EXPLORING BIOFLAVONOIDS AS NOVEL ANTIVIRALS AGAINST CHIKUNGUNYA VIRUS

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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne virus that recently has been classified as a Category C pathogen by National Institute of Allergy and Infectious Diseases (NIAID). This alphavirus causes several clinical features similar to dengue virus infection, except polyarthritis and tenosynovitis, where the similarities would usually cause misdiagnosis. CHIKV has caused many large outbreaks all over sub-Sahara Africa and tropical Asia including India and the Western Pacific. This could possibly turn into an emerging global pandemic if no effective preventive measures are taken. Since CHIKV spreads by increased global travels, immunologically naive populations such as in the United States (US) is at risk, since it is one of the non-endemic regions. Besides the US recently, there have been travel-associated CHIKV cases in Australia, Asia and European countries as well.

The challenge posed by CHIKV is there is no vaccine and antiviral treatment currently available for CHIKV infection. CHIKV infection is treated symptomatically by the administration of non-steroidal anti-inflammatory drugs or steroids, bed rest and fluids. In worst case scenarios, such as debilitating chronic CHIKV infection, corticosteroids is the only option. Available treatments such as chloroquine can only inhibit CHIKV cell-to-cell spread but not the replication of the infected cells. Research on vaccines and antivirals are still actively pursued to produce safer vaccines with longer protective effects and persistent antibodies. Live vaccines were produced, but with side effects including risks of producing chronic rheumatism. Most antiviral drugs that have been suggested are nucleoside analogues where they are potentially teratogenic, embryotoxic, carcinogenic and possess anti-proliferative activities.

Turning to organic sources may prove to be more beneficial in the search for anti-CHIKV compounds, such as natural bioflavonoids which can be derived from most herbal medicines and ordinary fruits. Bioflavonoids are phenolic compounds that possess anti-oxidant, anti-tumor, anti-proliferative, anti-inflammatory, antibacterial and antiviral activities. Thus, in this study, the main objective is to find non-toxic bioflavonoid compounds that could inhibit the CHIKV infection or at least reduce the CHIKV replication at *in vitro* level.

In order to meet the objectives of this study, various antiviral assays were performed including the CHIKV replicon cell line-based assay, immunofluorescence assay and western blotting analyses. The replication efficiency of CHIKV at each antiviral assay was determined by using the qRT-PCR assay with RNA copy number as the parameter. Statistical analysis was performed by using the Graph Pad Prism 5 software with suitable statistical analysis for each assay. Through this study, 4 out of 14 bioflavonoid compounds were identified to exhibit intracellular antiviral activity against CHIKV at different stages of CHIKV life cycle.

These compounds are baicalein, fisetin, quercetagetin and silymarin. These compounds were also able to suppress the accumulation of important CHIKV proteins such as pE2, E2, nsP1 and nsP3 proteins in addition to the ability to interfere with CHIKV replication cycle. This study is the first step towards finding a potent anti-CHIKV compound.

ABSTRAK

Virus Chikungunya (CHIKV) ialah virus bawaan nyamuk yang mutakhir ini dikelaskan sebagai patogen kategori C oleh *National Institute of Allergy and Infectious Diseases* (NIAID). Alphavirus ini menyebabkan beberapa ciri klinikal yang sama seperti virus denggi, kecuali *polyarthritis* and *tenosynovitis*, dan persamaan tersebut sering menyebabkan ketidaktepatan dalam kajian diagnostik. CHIKV telah menyebabkan banyak kes penularan sekitar Sahara Afrika dan Asia tropika termasuk India dan Pasifik barat. Hal ini boleh menyebabkan pandemik global yang baru jika tiada sebarang tindakan yang efektif diambil. Populasi yang naif immunologi seperti di Amerika Syarikat adalah berisiko untuk penularan CHIKV dengan meningkatnya pelancongan global memandangkan ianya bukan kawasan endemik. Malangnya, sudah terdapat beberapa kes CHIKV berkaitan pelancongan di Eropah, Australia, Asia dan baru-baru ini Amerika Syarikat.

Masalah yang membimbangkan sekarang ialah tidak ada sebarang vaksin dan rawatan antivirus yang terdapat bagi CHIKV. Jangkitan CHIKV dirawat berdasarkan gejala dengan ubatan anti-radang bukan steroid atau steroid, rehat dan cecair. Kortikosteroid digunakan bagi gejala yang lebih teruk seperti jangkitan CHIKV kronik yang melemahkan. Ubatan yang boleh didapati seperti chloroquine cuma boleh menyekat penyebaran dari sel ke sel dan bukan replikasi di dalam sel yang telah dijangkiti. Penyelidikan mengenai vaksin dan antivirus masih lagi diteruskan bagi mencari vaksin yang selamat dengan kesan perlindungan yang berpanjangan dan antibodi yang berterusan. Vaksin hidup merupakan salah satu calon akan tetapi risiko sakit sendi yang kronik tidak boleh diambil ringan. Kebanyakan antivirus yang dicadangkan adalah analog nucleoside dan ia mempunyai potensi teratogenic, embriotoksik, karsinogenik dan mempunyai aktiviti anti-proliferatif.

Adalah tidak salah meningkatkan peluang bagi mendapatkan antivirus yang sesuai dengan meluaskan bidang penyelidikan apatah lagi bila beralih kepada kompaun bioflavonoid semula jadi yang boleh didapati menerusi kebanyakan ubatan herba dan buah-buahan. Bioflavonoid merupakan kompaun fenolik yang mempunyai aktiviti anti-oksida, anti-tumor, anti-proliferatif, anti-radang, anti-bakteria dan antivirus. Maka, pada kajian ini, matlamat utama ialah bagi mencari kompaun bioflavonoid yang tidak toksik pada kepekatan tertentu boleh menyekat jangkitan CHIKV atau sekurang-kurangnya boleh mengurangkan replikasi CHIKV pada tahap *in vitro*.

Bagi mencapai matlamat tersebut, pelbagai jenis kajian antivirus telah dijalankan termasuk kajian berdasarkan sel replikon CHIKV, *immunofluorescence* dan analisis *western blotting*. Keberkesanan replikasi CHIKV pada setiap kajian antivirus ditentukan melalui *qRT-PCR* dengan menggunakan bilangan salinan RNA sebagai parameter. Analisis statistical telah dijalankan dengan menggunakan perisian Graph Pad Prism 5 dengan analisis yang sesuai bagi setiap kajian antivirus. Melalui kajian ini, 4 daripada 14 kompaun bioflavonoid telah dikenalpasti mempunyai aktiviti antivirus intraselular terhadap CHIKV pada tahap yang berbeza dalam kitar hidup CHIKV.

Kompaun tersebut merupakan *baicalein*, *fisetin*, *quercetagetin* dan *silymarin*. Kompaun ini juga boleh menyekat akumulasi protein-protein penting CHIKV seperti pE2, E2, nsP1 and nsP3 selain berkebolehan mengganggu kitar replikasi CHIKV. Semoga kajian ini menjadi langkah pertama ke arah mencari poten anti-CHIKV.

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"And pursue not that of which you have no knowledge; for surely the hearing, the sight, the heart, all of those shall be questioned of" (Quran, 17:36).

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-	Minus
%	Percentage
˚C	Degree Celsius
>	More than
÷	Divide
≥	More than or equal to
µg/ml	Microgram per milliliter
µM	Micro molar
Ae.	*Aedes* species
Akt	Protein kinase B
bp	Base pair
CA16	Coxsackie virus 16
CC_{50}	Half-maximal concentration exhibit cytotoxicity
CD4	Cluster of differentiation 4
CHIKV	Chikungunya virus
CPE	Cytopathic effect
DENV	Dengue virus
DMSO	Dimethyl sulfoxide

E1	Envelope protein 1
EC_{50}	Half-maximal showing effective respons
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EtOAc	Ethyl acetate
EV71	Enterovirus 71
H1N1	A strain of influenza virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HCVpp	Hepatitis C virus pseudoparticles
HIV	Human immunodeficiency virus
hpi	Hour post-infection
IC_{50}	Half-maximal treatment concentration showing inhibitory effect/untreated infected control
ID_{50}	Half-maximal dose causing infection
IE-1	Immediate-early protein 1
IE-2	Immediate-early protein 2
IFN-α	Interferon alpha
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-8	Interleukin 8
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
NC	Nucleocapsid
ng	Nano gram
nm	Nanometer
NS5B	Non-structural protein 5B
nsps	Non-structural proteins
ORF	Open reading frame
PBS	Phosphate buffer saline

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
ssRNA	Single-stranded ribonucleic acid
$TCID_{50}$	Half-maximal tissue culture inhibition dose
UV	Ultraviolet
µg/ml	Microgram per milliliter

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CHAPTER 1: INTRODUCTION

1.1 Chikungunya virus

The word 'chikungunya' was derived from Makonde language which means 'that which bends up', refers to the contorted posture of the infected person who is suffering from the severe joint pain (Mavalankar *et al*., 2008). Chikungunya virus (CHIKV) is classified under the *Togaviridae* family, in *Alphavirus* genus (Johnston & Peters, 1996). The transmission of the virus is through infected *Aedes aegypti*, the classical vector, and *Aedes albopictus* (Tsetsarkin *et al*., 2007) as well as the maternal-fetal transmission (Gerardin *et al*., 2008) which occurs in some cases of a recent epidemic (Schuffenecker *et al*., 2006).

Since the outbreak in La Réunion, *Ae. albopictus* has become one of the vectors for CHIKV (de Lamballerie *et al.,* 2008). The CHIKV has proven to adapt to geographic changes and temporal distribution of the vectors (Watts *et al.,* 1987), thus increasing replication efficiency and viral incubation period inside the vectors (Alto & Juliano, 2001). Thus, the changes in envelope protein (E1) helped it to achieve the adaptation (Gould & Higgs, 2009). The changes in E1 protein (class II viral fusion protein) assists the adaptation of CHIKV into a new vector (Gibbons *et al.,* 2003), *Ae. albopictus* in a way that it mediates the entry of the virus at a low pH and at once (Gibbons *et al.*, 2004), affect the viral fusion, assembly as well as the cell tropism (Kielian & Rey, 2006). Also, Ala226Val mutation in E1 protein was observed to be absent in the previous strains but exists in >90% of the CHIKV strains after the La Réunion outbreak (Schuffenecker *et al*., 2006). The ability of CHIKV to infect insect cells improved due to the cholesterol dependency which is affected

through the mutation by providing a preferable adaptation to the lipid composition of the cells and thus, ease the spread of CHIKV (Schwartz & Albert, 2010).

CHIKV virions exhibit the typical icosahedral shape of the alphavirus structure and they are made up of multiple arranged shells of molecules (Powers & Logue, 2007; Jose *et al.,* 2009). The icosahedral nucleocapsid (NC) is formed by the capsid proteins which encapsidates the genomic RNA. Its envelope is a host-derived lipid bilayer from the host cell membrane in which the viral glycoproteins are arranged in icosahedral lattice and 240 copies of E1 and E2 are embedded (Strauss & Strauss, 1994).

The CHIKV genome consists of linear, positive-sense, single-stranded RNA molecule of approximately 11.8 kb in size (Powers & Logue, 2007). The 5' two thirds of the CHIKV genome encoded the non-structural (NS) proteins (nsP1, nsP2, nsP3 and nsP4) required for virus replication and the 3' one third of the genome encoded the structural genes (Simizu *et al.,* 1984). The genome is capped with 7-methylguanosine at its 5' end while the 3' end is polyadenylated (Powers & Logue, 2007).

Phylogenetic analysis of the 837bp partial E1 CHIKV sequences (APPENDIX A) differentiated the CHIKV into 3 genotypes; West African, Central/East African and Asian genotypes, which were initially named based on the regions from the strains were first reported. The strains isolated from outbreak in Malaysia were the Central/East African genotype and Asian genotype (Sam *et al.,* 2009). The strain used in the present study is the Central/East African genotype (accession number: Malaysia08/MY/065/FN295485).

1.1.1 Epidemiology of chikungunya virus

CHIKV was first isolated in Tanzania from human and mosquitoes (*Ae. aegypti*) during the 1953's outbreak (Robinson, 1955). After 1954, occasional outbreaks occurred all over sub-Sahara Africa and tropical Asia (Hammon *et al*., 1960) including India and Western Pacific (Rao, 1971), and from Africa to Asia the outbreaks flourished in Philippines, Thailand, Indonesia, India, Sri Lanka, Vietnam, Cambodia as well as Myanmar (Thuang *et al*., 1975; Adesina & Odelola, 1991).

The studies done on differences between the transmission of CHIKV in Africa and Asia proposed that the transmission of CHIKV in Asia is based on mosquito-to-human transmission by the vector *Aedes aegypti* (Turrel *et al*., 1992), whereas, the transmission of CHIKV in Africa is only among human who lives in villages and rural areas (Diallo *et al*., 1999) and the main cycle maintained by non-human primates such as baboons and *Cercopithecus* monkeys (Adesina & Odelola, 1991). Massive CHIKV outbreaks befell the islands in Indian Ocean (the Comoros, Mauritius, Seychelles, Madagascar, Mayotte and Réunion) in 2006. About 265 000 clinical cases and 237 deaths were reported in Réunion island which has a population of only 770 000 (Charrel *et al*., 2007).

Among the first published studies in Malaysia on CHIKV was the serosurvey on anti-CHIKV antibody in Peninsular Malaysia by Marchete *et al*., 1978. Anti-CHIKV antibody was reported to be found in subjects older than 20 years and mainly live in the northern part of Malaysia including Perlis, Kedah and Kelantan (on the border with Thailand). In 1980, Marchete and colleagues again have reported that the presence of specific neutralizing antibody against CHIKV in a chicken in Kelantan and a pig in Kedah, and these further supported the CHIKV activity throughout the Malaysia-Thailand border.

The aforementioned studies showed that Malays who were mainly rural and aborigines, with the way of life as forest-dwellers, had high frequency of CHIKV antibody as their habitation was also inhabited by monkeys which are vertebrate hosts (Marchete *et al*., 1980). During the time the studies were performed, there were no recorded CHIKV outbreaks in Malaysia. The first CHIKV outbreak affected the residents in the suburb Klang, Selangor in 1998. Fever, rashes and joint pains were reported from the Klang outbreak and poor sanitation as well as unsatisfactory refuse disposal were cited as the cause (Lam *et al*., 2001).

In the early of 2006, second outbreak occurred in Bagan Panchor in Perak and the causal genotype was the Asian genotype, which is similar to the first outbreak (Kumarasamy *et al*., 2006). In another part of Perak state, Batu Gajah had an isolated case caused by Central/East African genotype. It was contracted from India in late 2006. Between April and September of 2008, southern part of Johor in Malaysia had the outbreak of more than a thousand suspected cases reported (Chew *et al*., 2009). From April 2008 to March 2010, over 10 000 cases without fatalities were reported nationwide (MOH, 2010).

The amplification of viral pools in wild rodents or birds has affected the ecology of arboviral species, and the zoonotic cycles which have been maintained in nearby forests and wetland are said to be associated with the large outbreaks (Weaver & Reisen, 2010; Pohjala *et al*., 2011). The rise of *Ae. albopictus* which has adapted to urban environment has changed the pattern of transmission through increased human-to-human transmission by feeding mosquitoes (Power & Logue, 2007).

1.1.2 Chikungunya virus genome organization

Once the approximately 11.8kb genome (**Figure 1.1**) is free and ready for translation, the non-structural proteins will be initially translated. This positive single-stranded RNA (ssRNA) genome encodes for two open reading frames (ORF) which are flanked by the 5' and 3' untranslated regions (Schwartz & Albert, 2010). Two precursors of non-structural proteins (nsPs) are translated from the 5' ORF of the genomic RNA by the cap-dependent mechanism. These precursors will then cleave into nsP1, nsP2, nsP3 and nsP4 which are responsible for the cytoplasmic RNA replication as well as the modulation of cellular antiviral responses (Tsetsarkin *et al.*, 2011). The roles of each non-structural protein are shown in the **Table 1.1**.

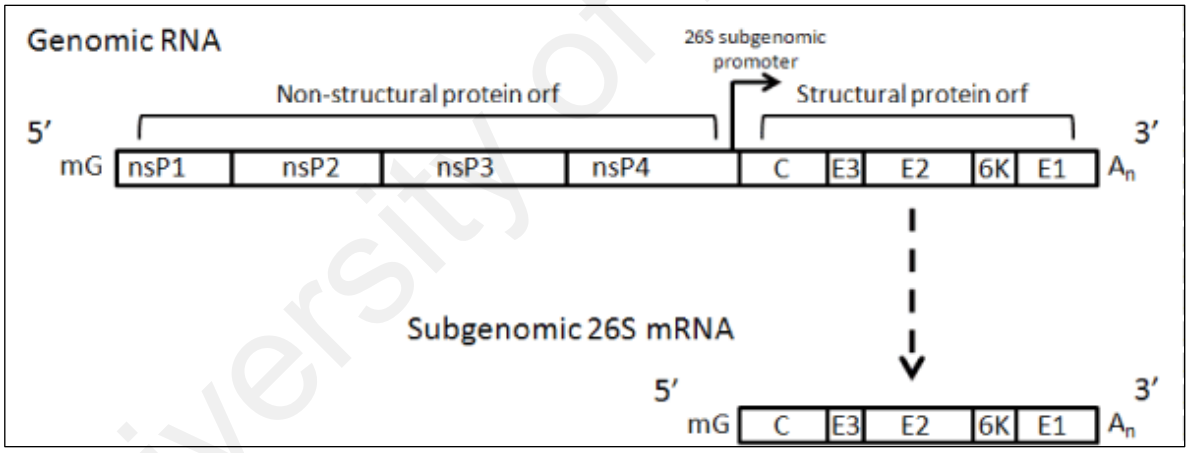

Figure 1.1: Chikungunya virus genome. Adapted from Spurgers and Glass, 2011.

Table 1.1: The roles of the non-structural proteins

Non-structural protein (nsP)	Roles
nsP1	Involved in the synthesis of the negative strand of viral RNA and and has RNA capping properties
nsP2	Displays RNA helicase, RNA triphospatase and proteinase activities and involved in the shut-off of host cell transcription
nsP3	Part of replicase unit
nsP4	Viral RNA polymerase

A full-length negative-strand RNA intermediate is synthesized by the viral replication complex made up of those proteins, and serves as the template for the synthesis of both subgenomic (26S) and genomic (49S) RNAs. The subgenomic RNA is translated from the 3' ORF, which is also capped, to yield three major structural virus proteins which are capsid, E2 and E1 glycoproteins (Schwartz & Albert, 2010). These three proteins are derived from the precursor polyprotein, C-pE2-6K-E1, through the processing by an autoproteolytic serine protease. The positive-strand and subgenomic RNA are synthesized exclusively later after infections and all the viral components synthesis are ready to assemble and leave the infected cells to infect others (Tsetsarkin *et al*., 2011).

1.1.3 The pathogenesis of chikungunya virus

Alphaviruses are classified into two subgroups based on their clinical pathogenesis which are; the New World viruses (those associated with encephalitis) (Weaver & Reisen, 2009) and the Old World viruses (those associated with polyarthritis and a rash) (Griffin, 2007). CHIKV is a member of arthritogenic alphaviruses, although both different manifestations can be seen due to its infection especially after the recent outbreak. Meningoencephalities (primarily in neonates) and haemorrhagic cases were documented from the recent outbreak (Gerardin *et al*., 2008). Despite infecting neurons like other

typical encephalogenic alphaviruses, CHIKV appears to infect the lining of the choroid plexus, specifically the stromal cells of the central nervous system.

Following a bite of infected *Ae. aegypti* and *Ae. albopictus*, CHIKV replicates in the skin (Talarmin *et al*., 2007) and spreads to the liver and joints (Robin *et al*., 2009), most likely through the blood (Couderc *et al*., 2008) and subsequently followed by a sudden onset of clinical manifestation after 2-4 days. Years of research on determining the pathogenesis of CHIKV has shown that CHIKV grows well and has tropism towards various human adherent cells including epithelial and endothelial primary cells and cell lines, fibroblasts and even towards the monocyte-derived macrophages (Sourisseau *et al*., 2007). Through this finding, Vero cells and BHK21 have been used in this study as CHIKV is highly cytopathic in mammal cell cultures and rapidly induces apoptotic cell death (Griffin, 2007).

1.1.4 The symptoms and diagnosis of chikungunya virus infection

During the acute phase of CHIKV infection, which typically lasts from a few days to a couple of weeks, the infected person might suffer from one or all of the symptoms such as; high fever, rigors, headache, photophobia and petechial rash or maculopapular rash (Mourya & Mishra, 2006). Unfortunately, the most unpleasant symptom of all is the incapacitating severe joint pain which recurs in 30-40% of those infected and last for even years after infection (Morrison, 1979). Routine chemistry and haematology profile of acute infection shows the viral load as high as 10^8 viral particles/ml of blood (Ng *et al*., 2009), 0.5-2 ng/ml of type 1 interferons (IFN) (Chirathaworn *et al*., 2010) as well as other pro-inflammatory cytokines and chemokines (Eckels *et al*., 1970). The laboratory diagnosis of CHIKV infection is done based on the detection of virus on early samples and /or anti-

CHIKV antibodies (after 5 days for IgM and few days later for IgG) on blood samples. Commercial diagnostic kits are available for both detection and occasionally come with excellent specificity and sensitivity (Presti *et al*., 2014).

1.1.5 Research and development on vaccine and antiviral drugs

As the CHIKV infection is symptomatic, the treatment offered for it also typically soothe the symptoms. The antipyretics and anti-inflammatory agents are used to combat the principal signs such as fever and joint pain. Various drugs have been tested and used including aspirin (Tesh., 1982), acetaminophen, ibuprofen (Brehin *et al*., 2009), steroid and non-steroidal drugs such as indomethacin and corticosteroids (Queyriaux *et al*., 2008). However, major drawbacks have been observed including hemorrhagic manifestation caused by the administration of aspirin and serious side effects after prolonged exposure to the later drugs (Powers & Logue, 2007). Fortunately, there are many natural or synthesized compounds and entities to be explored for their potency as antiviral agents. Chikungunya virus has all the features entitling it to be one of the targeted virus in the field of vaccine and antiviral drug development as it has high infection rates, broad geographical distribution and severe impact of morbidity. The availability of vaccine and antiviral drugs for CHIKV would open a whole new chapter in CHIKV treatment especially for the benefit of travelers to endemic areas and laboratory or medical workers with occupational risks.

The CHIKV vaccine development has started as early as in 1970s, which was initiated by Walter Reed (USA) with two modes of activation; formalin fixation and ether extraction. Both ways succeeded in inactivating the CHIKV and at the same time stimulating the neutralization antibodies, complements and haemagglutination (Eckels *et al*., 1970; Harrison *et al*., 1971). The results of the potency tests varied depending on the

dose, route of administration and the concentration. The formalin-fixed CHIKV vaccine harvested from green monkey kidney cells (GMKC) was also evaluated in human trials consisting of 16 US army recruits, and high levels of neutralizing antibodies were detected and no detectable viraemia was observed in the monkeys (Harrison *et al*., 1967).

The study went through a relatively slow progress. 30 years later, it was continued with Phase II for safety and immunogenicity and adverse events trials by using attenuated CHIKV strain from Thailand's 1962 outbreak (designated as 15561 strain). Both mice and human trials were performed and there were no adverse effects except mild to moderate joint pains in five subjects, and neutralizing antibodies developed with significant levels after 42 days (Edelman *et al*., 2000). The final form of the vaccine named CHIK 181/clone 25, gave promising results and was proposed for new drug application. However, the relevance of proceeding with the development of this vaccine was being questioned since no outbreaks occurred yet at that time. With the lack of commercial possibility and inadequate resources, the vaccine development could go nowhere but a Material Transfer Agreement signed by the United States Army Medical Research Institue for Infectious Diseases (USAMRIID) and The French National Institute of Health and Medical Research (Inserm) Transfert, Inserm's technology-transfer organization on 2006. There was no progress reported later but further clinical trials are still being held in the affected areas (Powers & Logue, 2007).

Despite unresolved vaccine development, investigations continued with another available option by studying antivirals. Most of the probable antivirals are still in preliminary stages and some of them have shown *in vivo* efficacy. The well-known broad spectrum antivirals such as chloroquine, ribavirin and IFN-α have shown potency as an anti-CHIKV. Unfortunately, the pathogenesis and biology of CHIKV as well as the life

cycle at molecular levels were poorly understood and remain unknown. These questions posed the greatest challenge towards finding an effective CHIKV antiviral. However, more studies and more antiviral candidates would broaden the probability of finding the solution. Some of the compounds and drugs which have been tested for anti-CHIKV activities were summarized **Table 1.2**.

Table 1.2: The compounds/drugs with their anti-CHIKV activities

Compounds/drugs	Anti-CHIKV activity/target	Reference (s)
Chloroquine	Inhibitors of viral entry	Khan *et al*., 2010
Arbidol		Delogu *et al*., 2011
10H-phenothiazines (chlorpromazine,perphenazine, Ethopropazine, thioridazine thiethylperazine and methdilazine)		Pohjala *et al*., 2011
RNA interference (siRNA & shRNA)	Inhibitors of viral protein translation	Dash *et al*.,2008; Lam *et al*., 2012
Harringtonine		Kaur *et al*., 2013
Homoharringtonine		
5,7-Dihydroxyflavones (apigenin, chrysin, naringenin and silybin)	Inhibitors of viral replicase	Pohjala *et al*., 2011
Prothipendyl		
Dapnane diterpenoids (Trigocherrins A, B &F and trigocherriolides A, B &C)		Allard *et al*., 2012
Ribavirin	Inhibitors of viral genome replication	Briolant *et al*., 2004
6-Azauridine		
Mycophenolic acid		Khan *et al*., 2011
IFN-α (2a and 2b)	Host immune response modulators	Briolant *et al*., 2004
Polyinosinic acid:polycytidylic acid [poly(I:C)]		Li*et al*., 2012
Decanoyl-RVKR-chloromethyl ketone	Inhibitors of viral glycoprotein maturation	Ozden *et al*., 2008.
Tigliane diterpenoids (Trigowiin A, Prostratin, 12-**O**-Tetradecanoylphorbol 13-acetate, 4α-12-O-Tetradecanoylphorbol 13-acetate)	Inhibitors with unknown targets	Bourjot *et al*., 2012

1.2 Bioflavonoids

Bioflavonoids, or flavonoids, are polyphenolic compounds that mainly can be found in plants. They are secondary metabolites with low molecular weight and are not essential for plant survival (Buer *et al*., 2010). Bioflavonoids exist ubiquitously in plants as aglycones, glycosides or methylated derivatives and serve as coloring agent (Mol *et al*.,

1998), auxin transport inhibitor (Peer & Murphy., 2007), allelopathy, defense (Bais *et al*., 2006), as well as UV protection in plants (Treutter., 2005). The basic structure of bioflavonoids are made up of 2-phenyl-benzo[α] pyrane or known as flavane nucleus (**Figure 1.2**), and 14 classes of bioflavonoids differ from each other based on their chemical nature as well as the substituents' position on the A, B and C rings (Brown, 1980).

