BIOLOGICAL ACTIVITIES OF SALIVA EXTRACT FROM THE MEDICINAL MALAYSIAN LEECH *HIRUDINARIA MANILLENSIS*

BY

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ABSTRACT

The work presented in this thesis describes selected biological activities of the salivary gland secretion of the medicinal Malaysian leech *Hirudinaria manillensis* (Lesson, 1842). A new device for leech feeding was developed using common laboratory tools consisting of a glass funnel wrapped with a parafilm membrane and filled with the phagostimulatory solution. Many phagostimulatory solutions were examined and only those containing sodium chloride and arginine were accepted by leeches. It was found that 71% of the tested leeches continued sucking for 15-45 min and 86% of them experienced 1-5 folds increments in body weight. For collection of a high quality and quantity LSE, we starved leeches for three weeks. A new method for LSE collection was described. The fed leeches were forced to regurgitate whatever they sucked by immersing them in ice containers. By using this method, leeches stayed alive and regain their activity after 15-30 min. The maximum concentration (A_{280} = 0.342) was provided within the first five minutes of sucking action. Gel electrophoresis (SDS-PAGE and Tricine-SDS-PAGE) methods revealed the existence of various peptides and proteins with molecular weights ranging from 2.3-250 kDa. Amidolytic activity assay showed that LSE inhibited thrombin-induced release of p-nitroanilide from the synthetic substrate S-2238 with IC_{50} of 49.391±2.219 µg/ml. LSE was shown to prolong thrombin time *in vitro*. The maximum antithrombin activity was obtained during the dry season (IC_{100} =16.081±0.079 µg/ml). A longer starvation period yielded a lower antithrombin activity. An optimization of lyophilization conditions revealed that pre-freezing at -20°C and a lyophilization cycle of 24 hours produced a dried leech saliva extract with antithrombin activity similar to that of the fresh extract. Findings also showed that the best stability was achieved when the lyophilized leech saliva extract was stored at -20°C in a dark place and glass tubes with loss of activity less than 10% during the study period of six months. Leech saliva showed an antibacterial activity against *Sal. typhi* and *S. aureus* with MIC of 78.353 µg/ml and against *E. coli* with MIC of 119.691 µg/ml. Disc diffusion test showed that leech saliva extracted from 16-week starved leeches exhibited zones of inhibition of 22 and 25 mm against *Sal. typhi* and *E. coli.* Starvation period was found to have a serious impact on the antimicrobial activity of leech saliva. No activity was observed against the fungal strains, *C. albicans* and *C. neoformans.* Dose responsive curve of the DPPH free radical scavenging method showed an antioxidant activity of LSE with IC_{50} of 7.282 µg/ml. The antidiabetic activity of subcutaneously injection of LSE (500 or 1000 µg/kg b.w) significantly reduced blood glucose in alloxan-induced diabetic rats. LSE at the mentioned doses had no toxic events. The concurrent injection of LSE (250 µg/kg b.w) and insulin (10 units/kg b.w) led to a good control in the glycemic states of the experimental rats with an instant decrease in blood glucose of 80-90% during the whole period of the study. Injection of LSE (250 µg/kg b.w) one hour prior to alloxanisation (160 mg/kg b.w) prevented diabetes induction in the experimental rats. Nevertheless, double doses of alloxan produced mild diabetic states. Leech saliva extract exhibited a cytotoxic activity against small cell lung cancer (SW1271 cell line) with IC_{50} of 119.844 µg/ml compared to the IC_{50} values of two reference standard drugs, Irinotecan (5.813 µg/ml) and Carboplatin (18.754 µg/ml). LSE reduced the IC_{50} of Carboplatin and Irinotecan by 65% and 11.5% respectively. Carboplatin reduced the IC_{50} of LSE by 4.6%. Irinotecan was found to decline IC_{50} of LSE by 57%.

