

Original Research Article

Comparative study of the analgesic effects of Bungarus fasciatus snake venom from Vinh Phuc and Tien Giang Provinces of Vietnam

Thien Vu Tran¹, Trang Thuy Thi Nguyen², Anh Ngoc Hoang³, Yuri N Utkin^{4*}

¹Tra Vinh University, Tra Vinh City, ²Faculty of Pharmacy, Nguyen Tat Thanh University, ³Institute of Applied Materials Science, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam, ⁴Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

*For correspondence: **Email:** utkin@ibch.ru; yutkin@yandex.ru

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Abstract

Purpose: To determine the analgesic activity of Bungarus fasciatus venoms and their fractions from two Vietnamese Provinces.

Methods: Male Swiss Albino mice were randomly divided into three groups containing 8 to 10 mice each. Control group was injected subcutaneously with normal saline, standard group received aspirin solution (50 mg/kg) perorally, and study group received a solution of crude venom or isolated fractions in physiological saline. To determine analgesic activity, acetic acid writhing and tail immersion tests were used. The venoms were separated by liquid chromatography and the analgesic activity of the fractions was analyzed.

Results: Both venoms showed analgesic effect in the acetic acid writhing test, but only the venom from Tien Giang showed analgesic effect in the tail immersion test. The bioactive fractions of Vinh Phuc and Tien Giang venoms were significantly different, with most of Vinh Phuc venom fractions being more active ($p < 0.05$). Thus, 35 min after the injection, the number of writhings decreased from 15 - 16 in the control to 0.85 ± 0.34 for the BF-4VS (Vinh Phuc) fraction compared to 2.67 ± 1.20 ($p < 0.05$) for the BF-4DT (Thien Giang) fraction. Two proteins with analgesic activity were isolated from Vinh Phuc venom, and one with greater activity matched the known B. fasciatus phospholipase A2.

Conclusion: The analgesic activity of two samples of B. fasciatus venom from two different provinces in Vietnam reveal that their pharmacological profiles differ. The isolates can be explored as leads in the development of new analgesic agents.

Keywords: Analgesic activity, Krait Bungarus fasciatus, Phospholipase A2, Venom

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INTRODUCTION

Elapidae is an extensive family of venomous snakes which include about 400 species, found in more than 60 genera. Cobras, mambas and

kraits are the most commonly known Elapidae genera. Elapids are highly venomous and their bites are usually fatal if left untreated. However, some Elapidae venoms may contain components that hold promise for the development of new

drugs. Thus, mambalgins isolated from mamba venom interact with acid-sensing ion channels which are involved in pain sensation [1]. Interaction of mambalgins with acid-sensing ion channels results in an analgesic effect similar to that of morphine, but without addiction [2]. Analgesic activity was also demonstrated for some cobra toxins. It has been shown that neurotoxin isolated from the venom of cobra *Naja naja atra* demonstrated an analgesic effect in mice [3]. Another venom peptide homologous to cobra cytotoxins from the same venom reduced pain by inhibition of the voltage-gated sodium channel Nav1.8 [4].

It was shown recently that venom of krait *Bungarus fasciatus* from India showed analgesic activity in mice [5]. This snake is commonly found in the Indian subcontinent, Southeast Asia, and Southern China. In Vietnam, *B. fasciatus* is distributed everywhere, from the plains to the mountainous regions. It is a well-established fact that the composition of venoms of the same species can vary significantly depending on the geographic origin of the snake. Several proteomic studies of *B. fasciatus* venoms from different regions revealed that the venom composition varied greatly depending on the origin [6-8]. However, phospholipases A2 (PLA2) were predominant practically in all venoms, and previous studies of *B. fasciatus* venom from Vietnam also showed that the main venom components are PLA2s [9]. It should be noted that krait venoms do not contain mambalgins and cytotoxins. This raises the question of what components possess analgesic activity in *B. fasciatus* venom. It has been demonstrated previously that *B. fasciatus* venom from North Vietnam (Vinh Son, Vinh Phuc Province) was similar to that from India in manifesting analgesic effect [10]. In order to compare the analgesic activity, evaluate differences at the molecular level, and identify the analgesic components, *B. fasciatus* venoms from two Vietnamese Provinces, Vinh Phuc (Northern Vietnam) and Tien Giang (South Vietnam) were studied.

