UNIVERSITI PUTRA MALAYSIA

IN VITRO INVESTIGATION OF THE EFFECT OF KYLLINGA BREVIFOLIA AS A POTENTIAL PHYTOTHERAPY IN FELINE PARVOVIRUS INFECTION

HANIZAH BINTI HASHIM

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HANIZAH BINTI HASHIM

204153

Dissertation submitted in partial fulfillment of the requirements for the course VPD4999 - Final Year Project in the Department of Veterinary Clinical Studies Universiti Putra Malaysia

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CERTIFICATION

It is hereby certified that we have read this project paper entitle" *In vitro* Investigation of The Effect of *Kyllinga Brevifolia* as a Potential Phytotherapy in Feline Parvovirus Infection" by Hanizah binti Hashim and in our opinion it is satisfactory in term of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project.

_____

ASSOCIATEPROFESSOR DR NOR YASMIN ABD RAHAMAN

DVM, PHD (UPM)

Associate Professor,

Faculty of Veterinary Medicine,

University Putra Malaysia

(Supervisor)

_____

DR NORFITRIAH MOHAMED SOHAIMI

DVM, PHD (UPM)

Senior lecturer,

Faculty of Veterinary Medicine,

University Putra Malaysia

(Co-Supervisor)

DR NUR FAZILA SAULOL HAMID

DVM (UPM), PHD (UNIVERSITY OF LIVERPOOL)

Senior lecturer,

Faculty of Veterinary Medicine,

University Putra Malaysia

(Co-Supervisor)

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ABBREVIATIONS

%	Percent
°C	Degree Celsius
µL	Microliter
ANOVA	Analysis of variance
CBC	Complete blood count
CO2	Carbon dioxide
COVID-19	Coronavirus disease 2019
CPE	Cytopathic effects
CPV	Canine Parvovirus
CRFK	Crandell Ress Feline Kidney Cell
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FPV	Feline Parvovirus
g	Gram
g	G-force

H_0	Null hypothesis
H_a	Alternative hypothesis
HI	Hemagglutination inhibition
HPI	Hours post-infection
Hrs	Hours
IDEEX	Immunodiagnostics
KB	*Kyllinga Brevifolia*
Kb	Kilobase
mg/kg	Milligram per kilogram
mg/ml	Milligram per milliliter
Mya-1	Feline T-lymphoblastoid cell line
NLFK	Norden Laboratory Feline Kidney
nm	Nanometer
NS	Non-structural
ORF	Open reading frames
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SNT	Serum neutralization test

SPSS	Statistical Package for Social Sciences
$TCID_{50}$	50% tissue culture infectious dose
VP	Viral proteins
W/V	Weight per volume

ABSTRAK

Abstrak daripada keras projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek

PENYIASATAN *IN VITRO* MENGENAI POTENSI KESAN *KYLLINGA BREVIFOLIA* SEBAGAI FITOTERAPI BAGI JANGKITAN FELINE PARVOVIRUS

Oleh

HANIZAH BINTI HASHIM

2023

Penyelia: Profesor Madya Dr Nor Yasmin Abd Rahaman

Penyelia Bersama: Dr Norfitriah Mohamed Sohaimi &

Dr Nur Fazila Saulol Hamid

ABSTRACT

Feline panleukopenia, yang disebabkan oleh parvovirus kucing (FPV), ialah penyakit yang mempunya kadar jangkitan dan kematian yang tinggi bagi kucing. FPV tidak bersampul dan merupakan virus DNA bebenang tunggal yang kental dan boleh bertahan di prsekitaran pada jangka masa yang panjang. Terkini, masih tiada rawatan untuk feline panleukopenia, menyebabkan pemilik haiwan di Malaysia memilih alternatif lain seperti memberi kucing mereka pokok *shortleaf spikesedge* atau dikenali sebagai, *Kyllinga brevifolia* (KB), kerana mereka percaya ia boleh merawat jangkitan FPV. Namun, tiada kajian saintifik yang menyatakan mengenai potensi antiviral KB untuk merawat jangkitan FPV. Justeru, untuk menyiasat hal ini, penyelidikan ini dijalankan bagi menyiasat potensi antiviral KB semasa

