ANTI-VIRAL ACTIVITY OF PIPER SP. EXTRACT (PIPERINE) AGAINST NEWCASTLE DISEASE VIRUS

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ANTI-VIRAL ACTIVITY OF Piper sp. EXTRACT (PIPERINE) AGAINST NEWCASTLE DISEASE VIRUS

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Anti-viral Activity of *Piper* spp. Extract (*Piperine*) Against Newcastle Disease Virus

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ABSTRACT

Newcastle Disease Virus (NDV) is an agent of Newcastle Disease (ND) which is a main disease in poultry industry. Poultry industry played an important role to supply food and economic source for the country. The main objective of this study was to determine an anti-viral activity of *Piper* spp. extract which was *piperine* against NDV. Vero cells cultured in Dulbecco’s Modified Eagle Medium (DMEM) were used to maintain the NDV on the cells. This virus activity had been studied by observing the cytopathic effects (CPEs) formation on the Vero cells after the virus was inoculated on the cells. By using an anti-viral activity procedure, the effect of *piperine* was assayed by Optical Absorbance Method using ELISA Reader. Result from this study was calculated by using a factorial design with SPSS software to determine the significant differences between all factors in this study.

Keyword: Newcastle Disease Virus, Piperine, Dulbecco’s Modified Eagle Medium, Cytopathic, ELISA.

ABSTRAK

Newcastle Disease Virus (NDV) adalah agen bagi Newcastle Disease yang merupakan penyakit utama dalam industri penternakan. Industri ini memainkan peranan penting untuk membekalkan makanan dan sumber kepada ekonomi negara. Tujuan utama kajian ini adalah untuk menentukan aktiviti ekstrak anti-virus tambah *piperine* terhadap NDV. Kultur sel-sel Vero dalam media Dulbecco’s Modified Eagle Medium (DMEM) digunakan untuk mengekalkan NDV di atas sel-sel tersebut. Aktiviti virus ini telah dikaafi dengan memerhatikan kesan-kesan sitofatik di atas sel-sel Vero selepas virus tersebut diinokulasi di atas sel-sel tersebut. Dengan menggunakan prosedur aktiviti anti-virus, kesan *piperine* telah dikaafi dengan menggunakan kaedah serapan optik yang diukur dengan alat ELISA Reader. Kesimpulan daripada kajian ini telah dihasilkan dengan menggunakan kaedah penjekoran dengan perisian SPSS untuk mengenalpasti perbezaan berteri antara faktor-faktor dalam kajian ini.

Kata kunci: Newcastle Disease Virus, Piperine, Dulbecco’s Modified Eagle Medium, Cytopathic, ELISA.
# List of Abbreviations

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<tr>
<td>LC$_{50}$</td>
<td>Lethal Concentration (Concentration of extract that cause 50% cell damage)</td>
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<td>TCID$_{50}$</td>
<td>Tissue Culture Infectious Dose Fifty Percent (Dilution that cause 50% infection to cell culture)</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunassay</td>
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<td>NaOH</td>
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1.0 Introduction

The first outbreaks of Newcastle Disease (ND) were recorded in 1926 in Newcastle, England and getting its name from the town. On the same year, the ND was also reported in Java, Indonesia and was initially found to be affecting the South Asia Region. In Malaysia, the disease was reported in 1934 and the vaccination of chickens against ND has been carried out since 1947 (Vegad, 2004; Zamri, 2006). Zamri (2006) defines Newcastle Disease Virus (NDV) as a contagious viral infection causing respiratory and nervous disorder in several species of fowl such as chicken, Japanese quail and turkeys. He also stated that the ND was caused by an avian paramyxovirus serotype 1 (APMV-1) and it could have considerable antigenic variation between different strains of NDV.

According to Wakamatsu et al. (2006) the NDV infected approximately 236 species of pets and free-living birds such as chicken, turkey, goose, duck and pigeon. They also stated that among poultry, chickens were most susceptible whereas ducks and geese were least susceptible.

A study conducted by Fatimah et al. (1993) found that this disease caused a big problem to the poultry industry because the industry plays an important role in an economy and food requirements of the Malaysian population. They also stated that the industry had transformed itself from subsistence farming to a highly modern and efficient production system.