Found in various plants and fruits, bioflavonoids are being consumed daily in significant quantities and they are one of the unavoidable components in our diet. Some of the bioflavonoids were also used in traditional eastern medicine in ancient years (Havsteen 1983; Grange & Davey,1990; Bosio *et al*., 2000). Bioflavonoids have been studied extensively in many fields of medical research. Many of them are known to have anti-oxidant (Williams *et al*., 2004), anti-tumor (Garcia-Mediavilla *et al*., 2007), anti-proliferative (Taylor & Grotewold., 2005), anti-inflammatory (Pandey *et al*., 2007) and pro-apoptotic activities (Sung *et al*., 2007). Nonetheless, they also exhibited anti-fungal (Wachter *et al*., 1999; Valsaraj *et al*., 1997; Zheng *et al*., 1996), antibacterial as well as antiviral activities (Tereschuk *et al*., 1997; Bosio *et al*., 2000; Pepeljnjak *et al*., 1982).

The antiviral activity of bioflavonoids against wide range of viruses is undeniable. Bioflavonoids such as baicalein, quercetin, kaempferol, luteolin and fisetin have shown their promising antiviral activity against dengue virus (Moghaddam *et al*., 2014), Japanese encephalitis virus (Zandi *et al*., 2012), enterovirus-71 (Evers *et al*., 2005), human cytomegalovirus (Mehla *et al*., 2011), herpes simplex virus (Xu *et al*., 2014) and even human immunodeficiency virus (Zhang *et al*., 2012).

There are many advantages of choosing bioflavonoid compounds as the candidates for anti-CHIKV, and some of them are; low-toxicity in animal, rarely have any side effects,

relatively long half-life, can easily be absorbed in the intestine, consumed daily and unlimited sources for extraction (Skibola *et al*., 2000; Middleton *et al*., 2000). Thus, in this study, 14 bioflavonoids from various groups were tested for possible anti-CHIKV activities *in vitro*, being selected based on previous published data on their antiviral activities against other viruses (please refer to the **Chapter 2: Literature review** for the comprehensive assessment of the relevant literature explaining the studies done on related bioflavonoids). The structure of the 14 bioflavonoids is as shown in **Figure 1.3**. The information of the 14 bioflavonoids with their chemical formula and molecular weight are available in the **Table 1.3**.

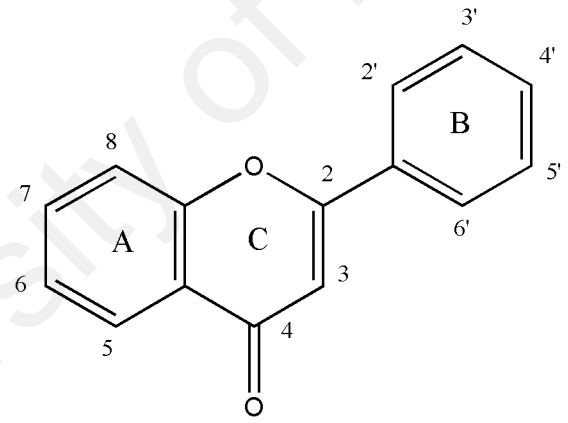

Figure 1.2. The basic structure of the bioflavonoids. Drawn by using ChemDraw 7.0.

Table 1.3: Bioflavonoids and their chemical formula and molecular weight

No.	Bioflavonoids	Chemical formula	Molecular weight (g/mol)
1	Baicalein	$C_{15}H_{10}O_5$	270.23
2	Glycitein	$C_{16}H_{12}O_5$	284.26
3	Isoflavone	$C_{15}H_{10}O_2$	222.23
4	Quercetagetin	$C_{15}H_{10}O_8$	318.23
5	Fisetin	$C_{15}H_{10}O_6$	286.23
6	Genistein	$C_{15}H_{10}O_5$	270.24
7	Luteolin	$C_{15}H_{10}O_6$	286.23
8	Flavanone	$C_{15}H_{12}O_2$	224.25
9	Kaempferol	$C_{15}H_{10}O_6$	286.23
10	Orientin	$C_{21}H_{20}O_{11}$	448.37
11	Apigenin	$C_{15}H_{10}O_5$	270.23
12	Diosmin	$C_{28}H_{32}O_{15}$	608.54
13	Silymarin	$C_{25}H_{22}O_{10}$	482.43
14	Quercetin	$C_{15}H_{10}O_7$	302.23

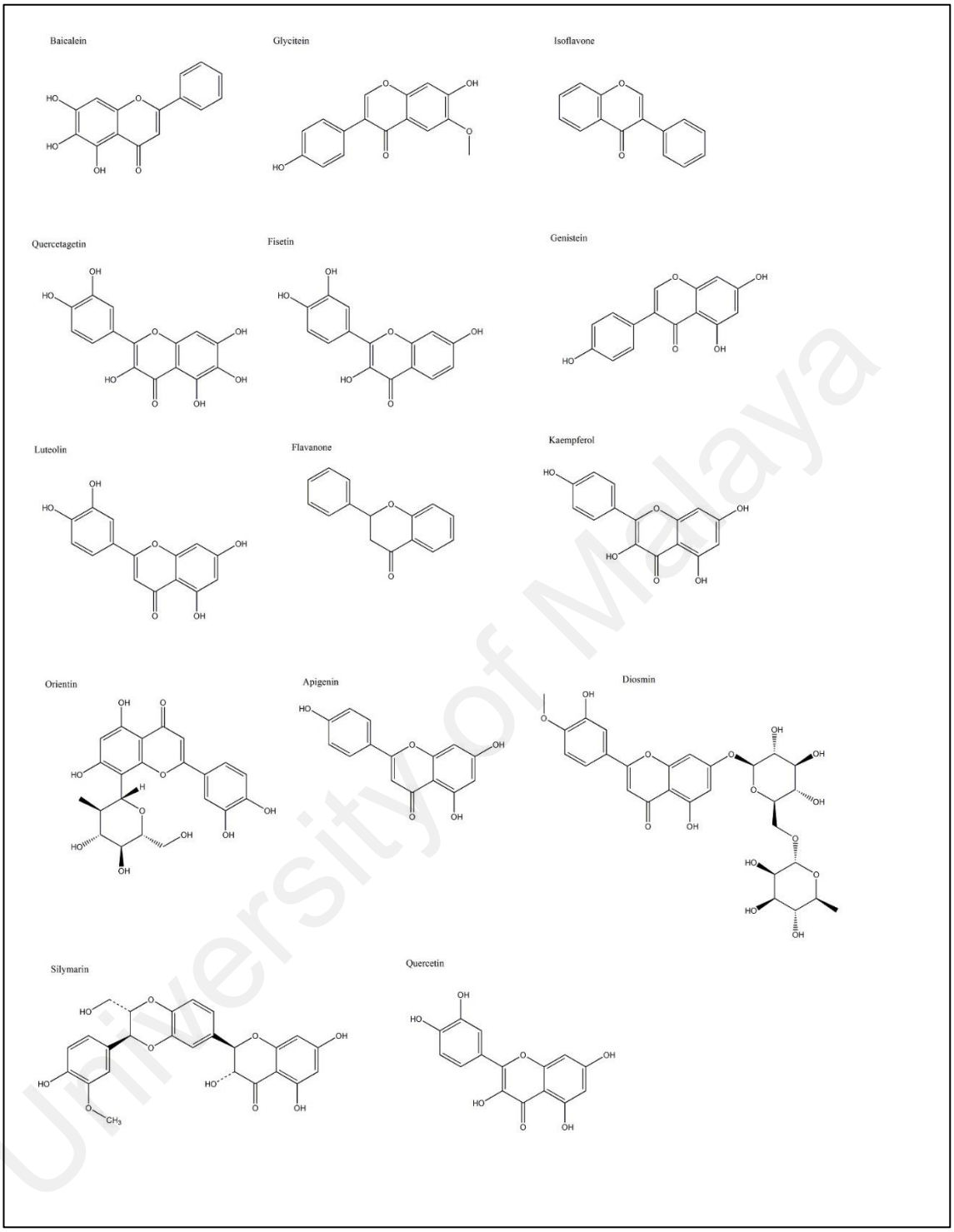

Figure 1.3: The chemical structure of bioflavonoid compounds used in this study. Drawn by using ChemDraw 7.0.

1.3 Research objectives

Hence, the main objective of this research is to;

1. Explore the antiviral activity of 14 different types of bioflavonoids against chikungunya virus replication in cell culture. The bioflavonoids were screened for their antiviral activity in the primary antiviral screening. The bioflavonoids that shown the antiviral activity in the primary antiviral screening were further analysed for their specific method of actions by performing specific antiviral assays.
2. The cytotoxicity of each bioflavonoid in cell culture will be determined in order to calculate the selectivity index. The cytotoxicity of the bioflavonoids on Vero cells and BHK-21 cells were determined by the cell viability assay (MTS assay).
3. The step(s) of viral replication cycle that can be interfered by effective compound(s) will also be investigated. The viral RNA copy number after the treatment with bioflavonoids, in each antiviral assay, were determined by qRT-PCR. The IC_{50} was calculated by comparing the treated CHIKV-infected sample with the CHIKV-infected sample without treatment.
4. The role of bioflavonoids on CHIKV protein expression and CHIKV genome will be studied. Immunofluorescence assay and immunoblot were performed to determine the effects of bioflavonoids treatment on the CHIKV protein accumulation.

CHAPTER 2: LITERATURE REVIEW

2.1 Chikungunya virus life cycle and replication

2.1.1 Entry (attachment, penetration and uncoating)

Like other alphaviruses, chikungunya virus enters the susceptible cells by engaging with a host receptor. Briefly during the process of entering, the virus E2 glycoprotein is responsible for receptor interaction and the E1 protein is related to the receptor engagement. The conformational changes occur in E2 and E1 glycoproteins as the engagement occurs between the viruses with the host receptors (Meyer & Johnston, 1993). Once they are bound to the receptor molecules, virions are endocytosed in a clathrin-dependent manner (Helenius *et al*., 1980). The pH of the virus-containing endosome turns acidic as it matures and this triggers the destabilization of the E1-E2 heterodimer (Lescar *et al*., 2001; Gibbons *et al*., 2003). Subsequently, the insertion of the fusion peptide into the late endosomal membrane occurs and trimerizes, leaving the mix of viral and endosomal membranes and the nucleocapsids deposited in the host cell cytoplasm through the fusion pores (Wahlberg *et al*., 1992). In the cytoplasm, the nucleocapsid disassembles and expose the encapsidated genome for translation.

2.1.2 Virus assembly and budding

The binding of the virus nucleocapsid to the genome RNA and the recruitment of membrane-associated envelope glycoproteins leads to the viral assembly. The icosahedral core-containing virion budding out from the plasma membrane of the host cells. Effective interaction between the nucleocapsid and the glycoproteins is crucial for this budding process (Schwartz & Albert, 2010). The binding of nucleocapsid with cdE2 (cytoplasmic

tail of E2) provides the free energy to propel the capsid across the plasma membrane and the mature infectious virion is free to infect other susceptible cells (Tsetsarkin *et al*., 2011).

2.2 Difficulties in developing antiviral agents

The majority of registered antiviral drugs are nucleoside analogues. Though they are undoubtedly effective, the shortcomings of these drugs are undeniable. Nucleoside analogue antiviral drugs are potentially teratogenic, embryotoxic, carcinogenic and possessed the anti-proliferative activities. The undesirable effects of these drugs are mostly involving the bone marrow depression and neurotoxicity. Some of them must be administered with care during pregnancy. One important point of establishing the antiviral drug is to inhibit viral replication without harming the cells or in this case, the virus machinery hosts (Morris, 1994).

A good potent antiviral candidate must possess certain characteristics through *in vitro* study to qualify it for further study towards antiviral drug development. The most important of these characteristics are; low toxicity towards the host cells, low half maximal inhibitory concentration (IC_{50}), high maximum non-toxic dose (MNTD), cheap and easy preparation as well as unlimited sources. However, in this study, all of these characteristics are present at the *in vitro* level, by selecting the compound of natural resources, bioflavonoids.

2.3 Bioflavonoids and its potential

Apart from its importance listed in **Chapter 1**, bioflavonoids have its own importance and role in antiviral research. All 14 bioflavonoids from various groups that are used in this study have already extensively discovered for their antiviral activity against various viruses.

2.3.1 Baicalein (5,6,7-trihydroxyflavone)

Baicalein was extracted from the plant named Huangchin (*Scutellaria baicalensis*) as well as *Scutellaria lateriflora*. It has been utilized for thousands of years in China's herbal medicine for its function to treat periodontal abscesses and infected oral wounds (Cushnie & Lamb, 2005). In 2012, Cotin and his colleague have reported the potential of baicalein as one of the antiviral candidates for human cytomegalovirus (HCMV) at the *in vitro* level. It was the most effective compound out of four antivirals against HCMV *in vitro* replication. They have shown that baicalein exhibited inhibitory effect against various stages of HCMV replication cycle with IC_{50}=2.2±0.5μM. They have found that baicalein has reduced the expression of the HCMV immediate early gene (IE-1) as well as total inhibition of IE-2 gene expression. Furthermore, baicalein was shown to inhibit the tyrosine kinase activity of the EGF receptor. Nevertheless, it inhibits the early stage of the viral cycle with the IC_{50} as low as 5μM (Cotin *et al*., 2012).

In the same year, Zandi and his colleagues discovered the dynamic roles of baicalein in inhibiting many stages of dengue virus type-2 (DENV-2) replication. Baicalein was not only shown the inhibitory effect against intracellular replication of DENV-2 with IC_{50}=6.46μg/ml; SI=17.8, but also interferes with early stages of DENV-2 replication cycle such as adsorption phase with IC_{50}=7.14μg/ml. It also possessed the direct virucidal acitivity against DENV-2 extracellular particles with IC_{50}=1.55μg/ml (Zandi *et al*., 2012).

A year after, Hour and his team demonstrated 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 H1N1 strain of influenza A virus was inhibited with the IC_{50} as low as 0.018µM (Hour *et al*., 2013).

Additionally baicalin, which is the main metabolite of baicalein, has also became one of the main antiviral candidates as it was shown to have the antiviral activity against different viruses including DENV-2 and Influenza A (H1N1/H3N2), both in cell culture and animal model as well (Moghaddam *et al*., 2014; Ding *et al*., 2014).

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

Isoflavones are differed from flavone in the location of their phenyl group. Apart from legumes, grains and vegetables at which the isoflavones can be found in the small amounts, soybeans has the highest concentration of isoflavones in human diet (Fletcher , 2003; Munro *et al*., 2003). Common isoflavones that are found in soy products (**Table 2.1**) are mainly in the form of glycosides and are not bioactive until fermentation or digestion process transforms it into a bioactive form called aglycone (Cassidy *et al*., 2006; Dixon, 2004; Cornwell *et al*., 2004).

Table 2.1: Isoflavone glycosides and its aglycones

Glycosides	Aglycones
Genistein	Genistin
Daidzein	Daidzin
Glycitein	Glycitin

In western countries, average consumption of isoflavones by adults was reported as 1-2mg/day which is lower compared to the Asian countries with 11-47mg/day. Genistein, as one of the isoflavones, was found to be able to reduce the infectivity of non-enveloped single-stranded RNA viruses such as coxsackie virus, poliovirus and echovirus; double-stranded RNA virus such as rotavirus; double-stranded DNA viruses including adenovirus, JC virus and Simian virus 40 (Andres *et al*., 2009).

In other studies, genistein exhibits anti-rotavirus activity and was suggested as a biologically active isoflavone as it shows better inhibitory activities compared to other isoflavones, which was individually tested in soy-based infant formula as well as by comparing isoflavones mixtures (MIX). MIX somehow lost its anti-rotavirus activity with the absence of genistein. Both genistein and MIX are able to reduce the rotavirus infectivity by 33-62% and 66-74% respectively as assessed by using focus forming unit assay. Genistein significantly reduces rotavirus infectivity in a dose-dependent manner at concentrations as low as 30µmol/L. Genistein acts by modulating the post-binding step, hence modulating the attachment of the virion to the host cells (Andres *et al*., 2007).

A cohort study was conducted from 1992 to 2008 involving 15, 607 women aged 35 years and above to find the association between soy and isoflavone intake with breast cancer incidence in the population. Women with higher intakes of soy and isoflavones were found to have lower relative risks of post-menopausal breast cancer compared to the women having lower intakes of soy and isoflavones (Wada *et al*., 2013). In the search for the broad-spectrum antiviral compound, isoflavones were tested in a high-throughput screening study against RNA viruses. The isoflavone compounds exhibit a highly potent activity against hepatitis C virus (HCV) and influenza virus by activating the interferon-

stimulated gene (ISG54) promoter and mediating the nuclear translocation of interferon regulatory factor (IRF-3) (Bedard *et al*., 2012).

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

On average, 5-10% of the total isoflavones in soy products are glycitein. In some soy supplements, glycitein content can be as high as 40% (Song *et al*., 1999). Although soy products are common in our diet, antiviral research on this compound is scarce. Most of the studies on glycitein mainly focused on the role of glycitein in the regulation of estrogen hormone. However, glycitein only possessed a weak estrogenic activity as it was reported to have low affinity towards the estrogen receptor at the *in vitro* level (Song *et al*., 1999; Molzberger *et al*., 2013). Roh and Sung have attempted to explore the possibility of antiviral activity in glycitein on the HCV. However, glycitein did not exhibit neither any antiviral effects nor inhibit the activity of viral protein NS5B (Roh & Jo., 2011).

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

Quercetagetin is mainly found in hydrolysates of the leaves from six *Eriocaulon* genus (Bate-Smith and Harborne, 1969). Recently, quercetagetin has atrracted attention from antiviral researchers. Though quercetagetin was found to inhibit HCMV replication, the activity was not efficient at various stages (Cotin S *et al*., 2012). It has turned out to be the most potent inhibitor of HCV RNA-dependent RNA polymerase (RdRp). The inhibition of RNA binding to the viral polymerase by quercetagetin is 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*., 2014).

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

Fisetin is usually found in many fruits and vegetables including strawberries, apples, onions and grapes (Fiorani and Accorsi, 2005; Maher *et al*., 2011; Arai *et al*., 2000). Lyu and the team have discovered the moderate inhibitory effects of fisetin against herpes simplex virus 1 (HSV-1) in 2005. In 2011, a research was initiated to evaluate 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*., 2011). A year later, fisetin was reported to also reduce enterovirus-A71-induced cytopathic effect and viral titer (Lin *et al*., 2012).

2.3.6 Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one)

Apart from the studies being discussed earlier in **2.2.2**, genistein, a tyrosine kinase inhibitor, was suggested to be able to reduce the level of HCMV DNA synthesis as well as early and late proteins through the mechanism assumed as blocking HCMV immediate-early protein functions (L. Evers *et al*., 2005). Genistein was also found to inhibit the infection or transduction in cells infected with severely fatal viruses such as Ebola virus, Marburg virus and Lassa virus in a study where the host cells were pre-treated with genistein and tyrphostin AG1478 (A. Kolokoltsov *et al*., 2012). In 2013, an important finding was made when the genistein was found to be able to inhibit the SDF-1-mediated chemotaxis and HIV infection of resting CD4 T cells. Genistein inhibited the viral DNA accumulation in resting CD4 T cells thereby interfering with SDF-1 and HIV-1 mediated actin dynamics in CD4 T cells (Guo *et al*., 2013).

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

In 2011, luteolin was found to be able to reduce the HIV-1 infection in the reporter cells and primary lymphocytes. However, the activity of luteolin was only at the viral entry level and unable to inhibit the reverse transcription of HIV-1 (Mehla *et al*., 2011). A year later, Calland *et al.* studied antiviral activity of luteolin against HCV infection in a cell-based assay system where NS5B polymerase was reported as the viral target with IC_{50} was 1.1 to 7.9µM (Calland *et al*., 2012). Luteolin was also listed as the most potent inhibitor of EV71 and CA16 infection through cell viability and plaque reduction assays with EC_{50} about 10µM. Luteolin targeted the post-attachment stage of EV71 infection and inhibited viral replication of CA16 (Xu *et al*., 2014). Murali *et al.* also have found the antiviral activity of luteolin against chikungunya virus. However, ethanolic extract of *Cynodon dactylon* was used, which differed from our study which used the commercial luteolin with ≥ 98% purity. The extract was able to reduce the viral mRNA synthesis and its CPE by 98% at a concentration of 50µg/ml (Murali et al., 2015).

2.3.8 Flavanone (2-Phenyl-2,3-dihydro-4H-chromen-4-one)

Flavanones are typical polyphenols in *Citrus* species. Naringenin, eriodictyol, isosakuranetin and hesperetin are among well-studied flavanones (Khan & Dangles, 2014). Flavanones draws attention mainly in the anti-inflammatory, anti-oxidant, anti-cancer and anti-microbial investigations, 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).

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

Kaempferol has shown about 40% of viral internal ribosome entry site (IRES) activity by interfering with EV71 virus replication and pseudotyped virus (Tsai *et al*., 2011). In addition, kaempferol is potent against the Japanese encephalitis virus (JEV) by inactivating the virus through its frameshift site RNA (Zhang *et al*., 2012). The inhibitory effect of kaempferol-7-O-glucoside on HIV-1 reverse transcriptase activity was more effective than the kaempferol itself (Behbahani *et al*., 2014). Also, it has more planar flavonol structure with only one C-4' phenolic hydroxyl group in the B ring, which is crucial for anti-influenza B virus activity in a structure-activity relationship study (Yang *et al*., 2014).

2.3.10 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)

There are not many studies involving the antiviral activity of orientin except about the antiviral activity of crude extracts from the flower *Trollius chinensis* which contains flavonoids, orientin, vitexin and proglobeflowery acid against the parainfluenza type 3. It was also shown that orientin has potent antiviral activity against parainfluenza virus type 3 (Li *et al*., 2002).

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

Recently, apigenin and enterovirus-71 (EV71) became closer partners in antiviral research. Apigenin is able to inhibit the EV71-mediated CPE, virus replication efficiency, viral protein expression and hindered EV71-induced cell apoptosis, intracellular reactive oxygen species (ROS) generation and cytokines up-regulation. Apigenin exhibited its activity very well after the viral entry and interfered with viral internal ribosome entry site (IRES) activity as well as modulating the (c-Jun N-terminal kinase) JNK pathway (Lv *et al*., 2014). Since EV71 infection requires the hnRNP proteins (trans-acting factor regulating the EV71 translation), apigenin is able to disrupt the viral RNA association with hnRNP A1 and A2 proteins selectively (Zhang *et al*., 2014). Foot-and-mouth disease virus (FMDV) translational activity was interfered as apigenin interrupts the IRES activity at the post-entry stage with no direct extracellular virucidal activity (Qian *et al*., 2015). Coincidentally, chikungunya virus was also targeted by apigenin. However, in this recent study, Murali and colleagues were using the apigenin-rich fraction from *Cynodon dactylon* rather than the pure apigenin compound. Apigenin and other major phytochemicals inhibit 98% of viral activity at the concentration of 50 µg/ml through the observation of CPE reduction as well as reduction in viral mRNA synthesis (Murali *et al*., 2015).

2.3.12 Diosmin (5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)- 7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy -6-[[(2R,3R,4R,5R,6S) -3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one)

Up to now, there is no report on antiviral activity of diosmin. Diosmin which is the alternative supplement taken in handling the venous diseases was found to have cardioprotective effects by free radical scavenging and anti-hyperlipidaemic activity as well

as protective effect on hepatic ischemia repefusion injury in rats (Queenthy and John, 2013; Tanrikulu *et al*., 2013).

2.3.13 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 complex of more than seven flavonolignans and one flavonoid that made up 65%-80% of milk thistle initial extract together with 20%-35% fatty acid. The seven flavonolignans are silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin and silydianin. In literature, silybin A and silybin B are also referred as silibinin A and silibinin B (Polyak *et al*., 2013). Silymarin is one of the most popular natural product consumed by the Western society especially for those who suffered from chronic hepatitis C (Polyak, Ferenci & Pawlotsky, 2013).

Silymarin was found to be able to inhibit the virus entry, RNA and protein expression and infectious virus production in hepatitis C virus cell culture infection. Silymarin did not block the binding of the virus to the cells but inhibited the entry of several viral pseudoparticles (pp) and fusion of HCVpp with liposomes, as well as inhibited microsomal triglyceride transfer protein activity, apolipoprotein B secretion and infectious virion production into culture supernatants. It is worth noting that the genotype 2a NS5B RNA-dependent RNA polymerase (RdRp) activity was inhibited by silymarin and not silibinin, one of its flavonolignan. Silymarin also blocked cell-to-cell spread of the virus (Wagoner *et al*., 2010). However, in a randomized controlled trial study, no evidence of salutary effects of oral silymarin has been reported although silymarin is very well-tolerated in chronic HCV-infected patients (Yang *et al*., 2014).

Apart from hepatitis C virus, silymarin also showed anti-influenza virus activity by reducing visible CPE by 98% at concentration of 100 μg/ml. It inhibited viral mRNA synthesis and also affect late viral RNA synthesis (Song & Choi, 2011).

2.3.14 Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one)

Quercetin is a well-studied flavonol. It was shown that it inhibited the DENV-2 *in vitro* replication with IC_{50}=35.7 μg/ml when the cells were treated after the virus adsorption, where the IC_{50} has dropped to 28.9 μg/ml when the cells were continuously treated for 5 hours before virus infection up to four days post-infection (Zandi *et al*., 2011). In research pertaining hepatitis C virus, quercetin was found to be able to inhibit the NS3 activity in a specific dose-dependent manner in an *in vitro* catalysis assay and confirmed to inhibit HCV RNA replication as analyzed in sub-genomic HCV RNA replicon system apart from the direct inhibition on HCV NS3 protease and infectious virus production in HCV cell culture system (Bachmetov *et al*., 2011). Quercetin is also able to improve the lung function in the rhinovirus-infected mice and reduced the expression of pro-inflammatory cytokines. The pre-treatment of the airway epithelial cells with quercetin decreased the Akt phosphorylation, viral endocytosis and IL-8 responses. Six hours after infection with rhinovirus, the IL-8 and IFN responses were decreased in airway epithelial cells (Ganesan *et al*., 2012).

2.4 Recent news regarding the spread of CHIKV in America

For about 62 years, CHIKV outbreaks have occurred occasionally in Africa, Asia, Europe as well as Indian and Pacific oceans. However, recently in late 2013, CHIKV was found for the first time in the Americas on islands in Carribean as reported by the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, U.S). The first local cases

were reported from St. Martin and the outbreak was quickly spread to other locations in Carribean and even beyond. In December 2013, over 750 travelers have returned to the United States infected with CHIKV and the transmission was predicted to be continuing throughout Americas. Since antiviral treatment has not yet existed, a symptomatic and supportive treatment was offered. Other than resting, acetaminophen or paracetamol are used to relieve fever. Also, ibuprofen, naproxen, or another non-steroidal anti-inflammatory agents (NSAID) are used to relieve the arthritic component of the disease.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell lines, virus and antibodies

The African green monkey kidney cell line or also known as Vero cells and the baby hamster kidney cell line (BHK-21 cells) were obtained from the American Type Culture Collection (ATCC, Virginia, 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 also 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). Both cell lines were incubated at 37°C in the presence of 5% CO_2 and were sub-cultured or cryopreserved as they reached to 80% confluency.

The CHIKV replicon BHK-21 cell line was contributed by Dr. Andres Merits from University of Tartu, Tartu, Estonia as published in Pohjala *et al*., 2011 (refer to **Appendix B**). 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. 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 also 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). The replicon cell line was incubated at 37°C in the presence of 5% CO2 and was sub-cultured or cryopreserved as they reached to 80% confluency.

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

3.1.1 CHIKV propagation in cell culture

In order to ensure the continuous supply of CHIKV for usage throughout the whole experiment, propagation of CHIKV was performed. Since CHIKV grows in a variety of non-human cell lines, including Vero cells and BHK-21 cells (Schwartz and Albert, 2010), 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.