EXPERIMENTAL

Materials

Krait *B. fasciatus* venoms were obtained from snake farms located in Dong Tam (Tien Giang Province) and Vinh Son (Vinh Phuc Province). The venoms were collected by farm staff, freeze-dried and kept at -20 °C until use. Four grams of venom was obtained from each farm and used in this study. Aspirin (VNB-0629-00) from Mekophar (Ho Chi Minh City, Vietnam) and morphine hydrochloride (VD-24315-16) from Vidipha (Ho

Chi Minh City, Vietnam) were also used. All other reagents of analytical grade or higher were obtained from local suppliers.

Animals

Male Swiss albino mice were acquired from Nha Trang Vaccine Institute (Vietnam) and kept for at least 2 days before testing at the Department of Pharmacology of the Nguyen Tat Thanh University (Ho Chi Minh). All the appropriate steps were taken to minimize discomfort to the mice. The ethical procedure was strictly followed under the Guide for the Care and Use of Laboratory Animals [11]. The Scientific Council of the Faculty of Pharmacy of Nguyen Tat Thanh University approved all the animal experiments described in this study (Protocol no. 1, dated January 19, 2017).

Acetic acid-induced writhing test

This test was performed as earlier described [10]. In brief, mice were randomly divided into three groups containing 8 - 10 mice each. Mice in control group were injected subcutaneously with physiological saline (0.9 % NaCl) in a volume of 0.1 mL per 10 g of mouse body weight. In the standard group, mice received aspirin solution (50 mg/kg) perorally. In the study group, mice were injected with a solution of crude venom or isolated fractions in physiological saline (in a volume of 0.1 mL per 10 g of mouse body weight) at a dose of 0.34 mg/kg corresponding to 1/10 of LD₅₀ for crude venom.

Thirty minutes after the drug administration, acetic acid (0.7 % solution, 10 µL/g body weight) was injected intraperitoneally and each mouse was observed in a separate cage. For every 5 min at times of 5 - 10 min, 20 - 25 min and 35 - 40 min after acetic acid injection, the number of writhes was counted and recorded.

Tail immersion test

This test was carried out as reported in previous studies [10]. In brief, mice were randomly divided into three groups containing 8 - 10 mice each. In the control group, mice were injected subcutaneously with physiological saline (0.9 % NaCl). In the standard group, mice were injected with a solution of morphine hydrochloride at a dose of 5 mg/kg and those in the study group with a solution of crude venom or isolated fractions in physiological saline at a dose of 0.34 mg/kg. A volume of 0.1 mL was injected per 10 g of mouse body weight.

After injection, the mice were fixed in cages with a free-hanging tail. The tail was immersed in hot water maintained at 55 ± 0.5 °C using a Water bath (Memmert GmbH + Co. KG, Schwabach, Germany). The reaction time of the mouse was registered from the moment when the mouse tail was immersed in water until the moment when the mouse pulled the tail out of the water. Before drug administration and at 30, 60, 90 and 120 min after drug administration, the mouse reaction times were recorded. If the mouse did not respond after 10 sec, then the tail was taken out of the water to prevent it from unintentional injury.

Separation of venom by liquid chromatography

The *B. fasciatus* venom was separated as described in a previous study [12]. The first step was high-performance gel-filtration on Superdex HR 75 (10 × 300 mm, Cytiva, Marlborough, MA, USA) in 0.1 M ammonium acetate buffer (pH 6.2) at a flow rate of 0.5 ml/min using a HP Agilent 1050 Series HPLC system equipped with Diode Array Detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The venoms were fractionated in several runs applying 30 mg of the venom per run. Before the further studies, the obtained fractions were freeze-dried.

Fraction BF-4VS obtained by gel-filtration of the venom from Vinh Son was further separated by reversed phase HPLC on Jupiter C18 column (4.6 × 250 mm, Phenomenex, USA) using gradient of acetonitrile concentration in water (from 15 to 45 % in 60 min) in the presence of 0.1 % trifluoroacetic acid. The flow rate was 1 ml/min. Before further use, the obtained fractions were freeze-dried. The molecular mass of the proteins was determined on an ESI-MS/MS high-resolution Agilent 6500 mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Statistical analysis

Differences between groups were analyzed by the Kruskal-Wallis method and then by the Mann-Whitney test using Minitab software Version 15.0. Significance level was set at $p < 0.05$, and the data are presented as the mean \pm standard error of mean (SEM).

