UNIVERSITI PUTRA MALAYSIA

IN VITRO EVALUATION OF ANTI-DENGUE ACTIVITY OF SELECTED SYNTHETIC SCHIFF BASES AND TRADITIONAL CHINESE MEDICINAL PLANTS EXTRACTS

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By

MARYAM MAQSOOD

Thesis Submitted to the Graduate School of Management Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This was and will always be for Raiha and Hamza

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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July 2018

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During the last few decades, dengue virus (DENV) has emerged as the major virus spread by mosquito that can cause life threatening disease. Recently, it has spread to more than a hundred countries around the globe and still lacks a specific treatable medication. Hospitals provide only supportive and symptomatic treatment hence the development of a safe and effective antiviral is an urgent need. To identify new antivirals against DENV, realms of both synthetic compounds and ethnomedicine were explored.

A library of eighty-five dithiocarbazate (DTC)-derived Schiff bases and their metal complexes including twelve plant extracts were analyzed in a primary antiviral evaluation. Hits displaying 50% or more anti-dengue activity were selected and evaluated through foci forming unit reduction assay, serotype based analysis and real time quantitative RT-PCR based time-of-addition analysis.

During antiviral evaluation of synthetic compounds, ten anti-dengue compounds were identified with promising antiviral activity. Secondary evaluation presented two lead Ni complexes with SI values 86.3 and 80.4. Ni complexes were the strongest contestants and inhibited all DENV serotypes equally. Lead compounds inhibited against all stages of virus replication cycle but the activity was strongest during early stages. It was demonstrated *in silico* that Ni complexes were binding at domain III of DENV E-glycoprotein involved during early stages of replication. In conclusion, Schiff bases and their metal complexes are a whole new horizon for anti-dengue development and can be studied further to develop safe, effective, stable, and affordable anti-dengue in future.

Discovery and development of modern medicine relies on long and rigorous clinical trials until they are available for public use. Meanwhile, new classes of chemical compounds are being explored for innovative anti-dengue options, Traditional Chinese Medicinal (TCM) plants are frequently being used by dengue endemic populations as Contemporary and Alternate Medicine (CAM). These traditional herbs will continue to be an alternative for dengue treatment as disease burden is rapidly increasing every year. Present study also evaluated the anti-dengue potential of twelve TCM plant extracts designated as cool herbs used for the diseases with high fever.

During antiviral evaluation of TCM plants, four anti-dengue plants were identified. Secondary evaluation proved two lead plants extract *Dryopteris crassirhizoma* (DC) and *Morus alba* (MA) with SI = 4.21 and 4.62 respectively, which inhibited DENV serotypes equally. In general, the plants were not equally inhibiting against all stages of viral replication cycle. DC was identified as potential anti-dengue plant which was active at late stages of virus replication. MA was also inhibiting but the dose was high, however its safety profile was better than DC and the plant was active at early stages of viral replication. Our research elucidated and identified the anti-dengue activity of the TCM plants in practice that have been time-tested to reduce the illness. Similar research can be planned for other traditional anti-dengue practices. The present times of integrated treatments brings together conventional medicine with safe and effective complementary medicine. Combined research approaches from different healthcare disciplines may lead to identification of new therapeutic options.

Abstrat tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN AKTIVITI ANTI-DENGGI *IN VITRO* DI KALANGAN BES SCHIFF SINTETIK DAN TUMBUHAN UBAT TRADISIONAL CINA

Oleh

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Dalam beberapa dekad yang lalu, virus denggi (DENV) yang disebarkan oleh nyamuk telah muncul sebagai virus utama yang boleh mengancam nyawa manusia. Virus ini telah merebak melebihi seratus buah negara di seluruh dunia dan masih tiada ubat yang boleh menyembuhkan penyakit ini. Hospital hanya boleh memberikan rawatan sokongan dan mengurangkan simtom pesakit. Justeru itu, pembangunan antivirus yang selamat dan berkesan terhadap DENV telah menjadi keperluan yang mendesak kini. Dalam kajian ini, kami telah menerokai kedua-dua sebatian sintetik dan etnoperubatan untuk tujuan ini.

Analisis telah dijalankan terhadap 85 jenis bes Schiff terbitan ditiokarbazat (DTC) dan kompleks logamnya serta 12 ekstrak tumbuhan untuk menilai aktiviti antivirus utama. Sebatian yang menunjukkan aktiviti anti-denggi melebihi 50% telah dipilih dan dinilai melalui cara *foci forming unit reduction assay*, analisis berasaskangolongan serotaip serta *real-time quatitative (qRT)-PCR based time-of-addition analysis* sehingga tahap perencatan virus.

