IDENTIFICATION OF ANTIVIRAL SECONDARY METABOLITES FROM SWEET LEMON GRASS, *CYMBOPOGON NARDUS* (L.) RENDLE, INHIBITING MEASLES VIRUS

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DECLARATION

I hereby declare that no portion of the work contained in this thesis has been submitted in support of any application for any other degree or qualification of this or any other university or institution of higher learning.

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Identification of antiviral secondary metabolites from sweet lemon grass, *Cymbopogon nardus* (L.) Rendle, inhibiting measles virus

ABSTRACT

An *in vitro* study was carried out to investigate the antiviral effects of sweet lemon grass (*Cymbopogon nardus* (L.) Rendle) on measles virus (MV). Crude extract of this plant were prepared using hexane and fractionation of this extract was done using column chromatography (CC). First CC produced a total of 20 combined fractions and further fractionation on selected fractions resulted in 77 combined subfractions. The toxicity of all fractions and subfractions towards Vero cells ranged from mildly toxic (50 μg/ml) to considerably non-toxic (600 μg/ml). In the antiviral test, majority of the fractions inhibited the production of MV-induced cytopathic effects (CPE) by more than 50%. Some of them were able to inhibit the CPE by more than 75%, which was similar to the activity showed by positive control, Ribavirin, which is 80%. Most of the subfractions conferred around 50% of protection to the cells, but majority of them had lower antiviral activity compared to their derived fractions.In an attempt to study the mode of antiviral action, pre-treatment and post-infection protocols were used. It was found that post-infection protocol was more effective among fractions, suggesting that the fractions may interfere with any of the steps in late stage of MV replication. However, the subfractions did not give any particular trend as to the most effective method of treatment. Active subfractions were then further purified using preparative thin layer chromatography (PTLC) and produced 11 isolated compounds. These compounds possess weak antiviral activity with majority of them inhibited less than 50% of virus CPE. Most of isolated compounds provided better protection to the cells in the pre-treatment

protocol, suggesting that the potential sites of activity may include inhibition of virus binding and/or entry which can be mediated by a number of cellular receptors such as CD46 present on Vero cells. Meanwhile for the synergistic assay, the combination of Ribavirin with isolated compounds at lowest concentration (0.01 LC_{50}) produced synergistic effect, either in one or both protocol. The clear synergistic tendencies displayed by these substances combination allows for the reduction of Ribavirin concentration, which minimizes toxicity and the probability of formation of resistance to this drug. This synergistic activity is probably connected to the different mechanisms of action of Ribavirin and isolated compound. Additional test, anti-proliferation assay, showed that the isolated compounds possess weak activity towards human papillary ovarian adenocarcinoma (Caov-3) cancer cells as they were able to inhibit less than 50% of the cells growth at highest concentration tested. Result also showed that they were toxic to normal cells as their cytotoxic values on cancer cells (>400 µg/ml) were much higher than that of the normal cells (150 to 270 µg/ml) and the reference drug, Tamoxifen (150 µg/ml). The GC-MS analysis revealed that majority of the active isolated compounds had more than one constituent that were accounted for their inhibitory activity. The constituents are made up of monoterpene, sesquiterpene and hydrocarbon that present commonly in the essential oil of *C. nardus*, such as methyl eugenol, citronellol and geraniol.

Keywords: *Cymbopogon nardus* (L.) Rendle, measles virus, cytotoxicity, antiviral, synergistic, anti-proliferation, GC-MS.

Pengenalpastian metabolit sekunder antivirus dari serai wangi, *Cymbopogon nardus* (L.) Rendle, yang merencat virus measles.

ABSTRAK

Satu kajian secara *in vitro* telah dilakukan untuk mengkaji aktiviti antivirusserai wangi (*Cymbopogon nardus* (L.) Rendle) terhadap virus measles. Ekstrak serai wangi disediakan menggunakan heksana dan pemfraksian menggunakan kromatografi turus (CC). Pemfraksian kali pertama menghasilkan 20 fraksi gabungan dan pemfraksian seterusnya ke atas fraksi-fraksi terpilih menghasilkan 77 subfraksi gabungan. Ujiantoksisiti ke atas fraksi dan subfraksi terhadap sel Vero menunjukkan bahawa nilai ketoksikan (LC_{50}) adalah di antara sederhana toksik (50 μg/ml) ke tidak toksik (600 μg/ml). Bagi ujian antivirus, kebanyakan fraksi berjaya menghalang pembentukan kesan sitopatik (CPE) virus lebih dari 50%. Terdapat juga fraksi yang mampu menghalang pembentukan CPE sehingga 75%, iaitu menghampiri peratusan yang ditunjukkan oleh kawalan positif, Ribavirin, iaitu sebanyak 80%. Kebanyakan subfraksi pula berjaya menghalang pembentukan CPE sebanyak 50%, namun majoriti subfraksi mempamerkan aktiviti antivirus yang lebih rendah berbanding dengan fraksi asal. Untuk mengkaji mod tindakan antiviral, protokol pra-rawatan dan pasca-rawatan telah dilakukan. Hasil yang diperolehi menunjukkan bahawa fraksi adalah lebih efektif dalam protokol pasca-rawatan dan ini mencadangkan bahawa fraksi mungkin mengganggu fasa akhir kitar replikasi virus. Walaubagaimanapun, tidak seperti fraksi, subfraksi tidak menunjukkan trend tertentu sebagai kaedah rawatan yang paling berkesan. Subfraksi yang aktif telah dipilih untuk proses penulenan selanjutnya menggunakan kromatografi lapisan nipis preparatif (PTLC) dan 11 sebatian terasing

telah terhasil. Sebatian terasing mempamerkan aktiviti antivirus yang lemah, di mana kebanyakannya berjaya menghalang pembentuakn CPE kurang dari 50%. Bagi mod tindakan, kebanyakan sebatian terasing memberikan perlindungan yang lebih baik kepada sel dalam protokol pra-rawatan. Ini mencadangkan bahawa sebatian ini berpotensi mengganggu proses perlekatan virus pada reseptor sel seperti CD46 yang hadir pada sel Vero. Sementara itu, bagi ujian sinergistik, kombinasi Ribavirin dengan sebatian terasing pada kepekatan terendah (0.01 LC_{50}) menunjukan kesan sinergi, sama ada bagi satu atau kedua-dua protokol. Kesan sinergi ini membolehkan pengurangan kepekatan Ribavirin yang digunakan sekaligus mengurangkan ketoksikan dan kebarangkalian pembentukan rintangan terhadap dadah ini. Kesan sinergistik ini mungkin disebabkan oleh mekanisme tindakan berbeza antara Ribavirin dan sebatian terasing. Ujian tambahan iaitu ujian antiproliferasi pula menunjukkan sebatian terasing mempunyai aktiviti yang lemah terhadap sel kanser ovari manusia (Caov-3) dimana ia hanya mampu merencat pertumbuhan sel ini kurang dari 50% pada kepekatan tertinggi. Hasil juga menunjukkan bahawa sebatian terasing mempunyai nilai toksik yang lebih tinggi ke atas sel kanser (>400 µg/ml) berbanding sel normal (150-270 µg/ml) dan kawalan positif, Tamoxifen (150 µg/ml). Analisis GC-MS menunjukkan bahawa kebanyakan sebatian terasing mempunyai lebih dari satu komponen yang menyumbang terhadap aktiviti biologi mereka. Sebatian terasing tersebut terdiri daripada monoterpena, susquiterpena dan hidrokarbon yang biasa hadir dalam minyak pati *C. nardus* seperti metil eugenol, citronellol dan geraniol.

Kata kunci: *Cymbopogon nardus* (L). Rendle, virus measles, sitotoksisiti, antivirus, sinergistik, antiproliferasi, GC-MS

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LIST OF ABBREVIATIONS

CC	Column chromatography
CC_{50}	Cytotoxic concentration (the concentration of drug that results in toxicity of 50% of the cells compared with untreated control cells)
CPE	Cytopathic effect
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
EtOH	Ethanol
EtoAc	Ethyl acetate
FBS	Foetal bovine serum
FH	Fraction of hexane
FIC	Fractional inhibitory concentration (value utilised to *define* the degree of synergy)
GC-MS	Gas Chromatography-Mass Spectrometry
IC_{50}	Inhibitory concentration (the concentration of test materials that inhibit the viral infection by 50%)
LC_{50}	Lethal concentration (the concentration of test materials which kill 50% of viable cells)
MeOH	Methanol
ml	Millilitre
mg	Milligram
MV	Measles virus

OD	Optical density
PTLC	Preparative thin layer chromatography
Rf	Retention factor
$TCID_{50}$	Tissue culture infectious dose (the amount of virus that produce pathological change in 50% of cell cultures inoculated)
TLC	Thin layer chromatography
µl	Microlitre

CHAPTER 1: INTRODUCTION

Measles is a highly contagious viral disease that infects nearly every susceptible person (Hilleman, 2001) and has been considered to be one of the leading causes of death among young children globally (World Health Organization [WHO], 2011). Despite the availability of an effective vaccine,an estimated 164,000 people have died from measles in 2008, mostly being children under age of five (WHO, 2009). This phenomenon has been attributed to two basic issues, which are, firstly, inadequate vaccination campaigns in underdeveloped and developing countries (Mulder *et al*., 2001), and secondly, undesirable patients' responses as described by Webster *et al*. (2005).

With regards to the first issue, Mulder *et al*. (2001) explained that the success of vaccination campaigns is perceived to be dependent on achieving coverage rates sufficient to interrupt disease transmission to infants who are at greatest risk for the life-threatening disease. However, achieving this goal on a global scale, particularly in underdeveloped and developing countries, is impractical because of limitations in the production, distribution and delivery of vaccines. The problem is further confounded by the undesirable patients' responses that can be attributed to the presence of maternal antibodies in infants, whereby measles vaccine may become less effective (Webster *et al*., 2005). This situation may result in the inhibition of successful vaccination and subsequent protection afforded by the vaccine against the disease. In addition, the measles vaccine may cause illness in immunocompromised patients, especially in areas of high prevalence of HIV/AIDS, which at the same time are most at risk from measles (Moss *et al*., 1999). Under these circumstances, the development of plant based complementary

and alternative medicine for measles is required to address these limitations and facilitate its eradication.

Plants have been used as the natural source of medicines for thousands of years and continue to provide mankind with new remedies for various ailments (Gurib-Fakim, 2006). Many important drugs such as aspirin, atropine, cocaine, codeine, digoxin, morphine and quinine are all discovered from plants (Wang, 2008). Today, about 25 percent of modern medicines are derived from secondary products of plants, which have been used in traditional medicine (WHO, 2003). According to Cseke *et al*. (2006), the medicinal properties of plants are usually conferred by their secondary metabolites, which act as defence elements against different organisms as well as for their survival in harsh and challenging environments. Studies have showed that these secondary metabolites possess various biological activities including antibacterial, antifungal, antiviral and anticancer (Ooi *et al*.,2004; Yarmolinsky *et al*., 2009).

Thus, there is relationship between the presences of biologically active compounds with the use of plants in traditional medicine. The search for biologically active compounds with medicinal properties from plants that are traditionally used for similar diseases form a very valuable shortcut to drug discovery (Lopez *et al*.,2001). The success of this approach is exemplified by the discovery of many new drugs and hundreds of pharmacologically active substances for synthetic modifications. With the abundance of plant resources connected to medicinal applications, it would be beneficial to use such plants for screening antiviral plant secondary products from such plants.

In Malaysia, sweet lemon grass (*Cymbopogon nardus* (L.) Rendle) has been widely used in Malay traditional medicine for treatment of various ailments such as skin cuts, diarrhoea (Burkill, 1966), joint pains, bronchitis and malaria (Perry & Metzar, 1980). Studies showed that the crude extracts and partially purified subfractionsare active against Newcastle disease virus (NDV), herpes simplex virus type-1 (HSV-1) and measles virus (MV) (Ahmad *et al*., 1992; Hanina, 2006; Nurul Aini *et al*., 2006). Some of the partially purified subfractions also showed synergistic effects in combination with the toxic antiviral drug, Ribavirin. However, the study did not identify active substances. Therefore, further studies to determine the active principles, most importantly with novel mechanisms of action, should be carried out.

Bioassay-guided fractionation, which involves serial fractionations of plant extract using standard chromatographic methods until the pure compounds or partially purified fractions are obtained (Freitas *et al*., 2009), has been widely applied in the screening for antiviral compounds from plants. Each step of fractionation will increase the purity of fractions while retaining the antiviral activity. During fractionation process, it is often encountered that biological activities exist in not just one or two fractions, but in several fractions at different relative strength (Nurul Aini *et al*., 2006; Adibah *et al*., 2010). It is possible that the low activity may be due to low concentration of the active compound in the fractions (Kim *et al*., 2009; Ianora *et al*., 2011). Thus, in this study, fractions with low activities were also selected for further fractionation.

In this study, two different protocols are used to determine the mode of antiviral action of fractions during the virus reproductive cycle, whether it occur before or after virus entry into the cells. In the pre-treatment protocol, cells are incubated with fractions which were discarded

before addition of virus. If some of the active compounds remained associated with the cells, it might still continue to protect the cell even after adding the virus (Wirotesangthong *et al*., 2006). In post-infection protocol, fraction is added to the cells after virus infection. The active compound may interfere with any steps in virus replication cycle such as uncoating, replication, transcription and translation (Roner *et al*., 2007).

Combinations of drugs with dissimilar mechanisms or modes of action may direct the effect against a single target with more effective therapeutic outcome (Chou, 2006). Besides, drug combinations may also offer increased antiviral efficacy while decreasing cytotoxicity by minimizing the required therapeutic doses (Barquero *et al*., 1997). The ability to complement the mechanism of action of the existing antiviral drugs such as Ribavirin is an important characteristic of a newly isolated antiviral substance.

Thus, the main aim of this study is to purify and identify substances which possess anti measles virus activity individually or in combination with Ribavirin.The specific objectives are:

1. To fractionate, purify and identify secondary metabolites from *C. nardus*.
2. To screen for the cytotoxicity and antiviral activity of fractions and secondary metabolites.
3. To determine presence of synergistic effect from combination of *C. nardus* fractions with Ribavirin against measles virus

CHAPTER 2: LITERATURE REVIEW

2.1 MEASLES

Measles is a deadly viral disease that can infect nearly every susceptible person through droplet or airborne exposure. For this virus to persist in a population, the size of that population should be at least 500,000 (Hilleman, 2001). This disease was first observed as early as the seventh century by the Hebrew physician, Al-Yehudi but the symptoms and signs of measles were first described in details by Persian physician, Al-Razi or Rhazes in his book entitled *Kitab al-Jadari wa 'I-Hasba* (Book on Smallpox and Measles) in the tenth century (Kaadan, 2007). Globally, measles affected more than 40 million people each year and causes approximately 500,000 deaths (Parker *et al*.,2007). In roughly the last 150 years, this disease has been estimated to have killed about 200 million people worldwide (Torey & Yolken, 2005). Despite the availability of an effective vaccine, measles is still a leading cause of death among children,especially in developing countries (Sonibare *et al*.,2009). It has been reported that more than 600 children die daily as a result of measles infection. In 2006 alone, approximately 242,000 children died from this disease, often from secondary complications related to pneumonia, diarrhoea and encephalitis.

2.1.1 The viral aetiology of measles

Measles is caused by the measles virus (MV). The virus is an enveloped, single-stranded, negative-sense RNA virus belonging to the genus *Morbillivirus* of the family Paramyxoviridae

(Jeulin *et al*., 2006). Measles is the only known of the genus that infect human, whereas other members, namely rinderpest virus, canine distemper virus, peste des petits ruminants virus and cetacean and porcine distemper virus infect animal hosts. In addition to human host, MV also infects non-human primates (Rota *et al*.,2009). Structurally, MV is highly pleomorphic with a diameter of 150 to 300 nm and consists of an outer lipid bilayer envelope that derived from the host cell (Wolfgang, 1988). MV genome which consists of 15,894 nucleotides codes for the six structural proteins, namely the nucleoprotein (N), phospoprotein (P), matrix (M), fusion (F), haemagglutinin (H), and large protein (L). The F and H proteins are responsible for fusion of virus with host cell membrane, allowing viral penetration and release of the nucleocapsid complexes into cytoplasm, where replication occur (Hilleman, 2001). MV is considered monotypic although genetic heterogeneity has been detected among wild-types strain, with 23 genotypes has been recognized circulating in different part of the world (Chironna *et al*.,2007). However, this slight genotypic variation has not affected the protective efficacy of live-attenuated measles vaccines (Abdelwahab, 2005).

Two cellular proteins have been identified as MV receptors, which are CD46 and signalling lymphocytes activation molecule (SLAM) (Bellini & Icenogle, 2007). CD46 is a regulator of complement activity. This protein is present on most cell types and appears to promote the entry of vaccine and certain wild type viruses. SLAM, also called CD150, is a regulator of antigen-driven T-cell responses and macrophage functions. It is used by both attenuated and wild-type MV for entry. The expression of SLAM is restricted to certain cells of the immune system, including activated B and T lymphocytes, mature dendritic cells and macrophages (Tahara *et al*.,2005).

2.1.2 Epidemiology

In temperate areas, the peak incidence of measles infection occurs during late winter and spring (Wolfgang, 1988), but it is not seasonal in the tropics (Rima, 2001). MV is known to be transmitted via direct contact with the contaminated respiratory droplets or through direct inhalation of aerosolised virus particles (WHO, 2005). It replicates in tracheal and bronchial epithelial cells during the incubation period and then spread to the lymph nodes where it is amplified and gives rise to primary viremia. A secondary viremia which occurs 5 to 7 days later resulting in the dissemination of virus to multiple organs including the skin, gastrointestinal tract, liver, central nervous system and thymus (Ohgimoto *et al*.,2006). The immune suppression that accompanies measles significantly enhances an individual's susceptibility to secondary infection which is the reason for the morbidity and mortality of the disease (Schneider *et al*., 2002).

The introduction of routine vaccination against MV has dramatically reduced the incidence rate of measles worldwide (Muller *et al*.,2003). Measles vaccine is live attenuated virus strain derived from the Edmonston strain of the virus isolated in 1954 and prepared in chick embryo fibroblast tissue culture (Rima, 2001). This vaccine is dispensed as a monovalent vaccine or as a trivalent vaccine together with mumps and rubella vaccines (MMR vaccine) (Dudgeon & Cutting, 1991). Prior to the introduction of vaccine, almost all members of a given population contracted measles disease by adolescence. Case reports have fallen by over 90% and some countries have achieves almost complete eradication after vaccination programmes have been carried out (Selina, 2008). However, measles still remains as a common disease in certain industrialized and developing countries (WHO, 2001). The importance of measles vaccination

can be perceived to the extent that failure in administrating at least one dose of measles vaccine to all children has been claimed to result in nearly 50 percent of the 1.7 million deaths of vaccine-preventable childhood diseases mortality.

In Malaysia, measles was endemic in the prevaccine era until the single dose monovalent measles vaccine was included in the Expanded Program of Immunization (EPI) in 1982, which was conducted by the Ministry of Health (MOH) and supported by the Institute for Medical Research (IMR) (Saraswathy *et al*., 2009). The incidence rate of measles cases in 1982 was 65.2 cases per 100,000 populations, but decreased dramatically with more areas were covered by the vaccination programme. From 1990 to 2000, the vaccination programme has covered up to 88.4 percent, thereby reducing the incidence rates to 1.51 and 5.87 cases per 100,000 populations. In 2002, the monovalent measles vaccine was replaced by trivalent MMR vaccine and it is given to children in two doses, once at 12 months and the second at 7 years of age.During this time, the measles vaccination coverage was above 85 percent and the estimated incidence of measles ranged from 22.3 cases (in 2004) to 2.27 cases (in 2006) per 100,000 populations.This vaccination strategy appears to have been successful in reducing the incidence of measles. However, in order to achieve the goal of measles elimination, continuing high vaccination coverage rates and ongoing measles surveillance are necessary.

2.1.3 Clinical features

The first sign of measles is a distinct prodrome, which begins about 10 to 12 days after exposure to the virus, and lasts 2 to 4 days (Centers of Disease Control [CDC], 2012). The prodrome is

characterised by fever, malaise, conjunctivitis (watery eyes), coryza (runny nose) and cough. Towards the end of this stage, the body temperature can rise to as high as 40°C and Koplik spots (red spots with bluish-white specks in the centre) appear on the mucous membrane of the oral mucosa. Koplik spots are considered to be pathognomonic for measles and it occurs 1 to 2 days before the onset of the rash, and last for 1 to 2 days after the onset of the rash. After several days, the erythematous and maculopapular rash appears, usually starts behind the ears and on the forehead (Rollag & Haukenes, 1989). Over about 3 days, the rash spreads to cover most of the body and often causing itching. It usually persists for 4 to 5 days, and is most confluent on the face and upper body. The rash then fades in order of appearance and transient brownish discolouration appears after that.

The entire course of measles usually lasts 7 to 10 days in patients with a healthy immune system (Sabella, 2010). The cough is usually the last symptom to resolve. Patients are contagious 2 to 4 days before the onset of the rash and remain so through 4 days after the onset of the rash.

2.1.4 Complications

Measles is generally mild or moderately severe in healthy person and people who recover from measles acquire lifelong immunity. Most measles-related deaths are associated with disease complications. According to the World Health Organization (WHO) (2011), complications are more common in children under the age of five or adults over the age of 20. Approximately 30% of reported measles cases have one or more complications including pneumonia, ear infections

and diarrhoea (CDC, 2013).These complications are more common among children under 5 years of age and adults over 20 years old.

Measles commonly involves the central nervous system (CNS) with as many as 50 percent of cases reported to have electroencephalogram abnormalities during the acute or convalescent phase of the illness (Bellini & Icenogle, 2007). Acute disseminated encephalomyelitis (ADEM) occurs approximately a week after rash onset, and is characterized by resurgence of fever and by headache, seizures, confusion and coma (Rollag & Haukenes, 1989). This form of complication occurs at rate of one per 1,000 cases. Death occurs in 10 to 20 percent of patients, but majority of those who survive the disease have neurological sequelae of various severities (Matsumoto *et al*.,2005). Subacute sclerosing panencephalitis (SSPE) is an uncommon degenerative CNS disorder that occurs several years after measles infection (Haase *et al*.,1985). It is believed to be due to persistent MV infection of the CNS that occurs in approximately one per 100,000 measles cases. The course of SSPE is highly variable but usually starts with general intellectual and behaviour deterioration before neurological symptoms appear. The illness lasts from one to three years and lead to death.

Immunocompromised patients may succumb to additional risks of complication, which are syndrome such as giant-cell pneumonia and measles inclusion body encephalitis (MIBE). Measles-induced giant-cell pneumonia is usually unrecognized due to the absence of rash (Hilleman, 2001). MIBE is similar to SSPE but has a shorter incubation time (Cattaneo & Rose, 1992). It arises as a result of the bodies inability to eliminate the virus-infected cells because the lack of cytotoxic T-cells in the patients with MIBE (Duke & Mgone, 2003). Infections during

pregnancy result in higher risk of spontaneous abortion, premature labour and low birth infants (Bellini & Icenogle, 2007). The possibility of transplacental transmission has been deduced from the observation that infants delivered during the mother's incubation period of measles often develop a rash simultaneously with the mother.

2.1.5 Diagnosis

In most cases, diagnosis is made clinically based on history of fever of at least three days together with cough, coryza or conjunctivitis (Smith & Ritchie, 1980). The presence of Koplik spots on the oral mucosa is also a diagnostic feature for measles. Laboratory diagnosis is made after onset of rash by demonstrating multinucleated giant cells or fluorescent antibody-staining cells in nasal secretions, urine and skin biopsies. Routinely, measles infection is diagnosed serologically by demonstration of measles specific serum IgM antibodies in the acute phase or by at least a four-fold rise in IgG antibodies between acute and convalescent sera (Enders, 1996). A radioimmunoassay has been developed to detect the presence of measles IgM in saliva but this non-invasive technique is less sensitive compared to serum IgM detection (Azat *et al*., 2003). Isolation of virus from nasal secretions, throat, conjunctiva, urine or lymphocytes is also important in diagnosis especially for molecular epidemiologic surveillance in order to identify the source and route of the virus transmission but it is difficult to perform and therefore not suitable for routine diagnosis (Numazaki, 2007). The detection of RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) can also be used in complications and unusual manifestations of measles (Freeman *et al*., 2004).

2.1.6 Prevention

Shortly after one year of age, a single dose of measles vaccine is administered to all healthy infants to induce protective and permanent immunity against this virus (Atkinson *et al*., 2011).. The second dose of vaccine is then given between the ages of four to six years in order to boost the antibodies or to induce immunity in children with negative respond to the first dose. However, several precautions have to be considered when giving measles vaccine. Firstly, pregnant women and persons with severe allergic reactions towards vaccine components such as egg protein, gelatin and neomycin should not be vaccinated with measles vaccine (Patja *et al*., 2001). Administration of measles vaccine towards immunosuppressed or immunodeficient persons will cause replication of the vaccine virus (CDC, 2011). Secondly, measles vaccine cannot be given to infants younger than 9 months. Infants are generally protected from measles for six to eight months after birth due to immunity that is passed on from their mothers and these maternal antibodies may interfere with live-attenuated measles vaccination (Rollag & Haukenes, 1989).

2.1.7 Treatment

There is currently no specific treatment or cure for measles infection as most patients with uncomplicated measles will recover with rest and supportive treatment (Lam, 2010). However, some measures can be taken to protect vulnerable individuals who have been exposed to the virus. For non-immunized people, including infants, they may be given the measles vaccination within 72 hours of exposure to the measles virus, to provide protection against the disease (CDC, 2012). This is calledpost-exposure vaccination. If measles still develops, the illness usually has

milder symptoms and lasts for a shorter time. For pregnant women, infants aged 6 months to 1 year, infants younger than 6 months who are born to mother without measles immunity and people with weakened immune systems who are exposed to the virus, they may receive an injection of specific immunoglobulin (Ig) (Selina, 2008). Ig that is given within the first 3 days of exposure usually prevents infection or modifies the course and the protective immunity lasts approximately three to four weeks. However, it is not recommended to administer measles vaccine within 5 to 6 months after Ig administration because it can decrease the immune response to live virus vaccine. Ig therapy is comparatively expensive as it requires sterile materials and an uninterrupted cold-chain during transportation. Therefore, it is not recommended for the control of large measles outbreaks (Plemper & Snyder, 2009).

Chemotherapy using synthetic drugs is an alternative treatment. Despite the effectiveness of Ribavirin, either after intravenous or oral administration, alone or in combination with immune serum globulin in patients (Grancher *et al*., 2004), no chemotherapeutic agents that have been approved as prophylaxis or treatment against measles (Santagati *et al*., 2003). Ribavirin has been used to treat severely affected and immunocompromised adults with acute measles or SSPE as MV is susceptible to ribavirin *in vitro*. However, because there are no controlled trials have been conducted, ribavirin is not approved by the United States Food and Drug Administration (FDA) for this indication. The toxicity of ribavirin can cause the decreasing of red blood cells and not suitable for pregnant women, children, old citizens and anemia and thalassemia patients (Ogbru, 2010).

Vitamin A has also been suggested as supplement for reduction of severity of measles

infection (Frieden *et al*., 1992). This suggestion was based on the observation that people with low levels of vitamin A are more likely to have a more severe case of measles. Supplementation of vitamin A in developing countries during acute measles significantly reduces the risks in morbidity and mortality by 50% (WHO, 2005). Children diagnosed with measles recommended receiving two doses of 200,000 units of vitamin A supplement given 24 hours apart. However, large doses of vitamin A may be teratogenic and thus contraindicated in pregnancy (Selina, 2008). Fatal risk that has been revealed in animal studies has not been studied in human.

2.1.8 The search for new antiviral drug

Considering the mixed reports of efficacy and the additional limitations associated with antiviral agents, the development of novel, safe and efficacious compounds against MV is required. According to Plemper and Snyder (2009), a variety of different antiviral strategies for MV inhibition have been considered including antisense molecules, peptidic inhibitors, small molecule compounds, nucleoside analogs and natural extracts. In recent years, there has been an increasing interest in the use of natural substances and some questions concerning the safety of synthetic compounds have encouraged more detailed studies of plant resources (Astani *et al*., 2009). Plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources are of interest as possible sources to control viral infection.

Many naturally occurring plants, either as extracts or as pure compounds, have been reported to exhibit anti-measles viral activity. Nazlina *et al*. (2008) have reported that *Melastoma*

*malabathricum*showed a pronounced antiviral activity against MV. A study by Nardiah Rizwana *et al*. (2009) has demonstrated that various methanolic extracts of leaves from plants collected from two different forest reserves in Malaysia possess antiviral activity against MV. In Uganda, a study was done to screen antiviral activity of *Zanthoxylum chalybeum* against MV. They found that seed extracts of *Z. chalybeum* posses positive antiviral activities against this virus (Olila *et al*., 2002). Ethanolic extracts of *Bambusa vulgaris* and *Aframomum melegueta*, the traditional Nigeria medicinal plants, also showed pronounced antiviral activities against MV (Ojo *et al*., 2009). Another study on ethnomedicinal plant has also been carried out by Cos *et al*. (2004a) who showed an interesting antiviral activity of the leaves extract of *Macaranga kilimandscharica* against MV.

2.2 PLANTS AS A SOURCE OF MEDICINE

Throughout the ages, humans have relied on nature for their basic needs for the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours, fragrances and not the least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies (Schmidt *et al*.,2008). Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years (Gurib-Fakim, 2006).

The first records on the use of plant products in medicine are from Mesopotamia and dated back to approximately 2600 BC (Spainhour, 2005). They were written on clay tablets in

cuneiform and are still in use until today for the treatment of inflammation, influenza, coughing and parasitic infestation. Systematic study of plants in the 18th and 19th centuries has been one of the major factors in the development of drug research (Rishton, 2008). Many important drugs such as morphine, atropine, cocaine, codeine, papaverine, pilocarpine, digoxin, ergotamine, reserpine and artemisinin were all discovered from plants in the last centuries (Wang, 2008).

There have been times when the use of natural plant medicines has declined with the development of synthetic drug industry, especially in the developed countries. However, in the developing countries where synthetic drugs are expensive, traditional plants medicines continues to play an important role as therapeutic resource. For example, the World Health Organisation (WHO) has reported that 80 percent of people in developing countries still rely on plant-derived medicines for their healthcare (Parvez & Yadav, 2008). Thus, plants continue to be a major source of new lead compounds as they offer a unique and renewable resource for the discovery of potential new drugs and biological entities (Cos *et al*., 2006). Furthermore, the renewed interests in the used of medicinal plant is due to the fact that a lot of synthetic drugs are potentially toxic and are not free of side effects on the host (Astani *et al*.,2009). Researchers all over the world have been looking for formulation of new antimicrobial agents and evaluation of the efficacy of natural plant products as a substitute for chemical antimicrobial agents (Mahidol *et al*., 2002).

2.2.1 Medicinal plants and ethnobotany

Ethnobotany is the study of relationships between plants andpeople (Davidson-Hunt, 2000). Plants have been used as a source of medicine throughout history and their derived products have

dominated the human life for thousands of years almost unchallenged (Schmidt *et al*.,2008). According to Janssens and Subramaniam (2005), there are around 300,000 plant species exists in this world. About 35,000 to 70,000 of them are used worldwide for medicinal purposes and only 30 percents have been investigated scientifically (Mukherjee & Wahile, 2005). It has been estimated that 10,000 to 11,250 medicinal plant species are traditionally used in China, 7500 in India, 2237 in Mexico and 2500 by North American Indians (Hamilton *et al*., 2003). The World Health Organization (WHO) has compiled a list of over 20,000 common medicinal plants used in different parts of the world and many of these are known for their properties in several countries for use against different diseases (Maregesi *et al*.,2008). India, Korea, Japan, China, and Malaysia are the leading countries in the world that are using traditional plants as medicine (Wondimu *et al*., 2007)

The use of plants as medicine has been extensively documented in traditional medicine systems and it laid the basis for the discovery of modern medicine (Wondimu *et al*.,2007). Much of the knowledge about plants that have useful properties comes from native peoples (Cseke *et al*., 2006). According to Wijesekera (1991), this knowledge was acquired by "trial and error" methodology and transmitted from generation to generation. Ethnobotany term was first used by American botanist, John Harshberger in 1896 as he defined ethnobotany as a study of plants used by primitive and aboriginal peoples (Gerique, 2006).This term has beenredefined during the 20th century as the practice of ethnobotany has changed from the natural history of plantuses by primitive peoples to a wide range ofinterests of plants in cultural and ecological contexts (Soejarto *et al*.,2005). The early examples of drug inspired by a natural product from plant preparation with a rich ethnobotanic history are quinine and aspirin. Quinine, the anti-malaria

drug has been isolated from bark of *Cinchona* species by the French pharmacists, Joseph Bienaimé Caventou and Pierre Joseph Pelletier in 1820 (Saad & Said, 2011). The plant had long been used by indigenous groups in the Amazon region for the treatment of fevers (Gurib- Fakim, 2006). The synthetic drug, Aspirin, is synthesized from salicylic acid isolated from willow bark by Arthur Eichengrün and Felix Hoffman in 1897 (Florian *et al*., 2007). People had used willow bark's extract to treat rheumatism and headache for centuries (Rishton, 2008).

Like quinine and aspirin, many drugs that are commonly used today, for example, ephedrine, ergometrine, tubocurarine, digoxin, reserpine and atropine, are derived from indigenous medicine that is produced from the bioscientific studies of plants used by people throughout the world(Gurib-Fakim, 2006). The examples of drugs derived from plants are listed in Table 1. According to WHO (2003), 25 percents of modern medicines today are made from plants first used traditionally. The numbers of plants that contribute as sources of important drugs are limited. There are over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 different plants (Spainhour, 2005).

Approximately 75 percent of these substances were discovered as a direct result of chemical studies focused on the isolation of active substances from plants used in traditional medicine (Elhardallou, 2011). Between the years 2000 and 2005, 5 of 23 new drugs launched on the market were discovered from plants or derived from medicinal plant metabolites. These drugs were Apomophine, Tiotroipium, Nitisinone, Galanthamine and Artheeter (Table 1). The

Table 1: Botanical drugs used in traditional medicine and which have given useful modern drugs

Botanical name	Common name	Traditional use	Uses in biomedicine	Biologically active compound	References
Adhatoda vasica	Malabar nut	Antispasmodic, antiseptic, insecticide	Antispasmodic, oxytocic, cough suppressant	Vasicin	Gurib-Fakim (2006)
Artemisia annua	Sweet wormwood	Fever, jaundice, headache, malaria	Acute malaria	Arteether	WHO (2006)
Atropa belladonna	Devil's berries	Anesthetic, arrow poison	Bronchospasm, pulmonary disease	Tiotroipium	Wang (2008)
Callistemon citrinus	Crimson bottlebrush	Haemorrhoid treatment	Human tyrosinaemia type-1	Nitisinone	Ali *et al*. (2011)
Chondrodendron tomentosum	Curare	Arrow poison	Muscular relaxation	D-Tubocurarine	Anonymous, 2000
Catharanthus roseus	Madagascar periwinkle	Diabetes, fever	Cancer chemotherapy	Vincristine, Vinblastine	Royal Botanic Gardens, KEW (2012)

Table 1: (cont.)

Botanical name	Common name	Traditional use	Uses in biomedicine	Biologically active compound	References
Galanthus nivalis	Snowdrop	Insecticide	Alzheimer's disease	Galanthamine	Heinrich & Teoh (2004)
Ginkgo biloba	Gingko	Asthma, anthelmintic (fruit)	Dementia, cerebral deficiencies	Ginkgolides	Ehrlich (2010)
Piper methysticum	Kava	Stimulant, tonic	Anxiolytic, mild stimulant	Kava pyrones	Gurib-Fakim (2006)
Papaver somniferum	Opium poppy	Asthma, cold	Parkinson's disease	Apomophine	Wang (2008)

advantages of drug produced from plant are that they provide patients with a complex of natural compounds, have smoother action and are better tolerated than synthetic drugs, and produce few allergic reactions (Lovkova *et al*.,2001). They also do not accumulate in the body, and therefore can be administered for a long time.

2.2.2 Antiviral active compounds from plants

The medicinal properties of plants are usually conferred by their secondary metabolites which are synthesised as defence elements against different organisms or for survival in often harsh and changing environment (Yarmolinsky *et al*., 2009). These groups of secondary metabolites can be classified into alkaloids, terpenoids, phenylpropanoids and the complexes of these metabolites (Shasany *et al*.,2008). Studies have shown that these secondary metabolites possess various biological activities such as antibacterial, antifungal, antiviral and anticancer (Ooi *et al*.,2004).

In the past years, many screening efforts have been made to find antiviral agents which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules from medicinal plants (Schmitt *et al*., 2001). According to Freitas *et al*. (2009), approximately 44 percents of the antiviral drugs approved between 1981 and 2006 were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural product. During the last 30 years, extracts from more than 4000 different plant species were studied and about 10 percents of them showed a significant antiviral activity *in vitro* (Glatthaar-Saalmuller *et al*., 2001).

Previous studies have shown that many plants from different families have been scientifically studied for their metabolites with antiviral activities. Notable finding include the plant listed in Table 2. One of the main interests in the study of antiviral compounds from plants is to find anti-HIV and those that can be used to treat viral infections to which vaccines have not been sufficiently effective against the diseases. The secondary metabolite of *Calophyllum lanigerum*, calanolide A, for example, can reduce the HIV disease progression by inducing syncytium formation and activate virus-induce cytopathic effects (Cos *et al*., 2004b). Betulinic acid, a triterpenoid isolated from *Syzigium claviflorum*, has been found to contain anti-HIV activity in lymphocytes (Spainhour, 2005). The quassinoside glycoside isolated from *Allanthus altissima* has been found to inhibit HIV replication. A novel phorbol ester isolated from *Excoecaria agallocba* has been reported to be a potent inhibitor of HIV-1 reverse transcriptase.

2.3 SWEET LEMON GRASS (*Cymbopogon nardus* (L.) Rendle)

Sweet lemon grass, also known as citronella grass, is an aromatic grass belonging to the genus *Cymbopogon* in Gramineae family (Lai *et al*.,2005). It is commonly known as ‘serai wangi’ in Malaysia and Indonesia. This plant is native to Sri Lanka and South India, but it can also be found growing wild in most tropical Asian countries, Central America, and Africa (Hazwan *et al*., 2014). Today, sweet lemon grass is grown commercially on a large scale in Sri Lanka, India, Burma, Indonesia and Java (Pangnakorn *et al*., 2011).