jangkitan FPV. Asai *cell titer blue* telah digunakan untuk menentukan kesitotoksian KB pada sel Crandell-Rees Feline Kidney Cell (CRFK). FPV pada kepekatan $1x10^5$ $TCID_{50}$ telah diinokulasi ke dalam CRFK cell lines dan dirawat menggunakan KB. Pada masa yang sama CRFK cells diinokulasi dengan hanya PBS dan FPV, digunakan sebagai kawalan negatif dan positif selama 48 jam pada suhu 37°C dengan 5% CO2. *Kyllinga Brevifolia* pada kepekatan 25% menghasilkan kadar peratusan kebolehhidupan sel yang tertinggi (86%) selepas dirawat. Kawalan positif dan CRFK yang telah dirawat dengan KB kedua-duanya menunjukkan kesan sitopati ke atas sel, namun kesan sitopati adalah kurang di dalam sel yang telah dirawat dengan KB. Tambahan pula, terdapat pengurangan sederhana titer FPV di dalam sel yang telah dirawat KB iaitu $1x10^{3.5}$ $TCID_{50.}$ Penemuan ini menunjukkan bahawa KB mengurangkan kesan sitopati dan titer virus pada sel CRFK yang telah dijangkiti FPV. Ia mencadangkan bahawa KB mempunyai potensi sebagai antiviral dalam jangkitan FPV. Namun begitu, kajian *In vivo* diperlukan untuk menentukan kesan antiviral KB terhadap FPV.

Keywords: antiviral; efek sitopati, parvovirus kucing; *Kyllinga Brevifolia*

ABSTRACT

An abstract of the project presented to the Faculty of Veterinary Medicine in Partial fulfillment of the course VPD 4999 - Project

IN VITRO INVESTIGATION OF THE POTENTIAL EFFECT OF *KYLLINGA BREVIFOLIA* AS A PHYTOTHERAPY IN FELINE PARVOVIRUS INFECTION

By

HANIZAH BINTI HASHIM

2023

Supervisor: Associate Professor Dr Nor Yasmin Abd Rahaman

Co- Supervisor: Dr Norfitriah Mohamed Sohaimi &

Dr Nur Fazila Saulol Hamid

Feline panleukopenia, caused by feline parvovirus (FPV), is a disease that has a high morbidity and mortality rate in cats. FPV is a non-enveloped single-stranded DNA virus that is resistant and can persist in the environment for a long time. Currently, there is no treatment available for feline panleukopenia, causing pet owners in Malaysia to choose other alternatives, such as feeding their cats the shortleaf spikesedge plant, also known as *Kyllinga brevifolia* (KB), as they believe it would treat FPV infection. However, there is no scientific report stating the antiviral potential of KB in treating FPV infection. In order to investigate the validity of the claim, this study was carried out to evaluate the potential antiviral effect of KB in FPV infection. Cell titer blue assay was used to determine the cytotoxicity of KB on the Crandell-Rees Feline Kidney Cell (CRFK) cell line. FPV at the concentration of $1x10^5$ $TCID_{50}$ was inoculated in CRFK cell lines and treated with KB,

whereas CRFK cells inoculated with PBS and FPV only served as negative and positive control, respectively for 48 hours at temperature 37°C with 5% CO2. *Kyllinga Brevifolia* at 25% concentration yields the highest percentage of cell viability (86%) after treatment. Positive control and KB-treated CRFK cells showed formation of CPE, but less CPE was observed in KB-treated cells. Additionally, moderate reduction of FPV titers was also observed in KB-treated cells to $1x10^{3.5}$ $TCID_{50}$. The finding shows that KB causes a reduction of CPE and viral titers on CRFK cell lines after infection with FPV, suggesting that it may have potential antiviral properties against feline parvovirus. However, further *in vivo* studies are required to confirm the antiviral properties of KB.

