# ISOLATION AND CHARACTERIZATION OF MALAYSIAN LEECH SALIVA EXTRACT

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Pharmaceutical Chemistry

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# ABSTRACT

Leech saliva contains biologically active compounds that are mainly proteins and peptides. In Malaysia leeches have been used for traditional medicine for a long time. However, there are scanty studies about the isolation and characterisation of Malaysian leeches' saliva. This study aimed to isolate and characterize leeches saliva extract. A modified and smooth extraction method of leeches' saliva without leeches' scarification is used. UV and Bradford assay protein methods showed that the saliva extract contains high concentrations of protein. RP-HPLC chromatogram revealed that more than 30 different peaks were there in leech saliva extract. Gel electrophoresis revealed the existence of protein and peptides with different molecular weights. The gel showed up to 25 different bands. Comparison of gel electrophoresis data with protein database revealed the closeness of many molecular weights to known proteins isolated from the *Hirudinaria* leech family. Other proteins detected by gel electrophoresis may be related to completely new biologically active proteins and peptides or to a modification (isoforms) of the existing ones. It was observed that the period of starvation has a vital role in the concentration of saliva proteins and 12 weeks of starvation gave the highest concentration of proteins in the saliva. It was found also that 4 weeks of starvation after first feeding is enough for leeches to recover 42% of their protein concentration. Two anticoagulant proteins (protein 1 and protein 2) were isolated from leeches saliva extract by using RP-HPLC, and their molecular weights were identified (6.289kDa and 14.255kDa) respectively using tricine SDS-PAGE. These two proteins increased the thrombin time by 29.11% and 44.13% respectively. In addition, they inhibited the amidolytic activity of thrombin, evaluated by measuring the conversion of the chromogenic substrate (S-2238). The result showed a decrease in the conversion of the substrate (S-2238) by 30.61% and 41.22 % respectively. Traces of heavy metals concentrations were investigated in the water (natural habitat of the leeches), leeches' tissues and leeches' saliva extracts. The concentrations of heavy metals in the leeches' habitat water were found high. Hence the water specification is a class IV (INWQS). Furthermore, traces concentrations of heavy metals were found in leeches' tissues as well as in their saliva extracts. Such concentrations of heavy metals may cause health hazard, especially when leeches from such contaminated environment are applied in treatment and therapy directly without any precautions. Clearing leeches and their saliva extracts from these traces is investigated in this study. It was found that these high concentrations can be mitigated by successive replacement of the lake water by clean and non-chlorinated or distilled water for three weeks. A significant decrease for certain heavy metals concentrations was achieved depending on the type of metal. For instance in the saliva extract, undetected level of cadmium (Cd) was observed while a marginal 7.3% decrease in the case of arsenic (As) was reported after washing. In the case of leeches' tissues, the concentration of cadmium (Cd) increased unexpectedly, while a decrease of 92.38% and 20.01% in the concentration of lead (Pb) and arsenic (As) were recorded respectively.