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 also 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). The cultured cells were incubated at 37°C in the presence of 5% CO_2 and infected CHIKV after attaining 80% confluency. The flask containing CHIKV-infected BHK-21 cells was placed on the rocker for 30 minutes prior to the incubation at 37°C in the presence of 5% CO_2.

Before harvesting the CHIKV, the infected cells were incubated in the same environment for three days until about 70%-80% cytopathic effect (CPE) shows up. The CPE of alphaviruses in cell culture was characterized as lytic infection or apoptosis. On the third day, the cells were scrapped by using the cell scraper, centrifuged at 4°C and 2000rpm

to isolate the cell debris. The CHIKV was then aliquoted into screw cap tubes and stored at -80°C for titration and further experiments.

3.1.2 CHIKV titration assay

The virus titration has been done based on Reed-Muench endpoint calculation method to determine the tissue culture 50% infectious dose ($TCID_{50}$) (Reed and Muench, 1938). A monolayer of $1x10^4$ Vero cells per 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 also 50 IU penicillin/streptomycin (Sigma-Aldrich, MO, USA). The cells were then incubated at 37°C in the presence of 5% CO_2 until it reached 80% confluency.

The CHIKV stock was then serially 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 respective wells. The plate was then incubated at the same condition as mentioned earlier for virus propagation. The plate was then examined daily for the presentation and recording of the CPE in infected wells and the recording of the CPE presentation was performed until the mock infected cells showed healthy morphology.

To titer the viral stock based on Reed-Muench endpoint calculation method, a score was given to each of the dilution according to the amount of the wells showing CPE. The interpolated value of $TCID_{50}$ was then calculated by Reed-Muench formula as stated in the following:-

Reed-Muench formula

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

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 western blotting was 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 western blotting was provided by Dr. Andres Merits from University of Tartu, Tartu, Estonia.

3.2 Bioflavonoids and nucleoside analogue

13 bioflavonoids except quercetagetin were purchased from Sigma-Aldrich, MO, USA. Quercetagetin was purchased from Indofine Chemical Co., NJ, USA. All of the bioflavonoids are of ≥ 95% purity. Ribavirin, 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 in the post-entry assay, and purchased from Sigma-Aldrich, MO, USA of ≥ 95% purity. The bioflavonoids 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 then stored in -20°C for further experiments.

3.2.1 Cytotoxicity assay

In order to determine the cytotoxicity of the bioflavonoids and ribavirin on Vero cells and BHK-21 cells, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed. Through this assay, the half maximal cytotoxic concentration (CC_{50}) and the maximum non-toxic dose (MNTD) of each compound can be determined. Basically, 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 colored formazan and this reflects the amount of viable cells in the assay. This assay was performed according to the manufacturer's protocol.

A monolayer of $1x10^4$ Vero cells and BHK-21 cells per well were prepared in a different 96-well cell culture plate (Nest Biotechnology Co., Ltd. Wuxi, China) and were treated with different concentrations of each compound in triplicate together with negative control (media containing 0.1% DMSO). The prepared bioflavonoid compounds were filtered using 0.2µm syringe filter unit (Merck Millipore Ltd., MA, USA). The plate was then incubated at 37 °C with 5% CO_2 for 48 hours before the MTS assay was performed. Treated and control cells were kept for two days at 37°C, under similar conditions and duration 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 multiplate reader (Tecan, Männedorf, Switzerland). All experiments were conducted in triplicate. The CC_{50} and MNTD for each compound was determined through this assay using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005).

3.3 *In vitro* antiviral assays

3.3.1 Antiviral screening assay using CHIKV replicon cell line

To screen the compounds for their activity against CHIKV replicase complex, a CHIKV BHK-21 replicon cell line has been used. This assay was modified and performed according to Pohjala *et al*., 2011. A monolayer of $1x10^4$ CHIKV replicon cells were prepared in 96-well white plate (Corning Inc., NY, USA) and treated with increasing concentrations of each compound starting from MNTD concentration of each compound. After 48 hours incubation at 37°C with 5% CO_2, 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 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 compound. The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the *Rluc* activity.

3.3.2 Continuous treatment assay

As another antiviral screening assay using infectious CHIKV, a continuous treatment assay was performed to determine any plausible antiviral activity of a compound against any stage of virus life cycle. This assay was modified and performed according to Zandi *et al*., 2014. A monolayer of $1x10^4$ Vero cells per well were prepared in 96-well cell culture plate. The cells then were treated with increasing concentrations of each compound

starting from MNTD concentration of each compound. Concurrently, cells were infected with CHIKV of MOI=1 and the plate was then incubated for 2 hours at 37°C in the presence of 5% CO_2. After 2 hours of incubation, the supernatants were removed from each well and the newly prepared bioflavonoid compound with the same concentrations as the previous stage was added into the respective wells.

The plate was then incubated for 48 hours at the same condition as previously described until the CPE appears in the virus control wells. The plate was examined daily for the presentation of CPE and after two days, the percentage of CPE score was given based on the degree of inhibition of the CPE. The MTS assay was also performed to confirm the results. The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number. The bioflavonoid compounds that show significant inhibition against CHIKV were selected to continue with further experiments which are necessary to perform to determine the extent of its inhibitory activities.

3.3.3 Time-of-addition assay

This assay was modified and performed according to Kaur *et al*., 2013. The time-of-addition study was performed to determine the best acting time for the compounds against the virus as well as to determine how long the addition of a compound could be postponed before it loses its antiviral activity (Daelemans *et al*., 2011; Pauwels *et al*., 1990). In this assay, the bioflavonoids that results in positive antiviral hits in the previous assays were tested using the highest concentration of each compound that can be achieved below their MNTDs.

A monolayer of Vero cells were prepared in 96-well cell culture plate using EMEM supplemented with 10% FBS. For the pre-treatment assays, which refers to the -2h and -1h pre-infection; the cell monolayers were treated with the three compounds respectively at 2h and 1h before the infection with CHIKV. For the co-treatment assay (0h), Vero cells were treated with each compound and being infected at the same time. For the post-treatment assay, Vero cells were infected with CHIKV (MOI=1) and subsequently the compounds were added at 2h, 3h, 4h and 5h. The CHIKV for the positive controls were added at the 0h and the negative controls were only containing EMEM with 2% FBS. The plate was then incubated for 48 hours at 37°C in the presence of 5% CO_2 until the CPE is shown.

The plate was then examined daily and after 2 days, the percentage of CPE score was given based on the degree of inhibition of the CPE. The supernatants from each well were collected and a viral yield assay were conducted using a specific quantitative real-time polymerase chain reaction (qRT-PCR) for CHIKV, which will be elaborated in another section. The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.4 Virucidal assay

This assay was modified and performed according to Johari *et al*., 2012. The virucidal assay was performed to identify whether there is a possibility that the bioflavonoids can inactivate the extracellular virus particles or in other words before infections of the cells. Hence, in this assay, increasing concentrations of the compounds ranges from 6.25 μg/ml to 100 μg/ml were mixed with the CHIKV ($TCID_{50}=10^{4.5}$ EID_{50}/ml) and incubated for 2 hours at 37°C.

The compounds were further diluted before the addition of the mixtures on Vero cells. Treated viral mixtures were added to prepared monolayer of $6x10^5$ Vero cells in 24-well cell culture plates in triplicates, where the virus controls the CHIKV without any compound and the negative controls only contained cell culture medium with 2% FBS. The plates were then incubated at 37°C in the presence of 5% CO_2 for 48 hours. The plates were examined daily for the presentation of CPE and after 2 days of incubation, the supernatants were collected from their respective wells and the qRT-PCR assay were performed to evaluate the virucidal activity of the compound. The IC_{50} was determined and the non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.5 Anti-adsorption assay

This assay was modified and performed according to Zandi *et al*., 2013. In order to identify whether the compounds of interest could interfere with the specific binding of the viral attachment proteins and the cellular receptors. A viral suspension from CHIKV with MOI=1 and descending concentrations of bioflavonoids ranges from 100 µg/ml to 6.25 µg/ml were prepared in EMEM supplemented with 2% FBS and overlaid simultaneously on monolayer of $6x10^5$ Vero cells per well in 24-well cell culture plates.

The plates were then incubated at 37°C in the presence of 5% CO_2 for and after 2 days of incubation, the supernatants were collected from their respective wells and the qRT-PCR assay were performed to confirm the results. The IC_{50} and the non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.6 Anti-entry assay

The procedure of the anti-entry assay was modified and performed according to Lee *et al*., 2013. Monolayers of Vero cells were grown in 96-well plate with EMEM supplemented with 10% inactivated FBS. The Vero cells were then infected with CHIKV and the plate was incubated for 1 hour at 4 °C. Non-adsorbed virus was then 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. The EMEM supplemented with 2% inactivated FBS was added into every wells and the plate was incubated for 48 hours at 37 °C with 5% CO_2. After 2 days of incubation, the supernatants were collected from their respective wells and the qRT-PCR assay were performed to confirm the results. The IC_{50} and The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number.

3.3.7 Post-adsorption assay

This assay was modified and performed according to Zandi *et al*., 2011. A monolayer of $6x10^5$ Vero cells per well were grown in 24-well cell culture plates using EMEM supplemented with 10% FBS. The Vero cells were then inoculated with CHIKV of MOI= 1 and incubated for 2 hours at 37°C with 5% CO_2 prior to the treatment with different concentrations of bioflavonoid compounds ranging from 100 µg/ml to 6.25 µg/ml were prepared in EMEM supplemented with 2% FBS.

After the treatment, the plates were then incubated for 48 hours at 37°C with 5% CO_2. The plates were examined daily for the CPE presentation and after two days, 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 was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order 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 CHIKV RNA was extracted from supernatants collected from previous *in vitro* antiviral assays with QIAamp Viral RNA Mini Kit (QIAGEN, Germany). 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 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, then unincorporated primers were digested with 20 U of Exonuclease I (New England Biolabs, MA, USA). The cDNA were stored at −80 °C.

3.4.2 qRT-PCR

A qRT-PCR assay was used to evaluate the virus yield reduction due to the treatments through quantifying the extracellular CHIKV RNA copy number. For this experiment, the amplification of 136 base region of nsP3 encoding sequence was performed as described by Chiam *et al*., 2013. The qRT-PCR was performed in the Step-One Plus Real-Time PCR System (Life Technologies, USA) using a 10 µL reaction volume containing 1×Power SYBR Green PCR Master Mix (Life Technologies, USA), 1 µmol/L sense and antisense primers, 1 µL of cDNA, and 3.8 µL of nuclease-free water. The qRT-PCR was performed with a Step-OnePlus Real-Time PCR System (Life Technologies, USA) following the manufacturer's protocol, and using serially diluted standards. Cycling parameters were 95ºC for 10 min, 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The amplified product was verified by melting curve analysis. The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005) in order to determine the correlation between the treatments and the CHIKV RNA copy number.

3.5 Immunofluorescence assay

In order to quantify the CHIKV antigen production as a confirmatory test for other antiviral assays, an immunofluorescence assay was modified and performed according to Kaur et al., 2013. A monolayer of Vero cells were prepared in 96-well cell culture plate using EMEM supplemented with 10% FBS. The monolayer of Vero cells were then infected with CHIKV of MOI= 1 and incubated for 2 hours at 37°C with 5% CO2 prior to the treatment with different concentrations of tested compounds ranging from 100 µg/ml to 6.25 µg/ml in respective wells. The plates were then incubated for 24 hours at 37°C with 5% CO_2. After the overnight incubation, the cells were fixed using 100 µl of 4%

paraformaldehyde for 30 minutes at room temperature. The cells were then 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 1 hour, the plate was 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 one 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 and dried. The immunofluorescence signals were then measured and analysed using Harmony Software Version 3.5.1 (Perkin-Elmer, Hamburg, Germany). The image acquisition was captured by using the high content screening system (Operetta, Perkin-Elmer, Hamburg, Germany).

3.6 Immunoblot assay

To determine the effects of compounds treatment on CHIKV protein expression, western blotting assay has been developed for different 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 EMEM supplemented with 10% FBS was added to the flask to support the Vero cells monolayer preparation. After Vero cell monolayer was established, each flask was infected with CHIKV inoculum with MOI= 1 and then incubated at 37°C with 5% CO_2 for 2h for virus attachment and internalization.

Then each flask was treated with different concentrations of compounds of interest ranging from 100 µg/ml to 6.25 µg/ml in respective flasks, where the virus control flasks were 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 virus control flask. Once CPE was observed, cells were then 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 for each sample.

A cell lysate containing 100 µg of protein was denatured using SDS-loading buffer and proteins were separated using SDS-PAGE in 12% gels. The gels were then 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 rinsed three times with 1X PBS Tween 20 before being incubated with primary anti-CHIKV nsP1, anti-CHIKV nsP3 or anti-CHIKV E2 rabbit polyclonal antibodies in 1% casein solution. The blots were then washed three times with 1X PBS Tween20 for 15 min each time.

This was followed by incubation 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. Membranes were washed three times with PBS containing Tween 20 for 15 minutes each time. For the loading control, 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 15minutes each time. Membranes were developed by the colorimetric method using appropriate substrates (Thermo Scientific, Rockford, IL).

3.7 Statistical analysis

All the statistical analysis appropriate for each result were performed by using the Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005) and all the data was presented as the mean with the standard error mean (±SEM). The non-parametric one-way ANOVA was performed by using the Graph Pad Prism 5 (Graph Pad Software Imn., San Diego, CA, USA, 2005). This test allows one 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 Dunnett's multiple comparison posttest was performed after the one-way ANOVA test. This test allows one to compare each time of treatment with the positive control.

CHAPTER 4: RESULTS

4.1 Cell viability on flavonoids treatment

The MTS assay was performed to determine the cytotoxicity of the bioflavonoids and ribavirin on Vero cells. The CC_{50} and MNTD obtained from the assay are as presented in the **Table 4.1**.

Table 4.1: The CC_{50} and MNTD values of the flavonoid compounds and nucleoside analogue on Vero cells

No.	Bioflavonoids	Chemical formula	CC_{50} (µg/ml)	MNTD (µg/ml)
1	Baicalein	$C_{15}H_{10}O_5$	503.7	183.2
2	Glycitein	$C_{16}H_{12}O_5$	>400.0	80.52
3	Isoflavone	$C_{15}H_{10}O_2$	844.1	237.3
4	Quercetagetin	$C_{15}H_{10}O_8$	770.2	136.8
5	Fisetin	$C_{15}H_{10}O_6$	803.1	717.8
6	Genistein	$C_{15}H_{10}O_5$	690.4	49.89
7	Luteolin	$C_{15}H_{10}O_6$	769.9	269.0
8	Flavanone	$C_{15}H_{12}O_2$	397.8	131.9
9	Kaempferol	$C_{15}H_{10}O_6$	>400.0	128.4
10	Orientin	$C_{21}H_{20}O_{11}$	>800.0	>400.0
11	Apigenin	$C_{15}H_{10}O_5$	580.7	223.2
12	Diosmin	$C_{28}H_{32}O_{15}$	1242.0	284.1
13	Silymarin	$C_{25}H_{22}O_{10}$	425.1	262.5
14	Quercetin	$C_{15}H_{10}O_7$	>1000.0	>400.0
15	Ribavirin	$C_8H_{12}N_4O_5$	>600	799.2

Since the Vero cells are viable at the concentration of 100 µg/ml and below for each compound, the concentration used for screening and subsequent antiviral assays starts at 100 µg/ml except for glycitein and genistein.

4.2 Four flavonoids show inhibitory activity against CHIKV on primary screening

Based on the continuous treatment assay results through the percentage of CPE inhibition assay result of 14 compounds (Figure 4.1) and MTS assay (Figure 4.2**)**, four compounds; baicalein, fisetin, quercetagetin and silymarin have shown promising antiviral activity against CHIKV. By referring to the **Figure 4.1**, baicalein ($P< 0.0001$), fisetin ($P< 0.0001$), quercetagetin ($P< 0.0001$) and silymarin ($P< 0.0001$), showed promising significant inhibition against CHIKV *in vitro* replication in a dose-dependent manner. The IC_{50} values can be achieved at the concentration of 36.39 μg/ml, 21.15 μg/ml, 22.70 μg/ml and 24.84 μg/ml for baicalein, fisetin, quercetagetin and silymarin respectively. The IC_{90} values were 148.6 μg/ml, 103.8 μg/ml, 82.68 μg/ml and 85.49 μg/ml for baicalein, fisetin, quercetagetin and silymarin respectively.

Referring to **Figure 4.2**, baicalein ($P< 0.0001$), fisetin ($P< 0.0001$), quercetagetin ($P< 0.0001$) and silymarin ($P< 0.0001$), showed promising significant inhibition against CHIKV *in vitro* replication in a dose-dependent manner by comparing the amount of viable cells in the treated wells and the non-treated CHIKV-infected wells or the positive controls. The 50% of the Vero cells are still viable at the concentration of 67.87 μg/ml, 45.14 μg/ml, 49.76 μg/ml and 44.79 μg/ml with the treatment of baicalein, fisetin, quercetagetin and silymarin respectively. From the interpolated data graph, 90% of the viable Vero cells can be achieved at the concentration of 443.2 μg/ml, 403.0 μg/ml, 334.1 μg/ml and 394.5 μg/ml by the treatment with baicalein, fisetin, quercetagetin and silymarin respectively. Since the compounds were showing potential inhibitory activity against CHIKV *in vitro* replication, it is presumably worthwhile to perform further evaluation on the specific antiviral assays.

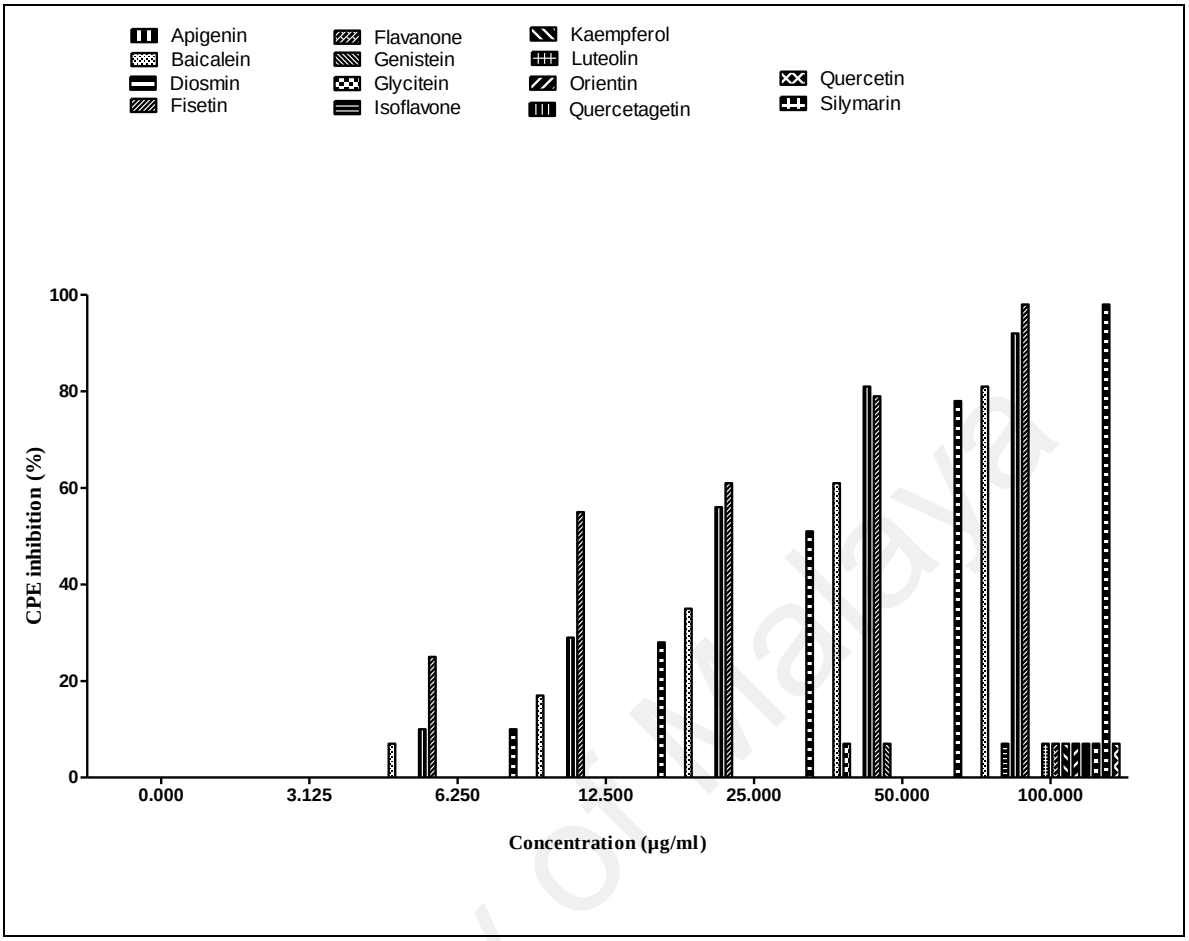

Figure 4.1: The bioflavonoids inhibited CHIKV-induced cytotoxic effect in continuous treatment. The results are normalized to the respective values obtained from the non-treated CHIKV-infected control cells ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Statistical significance is analyzed from a one-way ANOVA, $P<0.05$. Error bars represent standard errors of triplicate means.

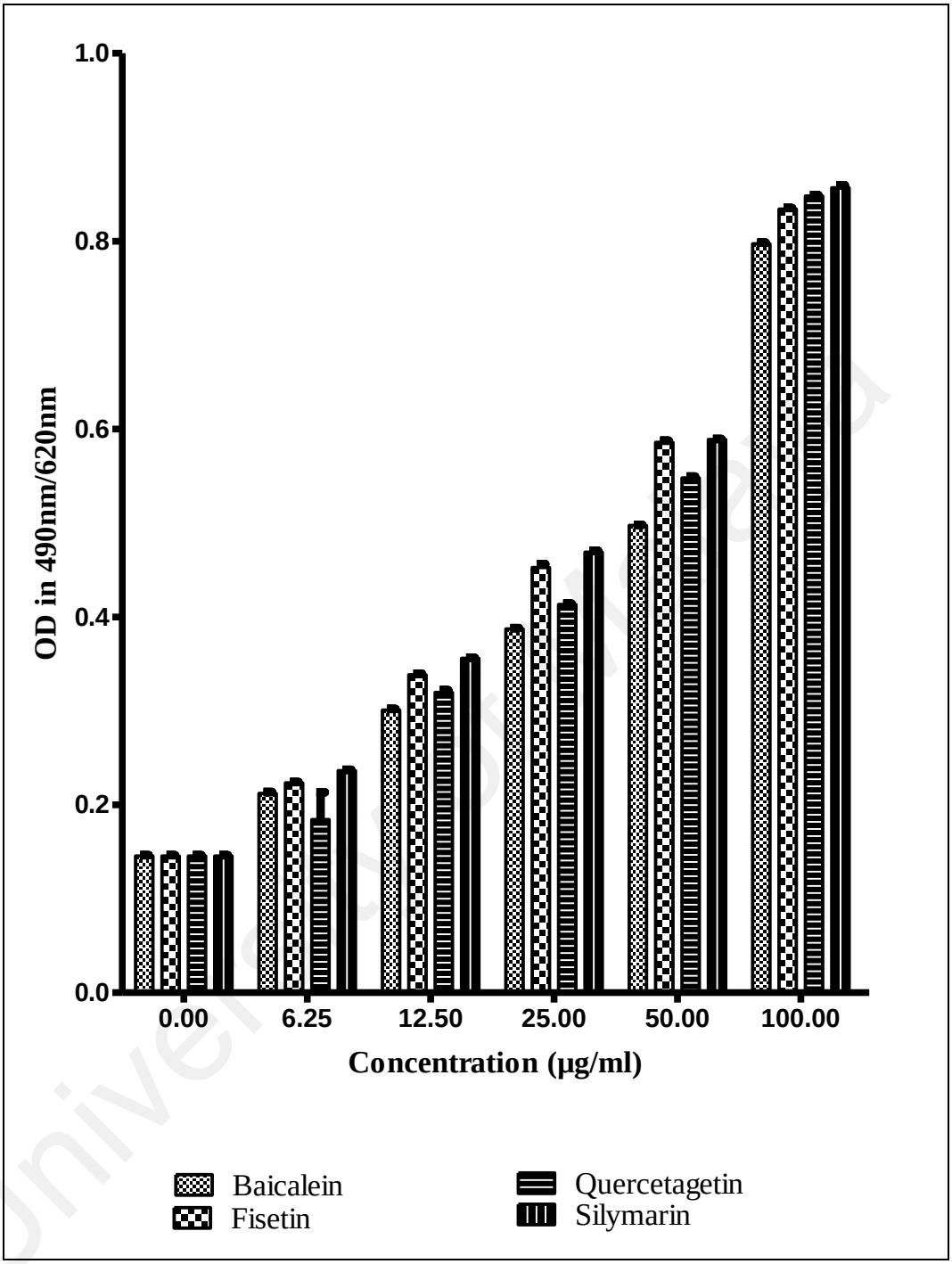

Figure 4.2: The cell viability results obtained through the MTS assay from the continuous treatment. The results are normalized to the respective values obtained from the non-treated CHIKV-infected control cells ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Statistical significance is analyzed from a one-way ANOVA, $P<0.05$. Error bars represent standard errors of triplicate means.

4.3 The inhibitory effect of selected flavonoids on CHIKV replicon cell line

As another reliable antiviral screening method, the effects of four selected compounds have been evaluated on CHIKV replicon cell line.The CHIKV replicon cell line-based assay offers the insight of the antiviral activity by providing the marker genes such as puromycin acetyltransferase, *EGFP* and *Renilla* luciferase marker genes together with the CHIKV replicase proteins. The cytotoxicity assay was performed on the BHK-21 cells to evaluate the MNTD and CC_{50} of the baicalein, fisetin, quercetagetin and silymarin before performing the CHIKV replicon cell-line based assay. The CC_{50} and MNTD values obtained from the cytotoxicity assay are shown in the **Table 4.2**.

Table 4.2: The CC50 and MNTD values of the flavonoid compounds on BHK-21 cells

Bioflavonoids	CC_{50} (µg/ml)	MNTD (µg/ml)
Baicalein	474.5	197.2
Fisetin	212.3	72.68
Quercetagetin	52.42	32.1
Silymarin	305.4	>150.0

Since the MNTD of each bioflavonoid on BHK-21 is different, the concentrations of the bioflavonoid used also varied. The result of baicalein treatment on CHIKV replicon cell line is shown in **Figure 4.3**. Baicalein, ranging from 6.25 µg/ml to 100 µg/ml significantly reduced the *Rluc* activity, $P< 0.0001$ and its IC_{50} value was 3.243 µg/ml. The result of fisetin treatment on CHIKV replicon cell line is shown in **Figure 4.4**. Fisetin, ranging from 6.25 µg/ml to 25 µg/ml also non-significantly reduced the *Rluc* activity, $P=0.7217$. The IC_{50} value is higher compared to baicalein at the concentration of 44.27 µg/ml. The result of quercetagetin treatment on CHIKV replicon cell line is shown in **Figure 4.5**. Quercetagetin, ranging from 6.25 µg/ml to 25 µg/ml, has significantly reduced the percentage of *Rluc* activity ($P=0.0029$) with an IC_{50} = 13.53 µg/ml.