Keywords: antiviral; cytopathic effect, feline parvovirus; *Kyllinga Brevifolia*

CHAPTER 1

|INTRODUCTION

1.1 Background

1.1.1 Feline Parvovirus

Feline Parvovirus (FPV) is a non-enveloped single stranded DNA virus, causes Feline Panleukopenia in cats and other *Felidae* families (Jiao et al., 2020). Infected cats shed the virus through feces, urine, and nasal secretions, contaminating the surroundings and resulting in potential exposure for uninfected cats. It is common for outbreaks of FPV to occur in populations of cats that are not vaccinated (Squires, 2023). According to Kruse (2010) Feline Panleukopenia has a more severe manifestation in kittens, with acute cases showing a mortality rate ranging from 25% to 90%, and peracute infections resulting in 100% mortality. FPV demonstrates a tropism for rapidly dividing cells such as cells in bone marrow, intestine and particularly in lymphoid tissue, leading to immunosuppression, and in the intestinal mucosa, resulting in severe diarrhea (Parrish, 1995).

Currently, no specific treatment is available for Feline Parvovirus infection. The treatments given are palliative and aim solely to reduce the clinical severity of the disease. This may involve measures such as administering fluids to address dehydration resulting from diarrhea and using antibiotics, especially in cases where cats are immunosuppressed, to mitigate the risk of secondary bacterial infections (Awad, 2018). Hence, vaccination is essential for inducing antibody production against FPV (Bergmann et al., 2017)

1.1.2 *Kyllinga Brevifolia*

According to information from the National Parks Board Singapore, *Kyllinga Brevifolia* (KB) belongs to the *Cyperaceae* family, representing a grass-like plant that thrives in tropical climates and is commonly found in both terrestrial and shoreline habitats. Malaysian pet owners have been promoting the shortleaf spikesedge plant, KB as a treatment for FPV on various social media platforms.

1.2 Justification

Currently, there is no established treatment for FPV; only palliative treatments are available. KB is commonly used as a phytotherapy for FPV infection among Malaysian pet owners. Some individuals are even selling this plant on online shopping platforms. However, limited research are available on KB and currently, there is no evidence supporting the claim that KB possesses antiviral properties against FPV infection. This emphasizes the need for more research to explore potential antiviral therapeutic options. Therefore, in order to investigate the validity of the claim, this study was carried out with the following objective:

1.3 Objective

The objective for this study was:

1. To investigate the antiviral effect of *Kyllinga Brevifolia* as a potential phytotherapy for feline parvovirus infection.

1.4 The following were the hypothesis of this study

Null hypothesis (H_0): There is no significant antiviral effect of using *Kyllinga Brevifolia* as a traditional remedy for feline parvovirus.

Alternative hypothesis (H_a): There is a significant antiviral effect of using *Kyllinga Brevifolia* as a traditional remedy for feline parvovirus.

CHAPTER 2

LITERATURE REVIEW

2.1 Feline Parvovirus

2.1.1 Etiology

2.1.1.1 Classification

Feline Parvovirus (FPV), also known as Feline Distemper Virus, is classified under the family of *Parvoviridae*, genus Parvovirus (Odend'hal, 1983). FPV is closely related, sharing the same viral genus with Canine Parvovirus type 2 (CPV-2), with both viruses capable of infecting hosts in the *Felidae* family (Hasırcıoglu et al., 2023).

