EXTRACTION AND ISOLATION OF CHEMICAL COMPOUND FROM *TABERNAEMONTANA DIVARICATA* (L.) R.BR. EX ROEM. & SCHULT. LEAVES WITH POTENTIAL ANTI-NEURAMINIDASE ACTIVITY

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2015

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BY

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Thesis submitted in fulfillment of the requirements for the degree of

Master of Science

SEPTEMBER 2015

ACKNOWLEDGEMENTS

Alhamdulillah…Thank you Allah for giving me courage and strength to complete my study. I owe my deepest gratitude to my supervisor, Prof. Dr. Habibah A. Wahab for her continuous supports, advises and guidance over the years. I would like to thank my co-supervisor, Dr. Maizan Mohamed for her encouragement and opinion in this study.

I would also like to express my sincere appreciation to Nur Kusaira for her help and guidance in conducting bioassay analysis. I would never forget the help I got from Maywan, Yusuf, Adila and all PhDS lab members while doing computational docking study. Thank you so much for your time and guidance. Special thanks to Dr. Che Puteh Osman for helping and guiding me in elucidating the compound structure.

Million thanks to Azimah, Norizamimie, Ilyana, Anuar, Faisalina and Zuraidah for their valuable helps, supports and continuous motivation throughout my study. I am pleased to thank everyone who involved and helped directly and indirectly along this memorable journey of mine.

Special dedication goes to my family members for their endless love, support, understanding and encouragement. To my dearest Ummi and Abah, thank you so much for your prayers and blessings.

TABLE OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: RESULTS AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATION

ADT	AutoDockTools
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATR-FTIR	Attenuated Total Reflectance - Fourier Transform Infrared
c	Concentration
C	Carbon
$CaCl_2$	Calcium chloride
CC	Column Chromatography
CF	Combine Fraction
$CDCl_3$	Deuterated Chloroform
cm	centimetre
CNS	central nervous system
COSY	Heteronuclear Correlation Spectroscopy
d	Doublet
dd	Doublet of Doublet
ddH_2O	deionised water
DEPT	Distortionless Enhancement by Polarization Transfer
dm	decimetre
DMSO	Dimethyl sulfoxide
DSC	Differential Scanning Calorimetry
ECG	electrocardiogram
EtOH	Ethanol
F	Fluorine
FANA	2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid
FEB	Free Energy of Binding
g	grams
Glu	Glutamic acid
H	Hydrogen
HA	haemagglutinin
HMBC	Heteronuclear Multiple-Bond Correlation

HMQC	Heteronuclear Multiple Quantum Coherence
Ile	Isoleucine
IPharm	Malaysian Institute of Pharmaceuticals and Nutraceuticals
IR	Infrared
kg	kilogram
L	Litre
LCMS QTOF	Liquid Chromatography-Mass Spectrometry Quadrupole-Time-of-Flight
m	multiplet
M	Molar
mL	millilitre
M2	Matrix protein
MeOH	Methanol
MES	2-(N-morpholino) ethanesulfonic acid
MHz	Megahertz
MIC	minimum inhibitory concentration
mm	millimetre
mM	millimolar
m.p	Melting Point
MUNANA	2’2-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid sodium salt hydrate
m/z	mass-to-charge-ratio
N	Nitrogen
NA	neuraminidase
NADI	Natural Product Discovery
NAI	Neuraminidase Inhibitor
NaOH	Sodium hydroxide
Neu5Ac	acetylneuraminic acid
Neu5Ac2en/DANA	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
NMR	Nuclear Magnetic Resonance
O	Oxygen
PDB	Protein Data Bank
q	quadruplet

RMSD	Root Mean Square Deviation
RNA	Ribonucleic acid
s	singlet
sd	Singlet of Doublet
Ser	Serine
SA	sialic acid
syn	synonym
t	Triplet
T	Temperature
TLC	thin layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
U	Unit
USM	Universiti Sains Malaysia
UV	Ultraviolet
3-D	3-Dimensional
Å	Angstrom
^{o}C	Degree Celsius
%	percentage
μL	microlitre
α	Specific rotation
λ	Wavelength
w/v	Weight per volume
l	Tube length
π	Pi

LIST OF APPENDICES

LIST OF PUBLICATION

1. IKRAM, N. K. K., DURRANT J. D., MUCHTARIDI, M., **ZALALUDIN, A. S.**, PURWITASARI, N., MOHAMED, N., RAHIM A. S., LAM, C. K., NORMI, Y. M., RAHMAN, N. A., AMARO, R. E. & WAHAB, H. A. (2015). A Virtual Screening Approach for Identifying Plants with Anti H5N1 Neuraminidase Activity. *Journal of Chemical Information and Modeling*, *55*(2), 308-316.

PENGEKSTRAKAN DAN PENGASINGAN SEBATIAN KIMIA DARI DAUN *TABERNAEMONTANA DIVARICATA* (L.) R.BR. EX ROEM. & SCHULT. YANG BERPOTENSI SEBAGAI AKTIVITI ANTI-NEURAMINIDASE

ABSTRAK

Virus influenza telah dikenal pasti sebagai salah satu jangkitan paling lazim yang menyerang sistem pernafasan manusia. Virus ini boleh menyebabkan penyakit serius yang boleh membawa kepada peningkatan kadar penyakit dan kematian. Neuraminidase (NA) yang turut dikenali sebagai sialidase ialah protein tetramer yang memainkan peranan penting dalam kehidupan virus influenza. Ini termasuk membantu pembebasan virus ke sel-sel yang berhampiran. Oleh itu, NA telah dikenal pasti sebagai sasaran untuk membangunkan strategi terapi bagi melawan jangkitan virus. Kajian ini menumpukan kepada pengekstrakan, fraksinasi dan pengasingan sebatian kimia alkaloid yang berpotensi sebagai aktiviti anti-neuraminidase dari *Tabernaemontana divaricata* yang telah dikenal pasti melalui penyaringan maya dari pangkalan data NADI diikuti dengan penilaian-penilaian *in vitro* dan *in silico* ke atas sebatian yang telah diasingkan bersama-sama dengan drug standard (DANA). Pengasingan sebatian dikenali sebagai voafilin (0.059 g/kg) telah diperoleh daripada salah satu daripada empat belas fraksi yang telah dikumpulkan melalui pengasingan turus kromatografi. Penilaian *in vitro* bagi DANA, ekstrak-ekstrak metanol dan alkaloid, fraksi-fraksi dan sebatian voafilin telah dicapai melalui asai MUNANA diikuti dengan penilaian *in silico* bagi DANA, voafilin dan enantiomernya (ervayunin) melalui kajian pendokan molekul yang telah dijalankan menggunakan perisian AutoDock 4.2. Asai perencatan neuraminidase (asai MUNANA) bagi