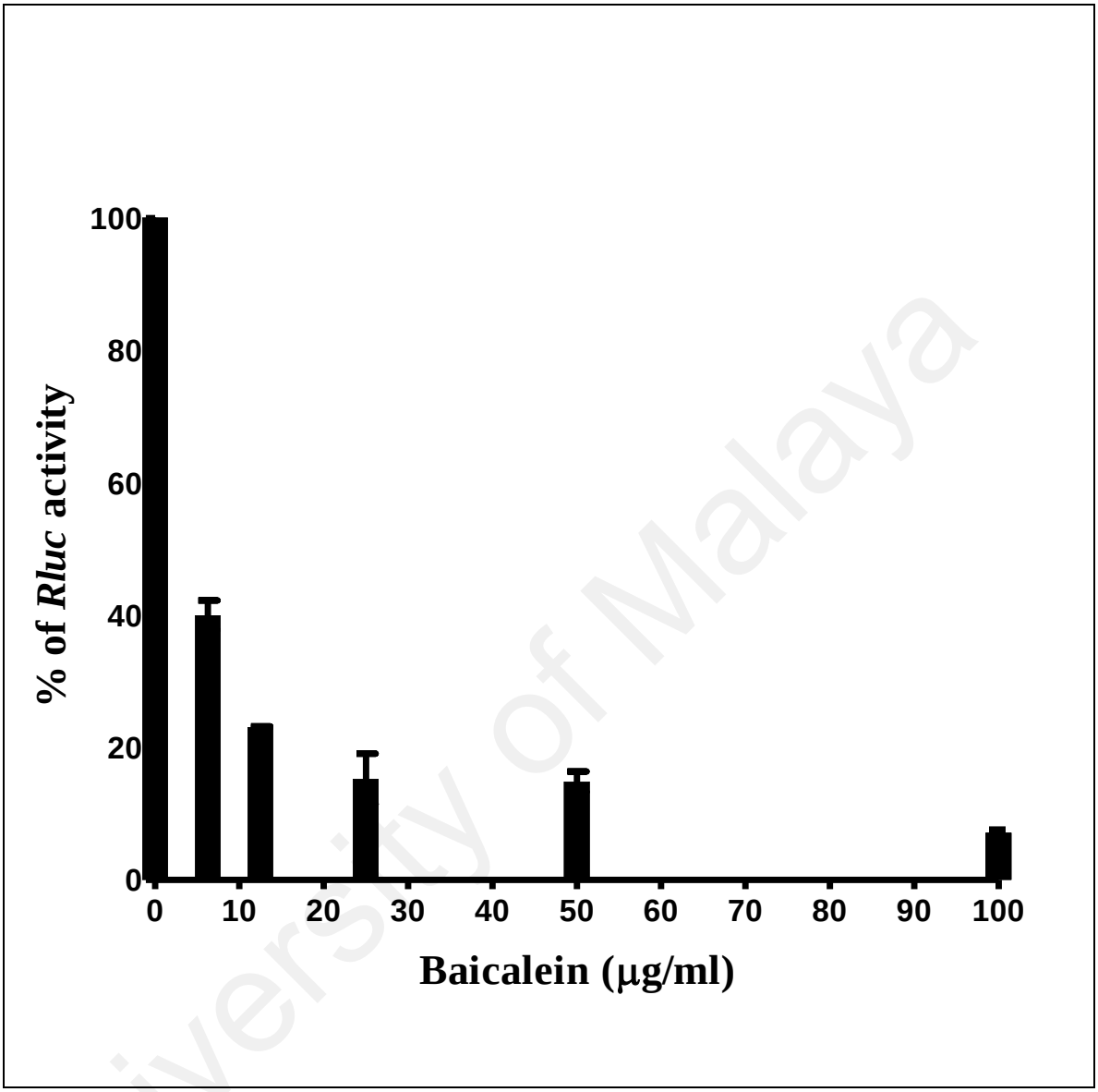

Figure 4.3: The *Rluc* activity has been reduced significantly by baicalein treatment on the CHIKV replicon cell line. Statistical significance was analyzed from a one-way ANOVA, $P<0.05$. Error bars represent standard errors of triplicate means.

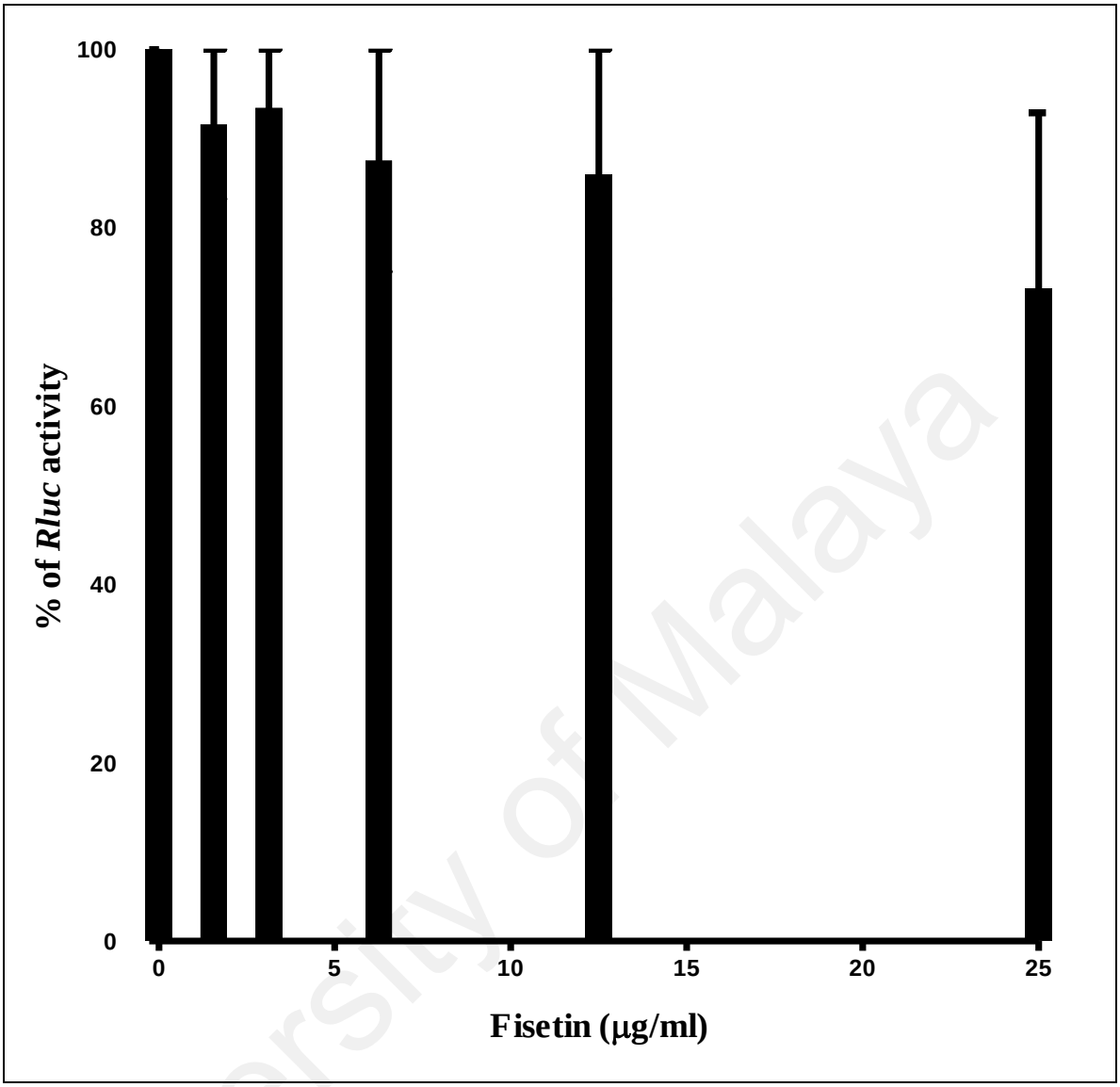

Figure 4.4: The *Rluc* activity is not reduced significantly at all concentrations of fisetin treatment in the CHIKV replicon cell line. Statistical significance was analyzed from a one-way ANOVA (Kruskal-Wallis test), $P<0.05$. Error bars represent standard errors of triplicate means.

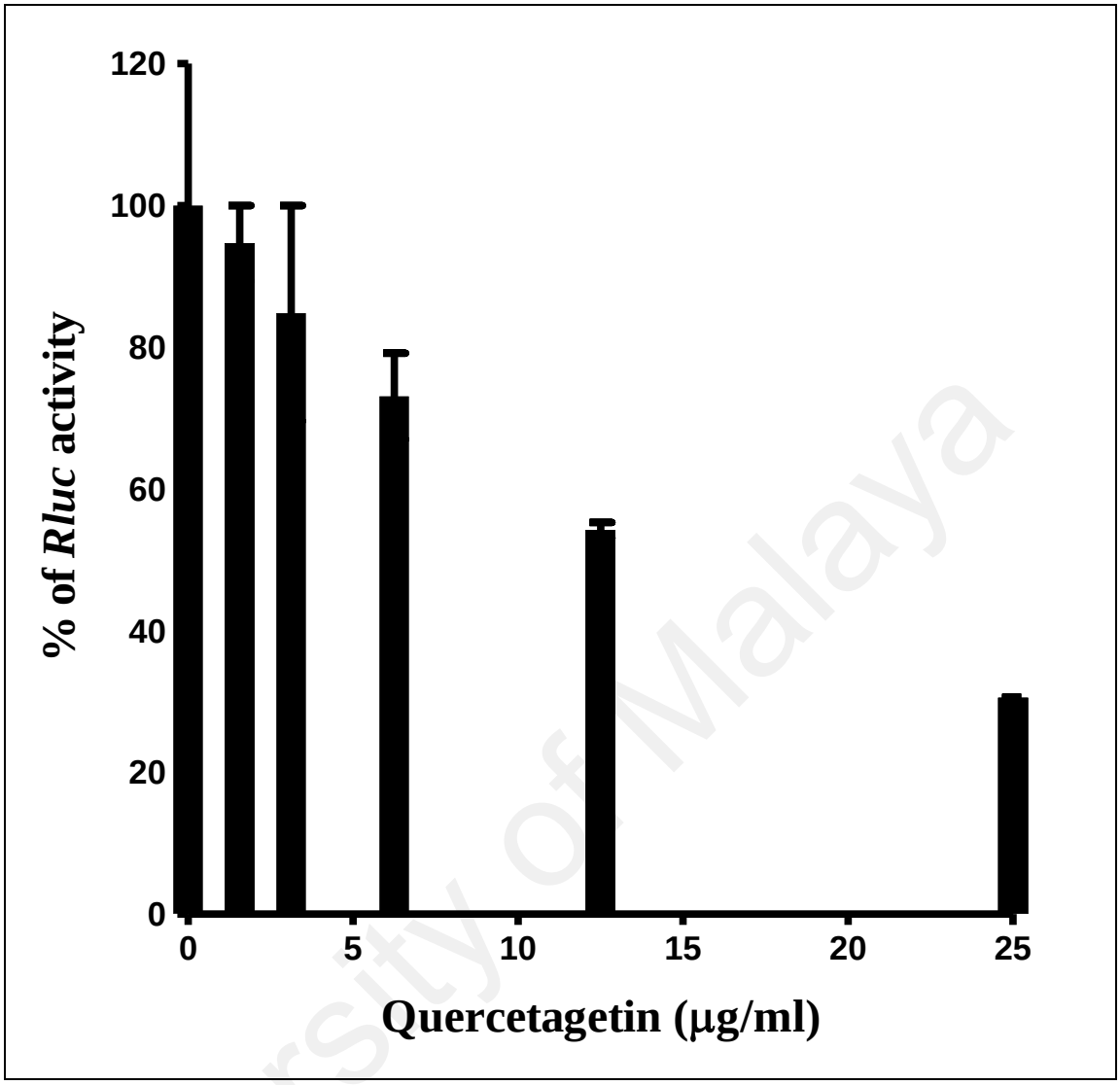

Figure 4.5: The *Rluc* activity reduced significantly by quercetagetin treatment on the CHIKV replicon cell line. Statistical significance was analyzed from a one-way ANOVA, $P<0.05$. Error bars represent standard errors of triplicate means.

The result of silymarin treatment on CHIKV replicon cell line is shown in **Figure 4.6**. Silymarin, ranging from 6.25 μg/ml to 100 μg/ml, has reduced the percentage of *Rluc* activity significantly, $P < 0.0001$ with an $IC_{50} = 21.08$ μg/ml.

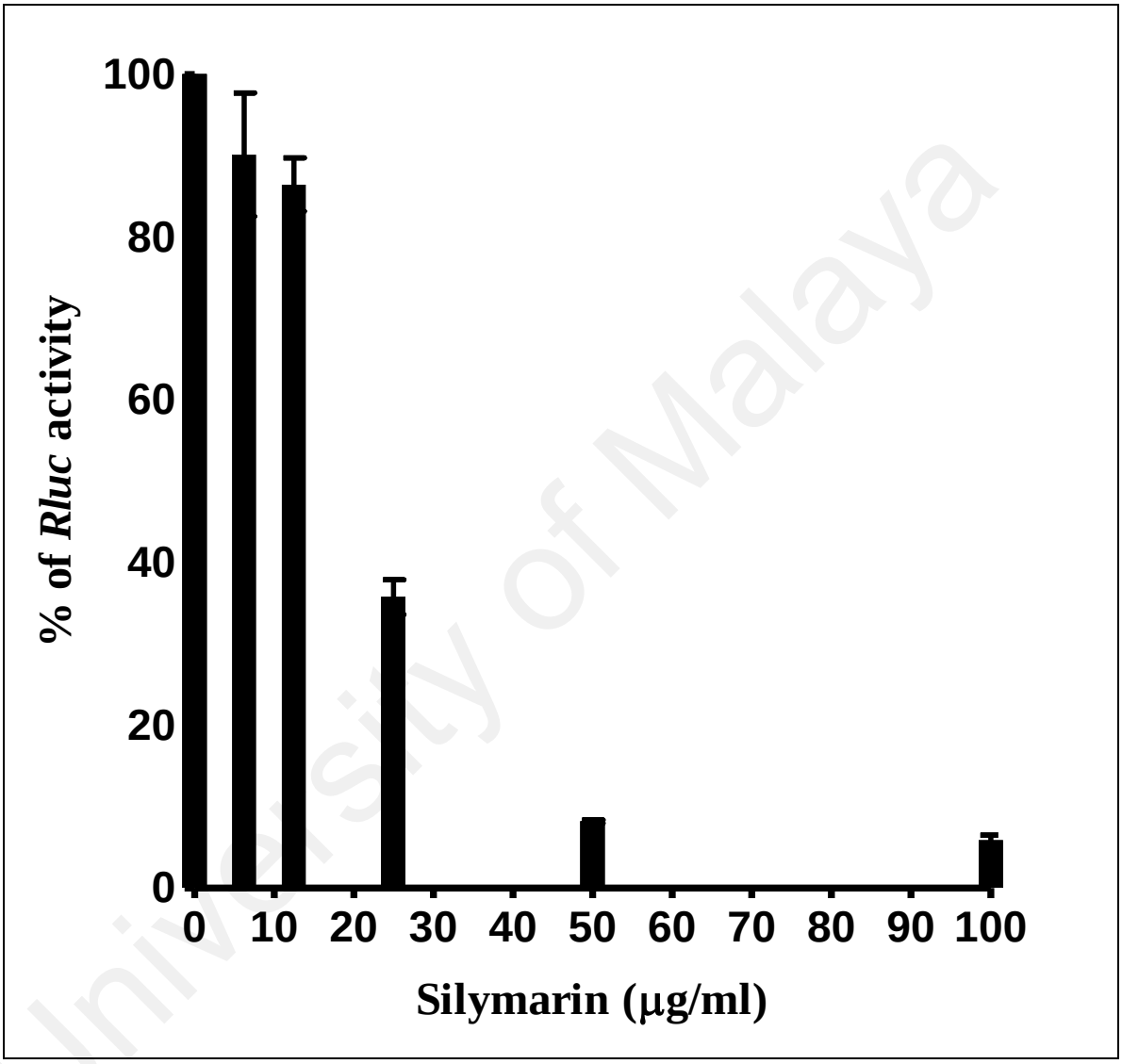

Figure 4.6: The *Rluc* activity has reduced significantly by silymarin treatment on the CHIKV replicon cell line. Statistical significance was analyzed from a one-way ANOVA, $P<0.05$. Error bars represent standard errors of triplicate means.

4.4 The window in the CHIKV replication cycle when the flavonoids exert its antiviral effect

Based on the time-of-addition assay results which were confirmed by qRT-PCR, almost all four tested compounds; baicalein (**Figure 4.7(a)**), fisetin (**Figure 4.7(b)**),

quercetagetin (**Figure 4.7(c)**) and silymarin (**Figure 4.7(d)**), showed good inhibitory effects at the early stage of the CHIKV life cycle considering the reduction of the of the RNA copy number during early hours of the treatment comparing to the non-treated CHIKV-infected Vero cells or the positive controls.

By referring to the **Figure 4.7(a)**, both baicalein treatment and the time of baicalein treatment do affect the CHIKV RNA copy number and the interactions are considered as significant. The treatment with baicalein at 100 μg/ml gave a significant reduction on CHIKV RNA copy number, $P< 0.0001$, as confirmed by one-way ANOVA test. As confirmed by using the Dunnett's multiple comparison posttest, at the time of treatment (-2, -1, 0, 2 and 3 hpi), the association between the time of baicalein addition and the reduction of CHIKV RNA copy number is very potent, ***, whereas at other hours of treatment the association is insignificant. This means that the best time of treatment with baicalein is up to 3 hours after CHIKV infection.

Figure 4.7(b) showed that, the treatment with fisetin at 100 μg/ml gave a significant reduction on CHIKV RNA copy number, $P< 0.0001$, as confirmed by one-way ANOVA test. As confirmed by Dunnett's multiple comparison posttest, all time of treatment used in this assay has significantly reduced the CHIKV RNA copy number. The association between the time of fisetin addition and the reduction of CHIKV RNA copy number is very strong, ***. This means that the effective time of treatment with fisetin is up to 5 hours after CHIKV infection.

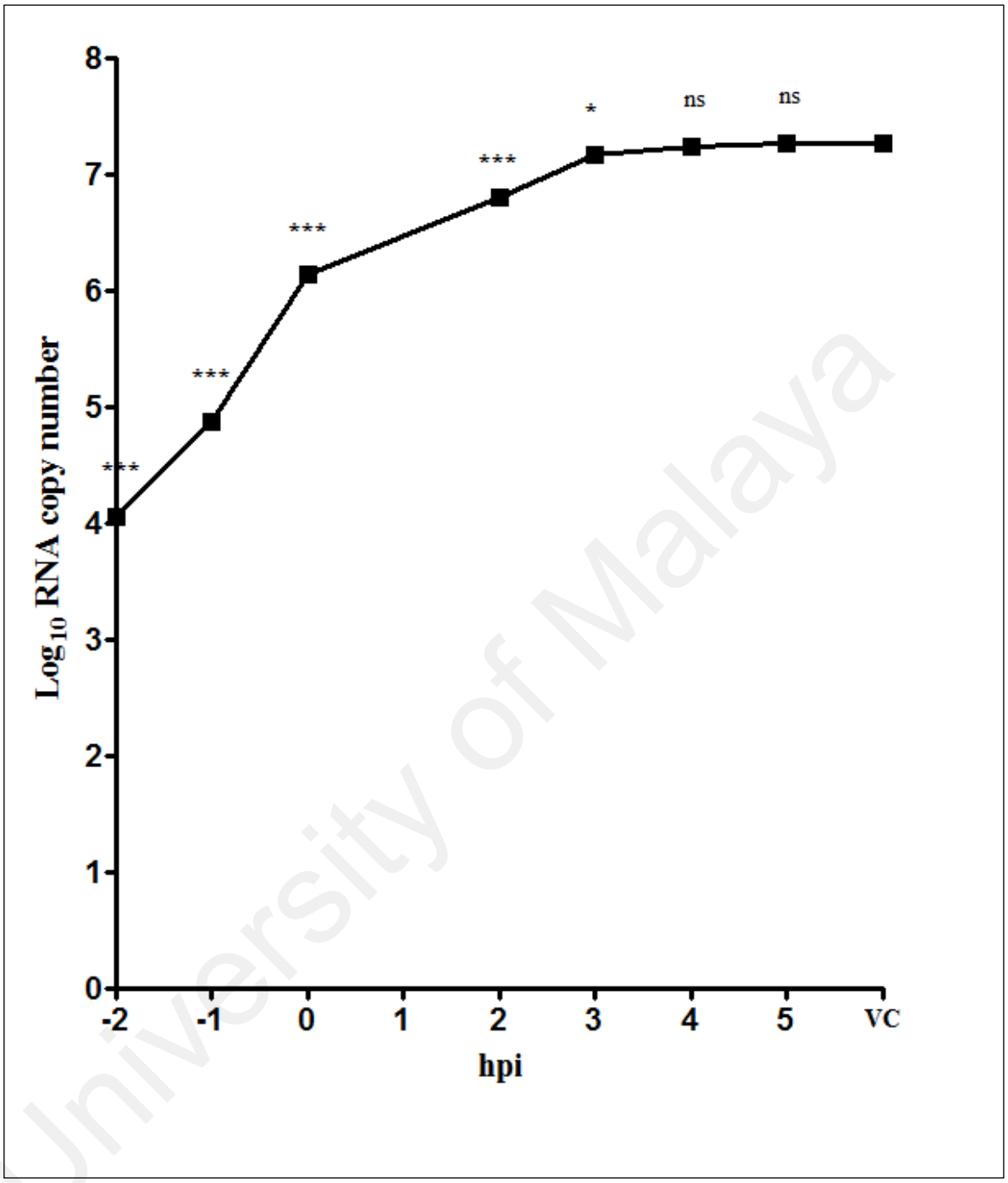

Figure 4.7(a): The RNA copy numbers of CHIKV reduced significantly at the early hours of baicalein treatment in the time-of-addition assay. "VC" is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA and Dunnett's multiple comparison posttest. ***, $P<0.05$. "ns" is referring to not significant. "hpi = hours post-infection". Error bars represent standard errors of triplicate means.

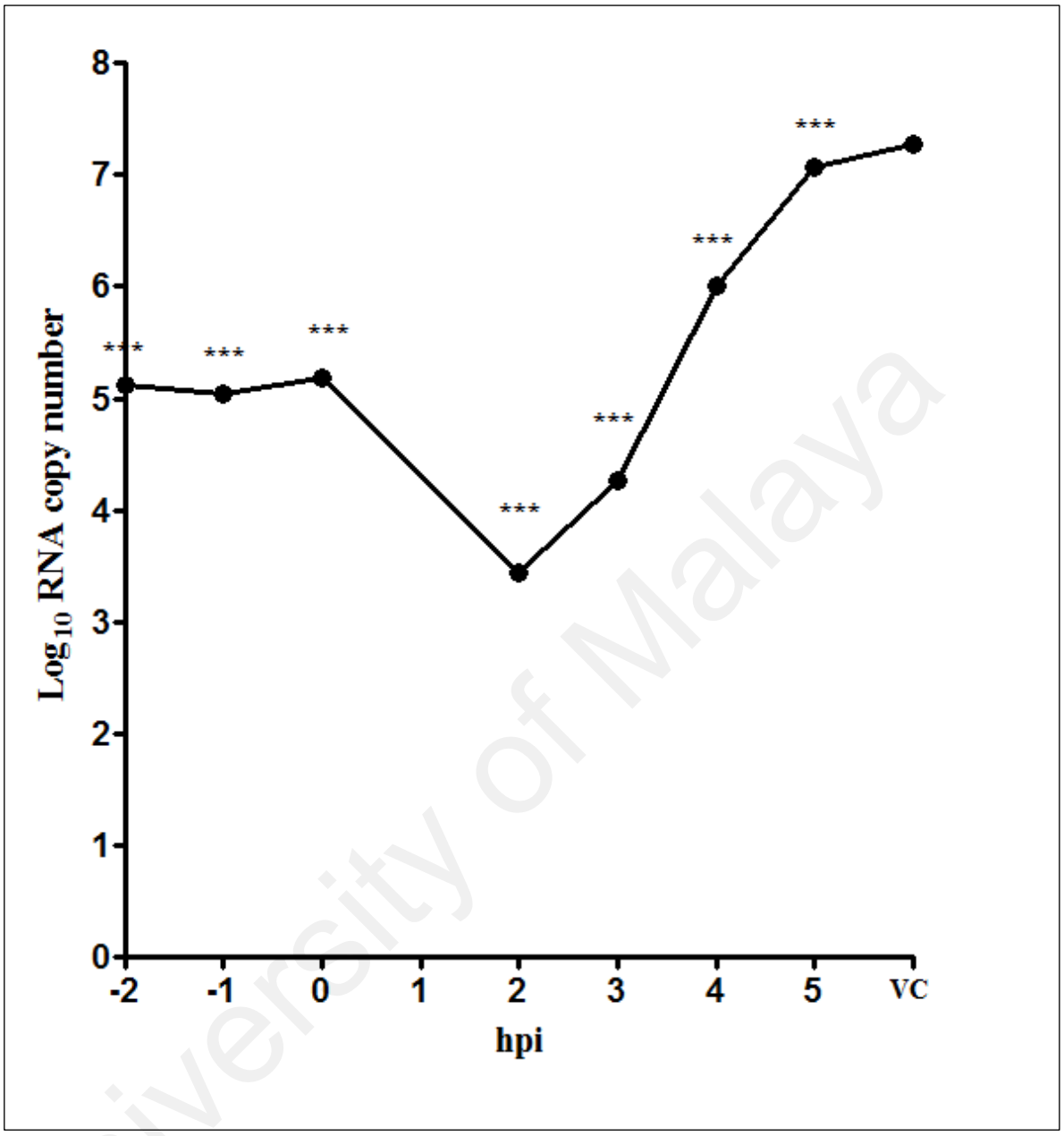

Figure 4.7(b): The RNA copy numbers of CHIKV reduced significantly at all time of treatment with fisetin. "VC" is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA and Dunnett's multiple comparison posttests. ***, $P<0.05$. "ns" is referring to not significant. "hpi = hours post-infection".Error bars represent standard errors of triplicate means.

Figure 4.7(c) showed that, quercetagetin at 100 µg/ml has showed a significant effect against CHIKV virus yield, $P< 0.0001$, as confirmed by one-way ANOVA test. As confirmed by Dunnett's multiple comparison posttest, at the time of treatment (-2, -1, 0, 2 and 3 hpi), the association between the time of quercetagetin addition and the reduction of

CHIKV RNA copy number is very strong, ***, whereas at other hours of treatment the association is insignificant. This means that the effective time of treatment with quercetagetin is up to 3 hours after CHIKV infection.