2.1.1.2 Morphology and Genetic Information

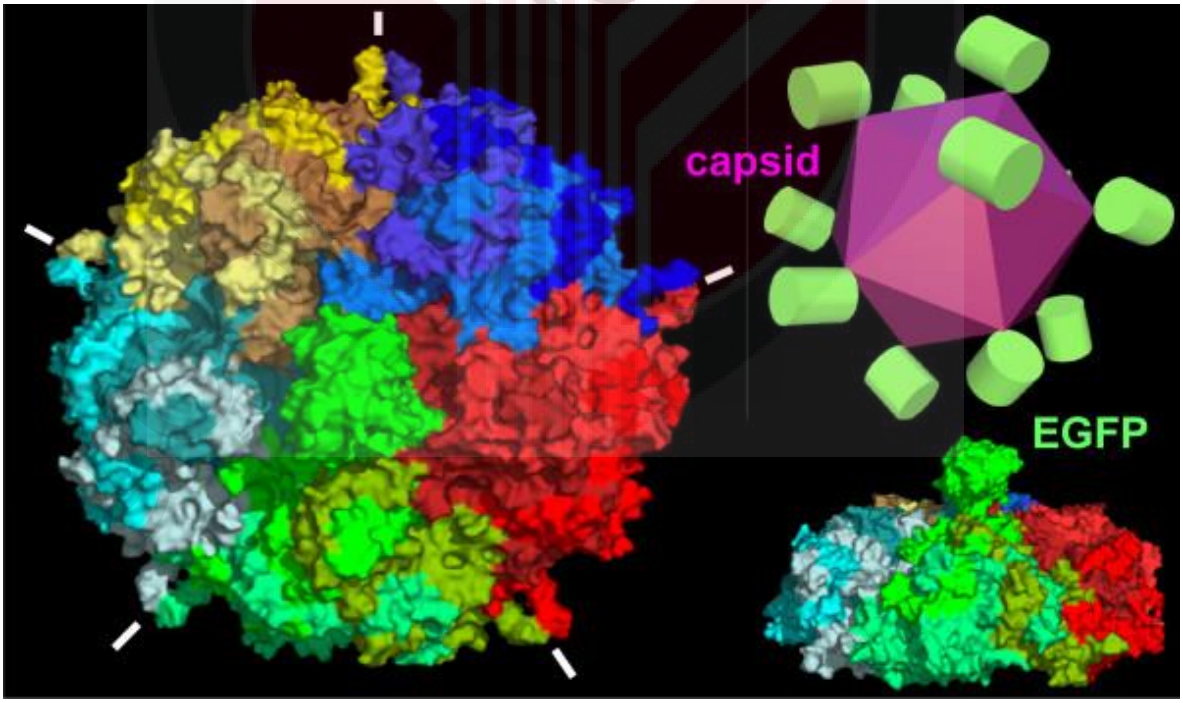

Figure 1. Parvovirus capsid protein. Source from Gilbert et al., (2006)

FPV, which causes feline panleukopenia in cats, is a non-envelope, icosahedral single-stranded DNA virus with linear genome (Figure 1). The genetic material of FPV spans approximately 5.1 kilobases (kb) and comprises two distinct regions known as open reading frames (ORFs); ORF1 and ORF2 (Liu et al., 2023). Within the genome of FPV, ORF1 is responsible for encoding the synthesis of non-structural proteins NS1 and NS2, while ORF2 is responsible for encoding the viral proteins, VP1 and VP2 (Capozza et al., 2021). According to Leal et al. (2020), FPV viral protein (VP) 2 plays an important role for the virus's ability to infect and replicate within a host cell. VP2 is responsible for determining the antigenic properties and host specificity of the virus, as VP2 facilitates transferrin receptor binding, allowing virus entry into the host cell (Govindasamy et al., 2003). VP2 is also crucial for detection of antibodies in the host as it triggers an immune response following infection or vaccination. (Yang et al., 2021).

2.1.2 Epidemiology

2.1.2.1 Mode of Transmission

Infected cats shed FPV in their secretions, including feces, urine, vomit, and saliva, contaminating the environment and exposing other susceptible cats to infection (Janke,et al., 2021). According to Squires (2023), the incubation period for FPV ranges from 2 to 7 days post-infection, and the virus continues to be shed from the infected hosts for up to 6 weeks even after recovery. The primary route of transmission is through the fecal-oral route or contact with contaminated objects (fomites), with intrauterine infection occurring only rarely (Truyen et al., 2009). FPV is highly infectious and resistant, maintaining stability and can persist in the environment for up to one year (Rehme et al., 2022)