DANA telah menunjukkan IC_{50} 27.76 ± 0.14, 4.25 ± 0.07 dan 12.05 ± 0.09 µg/mL terhadap neuraminidase dari *Clostridium perfringens*, H1N1 dan H5N1. Ekstrak-ekstrak metanol dan alkaloid, fraksi-fraksi dan sebatian yang diasingkan telah menunjukkan kesan perencatan yang ketara ($p<0.05$) berbanding kawalan positif di dalam asai perencatan neuraminidase terhadap ketiga-tiga jenis enzim neuraminidase. Kajian pendokan molekul bagi voafilin yang telah dijalankan melalui perisian AutoDock 4.2 telah menunjukkan bahawa voafilin, ervayunin dan DANA mempunyai tenaga pengikatan yang rendah iaitu masing-masing -7.31, -5.52 dan -6.29 kcal/mol dengan reseptor neuraminidase H1N1 (kod PDB: 3TI6) dengan penglibatan beberapa interaksi pengikatan telah diramalkan. Penyaringan *in silico* bagi sebatian-sebatian telah menunjukkan bahawa ramalan tumbuh-tumbuhan yang mengandungi sebatian kimia yang menarik bagi penyakit tertentu boleh dicapai melalui teknik pengkomputeran dengan pengesahan bagi aktiviti sebatian yang dijangka adalah tertakluk kepada penilaian uji kaji melalui asai *in vitro*.

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ABSTRACT

Influenza virus was identified as one of the most common pathogens that infect human respiratory system. The infection may cause serious illness which could lead to high morbidity and mortality rate. Neuraminidase (NA), also known as sialidase is a tetramer protein which plays important roles in the life of influenza virus. This includes facilitating the release of the virus to other neighbouring cells. Thus NA has been identified as the target to develop therapeutic strategies against the viral infection. This study is focused on the extraction, fractionation and isolation of alkaloid chemical constituent with potential anti-neuraminidase activity from *Tabernaemontana divaricata*, identified by virtual screening from NADI database followed by *in vitro* and *in silico* evaluations of the isolated compound along with standard drug (DANA). Isolation of compound known as voaphylline (0.059 g/kg) was obtained from one of the fourteen fractions collected via column chromatography separation. *In vitro* evaluations of DANA, methanol and alkaloid extracts, fractions and voaphylline compound were achieved via MUNANA assay followed by *in silico* evaluations of DANA, voaphylline and its enantiomer (ervayunine) via molecular docking study performed using AutoDock 4.2 software. Neuraminidase inhibition assay (MUNANA assay) of DANA showed IC_{50} of 27.76 ± 0.14, 4.25 ± 0.07 and 12.05 ± 0.09 µg/mL against neuraminidase from *Clostridium*

perfringens, H1N1 and H5N1. Methanol and alkaloid extracts, fractions and the isolated compound showed significant inhibitory effect ($p<0.05$) compared with positive control in neuraminidase inhibition assay against all three types of neuraminidase enzymes. Molecular docking study of voaphylline performed via AutoDock 4.2 software showed that voaphylline, ervayunine and DANA had low binding energy of -7.31, -5.52 and -6.29 kcal/mol, respectively with H1N1 neuraminidase receptor (PDB code: 3TI6) with a number of predicted bonding interactions involved. *In silico* screening of compounds had demonstrated that prediction of plants containing chemical compound of interest for a particular disease could be achieved via computational method with validation of the predicted compound's activity was subjected to experimental evaluation via *in vitro* assay.

CHAPTER ONE

INTRODUCTION

1.1 The Influenza Virus

Influenza or commonly known as flu is one of the most common but significant human respiratory infection due to its high morbidity and mortality rate. Influenza viruses may cause epidemics (seasonal infections) and pandemics (periodic and unpredictable infections) which lead to high number of infection and fatality (Taubenberger & Morens 2008). Younger and older generations with existing and chronic kidney failure, heart disease and respiratory problems as well as pregnant women are at higher risk for influenza infection (Douglas 1990; Woo 2010).

One or more symptoms are usually shown by people that have been infected by influenza virus. Among the most common symptoms includes sneezing or dry coughing, sudden high fever, headache, sore throat, body weakness and muscle pains (Taubenberger & Morens 2008; Woo 2010). Besides that, influenza infection could also be accompanied by other symptoms such as diarrhoea, poor or loss of appetite and photophobia (Laver & Garman 2002; Naffakh & Werf 2009). Severe influenza illness may cause other complication such as pneumonia (Gong *et al*. 2007).

Pneumonia is the most common complication that occur following influenza infection. The complication might occur due to either secondary bacterial pneumonia or primary viral pneumonia (Gong *et al.* 2007). Pneumonia complication could also occur due to both primary viral pneumonia and secondary bacterial pneumonia

(Douglas 1990). Besides that, other less frequent complications were also reported due to post-influenza virus infection such as secondary bacterial infection, instable or abnormal cardiac electrocardiogram (ECG) readings due to inflammation of heart muscle and brain inflammation which could lead to brain damage or disease (Gong *et al.* 2007).

There are three types of influenza virus; A, B and C which differ in the host range and ability in causing diseases. Influenza B and C are almost exclusively found in humans while Influenza A could be found in wider variety of mammals including birds, humans, pigs, horses and many other warm blooded animals (Suzuki 2005; Gong *et al.* 2007; Taubenberger & Morens 2008). Wild waterfowls were reported to be the source of all known influenza virus subtypes while pigs which usually have close contact with humans and birds serve as the intermediate host for mixing of genetic materials from both human and avian influenza viruses due to its capacity to be infected by Influenza A viruses from both species (Brown 2000; Laver & Garman 2002; Brown 2003).