Table 2: Plants that have been scientifically studied for their metabolites with antiviral activities

Botanical name	Common name	Origin	Antiviral activity	References
Acokanthera schimperi	Arrow-poison plant	Ethiopia	Methanolic extract of this plant inhibited coxsackie virus B3 and herpes simplex virus 1 (HSV-1)	Gebre-Mariam *et al*. (2006)
Artocarpus integrifolia L.	Jackfruit	Asia	Extract from this plant bark had antiviral activity against simian (SA-11) and human (HCR3) rotaviruses	Gonçalves *et al*. (2005)
Baccharis genistelloides	Carqueja	South America	Aqueous extracts of this plant was active against vesicular stoma virus (VSV)	Abad *et al*. (1999)
Codium fragile	Dead man's fingers	Korea	Methanolic extract from this plant was active against HSV-1, sindbis virus (SINV) and poliovirus	Hudson *et al*. (1999)
Dunbaria bella	-	Asia	A partially purified fraction from the dichloromethane-methanol extract of this plant was active against HSV-1 and HSV-2	Akanitapichat *et al*. (2006)

Table 2: (cont.)

Botanical name	Common name	Origin	Antiviral activity	References
Eleutherococcus senticosus	Siberian ginseng	Northeastern Asia	Liquid extract from this plant roots inhibited the productive replication of human rhinovirus (HRV), respiratory syncytial virus (RSV) and influenza A virus	Glatthaar-Saalmüller *et al*. (2001)
Lippia alba	Bushy matgrass	South America	Ethyl acetate extract of this plant showed anti-poliovirus activity	Andrighetti-Frohner *et al*. (2005)
Melia azedarach L.	Chinaberry	India	Ethyl acetate extract of leaves of this plant exhibited anti-VSV and anti-HSV-1	Alche *et al*. (2003)
Olea europaea	Olive tree	Mediterranean	Extract derived from leaf inhibited viral haemorrhagic septicaemia virus (VHSV)	Micol *et al*. (2005)
Phyllanthus urinaria	Chamberbitter	Eastern Asia	Acetone, ethanol and methanol extracts of this plant inhibited HSV infections	Cheng *et al*. (2009)
Piper aduncum	Bamboo piper	Tropical regions	Methanolic extracts of this plant was found active on poliovirus	Lohézic-Le Dévéhat *et al*. (2002)

Table 2: (cont.)

Botanical name	Common name	Origin	Antiviral activity	References
Plantago major L.	Greater plantain	Asia	Pure compound from aqueous extract of this plant exhibited potent antiviral activity against HSV-1 and adenovirus (ADV-3)	Chiang *et al*. (2002)
Polygonum punctatum	Water smartweed	North America	Aqueous extract of this plant showed antiviral activity against RSV	Kott *et al*. (1999)
Verbascum thapsus	Common mullein	Europe	Extract of this plant exhibited strong anti-influenza viral activity	Rajbhandari *et al*. (2007)
Zanthoxylum chalybeum	Knob wood	Uganda	Seed extracts of this plant possess activity against measles virus	Olila *et al*. (2002)

2.3.1 Biology

Sweet lemon grass is a perennial, coarse, clump-forming tropical grass that can grow up to a height of 1 to 1.5 meter (Barceloux, 2008). The leaves are arching and flat, and can grow to an average size of 60 cm long and 2.5 cm wide. The surface of the leaf-blade is smooth or scaberulous (small raised areas of roughness) and gray-green. The stems are red at the base and cane like. Sweet lemon grass grows in clumps that continually increase in size until mature (Hart, 2007). According to Gardener (1995), there are two types of sweet lemon grass, which are maha pengiri or old citronella grass (Java type) and lenabatu pangiri or lena batu (Ceylon type). They are distinguished from each other by different leaf morphology and chemical composition, whereby Java type has larger leaf and yield essential oil twice as much as Ceylon type.

Sweet lemon grassgrows very well in moist alluvial soil and its growth becomes retarded during the dry season. For the best growth, it needs a long and warm growing season, well drain soil and positioned in plenty of sun. It has been reported that sweet lemon grass grown on sandy soil have higher leaf oil content (Anonymous, 2008). The first harvest is usually six to eight months after planting, then three to six times annually. Harvesting must be done when the plants are dry, as wet plant materials will quickly ferment. The bush is cut from 7 to 25 cm above ground and it is usually harvested in the early morning.

2.3.2 Chemical composition

Chemical analysis of citronella oil, the essential oil from sweet lemon grass, indicated that it contain geraniol and a high content of monoterpene constituents such as citral, citronellol, citronellal, linalool, elemol, 1,8-cineole, limonene, geraniol, *β*-carophyllene, methyl heptenone, geranyl acetate and geranyl formate (Herath *et al*.,1979; Shasany *et al*.,2008). This oil is a complex natural mixture of volatile secondary metabolites and it is isolated from plants by hydro or steam distillation. Other compounds that are predominant in citronella oil include 4-terpinenol, α-felandrene, α- pinene, borneol, bournonene, β-bourbonene, β- kariofeline, camphene, chamfor, cis-osimene, citronellyl acetate, citronellyl butyrate, D-citronellal, D-citronellol acetate, D-citronellol-N-butyrate, elemol, ethanol, farnesol, furforol, geranyl acetate, geranyl butyrate, L-borneol, L-carvotanacetone, limonene, linalil acetate, linalool, methyleugenol, methyl isoeugenol, nerol, pasimene, trans-osimene, and tricyclene (Jaganath & Ng, 2000).

2.3.3 Uses in traditional medicine

Sweet lemon grass has long been used in many part of the world as traditional medicine. This plant is commonly used in folk medicine in China for treatment of rheumatic pain and arthritis (Soni, 2006). In Mozambique, it is used to reduce appetite and revitalize nerves (Anonymous, 2004). Meanwhile in South Africa, sweet lemon grass is used as natural remedies for intestinal worms and cold (Jaganath *et al*., 2000). The fomentation of sweet lemon grass leaves is widely used in India and Sri Lanka to treat minor wounds, abrasions and swelling (Weiss, 1997).In many

other countries, this plant is used to treat fever, intestinal parasites, digestive and menstrual problems (Abena *et al*., 2007).

In Malaysia, sweet lemon grasshas been used for treatment of various ailments. The entire plant can be boiled with ginger and sugar, and drink to treat abdominal pain, flatulence, diarrhoea and vomiting (Fasihuddin & Hasmah, 1993). The decoction of its root has been used for treatment of various ailments such as bladder dysfunction, menstrual cramps, sore throat and fever (Perry, 1980). Meanwhile, the decoction of its leaves has been used for treatment of rheumatic pain. According to Jaganath *et al*. (2000), the decoction of the leaves with those of banana (golden banana), Gandarusa (*Gendarussa vulgaris*) and betel-pepper is being used as an after childbirth wash. Besides that, the leaves extract of this plant can be used in treating skin sores, wounds (Burkill, 1966) and bladder dysfunction (Perry, 1980). The extract can also be used in hot bath to treat swelling and body odour. In addition, the leaves cooked with coconut oil can be used to overcome the bone pain, muscle sprain, insect bites and stomach bloating (Fasihuddin & Hasmah, 1993). Other than that, the essential oil obtained from fresh leaves of this plant can be given in small dose to relieve stomach discomfort, adding digestion and as antispasmodic agent (Lai *et al*., 2005). The essential oil is also used as traditional mosquito repellent and household fumigants (Barceloux, 2008).

2.3.4 Studies on biological activities

Based on the wide use of sweet lemon grass as ingredients of traditional medicine, studies on biological activities of the plant have been carried out. Nakahara *et al*. (2005) have reported that

sweet lemon grass showed the highest inhibitory effects on the growth of weevil in stored grains, compared to the other traditional insect repellant plants, which are lemon grass (*Cymbopogon citratus*), peels of pomelo (*Citrus grandis*) and rhizomes of fingerroot (*Boesenbergia pandurata*). A study by Hammer *et al*.(1999) has demonstrated that essential oil of sweet lemon grass (citronella oil) possess antibacterial activity against *Acinetobacter baumanii, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. Besides, citronella oil has also been reported to have antifungal activity against the fungus *Aspergillus niger* (Nakahara et al., 2003) and *Candida albicans* (Hammer *et al*., 1999). According to study conducted by Kumar & Dutta (1987), citronella oil possesses larvacidal properties, whereby it is effective against *Anopheles stephesi* larvae, a carrier of malaria fever. Meanwhile, study by Muhd Haffiz *et al*. (2013) demonstrated a strong antiparasite activity of citronell oil against *Trypanosoma brucei brucei*, the parasite that cause African trypanosomiasis, known also as sleeping sickness in humans. In addition, study by Ranasinghe *et al*. (2004) suggested that citronella oil exhibited inhibitory activity of polyphenol oxidase (tyrosinase) responsible for the hyper pigmentation in humans and could be used as naturally occurring inhibitors for this enzyme.

Biological activity studies of this plant using Malaysia sample of *C. nardus* showed that the crude extract and partially purified subfractionsare active against newcastle disease virus (NDV), herpes simplex virus type-1 (HSV-1) and MV (Ahmad *et al*., 1992; Hanina, 2006; Nurul Aini *et al*., 2006). Some of the partially purified subfractions also showed synergistic effects in combination with Ribavirin against MV (Nurul Aini *et al*., 2006). Fractions and partially purified

subfractions were shown to possess antibacterial activity against S. *aureus* and MRSA isolates (Hanina *et al*., 2002).

2.4 SCREENING FOR NATURAL PRODUCT FROM PLANTS

A typical protocol used to isolate the active compounds from plants is bioassay-guided fractionation (Gurib-Fakim, 2006). Bioassay-guided fractionation is a procedure of whereby extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system and only active fractions are retained and further fractionated. This method is commonly employed in drug discovery research due to its effectiveness to directly link the analysed extract and targeted compounds using fractionation procedure that is complemented with selective test to determine the existence of biological activity. There are four main steps involved in the bioassay-guided fractionation. They are extraction, isolation and purification, bioassay, and characterization and structure elucidation of active compounds.

2.4.1 Extraction

Extraction is the crucial first step in the analysis of medicinal plants since the desired chemical components should be extracted from the plant materials before further separation and characterisation could be done (Sasidharan *et al*., 2011). The extraction process involves separation of medicinally active fractions of plant tissue from inactive/inert components by using selective solvents and extraction technology (Das *et al*., 2010). The solvents diffuse into the solid

plant tissues and solubilise compounds of similar polarity. The quality of plant extract depends on plant material, choice of solvents and the extraction methods.

2.4.1.1 Plant material

Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many researchers had reported on plant extract preparation from fresh plant parts. The choices are based on the ethno-medicinal use of fresh plant parts by the traditional people. However, before extraction begins, the plant materials are usually air dried to a constant weight before extraction (Das *et al*., 2010). Other researchers dry the plants in the oven at about 40°C for 72 h. This quick drying avoids natural degradation by microbes (Gurib-Fakim, 2006). Once the plant materials have dried to constant weight, it is ground to smaller grains and powder. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction.

2.4.1.2 Choice of solvents

Successful determination of a biologically active compound from plant material is largely dependent on the type of solvents being used in the extraction step. Properties of a good solvent for plant extractions include low toxicity, ease of evaporation at mild heating, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate (Tiwari *et al*., 2011). In addition, the solvent should be non-toxic and

should not interfere with the bioassay, considering that the end product in extraction will contain traces of residual solvent. The choice will also depend on the targeted compounds to be extracted. Initial screening of plants for possible antimicrobial activities typically begins by using the crude or alcohol extractions and can be followed by various organic solvent extraction methods. Water is an universal solvent and is commonly used to extract plant products with antimicrobial activity. Although traditional healers use primarily water for the extraction of healing ingredients, plant extracts from organic solvents have been found to give more consistent antimicrobial activity than water extract (Parekh & Chanda, 2007).

2.4.1.3 The extraction methods

Extraction is the separation of medicinally active portions of plant tissues from the inactive or inert components by using selective solvents through standard procedures (Ncube *et al*., 2008). As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. This precaution must be taken because unsuitable method or solvent may result in the failure to extract or even degrade the active compounds. Various methods, such as sonification, heating under reflux, Soxhlet extraction and others are commonly used for the plant extraction (Sasidharan *et al*., 2011).

Alternatively, plant extracts are prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems. Another common method is serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol).

This procedure will result in the extraction of a wide polarity range of compound from the plant materials (Hora, 2010). A brief summary of the experimental conditions for the various methods of extraction is shown in Table 3.

The extracting solvents are then filtered, and filtrate is subsequently subjected to concentration under vacuum for large volumes or 'blown down' under nitrogen for small volumes. Some researchers however centrifuge the filtrate at 20,000 × g for 30 minutes for clarification of the extract (Das *et al*., 2009). The concentrated extract is stored at -20ºC as this low temperature reduces the degradation of the bioactive natural product.

Table 3**:** The experimental conditions for various methods of extraction for plants material

Condition	**Soxhlet extraction**	**Sonification**	**Maceration**
Common solvents used	Methanol, ethanol or mixture of alcohol and water	Methanol, ethanol or mixture of alcohol and water	Methanol, ethanol or mixture of alcohol and water
Temperature (ºC)	Depending on solvent used	Can be heated	Room temperature
Pressure applied	Not applicable	Not applicable	Not applicable
Time required	3-18 hr	1 hr	3-4 days
Volume of solvent required (ml)	150-200	50-100	Depending on the sample size

2.4.2 Isolation and Purification

Plant extracts usually contain various types of secondary metabolites, and the biologically active compounds being searched for are having different polarities. In such cases their separation need to be carefully conducted in order to isolate, identify and characterise the bioactive compounds (Sasidharan *et al*., 2011). It is a common practice in isolation of these bioactive compounds that a number of different separation techniques be used. Separation techniques such as thin layer chromatography (TLC), column chromatography and preparative thin layer chromatography (PTLC) are used in alternative steps until pure compounds are obtained. The pure compounds are then used for the determination of structure and biological activity.

2.4.2.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a widely used separation techniques, mainly performed in a qualitative or semi-quantitative manner (Molnar-Perl, 1998). It is simple, fast and inexpensive procedure that gives the researcher a quick answer as to how many components are in a mixture (Sasidharan *et al*., 2011). TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the retention factor (RF) of a known compound (Kapetanovic & Lyubimov, 2008). TLC employs glass or aluminium plates pre-coated with the sorbent, such as silica gel, to varying thickness depending on the amount of sample to be loaded. The compound mixture is loaded both in the preparative or analytical TLC plates at around 2 cm from the bottom and lowered in a tank containing the solvent. The latter migrates up the plates

and separates the compound mixture according to the polarity of the components. Several reagents are available for visualisation of the separated materials. The advantage of using TLC in comparison to other separation techniques is it a cost-effective qualitative technique, whereby large number of samples can be analysed or separated simultaneously (Bhawani *et al*., 2010).

2.4.2.2 Column Chromatography

Column chromatography is another common and useful separation technique in organic chemistry (Anonymous, 2012). This separation method involves the same principles as TLC, but it can be applied to separate larger quantities than TLC. The underlying mechanism by which the column chromatographic technique achieves the separation of different compounds is differential migration (Church, 2005). This is a measure of the tendency that each compound has to move through the column at a different rate, thus separating from one another. Those with lower affinity to stationary phase move faster and eluted out first while those with greater affinity move or travel slower and get eluted out last (Bheem, 2012). The time required for a compound to pass completely through the column is called the retention time and is unique for each compound under given separation conditions, such as flow rate, temperature, mobile phase composition and stationary phase, for a compound.

Solvent systems for use as mobile phases in for column chromatography can be determined from previous TLC experiments, the literature or experimentally (Williamsons & Masters, 2011). The separation usually begins by using non-polar or low polarity solvent,

allowing the compounds to adsorb to the stationary phase. Then the polarity of the solvent is slowly switching to desorbs the compounds and allow them to travel with the mobile phase. The polarity of the solvents is changed gradually during the chromatography process. During the entire chromatography process, the eluent is collected in a series of fractions (Nithya *et al*., 2011). Each fraction is further analysed for dissolved compounds using TLC, UV absorption or fluorescence. Fractions with similar patterns and colour of spot or RF values are combined and resulting mixtures are evaporated to dry.

2.4.2.3 Preparative Thin Layer Chromatography (PTLC)

The procedures and substances for PTLC are generally similar to TLC except the plate is thicker, so more sample can be deposited on it. PTLC is used for analytical separations of larger quantities of materials, ranging from 10 mg to greater than 1 g (Sherma & Fried, 1987).In PTLC, materials to be separated are often applied as long streaks rather than spots as in TLC. After development with suitable solvent system, specific component may be recovered by scraping the sorbent layer from the plate in the region of interest and eluting the separated material from the sorbent using a strong solvent. According to Poole (2003), the material that is recovered from the layer may require further purification by TLC or other chromatography methods, or the purity may be adequate for identification and structure determination by elemental analysis or spectrometry, for use in biological activity or chemical synthesis studies, or for use as standard reference material for comparison with unknown samples.

2.4.3 Bioassay - Antiviral assay

Bioassay to detect and assess the secondary product showing desired activity is a very critical step in screening for biologically active natural plant products (Gurib-Fakim, 2006). The evaluation of antiviral activity *in vitro* includes antiviral efficacy and cell toxicity. Before antiviral assay can be conducted, it is essential that plant extracts to be tested dissolve completely in the *in vitro* medium. As this may prove to be difficult for non-polar extracts (Soumyanath & Srijayanta, 2006), stock solution of the extracts or compounds could be prepared at high concentration in a minimum quantity of water-soluble organic solvent such as ethanol, methanol and dimethylsulphoxide (DMSO) (Wadhwani *et al*., 2009). The stock solution is then added to the bioassay medium in a volume so that the concentration of solvent is not detrimental to the assay. This concentration is determined by prior experimentation. For example, a number of cell-based assay are able to tolerate up to 3% DMSO (Soumyanath & Srijayanta, 2006). When the concentrated extract solution is added to the aqueous medium precipitation of the dissolved substances may occur.

Determining the appropriate antiviral screening approach is critical since different viruses behave differently in animal cell cultures. It is virtually impossible to design a single antiviral test that could be applied for all viruses and as a result, different approaches have been designed for viruses that grow in different cell systems (Naithani *et al*., 2010). Various cell-based assays have been successfully applied for the antiviral evaluation of single substances or mixtures of compounds (Table 4). The methods commonly used for evaluation of *in vitro*antiviral activity are based on the different abilities of viruses to replicate in cultured cells (Vlietinck *et al*., 1997).

Table 4: *In vitro* antiviral screening assays

Assays	Applications	References
Plaque inhibition or reduction assays	i) Suitable only for viruses which form plaques in suitable cell systems. ii) Titration of a limited number of viruses or residual virus infectivity after extracellular action of the test substance. iii) The test substance must be in a non-toxic dose, or cytotoxicity should be eliminated by dilution or filtration before the titration.	El Sayed, 2000
Inhibition of virus-induced cytopathic effect (CPE)	i) For viruses that induce CPE, but not readily form plaques in cell cultures. ii) Determination of virus-induced CPE in monolayers infected with a limited dose of virus and treated with a non-toxic dose of the test substance.	Vlietinck *et al*., 1997
Virus yield reduction assay	i) Determination of virus yields in tissue cultures when infected with a given amount of virus and treated with a non-toxic dose of the test substance. ii) Virus titration is carried out by the plaque test (PT) or the 50% tissue culture dose end point test (TCD_{50}), after virus multiplication has occurred.	Cos *et al*., 2006

Table 4: (cont.)

Assays	Applications	References
End point titration technique (EPTT)	i) Determination of virus titer reduction in the presence of 2-fold dilutions of test compounds. ii) This method has been especially design for the antiviral screening of crude extract.	Vlietinck *et al*., 1997
Assays based on measurement of specialised functions and viral products	i) For viruses that do not form plaques or induce CPE in cell cultures. ii) Determination of virus specific parameters, for example hemagglutination and hemadsorption test, inhibition of cell transformation and immunological tests detecting antiviral antigens in cell cultures.	Salmasi *et al*., 2011

Some cause cytopathic effects (CPE) such as plaques formation, while others are capable of producing specialized functions or cell transformation. Virus replication can also be monitored through detection of viral replication products, such as viral DNA, RNA or polypeptides.Antiviral agents interfere with one or more dynamic processes during virus biosynthesis (Cos *et al*., 2006). Based on the previous studies, there are three methods used to identify the possible sites of action of the antiviral compounds in a given cell culture. They are pre-treatment, virucidal and post-infection methods (Table 5).

Bioassay-guided approach is a popular choice in the screening of antiviral substances from plant. In this approach, serial fractionations of plant extract are carried out using standard chromatographic methods until the pure compounds or partially purified fractions are obtained

Table 5: Three methods used to identify the possible sites of action of the antiviral compounds in a given cell culture

Method	Description	References
Pre-treatment	i) Cells are pre-treated with antiviral compound before virus inoculation in order to determine if the compound induces protection or an 'antiviral state', by a process akin to interferon induction. ii) Example: *C. nardus* fractions were able to protect the Vero cells from measles virus infection.	Hudson *et al.*,1999; Nurul Aini *et al.*, 2006
Virucidal	i) Virus is incubated with the compound which is diluted by several orders of magnitude in order to reduce further effects due to the compound, and then added to the cells. ii) Virion is inactivated either by disruption of the virion or by interfering with its ability to initiate a replication cycle. iii) Example: *In vitro* antiviral activity of an aqueous extract from *Phaeophysciaorbicularis* might be partially due to a direct interaction with several animal viruses' particles.	del Barrio & Parra, 2000; Schnitzler *et al.*,2008a
Post-infection	i) Antiviral compound is added to the cells following virus inoculation, whereupon it may interfere with any of the steps in virus uncoating, intracellular localisation, replication, transcription, translation, processing and virion assembly, or secretion from the cell. ii) Example: The mode of action of *Plantago major*pure compounds on HSV-2 and ADV-2 was found to be at post-infection stage with SI values greater than 400, suggesting the potential use of this compound for treatment of the infection by these two viruses.	Chiang *et al.*, 2002

(Freitas *et al*., 2009). Each step of fractionation will increase the purity of fractions while retaining the antiviral activity. In some instances biologically active compound exist in not just one or two fractions, but in several fractions at different relative strength (Nurul Aini *et al*., 2006; Adibah *et al*., 2010). It is possible that the low antiviral activity may be due to low concentration of the active compound in the fractions (Kim *et al*., 2009; Ianora *et al*., 2011). If selection of active compounds is based only on high antiviral activity of the fractions and ignoring low activity fractions, compound that are naturally occurring in the plants, will not be isolated for testing.

2.4.4 Characterisation and structure elucidation of bioactive compounds

After the biological evaluation has been performed and the separation of the natural product has been achieved, the final goal is to determine the structure and composition of the bioactive compounds. Structure elucidation depends on classical spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Infra Red (IR), Gas Chromatography-Mass Spectrometry (GC-MS) and X-Ray analysis (Gurib-Fakim, 2006). GC-MS is the single most important tool for the identification and quantitation of volatile and semi-volatile organic compounds in complex mixtures (Hites, 1997). As such, it is very useful for the determination of molecular weights and sometimes the elemental compositions of unknown organic compounds in complex mixtures

GC-MS comprises of a gas chromatography (GC) coupled to a mass spectrometer (MS). In this equipment, complex mixtures of chemicals may be separated, identified and quantified. The GC works on the principle that a mixture will separate into individual substances when

heated (Anonymous, 2008). The heated gases are carried through a column with an inert gas, such as helium. As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule. A library of known mass spectra data, covering several thousand compounds, is stored on a computer. Mass spectrometry is considered the only definitive analytical detector. However, not all experiments lead to the discovery of a totally new chemical compound. Occasionally the structural probing reveals a known bioactive chemical, although its source might be novel (Owen & Hundley, 2004).

2.5 SYNERGISTIC EFFECTS FROM ANTIVIRAL DRUGS COMBINATION

Synergistic effect is a condition where combination of drugs with other component exerts high effects even though the dose is the same as monotherapy, compared when it is applied individually (Adibah, 2008). The combinatorial use of drugs aims to induce a response upon multiple targets, multiple subpopulations, or multiple diseases simultaneously (Harrastani *et al*., 2010). Ever since the earliest days of recorded history, drug combinations have been used for treating diseases and reducing suffering (Chou, 2006). The herbal medicines in traditional Chinese medicines have provided good evidence on its effectiveness. As the science of isolation technology and chemical synthetic capability advance, drug combinations have been more refined and their scope continues to broaden. During the past century, many attempts have been made to quantitatively measure the dose-effect relationships of each drug alone and its combinations, and to determine whether the combination would result the synergistic effect compared when it is applied individually (Adibah, 2008).

Although there is a need for continuous development of new antiviral agents, the use of combinations of presently existing antiviral drugs is becoming increasingly important (Prichard & Shipman, 1990). The most notable advantage of using drug combinations is the reduction on the possibility of selecting drug resistant mutants. Combinatorial use of multiple drugs with dissimilar mechanisms or modes of action may direct the effect against a single target or disease with a more effective therapeutic outcome. In addition, drug combinations which exploit synergistic interactions also may offer increased antiviral efficacy while decreasing cytotoxicity by minimizing the required therapeutic doses (Hussain et al., 2009). However, not all combinations of drugs synergistically inhibit viral replication. Some drugs when used in combination may even antagonise the individual antiviral effects (Chou, 2006).

Basically, there are three ways in applying antiviral drug combination. Firstly, is by combining two or more plant extracts or plant-derived compounds. For example, a study performed by Cheng *et al.* (2006) showed that the combination between two plant extracts, oxymatrine-baicalin was able to inhibit DNA replication of hepatitis B virus much more effective than treatment with oxymatrine alone. Oxymatrine was derived from *Saphora florescens*,while baicalin was derived from *Scutellaria baicalensis.* Second approach is by combining two or more synthetic drugs. An example of this approach is the study by Snoeck et al. (1992) who showed that the combination between synthetic analog hydroxyl-2-phosphonomethoxy propyl cytosine (HPMPC) with foscarnet, ganciclovir or acyclovir resulted in partial synergistic effects against cytomegalovirus (CMV). The combinations did not enhance the cytotoxicity of the drugs to HeLa cells. In another study on drugs combination, Petrera & Coto (2006) showed the synergistic effects between Interferon-α and Interferon-γ able to inhibit replication of herpes

simplex virus type 2 (HSV-2) in Vero cells. Third approach is by combining synthetic drug and plant extract or plant-derived compounds. Barquero et al. (1997) have shown that meliacine, a peptide isolated from leaves of *Melia azedarach* L., worked synergistically with acyclovir to repress the antigen expression of herpes simplex virus type 1 (HSV-1) in infected cells.

2.5.1 Calculation of combination index

Several publications (Chou & Talalay, 1984; Nduati & Kamau, 2006; Cheng et al., 2009) have been dedicated to determine the nature of effect due to combination treatments. Combinatorial effect from the combination of drugs against a virus infection can be analysed by using the isobologram method. Zhao *et al*. (2004) analysed the effect of combination between doxorubicin and suramin in cultured tumour cells by taking into consideration of integrating nonlinear regression and curve shift analysis, reduced potential errors in estimation of these effects

In the isobologram method described by Nduati & Kamau (2006) and Cheng *et al.* (2009), the $IC_{50,}$ which was determined fromdose-response graph, was used to calculate the fractional inhibitory concentration (FIC) according to the following formula:

$$FICs = [Ac] / [Ae] + [Bc]/ [Be] + ... + [Xc] / [Xe]$$

where [Ae] is the concentration of a drug that produces a specific effect(IC_{50}) when used alone. [Ac] is concentration of the same drug that produced the same effect when used in combination with another drug(s). By this calculation, the interaction between two or more drugs was

interpreted according to the combined FICs index. The interactions were categorised as follows: high synergy was said to be present if FICs less than 0.40. Those combinations with FICs greater than 0.40 but lower than 1, were classified as having moderate synergy, while those with a FICs approximately equal to 1 represented additive interactions, while those with FICs between 1 and 2 were categorized as partially additive/weak antagonism and those greater than two represented high antagonism.

2.5.2 Ribavirin

Ribavirin is a broad-spectrum antiviral nucleoside (guanosine) which can act on a wide variety of DNA and RNA viruses, such as adenovirus, herpes virus, measles virus, Newcastle disease virus and parainfluenza 1, 2 and 3 (Fernandez *et al*., 1986). The chemical name of Ribavirin is 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide. Ribavirin inhibits virus replication by causing a reduction of intracellular guanosine triphosphate (GTP), which is an important component in transcription, translation and replication of viruses. Absence of GTP will lead to incomplete capping of 5'-terminus of RNA, which result in accumulation of mRNA impaired in protein synthesis. Therefore, the virus replication is inhibited (Sidwell *et al*., 1985).

Chemotherapy using Ribavirin has a limited success due to toxicity and other severe side effects towards human (So *et al*., 2012). The toxicity of Ribavirin can decrease red blood cells, making it unsuitable for pregnant women, children, old citizens and anaemia and thalassemia patients. Ribavirin may also cause birth defects and/or death of the exposed fetus, thus its therapy is contraindicated for use in women who are pregnant or in men whose female partners are

pregnant (Anonymous, 2009).Some mild reactions, for instance lip and gingival swelling, conjunctival hyperemia, headache and lethargy can also be observed among the recipients (Anonymous, 2002). Therefore, researchers have considered combining this drug with other substances as to be able to be used at lower concentration. This action will reduce its toxicity level and yet retain effectiveness. The notable findings include the drug combination listed in Table 6. It can be concluded that combination therapy is an effective alternative ways to treat viral infection as well as to reduce the toxicity level of each drug when used as monotherapy. Combination therapy also helps in reducing resistance of patients towards antiviral drugs treatment (Witlink, 1992).

Table 6: The drug combination that have been scientifically studied for their effectiveness against viruses

Combination	Effectiveness	References
Ribavirin with selanozofurin	The combination was effective in inhibiting measles virus even when the dosage of each drug had been reduced	Kirsi *et al.*,1984
Ribavirin with cyclodextrins	The 50% inhibitory concentration (IC_{50}) of ribavirin against measles virus was improved after the combination with cyclodextrins	Grancher *et al.*, 2004
Ribavirin with 6-mercaptho-9-tetrahydro-2-furylpurine (6-MPTF)	The combined treatment of the drugs markedly suppressed the replication of dengue viruses in human peripheral blood leukocytes (PBL)	Koff *et al*., 1982
Ribavirin with amantadine or rimantadine	The inhibitory effects of ribavirin towards influenza virus are improved after the combination with amantadine or rimantadine	Sidwell *et al*.,1985
Ribavirin with rimantadine or amantadine	The combination improved antiviral activity against human influenza virus.	Hayden, 1999
Ribavirin with β-cyclodextrin	The combination decreased measles virus load in the brain of the tested animal compared to ribavirin use in monotherapy	Jeulin *et al.*, 2006

CHAPTER 3: MATERIALS AND METHODS

3.1 RESEARCH MATERIALS

3.1.1 Plant material

Sweet lemon grass (*Cymbopogon nardus* (L.) Rendle) was collected from the Ladang Integrasi Herba in Johor, Malaysia. The sample was taxonomically identified using morphological and anatomical techniques. The voucher specimen of the plant was deposited at the herbarium of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). The plant material was air dried at room temperature for two weeks and ground to fine powder using laboratory grinder.

3.1.2 Hexane extraction

Hexane was used as the extraction solvent based on its properties of a good solvent in plant extraction as it is low in toxicity and ease of evaporation at low heat. Two kilograms of the powdered sweet lemon grass were soaked in hexane (Merck, Germany) for 3 days at room temperature, followed by filtration using Whatman No. 3 filter paper. The same procedure was repeated three times.The extract of three successive extractions was collected, combined and concentrated in a rotary evaporator (Laborata 4000, Germany). The weight of concentrated extract was recorded for yield calculations, after which it was stored at 4°C prior to screening.

3.1.3 Cells and virus

Vero cells (African green monkey kidney cells) used in this study was obtained from Virology Laboratory, Universiti Kebangsaan Malaysia, Bangi, Malaysia. Cells were routinely cultivated at 37°C in a 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (FBS, Hyclone, USA), penicillin-streptomycin antibiotics (Amresco, USA) and amphostat B (Thermo, USA) (Appendix A). Human papillary ovarian adenocarcinoma cells (Caov-3) used in this study was provided by Universiti Putra Malaysia, Serdang, Malaysia. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (FBS, Hyclone, USA) and penicillin-streptomycin antibiotics (Amresco, USA), and incubated in a humidified 37°C incubator with 5% carbon dioxide (CO_2) (Thermo, USA).

Edmonston's strain of measles virus was a commercial preparation purchased from Serum Institute of India Ltd. The virus was diluted with 500µl sterile distilled water provided by the manufacturer to obtain 1000 units 50% tissue culture infective dilution ($TCID_{50}$).

3.2 FRACTIONATION AND PURIFICATION OF ACTIVE FRACTIONS

3.2.1 Fractionation process

Bioassay-guided serial fractionations of *C. nardus* extract were carried out using column chromatography (CC) and thin layer chromatography (TLC). This process was performed based

on the previous method described by Nurul Aini *et al.* (2006)and Hanina (2006). This process was carried out in two stages, which is the first stage column chromatographic fractionation I (CC-I) and followed by the secondstage column chromatographic fractionation II (CC-2). Each progressive step of fractionation is designed to increase the purity of fractions while retaining the antiviral activity.

3.2.1.1 First stage column chromatographic fractionation I (CC-I)

The first phase column chromatographic fractionation (CC-I) was carried out on hexane crude extract. The crude extract was separated into different fractions by column chromatography (CC) on silica gel 60 (Merck, 230-400 mesh) in a 60 cm x 3 cm column.The column was first filled with 60 g of silica gel and then slowly compacted to remove air pockets. After rinsing the silica gel column with hexane, slurry prepared by mixing 4.0 g of hexane crude extract, hexane and silica gel, dispensed into the top of the column. Elution process was then performed with increasing polarity of solvent systems, beginning with hexane, dichloromethane (DCM), chloroform, ethyl acetate and methanol in different ratios (Table 8) (Appendix B). About 25 ml of eluent was collected each time and all fractions obtained were subjected to thin layer chromatography (TLC) analysis as in section 3.2.1.2.

3.2.1.2 Thin layer chromatography (TLC) analysis of fractions

All fractions obtained from CC-I were analysed using thin layer chromatography (TLC). A small spot of sample was applied onto the TLC plate (Merck, 0.25mm thickness) using capillary tube

and dried. The TLC plate was dipped in suitable developed solvents with appropriate ratio in a covered beaker (Appendix B). It was then visualized under UV light or sprayed with vanillin and the positions of the components were marked. The pattern and colour of each spot were recorded and the retention factor, Rf value was determined. Rf value is calculated based the ratio of the distance moved by the compound and the distance moved by the the solvent along TLC plate, where both distances are measured from the point where the sample is initially spotted on the plate. Upon completion, fractions with similar profiles, as determined by Rf values, were combined together and tested for anti-measles activity assay and cytotoxicity assay. Then, fractions with anti-measles activities were subjected to second stage fractionation as described below. Fractions showing various degrees of effectiveness were chosen for the next stage of purification.

3.2.1.3 Second stage fractionation using column chromatography II (CC-II)

Based on the antiviral activity data (section 3.4), six fractionswere selected for the second stage fractionation (CC-II). The procedure was performed using the same method as previously described in 3.2.1.1, except that the column size was smaller, which was 30 cm x 1.5 cm.Elution process was performed with increasing polarity of solvent systems, starting from hexane, dichloromethane (DCM), chloroform, ethyl acetate (EtoAc) and methanol (MeOH) in different ratios (Table 7). About 5 ml of eluent was collected each time and all subfractions obtained were subjected to TLC analysis.

Table 7: Increasing polarity of solvent system in different ratio used for column chromatography I (CC I) and column chromatography II (CC II)

Solvent system	**Ratio**
Hexane	100%
Hexane:DCM	9:1
Hexane:DCM	3:1
Hexane:DCM	1:1
Hexane:DCM	1:3
Hexane:DCM	1:9
DCM	100%
DCM: Chloroform	9:1
DCM: Chloroform	3:1
DCM: Chloroform	1:1
DCM: Chloroform	1:3
DCM: Chloroform	1:9
Chloroform	100%
Chloroform: EtoAc	9:1
Chloroform: EtoAc	3:1
Chloroform: EtoAc	1:1
Chloroform: EtoAc	1:3
Chloroform: EtoAc	1:9
EtoAc	100%
EtoAc: MeOH	9:1
EtoAc: MeOH	3:1
EtoAc: MeOH	1:1
EtoAc: MeOH	1:3
EtoAc: MeOH	1:9
MeOH	100%

3.2.1.4 Thin layer chromatography (TLC) analysis of subfractions

All subfractions obtained from CC-II were analysed using thin layer chromatography (TLC) as previously described in section 3.2.1.2. The TLC plates were developed with suitable solvents with different polarity to determine solvent that give best separation. The pattern and colour of each spot were recorded and the Rf value was determined. Subfractions with similar Rf values were combined and dried. The dried fractions were subjected to the cytotoxic and antiviral activities as described in sections 3.3 and 3.4.

3.2.2 Preparative thin layer chromatography(PTLC)

Subfractions with antiviral activity were further purified using preparative thin layer chromatography (PTLC) and gas chromatography-mass spectrometry (GC-MS). Data from GC-MS was used for the detection of chemical compounds and the determination of the molecular mass of the isolated compound.

3.2.2.1 Purification of selected fractions and subfractions

One fraction (CC-I product) and three subfractions (CC-II products), which showed anti-measles activity, were selected for further fractionation in PTLC. A small spot of sample was applied onto 20 x 20 cm PTLC plate, which is covered with silica gel 60 F_{254}(1.0 mm thickness; Merck, Germany). The plate was then dipped in suitable developing solvents with appropriate ratio. After separated spots on plate were visualized under UV light, the desired bands obtained were marked

and scraped off. The compounds contained within the scrapped silica gel were then extracted out with DCM. Upon filtration through Whatman No. 1 filter paper, the filtrate was left to evaporate. The dried powder was analysed using gas chromatography-mass spectrometry (GC-MS)technique for identification.