# خلاصة البحث

يذكر أن لعاب العلق الطبي يحتوي على مركبات فعالة حيويا تتكون بشكل اساسي من البروتينات والببتيدات، وقد استخدم العلق في ماليزيا لوقت طويل في الطب الشعبي التقليدي لعلاج العديد من العلل والأمراض بشكل تقليدي دون فهم عملي لعمله. لا توجد دراسات علمية منشورة كثيرة حول استخلاص وتوصيف لعاب العلق الطبي الماليزي. لذا كان الهدف من هذه الدراسة هو العزل و الوصف الكيميائي لخلاصة لعاب العلق الطبي الماليزي. استحدثت في هذه الدراسة طريقة جديدة وآمنة لاستخلاص اللعاب من العلق بحيث لا تؤدي لقتل الدودة و يمكن استخدامها لمرات عديدة. اظهرت دراسة تركيز البروتين بطريقة الامتصاص للأشعة فوق البنفسجية وطريقة برادفورد تراكيزا عالية للبروتينات. بينت نتائج الدراسة بالاستشراب السائل العكسي عالي الانجاز وجود اكثر من ثلاثين قمة مختلفة في خلاصة لعاب العلق الطبي الماليزي. أما نتائج الرحلان الكهربائي الهلامي فأظهرت وجود اكثر من خمس وعشرين حزمة مختلفة. اجريت مقارنة بين الاوزان الجزيئية للبروتينات المعزولة في هذه الدراسة والاوزان الجزيئية للبروتينات المعزولة في الدراسات السابقة على العلق ووجدنا تطابق في العديد من الاوزان الجزيئية مما قد يشير الى وجود هذه المركبات الفعالة في لعاب العلق الطبي الماليزي. وبالنسبة لبقية البروتينات فقد تشير الى مركبات فعالة حيوية جديدة كليا او الى مركبات ناتجة عن التعديل في المركبات الحالية. دلت هذه الدراسة ان تجويع العلق لمدة اربعة اسابيع بعد الاستخلاص الاولي للعاب كانت كافية للعلق ليستعيد القدرة على انتاج بروتينات بنسبة 42% من التركيز البدائي. تم عزل مركبين فعالين من لعاب العلق الطبي الماليزي باستخدام تقنية الاستشراب السائل العكسي عالي الانجاز و قد سميت (بروتين 1 وبروتين 2) وحسبت أوزانهما الجزيئية بطريقة الرحلان الكهربائي الهلامي بوجود التريسين حيث تراوحت الأوزان الجزيئية لهما (6289 و 14255 دالتون) على التوالي. لدى اختبار هذين البروتينين تبين أنهما طاولا زمن تخثر الدم بنسبة 29.11% و 44.13% على التوالي. اضافة الى ذلك فقد ثبطا تحول الركيزة S2238 بنسبة 30.61% و 41.22% على التوالي. تم الكشف عن المعادن الثقيلة في الماء و هي البيئة الطبيعية للعلق وكذلك في انسجة وخلاصة لعاب العلق. وجدت بعض التراكيز العالية من المعادن الثقيلة في ماء البيئة الطبيعية للعلق مما استدعى تصنيفه تحت الصنف 4 حسب التصنيف الماليزي الوطني لنوعية الماء. إن وجود هذه الأنواع من المعادن الثقيلة في نسج ولعاب العلق قد يعد خطرا على الصحة العامةخصوصا إذا أخذنا بعين الاعتبار الاستخدام المباشر للعلق المأخوذ من هذه المناطق الملوثة على المرضى في العلاج دون أخذ الحذر. استحدثت طريقة جديدة للتخلص من المعادن الثقيلة في جسم ولعاب العلق عن طريق التبديل المتتالي لماء البحيرة بماء الصنبور الخالي من الكلور أو بالماء المقطر لمدة ثلاثة اسابيع. وقد حققنا نتائج ملحوظة من انخفاض لتراكيز المعادن تبعا لنوع المعدن. فعلى سبيل المثال تم التخلص من كافة كمية الكادميوم من خلاصة لعاب العلق بينما كان لم يتعد الانخفاض في تركيز الزرنيخ ال 7.3% بعد الغسل. اما في حالة نسيج جسم العلق فقد انخفض تركيز الرصاص بنسبة92.38% أما الزرنيخ فانخفض بنسبة 20.01%. توصي الدراسة بالاستمرار في البحث لكشف أسرار لعاب هذه الدودة الطبية والتي قد تكون ذات فائدة كبيرة في علاج الكثير من الأمراض التي تهدد حياة البشر وتهدر اقتصادهم.

