoxic venom. Since anti-venin which is commonly used for treating snakebite patients has a chance to cause allergic reaction that can lead to death, medicinal herbs have become the important alternative treatment. To find effective herbs, screening assays have been developed. However, each snake species has different venom component and activity which may cause different screening method. This study aims to develop suitable *In vitro* assays for screening medicinal herbs which have anti-hemotoxic activity against eastern Russell's viper venom. There are five assays corresponding to 5 important biological activities of venom. First, the phospholipase A_2 assay, venom caused a large clear zone around the dropping well in agar plate with yolk solution. Fibrinogenolytic activity caused the cleavage of alpha-fibrinopeptide in the SDS-PAGE. Coagulation assay shown higher OD in the venom-added group. However, the venom not show the significant result in the proteolytic and hemagglutination assay. This can be described by previous case reports which mainly shown the coagulopathy-involving symptom.

Keywords: snake venom; *Daboia siamensis*; *In vitro* assay

Poster Presentation

"19th International Federation of Associations of Anatomists Congress (IFAA)"

August 9-11, 2019

ExCeL London

London, UK

Vasculature Anatomy as a Marker for Hematotoxic Effect of the Russell's Viper *Daboia siamensis* Venom on Chick Embryo *Gallus gallus domesticus*

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INTRODUCTION

The Russell's viper, *Daboia siamensis*, is one of the important venomous snakes causing serious public health problems in Southeast Asia. Its venom contains a variety of enzymes and peptides that cause hematotoxic effects. Since antivenin commonly used for treating snakebite patients may lead to a lethal allergic reaction, medicinal herbs have become an important alternative treatment for snake bites. To prove the efficacy and safety of medicinal herbs, an in vivo assay for hemotoxic activities of the snake venom is needed so that the effective medicinal herbs against the venom can be screened. In this study, microanatomy of embryonic vasculature was examined and verified as a suitable marker for hematotoxic effect of the snake venom.

MATERIALS AND METHODS

Eggs were obtained at day 0 after laying, and kept in an incubator at 38 °C.

Eggs were cracked open to transfer embryos and yolk to a plastic wrap hammock at day 2.

Embryos were incubated until day 6.

Venom solutions were added to filter papers and laid on top of vitelline veins of embryo.

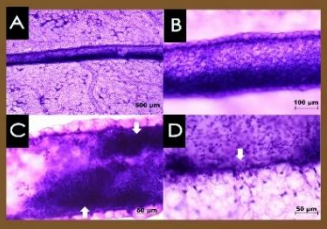
Blood vessels at the treated area were collected.

Two types of tissue processing and staining were used.

1) whole mount staining with hematoxylin and eosin (H&E)
2) cross-section staining with Periodic Acid–Schiff (PAS)

RESULTS

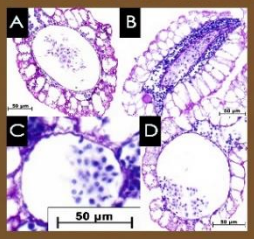
1. Whole mount staining



Representative micrograph of blood vessel of the chick embryo in the venom-treated group (H&E stain)

- An overview of blood vessel at low magnification (A), and high magnification (B).
- The clotted blood (white arrow; C).
- Sign of the rupture and leakage of erythrocytes (white arrow; D).

2. Cross-section staining



Representative micrographs of blood vessel of the chick embryo in the venom-treated group (PAS stain).

- Comparison was made between (A) the control group and the venom treated group (B, C and D).
- The pathological changes potentially related to hematotoxic effect of venom included blood clotting (B), membrane shrinkage or loss of membrane integrity (C), and the rupture of endothelial wall and leaking of erythrocytes and other components (D).

CONCLUSION & DISCUSSION

Microanatomy of embryonic vasculature was examined and verified as suitable markers for hematotoxic effect which were linked with biological activities of *Daboia siamensis* venom, including:

1. Blood clotting, potentially caused by enzymes from serine proteinases (RVV-V) and metalloproteinases (RVV-X) family [1].
2. Loss of membrane integrity or membrane shrinkage, potentially caused by phospholipase A_2 [2, 3].
3. Rupture of the blood vessel wall due to the destruction of connective tissue, potentially caused by phospholipase A_2 [4].

The results of this study could be applied to validate the potential use of chick embryo as an alternative assay in the toxicological study.