The ND can cause large economic losses to farmers when the disease infects the industry. These economic losses arise from high mortality, retarded growth, low fertility
and poor hatchability, low product quality and responsible for cost of any treatment or vaccination (Fatimah et al., 1993).

Although ND can be controlled by vaccination, but the control is not fully effective. Therefore, the main focus in this study was to determine an anti-viral activity by using Pepper sp. extract which was piperine against NDV. Piperine was categorized as a potential substance of phytochemical in black pepper (Piper nigrum). It had a bioavailability-enhancing activity for some nutritional substances and for some drugs (Khan et al., 2005).

Cowan (1999) categorized that piperine in an alkaloid class and had an activity as an anti-microbe against Lactobacillus, Micrococcus, E.coli, E. faecalis and fungi. He stated that the properties of alkaloid might be useful against Human Immunodeficiency Virus (HIV) infection as well as intestinal infections associated with Acquire Immunodeficiency Disease Syndrome (AIDS). He also stated that alkaloid had microbiocidal effects and potentially effective against trypanosomes and plasmodia.

Therefore, the objectives of this study are listed as below:

1. to determine an anti-viral activity of Piper spp. extract which a piperine against NDV.

2. to determine a tissue culture infectious dose of NDV on Vero cells culture by TCID₅₀ assay.

3. to observe the CPEs formation on the Vero cells culture after inoculation of NDV on the Vero cells.

4. to determine a lethal concentration of piperine by LC₅₀ assay.
2.0 Literature Review

2.1 Newcastle Disease Virus

NDV is classified in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus* (Wakamatsu et al., 2006). The NDV can cause ND which usually leads to acute and sudden death of many birds in the affected flocks.

NDV virus has single stranded, linear and negatively charge of RNA. The total genomic RNA of this virus is approximately 15,186 nucleotides with six major genes encoding the structural proteins in the 5' to 3' direction (Piacenti et al., 2006). According to Reynolds (2000), the virus has about the six major genes in the genomic RNA. The genome RNA of this virus encode six polypeptides which are L protein (polymerase), F (fusion) protein, HN (hemaglutinin-neuraminidase) protein, M (matrix) protein, P (phosphorylated nuclear protein) and NP (nucleoprotein). The F protein is known to be a major determinant of NDV virulence and the HN protein can contribute significantly to viral spread in the host (Piacenti et al., 2006).

According to Vegad (2004), NDV is categorizes as fatal viral disease affecting most species of birds without showing any symptoms. The NDV can be categorized into five strains based on its virulence. He stated that the NDV having low or no virulence is termed as lentogenic; medium pathogenic strain is termed as mesogenic; highly virulence is termed as velogenic; highly powerful virulence causing haemorrhage termed as viscerotropic velogenic; highly powerful virulence causing mortality followed by respiratory and nervous symptoms termed as neurotropic velogenic and lastly virus that
causes intestinal infection without showing any symptoms is termed as asymptomatic enteric virus.

This type of virus replicates in cytoplasm of the host cell after infection process. The replication process includes production of the new virus proteins and copies of the NDV genetic material. The progeny virus particles are released from infected cell by budding off from the cell (Peeples et al., 1992). Transmission process of NDV is direct contact between healthy cells and the infected cells. The HN and F proteins have been shown to be the viral antigens responsible for inducing neutralizing antibodies (Reynolds, 2000).

The NDV can survive for several weeks in a warm and humid environment on birds' feathers, manure, and other materials. It can survive for extremely long periods in frozen material. However, the virus is destroyed rapidly by dehydration and by the ultraviolet rays in sunlight (USDA, 2003).

The entry of enveloped virus into a susceptible cell is either by a engulfing process which the enveloped virus is enclosed by a membrane of susceptible cell into the interior of the cell or by a process of membrane interaction in which the viral envelop and cell membrane fuse together with resulting liberation of the nucleocapsid.
2.2 Newcastle Disease

A death rate of almost 100 percent can occur in unvaccinated poultry flocks caused by ND and this ND can also cause death even in vaccinated poultry. According to United State Department of Agriculture (USDA) (2003), approximately 932 infected premises were identified infected by ND and millions of birds were destroyed. The eradication effort cost was approximately USD168 million.

