ANTIVIRAL ACTIVITY OF HESPERETIN AND NARINGENIN AGAINST CHIKUNGUNYA VIRUS REPLICATION *IN VITRO*

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ABSTRACT

Chikungunya virus (CHIKV) is an emerging arbovirus, which has recently become globally important. It poses a progressive major impact on humankind in recent years, with possibly life-threatening and incapacitating arthritis. CHIKV infects human through the bite of mosquito vectors. National Institute of Allergy and Infectious Disease (NIAID) have classified CHIKV as Category C pathogen. It causes several clinical features similar to dengue virus infection, except that are associated with intense polyarthritis and tenosynovitis, where the similarities would usually cause misdiagnosis. Overall, millions of cases of CHIKV have been reported in over 50 countries.

Currently, there is no available effective antiviral drug or vaccine has been developed for treatment of CHIKV infection. Treatment is usually symptomatically, with bed rest, fluids, and medicines to relieve symptoms of fever and aching. Thus finding and developing of lead compounds with anti-CHIKV activity that could be further developed to a practical treatment is urgently required. Several studies have reported the wide-ranging antiviral activities of flavanones; however, an inhibitory effect of selected compounds yet to be shown against CHIKV. Flavanones are polyphenol specific of citrus fruits, where they are naturally present in high amounts and part of human diet almost exclusively.

In this study, we investigated the antiviral properties of two types of flavanones namely, naringenin and hesperetin against CHIKV *in vitro* replication. Our data have shown dose dependent inhibitory effects for naringenin and hesperetin against CHIKV intracellular replication using different assays including, CHIKV replicon cell line, time of addition and virus yield assay. MTS assay was performed to determine the cytotoxicity of hesperetin and naringenin on Vero and BHK cell lines. Antiviral

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activity of the compounds was further investigated by evaluation of CHIKV protein expression using quantitative immunofluorescence assay and Western blotting. Briefly, these compounds presented significant antiviral activity against CHIKV, reducing both CHIKV replication efficiency and down-regulating production of viral proteins involved in replication.

Naringenin with IC_{50}=6.818 µM (SI=80.27) and hesperetin with IC_{50}=8.500 µM (SI=23.34) inhibited the post entry stages of CHIKV replication activity. The replication efficiency of CHIKV at each antiviral assay was revealed by using the qRT-PCR assay with RNA copy number as guideline. In conclusion, data obtained from the current study suggest that naringenin and hesperetin could be potential candidates to be developed further as anti-CHIKV therapeutic agents. This study may have significant outcome to broaden the chance of discovering the effective antiviral agent for CHIKV infection.

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ABSTRAK

Chikungunya virus (CHIKV) merupakan arbovirus yang telah muncul baru-baru ini di peringkat global. Ia telah menimbulkan impak progresif besar pada manusia semenjak kebelakangan ini, dimana berkemungkinan akan mengancam nyawa dan melumpuhkan arthritis. CHIKV menjangkiti manusia melalui gigitan vektor nyamuk. Institut Alahan dan Penyakit Berjangkit (NIAID) telah mengklasifikasikan CHIKV sebagai patogen Kategori C. Ini adalah kerana ia mempunyai beberapa ciri-ciri klinikal yang sama kepada jangkitan virus denggi, kecuali yang berkaitan dengan polyarthritis lampau dan tenosynovitis, di mana persamaan kedua-dua penyakit menyebabkan ianya tersalah diagnosis. Secara keseluruhan, berjuta-juta kes CHIKV telah dilaporkan di lebih daripada 50 buah negara.

Pada masa kini, tiada lagi antiviral yang efektif atau vaksin berlesen yang dibangunkan untuk rawatan jangkitan CHIKV. Rawatan biasanya dirawat berdasarkan gejala, katil rehat, cecair, dan ubat-ubatan untuk melegakan gejala-gejala demam dan sakit. Oleh itu kajian berkaitan sebatian plumbum dengan aktiviti anti-CHIKV dapat dimajukan lagi untuk rawatan praktikal amatlah diperlukan. Beberapa kajian telah melaporkan bahawa aktiviti-aktiviti anti-virus yang meluas daripada flavanones; walaubagaimanapun, kesan membantutkan sebatian terpilih telah ditunjukkan terhadap CHIKV. Flavanones adalah polifenol tertentu buah-buahan sitrus, di mana ia hadir secara semulajadi dalam jumlah yang tinggi dan sebahagian daripada diet manusia hampir secara eksklusif.

Dalam kajian ini, kami telah mengkaji sifat-sifat anti-virus daripada dua jenis flavanones iaitu naringenin dan hesperetin terhadap CHIKV replikasi secara in vitro. Data kami menunjukkan kesan membantut dos bergantung bagi naringenin dan hesperetin terhadap replikasi CHIKV di dalam sel menggunakan kaedah yang berbeza

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termasuk, sel replikon CHIKV, masa tambahan dan kaedah hasil virus. Kaedah MTS dilakukan untuk menentukan ketahanan sel-sel Vero dan BHK terhadap toksik daripada hesperetin dan naringenin. Aktiviti sebatian antivirus telah ditentukan oleh ekspresi protein CHIKV dengan menggunakan kaedah kuantitatif immunofluorescence dan western blotting. Secara ringkas, sebatian ini menunjukkan kesan aktiviti antivirus yang ketara terhadap CHIKV, dimana ia mengurangkan replikasi CHIKV dan mengawalselia pengeluaran protein virus yang terlibat dalam replikasi.

Naringenin dengan IC50 = 6,818 mikron (SI = 80.27) dan hesperetin dengan IC50 = 8.500 mikron (SI = 23.34) membantut aktiviti replikasi CHIKV pada peringkat pos kemasukan. Kecekapan replikasi CHIKV pada setiap keadah antiviral ditunjukkan melalui qRT-PCR dengan nombor salinan RNA sebagai panduan. Kesimpulannya, data yang diperoleh dari kajian semasa dapat mencadangkan bahawa naringenin dan hesperetin dapat dijadikan calon yang berpotensi untuk dibangunkan sebagai agen terapeutik anti-CHIKV. Kajian ini juga mempunyai hasil yang besar untuk meluaskan peluang penemuan antivirus yang berkesan untuk jangkitan CHIKV.

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LIST OF SYMBOLS AND ABBREVIATIONS

-	Minus
%	Percentage
˚C	Degree Celsius
>	More than
÷	Divide
≥	More than or equal to
µg/ml	Microgram per milliliter
µM	Micro molar
Ae.	*Aedes* species
CC_{50}	Half-maximal concentration exhibit cytotoxicity
CD4	Cluster of differentiation 4
CHIKV	Chikungunya virus
CPE	Cytopathic effect
DENV	Dengue virus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	Envelope protein 1
EC_{50}	Half-maximal showing effective response
ED_{50}	Median effective dose
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EtOAc	Ethyl acetate
H1N1	A strain of influenza virus
HCMV	Human cytomegalovirus

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HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
hpi	Hour post-infection
IC_{50}	Half-maximal concentration showing inhibitory effect
ID_{50}	Half-maximal dose causing infection
IFN-α	Interferon alpha
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRES	Internal ribosome entry site
IV	Intravenous
JEV	Japanese encephalitis virus
kb	Kilo base
kDa	Kilo Dalton
mg	Milligram
mL	Milliliter
MNTD	Maximum non-toxic dose
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
ng	Nano gram
nm	Nanometer
NS5B	Non-structural protein 5B
nsps	Non-structural proteins
ORF	Open reading frame
PBS	Phosphate buffer saline

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pH	A figure expressing the acidity or alkalinity of a solution on a logarithmic scale on which 7 is neutral, lower values are more acid, and higher values more alkaline
qRT-PCR	Quantitative reverse transcription- polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDF-1	Stromal-derived factor 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean
SI	Selectivity index
$TCID_{50}$	Half-maximal tissue culture inhibition dose
UV	Ultraviolet
UTR	Untranslated region
µg/ml	Microgram per milliliter

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CHAPETR 1: INTRODUCTION

1.1 Chikungunya Virus

Chikungunya virus (CHIKV) is an arthropod-borne virus; belonging to the *Alphavirus* genus of the group IV *Togaviridae* family (Lumsden, 1955). There are more than 40 recognized members of *Alphaviruses* that cause diseases in humans and animals (Powers et al., 2001). These species of arboviruses have been classified into 7 antigenic complexes: Barmah Forest (BF), Eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SF), Venezuelan equine encephalitis (VEE), and Western equine encephalitis (WEE), which are widely distributed throughout the world. *Alphaviruses* can be broadly divided into New World and Old World viruses which have developed two different ways of interacting with their respective hosts and differ in their pathogenicity, tropism and interference with virus-induced immune responses (Peters et al., 2005). From a clinical prospective, predominantly New World viruses are associated with encephalitis, whereas poly-arthritis and rashes are predominantly associated with Old World *Alphaviruses* (Griffin, 2007; Powers et al., 2001). Serologically, Chikungunya virus is part of the Semliki Forest SF group of Old World *Alphaviruses* (Calisher et al., 1980). Although chikungunya virus is a member of the arthritogenic *Alphaviruses*, there are also cases of meningoencephalitis and haemorrhagic disease (Powers et al., 2001). Electron microscopy studies of CHIKV in green monkey kidney (Vero) cells demonstrated a characteristic *Alphavirus* morphology **Figure 1.1** (Simizu et al., 1984).

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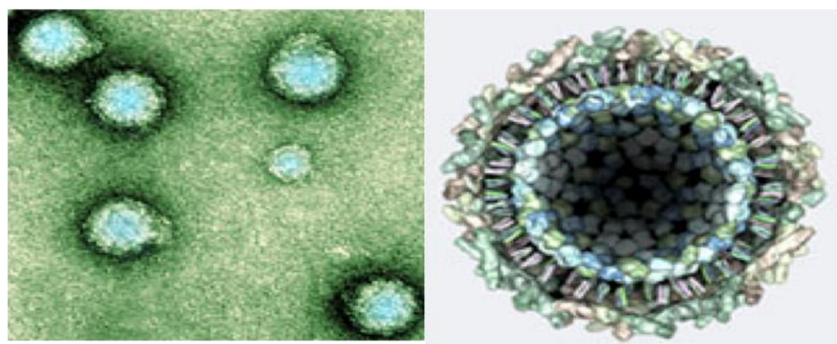

Figure 1.1 Chikungunya virus particle and electron microscopic picture of Chikungunya virions, which exhibit typical *Alphavirus* structure (Simizu et al., 1984)

1.1.1 CHIKV genomic organization

As a member of the *Alphavirus* genus, CHIKV is a small, spherical about 60–70 nm-diameter in neutral pH, with an icosahedral nucleocapsid enclosed in a lipid-protein envelope (Lumsden, 1955; Rashed et al., 2014; Strauss & Strauss, 1994). It is sensitive to desiccation and temperature of >58°C. *Alphaviruses* are among the simplest membrane-enveloped viruses. The genome consists of a linear, positive-sense, single-stranded RNA molecule of about 11,805 nucleotides long and is capped with 7-methylguanosine in 5′ end and has a polyA tail in the 3′ end **Figure 1.2** (Rashad et al., 2014).

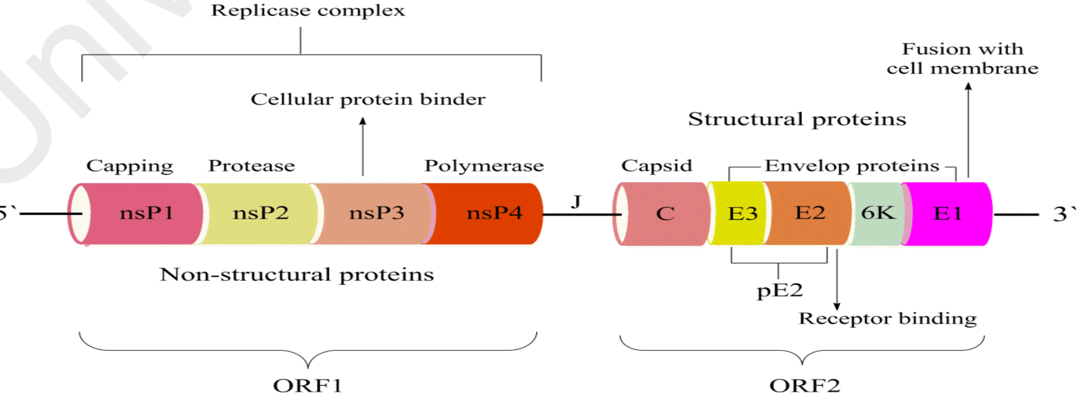

Figure 1.2 Schematic representation for the CHIKV genome showing the RNA sequence ORFs (Rashad et al., 2014)

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The genome structure includes two open reading frames (ORFs) that encodes for two polyproteins – the non-structural polyproteins consisting of four proteins (nsP1, nsP2, nsP3 and nsP4) and the structural polyproteins consisting of five proteins (Capsid, E3, E2, 6K and E1), which can be cleaved by viral and cellular proteases (A. H. Khan et al., 2002). Roles of each non-structural polyproteins are shown in the **Table 1.1**.

Table 1.1. The roles of non-structural polyproteins

Non-structural protein (nsP)	Roles
nsP1	Involved in the synthesis of the negative strand of viral RNA and and has RNA capping properties
nsP2	Act as protease and helicase and involved in closing down the host cell transcription
nsP3	Part of replicase unit and an accessory protein involved in RNA synthesis
nsP4	Viral RNA polymerase

These nsP123 and nsP4 polyproteins form the viral replication complex to synthesis a full-length negative stand RNA intermediate to serve as a template for synthesis of both subgenomic (26S) and genomic (49S) RNAs. The subgenomic RNA is translated from the 3′ end ORF, which is also capped and encodes the structural proteins, the capsid C, envelope glycoproteins E1 and E2 and two small cleavage products E3 and 6K. These proteins are derived from the polyprotein precursor, C-pE2-6K-E1 through its autoprotease activity. The E1 and E2 play significant roles during the early stages of CHIKV replication cycle, in which E1 mediates the fusion of virus envelope with the endosomal membrane during the entry of CHIKV into the host cell (Omar & Koblet, 1988), whereas the E2 glycoproteins facilitates receptor recognition and attachment of the virus to the surface receptors of the target cell (Voss

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et al., 2010). E3 mediates the folding of E2 precursor (pE2) into E2 as well as its subsequent association with the E1 glycoprotein to form a dimer (Snyder & Mukhopadhyay, 2012). In addition to this, E3 also protects the newly synthesized virus particles from the surrounding non-conducive pH environment during assembly and budding (Uchime et al., 2013). Capsid (C) protein is fundamental for the formation of a nucleocapsid core following its association with viral genomic RNA (Stefan W Metz et al., 2011; Stefan W. Metz et al., 2011). The exact function of 6K protein is yet to be known, however defective or altered 6K protein has been demonstrated to cause inefficient release of virions and the release of multi-cored mature virus particles (Gaedigk-Nitschko et al., 1990).

1.1.2 Virus Replication cycle

Chikungunya replication cycle is similar to the replication of other *Alphaviruses.* Virus enters the host cells following the interaction of the E2 envelope glycoproteins with surface receptors of the target cells. Upon the receptor binding the virus is internalized thorough pH dependent endocytosis in clathrin coated vesicles. The acidic environment of the endosome causes the conformational changes in the viral envelope glycoproteins, which enables the virus-host cell membrane fusion (Gould et al., 2010). This allows the virus particles to undergo disassembly, releasing the viral RNA genome into the cytoplasm of infected cells. Then, the viral genomes translate by the host cell machinery to generate the non-structural polyproteins, which is cleaved into the nsp1-4. These nsps interact with host proteins to form an unstable initial replication complex, which is able to synthesis the negative strand RNA (Solignat et al., 2009). The negative stand of RNA is then used as a template for synthesis of the positive stand of subgenomic (26S) mRNA and genomic (49S) RNA. The subgenomic

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(26S) mRNA is translated to polypeptide containing the structural viral proteins C-pE2-6K-E1 that is processed by an outoproteolytic serine protease to release the capsid into the cytoplasm (Gould et al., 2010). The remaining PE2-6k-E1 polypeptide is redirected in the endoplasmic reticulum for further post-translational modifications. The pE2 and E1 glycoprotein form the heterodimer complexes that migrate toward the cell membrane through Golgi complex, where pE2 is cleaved by cellular furin and furin like proteinase into mature E2 and E3. Lastly, mature virions assemble in the cytoplasm via binding of the viral nucleocapsid to viral RNA and bud out through the cell membrane acquiring a lipid bilayer envelope that contains the virus encoded glycoproteins E1-E2 (Gould et al., 2010).

