STUDIES ON MOLD EXTRACTS AS POTENTIAL THERAPEUTIC AGENTS FOR HEPATITIS C AND DENGUE DISEASES

by

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Sylvia Sandanamsamy
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degree celcius
µg/ml	Microgram / milliliter
µM	Micromolar
Abs	Absorbance
AD	Anno Domini
ADE	Antibody dependent effect
BHK	Baby hamster kidney cell
C	Capsid protein
C	Carbon
C≡C	Carbon triple bond
$CaCO_3$	Calcium carbonate
CC_{50}	Cytotoxicity concentration 50%
CH_2	Methylene
Cl	Chlorine
cm	Centimeter
CO_2	Carbon dioxide
COOH	Carboxyl
CPE	Cytopathic effect
CPM	Complex production medium
CPM W PAA	Complex production medium with phenylacetic acid
CPM W/O PAA	Complex production medium without phenylacetic acid
DAA	Direct acting antiviral

DC	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DENV	Dengue virus
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DHF	Dengue heamorraghic fever
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
E	Envelope protein
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FRET	Fluorescence resonance energy transfer
FT-IR	Fourier-transform infrared spectroscopy
g	Gram
GC-FID	Gas chromatography – flame ionization detector
GCMS	Gas chromatography- mass spectrometry
GFC	Glass Microfiber filters

hr	Hour
H	Hydrogen
H_2O	Water
HCV	Hepatitis C virus
He	Helium
HepG2	Hepatocellular carcinoma cell
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IC_{50}	Half maximal inhibitory concentration
IgG-HRP	Immunoglobulin G- horseradish peroxidase
ITS	Internal transcribed spacer
IU	International units
JEV	Japanese Encephalitis virus
KBr	Potassium bromide
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
M	Membrane protein
m	Meter
MCV	*Molluscum contagiosum* virus
MeOH	Methanol
mg/mL	Milligram / milliliter
$MgSO_4$	Magnesium sulfate
min	Minutes
mL	Milliliter

MNTD	Maximum nontoxic dose
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	Nitrogen
N_2	Nitrogen
NIST	National Institute Standard and Technology
nm	Nanometer
NS	Nonstructural protein
O	Oxygen
OH	Hydroxyl
PAA	Phenylacetic acid
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
Peg-IFNα	Peginterferon alpha
PFU	Plaque forming units
pNA	*Para*-nitroanilide
PS	Porcine skin cell
rDNA	Ribosomal DNA
R_f	Retention factor
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
RT	Retention time
sec	Second

SI	Selectivity index
TLC	Thin layer chromatography
UV	Ultra-violet
v/v	Volume per volume
WNV	West Nile virus
YFV	Yellow Fever virus

LIST OF APPENDICES

KAJIAN EKSTRAK KULAPUK SEBAGAI AGEN TERAPEUTIK YANG BERPOTENSI UNTUK PENYAKIT HEPATITIS C DAN DENGGI

ABSTRAK

Denggi (DENV) dan hepatitis C (HCV) adalah dua virus daripada famili Flaviviridae yang masing-masing mengakibatkan 50-100 juta kes demam denggi berdarah atau sindrom kejutan denggi dengan kematian melebihi 20,000 dan penyakit hati kronik kepada lebih kurang 150 juta orang masing-masing secara global. Sehingga kini, tiada antivirus untuk DENV manakala antivirus HCV tidak mencapai hasil yang memberangsangkan. Dalam pencarian antivirus untuk DENV dan HCV, ekstrak daripada pencilan kulapuk Malaysia telah diuji. Lima puluh lima pencilan kulapuk dikerjakan dalam tiga media berlainan; kaldu dekstrosa kentang (PDB), media kompleks tanpa asid fenilasetik (CM) dan media kompleks dengan asid fenilasetik (CM + PA) pada 30°C selama 5 hari. Supernatan bebas miselium diekstrak dengan metanol dan etil asetat (1:1) yang menghasilkan 165 ekstrak mentah metanolik dan 165 etil asetat. Protease HCV NS3/NS4A asai digunakan untuk menguji ekstrak mentah (73 etil asetat dan 61 metanolik) dan ekstrak yang menunjukkan aktiviti perencatan yang melebihi 95% telah dikelaskan menggunakan kromatografi turus. Penentuan kepekatan merencat 50% (IC_{50}), kesitotoksikan dan analisis Kromatografi Gas Spektrofotometer Jisim (GCMS) dijalankan ke atas pecahan subaktif yang terbaik. Pencilan kulapuk dikenalpasti menggunakan urutan 18S rDNA. Ekstrak-ekstrak mentah (45 etil asetat dan 37 metanolik) yang menunjukkan perencatan protease HCV yang melebihi 80% telah diuji serentak dengan aktiviti perencatan protease DENV NS2B/NS3. Ekstrak mentah yang terbaik telah difraksasi dan subfraksasi diuji dengan aktiviti perencatan protease DENV NS2B/NS3. Subfraksasi aktif telah disahkan

dengan ujian plak denggi seterusnya dengan ujian kesitotoksikan serta analisis GCMS. Tiga ekstrak metanolik dan lima ekstrak etil asetat menunjukkan aktiviti perencatan protease HCV yang melebihi 95% yang setanding dengan kuersetin (kawalan positif). Ekstrak etil asetat daripada *Trichoderma virens* P72 telah menunjukkan perencatan tertinggi terhadap protease HCV dalam cara berkeperluan dos dengan nilai IC_{50} = 64.63 µg/mL dan nilai kesitotoksikan CC_{50}=2000 µg/mL pada sel-sel Vero. Analisis GCMS telah menunjukkan sebatian utama dalam pecahan subfraksasi aktif adalah asid- asid heksadekenoik dan oktadekenoik, pirrolo [1,2-α] pirazina-1,4, dion, heksahidro-3-(2 metilpropil) dan asid adipik. Ekstrak dengan perencatan melebihi 80% dalam asai protease HCV diteruskan untuk kajian antivirus denggi. Asai protease DENV NS2B/NS3 menunjukkan bahawa dua ekstrak etil asetat memperlihatkan aktiviti perencatan melebihi 60%. Salah satu ekstrak dengan etil asetat dengan kod OPT 5 daripada spesis *Fusarium* telah dipencilkan lagi kepada sub pecahan aktif (R2) yang menunjukkan peratusan perencatan tertinggi sebanyak 95.8% pada 200 µg/mL terhadap akitiviti protease DENV dan nilai kesitotoksikan CC_{50} = 2000 µg/mL pada sel-sel Vero. Analisis GCMS menunjukkan sebatian utama dalam pecahan subfraksasi aktif (R2) adalah asid- asid heksadekenoik dan oktadekenoik, pirrolo [1,2-α] pirazina-1,4, dion, heksahidro-3-(2 metilpropil) dan ergotamina. Hasil kajian asai plak virus pada sel Vero yang dijangkiti DENV dengan rawatan pecahan subaktif R2 menunjukkan dos 250 µg/mL adalah tidak toksik terhadap sel Vero tetapi merencat 84.30% DENV. Kajian ini boleh dijadikan penentuan untuk aktiviti-aktiviti yang antivirus berkemungkinan bagi HCV, DENV dan virus lain dalam famili Flaviviridae.

