

VIRTUAL SCREENING AND ENZYMATIC
INHIBITION ANALYSIS OF BACTERIALLY
EXPRESSED LACTATE DEHYDROGENASE FROM
PLASMODIUM KNOWLESI FOR ANTIMALARIAL
DRUGS DEVELOPMENT

BY

NURHAINIS BINTI OGU SALIM

A thesis submitted in fulfilment of the requirement for the
degree of Master of Science (Biotechnology Engineering).

Kulliyyah of Engineering
International Islamic University Malaysia

SEPTEMBER 2021

ABSTRACT

Malaria is a mosquito-borne tropical disease caused by parasite of the Plasmodium genus. Globally, about 229 million cases of malaria with approximately 409 000 mortalities occurred in 2019. In Malaysia, *Plasmodium knowlesi* has now become the most common cause of malaria in the country. Disease management remains challenging due to malaria parasites resistance towards the current antimalarial agents, which is one of the factors that prevent the elimination of malaria. Hence, new drugs to treat malaria specifically caused by *P. knowlesi* should be a priority in malaria research. Selection of antimalarial candidates via virtual screening, which specifically targets enzymes such as lactate dehydrogenase (LDH) in glycolytic pathway of Plasmodium is a good strategy because it is the sole energy producer during erythrocytic cycle of the parasites. The aim of this study is to screen for novel antimalarial candidates *via virtual* screening and to evaluate the effects of the selected virtually screened compounds on the activity of LDH by inhibition studies. The potential compounds were screened *via* computational approach comprising Ligand-Based Drug Design using Ultra-Fast Shape Recognition with Atom Types (UFSRAT) and Ultra-Fast Shape Recognition with CREDO Atom Types (USRCAT), followed by structure-based drug design using Autodock4 programme. UFSRAT have resulted with similarity scores ranged from 0.832-0.914 for compounds that most resemble oxamate, a known LDH inhibitor. Meanwhile, USRCAT similarity scores for compounds that most resemble pyruvate (substrate) and lactate (product), ranged from 0.859 to 0.882 and 0.822 to 0.849, respectively. Structure-based drug design for analogues of oxamate, pyruvate and lactate have resulted with minimum binding energies ranged from -3.59 kcal/mol to -0.07 kcal/mol, -5.25 kcal/mol to -1.99 kcal/mol and -3.74 kcal/mol to -2.81 kcal/mol respectively. Meanwhile, cloning of *P.knowlesi* lactate dehydrogenase (*Pk*-LDH) gene into expression vector (pET21a) was performed, prior to protein expression in bacterial system. SDS-PAGE analyses revealed that a fusion protein of ~34 kDa was present in soluble fraction, which confirmed size of the bacterially-expressed *Pk*-LDH and then was subsequently purified to homogeneity using Immobilized Metal Ion Affinity Chromatography (IMAC) and Size Exclusion Chromatography (SEC). Protein characterization was performed to verify the identity of purified proteins using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry, results confirmed that bacterially-expressed *Pk*-LDH obtained in this study is similar to *P. knowlesi* LDH with 282 protein sequence coverage. The structural properties of *Pk*-LDH protein were investigated by using far UV Circular Dichroism (CD) Spectroscopy, where proper folding and correct disulfide linkages in the pure *Pk*-LDH enzyme were verified. The specific activity of the purified *Pk*-LDH enzyme was found to be 475.6 U/mg, confirming the presence of pure and active protein. Finally, selected compounds obtained from *in silico* screening were tested on purified *Pk*-LDH *via* enzymatic inhibition assay and the compound namely oxalic acid has shown 54.12% of inhibition with an IC₅₀ value of 0.6398 mM, the most potent compared to other compounds. This study has therefore resulted in the development of a reliable method for producing soluble *Pk*-LDH that is biologically-active and leads to further exploration of the potential compounds that can be developed as promising antimalarial drugs, specifically to treat malaria infections caused by *P. knowlesi*.