خلاصة البحث

تعتبر دودة العلق من الأدوات الطبية المستخدمة لعلاج الكثير من الأمراض البشرية منذ أقدم العصور وذلك لاحتواء لعابها على عدد كبير من المركبات الفعالة حيوياً, نذكر منها بشكل خاص البروتينات والببتيدات المضادة للتخثر. وقد احتل العلق حديثاً مكانة هامة في الجراحة التجميلية. تعيش دودة العلق في المناطق الدافئة والرطبة. يتألف جسمها من حلقات متتالية بالإضافة إلي محجمين أمامي (يستخدم لامتصاص الدم والتثبت على جلد الفريسة) وخلفي (يستخدم للتثبت والحركة). تتناول الأطروحة دراسة بعض التأثيرات العلاجية للعاب العلق الطبي الماليزي. لاستخلاص اللعاب من العلق بطريقة سهلة وكميات معتبرة تم تطوير جهاز جديد لتغذية العلق مؤلف من ورق البارافلم وقمع زجاجي مملوء بالسائل المغذي والذي تبين أنه يجب أن يحتوي على ملح كلور الصوديوم وحمض الأرجينين لكي يصبح غذاء مناسب للعلق. وجدنا خلال البحث أن 70% من العلق استمر بعملية الامتصاص مدة تتراوح 15-45 دقيقة وأن 81% منهم أظهر 1-5 أضعاف زيادة في الوزن بعد الامتصاص. لاستخلاص كميات معتبرة من اللعاب ذات نوعية عالية, تم تجويع العلق لفترة لا تقل عن ثلاث أسابيع. لأول مرة, تم استحداث طريقة علمية وآمنة للحصول على لعاب العلق عن طريق وضعهم في حمام ثلجي لإجبار العلق على الاستفراغ وهذه التقنية تستخدم لأول مرة في هذا البحث. تبين من خلال التجربة أن أكبر تركيز لمحتويات لعاب العلق تم الحصول عليه بعد 2-5 دقائق من بدء الامتصاص. أظهرت نتائج الرحلان الكهربائي للعاب العلق وجود عدد كبير من البروتينات والببتيدات ذات وزن جزيئي يتراوح من من 2.3-250 كيلو دالتون. بعد فحص الفعالية الحالة للرابطة الببتيدية, تبين أن لعاب العلق قد ثبط قدرة الثرومبين على تحرير بارانتروانيليد من الركيزة الصنعية وذلك عند تراكيز 2.219±49.391 ميكروغرام/مل. أظهرت النتائج أيضاً أن الفعالية العظمى للعاب العلق تم الحصول عليها خلال الفصل الجاف (0.079±16.081 ميكروغرام/مل) وأن فترات التجويع الطويلة أعطت لعاب ذو فعالية ضعيفة (0.553±45.371 ميكروغرام/مل بعد 26 أسبوع). أظهرت النتائج أيضاً أن لعاب العلق يجب ان يجمد في دراجة حرارة -20 درجة مئوية وأن يجفد لفترة لاتزيد عن 24 ساعة. فيما يتعلق بثباتية الخلاصة, تبين أن أفضل فعالية تم الحصول عليها عند حفظ العلق في درجة حرارة -20 درجة مئوية في إناء زجاجي بعيداً عن الضوء. كما تبين ان لعاب العلق يمتلك خواصاً مضادة لبعض السلالات الجرثومية كالسلمونيلا والزوائف الزنجارية (التركيز الأدنى الفعال 78.353 ميكروغرام/مل) والجراثيم القولونية (119.691 ميكروغرام/مل). وقد أثرت فترة التجويع على المكونات الفعالة في لعاب العلق حيت تبين أن اللعاب المستخلص بعد 26 أسبوع لا يمتلك خواصاً مضادة للجراثيم. وبالمقابل لم يظهر لعاب العلق أي فعالية تجاه السلالات الفطرة المستخدمة في التجربة. تم اختبار الفعالية المضادة للجذور الحرة عن طريق استخدام مولد للجذور الحرة, ووجد أن لعاب العلق يمتلك خواصاً مضادة للأكسدة عند تراكيز 7.282 ميكروغرام/مل. وللكشف عن فعالية لعاب العلق في مرض السكري تم حقن لعاب العلق في حيوانات تجربة أحدث فيها مرض السكري من النمط الاول مخبرياً بجرعات 500-1000 ميكروغرام/كغ تحت الجلد, فتبين أن له خواصاً خافضة لسكر الدم بدون تأثيرات سمية ملحوظة. وقد وجد أيضاً أن الإعطاء المتزامن للعاب العلق بجرعة 250 ميكروغرام/كغ تحت الجلد مع الأنسولين بجرعة 10 وحدة دولية/كغ تحت الجلد احدث ضبطاً جيداً لسكر الدم بانخفاض يتراوح من 80-90%. كما أن حقن لعاب العلق بجرعة 250 ميكروغرام/كغ تحت الجلد قبل حقن الألوكسان بجرعة 160 مغ/كغ في الجوف البريتواني قد منع حدوث مرض السكري عند حيوانات التجربة. أظهرت الدراسة ان لعاب العلق يمتلك تأثيراً قاتلاً للخلايا الصغيرة لسرطان الرئة عند تراكيز قاتلة لنصف الخلايا 119.844 ميكروغرام/مل. كما تبين أن لعاب العلق استطاع أن يخفض الجرعة الفعالة للكاربوبلاتين و الأيرينوتكان بمقدار 65% و 11.5% على التوالي. مما سبق يمكن القول أن لعاب العلق يمتلك خواصاً طبية علاجية ذات أهمية بالغة في علاج بعض الأمرض المزمنة والمهددة للحياة, لذا نقترح بإجراء دراسات مكثفة أكثر على لعاب العلق لاستخلاص المركبات الفعالة وتحديد بنيتها وخواصها العلاجية بدقة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Chemistry.