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ABSTRACT

Dengue (DENV) and hepatitis C viruses (HCV) are from the Flaviviridae family. DENV causes 50 -100 million dengue hemorrhagic fever or dengue shock syndrome with more than 20, 000 deaths globally. While HCV causes chronic liver diseases to 150 million people globally. Up to date there is no antiviral drug for DENV while HCV antivirals are performing below par. In the search for an antiviral drug for HCV and DENV, extracts from Malaysian fungal isolates were tested. Fifty five fungal isolates were fermented separately on three different media namely, potato dextrose broth (PDB), complex media without phenylacetic acid (CM) and complex media with phenylacetic acid (CM+PA) at 30°C for 5 days. Mycelia-free supernatants were extracted (1:1) using methanol and ethyl acetate which produced 165 methanolic and 165 ethyl acetate crude extracts. Crude extracts (73 ethyl acetate and 61 methanolic) were subjected to HCV NS3/NS4A protease assay and extract with more than 95% inhibition activity were further fractionated using column chromatography. Determination of inhibition concentration at 50% (IC_{50}), cytotoxicity and Gas Chromatography Mass Spectrometry (GCMS) analysis were performed on best active subfraction. Fungal isolate were identified using 18S rDNA sequencing. Crude extracts (45 ethyl acetate and 37 methanolic) that gave more than 80% HCV protease inhibition coherently were tested for DENV NS2B/NS3 protease inhibition activity. Most active crude extract was further fractionated and subfractions were tested using DENV NS2B/NS3 protease inhibition activity. Active subfraction was further confirmed with dengue plaque assay, cytotoxicity and GCMS analyses. Three

methanolic and five ethyl acetate extracts showed >95% HCV protease inhibition activities comparable with quercetin (positive control). Ethyl acetate extract from *Trichoderma virens* P72 showed highest inhibition activity against HCV protease in a dose-dependent manner with an IC_{50} = 64.63 µg/mL and cytotoxicity, CC_{50} = 2000 µg/mL on Vero cells. GCMS analysis revealed the major compounds in the active subfraction were hexadecenoic and octadecenoic acids, pyrrolo [1,2-α] pyrazine-1,4, dione, hexahydro-3-(2 methylpropyl) and adipic acid. Extracts with >80% inhibition with HCV protease assay were further proceeded for dengue antiviral study. DENV NS2B/NS3 protease assay revealed that two ethyl acetate extracts showed >60% inhibition activity. One of the extracts coded OPT5 PDB EA from *Fusarium* sp. was further fractionated in which active subfraction (R2) at 200 µg/mL showed 95.8% inhibition against DENV protease activity and cytotoxicity, CC_{50} = 2000 µg/mL on Vero cells. GCMS analysis revealed major compounds in subfraction R2 were hexadecenoic and octadecenoic acids, and pyrrolo [1,2-α] pyrazine-1,4, dione, hexahydro-3-(2 methylpropyl) and ergotamine. Results from viral plaque assay on DENV infected Vero cells with subfraction (R2) treatment showed that dose of 250 µg/mL was nontoxic to Vero cells but inhibited 84.30% of the DENV. This study could be the benchmark for possible antiviral for HCV, DENV and other viruses of Flaviviridae family.

CHAPTER 1

INTRODUCTION

1.1 Research background

Flaviviridae family has been one of the leading family that contributes to major illness to the global population. This family has been divided into several genus e.g. Flavivirus, Hepacivirus and Pestivirus (Xu et al., 2017).

Hepatitis C virus (HCV), placed under the genus Hepacivirus, remains a global threat with over 200 million carriers of this virus. HCV, a member of the Flaviviridae family (Paula et al., 2009), have become a burden in especially developing and under developed countries across the world as these countries are unable to access medication to treat patients with HCV.

Due to the presentation of HCV with many genotypes and sub genotypes, the development of vaccine is far away as the vaccine would need to match all the types. In the fight against HCV, antiviral agents serve as an immediate pathway for solving the emerging number of hepatitis C cases worldwide since there is no vaccine available for this disease. Drugs containing either combination of peg-interferon alpha (Peg-IFNα) with ribavirin or combination of ribavirin with boceprevir or telaprevir are currently being used as a standard procedure for treatment of patients with hepatitis C (Martinez et al., 2015).

Although the newly approved directly acting agents have been introduced, the treatment will take 24 to 72 weeks depending on HCV genotype, baseline viral load and the achievement of rapid virological response during therapy (Paula et al., 2009).

Unfortunately these drugs are not reaching the targeted group due to the cost of these medication and while failing to eradicate HCV (Paula et al., 2009). Hence, it is difficult to make these drugs available in government hospitals. According to physicians, diagnosis in detecting HCV is the last procedure as screening of other possible illness are done in the beginning.

Administration to patients with HCV type 1 was ineffective and half of the patients receiving these drugs suffer a side effect, most commonly anaemia (Huffman and Mounsey, 2014) and in some cases thrombocytopenia and pruritus (Wang et al., 2017) since the drugs are not compatible (Fujimoto et al., 2012). In addition, the treatment is burdensome as long periods and medication has to be taken before the disease can be treated and on top of that overall health of the patient is to be considered before administration (Rich and Taylor, 2010). Therefore, research on the development of a directly acting antiviral agent becomes the main focus of the pharmaceutical industry.

Another counterpart of HCV that shares same family is dengue virus, a top list vector-borne virus, has been transmitted by the *Aedes* mosquito. Dengue virus causes approximately 390 million infection annually (Hernandez-Morales et al., 2017) a leading cause of illness and death in the tropics and subtropics being endemic in more than 110 countries (Muhamad et al., 2010). Infection of the virus can be caused by one of the four serotypes of dengue virus (DENV).

