IN VITRO ACTIVITY OF LOCAL PLANTS FROM MALAYSIA AGAINST CHIKUNGUNYA VIRUS

By

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ABSTRACT

IN VITRO ACTIVITY OF LOCAL PLANTS FROM MALAYSIA AGAINST CHIKUNGUNYA VIRUS

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Chikungunya, is an acute febrile illness that has been identified in over 60 countries worldwide, and is associated with severe debilitating athralgias and rash. It is caused by Chikungunya virus (CHIKV), an arbovirus that is transmitted through the bite of an infected *Aedes* mosquito. So far there are no commercially available vaccines or antiviral drugs for the prevention and treatment. This study was conducted to investigate the activity of local plants namely *Ipomoea aquatica, Persicaria odorata, Rhapis excelsa, Rhoeo spathacea* and *Vernonia amygdalina* better known as 'kang kung', 'daun kesum', lady palm, boat lily and 'daun bismillah' respectively against CHIKV. The leave or aerial parts of the plants were selected and a total of 30 crude extracts were tested against the virus infected African green monkey kidney (Vero) cells in triplicates. Cytotoxic test was performed using the neutral red uptake (NRU) assay to determine the half-maximal cytotoxic concentration (CC_{50}) and the maximal non-cytotoxic concentration (MNCC) of the various plant extracts as a prerequisite for the antiviral assay. Based on the NRU assay, the highest cytotoxicity was found in the ethanol extract of *Rhapis excelsa* (CC_{50} = 51.67 ± 2.89 µg/mL), followed by ethyl acetate extract of *Vernonia amygdalina* (CC_{50} = 86.87 ± 2.89 µg/mL) and methanol extract of *Rhapis*

excelsa (CC_{50} = 91.67 ± 2.89 µg/mL). For the post-inoculation antiviral assay two-fold serial dilutions of each extracts were prepared from the MNCC. All tested extracts did not achieve the half-maximal effective concentration (EC_{50}). However, potential results were obtained from the water and hexane extracts of *Vernonia amygdalina* at the concentrations of 20 and 80 µg/mL. This study showed that all tested extracts had an activity potential below EC_{50}, and among them the water and hexane extracts of *Vernonia amygdalina* demonstrated potential antiviral activity on CHIKV. Further studies should be conducted to identify the plant bioactive compounds, phytochemistry as well as to quantify the viral load after the treatment.

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I thank Universiti Tunku Abdul Rahman through the Department of Biomedical Science for providing us the exposure of conducting and presenting our very own project. Lastly, I thank the Almighty God for giving me good health and allowing me to complete this work.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

ARVIND DEVAR RAMACHENDRIN

APPROVAL SHEET

This project report entitled "***IN VITRO* ACTIVITY OF LOCAL PLANTS FROM MALAYSIA AGAINST CHIKUNGUNYA VIRUS (CHIKV)"** was prepared by ARVIND DEVAR RAMACHENDRIN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by

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Date:_____

PERMISSION SHEET

It is hereby certified that **ARVIND DEVAR RAMACHENDRIN** (ID No: **13ADB00584**) has completed this final year project entitled **"*IN VITRO* ACTIVITY OF LOCAL PLANTS FROM MALAYSIA AGAINST CHIKUNGUNYA VIRUS"** supervised by Dr. SIT NAM WENG from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(ARVIND DEVAR RAMACHENDRIN)

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	Analysis of variance
CC_{50}	Half-maximal cytotoxic concentration
CHIKV	Chikungunya virus
CO_2	Carbon dioxide
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECSA	East-Central-South African
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
E1	Virus envelope 1
E2	Virus envelope 2
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IFN γ	Interferon gamma
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
LDH	Lactate dehydrogenase
MNCC	Maximal non-cytotoxic concentration
MOI	Multiplicity of infection

NHP	Non-human primate
NRU	Neutral red uptake
nSP	Non-structural protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
SD	Standard deviation
RT-PCR	Reverse transcription polymerase chain reaction
$TCID_{50}$	Tissue Culture Infectious Dose, 50%.
UTR	Untranslated region
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Chikungunya is a disease of viral origin that is characterised as a debilitating viral fever (Mudurangaplar, 2015), the etiological agent behind the disease is an alphavirus that is transmitted primarily to humans through the bite of an infected female *Aedes* mosquito (Raquin et al., 2015). The virus, Chikungunya (CHIKV) since its first isolation back in the year 1952 in Tanzania (LaBeaud et al., 2015) had caused massive outbreaks and is currently identified in over 60 countries and territories worldwide (Staples, Breiman and Powers, 2009). In India alone, it was approximated more than 1.4 million of its population would have been affected between the years of 2006 to 2008 (Weaver, 2014). Meanwhile, in Southeast Asia, the number of reported cases went up to an estimated 1.9 million patients from the year 2005 (Coffey, Failloux and Weaver, 2014). Following the autochronous transmission of the virus to the Western hemisphere, more than 1.2 million probable cases have been reported in the year 2014 (CDC, 2015; Javelle et al., 2015).

Notably in Malaysia it had caused several outbreaks following the 2005 epidemic in the Indian Ocean islands. A total of 6 314 confirmed cases were reported from the year 2006 to 2009 throughout the country with most incidence coming from Johor state contributing 43.6% to the total cases reported (Chua, 2010; Azami et al., 2013). The outbreak in Kenya in the year 2004 initiated the resurgence of the virus (Robinson et al., 2014) and during

this period the viral strain of East/Central/South African (ECSA) lineage was reported to have undergone a mutation on its E1 envelope glycoprotein (Ala-226-Val) that resulted in the transition of its principal transmission vector *Aedes aegypti* to *Aedes albopictus* and an efficient transmission of the disease to humans (Lee and Chu, 2015; Yoon et al., 2015). In spite of the increase in global incidence, to date there are no licensed vaccines or therapeutics available for its treatment and prophylaxis (Raquin et al., 2015). The therapy given is generally symptomatic with bed rest, rehydration and administration of analgesics (Hrnjakovic-Cvjetkovic et al., 2015).

Plant natural products, since the primordial days were used by all global traditions as the principal source of medicine. Presently, it is notable that around 80-85% of the world population depends on traditional herbal therapy for their health benefits and requirements (Rasingam, 2012; Prakash et al., 2013). Extracts from plants are an essential source of novel pharmacologically active products with many drugs being continuously acquired both directly and indirectly from plant sources (Veeresham, 2012). According to a study conducted in the year 2007 by Newman and Cragg, more than 44% of antivirals approved between the years of 1981 to 2006 were derived based on natural-product pharmacophores.

The inhibitory effects of medicinal plants extracts on the replication of viruses were reported in the past six decades (Liu and Du, 2012). Plant extracts have been shown to possess antiviral potential against viral strains that were resistant to conventional antiviral agents (Serkedjieva and Hay, 1998; Tolo et

al., 2006). The antiviral activities of natural products, including ingredients, fractions and extracts, has to be evaluated by various antiviral models, including *in vitro* and *in vivo* models (Liu and Du, 2012). With the advent of new and powerful screening assays and prediction tools, the idea of a drug to efficiently treat viral infections by blocking specific host functions has re-bloomed (Martinez et al., 2015).

This study which is a part of a screening project, was aimed to investigate the antiviral activity of extracts from five local plants namely *Ipomoea aquatica, Persicaria odorata, Rhapis excelsa, Rhoeo spathacea* and *Vernonia amygdalina.* A total of thirty plant extracts were obtained and examined for activity against CHIKV.

Therefore the objectives of this project are:

I) To perform cytotoxicity assay of the various plant extracts against Vero cell line in order to calculate the half-maximal cytotoxic concentration (CC_{50}) and the maximal non-cytotoxic concentration (MNCC) as a pre-requisite for the post-inoculation antiviral assay.

II) To analyse the antiviral activity of the prepared plant extracts against CHIKV using the post-inoculation antiviral assay and to calculate half-maximal effective concentrations (EC_{50}).

CHAPTER 2

LITERATURE REVIEW

2.1 Chikungunya Disease

Chikungunya is an emerging viral disease, which is often associated with acute febrile and sometimes eruptive polyarthritis (Javelle et al., 2015). The causative agent, Chikungunya virus (CHIKV) is an arbovirus that spreads through the bite of an infected *Aedes* mosquito (Aubry et al., 2015). The name 'Chikungunya' originates from the Swahili language which refers to the stooped posture developed as a result of the arthritic symptoms of the disease (Mudurangaplar, 2015). The disease has been reported in over 60 countries mainly from parts of Africa, Asia and recently from some parts of Europe (Hrnjakovic-Cvjetkovic et al., 2015; Khan et al., 2015).

2.1.1 History and Epidemiological Features

CHIKV was first isolated in Africa during an outbreak in the year 1952 from the Mokande Plateau of Tanzania (Stamm, 2015). In Asia, the disease was first recorded in the year 1958 following the isolation from Bangkok, Thailand (Moyen et al., 2014). Since then, the Asian genotype of CHIKV continued to cause several major outbreaks such as the outbreak that took place in South Asia from the year 1963 and 1964 in the city of Kolkata and Solapur, India (Mudurangaplar, 2015). Concurrently, the virus continued to be active in Thailand (Lam et al., 2001) and until then it had disappeared for three decades. Following the year 2004 the virus re-emerged causing an outbreak in Kenya

leading to the massive widespread of the disease in the Indian Ocean islands particularly in the French islands of Mayotte and La Re´union (Moyen et al., 2014; Robinson et al., 2014) with more than 1,400 000 to 6, 500 000 cases recorded in India and 300 000 cases in the island of La Re´union in the year 2006 (Hrnjakovic-Cvjetkovic et al., 2015). During this outbreak, the virus seems to have acquired mutations in glycoprotein E1, which is important for membrane fusion and virion assembly (Hamer and Chen, 2014). Travelers from India to Europe introduced chikungunya, resulting in local transmission in France and Italy with 207 confirmed cases reported from the year 2007-2010. However, in the year 2013, through autochronous transmission, CHIKV was reported in over 44 countries of the North, Central and South American countries and the viral strain responsible for the growing epidemic was reported to be the Asian genotype (Weaver, 2014).

In Malaysia, Chikungunya was first recorded in Port Klang between the year 1998 and 1999 (Lam et al., 2001; AbuBakar et al., 2007). Following the outbreak, the virus was silent for seven years till the next outbreak which took place in the coastal village of Bangan Panchor, Perak with a population of 200 villagers affected (AbuBakar et al., 2007), the third outbreak took place in Ipoh, Perak on the same year (Azami et al., 2013). Both of these outbreaks occurred synchronously with the ongoing epidemic then in the Indian Ocean islands (Paganin et al., 2006) however it was caused by the Asian genotype rather than the ECSA Indian Ocean lineage (Weaver, 2014). Later in the year 2008, fourth outbreak was reported from Johor state which then spread to other territories of Malaysia (Sam et al., 2009; Rozilawati et al., 2015). From then

there was a nationwide outbreak resulted in 10 000 cases, with no fatalities reported (Ministry of Health, Malaysia, 2010).

2.1.2 Chikungunya Virus (CHIKV)

2.1.2.1 Classification and Phylogenesis

CHIKV is a 12 kb positive sense single stranded RNA virus belonging to the genus alphavirus from the togaviridae family (Pun, Bastola and Shah, 2014; Yoon et al., 2015). It belongs to the antigenic complex IV (Semliki forest serocomplex) subgroup of the genus alphavirus (Pialoux et al., 2007; Hrnjakovic-Cvjetkovic et al., 2015). The phylogenetic study of CHIKV strains by Powers et al. (2000), identified three variant strains namely the West African (WAf), East/Central/South African (ECSA), and Asian genotypes (Volk et al., 2010; Vega-Rúa et al., 2015). Figure 2.1 illustrates the distribution of the three CHIKV strains.

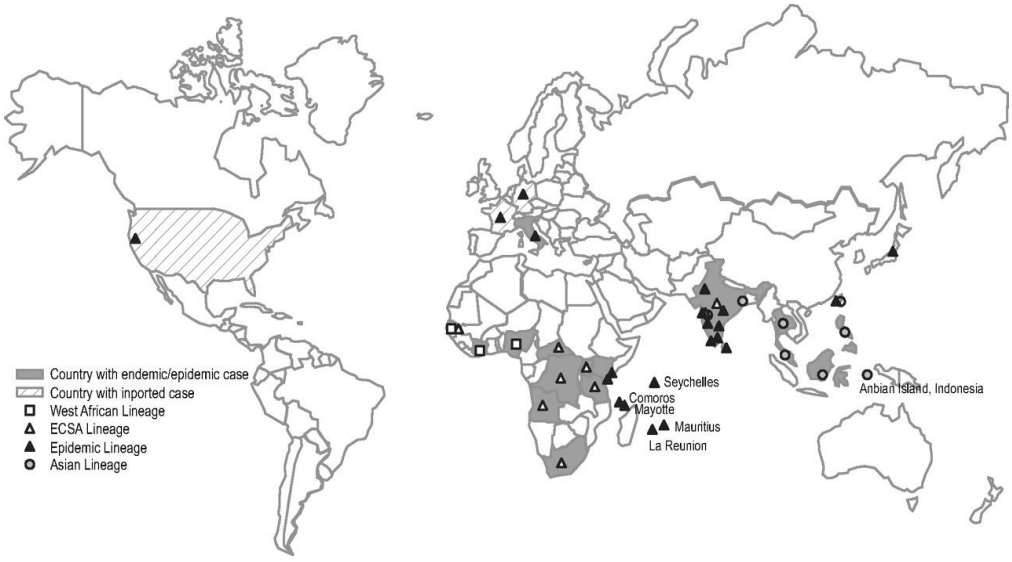


Figure 2.1: Global distribution of the three CHIKV strains (Volk et al., 2010).

2.1.3.2 Morphology and Genome Structure

CHIKV as shown in Figure 2.2, is a spherical-enveloped virus with a diameter of 60 to 70 nm that consists of its major structural proteins Capsid, E1 and E2 (Lim and Chu, 2014) and its viral genome shown in Figure 2.3 has two ORFs: structural and nonstructural that begins with a UTR at the 5' terminal end and followed by the coding regions for the nonstructural proteins (nsP1 to 4) which occupy two-third of its genome. Subsequently, the remaining one-third of the coding region encodes for the structural protein Capsid-E3-E2-6K-E, and a 3'-terminal poly-A-tail (Hussain and Chu, 2011). The nsPs are responsible for the different roles in viral replication where nsP1 is involved in viral RNA synthesis initiation and RNA capping (Ahola et al., 1997). The nsP2 possesses protease and RNA helicase activities (Gomez de Cedrón et al., 1999; Frolova et al., 2006) meanwhile the nsP3, composed of three domains, is required for the formation and localization of replication complexes. Lastly, nsP4 functions to possess RNA-dependent RNA polymerase (RdRp) activity which is important for replication and synthesis of the viral genome (Frolova et al., 2006; Tomar et al., 2006).

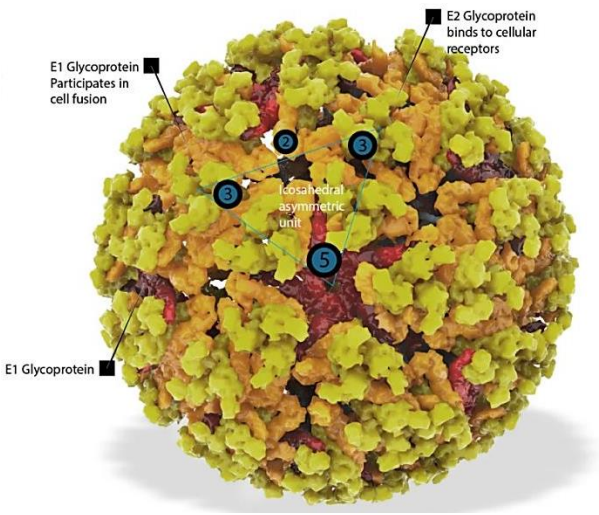


Figure 2.2: Illustration of CHIKV virion (3DCIENCIA.com, 2015).

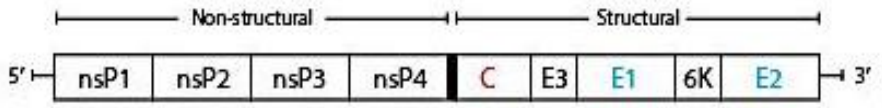


Figure 2.3: CHIKV genome organization (3DCIENCIA.com, 2015).

