THE EFFECTS OF *TAMARINDUS INDICA* SEED EXTRACT AS ANTI-SNAKE VENOM AGAINST *DABOIA RUSSELLI, NAJA KAOUTHIA* AND *OPHIOPHAGUS HANNAH* IN MICE

BY

SORAYA ISMAIL

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Medical Sciences)

Kulliyyah of Medicine
International Islamic University Malaysia

JUNE 2017

ABSTRACT

Globally, snakebite cases are estimated to be around 5 million annually affecting mainly the residents of poorer counties like Africa and Asia, and in 2009 WHO has categorised it as a 'neglected tropical disease'. Currently the standard treatment for snake envenomation is the use of anti-snake venom (ASV) therapy. However this is expensive and not readily available in smaller hospitals in the developing world. Herbal medicine has been and is still in use in some cultures for the treatment of snakebite and one such plant is *Tamarindus indica*. This plant is found in many countries where snake envenomation is also prevalent. This study was conducted to evaluate the potential of using *T. indica* seed extract (TSE) to inhibit the effects of snake venom of three snakes; namely *Naja kaouthia, Ophiophagus hannah* and *Daboia russelli*. The testa of tamarind seed was used and it underwent ethanolic soxhlet extraction to obtain TSE. The inhibition of the activity of the following enzymes i.e phospholipase A_2 (PLA_2), proteinase and phosphomonoesterase (PME) *in vitro* by the three snake venoms with TSE was studied. SDS-PAGE experiment was conducted to observe the effects of TSE on venom proteins. *In vivo* acute subcutaneous (SC) toxicity of TSE in ICR mice was conducted. Study on the inhibition of lethality was conducted on each of the three snake venoms when SC TSE was injected into mice. Venom concentration and site were fixed but TSE concentration, time and site of injection were manipulated. Findings from venom enzymatic inhibition studies showed that, TSE was able to significantly reduce ($p<0.05$) all three venom enzymatic activities i.e PLA_2, proteinase and PME. SDS-PAGE experiment showed that venom protein bands were disrupted when venom reacted with TSE. No signs of toxicity were observed over a period of 4 weeks when mice were exposed to SC TSE 60 mg/20 g body weight except for skin ulcers. Histological examination on liver, both kidneys and skin at the site of SC injection showed no changes compared to the control group injected with SC distilled water. TSE was able to increase the survival rate of ICR mice when exposed to each of the three snake venoms regardless of the site of injecting SC TSE. Mice injected with *N. kaouthia* or *D. russelli* venom, had increased 24 hour survival rate when SC TSE was given at 15 minutes; and of mice injected with *O. hannah* venom the 24 hour survival rate increased with higher TSE concentration when given sooner. In conclusion, SC TSE was safe to be injected up to 60mg/20 g and has the potential to delay the effects of venom from *N. kaouthia, O. hannah* and *D. russelli*.

