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Season Variation and Starvation Period Influence on the Antithrombotic Activity of Leech Saliva Extract From the Medicinal Malaysian Leech, *Hirudinaria Manillensis*

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Abstract

Leech therapy has been practiced for a wide range of therapeutical purposes since the extreme old ages. Nowadays, leech application in plastic and microsurgery has been considered as a promising tool. In Malaysia, traditional physicians have used the medicinal leeches as an effective remedy for bloodletting and many body disorders. Leech saliva extract (LSE) was collected after feeding leeches on the phagostimulatory solution through parafilm membrane. The total protein concentration was estimated using Bradford assay. The antithrombin activity was evaluated using the amidolytic assay of the synthetic substrate S-2238 and thrombin time assay *in vitro*. It was found that LSE could inhibit thrombin-mediated hydrolysis of the substrate. The extract effectively prolonged thrombin time of the citrated plasma in a linear dose-dependent manner. It was found that the extract collected during the dry season was more biologically active than those collected during the rainy season. Likewise, results revealed that the longer the starvation period, the lower the antithrombin activity. For effective utilization of leech therapy or leech products, we recommend to be used during the dry season and after a starvation period not more than 16 weeks.

Keywords: Amidolytic; Antithrombin; leeches; leech saliva; Thrombin time.

Abbreviations: BSA: Bovine serum albumin; LSE: Leech saliva extract; PHS: Phagostimulatory solution

Introduction

Physicians had used leeches since antiquity for a variety of therapeutic purposes as outlined in their paintings and manuscripts. Although, the medicinal usage of leech had experienced a diminishing era during the late 19th and the early 20th centuries, it has been recently used for reconstructive and cosmetic surgery [1].

Leech ability to overcome blood clotting while sucking and to keep it in a liquid state for a long period of storage in their crops had attracted the attention of traditional therapists, physicians and researchers as an effective remedy for diseases management especially coagulation disorders. Leeches have been the subject of many scientific researches to identify and characterize the blood-affecting constituents, especially peptides and proteins, in their salivary gland secretions [2-4]. A large number of antithrombin agents were identified from different leech species such as hirudin from the European leech *Hirudo medicinalis* [5,6], bufrudin from *Hirudinaria manillensis* [7] and theromin from *Theromyzon tessulatum* [8].

Scanty studies are available about the medicinal Malaysian leech, *Hirudinaria manillensis* [2]. However, some researchers characterized a hirudin-like peptide from the medicinal Malaysian leech body extract with antithrombin activity [9].

The aim of the present study is to evaluate the antithrombin activity of the salivary secretion of the medicinal Malaysian leech and to examine its effectivity in different seasons and after various starvation periods.

Materials and Methodology

Leech collection, maintenance and taxonomy

Leeches, *Hirudinaria manillensis*, were collected from Cheneh Lake, located in Trengganu, Malaysia. They were maintained in well-aerated plastic containers filled with un-chlorinated tap water. Water was regularly changed every two days. The collected leeches were kept under 12h: 12h light and dark cycle at the room temperature ($25 \pm 1^\circ\text{C}$).

Samples of the collected leeches were sent to Leeches Biopharm (UK) Ltd, for taxonomy and identification.

Chemicals and reagents

Thromboclotin[®] (thrombin reagent) and Control N[®] (control plasma) were purchased from Siemens Healthcare Diagnostic (Germany). Thrombin Substrate S-2238 (H-D-Phe-Pip-Arg-pNA) was from i-DNA Biotechnology Pte Ltd (USA). Sodium chloride, sodium phosphate and sodium azide were the product of Merck (Germany). Bovine serum albumin (BSA) and arginine hydrochloride were procured from Sigma Aldrich (Germany). Bradford reagent kit was purchased from Amresco Inc. (USA). Citrate tubes containing 0.5 ml

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of 0.11 mol/l sodium citrate was from BD Vacutainer Systems. Parafilm membrane was obtained from American Can Company (USA).