ND is caused by NDV (Vegad, 2004; Zamri, 2006). Velogenic strains of the virus can cause death to flock after infection without showing any sign of symptoms but for lentogenic and mesogenic virus, the disease lasts longer and has characteristic symptoms. For examples, ND is characterized by acute lethal infection with haemorrhagic lesions in the intestines is caused by viscerotropic velogenic NDV and another strain known as neuropathic velogenic NDV can cause high mortality following respiratory or neurological disease (Zamri, 2006).

A study conducted by USDA (2003) has characterized all the symptoms of infected bird by the ND. All the symptoms such as sneezing, gasping for air, nasal discharge, coughing, greenish, watery diarrhea, depression, muscular tremors, drooping wings, twisted of head and neck, circling, complete paralysis, partial to complete drop in egg production, production of thin-shelled eggs, swelling of the tissues around both eyes and neck and sudden death.

The ND is spread by direct contact between healthy birds with infected birds. This disease infects healthy birds through infected birds' droppings and secretion from nose,
mouth and eyes. Maintenance procedure by farmers also can spread the disease if the farmers have direct contact with infected birds then with health birds (APHIS, 2003).

The treatment for this disease is done by vaccination. The normal or usual vaccine is B1 and La Sota. These two vaccines are most successful and used widely by dropping into nostril, eye, drinking water and applied in spray form (Aini et al., 2006).

In Malaysia, the common vaccine used for vaccination is Lyophilised Newcastle Disease ‘F’ Vaccine, Australian Lentogenic V4 NDV and recently involved live and inactivated vaccines such as La Sota, Ulster 2C, NDV-6/10 and VG-VA. Other treatment includes the tunnel ventilated closed-house system in VG-GA vaccines (Aini et al., 2006).

2.3 Anti-viral Activity of Piperine

Piperine is an extract alkaloid compound found naturally from the plants in Piperaceae family. The examples Piper spp. in this family are Piper nigrum L., commonly known as black pepper and Piper longum L., commonly known as long pepper. This piperine comprises 1 to 99% of these plants (Badmacev et al., 1999).

Piperine is an active phenolic component of black pepper and long pepper. It is a potent inhibitor of mixed function oxygenase systems and a nonspecific inhibitor of p450 isoenzymes (Pradeep et al., 2002). It is also a weak base and tasteless but leaves a burning after taste. It belongs to the vanilloid family of compound and includes capsaicin. Its molecular formula is C₁₇H₁₉NO₃ and its molecular weight is 285.34 daltons. It is the
trans-trans stereoisomer of 1-piperoylpiperidine. It is also known as (E, E)-1-piperoylpiperidine (Atal et al., 1985).

Piperine has a bioavailability property. Bioavailability means enhancing activity for some nutritional substances and for some drugs. It has an anti-inflammatory activity and may have in promoting digestive processes (Majumdar et al., 1990). Demonstration on human and animal via in vitro, piperine shows the bioavailability of numerous drug and some nutritional supplements (Majeed et al., 1999).

2.4 Vero Cell Lines

According to Ahamed et al. (2004) since 1050s animal cells are used in culture for cultivation of virus growth. Vero cells are fibroblast like cells and anchorage dependent in culture. Vero cell lines are suitable host cell for NDV to adapt because the NDV is purposely adapted to alter growth and virulence characteristics. It is suitable for cultivation, mass propagation attenuation and genetic modification of NDV.

Vero cell lines are isolated from kidney epithelial cells of Africa Green Monkey (Cercopithecus aethiops). They were established by Yasumura and Kawakita at Chiba University in Chiba, Japan on 27th March 1962. Although the Vero cells are used widely in transfection and vaccine production, they are also used for detection of verotoxins (Yasumura et al., 1963).
2.5 Adaptation of NDV on Vero Cell Lines

The NDV can grow within different animal cells including primary cell culture and established cell lines. During adaptation, there may emerge variants capable of multiplying more efficiently in the host cells used than the original wild type virus. The infectivity of the virus to the Vero cell lines is increased but the virulence is decreased (Ahamed et al., 2004).

In order to adapt the NDV on the Vero cell lines, the Vero cells is incubated in the 25 cm² flask at 37°C until the formation of semi-confluent monolayer before the NDV is infected on the Vero cells (Ahamed et al., 2004). The optimal temperature of infectivity and reproducibility of NDV to any host is at 37°C (Foster et al., 1957).