RESULTS

It had previously been shown that the *B. fasciatus* venom obtained from Vinh Son farm (North Vietnam) manifested analgesic activity in mice [10]. Bearing in mind the fact that the

venom of snakes from different regions may differ in composition and activity, the analgesic activity of *B. fasciatus* venom collected at the Dong Tam farm (South Vietnam) was studied as well. In the previous study, the LD₅₀ for the venom from Dong Tam farm was determined to be 3.4 mg/kg body weight in mice [13]. During the LD₅₀ determination, no signs of toxicity was reported at 0.34 mg/kg (1/10 of LD₅₀). Therefore, this dose was chosen for the present study. The venoms from both regions showed equal analgesic effects in the acetic acid induced writhing test and their effects were comparable to that of aspirin (Figure 1). Interestingly, the Dong Tam venom was slightly more active at longer observation times. While the Dong Tam venom produced a noticeable effect in the tail immersion test, the Vinh Son venom manifested no analgesic effect in this test (Figure 2).

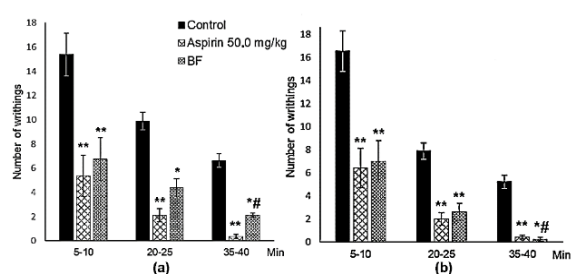


Figure 1: Influence of venom from (a) Vinh Son farm and (b) Dong Tam farm in acetic acid writhing in mice. * $P < 0.05$, ** $p < 0.01$ compared to control, # $p < 0.05$ compared to aspirin (50.0 mg/kg)

To identify the venom components possessing analgesic activity, the venoms from both locations were separated via gel-filtration chromatography (Figure 3). From each venom, five fractions were obtained and named BF-1VS to BF-5VS and BF-1DT to BF-5DT for Vinh Son and Dong Tam venom, respectively. The main difference between the venoms was in the content of fractions 4 and 5. Specifically, fraction 4 in Dong Tam venom was more abundant than in Vinh Son venom, while the content of fraction 5 was higher in Vinh Son venom (Figure 3).

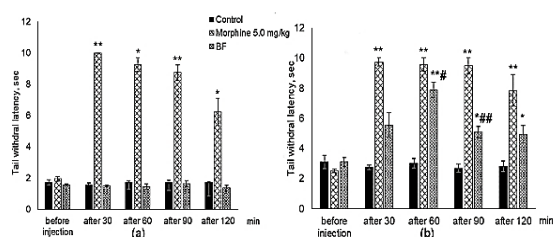


Figure 2: Analgesic effect of venoms in tail immersion test. The venoms are from (a) Vinh Son farm and (b) Dong Tam farm. * $P < 0.05$, ** $p < 0.01$ compared to control; # $p < 0.05$, ## $p < 0.01$ compared to morphine (5.0 mg/kg)

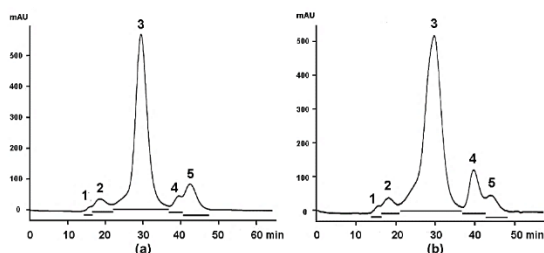


Figure 3: Gel filtration chromatograms of *B. fasciatus* venoms on Superdex 75 column. (a) The venom from Vinh Son farm, (b) the venom from Dong Tam farm. The horizontal bars indicate collected fractions

Furthermore, the pain-relieving effect of the fractions was studied. Since the composition of the fractions was not known, equal doses of 0.34 mg/kg for all fractions were used. In the acetic acid induced writhing test, the manifestation of spasmodic pain was observed during three-time intervals: 5 - 10, 20 - 25 and 35 - 40 min after acetic acid injection. It was found that the fractions manifested different antinociceptive effects.