خلاصة البحث

الملاريا مرض استوائي ينقله البعوض ويسببه طفيلي من جنس المتصورة. على الصعيد العالمي، حدثت حوالي 229 مليون حالة إصابة بالملاريا مع ما يقرب من 409,000 حالة وفاة في عام 2019م. وفي ماليزيا، أصبحت المتصورة النولسية الآن السبب الأكثر شيوعًا للملاريا في البلاد. لا تزال إدارة المرض صعبة بسبب مقاومة طفيليات الملاريا للعوامل المضادة للملاريا الحالية، والتي تعد أحد العوامل التي تمنع القضاء على الملاريا. ومن ثم، يجب أن تكون الأدوية الجديدة لعلاج الملاريا التي تسببها المتصورة النولسية على وجه التحديد أولوية في أبحاث الملاريا. يعد اختيار المواد المرشحة المضادة للملاريا عبر الفحص الافتراضي، والذي يستهدف على وجه التحديد الإنزيمات مثل اللاكتات ديهيدروجيناز (LDH) في مسار التحلل الجلدي للمتصورة، استراتيجية جيدة لأنه المنتج الوحيد للطاقة خلال دورة كريات الدم الحمراء للطفيليات. الهدف من هذه الدراسة هو الكشف عن مرشحات جديدة لمضادات الملاريا من خلال الفحص الافتراضي وتقييم تأثيرات المركبات المختارة عمليًا على نشاط LDH من خلال دراسات التثبيط. تم فحص المركبات المحتملة من خلال نذج حسابي يشتمل على تصميم دواء يعتمد على Ligand باستخدام التعرف على الشكل فائق السرعة مع أنواع الذرة (UFSRAT) والتعرف الفائق السرعة على الشكل باستخدام CREDO Atom Types (USRCAT)، متبوعًا بتصميم عقار قائم على الهيكل باستخدام برنامج Autodock4. نتج عن UFSRAT درجات تشابه تراوحت بين 0.832-0.914 للمركبات التي تشبه الأكسامات إلى حد كبير، وهو مثبط LDH المعروف. وفي الوقت نفسه، تراوحت درجات تشابه USRCAT للمركبات التي تشبه البيروفات (الركيزة) واللاكتات (المنتج) من 0.859 إلى 0.882 و 0.822 إلى 0.849 على التوالي. نتج عن تصميم الأدوية المعتمد على الهيكل لنظائر الأكسامات والبيروفات واللاكتات طاقات الربط الدنيا تتراوح من -3.59 كيلو كالوري / مول إلى -0.07 كيلو كالوري / مول، و -5.25 كيلو كالوري / مول إلى -1.99 كيلو كالوري / مول و -3.74 كيلو كالوري / مول إلى -2.81 كيلو كالوري / مول على التوالي. وفي الوقت نفسه، تم إجراء استنساخ جين *P.knowlesi* lactate dehydrogenase (*Pk-LDH*) إلى ناقل التعبير (pET21a)، قبل التعبير عن البروتين في النظام البكتيري. كشفت تحليلات SDS-PAGE أن بروتينًا اندماجياً يبلغ تقريبًا 34 كيلو دالتون كان موجودًا في جزء قابل للذوبان، مما أكد حجم *Pk-LDH* المعبر عنه بالبكتيريا ثم تمت تنقيته لاحقًا للتعانس باستخدام كروماتوجرافيا تقارب أيون معدني غير متحرك (IMAC) وكروماتوجرافيا استبعاد الحجم (SEC). تم إجراء توصيف البروتين للتحقق من هوية البروتينات المنقاة باستخدام مطياف كتلة التأين لوقت الطيران بمساعدة المصفوفة (MALDI-TOF)، وأكدت النتائج أن *Pk-LDH* المعبر عنه بالبكتيريا والذي تم الحصول عليه في هذه الدراسة يشبه *P.knowlesi* LDH مع 282 تغطية تسلسل بروتين. تم فحص الخواص الهيكلية لبروتين *Pk-LDH* باستخدام التحليل الطيفي ثنائي اللون الدائري (CD) للأشعة فوق البنفسجية البعيدة، حيث تم التحقق من الروابط الصحيحة للطي والثاني كبريتيد في إنزيم *Pk-LDH* النقي. وجد أن النشاط النوعي لإنزيم *Pk-LDH* المنقى هو 475.6 mg / U، مما يؤكد وجود البروتين النقي والنشط. أخيرًا، تم اختبار المركبات المختارة التي تم الحصول عليها من الفرز السيليكو على *Pk-LDH* المنقى عن طريق مقايسة التثبيط الإنزيمي وأظهر المركب وهو حمض الأكساليك 54.12% من التثبيط بقيمة IC50 تبلغ 0.6398 ملي مولار، وهو الأكثر فعالية مقارنة بالمركبات الأخرى. وبالتالي، أدت هذه الدراسة إلى تطوير طريقة موثوقة لإنتاج *Pk-LDH* القابل للذوبان والذي يكون نشطًا بيولوجيًا ويؤدي إلى مزيد من الاستكشاف للمركبات المحتملة التي يمكن تطويرها كأدوية مضادة للملاريا الواعدة، خاصة لعلاج عدوى الملاريا التي تسببها المتصورة النولسية.

APPROVAL PAGE

I certify that I have supervised and read this study and 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 Science (Biotechnology Engineering)

Fazia Adyani

.....
Fazia Adyani binti Ahmad Fuad
Supervisor

.....
Azura binti Amid
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 Science (Biotechnology Engineering)

.....
Azzmer Azzar bin Abdul Hamid
Internal Examiner

.....
Siti Hajar Noor Shaarani
External Examiner

This thesis was submitted to the Department of Biotechnology Engineering and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology Engineering)

.....
Mohammed Saedi Jami
Head, Department of
Biotechnology Engineering

This thesis was submitted to the Kulliyah of Engineering and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology Engineering)

.....
Sany Izan Ihsan
Dean, Kulliyah of Engineering

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.