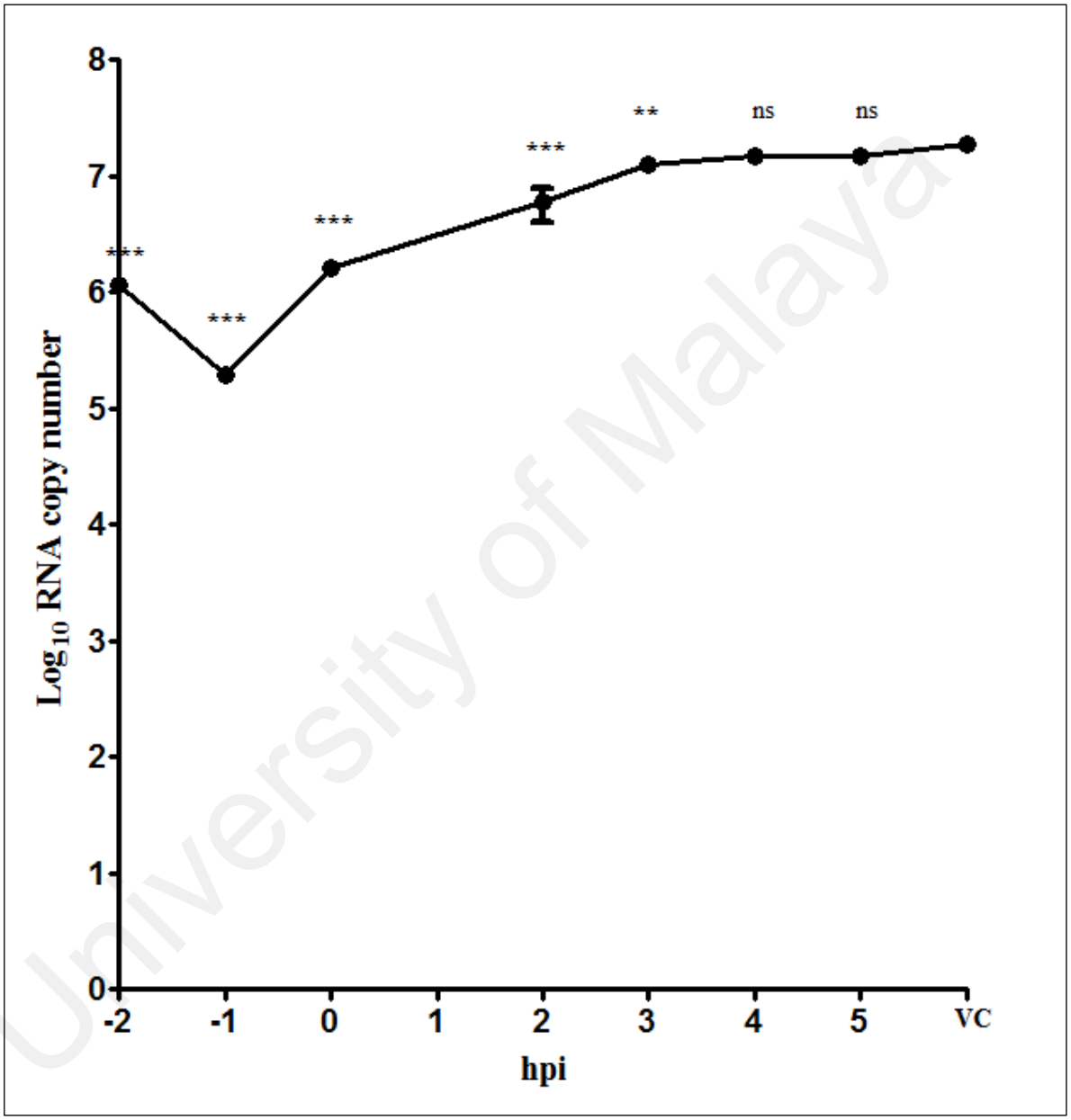

Figure 4.7(c): The RNA copy numbers of CHIKV reduced significantly up to 3 hpi of quercetagetin treatment in the time-of-addition assay. "VC" is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA and Dunnett's multiple comparison posttest. ***, $P<0.05$. "ns" is referring to not significant. "hpi = hours post-infection". Error bars represent standard errors of triplicate means.

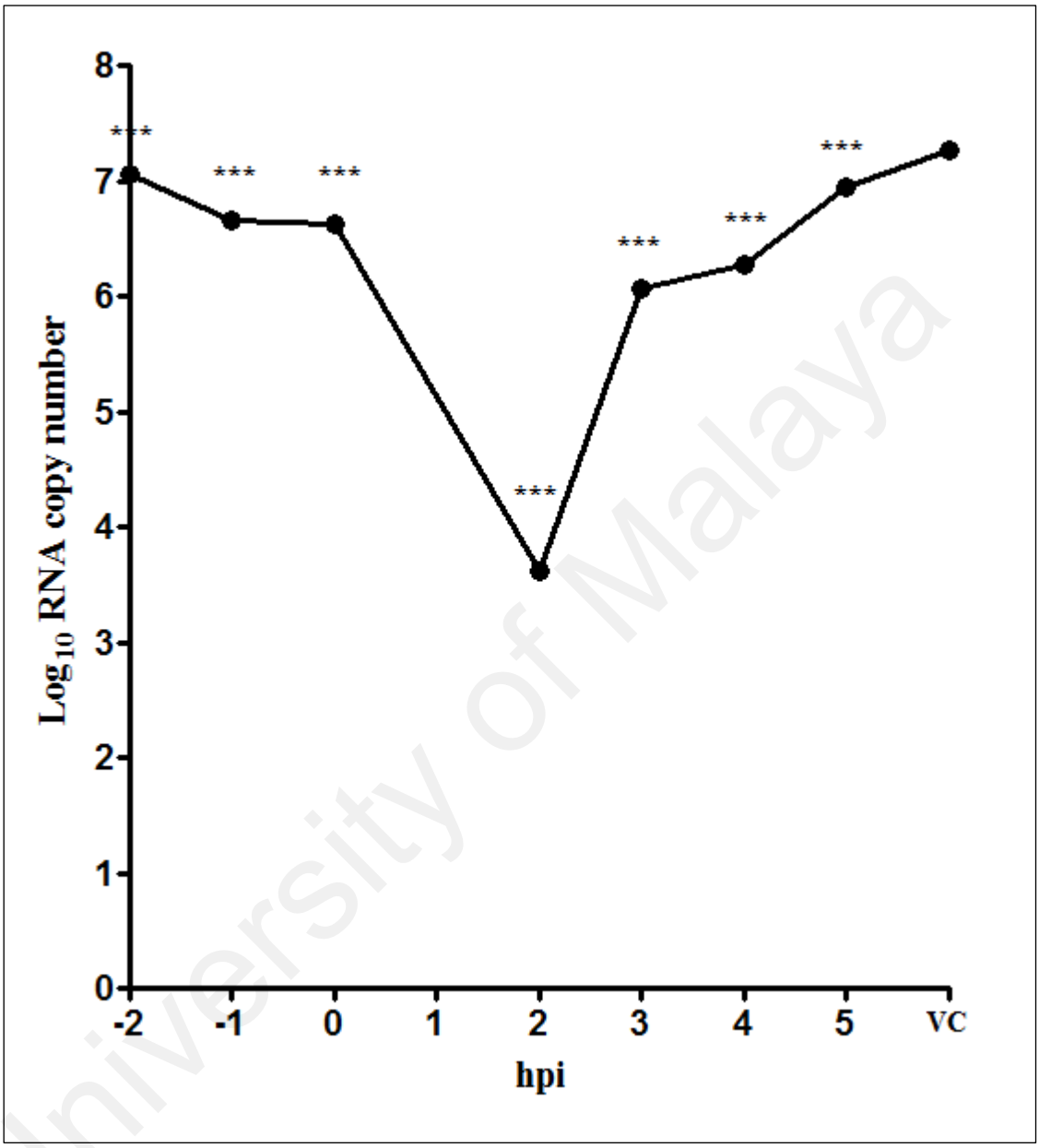

Figure 4.7(d): The RNA copy numbers of CHIKV reduced significantly at all time of silymarin treatment in the time-of-addition assay. "VC" is referring to the non-treated CHIKV-infected controls. Statistical significance was analyzed from a one-way ANOVA and Dunnett's multiple comparison posttest. ***, $P<0.05$. "ns" is referring to not significant. "hpi = hours post-infection". Error bars represent standard errors of triplicate means.

It was shown in **Figure 4.7(d)**, that silymarin at 100 μg/ml has significant anti-CHIKV activity, $P< 0.0001$, as confirmed by one-way ANOVA test. As confirmed by

Dunnett's multiple comparison posttest, all time of treatment used in this assay has significantly reduced the CHIKV RNA copy number. The association between the time of silymarin addition and the reduction of CHIKV RNA copy number is very strong, ***. This means that the effective time of treatment with silymarin is up to 5 hours after CHIKV infection. From the results of the time-of-addition assay, all four selected compounds seem to be able to inhibit CHIKV *in vitro* replication at its early stage of life cycle. Thus, individual antiviral assays were performed to determine at which stage of CHIKV life cycle does the bioflavonoid compounds affecting.

4.5 Selected flavonoids inactivate CHIKV particles

Based on the the results of virucidal assay shown in **Figure 4.8**, quercetagetin ($P< 0.0001$) and baicalein ($P< 0.0001$) exert potent dose-dependent effect against extracellular CHIKV particles with the IC_{50} values 9.934 μg/ml and 11.64 μg/ml respectively. Fisetin ($P< 0.0001$) and silymarin ($P< 0.0001$) also showed significant virucidal activity against CHIKV with the IC_{50} values of 52.71 μg/ml, 20.21 μg/ml respectively. The IC_{50} was calculated by comparing the treated CHIKV-infected sample with the CHIKV-infected sample without treatment.

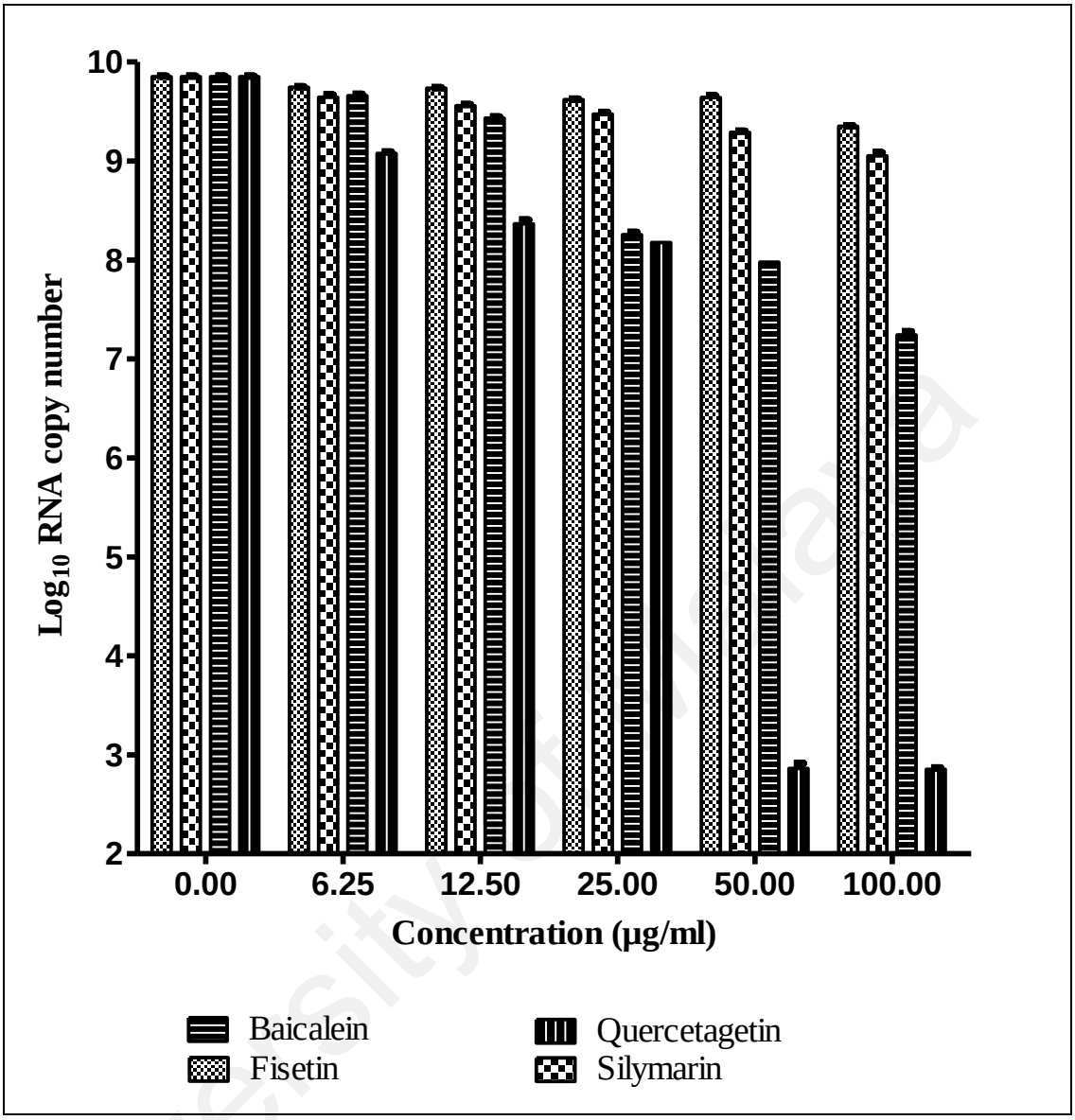

Figure 4.8: The reduction of the RNA copy number by the treatment of baicalein, fisetin, quercetagetin and silymarin in the virucidal assay. Statistical analysis was performed by using one-way ANOVA where $P<0.05$ is significant. ("0" on the X-axis is referring to the non-treated CHIKV-infected controls). Error bars represent standard errors of triplicate means.

4.6 Selected flavonoids interfere the adsorption of CHIKV on the cells

Based on the results of the anti-adsorption assay shown in **Figure 4.9**, baicalein ($P< 0.0001$), fisetin ($P< 0.0001$), silymarin ($P< 0.0001$) and quercetagetin ($P< 0.0001$) exert dose-dependent inhibition against CHIKV replication when they added during the virus attachment stage to the Vero cells. The IC_{50} values were 28.04 μg/ml, 176.0 μg/ml and 8.050 μg/ml for baicalein, silymarin and quercetagetin respectively.

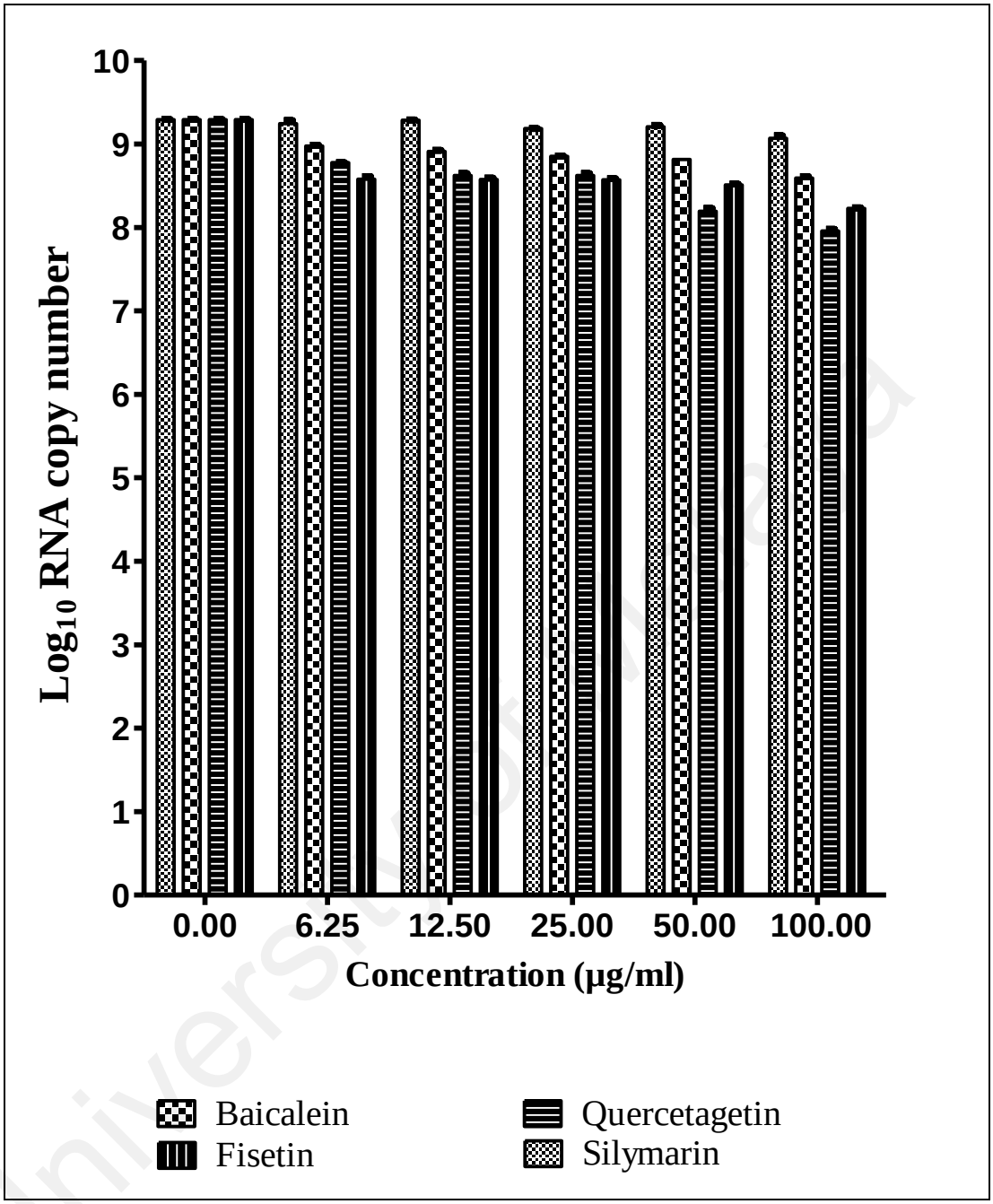

Figure 4.9: The reduction of the RNA copy number by the treatment of baicalein, fisetin, silymarin and quercetagetin in the anti-adsorption assay. Statistical analysis was performed by using one-way ANOVA where $P<0.05$ is significant. ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Error bars represent standard errors of triplicate means.

4.7 Selected flavonoids interfere the entry of CHIKV into the cells

It was shown in **Figure 4.10**, that baicalein ($P< 0.0001$), fisetin ($P< 0.0001$) and quercetagetin ($P< 0.0001$) exert significant dose-dependent inhibition against CHIKV after its adsorption to the Vero cells. Silymarin did show only moderate significant dose-dependent inhibition ($P=0.0319$) against CHIKV internalization. The IC_{50} values for baicalein, fisetin and quercetagetin were 21.01 μg/ml, 56.04 μg/ml and 64.21 μg/ml respectively. These results signified that baicalein is the strongest compound among the other tested compounds which could interfere with the entry of the CHIKV particles into Vero cells compared to the other three compounds.

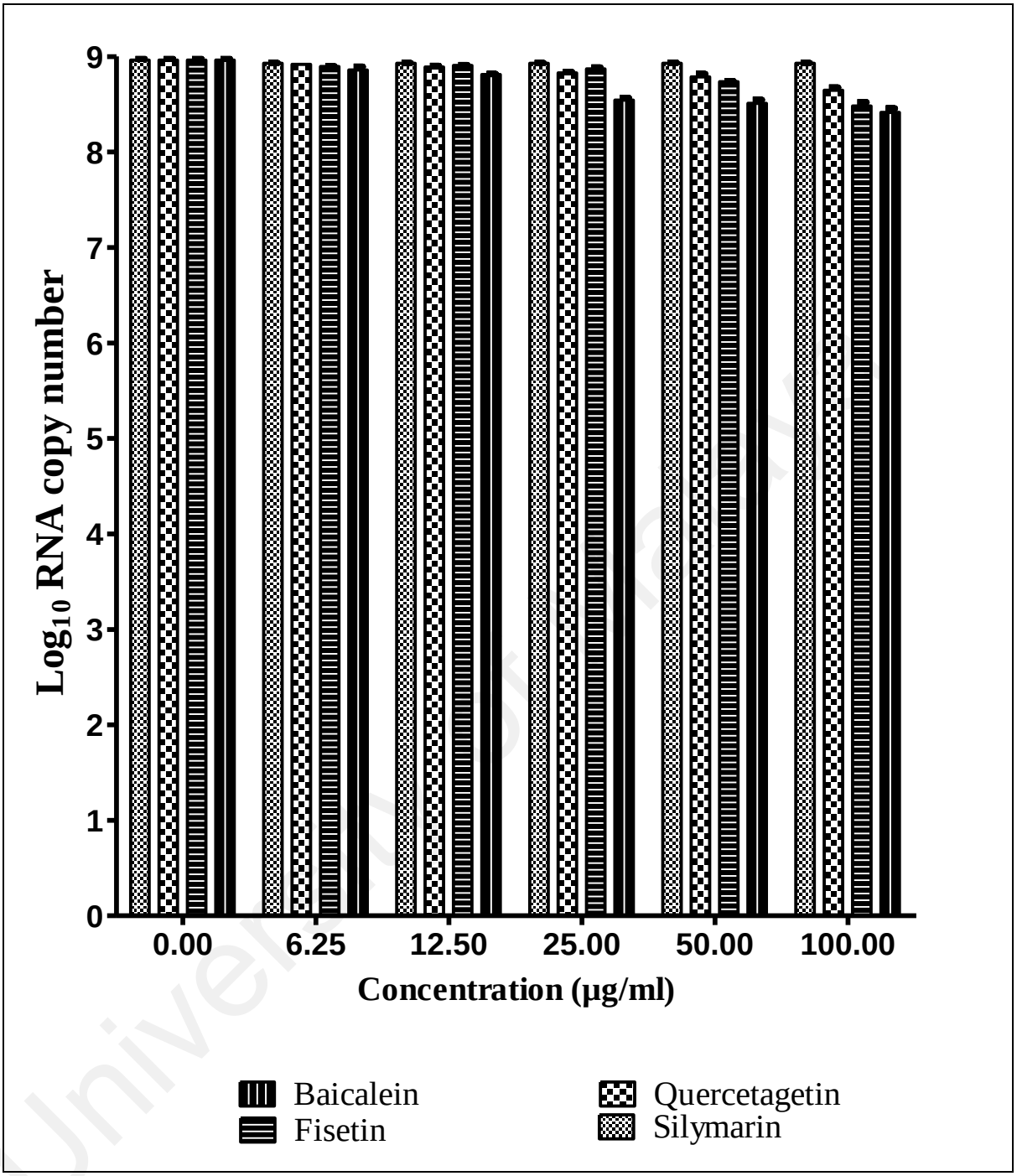

Figure 4.10: The reduction of the RNA copy number by the treatment of quercetagetin, baicalein, silymarin and fisetin in the anti-entry assay. Statistical analysis was performed by using one-way ANOVA lwhere $P<0.05$ is significant. ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Error bars represent standard errors of triplicate means.

4.8 Selected flavonoids interfere the intracellular CHIKV replication

Figure 4.11 showed that, baicalein ($P< 0.0001$), silymarin ($P< 0.0001$), quercetagetin ($P< 0.0001$), fisetin ($P< 0.0001$) and ribavirin ($P< 0.0001$) as a positive anti-CHIKV control exert significant dose-dependent inhibition against CHIKV *in vitro* replication when they were added after virus internalization. The IC_{50} values obtained were 1.891 μg/ml, 12.98 μg/ml, 13.85 μg/ml, 8.444 μg/ml and 11.07 μg/ml for baicalein, silymarin, quercetagetin, fisetin and ribavirin respectively. The IC_{90} values were 0.1812 μg/ml, 27.70 μg/ml, 142.7 μg/ml, 11.02 μg/ml and 16.63 μg/ml for baicalein, silymarin, quercetagetin, fisetin and ribavirin respectively. These results suggested that all four tested compounds could interfere with the specific binding of the viral attachment proteins and the cellular receptors.

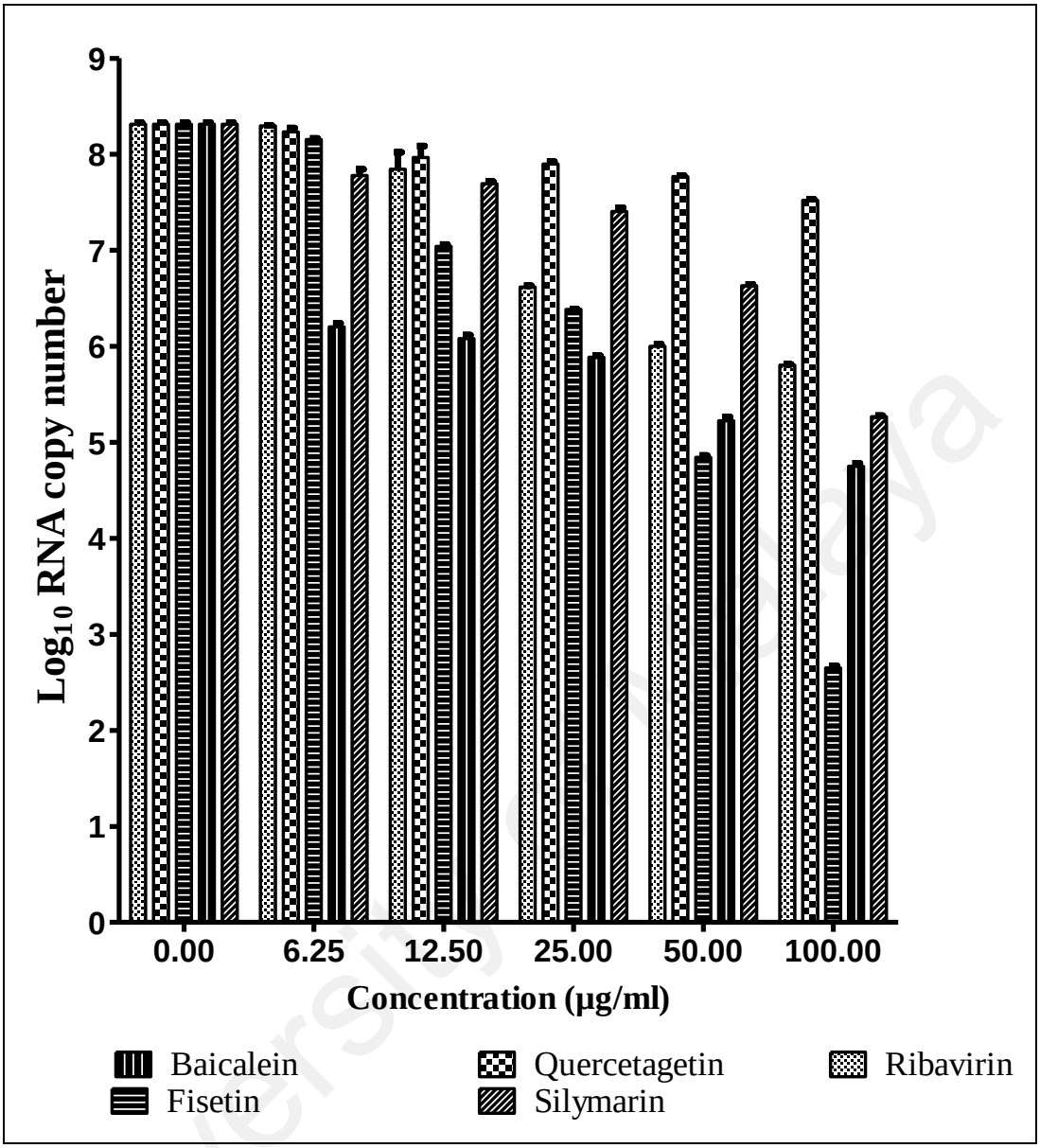

Figure 4.11: The reduction of the RNA copy number by the treatment of quercetagetin, baicalein, fisetin, silymarin and ribavirin at the post-adsorption assay. Statistical analysis was performed by using one-way ANOVA (Kruskal-Wallis test) where $P<0.05$ is significant. ("0" on the X-axis is referring to the non-treated positive CHIKV-infected controls). Error bars represent standard errors of triplicate means.

