Faculty of Resource Science and Technology

Antiviral Activity of *Cymbopogon nardus* SWB6 and SWB9 Extract Fractions against Newcastle Disease Virus

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Bachelor of Science with Honours

(Resource Biotechnology)

2008

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This Project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Resource Biotechnology Programme
Faculty of Resource Science and Technology
UNIVERSITY MALAYSIA SARAWAK

2008

ACKNOWLEDGEMENT

In the name of Allah the Almighty, I would like to express my gratitude to my supervisor Prof Madya Dr Awang Iskanderdzulkernain Bin Pengiran Rayari for his guidance and advices. Special appreciation and gratitude was also addressed to my co supervisor Prof Dr Ismail Bin Ahmad for his useful information, guidance, continuous support, material provided and time spent for us during the completion of this thesis. Not forgetting Dr. Andrew Wong and Dr. Ling Teck Yee for their assistance in doing SPSS.

I would like to thank my parents and my family members who keep continuing supporting me and always stand beside me in time I need no matter how hard and tough it is. Not forgetting the lab assistant Ms. Lim Tajai , Mr. Azis and Mr. Amin.

Lastly, I wish to express my deepest gratitude and sincere appreciation to those who has assisted me in conducting this study by giving material and moral support either directly or indirectly. Special thanks to my colleagues who give a lot of support and assistance during conducting this study especially to Nur Hidayah, Nur Ramadanilelawati, Faezah, Adibah, Frankie Thomas, Mohd Hafis Yuswan, Reagen Entigu, Mohd Iszuandy, Norzaitulrina, Iris Tia and Sura Illa.

To all my friends especially my course mate (Biotechnology Resource, 2005-2008) and members of ROTU (2005-2008) also the person who I have not mentioned, thank you for your support, kindness and help. I would like to apologize for not stating the names. Meaningful knowledge and skills was acquired during completion of this thesis. Hopefully through these experiences I can give valuable contribution in future.

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LIST OF ABBREVIATIONS

LC_{50}	Lethal Concentration (Concentration that lead to 50% of cells dying)
$TCID_{50}$	Tissue Culture Infective Dose Fifty Percent (Dilution that cause 50% infection to cell culture)
RBV	Ribavirin
SW	*Serai wangi*
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffered Saline
TCA	Trichloroacetic Acid
FBS	Foetal Bovine Serum
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunassay
NaOH	Natrium hydroxide

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ABSTRACT

Newcastle disease is a contagious viral infection among poultry. Poultry contribute a lot in livestock and economic sector. Therefore, many measures were taken in order to control this disease. This study was conducted to study the antiviral activity of SWB6 and SWB9 fractions of *Cymbopogon nardus* extract against Newcastle Disease Virus (NDV) on different level of fractions and virus concentration. Prior to addition of extract fractions, NDV was inoculated on the Vero cells and incubated for 10 minutes. The antiviral activity was determined by cell viability assay using ELISA reader. The significant difference among all treatment was analyzed by performing factorial design using SPSS. The result revealed that SWB6 showed higher cell viability than SWB9. In addition, both fraction and virus concentrations showed significant different in cell viability. However, there is no detectable association of interaction between the three factors.

Keyword: Newcastle Disease, Newcastle Disease Virus, *Cymbopogon nardus*, Vero cells, Cell Viability, ELISA analysis.

ABSTRAK

Penyakit Sapar Ayam adalah penyakit yang menular di kalangan ayam. Oleh kerana industri ini menyumbang kepada perkembangan ekonomi, pelbagai langkah diambil untuk mengatasi penyakit ini. Kajian ini dijalankan untuk mengkaji kesan fraksi SWB6 dan SWB9 ekstrak serai wangi terhadap aktiviti antivirus virus sapar ayam pada kepekatan fraksi dan virus yang berbeza. Fraksi ditambah selepas 10 minit masa pengeraman virus yang dikultur di atas vero sel. Aktiviti antivirus dinilai melalui asai kemandirian sel dengan menggunakan bacaan ELISA. Bagi mendapatkan perbezaan signifikan di antara rawatan yang diberi, rekabentuk faktorial digunakan. Melalui analisis statistik yang dijalankan, didapati fraksi SWB6 memberi nilai kemandirian sel yang tinggi berbanding SWB9 Selain itu, kepekatan fraksi dan kepekatan virus turut memberikan perbezaan yang ketara dalam purata kemandirian sel. Walaubagaimanapun, kaitan hubungan ketiga-tiga faktor ini adalah tidak ketara.

