SYNERGISTIC EFFECT OF RIBAVIRIN IN COMBINATION TREATMENT WITH LEMON GRASS (*CYMBOPOGON NARDUS* (L.) RENDLE) FRACTIONS SWA13 AND SWB6 TOWARDS MEASLES VIRUS

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Synergistic Effect Of Ribavirin in Combination Treatment With Lemon Grass 
(*Cymbopogon nardus* (L.) Rendle) Fractions SWA13 and SWB6 
Towards Measles Virus

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with Hounours 
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ABSTRACT

An *in vitro* study was carried out to investigate the antiviral activity in SWA13 and SWB6 fractions of *Cymbopogon nardus* (L.) Rendle and the synergistic effects from combination of the fractions with ribavirin towards measles virus. The antiviral activity was conducted by treating Vero/SLAM cells with two concentrations for *C. nardus* (0.1 LC$_{50}$ and 0.5 LC$_{50}$) and ribavirin (0.01CC$_{50}$ and 0.005CC$_{50}$). Two protocols were used in this study. Protocol I [(S+V)+A] was done by inoculating the virus (V) to the cells (S) before being treated with antiviral compounds (A). Protocol II [(S+A)+V], involved inoculation of the cells with virus after treatment with antiviral compounds. The antiviral activity was based on comparison of percentage of cells viability in viral inoculated cells with the control. Data was analyzed using one-way ANOVA and significant data was analyzed with Tukey’s Test. Results of monotherapy showed moderate antiviral activity of SWA13 in Protocol I but low activity in Protocol II. SWB6 exerted low antiviral activity in both protocols. Synergistic effect was observed from combination treatment of 0.005 CC$_{50}$ RBV + 0.1 LC$_{50}$ SWB6 in Protocol II with 80.23% cells viability. Further evaluation should be done to identify the active compounds and mechanisms of action of *C. nardus* extracts.

Keywords: *Cymbopogon nardus*, ribavirin, synergistic effect, combination treatment, monotherapy

ABSTRAK

Satu kajian secara in vitro telah dilakukan untuk mengkaji aktiviti antivirus fraksi SWA13 dan SWB6 daripada *Cymbopogon nardus* (L.) Rendle serta kesan sinergistik terhadap virus measles hasil gabungan fraksi *C. nardus* dengan ribavirin. Kajian aktiviti antivirus dilakukan dengan cara menguji sel Vero/SLAM menggunakan dua kepekatan berlainan *C. nardus* (0.1 LC$_{50}$ dan 0.5 LC$_{50}$) dan ribavirin (0.01CC$_{50}$ dan 0.005CC$_{50}$). Dua jenis protokol telah digunakan dalam kajian ini. Protokol I [(S+V)+A] dijalankan dengan menginokulat virus (V) ke atas sel (S) sebelum dirawat dengan antivirus ujian. Protokol II [(S+A)+V] melibatkan sel dirawat dengan antivirus ujian kemudian diinokulat dengan virus. Analisis aktiviti antivirus adalah berdasarkan perbandingan peratus kemandirian sel terinokulat dengan kawalan. Analisis data dilakukan menggunakan ANOVA sehala dan data yang signifikan dianalisis dengan ujian Tukey. Hasil ujian monoterapi menunjukkan kesan antivirus yang sederhana oleh SWA13 dalam Protokol I dan rendah dalam Protokol II. SWB6 menunjukkan kesan antivirus yang rendah untuk kedua-dua protokol. Kesan sinergistik diperoleh melalui gabungan perlakuan 0.005 CC$_{50}$ RBV + 0.1 LC$_{50}$ SWB6 dalam Protokol II. Peratus kemandirian sel 80.23%. Kajian selanjutnya harus difokus ke arah mengenalpasti komponen aktif dan mekanisma tindakan ekstrak *C. Nardus*.