Nurhainis binti Ogu Salim

Signature  Date 7 September 2021

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF
FAIR USE OF UNPUBLISHED RESEARCH**

**VIRTUAL SCREENING AND ENZYMATIC INHIBITION
ANALYSIS OF RECOMBINANT *PLASMODIUM KNOWLESI*
LACTATE DEHYDROGENASE FOR ANTIMALARIAL DRUGS
DEVELOPMENT**

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

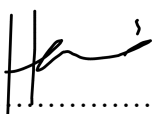
Copyright © 2021 Nurhainis binti Ogu Salim and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.
2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.
3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

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

Affirmed by Nurhainis binti Ogu Salim


.....
Signature

7 September 2021
.....
Date



ACKNOWLEDGEMENT

All glory is due to Allah, the Almighty, whose Grace and Mercies have been with me throughout the duration of my study. His Mercies and Blessings on me ease the enormous task of finishing this thesis.

I am most thankful to my supervisor, Asst. Prof. Dr. Fazia Adyani binti Ahmad Fuad, whose continuing compassion, diligence and friendship have facilitated the successful accomplishment of my work. I put on highest appreciation on her comprehensive remarks and inspiring queries which have significantly enhanced this thesis. Her brilliant grasp of the aim and content of this work led to her comments, ideas and queries which helped me a great deal. Despite her commitments, she took time to listen and attend to me whenever requested. The good support she gave me is a boost that helped me in structure and inscription the draft of this research work. I am also thankful to my co-supervisor, Prof. Dr. Azura binti Amid, whose provision and assistance contributed to the outcome of this work.

Lastly, my appreciation goes to my beloved parents, family, friends and colleagues in my workplace in Parasitology Unit, Institute for Medical Research especially to my Head of Unit, Dr. Shamilah Hisam, my colleagues Ms Adela Ida Jiram and Dr. Noor Azian Md Yusuf; for their prayers, understanding and endurance while I am working on this project. Thank you all.

Alhamdulillah.

TABLE OF CONTENTS

Abstract	ii
Abstract Arabic	iii
Approval Page.....	iv
Declaration	v
Copyright	vi
Acknowledgement	viii
Table of Contents	ix
List of Tables	xii
List of Figures	xiv
List of Equations	xvi
List of Symbols	xvii
List of Abbreviations	xviii
CHAPTER ONE: INTRODUCTION	1
1.1 Study Background.....	1
1.2 Problem Statement	4
1.3 Research Objective	7
1.4 Scope of Study	7
1.5 Thesis Organization	8
CHAPTER TWO: LITERATURE REVIEW	10
2.1 Introduction.....	10
2.2 Malaria	13
2.2.1 Malaria in the World	15
2.2.2 Malaria in Malaysia.....	17
2.2.3 <i>Plasmodium knowlesi</i>	18
2.3 Malaria Treatment.....	19
2.3.1 Current Antimalarial Drugs.....	19
2.3.2 Drug Resistance Issues.....	21
2.4 Drug Target for Treatment of Malaria	26
2.4.1 Metabolic Pathways in <i>Plasmodium</i> as Drug Targets.....	26
2.4.2 Glycolytic Pathway of Plasmodium.....	30
2.4.3 Enzymes in Glycolytic Pathways.....	32
2.4.4 Lactate Dehydrogenase	34
2.5 Inhibitors of Enzymes	37
2.5.1 Inhibitors of Lactate Dehydrogenase	37
2.6 <i>In Silico</i> Drug Design.....	39
2.6.1 Ligand-Based Drug Design (LBDD)	45
2.6.2 Structure-Based Drug Design (SBDD)	48
2.7 Inhibition Studies	52
2.8 Summary	52