Influenza virus was firstly isolated from bird species followed by the isolation of swine influenza virus in 1931 and human influenza virus in 1933. However, the identification of the first influenza virus isolated was only done in the year 1955. Another human influenza virus was isolated in 1940. However, this newly isolated influenza virus was genetically different from the human influenza virus isolated in 1933 (Gong *et al.* 2007).

It was reported that influenza viruses have unstable genetics which could lead to evolution of new viruses via either gene reassortment (antigenic shift) or point mutation (antigenic drift) (Taubenberger & Morens 2008). Influenza A which is the most virulent compared with the other two types of viruses, was reported to be the cause of epidemics and pandemics that occurred worldwide. Pandemics happened due to evolution of entirely new Influenza A virus subtypes via reassortment of its genome (antigenic shift) while epidemics occurred due to the changes of Influenza A and Influenza B surface antigens (Suzuki 2005; Gong *et al.* 2007; Bové *et al*. 2008). There are a number of influenza pandemic occurrences reported worldwide. However, the three influenza pandemics which occurred in 1918 (Spanish Influenza), 1957 (Asian Influenza) and 1968 (Hong Kong Influenza) had left significant impact due to the high number of deaths. Spanish Influenza (1918) had recorded a total of 50 million deaths worldwide while Asian Influenza (1957) and Hong Kong Influenza (1968) had caused 2 million and 1 million deaths, respectively (Oxford 2000).

1.2 Influenza Pandemic

Influenza A (H1N1) virus was the most common cause of human flu in 2009 which had caused a global panic over H1N1 flu. Since late April 2009, the outbreak of influenza A (H1N1) virus has raised concerns of the risk of a global flu pandemic. Pandemic, according to Taubenberger & Morens (2008) is the emergence of a new infectious disease that causes serious illness and spreads easily among humans across globally.

By June 11, 2009, nearly 30 000 of confirmed cases were reported by the World Health Organization (WHO) in more than 70 countries which led the organisation to raise the level of influenza pandemic alert to phase 6 which indicated that a global pandemic was underway (Halasa 2010; Woo 2010). Until the end of July 2010, more than 18 849 deaths from at least 214 countries with confirmed cases of influenza H1N1 were reported (WHO 2010a). After more than a year since the Influenza A H1N1 2009 pandemic, the number of reported cases have then slowed down and the world has moved from phase 6 of influenza A H1N1 pandemic to post-pandemic period (WHO 2010b). Although the Influenza A H1N1 2009 is already entering the post-pandemic period, it is expected that the virus will come back in future, considering the numbers and trend of pandemics that have occurred in the previous century.

The worst influenza A H1N1 pandemic recorded was in 1918 where approximately 50 million people are killed worldwide due to the virus infection (Halasa 2010). All influenza A pandemics that occur since 1918 were reported to have been originated from the 1918 virus strain. This includes the H1N1 viruses that had been isolated after the 1918 influenza pandemic and reassorted H2N2 as well as H3N2 viruses as reported by Kilbourne (2006). It was reported that H2N2 and H3N2 viruses consists of key genes from 1918 virus that were incorporated with avian influenza genes. According to Krause (2006) and Taubenberger & Morens (2006), other recent pandemics have also shown to be linked with the 1918 virus thus, making the 1918 influenza virus the mother of all pandemics. Therefore, intense studies and understanding of the genetics of the 1918 virus will certainly helpful in guiding the strategy to confront future influenza pandemics.

1.3 Influenza A Virus Structure

Influenza A viruses are a group of viruses from Mononegavirales order and Orthomyxoviridae family (Tambunan *et al.* 2012). The virus structure could be either in the form of round bodies (spherical or ovoid) or long filaments containing enveloped negative-sense, single-stranded and segmented RNA genome (Gong *et al*. 2007). Influenza A viruses are further subtyped according to the reactivity of their surface glycoproteins, namely haemagglutinin (HA) and neuraminidase (NA) (Qin *et al*. 2010; Woo 2010). These glycoproteins which cover the surface of the influenza virus play an important role in an influenza virus activity. Influenza A virus structure is illustrated in Figure 1.1.

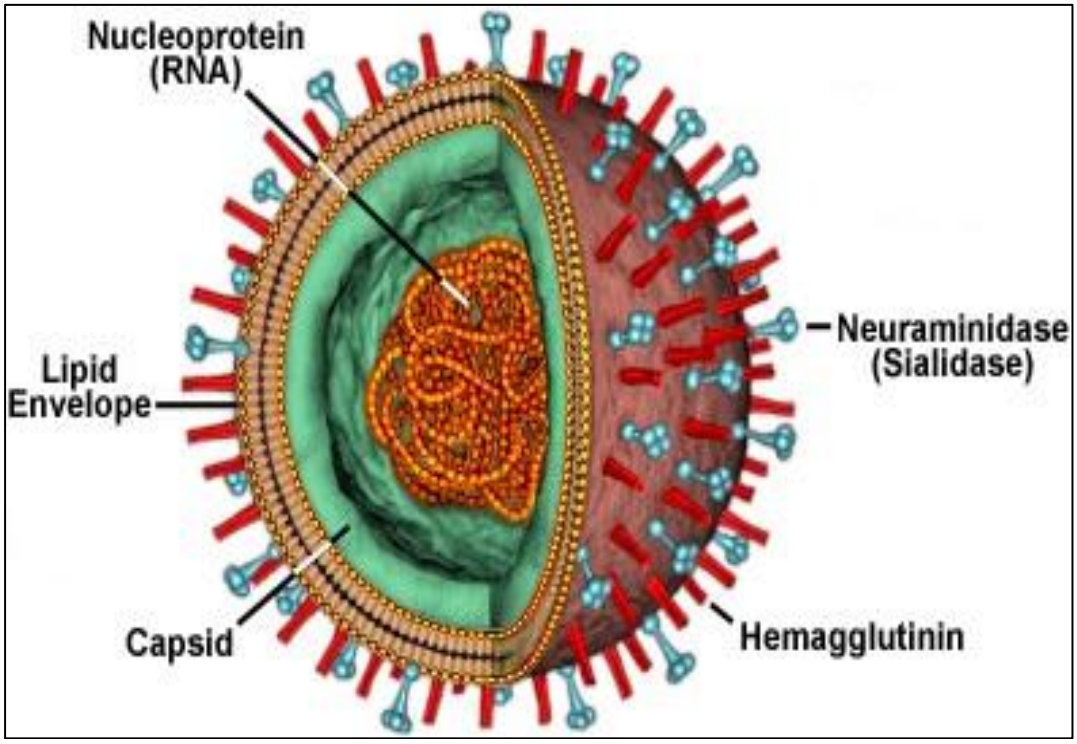

Figure 1.1: Influenza A virus structure (Davidson 2005)