3.2.2.2 Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS was set using Shimadzu QP-5000 series using DB-5 column (30 m x 0.25 mm). The initial temperature for the GC-MS was programmed at 50°C and increased to 320°C with rate of 6.5°C/min. The temperature of the injector and detector was set at 280°C and 320°C, respectively. About 2.0 mg of the samples were diluted in 200µl ethyl acetate and dichloromethane respectively. The diluted sample (1.0µl) was injected into GC-MS using splitless mode. The data analysis was carried out using GC-MS manufacturer's software. The QP-5000 uses a mass spectral (MS) data base which is based on data published by NIST (National Institutes of Standards and Technology).

3.3 CYTOTOXICITY TEST ON VERO CELLS

Fractions and subfractions were test for their cytotoxic effect on Vero cells by Eosin B assay as describe previously by Marini *et al.* (1998). This assay quantifies the amount of viable cells after their exposure to toxic substances by measuring the quantity of dye uptake by cells. The cytotoxicity was expressed as 50% lethal concentration (LC_{50}) which referred to the concentration of test materials that caused the reduction of viable cells by 50%.

3.3.1 Preparation of test materials

The fractions and subfractions obtained from CC-I and CC-II were dissolved in 10µl of dimethyl sulphoxide (DMSO, Sigma, USA), before adding Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% FBS to achieve a concentration of 4 mg/ml. Then, the solutions were sterilised by filtration using 0.22 µmmembrane filter (Minisart, USA) and used as the stock solution. Stock solution of each fraction was diluted 1:1 in DMEM supplemented with 5% FBS, resulting DMSO concentration to reduce at 0.5%. Then, two fold serial dilutions of fractions wereprepared in DMEM with 5% FBS and 0.5% DMSO.

3.3.2 Subculturing of cells

Aliquots of 100µl Vero cell suspension at concentration of 1×10^5 cells/ml were seeded into 96-wells microtiter plate (Nunclon, Denmark) (Appendix C) and the plates were then incubated overnight at 37°C in a 5% CO_2. Confluent monolayer cells were drained off of their growth medium and then washed with one change of sterile PBS. Two fold serial dilutions of fractions and subfractions (100µl) that were prepared separately in another microtiter plate at concentrations of 12.5, 25, 50, 100, 200 and 400 µg/ml were then apply onto cells, and plate was further incubated for 72 hours. Controls for this test were made up of cells treated with medium (mock treatment) and medium without cells. All controls always contained 0.5% DMSO in order to exclude any effect of DMSO on cells. The treatments were replicated four times.

3.3.3 Cells staining

After 72 hours of incubation, microtiter plate was processed using Eosin B uptake assay (Said *et al*., 2001) (Appendix D). Firstly, cells were fixed with 125µl of cold 25% trichloroacetic acid (TCA) at 4°C for an hour. Then, plate was washed with distilled water for five times and leave to dry at room temperature overnight. Next, the fixed cells were stained with 2% Eosin B (100µl) for an hour and then washed with 1% acetic acid (300µl) for five times to elute the excess dye. After two hoursdrying at 40°C, 5mM NaOH (200µl) was added into each well and incubated at room temperature for 20 minutes. Absorbance value of dye colour in each well was measured using ELISA reader (Lab Systems MTX Labs, USA) at 490nm wavelength.

3.3.4 Calculation of LC_{50}

The LC_{50} values were calculated as previously described by Marini *et al*. (1998). The concentrations of the test substances were plotted against their respective absorbance values. From the plot, the LC_{50} values of each substance was taken as the intermediate value between the LC_0 (0 percent cell death) and the LC_{100} (100 percent cell death).

3.4 ANTIVIRAL ASSAY AGAINST MEASLES VIRUS

The antiviral activity of *C. nardus* fractions and subfractions against measles virus (MV) was determined by dye uptake assay using Eosin B. This test was carried out according to the method previously described by Nurul Aini *et al*. (2006).

3.4.1 Preparation of test materials

Stock solution of each CC-I or CC-II fraction was prepared as previously described in cytotoxicity test (section 3.3.1), but the DMEM was supplemented with only 2% FBS. Fractions were then diluted to 1.0 LC_{50}, 0.1 LC_{50} and 0.01 LC_{50}, based on result from cytotoxicity test. The concentration of MV used for cell inoculation was fixed at 1000 $TCID_{50}$ (tissue culture infectious dose) unit, which is the virus dose that leads to the infection of 50% of the cells. Ribavirin (0.16 mg) was diluted in DMEM with 2% FBS to obtained a stock solution of 0.16 mg/ml, which equal to 50% cytotoxic concentration (CC_{50}) (Grancher *et al.,* 2004). This stock was sterilised by filtration using 0.22µm membrane filter (Minisart, USA).

3.4.2 Antiviral assay

Aliquots of Vero cells suspension (100µl) were seeded into 96-wells microtiter plate (Nunclon, Denmark) with a concentration of 1×10^5 cells/ml and incubated overnight at 37°C in a 5% CO_2 incubator to obtained confluent monolayer cells. The medium in each well was then removed and cells were washed with sterile PBS (pH 7.2). The mode of antiviral action of each fraction was determined by incubating cells and virus with test fraction at different stages during the viral infection cycle (Appendix E). Pre-treatment protocol involved pre-treating Vero cells with fraction before virus infection. On the contrary, post-infection protocol involved treatment of Vero cells after virus infection. The controls consisted of cells treated with fraction only, cells treated with virus only, cells treated with medium only, and medium only. All controls always

contained 0.5% DMSO in order to exclude any effect of DMSO on cells or virus and they were run simultaneously. Ribavirin was used as positive control in concentration of 0.1 and 0.01 CC_{50}.

3.4.2.1 Pre-treatment protocol

Aliquots of 100µl of diluted fractions were added to wells containing the Vero cells monolayer and then incubated at 37°C in a 5% CO_2. After 24 hours of incubation, wells were drained off of the fraction solutions and the cells that remain were washed twice with sterile PBS. Aliquots of 10µl of MV solution were then inoculated to the cells. This was followed by 1 hour incubation at 37°C for virus absorption and entry, and then by adding 100µl of DMEM to all wells. After 72 hours of incubation, the microtiter plate was then processed using the same method as previously described in cytotoxicity test (section 3.3.3).

3.4.2.2 Post-infection protocol

Aliquots of 10µl of MV solution were inoculated to the cells first, followed by 1 hour incubation at 37°C to allow the virus to absorb to and penetrate the cells. Subsequenly, 100µl of diluted fractions were added to the infected cells. The microtiter plate was incubated at 37°C in a 5% CO_2 incubator for 72 hours and then processed using the same method as previously described in cytotoxicity test (section 3.3.3).

3.4.3 Calculation of viral inhibition percentage

Absorbance value of dye colour in each well was measured using microplate reader (Lab Systems MTX Labs, USA) at 490nm wavelength. The percentage of MV-induced cytophatic effect (CPE) inhibition was calculated by the following formula (Semple *et al*. 1998; Chiang *et al.,* 2002; Jesus *et al.,* 2009) which is as follows:

$$\text{Percentage of MV-induced CPE inhibition} = (ODtv - ODcv) / (ODcd - ODcv) \times 100\%,$$

where ODtv is the absorbance of the test compounds with virus infected cells, ODcv is the absorbance of the virus control (cells treated with virus only) and ODcd is the absorbance of the cells control (cells treated with medium only), respectively.

3.5 ANTIVIRAL ASSAY OF ISOLATED COMPOUNDS AGAINST MEASLES VIRUS

CC-II subfractions which showed antiviral activity were further purified using preparative thin layer chromatography (PTLC) as previously described in section 3.2.2. The resulting subfractions, which also termed as isolated compounds, were screened for antiviral activity against measles virus. Preparation of stock solution and determination of LC_{50} values were similar to the assay method described for CC-I and CC-II fractions (sections 3.3.1, 3.3.3 and 3.4.1). Similarly, the antiviral assay was carried out using both protocols as described previously. Isolated compounds were diluted to 0.01 and 0.1 LC_{50}, based on result from cytotoxicity test.

3.6 COMBINATION TREATMENT OF ISOLATED COMPOUNDS AND RIBAVIRIN AGAINST MEASLES VIRUS

To assess the possible synergistic effect of *C. nardus* isolated compounds and Ribavirin on the inhibition of measles virus, the combination treatment was carried out. For this experiment, concentration ranges varied depending on the LC_{50} of each compound. The final concentration of DMSO in all assays was kept constant at 0.1% (v/v), at which the host cell was not affected by DMSO toxicity.

3.6.1 Preparation of assay materials

Isolated compounds were diluted from stock solution (section 3.5) to 0.01, 0.05 and 0.1 LC_{50}, based on result from cytotoxicity test. Meanwhile, Ribavirin was diluted to 0.01, 0.05 and 0.1 CC_{50} from stock solution as describe in section 3.4.1. All dilution were performed using DMEM with 2% FBS. The concentration of measles virus used for cell inoculation was fixed at 1000 $TCID_{50}$.

3.6.2 Synergistic activity assay

Combination treatment was performed as in antiviral assay previously described by Adibah *et al*. (2011) and is essentially following the antiviral assay as described in section 3.4.2. Aliquots of 100µl Vero cell suspension at concentration of 1×10^5 cells/ml were seeded into 96-wells microtiter plate (Nunclon, Denmark) and the plates were then incubated overnight at 37°C in 5%

CO_2. The resulting confluent cell monolayers were drained off of their growth medium and then subsequently washed with one change of sterile PBS.Aliquot (100µl) of both isolated compounds and Ribavirin were then simultaneously dispensed into the wells of microtitre plate. This combined treatment was done before virus inoculation (pre-treatment) or after virus inoculation (post-infection). After both measles virus inoculation and combined antiviral treatments were completed, the microtiter plate was returned to the CO_2 incubator for further 72 hours.

Microtiter plate processing to obtain absorbance values and hence, cell viability was done using the same method as previously described in cytotoxicity test (section 3.3.3). Seven controls were used in this assay. These were (i) cells treated with combination only, (ii) cells treated with virus and isolated compound, (iii) cells treated with virus and Ribavirin, (iv) cells treated with isolated compound only, (v) cells treated with Ribavirin only, (vi) cells treated with virus only, and (vii) cells treated with medium only. All controls had 0.5% DMSO in the growth media as in the test items.

3.6.3 Calculation of combination index

Thereafter, the combinatorial effect from the combination of Ribavirin and isolated compound against measles virus infection was analyzed by using the isobologram method. The $IC_{50,}$ which was determined fromdose-response graph, was used to calculate the fractional inhibitory concentration (FICs) according to the following formula (Nduati & Kamau, 2006; Cheng *et al.*, 2009):

$$FICs = [Cc] / [Ce] + [RBVc]/ [RBVe]$$

where Ce and RBVe were the concentrations of isolated compounds and Ribavirin, respectively, that produced a specified inhibition level (IC_{50}) when used alone. Cc and RBVc were the concentrations that produced the same inhibition levels when used in combination. By this calculation, the interaction between Ribavirin and isolated compound was interpreted according to the combined FICs index (FIC of isolated compound plus FIC of Ribavirin). The interactions were categorized as follows: high synergy was said to be present if FICs value is less than 0.40. Those combinations with FICs greater than 0.40 but lower than 1, were classified as having moderate synergy, while those with a FICs approximately equal to 1 represented additive interactions, while those with FICs between 1 and 2 were categorized as partially additive/weak antagonism and those greater than two represented high antagonism.

3.7 ANTIPROLIFERATIVE ACTIVITY OF SELECTED ISOLATED COMPOUNDS

In this anti-proliferative screening, 10 isolated compounds of *C. nardus* were tested for their anti-proliferative activity towards human papillary ovarian adenocarcinoma (Caov-3) cancer cell. This assay was done according to the method previously described by Nurmawati (2007).

3.7.1 Preparation of test materials

Isolated compounds were diluted from stock solution to 25, 50, 100 and 200 µg/ml. Positive control, Tamoxifen, was prepared in DMEM supplemented with 2% FBS plus 0.5% DMSO to obtain a stock solution of 0.40 mg/ml. This stock was sterilized by filtration using 0.22µm

membrane filter (Minisart, USA) and further diluted to 25, 50, 100 and 200 µg/ml. All dilutions were prepared using DMEM with 2% FBS plus 0.5% DMSO.

3.7.2 Cell proliferation assay

Aliquots of 100µl of Caov-3 cell suspension at concentration of 1.25×10^5 cells/ml were seeded into 96-wells microtiter plate (Nunclon, Denmark) and the plates were then incubated overnight at 37°C in 5% CO_2 (Appendix F). Confluent monolayer cells were drained off of their growth medium and then washed with one change of sterile PBS. Cells were then exposed to two-fold serial dilutions of isolated compounds (100µl) prepared at concentrations of 25, 50, 100, 200 and 400 µg/ml in quadruplicates, and further incubated for 72 hours. Controls for this test were made up of cells treated with medium and only medium. All controls were added with0.5% DMSO in the growth media as in the test items to exclude any effect of DMSO on cells.

3.7.3 Cells staining

After 72 hours of incubation, microtiter plate was processed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) assay as described by Nurmawati (2007) (Appendix G). Firstly, 20µl of 5mg/ml MTT were added to each well and the microtiter plate kept at 37°C for 4 hours to allow formation of the formazan crystals. The solution was then carefully discarded and the blue formazan crystals were dissolved in 100µl DMSO. The microtiter plate was shaken for 5 minutes and the absorbance of the solubilised blue formazan crystals was then measured using plate reader (Meter Tech, Taiwan) at 620nm wavelength. The effect of isolated compounds

on proliferation of Caov-3 cells was expressed as the percentage of cell viability using the following formula: percentage of cell viability = OD_{620} of treated cells / OD_{620} of control cells × 100% (Wang *et al.*, 2006).

3.8 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad, USA). One-way analysis of variance (ANOVA) and Student's *t*-test were used to compare data at a 95% confidence limit. A P value less than 0.05 ($P<0.05$) was regarded as significant. The LC_{50} and IC_{50} were determined from the line graphs where both curves crossed.

CHAPTER 4: RESULTS

4.1 EXTRACTION AND FRACTIONATION

From 2 kg of plant material that were extracted with hexane, 32.55 g of dried greenish crude extract was obtained. The percentage recovery (w/w) was calculated to be 1.63%. Fractionation of this extract was successfully done using column chromatography I (CC-I) with 200 fractions being obtained. Hence, the solvent system used was successful in fractionation process. All these fractions were subjected to thin layer chromatography (TLC) which was developed using suitable solvents. A total of 20 combined fractions were obtained as presented in Table 8.

4.2 CYTOTOXICITY AND ANTIVIRAL ACTIVITY ASSAY OF CC-I FRACTIONS

4.2.1 Cytotoxicity of fractions on Vero cells

The cytotoxicity values of all fractions were determined from graphical representation of cell survivor (absorbance) as a result of treated of Vero cells with the various concentrations of the fractions (Appendix H). Results in Table 9 and Figure 1 showed that these values ranged from 50 to 500 µg/ml. Most of the fractions were relatively non-toxic to the cells as they showed LC_{50} of more than 100 µg/ml. Only 4 fractions, namely FH11, FH12, FH19 and FH20, exhibited mild cytotoxicity on the cells with LC_{50} values between 50 to 100 µg/ml. All these LC_{50} values were used as the highest concentration of each fraction in the antiviral test.

Table 8: Combined fractions from column chromatography (CC I) of crude extract

Fractions	**Combined fractions**	**Weight (g)**	**Yield based on crude extract (%)**
1-9	FH1	0.00	0.00
10-24	FH2	0.03	0.75
25-27	FH3	0.04	1.00
28-37	FH4	0.30	7.50
38-40	FH5	0.35	8.75
41-48	FH6	0.30	7.50
49-72	FH7	0.10	2.50
73-96	FH8	0.47	11.75
97-115	FH9	0.11	2.75
116-122	FH10	0.69	17.25
123-124	FH11	0.13	3.25
125-128	FH12	0.32	8.00
129-139	FH13	0.05	1.25
140-147	FH14	0.04	1.00
148-152	FH15	0.07	1.75
153-170	FH16	0.05	1.25
171-177	FH17	0.01	0.25
178-180	FH18	0.01	0.25
181-192	FH19	0.03	0.75
193-200	FH20	0.04	1.00
Total weight		3.14	
Total % recovery			78.50

Table 9: The cytotoxicity of CC-I fractions on Vero cells as expressed in LC_{50} values

Combined fraction	LC_{50} (µg/ml)	Combined fraction	LC_{50} (µg/ml)
FH1	-[nd]	FH11	100
FH2	270	FH12	100
FH3	620	FH13	180
FH4	500	FH14	430
FH5	210	FH15	200
FH6	290	FH16	400
FH7	160	FH17	300
FH8	170	FH18	280
FH9	180	FH19	80
FH10	200	FH20	50

-[nd]: not determined

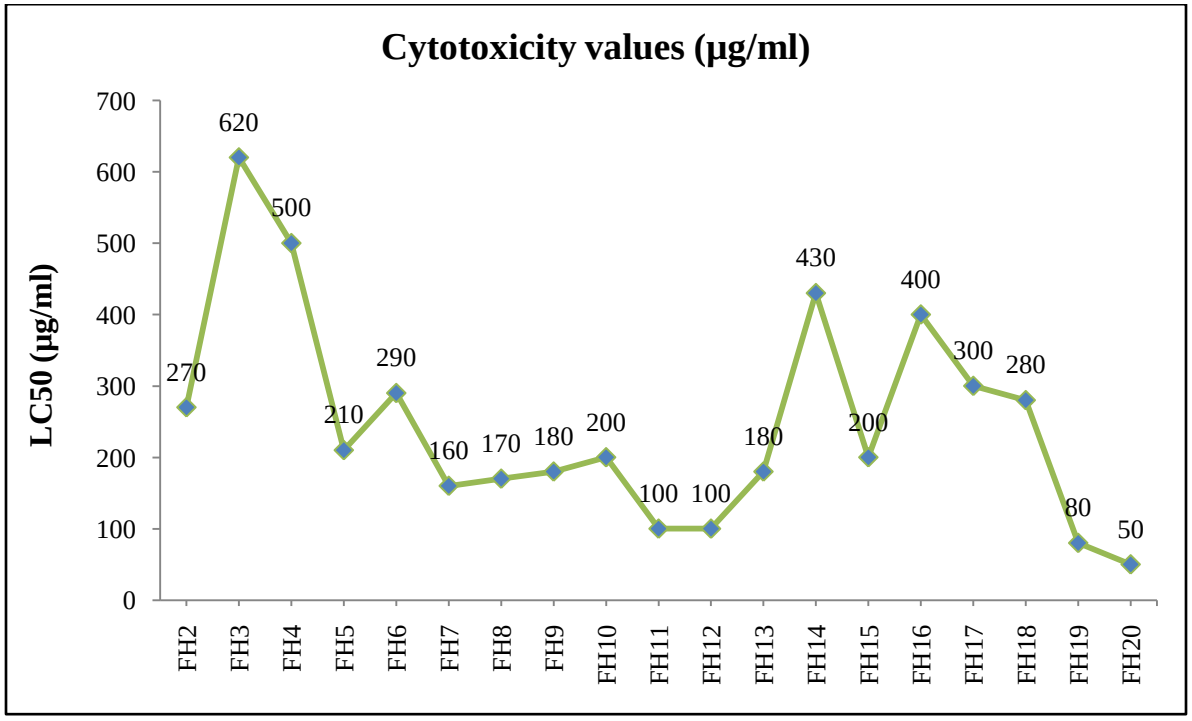

Figure 1: The LC_{50}values of CC-I fractions presented in graph

4.2.2 Antiviral activities of CC-I fractions against measles virus (MV)

The results of antiviral activities of each CC-I fraction against MV are presented in Figure 2 and Figure 3. The activities of these fractions were observed based on the percentage of MV-induced CPE inhibition, which previously described in methodology (section 3.4.3). All fractions significantly ($P<0.05$) reduced the CPE in all tested concentrations as compared with control (untreated cells) (Appendix I). Positive control, Ribavirin, showed 70% and 80% inhibition of virus at concentration of 0.1 CC_{50} (16.6 μg/ml) in pre-treatment and post-infection protocol, respectively. At lower concentration (0.01 CC_{50}), Ribavirin inhibited 40% and 50% of MV-induced CPE in each protocol.

Results showed that majority of the CC-I fractions exert good activity in the post-infection protocol. Of the 19 fractions tested, 9 fractions (FH2, FH4, FH5, FH12, FH13, FH14, FH16, FH19 and FH20) exhibited higher percentage of CPE inhibition in this protocol compared to the pre-treatment. Four of these fractions (FH4, FH5, FH12 and FH20) were able to inhibit MV infection by more than 75% at 1.0 LC_{50}, meanwhile the other 5 fractions (FH2, FH13, FH14, FH16 and FH19) showed the same activity at lower concentration (0.1 LC_{50}) as they were found to be cytotoxic at 1.0 LC_{50}. Statistical analysis showed that these results were significantly different from Ribavirin at concentration of 0.1 CC_{50} in the same protocol (Appendix I). The antiviral activity of these fractions could also be detected in the lower dilutions.

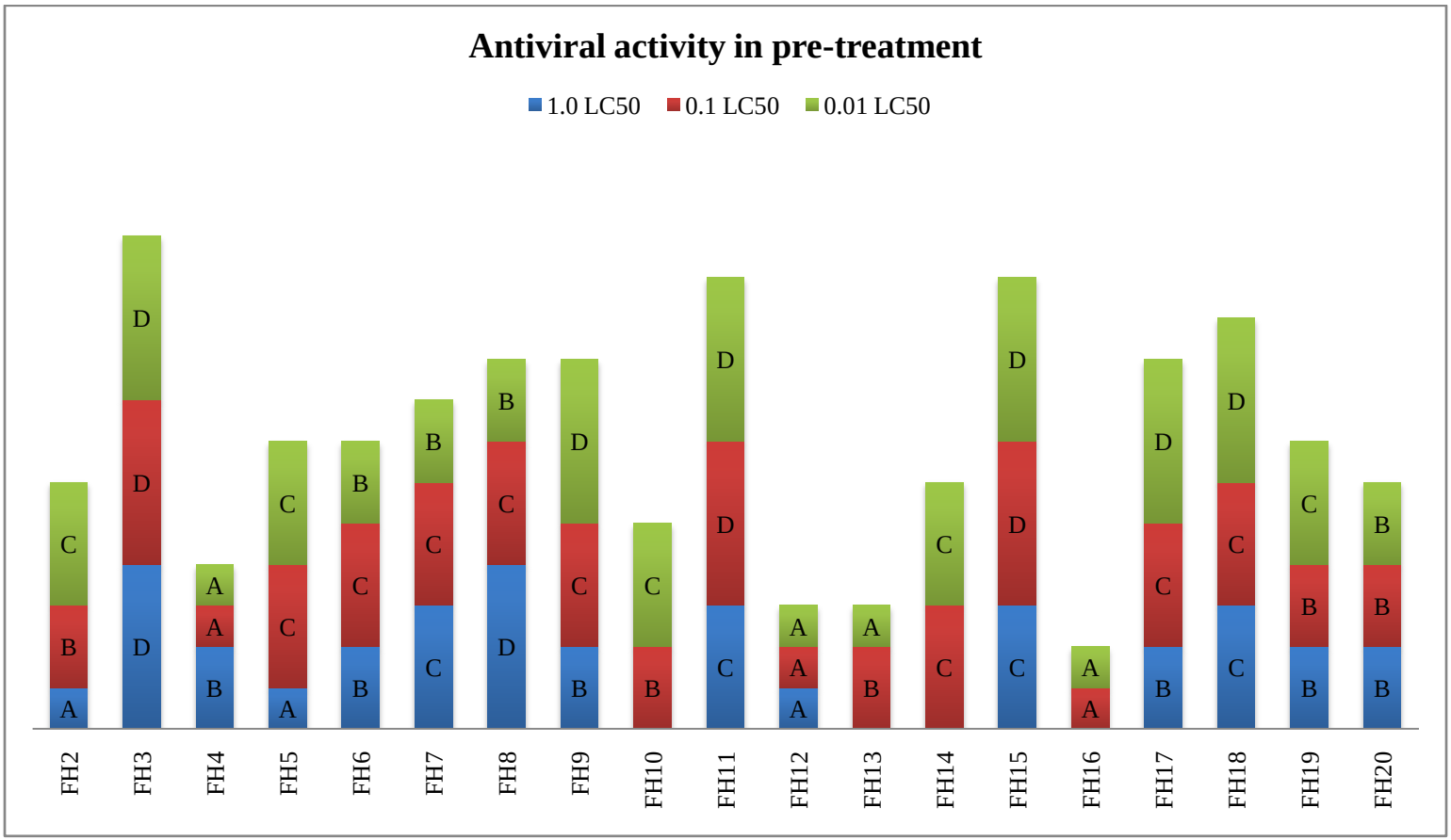

Figure 2: Antiviral activity of fractions at different concentration in pre-treatment protocol. Results are expressed as percentage of MV-induced CPE inhibition: A (≤25% inhibition); B (25% < χ < 50% inhibition); C (50% < χ < 75% inhibition); D (≥75% inhibition). Concentrations of fractions were at 0.01, 0.1 and 1.0 LC_{50} based on cytotoxocity values.

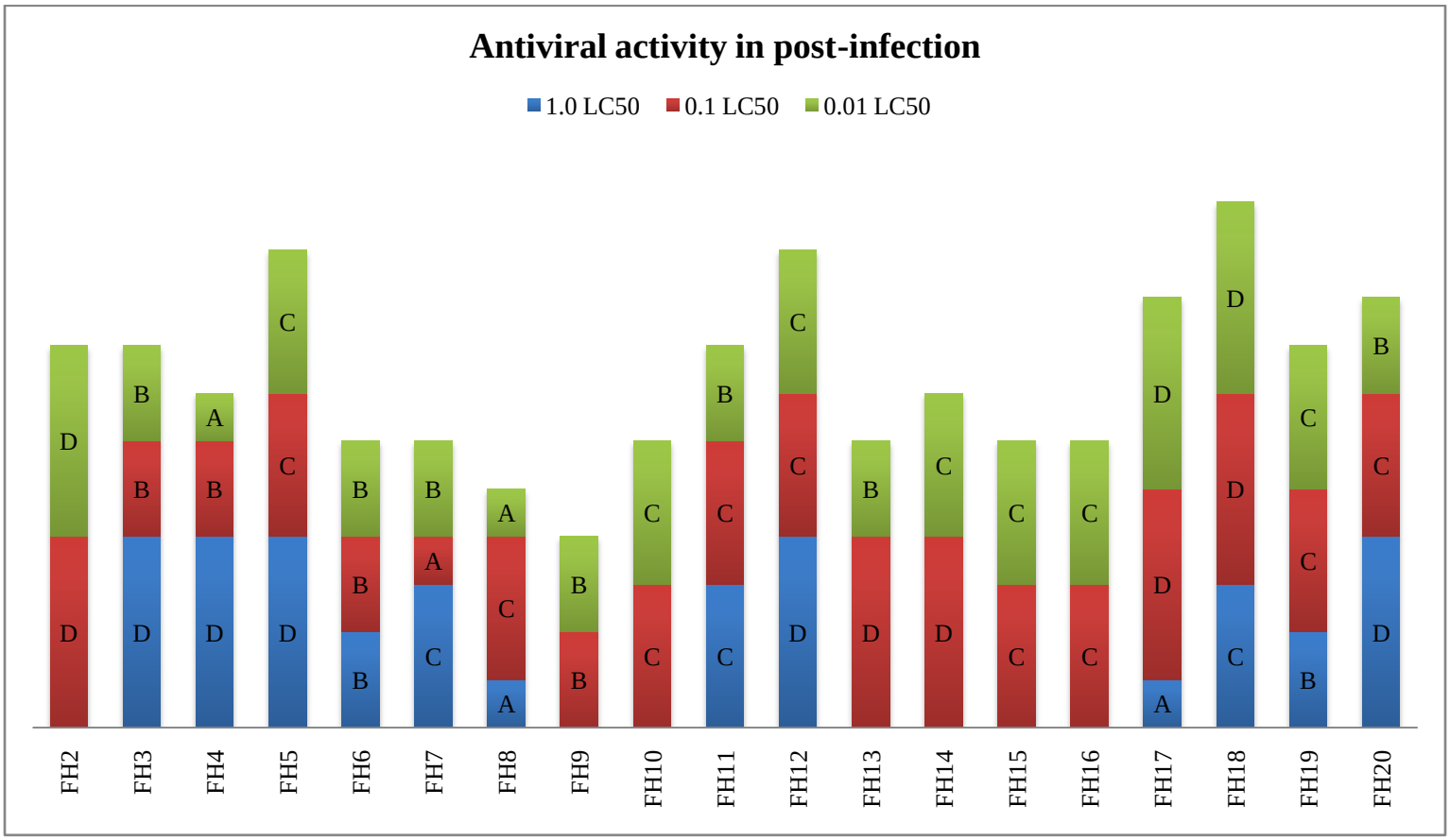

Figure 3: Antiviral activity of fractions at different concentration in post-infection protocol. Results are expressed as percentage of MV-induced CPE inhibition: A (≤25% inhibition); B (25% < χ < 50% inhibition); C (50% < χ < 75% inhibition); D (≥75% inhibition). Concentrations of fractions were at 0.01, 0.1 and 1.0 LC_{50} based on cytotoxocity values.

In the pre-treatment, only 3 fractions (FH8, FH9 and FH15) were found to be more effective against MV through this protocol. Fraction FH8 and FH15 were able to inhibit MV-induced CPE by more than 75% at 1.0 and 0.1 $LC_{50,}$ respectively.Both of these fractions showed weak antiviral activity in post-infection protocol at the same concentration evaluated, whereby they only able to inhibit less than 25% of CPE. Meanwhile for fraction FH9, the highest antiviral activity was obtained at the lowest dilution (0.01 LC_{50}), with more than 75% inhibition. These results were significantly different from Ribavirin at concentration of 0.1 CC_{50} (1.66 μg/ml) in the same protocol (Appendix I).

On the other hand, 7 fractions were found to be active in both pre-treatment and post-infection protocols. They were fraction FH3, FH6, FH7, FH10, FH11, FH17 and FH18. These fractions exhibited similar activity against MV at the same concentration tested in both protocols. For example at 1.0 LC_{50}, fraction FH3 was able to inhibit viral replication by more than 75%, fraction FH7, FH11 and FH18 showed higher than 50% of viral inhibition, fraction FH6 and FH17 possess less than 50% of viral inhibition, and fraction FH10 managed to inhibit the virus infection less than 25% but was increased to more than 50% in lower dilution (0.1 LC_{50}).

Due to the small amount of sample, only 8 active fractions (FH4, FH5, FH6, FH7, FH8, FH10, FH11 and FH15) were selected to further purification process using column chromatography II (CC-II).

4.3 ANTIVIRAL ACTIVITIES OF SELECTED CC-II SUBFRACTIONS

Pre-treatment and post-infection protocols were used to investigate the mechanism of antiviral action of each subfraction.The antiviral activity is presented as the inhibition percentages of MV-induced CPE and was determined at 50% inhibition. Positive control, Ribavirin, exhibited 70% and 85% inhibition of CPE at concentration of 0.1 CC_{50} (16.6 μg/ml) in pre-treatment and post-infection protocol, respectively. In lower concentration (0.01 CC_{50}; 1.66 μg/ml), this drug inhibited 40% and 50% of CPE in each protocol.

4.3.1 Fraction FH4

Fraction FH4 (0.30 g) was subjected to further fractionation process using CC-II. During storage, there was white powdery substance formed from this fraction and this substance was collected as it could indicate pure compound. This substance was labelled as FH4.01 and weighted 0.02g. The rest of the fraction was further separated through CC-II and a total of 204 subfractions were produced. Subfractions with similar profile were combined together after TLC, produced 11 combined subfractions. Results are summarised in Table 10. Due to the low weight of sample, subfraction FH4.11 was not further tested.

4.3.1.1 Cytotoxicity of FH4 subfractions on Vero cells

A total of 10 subfractions derived from fraction FH4 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all subfractions were ranging from 280 to 600 μg/ml (Table 11).

Table 10: Combined subfractions from CC-II of fraction FH4

Combined subfraction	Label	Weight (g)	Percentage of yield (%)
1-18	FH4.02	0.01	3.33
19-20	FH4.03	0.02	6.67
21-23	FH4.04	0.04	13.33
24-25	FH4.05	0.03	10.00
26-30	FH4.06	0.04	13.33
31-59	FH4.07	0.03	10.00
60-117	FH4.08	0.03	10.00
118-126	FH4.09	0.03	10.00
127-137	FH4.10	0.02	6.67
138-184	FH4.11	0.02	6.67
185-204	FH4.12	0.01	3.33
Total yield		0.28	
Total % recovery			93.33

Subfraction FH4.08 showed the lowest LC_{50} value, which was at 280 µg/ml. Meanwhile, subfraction FH4.07 showed highest LC_{50} value of 600 µg/ml. The other 9 subfractions showed LC_{50} values between 300 to 580 µg/ml. All subfractions were considered as not toxic to the cells and the LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

4.3.1.2 Antiviral activities of FH4 subfractions against measles virus

After determining their cytotoxicity concentration, the potential antiviral activities of FH4 subfractions against measles virus (MV) were investigated. Results of this assay are presented in

Table 11. Overall, majority of the subfractions were found to be cytotoxic at the highest concentration tested (1.0 LC_{50}) but not at tenfold dilution (0.1 LC_{50}).

Pre-treatment of Vero cells with FH4 subfractions significantly decreased MV-induced CPE ($P<0.05$) when compared with untreated cells (Appendix J). The strongest antiviral activity was found in subfraction FH4.01 and FH4.06, which exhibited antiviral activity at concentration ranging from 5.6 to 560 µg/ml (Table 11). These subfractions inhibited the development of CPE by more than 50% at 560µg/ml (1.0 LC_{50}). Similar activity was found in subfraction FH4.02 and FH4.04, which exhibited antiviral activity at concentration ranging from 4 to 400 µg/ml. In contrast, the weakest activity against MV was found in subfraction FH4.07 and FH4.09. These subfractions were able to inhibit the virus less than 25% at concentration of 0.1 LC_{50}. Statistical analysis using Student's *t*-test demonstrated a significant different between FH4.01 and 0.1 CC_{50} Ribavirin in the same protocol with $P<0.01$ (Appendix J). Meanwhile the antiviral activity showed by FH4.04 and FH4.05 were not significantly different from 0.1 CC_{50} Ribavirin in the same protocol.

In post-infection protocol, the subfractions significantly reduced the development of CPE ($P<0.05$) as compared with control (Appendix J). Subfraction FH4.01, FH4.02, FH4.04 and FH4.05 were found to have the greatest activity against measles virus, inhibiting the virus-induced CPE production by more than 50% at the highest concentration tested (1.0 LC_{50}), except for FH4.05 at 0.1 LC_{50},and manage to retain their activity in the lower dilutions (Table 11). There were no significant difference in reduction of CPE between these subfractions and 0.1 CC_{50}

Table 11: LC_{50} values and antiviral activities of FH4 subfractions

Subfractions	[a]LC_{50} (µg/ml)	Pre-treatment			Post-infection		
		[b]Antiviral activity	[c]Effective concentration range (µg/ml)	[d]Dilution factor	Antiviral activity	Effective concentration range (µg/ml)	Dilution factor
FH4.01	560	+++	5.6 - 560	2	+++	5.6 - 560	2
FH4.02	400	+++	4.0 - 400	2	+++	4.0 - 400	2
FH4.03	580	++	5.8 - 58	1	+	5.8 - 58	1
FH4.04	400	+++	4.0 - 400	2	+++	4.0 - 400	2
FH4.05	400	++	4.0 - 40	1	+++	4.0 - 40	1
FH4.06	560	+++	5.6 - 560	2	++	5.6 - 56	1
FH4.07	600	+	60	0	++	6 - 60	1
FH4.08	280	++	2.8 - 28	1	++	2.8 - 28	1
FH4.09	300	+	3.0 - 30	1	++	3.0	0
FH4.10	400	++	4.0 - 40	1	++	4.0 - 40	1

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

Ribavirin in the same protocol (Appendix J). Meanwhile, the weakest antiviral activity was found in subfraction FH4.03. This subfraction inhibited less than 25% of MV-induced CPE at concentration of 0.1 LC_{50} (58 µg/ml).

From the result, subfraction FH4.01, FH4.02 and FH4.04 were subjected to further purification process. Subfraction FH4.01 was further analysis using GC-MS, meanwhile subfraction FH4.04 was further purified using preparative thin layer chromatography (PTLC). However, FH4.02 was not further purified due to lack of sample.

4.3.2 Fraction FH5

Fraction FH5 (0.35 g) was further separated into different subfractions by CC-II. Elution process yielded a total of 151 subfractions. These subfractions were then analysed using thin layer chromatography (TLC) and subfractions with similar profile were combined together. Twelve combined subfractions were obtained and their weight was recorded as shown in Table 12. Subfraction FH5.01, FH5.02 and FH5.12 were not further used due to small weight of sample.

4.3.2.1 Cytotoxicity of FH5 subfractions on Vero cells

A total of 9 subfractions derived from fraction FH5 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all FH5 subfractions were ranging from 80 to 400 µg/ml (Table 13). Two subfractions, FH5.08 and FH5.10, exhibited mild cytotoxicity in Vero cells with LC_{50} of 80 to 90 µg/ml. Subfractions FH5.03 and FH5.05 were the less toxic to cells with LC_{50} of 400

μg/ml. The other 5 subfractions were considered not toxic to the cells as they showed LC_{50} higher than 100 μg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

Table 12: Combined subfractions from CC-II of fraction FH5

Combined subfraction	Label	Weight (g)	Percentage of yield (%)
1-13	FH5.01	0.01	2.86
14-25	FH5.02	0.01	2.86
26-30	FH5.03	0.03	8.57
31-38	FH5.04	0.03	8.57
39-50	FH5.05	0.04	11.43
51-54	FH5.06	0.05	14.29
55-67	FH5.07	0.05	14.29
68-94	FH5.08	0.04	11.43
95-108	FH5.09	0.03	8.57
109-120	FH5.10	0.03	8.57
121-134	FH5.11	0.02	5.71
135-151	FH5.12	0.01	2.86
Total yield		0.30	
Total % recovery			85.71

4.3.2.2 Antiviral activities of FH5 subfractions against measles virus

The potential antiviral activities of FH5 subfractions against measles virus (MV) are presented in Table 14. All subfractions were significantly ($P<0.05$) reduce the formation of MV-induced CPE in a dose-dependent manner compared to cells control (Appendix K).