Kata kunci: *Penyakit Sapar Ayam, Virus Penyakit Sapar Ayam, serai wangi, Sel-sel Vero, Kemandirian sel, analisis ELISA.*

1.0 Introduction

Newcastle disease (ND) is a contagious viral infection that is common among poultry and hitherto is endemic in Malaysia (Omar *et al.,* 2003). The causative agent of ND is Newcastle disease virus (NDV), which also known as avian paramyxovirus type-1 (de Leeuw and Peeters, 1999; Alexender, 2000). Since poultry is important in livestock sector and contributes a lot in food and economic, the disease may lead to large economic losses (Aini, 1993).

There is no specific treatment to eradicate ND. However, according to Lohmann Animal Health (2006), this disease is susceptible to common disinfectant. Vaccination is the only practical method to respond to this disease. Three types of vaccine available for ND are lentogenic, live mesogenic and inactivated vaccine. Effectively applied vaccine was derived from lentogenic virus such as B1, La Sota, F and V4 (Animal Health Australia, 2004). In addition, other vaccination program held to control this disease includes tunnel closed house system in VG-GA vaccines, the used of inactivated oil emulsion vaccines, and recently the used of recombinant fowl poxvirus vectored ND Vaccines (Aini *et al.*, 2006).

Although in most cases vaccination has been effective in controlling disease, there are some limitations for vaccine to be fully and successfully applied. For instance, commercial live ND vaccine is thermolabile, thus it has to be keep in a cold chain or otherwise will deactivated by heat (Aini *et al.*, 1992). Therefore, it is not applicable in village area. Furthermore, the vaccination program is costly; require suitable storage condition and technical expertise (Aini *et al.* 1990). Moreover, vaccination is only applicable to commercial system and not feasible due to the presence of maternal antibodies (Aini *et al.*, 2006). Thus, vaccination has to be accompanied by good management practice, biosecurity and hygienic control (Aini *et al.*, 2006; Vegad, 2004).

In order to increase the effectiveness in reducing disease occurrence, antiviral drug had been used. Several antiviral drugs that posses an antiviral activity against NDV had been reported such as kethoxal (McLimans *et al.*, 1957), chloroquine and flufenamic acid (Inglot, 1969), ribavirin (Huffman *et al.*, 1973) and halothane (Bedows *et al.*, 1984). However, this method is impractical mainly due to the weak antiviral activity and the toxicity effect to the cell. Prolong exposure of this drugs may also cause mutagenicity (Fernandez *et al*., 1986).

Consequently, screenings of new antiviral drugs have been carried out to obtain new drug that is feasible to control the disease. Plants had been used in traditional medication long time ago. There are five local herbs that are widely used in Malaysia (Mohamad Setefarzi & Mansor, 2000) and Citronella (*Cymbopogon nardus* (L.) Rendle) is one of the plants that have been the target of study due to its antimicrobial effect (Gimlette & Thomson, 1971).

Cymbopogon nardus (L.) Rendle has been known to have biological activity against wide range of organism. A study on the antiviral activity of *C.nardus* (L.) Rendle against NDV and measles virus was done by Ahmad *et al.*, (1993). In this study, chick embryo fibroblast (CET) was used as a host. The result obtained disclosed significant activity of antiviral among the cells treated with extract before virus inoculation. They suggested that the extract alter the virus receptor on CET that prevents the virus adsorption onto the cell surface.

This current study is conducted to determine the antiviral activity of *Cymbopogon nardus* extract fraction specifically SWB6 and SWB9 fractions against different concentration of fractions and NDV. Prior to addition of extract fractions, NDV was inoculated on the Vero cells and incubated for 10 minutes. The antiviral activity was determined by cell viability assay using

ELISA reader. In order to determine the significant different of each treatment, readings from ELISA was then further to statistical analysis. This study will provide more information on the possibility of *C. nardus* extract to be used in overcome ND.