1.1.3 Transmission

CHIKV is a mosquito-borne arbovirus. Its transmission involves the female mosquito vectors, mainly from the genus *Aedes*. *Aedes aegypti* and *Aedes albopictus* are the two most common vectors for transmission of CHIKV to humans, however, in the recent epidemic some cases were the result of maternal-fetal transmission (Gérardin et al., 2008). These species can also transmit other viruses, including dengue and zika virus. Human to human spread of CHIKV occurs when the mosquito vector feeds on the blood of an infected person, then transmitting the acquired virus to the next person whose blood they feed on. *Aedes albopictus* also known as Asian tiger mosquito was the main contributing vector for the transmission of CHIKV to new geographical regions where *Aedes aegypti* was absent, such as the Reunion Island, Mauritius and southern Europe. This was due to the improved molecular adaptability of CHIKV to its new vector as a result of a single E1 – A226V mutation, which was known as a most signification event in the history of CHIKV (Konstantin A Tsetsarkin

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et al., 2007). Successful isolation of CHIKV from mosquitoes *Culex ethiopicus* suggested that *Culex spp.* could contribute to CHIKV transmission in the community though the frequency of virus isolation from this species of mosquitoes was low (Diallo et al., 1999). Furthermore, a recent study in India has shown that *Anopheles stephensi*, an anthrophilic species of mosquito is capable of transmitting CHIKV (Yadav et al., 2003). However during epidemics, human are the primary reservoirs. Monkeys and other vertebrates such as rodents, birds and even small mammals have been identified as animal reservoirs (Diallo et al., 1999). The CHIKV commonly appears periodicity with occurrence of disease in the community with the latency intervals of 3-4 years, probably due to its cycle in monkeys (Barrett & Weaver, 2002; Diallo et al., 1999).

1.1.4 Pathogenesis and Clinical Manifestation

Following the transmission, CHIKV directly enters the subcutaneous capillaries where replication starts with some viruses infecting skin resident cells, such as dermal macrophages, fibroblast and endothelial cells (Couderc et al., 2008; Kam et al, 2009; Sourisseau et al., 2007). At this stage, viral replication restricted with the viruses being transported to secondary lymphoid organs, where new viruses produced from infected migratory cells to infect susceptible resident cells. Though the initial host immune response has been triggered at this stage, the virus rapidly disseminates into the lymph nodes via blood circulatory system (Kupper & Fuhlbrigge, 2004; Schilte et al., 2010). Once in the blood, the free virions will have access to the target organs, including the liver, muscle, joints and brain, developing the viremic phase of the disease. Virus can be easily transmitted to mosquitoes via blood meal during this phase (Kam et al., 2009). Once, CHIKV is replicated in these tissues, infection is associated with variety

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of cells including macrophages. The pathological manifestations within the tissues are normally subclinical in the liver as hepatocyte apoptosis and in lymphoid organs as adenopathy, whereas in the muscles and joints appears with very strong pain, with some patients experiencing arthritis (Dupuis-Maguiraga et al., 2012; Schwartz & Albert, 2010).

Earlier symptoms of CHIKV infection are similar to classical dengue fever including high fever, rigors, headache, photophobia and a petechial rash or maculopapular rash. Except, CHIKF is associated with intense arthralgia, which is strongly predictive of chikungunya (Morrison, 1979; Yazdani & Kaushik, 2007). The incubation period is typically short, lasting 2-4 days followed by a sudden onset of clinical symptoms. 'Silent' infection has been reported for around 15% of infected individual (Lemant et al., 2008).

During the acute phase of infection, symptoms may persist for 3-7 days, whereby the amount of CHIKV can increase to as high as 10^8 viral particles per mL of human blood. The symptoms in this phase are mostly due to the spread of infection on epithelial, endothelial as well as monocytes-derived macrophages and primary fibroblast. Consequently, the immune system is stimulated, leading to induction of pro-inflammatory cells like cytokines, chemokines on immune cells such as neutrophils, monocytes and macrophages (Chow et al., 2011). Type-1 interferon increases to the range of 0.5-2 ng per ml, which was suggested to play a role in causing severe CHIK fever (Couderc et al., 2008). The chronic and incapacitating arthralgia or myalgia may persist for weeks, months or even years (Brighton et al., 1983). Although CHIKF mortality rate is generally considered vey low, severe forms involving neurological and hepatological complications can occur (Pialoux et al., 2007; Simon et al., 2006).

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1.1.5 Phylogenetic and Epidemiology

Genetic analysis based on the E1 envelope glycoprotein sequences classified three distinct genotypes of CHIKV: the West African (WAf), the East-Central and South African (ECSA), and the Asian, which were named based on geographical origins **(Appendix A)** (Arankalle et al., 2007; Powers et al., 2000; Powers & Logue, 2007). Apparently, The CHIKV strains inhabited in these different geographic lineages circulate in regions that display different ecological backgrounds. The variance of each distinct lineage somehow reflected the path of worldwide transmission and periodic outbreaks. Several studies have shown that the genome length varied amongst and within the different geographic lineages. Removing the 20 nucleotides of both the 5' and 3' ends from sequencing, the genome of the ECSA lineage was found to be shortest among the three lineages (11,557 to 11,789 nucleotides) in comparison with the 11,843 to 11,881 nucleotides length of the WAf lineage and the Asian lineage, which possesses a genomic length of 11,777 to 11,999 nucleotides. Nucleotide differences in CHIKV genome from different lineages were due to the highly variable regions including 5' and 3' UTRs as well as the 26S junction region (Volk et al., 2010). Hence, over the years, CHIKV has adapted to vectors of different species aside from different environmental settings. To be noted that CHIKV's ORFs were also recognized as a highly conserved region within the virus genome.

CHIKV was first isolated from the serum of a febrile patient during an outbreak that occurred in the southern province of Tanzania (Makonde Plateau) in 1952-1953 (Robinson, 1955; Ross, 1956). The name "Chikungunya" is derived from the Mankone word meaning "to be bent over" relating to the stooped-over posture exhibited by individuals with the disease as a consequence of severe chronic incapacitating

arthralgias (Mavalankar et al., 2008). CHIKV likely originated in Africa, where the virus was found in 'sylvatic cycle' between wild primates and forest dwelling mosquito species. It was then introduced to Asia where it was transmitted from human to human mainly by both *Ae. aegypti* and *Ae.albopictus* through an urban transmission cycle (Jupp & McIntosh, 1988; Presti et al., 2012). Since its isolation, CHIKV caused several minor outbreaks in Africa and major epidemics in 1960s to 70s in India and Southeast Asia (Nimmannitya et al., 1969). In South-East Asia, epidemics have been documented in India, Pakistan, Sri Lanka, Myanmar, Thailand, Indonesia, the Philippines, Cambodia, Vietnam, Hong Kong, and Malaysia (Bhatia & Narain, 2009; Mohan, 2006; Pialoux et al., 2007). Subsequently only sporadic outbreaks were detected for the following 30 years with no major recurrence until a large outbreak in 2004 in Kenya (Njenga et al., 2008). Since 2005, ECSA strains from East Africa caused a massive outbreak in in the Islands of the Indian Ocean, which includes areas like Mauritius, La Reunion, Comoros, Madagascar and Seychelles, with more than 272,000 people infected (Beesoon et al., 2008; Josseran et al., 2006). The high incidence then spread to India when infected travellers returned from the Indian Ocean, whereby more than 1.5 million cases were reported (Lanciotti et al., 2007). Rapid spread of CHIKV in some locations may also be due to a mutation at residue 226 of the membrane fusion glycoprotein E1 (E1-A226V) that enhanced the replication and transmission efficiency in the *Ae. albopictus* mosquito. This mutation was detected in more than 90% of Chikungunya virus isolates from Reunion Islands after September 2005 (Konstantin A et al., 2007).

In 2010, a traveller who returned to France from India was diagnosed with CHIKV infection (Grandadam et al., 2011). Latter, CHIKV further led to clusters of autochthonous cases in Southern Europe like Italy (Angelini et al., 2007). Since late

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2013, a strain of CHIKV originating from Asia emerged in the Caribbean and spread widely to South, Central and North America (Campion et al., 2015; Weaver & Forrester, 2015). As of January 2015, 1.135 million of suspected cases were recorded in the Caribbean islands, Latin America countries and the U.S.A, with 176 deaths. Currently, chikungunya fever has been identified in nearly 40 countries. In 2008, chikungunya was declared as a US National Institute of Allergy and Infectious Diseases (NIAID) category C priority pathogen (Powers & Logue, 2007; Schwartz & Albert, 2010).

Based on earlier studies in Malaysia, seropositivity towards CHIKV have been found within the community in northern and eastern states of Peninsular Malaysia bordering Thailand (Marchette et al., 1980) as well as in East Malaysia (Bowen et al., 1975). This suggests that CHIKV has been present in Malaysia, but the transmissions were either rare or undiagnosed. The first outbreak of CHIKV was reported at Klang in 1991, where 51 infections were confirmed during the three-month period from December 1998 and February 1999 (SK Lam et al., 2001). After a hiatus of 7 years, a second outbreak occurred in Bagan Panchor, Perak in 2006, where at least 200 villagers were infected (AbuBakar et al., 2007). The re-emergence in Bagan Panchor coincides with the ongoing epidemic in the Indian Ocean and India which began in February 2005 (Paganin et al., 2006). Similarly, both outbreaks involve CHIKV of the Asian genotype (Ayu et al., 2010), whereas the ECSA lineage was identified in Batu Gajah in late 2006, isolated from a 44-year Indian man who have just returned from India. During the period of time, 108 cases were reported in 3 districts of Perak, namely Larut Matang Lama, Kerian and Kinta (Chua, 2010). The latest CHIKV outbreak in Malaysia was in the state of Johor which lasted from April till September 2008 (Zainah, Berendam, Rogayah, Khairul, & Chua, 2010). More than 1,000 cases

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were reported over the period of time. The index case was reported in Kampung Ulu Choh, Gelang Patah, prior to the spread throughout Johor. During the same period of time, high numbers of incidents were reported in Selangor, Melaka, Negeri Sembilan and Pahang. Since then, epidemics have occurred in other states such as Sarawak (2009) and Sabah (2009 & 2010), with urban areas such as Kuala Lumpur and Penang recording sporadic cases of infection.

1.1.6 Research and development on antiviral drugs and vaccine

Despite the recent threat posed by CHIKV, there is currently no vaccines or antivirals for the prevention or treatment of CHIKV infections. Up to now, treatment is purely symptomatic with analgesics, antipyretics (particularly paracetomal, ibuprofen and acetaminophen), often combined with non-steroidal anti-inflammatory drugs in addition to sufficient fluid and rest. Corticosteroids treatment may also be necessary to treat subacute and chronic symptoms (Staikowsky et al., 2008). During the La Reunion outbreak, chloroquine which was reported to be effective in treating certain forms of chronic arthralgia and may also inhibit *in vitro* CHIKV replication cycle, was administrated as a trial but it was found to be not effective (Brighton, 1984; Lamballerie et al., 2008). Ribavirin as an antiviral agent was given to patients to treat CHIKV induced arthritis, which was found to be helpful in resolving joint and soft tissue swelling (Ravichandran & Manian, 2008). Various substances that inhibit the chemokine pathways involved in recruiting monocytes and macrophages have also been suggested as a mode of relieving arthralgia in patients (Rulli et al., 2011).

Due to the high density of mosquito vectors in many parts of the world, which could serve as a vector for CHIKV, there is an urgent need for the development of safe and efficacious antivirals against CHIKV. Subsequently, with the re-emergence of

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CHIKV in recent years, countless studies have been made towards the development of antivirals against CHIKV. Although many antiviral compounds have been revealed to be effective in cell culture, barely few compounds have been considered in animal models. A summary of compounds/drugs that have shown anti-CHIKV activities is listed in **Table 1.2**.

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Table 1. 2 Major tested chemical compounds/drugs with their anti-CHIKV activities

Compounds/Drugs	Assay type	Anti-CHIKV activities/Targets	References
Arbidol	In vitro (primary human fibroblast and Vero cells)	Inhibitors of viral entry	(Delogu et al., 2011)
Chloroquine	*In vitro* (Vero cells)		(Delogu & de Lamballerie, 2011; Mohsin Khan et al., 2010)
10H-phenothiazines (chlorpromazine,perphenazine, Ethopropazine, thioridazine thiethylperazine and methdilazine)	*In vitro* (BHK-CHIKV-replicon cell line)		(Pohjala et al., 2011)
Homoharringtonine & Harringtonine	*In vitro* (BHK21 cells)	Inhibitors of viral protein translation	(Kaur et al., 2013)
RNA interference (siRNA & shRNA)	*In vitro* &*In vivo*		(Shirley Lam, Chen, Ng, & Chu, 2012)
Ribavirin	Human	Inhibitors of viral genome replication	(Ravichandran & Manian, 2008)
6-Azauridine	*In vitro* (Vero cells)		(Briolant et al., 2004)
Mycophenolic acid			(M. Khan et al., 2011)
IFN-α (2a and 2b)	*In vitro* (Vero cell)	Host immune response modulators	(Briolant et al., 2004)
Polyinosinic acid:polycytidylic acid [poly(I:C)]	*In vitro (*BEAS-2B cells)		(Y.-G. Li et al., 2012)
Decanoyl-RVKR-chloromethyl ketone	*In vitro* (human muscle cells)	Inhibitors of viral glycoprotein maturation	(Ozden et al., 2008)

Thus far, there is no licensed vaccine available. Scientists have made several approaches to identify effective vaccines against CHIKV infection. During 1980s the Walter Reed Army Institute of Research produced a vaccine containing an attenuated strain of CHIKV (181/25) obtained by serial passage in MRC-5 cells was administered in both mice and non-human primates. The virus managed to protect the animal against subsequent challenge with parent virus. However, during phase II trials, strain

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181/25 caused mild transient arthralgia in 8% of the vaccinees (Levitt et al., 1986).

Other promising vaccines candidates like formalin-inactivated vaccine (Tiwari et al., 2009), virus like particles (VLPs) (Akahata et al., 2010) and DNA vaccines have also been developed and tested (Muthumani et al., 2008). Immunization with VLPs and DNA vaccines produced high-titered neutralizing antibodies and protected the mice and non-human primates against viremia after CHIKV challenge. Wang and colleagues in 2008 developed chimeric *alphavirus* vaccine containing either Venezuelan equine encephalitis (VEEV) or Eastern equine encephalitis virus (EEEV), or Sindbis virus as a backbone and the structural protein genes of CHIKV. These vaccines produced robust neutralizing antibodies (Ab) and provided complete protection against disease after CHIKV challenge (E. Wang et al., 2008). Yet, these viruses still had the capability to infect potential mosquito vectors and attenuation was dependent on an intact murine interferon response. To overcome these limitations, another chimaeric vaccine containing VEEV strain TC-83 and encepahalomyocarditis virus (EMCV) IRES (internal ribosome entry site) in the subgenomic promoter was designed to diminish the transmission to mosquitoes. Basically, the EMCV IRES sequence cannot competently drive translation in arthropod cells. This vaccine was poorly immunogenic with no neutralizing antibody response. Hence, they developed a vaccine by introducing the EMCV IRES sequence into the CHIKV subgenomic promoter. This reduced the replication of the vaccine strain in mosquitoes and was highly immunogenic and efficacious in mice after a single dose. This vaccine is useful for predictable attenuation of any alphavirus. (Plante et al, 2011).

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Recently, a distinctive chimeric vaccine has been established using a vesiculostomatitis virus (VSV) backbone and CHIKV structural proteins (VSVΔG-CHIKV). This VSVΔG-CHIKV chimaeric virus induced a decent neutralizing antibody response and protected mice against CHIKV infection (Chattopadhyay et al., 2013). While vaccine development research against CHIKV is still ongoing, the world remains under the threat of rapidly spreading CHIKV infections and this underlines the importance of developing chemotherapeutics against the virus for controlling the existing infections in infected areas.

1.2 Flavonoids

Flavonoids, or bioflavonoids, are a group of natural compounds with diverse phenolic structures that can be found ubiquitously in plants as secondary metabolites (Tsuji & Walle, 2008). They are synthesized through phenylpropanoid pathways and encompasses series of reactions (Hwang et al., 2003). Their basic structure is a 15 carbon skeleton of diphenylpropane, two benzene rings (A and B, **Figure 1.3**) linked via a heterocyclic pyrane ring (C), which is referred to as C6-C3-C6 (Kumar, Mishra, & Pandey, 2013).