1.4 Haemagglutinin (HA)

HA which consists of sixteen subtypes (H1-H16) is a triangular rod-shaped molecule made up from three identical HA polypeptides embedded in the lipid membrane of influenza virus (Itzstein 2007). It is responsible for the binding of virus to the sialic acid (SA) receptor of the infected cells and to facilitate the fusion of the viral envelope with the host cell at the point of infection (Wiley & Skehel 1987; Russell *et al*. 2006). HA's binding sites are different between humans and birds. According to Oxford (2000) and Taubenberger & Morens (2006), influenza viruses in birds bind to the α(2,3) linked sugars while influenza viruses in humans bind to the α(2,6) linkages of sialic acid receptors of the host cell. The removal of sialic acid molecules from the host cell was proven not being able to stop infection from influenza virus (Gong *et al*. 2007). This suggests the existence of different receptor on the host cell which plays more important role in viral infection.

1.5 Neuraminidase Enzyme (NA)

NA is a major surface glycoprotein on a virus structure. It has been identified by Alfred Gottschalk as neuraminidase or sialidase (Gong *et al*. 2007) with its 3-D structure was discovered in 1983 (Kubinyi 1998). It is a tetramer protein which is made from four identical polypeptides embedded in the viral membrane of influenza virus and consists of 9 subtypes, N1-N9 (Itzstein 2007). Each polypeptide is composed of six 4-stranded anti-parallel β-sheets. Studies have shown that NA, which consists of nine identical subtypes (N1-N9) plays important role in the replication, maturity and release or delivery of influenza virus (Russell *et al*. 2006).

NA enzyme subtypes were divided into two distinct groups; group-1 neuraminidase and group-2 neuraminidase. Group-1 consists of N1, N4, N5 and N8 neuraminidase subtypes while group-2 consists of N2, N3, N6, N7 and N9 subtypes (Zhang *et al*. 2008; Guo *et al*. 2010). Group-1 neuraminidases are able to bind to neuraminidase inhibitor, oseltamivir in either 'open' or 'closed' 150-loop conformation. However, group-2 neuraminidases are only able to bind to neuraminidase inhibitor in 'closed' conformation. The 'open' and 'closed' 150-loop conformations help neuraminidase substrates to fit into the active site upon binding of ligands or inhibitors (Cheng *et al*. 2008; Vries *et al*. 2012).

Neuraminidase is an enzyme that facilitates movement of virus to and from the site of infection by catalysing the cleavages of (2,6)- or (2,3)-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue. The catalysing of cleavages are important in facilitating the movement of viruses, to prevent self-aggregation of viruses after being released from host cells, to prevent

inactivation of viruses and also to promote penetration of viruses into respiratory epithelial cells (Russell *et al*. 2006). Besides that, important role of NA influenza virus and *Streptococcus pneumonia* has also led to the secondary bacterial complication which was the main reason for influenza-associated death (Gong *et al*. 2007).

Both HA and NA recognise the same host cell molecule, SA or also known as *N*-acetylneuraminic acid (Neu5Ac) receptor. The functional balances between these two glycoproteins are important in virus replication (Wagner *et al*. 2002; Gong *et al*. 2007). As discussed by Gong *et al*. (2007), Itzstein (2007) and Taubenberger & Morens (2008), HA plays important role by facilitating the attachment of virus to the host cells. However, without NA, the virus cannot enter the cells as the cleavage of SA could not occur hence; the spread of the virus to other cells may be suppressed. Due to this reason, NA has been recognised as the main target for the design of anti-influenza agents. An intensive study and understanding of neuraminidase structure would be very useful in designing and synthesising neuraminidase inhibitors.

1.6 Neuraminidase Active Sites

Neuraminidase active sites consist of highly conserved amino acid residues, mainly of aspartic acid (Asp), glutamic acid (Glu) and arginine (Arg) amino acid residues. The active sites are divided into 5 main regions, namely Site 1 (S1), Site 2 (S2), Site 3 (S3), Site 4 (S4) and Site 5 (S5). S1 region is made up of Arg 118, Arg 292 and Arg 371 residues that bind to carboxylate group of inhibitor. S1 region provides suitable condition for binding of anionic substituents from inhibitors due to its positively charged and hydrogen-bonding environments. S2 region, which consists of Glu 119, Glu 227 and Asp 151 residues, on the other hand is a negatively charged region active site, which interacts with guanidino group of inhibitors. S3 region, which is a small hydrophobic region, consists of Ile 222 and Trp 178 that are adjacent to Arg 152 side chain. S4 on the other hand is the main hydrophobic region made of Ile 222, Ala 246 and Arg 224 amino acid residues. S5 region, which consists of the carboxylate group of Glu 276 and methyl group of Ala 246 is a region of mixed polarity (Stoll *et al*. 2003). Figure 1.2 illustrates neuraminidase binding sites with 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) inhibitor.