Sepuluh sebatian telah didapati mempunyai aktiviti anti-denggi yang baik semasa penilaian antivirus sebatian sintetik. Pengesahan kedua telah menunjukkan bahawa dua kompleks yang mengandungi nikel (Ni) dengan nilai SI 86.3 dan 80.4 masing-masing merupakan sebatian utama dalam aktiviti anti-denggi, di mana kedua-dua kompleks ini mempunyai aktiviti perencatan terhadap semua serotaip DENV yang paling ketara. Secara umum, kompleks-kompleks ini didapati merencatkan kitaran replikasi virus di semua peringkat tetapi perencatan yang paling ketara berlaku di peringkat awal kitaran replikasi virus. Kajian *in silico* telah mendapati bahawa kompleks-kompleks ini berikat pada domain III E-glikoprotein DENV yang terlibat semasa peringkat awal replikasi. Kesimpulannya, bes Schiff serta kompleks

logamnya merupakan ufuk yang baru dalam penyelidikan dan pembangunan anti-denggi yang selamat, berkesan, stabil, dan terjangkau pada masa depan.

Penemuan dan perkembangan perubatan moden bergantung pada ujian klinikal yang mengambil masa sehingga boleh digunakan oleh orang awam. Sementara itu, sebatian kimia kelas baru sedang dieksplorasi sebagai pilihan inovatif untuk anti-denggi. Perubatan tradisional Cina (TCM) yang berasaskan tumbuhan sering digunakan oleh penduduk endemik denggi sebagai perubatan kontemporari dan alternatif (CAM). Herba tradisional ini akan terus menjadi rawatan alternatif untuk penyakit denggi kerana beban penyakit denggi yang semakin meningkat setiap tahun. Penyelidikan ini juga menilai potensi anti-denggi untuk dua belas jenis ekstrak tumbuhan TCM yang digunakan secara tradisional sebagai herba penyejuk demam tinggi.

Empat jenis tumbuhan telah didapati mempunyai aktiviti anti-denggi. Melalui pengesahan kedua, ekstrak tumbuhan *Dryopteris crassirhizoma* (DC) dan *Morus alba* (MA) merupakan tumbuhan utama yang mempunyai aktiviti anti-denggi, di mana masing-masing mempunyai nilai SI = 4.21 dan 4.62. Ekstrak-ekstrak ini didapati merencatkan kesemua serotaip DENV sama sekali. Secara umumnya, ekstrak daripada tumbuhan-tumbuhan ini tidak merencatkan semua peringkat dalam kitaran replikasi virus. DC telah dikenal pasti sebagai tumbuhan anti-denggi yang berpotensi serta aktif di peringkat akhir kitaran replikasi virus. MA juga didapati merencatkan DENV tetapi hanya pada dos yang tinggi. Namun demikian, profil keselamatan MA adalah lebih baik daripada DC dan tumbuhan ini adalah efektif di peringkat awal kitaran replikasi virus. Penyelidikan kami telah menjelaskan dan mengenal pasti aktiviti tumbuhan TCM dalam melawan denggi. Rawatan bersepadu kini telah menggabungkan perubatan konvensional dengan perubatan komplementari yang selamat dan efektif. Penggabungan penyelidikan-penyelidikan dari disiplin berlainan akan membantu pengenalpastian pilihan terapeutik yang baru.

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I certify that a Thesis Examination Committee has met on 20 July 2018 to conduct the final examination of Maryam Maqsood on her thesis entitled "*In Vitro* Evaluation of Anti-Dengue Activity of Selected Synthetic Schiff Bases and Traditional Chinese Medicinal Plant Extracts" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AA	*Artemisia annua*
ADE	Antibody-dependent enhancement
AP	*Andrographis paniculata*
CAM	Complementary and alternative medicine
Cd	Cadmium
Co	Cobalt
C protein	Core Protein
CPE	Cytopathic effect
Cu	Copper
DC	*Dryopteris crassirhizoma*
DENV	Dengue Virus
DF	Dengue fever
DHF	Dengue Hemorrhagic fever
DMSO	Dimethyl sulfoxide
DTC	Dithiocarbazate
DSS	Dengue shock syndrome
E protein	Envelope protein
EMEM	Eagles minimal essential media
FBS	Fetal bovine serum
HCV	Hepatitis C virus
HPV	Human papilloma virus
IT	*Isatis tinctorial*
JEV	Japanese Encephalitis Virus
LCMS	Liquid chromatography –mass spectrometry
LE	*Lithospermum erythrorhizone*
LJ	*Lonicera joponica*
MA	*Morus alba*
MD	Molecular docking
MTT	3-(4,5-dimethylethiaza-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio

PBS	Phosphate buffer saline
PC	*Phellodendron chinense*
QSAR	Quantitative structure-activity relationship
RO	*Rheum officinale*
RT	Room temperature
RCM	*R*-camphor
RCQ	*R*-camphorquinone
RCV	*R*-carvone
SBDTC	*S*-benzyl dithiocarbazate
SI	Selectivity index
SG	*Smilax glabra*
SCM	*S*-camphor
SCQ	*S*-camphorquinone
SCV	*S*-carvone
SMDTC	*S*-methyl dithiocarbazate
SN	*Scrophularia ningpoensis*
TEM	Transmission electron microscopy
TCM	Traditional Chinese medicines
TCID50	Tissue culture infectious dose 50
VY	*Viola yedoensis*
WNV	West Nile Virus
Zn	Zinc

CHAPTER 1

INTRODUCTION

Dengue virus (DENV) is a positive single-stranded RNA virus of the *Flaviviridae* family that causes dengue fever (DF) with and without warning signs and severe dengue. It has four serotypes DENV 1, DENV2, DENV3, and DENV4 that are genetically and antigenically distinct and epidemiologically similar. Infection with one serotype leads to all-time protection against homologous re-infection but only brief protection against heterologous confront. DENV infection and immune system interactions may result in either immunopathology leading to severe forms of the disease or recovery from infection [1]. DENV is emerging as one of the biggest threats to the human population in the tropical and subtropical region, affecting 3.9 billion people worldwide every year. It is spreading rapidly due to global demographic changes, rapid and unrestrained urbanization, population growth and global ease of travelling [2]. In a recent study, the total cost for the management of the disease caused by DENV was estimated globally which turned out to be US$8·9 billion per year. The DENV transmission has also been shown in 141 countries around the globe [3]. Currently, there is no therapeutic medicine for DENV and according to WHO, vector management policies are the only alternative control [4]. Besides all the measures taken for the vector control, the increasing number of dengue cases around the globe shows the failure of vector management measures. Treatment is largely supportive and symptomatic, using common antipyretics and analgesics, oral or intravenous rehydration and in-patient monitoring. Appropriate medical attention reduces the fatality rate of severe disease to <1%. Currently, there are many dengue vaccine candidates and the leading candidate is CYD-TDV (Dengvaxia, Sanofi Pasteur), which manage to get licence from a few national regulatory authorities. However, DENV vaccine is intricate because of antibody-dependent enhancement (ADE) of the disease leading to Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSH) [5].

The disease has already grown around the globe and the symptomatic patients are increasing every year, so a suitable and effective antiviral is a better therapeutic solution for the ones who are sick and seeking treatment. Similarly, a prophylactic therapeutic option can help people living in high risk areas, to decrease dengue symptoms during dengue epidemics. Searching for a therapeutic solution for DENV is a matter of rigorous investigation and examining both synthetic and naturally available options. The advancement in high through-put target based approach for drug evaluation has led to the discovery of less number of approved drugs than the era of classical phenotypic target-free drug discovery approach [6].Therefore, many diseases are still lacking a specific treatment because the ideal disease target is yet to be identified. Consequently, phenotypic screening is gaining new momentum in drug screening. Phenotypic antiviral screening in resource-limited settings is convenient for the disease endemic countries because they can examine the locally infecting clinical isolates of the pathogen to identify effective antiviral options. Targets can then be identified for the potential *in vitro* or *in vivo* antiviral therapeutics.