In the pre-treatment protocol, the highest activity against MV was found in subfraction FH5.04, FH5.07 and FH5.09, which exhibited antiviral activity at concentration ranging from 2 to 200 µg/ml, 1.6 to 160 µg/ml and 1.5 to 150 µg/ml, respectively (Table 13). These subfractions inhibited the development of MV-induced CPE by more than 50% at the highest concentration tested (1.0 LC_{50}) and manage to retain their activity in the lower dilutions. Similar activity was found in subfraction FH5.08 and FH5.10, which exhibited antiviral activity at lower concentration ranging from 0.8 to 80 µg/ml and 0.9 to 90 µg/ml, respectively. Statistical analysis showed that these results were not significantly different from Ribavirin at concentration of 0.1 CC_{50} in the same protocol (Appendix K). On the other hand, the weakest antiviral activity was showed by subfraction FH5.03, FH5.05, FH5.06 and FH5.11. These subfractions possess less than 50% inhibition of CPE at highest concentration (1.0 LC_{50}).

Meanwhile in the post-infection protocol, subfraction FH5.04, FH5.06 and FH5.07 exhibited the strongest antiviral activity with more than 50% inhibition of MV-induced CPE at the highest concentration (1.0 LC_{50}) (Table 13). Their antiviral activity could also be detected in the two subsequent dilutions beyond. The active concentration of these subfractions ranged from 1.6 to 300 µg/ml. Statistical analysis demonstrated no significant difference between these subfractions with 0.01 CC_{50} Ribavirin in the same protocol (Appendix K). Meanwhile, the weakest activity was exhibited by subfraction FH5.03, FH5.05, FH5.08, FH5.09 and FH5.11. All of them were able to inhibit measles virus less than 25% at all concentrations evaluated.

From the result, subfraction FH5.04 and FH5.07 were subjected to further purification process using preparative thin layer chromatography (PTLC).

Table 13: LC_{50} values and antiviral activities of FH5 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH5.03	400	++	4.0 – 400	2	+	4.0 – 400	2
FH5.04	200	+++	2.0 – 200	2	+++	2.0 – 200	2
FH5.05	400	++	4.0 – 400	2	+	4.0 – 400	2
FH5.06	300	++	3.0 – 300	2	+++	3.0 – 300	2
FH5.07	160	+++	1.6 – 160	2	+++	1.6 – 160	2
FH5.08	80	+++	0.8 – 80	2	+	8.0 – 80	1
FH5.09	150	+++	1.5 – 150	2	+	1.5 – 150	2
FH5.10	90	+++	0.9 – 90	2	++	0.9 – 90	2
FH5.11	150	++	1.5 – 150	2	+	1.5 – 150	2

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media.

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

4.3.3 Fraction FH6

Fraction FH6 (0.30 g) was further separated into different subfractions by CC-II. A total of 125 subfractions were yielded after the elution process and these subfractions were analysed using thin layer chromatography (TLC). Subfractions with similar profile were combined together. Twelve combined subfractions were obtained and results were summarised in Table 14. Due to the low weight of FH6.12, this subfraction was not further tested in the subsequent experiment.

Table 14: Combined subfractions from CC-II of fraction FH6

Combined subfraction	**Label**	**Weight (g)**	**Percentage of yield (%)**
1-15	FH6.01	0.02	6.67
16-27	FH6.02	0.03	10.00
28-39	FH6.03	0.04	13.33
40-41	FH6.04	0.03	10.00
42-45	FH6.05	0.04	13.33
46-57	FH6.06	0.02	6.67
58-71	FH6.07	0.02	6.67
72-85	FH6.08	0.03	10.00
86-89	FH6.09	0.02	6.67
90-95	FH6.10	0.02	6.67
96-103	FH6.11	0.02	6.67
104-125	FH6.12	0.01	3.33
Total yield		0.30	
Total % recovery			100

4.3.3.1 Cytotoxicity of FH6 subfractions on Vero cells

A total of 11 subfractions derived from fraction FH6 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all FH6 subfractions were ranging from 100 to 400 µg/ml (Table 15). Subfraction FH6.07 showed the lowest LC_{50} value, which was at 100 µg/ml, and was considered as mildly cytotoxic. The highest LC_{50}value was showed by subfraction FH6.01 (400 µg/ml). The other 10 subfractions showed LC_{50} values between 150 to 300 µg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

4.3.3.2 Antiviral activities of FH6 subfractions against measles virus (MV)

After determining the cytotoxicity values, the potential antiviral activities of FH6 subfractions against MV were investigated. Results are summarised in Table 15. Overall, all subfractions were found to have statistically significant ($P<0.05$) anti-MV activities when compared with untreated cells (Appendix L).

In the pre-treatment protocol, the most pronounced antiviral activity was found in subfraction FH6.03, FH6.06 and FH6.10, which exhibited antiviral activity at concentration ranging from 3 to 300, 1.5 to 150 and 2 to 200 µg/ml, respectively (Table 15). These subfractions inhibited the development of MV-induced CPE by more than 50% at the highest concentration (1.0 LC_{50}). Statistical analysis revealed no significant difference in the reduction of CPE of infected cells treated with these subfractions compared to 0.1 CC_{50} Ribavirin in the same protocol (Appendix L). Meanwhile, the weakest activity against MV was found in subfraction FH6.02,

Table 15: LC_{50} values and antiviral activities of FH6 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH6.01	400	++	40 – 400	1	++	4 – 400	2
FH6.02	200	+	20	0	++	20 – 200	1
FH6.03	300	+++	3 – 300	2	++	3 – 300	2
FH6.04	300	++	3 – 300	2	++	3 – 300	2
FH6.05	200	+	2 – 20	1	++	20 – 200	1
FH6.06	150	+++	1.5 – 150	2	+++	15 – 150	1
FH6.07	100	++	10	0	++	1 – 100	2
FH6.08	200	+	2 – 200	2	++	2 – 20	1
FH6.09	270	++	27 – 270	1	+++	2.7 – 270	2
FH6.10	200	+++	2 – 200	2	++++	2 – 200	2
FH6.11	300	+	3 – 300	2	++++	3 – 30	1

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

FH6.05, FH6.08 and FH6.11. These subfractions were able to inhibit the virus less than 25% at all concentrations evaluated.

For the post-infection protocol, the strongest antiviral activity was showed by subfraction FH6.10 and FH6.11, which exhibited antiviral activity at concentration ranging from 2 to 200, and 3 to 30 µg/ml, respectively (Table 15). These subfractions were able to inhibit MV by more than 75% at highest concentration and manage to retain their activity in the lower dilution. There was no significant difference between these subfractions and 0.1 CC_{50} Ribavirin in the inhibition of MV-induced CPE in the same protocol (Appendix L). Meanwhile, the weakest activity against measles virus was found in subfraction FH6.01, FH6.02, FH6.03, FH6.04, FH6.05, FH6.07 and FH6.08. These subfractions only inhibited MV-induced CPE by more than 25% at highest concentration.

From the result, subfractions FH6.06 and FH6.10 were subjected to further purification process.

4.3.4 Fraction FH8

Fraction FH8 (0.47 g) was further separated into different subfractions by CC-II. Elution process produced a total of 160 subfractions and these subfractions were then analysed using thin layer chromatography (TLC). Seventeen combined subfractions were obtained and results were summarised in Table 16. Due to the low weight of sample, subfraction FH8.01, FH8.03, FH8.05, FH8.14, FH8.15 and FH8.17 were not further used in the subsequent experiment.

Table 16: Combined subfractions from CC-II of fraction FH8

Combined subfraction	Label	Weight (g)	Percentage of yield (%)
1-22	FH 8.01	0.01	2.13
23-28	FH 8.02	0.02	4.26
29-34	FH 8.03	0.01	2.13
35-38	FH 8.04	0.03	6.38
39-46	FH 8.05	0.01	2.13
47-50	FH 8.06	0.05	10.64
51-59	FH 8.07	0.04	8.51
60-75	FH 6.08	0.04	8.51
76-85	FH 8.09	0.03	6.38
86-97	FH 8.10	0.06	12.78
98-107	FH 8.11	0.03	6.38
108-111	FH 8.12	0.03	6.38
112-123	FH 8.13	0.03	6.38
124-131	FH 8.14	0.01	2.13
132-137	FH 8.15	0.01	2.13
138-149	FH 8.16	0.02	4.26
150-160	FH 8.17	0.01	2.13
Total yield		0.44	
Total % recovery			93.61

4.3.4.1 Cytotoxicity of FH8 subfractions on Vero cells

A total of 11 subfractions derived from fraction FH8 were screened for their toxicity on Vero cells. Overall, the cytotoxic concentrations of all subfractions were ranging from 60 to 360 µg/ml (Table 17). Six subfractions, namely FH8.09, FH8.10, FH8.11, FH8.12, FH8.13 and FH8.16,

exhibited mild cytotoxicity in Vero cells with LC_{50} ranging from 60 to 90 µg/ml. The other 5 subfractions were considered not toxic to the cells as they showed LC_{50} more than 100 µg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

4.3.4.2 Antiviral activities of FH8 subfractions against measles virus

The potential antiviral activities of each FH8 subfraction against measles virus (MV) are summarised in Table 17. Overall, all subfractions reduced the formation of MV-induced CPE in a dose-dependent manner, except for subfraction FH8.06 that were found to be cytotoxic at highest concentration (160µg/ml; 1.0 LC_{50}) but not at tenfold dilution (16µg/ml; 0.1 LC_{50}).

In the post-infection, all subfractions significantly reduced the development of MV-induced CPE ($P<0.05$) as compared with control (Appendix M). The strongest antiviral activity was found in subfraction FH8.08 and FH8.09, which exhibited antiviral activity at concentration ranging from 10 to 100 and 0.8 to 80 µg/ml, respectively (Table 17). These subfractions inhibited the CPE by more than 50% at highest concentration (1.0 LC_{50}). Similar activity was found in subfraction FH8.06 which exhibited antiviral activity at lower concentration ranging from 1.6 to 16 µg/ml. Statistical analyses demonstrated no significant differences between these subfractions with 0.01 CC_{50} Ribavirin in the same protocol (Appendix M). Meanwhile, the weakest antiviral activity was found in subfractions FH8.11. This subfraction was able to inhibit the virus less than 25% at highest concentration (80 µg/ml).

Table 17: LC_{50} values and antiviral activities of FH8 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH8.02	200	+	2	0	+	2 – 200	2
FH8.04	280	++	28 – 280	1	++	2.8 – 280	2
FH8.06	160	++	1.6 – 16	2	+++	1.6 – 16	2
FH8.07	360	++	3.6 – 360	2	++	3.6 – 360	2
FH8.08	100	++	10 – 100	1	+++	10 – 100	1
FH8.09	80	++	0.8 – 80	2	+++	0.8 – 80	2
FH8.10	70	++	0.7 – 70	2	++	7 – 70	1
FH8.11	80	++	8 – 80	1	+	80	0
FH8.12	90	++	9 – 90	1	+	9 – 90	1
FH8.13	70	++	0.7 – 70	2	++	0.7 – 70	2
FH8.16	60	++	0.6 – 60	2	++	0.6 – 60	2

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

From the result, subfractions FH8.06 and FH8.10 were subjected to further purification process.

4.3.5 Fraction FH10

Fraction FH10 (0.69 g) was further separated into different subfractions by CC-II. Elution process was performed with increasing polarity of solvent systems as described previously. A total of 121 subfractions were produced after the process and they were analysed using thin layer chromatography (TLC). Subfractions with similar profile were combined together. Six combined subfractions were obtained and results were summarised in Table 18.

Table 18: Combined subfractions from CC-II of fraction FH10

Combined subfraction	**Label**	**Weight (g)**	**Percentage of yield (%)**
1-13	FH10.01	0.06	8.70
14-29	FH10.02	0.10	14.49
30-71	FH10.03	0.18	26.08
72-84	FH10.04	0.13	18.84
85-94	FH10.05	0.10	14.49
95-121	FH10.06	0.08	11.59
Total yield		0.65	
Total % recovery			94.19

4.3.5.1 Cytotoxicity of FH10 subfractions on Vero cells

A total of 6 subfractions derived from fraction FH10 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all subfractions were ranging from 90 to 360 µg/ml (Table 20). The less cytotoxic subfraction was FH10.01 (370 µg/ml), while FH10.04 was the most cytotoxic subfraction with LC_{50} of 90 µg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

4.3.5.2 Antiviral activities of FH 10 subfractions against measles virus

After determining the cytotoxicity concentration of each subfraction on Vero cells, their potential activities against measles virus (MV) was investigated and results are summarised in Table 19. Overall, all subfractions possess significant ($P<0.05$) activities against MV when compared with untreated cells (Appendix N).

In the pre-treatment protocol, subfraction FH10.01, FH10.02, FH10.03 and FH10.04 possesses the highest activity against MV, with active concentration ranged from 3.7 to 370, 1.4 to 140, and 1.2 to 120 µg/ml, respectively. At highest concentration (1.0 LC_{50}), these subfractions inhibited the development of MV-induced CPE by more than 50%. Similar activity was also found in subfraction FH10.04 which exhibited antiviral activity at mildly cytotoxic concentration, ranging from 0.9 to 90 µg/ml. Statistical analyses showed that these results were not significantly different from antiviral activity exhibited by 0.1 CC_{50} Ribavirin in the same protocol (Appendix N).

Table 19: LC_{50} values and antiviral activities of FH10 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH10.01	370	+++	3.7 – 370	2	++++	37 – 370	1
FH10.02	140	+++	1.4 – 140	2	++	1.4 – 140	2
FH10.03	120	+++	1.2 – 120	2	++	1.2 – 120	2
FH10.04	90	+++	0.9 – 90	2	++++	0.9 – 90	2
FH10.05	180	++	1.8 – 180	2	+++	18 – 180	1
FH10.06	200	+	20 – 200	1	+++	2 – 200	2

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

Meanwhile, the weakest antiviral activity in this protocol was found in subfraction FH10.06. This subfraction was able to inhibit MV less than 25% at the highest concentration (200 µg/ml).

In the post-infection protocol, the highest antiviral activity was also found in subfraction FH10.01 and FH10.04. These subfractions possess more than 75% inhibition of CPE at the highest concentration (1.0 LC_{50}). However, unlike in the pre-treatment protocol, the activity of subfraction FH10.01 in this protocol was not retained in lowest dilution (3.7 µg/ml). Statistical analysis showed no significant difference in antiviral activity between these fractions and 0.1 CC_{50} Ribavirin in the same protocol (Appendix N). In contrast, the weakest antiviral activity was found in subfractions FH10.02 and FH10.03. These subfractions were managed to inhibit the development of virus CPE less than 50% at the highest concentration (1.0 LC_{50}).

From the result, subfractions FH10.01 and FH10.04 were subjected to further purification process.

4.3.6 Fraction FH11

Fraction FH11 (0.13 g) was further separated into different subfractions by CC-II. A total of 110 subfractions were produced after the elution process and they were analysed using TLC whereby subfractions with similar profile were combined together. Nine combined subfractions were obtained and results were summarised in Table 20. Due to the low weight of FH11.01, FH11.02 and FH11.03 samples, these subfractions were not further tested in the next experiment.

Table 20: Combined subfractions from CC-II of fraction FH11

Combined subfraction	Label	Weight (g)	Percentage of yield (%)
1-18	FH11.01	0.00	0.00
19-34	FH11.02	0.00	0.00
35-42	FH11.03	0.01	7.69
43-52	FH11.04	0.02	15.39
53-56	FH11.05	0.02	15.39
57-68	FH11.06	0.02	15.39
69-80	FH11.07	0.02	15.39
81-85	FH11.08	0.01	7.69
86-110	FH11.09	0.01	7.69
Total yield		0.11	
Total % recovery			84.62

4.3.6.1 Cytotoxicity of FH11 subfractions on Vero cells

A total of 6 subfractions derived from fraction FH11 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all subfractions were ranging from 70 to 250 µg/ml (Table 21). Subfraction FH11.06, FH11.08 and FH11.09 exhibited mild cytotoxicity in Vero cells with LC_{50} values of 80, 70 and 100 µg/ml, respectively. Other 3 subfractions were considered not toxic as they showed LC_{50} values of more than 100 µg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

4.3.6.2 Antiviral activities of FH11 subfractions against measles virus

The potential antiviral activities of each subfraction against measles virus (MV) are summarised in Table 21. Overall, all 6 subfractions possess antiviral activities in the dose-dependent manner, although 2 subfractions (FH11.05 and FH11.08) were found to be cytotoxic at 1.0 LC_{50}, but not at tenfold dilution (0.1 LC_{50}). Statistical analyses showed that the antiviral activities were significant ($P<0.05$) when compared with untreated cells (Appendix O).

In the pre-treatment protocol, the strongest antiviral activity was found in subfraction FH11.04 and FH11.09, which exhibited antiviral activity at concentration ranging from 1.5 to 150, and 0.7 to 70 µg/ml, respectively (Table 21). These subfractions were found to inhibit the development of MV-induced CPE by more than 50% at highest concentration and manage to retain their activity in the lower dilutions. Similar activity was found in subfraction FH11.08 which exhibited antiviral activity at lower concentration ranging from 1 to 10 µg/ml. These results were not significantly different from antiviral activity showed by 0.1 CC_{50} Ribavirin (Appendix O). On the other hand, the lowest antiviral activity was showed by subfraction FH11.05, FH11.06 and FH11.07. They only inhibited the virus CPE by more than 25% at highest concentration.

For the post-infection protocol, the strongest antiviral activity was found in subfraction FH11.04 and FH11.07. These subfractions inhibited MV-induced CPE by more than 50% at concentration ranging from 1.5 to 150, and 2.5 to 250 µg/ml, respectively (Table 22). Subfraction FH11.08 also possess the similar activity with active concentration ranging from 1 to 10 µg/ml.

Table 21: LC_{50} values and antiviral activities of FH11 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH11.04	150	+++	1.5 – 150	2	+++	1.5 – 150	2
FH11.05	180	++	1.8 – 18	1	++	1.8 – 18	1
FH11.06	80	++	0.8 – 80	2	+	0.8 – 80	2
FH11.07	250	++	2.5 – 250	2	+++	2.5 – 250	2
FH11.08	100	+++	1 – 10	1	+++	1 – 10	1
FH11.09	70	+++	0.7 – 70	2	++	7 – 70	1

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

Statistical analysis showed that the antiviral activity of subfraction FH11.04 and FH11.07 was not significant compared to 0.01 CC_{50} Ribavirin (Appendix O). Meanwhile, the antiviral activity showed by subfraction FH11.08 was not significantly different compared to 0.1 CC_{50} Ribavirin. In contrast, the weakest activity against MV was found in subfraction FH11.05, FH11.06 and FH11.09. These subfractions were able to inhibit virus less than 50% at highest concentration.

From the result, subfraction FH11.04 and FH11.08 were subjected to further purification process. However, due to small amount of sample, FH11.08 was not further purified.

4.3.7 Fraction FH15

Fraction FH15 (0.13 g) was further separated into different subfractions by CC-II. Elution process produced 85 subfractions and they were then analysed using thin layer chromatography (TLC), whereby subfractions with similar profile were combined together. Nine combined subfractions were obtained and results are summarised in Table 22. Due to the low amount of sample, subfractions FH15.01, FH15.02, FH15.03, FH15.08 and FH15.09 were not further tested in the next experiment.

4.3.7.1 Cytotoxicity of FH15 subfractions on Vero cells

A total of 4 subfractions derived from fraction FH15 were screened for their toxicity on Vero cells. Overall, the LC_{50} values of all subfractions were ranging from 70 to 400 µg/ml (Table 23). Subfraction FH15.04, exhibited mild cytotoxicity in Vero cells with LC_{50} values of 70 µg/ml.

Other 3 subfractions were considered not toxic as they showed LC_{50} values of more than 100 µg/ml. These LC_{50} values were used as the highest concentration of each subfraction in the antiviral test.

Table 22: Combined subfractions from CC-II of fraction FH15

Combined subfraction	Label	Weight (g)	Percentage of yield (%)
1-22	FH15.01	0.00	0.00
23-28	FH15.02	0.01	7.69
29-34	FH15.03	0.01	7.69
35-38	FH15.04	0.02	15.39
39-46	FH15.05	0.02	15.39
47-50	FH15.06	0.02	15.39
51-59	FH15.07	0.02	15.39
60-75	FH15.08	0.01	7.69
76-85	FH15.09	0.00	0.00
Total yield		0.11	
Total % recovery			84.62

4.3.7.2 Antiviral activities of FH15 subfractions against measles virus (MV)

After determining the cytotoxicity value of each subfraction, their potential antiviral activities against MV was investigated. Results are summarised in Table 23. Statistical analyses showed that treatment of Vero cells with FH15 subfractions not significantly decreased MV-induced CPE when compared with untreated cells, both in pre-treatment and post-infection protocols (P=0.45 and P=0.17, respectively) (Appendix P). Although not significant, these subfractions did show a

Table 23: LC_{50} values and antiviral activities of FH15 subfractions

Subfractions	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH15.04	170	++	1.7 – 17	1	++	1.7 – 17	1
FH15.05	400	+	4 – 400	2	++	4 – 400	2
FH15.06	270	++	2.7 – 270	2	++	2.7 – 270	2
FH15.07	70	++	0.7 – 70	2	++	0.7 – 70	2

[a]Cytotoxic concentration of subfraction that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

certain degree of activities against MV. Subfraction FH15.06 and FH15.07 inhibited the development of CPE by more than 25% at highest concentration (1.0 LC_{50}) in both protocols. Similar antiviral activities were also found in subfraction FH15.04 but at lower concentration, 0.1 LC_{50} (17 µg/ml), as this subfraction was found to be cytotoxic at 1.0 LC_{50} (170 µg/ml). Subfraction FH15.05 was able to inhibit MV more than 25% in post-infection, but exhibited less than 25% inhibition in pre-treatment, at the highest concentration, 400 µg/ml (1.0 LC_{50}). From the result, no subfractions were subjected to further purification process.

4.4 ANTIVIRAL ACTIVITIES OF SELECTED ISOLATED COMPOUNDS AGAINST MEASLES VIRUS

Results obtained in the previous screening justify continuing with the purification and isolation of active compounds for improving their potential as antiviral drugs and/or finding of new lead molecules. As in previous method, pre-treatment and post-infection protocols were used in order to identify the mode of antiviral action of the compounds. Untreated cells infected with virus were used as control. Untreated controls always contained 0.5% DMSO in order to exclude any effect of DMSO on cells or virus.

4.4.1 Purification of selected fraction and subfractions using preparative thin layer chromatography (PTLC)

Only fraction FH7 and subfractions FH4.04, FH6.06, FH6.10 and FH11.04 were further purified by extensive PTLC as other active subfractions were volatile during the storage period. Each

fraction and subfraction produced different number of bands on the PTLC plate under the UV light. For example, subfraction FH4.04 and fraction FH7 produced 5 bands, subfraction FH6.06 and FH6.10 produced 3 bands, and subfraction FH11.04 produced 2 bands. All bands were then scraped, extracted with a suitable solvent and filtered to give the isolated materials upon removal of the solvent. The description of isolated compounds was given in Table 24.

These isolated compounds were then further tested in antiviral assay, except for FH4.04.1, FH4.04.3, FH4.04.5,FH6.06.1 and FH11.04.1, due to the small amount of sample.

4.4.2 Cytotoxicity of isolated compounds on Vero cells

A total of 13 isolated compounds were screened for their toxicity on Vero cells and results are summarised in Table 26. From the results, the LC_{50} values of all compounds were ranging from 150 to 400 µg/ml. Compound FH11.04.2 exhibited the lowest LC_{50} value with 150 µg/ml, meanwhile the highest toxicity value was showed by compounds FH4.04.2 and FH4.04.4 (400 µg/ml). All these compounds were considered non-toxic to the cells as they showed LC_{50} of more than 100 µg/ml. These LC_{50} values were used as the highest concentration of each compound in the antiviral test.

4.4.3 Antiviral activity of isolated compounds against measles virus

The potential antiviral activities of each isolated compound against measles virus (MV) are summarised in Table 25. Overall, all isolated compounds possess antiviral activities with

Table 24: The isolated compounds obtained from purification process using PTLC

Fraction	Subfraction	Isolated compound	Weight (mg)	Description
FH4	FH4.04	FH4.04.1	2.0	Yellowish oil, showed yellow spot on PTLC
		FH4.04.2	3.0	Yellowish oil, showed light yellow spot on PTLC
		FH4.04.3	1.0	Yellowish oil, showed light yellow spot on PTLC
		FH4.04.4	3.0	Pale yellowish oil, showed pale yellow spot on PTLC
		FH4.04.5	2.0	Colourless oil, showed pale yellow spot on PTLC
FH 6	FH6.06	FH6.06.1	1.0	Yellowish oil, showed yellow spot on PTLC
		FH6.06.2	3.0	Yellowish oil, showed yellow spot on PTLC
		FH6.06.3	3.0	Yellowish oil, showed yellow spot on PTLC
	FH6.10	FH6.10.1	4.0	Yellowish oil, showed yellow spot on PTLC
		FH6.10.2	4.0	Yellowish oil, showed yellow spot on PTLC
		FH6.10.3	3.0	Yellowish oil, showed yellow spot on PTLC
FH7		FH7.01	3.0	Yellowish oil, showed yellow spot on PTLC
		FH7.02	4.0	Yellowish oil, showed pink spot on PTLC
		FH7.03	4.0	Colourless oil, showed green spot on PTLC
		FH7.04	3.0	Colourless oil, showed blue green spot on PTLC
		FH7.05	4.0	Colourless oil, showed light green spot on PTLC
FH11	FH11.04	FH11.04.1	1.0	Colourless oil, showed green spot on PTLC
		FH11.04.2	4.0	Colourless oil, showed green spot on PTLC

majority of them exhibited less than 50% inhibition of MV-induced CPE in both treatments Statistical analyses showed that the antiviral activities were significant ($P<0.05$) when compared with untreated cells (Appendix Q).

In the pre-treatment protocol, the strongest antiviral activity was found in compound FH11.04.2. This compound inhibited the formation of CPE by more than 50% at the highest concentration (40 µg/ml) and able to sustain the activity at lower concentration (4 µg/ml). Statistical analysis showed that the antiviral activity of this compound was significantly different compared to 0.01 CC_{50} Ribavirin in the same protocol (Appendix Q). Meanwhile, the weakest activity against MV was showed by compound FH7.05, which inhibited the development of CPE by zero percent.

In the post-infection protocol, the highest activity against MV was found in compound FH6.06.3, which exhibited antiviral activity at concentration ranging from 2.7 to 27 µg/ml. This compound inhibited the formation of MV-induced CPE by more than 50% at the highest concentration. This result was similar to the antiviral activity showed by 0.01 CC_{50} Ribavirin in the same protocol, and statistical analysis showed there were no significant difference between these substances (Appendix Q). Meanwhile, the weakest activity against MV was showed by compound FH7.01, which inhibited the development of CPE less than 25%.

Table 25: LC_{50} values and antiviral activities of isolated compounds

Compounds	**[a]LC_{50} (µg/ml)**	**Pre-treatment**			**Post-infection**		
		[b]Antiviral activity	**[c]Effective concentration range (µg/ml)**	**[d]Dilution factor**	**Antiviral activity**	**Effective concentration range (µg/ml)**	**Dilution factor**
FH4.04.2	400	++	4 – 40	1	++	4 – 40	1
FH4.04.4	400	++	4 – 40	1	++	4 – 40	1
FH6.06.2	270	++	2.7 – 27	1	++	2.7 – 27	1
FH6.06.3	270	++	2.7 – 27	1	+++	2.7 – 27	1
FH6.10.1	200	++	2 – 20	1	++	2 – 20	1
FH6.10.2	200	++	20	0	++	20	1
FH6.10.3	200	++	2 – 20	1	++	2 – 20	1
FH7.01	270	++	27	0	+	27	0
FH7.02	270	+	2.7 – 27	1	+	2.7 – 27	1
FH7.03	200	++	20	0	++	20	1

[a]Cytotoxic concentration of isolated compound that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

Table 25: (cont.)

Compounds	[a]LC_{50} (µg/ml)	Pre-treatment			Post-infection		
		[b]Antiviral activity	[c]Effective concentration range (µg/ml)	[d]Dilution factor	Antiviral activity	Effective concentration range (µg/ml)	Dilution factor
FH7.04	200	++	2 – 20	1	++	2 – 20	1
FH7.05	200	-	-		++	20	0
FH11.04.2	150	+++	1.5 – 15	1	++	1.5 – 15	1

[a]Cytotoxic concentration of isolated compound that reduced cell viability by 50% (LC_{50})

[b]Results are expressed as inhibition of virus at 1.0 LC_{50}: + (<25% inhibition); + + (25% < χ < 50% inhibition)); + + + (50% < χ < 75% inhibition inhibition); + + + + (≥75% inhibition). Results were confirmed in at least two duplicate experiments.

[c]The concentration range over which antiviral activity was observed, expressed as µg/ml in cell culture media

[d]The number of 10-fold dilutions beyond the highest concentration in which the detected antiviral activity was observed.

4.5 COMBINATION TREATMENT OF SELECTED ISOLATED COMPOUNDS AND RIBAVIRIN AGAINST MEASLES VIRUS

Combination therapy between selected isolated compounds of *C. nardus* and Ribavirin was carried out in order to investigate the ability of the isolated compound to increase the efficiency of antiviral therapy while using Ribavirin at a much lower concentration against measles virus (MV). The interaction between Ribavirin and isolated compound are described in terms of FICs index and the results were interpreted as high synergy (FICs $\leq$ 0.4), moderate synergy (0.4 < FICs < 1), additive (FICs = 1), partially additive/weak antagonism (1 < FICs < 2) and high antagonism (FICs $\geq$ 2).

Overall, all compounds (single and in combination) had no cytotoxic effect upon Vero cells at the concentrations used as assessed by cytotoxicity assay. Generally, cells viability was always above 90% (Appendix R). Statistical analyses on the reduction of the IC_{50} value for single Ribavirin treatment are summarised in Appendix S (pre-treatment protocol) and Appendix T (post-infection protocol).

4.5.1 Combination treatment of isolated compound FH6.06.2 with Ribavirin

Figure 4 and Table 26 showed the results of combining various concentrations of Ribavirin with fixed FH6.06.2 concentrations, which were 2.7 μg/ml (0.01 LC_{50}) and 13.5 μg/ml (0.05 LC_{50}).In the pre-treatment, analysis of the combinatorial effects of the combination using the IC_{50} data yielded FICs of 0.40, which suggest highly synergistic interaction between Ribavirin and

FH6.06.2 at the lowest concentration. The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 5.46 μg/ml to 1.47 μg/ml when combined with 2.7 μg/ml FH6.06.2. However, combinations at the higher concentration (13.5 μg/ml) yielded FICs of 1.61, which indicates partially additive interaction. Partially additive can also be referred as weak antagonistic interaction.

Meanwhile in the post-infection protocol, moderate synergistic interaction was also observed at the lowest concentration combination. The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.47 μg/ml when combined with 2.7 μg/ml FH6.06.2 (FICs=0.69). However, increases in the concentration of the compound to 13.5 μg/ml resulted in weak antagonistic interaction, with FICs index of 1.31.

From the results, it can be concluded that the synergistic interaction present at lower FH6.06.2 concentration, but indicate antagonism at higher concentrations in both protocols.

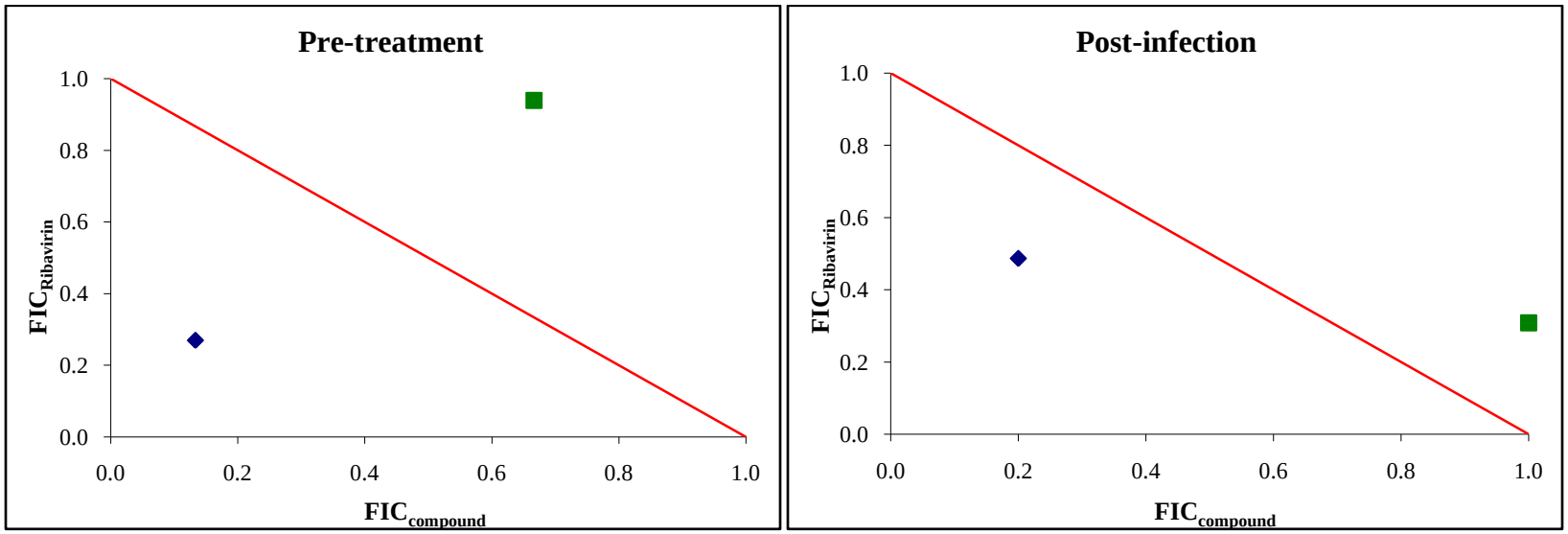

Figure 4: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH6.06.2 and Ribavirin. $FIC_{Ribavirin}$ represents the ratio of the IC_{50} of Ribavirin in the presence of a constant concentration of isolated compound to the IC_{50} of Ribavirin alone. The X-axis represents the ratio of the fixed concentration of compound to the IC_{50} of compound alone. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between 0.01 LC_{50} FH6.06.2 and Ribavirin produced synergistic interaction in the pre-treatment and post-infection protocol. However, combination at higher concentration (0.05 LC_{50}) yielded a partially additive interaction in both protocols.

Table 26: Inhibitory effects of FH6.06.2 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH6.06.2 only	20.25				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH6.06.2	1.47	0.13	0.27	0.40	High synergy
	Ribavirin + 0.05 LC_{50} FH6.06.2	5.13	0.67	0.94	1.61	Weak antagonism
Post-infection	FH6.06.2 only	13.5				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH6.06.2	1.47	0.20	0.49	0.69	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.06.2	0.93	1.0	0.31	1.31	Weak antagonism

4.5.2 Combination therapy of isolated compound FH6.06.3 with Ribavirin

Figure 5 and Table 27 showed the results of combining various concentrations of Ribavirin with fixed FH6.06.3 concentrations, which were 2.7 μg/ml (0.01 LC_{50}) and 13.5 μg/ml (0.05 LC_{50}).In the pre-treatment protocol, analysis of the combinatorial effects of the combinations using the IC_{50} data yielded FICs of 1.71 and 2.18, which suggest antagonistic interaction between Ribavirin and FH6.06.3 at all concentrations tested. The IC_{50} value for single Ribavirin treatment was increased from 5.46 μg/ml to 8.77 and to 8.92 μg/ml when combined with 2.7 and 13.5 μg/ml FH6.06.3, respectively.

However, in the post-infection protocol, combination of these substances produced moderate synergistic interactions in all concentrations tested. The IC_{50} values for single Ribavirin treatment were significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.07 μg/ml when combined with 2.7 μg/ml compound (FICs=0.47), and to 1.27 μg/ml when combined with 13.5 μg/ml compound (FICs=0.98).

From the results, it can be concluded that Ribavirin has a synergistic interaction with FH6.06.3, but only in post-infection treatment.

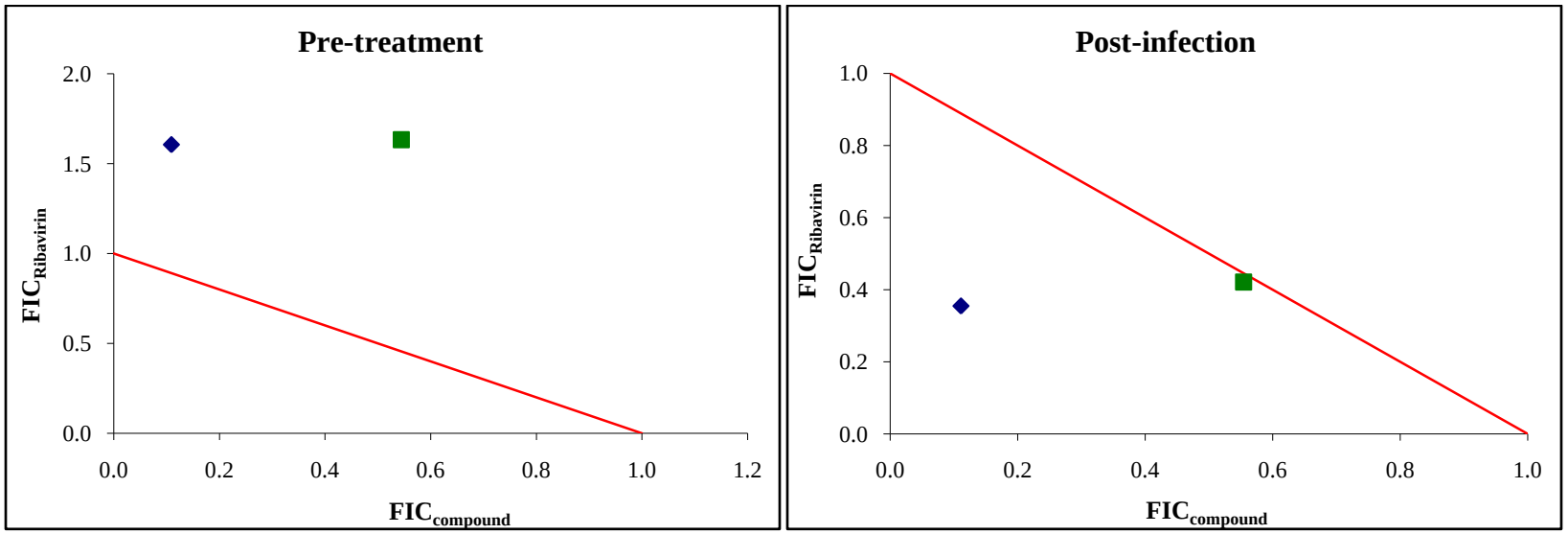

Figure 5: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH6.06.3 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between FH6.06.3 (0.01 LC_{50} and 0.05 LC_{50})and Ribavirin in the pre-treatment protocol produced antagonism interaction. Meanwhile in the post-infection protocol, combination between the substances yielded moderate synergy interactions.