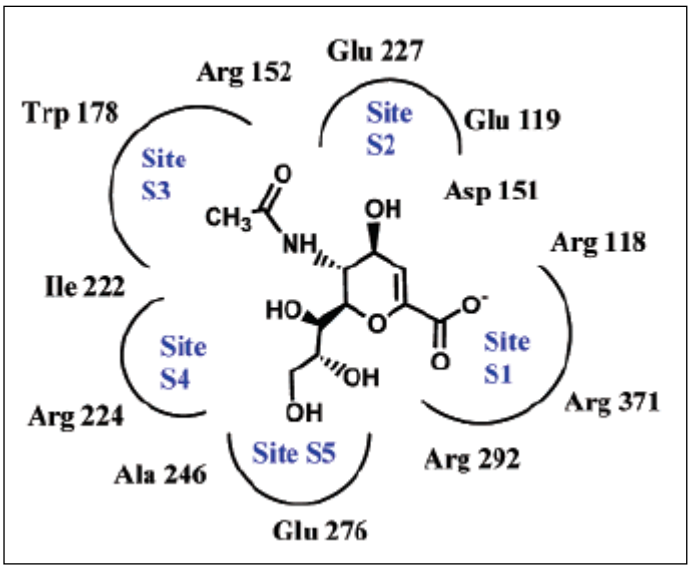

Figure 1.2: Illustration of five neuraminidase active sites (S1-S5) with Neu5Ac2en inhibitor (Stoll *et al*. 2003)

According to Lew *et al*. (2000), neuraminidase binding sites could also be divided into three major binding pockets: Pocket 1 (P1), Pocket 2 (P2) and Pocket 3 (P3). P1 consists of highly polar residues of Glu 276, Glu 277, Arg 292, Asn 294 and hydrophobic residue of Ala 246 which form interaction with glycerol moiety of SA. However, a study conducted had discovered that there was also high binding affinity by cyclohexene based neuraminidase inhibitors due to nonpolar interactions with amino acid residues in P1. P2 are made up by highly conserved amino acid residues which are Ile 222, Arg 224 and Ala 246. These amino acid residues are not involved in binding of SA but favourable hydrophobic interactions were reported to be made by the cyclohexene of neuraminidase inhibitors. P3 on the other hand is a large neuraminidase binding pocket which consists of hydrophobic and hydrophilic residues. This binding pocket is surrounded by Glu 119, Asp 151, Arg 152, Trp 178, Ser 179, Ile 222 and Glu 227 amino acid residues. Important amino acid residues with important binding pockets are shown in Figure 1.3. It is crucial to identify the binding pocket prior conducting docking study. Alternatively, original crystal

structure containing inhibitor or ligand could be used in identifying important binding sites.

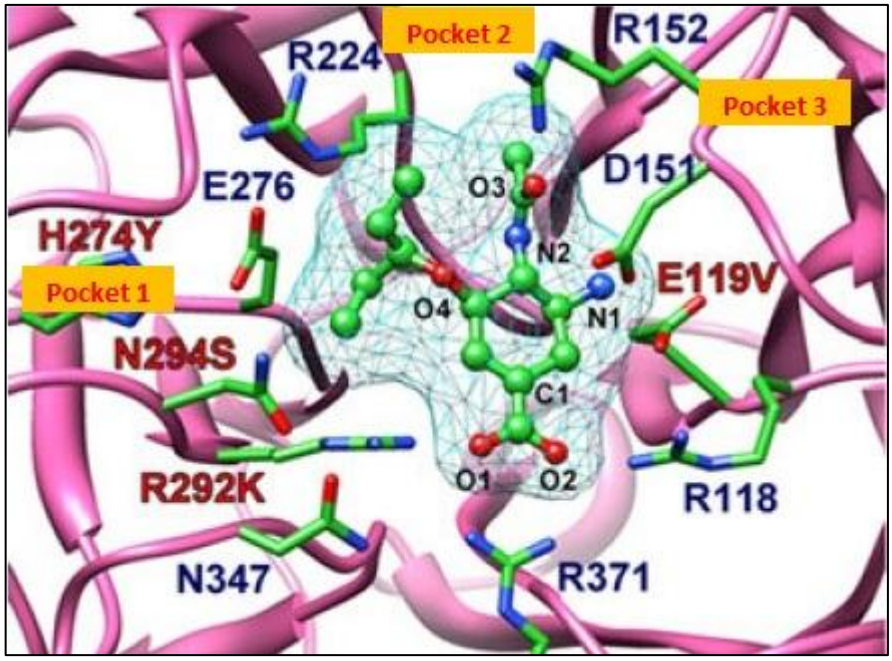

Figure 1.3: Illustration of oseltamivir bounds to the wild type strain of 2009-H1N1 influenza neuraminidase. This modelled structure was adapted and modified from Rungrotmongkol *et al*. (2010).

1.7 Neuraminidase Inhibitor and The Development of Antivirals

Neuraminidase enzyme plays critical roles in the life of influenza virus. This includes facilitating the release of the virus to other neighbouring cells by cleaving SAs from cellular receptor thus; NA has been identified as the target to develop therapeutic strategies against the viral infection. Based on the understanding of the enzyme's structure and functions, medicinal chemists have been designing and synthesising neuraminidase inhibitor (NAI) in order to overcome viral infection (Gong *et al*. 2007). Neuraminidase inhibitors mimic the natural substrate and bind to the active site, thus prevent the virus from reproducing and infecting other neighbouring host cells (Russell *et al*. 2006). Illustration of NA enzyme function in viral infection could be seen in Figure 1.4.