Kata kunci: *Cymbopogon nardus*, ribavirin, kesan sinergistik, kombinasi perlakuan, monoterapi
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1.0 Introduction

An average of 800,000 deaths per year worldwide is reported to have been caused by measles virus infection (Rager-Zisman et al., 2004). This occurs in spite of measles being a vaccine-preventable disease (WHO, 2001). Basically, there are two main obstacles pertaining to the issue. First, the vaccination campaigns in underdeveloped and developing countries are inadequate (Mulder et al., 2001). The second problem pertains to vaccine administration, in which immunosuppressed persons and children below a specified age may come down with vaccination-related problems (Atkinson et al., 2007; Tyring, 2005). These obstacles have made the eradication of measles infection through live attenuated vaccine difficult. Therefore, the use of antiviral drugs as therapeutic agents has the potential to be applied as an alternative to vaccination against measles (Tyring, 2005).

According to Hudson (1990), the mechanisms by which antiviral drugs inhibit viral replication are based on the interference with certain specific stage of the virus replication. These mechanisms include interference with the processes of attachment, penetration, uncoating, synthesis of DNA or RNA, or assembly of virion components. Ribavirin for instance, inhibits the replication of the viral genome. It is a broad-spectrum nucleoside analog which is known to be active against measles virus (Mims et al., 2004). Although the antiviral chemotherapy has reduced the number of deaths due to viral infections, the success of these synthetic drugs has been constrained by their adverse effects (Grancher, et al., 2004). At the effective concentrations, the drugs show cytotoxicity and teratogenicity in host and therefore, limit their usefulness in antiviral therapy.
Combination therapy is a procedure in which two or more drugs are given concurrently (Kirsi et al., 1984). In combination therapy, individual drugs are used at lower concentrations. Synergistic effects in combination therapies have been reported (Kirsi et al., 1984; Cheng et al., 2006). The aims of synergism are to maximize the drugs’ antiviral efficiency, to minimize the toxicity for the host and to reduce the possibility of the emergence of drug-resistant virus (Snoeck et al., 1992). Synergistic effect can be achieved by either combination of synthetic drugs (Petrera & Coto, 2006), combination of plant-based compounds (Cheng et al., 2006) and also combination between synthetic drug and plant-based compound (Barquero et al., 1997).

In recent years, several reports have been published on the screening for antiviral substances from plants (Chattopadhyay & Naik, 2007). This implies the greater interest among researchers on studies of plants for medicinal purposes. One of the reasons why these studies are so appealing is the high demand on new approach against viral infection (Cowan, 1999). Two different studies showed plant’s active compounds, Robustaflavone from *Garcinia multiflora* and Skimmianine from *Zanthoxylum chalybeum* are both effective against measles virus (Jassim & Naji, 2003; Olila et al., 2002). Recently, a study conducted by Nurul Aini et al. (2006) had also proved the effectiveness of *Cymbopogon nardus* (L.) Rendle fractions (serai wangi) towards measles virus. The fact that *C. nardus* is a medicinal plant which has been used by old folks to treat various illnesses has prompted the researchers to use extract from this plant in synergistic studies. In recent studies by Yip (2007) and Heah (2007), the synergistic effects from *C. nardus* sub fractions and ribavirin against measles virus have been detected.
In this study, *C. nardus* fractions of SWA13 and SWB6 were used against measles virus. Two different protocols were selected to determine the antiviral activity of SWA13 and SWB6 and synergistic effect from combination of SWA13 or SWB6 with ribavirin. The hypothesis is there will be a reduction in both antiviral activity and synergistic effect of the *C. nardus* fractions.

**Objectives of the Study**

1. To determine presence of antiviral activity in *C. nardus* fractions of SWA13 and SWB6 against measles virus

2. To determine existence of synergistic effect from combination of *C. nardus* fractions of SWA13 and SWB6 with ribavirin against measles virus.
2.0 Literature Review

2.1 Measles Virus

Measles virus is a single serotype virus with negative sense single stranded RNA genome and classified under genus *Morbilivirus* in the family of *Paramyxoviridae* (Rima, 2001). This virus has two important proteins on its envelope; fusion protein (F) for fusion of virus with host cell membrane, and also haemagglutinin protein (H) which facilitates the adsorption of viruses onto host cells (Atkinson *et al*., 2007). It is reported that human signaling lymphocytic activation molecule, SLAM (CD150) is the cellular receptor for measles virus. A study done by Ono *et al*. (2001) showed that Chinese hamster ovary (CHO) cells transfected with human SLAM produced cytopathic effect (CPE) whilst CHO cells expressing mouse SLAM did not show any sign of CPE. This study also proved the H protein ability to bind to human SLAM.