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Supervisor

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Co-Supervisor

I certify that I have read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Chemistry.

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Examiner

This thesis was submitted to the Department of Pharmaceutical Chemistry and is accepted as a fulfilment of the requirement for the degree of Master of Pharmaceutical Chemistry.

……..…….....….

Head of Department
Pharmaceutical Chemistry

This thesis was submitted to the Kulliyyah of Science and is accepted as a fulfilment of the requirement for the degree of Master of Pharmaceutical Chemistry.

……..…….....…….

Dean, Kulliyyah of Pharmacy

DECLARATION

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.

Abdualrahman M.Abdualkader

Signature……. Date..……

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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Affirmed by Abdualrahman M.Abdualkader

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Signature Date

To my beloved and respected family, thank you all for your kind support love and care. Special thank to you dear great mum, 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. Islamic motivations, and all such beautiful words that always come out of your mouths 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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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	antimicrobial peptide
AMPs	antimicrobial peptides
AMR	resistance to antimicrobial agent
APS	ammonium persulfate
ATIII	antithrombin III
b.w	body weight
BSA	bovine serum albumin
CAT	catalase
CFU	colony forming unit
CGM	complete growth media
CHD	coronary heart disease
CSII	continuous subcutaneous insulin infusion
CVDs	cardiovascular diseases
DM	diabetes mellitus
DTIs	direct thrombin inhibitors
DVT	deep venous thrombosis
FBG	Fasting blood glucose
FBS	fetal bovine serum
FDA	Food and Drug Administration
FIIa	activated coagulation Factor II (thrombin)
FXa	factor Xa
GLM	general linear model
GPx	glutathione dismutase
GSH	glutathione
5-HT	serotonin
HIT	heparin-induced thrombocytopenia
HMWH	high molecular weight heparin
i.p	intraperitoneal
IDDM	insulin-dependent diabetes mellitus
IDTIs	idirect thrombin inhibitors
IGT	impaired glucose tolerance
LMWh	low molecular weight heparins
LPS	lipopolysaccharides
10×LSE	ten times concentrated leech saliva extract
3×LSE	three times concentrated leech saliva extract
LSE	leech saliva extract
LSE-P/P	leech saliva extract peptides and proteins
MeOH	methanol
MHA	Mueller-Hinton agar

MHB	Mueller-Hinton broth
MIC	minimal inhibitory concentration
MIDD	maternally inherited diabetes and deafness
MODY	maturity-onset of diabetes of the young
NIDDM	insulin-idependent diabetes mellitus
OD	optical density
OGTT	oral glucose tolerance test
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PBS-BSA	bovine serum albumin- phosphate buffer saline
PDA	potato dextrose agar
PDB	potato dextrose broth
pen/strep	penicillin/streptomycin
10×PhS	ten times concentrated phagostimulatory solution
PhS	phagostimulatory solution
PhS1	phagostimulatory solution of 0.001M arginine in normal saline
PhSs	phagostimulatory solutions
PPAR-γ	peroxisome proliferator-activated receptor-gamma
R_f	mobility index
ROS	reactive oxygen species
s.c	subcutaneously/subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- Polyacrylamide Gel electrophoresis
SEM	standard error of the mean
SOD	superoxide dismutase
SPSS	statisitical Program for Social Sciences
T_{eut}	eutectic melting temperature
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TEMD	N,N,N´,N´-tetramethylethylenediamine
TFPI	tissue factor pathway inhibitor
T_g	glass transition temperature
TT	thrombin time
%TT	percentage increase in thrombin time
Tzds	thiazolidinediones
UFH	unfractionated heparin
vWF	von Will brand factor
WHO	World Health Organisation
β-ME	β-mercaptoethanol