2.1.2.3 Viral Replication

The transmission of CHIKV requires infection of a female mosquito through viremic blood meal and following a suitable extrinsic incubation period, transmission to another vertebrate host during subsequent feeding (Solignat et al., 2009). Upon transmission, virus entry is mediated through the clathrin-mediated endocytosis as soon as the virus binds to its receptors on the host cells (Kielian, Chanel-Vos and Liao, 2010). The viral particle then undergoes disassembly and releases its genomic RNA into the cytosol of the infected cell. The viral genome is then translated into structural and non-structural proteins (Glanville et al., 1976) and undergoes cleavage of its non-structural protein, during the process of cleavage; it forms the P123 and nsP4 (De Groot et al., 1990; Takkinen, Peranen and Kaariainen, 1991) peptides that causes the synthesis of negative strand RNA (Strauss and Strauss, 1994) by forming an unstable initiation complex. Soon after the cleavage of the non-structural proteins into nsP 1-4, the virus switches its synthesis of the negative-strand RNA to genomic and sub-genomic positive strand RNA synthesis (Iemm et al., 1994; Shirako, Strauss and Strauss, 2000). Upon the availability of C protein, it associates with the newly synthesized RNA and recognizes the specific signals for packaging in the 5' half of the genome, so that only RNA of full length is packaged (Owen and Kuhn, 1996; Weiss et al., 2015). The synthesized E2 and E3 glycoprotein interacts with each other and forms heterodimers which is then

transported to the cell surface via the Golgi complex from the endoplasmic reticulum. The cleavage of the precursor protein PE2 to generate mature E2 and E3 proteins causes conformational change and weakening of the interaction within the heterodimers (Wahlberg, Boere and Garoff, 1989) subsequently resulting in the priming of the fusion peptide for activation upon the exposure to a low pH. Through the interactions between C protein and the cytoplasmic domain of the E2 protein the budding process is initiated, with E1-E2 heterodimers forming an envelope around nucleocapsid-like particles (Ziemiecki, Garoff and Simons, 1980; de Curtis and Simons, 1988; Sariola, Saraste and Kuismanen, 1995). The virons acquire a phospholipid bilayed derived from the host cell membrane upon release from the host cell (Laine, Soderlund and Renkonen, 1973; Vogel et al., 1986; Fuller, 1987; Leung, Ng and Chu, 2011).

2.1.2.4 Antiviral Susceptibility

To date there are no therapeutics or vaccines available against CHIKV, however in the experimental conditions there are several chemical compounds as shown in Table 2.1 that can potentially minimize CHIKV activity in the biological condition (de Lamballerie, Ninove and Charrel, 2009; Kaur and Chu, 2013).

Table 2.1: Specific chemical compounds tested against CHIKV.

Chemical compound	Possible mode of action
Chloroquine	Disrupts endosome-mediated CHIKV internalization, possibly through the prevention of endosomal acidification.

(Parashar and Cherian, 2014)

Table 2.1(continued): Specific chemical compounds tested against CHIKV.

Chemical compound	Possible mode of action
Ribavirin	Can interact with the intracellular viral RNA production.
6-Azauridine	Inhibition of orotidine monophosphate decarboxylase, an enzyme involved in the de novo biosynthesis of pyrimidine, cytidine, and thymidine.
Arbidol	Inhibition of virus mediated fusion and blocking of the viral entry into the target cells through inhibition of glycoprotein conformational changes that are essential for the fusion process.
Harringtonine	Affects CHIKV RNA production inside the infected cell as well as viral protein expression such as the nsP3 and the E2 proteins.

(Parashar and Cherian, 2014)

2.1.3 Vector and Natural Reservoir

The strains from different geographical distributions relatively circulate in dissimilar ecological cycles. The strain in the African tropics exist in an enzootic cycle primarily between mosquitoes in the forest and non-human primates (NHP) which serves as the principle reservoir and amplication hosts in the cycle. Meanwhile in Asia and other continents affected, transmission primarily exists from the infected mosquitoe to humans (Eldridge and Edman, 2004; Weaver et al., 2012; Coffey, Failloux and Weaver, 2014). However, study conducted by Apandi et al. (2009) reported the possibility of the existence of such an Asian enzootic cycle. There are several mosquito species that can transmit CHIKV to humans; however the primary vectors in the large

human outbreaks were from the genus *Aedes* particularly *Ae.(Stegomyia) albopictus* and *Ae.(Stegomyia) aegypti* (Weaver et al., 2012; Parashar and Cherian, 2014; Tretyakova et al., 2014). These two urban species are globally spread and have drastically increased the incidence of viral spread to new regions where the environmental conditions were permissible for transmission (Vega-Rúa et al., 2015). Meanwhile, the 2005 to 2006 La Re´union outbreak, suggested the possibility of transmission vertically from an infected pregnant mother to her child, and the transmission is most likely to occur short before delivery (Thiboutot et al., 2010).

2.1.4 Pathogenesis

Following bite of an infected *Aedes* mosquito on the skin, CHIKV travels through the bloodstream and disseminates in the liver, muscles, lymph nodes and spleen where primary replication takes place (Schwartz and Albert, 2010; Miranda, Oliveira and Poian, 2013). As displayed on Figure 2.4 the incubation period for CHIKV is 2-4 days and is followed by the onset of clinical phases with no prodromal period. It is during this stage, infected individuals experience various life-debilitating symptoms which includes severe incapacitating joint pain. The acute phase of infection normally lasts days to weeks, and severity of this infection is solely based on the viral load (Roques and Gras, 2010). The onset of disease coincides with increasing virus titer and results in the activation of innate immune response with the characteristic production of type I interferons (IFN's) and pro-inflammatory cytokines and chemokines which includes IFN γ inducible protein 10, monocyte chemoattractant protein, and IL 8 are elevated (Sebastian, Lodha and Kabra,

2009). Eventually, the viral load will be cleared in a week time after the onset of infection and only during this period the cell-mediated immunity towards the virus is evident. However, some patients may experience a sequellae of long duration with athralgia and sometimes arthritis (Schwartz and Albert, 2010).

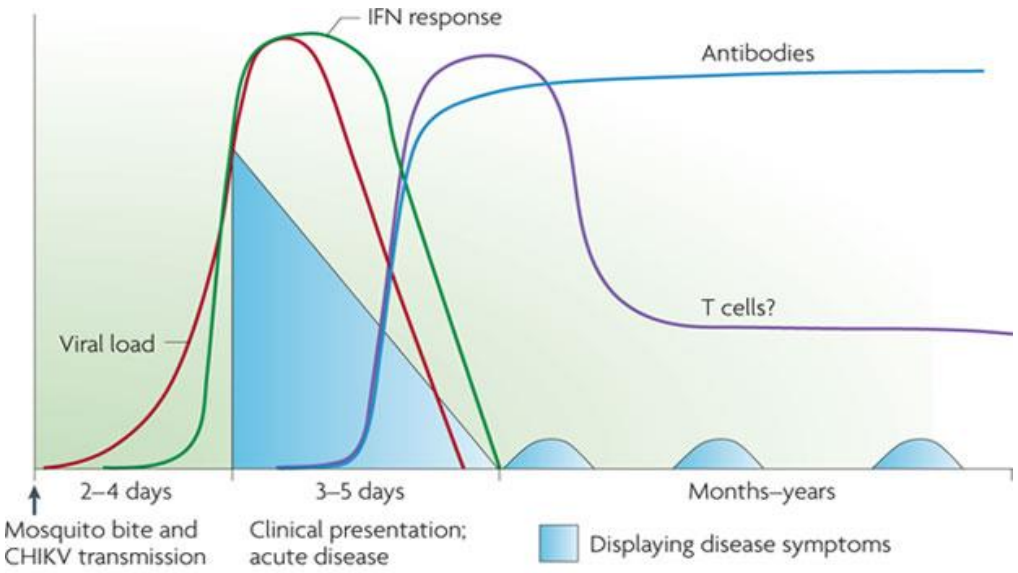


Figure 2.4: Pathogenesis of Chikungunya disease (Schwartz and Albert, 2010).

2.1.5 Clinical Features

The clinical manifestation of Chikungunya disease begins abruptly with fever headache, back pains, myalgia and arthralgia. Common sites of pain include the ankles, wrists and interphalangeal joints of hand (Hrnjakovic-Cvjetkovic et al., 2015). Other presentations reported consist of retro-orbital pain, rash and vomiting (Sissoko et al., 2010).

2.1.6 Diagnosis

The gold standard for detection of CHIKV disease is through virus culture and isolation (Pialoux et al., 2007; Powers and Logue, 2007; Chevillon et al., 2008; Simon, Savini and Parola, 2008; Sudeep and Parashar, 2008); which requires the collection of patient's blood samples that will be subjected to PCR for further downstream analyses (WHO, 2015). However due to its limited availability, other diagnostic methods are exercised. The standard diagnostic procedures recommended by the World Health Organization (WHO), other than virus isolation is serological testing. The serological test, Enzyme-linked immunosorbent assay (ELISA) is widely available and is relatively economical (Dash, Mohanty and Padhi, 2011). The principle is based on the detection of IgM and IgG antibodies against the CHIKV antigens. CHIK IgM antibodies become detectable during the fifth day of infection and remain the highest in patient's sera for 2-3 months period of illness (Pialoux et al., 2007; Hrnjakovic-Cvjetkovic et al., 2015). Meanwhile, the IgG antibodies are only evident during the 10^{th} -14^{th} day of disease (Dash, Mohanty and Padhi, 2011). Other diagnostic procedures that are currently used include indirect immunofluorescent, hemagglutination inhibition, neutralization techniques or genotyping (Lakshmi et al., 2008; WHO, 2009; Hrnjakovic-Cvjetkovic et al., 2015). Table 2.2 demonstrates the criteria's for a successful diagnosis of Chikungunya disease.

Table 2.2: Diagnostic criterias for Chikungunya Disease.

Diagnostic Criteria for CHIK Disease
Suspected case
A patient presenting with acute onset of fever usually with chills/rigors, which lasts for 3-5 days with multiple joint pains/swelling of extremities that may continue for weeks to months
Probable case
A suspected case (see above) with any one of the following:
History of travel or residence in areas reporting outbreaks
Ability to exclude malaria, dengue and any other known cause for fever with joint pains
Confirmed case
Any patient who meets one or more of the following findings irrespective of the clinical presentation
Virus isolation in cell culture or animal inoculations from acute phase sera
Presence of viral RNA in acute phase sera by RT-PCR
Presence of virus-specific IgM antibodies in single serum sample in acute or convalescent stage
Fourfold increase in virus-specific IgG antibody titer in samples collected at least three weeks apart

Footnote: RNA: Ribonucleic acid; RT-PCR: Reverse transcription polymerase chain reaction; IgM: Immunoglobulin M; IgG: Immunoglobulin G.

(Mohan et al., 2010)

2.1.7 Treatment and Prevention

The absence of therapeutics for the treatment of Chikungunya disease (WHO, 2015; Yoon et al., 2015) poses a challenge for physicians in identifying and administering the optimal treatment to prevent the progression and perpetuation of the infection into a possibly life-decapitating course of disease (Javelle et al., 2015).

Following the outbreak in the Re´union Island, analgesic drugs were the most preferred option in alleviating the debilitating arthralgia's associated with the infection, among these a combination of paracetamol and nonsteroidal anti-inflammatory drugs (NSAID's) was a regular choice. Corticosteroids were prescribed to some patients with incapacitating forms of the disease (Michault and Staikowsky, 2009). Prevention of disease transmission is mainly focused on the eradication of mosquito breeding sites and mobilization of those affected (Hamer and Chen, 2014; WHO, 2015) to prevent the transmission of disease to other healthy counterparts.

2.2 Plant of the Study: *Ipomoea aquatica*

2.2.1 Description

Ipomoea aquatic, shown in Figure 2.5 is also known as water spinach, swamp morning glory (USDA, 2015) or simply 'kangkung' in the Malay language. It is an aquatic plant and an important food crop belonging to the family Convolvulaceae (Austin, 2007) and is thought to have originated in China where it is consumed as a green leafy vegetable (Alkiyumi et al., 2012). The plant is a perennial herb that is widely distributed throughout Africa, Australia, South and Southeast Asia (Manvar and Desai, 2013) growing abundantly in muddy stream banks, freshwater pond and lakes (Hamid et al., 2011). *Ipomoea aquatica* is a vine that trails and creeps, moreover it has the ability to climb, overtop and twine around other plants. Its stems are usually thick and spongy, and rooting occurs at the nodes (Ogunwenmo and Oyelana, 2009). The leaves are 5-12.5 cm long and 3.2-7.5 cm broad, acute, cordate or hastate with rounded or acute lobes. Meanwhile, its flowers are infundibuliform, solitary

and consists of five free sepals, five united pale purple petals, unequal five stamens with spiny pollens, and two-celled glabrous ovary with two ovules in each cell. The fruits are capsuler with 1-4 seeds, 8 mm long capsules, ovoid, and minutely pubescent (Manvar and Desai, 2013).

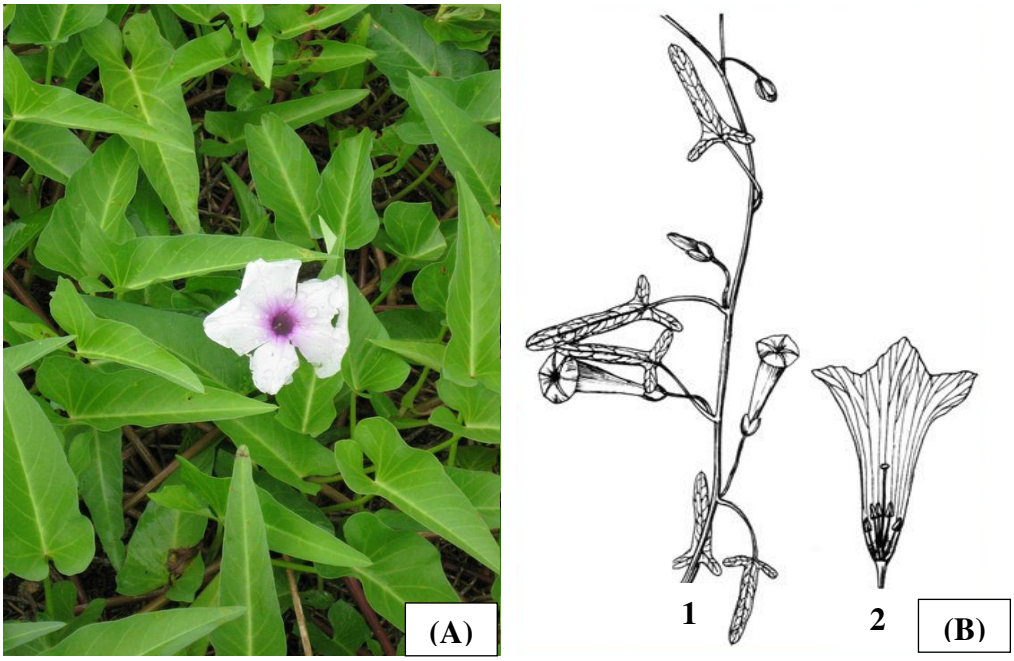


Figure 2.5: *Ipomoea aquatica*
(A) Leaves and flower (Medicalhealthguide.com, 2015).
(B) Branch[1] and disected flower[2] (Datta and Saha, 1974).

2.2.2 Chemical Constituent and Medicinal Uses

Phytochemical studies of the plant reported the presence of carotenes such as cryptoxanthin, lutein, lutein epoxide, violoxanthin and neoxanthin (Tee and Lim, 1991), flavonoids such as mycertin, quercetin, luteolin and apigenin (Daniel, 1989) and some alkaloids (Tofern et al., 1999). In addition, studies on the leaf parts of the plant was revealed the presence of adequate quantities of essential amino acids such as aspartic acid, glycine, alanine and leucine (Hamid et al., 2011). The plant is traditionally used in the treatment of nervous and general debility, piles, worm infections, leucoderma, leprosy, jaundice and

liver disorders (Alkiyumi et al., 2012). Furthermore, its leaf extracts are used to reduce blood sugar levels and as an antibiotic against *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* infections (Hamid et al., 2011).

2.3 Plant of the Study: *Persicaria odorata*

2.3.1 Description

The plant *Persicaria odorata*, synonymously known as *Polygonum odoratum* is a herb that has its origin in the tropics of Southeast Asia. It is a rampant member of the Polygonaceae families that are collectively named as smartweeds or pinkweeds, and is popularly known as 'Daun Kesum', laksa leaf or Vietnamese coriander among the locals (Seidemann, 2005; Starkenmann et al., 2006). As displayed in Figure 2.6, the plant is an evergreen stoloniferous soft-wooded perennial herb that grows best to a height of 30-35 cm (Orr, 2014) in the tropical and subtropical zones (Ridzuan, 2013). It has a characteristic pointed leaves with dark purple distinctive features on the centre, its top is dark green in colour and its bottom is in burgundy red, besides the plant's stem is joined at the leaf. The flowers are white and normally flowers during the periods of early to late summer. The plant is routinely used in cooking where the leaves of the plant are added into local dishes (Sasongko, Laohankunjit and Kerdchoechuen, 2011) mainly for its peppery and mint flavor as a replacement for the regular cilantro (Saad et al., 2014). The herb is usually propagated by cutting the parts of the plant, and the roots are developed simply from its nodes.