1.1 Research Objectives

i. Determine the infectivity of NDV.

ii. Determine the optimal concentration of SWB-6 and SWB-9 fraction for antiviral activity.

2.0 Literature Review

2.1 Newcastle Disease

Newcastle disease is first appears in 1926 in Java, Indonesia and had spread to many country within 10 years, predominantly South East Asia Region (Vegad, 2004). In Malaysia, the disease was first reported in 1934. It is believed that the movement of migratory wild birds, personnel and equipment are associated with the spread of this disease (Aini *et al.*, 2006).

Susceptibility to ND depend on species of birds (Palmer & Trainer, 1971), genetic difference in resistance, age of bird, method of management and numbers within area (Lancaster, 1966). This disease may spread through inhalation and ingestion besides transmission of virus by respiratory routes under crowded condition. The disease seems to be exacerbated due to the ability of the virus to survive in carcass or excretions (Vegad, 2004).

The severity of ND depends on the strain of virus, dose, route of entry, age of chicken and surrounding (Vegad, 2004). Unambiguously, infection by extremely virulent strain results in sudden death and high mortality without other signs. However, frequently observed signs are nasal discharge, excessive mucous in trachea, cloudy air sacs, casts or plugs in the air passage of the lungs and cloudiness in the cornea of the eye (Aini *et al.*, 2006).

Since vaccination is extensively practice, lentogenic virus vaccine known as B1 and La Sota types are the most successful and widely used (Animal Health Australia, 2004). These vaccines are drop into the nostril, eye, and drinking water or applied in spray form (LAH, 2006). In Malaysia, the first vaccine used is the lyophilised ND ‘F’ Vaccine. Subsequently, Australian Lentogenic V4 Newcastle Virus and recently involved live and inactivated vaccines such as La

Sota, Ulster 2C, NDV-6/10 and VG-GA. Other treatment includes the tunnel ventilated closed-house system in VG-GA vaccines (Aini *et al.*, 2006).

Breeders flock is vaccinated by inactivated oil-emulsion vaccines but this application is limited due to the presence of maternal antibodies. Later, recombinant fowl poxvirus vectored ND is introduced but not feasible in free range and backyard system. Therefore, food pellets NDV vaccine is introduced (Aini *et al.*, 2006). In corroboration, previous study done by Aini *et al.*, (1990) revealed food pellets contained heat resistant ND vaccine was practical to protect village chicken against NDV in tropical countries.

2.2 Newcastle Disease Virus

Newcastle Disease Virus (NDV) is a *paramyxovirus* with a single-strand non-segmented RNA (de Leeuw and Peeters, 1999) and membrane-enveloped virus (Seal *et al.,* 1999). It is classified as a member of *Mononegavirales*, family *Paramyxoviridae* and subfamily *Paramyxovirinae* (de Leeuw, 1999). However in 1993, NDV was classified within the genus *Rubulavirus* (Seal *et al.*, 1999). In 1999 de Leeuw and Peeters conduct a study on sequencing of NDV genome. They claimed that NDV should not be classified in the genus *Rubulavirus* since NDV did not contain small hydrophobic gene. Consequently, NDV had been classified to the new genus *Avulavirus* (Mayo, 2002).

NDV genome is approximately 15 kb and consists of six major genes which encodes the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large polymerase protein (L) (de Leeuw and Peeters, 1999; Seal *et al.,* 1999). NDV have two transmembrane glycoprotein which are HN and F that

form spike-like protrusions on the outer surface of the NDV's envelope virions. These proteins are responsible for initiation of infection by attachment of virus particle on sialic-acid containing receptors of the host cell (Seal *et al.,* 1999)

NDV infects variety of birds. Chicken is the most susceptible poultry whereas geese and ducks are less susceptible. Newcastle Disease Virus is inactivated at 56^{o}C/3 hours and 60^{o}C/30 minutes, acidic pH and by phenol and formalin disinfectants. Moreover, it is ether-sensitive and can survive for long period at ambient temperature, especially in feces. The mortality and morbidity rate is different among species, and virus strain. A carrier state may exist in some wild bird (WOFAH, 2006).