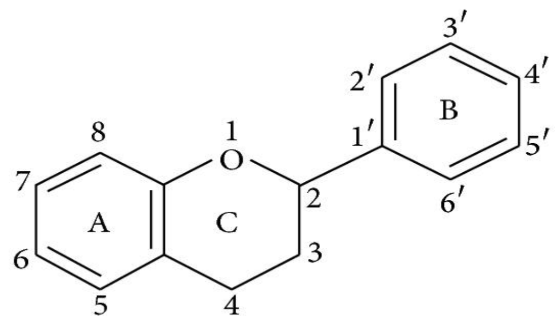

Figure 1.3 The general chemical structure of flavonoids

Flavonoids can be subdivided into different subgroups based on the carbon on

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the central pyrong ring, the C ring to which the B ring is attached, as well as the degree of unsaturation and oxidation of the C ring. Flavonoids in which the B ring is attached to position 3 of the C ring are called isoflavones and those B ring is attached to position 4, neoflavonoids whereas those in the which B ring is attached to position 2 can be further subdivided into different subgroups according to the molecular structure into flavones, flavonols, flavanones, flavanonols, flavanols or catechins and anthocyanins. Flavonoids with open C ring are also called chalcones **Figure 1.4** (Middleton Jr, 1998; Tsuchiya, 2010).

Flavones Flavonols Flavanones Flavanonols Flavanols Anthocyanidins Isoflavones Neoflavonoids Chalcones

Figure 1.4 Flavonoid subgroups (Middleton Jr, 1998; Tsuchiya, 2010)

Flavonoids structure configurations greatly determine their activities, whereby their chemical properties are influenced by their structural classes, metabolic processes, involving substitutions and conjugations, as well as the degree of hydroxylation and polymerization (Heim, Tagliaferro, & Bobilya, 2002). Flavonoids

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can exist either in the free form whereby sugar molecules are absent (aglycones), or in the form in which they are linked to one or more carbohydrates (glycosides) (Williamson et al., 2004). In plants as well as foods, flavonoids are commonly found in the form of glycosides, except for the flavanols such as catechins and proanthocyanidins. Upon ingestion, the bioavailability of flavonoids in humans depends on its physicochemical properties, which includes solubility, lipohilicity, configuration, molecular size and pKa, as well as structure, whereby aglycones are the more easily absorbed by the small intestine as compared to glycosides (Hollman et al., 1999). Following absorption in the small intestine, flavonoids are rapidly metabolised via the addition of methyl group, glucoronate or sulfation in the liver. Therefore, free flavonoids cannot be found in plasma or urine, except for catechins (Manach et al., 2004).

Flavonoids are extensively distributed among the plant kingdom and over 4,000 kinds have been characterized and classified so far (Cushnie & Lamb, 2005). They are found in vegetables, fruits, nuts, seeds, stem, flowers, tea, wine etc. Flavonoids truly form an important part of human diet (Cook & Samman, 1996; O'Prey, Brown, Fleming, & Harrison, 2003; Sahu & Gray, 1996). It has been estimated that the dietary intakes of flavonoid is about 1-2 g/day (Fernández et al., 2006). The average intake of flavonols and flavones is 23 mg/day, among which, flavonol quercetin contributed 16 mg/day (Heim et al., 2002). The flavonoids are one of the safest non-immunogenic agents because they are small organic compounds, which can naturally be absorbed by the human body. Flavonoids are recognized to exert a wide array of pharmacological activities: including anti-inflammatory, anti-cancer, anti-allergic, anti-microbial properties. The hydroxyl groups in flavonoids are involved in their antioxidant properties by metal ions chelation as well as scavenging of free radicals (Kumar et al.,

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2013). Damage to target biomolecules by free radicals can be prevented by flavonoid's metal chelation properties (Kumar et al., 2013; Leopoldini et al., 2006). Hence, flavonoids have been suggested to possess the potential protective effects against oxidative stress-causing diseases, such as the neurodegenerative diseases (Parkinson's disease and Alzheimer's disease) (Ramassamy, 2006). Besides, administration of flavonoids have been found to prevent the development of cognitive impairment because of ageing, oxidative stress and inflammation (Patil et al., 2003). However, due to the rapid metabolism of flavonoids in humans, it was found that the resulting flavonoid metabolites exert weaker antioxidant effects when compared to other antioxidants such as the ascorbic acid (Lotito & Frei, 2006; Williams, Spencer, & Rice-Evans, 2004). Flavonoids as well as the weaker flavonoid metabolites are capable of modulating the cell signalling pathways by selectively inhibiting the transcription and expression of kinases (Hou et al., 2004; Spencer, Rice-Evans, & Williams, 2003; Williams et al., 2004). As a result, it was suggested to possess anti-cancer properties by regulating the growth factors signalling pathway, hence able to preserve the normal cell cycle, inhibit excessive abnormal cell proliferation, induce apoptosis as well as tumor invasion and angiogenesis inhibition (Bagli et al., 2004; J. Chen, Ye, & Koo, 2004; M. H. Kim, 2003; Ramos, 2007; Sah et al., 2004; W. Wang et al., 2004). Moreover, via the regulation of cell signaling pathways, flavonoids have also been suggested as being able to prevent a person from developing cardiovascular disorders (Vita, 2003). This is made possible by the role of flavonoids in increasing the endothelial nitric oxide synthase (eNOS) activity as vasodilators, decreasing the expression of vascular cell adhesion molecules and via the inhibition of platelet aggregation (Vita, 2003). Many flavonoids have been found to exert antibacterial,

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antifungal as well as antiviral actions (Tereschuk et al., 1997; Wächter et al., 1999; Zheng et al., 1996).

Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication but baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase. Flavonoids including demethylated gardenin A and robinetin are also revealed to inhibit HIV-1 proteinase (Cushnie & Lamb, 2005). Zandi and colleagues studied the antiviral activities of daidzein quercetin, naringin, and hesperetin at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells (Zandi et al., 2011). Several flavonoids such as quercetin, morin, apigenin, baicalein, rutin, fisetin and hesperidine have been reported for their antiviral activities against dengue virus, Japanese encephalitis virus, herpes simplex virus, enterovirus-71, human cytomegalovirus and even human immunodeficiency virus (Moghaddam et al., 2014; Selway, 1986; Zandi et al., 2012a; Zandi et al., 2011).

In plants, flavonoids play vital roles in both the physical appearance and defense mechanisms. Flavonoids are essential pigments for providing flowers with various colours, which enable them to attract insects or animals as their pollinating agents (Nishihara & Nakatsuka, 2011). They were also found to regulate the expression of growth factors in plants such as auxin (Taylor & Grotewold, 2005). Flavonoids are located in the nuclei of mesophyll cells as well as the sites of free radicals generation in plants, hence playing an important role as a secondary antioxidant defense system against environmental stresses (Agati, Azzarello, Pollastri, & Tattini, 2012). Flavonoids have also been found to have inhibitory effects against infection and growth of microorganisms which could be pathogenic to plants, such as

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the *Fusarium oxysporum* fungi (Galeotti et al., 2008). Furthermore, flavonoids are also involved in photosensitization, respiration and photosynthesis control, sex determination, morphogenesis and energy transfer (Cushnie & Lamb, 2005).

Nowadays, many researchers and pharmaceutical companies have focused on dietary products as a potential resource for drug discovery and development because of their varied health benefits and therapeutic potential due to the presence of pharmacologically active compounds (Hossen, 2015). There are many advantages for choosing flavonoids as candidate against CHIKV infection. This include low toxicity in animal, low side effects, prolonged half-life, high accessibility in nature and consumed daily by human (Kwon et al., 2010; Middleton, Kandaswami, & Theoharides, 2000; Zandi et al., 2009). In this study, 2 flavonoids in the class of flavanone, hesperetin and naringenin (chemical structure in **Table 1.3**) were selected to examine their *in vitro* antiviral activities against the replication of CHIKV.

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Table 1.3 Structural characteristics and molecular weights of selected flavanones

No	Flavanone	Chemical Formula	Molecular Weight (g/mol)	Chemical Structure
1	Hesperetin	$C_{16}H_{14}O_6$	302.28	OH O HO O OH OCH3
2	Naringenin	$C_{15}H_{12}O_5$	272.257	OH O HO O OH

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1.3 Research objectives

The main objectives of this research are:

1. To explore the antiviral activities of hesperetin and naringenin against Chikungunya virus replication in cell culture.
2. To determine the step(s) of viral replication cycle that can be blocked by hesperetin and naringenin.

- To investigate the effect of hesperetin and naringenin on Chikungunya protein expression and genome.

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CHAPTER 2: LITERTURE REVIEW

2.1 Progress and pitfalls of antiviral drug development

Antiviral drugs are helpful in tackling viral disease where there is a lack of an effective vaccine or where infection has already taken place. Developing safe and effective antiviral drugs has been difficult, because most drugs that kill viruses also damage the host's cells, where the viruses hide and replicate. This makes it challenging to find targets for the drug that would inhibit the virus deprived of destroying the host cells. Besides, the major difficulty in developing vaccines and anti-viral drugs is because of viral variation. However, since the 1980s, with the emergence of HIV/AIDS, many researchers have focused on treating viral infections, and positive movement has been seen up to now. A number of antiviral drugs have been officially licensed and are widely used for the chemotherapy of specific viral infections. Other antiviral agents are also being developed which can be categorized into three classes: anti-herpesvirus, anti-retrovirus and anti-rhinovirus compounds. On the basis of targets, antivirals are classified into seven main groups: DNA polymerase inhibitors (DPIs), fusion inhibitors, reverse transcriptase inhibitors (RTIs), portmanteau inhibitors, protease inhibitors, integrase inhibitors and signaling inhibitors. These antiviral drugs rather than killing the viruses, block the stages in virus life cycle. E.g. the first types of effective antiviral drugs were nucleotide analogues (acyclovir and AZT), which are able to block the viral genome replication. Many other antiviral drugs may also stimulate the immune system so that the body can fight the viruses itself (Field & De Clercq, 2008).

Though, we live in a time of rapid development of antiviral drug, there are still some general limitations to overcome in antiviral chemotherapy, including: toxic side

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effects, inadequate antiviral activity spectrum, inefficiency against the latent phase of virus infection, and development of drug-resistant mutants (Baron & De Clercq, 1996; Bean, 1992).

In principle, a successful anti-viral drug should interfere with a virus-specific function or/and cellular function so as to prevent the virus replication. To be specific, the anti-viral drug must only kill virus-infected cells, which could be done by restricting drug activation to virus-infected cells. An ideal drug should be water soluble, stable in blood stream and easily absorbed by the cells and obviously shouldn't be toxic, carcinogenic, allergenic, mutagenic and teratogenic.

2.2 Flavonoids and their potentials

Apart from importance of flavonoids mentioned in **Chapter 1**, flavonoids have their own importance and role in antiviral research. Flavonoids with antiviral activity have been identified since the 1940s and there are many evidences on their antiviral activities that evolve around inhibition of various enzyme-associated with virus life cycle. The interaction of flavonoids with the different stages in the replication cycle of viruses was previously investigated. For example, some flavonoids work on the intracellular replication of viruses, whereas others inhibit the infectious properties of the viruses. Up to now, most studies of the effects on viruses were performed in vitro and little is known about the antiviral effect of flavonoids in vivo. Interestingly, there is a structure-function relationship between flavonoid and their inhibitory enzyme activity that influences the antiviral activity (Kaul, Middleton, & Ogra, 1985). The antiviral activity seems to be associated with non-glycosidic compounds, and hydroxylation at the 3-position is evidently a requirement for antiviral activity. The naturally occurring 4'-hydroxy-3-methoxyflavonoes have antiviral activity against

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rhino- and poliomyelitis viruses (Middleton et al., 2000). The anti-picornavirus activity of the methoxyflavones was associated with the 4'-hydroxyl and 3-methoxyl groups. These methoxyflavones, poly-substituted in the A-ring, display a better antiviral activity than do non-substituted compounds (De Meyer et al., 1991). There is also some evidence that flavonoids in their glycone form show greatest inhibitory effects on rotavirus infectivity than are flavonoids in their aglycone form (Bae et al., 2000). Furthermore, synergism has also been reported between flavonoids and other antiviral agents. Kaempferol and luteolin shown synergistic effects against herpes simplex virus (HSV). Quercetin is reported to enhance the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infections. Apigenin also enhances the antiviral activity of acyclovir against these viruses (Cushnie & Lamb, 2005).

2.2.1 Baicalein (5,6,7-trihydroxyflavone)

Baicalein is a flavone commonly extracted from a plant named Huangchin (*Scutellaria baicalensis*) and *Scutellaria lateriflora*. It has been used as a medication for thousands of years in China and Japan’s herbal medicine to treat different infectious disease, inflammations hyperlipidemia and hypertension (Cushnie T.P.T & Lamb A.J., 2005). In 2012, Johari and his colleagues studied the *in vitro* antiviral activity of baicalein against Japanese encephalitis virus (JEV) replication in Vero cells. They have shown that baicalein exhibited potent *in vitro* anti-JEV effects at all different stages of JEV infection compared to other tested compound. Baicalein exhibited significant direct virucidal activity (SI = 33.4) as well as intracellular anti-JEV activity (SI = 15.8) and anti-adsorption activity (SI = 15.8). They have found that the mechanism of action baicalein for intracellular anti-JEV activity is binding of

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baicalein to the viral RNA as well as its interaction with JEV structural and/or non-structural protein(s) (Johari et al., 2012). In the same year, baicalein as one of the promising antiviral candidates for human cytomegalovirus (HCMV) infection was reported. It was the most effective compound out of other tested antivirals against HCMV *in vitro* replication. It was shown that baicalein exhibited inhibitory effect against various stages of HCMV replication cycle with IC_{50}=2.2±0.5µM. It was discovered that baicalein reduced the expression of the HCMV immediate early gene (IE-1) as well as total inhibition of IE-2 gene expression. Moreover, baicalein was shown to inhibit the tyrosine kinase activity of the EGF receptor. However, it inhibits the early stage of the viral cycle with the IC_{50} as low as 5µM (Cotin et al., 2012).

Besides, Zandi and his colleagues studied the roles of baicalein in inhibiting different stages of dengue virus type-2 (DENV-2) replication. Baicalein displayed its inhibitory effects against intracellular replication of DENV-2 with IC_{50}=6.46µg/ml; SI=17.8, as well as its inhibition on the early stages of DENV-2 replication cycle such as adsorption phase with IC_{50}=7.14µg/ml. It also inhibited the direct virucidal activity against DENV-2 extracellular particles with IC_{50}=1.55µg/ml (Zandi et al., 2012b).

In 2013, Hour and his colleagues showed that baicalein extracted from *Scutellaria baicalensis* by using ethyl acetate (EtOAc) and chloroform has better antiviral activity against the pandemic 2009 H1N1 and seasonal Influenza A viruses compared to the one that being extracted by using the methanol. The baicalein extracted with EtOAc inhibit the viral neuraminidase activity with the IC_{50} ranges from 73.16 to 487.40µg/ml and the plaque reduction IC_{50} value ranges from 23.7 to 27.4µg/ml. The chloroform-extracted baicalein have shown the plaque reduction ranges from 14.16 to 41.49µg/ml. They have shown that the *in vitro* replication of

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H1N1 strain of influenza A virus was inhibited with the IC_{50} as low as 0.018µM (Hour et al., 2013).

2.2.2 Isoflavones (2-phenyl-*4H*-1-benzopyr-4-one)

Isoflavones are polyphenolic compounds which differed from flavones in their phenyl group location. Legumes, vegetables, grains and mostly soybeans, are the richest sources of isoflavones in the human diet (Fletcher, 2003; Munro et al., 2003). The major isoflavones found in soybeans are water-soluble glycosides (bound to sugar molecule), which are not bioavailable. Fermentation or digestion of soybeans results in the release of the sugar molecule from the isoflavone glycoside, giving an isoflavone aglycone that is bioactive. The soy isoflavone glycosides are named genistin, daidzin, and glycitin, while the aglycones are named genistein, daidzein, and glycitein (Cassidy et al., 2006; Dixon, 2004). Isoflavones are also known as phytoestrogens or plant hormones because they exert estrogen-like effect. Therefore, the consumption of soy generally has been considered beneficial, with a potentially protective effect against a number of chronic diseases including cardiovascular disorders, cancer and osteoporosis (Lampe, 2003).