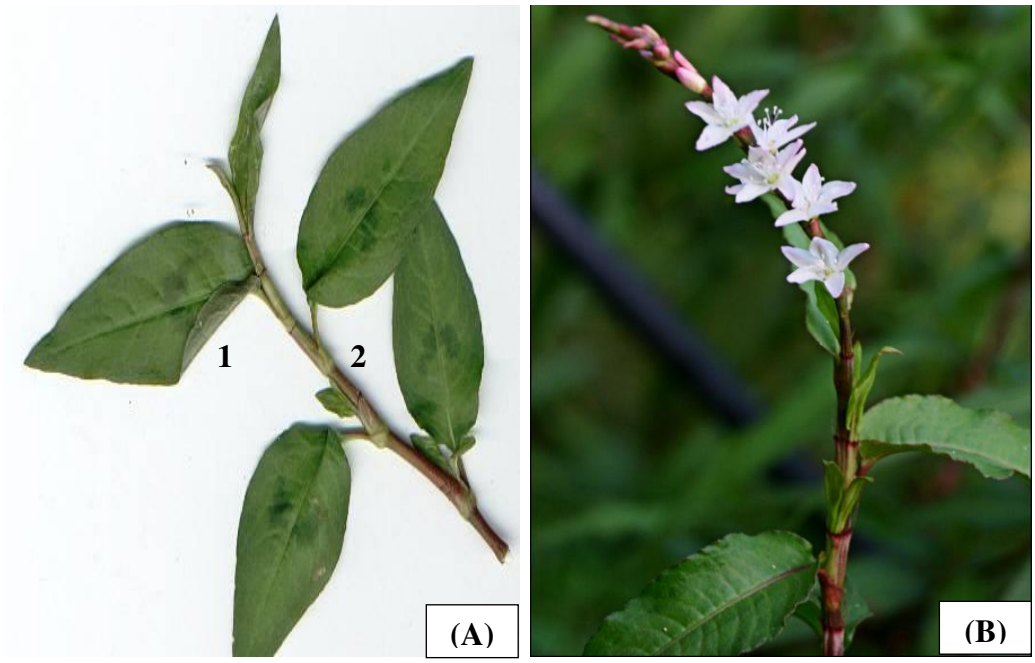


Figure 2.6: *Persicaria odorata*
(A) Leaf[1] and stem[2] (Saad et al., 2014).
(B) Flowers, leaves and stem (Daves, 2015).

2.3.2 Chemical Constituent and Medicinal Uses

The aerial parts of the plant were reported to contain various volatile compounds such as aldehydes, terpenes, and sesquiterpenes (Sasongko, Laohankunjit and Kerdchoechuen, 2011; Ridzuan, 2013). Among the aldehydes, (Z)-3-hexenal, (Z)-3-hexenol, 3-sulfanyl-hexanal, 3-sulfanyl-hexan-1-ol, decanal, undecanal, and dodecanal were most prevalently reported through Gas Chromatography analyses (Starkenmann et al., 2006; Quynh et al., 2009). Sesquiterpene lactones are part of a larger family of bioactive compounds that are present in the oils of the plant (Neerman, 2003). Studies on folk medicine reveal that sesquiterpene lactones were widely used as a form of treatment for various ailments including diarrhea, burns, influenza and neurodegeneration (Chadwick et al., 2013). Several other phytochemical studies reported the presence of defensins in the leaf extracts, defensins were said to be toxic to parasites, bacteria and fungus in a response to protect itself

from infections (Saad et al., 2014). The plant is widely regarded to have a variety of medicinal properties especially in managing fever and coughs, reducing thirst, application in stomach and lung injuries, diabetes mellitus and as a anti-inflammatory, insect antifeedant and antimicrobial activities (Shavandi, Haddadian and Ismail, 2015).

2.4 Plant of the Study: *Rhapis excelsa*

2.4.1 Description

Rhapis excelsa, shown in Figure 2.7 is commonly known as lady palm, broadleaf lady palm or raffia palm (da Luz, de Oliveira Paiva and Tavares, 2008). It is a garden and indoor ornamental plant from the Arecaceae family and is distributed from southern China to Southeast Asia (Dransfield and Uhl, 1998). *Rhapis excelsa* grows up to 4 m in height and 30 mm in diameter in multi-stemmed clumps with glossy leaves. It can be cultivated in a variety of soils and is represented as small under-growth palms of the tropical rainforest (Uhl and Dransfield 1998; Hastings 2003; Averyanov, Nguyen and Phan, 2006).

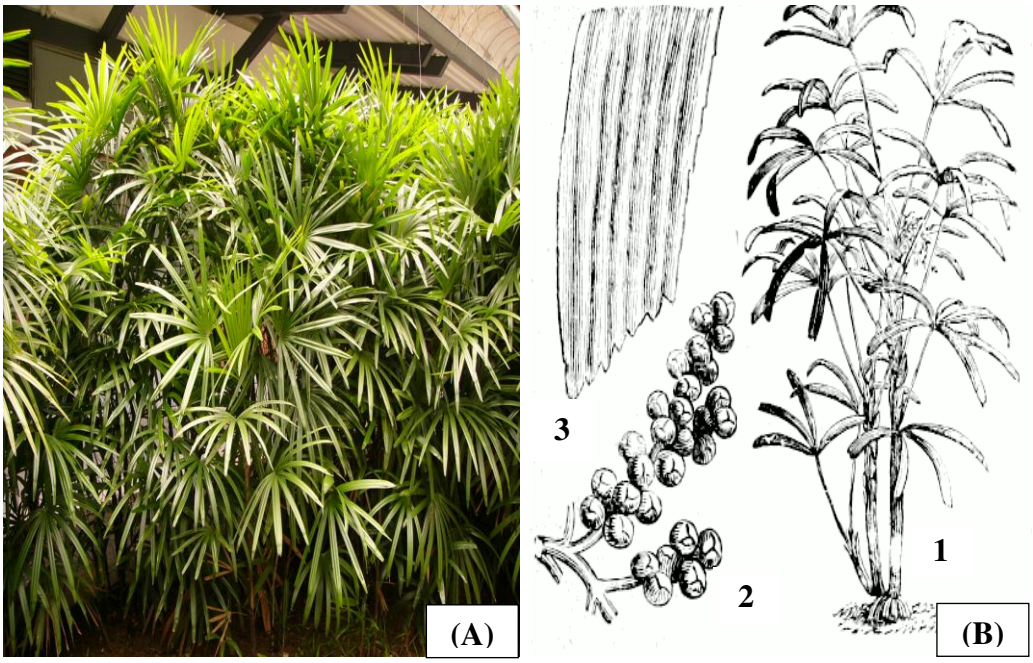


Figure 2.7: *Rhapis excelsa*
(A) Leaves (Floridata.com, 2015).
(B) Leaves[1], stem[1], seeds[2] and bark[3] (Dammer, 2008).

2.4.2 Chemical Constituent and Medicinal Uses

Little data are available on the phytochemical properties of *Rhapis excelsa*. Chromatographic fractionation of the leaf parts of the plant reported the presence of four flavonoids namely vitexin, vicenin-2, isoorientin and orientin. The leaves were reported to have antioxidant and antimicrobial activity against *Staphylococcus aureus* infections (Hassanein et al., 2015).

2.5 Plant of the Study: *Rhoeo spathacea*

2.5.1 Description

Rhoeo spathacea, shown in Figure 2.8 is a perennial herb from the Commelinaceae family of the Monocotyledonae class (USDA, 2015). It is commonly known as Boat lily, Moses in the cradle or oyster plant and has its origins in the West Indies and South America (Golczyk, 2013; Tan, Lim and Lee, 2014). The plant is generally used as a garden ornamental plant (Kumar,

Nagpal and Arun, 2011) and is grown widely in households of tropical countries like Malaysia. Parts of the plant are traditionally taken to treat various ailments meanwhile it is also popularly consumed as a favourite beverage in South American countries (Tan, Lee and Lim, 2013). Other synonyms of *Rhoeo spathacea* include *Tradescantia spathacea* and *Rhoeo discolor* (USDA, 2015). The plant is fleshy/succulent, short and can grow to a height of 20 cm; it has two colours on its leaves: green with yellow stripes on the top and purple on the bottom (Golczyk, 2013; Tan et al., 2014). They are large, imbricated and takes the shape of a lance with spiral pattern that closely overlaps (Parivuguna et al., 2008). The plant is immensely clumped with vibrant and lengthy leaves stemming out from its trunk (Bercu, 2013). The flowers are small, white and clustered within a folded bract protruding from the leaf axils with a short stalk; it has three petals and six stamens, produced throughout the year and adds features to the decorative elements of the leaves (National Parks, 2015)

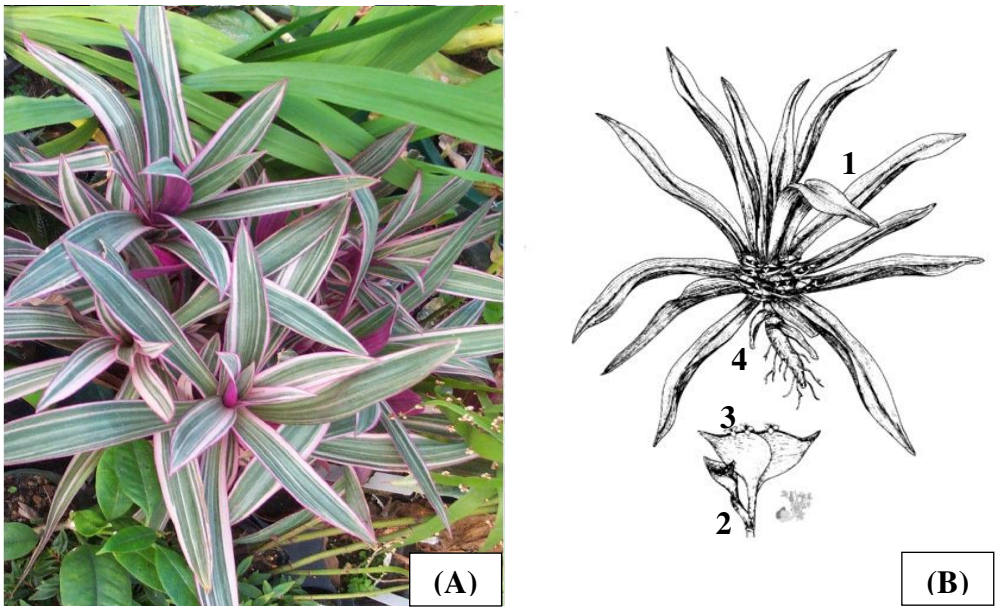


Figure 2.8: *Rhoeo spathacea*
(A) Leaves (USDA, 2015).
(B) Leaves[1], flowering branch[2], flower[3] and root[4] (Tramil.net, 2015).

2.5.2 Chemical Constituent and Medicinal Uses

Little data are available on the phytochemical properties of *Rhoeo spathacea*. A study by Parivuguna et al. (2008) through preliminary analysis on leaf extracts reported the abundance of alkaloids, flavonoids, steroids, saponins, cardiac glycoside, terpenoids, tannins, phenolic compounds and oil (Nikam et al., 2013). This plant has been traditionally used by communities in Mexico and Southeast Asia to treat cancer, superficial mycoses, coughs, colds, and dysentery (Rosales-Reyes et al., 2008; Joash et al., 2014). Furthermore, it was reported to have insecticidal, anti-inflammatory and anti-fertility properties (Siriwanthana et al., 2007).

2.6 Plant of the Study: *Vernonia amgydalina*

2.6.1 Description

Vernonia amygdalina as shown in Figure 2.9 is a short wooden shrub that grows to a height of 1 to 5 m and belongs to the family Asteraceae (Adesanoye et al., 2012). It originates in African tropics and is commonly used for culinary purposes (Ajibola, Eleyinmi and Aluko, 2011). It was reported being consumed by chimpanzees in the wild for self-deparasitization (Huffman, 2001; Nweze, Ogidi and Ngongeh, 2013). The plant is internationally recognized as African bitter leaf (Grubben, 2004) and locally as ‘daun bismillah’ (Mohd Abd Razak et al., 2014) which literally means sacred leaves in the local Malay language. The plant was only recently introduced in Malaysia, and is cultivated in many parts of the country for its medicinal values (Atangwho et al., 2013). The plant is moderately branched with a trunk up to the size of 40 cm in diameter, the barks are in grey to brown colour with a smooth texture and are fissured, and

however young branches are highly pubescent (Grubben, 2004). The leaves of the plant are simple, obovate-oblanceolate, display few lateral nerves and are finely glandular at the bottom. The flowers are white, fragrant, normally infested by bees and occur in copious corymbose panicles (Iwu, 2013). The stem and seeds of the plant were used for medicinal purposes whereas the leaves were used for both medicinal and cooking purposes (Mueller and Mechler, 2005). It can be harvested by simply by cutting the leafy shoots and allowing the new side shoots to develop, which normally takes a few weeks. Once the plant is established in the garden, the leaves and young shoots can be harvested for a period of 7 years (Grubben, 2004).

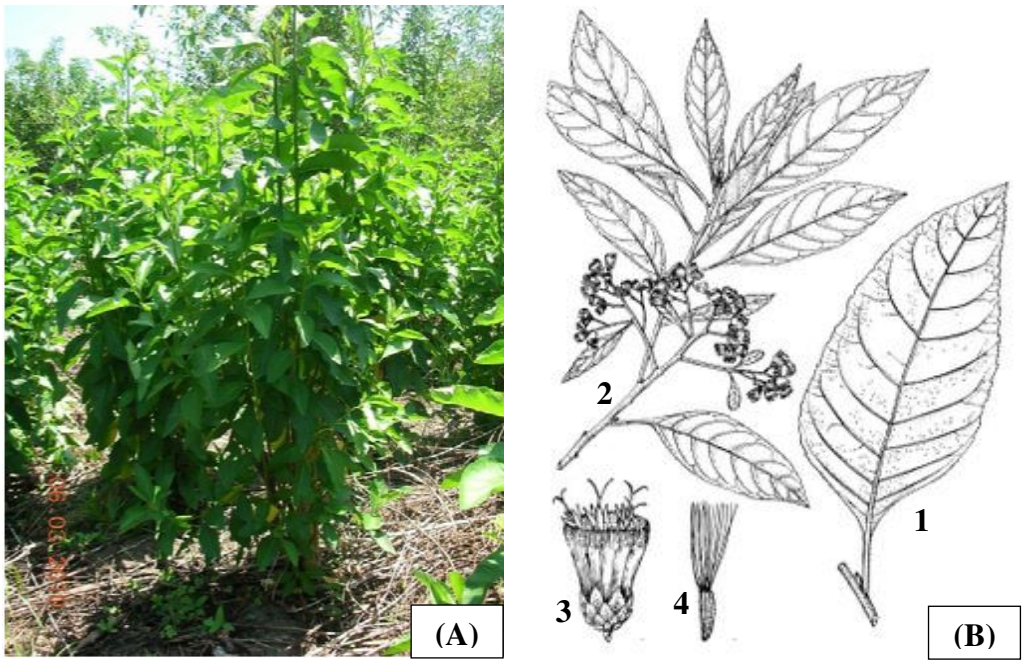


Figure 2.9: *Vernonia amygdalina*
(A) Leaves and Stems (Yeap et al., 2010).
(B) Leaf[1], flowering branch[2], flowering head[3] and fruit[4] (Grubben, 2004).

2.6.2 Chemical Constituent and Medicinal Uses

The characteristic bitter tastes of the leaves were ascribed to the presence of anti-nutritive constituents for instance alkaloids, glycosides, tannins and saponins (Adiukwu et al., 2013). However, the main constituents of the leaves

that contributes to its medicinal properties include saponin vernonin, sesquiterpene lactones such as vernodalin, vernolide, hydroxyvernolide, vernomydin and vernodal and the kaempferol flavonoid (Ademola and Eloff, 2010; Iwu, 2013). The pharmacological activities observed from this plant are attributed to the presence of various biologically active constituents in the leave. The vernonin from the plant is said to elicit strong antitumoral response in leukaemic cells. Meanwhile, the strong antioxidant activities of the plant are evident due to the presence of ubiquitous flavonoids (Adaramoye, 2008). Besides that, the antimalarial activity of *Vernonia amygdalina* can be related to the presence of flavonoids, saponins and alkaloids (Oyugi et al., 2009). The sequesterpene lactones and flavonoids produced by the leaf parts of the plant adds anti-phlogistic and anti-nociceptive effects to the plant's bioactive properties (Nangendo et al., 2002; Favi et al., 2008; Iroanya, Okpuzor and Mbagwu, 2010). In addition, the leaves of *Vernonia amygdalina* were highly reputed in the traditional African medicine to be effective in the treatment of gastrointestinal disorders, diarrhea and hepatitis (Wan, 2012; Nweze, Ogidi and Ngongeh, 2013).

2.7 Extraction of Medicinal Plants

Extraction is the pharmaceutical term used to define the approach used for the separation of the therapeutically desired compound from the other unwanted insoluble substances with the treatment of selective solvents (Kothari, Gupta and Naraniwal, 2012). Diverse solvent systems are available to extract bioactive compounds, selections are solely based on the target compounds, for instance an extraction of hydrophilic compound uses polar solvents such as

acetone, ethanol and methanol (Wendakoon, Calderon and Gagnon, 2012) whereas the extraction of a more lipophilic compound uses solvents like dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1.