Transmission of the virus may occur during active respiration and also through contaminated vaccine (Aini *et al.,* 2006) apart from direct contact with secretions of infected birds and also through contaminated feed, water, implements, premises and human clothing (WOFAH, 2006). The virus has different ability to cause disease or death. Some strain may cause little or no clinical sign which can only be detected through serological methods (McFerran *et al.,* 1968; Simmons, 1967).

Virus isolates are classified depending on pathogenicity which are velogenic, mesogenic and lentogenic. The velogenic virus spread rapidly and results in illness of short duration and peracute death. The mesogenic virus result in respiratory death, nervous symptom, decline in egg production and quality. The lentogenic virus results in mild respiratory symptom, low egg production and lethargic (Vegad, 2004; Lancaster, 1966).

2.3 Vero Cell

Vero cell is a fibroblast like cell from African green monkey (*Cercopithecus aethiops)* kidney which anchorage-dependant in culture (Macfarlene and Sommerville, 1969; Sheets, 2000). It is a continuous cell line, which is aneuploid and can grow indefinitely in culture (Sheets, 2000). Newcastle Disease Virus is purposely adapted on Vero cell line to alter growth and virulence characteristics so that it become suitable as laboratory host for cultivation, mass propagation, attenuation and genetic modification of NDV (Ahamed, 2004). These cell lines are recommended for use in WHO laboratory network (Ono *et al.*, 2001). Vero cell are not considered as hazardous since they are not persistently infected by virus thus, provides significant safety for laboratorians. In addition, Vero cells include heteroploid line which is easier to handle with minimum expense, easy to grow and formed monolayer besides susceptible to wide range of viruses and display clear cytopathic effect (Macferlane & Sommersville, 1969).

2.4 Virus Titration

A titer is defined as a given number of infectious virus units per unit volume. Tissue culture infective dose fifty percent ($TCID_{50}$) is used to titer the virus. According to Florence *et al.*, (1992), $TCID_{50}$ is a quantal assay that refers to the ability of certain virus dilution to infect fifty percent of the cell cultures inoculated. A $TCID_{50}$ unit can be calculated by using Karber method (Payment and Trudel, 1993) using the formula as below:

$$TCID_{50} = -\Delta-\delta\ (S-0.5)$$

Where, $\Delta = \log_{10}$ of the dilution with 100% positive cultures (with CPE)

$\delta = \log_{10}$ of dilution factor

S = sum of positive cultures per dilution including those at dilution with 100% infected cultures. This last dilution has a value of one and each other dilution a fraction of one.

Various dilution of virus is purposely added onto the confluent monolayer of host cells. The virus will subsequently replicate and infect the cell upon the addition. The cytopathic effect was allowed to develop for few days followed by staining. The $TCID_{50}$ titer provide qualitative information on dose or dilution that cause CPE in fifty percent of the sample instead of giving quantitative amount of virus infection that is present in a given sample .

Cytopathic effect (CPE) is degenerative changes in cells that are associated with the multiplication of certain viruses. The pattern of CPE depends greatly on the type of cell culture system used, properties of virus strain and the virus concentration (Florence *et al.*, 1992). In tissue culture, the spread of virus is restricted by an overlay of agar that leads to the formation of plaque. According to Ahamed *et al*. (2004), cytopathic effects are characterized by granularity in cytoplasm, rounding of infected cells, development of micro plaque, clustering of infected cells, intracytoplasmic bridge connecting those clusters, vacuolization in the cell system and the formation of syncytia.

Ahamed *et al*. (2004) has stated that, during first passage infection activity of wild NDV, there were no clear evidences and symptoms of cytopathic effects. This was because the NDV just started it adaptation process and its infectivity process was very low. However, after 24 hours of incubation following infection process in third passage, there were some changes developed in

monolayer Vero cell lines. Yet, the cytopathic effects of NDV on Vero cells still can not be found. At fourth and fifth passages, cytopathic effects was rapidly develop and consistent. Within 24 hours of infection, no cytopathic effects were found but after 24 hours of infection, the Vero cells' shape starts to change directly produced cytopathic effects.