All the fractions obtained from Vinh Son venom produced statistically significant decreases in the number of writhings ($p < 0.05$), the effect being

more pronounced about half an hour after fraction injection (Table 1). The fractions BF-1VS and BF-4VS were the most active. Not all the fractions of Dong Tam venom were as active as those of Vinh Son venom. However, the BF-1DT fraction was the most active, almost completely prevented writhing, and showed activity higher than that of aspirin (Table 2). All other fractions (BF-2DT to BF-5DT) were less active and none of them manifested activity comparable to aspirin. For example, fraction BF-4VS was significantly more active than fraction BF-4DT (Table 1 and Table 2).

The venom from Dong Tam showed analgesic activity in tail immersion test; therefore, the activity of the obtained fractions was checked in this test (Table 3). As seen in Table 3, fractions BF-3DT manifested very weak effect 120 min after injection. However, this effect was not analgesic but rather increased pain. So, the activity of crude Dong Tam venom may be caused by synergistic effects of several components which are separated by gel filtration.

After the separation of the fraction BF-4VS by reversed-phase HPLC, 5 main fractions have been collected (Figure 4).

Table 1: The effects of Vinh Son *B. fasciatus* venom and its fractions on the acetic acid induced writhings

Compound	Assay time interval (min)		
	5-10	20-25	35-40
Number of writhing			
Control (0.9% NaCl)	15.38±1.93	9.88±1.68	6.63±1.16
Standard (aspirin, 50 mg/kg)	5.38±1.48 ²	2.13±0.58 ²	0.38±0.18 ²
Vinh Son venom	6.75±2.02 ²	4.38±1.18 ¹	2.13±0.58 ^{1,3}
BF-1VS	3.50±1.35 ²	3.13±0.99 ²	0.63±0.26 ²
BF-2VS	6.29 ±1.19 ²	4.43±1.17 ¹	1.29±0.52 ²
BF-3VS	10.25±2.24	3.25±1.05 ²	1.75±0.70 ²
BF-4VS	2.43±1.45 ²	1.00±0.54 ²	0.85±0.34 ²
BF-5VS	7.25±1.97 ¹	4.13±1.04 ¹	2.38±1.12 ¹

¹ $P < 0.05$, ² $p < 0.01$ compared to the control; ³ $p < 0.05$ compared to the standard

Table 2: The effects of Dong Tam *B. fasciatus* venom and its fractions on the acetic acid induced writhings

Compound	Assay time interval (min)		
	5-10	20-25	35-40
Number of writhing			
Control (0.9% NaCl)	16.56±1.76	7.89±0.72	5.22±0.55
Standard (aspirin, 50 mg/kg)	6.44±1.68 ¹	2.00±0.53 ¹	0.44±0.18 ¹
Dong Tam venom	7.00±1.78 ¹	2.63±0.75 ¹	0.25 ±0.16 ^{1,2}
BF-1DT	0.00±0.00 ^{1,3}	2.17±1.11 ^{1,2}	0.00±0.00 ^{1,3}
BF-2DT	16.00±2.50	5.17±0.65 ⁴	2.00±0.58 ⁴
BF-3DT	10.25±2.24	4.50±1.26 ^{2,4}	2.50±0.43 ⁴
BF-4DT	9.67±2.51 ⁴	5.50±0.92 ⁴	2.67±1.20 ⁴
BF-5DT	10.33±3.22 ⁴	3.67±0.92 ^{1,2}	5.33±1.12

¹ $P < 0.01$ ⁴ $p < 0.05$ compared to the control; ² $p < 0.05$, ³ $p < 0.01$ compared to the standard

Table 3: The effects of Dong Tam *B. fasciatus* venom and its fractions on the tail immersion in mice