Conventional methods of bioactive compound extraction techniques are heating, maceration, refluxing, solid liquid extraction (Soxhlet) and steam distillation or cold press (Martins and da Conceição, 2015) which principally relies on the correct use of organic solvent and the use of agitation and/or heat to intensify the solubility of the bioactive compounds. However, these methods produces lower product yields and have minimal selectivity. Besides that, it uses large volumes of organic solvents which poses potential environmental and health risks (Abdel-Azim et al., 2013). The demand for a more efficient extraction process with maximal purity and with no loss of activity has led to the augmentation of newer extraction processes (Santos, Vardanega and De Almeida, 2014) collectively named as modern extraction techniques which includes the microwave assisted extraction (MAE), solid phase micro extraction (SPME) and Soxhwave ultrasonification assisted extraction (UAE) (Gupta, Naraniwal and Kothari, 2012). These modern sample preparation techniques resulted in the significant reduction in the consumption of organic solvent and in minimizing degradation of samples (Kothari, Gupta and Naraniwal, 2012) and are therefore advantageous over the conventional methods of extraction.

2.8 Vero Cell Line

2.8.1 Description

The Vero cell line is a continuous highly anchorage-dependent cell line that is derived from the African green monkey (*Cercopithecus aethiops*) kidney epithelium by two scientists Y. Yasumura and Y. Kawakita from Chiba University, Japan on March 27, 1962 (Osada et al., 2014). It is widely used for the production of vaccines and determination of virus contaminations, primarily due to its production of clear cytopathic effect (CPE) and wide range of susceptibility to human viruses (Cao et al., 2013). Besides that, the cell line is also used to evaluate mammalian cell susceptibility to bacterial toxins and in the study of intracellular bacterial cell propagation (Ammerman, Beier-Sexton and Azad, 2005). The Vero cell line is considered advantageous over other cell lines because they are widely available, require no extensive culture conditions and grow faster in cultures; also they can be used in suspension and microcarrier cultures for large scale production in bioreactors (Chen and Chen, 2009).

2.8.2 Morphology and Structure

The Vero cells as shown in Figure 2.10 have a cuboidal epithelial morphology growing in monolayer on a glass or treated plastic surface. Vero cells possess a pseudo-diploid karyotype (ATCC, 2015) which refers to a cell that has 46 chromosomes with one or more structural abnormalities, gain-loss of the whole chromosome, or both types of abnormality patterns (Williams et al., 1982) and they are non-oncogenic when a cell passage was not prolonged (Osada et al., 2014).

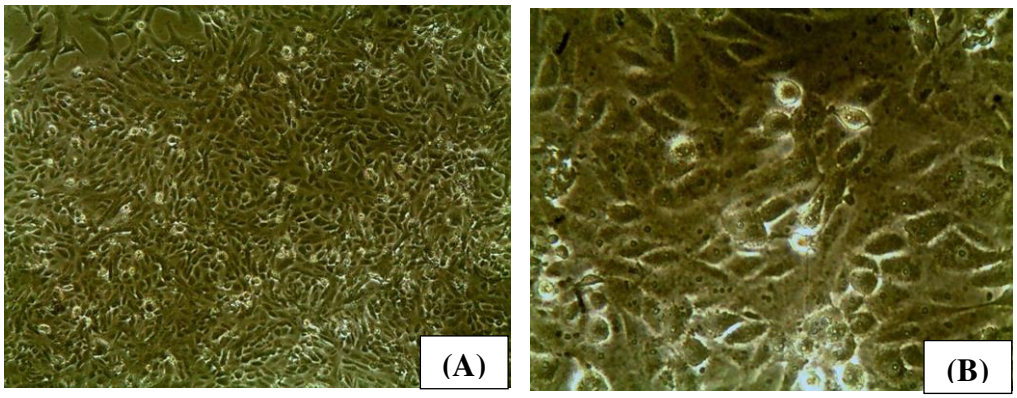


Figure 2.10: Vero Cell Line
(A) 100x Microscopy image of Vero cell.
(B) 400x Microscopy image of Vero cell.

2.8.3 Susceptibility and Resistance

The Vero cells have been classified as the most extensively used cell line in the culture of viruses. Table 2.3 shows list of viruses that are susceptible and resistant to the cell line.

Table 2.3: Virus species susceptible and resistant to Vero cell line.

Susceptibility/ Resistance	Viral Species
Virus susceptibility	Chikungunya virus
	Human poliovirus 1, 2, 3
	Getah virus
	Pixuna virus
	Ross River virus
	Semliki Forest virus
	Kokobera virus
	Modoc virus
	Guaroa virus
	Tacaribe virus , Tacaribe virus
	SV-5 (parainfluenza type 2), SV40
	Measles virus

(ATCC, 2015)

Table 2.3 (continued): Virus species susceptible and resistant to Vero cell line.

Susceptibility/ Resistance	Viral Species
Virus susceptibility	Rubella virus , Rubella virus
	Reovirus type 2, 3
	Simian adenovirus 3, 17, 11, 1 , 20, 18, 16, 8, 17, 19, 21, 25, 22, 23, 38, 37, 27, 39, 32, 34, 31, 33, 36
Virus resistance	Apeu; Ossa virus

(ATCC, 2015)

2.9 Cytotoxicity Assay

Cytotoxicity testing provides useful information in understanding the actions of chemicals on cells (Li et al., 2013). They utilize various parameters associated with proliferation and cell death, of them the most common is the Neutral red (3-amino-m-dimethylamino-2-methylphenazine hydrochloride) uptake assay which was developed in Rockfeller University as a tool for cell viability chemosensitivity determination, the assay quantifies the number of uninjured and viable cells through its ability to incorporate the supravital dye in its lysosomes (Repetto, del Peso and Zurita, 2008). Other cytotoxicity assays commonly in practice include the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, a measure of the reductive activity of dehydrogenase enzyme present in the mitochondria of viable cells and its ability to convert the reactant tetrazolium compound into a water insoluble formazan crystals (van Tonder, Joubert and Cromarty, 2015). The Lactate dehydrogenase (LDH) activity assay is based on the integrity of cell plasma

membrane. It is a measure of the cytoplasmic enzyme, LDH activity released by cells upon the damage of cell membrane (Weyermann, Lochmann and Zimmer, 2005).

2.9.1 Neutral Red Uptake (NRU) Asssay

Neutral red dye is frequently used in the evaluation of cell viability in cytotoxic assays, the principle behind the staining of cells by neutral red lies in the ability of the dye to penetrate the cell membrane through a non-ionic passive diffusion, it concurrently accumulate within the lysosomes by binding with anion and phosphate groups of the lysosomal matrix through electrostatic hydrophobic bonds. Further, evaluation of its mechanism reveals that its uptake principally relies on the capacity of the cell to establish equilibrium of its pH gradients through the production of ATP. At a pH of 7.4, the dye's net charge is maintained near zero; which enables the dye to penetrate the cell membrane. Meanwhile within the lysosomes, the action of a proton gradient lowers its pH to that of the cytoplasm causing the dye to be charged and retained within the lysosomes (Repetto, del Peso and Zurita, 2008). The dye retained can be extracted out from the cells via an acidified ethanol solution, and the amount of accumulated dye can be quantified by reading its absorbance at a wavelength of 540 nm (Fotakis and Timbrell, 2006). On the contrary if a cell dies or when the pH gradient was reduced, the dye will not be retained within the lysosomes.

2.10 $TCID_{50}$ Assay

The median tissue culture infective dose ($TCID_{50}$) also known as the endpoint dilution assay is one of the two most widely used methods of virus

quantification, the other is the plaque forming units (PFU) assay. These methods are built on serial dilutions of the virus samples and observation of the development of a cytopathic effect (CPE) in a cell monolayer (Grigorov et al., 2011). The $TCID_{50}$ assay is a quantal assay which determines the dilution of the sample at which 50% of the fractions have infectious virus whereas the PFU assay is a quantitative assay which determines the figure of infectious units of virus of interest in a sample (Lee et al., 2014). $TCID_{50}$ virus titers can be calculated by one of the two methods available namely, the Spearman-Kaerber and Reed–Muench calculation methods. Considering the $TCID_{50}$ method which is laborious and time consuming, newer variation of virus quantification techniques have been developed to replace the conventional cell culture based techniques, of these the real-time PCR technique is on the height due to its rapid and efficient quantification (Jonsson, Gullberg and Lindberg, 2009). Furthermore, it is also being used extensively in studies involving virus isolation, amplification and characterization. Other modern techniques for virus quantification include flow cytometry, tunable resistive pulse sensing (TRPS), and enzyme-linked immunosorbent assay (ELISA) (Pankaj, 2013).

2.11 Antiviral Assay

The conventional methods to analyze antiviral activity of a sample include both *in vitro* and *in vivo* techniques (Jassim and Naji, 2003; Esimone et al., 2005). *In vitro* studies to evaluate compounds with antiviral potential usually involve the methods such as the plaque inhibition assay, plaque reduction assay, inhibition of virus-induced cytopathic effect, virus yield reduction assay, end point titer determination assay, reduction or inhibition of the synthesis of virus-specific

polypeptides, immunological assays detecting viral antigens and viral enzyme inhibition-based assays (Vlietinck and Vanden Berghe, 1991; Cowan, 1999). In the *in vivo* techniques, the samples were tested on laboratory mice, ferrets, cotton rats and chickens to measure a few parameters to the extent of inhibition of the infection. There are two methods of propagation and isolation of virus namely the embryonated egg method (Härtl et al., 2004; Wang et al., 2008) and the cell culture based (Nwodo et al., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

Five plants were used in this study as listed in Table 3.1. The six crude extracts for each of the five plants were prepared by sequential solvent extraction process and were provided by supervisor Dr. Sit Nam Weng.

Table 3.1: Details of the tested plant sample.

Plant	Parts used
Ipomoea aquatic	Aerial
Persicaria odorata	Aerial
Rhapis excels	Leaf
Rhoeo spathacea	Leaf
Vernonia amygdalina	Leaf

3.1.2 Cell Line

Vero cell line (CCL-81™) was used in this study; it was purchased from American Type Culture Collection (ATCC). The cells were cultured and maintained from cryostorage with 5% Fetal Serum Bovine (FBS) supplemented Dulbecco's Modified Eagle Medium (DMEM).

3.1.3 Chikungunya virus (CHIKV)

The CHIKV used in this study is a clinical isolate belonging to the Asian genotype, and was provided by Professor Sharmala Devi (Department of Medical Microbiology, Faculty of Medicine, University of Malaya). The viral agent was cultivated in Vero cell culture and stored below -80 ^{o}C to maintain infectivity.

3.1.4 Chemical Reagents

The chemicals and reagents used in this study are listed in Table 3.2

Table 3.2: List of chemicals and reagents.

Chemical/Reagent	**Manufacturer**
Ethanol 95%	PROCHEM Chemicals, USA
Chloroquine	MP Biomedicals, USA
Dimethyl Sulfoxide (DMSO)	Merck Millipore, USA
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, China
Fetal Bovine Serum (FBS)	Biowest, USA
Glacial Acetic Acid	Bendosen, Norway
Hydrochloric Acid (HCl)	Merck Millipore, USA
Phosphate Buffer Saline (PBS)	Sigma-Aldrich, China
1% Penicillin-Streptomycin Solution	Biowest, USA
Neutral Red Solution	Sigma-Aldrich, China

Table 3.2 (continued): List of chemicals and reagents.

Chemical/Reagent	Manufacturer
Sodium Bicarbonate ($NaHCO_3$)	Merck Millipore, USA
Trypan blue	Thermo Fisher Scientific, USA
0.25% Trypsin:EDTA	Sigma-Aldrich, China

3.1.5 Equipment and Labwares

The equipment and labwares used in this study are listed in Table 3.3.

Table 3.3: List of equipment and labwares.

Equipments/Labwares	Manufacturer
Aluminium foil	DIAMOND
Autoclave machine	HICLAVE^{TM}HVE-50, HIRAYAMA
Bench-top centrifuge machine	Sigma-Aldrich, USA
Centrifuge tube	CELLSTAR®
Class IIB biological safety cabinet	TELSTAR®, Spain
Cryovial	Greiner Bio-one, USA
Freezer	HAIER®, China
Hemacytometer	HIRSCHMANN®, Germany
Incubator	Binder, Germany
Inverted microscope	OLYMPUS®, Japan
Laboratory film	Parafilm ―M‖ ®, Pechiney Plastic Packaging

Table 3.3 (continued): List of equipment and labwares.

Equipments/Labwares	Manufacturer
Laboratory oven	Memmert, Germany
Microcentrifuge tubes	AXYGEN, INC., Union City, USA
Micropipette	WATSON BioLab, Japan
Micropipette tips	AXYGEN, INC., Union City, USA
Microplate reader	TECAN M200®, USA
Multichannel pipette	Gilson, France
Pasteur pipette	AXYGEN, INC., Union City, USA
Portable bunsen	CAMPINGAZ® LABOGAZ 206
Refrigerator	TOSHIBA®, Japan
Sample vial	SAMCO®, United Kingdom
Glass bottle	SCHOTT DURAN®, Germany
Serological pipettes	Greiner bio-one, Austria
Serological pipetters	Thermo Fischer Scientific, USA
Syringe (3 mL/5 mL)	Terumo Medical Corporation, Japan
Syringe filter (0.45 μm)	Sartorius Minisart®
Flat-bottomed polystyrene 96-well plate	Thermo Fisher Scientific, USA
Tissue-culture flask	SPL Life Science, South Korea
Vacuum pump	Eppendorf, Concentrator plus, Germany
Vortex mixer	VELP® SCIENTICA, Europe
Weighing scale	KERN & SOHN®, Germany

3.2 Methodology

3.2.1 Preparation of Plant Extract

In order to prepare plant extract stock solution, 0.1024 mg of each extract was dissolved in 400 µL of DMSO: ethanol (6:4, v/v) to achieve the concentration of 256 mg/mL. The extract solutions were then filtered using a 0.45 µm syringe filter into sample vials and stored at 4 ^{o}C until use.

3.2.2 Preparation of Reagents

3.2.2.1 Phosphate Buffered Saline (PBS)

To prevent contamination, all the subsequent steps were performed within Class IIB biological safety cabinet. Phosphate Buffered Saline (PBS) solution was prepared by dissolving 4.8 g of powdered PBS into 500 mL distilled water. The solution was autoclaved at 121 ^{o}C for 20 minutes and stored at 4 ^{o}C prior usage.

3.2.2.2 Cell Freezing Medium

Cell freezing medium was used for the cryopreservation of Vero cells. For its preparation, 150 mL of FBS and 50 mL of DMSO was added into 300 mL serum negative DMEM. The medium was then sealed tightly and stored at 4 ^{o}C prior usage

3.2.2.3 1% and 5% FBS Supplemented DMEM

The 1% and 5% FBS supplemented DMEM was intended to be used in assays and for the maintenance of Vero cells respectively. For its preparation 13.4 g of powdered DMEM and 3.7 g of sodium bicarbonate was weighed and

transferred into a 1 L glass bottle, then 500 mL of distilled water was added to dissolve the powder. The solution was made up to 1 L with distilled water. The pH of the solution was adjusted between 7.40 to 7.49 by adding 1M Hydrochloric acid solution. Next, 10 mL of 1% Penicillin-Streptomycin solution was added and the medium was filtered using 0.45 µm filter unit. A volume of 5 mL (1% FBS supplemented DMEM) or 25 mL (5% FBS supplemented DMEM) FBS was added into the prepared medium depending on the type of growth medium being prepared. The occurrence of contamination was examined by aspiring 2 mL of the prepared medium into a petri plate. The plates were incubated at 37 ^{o}C for 72 hours in a CO_2 humidified incubator. Finally, the glass bottle containing prepared medium was sealed tightly with parafilm and stored at 4 ^{o}C prior usage.

3.2.2.4 Low Glucose Medium

Low glucose medium was intended to be used in the NRU assay and was prepared similarly as the 1% and 5% FBS supplemented DMEM (as mentioned in section 3.2.2.3, page 36), however a low glucose formulation of DMEM was used and the medium was supplemented with 5 mL of FBS.

3.2.2.5 Chloroquine

Chloroquine which served as the positive control was prepared by dissolving chloroquine powder into 100 mL of distilled water to achieve the concentration of 62 mg/mL. The solution was filtered using 0.45 µm syringe filter and stored away from light at 4 ^{o}C prior usage.

3.2.2.6 Neutral Red

In order to prepare Neutral red (NR) solution, 582 µL of NR stock solution (3.3 g/L, w/v) was diluted into 47.42 mL of low glucose medium to achieve the concentration of 40 µg/mL. The solution was stored away from light at room temperature prior usage.

3.2.2.7 Neutral Red Destain Solution

Neutral red destain solution was prepared by adding 10 mL of glacial acetic acid and 500 mL of 95% ethanol into 490 mL of distilled water. The solution was stored at room temperature prior usage.