CHAPTER THREE: RESEARCH METHODOLOGY	54
3.1	Introduction..... 54
3.2	Flow Chart 57
3.3	Chemicals And Reagents 58
3.4	Softwares And Websites For <i>In Silico</i> Studies 59
3.5	<i>In Silico</i> Screening 60
3.5.1	Ligand-Based Drug Design..... 60
3.5.2	Compound Toxicity Test..... 63
3.5.3	Sequence Alignment Of Lactate Dehydrogenase From <i>P. Knowlesi</i> With Lactate Dehydrogenase From Other <i>Plasmodium Spp.</i> 64
3.5.4	Physico-Chemical Characteristics Of <i>Pk-Ldh</i> And Analysis Of Secondary Structure Of <i>Pk-Ldh</i> 65
3.5.5	Modelling Of <i>Pk-Ldh</i> Structure 66
3.5.6	Structure Refinement Of <i>Pk-Ldh</i> Model..... 66
3.5.7	Verification And Validation Of The <i>Pk-Ldh</i> Model Structure 67
3.5.8	Structure-Based Drug Design 67
3.6	Construction Of Recombinant Plasmid <i>Pk-Ldh</i> 70
3.6.1	Amplification Of <i>Pk-Ldh</i> Gene..... 70
3.6.2	Ligation 71
3.6.3	Transformation Of Recombinant <i>Pk-Ldh</i> Gene Into <i>E. Coli</i> B121 (De3) 71
3.7	Development Of Expression Of <i>Pk-Ldh</i> In Bacterial System 72
3.7.1	Optimization Of Expression Condition For Recombinant <i>Pk-Ldh</i> 72
3.7.2	Analysis By Sds-Page 73
3.7.3	Large-Scale Protein Expression 75
3.7.4	Cell Lysis 75
3.8	Purification Of Recombinant <i>Pk-Ldh</i> 76
3.8.1	First Step: Immobilised Metal Affinity Chromatography (Imac)..... 76
3.8.2	Second Step: Size Exclusion Chromatography (Sec) 77
3.9	Protein Concentration And Quantitation 78
3.10	Protein Determination And Characterisation 78
3.10.1	Matrix-Assisted Laser Desorption Ionisation-Time Of Flight Mass Spectrophotometry (Maldi-Tof) 78
3.10.2	Circular Dichroisms..... 79
3.11	<i>Pk-Ldh</i> Enzymatic Assay..... 79
3.12	Enzymatic Inhibition Assay 80
3.12.1	Inhibition Analysis..... 80
3.13	Summary 81
CHAPTER FOUR: RESULT AND DISCUSSION	83
4.1	Introduction..... 83
4.2	<i>In Silico</i> Screening 84
4.2.1	Ligand-Based Drug Design (Lbdd)..... 84
4.2.1.1	Identification Of Small Molecule Analogues Of Oxamate By Ufsrat 84

4.2.1.2	Identification Of Small Molecule Analogues Of Pyruvate And Lactate By Usrcat.....	86
4.2.2	Toxicity Estimation Test	91
4.2.3	Physico-Chemical Characteristics And Secondary Structure Of <i>Pk-Ldh</i> Protein	96
4.2.4	Comparisons Of <i>Pk-Ldh</i> With Lactate Dehydrogenase From Other Malaria Parasites And Sequence Alignment Analysis	99
4.2.5	Generation Of <i>Pk-Ldh</i> Protein Structure Model	101
4.2.6	Predicted Model Refinement, Validation Of <i>Pk-Ldh</i> Model And Identification Of Protein Binding Site	102
4.2.7	Molecular Docking For Structure-Based Drug Design.....	107
4.2.7.1	Validation Of Docking Procedure.....	107
4.2.7.2	Molecular Docking For Compounds Obtained From Ufsrat And Usrcat.....	110
4.3	Cloning Of <i>Pk-Ldh</i> Into Pet21a Expression System.....	120
4.3.1	Gene Sequence Of Lactate Dehydrogenase (Ldh) From <i>P. Knowlesi</i>	120
4.3.2	Sequence Analysis Of Synthetic <i>Pk-Ldh</i>	120
4.3.3	Cloning Of <i>Pk-Ldh</i> Into <i>E. Coli</i> Expression System	121
4.4	Expression Of Recombinant <i>Pk-Ldh</i> In <i>E. Coli</i> BL21	(De3) 127
4.4.1	Determination Of Optimum Parameters For Protein Expression	127
4.5	Purification Of Recombinant <i>Pk-Ldh</i>	130
4.6	Protein Determination And Characterization.....	133
4.6.1	Determination Of Protein Concentration And Quantitation	133
4.6.2	Ldh Enzymatic Assay And Kinetics	134
4.6.3	Matrix-Assisted Laser Desorption Ionisation-Time Of Flight Mass (Maldi-Tof) Of Bacterially-Expressed <i>Pk-Ldh</i>	138
4.6.4	Circular Dichroisms Of Bacterially-Expressed <i>Pk-Ldh</i>	139
4.7	Inhibition Of <i>Pk-Ldh</i> Enzyme Activity By Compound O4, O6, P4 And L3	140
4.7.1	Ic ₅₀ For Compound O4.....	143
4.8	Summary	144
CHAPTER FIVE: CONCLUSIONS.....		146
REFERENCES.....		150
APPENDICES.....		171
	Appendix I List of Equipments and Materials	171
	Appendix Ii Experimental Work.....	172
	Appendix Iii Articles.....	183
	Appendix Iv: Gantt Chart.....	185