# APPROVAL PAGE

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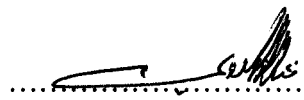

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Abbas Mohammad Ghawi
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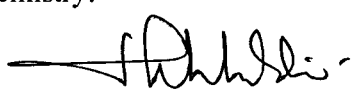

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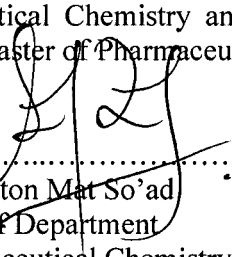

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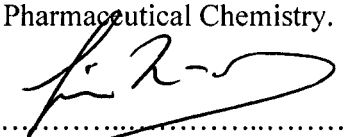

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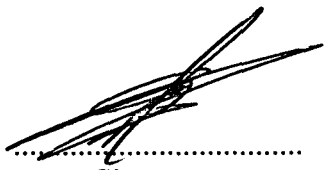

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Date

*To my beloved and respected family of Alaama, thank you all for your kind support love and care. Special thanks to you dear great mom, your selfless spiritual imparted moral support have always been with me helping me in achieving my goals. Tons of thanks to my beloved dad, you've sacrificed both personally and professionally for me to chase down my dreams. I have to express my feelings of appreciations to you my dear wife, even though my tongue is unable to pronounce enough words to praise for your wonderful spiritual advices, most especially if I remember your love towards me, advice, Islamic motivations, and all such beautiful words that always come out of your mouth that stir the love of Allah Almighty and His Most beloved Messenger Muhammad (SAW) in the depth of my heart, I hope you know how much it has meant to me and how important you are to me, blessings of Allah (SWA) be always with your souls of the lovers of Prophet Muhammad SAW.*

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Mohamed ALAAMA.

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| | |
|---|---|
| AAS | Atomic Absorption Spectroscopy |
| AB30 | Acrylamide/Bisacrylamide (29.2, 0,8) ratio |
| AEC | Anion Exchange Chromatography |
| APS | Ammonium Persulfate |
| Arg | Arginine |
| As | Arsenic |
| BCA | Bicinchoninic Acid |
| BSA | Bovine Serum Albumin |
| CCC | Criterion Continuous Concentration |
| CCME | Canadian Council of Ministers of the Environment |
| Cd | Cadmium |
| CE | Capillary Electrophoresis |
| CEC | Cation Exchange Chromatography |
| CEQG | Canadian Environmental Quality Guidelines |
| Cr | Chromium |
| DOE | Department of Environment |
| EPA | Environmental Protection Agency |
| FAAS | Flame Atomic Absorption Spectroscopy |
| Fe | Iron |
| GF AAS | Graphite Atomic Absorption Spectroscopy |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| INWQS | Interim National Water Quality Standards |
| LSE | Leeches Saliva Extract |
| Mn | Manganese |
| Ni | Nickel |
| Pb | Lead |
| Pi | Isoelectric point |
| RP-HPLC | Reversed Phase High-Performance Liquid Chromatography |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SEC | Size Exclusive Chromatography |
| Se | Selenium |
| TCA | Trichloroacetic acid |
| TEMED | Tetramethylethylenediamine |
| TFA | Trifluoroacetic acid |
| UV | Ultra Violet |
| Zn | Zink |

# CHAPTER ONE

# INTRODUCTION

## 1.1 LEECHES

Leeches are segmented worms in the subclass *Hirudinea* which are very important in the food web of aquatic ecosystem or in their habitat system acting both as predators, prey, and parasites (Russell, Paul, & Beverly McMillan, 2007). There are about 700-1000 species of leeches all over the world (Siddall, Bely, & Borda, 2006).

Leeches are hermaphrodites' worms and they don't live long after they have reproduced once or twice.

Some leeches are carnivorous; they prey on invertebrates, and feed on insect larvae, snails, crustaceans and other worms. Their digestive enzymes process their food within a few days. Other leeches are sanguivorous and they feed on the blood of vertebrates such as amphibians, birds, reptiles, fish and mammals including humans. Leeches need symbiotic bacteria to help in digesting their blood meals. (Parker, 2006; Sawyer, 1986b; Siddall, et al., 2006).

*Hirudinaria manillensis* exist widely in tropical areas especially in South East Asia (Mann, 1962). Malaysian leeches belong to the *Hirudinaria manillensis* species known also as buffalo leeches (Govedich, Moser, & Davies, 2004; Sawyer, 1986b). There are four families and seventeen species of *Euhirudinaria* in Malaysia distributed on the entire Malaysian ecosystem (Govedich, et al., 2004).