2..1.3 Pathogenesis

Feline Parvovirus enters the susceptible host via fecal-oral route. FPV exhibits a tropism for rapidly dividing cells, particularly in lymphoid tissues and intestinal mucosa. FPV infection in the bone marrow causes destruction of erythroid and myeloid colony progenitors, resulting in panleukopenia (Parrish, 2006). Panleukopenia is a condition characterized by a significant reduction in the number of all white blood cells in the body. The compromised ability of the bone marrow to produce white blood cells leads to immunosuppression, increasing the risk of death from secondary bacterial infections. FPV, which also has tropism for the intestinal mucosa, can cause acute gastroenteritis, resulting in diarrhea that may be bloody, leading to severe dehydration and acute death in affected cats (Inthong et al., 2019). When Feline Panleukopenia Virus (FPV) infects pregnant cats, it can result in the transmission of the infection to the neonatal kittens. 'In pregnant cats, FPV infection may lead to abortion or congenital anomalies. Additionally, FPV can cause destruction of Purkinje cells and granule precursor cells situated in the cerebellar external granular layer during the development of the fetus, leading to cerebellar hypoplasia in newborn kittens (Sykes, 2014).

2.1.4 Clinical signs

Clinical signs of FPV infection, such as inappetence, lethargy, fever, and dehydration, are nonspecific and can be observed in other infections. In cases of FPV infection, the clinical manifestations are typically acute, leading to pronounce symptoms like severe dehydration, loss of appetite, and the occurrence of enteritis-associated bloody diarrhea in affected cats (Abdel-Baky et al., 2023). Neurological signs such as ataxia and incoordination, associated with cerebellar hypoplasia can also be observed in newborn kittens whose mothers were exposed to FPV infection during pregnancy (Sykes, 2013).

2.1.5 Pathology

2.1.5.1 Gross lesions

According to Sykes (2013), the intestines of infected cats will appear hemorrhagic, accompanied by lymphadenomegaly in the surrounding oedematous mesenteric lymph nodes. In addition, bone marrow hypoplasia is also evident in these infected cats. A neonatal kitten infected during fetal development may present with a distinctly smaller cerebellum during post mortem (Squires, 2023).

2.1.5.2 Histological lesions

There will be blunting and shortening of the intestinal villi with evidence of enteritis due to destruction of intestinal crypt cells by FPV (Stokes, 2012). FPV, when it infects secondary lymphoid tissues, will also induce destruction and necrosis of lymphocytes (Carlson et al., 1978). Histologically, in the brain, a reduced number of Purkinje cells can be observed, along with indications of segmental loss in both the internal and external granular layers of the cerebellum (Akhtardanesh et al., 2014).

2.1.6 Diagnosis

2.1.6.1 Complete blood count (CBC)

Blood parameters will indicate evidence of panleukopenia, characterized by a decrease in white blood cells, neutropenia, and lymphopenia (Squires, 2023). This is further supported by Stuetzer and Hartmann (2014), that stated, the destruction of bone marrow caused by FPV, will lead to neutropenia and lymphopenia that can be observed in the blood parameters.

2.1.6.2 Rapid test kit

FPV infection is commonly diagnosed using commercially available rapid test kits that have been developed for detecting FPV antigen. For example, SensPERT® Feline Parvovirus Test Kit by Rhone Ma and VETSCAN ® Parvo Rapid Test by Zoetis. A study conducted by Jacobson et al. (2021) found that the IDEXX SNAP Parvo test kit, initially designed for CPV-2 antigen detection, also has high specificity to detect FPV antigen.

2.1.6.3 Molecular test

PCR can also be used for the detection of FPV viral nucleic acid using serum and fecal samples, with real-time PCR providing more advantage due to its capability to quantify the FPV viral load (Streck et. al., 2013) Samples such as blood and feces can be used for PCR for viral detection. (Sykes, 2014). A study by Jacobson et al. (2021), defined a positive PCR outcome characterized by a cycle threshold (Ct) value of 26 or lower, indicating the nucleic acid is present in substantial amount.

2.1.6.4 Serological test

A serological test for diagnosing FPV infection lacks reliability as it is unable to differentiate between antibody production resulting from the actual infection or by vaccination (Truyen, 2009). Detection of antibodies in serum is primarily useful for assessing the production of antibodies following vaccination, rather than being a diagnostic tool for confirming infection. According to Soma et al. (2019), serological tests such as hemagglutination inhibition (HI) and serum neutralization tests (SNT) can be used to detect antibodies against FPV, with SNT demonstrating higher sensitivity.