Thrombin time assay was recorded using Sysmex CA-50 coagulometer (Japan). Centrifugation was done using Universal 32R Centrifuge produced by Hettich ZenTrifugen (Germany). Microplate reader model Infinite M200, NanoQuant TECAN was the product of TECAN Corporation (USA).

Leech saliva extract collection

Leech saliva extract was collected from the starved leeches using a modified method for leech feeding. Briefly, leeches were fed on the phagostimulatory solution (PHS) of 0.001 M arginine in 0.15 M sodium chloride [10] which was filled in a glass funnel wrapped by parafilm membrane [11,12]. Then, the fed leeches were forced to regurgitate whatever they sucked by immersing them in ice containers [12]. Only the colorless salivary fluids were pooled and centrifuged at +4°C and 2500 rpm for 10 min [10], and the supernatant was filtered using 0.45 µm Sartorius® filter paper and termed as leech saliva extract (LSE) to be used during the following experimental procedures.

Total protein estimation

The total protein assay was performed according to the standard protocols of Bradford assay [13,14] and the instruction provided with Amresco Bradford reagent kit using BSA as a standard protein. The PHS was used as a blank.

The antithrombin activity of leech saliva extract

The antithrombin activity of LSE was ascertained using amidolytic assay and thrombin time (TT) assay *in vitro*.

Amidolytic activity: All reagents used for this experiment were prepared in phosphate buffered saline- bovine serum albumin buffer (PBS-BSA, pH 7.4) which contains 0.12 M NaCl, 0.01 M sodium phosphate, 0.01% sodium azide and 0.1% bovine serum albumin. Then, thrombin reagent and thrombin substrate S-2238 were prepared in PBS-BSA to a final concentration of 0.6NIHU thrombin/ml and 100 µM, respectively [15].

Amidolytic assay was carried out by adding volumes of 50 µl of thrombin reagent to equal volumes of different dilutions of LSE in the 96-well plate. The plate was shaken gently and incubated for 10min at 25°C in the microplate reader. Thereafter, 100 µl of the substrate was pipetted and the mixture was agitated. The absorbance at 405nm (A_{405}) was monitored for eight hours at 5-minute intervals [15]. Same procedures were repeated using the PHS as a negative control. Reaction buffer PBS-BSA was considered as a control reaction.

The percentage inhibition (% inhibition) was found out from the following equation:

$$\% \text{ inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of LSE}}{\text{Absorbance of control}} \right) \times 100$$

The protein concentration of LSE that inhibits 50% of the enzyme activity (IC_{50}) was determined from the curve resulted from plotting % inhibition against protein saliva concentrations.

Thrombin time assay *in vitro*: Thrombin time assay were fulfilled according to the standard protocols provided with Thromboclotin® reagent and the coagulometer manual [12,16] as the following:

Citrated plasma was always prepared from fresh human blood taken by venipuncture immediately prior to the experiment. Fresh human blood was mixed with sodium citrate in the citrate tube (9 parts of blood: 1 part of sodium citrate). Then, the mixture was centrifuged at 25°C for 10 min at 3000 rpm. The resultant supernatant plasma was taken and kept at room temperature (25°C) to be used within four hours of preparation.

Each vial of thrombin reagent was reconstituted with 10.0 ml distilled water. The resulted solution contained 2.5NIHU thrombin/ml. Thrombin solution was stable for one week when stored at 2-8°C according to the producer instruction.

Thrombin time assay was performed by pipetting aliquots of 100 µl of the freshly prepared citrated plasma into the pre-warmed coagulation tubes provided with the coagulometer and subsequently incubated at 37°C in the coagulation analyzer well for 3 min. Then, 100 µl of the reconstituted thrombin reagent was added and the time until coagulation starts was measured by the coagulometer. Different dilutions of the fresh LSE were mixed with the freshly prepared citrated plasma to yield a final volume of 100 µl and TT values of the mixtures were measured. The PHS was used as a negative control.