Leeching and leech saliva extracts have received much attention because of their extensive uses in many traditional & conventional medical fields (Sawyer, 1986b). Nowadays, leeches are being used for several medical ailments like: arthritis,

blood – clotting disorders, eyes diseases, post-operative venous congestion, and even in modern plastic reconstructive surgery (Govedich & Bain, 2005; Michalsen, Roth, & Dobos, 2007).

Leech's saliva contains many active compounds which have several biological activities, as anticoagulants, vasodilatants, anaesthetics, thrombolytics, antibiotics, analgesics, anti-metastasis and anti-inflammatory agents (Kraemer, Korber, Aquino, & Engleman, 1988; Michalsen, et al., 2007). While the anticoagulant compounds help to keep the host blood in the liquid statue during sucking (Govedich & Bain, 2005; Markwardt, 1957; Salzet, Chopin, Baert, Matias, & Malecha, 2000), as well as during the storage of it in leeches digestive system making its digestion easier (Mann, 1962; Sawyer, 1986b).

These anticoagulants are mainly proteins and peptides secreted by the leech's salivary glands. The anticoagulant activity of the extract of the head of *Hirudo medicinalis* leeches was for the first time described (Haycraft, 1884). This anticoagulant compound was found to be a protein in nature and it was named *hirudin* (Bodong, 1905). Later, *hirudin* was isolated in the pure state and was fully characterised (Bagdy, Barabas, Gráf, Petersen, & Magnusson, 1976; Markwardt, 1957). On the other hand, the saliva constituents of other species of leeches have been studied and number of proteins and peptides has been found (Faria, Kelen, Sampaio, Bon, Duval & Chudzinski-Tavassi, 1999; Nutt, Gasic, Rodkey, Gasic, Jacobs, Friedman, & Simpson, 1988; Salzet, et al., 2000).

Because it is highly atractive, several studies have been conducted aimed to isolate the active constituents of leeches' saliva extract using several types of analytical techniques. *Hirudin* for example was isolatedin pure form at the first time by organic solvents precipitation (Markwardt, 1957). In addition, electrophoritic

methods like SDS-PAGE were also used to isolate other proteins (Baskova, Cherkesova, & Mosolov, 1976). Chromatographic methods like gel filtration and ion-exchange chromatography were extensively used to prepare large amount of hirudin (Bagdy, Barabás, & Gráf, 1973) as well as HPLC methods were also used to isolate proteins from leeches saliva extract (Steiner, Knecht, Gruetter, Raschdorf, Gassmann, & Maschler, 1990). Nowadays the following procedures are used to isolate proteins from leeches saliva extract:

A. Concentration of the saliva extracts by lyophilization,
B. Crude separation via gel filtration and chromatography on anion exchangers.
C. Final step of purifiaction by HPLC
D. Molecular weights assessed using gel electrophoresis or mass spectroscopy (Wallis, 1996).

Some examples of the anticoagulant peptides and proteins that have been isolated from leech species *Hirudinaria manillensis* are: *bufridin* (Electricwala, Sawyer, Jones, & Atkinson, 1991) and *hirullin P6* and *hirullin P18* (Steiner, Knecht, Bornsen, Gassmann, Stone, Raschdorf & Maschler, 1992).

In Malaysia leeches are used in traditional medicine for treatment of many human disorders like arthritis. Direct leeching is used in Malaysian traditional medicine without considering the pollution which may result from the environment where leeches are having their habitat. For instance, many pollutants may contaminate the body and/or the saliva of the leech especially those pollutants from traces of heavy metals which have an impact on public health.

In the literature, there are scanty information about the constituent of local Malaysian leeches' saliva extract, their biological activity as well as the safety of using direct leeching.