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International Federation of Associations of Anatomists

19th Congress Abstracts

9th – 11th August 2019

London, UK

Hosted by the Anatomical Society

Symposium communications

The anatomy of our anatomy: how humans and their relatives came to be

S001

Nature's great experiment: The development and evolution of the human larynx

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[1]*Icahn School of Medicine at Mount Sinai, New York, United States*

While all people are created equal, all body parts are not. Simply put, some are more important than others both to an individual's development and to a species success. Amongst the most crucial of these is the larynx. As obvious as the lump in your throat, this invaluable structure receives relatively little public attention. Yet the larynx is integral in, and sits at the crossroads of, our breathing, swallowing, pressure-control, aeration, olfactory, and speech generating pathways. Its coordinated neural and structural functioning is integral for proper aerodigestive tract activities. Multi-disciplinary data have shown that humans exhibit many highly specialized, potentially unique, features of the larynx and its milieu that seminally affect our development, and that miscues in development may relate to species-specific clinicopathologies such as the Sudden Infant Death Syndrome. Similarly, comparative anatomical/physiological and paleontological research has allowed for glimpses into the role of laryngeal evolution in our ancestors, and the effect that this has had on who amongst our ancestors has prevailed. Indeed, the unique evolutionary trajectory of our larynx has played a major role in the acquisition of the human vocal tract and our speech capabilities. While much still remains unknown regarding the larynx, its centrality in both development and evolution is becoming ever clearer.

S002

The Atapuerca sites, at the crossroads of European prehistory

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The Sierra de Atapuerca (Burgos, Spain) contains an extensive karstic system in which a group of sites covering all of European prehistory is located, from the first settlement of the continent to the end of the Bronze Age. Human fossils have

coronary artery. They were fixed in 10% formalin for 10 days.
Two (2) cases were observed where the right coronary artery originated as a collateral branch of the left coronary artery, one ran between the aorta and the pulmonary artery; the other anterior to the pulmonary trunk, replacing the coronary artery. Coronary arteries had a diameter of 6.05 mm and 2.5 mm; a 90.4 mm path was recorded reaching the right margin and diaphragmatic surface of the heart.

P2-V21

Incidence of orthopaedic conditions: A Radiographic study of Feline patients at UCD Veterinary Hospital

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Caseloads in orthopaedic practices are often dominated by dogs, with cats being underrepresented. Several studies have been done to investigate the incidence and types of orthopaedic conditions commonly seen in canine patients, but little comparable research has been done for feline patients. This study aimed to investigate this by examination of radiographs (n=180) of feline orthopaedic cases which were admitted to UCD Veterinary Hospital between January 2010 and December 2017. This consisted of both first opinion patients that received primary care (including emergency admissions) and referral cases. The caseload data were analysed and patients were categorised based on breed, age, sex, weight, the presenting complaint and the type of orthopaedic condition present (both major and incidental findings), the latter being diagnosed by an experienced radiographer. Four main condition categories were created: Degenerative conditions (16); Fractures (91); Tumours (30); Others (43). A large proportion of patients belonged to the Domestic Short Hair/Long Hair breeds, the major representatives of the local feline population. There was no significant evidence of breed predisposition to orthopaedic disease in cats. However, it was observed that trauma was an important precursor to conditions in younger animals, while older animals were more prone to degenerative diseases and conditions that occurred as a secondary consequence of a separate, primary disease.
The findings from this study may be useful to those interested in feline medicine or for use in clinical teaching institutions as part of the curriculum.
A larger sample size incorporating a greater variety of breeds would better determine the incidence and types of orthopaedic conditions occurring in feline patients. It should also be noted that the hospital population may not be representative of the general population. Ethical approval was granted for this work.

P2-V22

Vasculature anatomy as a marker for hematotoxic effect of the Russell's viper Daboia siamensis venom on chick embryo Gallus gallus domesticus

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The Russell's viper, Daboia siamensis, is one of the important venomous snakes causing serious public health problems in Southeast Asia. Its venom contains a variety of enzymes and peptides that cause hematotoxic effects. Since antivenin commonly used for treating snakebite patients may lead to a lethal allergic reaction, medicinal herbs have become an important alternative treatment for snake bites. To test for efficacy of an herbal extract against snake venom, an effective screening method is needed. Prior studies showed that chick embryos can be used as an alternative model for in vivo screenings of