Isoflavones exert antiviral properties *in vitro* and *in vivo* against a wide variety of viruses (Andres, Donovan, & Kuhlenschmidt, 2009). Up to now, genistein is the most studied soy isoflavone in this regard, and it has been revealed to inhibit the infectivity of enveloped or non-enveloped viruses, as well as single-stranded or double-stranded DNA or RNA viruses. Flavonoids including genistein, at concentrations ranging from physiological to supraphysiological (3.7–370 µM) has been exhibited to reduce the infectivity of a variety of viruses affecting humans and animals **Table 2.1**. Isoflavones have been reported to affect virus binding, entry,

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replication, viral protein translation and formation of certain virus envelope glycoprotein complexes (Andres et al., 2009).

Table 2.1 Viruses inhibited by Genistein.

Viruses	Genistein Inhibitory dose	Model	References
Adenovirus	5–20 µM	SW480 cells	(E. Li et al., 2000)
Arenaviruses	100 µM	Vero cells	(Vela et al., 2008)
BHV-1	25 µM	MDBK cells	(Akula et al., 2002)
Bovine viral diarrhea virus	185–370 µM	MDBK cells	(Lecot et al., 2005)
EBV	30–50 µM	30–50 µM	(Fukuda & Longnecker, 2005)
HSV-1	5–25 µM	Vero cells	(Yura, Yoshida, & Sato, 1993)
HSV-2	50 µM	Vero cells	(Lyu, Rhim, & Park, 2005)
Human CMV	50 µM	HEL 299 cells	(Evers et al., 2005)
HIV	3.7–37 µM	Primary macrophages	(Stantchev et al., 2007)
Respiratory syncytial	25–50 µM	Vero cells	(Rixon et al., 2005)
Rotavirus	33 µM	MA-104	(Andres et al., 2007)
SV40 virus	200 µM	CV-1 cells	(Dangoria et al. , 1996)
MoMLV	62–92 µM	XC cells	(Kubo, Ishimoto, & Amanuma, 2003)

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2.2.3 Glycitein (4',7-Dihydroxy-6-methoxyisoflavone)

Glycitein is an O-methylated isoflavone. It comprises less than 10% of the total isoflavones in soy and soy food products, however, comprises about 50% of the isoflavone mass in soy germ. This phytoestrogen displays weak estrogenic activity in comparison to other soy isoflavones because it has low affinity towards the estrogen receptor at the *in vitro* level (T. Song et al., 1998). Although soy products are extensively used in human diet, there is an inadequate antiviral study but most of the studies mainly focused on the function of glycitein in regulation of estrogen hormone. In 2011, Roh studied the antiviral activity of glycitein on HCV infection. They revealed that glycitein did not show any antiviral activity nor inhibit the activity of HCV viral protein NS5B (Roh, Kim, & Jo, 2011). Later in 2015, another study was performed to investigate the activity of glycitein on HSV replication mainly associated with skin and epithelial mucosa infections. Glycitein showed no antiviral effects out of other selected compounds (Argenta et al., 2015).

2.2.4 Fisetin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one)

Fisetin is a flavonol, it can be found in wide variety of plants where it serves as colouring agents. Many vegetables and fruits also contain fisetin, including strawberries, apples, cucumbers and onions (Arai et al., 2000; Maher et al., 2010). Fisetin like other polyphenols has many biological activities including anti-inflammation, anti-depressant, anti-cancer, antioxidant and antiviral activity. In 2005, Lyu and colleagues carried out a study on anti-herpatic activity of 18 flavonoids against Herpes simplex virus1 (HSV-1) and type-2 (HSV-2) *in vitro*. They discovered that fisetin only showed moderate inhibitory effects against herpes simplex virus 1 (HSV-1) (Lyu et al., 2005).

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In 2011, Zandi and his colleagues evaluated the activity of fisetin against dengue virus. It was shown that fisetin can significantly interfere with dengue virus replication at the *in vitro* level with the treatment after virus adsorption (IC50 = 55 μg/ml) and treatment at 5 hours before virus infection (IC50 = 43.12 μg/ml) (Zandi et al., 2014). In 2012, the antiviral effect of fisetin against enterovirus-A71 was reported. Fisetin significantly reduced the EV-A71-induced cytopathic effects and viral plaque titers with IC50 values of 85 μM *in vitro*. This study suggested that fisetin is a protease inhibitor of EV-A71(Y.-J. Lin et al., 2012).

2.2.5 Quercetagetin (2-(3,4-dihydroxyphenyl)-3,5,6,7-tetrahydroxychromen-4-one)

Quercetagetin is a characteristic yellow flavone that has been identified in hydrolysates of leaves of six *Eriocaulon* species (Bate-Smith & Harborne, 1969). It has an additional 6-OH group based on the molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone). Like many other flavonoids, quercetagetin exert many effects, including antioxidant, antifungal and antibacterial (Céspedes et al., 2006; Schmeda-Hirschmann et al., 2004). There are recent reports that quercetagetin has exhibited potent antiviral activities against different viruses. In 2012, quercetagetin was shown to inhibit HCMV replication although the activity was less effective at various stages compared to other selected flavonoids (Cotin et al., 2012). In 2014, it appeared to be the most potent inhibitor of HCV RNA-dependent RNA polymerase (RdRp) activity. The screening identified the inhibition of RdRp was through inhibition of RNA binding to viral polymerase which was considered as the mechanism with broad genotypic activity and high barrier to resistance either by site-directed mutagenesis or long-term selection experiments (Ahmed-Belkacem et al.,

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2014). In 2016, Lani and her colleagues studied the antiviral activity of quercetagetin against Chikungunya virus. Quercetagetin displayed potent inhibitory activity against extracellular CHIKV particles with IC50 of 13.85 mg/ml (43.52 mM) with minimal cytotoxicity against Vero cell line. The analysis demonstrated that quercetagetin affects CHIKV RNA production and viral protein expression (Lani et al., 2016).

2.2.6 Luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone)

Luteolin is a flavone with a yellow crystalline appearance and occurs in many types of plants including fruits, vegetables and medicinal herbs. It has been used in Chinese traditional medicine for curing different diseases such as inflammatory disorders, hypertension and cancer. It is well known for its multiple biological activities, which are functionally related to each other (Y. Lin, Shi, Wang, & Shen, 2008). Luteolin has been discovered to have anti-HIV-1 activity by inhibiting HIV-1 integrase (IN), an enzyme essential for viral replication (Tewtrakul et al., 2003). Later in 2011, it was found that luteolin is capable of reducing the HIV-1 infection in the reporter cells and primary lymphocytes. The inhibitory activity of luteolin was only seen at the viral entry level but it wasn't capable of inhibiting the reverse transcription of HIV-1 (Mehla et al., 2011). In 2012, the antiviral activity of luteolin against HCV infection in a cell-based assay system was examined. It was found that luteolin inhibited the NS5B polymerase as the viral target with IC_{50} value of 1.1 to 7.9μM (Calland et al., 2012).

In 2014, luteolin was identified as the most potent inhibitor of EV71 and CA16 infection through cell viability and plaque reduction assays with EC_{50} value of about 10μM. Luteolin largely targeted the post-attachment stage of EV71 and CA16 infection by inhibiting the viral RNA replication (Xu et al., 2014). In 2015, the

antiviral activity of luteolin against Chikungunya virus was studied. It was revealed that luteolin ethanolic fraction from *C. dactylon* could be utilized as a potential therapeutic agent against CHIKV infection as the fraction did not show cytotoxicity while inhibiting the virus. Remarkably, the extract was able to reduce the viral mRNA synthesis with no CPE at a concentration of 50µg/ml (Murali et al., 2015). More recently in 2016, the antiviral activity of luteolin against Japanese encephalitis virus was investigated by a group of researcher. They found that luteolin has a potent antiviral activity against JEV replication in A549 cells with $IC_{50} = 4.56$ µg/mL. Luteolin showed great inhibition in extracellular virucidal activity on JEV (Fan, Qian, Qian, & Li, 2016).

2.2.7 Orientin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-8-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one)

Orientin is a water-soluble flavonoid *C*-glycoside. It is commonly extractable from medicinal plants, which is a crucial step in producing plant-derived drugs. Orientin often used in the studies due to its medicinal and therapeutic properties. The antiviral activities of orientin are beneficial for the future antibiotics development. It has been evidenced that Orientin has moderate or potent antiviral activity against parainfluenza type 3 (Para 3) virus (Y.-L. Li et al., 2002; Q. Lin et al., 2003). The mixture of flavonoids comprising orientin, rutin, kaempferol and quercetin at the maximum nontoxic dose of 0.048 μg/mL inhibited Herpes simplex virus type 2 (HSV-2) of different viral titre (1,10,100 TCID50) on Hep-2 cells (Boominathan et al., 2014).

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2.2.8 Apigenin (5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one)

Apigenin is a naturally occurring plant flavone and present in vegetables and fruits and some herbs. Apigenin possesses various pharmacological effects, including antioxidant, anticancer, anti-inflammatory and antiviral activities. Apigenin has been reported to inhibit the EV71-mediated CPE, virus replication efficiency, viral protein expression and prevented EV71-induced cell apoptosis, intracellular reactive oxygen species (ROS) generation and cytokines up-regulation. Apigenin displayed its inhibitory activity following the viral entry and interfered with viral internal ribosome entry site (IRES) activity and modulating the (c-Jun N-terminal kinase) JNK pathway, which is fundamental for viral replication (Lv et al., 2014). Because EV71 infection commonly requires the hnRNP proteins (trans-acting factor regulating the EV71 translation), apigenin block EV71 infection by interfering the viral RNA association with hnRNP A1 and A2 proteins with CC50 value of 79.0 µM (W. Zhang et al., 2014). Apigenin can inhibit the foot-and-mouth disease virus (FMDV) replication *in vitro*. It was demonstrated that The FMDV-translational activity was inhibited effectively because apigenin disrupts the IRES activity at the post-entry stage with no direct extracellular virucidal activity (Qian et al., 2015).

Apigenin is known as an inhibitor of maturation of a subset of miRNAs and potently inhibits HCV replication *in vitro*. Apigenin considerably reduced the expression levels of mature miR122 without disturbing the cell growth. Because supplementation of synthesized miR122 oligonucleotides or overexpression of constitutively active TRBP blocked these effects, the inhibitory effects of apigenin on HCV replication seemed to be dependent on the reduction of mature miR122 expression levels through inhibition of TRBP phosphorylation. Hence, apigenin intake

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either through supplements or regular diet has been strongly recommended because it can decrease HCV replication in chronically infected patients (Shibata et al., 2014).

2.2.9 Kaempferol (3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one)

Kaempferol is a polyphenol antioxidant found in plant-derived foods and in plants or botanical products commonly used in traditional medicine. Many studies have described the beneficial effects of dietary kaempferol in reducing the risk of chronic diseases, especially cancer. Research on kaempferol is scarce in humans in supplemental form, but in vitro research seems promising. Kaempferol has shown anti-viral activity against several viruses, including herpes simplex virus (Amoros et al., 1992), influenza virus (Jeong et al., 2009), cytomegalovirus (Mitrocotsa et al., 2000), and human immunodeficiency virus (HIV) (Mahmood et al., 1996; Min et al., 2001).

There are some reports that kaempferol can inhibit the enzyme reverse transcriptase and it has been suggested that the hydroxyl groups at C3 and C4' are vital for the inhibitory activity of this enzyme.(Chu, Hsieh, & Lin, 1992; Mahmood et al., 1996; Min et al., 2001) This inhibitory activity together with its capability to inhibit viral proteases and binding of gp120 to lymphocytes CD4 may result in the anti-HIV activity of Kaempferol (Mahmood et al., 1996).

In 2000, Mitrocotsa and colleagues studied the antiviral activity of seven kaempferol derivatives against human cytomegalovirus and observed the presence of acyl substituents significantly increased the activity and the most active compound was kaempferol-3-O-a-L-(2",3"-di-E-p-acetylcoumaroyl)-rhamnopyranoside (Mitrocotsa et al., 2000).

Jeong and colleagues (2009) showed that kaempferol isolated from Rhodiola rosea

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inhibited neuraminidase activity with IC_{50} values ranging from 0.8 to 56.9 μM. Kaempferol exhibited high inhibitory activity against the influenza viral strains H1N1 and H9N2 EC50 values of 30.2 and 18.5 μM, respectively. It was believed that its activity depended on the position and number of hydroxy groups on the flavonoids backbone (Jeong et al., 2009). In addition, kaempferol was also reported as a potent inhibitor of Japanese encephalitis virus (JEV) by inactivating the virus by binding with its frameshift site RNA (T. Zhang et al., 2012).

2.2.10 Silymarin ((2R,3S)-3,5,7-Trihydroxy-2-[(2R)-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4H-chromen-4-one)

Silymarin is a unique mixture of flavonolignans extracted from the milk thistle and has been the subjects of research into their beneficial properties. The main component of silymarin is silibinin (a mixture of Silybin A and Silybin B); the remaining components are silydianin, silycristin, isosilybin A, isosilybin B, taxifolin and isosilycristin. Silymarin is one of the most popular natural products consumed by western society, additionally is the most commonly consumed botanical medicine identified in patients with chronic hepatitis C (Polyak, Ferenci, & Pawlotsky, 2013; Seeff et al., 2008).

Silymarin was discovered to inhibit hepatitis C virus (HCV) infection, both *in vitro* and *in vivo*. Silymarin exhibited its antiviral properties against hepatitis C virus cell culture (HCVcc) infection by variably inhibiting the virus entry, RNA and protein expression, and infectious virus production. Silymarin did not inhibit HCVcc binding to cells but blocked the entry of viral pseudoparticles (pp), plus fusion of HCVpp with liposomes. Interestingly, silymarin inhibited genotype 2a NS5B RNA-dependent RNA

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polymerase (RdRp) activity at concentrations higher than required for anti-HCVcc effects. Silymarin blocked microsomal triglyceride transfer protein activity, apolipoprotein B secretion, and infectious virus production into culture supernatants. Silymarin could also block cell-to-cell spread of virus (Wagoner et al., 2010).

Recently, Lani and colleagues (2015) have studied the antiviral activity of silymarin against Chikungunya virus replication. They discovered that silymarin has potent *in vitro* antiviral activity against CHIKV. It inhibited the post-entry stages of CHIKV infection and most likely CHIKV RNA replication in a dose dependent manner with the IC_{50} of 16.9 µg/ml (Lani et al., 2015). Silymarin also displayed anti-influenza virus activity by reducing the formation of a visible CPE by 98% at concentration of 100 µg/ml with no cytotoxicity. Silymarin inhibited viral mRNA synthesis and also disturbed late viral RNA synthesis (J. H. Song & Choi, 2011).

2.3 Flavanones derivatives and their potential

Flavanones comprise the majority of flavonoids in citrus (Rutaceae), and it is estimated that about 95% of the flavanones comes from citrus fruits such as grapefruit, orange and lemon and in some aromatic herbs like mint. Citrus fruits are widely consumed as part of human diet. Through extraction and analysis of flavanones in these fruits, hesperetin and naringenin were found as the most abundant natural flavanone (Kawaii et al., 1999). These flavanones form glycones as hesperidin and naringin, which enhances intestinal absorption (Haidari et al., 2009). Citrus flavanones like hesperetin and naringenin have been a subject of great interest for scientific research because they exert a variety of biological activities such as anti-mycobacterial (Prado et al., 2007), anti-microbial (Vatkar et al., 2010), anti-bacterial (Mughal et al., 2006), anti-fungal (Dandia, Singh, & Khaturia, 2006), anti-lung cancer (Hammam et

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al., 2005), antiproliferative (I. L. Chen et al., 2008), anti-arrhythmic (Koufaki et al., 2006), anti-Tuberculosis (Y.-M. Lin et al., 2002), anti-viral (Pandey et al., 2004), anti-oxidant, anti-inflammatory and anti-hypertensive (Tapas, Sakarkar, & Kakde, 2008).

2.3.1 Antiviral activity of flavanones (2-Phenyl-2,3-dihydro-4H-chromen-4-one)

Naringenin and hesperetin are among well-studied flavanones, but least known in the area of the antiviral research. Hesperidin, the glycosides of hesperetin, was found to exhibit antiviral activity against herpes simplex virus, poliovirus and parainfluenza virus (Kaul et al., 1985). Hesperitin and narigenin showed antiviral activity against Sindbis neurovirulent strain virus (NSV). In a dose-dependent manner, a decrease of viral plaque formation for both used flavanones, scored the ID_{50} as low as 20.5 and 14.9µg/ml respectively. Beyond hesperetin with only 50% of viral replication inhibition at 25µg/ml, naringenin is the most effective anti-sindbis NSV with up to 80% viral replication inhibition at the same concentration (Paredes et al., 2003). Zandi and colleagues (2011) showed that naringin exhibited anti-adsorption stage against DENV-2 replication with IC50 = 168.2 µg mL-1 and its related SI was 1.3 (Zandi et al., 2011).