4.9 Selectivity index value

The calculation of the selectivity index (SI) value is essential to determine the potency of the treatment by dividing the concentration that reduces the cell viability by 50% to the concentration that offered the inhibitory effect by 50% (CC_{50}/IC_{50}). The selectivity index value for the four selected compounds at each antiviral assays were summarized in **Table 4.3**.

Table 4.3: Selectivity index of compounds for each antiviral assays.

Bioflavonoids	Antiviral assay			
	Virucidal	Adsorption	Post-adsorption	Entry
Baicalein	43.27	17.96	266.37	25.26
Fisetin	15.24	Undetermined	95.11	14.33
Quercetagetin	77.53	95.68	55.61	11.99
Silymarin	21.03	2.42	32.75	Undetermined

4.10 Selected flavonoids reduced the success rate of CHIKV infection

The immunofluorescence assay was performed to detect the alphaviral envelope protein as an indication of successful CHIKV infection and replication. Ribavirin has previously been shown to inhibit CHIKV replication (Briolant *et al.*, 2004) and was selected to serve as a positive control, as well as to assess the robustness of the assay.The result of immunofluorescence assay for baicalein treatment is shown in **Figure 4.12**.

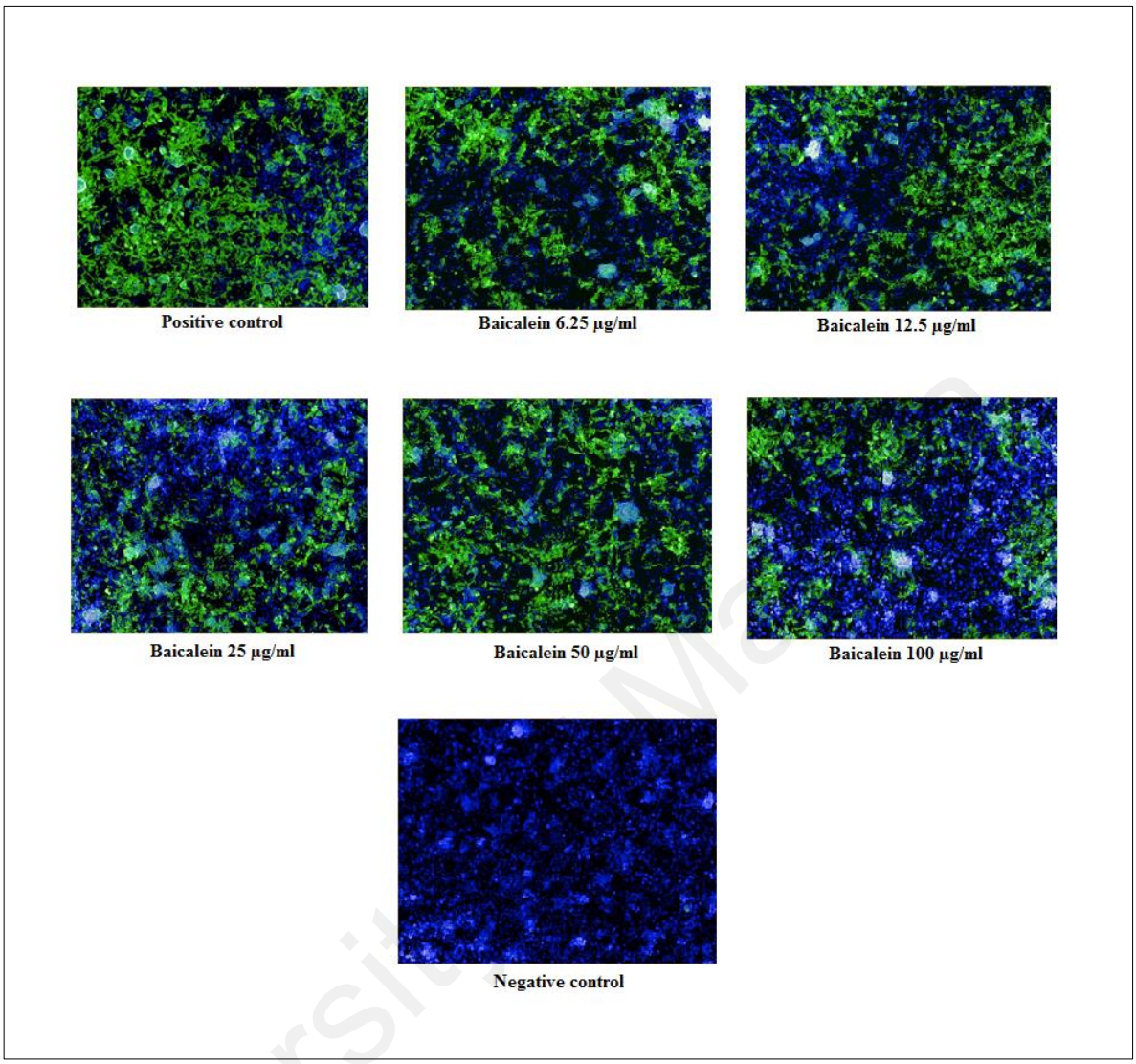

Figure 4.12: Dose-dependent inhibition of baicalein on CHIKV infectivity shown using immunofluorescence assay. Immunofluorescence detection of alphavirus envelope protein is used as an indication of CHIKV infection. CHIKV infection of Vero cells is compared untreated cells. Cell nuclei are stained with DAPI (blue), and CHIKV infection is indicated by Alexa Fluor (green) staining. Mock-infected cells were stained with both primary and secondary antibodies, as well as DAPI.

By referring to the **Figure 4.12,** dose-dependent reduction of viral antigen-positive cells for the different baicalein concentrations is observed from immunofluorescence images. Baicalein with 100 µg/ml concentration exerted about 70% inhibition against CHIKV antigen presentation. The results of immunofluorescence assay for fisetin treatment is shown in **Figure 4.13.**

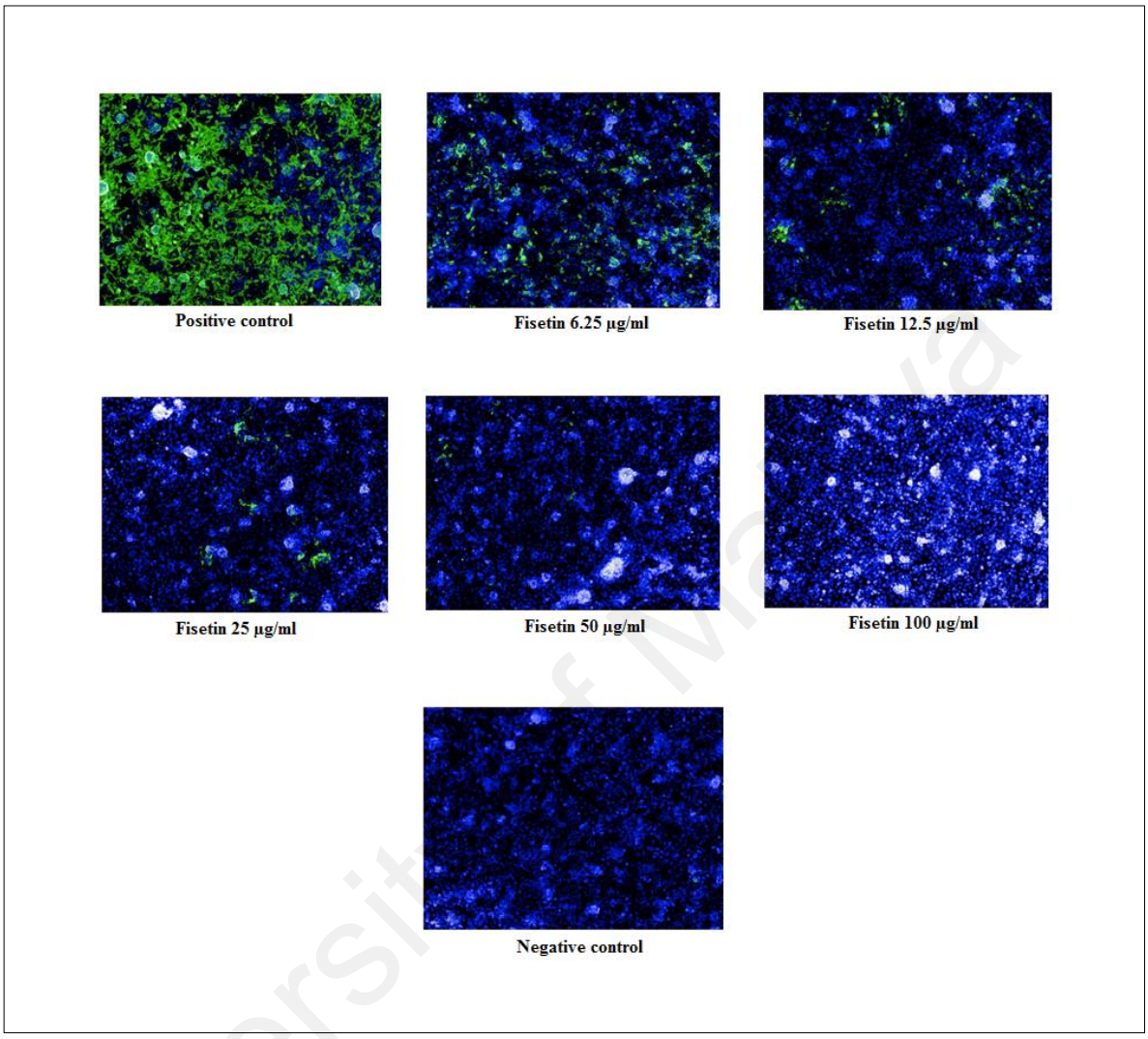

Figure 4.13: Dose-dependent inhibition of fisetin on CHIKV infectivity shown using immunofluorescence assay. Immunofluorescence detection of alphavirus envelope protein is used as an indication of CHIKV infection. CHIKV infection of Vero cells is compared untreated cells. Cell nuclei are stained with DAPI (blue), and CHIKV infection is indicated by Alexa Fluor (green) staining. Mock-infected cells were stained with both primary and secondary antibodies, as well as DAPI.

As shown in **Figure 4.13**, fisetin has successfully inhibited the CHIKV antigen production even by the lowest concentration. A dose-dependent reduction of viral antigen-positive cells for the different fisetin concentrations is observed from immunofluorescence images.. The lowest concentration of fisetin treatment, 6.25 µg/ml, offered more than 90% protection against CHIKV infection.

The results of immunofluorescence assay for quercetagetin treatment is shown in **Figure 4.14**. A dose-dependent reduction of viral antigen-positive cells for the different quercetagetin concentrations is observed from immunofluorescence images which indicates successful inhibition of CHIKV antigen production. Quercetagetin with 100 µg/ml inhibited the CHIKV antigen production for more than 80%.

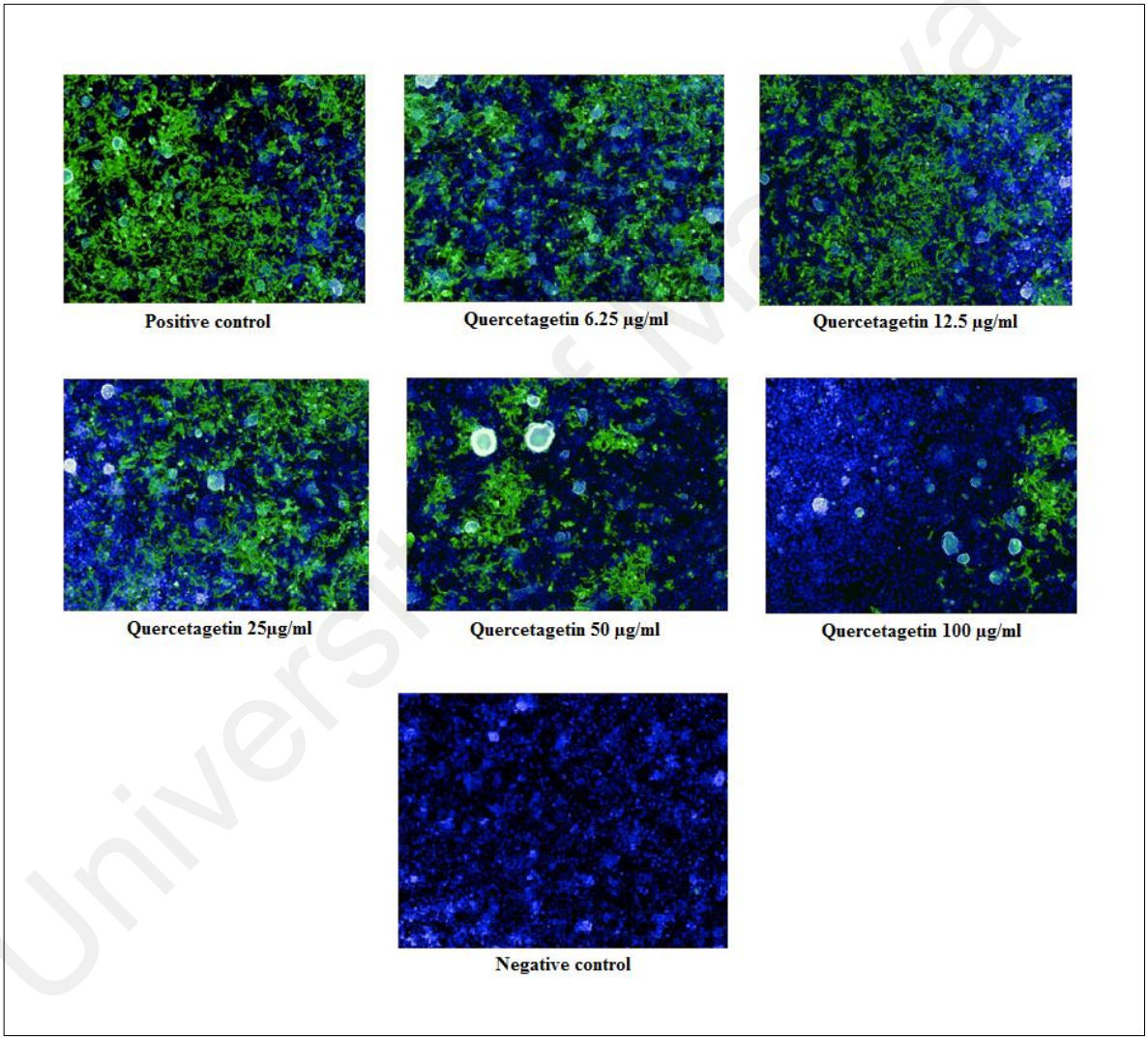

Figure 4.14: Dose-dependent inhibition of quercetagetin on CHIKV infectivity shown using immunofluorescence assay. Immunofluorescence detection of alphavirus envelope protein is used as an indication of CHIKV infection. CHIKV infection of Vero cells is compared untreated cells. Cell nuclei are stained with DAPI (blue), and CHIKV infection is indicated by Alexa Fluor 488 (green) staining. Mock-infected cells were stained with both primary and secondary antibodies, as well as DAPI.

The results of immunofluorescence assay for silymarin treatment is shown in **Figure 4.15**. A dose-dependent reduction of viral antigen-positive cells for the different silymarin concentrations is observed from immunofluorescence images.which indicates successful inhibition of CHIKV antigen production. At concentration of 100 µg/ml, silymarin offered more than 80% inhibition against CHIKV antigen production.

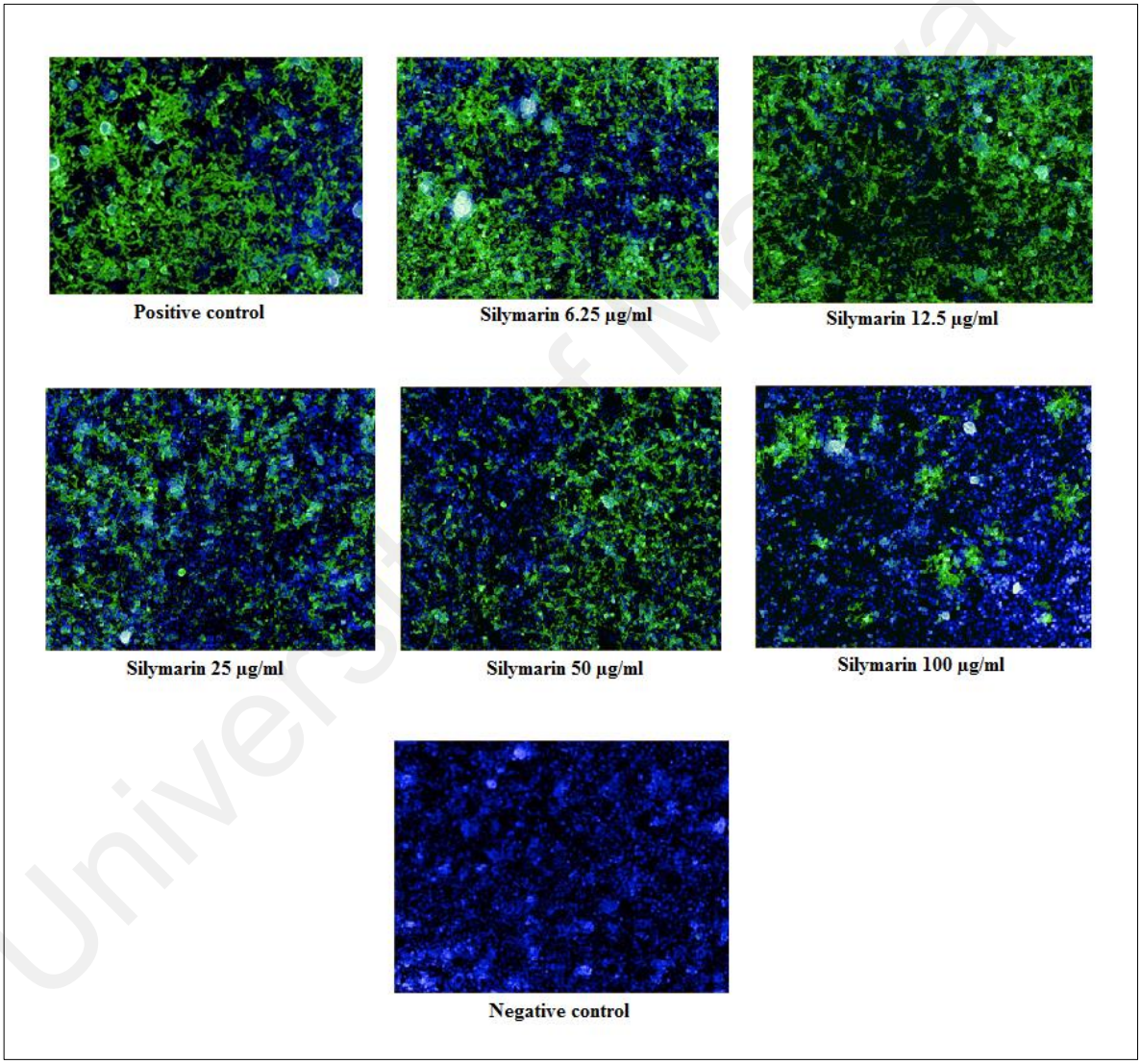

Figure 4.15: Dose-dependent inhibition of silymarin on CHIKV infectivity shown using immunofluorescence assay. Immunofluorescence detection of alphavirus envelope protein is used as an indication of CHIKV infection. CHIKV infection of Vero cells is compared untreated cells. Cell nuclei are stained with DAPI (blue), and CHIKV infection is indicated by Alexa Fluor 488 (green) staining. Mock-infected cells were stained with both primary and secondary antibodies, as well as DAPI.

The results of immunofluorescence assay on the nucleoside analogue anti-CHIKV control, ribavirin, is shown in **Figure 4.16**. A dose-dependent reduction of viral antigen-positive cells for the different baicalein concentrations is observed from immunofluorescence images.

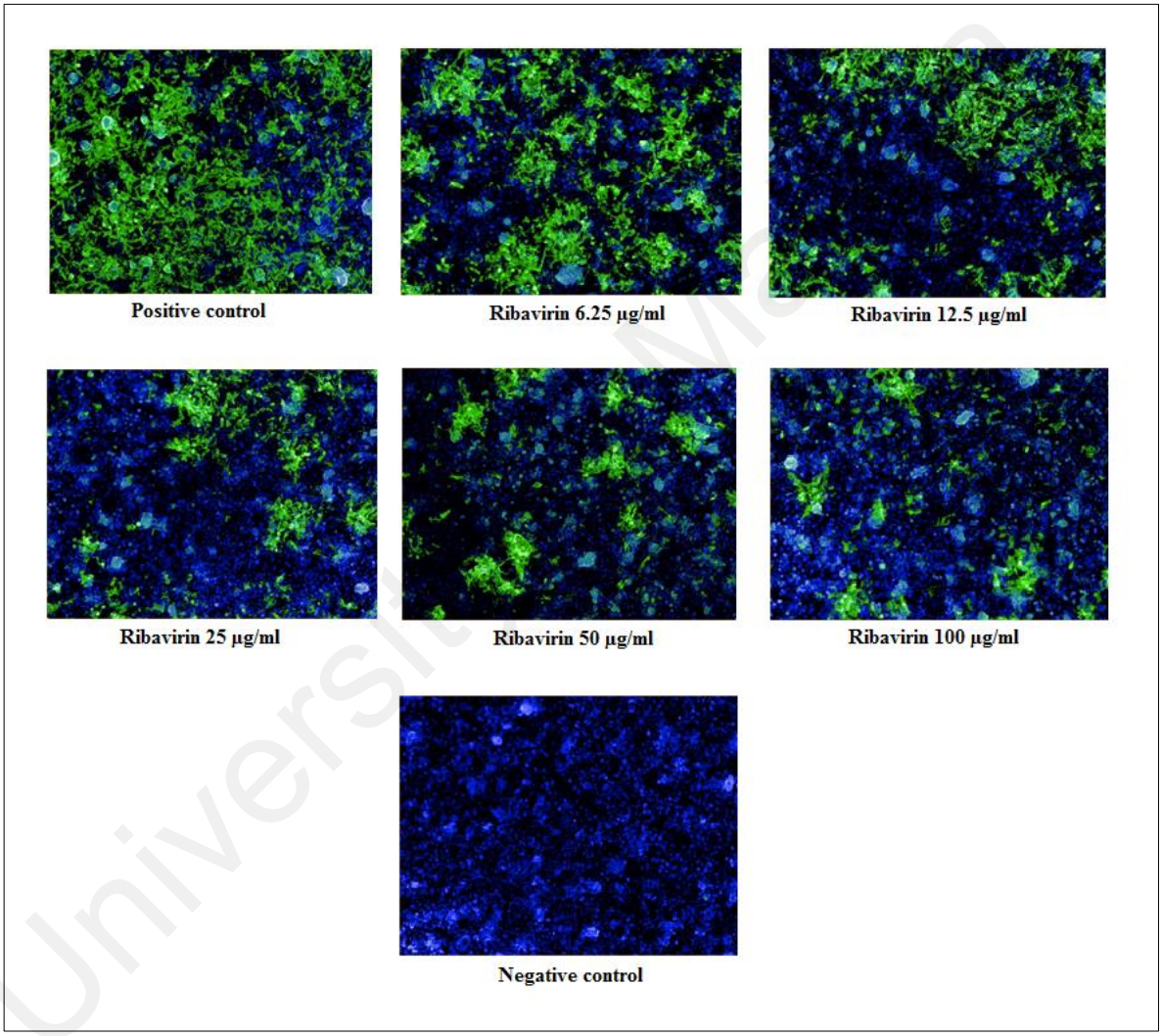

Figure 4.16: Dose-dependent inhibition of ribavirin on CHIKV infectivity shown using immunofluorescence assay. Immunofluorescence detection of alphavirus envelope protein is used as an indication of CHIKV infection. CHIKV infection of Vero cells is compared untreated cells. Cell nuclei are stained with DAPI (blue), and CHIKV infection is indicated by Alexa Fluor 488 (green) staining. Mock-infected cells were stained with both primary and secondary antibodies, as well as DAPI.

4.11 Selected flavonoids reduced the efficiency of CHIKV proteins accumulation

In order to determine the effect of the bioflavonoid compounds on CHIKV proteins synthesis, western blot analyses were performed. CHIKV proteins such as; pE2 (65 kDa), E2 (50 kDa), nsP1 (59 kDa) and nsP3 (76-78kDa) were detected by using the colorimetric method. β-actin was used to as a loading control in the experiment, as well as to ensure that the concentrations of the tested compounds did not affect the synthesis and expression of host cellular proteins, and also the integrity of the treated cells. The detection of the target CHIKV proteins would reflect the effect of the selected compounds on the process of CHIKV *in vitro* successful replication.

The results of western blotting upon baicalein treatment is as shown in **Figure 4.17**. Baicalein with concentration of 100 μg/ml showed significant inhibition against nsP1 and nsP3 protein accumulation. The E2 protein faded at the same concentration. By referring to the relative band intensity, the sudden drop in the band intensity of all three proteins were shown. This results reflect the ability of baicalein to suppress the efficiency of the process of CHIKV *in vitro* replication.

The results of western blotting upon fisetin treatment is shown in **Figure 4.18**. The nsP1 band intensity has been strongly affected by the treatment of fisetin with concentrations of 12.5 μg/ml and above. The nsP3 protein faded and totally disappeared at concentrations of 25 μg/ml and above. The E2 protein remained producing efficiently and was not affected by fisetin treatment. By referring to the relative band intensity, the sudden drop in the band intensity of nsP1 and nsP3 proteins were shown. This results reflect the ability of fisetin to suppress the CHIKV *in vitro* replication.