2.1.6.5 Virus isolation and identification

Based on an *in vitro* study by Miyazawa et al. (1999), FPV can be isolated from either feces or peripheral blood mononuclear cells of an infected cat. Tissue samples, such as the

intestine from an infected cat, can also be used for FPV isolation (Pacini et al., 2023). FPV can be cultured and propagated using feline T lymphoblastoid cells, such as the MYA-1 cell line, or more commonly, the Crandell-Rees Feline Kidney (CRFK) cell line (Miyazawa et al., 1999). Cell lines derived from CRFK, Norden Laboratory feline kidney (NLFK) can also be used for FPV isolation (Kurtzman et al., 1989). According to the findings by Pacini et al. (2023), the cytopathic effect (CPE) on CRFK cell lines becomes noticeable at 48 hours post-infection (hpi), marked by distinctive cellular changes including rounding, elongation, and detachment. CRFK will also showed increased granularity after infection with FPV (Parthiban et al., 2011). Polymerase chain reaction (PCR) is an effective method for confirming the isolation of FPV. (Streck et al., 2013).

2.1.7 Control and prevention

To prevent FPV infection, it is important to administer immunization to all cats, particularly in high-risk areas such as shelters. To establish immunity against FPV, vaccination is recommended at 8-9 weeks of age, with a subsequent dose administered 3-4 weeks later and annual booster to maintain optimal antibody titers (Truyen et al., 2009). Cats that are tested positive for FPV should be isolated from susceptible cats, particularly those that are unvaccinated. Infected cats will shed the virus into the environment through their secretions; therefore, proper disinfection is essential to prevent the spread of the virus. Alcohol is insufficient for effectively killing FPV; household bleach, hydrogen peroxide, or potassium peroxymonosulfate are recommended as suitable disinfectants (Sykes, 2014). Bleach, which contains Sodium Hypochlorite, diluted at a ratio of 1:32 is an effective disinfectant to inactivate FPV (Jaeger, 2001).

2.2 *Kyllinga Brevifolia*

2.2.1 Properties of KB

Figure 2. Shortleaf spikesedge plant, *Kyllinga Brevifolia*

Kyllinga Brevifolia (KB) belongs to family *Cyperaceae* and is classified under grass & grass-like plant that is native to pantropical region according to data from the National Parks Board Singapore (Figure 2). Within the Paraguayan immigrant community, KB is employed as a traditional medicinal remedy, with the community members attributing it with various beneficial properties such as diuretic, sedative and antispasmodic (Helliön-Ibarrola et al., 2016). According to the study by Helliön-Ibarrola et al. (2016), the crude hydro-ethanolic extract of KB influences the central nervous system and exhibits antidepressant-like effects in experiments conducted on mice.

2.2.2 Antiviral properties of KB

Although research on the antiviral properties of Kyllinga is limited, a study has suggested that it may have potential antiviral effects (Leng et al., 2021; Zarina et al., 2023). KB possesses antiviral properties against coronavirus, which causes COVID-19, a pandemic that leads to global lockdown in 2019. According to Leng et al. (2021), Kyllinga demonstrates the ability to inhibit cell death and competitive inhibitory effect to block cellular receptors for

the coronavirus, thereby preventing viral infection. Another study has found that methanolic extract from a root of Kyllinga Nemoralis, which belongs to the same species as KB, has demonstrated the ability to inhibit the binding of Herpes Simplex Virus Type 1 to the receptors of Vero cells based on an *in vitro s*tudy (Ganasen et al., 2022).

Limited research exists that proves the antiviral properties of KB and the available studies primarily concentrate on enveloped viruses like coronavirus.

2.2.3 Method of KB sterilization

Plant extract can be sterilized by using autoclave at 121°C for 15 min (Harjanti et al., 2020). Sterilization can also be done by dry heat, by using oven (Darmady et al., 1961). Both methods required KB to be exposed to high temperatures. There is no available study that states exposing KB to high temperature will disrupt its active compound. However, a study by Monalisa et. al., (2019) found that boiling pumpkin for 20 minutes will cause significant reduction of active compound and antioxidant activity. For this study, sterilization of KB stock solution is by using 0.22 µm membrane filters to remove contaminants (Wickramasinghe et al., 2010).