2.5 Antiviral from Plant Extract

According to WHO (2005), 80% of world population depend on traditional medicine and this percentage is always increase eventhough among the youth. There are 500,000 species of plants had been studied and 35,000 of it is proven effective for treatment. More than 2,000 species of that plant had been found in Malaysia.

Cymbopogon nardus (L.) Rendle is a plant in *Graminae* family. It is a type of grass plant that grows tall and thickly. According Jaganath & Ng (2000) *C.nardus* is the main source of Citronella extract in India and Ceylon. *C. nardus* is usually used for traditional medicine. Each part of the plant has specific function where the root has diuretic, diaphoretic and antipyretic characteristics that able to cure bronchitis. Grass part of the plant is used as massage oil to overcome insects bite, numbness and headache. Apart from that, it is also use for post partum treatment.

C. nardus is one of plants that have been used in traditional medicine (Ahmad *et al.*, 1993; Gimlette & Thompson, 1971). Several studies had been conducted to test the potential compound that is able to cure disease because some plants extract posses an antiviral activity. A study conducted by Nurul Aini *et al*. (2006) showed that the sub fraction of *C. Nardus* (L.) Rendle has the ability to protect the Vero cells from entrance of measles virus.

Studies done by Ahmad *et al.* (1993) revealed that Citronella extract exert antiviral activity against Newcastle disease but not poliovirus. This showed that, the antiviral activity of citronella fraction is related to the presence of lipoprotein that surrounds the NDV but not poliovirus. Further research by Nurul Aini *et al.* (2006) revealed that citronella fraction was successfully in preventing the infection of membrane virus.

2.6 Antiviral Activity Assay

In previous study done by Hanina (2006), three protocols have been used. This study was based on a study done by Rao *et al.* (1968). In the first protocol, Vero cell (C) was inoculated with herpes simplex virus (V) prior to treatment with extract (E) which is simplified as [C+V] +E. In the second protocol, virus was added after the cell was treated with the extract [C+E] +V. In the third protocol, the virus and extract was added simultaneously into the cell culture [E+V] +C.

The first protocol was done to test the ability of extract to treat the infected cell. The second protocol was intended to test the ability of extract to prevent virus infection on the extract-containing cells. The third protocol was done to test the ability of virus to infect the cell before treatment with extract (Hanina, 2006). This study revealed that first protocol is the least effective among the three protocols.

3.0 Materials and Methods

3.1 Vero Cell Culture Preparations

This method was modified from a protocol done by Hanina (2006). The cells were obtained from Universiti Putra Malaysia (UPM). Firstly, the cells were observed under inverted microscope to determine the formation of cell confluent. The growth medium from the 25 cm^3 flask was discarded. Next, the cells were washed twice with 0.5 ml of sterile PBS pH 7.2. Prior to incubation at 37°C with 5% CO_2 for two minutes, 0.3 ml trypsin (0.25%) was added to disaggregate the confluent cells. Subsequently DMEM with 10% FBS was added to stop the trypsinization. Later, 1 ml of the cell suspension was put into another flask and DMEM was added to final volume of 5 ml. The flasks were labelled and incubated at 37ºC with 5% CO_2 and the cells were observed once a day until it was confluent and ready to subculture.

3.2 Newcastle Disease Virus Preparation

The virus was supplied by Prof. Aini Ideris from Universiti Putra Malaysia (UPM). The V4 strained of NDV was used in this study. The virus stock was diluted by adding 100 µl of the virus stock into 900 µl Dulbecco's modified Eagle medium (DMEM) with 2% fetal calf serum (FBS) to obtain 10 times dilution. The virus prepared was diluted 10-fold to obtain a series of dilution ranges from 10^{-1} to 10^{-6} of original stock concentration.