The portal of entry of this virus is respiratory system where water droplets containing measles virus will infect the respiratory epithelium of the nasopharynx. The virus remains latent from 10 to 14 days before showing early symptoms such as fever, dry cough and conjunctivitis (Rima, 2001). Appearance of Koplik spots, red dots with blue-white spots, implies the infection of measles virus (Atkinson *et al*., 2007). This is followed by appearance of measles rash. The rash is a maculopapular eruption which occurs mostly on the face and upper neck. This symptom persists for about 5 to 6 days after which it begins to fade (Numazaki, 2007).
Infection of measles virus can bring several complications, especially with malnourished children with poor medical care available. Subacute Sclerosing Panencephalitis (SSPE) is a rare degenerative central nervous system disease which is caused by persistent infection of measles virus in the brain. The virus remains dormant and secondary infection develops from 1 to 10 years after recovery from acute infection (Mims et al., 2004). Besides SSPE, measles infections can also cause common bacterial superinfection which includes otitis media and pneumonia, primary measles virus pneumonia and measles encephalomyelitis. Measles encephalomyelitis can cause epilepsy and has mortality rate of nearly 20% (Madigan & Martinko, 2006).

2.2 Measles Infection

Measles virus is a potentially lethal virus among those Paramyxoviruses which can cause human infection. It is a major cause of death in childhood, especially children of malnourished and has poor medical care. The death toll can be up to one million per year worldwide (Mims et al., 2004). According to World Health Organization (2001), it is estimated that 45.5% of 1.7 million annual deaths is due to childhood vaccine-preventable diseases, where one of the diseases is measles.

There are several attempts to reduce the crisis. Early treatment of intravenous vitamin C, treatment of prophylactic antibiotics and adequate uptake of vitamin A were some of the solutions taken before the introduction of live attenuated vaccine. To date, the vaccine which are commonly used are the Edmonston-Enders strains and they are given together with mumps and rubella vaccine, collectively known as MMR. Measles infection is highly
contagious, that measles infection will persist in a community if the susceptible population exceeds 3-7% (Lee & Choo, 2001). A reduction of global measles vaccination coverage from 79% to 72% has also occurred in 1998, with Africa and South East Asia having the lowest coverage rate (Mulder et al., 2001). The problems of controlling measles are furthered weighed with recurrence of outbreak. As children are the reservoir for measles virus, the increase in travels among children into other countries has allowed the virus to be reintroduced into susceptible populations, leading to recurrences of outbreak (Rima, 2001).

2.3 Vaccination toward Measles Virus

Control of measles infection has successfully achieved with the use of live attenuated vaccine (Madigan & Martinko, 2006). Combination vaccination of measles, mumps and rubella (MMR) was introduced into the national Expanded Program of Immunization of Malaysia in 2002, replacing the monovalent measles and rubella vaccines (Anon., 1998). Currently, there are three live attenuated measles vaccine strains available in Malaysia; Moraten strain, Schwarz strain and Edmonston-Zagreb strain (Lee & Choo, 2001). Administration of MMR vaccine is done twice; once during the age of 12-15 months and once between 4 to 6 years old.

However, there are several precautions have to be considered when giving MMR vaccine. Firstly, persons with severe allergic reactions towards vaccine components and pregnant women should not be vaccinated with MMR. Administration of MMR towards immunosuppressed or immunodeficient persons will cause replication of the vaccine virus (Atkinson et al., 2007). Secondly, MMR vaccination can not be given to a child younger than 9 months in order to avoid interference of maternal antibodies. By this time, the maternal
antibodies decrease to a low level, thereby increasing the risk of measles virus infections before the vaccine is given at a proper age of 12 months (Bouche, et al., 2005). Since measles vaccine used in MMR is a live attenuated virus, there are a number of safety problems such as probability of the virus to revert into wild strain, contamination with other viruses and fetal damage (Mims et al., 2004). These obstructions have caused a limitation in giving vaccination. Therefore, improvement of vaccines or finding new antiviral drugs which gives no or less adverse effects has gained greater importance in recent years.