Control plasma test was always run before the experiment to evaluate the precision and accuracy of the reagents used and the coagulometer. Each vial of Control N® was dissolved in 1.0 ml distilled water, shaken gently and let to stand for 15 min at room temperature. The reconstituted control plasma was kept at -20°C for a maximum period of four weeks according to Control N® manual.

The percentage increase in thrombin time (% TT) was calculated from the equation:

$$\% \text{TT} = \left(\frac{\text{TT of the sample} - \text{TT of the citrated plasma}}{\text{TT of the citrated plasma}} \right) \times 100$$

The protein concentration of LSE which can increase TT by double folds (IC_{100}) was estimated from plotting % TT values against saliva protein concentrations that were mixed with the citrated plasma.

The effect of season variation on the antithrombin activity of LSE: The antithrombin activity (TT assay *in vitro*) of LSE was monitored for the fresh extracts LSE₁ and LSE₂ collected during the dry season (May) and the rainy season (November), respectively. The IC_{100} of each extract was estimated and compared with the other.

The impact of starvation period on the antithrombin activity of LSE: The antithrombin activity (TT assay *in vitro*) of LSE was monitored for many fresh extracts LSE₂, LSE₃ and LSE₄ collected during the rainy season after different starvation periods 16, 22 and 26 weeks corresponding to November, December and January, orderly. The IC_{100} of each extract was estimated and compared with the others.

Statistical analysis

All measurements were repeated in triplicates, the results were expressed as the mean ± the standard error of the mean (SEM) and analyzed by One-Way ANOVA using the Statistical Package for the Social Sciences SPSS 18.0 software. $P \leq 0.05$ was considered statistically significant.

Results

Leech taxonomy

Scientific identification confirmed that the used leeches were related to the species *Hirudinaria manillensis* (Lesson, 1842).

Total protein concentration

Bradford assay revealed that LSE₁, LSE₂, LSE₃ and LSE₄ contained total protein concentrations of 61.966 ± 2.017, 119.691 ± 6.025, 62.682 ± 1.705 and 80.549 ± 2.976 µg/ml, respectively.

Amidolytic activity

Results revealed that the collected LSE₁ effectively inhibited thrombin-mediated release of the p-nitroanilide from the synthetic substrate S-2238. The maximum duration period of the amidolytic activity was 270 min. On the other hand, it was found that the amidolytic activity of LSE was a linear function with protein concentration with IC₅₀ value of 49.391 ± 2.219 µg/ml (Figure 1).

Thrombin time assay

Results presented in Table 1 show that the fresh LSE significantly prolonged thrombin time in a dose dependent manner. Results revealed that the antithrombotic activity of LSE was a linear function with the protein concentration in plasma (Figure 2). Consequently, it was found that IC₁₀₀ values of LSE₁, LSE₂, LSE₃ and LSE₄ were 16.081 ± 0.079, 21.253 ± 0.789, 31.043 ± 1.147 and 45.371 ± 0.553 µg/ml, respectively.

The effect of season variation on the antithrombin activity of LSE

Findings exhibited a significant difference (p<0.05) in the antithrombin activity of LSE collected in different seasons (Figure 3). The IC₁₀₀ value of LSE₁ collected during the sunny season was 16.081 ± 0.079 µg/ml, whereas the IC₁₀₀ of LSE₂ which was extracted during the rainy season was 21.253 ± 0.789 µg/ml.

The impact of starvation period on the antithrombin activity of LSE

Results showed that starvation period significantly (p<0.001) affected the antithrombotic activity of LSE (Figure 3). The estimated IC₁₀₀ values of LSE₂, LSE₃ and LSE₄ were 21.253 ± 0.789, 31.043 ± 1.147 and 45.371 ± 0.553 µg/ml, respectively.