3.2.3 Vero Cell Culture

3.2.3.1 Propagation from Frozen Stock

For the propagation of Vero cells from frozen stock, the cells were removed from liquid nitrogen tank and thawed in water bath at 37 ^{o}C with gentle swirling. The thawed cells were diluted with 5% FBS supplemented DMEM to remove the cryopreservant, prior to the transfer into a 75 cm^2 tissue culture flask containing 6 mL of 5% FBS supplemented DMEM.

The cells were then incubated for 6 hours at 37 ^{o}C in a 5% CO_2 humidified incubator. After that, the cell suspension was transferred into a 15 mL centrifuge tube and spinned at (1, 500) rpm for 5 minutes at room temperature. The resulting pellet was re-suspended in 5 mL of 1% FBS supplemented DMEM and seeded into a new 75 cm^2 tissue culture flask containing 10 mL of

5% FBS supplemented DMEM. The cells were incubated, and monitored daily to obtain 70-80% cell confluency.

3.2.3.2 Maintenance of Cells

Vero cells were maintained by replenishing the exhausted cell culture medium 2 to 3 times in a week (ATCC, 2015) depending on the cell confluency. The cells require regular inspection under an inverted phase-contrast microscopy to ensure it is free from any forms of contamination.

3.2.3.3 Subculture of Cells

Vero cells are normally subcultured when it reaches a percentage of 70-80% cell confluency (ATCC, 2015). In order to subculture Vero cells, the cell culture medium in the culture flask was removed. Then, the adherent cells in culture flask were washed twice with 8 mL of prepared PBS (as mentioned in section 3.2.2.1, page 36) and discarded after washing. These steps were performed to remove traces of FBS which contains trypsin inhibitors that may interfere in the process of enzymatic cell disaggregation (Ammerman, Beier-Sexton and Azad, 2005). Next, 1.5 mL of 0.25% Trypsin: EDTA solution was added and the cells were incubated at 37 ^{o}C in a 5% CO_2 humidified incubator for 5 minutes.

After incubation, gentle shaking and tapping was performed to aid in cell detachment. The degree of cell detachment was examined under an inverted phase contrast microscope. Then, to inactivate the activity of trypsin: EDTA, an equivalent quantity of 1% FBS supplemented DMEM was added. The cell suspension in the flask was transferred into a 15 mL centrifuge tube and

centrifuged at (1, 500) rpm for 5 minutes at room temperature. Once centrifugation was complete, the supernatant was discarded and the obtained pellet was re-suspended with 4 mL of 1% FBS supplemented DMEM. The suspension was divided evenly into two tissue culture flask and topped up with 10 mL of 5% FBS supplemented DMEM. The flasks containing the cells were incubated at 37 ^{o}C in a 5% CO_2 humidified incubator and monitored daily to ensure it is free from any forms of contamination.

3.2.3.4 Cell Count

Vero cell concentration in a cell suspension was established by cell count procedure using a hemacytometer. Following the re-suspending of cell pellet with 1% FBS supplemented DMEM (as mentioned in section 3.2.3.3, page 39), 10 µL of cell suspension was pipetted into a 1.5 mL microcentrifuge tube and thoroughly mixed with 10 µL of 0.4% trypan blue. Next, 10 µL of the mixed suspension was loaded onto a haemacytometer and via an inverted phase contrast microscope at 100x magnification, the cells which were located in the counting grids as shown in Figure 3.1 were enumerated carefully. Based on the formula given below, the cell suspension concentration (cells/mL) was calculated.

$$\text{Cell concentration (cells/mL)} = \left(\frac{A + B + C + D}{4}\right) \text{x } 2 \text{ x } 10^4 \text{ x } V \text{ ml}$$

Where, V is the volume of 1% FBS supplemented DMEM used to re-suspend the pellet.

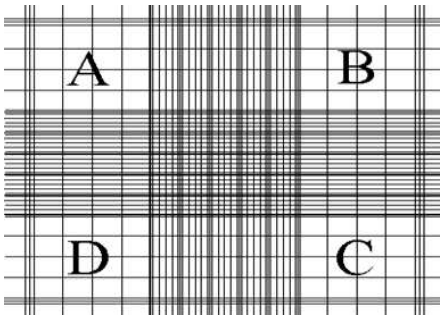


Figure 3.1: Four cell counting grids where viable cells were enumerated.

3.2.3.5 Cryostorage of Cells

Maintenance of frozen cell stocks is essential during the culture of cell lines. Frozen cell stocks were routinely prepared shortly after the initiation of cultures from the previously frozen stocks (Ammerman, Beier-Sexton and Azad, 2005). For the cryopreservation of Vero cells, cells from the culture flask were trypsinized and transferred into a 15 mL centrifuge tube. Next, it was centrifuged at (1, 500) rpm for 5 minutes at room temperature. Once centrifugation was complete, the supernatant was discarded and the obtained pellet was re-suspended with 1 mL of prepared cell freezing medium. The suspension was transferred into a 1.5 mL cryovial and frozen slowly to -80 ^{o}C for 24 hours. On the following day, the cryovial containing the suspension was transferred into a liquid nitrogen tank for long term storage at -196 ^{o}C.

3.2.4 CHIKV Culture

3.2.4.1 Thawing of CHIKV

Thawing of CHIKV stock was performed prior to virus titer assessment and antiviral assays primarily to maintain stability of the virus. The cryovial containing the virus stock was removed from ultrafreezer and thawed on a 37 ^{o}C water bath with gentle agitation.

3.2.4.2 Cultivation and Storage of CHIKV

To cultivate CHIKV through Vero cell culture, Vero cells were passaged into two 25 cm^2 tissue culture flasks containing 6 mL of 5% FBS supplemented DMEM. The cells were incubated, and monitored daily to obtain 70-80% cell confluency. Meanwhile, the thawed virus stock was diluted to a ratio of 1:10 with 1% FBS supplemented DMEM. Once the desired confluency was obtained, 500 µL of the diluted virus suspension was inoculated into the two culture flasks. The flasks were incubated at 37 ^oC in a 5% CO_2 humidified incubator. Next, Vero cells were examined daily under an inverted phase contrast microscope for the development of cytopathic effect (CPE). Once the desired degree of CPE was produced, the cell-virus suspension in the flask was transferred into a 15 mL centrifuge tube and centrifuged at (1, 500) rpm for 5 minutes at room temperature. The resulting supernatant which contains the new virus stock was transferred into 1.5 mL cryovials and stored in an ultrafreezer at -80 ^oC prior to virus titer determination (Lennette and Schmidt, 1979; Rovozzo and Burke, 1982; Burleson, Chambers and Wiedbrauk, 1992).

3.2.5 NRU Cytotoxicity Assay

In order to perform the NRU cytotoxicity assay, all sterile 96-well plates were labeled correctly as shown in Figure 3.3. Following the cell count procedure (as mentioned in section 3.2.3.4, page 40), 100 µL of cell suspension consisting of 4 x 10^4 cells/mL were seeded into the test and cell control wells of 96-well plates. All plates were covered and sealed properly with parafilm and incubated at 37 °C in a 5 % CO_2 humidified incubator for 24 hours.

On the next day, a two-fold serial dilution of each plant extract stock solutions were performed as illustrated in Figure 3.2, by dissolving 5 µL of each extract with 995 µL of 1% FBS supplemented DMEM and consecutively transferring 500 µL into the subsequent tubes consisting 500 µL of 1% FBS supplemented DMEM. After serial dilution, 500 µL of solution was discarded from the final tube

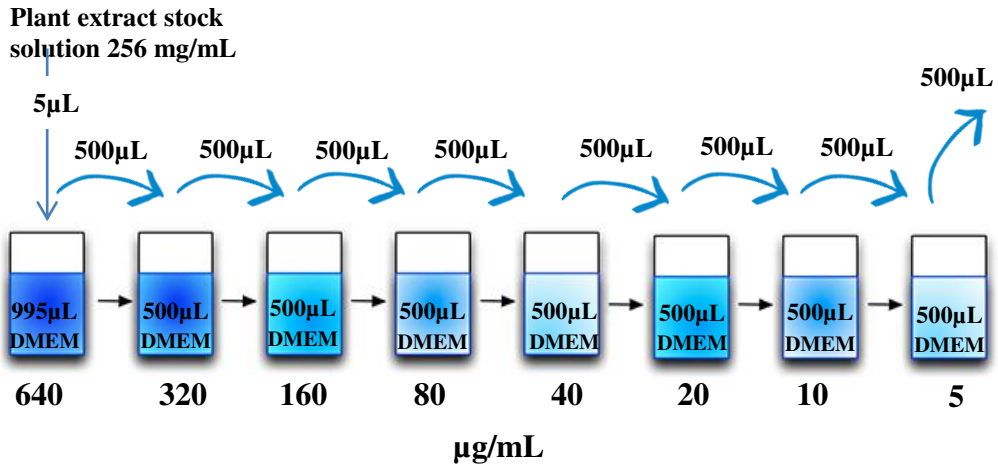


Figure 3.2: Illustration of two-fold serial dilution of each plant extract stock solution for NRU cytotoxicity assay.

Next, 100 µL and 200 µL of 1% FBS supplemented DMEM was pipetted into each cell control and medium control wells respectively. After that, 100 µL of each extract was pipetted into the test wells accordingly. All the plates were then covered and sealed properly with parafilm and incubated at 37°C in a 5% CO_2 humidified incubator for 72 hours.

After incubation, solutions within the wells were discarded and they were subjected to washing with 150 µL of PBS followed by gentle shaking. Next, the solutions were discarded and 100 µL of NR solution was added into the wells. The plates were covered and incubated at 37°C in a 5% CO_2 humidified

incubator for 2 hours. Once incubation was complete, NR solution were replaced with 150 µL of NR destain solution for colour development. The wells that were stained red indicate the presence of viable cells. The observations were recorded and the absorbances of the all wells were read using a multiplate reader at a wavelength of 540 nm. The NRU cytotoxicity assay was repeated in quadruplicate for each plant extract.

Plate 1

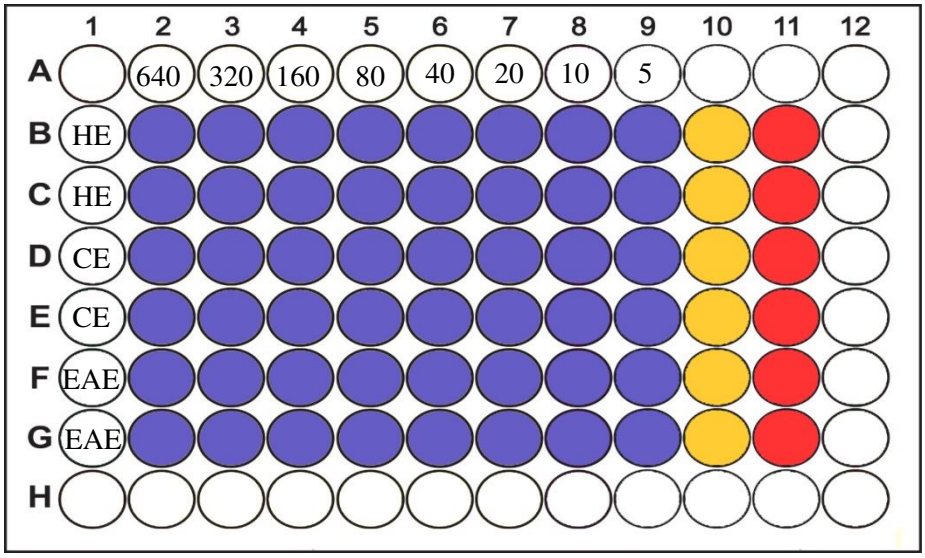
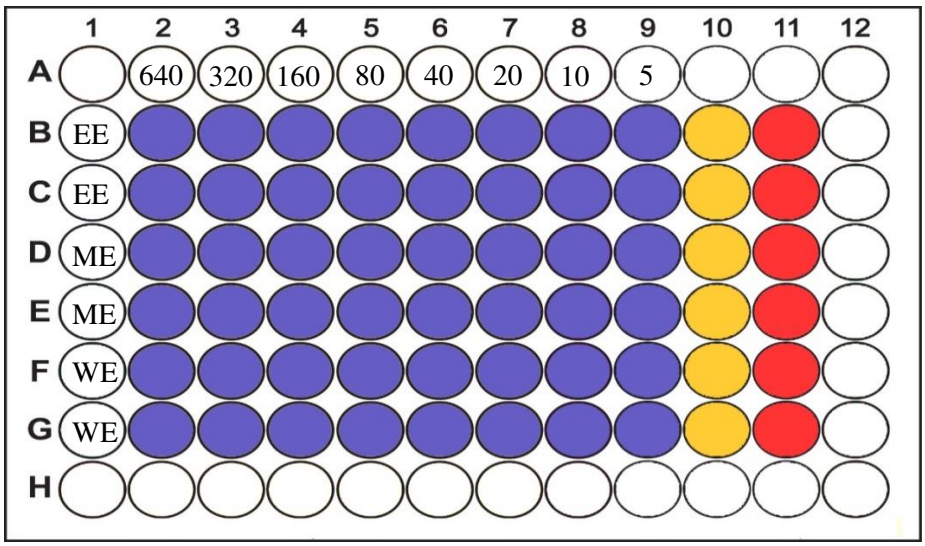


Plate 2



Test wells [consist of 100 µL of cells and 100 µL of plant extract]

Cell control [consist of 100 µL of cells and 100 µL of medium]

Medium control [consist of 200 µL of medium]

Footnote: HE=Hexane extract; CE=Chloroform extract; EAE=Ethyl acetate extract; EE=Ethanol extract; ME=Methanol extract; WE=Water extract; Row 1= Plant extract concentration used in the wells of the respective column (µg/mL).

Figure 3.3: Layout of 96-well plates for NRU cytotoxicity assay.

3.2.6 $TCID_{50}$ Assay

The tissue culture infectious dose ($TCID_{50}$) assay was used to determine the titer of CHIKV stock suspension (as mentioned in 3.2.4.2, page 41). For $TCID_{50}$ assay, all sterile 96-well plates were labeled correctly as shown in Figure 3.5. Following the cell count procedure (as mentioned in section 3.2.3.4, page 40), 100 µL of cell suspension consisting of 4 x 10^4 cells/mL were seeded into the test and cell control wells of 96-well plates. All plates were covered and sealed properly with parafilm and incubated at 37 °C in a 5% CO_2 humidified incubator for 24 hours.

On the next day, the thawed virus stock was diluted to a ratio of 1:10 by dissolving 5 µL of the thawed CHIKV suspension with 995 µL of 1% FBS supplemented DMEM and consecutively a two-fold serial dilution of CHIKV suspension was prepared as illustrated in Figure 3.4, by dissolving 100 µL of the diluted suspension with 900 µL of 1% FBS supplemented DMEM to achieve CHIKV dilutions of 10^{-1} to 10^{-10}.

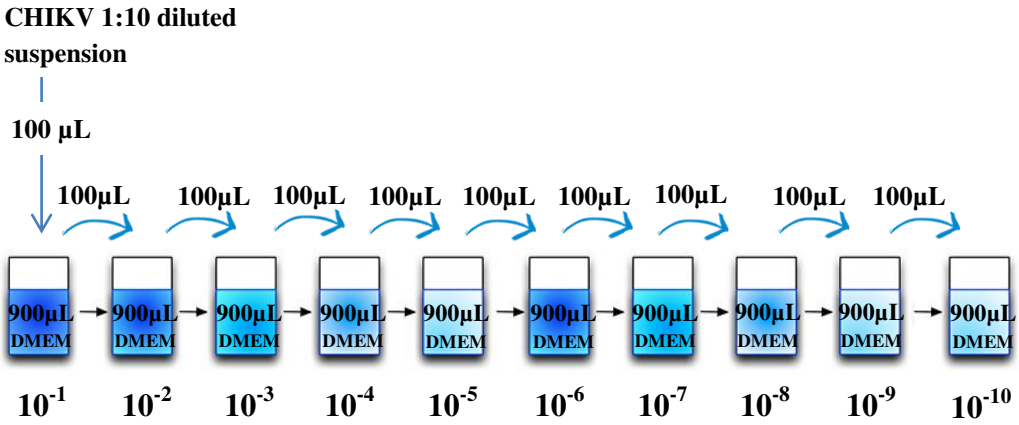
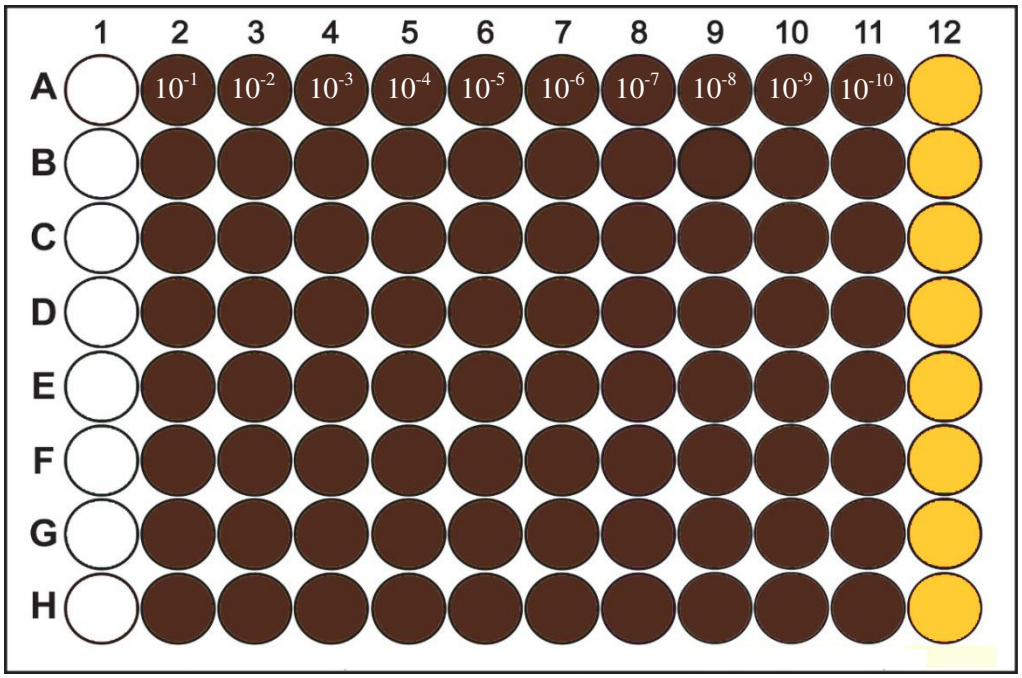


Figure 3.4: Illustration of two-fold serial dilution of CHIKV stock suspension for $TCID_{50}$ assay.