2.4 Antiviral Drugs Chemotherapy

Chemotherapy using antiviral drug is considered as an alternative after vaccination due to its effectiveness when it is used at optimum concentration (Tyring, 2005). Ribavirin has been approved by Food and Drug Administration (FDA) of United States, and is currently approved for treatment towards Respiratory Syncytial Virus (RSV).

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 virus, and Parainfluenza 1,2 and 3 (Fernandez et al., 1986). It appears that 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. 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).
In spite of its usefulness, ribavirin chemotherapy produces adverse effects. Some mild reactions, for instance lip and gingival swelling, conjunctival hyperemia, headache and lethargy can be observed among the recipients. A study conducted by Hosoya and colleagues (2004) proved the effectiveness of ribavirin in chemotherapy through intraventricular administration. However, prolonged administration is required for patients with short ribavirin half-life in cerebrospinal fluid. A prolonged treatment may lead to anemia. Furthermore, a study using rodent as an in vivo model showed ribavirin able to cause teratogenicity, a capability to induce malformation (Fernandez et al., 1986).

2.5 Antiviral Activity from Plants’ Extracts

Medicinal plants has been used traditionally as therapeutic agents decades ago, and this has become a centre of attention among scientists to uncover the secrets that makes medicinal plants so valuable (Cragg et al., 1997). Although a number of medicinal plants have been developed as therapeutic agents, Chattopadhyay (2006) stated fewer plants are explored for antiviral since there is lack of specific targets for natural molecules to interact with. Nevertheless, laboratory investigations of medicinal plants showed the possibility of these plants to exert antiviral activity.

World Health Organization (1999) has listed Chamomilla rectita, Ephedra sinica, Glycyrrhiza glabra and several Echinacea species as traditional medicine for treating viral infection. Scientific evidence has proved Acokanthera schimperi, Euclea schimperi and Inula confertiflora to possess antiviral activity against Coxsackievirus B3, Influenza A virus and Herpes Simplex virus Type-1 Kupka. These plants are used traditionally by Ethiopians for
skin diseases of viral origin (Gebre-Mariam et al., 2006). Rajbhandari and colleagues (2007) have found that extracts of some Nepalese traditional medicine were effective against Herpes Simplex virus and influenza virus. Antiviral activity has also been detected in Ancistrocladus tectorius (Said, et al., 2001). The cell death caused by HSV-1 was reduced by increasing the A. tectorius extract before inoculation of the virus.

2.6 Antiviral Screening

Screening for antiviral activity in plant extract has become a trend among scientists lately. One of the plants which are currently being studied is Citronella grass (Cymbopogon nardus (L.) Rendle), or locally known as serai wangi (sweet lemon grass). Serai wangi is closely related to Citronella citratus which grows wildly across tropical countries of Asia (Anon., 2003).

Serai wangi has coarse, clump-forming leaves that can grow up to 5-6 feet and gives out aromatic smell, which is the reason why it is commonly used as essential oil. Traditional medicine also used this grass as after-childbirth wash, to comfort stomach upset and as insect repellent. According to Lewis and Elvin-Lewis (1977), the primary components of C. nardus are citral (geranial) and citronellal.

Early studies by Ahmad et al. (1992; 1993) have shown antiviral activity of C. nardus towards measles virus and Newcastle disease virus. A study conducted by Nurul Aini et al. (2006) showed that the sub fractions of Cymbopogon nardus (L.) Rendle able to protect the Vero cells from entrance of measles virus. Besides having the ability to inhibit RNA virus, a
preliminary study has also proved that *C. nardus* was effective against Herpes Simplex virus, a DNA virus (Hanina, 2006).

### 2.7 Synergistic Effects from Antiviral Drugs Combination

Chemotherapy using antiviral drugs has a limited success due to toxicity, mutagenicity and other severe side effects towards human (Tyring, 2005). Therefore, researchers have come up with an idea of combining the drugs with effects obtained threefold. In other words, the drugs will work synergistically against viruses. As virus replication comprises of several stages (Ahmad, 1993), each individual drugs of lower concentration will act at different stages (Chou, 2006). Interference of more than one stage of virus replication increases the inhibition efficiency against the virus replication. This type of combination is reported to be able to maximize the antiviral efficiency, minimize the toxicity and reduce the possibility of emergence of drug-resistant virus strains (Snoeck *et al.*, 1992). This conclusion was made based on their study, where combination of synthetic analog HPMPC with foscarnet, ganciclovir or acyclovir resulted in partial synergistic effects against Cytomegalovirus (CMV). The combinations also did not enhance the cytotoxicity to the HEL cells.