More recently, it has been shown that hesperetin and naringenin and their glycosides forms significantly inhibited the replication of the 17D strain of yellow fever virus at the highest concentration employed (0.092 µM for naringenin and 0.083 µM for hesperetin, P=0.003). Both naringenin and hesperetin were able to reduce the number and size of viral plaques and their ED_{50} of 0.0013 µM and 0.01 µM respectively (Castrillo et al., 2015).

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CHAPTER 3: MATERIALS AND METHODS

3.1 Cell lines, virus and antibodies

The African green monkey kidney cell line known as Vero cells and the baby hamster kidney cell line (BHK-21 cells) were purchased from the American Type Culture Collection (ATCC, USA). Both cell lines were cultured and maintained in the Eagle's Minimum Essential Medium (EMEM) (Thermo-Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Bovogen Biologicals, Vic, Australia), 2 mM L-glutamine (HiMedia Laboratories, Mumbai, India), 1x non-essential amino acid (NEAA) (HiMedia Laboratories, Mumbai, India) and 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). Both cell lines were incubated at 37°C in 5% CO_2 and were sub-cultured once they reached to 80% to 90% confluency.

The BHK-CHIKV replicon cell line was kindly contributed by Dr. Andres Merits from (University of Tartu, Tartu, Estonia) and stored in liquid nitrogen tank (-196°C). The CHIKV replicon containing the virus replicase proteins together with puromycin acetyltransferase, *EGFP* and *Renilla* luciferase marker genes was transfected into BHK-21 cells to yield a stable cell line **(Appendix B)** (Pohjala et al., 2011). The CHIKV replicon cell line was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo-Fisher Scientific, MA, USA) supplemented with 8% FBS (Bovogen Biologicals, Vic, Australia), 2% tryptose-broth phosphate (Sigma-Aldrich, MO, USA), 2 mM L-glutamine (HiMedia Laboratories, Mumbai, India) and 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). The replicon cell line was incubated at 37°C in 5% CO_2 and sub-cultured once they reached to 80% to 90% confluency.

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The CHIKV of ECSA genotype (accession number: MY/065/08/FN295485) was used throughout the experiment. The virus was isolated in Malaysia in 2008 and was kindly provided by Assoc. Prof. Jamal I-Ching Sam from University of Malaya (Sam et al., 2009).

3.1.1 Propagation and harvesting of CHIKV

Since CHIKV cultivates in a variety of non-human cell lines, including Vero cells and BHK-21 cells (Schwartz & Albert, 2010). In this study, the propagation and harvesting of the CHIKV were performed using BHK-21 cells and the subsequent antiviral assays were performed by using the Vero cells. CHIKV stock was diluted at 1:2.5 in EMEM containing 2% FBS, 1 x non-essential amino acid, 2mM L-glutamine and 50 IU penicillin/streptomycin at room temperature.

Following the dilution steps, a monolayer of the BHK-21 cells were cultured in 75 cm^2 cell culture flask using Eagle's Minimum Essential Medium (EMEM) (Thermo-Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Bovogen Biologicals, Vic, Australia), 2 mM L-glutamine (HiMedia Laboratories, Mumbai, India), 1x non-essential amino acid (NEAA) (HiMedia Laboratories, Mumbai, India) and 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA) and incubated at 37°C in the presence of 5% CO_2. Then cells were treated with CHIKV inoculum after attaining 80% confluency. The flask containing CHIKV-infected BHK-21 cells was placed on the rocker at room temperature for 30 minutes to allow virus adsorption prior to the incubation at 37°C in 5% CO_2.

Prior to harvesting the CHIKV, the infected cells were incubated in the same environment for three days until about 70%-80% of cytopathic effects (CPE) appeared. To be noted that the CPE of alphaviruses in cell culture was characterized as

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lytic infection or apoptosis. On day 3, the cells were scrapped by cell scraper, centrifuged at 4°C and 2000rpm to remove the cell debris. Finally, the CHIKV was aliquoted into sterile screw cap tubes and stored at -80°C for titration and further experiments.

3.1.2 CHIKV titration assay

The virus titration was performed based on Reed-Muench endpoint calculation method to determine the tissue culture 50% infectious dose ($TCID_{50}$) (Reed & Muench, 1938). A monolayer of $1x10^4$ Vero cells in each well were grown in a 96-well plate in the growth media made up of the HyClone™ Eagle's Minimum Essential Medium (EMEM) (Thermo-Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Bovogen Biologicals, Vic, Australia), 2 mM L-glutamine (HiMedia Laboratories, Mumbai, India), 1x non-essential amino acid (NEAA) (HiMedia Laboratories, Mumbai, India) and 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). The cells were then incubated at 37°C in 5% CO_2 until it reached 80% confluency.

The stock of CHIKV was then diluted tenfold EMEM (Thermo-Fisher Scientific, MA, USA) supplemented with 2% fetal bovine serum (FBS) (Bovogen Biologicals, Vic, Australia) and each dilution was used to infect the cells in separate wells. The plate was incubated in a similar condition to virus propagation. The plate was examined everyday for the appearance and record of CPE in infected wells. The CPE appearance was recorded until the infected cell indicated its healthy morphology.

In order to titerate the viral stock according to Reed-Muench endpoint calculation technique, a score was given to each of the dilution according to the

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amount of the wells showing CPE. The interpolated value of $TCID_{50}$ was calculated by Reed-Muench formula as the following: -

Reed-Muench formula

$TCID_{50/ml} = 10^{\text{log total dilution above 50\% - (}\alpha\text{ x log dilution factor)}}$

Where α = (% of wells infected at dilution above 50% - 50%) ÷ (% of wells infected at dilution above 50% - % of wells infected at dilution below 50%)

3.1.3 Anti-CHIKV antibodies

The monoclonal rabbit anti-E2 CHIKV antibody used in the immunofluorescence assay and immunoblotting was kindly provided by Dr. Justin Chu Jang Hann from National University of Singapore. The monoclonal anti-nsP1 CHIKV and anti-nsP3 CHIKV used in the immunofluorescence assay and the immunoblotting was provided by Dr. Andres Merits from University of Tartu, Tartu, Estonia.

3.2 Bioflavonoids and nucleoside analogue

Two types of flavanones, hesperetin and naringenin were purchased from Sigma-Aldrich, MO, USA. Ribavirin of ≥ 95% purity, a nucleoside analogue known to inhibit the CHIKV viral genome replication (Briolant et al., 2004) was used as the control for positive anti-CHIKV activity was purchased from Sigma-Aldrich, MO, USA. The flavanones and ribavirin stock solutions were prepared in 0.1% dimethyl sulfoxide (DMSO) or methanol according to the solvent suggested by the manufacturer. The stock solutions were stored in -20°C for further experiments.

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3.2.1 Cytotoxicity assay

The cytotoxicity of the flavanones and ribavirin on Vero cells and BHK-21 cells were determined by using the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. The half maximal cytotoxic concentration (CC_{50}) and the maximum non-toxic dose (MNTD) of each compound can be determined by this assay. Mainly, in the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, WI, USA), the MTS solution and an electron coupling reagent (phenazine ethosulfate; PES) were combined to form a stable solution. The NAD(P)H-dependent cellular oxidoreductase enzymes in the viable cells will reduce the dye to its coloured formazan, which reflects the actual amount of viable cells in this assay. This assay was performed according to the manufacturer's protocol.

To perform the assay, a monolayer of Vero cells and BHK-21 cells were seeded in a 96-well cell culture plate (Nest Biotechnology Co., Ltd. Wuxi, China) and were treated with different concentrations of each compound in triplicate plus negative control (media containing 0.1% DMSO). The flavanone compounds were filtered using 0.2µm syringe filter unit (Merck Millipore Ltd., MA, USA). The plate was then incubated at 37 °C in 5% CO_2 for 48 hours before the MTS assay was performed. Both treated and control cells were maintained for two days at 37°C, in similar conditions and durations until used for antiviral activity assay. After two days post-treatment, MTS solution was added to the cells and incubated for 4 hours at 37°C with 5% CO_2 prior to absorbance detection at 495 nm wavelength using Infinite 200 Pro multi-plate reader (Tecan, Männedorf, Switzerland). Typically, all experiments were conducted in triplicate. The CC_{50} and MNTD for each compound were determined through this

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assay using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005).

3.3 *In vitro* antiviral assays

3.3.1 Primary screening using CHIKV replicon cell system

CHIKV BHK-21 replicon cell line was used to screen the compounds for their activity against CHIKV replicase complex. This assay was modified and performed according to (Pohjala et al., 2011). BHK-CHIKV replicon cells were seeded onto opaque-white 96-well plate with clear bottom at a confluency of approximately 80 to 90 % (Corning Inc., NY, USA). The cells were exposed to different concentrations of each compound for 48 hours at 37°C with 5% CO_2. After the incubation period, the activity of the *Renilla* luciferase (Rluc) expressed by CHIKV replicon was detected by using *Renilla* luciferase assay (Promega, WI, USA). The assay was performed according to the manufacturer's protocols. The resulting luminescence signal was then measured by using the GloMAX 20/20 Luminometer (Promega, WI, USA). The signal was plotted against the log transformation of the concentrations of the compounds and a sigmoidal curve fit with variable slope was created to obtain the half maximal inhibitory concentration (IC_{50}) value for each flavanone compound. The non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the *Rluc* activity.

3.3.2 Time-of-addition assay

The time-of-addition study was performed to determine the effects of varying the time of addition of compound addition on CHIKV replication as well as to

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determine how long the addition of a compound could be postponed before it loses its antiviral activity (Daelemans et al., 2011). The confluent monolayer of Vero cells were prepared in 96-well cell culture plate using EMEM supplemented with 10% FBS. For the pre-treatment assays, which refer to the -2h and -1h pre-infection; Vero cell were treated with each compound followed by infection after 2 hours. For the co-treatment assay (0h), Vero cells were treated with each compound and infected together. For the post-treatment assay, Vero cells were infected with CHIKV (MOI=1) and then the compounds were added at 1h, 2h and 4h to 12h. In positive control, CHIKV were added at the 0h and the negative controls were only treated with EMEM and 2% FBS. The plate was then incubated for 48 hours at 37°C in the presence of 5% CO_2 until the CPE appeared.

The plate was examined daily and on day 2; the percentage of CPE score was given according to the degree of the CPE inhibition. The supernatants in each well were collected and a viral yield assay was conducted using a specific quantitative real-time polymerase chain reaction (qRT-PCR) for CHIKV. The non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.3 Direct virucidal assay

The virucidal assay was performed to identify whether there is a possibility that the selected flavanones can inactivate the free extracellular virus particles outside the cells or before infection activity. In this assay, the increasing concentrations of the compounds were mixed with the CHIKV and incubated for 2 hours at 37°C. This assay was modified and performed according to (Johari et al., 2012). The flavanone

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compounds were further diluted before the addition of the mixtures on Vero cells. Treated viral mixtures were added to monolayer of $6x10^5$ Vero cells in 24-well cell culture plates in triplicates and placed on a rocker at room temperature for 30 minutes. The plates were then incubated at 37°C in the presence of 5% CO_2 for 48 hours. The positive control wells were treated with CHIKV only whereas the negative control wells only contained cell culture medium with 2% FBS.

The plates were examined daily for the presentations of CPE and after 2 days of incubation, the supernatants were collected from each wells and the qRT-PCR assay was performed to evaluate the virucidal activity of the compound. The IC_{50} was determined and the non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005 to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.4 Anti-adsorption assay

In order to identify whether these compounds could interfere with the attachment of CHIKV onto specific receptors on the surface of the cells. This assay was modified and performed according to (Zandi et al., 2013). A viral suspension of CHIKV with MOI=1 and descending concentrations of flavanones were prepared in EMEM supplemented with 2% FBS and overlaid on monolayer of $6x10^5$ Vero cells in 24-well cell culture plates. The plates were then incubated at 37°C in 5% CO_2 for 2 days. After the incubation period, the supernatants were collected from each wells and the qRT-PCR assay was performed to confirm the results. The IC_{50} and the non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph

Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.5 Anti-entry assay

This assay aims to examine the effect of selected flavanones on inhibiting the entry of CHIKV into the cells. The procedure of the anti-entry assay was modified and performed according to Lee et al., (2013). Monolayers of Vero cells were grown in 24-well plate with EMEM supplemented with 10% inactivated FBS. The Vero cells were then infected with CHIKV and incubated for 1 hour at 4 °C. Non-adsorbed virus was washed with 1xPBS. Tested compound was added in different concentrations and incubated at 37 °C with 5% CO_2 for 2 hours. The plate was again washed with 1xPBS and treated with citrate buffer (pH = 3) to inactivate the non-internalized virus, before the plate was again washed with 1xPBS. EMEM supplemented with 2% inactivated FBS was added into each well and incubated for 48 hours at 37 °C with 5% CO_2. After 2 days of incubation, the supernatants were collected from each well and the qRT-PCR assay was performed to confirm the results. The IC_{50} and the non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.6 Post-adsorption assay

This assay was designed to investigate the antiviral activity of selected flavanones on inhibiting the intracellular replication of CHIKV. This assay was modified and performed according to Zandi et al., (2011). A monolayer of $6x10^5$ Vero cells were cultured in 24-well cell culture plates using EMEM supplemented with 10%

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FBS. The Vero cells were inoculated with CHIKV of MOI= 1 for 2 hours at 37°C with 5% CO_2 prior to the treatment with different concentration of compounds in EMEM supplemented with 2% FBS and incubated for 48 hours at 37°C with 5% CO_2. The plates were examined daily for the CPE and after two days, the degree of CPE was determined according to the virus replication inhibition, which was expressed as percent yield of virus control (% virus control = CPE experimental group/CPE virus control × 100). Additional assays such as the MTS assay (Promega, WI, USA) and the quantitative real-time polymerase chain reaction (qRT-PCR) assay were performed to confirm the results. The IC_{50} and the non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the CHIKV RNA copy number.

3.4 Virus yield assay using quantitative reverse transcription PCR (qRT-PCR)

3.4.1 CHIKV RNA extraction and the generation of cDNA

The extracellular CHIKV RNA was extracted from supernatants collected from wells of previous *in vitro* antiviral assays with QIAamp Viral RNA Mini Kit (QIAGEN, Germany). The experiment was performed according to the manufacturer's recommended protocol. The nsP3 primers of 136bp were nsP3-F (5'-GCGCGTAAGTCCAAGGGAAT-3') and nsP3-R (5'-AGCATCCAGGTCTGACGGG-3') as described by Chiam and colleagues (Chiam et al., 2013). The nsP3-R primer was used to generate nsP3 positive-strand cDNA. The mixture of 500 nmol/L of primers, 50 nmol/L of dNTP mix (Promega, WI, USA), and 1 µL of RNA was incubated at 65°C for 5 min and placed on ice for 4 min. The cDNA was synthesized with 200 U of Superscript III Reverse Transcriptase (Life

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Technologies, USA), 0.1 mol/L DTT (Life Technologies, USA), 40 U of RNaseOUT (Life Technologies, USA), and 1× First Strand buffer (Life Technologies, USA) at 50 °C for 60min. Reverse transcriptase enzyme was inactivated at 70°C for 15 min, unincorporated primers were digested with 20 U of Exonuclease I (New England Biolabs, MA, USA). Lastly, the cDNA were stored at −80 °C.

3.4.2. Quantitative RT-PCR

Quantitative RT-PCR was performed to determine the effects of selected flavanones on CHIKV replication by quantifying genomic RNA copies. In this experiment, the amplification of 136 base region of nsP3 encoding sequence was performed as described by Chiam et al., (2013). This assay was performed in the Step-One Plus Real-Time PCR System (Life Technologies, USA) following the manufacture's recommended protocol. Consumed a 10 µL reaction volume containing 1×Power SYBR Green PCR Master Mix (Life Technologies, USA), 1 µL of cDNA, 1 µmol/L sense and antisense primers, and 3.8 µL of nuclease-free water. The cycling parameters were 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The melting curve analysis confirmed amplified product. The non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) to determine the correlation between the treatments and the CHIKV RNA copy number.

3.5 Immunofluorescence assay

To quantify the CHIKV antigen production as well as to confirm the results of the employed antiviral assays, an immunofluorescence assay was modified and performed according to Kaur & Chu, (2013). A monolayer of Vero cells were

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prepared in 96-well cell culture plate using EMEM supplemented with 10% FBS. The cells were infected with CHIKV (MOI= 1) and incubated for 2 hours at 37°C with 5% CO2 prior to the treatment with different concentrations of compounds in each wells. Following, the plates were incubated for 24 hours at 37°C with 5% CO_2. After the incubation period, the cells were fixed using 100 µl of 4% paraformaldehyde for 30 minutes at room temperature. The cells were washed three times with 100 µl 1xPBS prior to the addition of 40 µl of monoclonal rabbit anti-E2 antibody (diluted to 1:300) and incubated for 1 hour at 37ºC.