CHAPTER 3

MATERIALS AND METHOD

3.1 Crandell-Rees Feline Kidney cell lines preparation

Crandell-Rees Feline Kidney Cell (CRFK) cell line is an immortalized cell line derived from feline kidney cells. CRFK in Dulbecco's Modified Eagle's Medium (DMEM) with dimethyl sulfoxide (DMSO) was revived from cryopreservation in a liquid nitrogen tank at a temperature below -130 °C. Following the thawing process, CRFK cell lines were cultured in incubator at (Biobase, China) at 37 °C with 5% CO_2 in a T75 flask (Corning, USA) containing 15 mL of DMEM with low glucose (1g/L) (Gibco, USA) media supplemented with 10% fetal bovine serum (FBS) (Biowest, France) and 1% penicillin (HiMedia, USA). DMEM media was changed after 24 hrs to remove Dimethyl sulfoxide (DMSO) Gibco, USA) which can be toxic to the cells. CRFK cell line was closely monitored for both growth and confluency.

3.2 Sample Preparation, *Kyllinga Brevifolia*

Kyllinga Brevifolia (KB) were air dried for a week to remove its water content. Dried KB were then grounded to form fine powder. To produce stock solution, grounded powder will be mixed with deionized distilled water at a ratio of 1:10 (W/V). After 24 hrs, the mixture will be centrifuged at 700 *g* for 5 mins (Eba, Germany). Supernatant then will be filtered using conventional filter bags then filtered again using Whatman filter paper no 2 (CSI, Malaysia). To sterilize the solution, 0.22 µm membrane filters (Membrane Solutions, United State of America) were used to filter the solution. Stock solution obtained is considered to be at 100% concentration and will be refrigerated at temperature 4 °C (Biobase, China) until further use.

3.2.1 Cytotoxicity testing

KB cytotoxicity on CRFK cells were assessed using CellTiter-Blue® Cell Viability Assay (Promega, USA). 100% stock solutions of KB were diluted to 75%, 50% and 25% concentration using DMEM media. CRFK cells were counted using a haemocytometer (IMS, USA) and 10,000 cells were seeded in each well on a 96-well plate (Corning, USA). 50 µL of samples at different concentrations were then added in 100 µL media with 10,000 CRFK cells. After 48 hrs incubation, media was removed and 20 µL resazurin dye (Promega, USA) were added. Cells were then incubated for another 4 hrs and cell viability at different KB concentrations was measured using a plate reader (Thermo Scientific, Malaysia) at wavelength 570 nm and 600 nm. Statistical analysis was done by using software SPSS by using Analysis of Variance (ANOVA) to test the significant difference between cell viability.

3.3 Feline Parvovirus stock preparation

For viral stock preparation, 10 mL of Phosphate buffer saline was added into 1 g of an intestine of a cat that was previously tested positive for FPV (Sykes, 2013). Mortar and pestle with sterile sand were used to grind the tissue to release viruses from the cell. The mixture was then centrifuged at 1008 g for 10 mins (Eba, Germany). Supernatant was filtered using a 0.22 µm membrane filter, allowing only virus particles to pass the membrane pores. Virus was further propagated in a CRFK cell line that was cultured with 4 mL DMEM containing 2% FBS in a T25 flask (Corning, USA). 200 µL of virus stock were added in each T25 flask. CRFK infected with FPV was then incubated at temperature 37 °C with 5% $CO_2$and CPE was observed after 48 hpi.