Another study on drugs combination has also been carried out by Petrera and Coto (2006) who showed the synergistic effects between Interferon-α and Interferon-γ able to inhibit replication of Herpes Simplex Virus Type 2 in Vero cells. Oxymatrine-baicalin combination, both of which were derived from plant extracts was shown to inhibit DNA replication of Hepatitis B virus much more effective than treatment with oxymatrine alone (Cheng *et al.*, 2006). Oxymatrine was extracted from *Saphora florescens* while Baicalin was
extracted from *Scutellaria baicalensis*. Besides applying combination between synthetic drugs or between plant extracts, studies on combination between synthetic drug and plant extract have also been carried out. Barquero *et al.* (1997) for instance, 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 in infected cells.

### 2.8 Animal Cell Culture in Antiviral Screening

Antiviral screening in animal cell culture is considered as an *in vitro* system in determining the antiviral activity and toxicity of the potential antiviral compound. This preliminary step is important before the potential antiviral compound can be tested *in vivo* (Ahmad, 1993). Different cell types have been used in studies involving viruses. The cells being used are based on the purpose of the study. In antiviral screening, observations of cytopathic effect (CPE) on inoculated cells are virus-specific. For instance, Vero cells inoculated with HSV will produce grape-like clusters of round refractile cells after 24 hours incubation. However, when Vero cells are inoculated with measles virus the CPE produced consist of large syncytia (Gray, 1999).

Vero cell is a type of epithelial cell line derived from kidney epithelium of African Green monkey, *Cercopithecus aethiops* (Macfarlane & Sommerville, 1969). Several studies which have used Vero cells as host in antiviral screening include combination therapy of interferon against Herpes Simplex virus (Petrera & Coto, 2006), anti-Herpevirus activity of plant volatile oil (Saddi *et al.*, 2007), biological activity of plant extracts against measles virus (Olila *et al.*, 2002) and inhibitory effect of essential oils against Herpes Simplex virus Type-1
(Minami et al., 2003). Vero cells are favoured because they are not persistently infected with virus, thus providing safety to laboratorians (Centres of Diseases Control and Prevention, 2007).

Earlier report by Macfarlane & Sommerville (1969) had shown that measles virus can be grown on Vero cells with cytopathic effect development without prior adaptation. Following the identification of SLAM as the measles virus receptor (Ono et al., 2001), Vero cells have been transfected with human SLAM receptor and recommended for laboratory use. Vero/SLAM cells have to be cultured in media containing geniticin in order to maintain the SLAM expression. However, they can still be passaged for up to 15 times and used for virus inoculation even without geniticin (Centres of Diseases Control and Prevention, 2007).

3.0 Material and Method

3.1 Source of Materials

*Cymbopogon nardus* (*serai wangi*) fractions used were obtained from research done by Hanina (2006). The fractions were prepared via Flash Chromatography I and II.

Vero/SLAM cell culture was obtained from Institute of Medical Research (IMR) Kuala Lumpur. The cells have been transfected with plasmid encoding human SLAM protein. SLAM protein is the receptor for attachment of measles virus, both wild and Edmonston strain.
Measles virus used in this study was obtained commercially (Edmonston strain, 1000 TCID\textsubscript{50}, Cambrex Company). The virus was diluted in 0.5 mL sterile solvent before use in order to get a virus concentration of 1000 TCID\textsubscript{50}. Dilution was done with aseptic techniques.

Ribavirin used in this study was bought from Sigma-Aldrich, Inc. US and kept at -4°C. The concentrations of ribavirin used in this study were 0.1, 0.01 and 0.005 CC\textsubscript{50}.