After incubation, 100 µL of 1% FBS supplemented DMEM was pipetted into the cell control wells which serve as the negative control. Then, 100 µL of prepared CHIKV dilutions were introduced into the test wells accordingly. All plates were covered and sealed properly with parafilm and incubated at 37 ^{o}C in a 5% CO_2 humidified incubator for 72 hours.

Once incubation was complete, Vero cells were examined under an inverted phase contrast microscope for the development of CPE, the observation were recorded and the resulting virus titer and MOI was determined using the Reed-Meunch method. The $TCID_{50}$ assay was repeated in quadruplicate for CHIKV stock suspension in each cryovial. Based on the formula given below, the CHIKV titer was calculated (Reed and Meunch, 1938).

$$X = \log_{10} \text{dilution factor} \left(\frac{\text{\% infection at next dilution above 50\%-50\%}}{\text{\% infection at next dilution above 50\% - \% of infection at next dilution below 50\%}}\right)$$



Test wells [consist of 100 µL of cells and 100 µL of virus inoculum]

Cell control [consist of 100 µL of cells and 100 µL of medium]

Footnote: Row 1=CHIKV dilutions used in the wells of the respective column.

Figure 3.5: Layout of 96-well plate for $TCID_{50}$ assay.

3.2.7 Post-Inoculation Antiviral Assay

In order to perform the post-inoculation antiviral assay, all sterile 96-well plates were labeled correctly as shown in Figure 3.7. Following the cell count procedure (as mentioned in section 3.2.3.4, page 40), 50 µL of cell suspension consisting of 4 x 10^4 cells/mL were seeded into the test, antiviral control, virus control, cell control and cytotoxic control wells of 96-well plates. All plates were covered and sealed properly with parafilm and incubated at 37 °C in a 5% CO_2 humidified incubator for 24 hours.

On the next day, CHIKV dilutions were performed with 1% FBS supplemented DMEM based on the calculated virus titer from the $TCID_{50}$ assay (as mentioned in 3.2.6, page 45). After incubation, 100 µL of virus suspension was pipetted into the test, antiviral control and virus control wells. The plates were covered and sealed properly with parafilm and incubated at 37 ^{o}C in a 5% CO_2 humidified incubator for 1 hour. Meanwhile, during the incubation period a two-fold serial dilution (similar to Figure 3.1, page 43) of each plant extract stock solutions were performed from the calculated MNCC (as shown in Table 4.1, page 57). Also, a two-fold serial dilution of Chloroquine stock solution were prepared as illustrated in Figure 3.6, by dissolving 500 µL of 62 mg/mL Chloroquine stock solution with 500 µL of 1% FBS supplemented DMEM and consecutively transferring 500 µL into the subsequent tubes consisting 500 µL of 1% FBS supplemented DMEM to achieve the concentrations of 1240, 620, 310, 155, 77.5 and 38.8 µg/mL. After serial dilution, 500 µL of solution was discarded from the final tube.

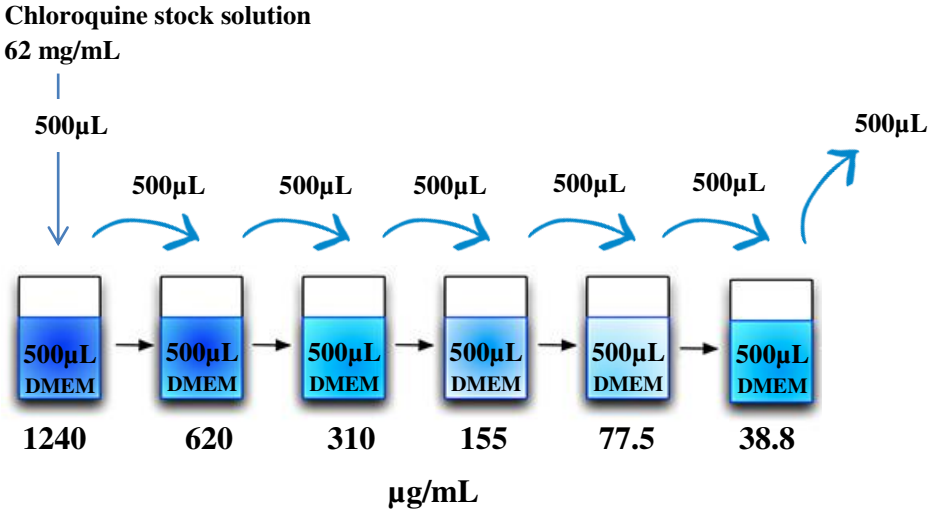


Figure 3.6: Illustration of two-fold serial dilution of Chloroquine stock solution for post-inoculation antiviral assay.

Once incubation was complete, 100 µL of each extract and 100 µL of the prepared Chloroquine solutions were pipetted into the test and antiviral control wells accordingly. Finally, 100 µL, 250 µL, 200 µL and 100 µL of 1% FBS supplemented DMEM was pipetted into the virus control, medium control, and cell control, and cytotoxic control wells respectively. All the plates were then covered and sealed properly with parafilm and incubated at 37 ^{o}C in a 5% CO_2 humidified incubator for 72 hours.

After incubation, the plates were subjected to washing and measurement of cell viability at 540 nm using NRU assay (as mentioned in section 3.2.5, page 42). The post-inoculation antiviral assay was repeated in triplicate for each plant extract.

Plate 1

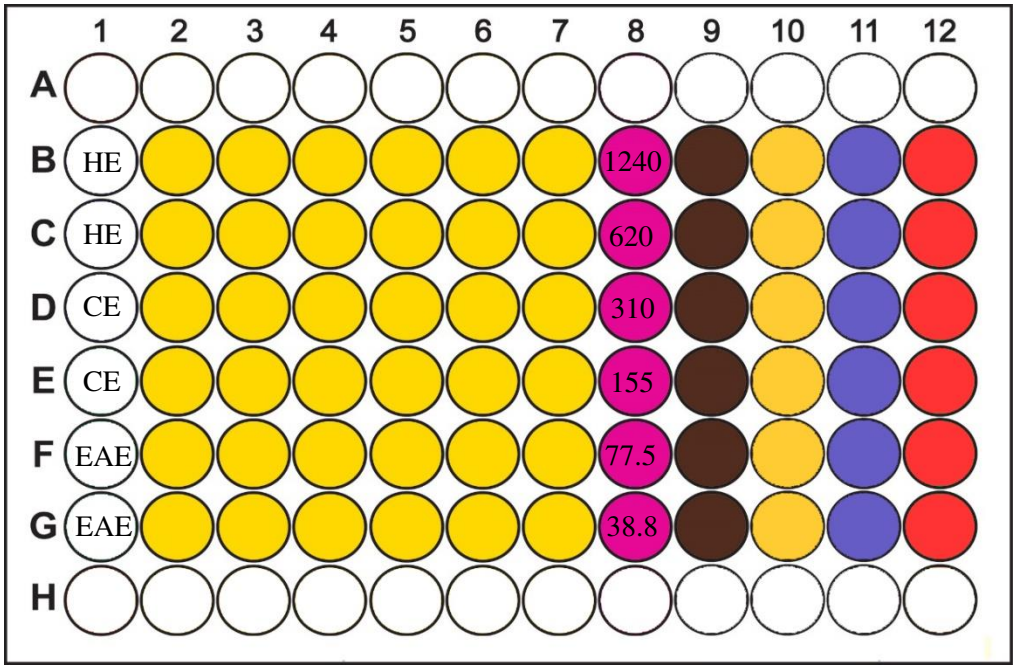
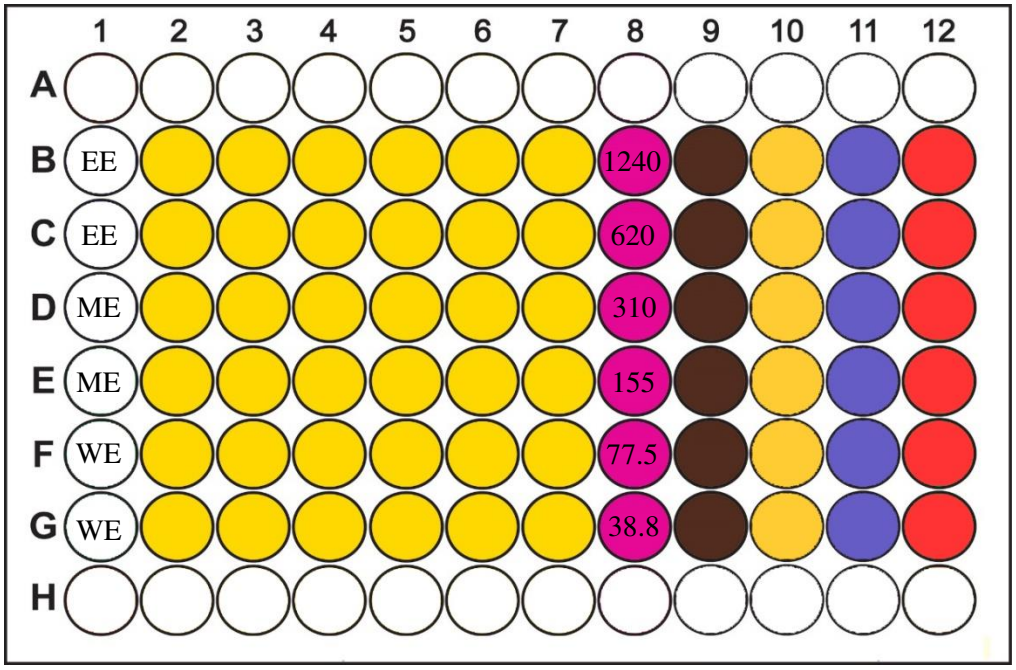


Plate 2



Test wells [consist of 50 µL of cells, 100 µL of virus inoculum and 100 µL of plant extract]

Antiviral control [consist of 50 µL of cells, 100 µL of virus inoculum and 100 µL of Chloroquine]

Virus control [consist of 50 µL of cells, 100 µL of virus inoculum and 100 µL of medium]

Cell control [consist of 50 µL of cells and 200 µL of medium]

Cytotoxic control [consist of 50 µL of cells, 100 µL of plant extract and 100 µL of medium]

Medium control [consist of 250 µL of medium]

Footnote: HE=Hexane extract; CE=Chloroform extract; EAE=Ethyl acetate extract; EE=Ethanol extract; ME=Methanol extract; WE=Water extract; Column 8= Chloroquine working solution concentration used in the respective wells (µg/mL)

Figure 3.7: Layout of 96-well plate for post-inoculation antiviral assay.

3.3 Data Analysis

Results obtained in both the NRU cytotoxicity and post-inoculation antiviral assay were expressed as mean cell viability percentage ± SD (standard deviation). Statistical analyses were carried out using the IBM® SPSS® software version 20. Tukey honestly significant difference (HSD) and Dunnets T3 tests were used to compare all pairs of groups via the One-way ANOVA test and $p < 0.05$ was considered statistically significant.

3.3.1 NRU Cytotoxicity Assay

The Vero cell viability percentage for NRU assay was calculated based on the formula given below.

$$\text{Cell viability}(\%) = \frac{\text{Absorbance of sample-Absorbance of medium control}}{\text{Absorbance of cell control-Absorbance of medium control}} \text{ X } 100$$

3.3.2 Post-Inoculation Antiviral Assay

The Vero cell viability percentage for post-inoculation antiviral assay was calculated based on the formula given below.

$$\text{Cell viability}(\%) = \frac{\text{Absorbance of sample-Absorbance of virus control}}{\text{Absorbance of cell control-Absorbance of medium control}} \text{ X } 100$$

CHAPTER 4

RESULTS

4.1 NRU Cytotoxicity Assay

In order to distinguish antiviral activity from cytotoxic effect, the concentrations of tested extracts that reduced viability of Vero cells by half (CC_{50}) and the maximal non-cytotoxic concentration (MNCC) were determined via the neutral red uptake (NRU) assay. Figures 4.1 - 4.5, display cytotoxic activity of *Ipomoea aquatica*, *Persicaria odorata*, *Rhapis excelsa*, *Rhoeo spathacea* and *Vernonia amygdalina* extracts at varying concentrations of 5, 10, 20, 40, 80, 160, 320 and 640 µg/mL on Vero cells in a 72 hour NRU assay. Based on Figures 4.1 to 4.5, Vero cells treated with ethanol extract of *Rhapis excelsa* between the concentrations of 80-640 µg/mL were demonstrated to be the most toxic, and exhibited the lowest cell viability percentage following 72 hours of incubation, ranging from 8.86% ± 0.02 to 2.24% ± 0 (CC_{50} = 51.67 ± 2.89 µg/mL), this was followed by ethyl acetate extract of *Vernonia amygdalina* (CC_{50} = 86.87 ± 2.89 µg/mL) and methanol extract of *Rhapis excelsa* (CC_{50} = 91.67 ± 2.89 µg/mL) from the concentrations of 320 to 640 µg/mL and 160 to 640 µg/mL correspondingly. The NRU assay further reported that Vero cells with the treatment of chloroform and ethyl acetate extracts of *Ipomoea aquatica* and *Vernonia amygdalina* resulted in more than 50% and 90% cell death respectively between the concentrations of 320 to 640 µg/mL. On the other hand, treatment of hexane, chloroform and ethyl acetate extracts of *Persicaria odorata* exhibited more than 70% cell death between the

concentrations of 320 to 640 µg/mL. Meanwhile, the MNCC of ethanol and methanol extracts of *Rhapis excelsa* in conjunction with ethyl acetate, chloroform and water extract of *Vernonia amygdalina* were demonstrated to be the lowest at 20 µg/mL, as shown in Table 4.1.

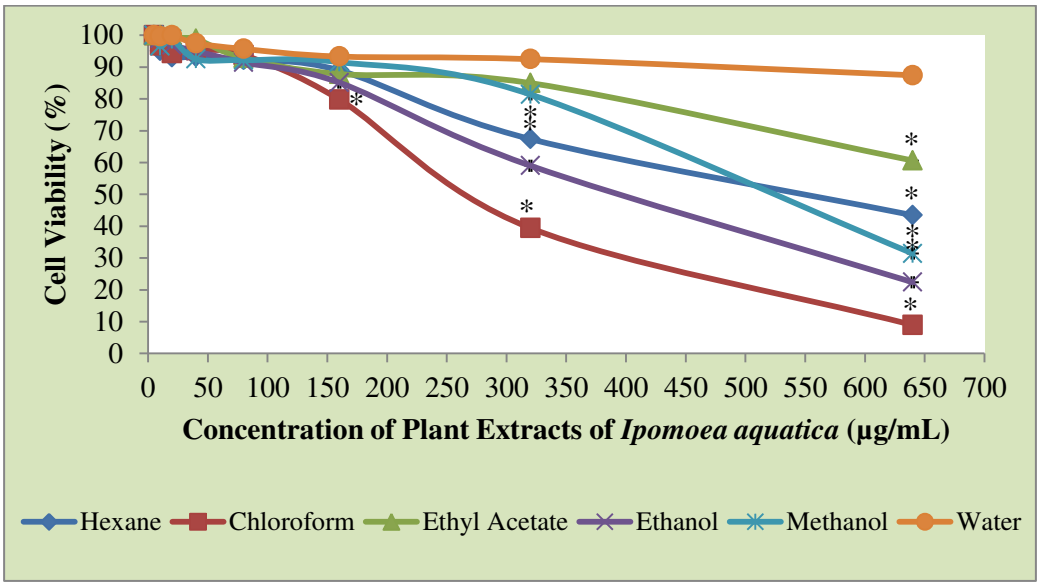


Figure 4.1: Cytotoxic activity of *Ipomoea aquatica* extracts on Vero cells at different concentrations in 72 hour NRU assay.
*Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test.