Advances in the field of scientific research in the recent years have given a whole new look to the pathogenesis and the approaches that could be taken in developing the antiviral compounds and dengue vaccines (Wilder-Smith et al., 2010). Unfortunately, there are no approved drugs available for the treatment of this disease. Dengvaxia, a newly approved vaccine is only available in some countries but the

outcome does not seem to be satisfactory. Ministry of Health Malaysia reported that dengue is ever increasing in incidences and mortality in a linear trend. In 2015, there were 21,310 cases reported and 54 deaths of dengue patients (Ministry of Health Malaysia, 2019).

Nature has benefited the human race in many different ways. Beginning from the discovery of penicillin a new industry has been initiated that helps in the discovery of drug from fungi which has a major centre of attention for scientists and public (Joel and Bhimba, 2013a). According to Shoeb (2006), secondary metabolites from natural sources pose characteristics that are potential for drug development because they are derived from the living ecosystems in which they can show close similarities to drugs and be bio-friendly than totally synthetic drugs.

Fungi have been given much attention due to their presence of bioactive metabolites that are stimulated in adverse conditions. Many types of secondary metabolite are produced from fungi (Calvo et al., 2002). Antiviral properties are also owned by the secondary metabolites that are found in fungi. For example, the *Coriolus versicolor*, commonly known as turkey tail fungus, was found to have antiviral effect on human immunodeficiency virus (HIV) *in vitro* (Jordan, 2004). According to Walder *et al.* (1995) the aqueous extracts of *Fomitella supina*, *Phellinus rhabarbarinus*, *Trichaptum perrottotti*, and *Trametes cubensis*, showed strong anti-HIV-1 activity without exhibiting toxicity towards lymphocytic cells.

Research conducted by Hwang *et al.* (1999) had shown that Sansalvamide A, a cyclicdepsipeptide isolated from the marine fungus *Fusarium* sp. was found to inhibit the topoisomerase of the pathogenic poxvirus *Molluscum contagiosum* (MCV) by inhibition of topoisomerase catalyzed DNA relaxation, DNA-binding and covalent complex formation (IC_{50}=124 μM).

1.2 Problem statement

Hepatitis C virus (HCV) have become a burden especially in developing and under developed countries across the world as these countries are unable to access medication to treat patients with HCV. In Malaysia, the prevalence of HCV is getting high on the charts while treatment cost is also on the rise. Though there are standard approved drugs for the treatment of HCV, but those treatment are not effective for all patients and has many side effects.

Although the newly approved directly acting agents have been introduced to reduce and altogether eliminate the usage the ribavirin and boceprevir together with Peg-IFNα as current standard procedure, unfortunately these drugs are not reaching the targeted group due to the cost of these medication hence making them difficult to be available in government hospitals as these is the only avenue for the patients to get their medication. Certain reports have shown that HCV are not only developed with the most common mode of infection, (ex: liver transplant blood transfusion), HIV related and other, but, it seems to be associated with the occurrence of HCV with the same member of the family, dengue.

Dengue disease is endemic many countries presenting 2.5 billion people being at risk of infection globally (Panraksa et al., 2017) and causing pandemics in non-tropical countries. Statistically, it is estimated that 50–100 million cases of dengue occur in tropical and subtropical regions, in which 500,000 results in the dengue heamorraghic fever (DHF)/dengue shock syndrome (DSS), resulting with more than 20,000 deaths (Guzmán and Kourí, 2002). Dengue disease has a unique but dangerous effect where primary infection with any of the serotype gives a life long immunity but things are towards warning side when the patients are infected with a secondary

infection with a different serotype. Antibody dependent effect (ADE) starts to take place when heterotypic non-neutralizing anti-dengue antibodies mediating infection of Fc receptor bearing cells and this causes DHF and DSS (Panraksa et al., 2017) with main systems like the vascular, muscular and haematological being effected (Seneviratne et al., 2006). Vaccine development are hindered by the ADE. However, Dengvaxia a newly introduced dengue vaccine was limited to certain countries and results are suboptimal (Panraksa et al., 2017). Therefore, antiviral drugs are in top priority when it comes to treatment of dengue.

Secondary metabolites which are also known as bioactive compounds that are produced from microbial cultures have been widely explored. These bioactive substances are produced by the microbes when they are subjected to conditions that are not suitable for growth or in defence mechanism. Although many anti-viral studies have been performed on other viruses such as HIV virus and etc., there are very little data on study conducted on the fungal extracts especially Malaysian fungal isolates extracts which contain these bioactive substances against hepatitis and dengue virus.

In Malaysia, hepatitis and dengue drug discovery has not been made into a more concrete effort, encompassing approaches from computational chemistry, biology, organic chemistry and natural products. As Malaysian incidence of infectious diseases (hepatitis C and dengue) is increasing annually, with the country being endowed with natural mega-biodiversity, this serves as a great opportunity for this study to find the cure for this infectious disease.

1.3 Importance of study

Given the increasing number of hepatitis and dengue cases annually in Malaysia or other tropical countries for that matter, urgency to find and discover cure for both of these diseases remains unshaken. Both of these diseases have claimed many lives and still remains to do the same if there are no effective drugs or antiviral to cure or treat both diseases.

The significant importance of this study is that this research provides important data regarding bioactive compounds from Malaysian fungal cultures that can act as anti-hepatitis C and/or anti-dengue agents. As Malaysia is blessed with mega biodiversity, there are higher chances for the discovery of anti-dengue and anti-hepatitis drugs commercially available for the use of the people. With sustainable and demanding market for anti-dengue and anti-hepatitis drugs, it is worthwhile to search and develop this new source of treatment or cure.

In this study, it was aimed to discover bioactive metabolites that are obtained from fermentative procedures of fungal cultures that can be used as antiviral agent for the possible treatment of hepatitis C and dengue. Thus, this brings to the main objectives of this project that are to discover potential therapeutic anti-hepatitis and anti-dengue agents from Malaysian fungal isolates to combat hepatitis C and dengue diseases, two infectious diseases which are of high burden and national priority.