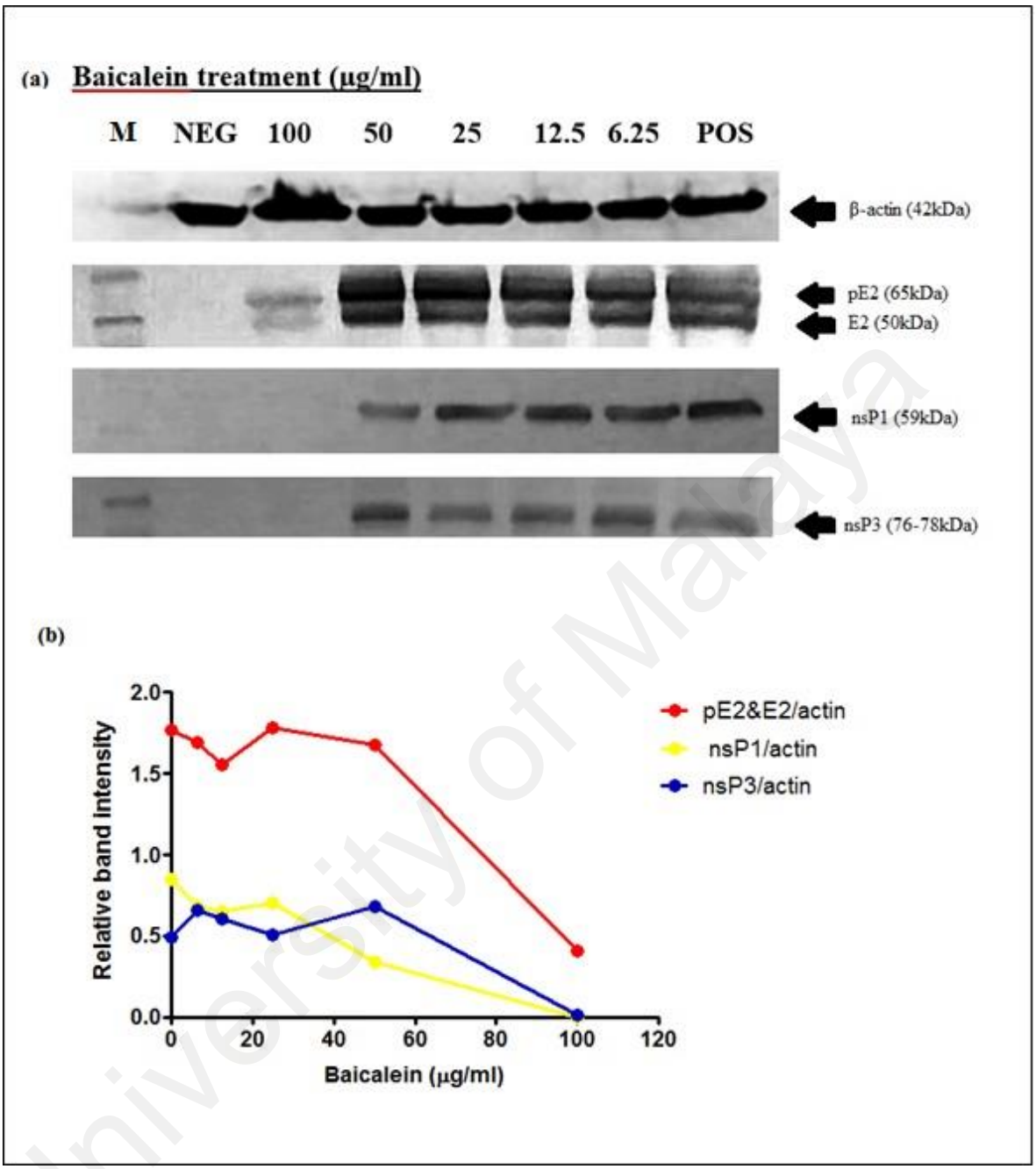

Figure 4.17: Baicalein suppressed the accumulation of CHIKV-encoded proteins.
(a) Bands of the specific proteins as observed on the blot. A dose dependent reduction of CHIKV nsP1, nsP3 and pE2/E2 proteins were observed upon baicalein treatments for 48 h.
(b) The above mentioned observation confirmed by calculating the relative band intensity (calculated from the band intensity of sample/band intensity of β-actin loading control) using Image J. β-actin is used as a loading control for each set of samples.

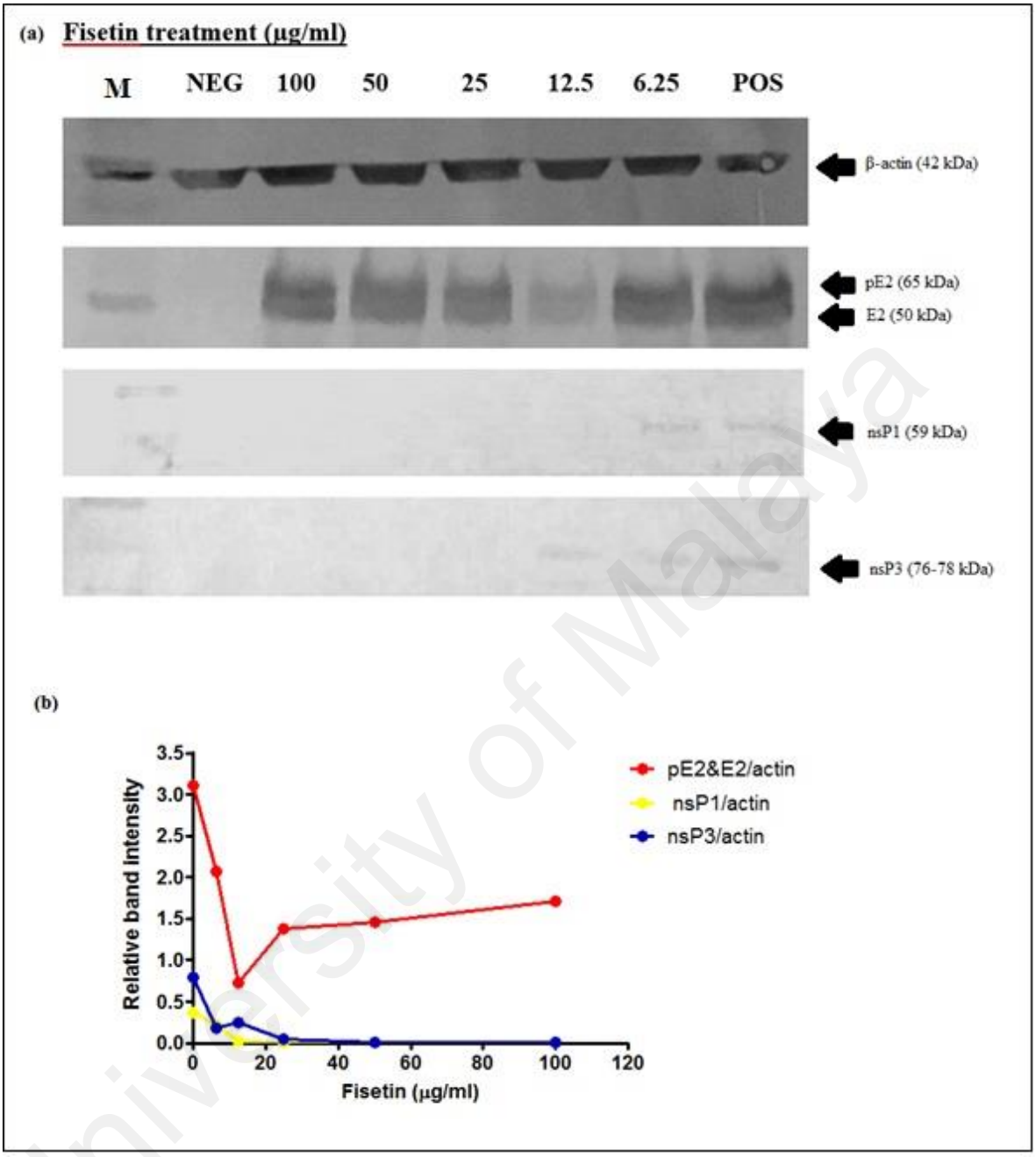

Figure 4.18: Fisetin suppressed the accumulation of CHIKV-encoded proteins.
(a) Bands of the specific proteins as observed on the blot. A dose dependent reduction of CHIKV nsP1 and nsP3 proteins were observed upon fisetin treatments for 48 h.
(b) The above mentioned observation confirmed by calculating the relative band intensity (calculated from the band intensity of sample/band intensity of β-actin loading control) using Image J. β-actin is used as a loading control for each set of samples.

The results of western blotting upon quercetagetin treatment is as shown in **Figure 4.19**. Quercetagetin, at all concentrations used, did not affect the accumulation of all targeted CHIKV proteins. The intensity of CHIKV proteins bands are equivalent to the positive controls. This result reflects the inability of quercetagetin to suppress the efficiency of CHIKV negative-strand synthesis and CHIKV replicase unit functions but not the process of glycoprotein maturation and transportation. These results indicate a possibility of the antiviral activity of quercetagetin at the later stage of CHIKV life cycle such as the assembly of the nucleocapsid core and genomic RNA packaging or even virus budding.

Whereas for silymarin (results shown in **Figure 4.20)**, did affect the CHIKV proteins accumulation in a dose-dependent manner. At silymarin concentration of 100 µg/ml, total disappearance of the nsP1 band can be seen after a dose-dependent reduction of the protein accumulation. The E2 and nsP3 proteins faded at the same concentration. By referring to the relative band intensity, sudden drop in the band intensity of all three proteins were shown. This results reflect the ability of silymarin to suppress the efficiency of the process of glycoprotein maturation and transportation, CHIKV negative-strand synthesis and CHIKV replicase unit functions.

All the results supported the assumptions that four selected compounds; baicalein, fisetin, quercetagetin and silymarin were able to inhibit and interfere CHIKV infection and replication efficiency at various stages of CHIKV *in vitro* life cycle efficiently.

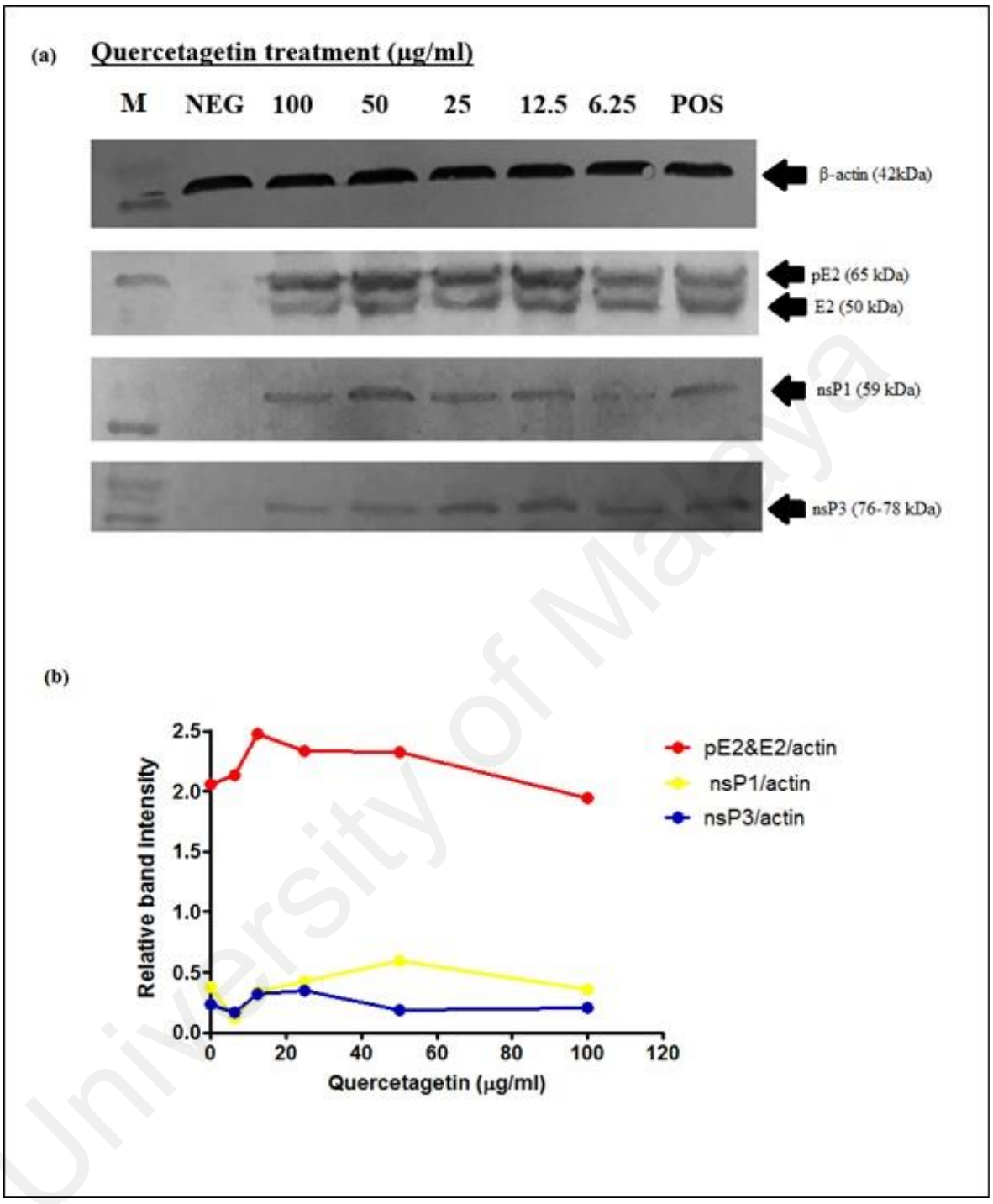

Figure 4.19: Quercetagetin suppressed the accumulation of CHIKV-encoded proteins.
(a) Bands of the specific proteins as observed on the blot. No dose dependent reduction of CHIKV pE2/E2, nsP1 and nsP3 proteins were observed upon quercetagetin treatments for 48 h.
(b) The above mentioned observation confirmed by calculating the relative band intensity (calculated from the band intensity of sample/band intensity of β-actin loading control) using Image J. β-actin is used as a loading control for each set of samples.

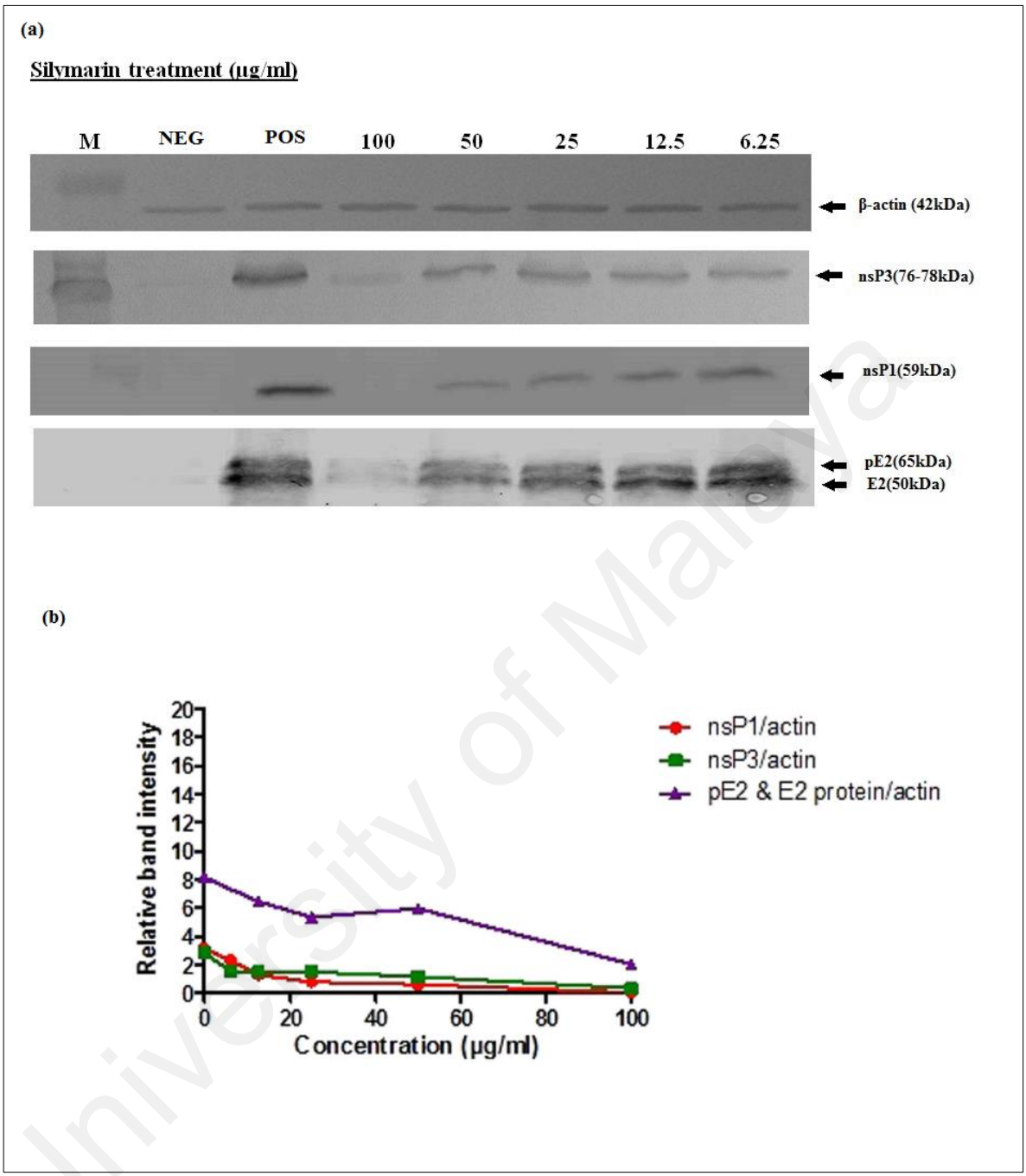

Figure 4.20: Silymarin suppressed the accumulation of CHIKV-encoded proteins.
(a) Bands of the specific proteins as observed on the blot. A dose dependent reduction of CHIKV nsP1, nsP3 and pE2/E2 proteins were observed upon silymarin treatments for 48 h. (b) The observation above is confirmed by calculating the relative band intensity (calculated from the band intensity of sample/band intensity of β-actin loading control) using Image J. β-actin is used as a loading control for each set of samples.

CHAPTER 5:DISCUSSION

The year of 2005 remarked the importance of research regarding CHIKV antivirals since the chikungunya fever epidemic in La Réunion, which was the worst in history. The fatal outbreak was also accompanied by the mutation of alanine to valine in the CHIKV E1 glycoprotein (A226V). Subsequently, CHIKV was transmitted by Aedes albopictus, the native mosquito vector in temperate regions (Schuffenecker *et al*., 2006). Many important medical factors contributed to the urge of finding the antiviral for CHIKV. CHIKV has re-emerged in recent years rapidly due to the efficiency of the mosquito vectors in establishing the CHIKV infection to the wide geographical regions. Approved antivirals are needed to control symptoms and minimize the complications in future epidemics (Kaur & Chu., 2013).

The best treatments that can be offered now are usually symptomatic by the administration of non-steroidal anti-inflammatory drugs or corticosteroids to soothe the arthralgia and myalgia (Queyriaux *et al*., 2008). In order to cope with increased global travels, wide distribution of vectors in many geographical regions as well as the risk of future epidemic, active investigations are continuously conducted to find effective antivirals.

Most antivirals such as harringtonine, arbidol, mycophenolic acids and many more are only in the preliminary stages of antiviral discovery. Harringtonine inhibited the production of CHIKV nsP3 and E2 proteins as well as positive- and negative-sense CHIKV RNA (Kaur *et al*., 2013). Arbidol, the broad-spectrum antiviral, prevented the CHIKV adsorption to target cells thus inhibited the viral entry. Mycophenolic acids inhibited CHIKV replication in a similar mechanism to that of the nucleoside analogue, ribavirin.

However, a number of these preliminary antiviral studies have provided an insight towards understanding the biology of CHIKV.

Well-known broad-spectrum antivirals such as chloroquine, ribavirin and IFN-α have shown the efficacy *in vivo*. Upon administration of ribavirin into patients experiencing arthritis and lower limb pains after CHIKV infection, they were found to have reduced soft tissue swelling and joint pains. However, a lot remains unknown regarding CHIKV biology *in vivo* (Kaur & Chu., 2013). Furthermore, the shortcomings of nucleoside analogues or synthesized drugs cannot be underestimated since they are potentially teratogenic, embryotoxic, carcinogenic and possessed the anti-proliferative activities (Morris D.J., 1994).

The undesirable side effects should be taken into account before a compound could be considered an antiviral candidate. Continuous research is required to establish a suitable and effective antiviral and diversifying potential compounds would broaden the chances of getting one. In doing so, many researchers turn to natural products for antivirals because it guarantees continuous supply, ease of preparation, relatively low toxicity and low side effects. In this case, bioflavonoids have shown high degree of potential for anti-CHIKV research.

Although these phenolic compounds are nonessential for plant survival, their potentials in medical research fields should not be overlooked. Bioflavonoid compounds played roles in plants such as defense (Mol *et al*., 1998), allelopathy (Peer & Murphy., 2007), providing flower coloring to attract pollinators (Bais *et al*., 2006), modulating the levels of reactive oxygen species and influenced the transport of the auxin (Treutter., 2005). In medical research fields, bioflavonoids compounds were found to have anti-oxidant (Williams R.J *et al*., 2004), anti-tumor (Garcia-Mediavilla *et al*., 2007), anti-proliferative

(Taylor & Grotewold., 2005), anti-inflammatory (Pandey *et al*., 2007), anti-fungal (Sung *et al*., 2007), anti-bacterial (Wachter *et al*., 1999) and even antiviral activity against many viruses (Valsaraj *et al*., 1997; Zheng *et al*., 1996; Li *et al*., 2000; Ono *et al*.,1989). While going through the research for antivirals against CHIKV, a number of researchers have already started roaming across the library of bioflavonoid compounds regardless whether they are pure compounds or a mixture of compounds. Among the bioflavonoid compounds were naringenin, chrysin, silybin and apigenin, also investigated in this study.

Naringenin possessed anti-Sindbis virus activity which is an alphavirus similar to CHIKV (Paredes *et al*., 2003). Thus predictably, naringenin (IC_{50}=30.0 μM) was also studied by using a stable CHIKV replicon cell line, together with silybin (IC_{50}=59.8 μM), chrysin (IC_{50}=50.2 μM) and apigenin (IC_{50}=28.3 μM), and was able to suppress the *Rluc* marker gene activity expressed by CHIKV replicon (Pohjala *et al*., 2011). However, the study did not involve infectious CHIKV. Silybin, on the other hand, is a semi-purified, commercially available fraction of silymarin, the bioflavonoid that has also been investigated in this study.

Though silybin exhibited antiviral activity against CHIKV, we recently published a study suggesting that silymarin, as a whole complex of more than 7 flavonolignans including silybin, possessed better activity against CHIKV. Moreover, in a previous study IC_{50} of silybin, one of the major components of silymarin, was estimated as 59.8 μM (approximately 30 μg/ml). Compared to this, silymarin was somewhat more efficient as nearly three-fold inhibition was observed at 25 μg/ml indicating that other components of silymarin likely contributed to its anti-CHIKV activity. It has been concluded that other components of silymarin have enhanced its anti-CHIKV activity (Lani *et al*., 2015).

In this study, 14 bioflavonoids were screened for any possible antiviral activity against CHIKV. However, after performing continuous treatment as a screening method, only four showed potential inhibition activity against CHIKV. A qualitative assay, CPE inhibition assay was performed to validate the screening result. A quantitative assay, MTS assay confirmed that the four compounds can inhibit CHIKV infection significantly from the screening. Those four compounds were baicalein, fisetin, quercetagetin and silymarin. Interestingly, these four compounds are of different chemical structure based on the flavone ring. Silymarin is a flavonolignan which is a mixture of the flavonoid and lignan, baicalein is a flavone whereas both fisetin and quercetagetin are flavonol. Their antiviral activities against CHIKV are varied based on the antiviral assays that has been performed. These four bioflavonoids interfere with different stages of CHIKV *in vitro* replication in Vero cells. The findings of this study are summarized in **Table 5.1**.

Table 5.1: Summary of the result obtained from various assays performed in the current study.

Bioflavonoids	T.O.A	Antiviral assays				Western blotting		
	Effective time	Virucidal	Adsorption	Post-adsorption	Entry	Proteins		
						E2	nsP1	nsP3
Baicalein	-2 to 3 hpi	+	+	+	+	+	+	+
Fisetin	All time of treatment hpi	+	+	+	+	+	+	+
Quercetagetin	-2 to 3 hpi	+	+	+	+	-	-	-
Silymarin	All time of treatment	+	+	+	+	+	+	+

Note that, "T.O.A: time of addition assay", "hpi: hours post-infection", "+: significant inhibition activity" and "-: non-significant inhibition activity".

In this study, a positive strand qRT-PCR has been performed to evaluate the virus yield reduction as described by Chiam *et al*., 2013. The target gene was the nsP3 gene by

using the designed nsP3 primer (136bp). The positive strand qRT-PCR assay was chosen because the previous study showed that it was specific for CHIKV, possessed more optimum high coefficients of determination (R^2), slope, and efficiency. The positive strand qRT-PCR was able to detect CHIKV RNA with highest sensitivity and appeared to be more suitable for the detection of the high viral loads as expected in this study (Chiam *et al*., 2013).

Focusing on the ability of the bioflavonoid compounds to interfere the CHIKV replication, a CHIKV cell line-based replicon was chosen to depict the assumption. CHIKV replicon cell lines offer a screening-friendly approach. By constructing replicon containing CHIKV replicase proteins with puromycin acetyltransferase, EGFP and Renilla luciferase marker genes was constructed for this objective. This enabled us to identify the potential antiviral candidates for alphavirus entry and replication phase inhibitiors (Pohjala *et al*., 2011). Through screening using CHIKV replicon, baicalein showed the best inhibitory activity with IC_{50}=3.243 μg/ml. However, the result was different from the antiviral assay performed with infectious CHIKV most probably due to the structural glycoproteins of CHIKV which also facilitated anti-CHIKV activity of the compounds.

In the time-of-addition assay, bioflavonoids were added to the CHIKV-infected Vero cells as the pre-treatment (-2 and -1 hpi), co-treatement (0 hpi) and post-treatment (2, 3, 4, and 5 hpi) assays. Through this assay, the best time for the most potent anti-CHIKV effects due to treatment with selected bioflavonoids have been investigated. The half-life of a compound would probably be one of the ultimate reasons that would cause the effective time for baicalein and quercetagetin to exert its antiviral effect up to 3 hpi, while fisetin and silymarin performed better up to 5 hpi. Almost 100% of CHIKV replication efficiency can be inhibited by baicalein and quercetagetin at -2 to 2 hpi whereas fisetin and silymarin at all

time of treatment.It is highly certain that these bioflavonoid compounds acted at the early hours and most possibly early stages of CHIKV infection. Thus, in the antiviral assays composed of the assays that will portrayed the actions of bioflavonoids on early events of CHIKV infections, the probable mechanism could be determined.

In virucidal assays, the four compounds have successfully inactivated the CHIKV prior to the infection of the Vero cells. The reduction of CHIKV RNA yield in this assay reflects that most of treated CHIKV particles were unable to infect the Vero cells. All four bioflavonoids showed effective virucidal effect against CHIKV (the effectivity order as quercetagetin>baicalein>silymarin>fisetin), but the most potent bioflavonoid was quercetagetin with IC_{50}=9.934 μg/ml and SI=77.53. However, there are no reported virucidal activity of quercetagetin against other viruses. Baicalein showed virucidal activity against JEV (IC_{50}=3.44 μg/ml) and DENV-2 (IC_{50}=1.55 μg/ml) (Johari *et al*., 2012; Zandi *et al*., 2012). Fisetin, in the other hand, did not exhibit any virucidal activity against DENV-2 and EV-71 (Zandi *et al*., 2011; Lin *et al*., 2012). Silibinin, the primary active component of silymarin, showed virucidal activity against herpes virus with IC_{50}=5.0 μg/ml (Cardile & Mbuy., 2013).

We have found that all four selected bioflavonoids in this study showed more anti-adsorption activity against the adsorption of CHIKV particles to the Vero cells. The proteins that are most likely to be involved were E1 and E2 glycoproteins since these are the proteins crucial for the host-receptor interaction during CHIKV adsorption (Jose, Snyder & Kuhn., 2010). The reduction of the CHIKV adsorption efficiency can be interpreted through the reduction of the CHIKV yield in the respective assay. Quercetagetin again exhibited the best anti-adsorption activity against CHIKV with IC_{50}=8.050 μg/ml and SI=95.68. Quercetagetin has successfully interfered the interactions between CHIKV

glycoproteins and host receptors. Among four bioflavonoids, baicalein showed anti-adsorption activity against JEV (IC_{50}=7.27 μg/ml) and DENV-2 (IC_{50}=7.14 μg/ml) in previously reported studies (Johari *et al*., 2012; Zandi *et al*., 2012).