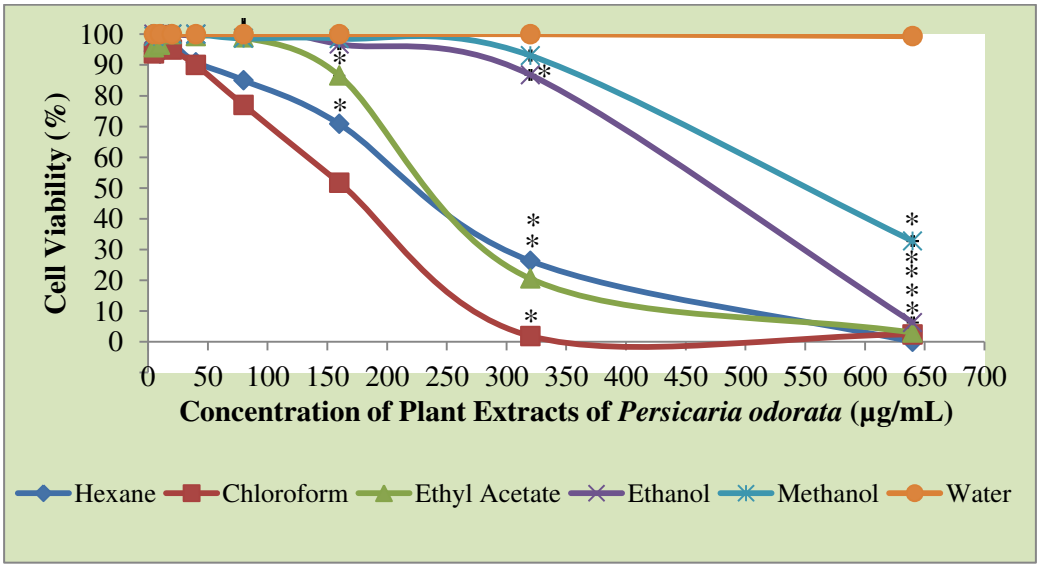


Figure 4.2: Cytotoxic activity of *Persicaria odorata* extracts on Vero cells at different concentrations in 72 hour NRU assay.
*Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test.

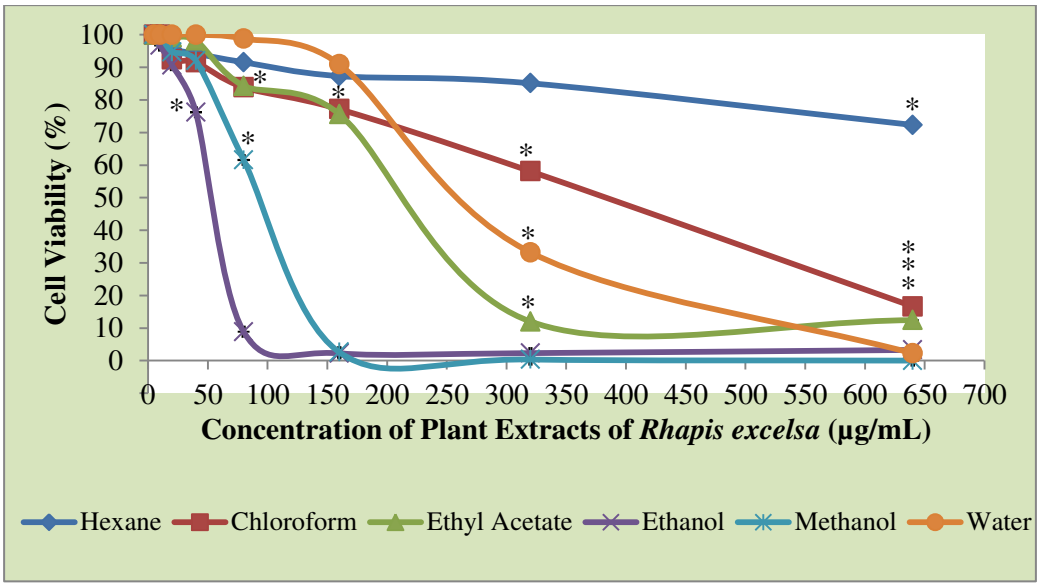


Figure 4.3: Cytotoxic activity of *Rhapis excelsa* extracts on Vero cells at different concentrations in 72 hour NRU assay.
*Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test.

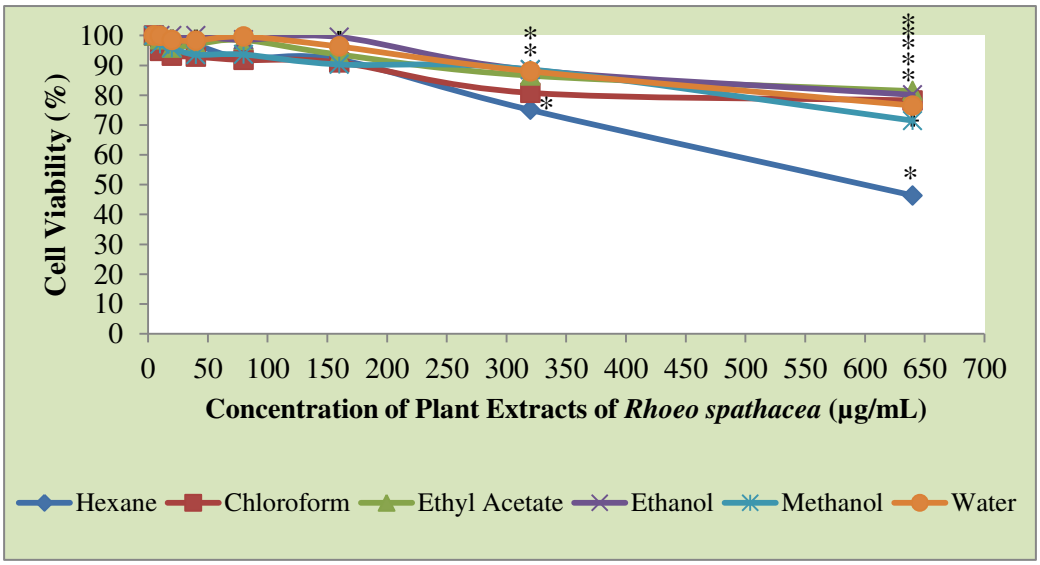


Figure 4.4: Cytotoxic activity of *Rhoeo spathacea* extracts on Vero cells at different concentrations in 72 hour NRU assay.
*Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test.

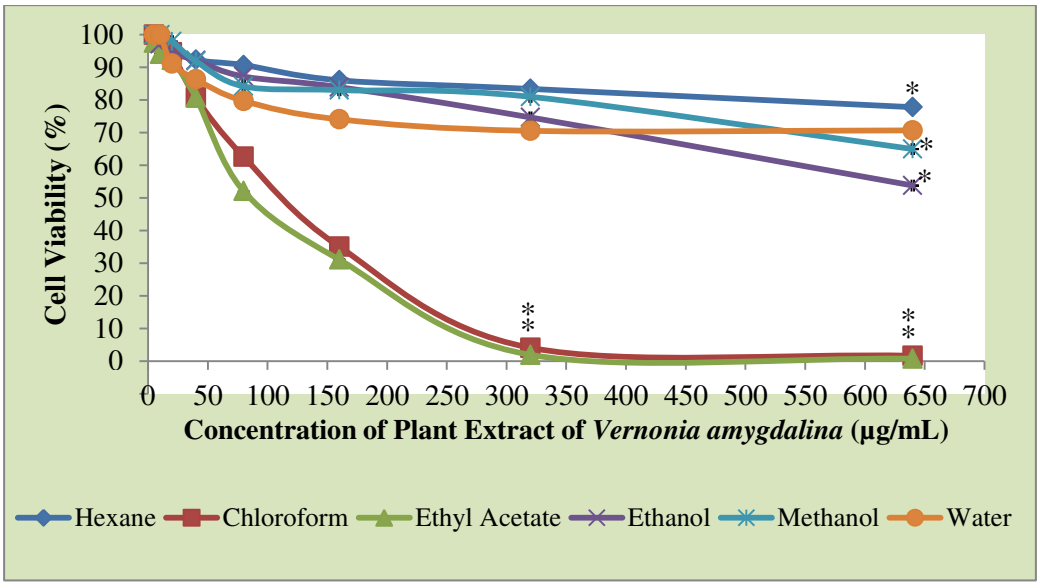


Figure 4.5: Cytotoxic activity of *Vernonia amygdalina* extracts on Vero cells at different concentrations in 72 hour NRU assay.
*Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test.

Table 4.1: Cytotoxicity of *Ipomoea aquatica*, *Persicaria odorata*, *Rhapis excelsa*, *Rhoeo spathacea* and *Vernonia amygdalina* plant extracts on Vero cells expressed as CC_{50} and MNCC obtained in 72 hour assay NRU assay.

Plant species	**Extracts**					
	Hexane	**Chloroform**	**Ethyl Acetate**	**Ethanol**	**Methanol**	**Water**
Ipomoea aquatica						
CC_{50}	542 ± 3.46	260 ± 0	NA	329.33 ± 4.04	521.67 ± 4.93	NA
MNCC	80	80	80	80	160	320
Persicaria odorata						
CC_{50}	220 ± 0	163.67 ± 3.21	233.33 ± 3.06	473.33 ± 3.06	552.67 ± 4.62	NA
MNCC	40	40	80	160	320	640
Rhapis excelsa						
CC_{50}	NA	380 ± 0	211.67 ± 2.89	51.67 ± 2.89	91.67 ± 2.89	257 ± 1.73
MNCC	80	40	40	20	40	160
Rhoeo spathacea						
CC_{50}	590.33 ± 4.73	NA	NA	NA	NA	NA
MNCC	160	160	160	160	160	160
Vernonia amygdalina						
CC_{50}	NA	110 ± 5	86.67 ± 2.89	NA	NA	NA
MNCC	80	20	20	40	40	20

Mean ± SD (Standard deviation, n=3)
CC_{50}: Half-cytotoxic concentration; NA: CC_{50} beyond the concentration range of study
MNCC: Maximal non-cytotoxic concentration (µg/mL)

4.2 Post-Inoculation Antiviral Assay

Antiviral activity of the various test extracts were determined via the post-inoculation antiviral assay, where the maximal non-cytotoxic concentration (MNCC) as indicated in Table 4.1 (page 57) was used as the initial concentration for the assays and were incorporated after the inoculation of CHIKV. The antiviral activity of each extract is displayed in Figures 4.6-4.10.

Based on Figures 4.6 to 4.10, all tested extracts did not achieve the half-maximal effective concentration (EC_{50}) with cell viability percentage ranging from 1.00 to 23.13%. Among these, the water and hexane extracts of *Vernonia amygdalina* exhibited potential activity against CHIKV infection at the concentrations of 20 µg/mL and 80 µg/mL respectively with a mean cell viability percentages of 23.13 ± 0 and 19.09 ± 0 correspondingly. Moreover, among the various extracts of plants studied, hexane extract of *Ipomoea aquatica* and *Rhapis excelsa* together with hexane, ethyl acetate, and chloroform extracts of *Persicaria odorata* had notable activity against CHIKV infection at MNCC among the respective plant groups.

As for the antiviral control group, the drug Chloroquine displayed highest activity against CHIKV at the concentrations of 620 to 1240 µg/mL with mean cell viability percentage ranging from 65.47 ± 0.02 to 80.87 ± 0.06. Table 4.2 shows the Vero cell viability percentage after the treatment with Chloroquine following the inoculation of CHIKV in 72 hours post-inoculation antiviral assay.

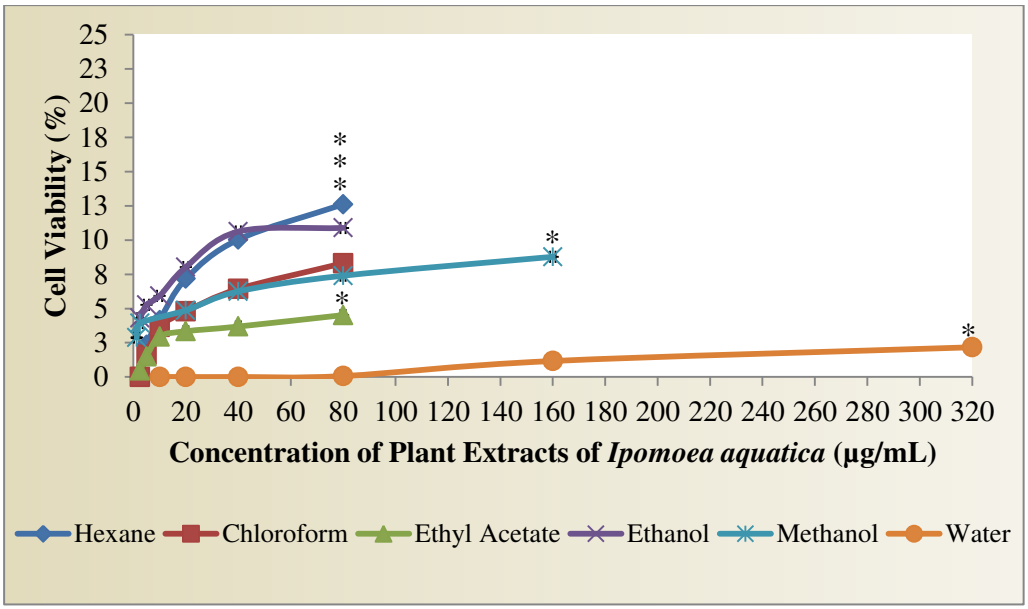


Figure 4.6: Antiviral activity of *Ipomoea aquatica* extracts at different concentrations against CHIKV in 72 hour post-inoculation antiviral assay. *Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test; Multiplicity of infection = 1

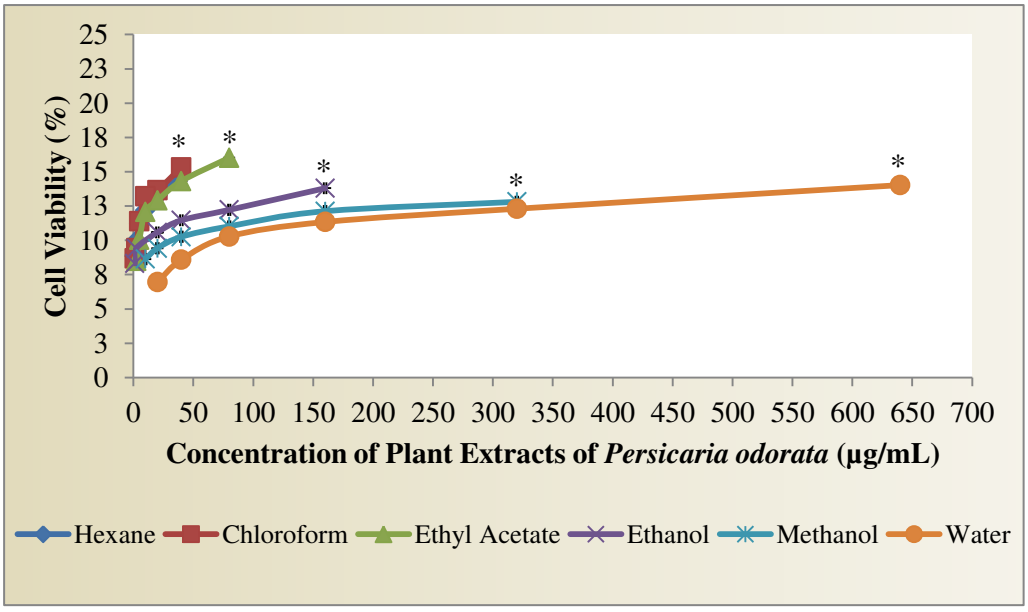


Figure 4.7: Antiviral activity of *Persicaria odorata* extracts at different concentrations against CHIKV in 72 hour post-inoculation antiviral assay. *Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test; Multiplicity of infection = 1

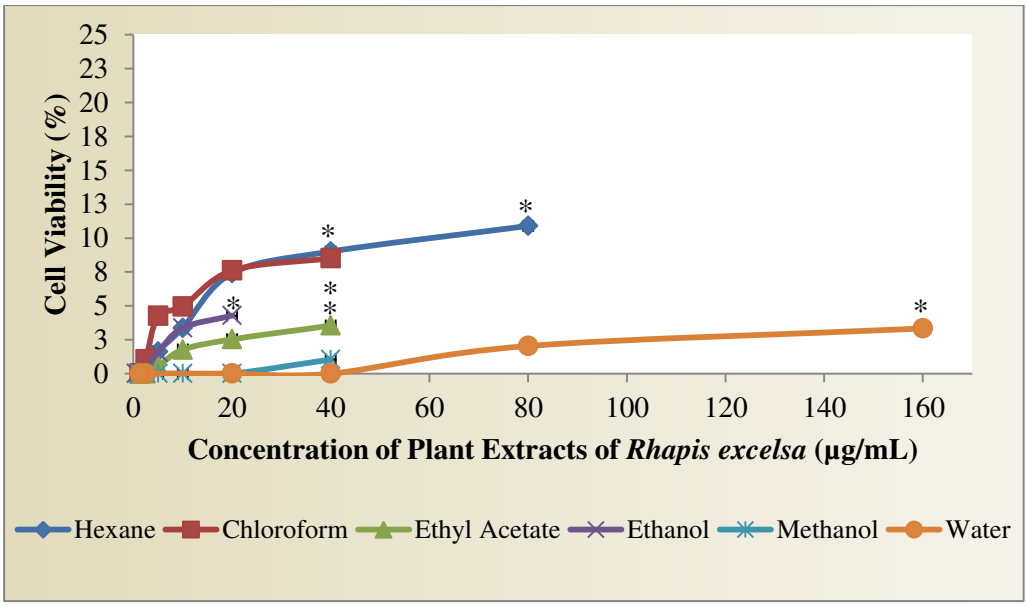


Figure 4.8: Antiviral activity of *Rhapis excelsa* extracts at different concentrations against CHIKV in 72 hour post-inoculation antiviral assay. *Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test; Multiplicity of infection = 1