3.3 Tissue Culture Infective Dose Assay ($TCID_{50}$)

A volume of 100 µl cell suspension ($1.25x10^5$ cells/ml) was subcultured into a microtiter plate and incubated for 24 hours. Next, the microtiter plate was washed with PBS medium. A volume

of 10 µl virus was added into each well. Subsequently, 90 µl of maintenance medium was added into each well. The microtitre plate was sealed and incubated in CO_2 incubator. The cytophatic effect was observed everyday.

3.4 Citronella Extract Fractions Preparation

C. nardus extract fraction was obtained from Faculty of Science and Technology of Universiti Kebangsaan Malaysia. These fractions were prepared by Hanina (2006) through a flash chromatography technique. In this study, SWB6 and SWB9 fractions were used.

A weigh of 5 mg extract was suspended into 100 µl DMSO and homogenized using a Crest™ sonicator for half hour. Next, 900 µl of 2% DMEM was added. The test concentration of 0.1 LC_{50} and 0.5 LC_{50} were prepared according to Yip (2007) using the LC_{50} value of 200 µl/ml for both extract. Thus, 0.06 ml of stock was mixed with 2% DMEM to final volume of 1.5 ml. The value for 0.5 LC_{50} is 100 µl/ml, thus 0.75 ml solution from LC_{50} was mixed with DMEM to final volume of 1.5 ml. The value for 0.1 LC_{50} is 20 µl/ml, thus 0.15 ml solution from LC_{50} was mixed with DMEM to final volume of 1.5 ml.

3.5 Antiviral Activity Assay

The antiviral activity assay was based on antiviral activity assay protocol reported by Hanina (2006) with some modifications. Three controls were used in the antiviral activity assays which are C1 (cell culture + medium), C2 (medium) and C3 (cell culture + medium + ribavirin + NDV). The extract fractions were tested on two levels of concentration and five replicates were used.

A volume of 100 µl of cells (1.25 x 10^5 cells/ml) were aliquot into each of the wells of the 96-wells plate and incubated at 37°C with 5% CO_2 for 24 h. The cells were then washed twice with 200 µl PBS. A volume of 10 µl virus was added into each well and then incubated for 10 minutes. Finally, a volume of 200 µl extract was added and incubates for 24 h prior to first observation under inverted microscope.

After 48 h, plate processing was done according to the method described by Hanina, (2006). The medium in each well was discarded. A volume of 125 µl 25% cold TCA was added into each well and subsequently kept in -4°C for 1 h. Next, the TCA was decanted and 125 µl of 0.25 % crystal violet dye was added into each well and the plate was left in the dark. After 15 minutes, the plate was washed under tap water to remove the dye. Prior analyzing using ELISA, 0.1 µl of 10% acetic acid was added into each well.

4.0 Results and Discussions

4.1 $TCID_{50}$ Assay

Attempt was made to determine fifty percent TCID of the stock NDV culture. This was intended to obtain the range of virus that is required to infect fifty percent of the cell culture. The range of 10^{-1} to 10^{-6} virus dilution was done to determine the best virus concentration to further this study. Virus concentration range from $0.5x\ 10^{-2}$ to $0.5x10^{-6}$ was included to narrow down the observation of CPE. However, $TCID_{50}$ done from this study did not give satisfactory result due to some limitations as discussed in Section 4.6.

Virus replication has a strong interaction with the host metabolism during incubation period. It is difficult for the antiviral to work without affecting the host cell (Klein *et al.*, 1998). The CPE was allowed to develop for up to five days before staining since CPE for paramyxovirus may take a long time to develop. However the incubation period of five days was too long for the cell culture. It is assumed that the cells were overgrown and probably unhealthy which directly led to the cells to be detached from the well during staining process. Failure of $TCID_{50}$ determination has led to the used of 10^{-3} and 10^{-6} virus dilution to test the antiviral activity in this study.

In this study, seeding concentration of cells was 1.25×10^5. Similar experience was recorded in a previous study done by Yip (2007), seeding concentration of 2.25×10^5 was initially used. However, the cell density was then reduced to 1.25×10^5 because denser amount of cells can lead to over confluent and detachment of cell after 24 h incubation period. Therefore, the