LIST OF TABLES

Table 2.1:	Drugs for treating malaria infection according to the species. Table adapted from Management Guidelines for Malaria in Malaysia by Ministry of Health, Malaysia.	20
Table 2.2:	Specific regions where antimalaria drug resistance has been reported with their respective molecular markers.	25
Table 2.3:	Plasmodial Enzymes in Metabolic Pathways as Therapeutic Targets.	27
Table 2.4:	List of research related to <i>Pk</i> -LDH since 1985 until 2019.	36
Table 2.5:	Implementation of SBDD and LBDD approaches to find new inhibitors in malaria drug discovery	44
Table 3.1:	List of softwares and websites used in this study for <i>in silico</i> screening.	59
Table 3.2:	Forward and reverse primers used in PCR amplification.	71
Table 3.3:	The parameters used in the protein expression trial.	73
Table 4.1:	The similarity scores (S _{qc}) and 2D structure for compounds similar to Oxamate obtained from UFSRAT.	86
Table 4.2:	The similarity scores (S _{qc}) and 2D structure for compounds similar to pyruvate obtained from USRCAT.	89
Table 4.3:	The similarity scores (S _{qc}) and 2D structure for compounds similar to lactate obtained from USRCAT.(Shave et al., 2015)	90
Table 4.4:	Estimation of toxicity values for compounds similar to oxamate using Quantitative Structure Activity Relationships (Hierarchical method)	93
Table 4.5:	Estimation of toxicity values for compounds similar to pyruvate using Quantitative Structure Activity Relationships (Hierarchical method)	94
Table 4.6:	Estimation of toxicity values for compounds similar to lactate using Quantitative Structure Activity Relationships (Hierarchical method)	95
Table 4.7:	The primary structure analysis of <i>Pk</i> -LDH	98
Table 4.8:	The secondary structure analysis of the sequence of <i>Pk</i> -LDH	99

Table 4.9:	Amino acid sequence alignment of <i>Pk</i> -LDH gene with other parasites LDH.	101
Table 4.10:	Binding energy from structure-based drug design and physicochemical and pharmacophore properties for all compounds.	114
Table 4.11:	The optimized expression conditions for <i>Pk</i> -LDH	129
Table 4.12:	Protein concentration for each sample	134
Table 4.13:	The purification profile of the protein obtained through IMAC and SEC.	136



LIST OF FIGURES

Figure 2.1:	The life cycle of malarial parasites, <i>Plasmodium spp.</i>	14
Figure 2.2:	Incidence of Malaria.16	16
Figure 2.3:	Annual reported malaria cases and deaths in Malaysia 2000 2018.	18
Figure 2.4:	Greater Mekong Subregion.	23
Figure 2.5:	General overview of metabolism pathways and potential target for novel inhibitor in <i>Plasmodium spp.</i>	30
Figure 2.6:	The glycolytic pathway, which is the metabolic pathway that converts glucose into pyruvate.	31
Figure 2.7:	Drug design and discovery workflow starting from target identification until drug approval.	40
Figure 2.8:	Virtual screening workflow.	41
Figure 2.9:	Ligand-based virtual screening methods.	46
Figure 2.10:	Docking and scoring in structure-based drug design.	49
Figure 3.1:	The flow chart of experiment conducted in this study.	57
Figure 4.1:	The query molecule which were used in UFSRAT.	85
Figure 4.2:	The query molecules which were used in USRCAT.	88
Figure 4.3:	Overlay of the model of <i>Pk</i> -LDH based on the crystal structure of <i>Pf</i> -LDH.	102
Figure 4.4:	Results from verified 3D.	103
Figure 4.5:	ERRAT interpreted the quality of <i>Pk</i> -LDH model with 92.2% score.	103
Figure 4.6:	Ramachandran Plot analysis of <i>Pk</i> -LDH.	105
Figure 4.7:	Modelled <i>Pk</i> -LDH structure with active site (red) selected for docking.	106
Figure 4.8:	Crystal structure of <i>Pf</i> -LDH-Oxamate complex.	109