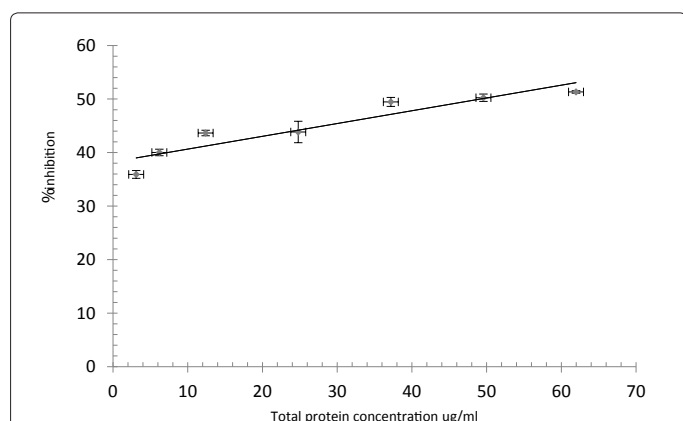


Figure 1: Dose-dependent amidolytic activity of LSE from the medicinal Malaysian leech. $Y=2.28X+38.26$, where: $Y=\%$ inhibition and $X=$ protein concentration (µg/ml), $R^2=0.878$. All measurements were the mean of triplicates ± SEM.

Sample	Protein concentration (µg/ml plasma)	TT (sec)	%TT
Control N ^o		17.10 ± 1.14	
Citrated plasma	--	18.30 ± 0.61	0
LSE ₁	12.393	28.37 ± 6.66	55.01 ± 3.59 ^a
	18.589	38.83 ± 7.5	112.20 ± 4.11 ^c
	21.688	53.03 ± 0.59	189.80 ± 3.24 ^a
	24.786	64.70 ± 1.36	253.55 ± 7.44 ^a
Citrated plasma	--	17.43 ± 1.01	0
LSE ₂	11.969	26.50 ± 0.46	52.01 ± 2.65 ^a
	23.938	34.50 ± 1.21	97.90 ± 6.92 ^a
	29.9227	41.23 ± 2.01	136.55 ± 6.67 ^a
	35.907	47.17 ± 1.01	170.55 ± 3.33 ^a
Citrated plasma	--	22.70 ± 0.13	0
LSE ₃	12.536	23.50 ± 0.50	3.52 ± 2.22
	18.804	29.43 ± 0.79	29.66 ± 3.46 ^β
	25.072	36.97 ± 1.07	62.85 ± 4.73 ^a
	31.341	47.03 ± 1.62	107.20 ± 7.15 ^a
Citrated plasma	--	17.1 ± 1.14	0
LSE ₄	24.164	22.43 ± 1.11	31.19 ± 6.49 ^β
	40.274	30.57 ± 1.58	78.75 ± 9.22 ^a
	48.329	35.17 ± 0.52	105.65 ± 3.04 ^a
	56.384	41.43 ± 0.79	142.30 ± 4.60 ^a

LSE₁: leech saliva extract collected from starved leeches in May (sunny season). **LSE₂**: leech saliva extract collected from starved leeches starved in November (rainy season). **LSE₃**: leech saliva extract collected from starved leeches in December (rainy season). **LSE₄**: leech saliva extract collected from starved leeches in January (rainy season). **TT**: thrombin time determined using thrombin time assay *in vitro*. **%TT**: the percentage increase in thrombin time. All results are expressed as the mean of triplicates ± SEM and analyzed by One-Way ANOVA using SPSS 18.0 software.

^ap<0.001 when compared with %TT of the citrated plasma (zero point). ^β p<0.05 when compared with %TT of the citrated plasma (zero point).

Table 1. The antithrombin activity of leech saliva extract from the medicinal Malaysian leech.