1.4 Objectives of study

The main objectives of the research; to discover a potential therapeutic agent from Malaysian fungal isolates for hepatitis C and dengue diseases. Thus, few specific objectives were aimed to achieve as follows:

A) To discover a potential therapeutic agent for hepatitis C disease

   i. To determine crude fungal extracts that are non-cytotoxic for further assays from 165 methanolic and 165 ethyl acetate crude extracts,

   ii. To determine the best fungal extract for anti-HCV protease activity from 73 ethyl acetate and 61 methanolic crude extracts,

   iii. To determine the best solvent system for column chromatography from 6 different solvent system for separation of bioactive metabolites with anti-HCV property,

   iv. To identify the bioactive metabolites with highest inhibition activity against HCV protease activity, and

   v. To identify the fungal isolate that produce bioactive metabolites with highest inhibition activity against HCV protease activity.

B) To discover a potential therapeutic agent for dengue disease

   i. To determine best fungal extract for anti-dengue protease activity from 45 ethyl acetate and 37 methanolic crude extracts,

   ii. To determine best solvent system for column chromatography from 6 different solvent system for separation of bioactive metabolites with anti-dengue property,

   iii. To identify the bioactive metabolites with highest inhibition activity against DENV protease activity,

iv. To determine anti-dengue property of the bioactive metabolites *in vitro* using dengue virus plaque immunostaining assay, and

v. To identify fungal isolate that produce bioactive metabolites with the highest inhibition activity against dengue.

CHAPTER 2

LITERATURE REVIEWS

2.0 Introduction to family of Flaviviridae

Viruses in general are a unique pathogen incapable of replicating without a host, however, unfortunately the designing of treatment to attack the virus or inhibit its replication maybe difficult but not an impossible task due to the unique characteristics of the virus (Kitazato et al., 2007). Viruses replication cycle are similar which includes attachment, entry, transcription, replication, assembly and budding as mature virus particles irrespective of whether the virus is enveloped or not or either possessing different genetic material (DNA or RNA) (Kitazato et al., 2007).

Flaviviridae family is one of the virus families that contributes to most of the diseases in the world. This family consists of the genera *Flavivirus*, *Pestivirus* and *Hepacivirus. Flavivirus* includes Dengue virus (DENV), Yellow Fever virus (YFV), West Nile virus (WNV), Japanese Encephalitis virus (JEV) and Tick-borne Encephalitis virus with the latest addition to the show would be Zika virus (Xu et al., 2017). Hepatitis C virus (HCV) and Bovine virus diarrhea are characterized under the genus of *Hepacivirus* and *Pestivirus*, respectively (Efferth et al., 2008).

This literature review will discuss on two viruses, DENV and HCV which are the main focus of this study. Fundamental characteristics of the viruses which includes viruses structure, mechanism of infection, global view on the viruses, current available antivirals (HCV) or antivirals under progress (DENV), similarities between both the viruses and using the knowledge from HCV antiviral discovery and repurposing for

the discovery of DENV antiviral drugs. This review will also include the antivirals assays for both HCV and DENV and instrumentation to identify bioactive metabolites.

2.1 Hepatitis C

Hepatitis C virus (HCV), an enveloped virus, has a positive-sense single strand RNA (Wang et al., 2017) with 9.6 kb genome that contains a single open reading frame which encodes a polyprotein of 3010 to 3033 amino acids (Berdichevsky et al., 2003). HCV polyprotein is posttranslationally processed into structural (C, E1, and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Rehman et al., 2011) which are found in the genome of the virus (Figure 2.1). Nonstructural (NS) proteins found in the endoplasmic reticulum membrane are essential for the replication and assembly of the viral RNA (Ye, 2012).

HCV has been classified into seven genotypes and with more than 70 subtypes (Wahyuni et al., 2013) which significantly varies in geographical distribution (Brechot, 1996) and hampers vaccine development (Potisopon et al., 2014). HCV could be either acquired by intravenous drug users or during blood transfusion process (Brechot, 1996). Hepatitis C is one of the leading causes of liver transplant (Lin et al., 2009) and in another extent causing chronic liver disease, and eventually causes hepatocellular carcinoma (primary liver cancer) (Efferth et al., 2008).

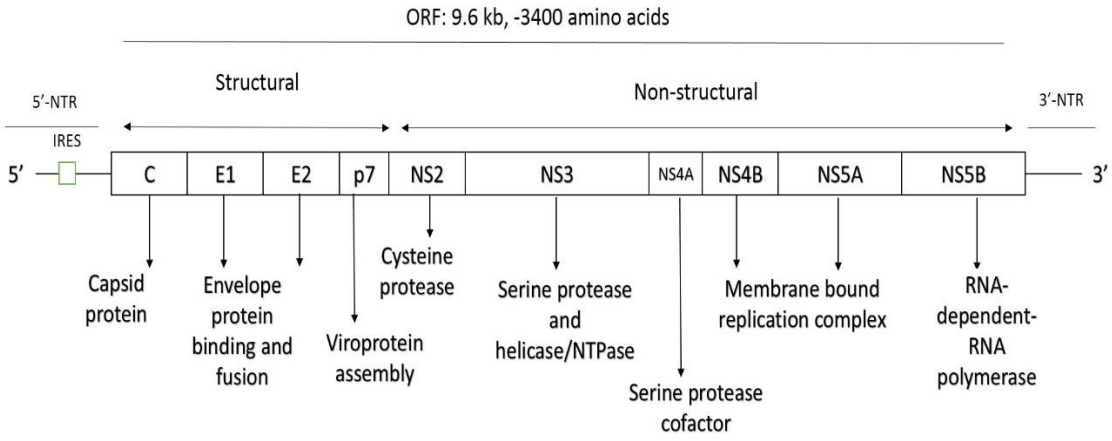

Figure 2.1: Structure of hepatitis C virus genome contains at each 5'and 3'NTR's with open reading frame (ORF). (Source: Potisopon et al., 2014)

2.2 Life cycle of HCV

Replication cycle of the HCV (Figure 2.2) are known to be similar to many positive strain RNA (Kwo and Vinayek, 2011). Entry of HCV into the cells are seem to be associated with several host-generated molecules including low density lipoprotein receptors, glycosaminoglycans, high density lipoprotein receptor scavenger receptor class B type I (Wang et al., 2017).

Following that, specific interaction between the virus envelope and the host mediates the entry of HCV via clathrin-mediated endocytosis, further progresses into translational at the endoplasmic reticulum giving rise to a single polyprotein that is cleaved in 10 proteins (Jardim et al., 2018).