After incubation, the plates were washed for 3 times with 1xPBS prior to the addition of 40 µl secondary antibody (diluted to 1:1000), the anti-rabbit IgG Fab2 conjugated with Alexa Fluor (RT) 488 (Cell Signaling Technology, MA, USA) and incubated for 1 hour at 37ºC. Nuclei were stained with 40 µl of DAPI (Thermo Scientific, MA, USA) for 15 minutes at room temperature before being washed again for 3 times with 1xPBS. Accordingly, the immunofluorescence signals were measured and analyzed using Harmony Software Version 3.5.1 (Perkin-Elmer, Hamburg, Germany). The images were taken by using the high content screening system (Operetta, Perkin-Elmer, Hamburg, Germany).

3.6 Immunoblot assay

In order to determine the effects of flavanone compounds on CHIKV protein expression, immunoblotting assay was performed on structural and non-structural proteins of CHIKV. Vero cells at the density of $3x10^6$ cells were seeded into a $75cm^2$ cell culture flask and supplemented with EMEM and 10% FBS. Once the flask were confluent enough, cells were infected with CHIKV (MOI= 1) and incubated at 37°C with 5% CO_2 for 2h to allow virus attachment and internalization. The flasks were

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treated with different concentrations of compounds, where the control flasks were only treated with working medium containing 0.1% DMSO. All the flasks were then incubated at 37°C with 5% CO_2 until the CPE presentation appeared in the control flask. Once CPE was observed, cells were scraped, washed with PBS and lysed using 300 µl of 1% Triton X100 (Sigma-Aldrich, St. Louis, MO, USA) containing complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 45 min. Cellular debris was pelleted out by centrifugation at 16,000 × g for 5 min. A Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL) was used to quantify the protein concentration.

The cell lysates containing 100 µg of protein were denatured using SDS-loading buffer and proteins were separated using 12% SDS-PAGE. The gels were equilibrated in Towbin buffer (0.025 M Tris, 0.192 M glycine 20% methanol) for 10 min and proteins were transferred to a PVDF membrane using the Bio-Rad wet transfer system (Bio Rad, San Francisco, CA). For detection of nsP1 and nsP3, membranes were blocked with 1X PBS 1% Casein Blocker (Bio Rad, San Francisco, CA) for an hour at room temperature on a shaker. The blots were washed three times with 1X PBS Tween 20 for 15 min each time before being incubated with primary anti-CHIKV nsP1, anti-CHIKV nsP3 rabbit polyclonal antibodies in 1% casein solution. The blots were washed with 1X PBS Tween 20 for 15 min each time before being incubated with the secondary goat anti-rabbit IgG (Abcam, Cambridge, UK) antibodies conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature on an orbital shaker. The membranes were washed three times with PBS containing Tween 20 for 15 min each time. Separate blots containing the same samples were incubated with primary anti-β-actin mouse monoclonal antibody conjugated with HRP (Cell Signaling Technology, MA, USA) dissolved in 1% Casein

for 1 hour at room temperature on shaker. The blots were then washed three times with 1XPBS Tween 20 for 15 min each time. Membranes were developed by the colorimetric method using appropriate substrates (Thermo Scientific, Rockford, IL).

3.7 Statistical analysis

In this study, the statistical analysis for each assay were performed by using the Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005) and the data was presented as the mean with the standard error mean (±SEM). The non-parametric one-way ANOVA (Kruskal-Wallis test) was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005). This will allows to determine if there are statistically significant differences between two or more groups of an independent variable on a continuous or ordinal. In order to determine the effective time of treatment in the time-of-addition assay, a Dunn's multiple comparison posttest was performed after the Kruskal-Wallis test. This allows comparing the treatments with the positive control.

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CHAPTER 4:RESULTS

4.1 Determination of potential cytotoxic activity of compounds

The cytotoxic effects of compounds on Vero cells and/or BHK-21 cells were first evaluated using MTS assay and microscopic observations. **Table 4.1** showed the 50% cytotoxic concentration values (CC_{50}) of each compound against Vero and BHK-21. 0.1% DMSO as vehicle control, did not show any cytotoxicity against the cells.

Table 4.1 The CC_{50} values of tested flavanones and nucleoside analogue on Vero cells and BHK-21 cells.

Compounds	Chemical Formula	Vero Cells		BHK-21 Cells	
		CC50 (µM)	MNTD (µM)	CC50 (µM)	MNTD (µM)
Hesperetin	$C_{16}H_{14}O_6$	198.4	>160	>125	>125
Naringenin	$C_{15}H_{12}O_5$	547.3	283.6	831.3	425
Ribavirin	$C_8H_{12}N_4O_5$	>500	>500	>500	>500

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4.2 Primary screening assays- CHIKV replicon cell line-based assay

CHIKV replicon cell line was used to study the antiviral activity of the compounds. The replicon was constructed from non-infectious replicon of CHIKV as well as the virus replicase proteins together with puromycin acetyltransferase, *EGFP* and *Renilla* luciferase marker genes (Pohjala et al., 2011). The replicon was transfected into BHK cells to yield a stable cell line. The *Rluc* activity in CHIKV replicon was proportional to viral replicon RNA replication. In this experiment, it was found that hesperetin and naringenin inhibited the *Rluc* activity with the IC_{50} = 85.0 and 93.27μM, respectively, **Figure 4.1**.

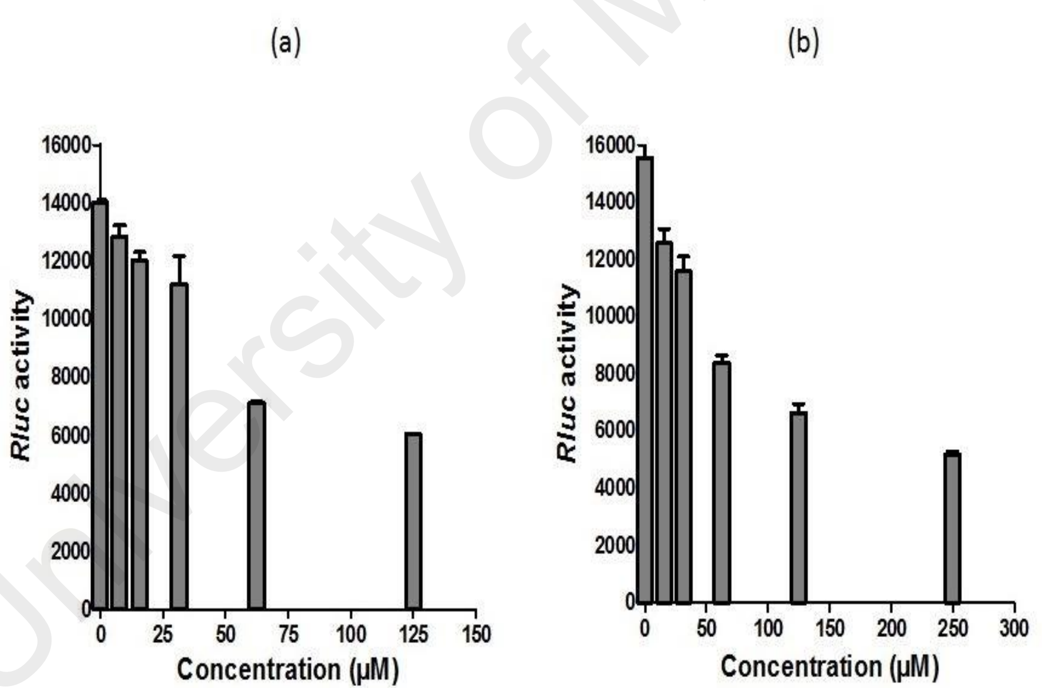

Figure 4.1 Evaluation of anti-CHIKV activity of selected flavanone using CHIKV replicon cell line. Both flavanone, Hesperetin (a) and Naringenin (b) are able to reduce the percentage of *Rluc* activity produced by the CHIKV replicon in a dose-dependent manner. The *Rluc* activity was measured at 48 h post treatment. Vehicle–treated (0.1% DMSO) cells were used as control ("0") concentration). Data from triplicate assays were plotted and analyzed using one-way ANOVA (Kruskal-Wallis test), Graph Pad Prism Version 5, Graph Pad Software Inc., San Diego, CA), $P<0.05$. Error bars represent standard errors of triplicate means.

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Hesperetin (P=0.0027) and Naringenin (P=0.0014) showed moderate inhibitory effects against CHIKV replicon, which validate of both compounds affect of post-entry steps of CHIKV infection. These data suggested that the compounds could interfere with CHIKV RNA replication by affecting the viral replicase system. Since these compounds showed inhibitory activity against CHIKV replication *in vitro*, it was worthwhile to implement further evaluation on different antiviral assays.

4.3 Time of addition study

A time of addition assay was performed to determine the effects of varying the time of compounds addition against CHIKV replication as well as to determine how long the addition of a compound could be postponed before it loses its antiviral activity. In this assay, the flavanones that result in positive antiviral hits in the previous assays were tested using the highest concentration of each compound. Hesperetin, naringenin and ribavirin (as a control) were added at different time points before and after infection of Vero cells with CHIKV. Significant inhibition of virus replication was observed once hesperetin and naringenin were added 2h before the viral infection.

Hesperetin exerted its antiviral activity when added prior to the virus infection until 4 hour of post infection while naringenin possessed its antiviral activity until 12h of post infection **Figure 4.2**. Ribavirin also showed its anti-viral activity at 2h prior of infection until 2h of post infection. The antiviral activities of ribavirin at these hours were predicated beforehand since it acted as a RNA replication inhibitor. The results suggested that hesperetin and naringenin may inhibit the early to middle stages of CHIKV replication. Yet, narigenin displayed its potent antiviral effects in comparison to hesperetin. These two compounds were then selected for further analysis of their

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anti-CHIKV activity due to their significant inhibition of CHIKV infection and minimal cytotoxicity, as well as its novel status as antiviral agents. The following antiviral assays were organized to determine the effect of compounds on different stages of CHIKV life cycle.

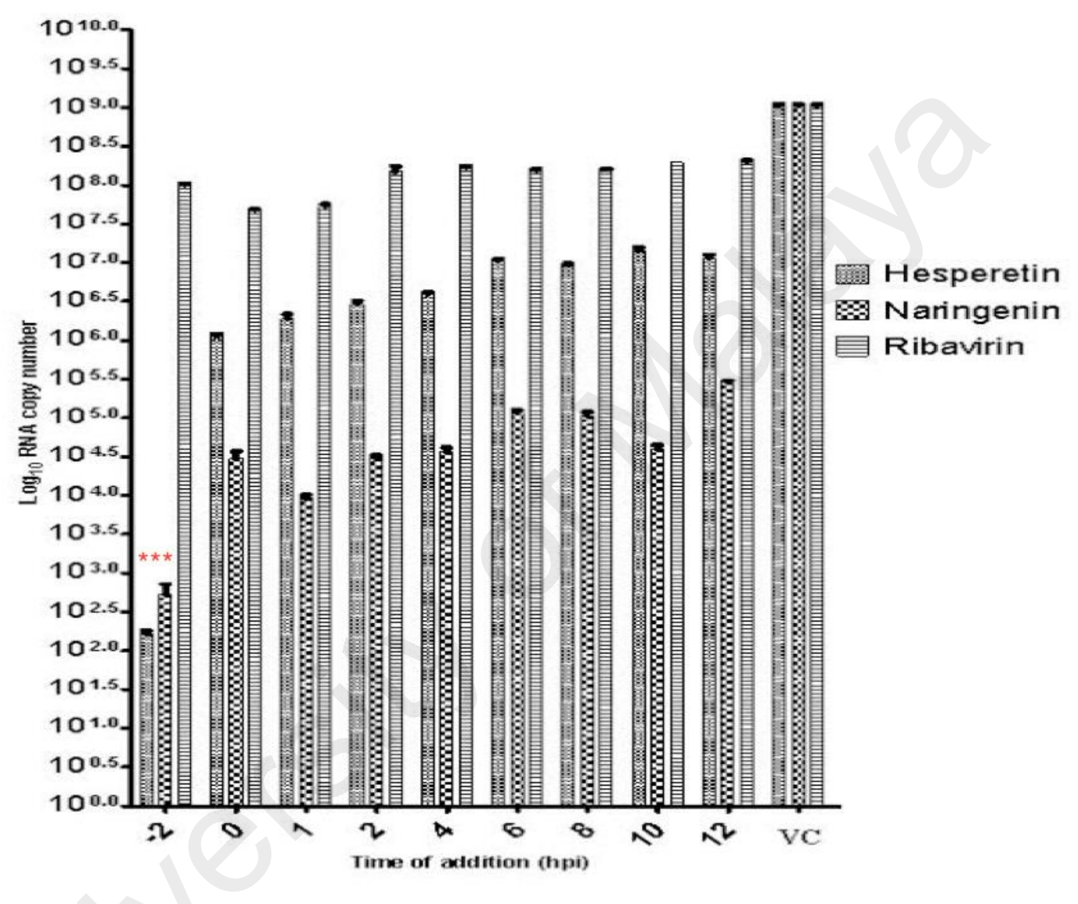

Figure 4.2 Effects of time-of-addition of selected flavanones on CHIKV RNA copy number. CHIKV viral load reduced significantly at the early hour (-2 hpi) of hesperetin, naringenin and ribavirin treatment in the time-of-addition assay. “VC” is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA (Kruskal-Wallis test) and Dunn’s multiple comparisons post-test.

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4.4 Post-adsorption assay

Given that hesperetin and naringenin were most likely affecting CHIKV intracellular replication of CHIKV, their activities on post entry stages of CHIKV infection were investigated. Hesperetin (*P*=0.0090) with IC_{50}=8.500 μM, naringenin (*P*=0.0053) with IC_{50}=6.818 μM showed potent intracellular anti-CHIKV activity **Figure 4.3**. However, compared to Ribavirin (*P*=0.0061) with IC_{50}=17.68 μM as a positive control with defined anti-CHIKV activity, it could be concluded that the tested compounds exerted stronger intracellular anti-CHIKV activity than ribavirin. Hesperetin and naringenin could interfere with specific binding of the viral attachment proteins and the cellular receptors.

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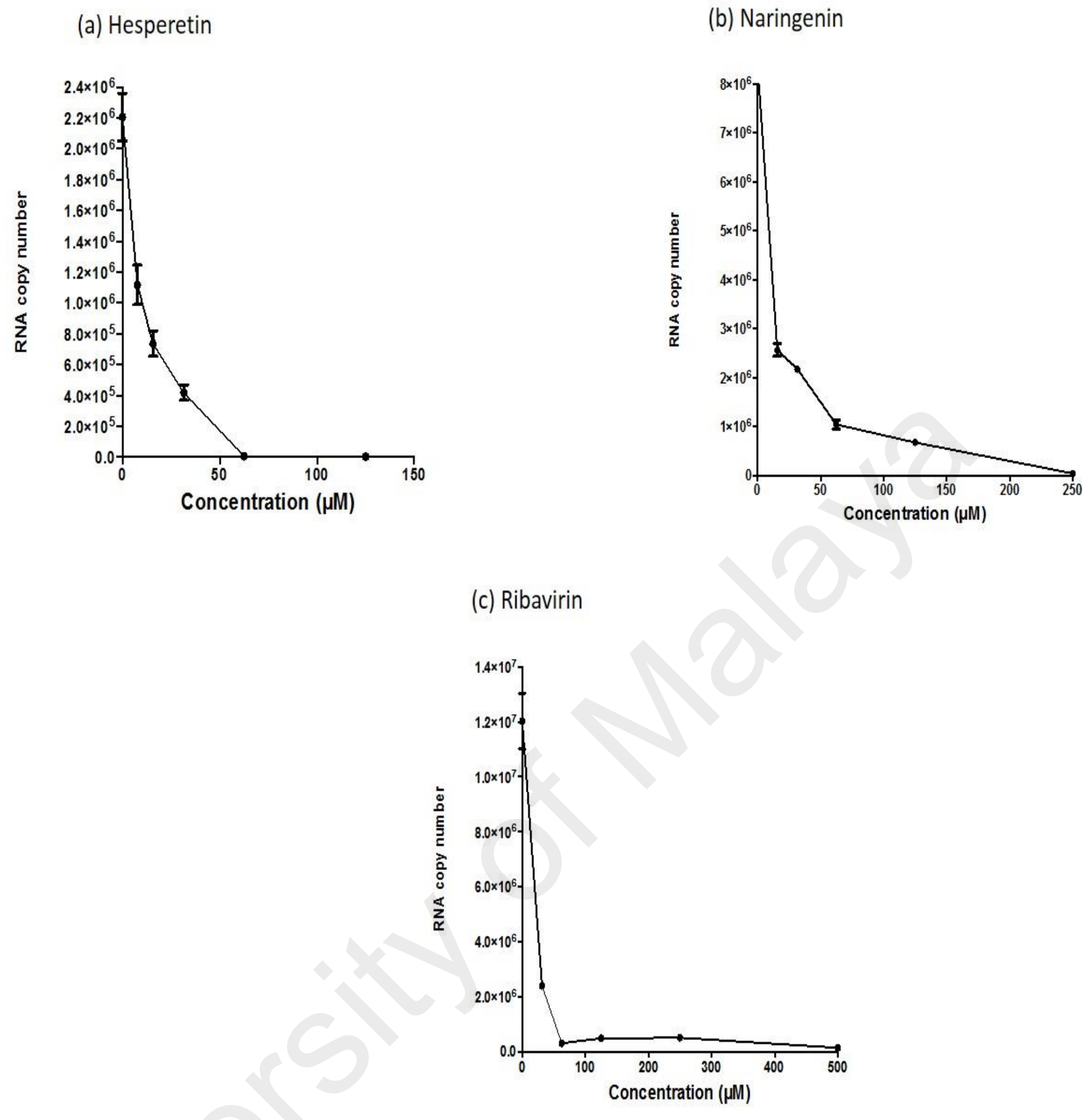

Figure 4.3 Evaluation of selected flavanone and ribavirin againt post–entry stages of CHIKV infection. Hesperetin, naringenin and ribavirin displayed potent antiviral activity at the post-entry stage of CHIKV infection**.** The GraphPad Prism 5 used to plot the graph. Error bars represent standard errors of triplicate means.