Taking all previous issues in mind, this study was carried out to ensure the following objectives:

## 1.2 OBJECTIVES

- To develop a method of saliva extraction in reasonable quantities without scarifying the leeches.
- To optimize storage conditions for the saliva extract.
- To assay the biological activities of the Malaysian leeches' saliva extract, and prove its anticoagulant activity.
- To describe and characterize Malaysian leeches' saliva extract using all the available analytical methods. To find a relationship between the quality and quantity of proteins in leech saliva extract and their starvation period.
- To assess the trace amounts of heavy metals in leech's tissues and their saliva extract which are considered as an environmental contaminants with an impact on public health.
- To optimize methods of minimizing the concentration of heavy metals in leeches' tissues and/or their saliva extract if there is any contamination by heavy metals.

## 1.3 STATMENT OF THE PROBLEM

Leeches' saliva analysis for its biologically active components was the target of many researchers. However, in Malaysia scanty scientific reports are available regarding the biological activity of medical Malaysian leeches' saliva components. Hence, the main goal of this study is to isolate and characterise the active components in Malaysian leeches' saliva extract.

## 1.4 RESEARCH HYPOTHESIS

The Malaysian medical leech family is similar to its European counterpart, for which an abundant literature describing active components in their saliva extracts and the associated biological activities is available. In the contrary very few studies were undertaken for the Malaysian leeches. Developing a smooth method of leech extraction and characterisation could reveal very interesting and useful compounds that still unknown.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 LEECHES

Leeches (Figure 2.1) are segmented worms in the Subclass Hirudinea that are usually ectoparasitic (Basu & Chandra, 2004; Russell et al., 2007). They are included in the Class Clitellata, Subclass Hirudinida, and Superorder Euhirudinea (Mann, 1962; Sawyer, 1986a).

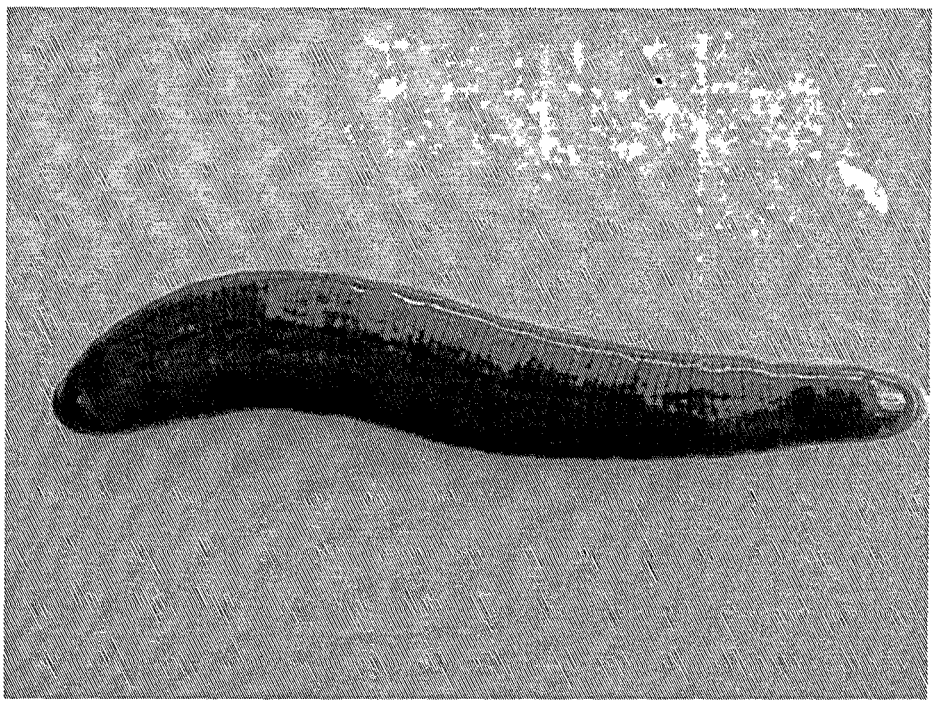

Figure 2.1: Photo of a leech.

Leeches usually live up to 1-3 years. From the reproductive view, leeches are hermaphrodites which mean that each leech has both male and female reproductive organs. The typical life cycle of leeches consists of egg which is laid usually at the