Next, membranous structures induced by virus-induced perturbations of the endoplasmic reticulum is the site of the genome replication in double-membrane vesicle replication factories (Villareal et al., 2015). HCV associates with lipids droplet during assembly egress and further progresses as mature virions to be secreted through plasma membrane after being trafficked to the Golgi (Villareal et al., 2015).

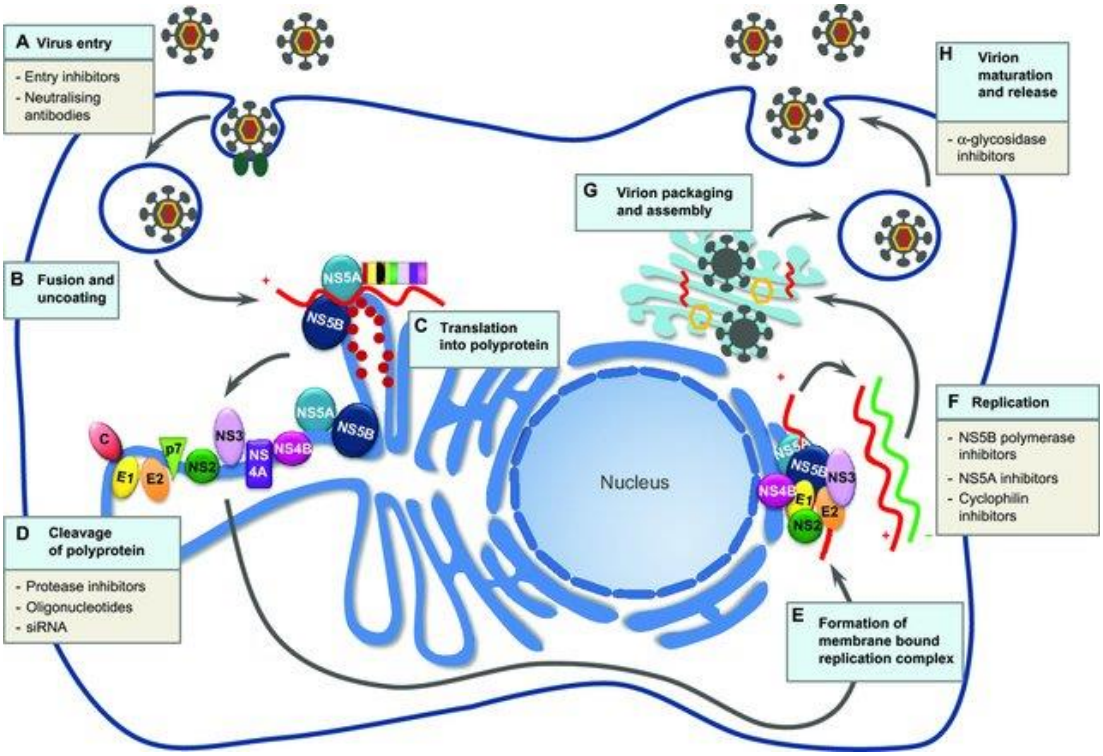

Figure 2.2: Life cycle of the Hepatitis C virus upon infection where several processes similar to Flavivirus takes place. The main processes include entry, fusion, genome replication, assembly and egress, budding and secretion of mature virion.
(Source: Holmes and Thompson, 2015)

2.3 Hepatitis C in a world view

Hepatitis C virus (HCV) remains a global threat around the world (Figure 2.3) being one of the world health problems (Jardim et al., 2015) with over 170 million carriers of this virus. Figure 2.3 shows the spread of HCV globally from the data collected by WHO in 2002, where almost half of the world affected. More 4 countries are in the red zones representing 10% of the population being infected while the other countries have the presence of HCV but at a lower percentage. Unfortunately, there are only a handful of countries have no or unknown presence of HCV. Eventually patients are prone to develop cirrhosis of the liver or hepatocellular carcinoma after

having chronic infection in which 4% of the global population are chronically infected (Tsai et al., 2017).

HCV, a member from the Flaviviridae family, have become a burden in developing and under developed countries across the world as these countries are unable to access medication to treat patients with HCV. Screening of donated blood for HCV was not done till the 1990's until Food and Drug Administration (FDA) authorized the screening as it was believed to be the main route of transmission of HCV (Jardim et al., 2018).

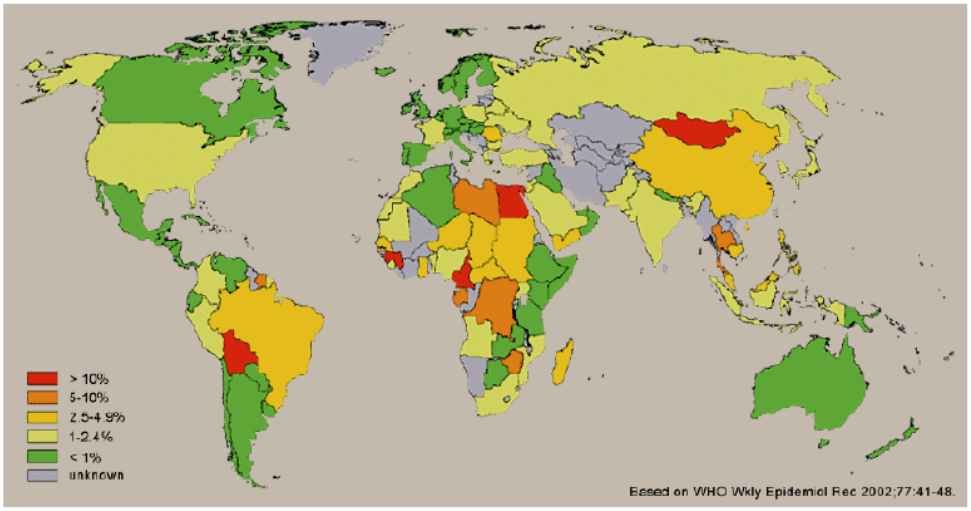

Figure 2.3: Worldwide spread of Hepatitis C infection. The map depicts the prevalence of HCV worldwide in which almost 90% of the globe seems to be affected by this virus. (Source: World Health Organization (WHO), 2002)

2.4 Current treatment of hepatitis C

Eradication of HCV seems to have a dark side as there are no vaccine available (Jardim et al., 2015). Although the newly approved DAA using drugs containing either combination of peg-interferon alpha (Peg-IFNα) with ribavirin or combination of ribavirin with boceprevir or telaprevir are currently being used (Martinez et al., 2015), however, unfortunately, these drugs are not reaching the targeted group due to the cost of these medication (Paula et al., 2009). Hence, it is difficult to make these drugs available in government hospitals even though it is the only avenue for the patients to get their medication.