Schiff bases have been long known for their remarkable biological activities such as antimicrobial, antihistaminic, antipyretic, antifungal, anthelmintic, antiviral as well as they have been widely explored for their industrial applications. Schiff bases are the huge class of compounds and are easily derived from a wide range of derivatives while based on their types of derivative they may have varied biological activity. Schiff base complexes are among the most important stereochemical models and metal coordination chemistry due to their preparative accessibility and structural variety, and the metal complexes have enhanced biological activities [7]. Schiff bases and metal complexes are widely studied owing to their ease of preparation and diverse pharmacological potential. Medicinal chemists across the world have done immense work on Schiff bases and developed agents with better medicinal activity and low toxicity profiles. However, the antiviral activity of these compounds deserves further investigation. Present study focuses on S-substituted dithiocarbazate (DTC)-derived Schiff bases and their metal complexes for their antiviral potential. Other than the fact that these compounds were easily and economically synthesized locally by the Department of Chemistry, Universiti Putra Malaysia, selection of this compound library was for two main reasons;

1. This synthetic compound library is proved to have anticancer properties while cancers are sometimes found associated with virus. Therefore, it was suggested that if these compounds are anticancer they might have antiviral potential as well.
2. Metal complexes were aimed for the metallotherapeutics, the principle of metal based therapeutics is that metal ions can alter the virus structure by metal chelation because virus nucleic acid and proteins are effective chelating agents.

The whole compound library was a series of structurally related compounds and their respective metal complexes, which were systematically subjected to *in vitro* antiviral evaluation to find out any pattern of viral inhibition that could be related to the structure or the addition of the metal atom to the compounds.

Medicinal herbs are being progressively documented as useful complementary treatments for many ailments. Many studies have reported the beneficial as well as safer effects of herbal medicines on patients suffering from common to life threatening diseases. Studies even support the idea of enhanced therapeutics when these herbal medicines are used in combination with conventional antiviral medicines [8]. For diseases like dengue, traditional herbs are a safe alternative approach as modern medicine has not managed to solve the problem yet. There are many herbs that are being used in routine as practical and clinical alternative medicine against DENV infection by the local communities, that have been time-tested to reduce the illness [9]. These traditional herbs will continue to be an alternative for dengue treatment as the discovery and development of modern medicine relies on long and rigorous clinical trials to test the effectiveness of a drug. This process may involve years of research, meanwhile, if the scientific research proves the effectiveness of these medicinal plants, patients can always benefit from

them. Physicians may recommend alternative therapies to their patients for the diseases lacking specific medicinal treatment which might work better than expensive drugs at treating life threatening diseases like dengue. Chinese Herbal Medicine (CHM) is one of the great herbal systems of the world and it is the most important components of Traditional Chinese Medicine (TCM). It has been reported to cure infectious diseases, in the form of hot water extracts, for almost 2,000 years with 100,000 recorded recipes for 10,000 herbal medicines in ancient TCM literature [10]. These TCM and other Complementary and Alternative Medicine (CAM) with their plants and plant-based products will continue to provide treatments for many diseases including dengue for which modern medicine cannot cater. Therefore, present study also focused on the anti-dengue potential of TCM herbal medicines.

1.1 Problem statement

Over the past few decades, there has been a dramatic increase in cases of dengue infection thus, proving that vector management policies alone are not enough to curb the problem. Though vaccine is a better option for any viral disease but dengue vaccine is complicated because of antibody-dependent enhancement which compromises its efficacy. Dengue is a rapidly increasing health problem contributing immensely toward health economics in endemic countries and patient with symptomatic cases are on the rise, seeking medical treatment. Whereas, need for the development of effective dengue therapeutic and prophylactic is still not fulfilled.

1.2 Significance of the study

This study focuses on both the naturally existing and synthetic therapeutic options for the development of safe and effective dengue therapeutics. The natural options are the medicinal plants used as Traditional Chinese Medicine (TCM) for centuries to treat numerous common illnesses as well as dengue fever. Synthetic library includes perceptively designed compound scheme of Schiff bases for chemotherapeutics and their metal complexes for more recent metallotherapeutics. Prospective antivirals evaluated through a blind target-free phenotypic analysis method, an original drug evaluation paradigm, for the hit identification. Various virological methods employed for hit-to-lead and earlier lead optimization of the drug discovery process. The leading prospective anti-dengue agents can be made available for future *in vivo* studies and clinical trials.

1.3 Objectives

1.3.1 General objectives

To evaluate potential anti-dengue activity of synthetic compounds and crude plant extracts and make them available for further research.

1.3.2 Specific objectives

1. To systematically evaluate synthetic compounds and crude plant extracts against DENV 2 to identify the anti-dengue "hits".
2. To determine the cytotoxic dose (CC50) of the potential anti-dengue compounds and plant extracts.
3. To optimize the "lead" through Foci Forming Unit Reduction Assay (FFURA) and to measure the difference of inhibition against four serotypes of DENV.
4. To perform time-of-addition analysis by measuring the reduction in DENV RNA copy number through quantitative real-time RT-PCR and deduce stage of virus inhibition.

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