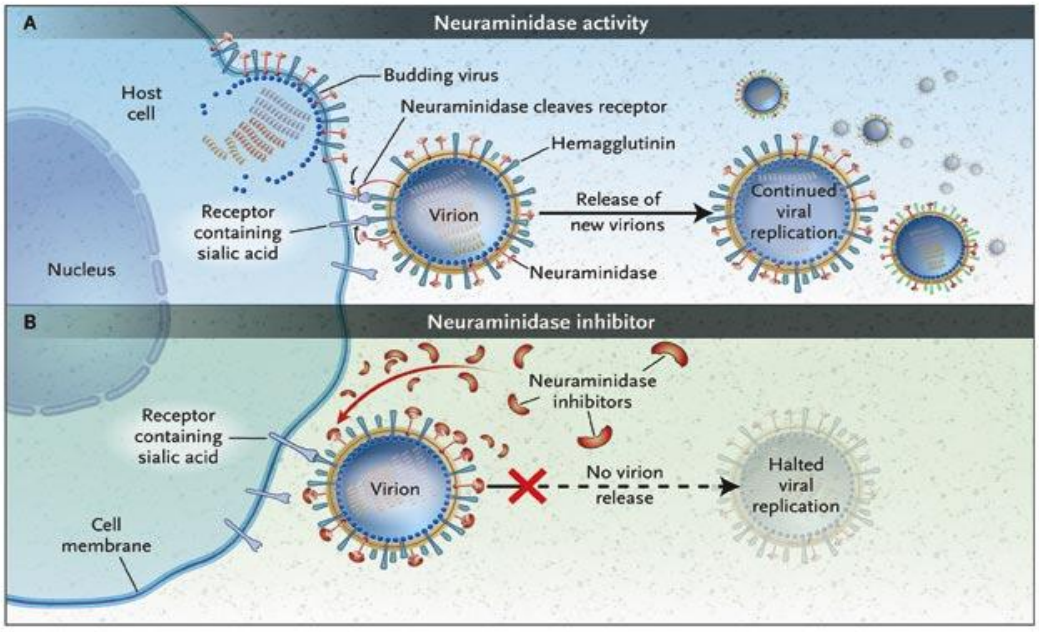

Figure 1.4: Mechanism of action of neuraminidase inhibitors.
A: Replication and release of virus to new host cells continues as neuraminidase enzyme cleaves the receptor containing sialic acid that binds with haemagglutinin
B: Viral replication discontinued as neuraminidase inhibitor binds to the active site (Moscona 2005)

1.7.1 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en/DANA) and 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA)

The first neuraminidase enzyme inhibitors discovered were dehydrated and fluorinated sialic acid, DANA and FANA (Figure 1.5). These two inhibitors, which were discovered by Peter Palese and his colleagues in 1970, are very effective against influenza neuraminidase. Unfortunately, these two inhibitors are found to be and only effective in cell culture (*in vitro*). Therefore, the search for neuraminidase inhibitor which could inhibit neuraminidase activity *in vivo* are intensified (Laver & Garman 2002).

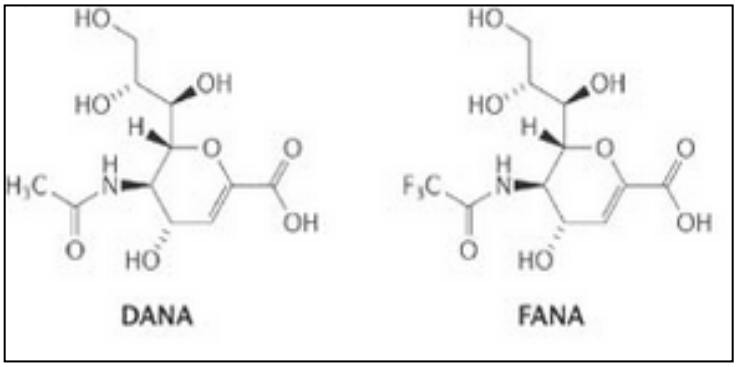

Figure 1.5: Molecular structures of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) and 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) (Clercq 2006)

1.7.2 Adamantane Derivatives

Several anti-influenza drugs or vaccines have been designed to combat influenza viruses based on intensive study on the structure of influenza virus. The first two drugs available to treat influenza in human and infected animals were adamantane derivatives; rimantadine and amantadine (Gong *et al*. 2007; Itzstein 2007). The derivatives were firstly found in petroleum in 1933 by Landa and Machacek. Eight years later (1941), Prelog and Seiwerth have successfully synthesised adamantane derivatives for the first time (Guo *et al*. 2010). These two drugs (Figure 1.6) act by interfering with the M2 protein ion channel function that is only found in influenza A virus (Laver & Garman 2002; Taubenberger & Morens 2008).

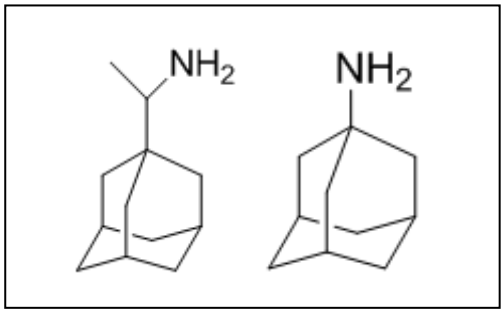

Figure 1.6: Molecular structures of M2 channel inhibitors; rimantadine (left) and amantadine (right) (Liu *et al*. 2010)

M2 protein plays important role during viral replication as it equilibrates pH of the viral membrane during viral maturation (Qin *et al*. 2010). The blocking of M2 ion channel activity will inhibit the uncoating of influenza viruses in the infected cells hence, will prevent the replication of the virus' RNA (Suzuki 2005). However, these two drugs have been reported to cause side effects on the central nervous system [CNS] (Itzstein, 2007). Adamantane derivatives were also reported to be effective against influenza A viruses only. Besides that, the derivatives have also caused nausea, dizziness, depression, hallucinations and anxiety in patients undergone treatment (Bové *et al*. 2008). Rapid emergence of drug resistance from the use of

these two drugs has also been reported (Laver & Garman 2002). During H1N1 influenza pandemic in 2009, 99.8% of patients in the US were reported to be resistance against both rimantadine and amantadine (Woo 2010).

1.7.3 Oseltamivir and Zanamivir

Two newer antiviral class of neuraminidase inhibitors available in the market are zanamivir (Relenza) and oseltamivir (Tamiflu). Structures of these two drugs are shown in Figure 1.7. Zanamivir and oseltamivir are effective against both influenza A and B viruses by interfering with the release of the virus from the infected cells (Suzuki 2005; Taubenberger & Morens 2008).

Zanamivir or commercially known as Relenza by GlaxoSmithKline (GSK) was approved as anti-influenza drug in 1999 and is marketed as inhaled anti-influenza drug which could directly deliver the drug to the site of infection (Itzstein 2007; Grienke *et al*. 2010). According to Itzstein (2007), the drug is marketed as inhaled formulation due to its limited bioavailability as the drug has high polarity in nature and the compound is rapidly excreted from the body.

