Preliminary study on leech crude extract as an anti-coagulant agent

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Abstract

Hirudin is the generic name for a family of closely related homologous peptides that are all found in the cranial salivary glands of medicinal leech (Wallis, 1996). In 1984, the blood-thinning agent (anticoagulant) hirudin was isolated for the first time from leech salivary glands (Weinnberg, 1994). Today, hirudin is the most specific and active of known thrombin inhibitors not influencing on other peptidases (Hofsteenge and Stone, 1987). *Hirudinea* sp. (Buffalo Leech) was used for the isolation of hirudin for anti-coagulant application. The leech crude extract was prepared by using conventional method and tested to fresh rabbit blood. The time of blood clotting was recorded by using a drop method (Prakasam *et. al*., 2005). Three replicates were applied for each treatment. The results showed that the rabbit blood treated with leech crude extract gave the longest time of blood to clot with average and standard deviation values of 15.33±0.6 minutes when compared to treatment with distilled water and that on blood only (control) with an average and standard deviation values of 2.83±0.3 and 0.59±0.01 minutes, respectively. These results revealed that crude extract of leeches contain an anti-coagulant agent to prevent blood from clotting. The study on leech crude extract will be extended to include purification of hirudin for applications as anti-coagulant agent.

Introduction

Traditionally, leeches are widely used as a model animal in toxicological, physiological, neurobiological, biochemical, histological and many other studies (Mann, 1962; Flerov and Lapkina, 1976; Lapkina and Flerov, 1979; Sawyer, 1986; Lapkina, 1992; Huguet and Molinas, 1992, 1996; Blackshaw and Nicholls, 1995; Petrauskienė, 2001). The use of leeches for clinical or medicinal purposes (e.g. blood-letting) has occurred since the 5th century BC, with considerable usage in the 19th century, followed by a decline in the early 20th century (Kasparek *et al*., 2000). However, in more recent years, the medically beneficial usage of leech is once again becoming more sought after (Baskova et al., 1983, 1992). For example, *H. medicinalis* is used by plastic surgeons to restore venous circulation in tissue grafts where blood stagnation is a problem (Sawyer, 1986; Rigbi *et al*., 1987; Roters and Zebe, 1992; Whitaker *et al*., 2004; Huang *et al*., 2006b).

Extracts from this species have been shown to have an important thrombolytic effect on experimental thrombosis (Tan and Liu, 2002; Huang *et al*, 2006a, 2006b; Li *et al*., 2006). There has been increasing collection of this species for medicinal purposes in the 20th century, and this, combined with a general loss and pollution of wetland habitats have caused a dramatic decline of *H. manillensis* throughout its geographical range (Steiner *et al*., 1990; Electricwala *et al*., 1993; Singhal and Davies, 1996).

In this country, it is not known or proven conclusively that the locally named Buffalo Leech is not of *H. manillensis.* Local taxonomists have not been able to identify the species used those for medical purposes and would rather refer to its genus only as *Hirudinea* sp.

Material and Methods

Sample Preparation

Hirudinea sp. used in the study was provided by PT Dynamic Consultant Co., Kota Bharu, Kelantan. The non-chlorine freshwater in the aquarium tank placed indoor was aerated, with 50% of the water changed once every 3 days. The temperature, pH and light intensity were maintained at room temperature (25 °C -27 °C), 6-8, and 0 - 100 lux, respectively. The leeches were starved before starting this study.

Experimental Method

For the experiment, 10 gram of leeches was taken and the crop emptied of the blood by placing the leeches in a container filled with crystals of sodium chloride for 15 minutes and then washed with distilled water. They are then cut into small pieces and grinded using a blender. The extraction was made with a volume of distilled water equal to six times the weight of the pulp. After centrifugation at 1500 rpm for 60 minutes at 4 °C, the supernatant fluid was filtered through coarse paper. Finally, the crude extract was stored at 4°C for 24 hours.

Precipitation

A 0.5 % copper sulfate solution was added drop by drop to the prepared extract until the reagent no longer produced further precipitation. After removal of the precipitate by filtration on course paper, the clear solution was placed in visking tubing and dialysed.

Dialysis

Dialysis was done by putting 20 ml sample of the leech extract supernatant into visking tubing and dialysed against cold distilled water at 4°C on the shaking machine for eight hours at 100 rpm. The leech extract was stored at 4 °C for 24 hours.

Testing anti-coagulant activity of leech extract on blood

The crude extract was tested on their anti-coagulant activity by adding 0.5 ml of the extract to 2 ml of fresh rabbit blood and the time of clotting recorded.