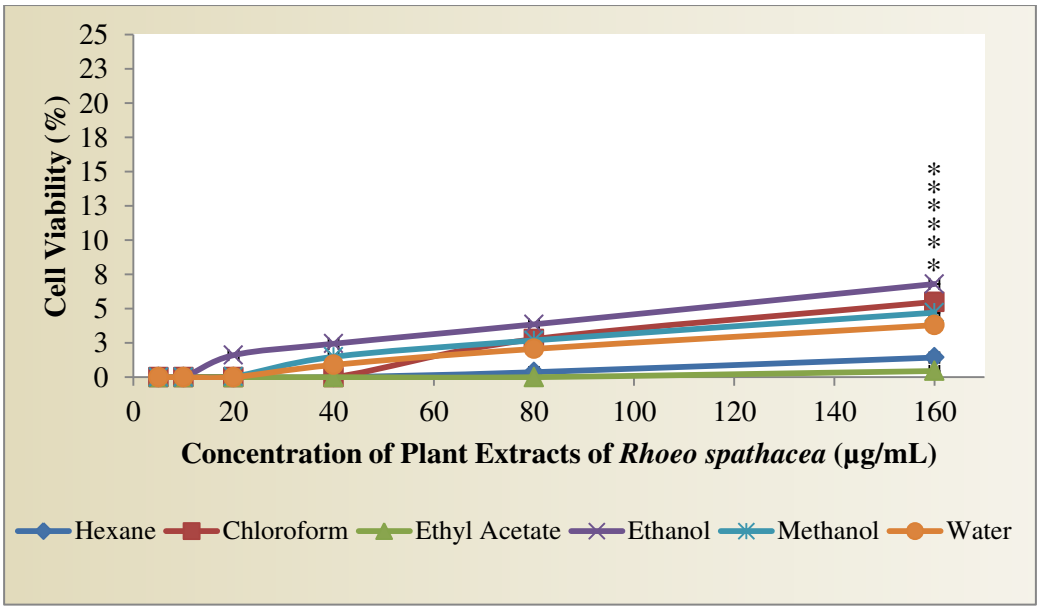


Figure 4.9: Antiviral activity of *Rhoeo spathacea* extracts at different concentrations against CHIKV in 72 hour post-inoculation antiviral assay. *Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test; Multiplicity of infection = 1

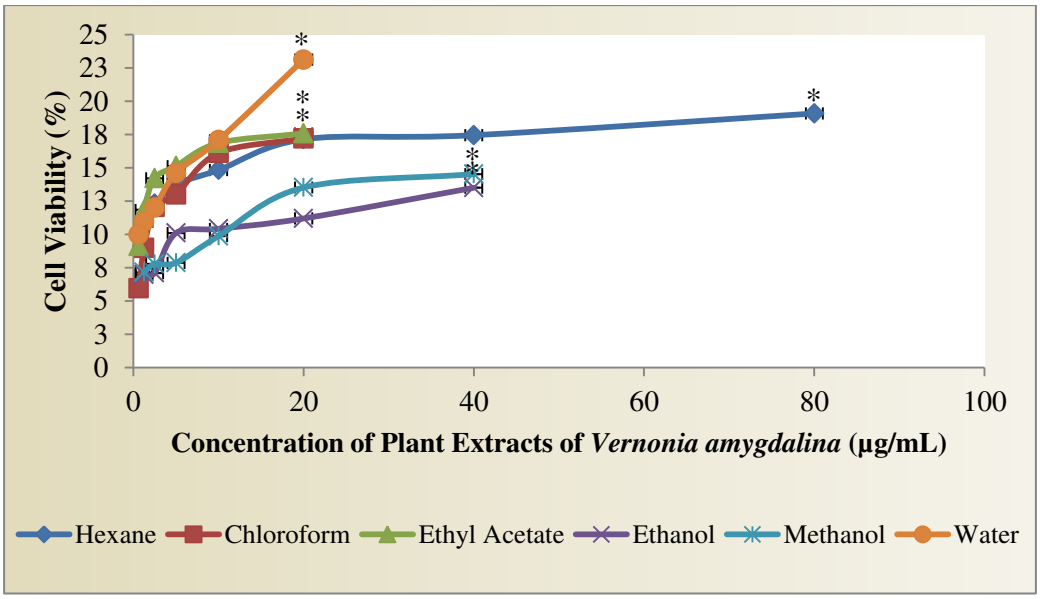


Figure 4.10: Antiviral activity of *Vernonia amygdalina* extracts at different concentrations against CHIKV in 72 hour post-inoculation antiviral assay. *Significant differences at $p < 0.05$ among different concentrations using One-way ANOVA test; Multiplicity of infection = 1

Table 4.2: Antiviral activity of Chloroquine against CHIKV in 72 hour post-inoculation antiviral assay.

Concentration (µg/mL)	**Mean cell viability (%)**
1240	80.87 ± 0.03
620	65.47 ± 0.02
310	49.94 ± 0.05
155	19.92 ± 0.04
77.5	NVC
38.8	NVC

Mean ± SD (Standard deviation, n=3)
NVC: No viable cell

CHAPTER 5

DISCUSSION

5.1 Preparation of Plant Extract

Most biological agents are poorly soluble in water and tend to be more soluble in lipophilic solvents (Kelava, Cavar and Culo, 2011). The stock solutions of various plant extracts were prepared by diluting crude extracts into the hydrophobic solvents DMSO:Ethanol at a ratio of 6:4, v/v. The solvents ethanol and DMSO are extensively applied both *in vitro* and *in vivo* assays as carrier solvents for hydrophobic chemicals and to a lesser extent hydrophilic chemicals (Adefolaju, Theron and Hosie, 2015). Studies suggests that the solvent DMSO has tendencies to increase the diffusion of pharmacologically active compounds through cell membrane by disruption of cell barrier function. This phenomenon can be explained through the aprotic interactions that DMSO mediate with the intercellular lipids and through reversible distortion of lipid head groups of the phospholipid bilayer in order to produce a greater permeable packing arrangement (Capriotti and Capriotti, 2012).

Inspite the fact that DMSO is comprehensively employed in cell-based assays, this solvent however does not dissolve all compounds and frequently, other solvents are used in combination to achieve sufficient solubility. Ethanol is a principal carrier in pharmacology and biochemistry (Maes et al., 2012). Studies shows that its lipophilic carbon tail, has the capacity to concentrate at the interface between lipid and surrounding water forming hydrogen bonds with

the hydrophilic head groups of bilayer giving rise to an increase in area per lipid, contributing to overall fluidity of the membrane to deliver hydrophilic chemicals more easily in unison with DMSO (Patra et al., 2006; Gurtovenko and Anwar, 2009).

Studies on the effects of DMSO:ethanol on Vero cells by Chan (2013) revealed that at the concentration of 1% and below, cytotoxic effects on the Vero cells was not apparent and cell viability remained at 100%. In this study, the concentration of DMSO:Ethanol used on Vero cells were 0.5% and below.

5.1 NRU Cytotoxicity Assay

According to Betancur-Galvis et al. (2002), the half-maximal cytotoxic concentration (CC_{50}) is the concentration of test extracts that reduces the absorbance value at 540 nm of treated uninfected cells to 50% of that of untreated uninfected cells. According to Anyango (2011), definition of cytotoxicity on Vero cells is as follows: CC_{50} < 10 µg/ml, high toxicity; CC_{50} 11-50 µg/ml, moderate toxicity; CC_{50} 51-100 µg/ml, mild toxicity; and CC_{50} > 100 µg/ml not toxic.

In this study, none of the extracts tested was highly toxic though mild toxicity was observed in the ethanol and methanol extract of *Rhapis excelsa* and ethyl acetate extract of *Vernonia amygdalina*. No previous studies on all tested plants were recorded against Vero cells. However, a search through the literatures showed that the ethanol extract of *Calamus gibbsianus* belonging to the same family as *Rhapis excelsa* (Arecaceae) demonstrated moderate to high

toxicity against Vero cells. Meanwhile, ethyl acetate extract of the plant *Vernonia zollingerianoides* belonging to same genus as *Vernonia amygdalina* (Asteraceae) revealed moderate toxicity on Vero cells (Siti et al. 2012; Roshida, 2014) .

On the contrary, MNCC of test extracts is defined as the highest concentration tolerated by the treated uninfected cells without toxic symptoms (Orsine et al., 2012). The ethanol extract of *Rhapis excelsa* together with chlorofom, ethyl acetate and water extract of *Vernonia amygdalina* demonstrated the lowest MNCC value of 20 µg/mL. However, no previous studies were reported on the MNCC of these plant extracts on Vero cells.

5.2 Post-Inoculation Antiviral Assay

By comparing the Figures 4.6 to 4.10 (page 60-62) it is apparent that all 30 tested extracts from the various plants of study did not achieve the half-maximal effective concentration (EC_{50}). However, the water and hexane extracts from the leaves of *Vernonia amygdalina* has a potential activity against CHIKV than the aerial and leaf extracts of all other tested plants. This was assumed to be due to presence of plant-derived secondary metabolites with various viral targets ranging from adsorption of the virus to the host cell to release of the virus from the cells. Some common plant metabolites in this category include alkaloids, flavonoids, and steroid saponins (Wink, 1999).

Flavonoids are polyphenolic compounds that are present in different plants. They are well known for various biological properties including: antimicrobial

activity, anti-inflammatory activity, anti-allergic activity, and antitumor activity (Lani et al., 2015). Phytochemical screenings of leaf parts of the plant *Vernonia amygdalina* and *Ipomoea aquatica* revealed the presence of flavonoids especially luteolin, luteolin 7-O-glucosides and luteolin 7-O-glucuronide in *Vernonia amygdalina* (Farombi and Owoeye, 2011) and mycertin, quercetin, luteolin and apigenin in aerial parts of the plant *Ipomoea aquatica* (Igwenyi et al., 2011). According to Zandi et al. (2011), antiviral activities of flavonoids have been demonstrated against numerous viruses including human cytomegalovirus (HCMV), Herpes Simplex Virus-1 and 2 and some types of human adenoviruses. A recent study conducted by Murali et al. (2015) on the activity of luteolin and apigenin rich fractions from ethanolic extract of *Cynodon dactylon* against CHIKV revealed potent, 98% virus inhibition activity at the concentration of 50 µg/mL. The mechanisms of antiviral activity were reported to revolve mainly in interfering with viral nucleic acid synthesis by binding to the viral polymerases, preventing viral entry and enhancing activity of interferon (Lani et al., 2015; Murali et al., 2015).

Alkaloids are important chemical compounds that serve as a rich reservoir for drug discovery. Alkaloids such as nortropane alkaloids constitute as the major secondary metabolite in the aerial parts of the plant *Ipomoea aquatica* (Malakar and Choudhury, 2015). Studies shows that nortropane alkaloids possess glycosidase inhibitory activity and thus has a potential utility as an antiviral agent (Rahman, 2012). The viral envelope glycoproteins are often essential for virion assembly and secretion and infectivity. Compounds that

interfere with the glycosylation processes of viral glycoproteins such as nortropane alkaloids may have significant effects in the inhibition of infectivity (Asano, 2003). Alkaloids were also reported as a major constituent of the leaf parts of the plants *Vernonia amygdalina* and *Rhoeo spathacea* (Farombi and Owoeye, 2011; Nikam et al., 2013). However, limited studies been reported on the type of alkaloid present in these two plants.

The presence of terpenes such as triterpene lupenone in *Rhoeo spathacea* was revealed by Nikam et al. (2013). Leyssen et al. (2014) reported that triterpene lupenone had moderate to significant antiviral activity against CHIKV and other alphavirus, the mode of action revolves mainly in the inhibition of viral replications. Other plant secondary metabolites that are reported to possess potent antiviral activity include sesquiterpene lactones that is demonstrated in *Persicaria odorata* (Sasongko, Laohankunjit and Kerdchoechuen, 2011; Ridzuan, 2013) and saponins such as vemoniosides D and E that are present in *Vernonia amygdalina* which exhibits virucidal mechanism especially by coating the viral binding sites and preventing attachments in Rotavirus infections (Adiukwu et al., 2013; Arthanari et al., 2013). Meanwhile, sesquiterpene lactones such as chlorohyssopifolin A, chlorojanerin and 13-acetyl solstitialin A were reported to have activity against herpes simplex virus type 1 (HSV-1) (Özçelik et al., 2009). However, the mechanism may not be similar in CHIKV and other alphaviruses since their genome is composed of a positive sense single stranded RNA virus (Pun, Bastola and Shah, 2014).

Our results of Chloroquine matched the data reported in the study conducted by Khan et al. (2010). Chloroquine being a weak base is thought to possess multiple mechanism of action that differs predominantly based on the type of pathogen being targeted (Farias et al., 2014). In the case of viral infection, its therapeutic value lies in its ability to accumulate in the acidic vesicles of a cell such as the endosome, lysosome vesicles and the Golgi apparatus consequently increasing the pH therein. The increase of pH prevents the low pH-induced fusion of viral envelope and cell endosome membranes, successfully blocking the entry of viruses into the cytosol (Farias, Machado and da Fonseca, 2013; Farias et al., 2014).

Chloroquine is a highly lipophilic drug in nature (Day, 2005). According to Adams and Merluzzi (1993) hydrophobicity of compounds is an essential criterion to achieve high antiviral activity. The hydrophobic property of chloroquine explains its ability to penetrate the lipid membrane and accumulate in the acidic vesicles of infected cells. In this study, the notable activity demonstrated by the less polar extracts such as the hexane extract of *Ipomoea aquatica* and *Rhapis excelsa* and hexane, chloroform and ethyl acetate extracts of *Persicaria odorata* was assumed due to their non-polar nature. However, this finding contradicts with studies conducted by Sangeetha and Rajarajan (2015) on five medicinal plants from the genus alpinia, andrographis and azadirachta against CHIKV that reports polar extracts such as water and ethanol had more prominent activity against the virus. However, the activity demonstrated by certain plants at a more polar solvent might not be the same for all other plants (Anyango, 2011).

5.3 Limitations

Despite the presence of various secondary metabolites with significant antiviral activity, all tested plant extracts did not achieve the half-maximal effective concentration (EC_{50}). This may be due to limitations on crude extract, which often contain various compounds that may interfere with the bioassay antagonistically.

5.4 Future Perspectives

Other parts of the plants such as the roots and stem of *Ipomoea aquatica* and *Persicaria odorata* and the fruit, roots and stem of *Rhapis excelsa, Rhoeo spathacea* and *Vernonia amygdalina* can be used for screenings against CHIKV in future. The various extracts can also be assessed on other viruses such as dengue virus, an endemic virus in Malaysia.

Meanwhile, the active secondary metabolites in the water and hexane extracts of *Vernonia amygdalina* can be isolated and purified using the high performance liquid chromatography, column chromatography and thin layer chromatography. Furthermore, CHIKV viral load can be measured using the real-time polymerase chain reaction technique in order to study the mode of action of the tested extracts.

CHAPTER 6

CONCLUSION

The NRU assay demonstrated the lowest CC_{50} for ethanol (51.67 ± 2.89 µg/mL) extract of *Rhapis excelsa* followed by ethyl acetate (86.87 ± 2.89 µg/ mL) extract of *Vernonia amygdalina* and methanol (91.67 ± 2.89 µg/mL) extract of *Rhapis excelsa*. Moreover, all the three extracts together with chlorofom and water extracts of *Vernonia amygdalina* were reported to have the lowest MNCC at 20 µg/mL.

In addition, all tested extracts did not achieve the half-maximal effective concentration (EC_{50}), with cell viability percentage ranging from 1.00 to 23.13%. Among them the water and hexane extracts of *Vernonia amygdalina* exhibited potential activity against CHIKV infection at the concentrations of 20 µg/mL and 80 µg/mL with a mean cell viability percentage of 23.13% ± 0 and 19.09% ± 0 respectively.

Furthermore, among the various extracts of plants studied, hexane extract of *Ipomoea aquatica* and *Rhapis excelsa* together with hexane, ethyl acetate and chloroform extracts of *Persicaria odorata* had notable activity against CHIKV at MNCC.

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