خلاصة البحث

تقدر حالات لدغ الأفاعي على الصعيد العالمي بحوالي 5 ملايين سنويا، مما يؤثر بشكل كبير على سكان البلدان الفقيرة مثل افريقيا وآسيا. وقد صنفت منظمة الصحة العالمية عام 2009 لدغ الأفاعي ضمن "أمراض المناطق الاستوائية المهملة". وحاليا فإن المعيار القياسي للعلاج من حالات التسمم من اللدغات السامة يكمن في استخدام ترياق مضاد لسموم الأفاعي . ولكن هذا أمر مكلف وغير متوفر بسهولة في المستشفيات الصغيرة في العالم النامي. وقد استخدمت الأدوية العشبية في الماضي لعلاج لدغات الأفاعي ومن بينها نبات التمرهندي كأحد هذه الوسائل. وقد تم العثور على هذا النبات في العديد من البلدان حيث التأثر بزعاف الحشرات السامة لا تزال سائدة أيضا. وقد أجريت هذه الدراسة لمعرفة إمكانية استخدام مستخلص بذور التمرهندي لمنع آثار التسمم من ثلاثة ثعابين وهي: الأُميوفيجَس هانا، والناجاكوثيا والدابيا روسلي. لقد استخدم غلاف بذور التمرالهندي والذي تم استخراجه عن طريق السوكسليت المثيلي وذلك للحصول على مستخلص التمرهندي. ولقد استخدم في المختبر مستخلص بذور التمرهندي لدراسة تثبيط نشاط انزيمات الفوسفوليبد أ2، بروتيوليتِك، والفوسفومونواستيريز وذلك لسموم ثلاثة أفاعي. وقد أُجري الفصل الكهربائي لهلام متعدد الأكريلاميد لملاحظة تأثير مستخلص التمر هندي على بروتينات سموم الأفاعي. هذا وقد أُجريت دراسة التأثير الحاد لحقن الفئران تحت الجلد بمستخلص التمرهندي.
وقد تمت دراسة تثبيط فتك سمية ثلاثة أفاعي بحقن مستخلص التمرهندي تحت جلد فئران التجارب.. وقد ثُبتت جرعات سموم الأفاعي وكذلك أماكن حقنها؛ مع تغيير جرعات، ووقت وأماكن حقن مستخلص التمرهندي. وأظهرت نتائج تثبيط فاعلية انزيمات سموم الأفاعي على مقدرة مستخلص التمرهندي على اضعاف تأثير سُمّية انزيمات الأفاعي الثلاثة (انزيمات الفوسفوليبد أ2، بروتيوليتِك، والفوسفومونواستيريز). وقد أظهرت تجارب فصل الرحلان الكهربائي لهلام الأكريلاميد عُطل رحلان سموم الأفاعي عند استعمال مستخلص التمرهندي. ولمدة 4 أسابيع لم يُلاحظ ظهور أعراض لتمسمم الفئران المحقونة بمستخلص التمر هندي بجرعة 60 ملج/20ملج باستثناء التقرحات الجلدية. ولم يُظهر الفحص النسيجي للكبد والكُليتن ومواضع الحقن تحت الجلد أي تغييرات بالمقارنة بفئران مجموعة التحكم والتي حقنت بالماء المُقطّر. وقد رفع مستخلص التمرهندي معدل بقاء الفئران على قيد الحياة عند تعرضها لسموم كل من الثلاثة أفاعي بغض النظر عن موقع الحقن تحت الجلد. ارتفع معدل بقاء الفئران على قيد الحياة لمدة 24 ساعة التي حُقنت بسموم أفعى الأُميوفيجَس هانا وذلك عند التعجيل بحقنها بجرعة عالية من مستخلص التمرهندي. أما الفئران التي حُقنت بسموم أفعى الناجاكوثيا والدابيا روسلي فقد زاد معدل بقائها على قيد الحياة لمدة 24 ساعة عند حقنها بمستخلص التمرهندي كل 15 دقيقة. واستنتجت الدراسة أن لدى مستخلص التمرهندي قدرة على تأخير آثار سموم أفاعي الأُميوفيجَس هانا، والناجاكوثيا والدابيا روسلي عند حقنها تحت الجلد بمستخلص التمرهندي بجرعات تتراوح بين 60 ملج الى 20 ملج.

APPROVAL PAGE

The thesis of Soraya binti Ismail has been approved by the following:

_____
Pakeer Oothuman Syed Ahamed
Supervisor

_____
Khin Maung Maung
Co-Supervisor

_____
M. Taher Bakhtiar
Internal Examiner

_____
Stephen Ambu
External Examiner

_____
Rafidah Hanim Mokhtar
External Examiner

_____
Wan Mohd Azizi Wan Sulaiman
Chairman

DECLARATION PAGE

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Soraya binti Ismail

Signature …… Date ……...……..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

THE POTENTIAL OF *TAMARINDUS INDICA* SEED EXTRACT AS ANTI-SNAKE VENOM AGAINST *DABOIA RUSSELLI, NAJA KAOUTHIA* AND *OPHIOPHAGUS HANNAH* IN MICE

I declare that the copyright holders of this thesis are jointly owned by the student and IIUM.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Soraya binti Ismail

……..…….. ……..
Signature Date

This thesis is dedicated to my loving family;

To my respectful and loving parents; Haji Ismail Bazain and Allahyarharmah Hajjah Khadijah Haji Mohd Said, thank you very much for allowing me to pursue my dreams and supporting me all the way. Without your blessing I will never be where I am today. Thank you for believing in me.

To my sweet husband; Dr. Ahmad Faidzal Othman, in you soft embrace I find the strength; and in your kind words I find the wisdom. I am blessed to have you in my life.

To my adorable children; Ahmad Nadzrein Iskandar, Ahmad Faredz Idham & Ahmad Redza Iezlan, you are my pride and joy. Soar the sky and live your dream.

In all of you I find love and sincerity, warmth and comfort, peace and serenity. Thank you for being part of the worderful journey of my life. I love you all.

ACKNOWLEDGEMENT

In the name of Allah, most gracious, most merciful.