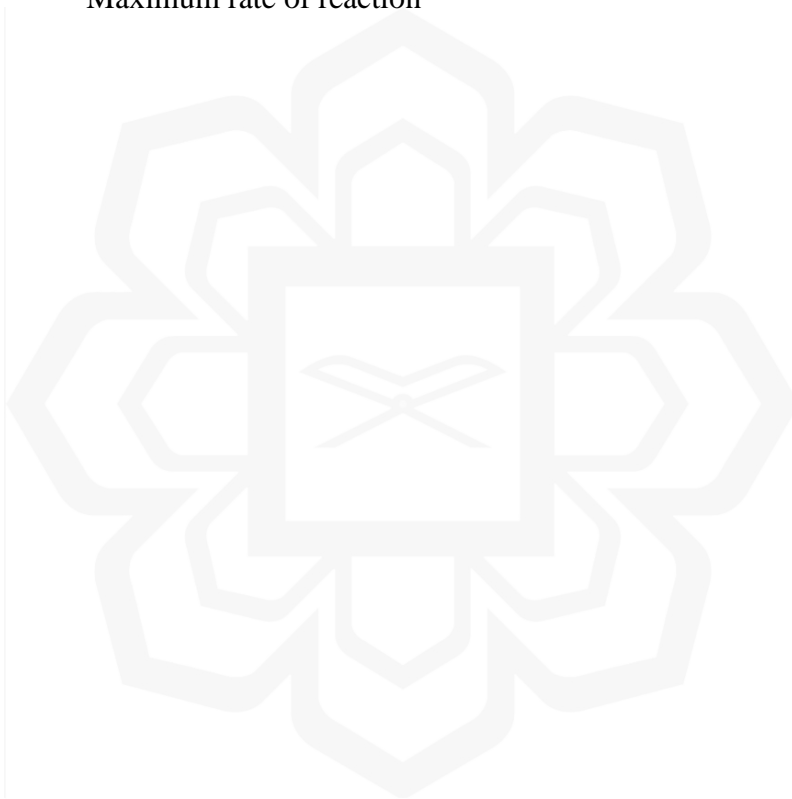
Figure 4.9:	Docking analysis of compound O4.	118
Figure 4.10:	The gene sequence of <i>Pk</i> -LDH.	120
Figure 4.11:	Amplification of <i>Pk</i> -LDH gene by PCR from pUC57 vector using GoTaq DNA Polymerase (Promega).	121
Figure 4.12:	<i>Pk</i> -LDH_pET21a map show the <i>Pk</i> -LDH gene insert enzyme restriction sites and digestion of PCR product (<i>Pk</i> -LDH gene) and expression vector pET21a	123
Figure 4.13:	Verification of transformants by PCR colony.	124
Figure 4.14:	Verification of transformants by restriction enzymes analysis.	125
Figure 4.15:	Verification of transformant by DNA sequencing.	126
Figure 4.16:	SDS page images of the first trial of protein expression	128
Figure 4.17:	SDS page images of the second batch of protein expression trial.	128
Figure 4.18:	The elution profile of <i>Pk</i> -LDH from on 5 ml IMAC HisTrap HP column.	132
Figure 4.19:	The elution profile of <i>Pk</i> -LDH protein showed on gel filtration Superdex TM 200 GL 10/300 column	132
Figure 4.20:	The purified protein based on 12% SDS-PAGE analysis.	133
Figure 4.21:	Michaelis Menten Plot <i>Pk</i> -LDH with pyruvate as substrate and Lineweaver and Burk Plot for <i>Pk</i> -LDH	137
Figure 4.22:	Diagram of CD spectrometry far-UV analysis.	140
Figure 4.23:	Specific activity of <i>Pk</i> -LDH in the presence of compounds	142
Figure 4.24:	The IC ₅₀ value of compound O4.	144

LIST OF EQUATIONS

Equation 3.1	USRCAT Equation	61
Equation 3.2	UFSRAT Equation	62
Equation 3.3	<i>P</i> <i>k</i> -LDH Activity Equation	80
Equation 3.4	Specific Activity Equation	80
Equation 3.5	Linear Equation	81
Equation 4.1	Extinction Coefficient Equation	96
Equation 4.2	Protein Absorbance Equation	97
Equation 4.3	Instability Index Equation	97
Equation 4.4	Aliphatic Index Equation	98
Equation 4.5	AutoDock Scoring Function Terms	107
Equation 4.6	AutoDock Scoring Function	107
Equation 4.7	Protein Concentration Determination	133
Equation 4.8	Michaelis Menten Equation	134
Equation 4.9	Lineweaver-Burk Equation	135

LIST OF SYMBOLS

K_m	Michaelis constant
s	Substrate concentration
U	Units
v	Reaction rate
V_{max}	Maximum rate of reaction



LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AL	Artemether-lumefantrine
ATP	Adenosine triphosphate
APS	Ammonium Persulfate
CADD	Computer aided drug design
CAS	Chemical Abstracts Service
CD	Circular Dichroisms
CGI	Common gateway interface
cDNA	Complementary DNA
CV	Column volumes
DHFR	Dihydrofolate reductase
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDULISS	EDinburgh University Ligand Selection System
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G3P	Glyceraldehyde-3-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GRAVY	Grand average hydropathicity
hGAPDH	Human Glyceraldehyde 3-phosphate dehydrogenase
HTS	High Throughput screening
IC ₅₀	50% inhibitory concentration
IMAC	Immobilised Metal Affinity Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K13-propeller	Kelch propeller
LB	Luria Bertani
LBDD	Ligand-based drug design
LDH	Lactate dehydrogenase
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation-Time of Flight
MDL	Molecular Design Limited
MgCl ₂	Magnesium Chloride
MVV	Medicine for Malaria Ventures
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NaCl	Sodium Chloride
NaH ₂ PO ₄	Monosodium phosphate
NCBI	National Center for Biotechnology Information
OXM	Oxamate
QSAR	Quantitative Structure Activity Relationships
PCR	Polymerase chain reaction
PDB	Protein Database
<i>Pf</i> -LDH	<i>Plasmodium falciparum</i> lactate dehydrogenase
<i>Pk</i> -LDH	<i>Plasmodium knowlesi</i> lactate dehydrogenase
<i>p</i> LDH	<i>Plasmodium</i> lactate dehydrogenase