Table 27: Inhibitory effects of FH6.06.3 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH6.06.3 only	24.82				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH6.06.3	8.77	0.11	1.61	1.71	Weak antagonism
	Ribavirin + 0.05 LC_{50} FH6.06.3	8.92	0.54	1.63	2.18	High antagonism
Post-infection	FH6.06.3 only	24.35				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH6.06.3	1.07	0.11	0.35	0.47	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.06.3	1.27	0.55	0.42	0.98	Moderate synergy

4.5.3 Combination therapy of isolated compound FH6.10.1 with Ribavirin

Figure 6 and Table 28 showed the results of combining various concentrations of Ribavirin with fixed FH6.10.1 concentrations, which were 2.0 μg/ml (0.01 LC_{50}) and 10 μg/ml (0.05 LC_{50}). In the pre-treatment protocol, analysis of the combinations of Ribavirin and FH6.10.1 at concentration of 2.0 and 10 μg/ml yielded FICs of 0.99 and 0.80, which indicate moderate synergistic interaction. The IC_{50} values for single Ribavirin treatment were significantly ($P<0.05$) reduced from 5.46 μg/ml to 4.80 and 1.47 μg/ml, respectively.

Meanwhile in the post-infection protocol, moderate synergistic interaction was observed in the lowest concentration (2.0 μg/ml). The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.6 μg/ml when combined with FH6.10.1 (FICs=0.65). However, combination at higher concentration (10 μg/ml) yielded FICs of 3.53, which indicates a highly antagonistic interaction.

From the results, it can be concluded that the combination of Ribavirin and FH6.10.1 at low concentration produced moderate synergistic interaction in both protocols. However, when the concentration of compound was increased, synergistic interaction only occurred in the pre-treatment protocol.

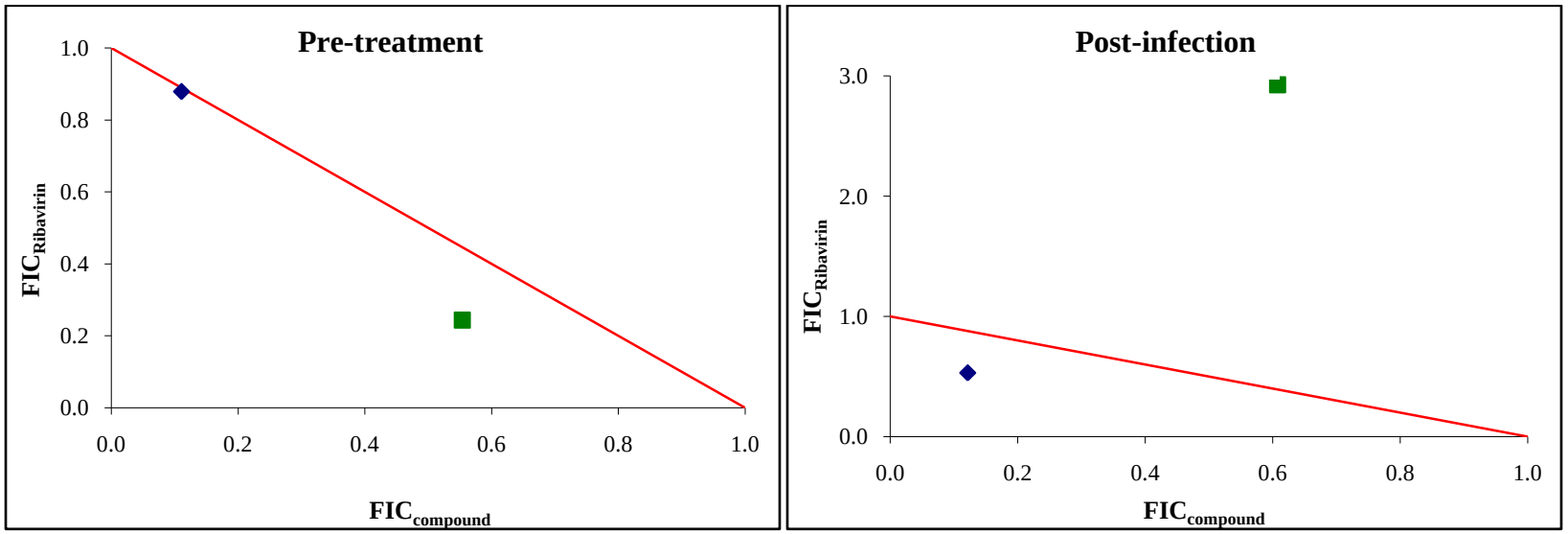

Figure 6: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH6.10.1 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between 0.01 LC_{50} FH6.10.1 and Ribavirin produced moderate synergy interactions in both protocols. However, when the concentration of compound was increased to 0.05 LC_{50}, synergistic interaction occurred only in pre-treatment protocol.

Table 28: Inhibitory effects of FH6.10.1 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH6.10.1 only	18.06				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH6.10.1	4.80	0.11	0.88	0.99	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.10.1	1.33	0.56	0.24	0.80	Moderate synergy
Post-infection	FH6.10.1 only	16.46				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH6.10.1	1.60	0.12	0.53	0.65	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.10.1	8.83	0.61	2.93	3.53	High antagonism

4.5.4 Combination therapy of isolated compound FH 6.10.2 with Ribavirin

Figure 7 and Table 29 showed the results of combining various concentrations of Ribavirin with fixed FH6.10.2 concentrations, which were 2.0 μg/ml (0.01 LC_{50}) and 10 μg/ml (0.05 LC_{50}). In the pre-treatment, analysis of the combinatorial effects of the combinations using the IC_{50} data yielded FICs of 1.74 and 1.23, which suggest weak antagonistic interaction between Ribavirin and FH6.10.2. The IC_{50} value for single Ribavirin treatment was increased from 5.46 μg/ml to to 8.93 μg/ml when combined with 2.0 μg/ml compound.

Meanwhile in the post-infection protocol, moderate synergistic interaction was observed in lowest concentration of compound (2.0 μg/ml). The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.50 μg/ml when combined with 2.0 μg/ml FH6.10.2 (FICs=0.60). However, at higher concentration (10 μg/ml), the IC_{50} data yielded FICs of 3.65 which indicates highly antagonistic interaction.

From the results, it can be concluded that the synergistic interaction only presents in post-infection protocol, but only at lowest FH6.10.2 concentration.

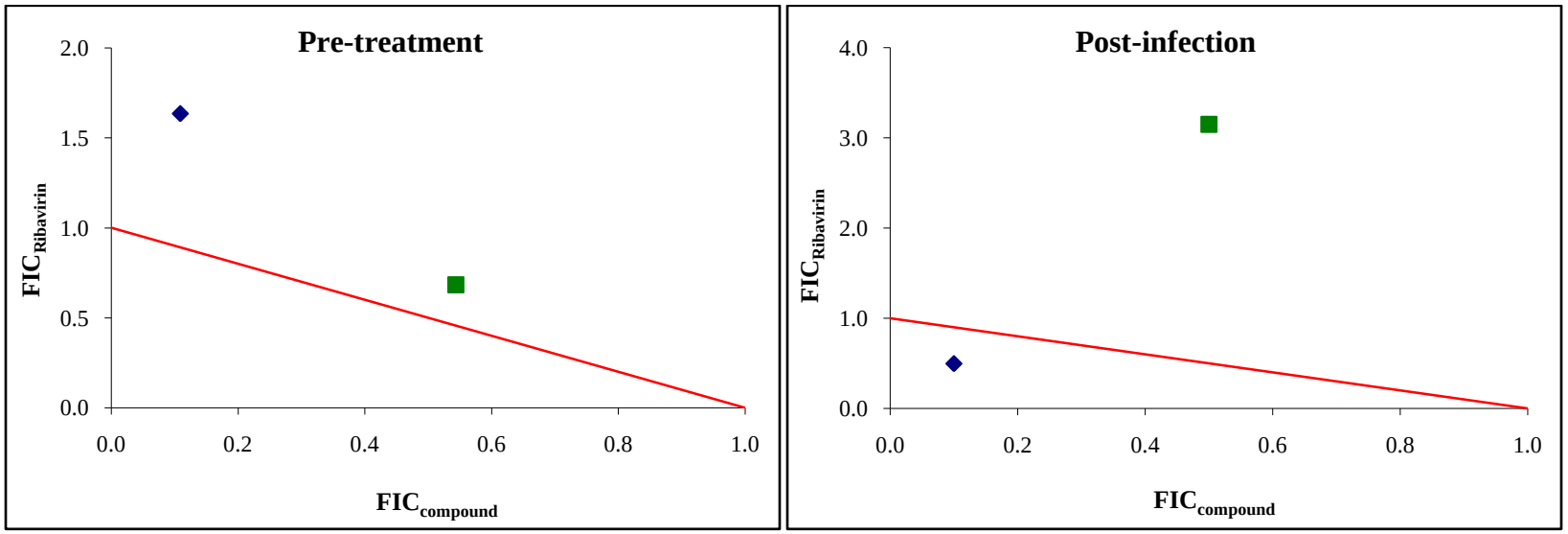

Figure 7: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH6.10.2 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between FH6.10.2 and Ribavirin produced antagonistic interactions in the pre-treatment protocol. In the post-infection protocol, the combination produced synergistic interaction at low concentration of compound, but turned to antagonistic when concentration was increased to 0.05 LC_{50}.

Table 29: Inhibitory effects of FH6.10.2 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH6.10.2 only	18.39				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH6.10.2	8.93	0.11	1.64	1.74	Weak antagonism
	Ribavirin + 0.05 LC_{50} FH6.10.2	3.73	0.54	0.68	1.23	Weak antagonism
Post-infection	FH6.10.2 only	20.00				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH6.10.2	1.50	0.12	0.53	0.60	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.10.2	9.50	0.61	2.93	3.65	High antagonism

4.5.5 Combination therapy of isolated compound FH6.10.3 with Ribavirin

Figure 8 and Table 30 showed the results of combining various concentrations of Ribavirin with fixed FH6.10.3 concentrations, which were 2.0 μg/ml (0.01 LC_{50}) and 10 μg/ml (0.05 LC_{50}).In the pre-treatment protocol, examination of the combinatorial effects by standard isobologram analysis exhibitedmoderate synergistic interaction in all concentrations tested.Combinations of Ribavirin with FH6.10.3 at concentration of 2.0 and 10 μg/ml were significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 5.46 μg/ml to 3.47 μg/ml (FICS=0.76) and 1.33 μg/ml (FICs=0.85), respectively.

Meanwhile in the post-infection protocol, moderate synergistic interaction was also observed in the lowest concentration (2.0 μg/ml). The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.0 μg/ml when combined with FH6.10.3 (FICs=0.56). However, combination at higher concentration (10 μg/ml) yielded FICs of 3.90, which indicates a highly antagonistic interaction.

From the results, it can be concluded that the moderate synergistic interaction present at low concentration of FH6.10.3 in both protocols. At higher concentration, synergistic interaction only happened in pre-treatment protocol.

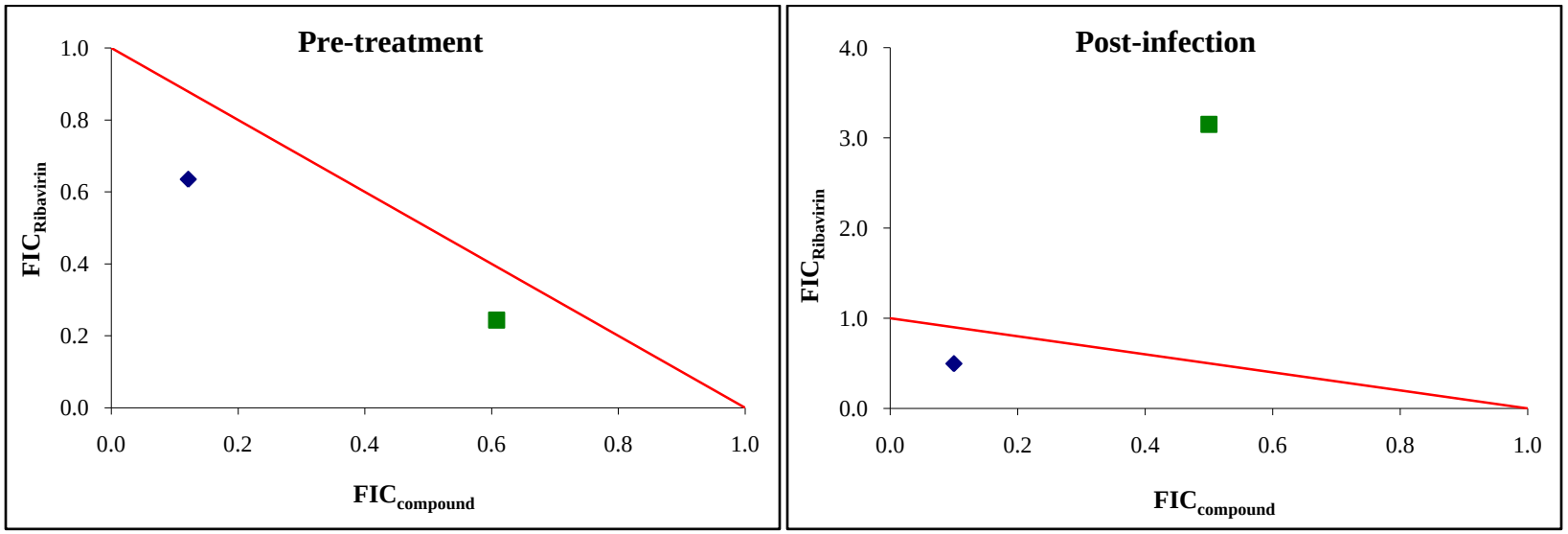

Figure 8: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH6.10.3 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between FH6.10.3 and Ribavirin produced antagonistic interactions in the pre-treatment protocol. In the post-infection protocol, the combination produced synergistic interaction at low concentration of compound, but turned to antagonistic when concentration was increased to 0.05 LC_{50}.

Table 30: Inhibitory effects of FH6.10.3 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH6.10.3 only	16.45				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH6.10.3	3.47	0.12	0.64	0.76	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.10.3	1.33	0.61	0.24	0.85	Moderate synergy
Post-infection	FH6.10.3 only	8.75				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH6.10.3	1.00	0.23	0.33	0.56	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH6.10.3	8.33	1.14	2.76	3.90	High antagonism

4.5.6 Combination therapy of isolated compound FH7.01 with Ribavirin

Figure 9 and Table 31 showed the results of combining various concentrations of Ribavirin with fixed FH7.01 concentrations, which were 1.6 μg/ml (0.01 LC_{50}) and 8.0 μg/ml (0.05 LC_{50}). In the pre-treatment protocol, the combination of Ribavirin and 1.6 μg/ml FH7.01 exhibited a high level of synergy (FICs=0.38). It significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 5.46 μg/ml to 1.49 μg/ml. However, when the concentration of FH7.01 was increased to 8.0 μg/ml, the interaction was changed to antagonistic with FICs of 1.42.

Meanwhile in the post-infection protocol, a moderate synergistic interaction was observed for all tested combinations of Ribavirin and FH7.01 with FICs of 0.52 and 0.66. The IC_{50} value for single Ribavirin was significantly ($P<0.05$) reduced from 3.02 μg/ml to 1.40 and 1.20 μg/ml when combined with 1.6 and 8.0 μg/ml FH7.01, respectively.

From the results, it can be concluded that FH7.01 was able to increase the efficiency of antiviral therapy against MV in both protocols as it exhibited synergistic interaction with Ribavirin in the lowest concentration tested.

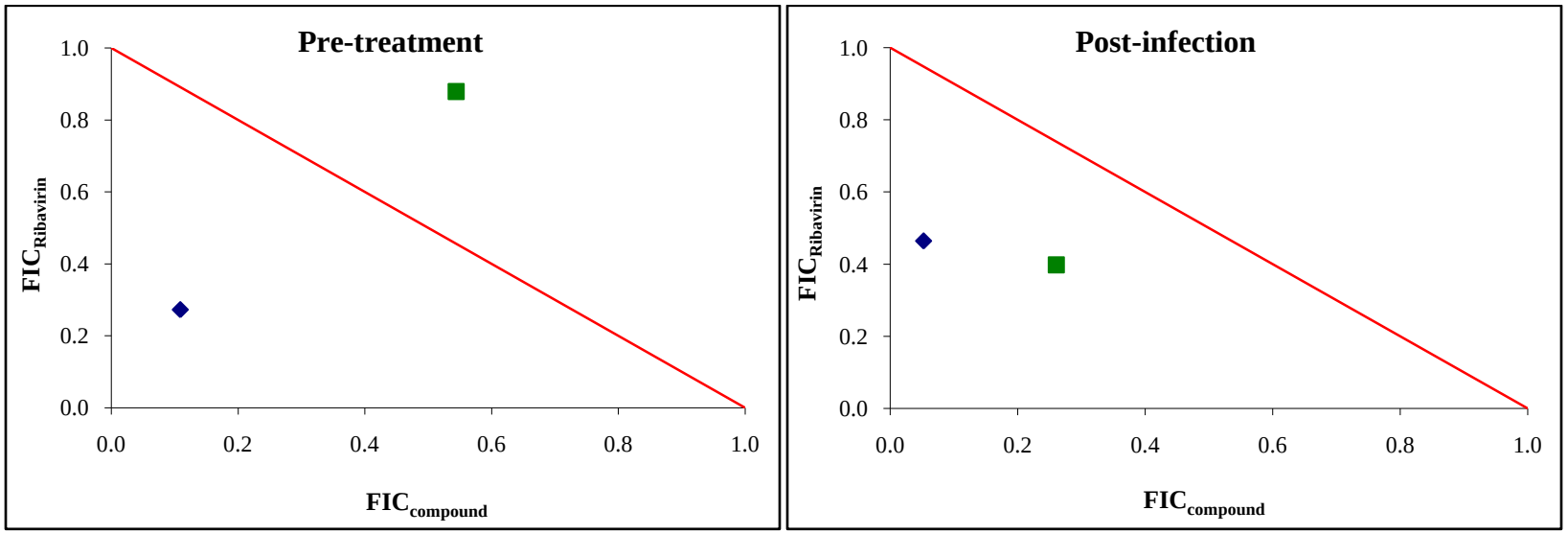

Figure 9: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH7.01 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between FH7.01 and Ribavirin produced synergistic interactions in the post-infection protocol. Synergistic effect was also recorded in the pre-treatment protocol, but only at low concentration of compound.

Table 31: Inhibitory effects of FH7.01 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH7.01 only	14.71				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH7.01	1.49	0.11	0.27	0.38	High synergy
	Ribavirin + 0.05 LC_{50} FH7.01	4.80	0.54	0.88	1.42	Weak antagonism
Post-infection	FH7.01 only	8.75				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH7.01	1.40	0.05	0.46	0.52	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH7.01	1.20	0.26	0.40	0.66	Moderate synergy

4.5.7 Combination therapy of isolated compound FH7.02 with Ribavirin

Figure 10 and Table 32 showed the results of combining various concentrations of Ribavirin with fixed FH7.02 concentrations, which were 1.6 µg/ml (0.01 LC_{50}), 8.0 µg/ml (0.05 LC_{50}) and 16.0 µg/ml (0.1 LC_{50}). In the pre-treatment protocol, a moderate synergistic interaction was observed for all concentrations tested, with FICs of 0.94, 0.60 and 0.81. The IC_{50} values for single Ribavirin were significantly ($P<0.05$) reduced from 5.46 µg/ml to 4.80, 1.70 and 0.80 µg/ml when combined with 1.6, 8.0 and 16.0 µg/ml FH7.02, respectively.

Meanwhile in the post-infection protocol, the combination at lowest FH7.02 concentration (1.6 µg/ml) exhibited moderate synergistic interaction with FICs of 0.60. It significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 3.02 µg/ml to 1.60 µg/ml. However, interactions at higher concentrations (8.0 and 16.0 µg/ml) yielded FICs of 2.46 and 3.33, which indicate high antagonistic interactions.

From the results, it can be concluded that FH7.02 was able to increase the efficiency of antiviral therapy against MV in both protocols as it exhibited synergistic interaction with Ribavirin in the lowest concentration tested.

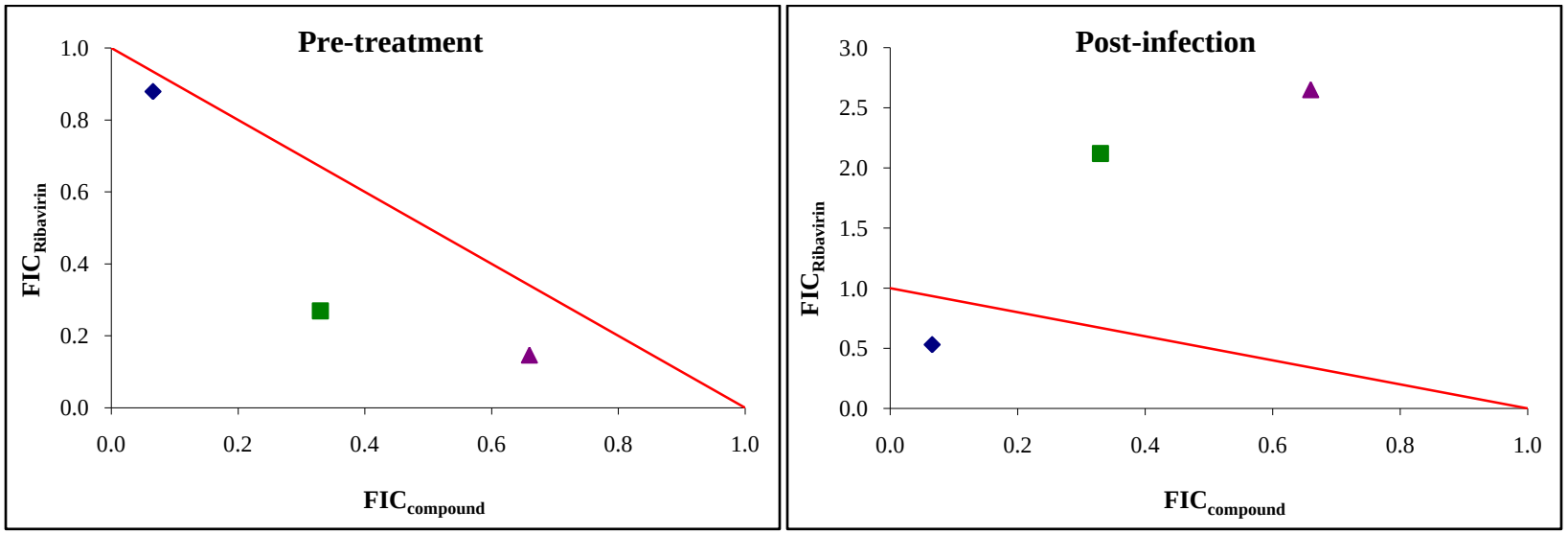

Figure 10: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH7.02 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆), 0.05 LC_{50} (■) and 0.1 LC_{50} (▲). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, the combination between FH7.02 and Ribavirin produced synergistic interactions in the pre-treatment protocol. Synergistic interaction was also recorded in the post-infection protocol, but only at the lowest concentration of compound.

Table 32: Inhibitory effects of FH7.02 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH7.02 only	24.25				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH7.02	4.80	0.07	0.88	0.94	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH7.02	1.47	0.33	0.27	0.60	Moderate synergy
	Ribavirin + 0.1 LC_{50} FH7.02	0.80	0.66	0.15	0.81	Moderate synergy
Post-infection	FH7.02 only	23.53				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH7.02	1.60	0.07	0.53	0.60	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH7.02	6.40	0.33	2.12	2.46	High antagonism
	Ribavirin + 0.1 LC_{50} FH7.02	8.00	0.66	2.65	3.33	High antagonism

4.5.8 Combination therapy of isolated compound FH 7.03 with Ribavirin

Figure 11 and Table 33 showed the results of combining various concentrations of Ribavirin with fixed FH7.03 concentrations, which were 1.6 μg/ml (0.01 LC_{50}) and 8.0 μg/ml (0.05 LC_{50}). In the pre-treatment protocol, a moderate level of synergy was demonstrated with the combination of Ribavirin plus 8.0 μg/ml FH7.03 (FICs=0.77). The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 5.46 μg/ml to 1.40 μg/ml. However, combination at lower concentration (1.6 μg/ml) of FH7.03 only produced partially additive interaction, with FICs of 2.46.

Meanwhile in the post-infection protocol, combination at the lowest FH7.03 concentration (1.6 μg/ml) exhibited moderate synergistic interaction with FICs of 0.61. It significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 3.02 μg/ml to 1.33 μg/ml. The other combination showed antagonism interactions with FICs of 1.17.

From the results, it can be concluded that FH7.03 was able to increase the efficiency of antiviral therapy against MV in both protocols as it exhibited synergistic interaction with Ribavirin.

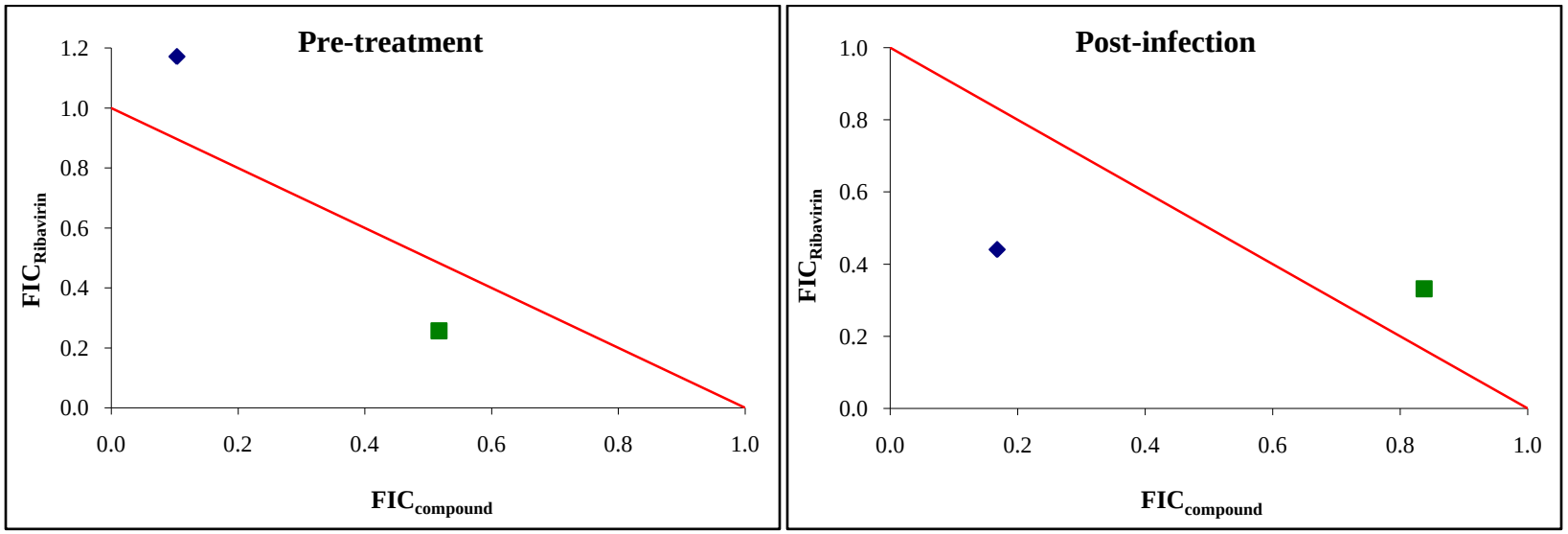

Figure 11: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH7.03 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, synergistic interactions occur in both protocols, but at different concentration of compound.

Table 33: Inhibitory effects of FH7.03 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH7.03 only	15.48				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH7.03	6.40	0.10	1.17	1.27	Weak antagonism
	Ribavirin + 0.05 LC_{50} FH7.03	1.40	0.52	0.26	0.77	Moderate synergy
Post-infection	FH7.03 only	9.55				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH7.03	1.33	0.17	0.44	0.61	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH7.03	1.00	0.84	0.33	1.17	Weak antagonism

4.5.9 Combination therapy of isolated compound FH 7.04 with Ribavirin

Figure 12 and Table 34 showed the results of combining various concentrations of Ribavirin with fixed FH7.04 concentrations, which were 1.6 μg/ml (0.01 LC_{50}) and 8.0 μg/ml (0.05 LC_{50}). In the pre-treatment protocol, analysis of the combinatorial effects of all combinations using the IC_{50} data yielded FICs of 0.91 and 1.72. The combination of Ribavirin and FH7.04 at lowest concentration (1.6 μg/ml) exhibited a moderate level of synergy (FICs=0.91). This combination significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 5.46 μg/ml to 4.32 μg/ml. However, when the concentration of FH7.04 was increased to 8.0 μg/ml, the interaction was changed to weak antagonistic with FICs of 1.72.

Meanwhile, in the post-infection protocol, analysis of the combinatorial effects showed no reduction in IC_{50} values compared with the single Ribavirin treatment at every concentration of combinations. Analysis using the IC_{50} data yielded FICs of 3.57 and 2.88, which suggest highly antagonistic interaction.

From the results, it can be concluded that FH7.04 was able to increase the efficiency of antiviral therapy against MV only in pre-treatment protocol as it exhibited synergistic interaction with Ribavirin at low concentration.

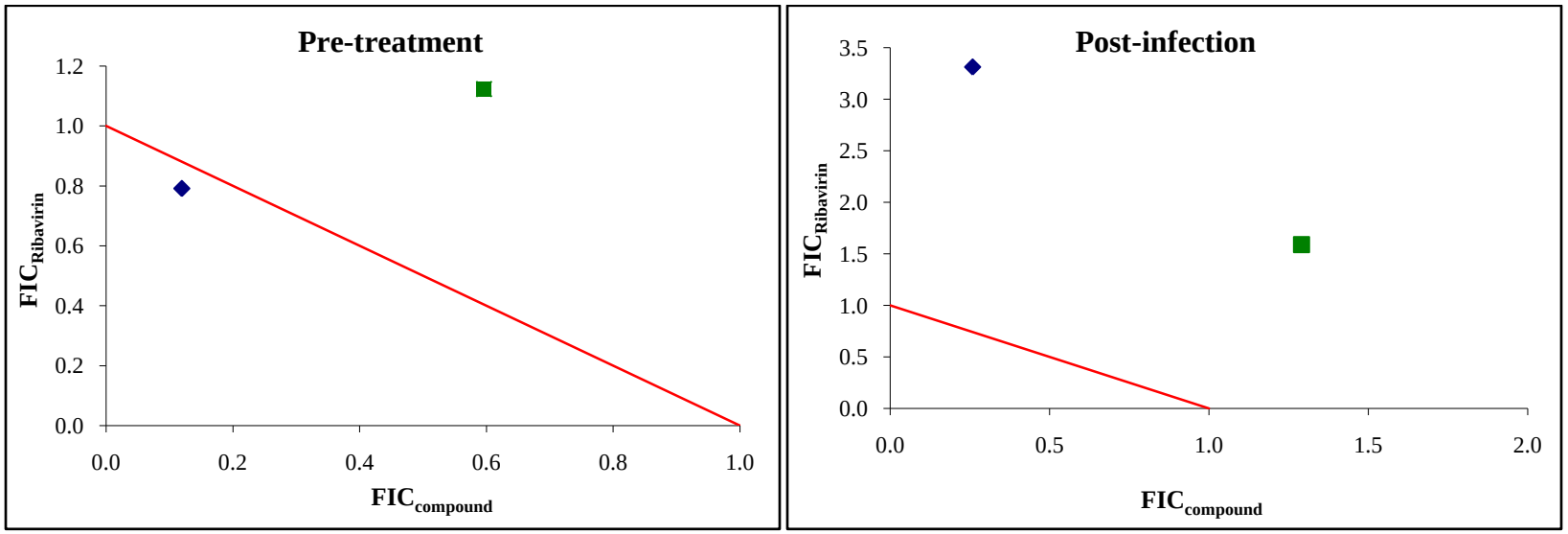

Figure 12: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH7.04 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, none of these combinations displayed a synergistic activity against MV in the post infection protocol. Moderate synergistic effect only present in the pre-treatment protocol at concentration of 0.01 LC_{50}.

Table 34: Inhibitory effects of FH7.04 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH7.04 only	13.42				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH7.04	4.32	0.12	0.79	0.91	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH7.04	6.13	0.60	1.12	1.72	Weak antagonism
Post-infection	FH7.04 only	6.20				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH7.04	10.00	0.26	3.31	3.57	High antagonism
	Ribavirin + 0.05 LC_{50} FH7.04	4.80	1.29	1.59	2.88	High antagonism

4.5.10 Combination therapy of isolated compound FH 7.05 with Ribavirin

Figure 13 and Table 35 showed the results of combining various concentrations of Ribavirin with fixed FH7.05 concentrations, which were 1.6 μg/ml (0.01 LC_{50}) and 8.0 μg/ml (0.05 LC_{50}).In the pre-treatment protocol, analysis of the combinatorial effects of the combination at lowest concentration (1.6 μg/ml) of FH7.05 yielded FICs of 0.34, which suggest highly synergistic interaction. The combination significantly ($P<0.05$) reduced the IC_{50} value for single Ribavirin treatment from 5.46 μg/ml to 1.27 μg/ml. However, combinations at higher concentration (8.0 μg/ml) showed highly antagonistic interactions, with FICs of 2.17.

Meanwhile in the post-infection protocol, no reduction in IC_{50} values compared with the single Ribavirin treatment was detected at every concentration of combinations. Analysis of the combinatorial effects using the IC_{50} data yielded FICs of 3.57 and 2.88, which suggest highly antagonistic interaction.

From the results, it can be concluded that FH7.05 was able to increase the efficiency of antiviral therapy against MV only in the pre-treatment protocol as it exhibited synergistic interaction with Ribavirin, but only at low concentration.

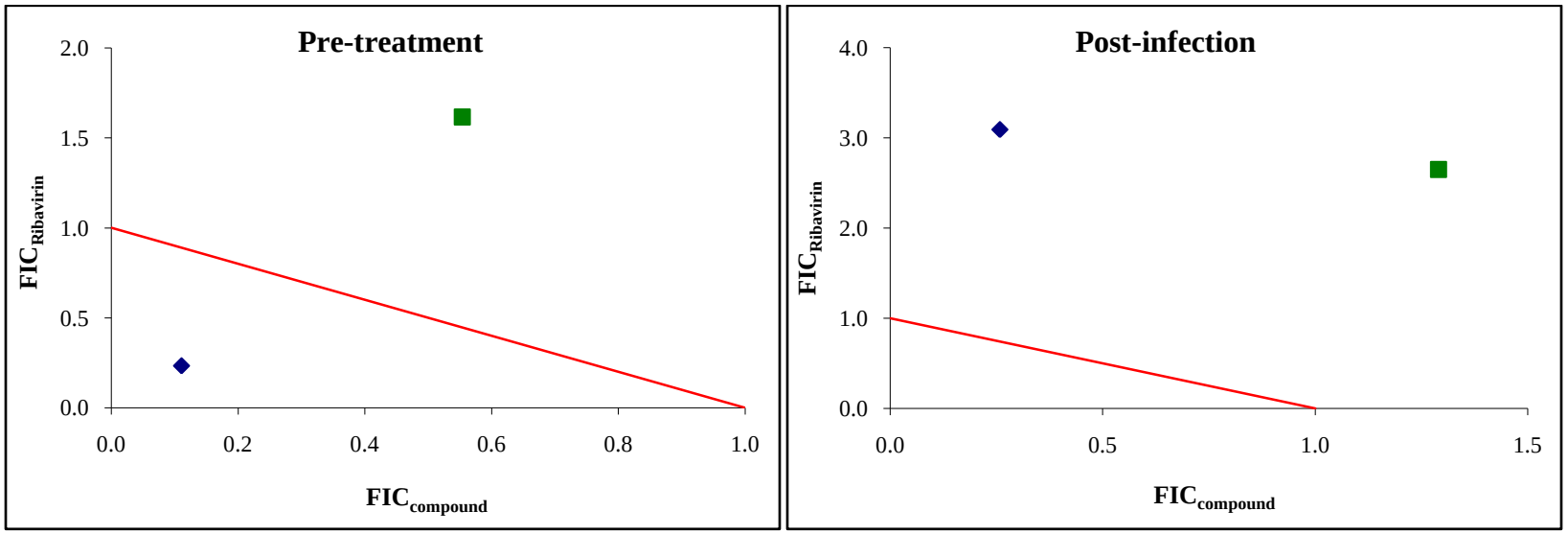

Figure 13: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH7.05 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, FH7.05 was able to increase the efficiency of antiviral therapy against MV only in the pre-treatment protocol as it exhibited highly synergistic interaction with Ribavirin, but only at low concentration.