2.6 Cytopathic Effects

During first passage infection activity of wild NDV, wild NDV do not show any symptoms or no clear evidences of cytopathic effects. This is because the NDV just started it adaptation process and its infectivity process is very low (Ahamed et al., 2004).

After 24 hours of incubation following infection process in third passage, there are some changes developed in monolayer Vero cell lines. Unfortunately, the cytopathic effects of NDV on Vero cells still cannot be found (Ahamed et al., 2004).

During fourth and fifth passages, cytopathic effects rapidly develops and consistent. Within 24 hours of infection, no cytopathic effects are found but after 24
hours of infection the Vero cell starts to change in shape to produce cytopathic effects (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).
3.0 Materials and Methods

3.1 Source of Piper sp. Extract (Piperine)

The extract that had been used in this study was a piperine which was extracted from black pepper (Piper nigrum L). There were two types of extract that had been used, first was a commercial extract ordered from Acros Organics Company, New Jersey, USA with product no. 381450050 and the second one was a crude extract obtained from Pepper Board Malaysia, Kuching. These two types of extract were stored at a room temperature in the Virology Laboratory, Faculty of Resource Science and Technology (FRST), Universiti Malaysia Sarawak (UNIMAS).

3.2 Source of Vero Cells Culture

Vero cells culture from African Green Monkey Kidney were used in this study. These cells were fibroblast-like cells and had fast growth rate, thus the medium replacement was done after two or three days. These Vero cell cultures were obtained from Virology Laboratory, Faculty of Veterinary Medicine and Animal Science, Universiti Putra Malaysia (UPM). The cells were incubated at temperature 37°C with 5% of CO₂. The cells in the T-flask were observed frequently in order to maintain the growth medium and prevent the Vero cells from becoming confluent. The sub-culture process was done when the Vero cells were too confluent.
3.3 Source of NDV

NDV was supplied by Virology Laboratory, Faculty of Veterinary Medicine and Animal Science, UPM. This NDV was stored in freezer at -4°C for short term storage and -80°C for long term storage in. Before using this virus, the virus was diluted to $10^3$ TCID$_{50}$ and $10^6$ TCID$_{50}$ dilution. The dilution procedure was done via aseptic tools in laminar fume hood and all the safety aspect was considered first as a prevention way for all possibility.

3.4 Tissue Culture Infectious Dose (TCID$_{50}$) Assay

A volume of 100 µl of cells suspension ($1.25 \times 10^5$ cell/ml) was subcultured into a micro-titer plate and was incubated for 24 hours. The microtitre plate was then washed by PBS after discarded the previous medium of each well. 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 cytopathic effect was observed every day.

3.5 Preparation of Piper sp. Extract (Piperine)

There were two types of piperine that had been used in this study which a crude and commercial piperine. This extract preparation was based on Yip (2007) method with some modifications to prepare 0.1 and 0.01 LC$_{50}$ concentration. To prepare a stock solution, 5 mg extract was suspended into 100 µl DMSO and homogenized using a Crest™ sonicator for 30 minutes followed by adding 900 µl of DMEM with 2% fetal bovine serum (FBS). Subsequently, for the working extract of 0.1 LC$_{50}$ concentrations,
0.03 ml of stock was transferred into new vial and mixed with DMEM with 2% FBS until the volume reached 1.5 ml. For preparing the working concentration of extract 0.01 LC₅₀ concentrations, 0.15 ml solution from 0.1 LC₅₀ was mixed with 2% DMEM to final volume of 1.5 ml.

3.6 Preparation of Vero Cells Culture

Preparation of Vero cells culture was based on Heah (2007) method with some modifications. Vero cells were cultured on the Dubcco’s Modified Eagle Medium (DMEM) with 10% of Fetal Calf Serum (FCS) for overnight at temperature 37°C and 5% of CO₂. After cell monolayers were obtained, the growth medium was discarded and the cells were washed with 5.0 ml of sterile Phosphate Bovine Saline (PBS), pH 7.2. This step was repeated twice before the cells were trypsinized by adding several drops of trypsin.