Certain reports have shown that HCV are not only developed with the most common mode of infection, (ex: liver transplant blood transfusion), HIV related and other, but, it seems to be associated with the occurrence of HCV with the same member of the family, Dengue. In Malaysia, the prevalence of HCV is getting high on the charts while treatment cost is also on the rise. In a medical setting, diagnosis in detecting HCV is the last procedure as screening of other possible illness is done in the beginning. Due to the presentation of HCV with many genotypes and sub genotypes, the development of vaccine is far away as the vaccine would need to match all the types.

In the fight against HCV, antiviral agents serve as an immediate pathway for solving the emerging number of hepatitis C cases worldwide since there is no vaccine available for this disease. Half of the patients receiving these drugs suffer a side effects, most commonly anaemia (Huffman and Mounsey, 2014), since the drugs are not compatible (Fujimoto et al., 2012). Telaprevir contribute to gastrointestinal effects including diarrhea, rash, pruritus and anemia, while on the other hand boceprevir leads to anemia and dysguesia (Kwo and Vinayek, 2011). In addition, administration of

these expensive drugs to patients is burdensome and overall health of the patient is to be considered before administration (Rich and Taylor, 2010). On top of that, the prompting in transient virologic reaction was related with expanded symptoms when higher doses and delayed treatment was done. (Rehman et al., 2011).

Therefore, research on the development of a directly acting antiviral (DAA) agent becomes the main focus of the pharmaceutical industry. DAA targets for the nonstructural proteins that are translated after the viral RNA entry into the host cell. The DAA agents defined by the mechanism of action and therapeutic target are grouped into 4 classes; nonstructural protein NS3/4A protease inhibitors, NS5B nucleoside polymerase inhibitors, NS5B non-nucleoside polymerase inhibitors and NS5A inhibitors (Pockros, 2010).

2.5 Dengue virus

Dengue virus, an arthropod-borne human pathogen (Xu et al., 2017) poses a major health crisis especially in the tropics area when it emerged in the 1990's (Se-Thoe et al., 1999). It is a RNA virus, and consists of 4 serotypes ranging from DENV 1-4 (Seneviratne et al., 2006). DENV are usually are spherical in shape (Lim et al., 2013) enveloped, single-stranded, positive-sense RNA virus of approximately 11 kb long. DENV contains a single open reading frame that encodes a polyprotein precursor of about 3,400 amino acids (Hsu et al., 2012) which is processed by viral and host cell proteases to produce three structural proteins, capsid (C), premembrane/membrane (prM/ M) and envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Costa et al., 2007).

Genome of the dengue (Figure 2.4) is an important factor in the discovery of drugs that can exhibit antiviral activity against dengue. It is said so because each part of the genome plays a vital role in each step of the virus life cycle. Severing or disfunctioning one of this structures can stop the multiplication of the dengue virus.

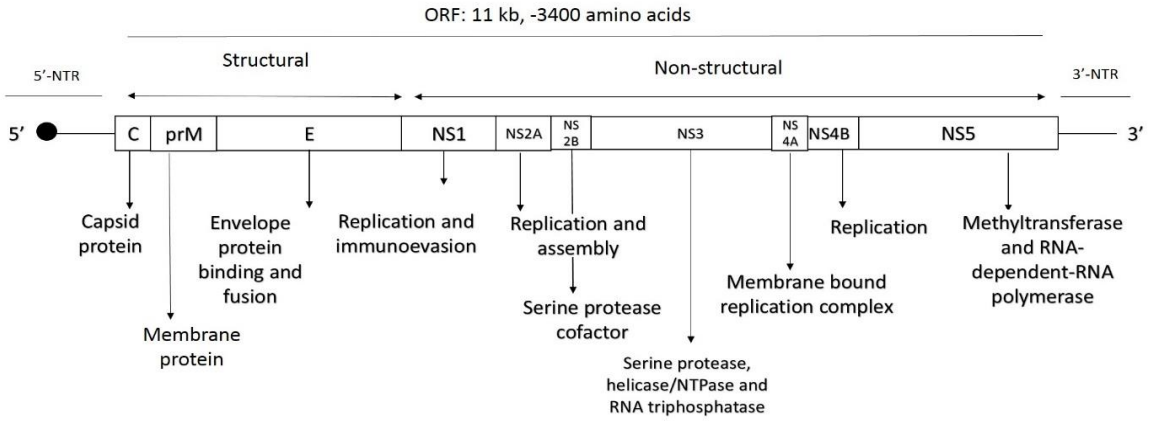

Figure 2.4: Structure of dengue virus genome contains at each 5'and 3'NTR's with open reading frame (ORF). (Source: Potisopon et al., 2014)

2.6 Dengue in a world view

Dengue is a vector borne disease (Machain-Williams et al., 2014) that keeps on increasing in magnitude and poses a worldwide risk (Rattanaburee et al., 2015). Dengue is also known as break bone fever, due to the severity of the fever with causing pain mimicking bone breaking pain (Lim et al., 2013), is transmitted by an infected female *Aedes* mosquito (*Aedes aegypti* and *Aedes albopictus*) (Hernandez-Morales et al., 2017) by biting humans (Hidari et al., 2013). Dengue which has not only become a threat by being one of important cause of morbidity in the Southeast Asia but has expanded across the globe (Lim et al., 2013).

Lim *et al.* (2013) reported that annually among the 50-100 million cases that occur in the tropical and subtropical regions in which 500,000 cases result in the

Dengue Haemorrhagic Fever (DHS)/Dengue Shock Syndrome (DSS). More than 20,000 deaths are reported from countries that have limited healthcare facilities (Hidari and Suzuki, 2011).

Although the most stringent efforts have been adopted to control the disease (vector control) yet dengue remains as a major global public health problem that affects 2.5 billion people in urban and rural areas of the tropics and subtropics (Whitehorn and Farrar, 2010). Dengue does not only lead to a public health crisis but its penetration has affects on the economy of the country (Kannabirran et al., 2012) in the form of lost wages, increase in care-giving expenses and medical expenses (Moghaddam et al., 2014).