Table 35: Inhibitory effects of FH7.05 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH7.05 only	14.45				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH7.05	1.27	0.11	0.23	0.34	High synergy
	Ribavirin + 0.05 LC_{50} FH7.05	8.83	0.55	1.62	2.17	High antagonism
Post-infection	FH7.05 only	6.20				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH7.05	9.33	0.26	3.09	3.35	High antagonism
	Ribavirin + 0.05 LC_{50} FH7.05	8.00	1.29	2.65	3.94	High antagonism

4.5.11 Combination therapy of isolated compound FH 11.04.2 with Ribavirin

Figure 14 and Table 36 showed the results of combining various concentrations of Ribavirin with fixed FH11.04.2 concentrations, which were 1.5 µg/ml (0.01 LC_{50}) and 7.5 µg/ml (0.05 LC_{50}). Moderate synergistic interaction was observed in combination at lowest concentration (1.5 µg/ml) of FH11.04.2 in both protocols. The IC_{50} value for single Ribavirin treatment was significantly ($P<0.05$) reduced from 5.46 to 1.27 µg/ml in pre-treatment (FICs=0.45), and from 3.02 to 1.60 µg/ml in post-infection (FICs=0.76). However, when the concentration of FH11.04.2 was increased to7.5 µg/ml, the IC_{50} data yielded FICs of 1.35 and 3.91, which indicate antagonism interaction.

From the results, it can be concluded that FH11.04.2 was able to increase the efficiency of antiviral therapy against MV in both protocols as it exhibited synergistic interaction with Ribavirin at low concentration.

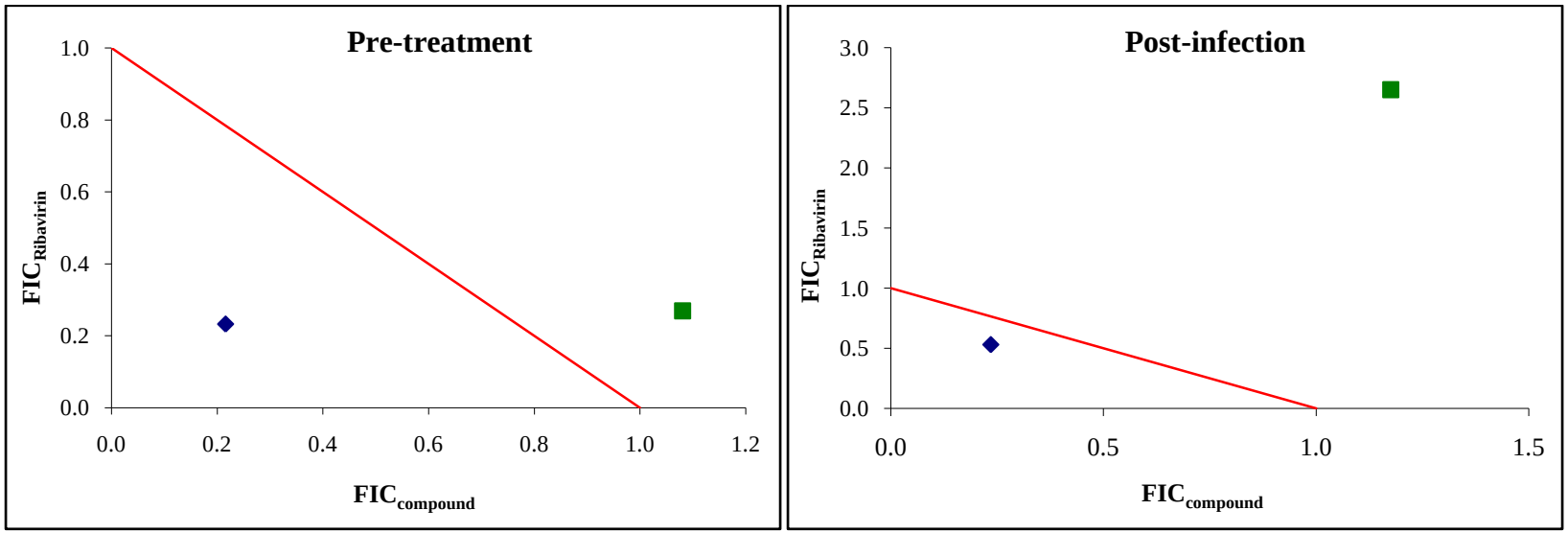

Figure 14: The IC_{50} values were used to construct the isobologram of antiviral interactions between FH11.04.2 and Ribavirin. The experimental data points correspond to different compound concentration: 0.01 LC_{50} (◆) and 0.05 LC_{50} (■). Values above, on orbelow the theoretical line indicate antagonism, additive or synergy, respectively. Based on the graph, FH11.04.2 was able to increase the efficiency of antiviral therapy against MV in both protocols as it exhibited synergistic interaction with Ribavirin at low concentration.

Table 36: Inhibitory effects of FH11.04.2 in combination with Ribavirin against the infection of measles virus in Vero cells using pre-treatment and post-infection protocol

Protocol	**Combination**	**IC_{50} (µg/ml)**	**$FIC_{compound}$**	**$FIC_{Ribavirin}$**	**$FIC_{compound}$ + $FIC_{Ribavirin}$**	**FIC index interpretation**
Pre-treatment	FH11.04.2 only	6.94				
	Ribavirin only	5.46				
	Ribavirin + 0.01 LC_{50}FH11.04.2	1.27	0.22	0.23	0.45	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH11.04.2	1.47	1.08	0.27	1.35	Weak antagonism
Post-infection	FH11.04.2 only	6.38				
	Ribavirin only	3.02				
	Ribavirin + 0.01 LC_{50}FH11.04.2	1.60	0.24	0.53	0.76	Moderate synergy
	Ribavirin + 0.05 LC_{50} FH11.04.2	8.00	1.18	2.65	3.91	High antagonism

4.6 ANTI-PROLIFERATIVE ACTIVITY OF SELECTED ISOLATED COMPOUNDS

In this screening, the cytotoxic effect of selected isolated compounds of *C. nardus* against human papillary ovarian adenocarcinoma (Caov-3) cancer cells was evaluated using the MTT assay. The cytotoxic effect against this cell line is considered as a predictive anti-proliferative activity indicator. The percentage of growth inhibition of each compound on Caov-3 cells was determined as the percentage of viable treated cells in comparison with viable cells of untreated controls. All controls always contained 0.5% DMSO in order to exclude any effect of DMSO on cells. The LC_{50} value for positive control drug, Tamoxifen, was 150µg/ml.

From the results, the relative cell survival progressively decreased in a dose-dependent manner when the cells were treated for 72 hours with 0, 25, 50, 100, 200 and 400 µg/ml of isolated compounds, as shown in Figure 15 and Appendix U. Statistical analysis showed that the activity was significant ($P<0.05$) when compared with untreated cells (Appendix V). None of the 10 isolated compounds had better than weak activity towards Caov-3 cells as they inhibited less than 50% of Caov-3 cell growth at the highest concentration evaluated (400µg/ml) (Table 37). Compound FH6.10.3 was the most active compound in this assay, exhibiting the highest percentage of cells growth inhibition (41.7%) when compared to untreated control. Meanwhile, the less active compound was FH6.06.2 with only 19.3% of cells growth inhibition. The other 8 compounds only produced 20 to 40 percents of growth inhibition in Caov-3 cells at the same concentration. DMSO used as delivery vehicle did not affect the cell growth when treated for the same time period (Appendix W).

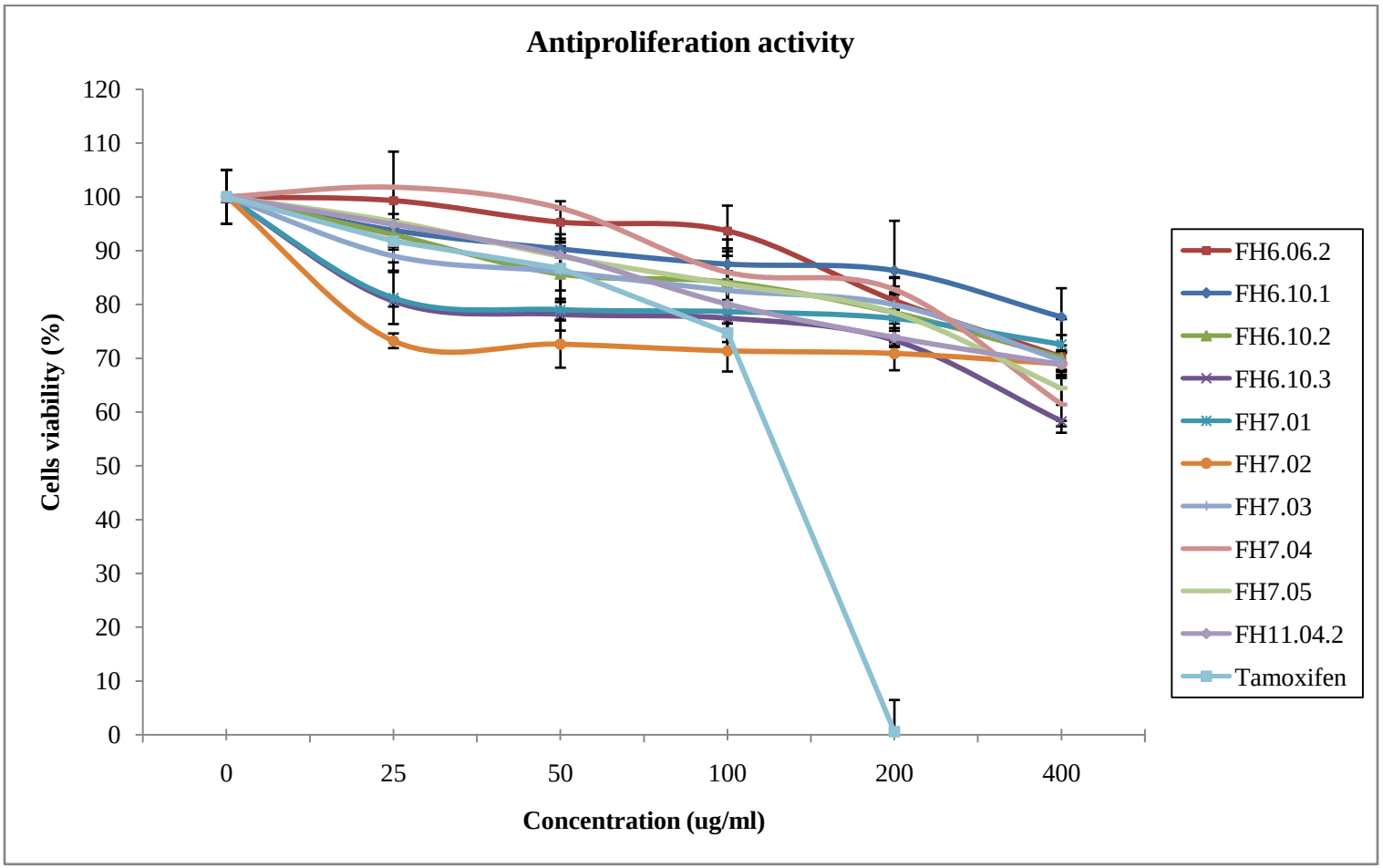

Figure 15: The Caov-3 cells survival progressively decreased in a dose-dependent manner when cells were treated with 0, 25, 50, 100, 200 and 400 µg/ml of isolated compounds

Table 37: LC_{50} values of selected isolated compoundson Caov-3 cells at the highest concentration tested (400µg/ml)

Isolated compound	**LC_{50} (µg/ml)**		**[c]Growth inhibition (%)**
	[a]Vero cell	**[b]Caov-3 cell**	
FH6.06.2	270	>400	19.3 ± 3.0
FH6.10.1	200	>400	23.0 ± 5.3
FH6.10.2	200	>400	29.3 ± 1.1
FH6.10.3	200	>400	41.7 ± 2.9
FH7.01	270	>400	27.5 ± 4.7
FH7.02	270	>400	31.1 ± 2.0
FH7.03	200	>400	30.4 ± 1.9
FH7.04	200	>400	38.6 ± 3.1
FH7.05	200	>400	35.6 ± 4.1
FH11.04.2	150	>400	31.2 ± 2.2
Tamoxifen	200	150	50.0 ± 5.8

[a]Cytotoxic concentration of isolated compound that reduced Vero cells viability by 50%

[b]Cytotoxic concentration of isolated compound that reduced Caov-3 cells by 50%

[c]Growth inhibition of treated Caov-3 cells compared to untreated control

As also shown in Table 37, the cytotoxic values of all isolated compounds were higher in Caov-3 cells compared to normal cells (Vero cells). The LC_{50} values for all isolated compounds were ranged between 150 to 270 µg/ml in normal cells, but concentration needed to kill cancer cells is higher, which is more than 400µg/ml. This suggest that during the fraction treatment, the compound not only kill the cancer cells, but also the normal cells, as the concentration used to kill the cancer cells was way much higher than concentration that can be tolerate by normal cells. In addition, according to the criteria of the American National Cancer Institute, the cytotoxic limit to consider a crude extract promising for further purification is lower than 30µg/ml (Costa-Lotufo *et al*.,2005; Kheng *et al*., 2010). Thus, none of these compounds could be considered as potential source of anticancer compounds.

4.7 STRUCTURE ELUCIDATION OF SELECTED ISOLATED COMPOUNDS USING GC-MS

Selected isolated compounds, namely FH4.01, FH4.04.4, FH6.06.2, FH6.06.3, FH6.10.1, FH6.10.2, FH7.03 and FH11.06.2 were further analysed with gas chromatography-mass spectroscopy (GC-MS). GC-MS was used to determine the chemical compound and the molecular mass of these selected isolated compounds. About 2.0 mg of each isolated compound was diluted in 200µl dichloromethane or ethyl acetate and these samples were then injected into GC-MS using splitless mode.

4.7.1 Compound FH4.01

Compound FH4.01 was isolated as a white powdery substance from fraction FH4. The chromatogram from GC-MS analysis of this compound is shown in Figure 16. Based on the chromatogram, one sharp peak was detected at retention time of 29.91 min. The molecular mass of compound is 534 $gmol^{-1}$ which corresponded to molecular formula of $C_{36}H_{54}OS$ (Table 38).

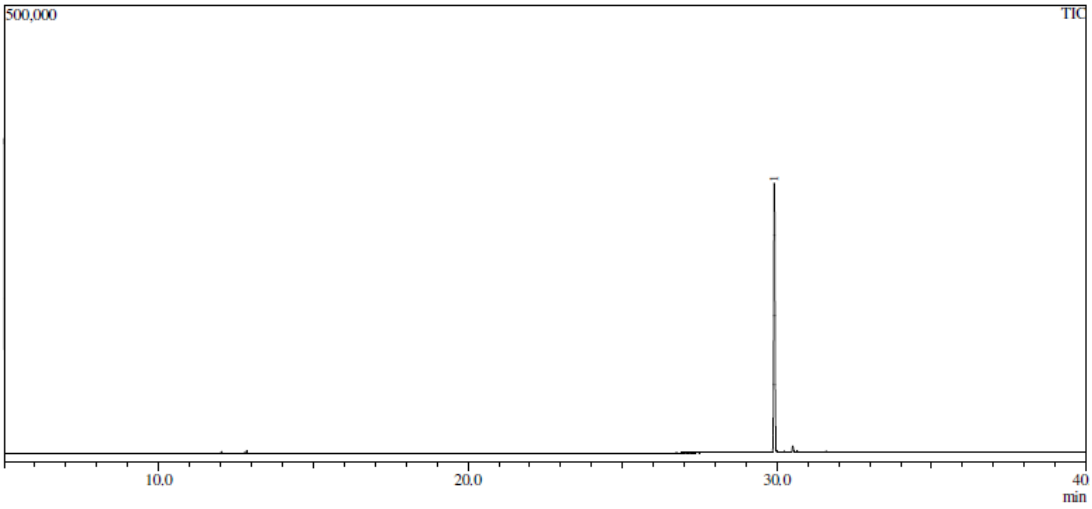

Figure 16: Chromatogram from GC-MS analysis of compound FH4.01

Table 38: Compound present in FH4.01 analysed using MS-spectra

Peak	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	78	$C_{36}H_{54}OS$	534	29.91	17-(1,5-Dimethyl-3-phenylthiohex-4-enyl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopent(a)phenanthren

4.7.2 Isolated compound FH4.04.4

Compound FH4.04.4 was isolated as pale yellowish oil from subfraction FH4.04. The chromatogram from GC-MS analysis is shown in Figure 17. Based on the chromatogram, there was one sharp peak detected at retention time of min 12.64 min. The molecular mass of compound is 178 $gmol^{-1}$ which corresponded to molecular formula of $C_{11}H_{14}O_2$ (Table 39).

Table 39: Compound present in FH4.04.4 analysed using MS-spectra

Peak	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	78	$C_{11}H_{14}O_2$	178	12.64	Methyl eugenol

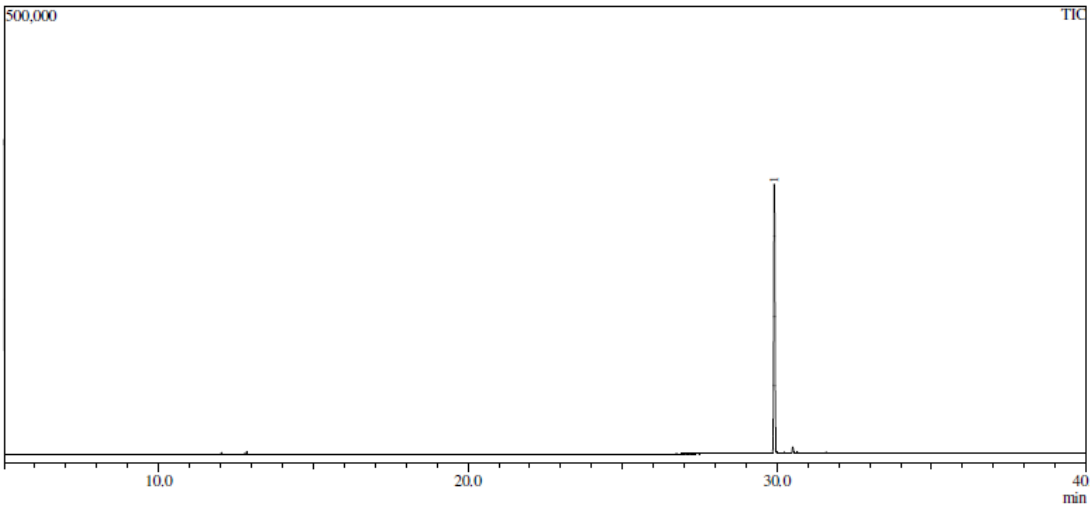

Figure 17: Chromatogram from GC-MS analysis of compound FH4.04.4

4.7.3 Isolated compound FH6.06.2

Compound FH6.06.2 was isolated as yellowish oil from subfraction FH6.06. The chromatogram from GC-MS analysis is shown in Figure 18. Based on the chromatogram, there were nine sharp peaks detected at retention time of min 15.42 min (peak 1), 16.47 min (peak 2), 16.57 min (peak 3), 16.61 min (peak 4), 16.76 min (peak 5), 16.83 min (peak 6), 17.91 min (peak 7), 19.95 min (peak 8) and 21.81 min (peak 9). The molecular mass and molecular formula of each peak were summarised in Table 40. From the result, compound FH6.06.2 can be considered not pure enough and further purification can be carried out. However, due to small amount of sample, no further purification was carried out.

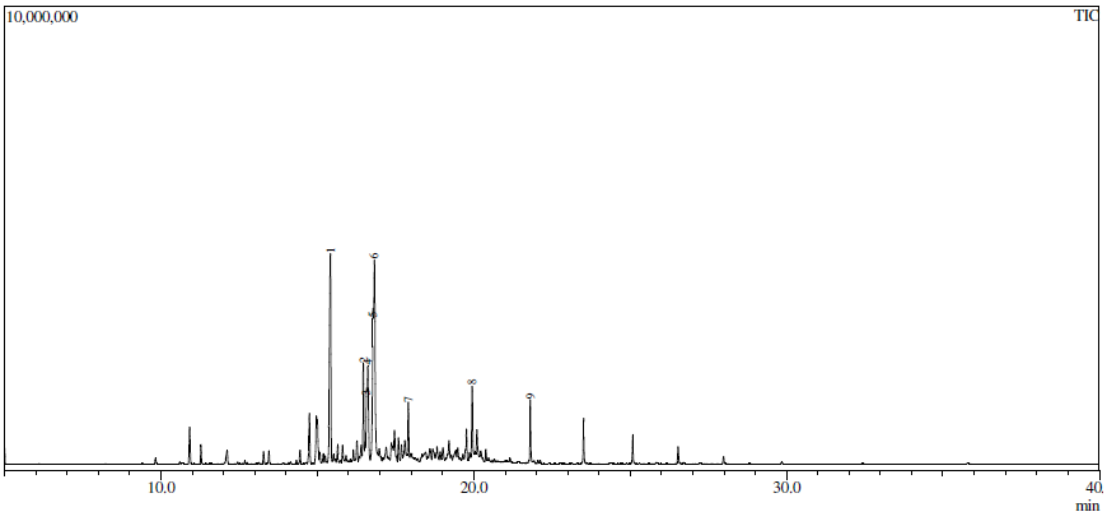

Figure 18: Chromatogram of GC-MS analysis of compound FH6.06.2

Table 40: Compound present in FH6.06.2 analysed using MS-spectra

Peaks	**Similarity**	**Formula**	**Molecular weight ($gmol^{-1}$)**	**Retention time (min)**	**Compound name**
Peak 1	95	$C_{15}H_{26}O$	222	15.42	Cyclohexanemethanol
Peak 2	94	$C_{15}H_{26}O$	222	16.47	2-Naphthalenemethanol
Peak 3	84	$C_{15}H_{24}$	204	16.57	(+)-Epi-bicyclosesquiphellandrene
Peak 4	84	$C_{15}H_{26}O$	222	16.61	alpha.-Cadinol
Peak 5	79	$C_{15}H_{26}O$	222	16.76	alpha.-Cadinol
Peak 6	94	$C_{15}H_{26}O$	222	16.83	2-Naphthalenemethanol
Peak 7	95	$C_{19}H_{38}$	266	17.91	1-Nonadecene
Peak 8	94	$C_{20}H_{40}$	280	19.95	1-Eicosene
Peak 9	94	$C_{26}H_{45}F_7O_2$	522	21.81	Docosyl heptafluorobutyrate

4.7.4 Isolated compound FH6.06.3

Compound FH6.06.3 was also isolated as yellowish oil from subfraction FH6.06. The chromatogram from GC-MS analysis is shown in Figure 19. Based on the chromatogram, four sharp peaks were detected at retention time of min 17.06 (peak 1), 19.09 min (peak 2), 20.95 min (peak 3) and 22.63 min (peak 4). The molecular mass of compound 1, compound 2 and compound 3 are the same which is 266 $gmol^{-1}$ and they corresponded to molecular formula of $C_{19}H_{38}$ (Table 41). Meanwhile the molecular mass of compound 4 is 394 $gmol^{-1}$ which corresponded to molecular formula of $C_{22}H_{41}F_3O_2$. From the result, compound FH6.06.3 can be considered not pure enough and further purification can be carried out. However, due to small amount of sample, no further purification was carried out.

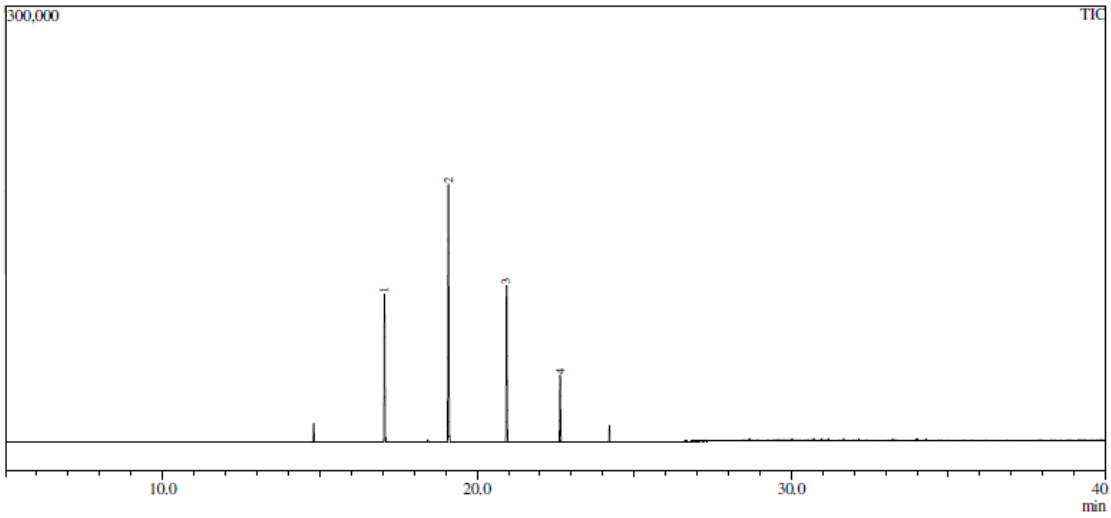

Figure 19: Chromatogram of GC-MS analysis of compound FH6.06.3

Table 41: Compound present in FH6.06.3 analysed using MS-spectra

Peaks	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	94	$C_{19}H_{38}$	266	17.06	1-Nonadecene
Peak 2	95	$C_{19}H_{38}$	266	19.09	1-Nonadecene
Peak 3	93	$C_{19}H_{38}$	266	20.95	1-Nonadecene
Peak 4	87	$C_{22}H_{41}F_3O_2$	394	22.63	Eicosyl trifluoroacetate

4.7.5 Isolated compound FH6.10.1

Compound FH6.10.1 was isolated as yellowish oil from subfraction FH6.10. The chromatogram from GC-MS analysis is shown in Figure 20. Based on the chromatogram, one sharp peak was detected at retention time of 19.09. The molecular mass of the compound is 196 $gmol^{-1}$ which corresponded to molecular formula of $C_{13}H_{24}O$. Compound FH6.10.1 can be considered not pure enough as the chromatogram still showed many minor peaks and further purification can be carried out. However, due to small amount of sample, no further purification was carried out.

Table 42: Compound present in FH6.10.1 analysed using MS-spectra

Peak	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	81	$C_{13}H_{24}O$	196	19.09	1-Tridecyn-4-ol

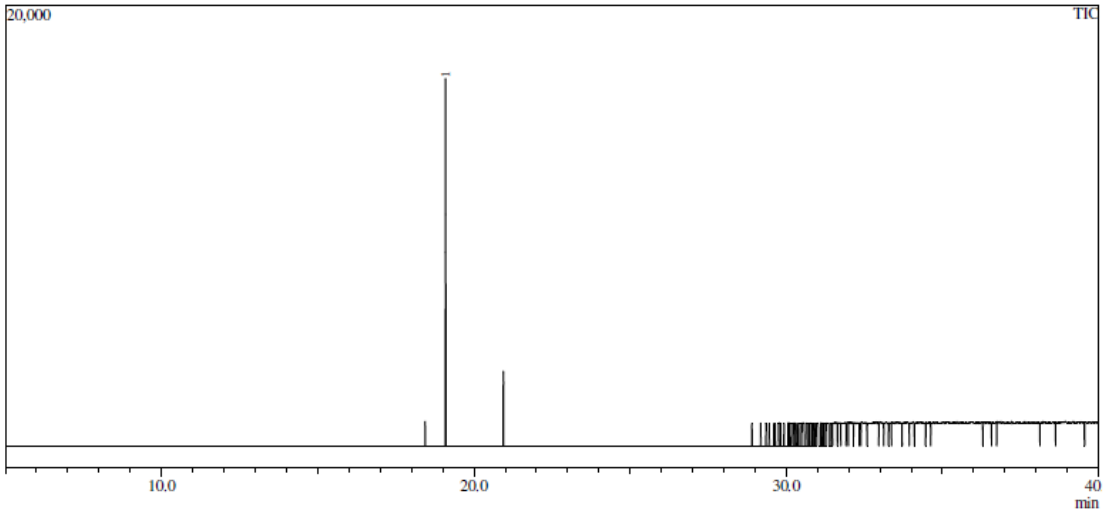

Figure 20: Chromatogram of GC-MS analysis of compound FH6.10.1

4.7.6 Isolated compound FH6.10.2

Compound FH6.10.2 was isolated as yellowish oil from subfraction FH6.10. The chromatogram from GC-MS analysis is shown in Figure 21. Based on the chromatogram, there were seven sharp peaks detected at retention time of min 17.91 (peak 1), 19.95 min (peak 2), 21.81 min (peak 3), 23.51 min (peak 4), 25.08 min (peak 5), 26.54 min (peak 6) and 27.99 min (peak 7). The molecular mass and molecular formula of each peak were summarized in Table 43. From the result, compound FH6.06.2 can be considered not pure enough and further purification can be carried out. However, due to small amount of sample, no further purification was carried out.

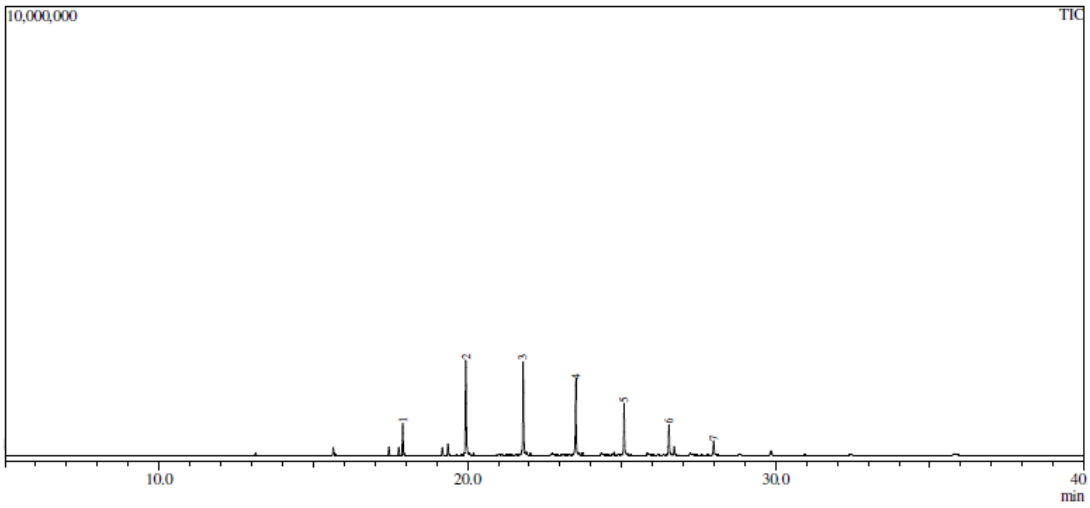

Figure 21: Chromatogram of GC-MS analysis of compound FH6.10.2

Table 43: Compound present in FH6.10.2 analysed using MS-spectra

Peaks	**Similarity**	**Formula**	**Molecular weight ($gmol^{-1}$)**	**Retention time (min)**	**Compound name**
Peak 1	96	$C_{19}H_{38}$	266	17.91	1-Nonadecene
Peak 2	94	$C_{22}H_{41}F_3O_2$	394	19.95	Eicosyl trifluoroacetate
Peak 3	94	$C_{26}H_{45}F_7O_2$	522	21.81	Docosyl heptafluorobutyrate
Peak 4	94	$C_{31}H_{55}F_7O_2$	592	23.51	Heptacosyl heptafluorobutyrate
Peak 5	93	$C_{31}H_{55}F_7O_2$	592	25.08	Heptacosyl heptafluorobutyrate
Peak 6	93	$C_{31}H_{55}F_7O_2$	592	26.54	Heptacosyl heptafluorobutyrate
Peak 7	93	$C_{31}H_{55}F_7O_2$	592	27.99	Heptacosyl heptafluorobutyrate

4.7.7 Isolated compound FH7.03

Compound FH4.01 was isolated as colourless oil from fraction FH7. The chromatogram from GC-MS analysis is shown in Figure 22. Based on the chromatogram, two sharp peaks were detected at retention time of 10.09 min (peak 1) and 10.45 min (peak 2). The molecular mass of compound 1 is 156 $gmol^{-1}$ which corresponded to molecular formula of $C_{10}H_{20}O$and the molecular mass of compound 2 is 154 $gmol^{-1}$ which corresponded to molecular formula of $C_{10}H_{18}O$(Table 44).

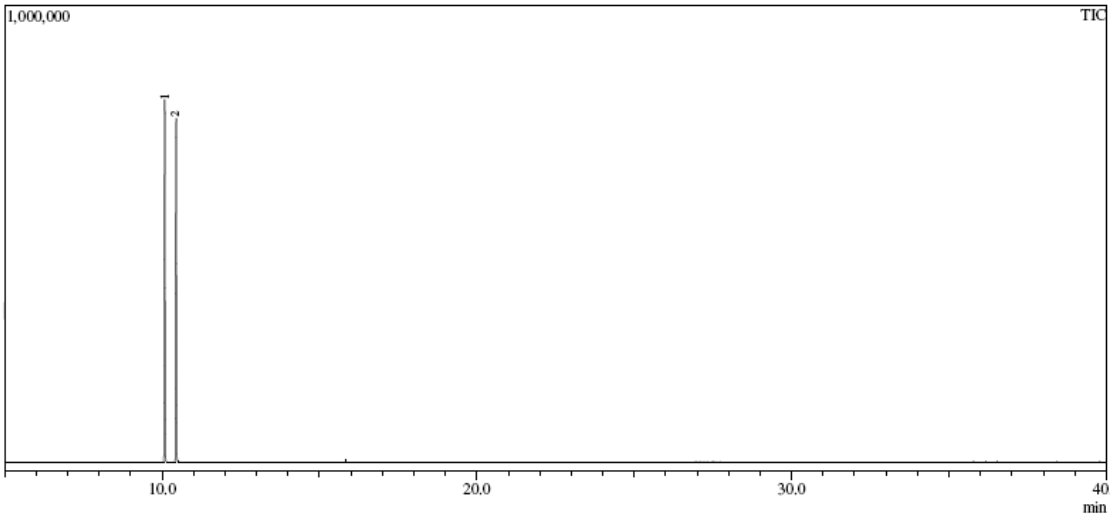

Figure 22: Chromatogram of GC-MS analysis of compound FH7.03

Table 44: Compound present in FH7.03 analysed using MS-spectra

Peaks	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	98	$C_{10}H_{20}O$	156	10.09	Citronellol
Peak 2	96	$C_{10}H_{18}O$	154	10.45	Geraniol

4.7.8 Isolated compound FH11.04.2

Compound FH11.04.2 was isolated as colourless oil from subfraction FH11.04. The chromatogram from GC-MS analysis is shown in Figure 23. Based on the chromatogram, there were six sharp peaks detected at retention time of min 17.91 (peak 1), 18.65 min (peak 2), 19.93 min (peak 3), 21.79 min (peak 4), 23.49 min (peak 5) and 25.03 min (peak 6). The molecular mass and molecular formula of each peak were summarised in Table 45. From the result, compound FH11.04.2 can be considered not pure enough and further purification can be carried out. However, due to small amount of sample, no further purification was carried out.

Table 45: Compound present in FH11.04.2 analysed using MS-spectra

Peaks	Similarity	Formula	Molecular weight ($gmol^{-1}$)	Retention time (min)	Compound name
Peak 1	96	$C_{19}H_{38}$	266	17.91	1-Nonadecene
Peak 2	95	$C_{27}H_{56}O$	396	18.65	1-Heptacosanol
Peak 3	94	$C_{26}H_{45}F_7O_2$	522	19.93	Docosyl heptafluorobutyrate

Table 45: (cont.)

Peaks	**Similarity**	**Formula**	**Molecular weight ($gmol^{-1}$)**	**Retention time (min)**	**Compound name**
Peak 4	94	$C_{31}H_{55}F_7O_2$	592	21.79	Heptacosyl heptafluorobutyrate
Peak 5	94	$C_{31}H_{55}F_7O_2$	592	23.49	Heptacosyl heptafluorobutyrate
Peak 6	92	$C_{22}H_{39}F_5O_2$	430	25.03	Nonadecyl pentafluoropropionate

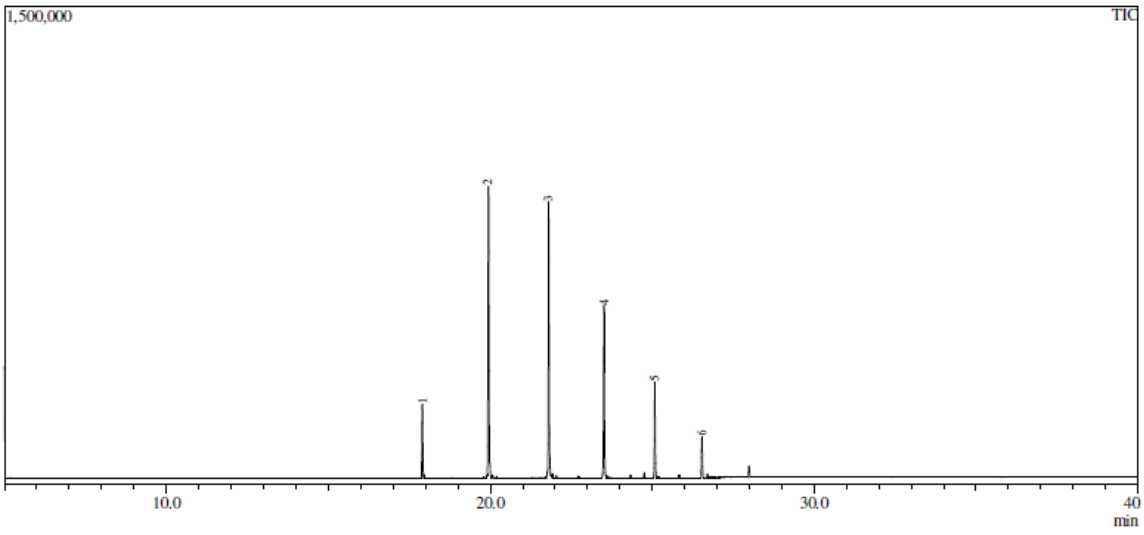

Figure 23: Chromatogram of GC-MS analysis of compound FH11.04.2

CHAPTER 5: DISCUSSION

Plants have a long evolutionary history with respect to developing resistance against viruses and are increasingly drawing attention as potential sources for development of antiviral drugs (Yu *et al*., 2009). In the past years, many screening efforts have been made to find antiviral agents which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules from medicinal plants (Schmitt *et al*., 2001). During the last 30 years, extracts from more than 4000 different plant species were studied and about 10 percents of them showed a significant antiviral activity *in vitro*(Che, 1991). Sweet lemon grass (*Cymbopogon nardus* (L.) Rendle) has long been used in many part of the world as traditional medicine. In Malaysia, this planthas been used widely in Malay traditional medicine for treatment of various ailments such as skin cuts, diarrhoea (Burkill, 1966), joint pains, bronchitis and malaria (Perry & Metzer, 1980).