Discussion

The present results confirmed that the fresh saliva collected from the medicinal Malaysian leech *H. manillensis* possessed a remarkable antithrombin activity at low concentrations. These findings were in a good agreement with what had been reported about leech saliva biological activities [17]. Some Malaysian researches who studied the medicinal Malaysian leeches argued that the whole leech body extract showed an antithrombin activity. They characterized a direct thrombin inhibitor, called hirudin-like peptide with a molecular weight of about 7kDa [9]. Outside Malaysia, many other scientists indicated that the extract of the leech species *H. manillensis* contained an antithrombin agent, named bufrudin [7,18].

The results of this research indicated clearly that LSE collected from the medicinal Malaysian leeches, *H. manillensis*, possessed an amidolytic activity, since it effectively inhibited the ability of thrombin to release the chromogenic compound, p-nitroanilide, from the synthetic substrate S-2238. Many previous studies which were undertaken about the amidolytic activity of the salivary gland secretions of *H. medicinalis* revealed the existence of an antithrombin agent, hirudin, which can bind thrombin very tightly [15].

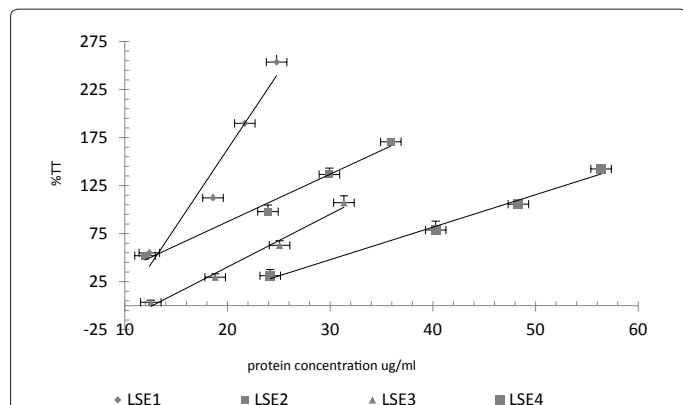


Figure 2: Dose-dependent antithrombin activity of LSE from the medicinal Malaysian leech. X= protein concentration ($\mu\text{g/ml}$) and Y= percentage increase in TT. **LSE₁**: leech saliva extract collected from starved leeches in May (sunny season). $Y=16.01X-157.5$, $R^2=0.948$. **LSE₂**: leech saliva extract collected from starved leeches starved in November (rainy season). $Y=4.953X-11.73$, $R^2=0.984$. **LSE₃**: leech saliva extract collected from starved leeches in December (rainy season). $Y=5.491X-69.66$, $R^2=0.986$. **LSE₄**: leech saliva extract collected from starved leeches in January (rainy season). $Y=3.382X-53.56$, $R^2=0.989$. All results are expressed as the mean of triplicates \pm SEM.

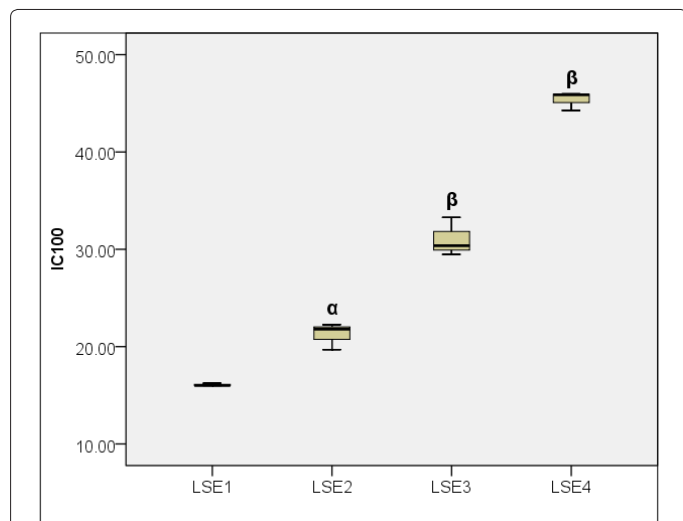


Figure 3: The effect of starvation period and season different on the antithrombotic activity of LSE from the medicinal Malaysian leech. **LSE₁** was collected during the sunny season (May). **LSE₂**, **LSE₃** and **LSE₄** were collected during the rainy season (November, December and January).