herbs. In this study, microanatomy of embryonic vasculature was examined and verified as a suitable marker for hematotoxic effect of the snake venom. Gallus gallus domesticus (white leghorn) eggs were obtained at day 0 after laying, and kept in a microprocessor controlled incubator to increase the survival rate. Eggs were cracked open to transfer embryos and yolk to a plastic wrap hammock at day 2, and embryos were incubated until day 6. Venom solutions in phosphate buffered saline were added to filter papers and laid on top of vitelline veins of embryo. After 4 hours of exposure, blood vessels at the treated area were collected, fixed, processed through paraffin method, and stained with Periodic acid–Schiff. Microscopic examination for change at the basement membrane, presence of clotted blood in the vessel and loss of membrane integrity was carried out. Overall results confirm the hematotoxic effect of D. siamensis venom on chick embryonic vasculature, and provided a reference guide for testing efficacy of herbal extracts against snake hematotoxin. The results from this study could be applied to validate the potential use of chick embryo as an alternative assay in toxicological studies.

P2-V24

Skull Anatomy of Forest Skinks (Sphenomorphus Fitzinger, 1843) Living in Different Habitats of Java and Sumatra Islands, Indonesia

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Skink is one of the most diverse groups of reptile. Conversely several species have generalized external morphology. In Southeast Asia, the forest skinks in the genus Sphenomorphus are widely distributed and living in various habitats. In addition, the taxonomy of these skinks is still controversial. Java and Sumatra Islands are located in the Sunda Land biodiversity hotspot. The skinks that live in these areas are considered as having a cryptic lineage. Following herpetological surveys in montane forest of Java and Sumatra, the taxonomy of these skinks have been revised under the humane approved protocols number UTA IACUC A12.004 from the University of Texas at Arlington. Skull comparative anatomy had been conducted among various groups of the forest skinks including Sphenomorphus cameronicus, S. melanopogon, S. cyanolaemus, S. scotophilus and S. sungaicolus. Micro-computed tomography scans were obtained from these specimens by using inspeXio SMX-100CT, Shimadzu. The results indicated that there are different skull characteristics among species especially for the species that live under leaf litter. For example, S. cameronicus lives under leaf litter and has a smaller optic cavity compared to other species that live above the leaf litter. The skull of S. cameronicus is more compact and the eyes are recessed in the optic cavity, they do not project dorsally as in the other species scanned. The epipterygoid is at right angle with sagittal body plane which is different from other species having obtuse angle. Results indicate that skull comparative anatomy plays a role in the taxonomic study of skink.

P2-V26

Gross anatomy of the brachial plexus in three species of wild carnivorans (Lontra longicaudis, Procyon cancrivorus and Potos flavus)

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The order Carnivora has a high diversity of species with different kinds of locomotion, as such their thoracic limbs have different morphological

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Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of in vitro assays for hematotoxic activity of the Russell's viper Daboia siamensis venom. AIP Conference Proceedings, 2120(1), 070012. [SCOPUS]

ABSTRACT:

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2018) Development of screening assays for Thai herb with anti-hemotoxic activities against the eastern Russell's viper Daboia siamensis (Smith, 1917) venom. Young Scientist Award 2018 (YSA), March 29-30, 2018, Pathum Thani, Thailand. [poster presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of in vitro assays for hematotoxic activity of the Russell's viper Daboia siamensis venom. AIP

Conference Proceedings, 2120(1), 070012. International Conference on Biology and Applied Science 2019 (ICOBAS), March 20-21, 2019, Malang, Indonesia. [oral presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2019). Development of in vitro assays for hematotoxic activity of the Russell's viper Daboia siamensis venom. 18th Biological Sciences Graduate Congress (BSGC), December 18-20, 2018, Bangkok, Thailand. [poster presentation]

Sittishevapark, P., Kitana, J., Sukrong, S., & Kitana, N. (2020). Vasculature anatomy as a marker for hematotoxic effect of the Russell's viper Daboia siamensis venom on chick embryo Gallus gallus domesticus. Journal of Anatomy 236 (Supplement I): 288-289. [Scimago Journal Rank 2019: Q1] Meeting Abstract P2-V22, the 19th International Federation of Associations of Anatomists Congress (IFAA), August 9-11, 2019, London, UK. [poster presentation]

AWARD RECEIVED

2016-2019 The Science Achievement Scholarship of Thailand (SAST)

2019: The 90th Anniversary of Chulalongkorn University Scholarship from the Graduate School, Chulalongkorn University

2019: Overseas Academic Presentation Scholarship for Graduate Students from the Graduate School, Chulalongkorn University