After adsorption of CHIKV to the Vero cells, the host receptors induce conformational changes in the E1 and E2 glycoproteins. This stage is known as the entry stage. From the anti-entry assay, the reduction in the CHIKV RNA copy number can be seen after the treatment with all compounds. These compounds are hypothesized to interfere the conformational changes. The CHIKV bound to the receptor molecules were then endocytosed in clathrin-dependent manner. In order to interfere this stage, a compound must be able to either block the clathrin-coated pits or influence cells to ablate the ability of forming functional clathrin-coated pits. The CHIKV yield has been reduced since CHIKV cannot successfully enter Vero cells upon treatment with these three bioflavonoids. Three compounds in the effectivity order as baicalein>fisetin>quercetagetin showed anti-entry activity against CHIKV, baicalein being the most effective compound with IC_{50}=21.01 μg/ml and SI=25.26. Silymarin did not exhibit any anti-entry activity against CHIKV.

The post-adsorption or post-entry assay is crucial to determine the intracellular antiviral activity of compounds against CHIKV. Ribavirin was only relevant as anti-CHIKV positive control in this assay because it is the RNA replication inhibitor and it acts intracellularly. All selected compounds showed activity against post-entry activity of CHIKV with the effectivity order baicalein>fisetin>ribavirin>silymarin>quercetagetin. The best compound which exhibited post-entry antiviral activity against CHIKV was baicalein with IC_{50}=1.891 μg/ml and SI=266.37. Baicalein, fisetin, quercetagetin and silymarin have shown promising CHIKV inhibitory results, comparable to the performance of ribavirin.

These four compounds are highly recommended for further *in vivo* research since ribavirin was able to reduce joint pain in a few *in vivo* studies.

The effects of the bioflavonoids' treatment on production of the CHIKV E2 antigen was determined using the immunofluorescence assay. The effectivity order of the treatment determined from the intensity and quantity of the stained antigen is designated as fisetin>quercetagetin>silymarin>baicalein. Fisetin showed 90% reduction in the intensity and quantity of the antigen at even the lowest concentrations. This shows that the bioflavonoids 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 study will be more beneficial if the production of the CHIKV proteins upon treatment with bioflavonoid compounds can be determined. Thus, the western blotting analyses were performed. All three of the compounds except quercetagetin, were able to suppress the production or accumulation of the targeted proteins, pE2/E2, nsP1 and nsP3. This effect could be due to inhibition of CHIKV RNA replication and/or transcription. However, as in virus expression and replicon cell lines the synthesis of viral RNAs and proteins are coupled further study is necessary to evaluate the direct effect of the bioflavonoids on inhibition of newly synthesized CHIKV proteins.

It is possible that some of non-structural proteins of CHIKV represents direct target for the bioflavonoids. The possibilities include nsP1 protein, which is involved in the synthesis of the negative strand of viral RNA and RNA capping, and nsP3 protein, that is another component of the viral replicase complex. Down regulation of E2 expression may represent consequence of suppression of replication (directly or via inhibition of ns-protein(s)). However, this down regulation is clearly relevant from point of view of

development of effective antiviral as E2 protein is one of the important virion glycoproteins and is essential for receptor binding

Baicalein, fisetin and silymarin were able to interfere the accumulation of CHIKV proteins involved in negative-strand synthesis, replicase unit and the process of glycoprotein maturation and transportation. Although quercetagetin did not show any suppression, this result actually show that quercetagetin might interfere with the later stage of the CHIKV life cycle which is the assembly of the nucleocapsid core (together with its genomic RNA packaging) with the processed glycoproteins at the plasma membrane before budding. It is worth noting that these four bioflavonoid compounds exhibited antiviral activity against CHIKV with different strategies.

CHAPTER 6: CONCLUSION

Throughout this study, all the objectives were achieved. The cytotoxicity assay of 14 bioflavonoid compounds towards Vero cells were determined. Four out of 14 bioflavonoid compounds; baicalein, fisetin, quercetagetin and silymarin exhibited significant antiviral activity against CHIKV at different stages of CHIKV life cycle. Baicalein, fisetin and silymarin interfered with the CHIKV proteins involved in negative-strand synthesis and replicase unit.

Whereas quercetagetin, most probably interfered the later stage of CHIKV life cycle which is the assembly of nucleocapsid core together with RNA genomic packaging and processed glycoproteins. However, we have also successfully shown that quercetagetin can interfere with the early stages of CHIKV replication cycle especially with virus attachment to the Vero cells. These results are in conjunction with the ability of baicalein, fisetin and silymarin to suppress the accumulation or production of pE2/E2, nsP1 and nsP3 proteins. The selectivity index value for each effective compound in each antiviral assay have also been identified.

In future, further investigations should be carried out to determine the relationship between the differences of these four bioflavonoids chemical structure and the means of their antiviral activity against CHIKV. Performing molecular docking analysis would provide the prediction on predominant binding mode(s) of the compounds with a protein of known three-dimensional structure as well as providing its mechanism of actions. Performing the *in vivo* research by using animal models would be the second step towards understanding the outcomes of the the bioflavonoids treatment, side effects and suitable dosage in more complex organisms.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. Lani, R., Moghaddam, E., Haghani, A., Chang, L. Y., AbuBakar, S., and Zandi, K. (2014). Tick-borne viruses: a review from the perspective of therapeutic approaches. Ticks and tick-borne diseases, 5(5), 457-465.
2. Moghaddam, E., Teoh, B. T., Sam, S. S., Lani, R., Hassandarvish, P., Chik, Z., Yueh, A., AbuBakar, S., and Zandi, K. (2014). Baicalin, a metabolite of baicalein with antiviral activity against dengue virus. Scientific reports, 4.
3. Marlina, S., Radzi, S. F. M., Lani, R., Sieng, K. C., Rahim, N. F. A., Hassan, H., Chang, L.Y., AbuBakar, S., and Zandi, K. (2014). Seroprevalence screening for the West Nile virus in Malaysia's Orang Asli population. Parasites & vectors, 7(1), 597.
4. Shukri, M. M., Kho, K. L., Kisomi, M. G., Lani, R., Marlina, S., Radzi, S. F. M., Tay, S.T., and Zandi, K. (2015). Seroprevalence report on tick-borne encephalitis virus and Crimean-Congo hemorrhagic fever virus among Malaysian's farm workers. BMC public health, 15(1), 704.
5. Lani, R., Hassandarvish, P., Chiam, C. W., Moghaddam, E., Chu, J. J. H., Rausalu, K., Merits, A., Higgs, S., Vanlandingham, D., AbuBakar, S., and Zandi, K. (2015). Antiviral activity of silymarin against chikungunya virus. Scientific reports, 5.
6. Lani R, Mohd Rahim NF, Hassan H, Yaghoobi R, Chang LY, AbuBakar S, Zandi K. (2015) First report on the seroprevalence of the Crimean-Congo haemorrhagic fever virus, a tick-borne virus, in Malaysia's Orang Asli population. Eur Rev Med Pharmacol Sci., 19(3):461-6.

Papers Presented

1. "Evaluation of *in vitro* antiviral activity of silymarin against chikungunya virus" was presented at Chikungunya 2013, Langkawi, Malaysia on 28 to 30 October 2013.
2. "The *in vitro* study of the antiviral activity of silymarin, quercetin and kaempferol against chikungunya virus" was presented at InPRAS, University of Malaya, Malaysia on 10 until 11 December 2014.
3. "Evaluation of in vitro antiviral activity of fisetin against chikungunya virus" was presented at ICBHSR, Putrajaya, Malaysia on 25 until 27 February 2015.

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Review Article

Tick-borne viruses: A review from the perspective of therapeutic approaches

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ABSTRACT

Several important human diseases worldwide are caused by tick-borne viruses. These diseases have become important public health concerns in recent years. The tick-borne viruses that cause diseases in humans mainly belong to 3 families: Bunyaviridae, Flaviviridae, and Reoviridae. In this review, we focus on therapeutic approaches for several of the more important tick-borne viruses from these 3 families. These viruses are Crimean-Congo hemorrhagic fever virus (CCHF) and the newly discovered tick-borne phleboviruses, known as thrombocytopenia syndromevirus (SFTSV), Heartland virus and Bhanja virus from the family Bunyaviridae, tick-borne encephalitis virus (TBEV), Powassan virus (POWV), Louping-ill virus (LIV), Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), and Alkhurma hemorrhagic fever virus (AHFV) from the Flaviviridae family. To date, there is no effective antiviral drug available against most of these tick-borne viruses. Although there is common usage of antiviral drugs such as ribavirin for CCHF treatment in some countries, there are concerns that ribavirin may not be as effective as once thought against CCHF. Herein, we discuss also the availability of vaccines for the control of these viral infections. The lack of treatment and prevention approaches for these viruses is highlighted, and we hope that this review may increase public health awareness with regard to the threat posed by this group of viruses.

Contents

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Baicalin, a metabolite of baicalein with antiviral activity against dengue virus

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Baicalin, a flavonoid derived from *Scutellaria baicalensis*, is the main metabolite of baicalein released following administration in different animal models and human. We previously reported the antiviral activity of baicalein against dengue virus (DENV). Here, we examined the anti-DENV properties of baicalin *in vitro*, and described the inhibitory potentials of baicalin at different steps of DENV-2 (NGC strain) replication. Our in vitro antiviral experiments showed that baicalin inhibited virus replication at IC_{50} = 13.5 ± 0.08 μg/ml with SI = 21.5 following virus internalization by Vero cells. Baicalin exhibited virucidal activity against DENV-2 extracellular particles at IC_{50} = 8.74 ± 0.08 μg/ml and showed anti-adsorption effect with IC_{50} = 18.07 ± 0.2 μg/ml. Our findings showed that baicalin as the main metabolite of baicalein exerting *in vitro* anti-DENV activity. Further investigations on baicalein and baicalin to deduce its antiviral therapeutic effects are warranted.

Dengue virus (DENV) is an enveloped RNA virus belonging to the *Flaviviridae* family. There are four distinct serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. Dengue virus can cause a range of diseases from asymptomatic infection to mild dengue fever (DF) or severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[1,2]. DENV is transmitted principally in a cycle that involves humans and mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. All DENV serotypes are widespread geographically in tropical and subtropical regions of the world, causing life-threatening disease imposing considerable health and economic burden. Currently, there is no approved vaccine or antiviral agents against clinical dengue necessitating prompt strategies to design effective antiviral strategies against this infection.

In recent years, many investigators focus on plants and their derivatives to develop new antiviral drugs[3–5]. Some phytochemicals have been shown to have therapeutic applications against genetically and functionally diverse viruses[6,7]. Flavonoids are polyphenolic plant metabolites with numerous biological activities and low toxicity. More than 5000 natural flavonoids have been identified in plants or various dietary sources that are presumed to have potential health benefits[8]. Antiviral properties of flavonoids, a group of plant polyphenolics have been reported against different viruses including DENVs[9–21]. Recently, we showed that baicalein, a flavonoid belonging to the flavones subgroup (Figure 1A) exhibited significant antiviral effects against *in vitro* replication of DENV-2 in Vero cells, functioning at different stages of virus replication[9]. Baicalein (5,6,7-trihydroxyflavone) is a flavonoid originally isolated from *Scutellaria baicalensis*, a Chinese medicinal plant with various biological properties. Baicalin (5,6-dihydroxy-7-O-glucuronide flavone) is also a flavonoid (Figure 1B) presents in the roots of *S.baicalensis*. Baicalein is metabolized and converted mainly to baicalin following intake to animals and humans[22–26].

It has also been shown that ~90% of baicalein administered is metabolized to baicalin[17], and hence, it is necessary to investigate the role of baicalin in any particular medical condition.

Here, we determined the antiviral activities of baicalin at different stages of DENV replication in Vero cells and on DENV replicon cell line. We showed that baicalin interferes and inhibits DENV-2 *in vitro* replication at various stages of the virus replication cycle.

Results

Cytotoxic activity of baicalin. Cytotoxicity assay was performed to determine the non-toxic concentrations of baicalin against Vero cells using the MTS assay. We found that the half maximal cytotoxic concentration (CC_{50})

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RESEARCH Open Access

Seroprevalence screening for the West Nile virus in Malaysia's Orang Asli population

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Abstract

Background: West Nile virus (WNV) infection is an emerging zoonotic disease caused by an RNA virus of the genus *Flavivirus*. WNV is preserved in the environment through cyclic transmission, with mosquitoes, particularly *Culex* species, serving as a vector, birds as an amplifying host and humans and other mammals as dead-end hosts. To date, no studies have been carried out to determine the prevalence of the WNV antibody in Malaysia. The aim of this study was to screen for the seroprevalence of the WNV in Malaysia's Orang Asli population.

Methods: Serum samples of 742 Orang Asli were collected in seven states in peninsular Malaysia. The samples were assessed to determine the seroprevalence of WNV immunoglobulin (Ig)G with the WNV IgG enzyme-linked immunosorbent assay (ELISA) method. For each individual, we documented the demographic factors. Anti-dengue and anti-tick-borne encephalitis virus IgG ELISA were also performed to rule out a cross reaction. All statistical analyses were performed using the GraphPad Prism 6 (GraphPad Software, Inc.); p values of less than 0.05 were considered significant.

Results: The serosurvey included 298 men (40.16%) and 444 women (59.84%) of Malaysia's Orang Asli. Anti-WNV IgG was found in 9 of the 742 samples (1.21%). The seroprevalence was 0.67% (2 of 298) in men and 1.58% (7 of 444) in women. The presence of anti-WNV IgG was found not to be associated with gender but, however, did correlate with age. The peak seroprevalence was found to be 2.06% (2 of 97) in individuals between 30 to 42 years of age.

Conclusions: No previous studies have examined the seroprevalence of the WNV antibody in the human population in Malaysia, and no clinical reports of infections have been made. Screening for the WNV seroprevalence is very significant because of many risk factors contribute to the presence of WNV in Malaysia, such as the abundance of *Culex* mosquitoes as the main vector and a high degree of biodiversity, including migratory birds that serve as a reservoir to the virus.

Keywords: West Nile virus, *Culex* mosquitoes, Malaysia's Orang Asli, Seroprevalence, Migratory birds, Vector

Background

The WNV is a member of the virus family Flaviviridae, which belongs to the Japanese encephalitis virus (JEV) serogroup of flaviviruses and is closely associated with other human pathogens such as dengue virus (DENV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV) [1]. The flaviviruses are positive sense, single-stranded RNA viruses [1]. Murray Valley encephalitis viruses (MVEV), St. Louis encephalitis virus (SLEV) and Usutu virus (USUV) are also included in the JEV serogroup [1,2]. The WNV species also contains the Kunjin virus (KUNV) subtype that is endemic in Australia and Malaysia [3]. The flaviviruses of the JEV serocomplex are the prominent cause of arboviral encephalitis in vertebrate hosts, including humans [2].

Phylogenetic lineage studies show that approximately 1000 years ago, WNV emerged as a distinctive virus and had developed into two distinct lineages [4]. Lineage 1 was found to be the source of epidemic transmission in Africa and throughout the world, whereas lineage 2 was discovered in horses in sub-Saharan Africa and Madagascar [5]. The West Nile virus was first isolated in a woman in the West Nile district of Uganda in 1937 [6].

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BMC Public Health

RESEARCH ARTICLE Open Access

Seroprevalence report on tick-borne encephalitis virus and Crimean-Congo hemorrhagic fever virus among Malaysian's farm workers

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Abstract

Background: Tick-borne encephalitis virus (TBEV) and Crimean-Congo haemorrhagic fever virus (CCHFV) are important tick-borne viruses. Despite their wide geographical distribution and ease of acquisition, the prevalence of both viruses in Malaysia is still unknown. This study was conducted to determine the seroprevalence for TBEV and CCHFV among Malaysian farm workers as a high-risk group within the population.

Methods: We gave questionnaires to 209 farm workers and invited them to participate in the study. Eighty five agreed to do so. We then collected and tested sera for the presence of anti-TBEV IgG (Immunoglobulin G) and anti-CCHFV IgG using a commercial enzyme-linked immunosorbent assay (ELISA) kit. We also tested seroreactive samples against three other related flaviviruses: dengue virus (DENV), West Nile virus (WNV) and Japanese encephalitis virus (JEV) using the ELISA method.

Results: The preliminary results showed the presence of anti-TBEV IgG in 31 (36.5 %) of 85 sera. However, when testing all the anti-TBEV IgG positive sera against the other three antigenically related flaviviruses to exclude possible cross reactivity, only five (4.2 %) sera did not show any cross reactivity. Interestingly, most (70.97 %) seropositives subjects mentioned tick bite experience. However, there was no seroreactive sample for CCHFV.

Conclusions: These viruses migrate to neighbouring countries so they should be considered threats for the future, despite the low seroprevalence for TBEV and no serological evidence for CCHFV in this study. Therefore, further investigation involving a large number of human, animal and tick samples that might reveal the viruses' true prevalence is highly recommended.

Keywords: Tick borne viruses, TBEV, CCHFV, Seroprevalence, Farmer workers, Malaysia

Background

Ticks are important and prevalent vectors for several animal and human infectious diseases, carrying harmful pathogens such as Borrelia spp, Rickettsia spp, Babesia spp, and various viruses including TBEV and CCHFV. TBEV is a member of the genus Flavivirus within the Flaviviridae family. This etiologic agent of tick-borne encephalitis can cause a potentially fatal neurological infection affecting the human central nervous system. TBEV has three subtypes: European (TBEV-EU), Far Eastern (TBEV-Fe) and Siberian (TBEV-Sib) [1]. *Ixodes ricinus* is the main vector for TBEV-EU, while the other two subtypes are transmitted mainly by I.persulcatus [2]. The vector facilitates virus transmission to other vertebrates, which also act as a reservoir for the virus. Ixodes ticks acquire TBEV by feeding on viraemic animals, especially small rodents that serve as main vertebrate hosts and virus reservoirs [3]. The virus will be carried by infected ticks for life and maintained transstadially

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Antiviral activity of silymarin against chikungunya virus

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The mosquito-borne chikungunya virus (CHIKV) causes chikungunya fever, with clinical presentations such as severe back and small joint pain, and debilitating arthritis associated with crippling pains that persist for weeks and even years. Although there are several studies to evaluate the efficacy of drugs against CHIKV, the treatment for chikungunya fever is mainly symptom-based and no effective licensed vaccine or antiviral are available. Here, we investigated the antiviral activity of three types of flavonoids against CHIKV *in vitro* replication. Three compounds: silymarin, quercetin and kaempferol were evaluated for their *in vitro* antiviral activities against CHIKV using a CHIKV replicon cell line and clinical isolate of CHIKV of Central/East African genotype. A cytopathic effect inhibition assay was used to determine their activities on CHIKV viral replication and quantitative reverse transcription PCR was used to calculate virus yield. Antiviral activity of effective compound was further investigated by evaluation of CHIKV protein expression using western blotting for CHIKV nsP1, nsP3, E1 proteins. Briefly, silymarin exhibited significant antiviral activity against CHIKV, reducing both CHIKV replication efficiency and down-regulating production of viral proteins involved in replication. This study may have important consequence for broaden the chance of getting the effective antiviral for CHIKV infection.

Malaysia encompasses tropical rainforests and swamps, which ~~are the~~ a niche for several mosquito-borne viruses. However, due to massive urbanization and deforestation several of these viruses were easily introduced to our human population including chikungunya virus (CHIKV). CHIKV is a member of *Togaviridae* family (genus Alphavirus) and causes chikungunya fever in humans. It was first isolated from the dengue-like outbreak in Tanzania (East Africa)[1–3]. Apart from the usual clinical manifestations such as fever, headache, lymphadenitis and rashes, the most prominent complaint from infected person is the chronic pain and stiffness due to arthritis or swelling of small joints which remains for weeks or even years, as define in the Makonde language in which 'Chikungunya' means 'that which bends up,[4–6].

Recently CHIKV infections emerged in Kenya and then were recorded in Comoros during 2004 with 5000 cases being reported. La' Reunion in France (2005–2006), together with neighboring islands in the Indian Ocean, Seychelles, Madagascar, Mauritius and Mayotte; experienced an explosive outbreak with 300,000 cases and 237 deaths. CHIKV subsequently spread to India and Southeast Asia causing large-scale epidemics. In 2008, CHIKV was categorized as a Category C priority pathogen in the list of US National Institute of Allergy and Infectious Diseases (NIAID). To this date, more than 50 countries have been identified to be at risk[2,7].

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First report on the seroprevalence of the Crimean-Congo haemorrhagic fever virus, a tick-borne virus, in Malaysia's Orang Asli population

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Abstract. – OBJECTIVE: The Crimean-Congo haemorrhagic fever virus (CCHFV), which is transmitted by the ticks of *Hyalomma spp.* in general and *H. marginatumin* particular, can cause severe disease in humans, with mortality rates of 3-30%. Other than from the bites of infected ticks, CCHFV can also be transmitted through contact with patients with the acute phase of infection or contact with blood or tissues from viraemic livestock. Outbreaks of human cases of haemorrhagic manifestations have been documented since 1945 and described in parts of Africa, Asia, Eastern Europe and the Middle East and most recently India in 2011. In addition, serological evidence of the disease has been reported in some countries where no human cases were reported. As regional neighbours China and India have been affected by this virus, this study was conducted to determine the seroprevalence of CCHFV among Orang Asli population of Malaysia as the most at risk people who residing in the deep forests.

PATIENTS AND METHODS: A total of 682 serum samples were collected from the Orang Asli population residing in eight states in peninsular Malaysia and analysed for the presence of anti-CCHFV immunoglobulin G (IgG) using a commercial enzyme-linked immunosorbent assay kit.

RESULTS: The study subjects comprised 277 (40.6%) men and 405 (59.4%) women. However, anti-CCHFV IgG was detected in only one female serum sample (0.1%). The presence of anti-CCHFV IgG could not be correlated to age or sex from these findings.

CONCLUSIONS: The results of this screening survey showed that the seroprevalence of the anti-CCHFV IgG among Malaysia's Orang Asli population is too low for detection or totally negative compared with that in neighbouring countries, such as India and China.

Key Words:
Tick-borne virus, Crimean-Congo hemorrhagic fever virus, Seroprevalence, Orang Asli, Malaysia.

Introduction

The Crimean-Congo haemorrhagic fever virus (CCHFV) was first characterised when severe haemorrhagic fever occurred in Crimea during 1944. In 1969, an antigenically identical virus was recognised after being isolated from a febrile patient in the Congo, from which the virus received the nomenclature by which it is known today. CCHFV is maintained in nature by the family of Ixodidae (hard ticks), in particular *Hyalomma spp.*, which acts as both reservoir and vector. Ticks of other genera such as *Rhipicephalus*, *Boophilus* and *Dermacentor* have also been found to contribute to the ecological cycle of the virus, which is passed through trans-stadial and trans-ovarial transmission within the vector population.

A wide range of wild and domestic mammals are known to be the amplifying hosts, and the major concern is livestock that live closer proximity to human populations. In addition to vector-to-human transmission from infected tick bites and crushing the infected ticks, human-to-human transmission can occur through direct contact with infectious animal blood or body fluids and also by drinking unpasteurised milk from infected animals. Nosocomial outbreaks have mostly occurred through unprotected contact with highly infectious blood or body fluids from patients during the acute phase of infection as

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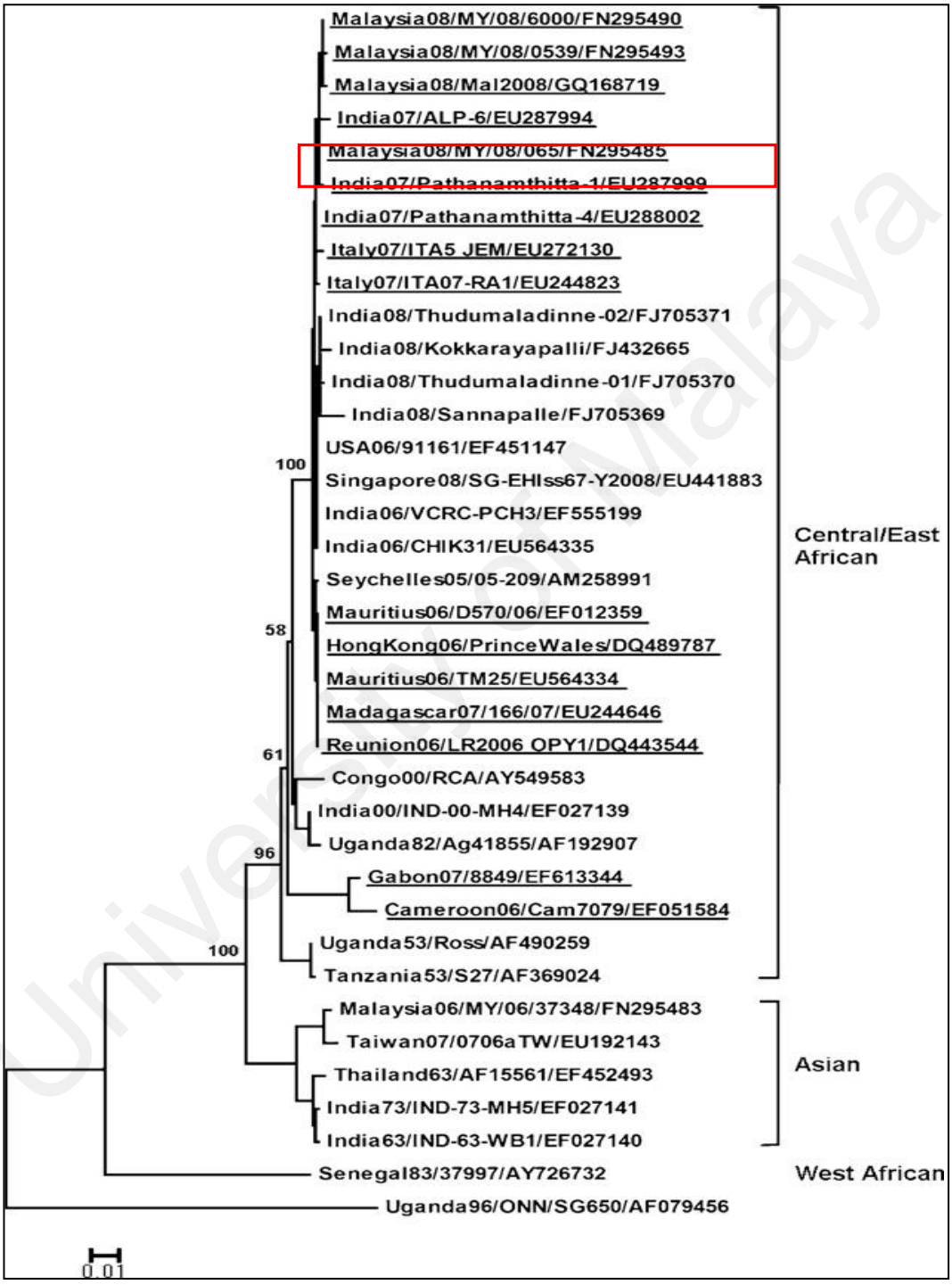
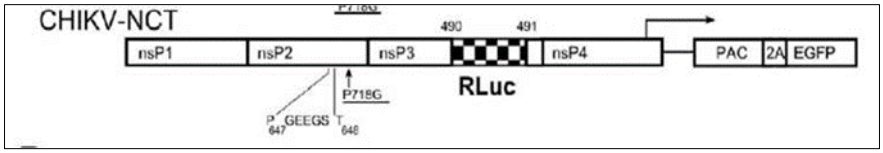
APPENDICES

APPENDIX A



Reproduce from Sam *et al.*, 2009. **The phylogenetic analysis of 837bp partial E1 CHIKV sequences.**

APPENDIX B



Reproduce from Pohjala *et al*., 2011. **Schematic representation of the used CHIKV replicon.**