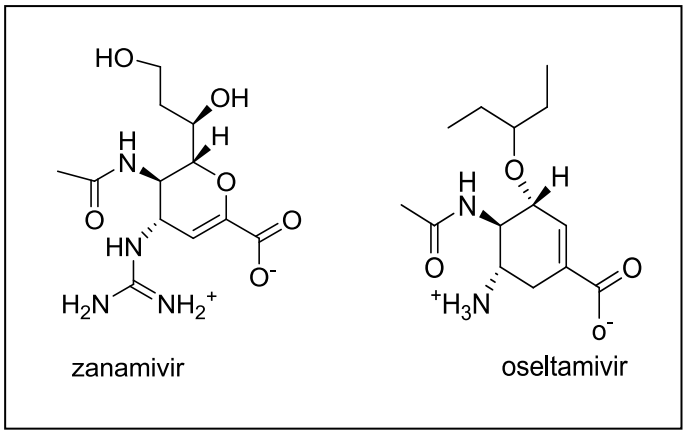

Figure 1.7: Molecular structures of neuraminidase inhibitors; zanamivir and oseltamivir (Liu *et al*. 2010)

Oseltamivir on the other hand is the first orally active sialidase inhibitor that has been approved for treating influenza (Itzstein 2007). The drug which is commercially known as Tamiflu is marketed by Gilead/Roche. However, rapid emergence of drug-resistance has been reported against the use of oseltamivir as anti-influenza drug. Russell *et al*. (2006) reported that mutations had occurred at the active sites of neuraminidase enzyme which led to inhibitor resistance. Since neuraminidase continues to be attractive for the development of new virus inhibitors, the search for novel neuraminidase inhibitors against influenza virus still remains as a worldwide priority.

1.8 Anti-Influenza from Natural Product

Currently, a number of studies on natural products have been conducted by other researchers to isolate chemical compounds with potential antiviral activities. For instance, an experimental study of propolis (bee glue) was conducted to investigate the potential use of propolis extract as antiviral. From this study, diethyl ether fraction of propolis extract showed inhibitory effect on the activity of H1N1 and H3N2 viruses (Serkedjieva *et al*. 1992). On the other hand, an *in vitro* study conducted by Watanabe *et al*. (2014) showed that manuka honey has high activity in inhibiting influenza virus replication in MDCK cells.

Inhibition of human influenza A/Hong Kong (H3N2) replication in MDCK was also reported by Haidari *et al*. (2009) in their study on polyphenol extract of pomegranate or scientifically known as *Punica granatum*. Another study conducted by another research group on the potential use of plant-derived isoquercetin as influenza virus inhibitor showed that the compound was capable of inhibiting the replication of influenza A and B viruses (Kim *et al*. 2010). Besides that, reduction of viral replication (*in vitro*) was also reported in a study of double treatment of isoquercetin and amantadine. Study on water extracts of five traditional Chinese medicines which are *Duchesnea indica* Andr., *Liquidambar formosana* Hance., *Lithospermum Erythrorhizon* Sieb. et Zucc., *Melia toosendan* Sieb. et Zucc. and *Prunella vulgaris* L. had also showed reduction of virus yield in MDCK cells as reported by Tian *et al*. (2011).

Experimental study on water extract of *Psidium guajava* Linn. (guava) tea conducted by Sriwilaijaroen *et al*. (2012) on the other hand showed growth inhibition of H1N1 influenza virus isolated from the 2009 pandemic strain. Similarly, β-santalol compound isolated from hexane extract of dried sandalwood (*Santalum album* L.) showed high antiviral activity with 86% inhibition at 100 µg/mL concentration against influenza A H3N2 virus in MDCK cells (Paulpandi *et al*. 2012). Studies on methanolic extract of Saudi plants (*Adenium obesum* and *Tephrosia nubica*) and ethanolic extract of *Peperomia sui* Lin and Lu, an endemic species of plant in Taiwan conducted by Kiyohara *et al*. (2012) and Yang *et al*. (2014), respectively have also reported inhibition of antiviral activity in cells.

The local plant selected in this project was identified by *in silico* screening from Natural Product Discovery (NADI) system conducted by the other member of our group. The pharmacological properties of the natural compound from the best docking results were evaluated via enzyme assay (neuraminidase inhibition assay). From the neuraminidase inhibition assay, methanol extract of *Tabernaemontana divaricata* leaves gave one of the highest percentages of neuraminidase inhibition thus, this plant was selected for extraction, isolation and elucidation of its chemical constituent with potential anti-neuraminidase activity.

1.8.1 *Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult.

Kingdom	: Plantae (Plants)
Subkingdom	: Tracheobionta (Vascular plants)
Superdivision	: Spermatophyta (Seed plants)
Division	: Magnoliophyta (Flowering plants)
Class	: Magnoliopsida (Dicotyledons)
Subclass	: Asteridae
Order	: Gentianales
Family	: Apocynaceae
Genus	: *Tabernaemontana* (L.)
Species	: *Tabernaemontana divaricata* (L.)

Source taken from USDA, NRCS (2011) website, retrieved 20th August 2011.

Pictures of *T. divaricata* plant and list of the plant's synonyms are shown in Figure 1.8 and Table 1.1, respectively.

Figure 1.8: Pictures of *Tabernaemontana divaricata* plant, taken from sampling site

Table 1.1: Synonyms of *Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. obtained from The Plant List website (2013)