α $p < 0.05$ when compared with **LSE₁**. β $p < 0.001$ when compared with **LSE₂**. Data were analyzed by One-Way ANOVA using SPSS 18.0 software.

Studies about the mechanism by which hirudin inactivates thrombin suggested a two-step binding mechanism. In the first step, hirudin will bind to thrombin at a site different from the active site forming a weak complex which later undergoes a rearrangement reaction to produce a tight hirudin-thrombin complex in which hirudin is bound to the active site of thrombin. The second step involves another binding site which could be on either thrombin or hirudin. The substrate will compete with the inhibitor (hirudin) by binding to the second site rather than the first one [19].

As far as we know, it is reported here for the first time that the intensity of the antithrombotic activity of LSE from the medicinal Malaysian leech was affected by both starvation period and seasonal

variation. The LSE with the maximal antithrombin activity was collected during the sunny season (May) whereas less antithrombin activity was observed during the rainy season (November to January). Thus, leeches may produce salivary secretions with a higher anticoagulant content during the dry season. To explain this difference in activity, we can hypothesize a season-dependent distribution of the antithrombotic agents in the salivary secretions of the medicinal Malaysian leech. Noteworthy, some studies speculated that the tropical leeches go through a hibernation stage during the dry (sunny) season in the regions with dry and humid periods [2]. Thus, we can suggest that leeches would store a large amount of nutrients in their body enough to pass this dormant stage in their life cycle. Consequently, a large amount of stored blood requires a higher concentration of anticoagulant agents including antithrombin peptides. Similarly, some researchers reported that there is a seasonal difference in hirudin content of the European leech *H. medicinalis* salivary gland secretion. They found that the maximal antithrombin activity could be achieved in winter (rainy) and hirudin could be detected only in saliva collected during this season [20].

The current results showed also that the longer the starvation period, the higher the IC_{100} value and then the lower the antithrombin activity. In other words, the IC_{100} of the **LSE₄** collected from leeches starved for 26 weeks is greater by more than two folds than that of the **LSE₂** collected after 16 weeks of starvation. Consequently, we can conclude that the content of the actual antithrombin agent in **LSE₂** is higher than in **LSE₄**. These results could be ascribed according to the following suggestions:

It had been stated that the ingested blood can be stored in leech body for up to 18 months. During this storage period, the blood will not clot because of the anticoagulant proteins produced by leeches, and the ingested blood will undergo a very slow digestive process including haemolysis of erythrocytes and haemoglobin [4]. Consequently, we can suggest that the slightly breakdown of the nutrients would result in a moderate decline in the amount of blood clotting elements which requires less concentration of the anticoagulant enzymes (proteins) to be secreted.

Additionally, we can suppose that a long starvation period is a time consuming because leeches will get exhausted and aged. Logically, a starvation period of more than six months is enough for most of the collected leeches to reach sexual maturity or even the end of their life cycle. However, their ages when they had been collected was not defined.

Nevertheless, the actual reason still obscure and more studies about leech feeding behavior and their nutrition biology should be undertaken. In addition, clinical experiments should be undertaken to illustrate the seasonal prevalence of hirudotherapy.

Conclusion

It is stated here that leech saliva extract from the medicinal Malaysian leech *H. manillensis* displayed a thrombin inhibitory activity. The biological activity of LSE was remarkably influenced by starvation period and seasonal variation. Considering all, we recommend collecting LSE during the dry season after 16 weeks of starvation for more successful therapeutic usage and scientific research. Based on what mentioned above, physicians who use leeches for treatment are preferentially advised to consider season when using hirudotherapy.

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