Figure 2.5 shows the global incidence of dengue where almost half of the globe has been affected. Number of cases that are reported are in millions over the globe which indicates the severity of this disease and the high probability of fatalities that could occur that has been affecting many countries that has been indicated as red. Herricks *et al.* (2017) reported Asia, Africas and Americas are experiencing a 610% increase in the occurrence of dengue cases beyond imagination as the global population demography continues to have a drastic change continuesly.

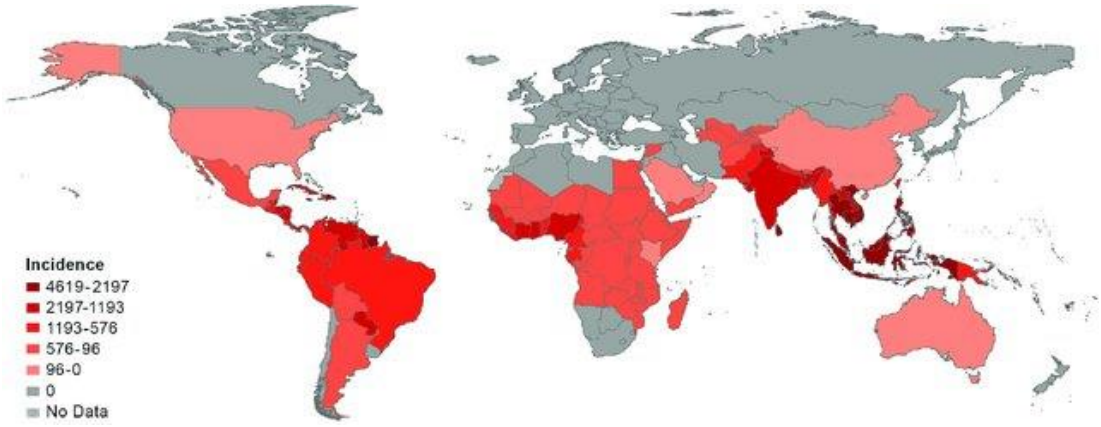

Figure 2.5: Global dengue incidence. The data was reported as per 100,000 person in 2013. (Source: Herricks et al., 2017)

2.7 Epidemiology of the dengue virus

Dengue virus was originally a monkey virus in non-human primates in Africa and Asia but independent cross-species transmission of all the four serotypes had occurred sharing around 65% of the genome and causes almost identical syndrome in humans (Alen and Schols, 2012a). Chin Dynasty (265 to 420 AD) has recorded dengue virus as "water poison" in ancient Chinese medical literatures due to its association with flying insects bred in clean water (Lim et al., 2011). Dengue predated back to a long time ago while being posing threats not only then but currently too.

2.8 Effects of DENV infection in humans

Infection with DENV can lead to mild flu like symptoms appearing after 5 to 7 days and can progress to a more severe condition like DHF characterized by coagulopathy, increased vascular fragility and permeability. Patients with DHF have higher chances to encounter DSS and hypovolemic shock (Xu et al., 2017). Hallmark

of the disease was known to be vascular leakage (Noble and Shi, 2013). Halsey *et al.* (2012) explained well in terms of the spread of DENV infection which covers dermatologic, neurologic, respiratory and gastrointestinal organs. Further study on this matter by Halsey *et al.* (2012) showed that DENV-3 had exhibited musculoskeletal and gastrointestinal manifestations, while DENV-4 had cutaneous and respiratory manifestations with DENV-1 showed rhinorrhea and DENV-2 exhibiting malaise.

Another research finding revealed that brain cells can be affected by the spreading of the DENV causing severe side effects to the central nervous system denoted by neurological complications including dengue encephalopathy, encephalitis, neuromuscular complications and neuro-ophthalmic involvement (Jhan et al., 2017).

In a hospital setting, physicians are battling the challenges of clinical diagnosis of dengue patients as other pathogenic agents exacerbate clinical sympotoms in dengue patients (Machain-Williams et al., 2014). However, in another setting, DENV transmission was speculated to be via vertical and sexual transmission as high viral loads were present in breast milk and saliva (Boldescu et al., 2017).

Researchers have been extensively conducting experiments on the effects of infection with dengue virus to deliver important information and at the same time to warn the public that dengue should not be taken lightly as the effects can be severe and even causing fatalities. Even though extensive research has been done throughout the ages, however, there are still unachieved objectives as there are no antiviral drug nor vaccine for DENV.

2.9 Dengue vaccine development and obstacles encountered

An ideal dengue vaccine has to provide full immunity against all 4 serotypes of DENV making this characteristic to become one of the hindering blocks in the development of a dengue vaccine (Muhamad et al., 2010). Other challenges that arises with the developement of DENV vaccine would be that the vaccine has to be intravenous dosing instead of oral dosing and low potential barrier of resistance emergence (Gu and Shi 2014).

Principal challenge in the development of dengue vaccine would be the antibody dependent enhancement (ADE) effect. This effect takes place as Fc-mediated uptake of subsequent infecting heterologous strain by Fc-receptor positive cells are being facilitated by the cross-reactive antibodies (Julander et al., 2011). These antibodies that were produced upon the primary infection with a different serotype leading to severe pathological response which have been observed in secondary infection of dengue (Julander et al., 2011). Boldescu *et al.* (2017) states that increased viral entry to the host cells and inhibiton of viral neutralization was predominantly caused by the antibodies against pre-membrane (prM) glycoprotein.

Although approved in only several countries, tetravalent vaccine, Dengvaxia, (Tarasuk et al., 2017) has questionable efficiency (Peng et al., 2017). Low *et al.* (2017) points out that the vaccine had lower efficiency for DENV-1 and DENV-2 as compared to DENV-3 and DENV-4 with different effectiveness was observed varied by age and serostatus of the vaccine recipient at baseline.

The truth of the matter is that there is a growing need for antiviral therapies as there are many individuals that are unable to receive the vaccine and also for unvaccinated patients (Frabasile et al., 2017). On top of that, one of the leading causes

of child death and hospitalization is due to dengue virus but many of these countries being infected have a limited financial aid (Blaney et al., 2005). Therefore, the need for an economical and fast acting antiviral which also cures the disease is on the top priority listing.

2.10 Mechanism of dengue virus infection

Life cycle of the dengue virus is shown in Figure 2.6. Infection of the dengue virus begins with the *Aedes* mosquito (vector) feeds on a blood meal and introduces the virus into the host (Clyde et al., 2006). An average incubation period of 4 to 7 days occurs in the body of the infected individual upon primary infection with dengue virus (Kannabirran et al., 2012). Then, viral RNA is translated into a polyprotein which is pre and post translationally cleaved by cellular and viral proteases into the structural proteins C, prM and E and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Welsch et al., 2009). Legitimate cleavage of the viral polyprotein, including preparing 8 of the 13 substrate cleavage sites by dengue virus protease NS2B/NS3 will ensure maturation of DENV particles for the replication process (Lin et al., 2017).