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4.5 Anti –Entry assay

Subsequent experiments were performed to evaluate the potential of compounds against the early stages of CHIKV life cycle, namely anti-entry (I), anti-adsorption (II) and direct virucidal (III). The inhibitory activity of flavanones against the internalization of CHIKV into Vero cells were tested by performing compounds treatment on CHIKV-infected cells at the stage of viral entry. Based on the results of the anti-entry assay shown in Figure 4.4, hesperetin and naringenin did not show inhibition against CHIKV internalization into the Vero cells. Therefore, it can be concluded that both compounds are not able to affect the early stages of CHIKV *in vitro* replication.

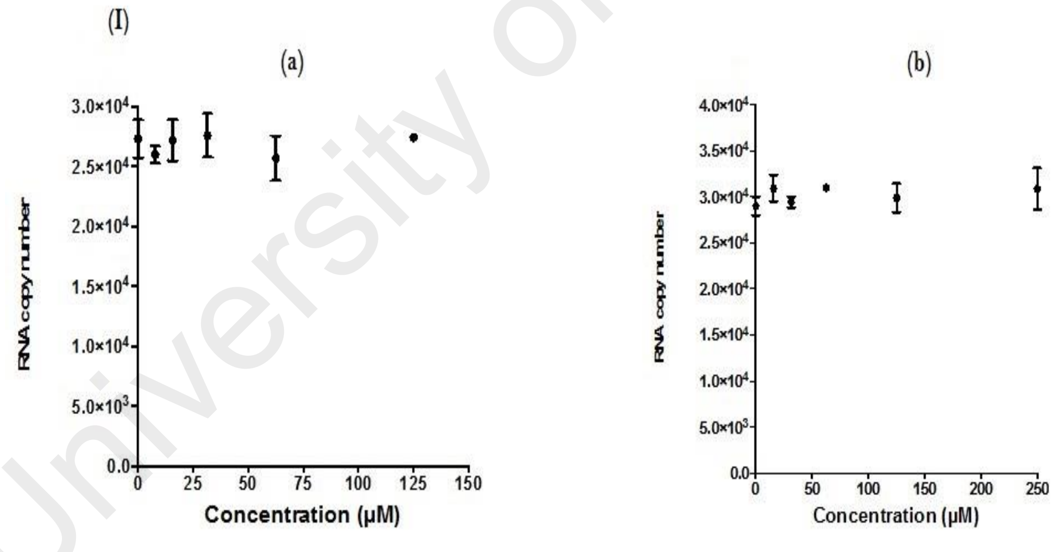

Figure 4.4 Effect of hesperetin and naringenin against anti-entry stage of CHIKV replication. There were no antiviral activities of hesperetin (a) and naringenin (b) against anti-entry stage of CHIKV replication cycle. The GraphPad Prism 5 used to plot the graph. Error bars represent standard errors of triplicate means

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4.6 Anti-adsorption assay

The activity of hesperetin and naringenin on inhibiting of CHIKV into the cells' surface receptors were determined by carrying out the anti-adsorption assay, in which cells were infected with CHIKV simultaneously with the addition of compounds. Based on the results of the anti-adsorption assay shown in **Figure 4.5**, hesperetin and naringenin did not exert inhibition against CHIKV replication when they were added during the virus attachment stage to the Vero cells. Therefore, it can be determined that there is no antiviral activity of these two compounds against virus adsorption to the cell surface.

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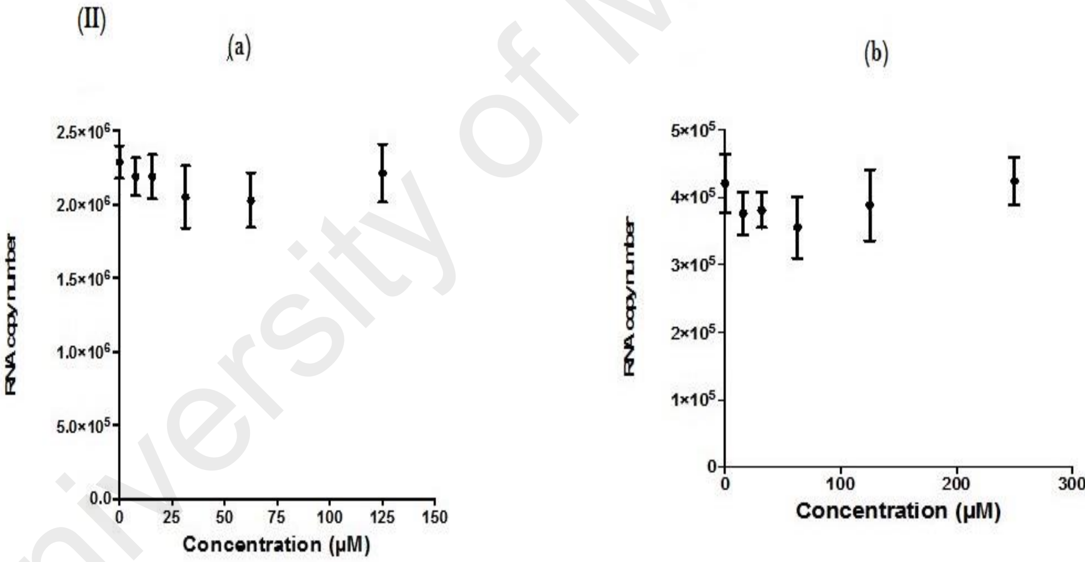

Figure 4.5 Effect of hesperetin and naringenin against anti-adsorption stage of CHIKV replication. There were no antiviral activities of hesperetin (a) and naringenin (b) against anti-adsorption stages of CHIKV replication cycle. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where $P<0.05$ is significant. "0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

4.7 Direct virucidal assay

Antiviral activity of compounds against extracellular CHIKV particles were examined by first treating the CHIKV suspension with different concentrations of compounds prior to infection the Vero cells. Based on the results of virucidal assay shown in **Figure 4.6**, hesperetin and naringenin did not show any inhibitory effects against extracellular CHIKV particles either. These results suggest that both compounds could not inactivate free CHIKV particles and neutralize their infectivity attributes once they were added to virus suspension directly. In this study, it can be concluded that hesperetin and naringenin did not affect the early stages of CHIKV replication *in vitro*.

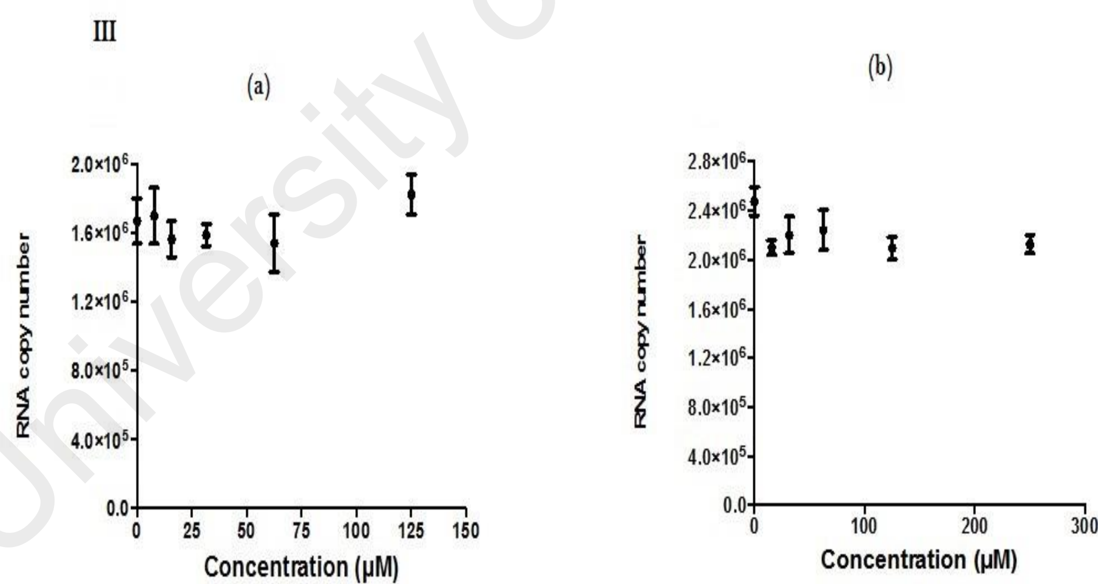

Figure 4.6 Effect of hesperetin and naringenin against direct virucidal stages of CHIKV replication. There were no antiviral activities of hesperetin (a) and naringenin (b) against direct virucidal stages of CHIKV replication cycle. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where $P<0.05$ is significant. "0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means

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4.8 Immunofluorescence assay

The immunofluorescence assay was conducted to distinguish the CHIKV antigen presentation as an indicator for the successful replication and infection of CHIKV as well as to discover the degree of inhibition by tested compounds. All tested compounds displayed a dose-dependent inhibition of CHIKV infection compared to the vehicle control **Figure 4.7**. The appearance of the CHIKV E2 stained with Alexa Fluor 488 (green colour) reduced in terms of intensity and amount as the concentration of the both compounds were increased. According to automated calculation by HTS machine, hesperetin and naringenin at concentration of 125 and 250 µM, respectively, were proficient to inhibit ≥90% CHIKV antigen presentation. Therefore, this screening platform was found to be beneficial and consistent for showing the compounds antiviral effects effectively and revealing the potential CHIKV inhibitors.

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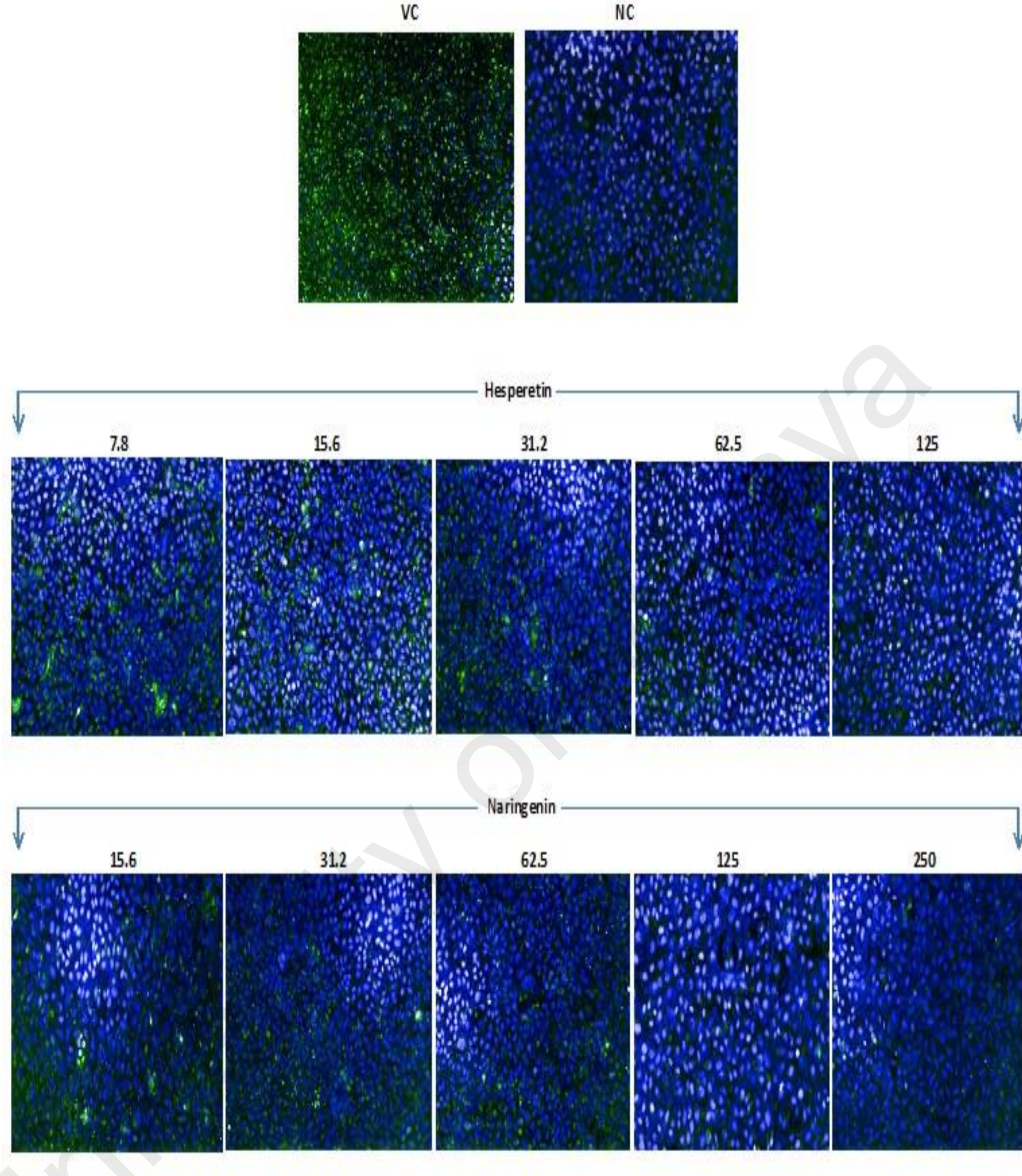

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Figure 4.7 Evaluation of flavanones on CHIKV activity using immunofluorescence assay. Dose-dependent inhibition of hesperetin and naringenin on the CHIKV infectivity has been shown. Detection of the CHIKV E2 protein is used as the indication of the successful CHIKV infection. Cell nuclei are stained with DAPI (blue) and CHIKV infection is indicated by Alexa Fluor 488 (green) staining.

4.9 Immunoblotting

To determine the effects of hesperetin and naringenin on CHIKV protein synthesis, Western blot analyses were performed. CHIKV Proteins including nsp1 (59 kDa) and nsp3 (76-78kDa) proteins were distinguished by using the colorimetric Method. The detection of the target CHIKV proteins would reveal the effects of the selected compounds on the process of CHIKV replication *in vitro*. A dose-dependent reduction of CHIKV, nsp1 and nsp3 following hesperetin and naringenin treatment showed **Figure 4.8**. Hesperetin at concentrations of 125 and 62.5 μM and naringenin at 250 and 125 μM, were showed minimal amounts of nsP3 and nsp1 proteins. This suggested that hesperetin and naringenin might inhibit CHIKV protein production, leading to a decrease in infectious virus titers as seen above. Nevertheless, there was a general trend of dose-dependent inhibition of CHIKV infection by hesperetin and naringenin, supporting the results obtained with the different experimental approaches reported above. β-Actin was used as a loading control in the experiment, as well as to ensure that the concentration of both compounds used in this study did not affect the synthesis and expression of host cellular proteins.