<i>Pfprt</i>	<i>P. falciparum</i> chloroquine resistance transporter
<i>PfK13</i>	<i>P. falciparum</i> Kelch-13 propeller domain
<i>PfGAPDH</i>	<i>P. falciparum</i> glyceraldehyde-3-phosphate dehydrogenase
<i>PfMDR1</i>	<i>P. falciparum</i> multidrug resistance 1
PSI	Protein-Specific Iterative
RBC	Red blood cells
RMSD	Root Mean Square Deviation
SAVES	Structure Analysis and Verification Server
SBDD	Structure-based drug design
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SOPMA	Self-Optimise Prediction Method with Alignment
SP	Sulphadoxine–pyrimethamine
TB	Terrific broth
TCP	Target Candidate Profile
TEMED	Tetramethylethylenediamine
TEST	Toxicity Estimation Software
UFSRAT	Ultra-Fast Shape Recognition with Atom Types
USR	Ultrafast Shape Recognition
USRCAT	Ultra-Fast Shape Recognition with Credo Atom Type
UV-VIS	Ultraviolet–visible spectrophotometry
WHO	World Health Organisation
1,3-BPG	1,3-biphosphoglycerate

CHAPTER ONE

INTRODUCTION

1.1 STUDY BACKGROUND

The World Malaria Report estimates an occurrence of 229 million cases in 2019 over the world (WHO, 2020). In Malaysia, a total of 4630 malaria cases was reported in 2018, in which 4131 cases were mainly caused by *Plasmodium knowlesi* infection and another 499 cases were caused by other Plasmodium species that are known to infect human, such as *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale* (Chin et al., 2020). Malaysia is located at the equatorial zone with high temperatures and humidity, which is deemed important for transmission of malaria, causing the disease to remain as a public health treat in Malaysia (Jamaiah et al., 2005). In most parts of Peninsular Malaysia, the disease is still under control, but the problem remains persistent in Sabah and Sarawak, where the disease occurred in areas such as in the aboriginal settlements and tribal villages that are mostly found in cleared hilly jungles. Deforestation and associated environmental and population changes have been hypothesized as main drivers of this apparent emergence (Fornace et al., 2016).

There are four species of malaria parasites that are normally associated with human, which are *P.falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. In Malaysia, there is a rise of zoonotic malaria infections among human caused by *P. knowlesi*, which is the species previously known to cause malaria in primates. The evidence of human infection was recorded in the discovery of naturally-acquired *P. knowlesi* infection among people in Kapit Division of Sarawak, Malaysia (Singh et al., 2004). *P. knowlesi* asexual erythrocytic cycle is about 24 hours in human, causing symptoms such as fever that typically occurs at the same frequency and can rapidly develop from febrile illness

into severe malaria, and sometime can lead to death in case of late treatment (Singh et al., 2013) . Severe malaria is life- threatening, due to vital organs dysfunction, for example debilitated lungs, kidneys, liver and brain (Carter & Mendis, 2002). In order to avoid severe illness or casualty, appropriate management of malaria is important.

The management of malaria includes diagnosis and treatment of the disease. The diagnosis of malaria depends upon the summarization of information on clinical observation, previous medical history and laboratory identification of malaria parasites. The treatment of malaria includes drug therapy given to malaria infected patients according to the standard treatment recommended by World Health Organisation (WHO). Artemisinin Combination Therapy (ACT) is the first line treatment for malaria to ease the symptoms, prevent lapse and spread of the disease. Drugs such as chloroquine, malarone, artemether-lumefantrine, mefloquine, quinine and quinidine are also included in treatment of malaria. These drugs are active against the parasites in the blood (CDC, 2015).

Currently, there is no specific drug targeting *P. knowlesi* infection in human. At the moment, the treatment for malaria infection in human that is caused by *P. knowlesi* is built upon drugs that are used to treat malaria infections by other *Plasmodium* species that infect human. Unfortunately, the current drug used is facing major difficulty, due to drug resistance issue, which has been reported in many parts of the world. The emergence of *P. falciparum* resistance towards artemisinin has been reported by WHO in 2013 in five countries near the Great Mekong River such as Cambodia, Laos, Myanmar, Thailand and Vietnam (WHO, 2013).

In Malaysia, there is no report regarding *Plasmodium* resistance towards artemisinin. However, drugs such as mefloquine has been reported to be less sensitive in a study of susceptibility towards antimalarials for malaria patients infected with *P.*

knowlesi, who sought for treatment at the hospitals in Sarawak. It is noteworthy to note that in Malaysia, approximately 70% of malaria cases occurred in Borneo Island, where drug resistance is an emerging problem (Fatih et al., 2013; Jamaiah et al., 2005). Therefore, persistent research for new, non-toxic and functional antimalarial drugs is vital to treat malaria in patients infected with *P. knowlesi*.