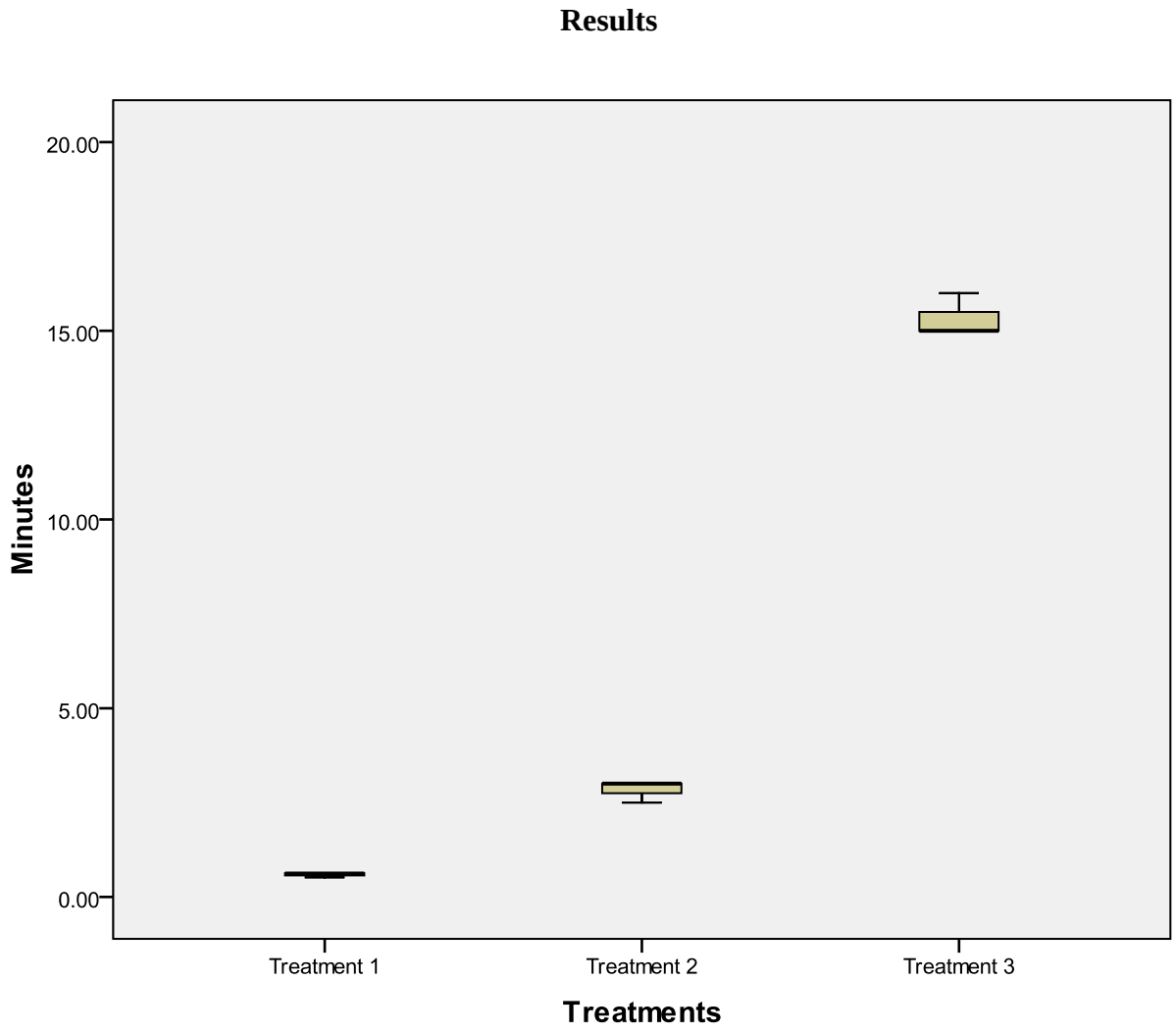

Figure 1: Blood clotting time under different treatments.

Figure 1 shows the time for blood to clot for different treatments. Analysis carried out using the One-Way ANOVA showed significant differences among the different treatments (p=0.00). Treatment 3 gave the highest result with mean and standard deviation values of 15.33±0.6 compared with treatment 1 gave the lowest result with mean and standard deviation values of 0.59±0.01.

Treatment	Time of Blood Clotting
(1) Rabbit blood only (Control)	0.59±0.01
(2) Rabbit blood + distilled water	2.83±0.3
(3) Rabbit blood + crude extract	15.33±0.6

Table 1: Time taken for blood to clot under three treatment regimes. Data in the table were means and standard deviations (mean±S.D.)

Table 1 shows treatment 3 (Rabbit blood+crude extract) gave the longest time to clot with mean and standard deviation values of 15.33±0.6 when compared with treatments 1 (Control) and 2(Rabbit blood+distilled water) with mean and standard deviation values of 0.59±0.01 and 2.83±0.3, respectively.

Discussion

This experiment was conducted to determine the anti-coagulant activity of the buffalo leech extract. The anti-coagulant activity was evaluated by testing the crude extract on fresh rabbit blood and recording the time of clotting. The fact that a strong anti-coagulant activity was exhibited in the local buffalo leeches indicates that hirudin was present in the crude extract. Generally, hirudin is the generic name for a family of closely related homologous peptides that are all found in the cranial salivary glands of medicinal leech (Wallis, 1996). In 1984, the blood-thinning agent (anticoagulant) hirudin was isolated for the first time from leech salivary glands (Weinnberg, 1994). Today, hirudin is the most specific and active of known thrombin inhibitors not influencing on other peptidases (Hofsteenge and Stone, 1987).

In this study there was a marked difference in the clotting time for the treatments employed with that treated on rabbit blood gave the highest values. The result of this preliminary study revealed that crude extract of local leeches contained an anti-coagulant to prevent blood from clotting.

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