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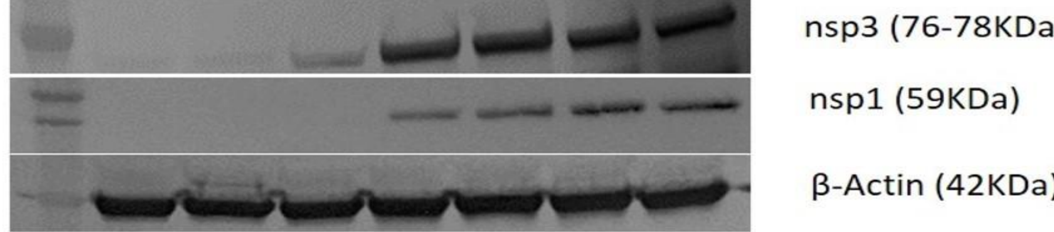

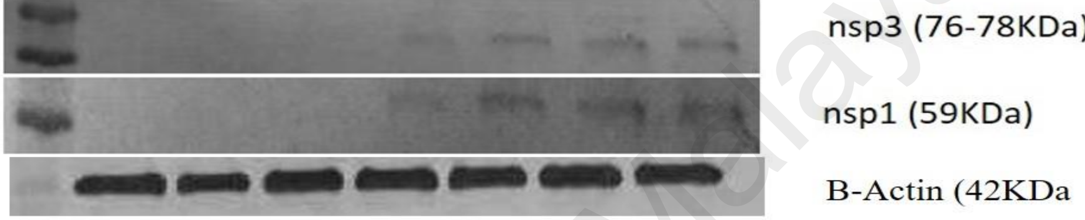

Figure 4.8 Hesperetin and naringenin inhibited the accumulation of CHIKV-encoded proteins. Western blot analyses were performed to determine the effect of both compounds on production of nsp1 and nsp3 proteins**.** Reduction of CHIKV nsp1 and nsp3 proteins observed upon naringenin and hesperetin treatment. β-Actin is used as a loading control for each compound.

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CHAPTER 5:DISCUSSION

5.1 Experimental findings

In recent years, the number of reported CHIKV occurs has severely increased. The most drastically outbreak to date was documented between 2005–2006 in La Réunion (Her et al., 2009; Keren et al., 2006). Originating from Africa, CHIKV spread to Asia, emerged in Southern Europe and reached the Americas (Weaver & Forrester, 2015). CHIKV causes Chikungunya fever, an acute infections which usually associated with a low mortality rate, but can proceed into a painful chronic stage, during which patients can suffer from polyarthralgia and joint stiffness for weeks and even several years. There are no licensed vaccines or antiviral drugs available for the prevention or treatment of CHIKV infections. Current therapy is solely with administration of analgesics, antipyretics, and non-steroidal anti-inflammatory agents to relieve symptoms. The increasing incidence of chikungunya outbreaks has made huge efforts to discover an effective antiviral drugs and vaccines to manage CHKV infections (Thiboutot et al., 2010).

Until recently, only several studies aiming at CHIKV antiviral drugs have been reported. Arbidol, a small indole-derivative, antiviral drug originally used for treatment of influenza and other respiratory viruses was reported to have potent antiviral properties against CHIKV *in vitro*. It was found to exert its anti-CHIKV effect by binding to the E2 domain of the viral envelope protein and interfering with viral attachment on host cell receptor (Delogu et al., 2011). The chloroquine phosphate, a quinolone-containing drug used as an antimalarial drug was previously suggested for both therapeutic and prophylactic effects of CHIKV-associated arthralgia (Bloland et al., 1993). Chloroquine demonstrated its inhibitory properties against CHIKV infection *in vitro*, which has a narrow selectivity index in cell cultures

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(Sourisseau et al., 2007). However, in clinical trial for treatment of acute chikungunya infections in La Réunion with chloroquine did not show any significant benefits in the use of this drug (Lamballerie et al., 2008).

Ribavirin, a nucleoside analogue prodrug that has been widely used as an antiviral for several DNA and RNA viruses, was also recommended as a therapeutic drug to treat Chikungunya induced arthritis (Ravichandran & Manian, 2008; Wu, Lin, & Hong, 2003). However, no further follow-up studies were conducted. Recently, a distinctive method to identify inhibitors of alphavirus entry and replication was studied (Pohjala et al., 2011). The assays performed in this particular screening involved the use of a non-cytotoxic CHIKV replicon expressing *EGFP* and *Rluc* and a SFV surrogate model. The study reported several active 5,7-dihydroxyflavones (e.g. apigenin, chrysin, naringenin and silybin) from a collection of natural products that suppresses CHIKV and SFV replication, suggesting these molecules may be good antiviral candidates against alphavirus infections.

Since several compounds with anti-CHIKV drugs didn't support any beneficial effects in clinical cases so far, there is an urgent need to study the possibility of searching antiviral compounds with high viral inhibitory activities, low toxicity and high efficiency. Lately, there has been increasing interest in the research on bioflavonoids as natural organic products as antiviral agents because it guarantees continuous supply, ease of preparation, relatively minimal toxicity and side effects. However, these phenolic compounds do not play vital role for plant survival and growth, however, many of them are shown to have numerous medicinal properties including, anti-allergic, anti-diabetic, anti-bacterial, antiviral, anti-inflammatory, anti-proliferative, anti-thrombotic, anti-mutagenic anti-carcinogenic, oestrogenic, hepatoprotective, insecticidal, and antioxidant activities (Tringali, 2003).

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The current study was designed to evaluate the anti-CHIKV activity of selected flavanones; hesperetin and naringenin, which are well known to exhibit various medicinal properties encompassing antiviral activity. Several studies have reported that both flavanones were able to block the replication of variety of other viruses. Hesperetin was shown to inhibit the replication of poliovirus type 1, herpes simplex virus type 1, parainfluenza virus type 3, influenza, respiratory syncytial virus and sindbis virus. Naringenin was also shown to have a great inhibition on hepatitis C virus (HCV) and the CHIKV replicon cell system, along with a slight inhibition of HIV-1 virus. More recently, it has also been reported that hesperetin and naringenin also inhibit the replication of yellow fever virus. (Castrillo et al., 2015; Kaul et al., 1985; H. K. Kim, Jeon, & Ko, 2001; Paredes et al., 2003).

It has also been reported that both flavanones are able to reduce the activity and the expression of the microsomal triglyceride transfer protein and the acyl-coenzyme A cholesterol acyltransferase 2, which modulate the apolipoprotein B (apoB) secretion that is vital for HCV budding from human hepatoma cell line (Castrillo et al., 2015; Wilcox, Borradaile, de Dreu, & Huff, 2001). Naringenin were discovered to inhibit virion assembly and more recently docking studies of HCV nonstructural protein (NS2) protease as targets indicated that naringenin was a good inhibitor of the virus replication (Khachatoorian et al., 2012; Lulu et al., 2015).

In this study, Vero cell line was used due to the high tropism of CHIKV towards these cells (Sourisseau et al., 2007). Cytotoxicity assay was first performed using MTS assay, to determine the level of cytotoxicity of each compounds together with the vehicle control, 1% DMSO on Vero cells. Cytotoxicity assay showed that hesperetin and naringenin up to 125 and 250μM concentrations could be used to examine the antiviral activity without affecting the cell viability. The degree of

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cytotoxicity of compounds and 1% DMSO were needed prior to the subsequent screening and antiviral assays to prevent from obtaining false positive results due to deaths of Vero cells as a result of the toxicity of the compounds or the vehicle control itself.

After determining the level of toxicity of selected flavanones, the compounds were screened for inhibitory effects on CHIKV replication. The first screening assay performed was the luciferase assay, which involves the BHK-CHIKV replicon cell line only. This cell line system was chosen to test our hypothesis plus it also offers a screening-friendly approach. The replicon was constructed from a non-infectious replicon of CHIKV as well as the virus replicase proteins with puromycin acetyltransferase, EGFP and Renilla luciferase marker genes for this objective. Mainly, the BHK-CHIKV replicon cells lack the nucleotide sequence coding for structural proteins of CHIKV, so any inhibitory effect would be targeted against the non-structural protein regions of the virus. Upon the addition of the lysis buffer, the cells were lysed and CHIKV replicons were released. The amount of CHIKV replicons in the media were quantified according to the luciferase signal in the form of luminescent, resulting from the reaction between the luciferase enzymes encoded by the CHIKV replicons with subsequent addition of luciferase substrate. This allowed us to identify the potential antiviral candidates for alphavirus entry and replication phase inhibitors. Throughout the screening procedure, the level of luciferase signals was shown to decrease as the concentration of hesperetin and naringenin increased. It was found that hesperetin and naringenin inhibited the *Rluc* activity with IC_{50}=85.0 with IC_{50}=93.27 μM, respectively. From these readings, it can be assumed that hesperetin and naringenin moderately displays an inhibitory effect on CHIKV replication. Recently, it has been reported that naringenin considerably reduced the expression of

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viral RNA levels by utilizing a CHIKV replicon system and in the same study, the authors examined different compounds using a different replicon system, showed naringenin was also capable of inhibiting the viral entry of Semliki Forest Virus (Pohjala et al., 2011). However, any related result on CHIKV-infected Vero cells was yet to be known.

Hence, the subsequent screening assay was carried out using the Vero cell line. This was achieved by carrying out time of addition assay, to identify the stages of CHIKV replication cycle at which hesperetin and naringenin exerts their inhibitory activities. Vero cells were treated with hesperetin, naringenin and ribavirin (as a control) at different time points, whereby each time point represents different stages of CHIKV replication cycle. As shown in Figure 4.2, CHIKV viral load reduced significantly at the early hour (-2, 0 hpi) of hesperetin, naringenin and ribavirin treatment. This shows that hesperetin and naringenin exerts their anti-CHIKV effects mainly during the early to middle stages of CHIKV replication cycle. The half-life of compounds would possibly be one of the ultimate reasons that would cause the effective time for both flavanones to exert its antiviral effect at -2 hpi. Ribavirin has shown the most potent antiviral activity up to 1 hour post infection but like other tested compounds it has shown anti-CHIKV activity up to 12 hours post infection which was consistent with previous findings (Rothan et al., 2015). In addition, both flavanones could possess prophylactic activity towards the infection of CHIKV as well.

The results from time of addition assay were further clarified by different antiviral assays, namely post-adsorption, anti-entry, anti-adsorption and direct virucidal assays, which study the affectivity of compounds against different stages of CHIKV replicative cycle.

The post-adsorption or post-entry assay is central to conclude the intracellular antiviral activity of compounds against CHIKV. Both flavanone compounds showed activity against the post entry activity of CHIKV. It was shown that hesperetin with IC_{50}=8.500 μM and naringenin with IC_{50}=6.818 μM possess significant antiviral effects that act by inhibition of viral replication in post-entry assay. Interestingly, the result was consistent with our primary data from the time of addition study where the virus yield was significantly reduced in CHIKV-infected cells upon treatment with hesperetin and naringenin shortly before and through few hours of infection (-2,0 and 2hpi). These findings were also consistent with CHIKV replicon cell system, where no virus entry or exit takes place. Taken together, the obtained results were extensively explained by remaining antiviral assays where there is no inhibition in anti-entry, anti-adsorption, direct virucidal assay correspondingly.

To further confirm the potent anti-CHIKV activity of flavanones, we examined the effects of the tested compounds against viral antigen synthesis in CHIKV infected cells through a quantitative immunofluorescence assay using a monoclonal antibody against envelope protein of CHIKV together with immunoblotting which showed the significant reduction in structural and non-structural protein synthesis. In immunofluorescence assay, hesperetin and naringenin showed 90% reduction in the intensity and quantity of the antigen even at the lowest concentrations, Figure 4.7. This confirms that the flavanones were able to interfere with the processing and maturation of the E2 antigen as well as the transportation of the E2 antigen to the plasma membrane. This observation could be due to inhibition of CHIKV RNA replication and/or transcription or even inhibition of viral protein synthesis and processing. It is most likely that some of non-structural proteins of CHIKV become possible target for hesperetin and naringenin.

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The possible targets may include nsP1 protein, which participates in the synthesis of the negative strand of viral RNA and RNA capping, as well as nsP3 protein, that is another key component of the viral life cycle such as viral RNA synthesis, virulence and protein-protein interactions. Since, it has been reported that the nsP3 proteins is the only non-structural protein that contains a large number of serine and threonine residues in order to be phosphorylated by activated host kinases (Jose, Snyder, & Kuhn, 2009), both flavanones may target the phosphorylation status of nsP3 which led to restriction of viral replication and increase host survival.

Reduction of E2 protein expression is possibly due to suppression of replication directly or via inhibition of ns-protein(s). This viral protein reduction is applicable from point of view of development of effective antiviral as E2 protein plays an important role in viral glycoproteins and is essential for receptor binding. Nevertheless, to confirm the direct effect of the compounds against protein synthesis and processing further experiments are needed, which could be considered for future studies. Nevertheless, the molecular size and the solubility of hesperetin and naringenin that might lead to the significant penetration through the biological membrane and entering to the cytoplasm or other subcellular compartments could nominate them as noble candidates for further investigation towards antiviral drug development (Castrillo et al., 2015).

The statistical analysis showed that all experimental qRT-PCR results were statistically significant ($p < 0.05$) which show that all antiviral activities observed were due to the treatment with hesperetin and naringenin. The qRT-PCR results supported the cytopathic effects (CPE) observed in the infected Vero cells on 2nd day of all experiments.

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5.2 Limitations and Recommendations

Although this study has achieved its aims, there were some unavoidable limitations and difficulties. At the beginning of practical work, inaccurate and undesirable results were obtained due to limited skills in performing certain procedures and methods. For instance, while performing the cytotoxicity and different antiviral assays, the % of cell viability and the CHIKV RNA levels obtained were different significantly due to possible errors made once diluting the compounds, pipetting errors while working on duplicates or triplicates and variations in the amount of cells seeded into each well. Eventually, we overcame this challenge by frequent practices. Moreover, as the Vero cell is a fast growing cell line, the confluency of the cell culture had to be monitored daily to avoid the cells from being too confluent, which could cause cell deaths due to the lack of nutrients and space to grow.

Performing molecular biology techniques works such as RNA extraction, cDNA synthesis and qRT-PCR required well-trained skills since they are highly sensitive to errors and contamination, so it was needed to improve my abilities and stay focused while carrying out these experiments. Contamination could be avoided by wiping the hood or workplace with alcohol, as well as using RNase inhibitor for cDNA and qRT-PCR works, before and after the experiments. It was also important to avoid light-sensitive materials such as the MTS reagent and compound dissolved in 1% DMSO from directly exposed to light. Hence, when working with these materials, the light in the hood and the workplace had to be switched off.

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CHAPTER 6: CONCLUSION

In summary, the objectives of this study, which were achieved accordingly. We convincingly showed that hesperetin and naringenin exhibited significant anti-CHIKV activities probably by interfering with virus intracellular replication. Both flavanones suppressed post-entry stages of viral replication in a dose dependent manner. Coherent with this the expression of proteins, needed for RNA replication, and also expression of viral structural E2 protein were down regulated. These findings warrant future mechanistic, *in vivo* anti-viral, toxicity and pharmacokinetic studies as part of the process to evaluate hesperetin and naringenin as potential therapeutic candidates against CHIKV. *In vivo* studies are essential for the development of an antiviral drug as the affectivity of the compounds need to be proven in animals as well as cell lines to enhance the confidence of the community on this compound's ability in treating CHIKV infection in humans. Another approach for inhibiting the CHIKV infection is by targeting cellular factors such as protein kinases and other cellular factors, which are involved in CHIKV replication and/or in another way by the induction of immune-based cellular enzymes that possess antiviral activity.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Azin Ahmadi, Pouya Haasandarvish, Rafidah Lani, Pedram Yadollahi, Amin Jokar, Sazaly Abu Bakar and Keivan Zandi "Inhibition of chikungunya virus replication by hesperetin and naringenin" RSC Advances, 2016, 6, 69421-69430.
2. Azin Ahmadi, Soheil Zorofchian Moghadamtousi, Sazaly Abubakar and keivan Zandi "Antiviral Potential of Algae Polysaccharides Isolated from Marine Sources: A Review" Journal of synthesis. 2015, 41:42.

Papers Presented

1. Azin Ahmadi, Pouya Haasandarvish, Rafidah Lani, Pedram Yadollahi, Amin Jokar, Sazaly Abu Bakar and Keivan Zandi "Inhibition of chikungunya virus replication by hesperetin and naringenin" Poster presentation delivered at Malaysian Society of Pharmacology and Physiology (MSPP) at Putrajaya, Malaysia, 15th& 16th August, 2016.

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