Compound	Assay time, min				
	Before injection	After 30 min	After 60 min	After 90 min	After 120 min
	Tail withdrawal latency/Reaction Time (sec)				
Control (0.9% NaCl)	3.11±0.29	2.74±0.16	3.01±0.31	2.68±0.27	2.81±0.34
Standard (morphine, 5 mg/kg)	2,50±0,12	9.74±0.26 ¹	9.57±0.43 ¹	9.49±0.51 ¹	7.85±1.04 ¹
Dong Tam venom	3,11 ±0,30	5.56 ±0.80	7.88 ±0.51 ¹	5.09 ±0.38 ²	4.94 ±0.60 ²
BF-1DT	2.61±0.06	2.63±0.27	2.18±0.29	2.29±0.31	1.90±0.25
BF-2DT	2.57±0.16	2.40±0.10	2.62±0.18	2.40±0.14	2.27±0.19
BF-3DT	2.37±0.09	2.35±0.18	2.27±0.22	2.13±0.10	1.93±0.13 ²
BF-4DT	2.77±0.33	2.54±0.18	2.36±0.17	2.39±0.23	2.65±0.27
BF-5DT	2.84±0.29	2.56±0.25	2.43±0.08	2.87±0.07	2.63±0.38

¹p < 0.01 ²p < 0.05 compared to the control

Table 4: Effects of the fractions obtained via reversed-phase chromatography on the acetic acid induced writhings

Compound	Assay time interval (min)		
	5-10	20-25	35-40
	Number of writhing		
Control (0.9% NaCl)	19.33±1.76	8.67±1.14	5.11±0.79
Standard (aspirin, 50 mg/kg)	12.63±1.58 ¹	7.38±1.03	4.63±1.41
BF-4.7	14.13±3.46	4.25±1.19 ¹	2.00±0.50 ¹
BF-4.11	12.11±3.17	3.67±1.26 ^{1,2}	2.22±0.68 ¹
BF-4.12	7.44±2.95 ¹	1.33±0.41 ^{3,4,5}	0.67±0.24 ^{3,4,5}
BF-4.14	10.75±3.17	6.50±1.68	3.38±0.80
BF-4.15	12.00±2.63 ¹	4.78±1.10 ¹	2.33±0.78 ¹

¹P < 0.05, ³p < 0.01 compared to the control; ²p < 0.05, ⁴p < 0.01 compared to the standard; ⁵p < 0.01 compared to BF-4.11

The analgesic activity of obtained fractions was determined by the acetic acid induced writhing test (Table 4). All the fractions were found to possess analgesic effect. The most active was fraction BF-4.12, the activity of which exceeded that of the standard. The fraction BF-4.11 also showed analgesic activity, but was less active than BF-4.12 and its activity did not exceed that of standard.

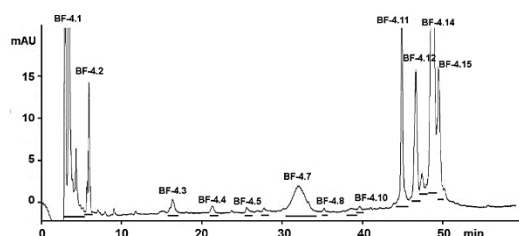


Figure 4: Reversed phase HPLC of fraction BF-4VS using Jupiter C18 column (4.6 x 250 mm, Phenomenex, USA) and gradient of acetonitrile concentration in water (from 15 to 45 % in 60 min) in the presence of 0.1 % trifluoroacetic acid. A flow rate is 1 mL/min

Since fractions BF-4.11 and BF-4.12 produced the best analgesic effect, they were further purified and analyzed via high resolution mass spectrometry. The molecular masses determined were equal to 13037.33 and 13035.32 Da for BF-

4.11 and BF-4.12, respectively. In the venom of *B. fasciatus*, only PLA2s have molecular masses in the range of 13 - 14 kDa [9]. The complete amino acid sequences for about two dozen PLA2s from *B. fasciatus* are known. One of them, basic PLA2 BFPA (UniProtKB accession number A6MEY4), possesses the molecular mass of 13035.69 Da. Within the measurement error, this value coincides with the value determined for the BF-4.12. Therefore, it can be considered that fraction BF-4.12 contains basic PLA2 BFPA. The PLA2s form one of the most abundant families in snake venoms. Single venom may contain several PLA2 isoforms. The difference between isoforms may be minimal, therefore it is believed that the fraction BF-4.11 also contains PLA2.