5.1 EXTRACTION, FRACTIONATION AND PURIFICATION OF *C. NARDUS* (L.) RENDLE

5.1.1 Extraction

Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures (Tiwari *et al*., 2011). During extraction, solvents diffuse into the solid plant tissues and solubilise compounds with similar polarity. The dried leaves of sweet lemon grass were used as a source for the extraction of secondary plant components. Many researchers working on the chemistry of secondary plant componentshave tended to use dried

plant material over fresh plant tissues (Das *et al*., 2010). This is due to the differences in water content within different plant tissues which may affect solubilityof subsequent separation by liquid-liquid extraction. Furthermore many plants are used inthe dry form or as an aqueous extract by traditionalhealers. The dried plant material was ground up to smaller particles using grinder before extraction. The objective of grounding is to rupture the plant tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent (Tiwari *et al*., 2011). Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent. A study by Eloff in 1998 showed that 5 minutes extractions of very fine particles of diameter 10µm gave higher quantities than values obtained after 24 hours in a shaking machine with less finely ground.

In this study, hexane was used as the extraction solvent based on previous studies by Nurmawati (1995), Hanina (2006) and Nurul Aini *et al*. (2006) who reported the presence of antibacterial and antivirus substances in the hexane extract of *C. nardus*. Hexane has properties of a good solvent in plant extraction as it is low in toxicity and ease of evaporation at low heat (Ohler, 1999). From the result, only a small amount of crude extract were obtained because hexane is a non-polar solvent that able to extract only non-polar compounds such as fat, terpenes, chlorophyll (Hanina, 2006), fatty acids, terpenoids, alkaloids, terpenic alcohols, terpenic aldehydes and ketones (Nor Afifah *et al*., 2010). Although hexane only extracted the non-polar compounds, there are numerous researchers have found the hexane extracts from various plants to possess antiviral activity against many viruses such as herpes simplex virus type 2 (HSV-2) (Akanitapichat *et al*., 2006), herpes simplex virus type 1 (HSV-1), Parainfluenza-2 virus (PI-3)

(Orhan *et al.*, 2009), vesicular stomatitis virus (VSV) and Semiliki forest A7 virus (SF A7) (Maregesi *et al*., 2008),

5.1.2 Fractionation

Plant extracts are usually complex mixtures containing hundreds or thousands of different constituents. The presence of other plant constituents in a crude extract makes the isolation and measurement of active constituents difficult and powerful separations techniques with high efficiency and sensitivity are required. In this study, bioassay-guided fractionation was used to separate the active compounds. Bioassay-guided fractionation is a procedure of whereby extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated (Ernawita, 2008). Each fraction produced during the fractionation process is evaluated in a bioassay system and only active fractions are fractionated. Chromatographic techniques involved in this procedureare column chromatography (CC), thin layer chromatography (TLC)and preparative thin layer chromatography (PTLC).

Fractionation of crude extract using column chromatography I (CC I) with increasing polarity of solvent systems, starting from hexane, dichloromethane (DCM), chloroform, ethyl acetate and methanol in different ratios, produced 20 combined fractions labelled as FH1 to FH20. Based on yield percentage, it can be concluded that fractions eluted from moderately polar solvent system have higher percentage. Meanwhile, fraction eluted from the polar solvent, which is methanol, have lowest percentage. All of these combined fractions were then tested for antiviral activity. Seven fractions, namely FH4, FH5, FH6, FH8, FH10, FH11 and FH15, were

selected for second column chromatography (CC II) as they exhibited promising antiviral activity against measles virus. A total of 77 combined subfractions were obtained and they were further tested in antiviral assay. White powdery substance that formed from fraction FH4 before the fractionation process was also tested in antiviral assay.

5.1.3 Purification

Fraction and subfractions with pronounced antiviral activity were subjected to purification process using preparative thin layer chromatography (PTLC). Before PTLC was conducted, all selected fraction and subfractions were re-subjected to TLC again, in order to determine if compound in fraction or subfraction was volatile during storage. Results showed that nine subfractions were volatile as they showed less spots on TLC sheet compared to previous TLC. According to Pezzuto (1996), the activity of about 50 percent of the samples will be lost during the process of bioassay-directed fractionation causes by the lack of chemical stability and compounds lacking chemical stability are rarely isolated. Therefore these subfractions were not further purified and eliminated from antiviral test.Purification of fraction FH7 and subfractions FH6.06, FH6.10 and FH11.04 produced different number of bands on the PTLC plate under the UV light. For fraction FH7, five bands were observed on the PTLC plate, meanwhile three bands were produced by subfractions FH6.06 and FH6.10.Only one band was obtained from subfraction FH11.04 on the PTLC plate. All bands were then scraped, extracted with a suitable solvent and filtered to give the isolated materials upon removal of the solvent. These isolated compounds were then further tested in antiviral assay.

5.2 ANTIVIRAL ACTIVITY OF *C. NARDUS* (L.) RENDLE AGAINST MEASLES VIRUS

In the past years, many screening efforts have been made to find antiviral agents which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules from medicinal plants (Schmitt *et al*., 2001). According to Khan *et al*. (2005), approximately 44 percents of the antiviral drugs approved between 1981 and 2006 were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural product.

In this study, the fractions, subfractions and isolated compounds of *C. nardus* exhibited antiviral activity in cell culture against measles virus. This result is consistent with previous studies by Ahmad *et al*. (1992), Hanina (2006) and Nurul Aini *et al*. (2006) which reported that the crude extract, fractions and partially purified subfractionsof this plant are active against Newcastle disease virus (NDV), herpes simplex virus type 1 (HSV-1) and measles virus (MV). The evaluation of antiviral activity in this study includes antiviral efficacy and cell toxicity. Vero cell (African green monkey kidney cell) was used as the host cells system for virus infection. This cell line is suitable for use in routine diagnostic virology as it grow to monolayers as rapidly as other heteroploid cell lines, but will maintain as usable monolayers in conventional maintenance medium for a significantly longer time (Macfarlane & Sommerville, 1969). Besides that, Vero cell support the growth of a very wide range of viruses with development in most instances of a characteristic cytopathic effect. For preparation of stock solutions, fractions, subfractions and isolated compounds were pre-dissolve in dimethylsulphoxide (DMSO) before added to the bioassay medium (DMEM) in a volume so that the concentration of DMSO was not detrimental to the assay (Soumyanath & Srijayanta, 2006). According to Cos *et al.* (2006), there

are two advantages of preparing stock solutions in DMSO. Firstly, the elimination of microbial contamination that reduce the need for sterilisation by autoclaving or other strenuous methods which may affect the quality of the test sample. Secondly, the good compatibility with test automation and integrated screening platforms, assuring for example good solubility during the serial dilution procedures. However, DMSO is potentially toxic for cells. To avoid later interference in the biological test systems, the in-test concentration of DMSO should not exceed 1% (Naidoo, 2007). In this study, DMSO was used at concentration of .0.5%. At this concentration, DMSO did not affect the growth of Vero cell.

5.2.1 Cytotoxicity of fractions, subfractions and isolated compounds of *C. nardus* on Vero cells

Before the evaluation of the antiviral activity, the cytotoxic effects of the fractions, subfractions and isolated compounds on Vero cells were investigated. According to Simmonds & Howes (2006) and Yucharoen *et al*. (2011), evaluation of cytotoxicity is an important part of the assessment of a potential antiviral agent since the beneficial extracts should be selective for virus-specific processes with little or no effects on the metabolism of host cells. Therefore, it is critical to perform cytotoxicity test although most tests only measures acute toxicity, or the result obtained using cell culture may not be extrapolate directly to the whole animal situation, but it is fairly certain that if some products produce deleterious effects on cell, some bad effect may be expected if the same products are applied to a whole animal (Marini *et al.*, 1998). The accuracy of cytotoxicity analysis is very important to avoid mistakes during antiviral screening (Nardiah Rizwana *et al.*, 2010). If the fractions, subfractions or isolated compounds are too toxic, the

antiviral results are not valid because it will have a low therapeutic value (treatment value: toxicity level ratio).

The cytotoxicity, measured in term of 50% lethal concentration (LC_{50}), of different fractions, subfractions and isolated compounds ranged from highly toxic (0 to 25 µg/ml) to considerably non-toxic (above 100µg/ml). The LC_{50} of all fractions ranged from 50 to 600 µg/ml. Four fractions exhibited mild toxicity with LC_{50} ranging from 50 to 100 µg/ml, while rest of the fractions were non-toxic at above 100µg/ml. Meanwhile for the subfractions, the LC_{50} were ranged from 60 to 600 µg/ml. Fifteen out of 77 subfractions showed mild toxicity with LC_{50} between 60 to 100 µg/ml and the other 62 subfractions demonstrated no toxicity on the cells. On the other hand, all of the isolated compounds showed no toxicity on Vero cells with LC_{50} ranging from 150 to 400 µg/ml. Overall, the cytotoxicity of each tested compound decreases at every level of purification. According to Yarmolinsky *et al*. (2011) and Wildman (2003), separation of plant extract into different fractions decreased the cytotoxicity of the fractions compared to the crude extract. Varied responses of the cells to the chemical agents have been correlated with the capability of the cells to multiply, and differences in the cells growth rate, cell size and cell defences (Nurul Aini *et al*., 2006).

5.2.2 Antiviral activity of fractions, subfractions and isolated compounds of *C. nardus* against measles virus

One of the inherent drawbacks of *in vitro*antiviral testing is the environmental sensitivity of animal cells in culture. Preparations which exert antiviral effects *in vivo* may not be detected in *in*

vitro assays because of the extremely low concentrations of extract tolerated by cells in the artificial system (McCutcheon *et al.,* 1995). Even with this limitation, 14 of the 20 fractions screened exhibited promising antiviral activity against measles virus. Five of these active fractions inhibited the production of virus induced cytopathic effects (CPE) by more than 75% at the non-cytotoxic concentrations tested and completely inhibited visible signs of CPE. The antiviral activity of the 14 active fractions could also be detected in the two subsequent dilutions beyond the non-cytotoxic concentration. Cos *et al*. (2002) have suggested that antiviral activity should be detectable in at least two subsequent dilutions of the test substance to ensure that the activity is not directly correlated with its toxicity or the activity is only virucidal.

According to Rajbhandari *et al.* (2007), the extracts that exhibited only medium activity could also be the source of potential antiviral drugs because the bioactive compounds may be present in too low concentrations to show effective antiviral activity at non-toxic concentration and further process may reveal potent antiviral activity. Therefore, seven fractions, namely FH4, FH5, FH6, FH8, FH10, FH11 and FH15, were selected for further purification using column chromatography and a total of 77 combined subfractions were obtained. Most of the subfractions tested conferred some degree of protection to the cells against measles virus infection and majority of them had lower antiviral activity compared to their derived fractions. These results are in line with previous studies by Hanina (2006) and Nurul Aini *et al*. (2006) who also demonstrated that the antiviral activity of *C. nardus* subfractions was lost or reduced as compared to the activity shown by the fractions from which they were derived. According to Cos *et al.* (2006), fractionation frequently leads to a reduction or loss of biological activity by compound break-down or loss of additive or synergistic effects between analogue constituents. Isolated

compounds also showed a lower antiviral activity compared to their derived subfractions. All 11 compounds possess weak to moderate antiviral activity against measles virus at each concentration evaluated compared to Ribavirin, with majority of them exhibited not more than 40% of viral inhibition. Among 11 isolated compounds tested, only two compounds showed to possess the major activity against virus infection.

The reference drug in this assay was Ribavirin, a broad-spectrum antiviral drug with inhibitory activity against many RNA viruses including measles virus (Hosoya *et al*., 2004). Ribavirin has the capability of inhibiting the virus replication by phosphorylating into ribavirin triphosphate causing a reduction of intracellular guanosine triphosphate (GTP) which is an important component in transcription, translation and replication of viruses. The absence of GTP will lead to incomplete capping of 5'-terminus of RNA, which resulted in the accumulation of mRNA in impaired protein synthesis, thus inhibited virus replication (Sidwell *et al*., 1985). In the early screening, the 50% cytotoxic concentration (CC_{50}) of Ribavirin on Vero cells was 160µg/ml. Therefore, Ribavirin at concentrations of 0.01 (1.6µg/ml), 0.1 (16µg/ml) and 1.0 (160µg/ml) CC_{50} were used as the positive control in all antiviral assays. At these concentrations, Ribavirin was able to inhibit virus induced CPE by more than 70% and 80%, respectively.

5.2.3 Mode of antiviral activity of fractions, subfractions and isolated compounds of *C. nardus*

The purpose of using several treatments in this study is to screen the possible phase of action of the antiviral compounds in the cell culture.Some studies have revealed compound that act at the

pre-infection stage by protecting the cell through a process akin to interferon induction from subsequent virus infection (Hudson *et al.*, 1999). This action impairing the ability of virus to adsorb to and penetrate into the host cells. Study by Yucharoen *et al*. (2011) showed that green monkey kidney cells were protected from HSV-2 infection by the dichloromethane extract of *O. americanum* L. and the methanol extract of *O. sanctum*, when the cells were treated before viral infection. Reichling *et al*. (2009) reported that the infectivity of HSV-1 on RC-37 cells was reduced by 50% when the cells were pretreated with the *Rhus aromatica* extract for one hour prior to virus infection.

Other than that, studies also showed that plant compound may interfere with any of the steps in virus uncoating, intracellular localization, replication, transcription, translation, processing and virion assembly, or secretion from the cell. Thus, the yield of infectious virus per cell or the number of plaque would be reduced. Study by Chiang *et al*. (2002) concludes that the mode of action of *Plantago major*pure compounds against HSV-2 and ADV-3 was found to be at post-infection stage, whereby the inhibition of ADV-3 occurred during 0-2 hours after infection while HSV-1 between 0-12 hours. Glatthaar-Saalmuller *et al*. (2001) demonstrate that the *Eleutherococcus senticosus* extract showed a strong antiviral activity in post-infection treatment by inhibiting the productive replication of human rhinovirus (HRV), respiratory syncytial virus (RSV) and influenza A virus in cell cultures infected with these viruses. Many research also revealed plant compound that act directly on the virus and this effect is called the virucidal effect. Virucidal effect resulted in the inactivation of the virion, either by disruption of the virion or by interfering with its ability to initiate a replication cycle. Study by del Barrio & Parra (2000) suggested that *Phyllanthus orbicularis* antiviral activity on bovine herpesvirus type 1 (BHV-1)

and HSV-2 could be due to an interaction with the viral membranes, since no antiviral activity was observed against adenovirus type 7 (Ad7) and mengovirus, the non-enveloped viruses, in the same experiment. Another study by Schnitzler *et al.* (2008b) showed that *Melissa officinalis* essential oil exerting a direct antiviral effect on HSV-1 and HSV-2 before adsorption of the virus into the RC-37 cell.

In order to understand on how the fractions, subfractions and isolated compounds inhibit the viral replication, the pre-treatment and post-infection effect of these compounds was investigated. For fractions, the dominant effect observed was post-infection, suggesting that the fractions may interfere with any of the steps in virus uncoating, intracellular localization, replication, transcription, translation, processing and virion assembly, or secretion from the cell, thus reduced the yield of infectious virus per cells. In the post-infection protocol, fractions were added to the cells which have been infected with measles virus. A common change in infected cells is an increased permeability in the cell membrane, which renders the cell more susceptible to ionic disturbances and possible leakage of vital molecules and macromolecules (Cos *et al.*, 2006). It has been suggested that this increased permeability could be exploited to increase the intracellular concentration of an antiviral or even to allow access to an antiviral which could not normally gain entry. This result is in contrast with those obtained by Nurul Aini *et al*. (2006) who showed thatthe fractions of *C. nardus* were more effective in protecting Vero cells against measles virus infection. The different performances can be linked to the different chemical compositions of the fractions as they use methanol, a very polar solvent, in the extraction process.

For the subfractions, results showed that certain subfractions exhibited the same mode of action as their derived fractions, meanwhile certain subfractions were not. This might be due to compound break-down during the fractionation process. According to Ooi *et al*.(2004), further purification of the active principles would clarify the chemical nature and mode of action of the bioactive components.Some of the subfractions were more effective in pre-treatment protocol, suggesting that subfractions inhibited virus replication by interfering with virus attachment to the cells (Roner *et al*., 2007). This could be explained either by a strong or maybe irreversible interaction between the subfraction and the cell membrane. Meanwhile, some subfractions showed better activity in post-infection protocol, suggesting that subfractions masking viral compounds which are necessary for adsorption or penetration into the host cells interrupt the viral replication process (Schnitzler *et al*., 2008b). This inhibition could also be a result of preventing re-infections with the newly produced viruses by the subfractions. Interestingly, some subfractions did not give any particular trend as to the most effective method of treatment, suggesting that different mechanisms or combinations of mechanisms participate in the antiviral activity (Parker *et al*., 2007).

Meanwhile, majority of the isolated compound provide better protection to the cells against measles virus infection in the pre-treatment protocol, suggesting that the potential sites of activity may include inhibition of virus binding and/or entry which can be mediated by a number of cellular receptors such as CD46 present on Vero cells (Parker *et al*., 2007). In this protocol, the isolated compounds were aspirated from the wells before adding virus and if some of the active compound remaining associated with the cells, it might still continue to function after adding the

virus. Interferon induction can be ruled out in this result because Vero cells are known to be incapable of producing interferon (Hudson *et al.*, 1999).

5.3 SYNERGISTIC ACTIVITY OF SELECTED ISOLATED COMPOUNDS OF *C. NARDUS* (L.) RENDLE AND RIBAVIRIN AGAINST MEASLES VIRUS

Ever since the earliest days of recorded history, drug combinations have been used for treating diseases and reducing suffering (Chou, 2006). The herbal medicines in traditional Chinese medicines are the vivid examples. Herbal practitioners have always believed that effective phytomedicines acquire their therapeutic efficacy via synergistic interactions between the components of individual or mixtures of herbs. The use of drug combinations is not confined to herbal products alone. Cancer chemotherapy, the treatment of HIV, neurological disorders and hypertension routinely employs drug combinations consisting of two or more substances. The combinatorial use of drugs aims to induce a response upon multiple targets, multiple subpopulations, or multiple diseases simultaneously (Harasstani *et al*., 2010). Furthermore, combinatorial use of multiple drugs with dissimilar mechanisms or modes of action may also direct the effect against a single target or disease with a more effective therapeutic outcome. The possible favourable outcomes for synergism include increased therapeutic efficacy, decreased dosage and toxicity while increasing or maintaining the same efficacy, and reduction of the development of drug resistance (Snoeck *et al*., 1992).

The results of this study demonstrate that the combination of *C. nardus* isolated compounds and Ribavirin were effective in inhibiting the production of virus induced CPE in

Vero cells. All isolated compounds at lowest concentration (0.01 LC_{50}) showed synergistic effect when combined with Ribavirin, either in one or both assay. Synergistic refers to the phenomenon where the combination of drugs generates an effect that is greater than the sum of the effects produced by each of the components alone (Pietschmann *et al*., 2009). The clear synergistic tendencies displayed by these two substances combination allows for the reduction of the Ribavirin concentration, which minimizes toxicity and the probability of formation of resistance to this drug. This synergistic activity is probably connected to the different mechanisms of action of Ribavirin and isolated compound. While Ribavirin inhibits viral RNA synthesis (Sidwell *et al*., 1985), the isolated compounds are thought to exert their inhibitory action at a very early stage in the viral infection cycle that is virus adsorption onto and/or penetration into the cell.

Effective combination therapy of Ribavirin with other substances also depending on the ability of those substances to phosphorylate Ribavirin into active forms which are, Ribavirin monophosphate, Ribavirin diphosphate and Ribavirin triphosphate (Jim *et al.,* 2005). According to this study, the active form of Ribavirinis able to interrupt at different stages of viral replication. Active form of Ribavirin will subsequently incorporate into viral genome and induce viral mutation. Frequent mutation of viral genomes would result in error catastrophe (Crotty *et al.,* 2002). Error catastrophe is a condition where RNA viruses already achieve the maximum number of mutation in their genome which resulted in losing their genetic information. In agreement with these findings, previous results in antiviral assay showed that *C. nardus* fractions tested exert their antiviral effect mainly by blocking virus access to the host cells. It seems probable that this effect is caused either by a strong interaction between the cells and the compounds, the effect being maintained after several serial dilutions. These results are in agreement with those obtained

by Heah (2007), Yip (2007) and Adibah (2008) who showed that the combination of fractions of *C. nardus* and Ribavirin was more effective than fractions alone or Ribavirin alone in treating measles infection. However, when higher concentrations of isolated compounds (0.1 and 1.0 LC_{50}) were used, only additive or antagonism interaction was produced. These results might be due to the toxicity that is produced when two substances were combined.

Several studies have been made to eradicate the problem related to toxicity effects of monotherapy drug through combination therapy. Betoni *et al.* (2006) showed that *C. citratus* exert highest synergistic effects against *S. aureus* when combined with antimicrobial drugs, such as tetracycline. Other than that, combination of Ribavirin and selanozofurin was effective in inhibiting measles virus even when the dosage of each drug is reduced (Kirsi *et al.*, 1985). Another study done by Grancher *et al.* (2004) claimed that 50% inhibitory concentration (IC_{50}) of Ribavirin against measles virus is improved after the combination of Ribavirin with cyclodextrins. Combination between Ribavirin with 6-mercaptho-9-tetrahydro-2-furylpurine produces synergistic effects against dengue virus. Furthermore, inhibitory effects of Ribavirin towards influenza virus are improved in combination with amantadine or rimantadine (Sidwell *et al.*, 1985). Similarly, a study carried out by Hayden (1999) revealed that the combination of Ribavirin with rimantadine or amantadine improved antiviral activity against human influenza virus. The antiviral activity of Ribavirin in combination with β-cyclodextrin against measles was tested *in vivo* (Jeulin *et al.*, 2007). They found that viral load in the brain of the tested animal decreases compared to Ribavirin use in monotherapy. Thus, combination therapy is an effective alternative ways to treat viral infection as well as to reduce the toxicity level of each drug when

used as monotherapy. Combination therapy also helps in reducing resistance of patients towards antiviral drugs treatment (Witlink, 1992).

5.4 ANTI-PROLIFERATION ACTIVITY OF SELECTED ISOLATED COMPOUNDS OF *C. NARDUS* (L.) RENDLE

Plants have a reputable history of use in the treatment of cancer. In a review by Hartwell in 1982, more than 3000 plant species are listed that have reportedly been used in the treatment of cancer (Kamatou *et al*., 2008). Over 60 percent of currently used anticancer agents are derived in one way or another from natural sources including plants. Several potential lead molecules such as camptothecin, vincristine, vinblastine, taxol, podophyllotoxin, and combretastatins have been isolated from plants and are in clinical use all over the world (Srivastava *et al*., 2005). A number of promising agents such as flavopiridol, roscovitine, combretastatin A-4, betulinic acid and silvestrol are in clinical or preclinical development (Shoeb, 2006). Many studies have been carried out on the anticancer activity of various plant species with some encouraging results. Study by Costa-Lotufo *et al*. (2005) showed that the extract of *Oroxylum indicum* exhibited high toxicity on B-16 (murine melanoma), HCT-8 (human colon carcinoma), CEM and HL-60 (leukemia) tumor cell lines, with an IC_{50} of 19.6µg/ml for CEM, 14.2µg/ml for HL-60, 17.2µg/ml for B-16 and 32.5µg/ml for HCT-8. Another study by Ju *et al*. (2004) showed that the treatment with *Betula platyphylla* var. *japonica* extract induced cytotoxicity and apoptosis in HL-60 cells, and gradually increased the expression of pro-apoptotic Bax and led to the activation of caspase-3 and cleavage of PARP. Meanwhile, Kheng *et al*. (2010) showed that the chloroform extract of *Physalis minima* exerted anticancer effect due to a combination of apoptotic and autophagic cell

death mechanisms on Caov-3 cells. The induction of these programmed cell deaths was mediated via c-myc, p53 and caspase-3 dependent pathway.

In this study, ten isolated compounds of *C. nardus* were additionally tested against human papillary ovarian adenocarcinoma (Caov-3) cancer cell. Although there is no evidence that *C. nardus* has been used as anticancer agent, several medicinal plants belonging to this genus are found to have antitumor activity, for example *C. citratus* D.C.(Manosroi *et al*., 2006). Study by Sharma *et al*. (2009) indicate that the essential oil from *C. flexuosus* has a promising anticancer activity against twelve human cancer cell lines and causes loss in tumor cell viability by activating the apoptotic process. The result of the present study is the first report of anticancer activity of *C. nardus*. This study revealed that all ten isolated compounds from *C. nardus* presented some anticancer activity in the MTT assay, indicating the presence of cytotoxic substances. When Caov-3 cell was treated for three days with 0, 25, 50, 100, 200 and 400 µg/ml of the compounds, the relative cell survival progressively decreased in a concentration-dependent manner. However, none of the compounds had better than weak activity towards Caov-3 cells as they inhibited less than 50 percent of Caov-3 cell growth. According to Parekh & Chanda, (2007), negative results do not mean absence of bioactive constituents nor is that the plant inactive. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed (Taylor *et al*., 2001).Lack of activity can thus only be proven by using large doses (Farnsworth, 1993). Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents (Jager et al., 1996).

On the other hand, anticancer drugs are expected to have effect against the cancer cells without producing cytotoxicity in normal cells. Therefore, as the LC_{50} values of the compounds varied in the range of 150 to 270 µg/ml in normal cells (Vero cells), the cytotoxicity of the compounds should be lower than those of references in Caov-3 cells. In this study, isolated compounds were found to be toxic on normal cells. This is because in order to obtain 50 percent Caov-3 cell death (LC_{50}), a concentration of above 400 µg/ml of isolated compounds was required. At this concentration, over 50 percent of normal cell died when treated with these compounds as their LC_{50} values on this cell ranged from 150 to 270 µg/ml. This cytotoxic value is also much higher than that of the reference drug, Tamoxifen (LC_{50}=150 µg/ml). According to Sharma *et al*. (2009), a successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. In addition, the American National Cancer Institute stated that the cytotoxic limit to consider a crude extract promising for further purification is lower than 30µg/ml (Costa-Lotufo *et al*.,2005; Kheng *et al*., 2010). Therefore, these results suggest that none of these compounds could be considered as potential sources of anticancer compounds.

5.5 DETERMINATION OF ACTIVE COMPOUNDS OF *C. NARDUS* (L.) RENDLE USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

After the biological evaluation has been performed and the separation of the natural product has been achieved, the final goal is to determine the structure and composition of the compound (Hites, 1997). In the present study, the main structure-determining technique used was GC-MS. GC-MS analysis has been proved to be of great utility in the analysis of compound (Yusuf

&Bewaji, 2011). This technique can accurately analyse small sample (less than 5 mg) and is particularly suited to natural compound that is often present in small quantity in plants (Hanina, 2006).GC-MS comprising a gas chromatography (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas, such as helium. As the separated substances emerge from the column opening, they flow into the MS. MS identifies compounds by the mass of the analyte molecule. A library of known mass spectra, covering several thousand compounds, is stored on a computer.

The GC-MS analysis showed that the active compounds of *C. nardus* are made up of monoterpene, sesquiterpene and hydrocarbon. The result presented here are in agreement with those from Hanina (2006) who reported that the majority compositions in *C. nardus* are monoterpene and sesquiterpene. Terpenes are secondary metabolites, represented by hemiterpenes, monoterpenes, sesquiterpenes and their terpenoid derivatives (Echeverrigaray *et al*., 2008). Terpenes are widespread in nature, and are the most important component of the essential oil of many higher plants (Tozoni *et al*., 2010). These compounds are built up by isoprene units in cyclic or acyclic form and can be functionalised by carbonyl, hydroxyl or carboxyl groups and by presence of additional carbon-carbon double bonds (terpenoids) (Förster-Fromme & Jendrossek, 2010). Terpenes are important materials for a plant as components of its body and as a material to protect it from external enemies (Paduch *et al*., 2007).Terpenes in plants are also believed to play ecological roles mainly, serving as herbivore-feeding deterrents, antifungal defenses and attractants for pollinators (Adinee *et al*., 2008).

The GC-MS analysis also revealed that only FH4.01, FH4.04.4, FH6.01 and FH7.03 were pure as only one compound presence in these subfractions. The GC-MS analysis of FH4.01 showed the present of 17-(1,5-Dimethyl-3-phenylthiohex-4-enyl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopent(*α*)phenanthren-3-ol. There is just a little information provided about this compound, but it is believed to be one of several phytosterols (plant sterols) with chemical structures similar to that of cholesterol, just like β-Sitosterols (17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol). This compound has the same criteria like sitosterols which are white, waxy powders with a characteristic odour.

Meanwhile, GC-MS analysis of FH6.10.1 indicated that it contain 1-Tridecyn-4-ol, an alkynes compound. Alkynes are hydrocarbons that have a triple bond between two carbon atoms and they are not as common in nature as alkenes, but some plants do use alkynes to protect themselves against disease or predators. Study by Pokharkar *et al*. (2011)showed that the oil extract of *Abrus precatorius*, a medicinal plant in India that used to treat many illnesses such as snake bite, ringworm, mouth ulcers, throat sore and anthrax, contains 1-Tridecyn-4-ol. NTP (2008) also showed that extract of Dong quai (*Angelica sinensis*) which has been used in traditional Chinese, Korean and Japanese medicine to treat a variety of ailments including lumbago, hypertonia, nervous disorders, menopausal symptoms, neuralgia, insomnia, and arthritis, also contain 1-Tridecyn-4-ol. GC-MS analysis of FH4.04.4 revealed the present of methyl eugenol, a phenylpropene compound that present in many essential oils. According to Hanina (2006), methyl eugenol is one of the predominant compounds in *C. nardus*. GC-MS analysis of FH7.03 showed it contains citronellol and geraniol, the common monoterpenes

alcohol compounds that present in essential oil of *C. nardus*(Herath *et al.,* 1979; Shasany *et al.,* 2008).

On the other hand, the GC-MS analysis of FH6.06.2, FH6.06.3, FH6.10.2 and FH11.04.2 showed that the subfractions were not pure enough as more than one compound was detected. Certain of the compounds such as 1-Nonadecene, 1-Heptacosanol, Eicosyl trifluoroacetate, Docosyl heptafluorobutyrate and Heptacosyl heptafluorobutyrate were common in the subfractions. Other compounds that were present in these subfractions were α-Cadinol, Cyclohexanemethanol, 2-Naphthalenemethanol, (+)-Epi-bicyclosesquiphellandrene, 1-Eicosene and Nonadecyl pentafluoropropionate. Most of these compounds are present naturally in many essential oils. For example, 1-Nanodecane was identified from essential oil from *Gongronema latifolium*, a tropical rain forest plant in Nigeria which primarily used as spice and vegetable in the traditional folk medicine (Adeleye *et al*., 2011). Meanwhile, α-Cadinol, an aromatic sesquiterpene, present in a number of plants including yarrow, jasmine, many species of juniper and some species of St. Johns Wort (Anonymous, 2011). Study by Chang *et al*. (2001) showed the present of α-Cadinol in the *Taiwania cryptomerioides* heartwood essential oil and it possessed the strongest antimite activity compared with other components of the plant.

5.6 DETERMINED COMPOUNDS AND ANTIVIRAL ACTIVITY: CONNECTION

Nowadays rapid development is continuously to happen in the field of chemistry of medicinal research. Despite this rapid development, many plant derived drugs are still cannot be synthetically produced. There are two main reason stands behind the statements. Some

compounds such as atropine and reserpine are still too expensive to be synthesized, and many useful drugs also still cannot be synthesized such as morphine, cocaine, ergotamine and digitalis (Ernawita, 2008). Thus, the isolation of plant derived drugs still holds important rules in drug discovery. Once plant derived drugs has been isolated, it can act as the lead compound which is a good starting point in developing new drug. It can allow the design and rational planning of the new drugs as well as biomimetic synthesis development and discovery of new biological activity not yet related to the known compounds (Archana *et al*., 2011). One example is salicylic acid that originally synthesized to find replacement for phenol as antiseptic. Further finding then reported the antypiretic and antirheumatic activities.

In the present study, *C. nardus* exhibited antiviral activity against measles virus. Solvent extraction, fractionation and bioassay led to the isolation of the group of compounds showing antiviral activity, which was characterise by GC-MS. There is no prevailing compound identified in the whole plant hexane extract of *C. nardus* as all the compounds was reported in other study. Most of the identified compounds were terpenes, a huge group of natural compounds characterised by their predominantly pleasant smell. A broad range of the biological properties of terpenes is described, including cancer chemopreventive effects, antihyperglycemic, anti-inflammatory (Paduch *et al*., 2007), antimicrobial, antifungal, antiviral and antiparasitic activities (Echeverrigaray *et al*., 2008). The action of terpenes is not fully understood but is speculated to involve membrane modifications resulting in alterations of membrane permeability and in leakage of intracellular materials (Echeverrigaray *et al*., 2008). According to Paduch *et al*. (2007), terpenes are presented as skin penetration enhancers and agents involved in the prevention and therapy of several inflammatory diseases. The results showed that majority of the

isolated compounds affected measles virus before or during absorption into the cells. Therefore, these compounds might be suitable as therapeutic agent as they could penetrate into the skin and protect the cells by preventing cell-to-cell virus spread in infected cells.

Citronellal and geraniol, the common monoterpene alcohol in essential oil of *C. nardus*, have been found to exhibit antiviral activity against measles virus. Citronellol is among the main constituent of the essential oils of several plants, like citrus, roses, basil and lemon eucalyptus, being responsible for the aromatic and biological properties, including antibacterial activity, of these oils (Tozoni *et al*., 2010). Geraniol is also found in many essential oils and it possesses lipophilic characteristic, whereby it is thought to be absorbed in tissue (Shoji *et al*., 1998). The individual component citronellol and geraniol have been reported to possess antibacterial, antiviral and antispasmodic effects. *Melissa officinalis* L. essential oil can inhibit the replication of HSV-2 due to the presence of citral and citronellal (Allahverdiyev *et al*., 2004). Geraniol elicited a dramatic reduction in the amounts of thymidylate synthase (TS) and thymidine kinase (TK) expression in colon cancer cells (Edris, 2007). Meanwhile, methyl eugenol is a monoterpenoid alcohol which naturally present in many essential oil and fruits. It is used as a fragrance in cosmetics and as a flavor additive in the food industry (Sudhakar *et al*., 2009), which is on the Food Manufacturers Generally Regarded as Safe (GRAS) list (Brennan *et al*., 1996). Methyl eugenol has also been used in agriculture as an insect attractant in eradication programs and as an anesthetic in rodents (NTP, 2000). Unfortunately, no literature was found concerning the antiviral applications of methyl eugenol against viruses, but the promising results illustrated here may promote further investigations in this area.

CHAPTER 6: CONCLUSION

In conclusion, the results of this study were encouraging, although clinical controlled studies are needed to define the real efficacy and possible toxic effects *in vivo*. This study suggests that secondary metabolites of *C. nardus* exhibited potent antiviral activity against measles virus (MV) *in vitro* and displayed synergistic tendencies when combined with Ribavirin, the presently clinically used drug for measles.GC-MS analysis showed that the active compounds of *C. nardus* are made up of monoterpene, sesquiterpene and hydrocarbon. Among them, the monoterpene alcohol compounds, citronellol and geraniol, were found to possess the strongest antiviral activity. Due to the lack of approved drugs in treating MV infection, citronellol and geraniol might be a potential alternative medicine for treating measles. As indicated by the low cytotoxic values (200 to 300 μg/ml), these two compounds are considered to be less toxic than Ribavirin (160μg/ml). Therefore, the potential of citronellol and geraniol for use in treating MV infection merit a greater attention.

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Appendix A

1) Preparation of Dulbecco's modified Eagle medium (DMEM)

1. 900ml of sterile distilled water (dH_2O) is measured using graduated cylinder and put into sterile bottle.
2. DMEM powder is added to dH_2O with gentle stirring. Remaining powder inside the package is rinsed and added into the bottle.
3. 3.7g of natrium bikarbonat ($NaHCO_3$) is added into the medium.
4. Another 100ml of dH_2O is added and the medium is stirred until all powders are dissolved.
5. The pH of the medium is measured and adjusted to pH 7.0 using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl).
6. The medium is then sterilized using 0.22µm membrane filter and stored at 4 ºC.

2) Preparation of phosphate buffered saline (PBS)

Chemicals:NaCl (8.00g),$K_2HPO_4.3H_2O$ (1.34g),KH_2PO_4 (0.34g)

1. All the chemicals above are dissolved in 1 litre of distilled water (dH_2O).
2. The pH is measured and adjusted to pH 7.2 using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl).
3. The solution is sterilised at 121^{o}C for 15 minutes.