Alhamdulillah, praise be to Allah the Almighty, for His will this thesis is able to be completed. My sincere appreciation to my respected supervisor; Professor Dr. Pakeer Oothuman b Syed Ahamed and co-supervisor; Dr. Khin Maung Maung. Thank you very much for the knowledge shared and guidance given in seeing me through PhD journey. My sincere appreciation too to Assoc. Professor Imad Matloub Dally Al Ani for assisting me with my histopathological examination and Assoc. Professor Dr. Emad Mohamad Nafie Abdel Wahab for helping write the Arabic translation of the abstract.

Many thanks to Encik Ramali b Awang; Director, Department of Landscape, Kuantan Municipal Council and Teruntum Mini Zoo staff for allowing and facilitating the milking of snake venom from the snakes available. Many thanks too to the laboratory staff from Kulliyyah of Pharmacy, Kulliyyah of Science and Kulliyyah of Medicine for assisting in laboratory work conducted.

Many thanks to the Research Management Center, IIUM for funding the study (EDW B 14-211-1096). Lastly, my warmest appreciation to all my lecturers, staff, fellow postgraduate students and friends alike who have taught me and supported me along the way. May Allah bless all of you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMSEM	Advanced workshop on marine and snake envenomation management
H & E	Haematoxylin - Eosin
HPLC	High performance liquid chromatography
IM	Intramuscular
IV	Intravascular
LD_{50}	50% of lethal dose
LD_{100}	100% of lethal dose
MHD	Minimum haemorrhagic dose
MPT	*Mimosa pudica* extract (*Mimosa pudica* tannin)
OECD	Organization for Economic Co-operation and Development
PLA_2	Phospholipase A_2
PME	Phosphomonoesterase
rpm	Revolution per minute
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMVPs	Snake venom metalloproteinases
TSE	Tamarind seed extract

LIST OF DEFINITIONS

Antivenom
A purified fraction of immunoglobulins or its fragments fractionated from the plasma of animals that have been immunized against venom from one or several species of snakes

Venom
The toxic secretion of a specialized venom gland which in the case of the snake, it is delivered through the fangs and provokes deleterious effects. Venoms usually comprise many different protein components of variable structure and toxicity. Venom enters the victims via 'injection'.

Toxin
A toxic substance, especially a protein, which is produced by living cells or organism and is capable of causing disease when introduced into the body tissues. It is also often capable of introducing neutralizing antibodies or antitoxins. Toxin enters the victims via ingestion.

Calloselasma rhodostoma
Synonym to i) *Agkistrodon rhodostoma*
Common name: Malayan pit-viper

Daboia russelli
Synonym to: *Vipera russelli*
Common name: Russell's viper, Chain viper

Daboia russelli siamensis
Synonym to: *Vipera russelli*
Common name: Eastern Russell's viper, Siamese Russell's viper

Naja kaouthia
Synonym to: *Naja naja kaouthia, Naja-naja*
Common name: Monocled cobra
It was formerly treated as a subspecies of Indian cobra *(Naja naja)*

Naja sumatrana
Synonym to: *Naja naja sputatrix*
Common name: Spitting cobra

Ophiophagus hannah
Synonym to: Hamadryad
Common name: King cobra

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Snakes have been the subject of fascination, fear and myths all through history. In ancient Egyptian times, the cobra was worshipped and its replica was used to decorate the crowns of the Pharaohs (Belluccio & Johnson, 1992). In ancient Greek too, Asclepius the demi-god of medicine carried a pole with two snakes entwined on it (Wilcox & Whitham, 2003). The Greek mythology associates snake with being deadly and dangerous. The infamous Medusa had snakes in place of hair (Graves, 1990). In Hindu mythology, the cobra occupies the revered position i.e around Lord Shiva's neck and is protected from enemy, eagle or garuda (Lochtefeld, 2002). In ancient Chinese astrology, the snake represents a month in the Chinese zodiac calendar (Eberhard, 2006).

The scientific classification of snake is shown below in Figure 1.1. The pylogeny of snakes have gone through many updates through the years. The latest update uses snake classifications by Pyron et al (2011). This classification is available in Appendix A.

Globally, over 3400 species of snakes have been documented (Uetz & Hosek, 2015a). From that, peninsular Malaysia has over 141 known species of land and sea snakes. Of these, 26 species of land snakes (Das, Ahmed, & Liat, 2015) and 21 species of sea snakes are venomous (Lim, 1982). The medically significant venomous snakes