3.2 Continuous Cell Culture Preparation

Cell culture was grown in Dulbecco’s Modified Eagle medium, DMEM (Sigma Chemical Company, US) with 10% of foetus bovine serum (PAA Company) for overnight at 37°C and 5% CO\textsubscript{2}. After a monolayer of cells was obtained, the growth medium was taken out and the cells were washed with sterile PBS (phosphate buffered saline) pH 7.2 twice before disaggregated by addition of 2-3 drops of trypsin-verseine solution (0.25-0.018%).

3.3 Serai Wangi Fractions Preparation

Each fraction was dissolved in 100 µL DMSO and diluted with 900 µL DMEM medium 2% FBS in order to obtain a stock solution with concentration of 5 mg/mL. The extract was sonicated (CREST OITG36305) until it mixed well. The concentration of stock fraction then diluted into 200 µg/mL with growth medium DMEM 2% FBS. This is the LC\textsubscript{50} value for the fraction (Hanina, 2006). The fraction was further diluted into 0.1 LC\textsubscript{50} and 0.5 LC\textsubscript{50} for antiviral activity screening.
3.4 Combination of Antivirus Preparation

The combination of *serai wangi* fractions and ribavirin (RBV) were prepared for antiviral activity screening. Four combinations used in this study were; (0.01 CC$_{50}$ RBV; 0.1 LC$_{50}$ SW, 0.01), (0.01 CC$_{50}$ RBV; 0.5 LC$_{50}$ SW), (0.005 CC$_{50}$ RBV; 0.1 LC$_{50}$ SW) and (0.005 CC$_{50}$ RBV; 0.5 LC$_{50}$ SW). Every combination used 50 µL ribavirin and 50 µL *serai wangi* at the concentrations mentioned above.

3.5 Antiviral Activity Screening

Antiviral activity screening on *serai wangi* fractions, ribavirin, and combination of *serai wangi* with ribavirin were done according to the method explained by Ahmad *et al.* (1993) and Hanina (2006). 100 µL cells at concentration of 1.25 x 10$^5$ cell/mL were put into a sterile 96-wells microtiter plate (Nunclon®). Next, the plate was incubated at 37°C, 5% CO$_2$ overnight to achieve 80% confluent monolayer cells. There were two protocols to determine the antivirus activity. In Protocol I, [(S+V)+A], antivirus (A) was added after cells (S) were inoculated with virus (V). In Protocol II, [(S+A)+V] the antivirus was added before the cells were inoculated with virus (Ahmad *et al.*, 1993). Four controls were used in this study; i) cells culture inoculated with virus only, ii) cells culture added with ribavirin only at concentration 0.1 CC$_{50}$, iii) cells culture added with medium only, and iv) medium only.
3.5.1  Treatment Using Protocol I

Before inoculation of virus, the media were decanted and the cells were washed with PBS two times to remove any traces of FBS. Protocol I, [(S+V)+A] began with inoculation of 5 µL measles virus (1000 TCID$_{50}$) to cells. After 20 minutes of incubation at 37°C, 100 µL of antivirus compounds (serai wangi fractions at concentration 0.1 LC$_{50}$ and 0.5 LC$_{50}$, ribavirin at 0.01 CC$_{50}$ and 0.005 CC$_{50}$ together with the four combinations mentioned above) were added to cells. The plate was incubated for 48 hours with 5% CO$_2$ at 37°C. After that, the plate was processed and data was collected at wavelength 620 nm.

3.5.2  Treatment Using Protocol II

Protocol II, [(S+A)+V] was done by adding 100 µL of antivirus compounds to the cells and the plate was incubated at 37°C with 5% CO$_2$ for 24 hours before being inoculated with virus. Cells were washed with PBS and then inoculated with 10 µL measles virus (100 TCID$_{50}$) except for the control wells. The plate was incubated for 20 minutes (37°C, 5% CO$_2$) for virus adsorption. Then, each well was filled with 200 µL growth medium (DMEM with 2% FBS) before incubated at 37°C with 5% CO$_2$ for 48 hours.

3.6  Plate Processing using Optical Absorbance

Plate processing was based on Hanina (2006) with slight modifications. The cell culture was fixed by 125 µL trichloroacetic acid (TCA) 25% and incubated at 4°C for 1 hour. After incubation, all the solution in the wells was discarded. Cells at the base were washed five