Appendix B

Solvent system for column chromatography (CC) and thin layer chromatography (TLC)

Fractions	Combined fractions	Solvent for CC	Solvent for TLC
1-9	FH1	Hexane	Hexane: DCM
10-24	FH2	Hexane: DCM (9:1)	(4 :1)
		Hexane: DCM (3:1)	
25-27	FH3	Hexane: DCM (1:1)	
28-37	FH4	Hexane: DCM (1:1)	
		Hexane: DCM (1:3)	
38-40	FH5	Hexane: DCM (1:3)	Hexane: Chloroform: DCM
41-48	FH6	Hexane: DCM (1:9)	(2 : 1: 0.5)
49-72	FH7	DCM	Hexane: Chloroform: DCM
		DCM: Chloroform (9:1)	(3 : 2 : 0.5)
		DCM: Chloroform (3:1)	
73-96	FH8	DCM: Chloroform (1:1)	Hexane: Ethyl Acetate: DCM
		DCM: Chloroform (1:3)	(4 : 1 : 0.5)
		DCM: Chloroform (1:9)	
		DCM: Chloroform (1:9)	
97-115	FH9	Chloroform	DCM: Chloroform: Ethyl Acetate
		Chloroform: EtoAc (9:1)	(3 : 2 : 0.5)
		Chloroform: EtoAc (3:1)	
116-122	FH10	Chloroform: EtoAc (3:1)	DCM: Chloroform: Ethyl Acetate
		Chloroform: EtoAc (1:1)	(3 : 2 : 0.5)

			Chloroform: Ethyl Acetate (4 : 1)
123-124	FH11	Chloroform: EtoAc (1:1)	Chloroform: Ethyl Acetate
125-128	FH12	Chloroform: EtoAc (1:1)	(4 : 1)
129-139	FH13	Chloroform: EtoAc (1:3)	Chloroform: MeOH
		Chloroform: EtoAc (1:9)	(7 : 1)
140-147	FH14	Chloroform: EtoAc (1:9)	
		EtoAc	
148-152	FH15	EtoAc	
153-170	FH16	EtoAc: MeOH (9:1)	Chloroform: MeOH
		EtoAc: MeOH (3:1)	(11 : 1)
		EtoAc: MeOH (1:1)	
171-177	FH17	EtoAc: MeOH (1:1)	Chloroform: MeOH
		EtoAc: MeOH (1:3)	(11 : 1)
			Chloroform: MeOH
			(10 : 1)
178-180	FH18	EtoAc: MeOH (1:3)	Chloroform: MeOH
181-192	FH19	EtoAc: MeOH (1:3)	(10 : 1)
		EtoAc: MeOH (1:9)	
193-200	FH20	MeOH	Chloroform: MeOH
			(7 : 1)

Appendix C

Procedures in cells culture

1) Cells counting using haemacytometer

1. 100µl of cells suspension is mixed with 200µl of trypan blue dye
2. 50µl of the mixture is put onto the haemacytometer slide using micropipette.
3. After 2 minutes (to allow the cells to sediment), counting chamber containing stained cells is observed under microscope using 10x objective lens. Power of objective lens is increased when the counting area identified.
4. Cells are counted from right to left.
5. Characteristic of cells which are followed to obtained accurate counting include :
    i. Single cell was counted as one
    ii. For a group of cells, the number of cells counted was the one which display clear nucleus and cytoplasm
    iii. If the group of cells are not visibly different, the cells are considered as one
6. Cells suspension inside the original flask is counted and diluted to obtain the desired cells concentration.

2) The subculture procedure

1. Medium in flask is decanted.
2. The cells are washed with PBS thoroughly for about 5 seconds. Several washes are required for bigger flask (75 cm^2).
3. The cells are disaggregated using trypsin. For 25 cm^2 flask, 1 drop (100 µL) of trypsin is added while for 75 cm^2 flask, 300 µL of trypsin is added. More trypsin can be added if it is not enough but should not exceed 7 drops. The trypsin is spread evenly and the flask is knocked on the palm a few times to improve cell detachment.

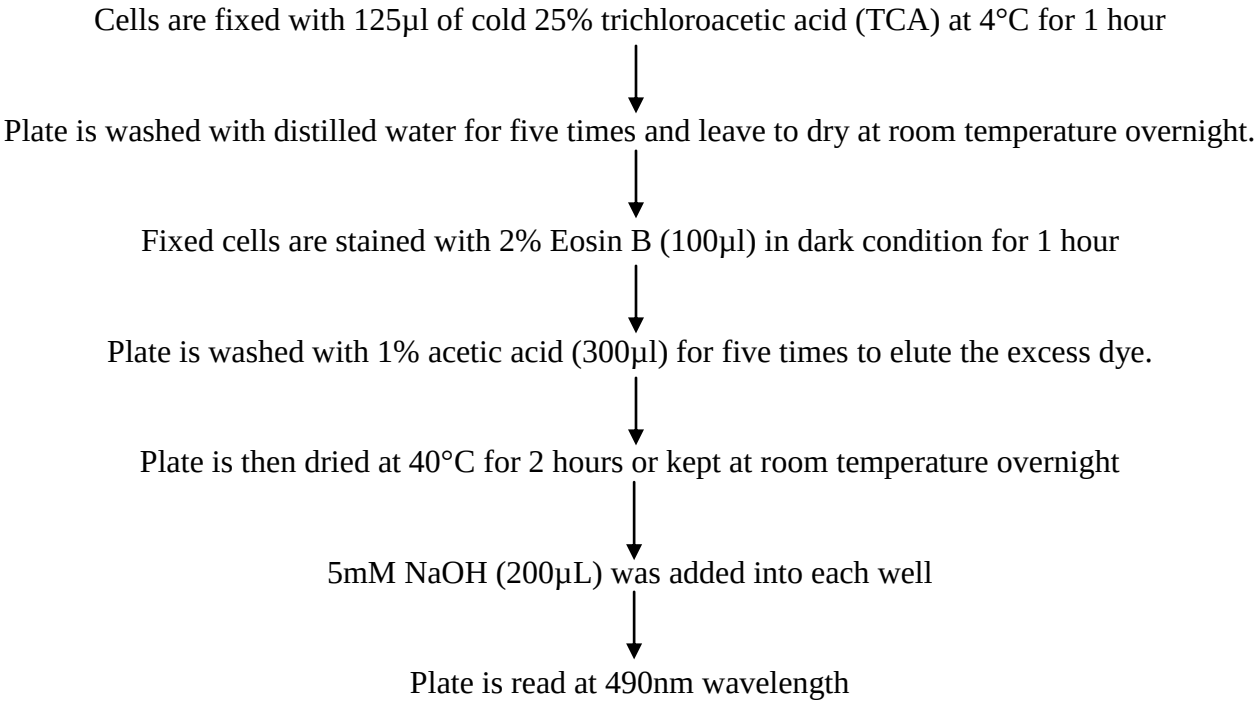
4. 2-3 drops of medium is added to stop the trypsin activity and within 5 minutes, the cells are checked under inverted microscope.
5. Medium is added into the flask up to 2ml.
6. The medium containing detached cells are segregated into several flasks.
7. Growth medium (DMEM with 10% FBS) is added into each flask.
8. The flask cap is loosen 1/3 for CO_2 aeration before proceed to incubation in CO_2 incubator.
9. Aseptic techniques are applied throughout the procedure.

3) Vero cells preservation

1. After cells disaggregation, freezing medium is added into the flask. Freezing medium contains 70% DMEM, 20% FBS and 10% DMSO.
2. Cells suspension is then transferred into sterile cryo vials.
3. The vials are sealed with parafilm and put into Cryo 1°C freezing container before store it in -80ºC freezer for 24 hours.
4. After that, the vials are moved into cryo vial box and store in -80ºC freezer.
5. Aseptic techniques are applied throughout the procedure.

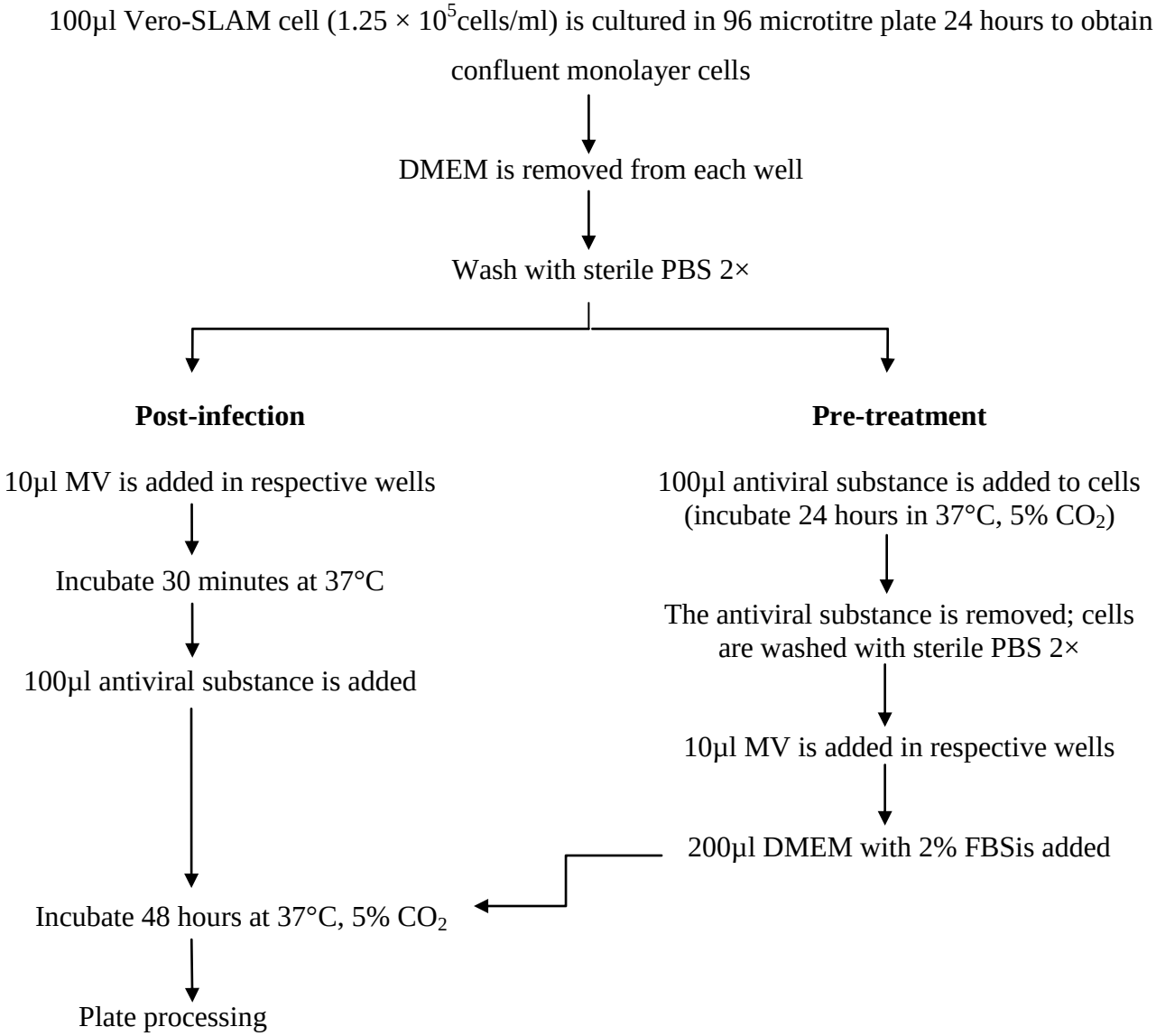
Appendix D

Cells staining procedure



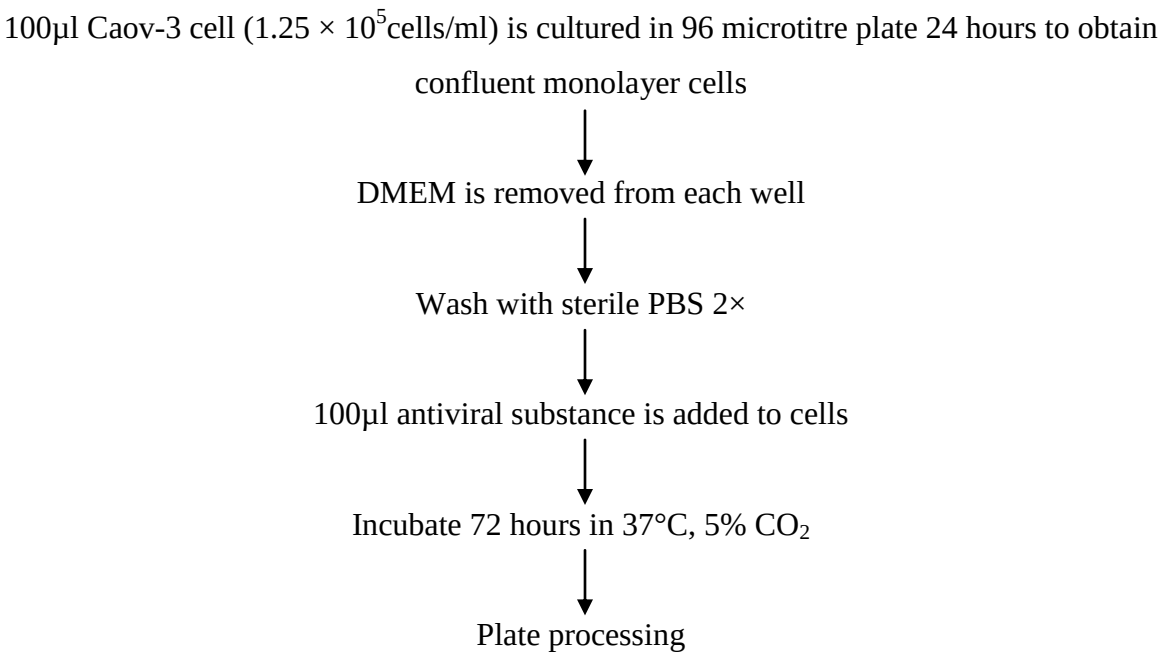
Appendix E

Antiviral assay



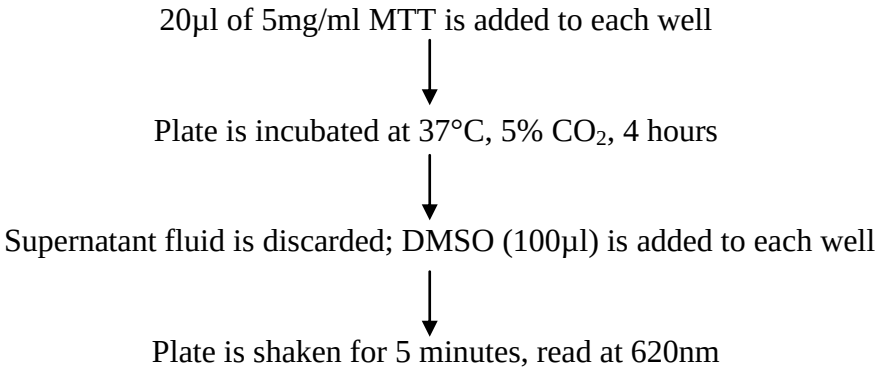
Appendix F

Anti-proliferative assay

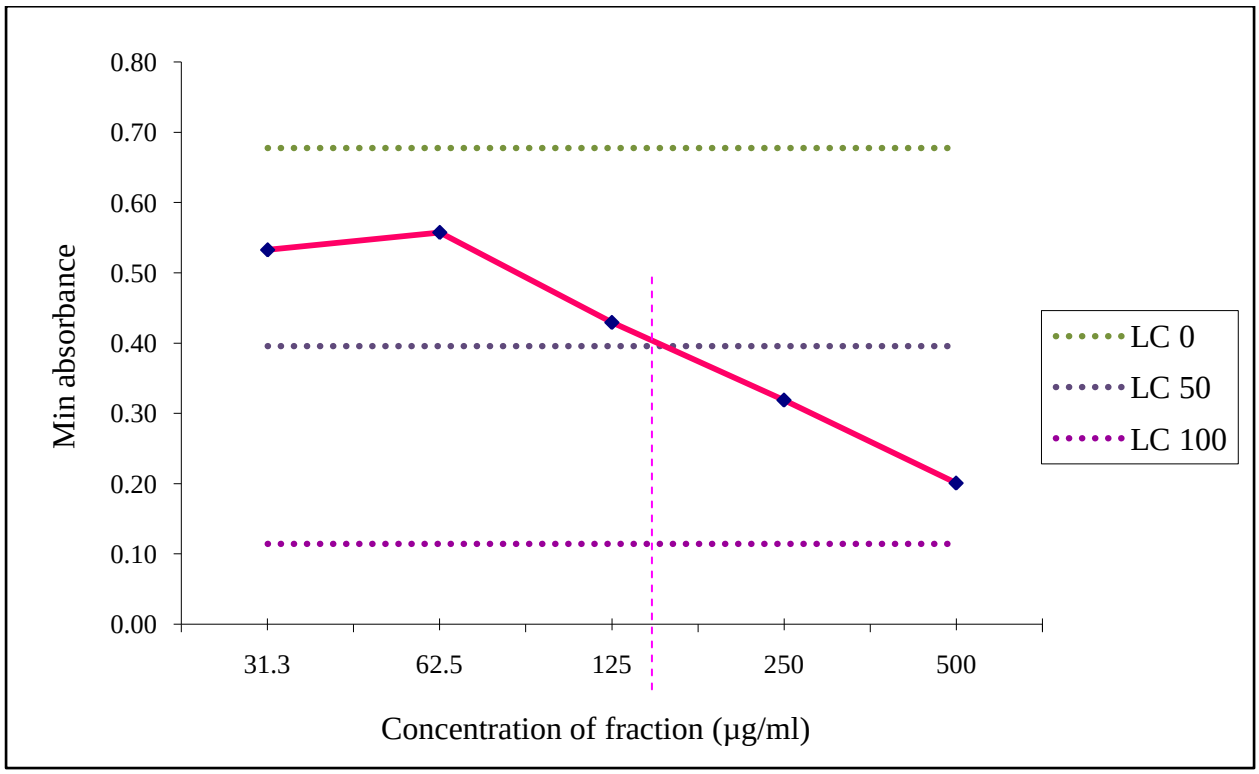


Appendix G

Cells staining procedure for antiproliferative assay



Appendix H



The LC_{50} value is obtained from plotted graph of min absorbance value against fraction concentration (µg/ml). The LC_{50} value is taken as intermediate value between the positive control, LC_0 (no cell death) and the negative control, LC_{100} (all cell death). Based on this graph, the LC_{50} value for fraction is 180 µg/ml.

Appendix I

1) Statistical analysis of CC-I fractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	6.773				
P value	0.0072				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.5094				
R square	0.2734				
Was the matching effective?					
F	21.47				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.8387				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.3317	3	0.1106	F (1.528, 27.51) = 6.773	P = 0.0072
Individual (between rows)	6.309	18	0.3505	F (18, 54) = 21.47	P < 0.0001
Residual (random)	0.8815	54	0.01632		
Total	7.523	75			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	19				

Student's *t*-test

Table Analyzed	Repeated measures one-way ANOVA data
Column E	Ribavirin 0.1
vs.	vs.
Column A	1 LC_{50}
Paired t test	
P value	0.0006
P value summary	***
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.133 df=18
Number of pairs	19
How big is the difference?	
Mean of differences	0.2181
SD of differences	0.2300
SEM of differences	0.05277
95% confidence interval	0.1072 to 0.3290
R square	0.4869
How effective was the pairing?	
Correlation coefficient (r)	0.8240
P value (one tailed)	< 0.0001
P value summary	****
Significant correlation? (P > 0.05)	No

2) Statistical analysis of CC-I fractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	11.87				
P value	0.0003				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.5582				
R square	0.3974				
Was the matching effective?					
F	2.599				
P value	0.0035				
P value summary	**				
Is there significant matching (P < 0.05)?	Yes				
R square	0.3430				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.5826	3	0.1942	F (1.675, 30.14) = 11.87	P = 0.0003
Individual (between rows)	0.7653	18	0.04252	F (18, 54) = 2.599	P = 0.0035
Residual (random)	0.8833	54	0.01636		
Total	2.231	75			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	19				

Student's *t*-test

Table Analyzed	Repeated measures one-way ANOVA data
Column J	Ribavirin 0.1
vs.	vs.
Column F	1 LC_{50}
Paired t test	
P value	0.0012
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.830 df=18
Number of pairs	19
How big is the difference?	
Mean of differences	0.1725
SD of differences	0.1964
SEM of differences	0.04505
95% confidence interval	0.07788 to 0.2672
R square	0.4490
How effective was the pairing?	
Correlation coefficient (r)	0.4473
P value (one tailed)	0.0274
P value summary	*
Significant correlation? (P > 0.05)	No

Appendix J

1) Statistical analyses of FH4 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	4.483				
P value	0.0503				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4222				
R square	0.3591				
Was the matching effective?					
F	21.47				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.8387				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.3317	3	0.1106	F (1.528, 27.51) = 6.773	P = 0.0072
Individual (between rows)	6.309	18	0.3505	F (18, 54) = 21.47	P < 0.0001
Residual (random)	0.8815	54	0.01632		
Total	7.523	75			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	19				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH 4.01	*	0.000951255	1.352	0.976	0.376	0.0739926	5.08159	8.0
FH4.02		0.314527	1.118	0.976	0.142	0.132327	1.0731	8.0
FH4.04		0.0780617	0.7788	0.976	-0.1972	0.097625	2.01997	8.0
FH4.06	*	0.00280395	0.6586	0.976	-0.3174	0.0747045	4.24874	8.0

2)Statistical analyses ANOVA of FH4 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	13.75				
P value	0.0014				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4674				
R square	0.6045				
Was the matching effective?					
F	3.560				
P value	0.0050				
P value summary	**				
Is there significant matching (P < 0.05)?	Yes				
R square	0.3194				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.791	3	0.5970	F (1.402, 12.62) = 13.75	P = 0.0014
Individual (between rows)	1.391	9	0.1545	F (9, 27) = 3.560	P = 0.0050
Residual (random)	1.172	27	0.04340		
Total	4.353	39			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	10				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH4.01		0.171387	0.884	0.963	-0.079	0.0525814	1.50243	8.0
FH4.02		0.106248	0.7888	0.8664	-0.0776	0.0426368	1.82002	8.0
FH4.04		0.606681	1.4404	1.4888	-0.0484	0.0903355	0.535781	8.0
FH4.05		0.0948338	1.559	1.7376	-0.1786	0.0942956	1.89404	8.0

Appendix K

1) Statistical analysis of FH5 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	88.70				
P value	<0.0001				
P value summary	****				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.6013				
R square	0.9173				
Was the matching effective?					
F	42.32				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.5385				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2487	3	0.0829	F (1.804, 14.43) = 6.773	P < 0.0001
Individual (between rows)	0.3164	8	0.0395	F (8, 24) = 42.32	P < 0.0001
Residual (random)	0.0224	24	0.0009		
Total	0.5876	35			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	9				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH5.04		0.781767	1.18825	1.2114	-0.02315	0.0804121	0.287892	7.0
FH5.07		0.939417	1.0714	1.0775	-0.00610	0.0774368	0.0787742	7.0
FH5.08		0.525946	0.9818	1.0444	-0.0626	0.094414	0.663038	8.0
FH5.09		0.851713	1.0278	1.0444	-0.0166	0.0859773	0.193074	8.0
FH5.10		0.12501	0.9506	1.0506	-0.1	0.0583671	1.71329	8.0

2)Statistical analyses ANOVA of FH5 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	26.01				
P value	<0.0001				
P value summary	****				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.5903				
R square	0.7648				
Was the matching effective?					
F	120.4				
P value	<0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.9042				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.1254	3	0.04181	F (1.771, 14.17) = 26.01	P < 0.0001
Individual (between rows)	1.548	8	0.1935	F (8, 24) = 120.4	P < 0.0001
Residual (random)	0.03858	24	0.001607		
Total	1.712	35			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	9				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH5.04		0.0866952	1.13825	1.2574	-0.11915	0.0598283	1.99153	7.0
FH5.06		0.0243309	0.6562	0.7292	-0.073	0.0263625	2.76909	8.0
FH5.07		0.257158	0.684	0.7292	-0.0452	0.0370451	1.22013	8.0

Appendix L

1) Statistical analysis of FH6 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	5.429				
P value	0.0095				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.7596				
R square	0.3519				
Was the matching effective?					
F	13.53				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.7451				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.0656	3	0.0219	F (2.279, 22.79) = 5.429	P = 0.0095
Individual (between rows)	0.5451	10	0.0545	F (10, 30) = 13.53	P < 0.0001
Residual (random)	0.1209	30	0.0040		
Total	0.7317	43			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	11				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.03		0.463505	0.6655	0.728	-0.0625	0.0806066	0.775371	7.0
FH6.06		0.653215	0.90925	0.9464	-0.03715	0.0791836	0.469163	7.0
FH6.10		0.814435	0.9208	0.9422	-0.0214	0.0882189	0.242579	8.0

2) Statistical analysis ANOVA of FH6 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	3.885				
P value	0.0420				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.6147				
R square	0.2798				
Was the matching effective?					
F	2.015				
P value	0.0676				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.3260				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.7419	3	0.2473	F (1.844, 18.44) = 3.885	P = 0.0420
Individual (between rows)	1.283	10	0.1283	F (10, 30) = 2.015	P = 0.0676
Residual (random)	1.910	30	0.06366		
Total	3.934	43			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	11				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.10		0.96358	1.56	1.566	-0.00600001	0.12605	0.0476002	6.0
FH6.11		0.393153	1.575	1.605	-0.03	0.032118	0.934055	5.0

Appendix M

1) Statistical analysis of FH8 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	1.812				
P value	0.1898				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.6242				
R square	0.1414				
Was the matching effective?					
F	2.783				
P value	0.0113				
P value summary	*				
Is there significant matching (P < 0.05)?	Yes				
R square	0.4434				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.1259	3	0.04198	F (1.873, 20.60) = 1.812	P = 0.1898
Individual (between rows)	0.7095	11	0.06450	F (11, 33) = 2.783	P = 0.0113
Residual (random)	0.7647	33	0.02317		
Total	1.600	47			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	12				

2) Statistical analysis ANOVA of FH8 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	6.106				
P value	0.0170				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4770				
R square	0.3569				
Was the matching effective?					
F	5.276				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.5307				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2728	3	0.09094	F (1.431, 15.74) = 6.106	P = 0.0170
Individual (between rows)	0.8645	11	0.07859	F (11, 33) = 5.276	P < 0.0001
Residual (random)	0.4915	33	0.01489		
Total	1.629	47			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	12				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH8.05		0.0780617	0.7788	0.976	-0.1972	0.097625	2.01997	8.0
FH8.06		0.411015	1.123	1.22467	-0.101667	0.110865	0.917028	4.0
FH8.08		0.504874	1.12375	1.22467	-0.100917	0.140545	0.71804	5.0
FH8.09		0.9621	0.726	0.72175	0.00424999	0.0857957	0.0495362	6.0

Appendix N

1) Statistical analysis of FH10 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	32.74				
P value	0.0003				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.5062				
R square	0.8675				
Was the matching effective?					
F	6.730				
P value	0.0018				
P value summary	**				
Is there significant matching (P < 0.05)?	Yes				
R square	0.2291				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.3019	3	0.1006	F (1.518, 7.592) = 32.74	P = 0.0003
Individual (between rows)	0.1034	5	0.02069	F (5, 15) = 6.730	P = 0.0018
Residual (random)	0.04611	15	0.003074		
Total	0.4515	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH10.01		0.722361	1.0165	0.9938	0.0227	0.0613602	0.369947	7.0
FH10.02		0.246639	0.91	0.9938	-0.0838	0.0652876	1.28355	6.0
FH10.03		0.805992	0.9835	0.9984	-0.0149	0.0584127	0.255081	7.0
FH10.04		0.725786	0.97375	0.9984	-0.02465	0.0675071	0.365146	7.0

2) Statistical analysis ANOVA of FH10 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	115.0				
P value	0.0001				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.3425				
R square	0.9583				
Was the matching effective?					
F	1.083				
P value	0.4089				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.0148				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2927	3	0.09757	F (1.028, 5.138) = 115.0	P = 0.0001
Individual (between rows)	0.004596	5	0.0009192	F (5, 15) = 1.083	P = 0.4089
Residual (random)	0.01273	15	0.0008487		
Total	0.3100	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH10.01		0.645255	0.8348	0.8602	-0.0254	0.0531082	0.478269	8.0
FH10.04		0.659405	0.830333	0.8642	-0.0338667	0.0730817	0.463408	6.0

Appendix O

1) Statistical analysis of FH11 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	4.758				
P value	0.0391				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.6250				
R square	0.4876				
Was the matching effective?					
F	1.507				
P value	0.2461				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.2047				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.06555	3	0.02185	F (1.875, 9.376) = 4.758	P = 0.0391
Individual (between rows)	0.03460	5	0.006920	F (5, 15) = 1.507	P = 0.2461
Residual (random)	0.06889	15	0.004592		
Total	0.1690	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH11.04		0.351503	0.8926	0.828	0.0646	0.0653005	0.989272	8.0
FH11.08		0.42167	0.8806	0.828	0.0526	0.0621101	0.846883	8.0
FH11.09		0.991978	0.8276	0.828	-0.0003	0.0385637	0.0103723	8.0

2) Statistical analysis ANOVA of FH11 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	7.718				
P value	0.0289				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4017				
R square	0.6069				
Was the matching effective?					
F	0.6163				
P value	0.6894				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.0747				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.364	3	0.4546	F (1.205, 6.025) = 7.718	P = 0.0289
Individual (between rows)	0.1815	5	0.03630	F (5, 15) = 0.6163	P = 0.6894
Residual (random)	0.8835	15	0.05890		
Total	2.429	23			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	6				

Multiple *t*-tests: Subfractions & 0.01 CC_{50} Ribavirin

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH11.04		0.851713	1.0278	1.0444	-0.0166	0.0859773	0.193074	8.0
FH11.07		0.0203984	1.247	1.3665	-0.1195	0.0320868	3.72427	4.0

Multiple *t*-tests: Subfraction & 0.1 CC_{50} Ribavirin

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH11.08		0.0306977	1.20625	1.44675	-0.2405	0.0855431	2.81145	6.0

Appendix P

1) Statistical analysis of FH15 subfractions for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	0.7521				
P value	0.4574				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.3642				
R square	0.2004				
Was the matching effective?					
F	5.864				
P value	0.0168				
P value summary	*				
Is there significant matching (P < 0.05)?	Yes				
R square	0.6098				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.07722	3	0.02574	F (1.092, 3.277) = 0.7521	P = 0.4574
Individual (between rows)	0.6021	3	0.2007	F (3, 9) = 5.864	P = 0.0168
Residual (random)	0.3080	9	0.03423		
Total	0.9874	15			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	4				

2) Statistical analysis ANOVA of FH15 subfractions for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	2.959				
P value	0.1724				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.3910				
R square	0.4965				
Was the matching effective?					
F	1.040				
P value	0.4205				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.1487				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2835	3	0.09449	F (1.173, 3.519) = 2.959	P = 0.1724
Individual (between rows)	0.09969	3	0.03323	F (3, 9) = 1.040	P = 0.4205
Residual (random)	0.2874	9	0.03194		
Total	0.6706	15			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	4				

Appendix Q

1) Statistical analysis of isolated compounds for pre-treatment protocol

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	7.847				
P value	0.0053				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.7899				
R square	0.3954				
Was the matching effective?					
F	4.221				
P value	0.0013				
P value summary	**				
Is there significant matching (P < 0.05)?	Yes				
R square	0.5607				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.1927	2	0.09637	F (1.580, 18.96) = 7.847	P = 0.0053
Individual (between rows)	0.6221	12	0.05184	F (12, 24) = 4.221	P = 0.0013
Residual (random)	0.2947	24	0.01228		
Total	1.110	38			
Data summary					
Number of treatments (columns)	3				
Number of subjects (rows)	13				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH11.04.2	*	0.00444543	1.19325	0.944	0.24925	0.0507896	4.9075	5.0

2) Statistical analysis ANOVA of isolated compounds for post-infection protocol

One-way ANOVA

Table Analyzed	Data 2				
ANOVA summary					
Assume sphericity?	No				
F	24.92				
P value	< 0.0001				
P value summary	****				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.6347				
R square	0.6750				
Was the matching effective?					
F	2.202				
P value	0.0482				
P value summary	*				
Is there significant matching (P < 0.05)?	Yes				
R square	0.2635				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2606	2	0.1303	F (1.269, 15.23) = 24.92	P < 0.0001
Individual (between rows)	0.1381	12	0.01151	F (12, 24) = 2.202	P = 0.0482
Residual (random)	0.1255	24	0.005228		
Total	0.5242	38			
Data summary					
Number of treatments (columns)	3				
Number of subjects (rows)	13				

Multiple *t*-tests

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.06.3		0.0773001	0.774333	1.0105	-0.236167	0.106466	2.21823	5.0

Appendix R

Cytotoxic of all isolated compounds and Ribavirin alone and in combination to Vero cells

Compound	Viable cells with respect to controls (%)							
	Control	Pre-treatment				Post-infection		
		Compound alone	Ribavirin alone	Comb. 1	Comb. 2	Compound alone	Ribavirin alone	Comb. 1	Comb. 2
FH6.06.2	100	93.97	92.51	93.82	93.23	95.13	93.44	91.34	90.40
FH6.06.3	100	94.60	92.51	91.68	93.37	95.66	93.44	92.92	93.98
FH6.10.1	100	93.95	92.51	96.26	94.73	90.15	93.44	94.14	96.71
FH6.10.2	100	93.39	92.51	91.72	92.55	93.71	93.44	92.62	90.68
FH6.10.3	100	93.61	92.51	94.12	96.74	94.57	93.44	95.49	92.17
FH7.01	100	94.47	92.51	95.42	92.62	93.70	93.44	93.83	92.67
FH7.02	100	95.17	92.51	93.39	90.40	96.62	93.44	92.92	95.81
FH7.03	100	91.97	92.51	95.65	93.66	93.91	93.44	95.41	92.40
FH7.04	100	95.53	92.51	96.98	95.63	90.26	93.44	95.59	90.26

FH7.05	100	95.98	92.51	94.93	98.60	95.69	93.44	90.74	91.90
FH11.04.2	100	95.84	92.51	93.79	92.94	90.18	93.44	94.87	90.33

Appendix S

Multiple *t*-tests of the IC_{50} value for single and in combination Ribavirintreatment in pre-treatment protocol

1) Ribavirin alone vs Ribavirin+0.01LC_{50} compound

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.06.2	*	< 0.0001	5.455	1.465	3.99	0.00707115	564.265	2.0
FH6.10.1	*	0.00011477	5.455	4.795	0.66	0.00707123	93.3359	2.0
FH6.10.3	*	< 0.0001	5.455	3.465	1.99	0.00707115	281.425	2.0
FH7.01	*	< 0.0001	5.455	1.485	3.97	0.00707115	561.437	2.0
FH7.02	*	0.00011477	5.455	4.795	0.66	0.00707123	93.3359	2.0
FH7.04	*	< 0.0001	5.455	4.315	1.14	0.00707123	161.217	2.0
FH7.05	*	< 0.0001	5.455	1.265	4.19	0.00707115	592.549	2.0
FH11.04.2	*	< 0.0001	5.455	1.265	4.19	0.00707115	592.549	2.0

2) Ribavirin alone vs Ribavirin+0.05LC_{50} compound

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.10.1	*	0.00011477	5.455	1.333	4.122	0.00707123	93.3359	2.0
FH6.10.3	*	< 0.0001	5.455	1.330	4.125	0.00707115	281.425	2.0
FH7.02	*	0.00011477	5.455	0.800	4.655	0.00707123	93.3359	2.0
FH7.03	*	< 0.0001	5.455	1.405	4.050	0.00707123	132.933	2.0

Appendix T

Multiple *t*-tests of the IC_{50} value for single and in combination Ribavirin treatment in post-infection protocol

1) Ribavirin alone vs Ribavirin+0.01LC_{50} compound

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.06.2	*	< 0.0001	3.01	1.47	1.54	0.00707123	490.721	2.0
FH6.06.3	*	< 0.0001	3.01	1.07	1.94	0.00707123	468.094	2.0
FH6.10.1	*	0.00011477	3.01	1.60	1.41	0.00707123	93.3359	2.0
FH6.10.2	*	< 0.0001	3.01	1.50	1.51	0.00707123	490.721	2.0
FH6.10.3	*	< 0.0001	3.01	1.00	2.01	0.00707115	281.425	2.0
FH7.01	*	< 0.0001	3.01	1.40	3.97	0.00707115	561.437	2.0
FH7.02	*	0.00011477	3.01	1.60	0.66	0.00707123	93.3359	2.0
FH7.03	*	< 0.0001	3.01	1.33	-0.94	0.00707123	132.933	2.0
FH11.04.2	*	< 0.0001	3.01	1.60	4.19	0.00707115	592.549	2.0

2) Ribavirin alone vs Ribavirin+0.05LC_{50} compound

	Significant?	P value	Mean1	Mean2	Difference	SE of difference	t ratio	df
FH6.06.3	*	< 0.0001	3.01	1.270	1.74	0.00707123	468.094	2.0
FH7.01	*	< 0.0001	3.01	1.200	1.01	0.00707115	561.437	2.0

Appendix U

Caov-3 cells viability when treated with 0, 25, 50, 100, 200 and 400 µg/ml of isolated compounds

Isolated compounds	**Cells viability (%)**					
	0	25	50	100	200	400
FH6.06.2	100	99.30	95.30	93.70	80.70	70.30
FH6.10.1	100	93.80	90.30	87.50	86.30	77.70
FH6.10.2	100	92.96	85.66	84.10	78.49	70.12
FH6.10.3	100	80.60	78.20	77.40	73.40	58.30
FH7.01	100	81.20	79.00	78.70	77.40	72.50
FH7.02	100	73.20	72.64	71.40	70.90	68.90
FH7.03	100	89.00	86.06	82.60	79.95	69.59
FH7.04	100	101.80	97.90	86.00	82.80	61.40
FH7.05	100	95.40	88.96	83.78	78.45	64.39
FH11.04.2	100	94.90	89.20	80.10	73.90	68.80
Tamoxifen	100	91.77	86.74	74.75	0.62	0

Appendix V

Statistical analysis of isolated compounds for antiproliferative assay

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	14.10				
P value	0.0014				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.2597				
R square	0.5851				
Was the matching effective?					
F	2.392				
P value	0.0209				
P value summary	*				
Is there significant matching (P < 0.05)?	Yes				
R square	0.1656				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.031	5	0.2061	F (1.299, 12.99) = 14.10	P = 0.0014
Individual (between rows)	0.3496	10	0.03496	F (10, 50) = 2.392	P = 0.0209
Residual (random)	0.7308	50	0.01462		
Total	2.111	65			
Data summary					
Number of treatments (columns)	6				
Number of subjects (rows)	11				

Appendix W

Statistical analysis on Caov-3 cells growth with and without DMSO

One-way ANOVA

Table Analyzed	Data 1				
ANOVA summary					
Assume sphericity?	No				
F	30.51				
P value	0.1140				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.3333				
R square	0.9683				
Was the matching effective?					
F	8.448				
P value	0.0622				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.08203				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.7210	3	0.2403	F (1.000, 1.000) = 30.51	P = 0.1140
Individual (between rows)	0.06654	1	0.06654	F (1, 3) = 8.448	P = 0.0622
Residual (random)	0.02363	3	0.007876		
Total	0.8112	7			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	2				