DISCUSSION

To study the analgesic activity of *B. fasciatus* venoms and fractions, two pain models were used: the acetic acid induced writhing test and tail immersion test, which are realized through the peripheral and central nervous system mechanisms, respectively. Aspirin was used as positive control in the acetic acid induced writhing test and morphine in tail immersion test. The antinociceptive effect of morphine was achieved through the central nervous system,

because it has a central antinociceptive effect, while the aspirin effects occurred in the peripheral nervous system and it is considered as peripheral analgesic agent [14]. In mice, aspirin and similar drugs block the delayed stretching response induced by an intraperitoneal injection of dilute acetic acid [15]. However, aspirin and similar drugs are not effective against nociception induced by stimulating the tail (e.g., tail immersion test) while morphine is very effective in these tests.

The venoms from two Vietnam regions showed equal analgesic activity effect in the acetic acid induced writhing test and their effect was comparable to that of aspirin. While the Dong Tam venom produced noticeable effect in tail immersion test, the Vinh Son venom manifested no analgesic effect in this test. Thus, the crude venoms from different regions differed in analgesic activity.

To investigate the reason for the observed difference in analgesic activity, venoms were separated using gel-filtration and the effect of the various fractions were analyzed. Although practically all the fractions obtained from both venoms produced statistically significant decrease in the number of writhings, this effect was more pronounced for the fractions from Vinh Son venom. In this venom, the fractions BF-1VS and BF-4VS were the most active. Acetic acid-induced writhing is considered as a typical model for inflammatory pain and as test for peripheral antinociceptive effects. The observed peripheral antinociceptive activities of the venoms and fractions may be related to the reduced concentration of inflammatory mediators and/or blockage of their receptors.

Although the venom from Dong Tam showed an analgesic effect in the immersion test, its fractions were practically inactive in this test. The activity of crude Dong Tam venom may be caused by synergistic effects of several components which are separated with gel filtration. Synergism between different toxins which results in toxicity potentiation is a well-known phenomenon in snake venoms [16].

The results obtained from this study showed that the fractions BF-4VS and BF-1DT manifested a better peripheral analgesic effect than the crude *B. fasciatus* venom and aspirin. Gel-filtration chromatography demonstrated that fraction BF-1DT contain compounds with high molecular masses, while BF-4VS comprise the proteins with much lower molecular masses. Such proteins may be considered as templates for the

design of new analgesics. Taking into account these considerations, the fraction BF-4VS was chosen to isolate a compound with analgesic properties. This fraction was further separated with reverse phase chromatography and the analgesic effects of obtained fractions were analyzed. The analysis of the fractions BF-4.11 and BF-4.12 possessing the best analgesic effect by mass spectrometry showed that they contain PLA2s. It is well known that single snake venom may contain multiple PLA2 isoforms. For example, seven isoforms were isolated from single *B. fasciatus* venom of Indian origin [17]. The important role of these enzymes in inflammation and pain has been demonstrated by numerous studies. Among the snake venom PLA2s, only dimeric PLA2 crotoxin was shown to possess analgesic activity [18]. Thus, the PLA2 from *B. fasciatus* identified in this study is the first example of monomeric snake PLA2 manifesting analgesic activity.

CONCLUSION

The analgesic activities of two samples of *B. fasciatus* venom originating from different Vietnam provinces have been compared; their pharmacological profiles are different. This work demonstrates the differences in analgesic activities of these venoms, their isolates and sub-fractions. These isolates can be further investigated as leads in the discovery of new and more potent analgesic agents.

DECLARATIONS

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Ethical approval

The Scientific Council of the Faculty of Pharmacy of Nguyen Tat Thanh University approved all the animal experiments described in this study (Protocol no. 1, dated January 19, 2017).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Anh Ngoc Hoang and Yuri N Utkin conceived and designed the study, Thien Vu Tran, Trang Thuy Thi Nguyen and Anh Ngoc Hoang collected and analyzed the data, Thien Vu Tran, Trang Thuy Thi Nguyen, Anh Ngoc Hoang and Yuri N Utkin wrote the manuscript. All authors read and approved the manuscript for publication.

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Composition study on toxins of Vietnamese krait