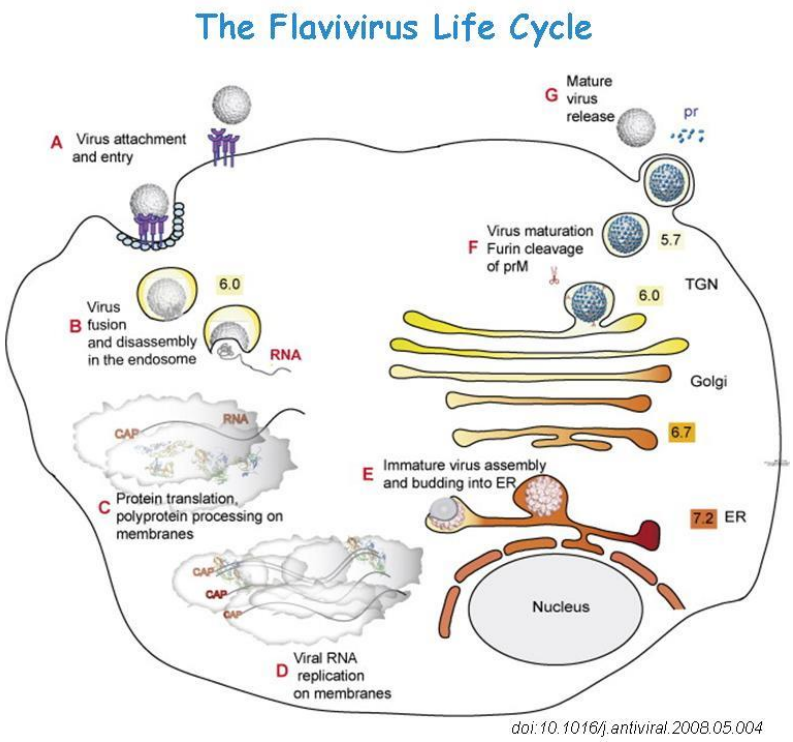

Figure 2.6: Lifecycle of a Flavivirus (Dengue virus). Figure depicts the stages of virus particle as it enters the cell of living organism and all the processes involved to produce a mature virus. (Source: Raphaella et al., 2017)

2.10.1 Receptor-mediated entry of dengue virus

Cellular tropism of dengue virus involves two phases, adsorption and entry. Receptor mediated endocytosis is the salient mode of entry of virus into the host cells (Clyde et al., 2006). Nucleocapsid are released into the cytoplasm upon fusion of viral envolope with host endosomal membrane facilitated by the low pH in the endosomes (Peng et al., 2017).

Firstly, primary replication site of the virus is at the monocular phagocyte lineage (Clyde et al., 2006) such as the dendritic cells which are in close proximity to the bite. DENV infection starts at the dendritic cells and spreades to lymph nodes as theses sites are amplification sites of the virus (Noble and Shi, 2013). Hidari and Suzuki (2011) reported that in the initial infection stage carbohydrate-binding proteins

on the dendritic cells (DCs) and macrophages plays an important role. Interaction between DC-SIGN and the virus is through the carbohydrate moieties on E protein which is similar to the glycosylation that occurs in insects cells.

Reaction can be promoted to a faster rate with the addition of high-mannose glycans residue N67 (Hidari and Suzuki, 2011). Mode of entry of DENV via receptors has been proposed to involve two or more receptors where DC-SIGN is being the ubiquitous lower affinity receptor which increases the local concentration and another being the high affinity receptor which mediates the internalization of the virion (Clyde et al., 2006).

Upon infection, viral particles are assembled in the endoplasmic reticulum where host factors are required in the glycosylation machinery in order to modify the envelope and membrane glycoproteins for the formation of immature viral particles (Plummer et al., 2015). Enveloped with the prM-E heterodimers,are immature progeny which are assembled and trafficed to Golgi where mature virus particles produced by the prM cleavage by host proprotein convertase furin within the *trans*-Golgi network (Peng et al., 2017).

Exocytosis releases mature viral particles to infect other cells (Peng et al., 2017) and does not only stop there when it starts infecting macrophages and lymphocytes and numerous human cells such as B cells, T cells, endothelial cells, hepatocytes, and neuronal cells, as well as a number of cell lines used for viral propagation (Clyde et al., 2006) and finally the bloodstream (Kannabirran et al., 2012).

In the human body, dendritic cells are antigen-presenting cells that are integral to inducing an immune response (Kannabirran et al., 2012). In previous researches that have been conducted where by inoculation of the human skin *in vivo* explants with dengue virus with 60-80% DENV antigens, only the immature cells (mixture of

langerhans cells and interstitial dendritic cells) were infected, which shows that the dengue virus prefers the dendritic cells, specifically monocyte-derived dendritic cells (resembling interstitial dendritic cells) and human skin Langerhans cells (Clyde et al., 2006; Kannabirran et al., 2012). Another study also showed that the dengue virus was detected in B cells in peripheral blood from naturally infected patients (Clyde et al., 2006).

2.11 Dengue antiviral agents

Antivirals for dengue have been explored in various sources be it from plant, chemically derived or any other types for that matter (Table 2.1). In the combat against dengue disease, the approach of fighting the virus with an antiviral is the step taken by researches as a vaccine candidate for dengue seems to be potentially low. Different phases in the virus life cycle are used as targets to inhibit the virus from developing further. However, researches have more to understand on the mechanism of actions of these antivirals against dengue (Muhamad et al., 2010). Unfortunately, though many research are being done, there are no approved treatment for dengue disease.

Principal aim of an antiviral therapy is to prevent the patients with dengue fever from developing DHF or DSS (Yin et al., 2009) by quickly reducing the viremia by 10-fold (Lim et al., 2013) as high level of viremia have been often seen in severe dengue (Zandi et al., 2011). DENV antivirals has to be drugs that are be able to enter systemic circulations and be distributed to many replication sites (Noble and Shi, 2013).

Significant development of an antiviral agent is that this compound or substance is inhibiting any steps of the viral life cycle and antagonists of viral enzymes