3.7 Cells Plating Process

Cells plating process was based on the Heah (2007) method with some modifications. Vero cells suspension in the growth medium (DMEM) that contained 10% FBS was mixed completely first before divided to micro-titer plate. The live cells were counted by using hemacytometer. Subsequently, the cells were diluted by particular volume of medium until the amount of live cells was $1.25 \times 10^5$ cell/ml. After dilution, cells suspension was cultured in DMEM with 5% of FBS at temperature 37°C. All the steps were done under sterile condition in laminar fume hood.
3.8 Anti-viral Activity Test

Anti-viral activity was based on Ahmad et al. (1993) and Heah (2007) methods with some modifications. The anti-viral activity test was done toward two types of piperine which a crude and commercial extract. A volume of 100 µl of cells suspension (1.25 x 10^8 cell/ml) were put into sterile micro-titer plate and was incubated for overnight at temperature 37°C with 5% of CO₂. The medium was discarded when the cells achieved 70-80% confluent and was washed twice with 200 µl PBS before either extract or virus was added into each well. The anti-viral activity was done by following the [(C+V) + E] protocol. The flow chart about the protocol was shown as in appendix 6.6. This test was done by four replicates for each eight tested extract and ten controls were used for this study. All the description about the controls is listed in the Appendix 6.6.

3.8.1 [(C+V) + E] Protocol

For this protocol, piperine extract (E) was added after cells (C) was inoculated first with virus (V). This protocol was started by inoculation of 10 µl of NDV (10^7 and 10^6 TCID₅₀) on the cells into specific well as shown in the Appendix 6.11. After an hour of incubation at temperature 37°C, a volume of 100 µl of anti-viral extract which a piperine at 0.01 LC₅₀ and 0.1 LC₅₀ concentrations was added into specific well as shown in the Appendix 6.11. The microtiter plate was incubated for 48 hours with 5% of CO₂ at 37°C. Subsequently, the plate was proceeded to plate processing.
3.9 Plate Processing

The plate processing was based on Ahmad et al (1993), Marini (1996) and Heah (2007) methods with some modifications. The growth medium was discarded and the cells culture were fixed by adding 125 µl cold trichloroacetic acid (TCA) (25%) before were incubated in fridge for an hour. Subsequently, the TCA was discarded and 100 µl of crystal violet (25%) were added into each well. The micro-titer plate was left on the bench for 15 minutes at room temperature. The crystal violet was discarded by running the tap water on the plate gently. The micro-titer plate was air-dried at the room temperature and was left in dark before being analyzed by Optical Absorbance Method using ELISA Reader.

3.10 Statistical Analysis

All the data obtained from ELISA assay was analyzed by using Factorial Design (FD) via SPSS software to determine the significant differences between three factors in this study. The three factors in this study were (i) type of piperine extract either crude or commercial (ii) two concentrations of LC₅₀ for piperine either 0.91 LC₅₀ or 0.1 LC₅₀ concentration and (iii) two concentration of TCID₅₀ for virus either 10⁻³ TCID₅₀ or 10⁻⁹ TCID₅₀ dilution. The value at P<0.005 level was assumed as significance.
4.0 Result and Discussion

4.1 Tissue Culture Infectious Dose (TCID<sub>50</sub>) Assay for NDV

In this study, the CPEs formation was noticed after five days inoculation. The CPEs should be formed on the Vero cells after inoculation at least after 24 hours (Ahamed <i>et al.</i>, 2004). Unfortunately, there were no CPEs seen on the Vero cell on the first day (Figure 1).

From the results, Vero cells showed the CPEs including the control well (Figure 2). The control well should not show any CPEs because the well is free from the virus. From the observation, it could be considered that the suitable dilutions for TCID<sub>50</sub> concentration were 1 x 10<sup>-3</sup> (Figure 3) and 1 x 10<sup>-6</sup> dilution (Figure 4).

According to Burleson <i>et al.</i> (1992), they defined TCID<sub>50</sub> as a dilution of virus required to infect 50% of the cell culture inoculated. They also stated that the rate of cellular changes and the patterns of CPE induced by different viruses depend upon the type of cell culture system, the properties of a given virus strain and the concentration of virus in the specimen.

Dilutions of NDV were in range of 1 x 10<sup>-1</sup> to 1 x 10<sup>-6</sup> dilution. The cytopathological damage was allowed and the cells were stained then observed microscopically to see the CPEs. CPEs were characterized by formation of syncytium, giant cell, dendritic-shaped cell and finally plaque (Ahamed <i>et al.</i>, 2004).