Species	Synonyms
Tabernaemontana divaricata (L.) R.Br. ex Roem. & Schult.	*Ervatamia coronaria* (Jacq.) Stapf
	Ervatamia divaricata (L.) Burkill
	Ervatamia divaricata var. *plena* (Roxb. ex Voigt) M.R.Almeida
	Ervatamia flabelliformis Tsiang
	Ervatamia recurva (Roxb. ex Lindl.) Lace
	Ervatamia siamensis (Warb. ex Pit.) Kerr
	Jasminum zeylanicum Burm.f.
	Kopsia cochinchinensis Kuntze
	Nerium coronarium Jacq.
	Nerium divaricatum L.
	Nyctanthes acuminata Burm.f.
	Reichardia grandiflora Dennst.
	Reichardia jasminoides Dennst.
	Taberna discolor (Sw.) Miers
	Tabernaemontana citrifolia Lunan
	Tabernaemontana coronaria (Jacq.) Willd.
	Tabernaemontana coronaria var. *plena* Roxb. ex Voigt
	Tabernaemontana discolor Sw.
	Tabernaemontana flabelliformis (Tsiang) P.T.Li
	Tabernaemontana gratissima Lindl.
	Tabernaemontana indica Willd. ex Roem. & Schult.
	Tabernaemontana lurida Van Heurck & Müll.Arg.
	Tabernaemontana recurva Roxb. ex Lindl.
	Tabernaemontana recurva Roxb.
	Tabernaemontana siamensis Warb. ex Pit.
	Testudipes recurva (Roxb. ex Lindl.) Markgr.
	Vinca alba Noronha

T. divaricata is one of more than 100 species of flowering plants that belongs to the family of Apocynaceae. The plant is also known as Crepe Jasmine, Pinwheel flower, Coffee rose and Spiral gardenia. Locally, *T. divaricata* plant is known as "pokok akar susun kelapa" and "pokok bunga cina". The plant is a multi-branched evergreen shrub which could reach up to 6-feet high. Its thick, dark green leaves are arranged opposite or sub-opposite on the stem and are fairly large. The 5 petal white flowers have sweet scent and are arranged in groups from the stems growing point. There is also another variety of flower called "Flore Pleno" which is a double petal variety and has more ruffled wavy petals (Pratchayasakul *et al*. 2008).

The uses of plants for medicinal purposes are very important since the ancient times due to the limited alternative therapy available for treatment of various diseases. Chinese, Indian and Thai folks have widely used *Tabernaemontana* plants for a number of treatments including fever, pain and dysentery which is an inflammatory disorder in the intestines due to infection (Henriques *et al*. 1996; Rumzhum *et al*. 2012). The genus could be found abundance is many tropical countries such as Brazil, Egypt, India, Sri Lanka, Malaysia, Vietnam and Thailand.

1.8.2 Traditional Use of *Tabernaemontana divaricata* Plant Parts

The plant extract was found to possess analgesic and CNS depressant effects, cytotoxic activity and is also found to be useful in the treatment of cancer during the ancient times (Hsu 1967). In addition to that, various parts of this plant are traditionally used in India for a number of treatments. The plant material is widely used to treat diarrhoea, as a purgative and tonic, especially to the brain, liver and spleen. The roots of the plant are chewed to relieve tooth-ache while the plant's latex on the other hand has a cooling effect and could be applied to wounds to prevent inflammation. The latex could also be mixed with oil and applied to head to relieve headaches, eye pressure and corneal inflammation (Kirtikar & Basu 1987).

1.8.3 Recent Study on *Tabernaemontana divaricata* Plant

In vitro and *in vivo* study on methanol extract of *T. divaricata* leaves showed potential anti-acetylcholinesterase activity to treat Alzheimer disease. Acetylcholinesterase is the main enzyme in nervous system that affects neurotransmission which results in memory and behaviour impairment in people with Alzheimer disease (Ingkaninan *et al*. 2003; Chattipakorn *et al*. 2007). Besides that, methanol extract of *T. coronaria* (syn: *T. divaricata*) leaves had also showed high anti-tuberculosis activity against *Mycobacterium tuberculosis* strain with minimum inhibitory concentration (MIC) reported to be 800 µg/mL (Mohamad *et al*. 2011). In addition, experimental studies on five different type (ethanol, petroleum ether, diethyl ether, methanol and aqueous) of extracts obtained from *T. divaricata* leaves proved the effectiveness of these extracts against a number of infectious pathogens including *Staphylococcus saprophyticus, Staphylococcus aureus, Staphylococcus pyogenes, Enterococus facealis, Streptococcus agalactae, Salmonella typhi, Escherichia coli, Shigella boydii, Shigella dysenteriae* and *Pseudomonas aeruginosa* (Ashikur *et al*. 2011).

Furthermore, antidiabetic activity of methanol extract obtained from *T. divaricata* leaves was also reported by Masudur Rahman *et al*. (2012). In this study, methanol extract of *T. divaricata* leaves showed its effectiveness in lowering blood sugar level in diabetic mice model. Methanol and aqueous flower extract of *T. divaricata* on the other hand showed anti-fertility effect in rats. Study conducted by Mukhram *et al*. (2012) showed that methanol flower extract of *T. divaricata* could lead to early abortion due to its anti-implantation activity while both methanol and aqueous flower extracts of *T. divaricata* showed significant estrogenic activity at 500 $mg \cdot kg^{-1}$.

Study on anti-diarrhoeal for *T. divaricata* leaves was reported for the first time in 2013. Hydroalcoholic and aqueous extract of the plant leaves showed significant activity in rats hence; validating the traditional use of this plant part in treating diarrhoea (Raj *et al*. 2013). On the other hand, Jain *et al*. (2013) had proved the use of *T. divaricata* leaves as anti-inflammation agent in India. Experimental study showed that hexane fraction obtained from *T. divaricata* leaves showed high inhibition (42.1% inhibition at 0.7 $\mu g \cdot cm^{-2}$) on male albino mice.

1.8.4 Alkaloid Chemical Constituents from *Tabernaemontana divaricata*

The phytochemistry of *T. divaricata* has been extensively studied due to its wide range of medicinally important application. Chemical constituents isolated from various part of this plant were classified as alkaloids, sterols, triterpenoids, steroids, flavonoids, phenyl propanoids, phenolic acids and enzymes (Gupta *et al*. 2004; Pratchayasakul *et al*. 2008). Generally, the classification of chemical constituents from plants was done according to their biosynthetic origin, solubility properties and the presence of certain key functional groups in the isolated compounds.

Based on the *in silico* screening from NADI database conducted by the other group of this project, compound(s) of interest to be isolated from this plant are classified as alkaloids. Alkaloids are the largest secondary plant metabolites with over 27,000 compounds have been successfully isolated and identified for various studies (Amirkia & Heinrich 2014). It has basic chemical property due to the presence of at least one nitrogen atom in each compound.