One of approaches to solve this issue is by finding substances that are able to target the source of energy for *Plasmodium*, such as glycolysis. *Plasmodium* obtained its energy during the intra-erythrocytic stage in mammalian host for its survival through glycolysis. For this reason, it was postulated that the glycolytic pathway is an ideal target for drug design against *Plasmodium*. In the glycolytic pathway, the final enzyme, which is lactate dehydrogenase (LDH) has been shown to be a potential immune genetic marker besides a good target for chemotherapy (Singh et al., 2012). The fact that *P. falciparum* LDH (*Pf*-LDH) and *P. knowlesi* LDH (*Pk*-LDH) are almost similar in terms of their gene sequences, it is postulated that by targeting that same enzyme in *P. knowlesi* may aid in the treatment of malaria patients and later prevents the spread of the disease.

As the final enzyme in glycolysis, LDH converts pyruvate to lactate, apart from converting NADH to NAD⁺ in the final stage of the pathway, hence providing energy for the parasite. Inhibition are defined by the binding affinity in which an inhibitor binds to the free form of the enzyme, or to the enzyme–substrate binary complex or to species that form subsequent to the enzyme–substrate complex along the reaction coordinate of the enzyme, or to both (Buker et al., 2019). Therefore, finding compound that have high binding affinity towards *Pk*-LDH enzyme, could lead to inhibition of the enzyme, hence, lead to parasites cell death It has been proven that compounds that compete with NADH for the active sites of LDH have potentials to inhibit the activity of the enzyme,

thus diminishing the parasites (Penna-Coutinho et al., 2011). Furthermore, the crystal structures of both human LDH and *Pf*-LDH showed two significant variances, which are the location of the NADH factor and the secondary structure of a loop region that closes down on the active site during catalysis (Cameron et al., 2004). No comparison study of human LDH and *Pk*-LDH was performed as the crystal structure of *Pk*-LDH is not yet available. The kinetic differences between human and *Pf*-LDH are very high that the observed LDH activity can be used as an indication of *in vivo* parasitaemia (Alam et al., 2014).

Due to distinctive structural and kinetic properties between human and the malaria parasites' enzymes, targeting LDH enzyme in *P. knowlesi* may aid in the treatment of malaria patients and later prevent the spread of the disease. The challenges deliberated above in fighting malaria due to infection with *P. knowlesi* emphasise the importance of developing novel antimalarial drugs, which may overcome the parasites' resistance issue. The focus of this work is to identify and validate potential compounds that specifically target LDH from *P. knowlesi* (*Pk*-LDH), which can further be developed as antimalarial drugs.

1.2 PROBLEM STATEMENT

Malaria was reported in 97 countries with ongoing malaria transmission. The severity of the disease is exhibited by the number of people developing the symptomatic disease, where in 2019, WHO estimates that 229 million cases occurred around the world and 1819 estimated deaths were recorded in South East Asia (WHO, 2020). According to Management Guideline of Malaria in Malaysia, the incidence rate of malaria in Malaysia over a decade period (1990-2000) ranges from 1.6 to 29.7 per 10,000 person at risk, in a declining trend. The actual number of malaria cases has dropped from nearly

60,000 cases in 1995 to 4725 cases in 2012. Overall, Sabah, Sarawak and Perak states remain stratified as “problematic” malaria areas. The objectives of the National Malaria Control Program are to reduce the national malaria incidence rate to less than 1 per 1,000 population by year 2015 for Peninsular Malaysia and by year 2017 for Sabah and Sarawak. One of the identified strategies to achieve the objective is to provide early case detection and early treatment.

In relation to malaria species, in 2010, *P. vivax* remains the most prevalent parasite species (56%), followed by *P. falciparum* (28%), *P. malariae* (7%) and mixed infections (3%). There is also 6% of malaria species identified as *P. knowlesi*. To date, almost half of malaria cases reported is due to *P. knowlesi* infection. Despite extensive studies in Malaysia on *P. knowlesi* in the 1960s, no additional reports appeared until 2004, when 120 cases of naturally-acquired *P. knowlesi* infection in humans were described in Malaysian Borneo (Singh et al. 2004). It was uncertain whether natural infection of *P. knowlesi* could take place and thus could be a zoonosis. The zoonotic potential of *P. knowlesi* has, until recently, seemed limited, with only sporadic case reports of human infection. Unfortunately, until now, no drugs have been introduced to specifically treat malaria due to *P. knowlesi* (knowlesi malaria) and an effective vaccine is still under development. Hence, drug development against knowlesi malaria is a great priority in managing malaria disease in this country.

Currently, the treatment for knowlesi malaria is based on drugs used to treat malaria caused by other human malaria parasites such as *P. falciparum* and *P. malariae*. Effective treatment of the disease is increasingly compromised by rising resistance of malaria parasites to currently available antimalarials (Turgut-balik et al., 2004). The treatment failure may well occur, and this is mainly due to other factors such as incorrect dosing, inaccuracy on the duration of treatment, misdiagnosis and poor drug quality.