3.3.1 $TCID_{50}$ viral titer determination

50% tissue culture infectious dose, $TCID_{50}$ was done to measure the infectious viral titer of FPV based on Reed and Muench method (Lei et al., 2020) Virus stock was diluted with DMEM using serial dilution until dilution 10^{-11}. 100 µL of media with each viral dilution was added into 96-well plates that were seeded with CRFK cell lines. Six replicates were done for each viral diluent and uninfected CRFK cell lines were used as a negative control. After 48 hrs incubation, each well was observed for the presence of CPE

3.4 Testing the potential antiviral properties of *Kyllinga Brevifolia*

The viral stock with a titer of $10^{8.9}$ was diluted using DMEM media to viral titer 10^5 for infection. By using 96-well plates (Corning, USA), 50 µL of KB were added into CRFK in 100 µL of media infected with FPV. 10 replicates were done for the treatment group, CRFK + KB as negative control and CRFK + FPV as negative control. CRFK cell lines infected with FPV were incubated at temperature 37 °C with 5% CO_2. After a 48-hour incubation, the media containing FPV-infected CRFK cells was pipetted out (Eppendorf, Germany). The collected media was then centrifuged at 1008 *g* for 10 mins. Subsequently, the supernatants were carefully collected. The viral stock, post-treatment with KB, underwent serial dilution until viral dilution of 10^{-11}. This process was carried out to determine the new TCID50 of FPV after treatment with KB. Table 1 shows interpretation for log_{10} viral titer reduction:

Table 1: Log_{10} reduction factors in viral clearance studies by Ruppach, (2014)

$\leq$ 1 log_{10}	**Not significant**
1 log_{10} 2 log_{10}	Indicative/contributable
2 log_{10} 4 log_{10}	Moderate
> 4 log_{10}	High

CHAPTER 4

RESULTS

4.1 Characterisation of Feline Parvovirus

4.1.1 Feline Parvovirus cytopathic effects

FPV caused formation of CPE on CRFK 48 hpi, characterized by shrunken, elongated, rounded, and detached cells as shown in Figure 3.

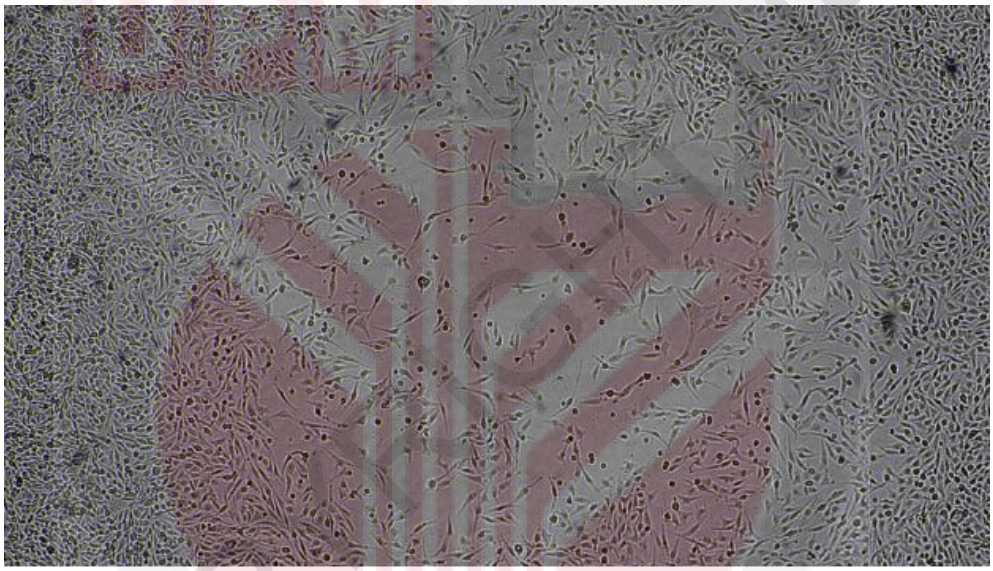

Figure 3. CPE formation caused by FPV. CPE, characterized by shrunken, elongated, rounded, and detached cells, 48 hpi, 10X magnification

4.1.2 $TCID_{50}$ of Feline Parvovirus stock

The FPV viral titer stock was assessed prior to treatment with 25% KB. Table 1 displays the $TCID_{50}$ of the FPV viral stock, $10^{8.9}$. The calculation of the $TCID_{50}$ viral titer followed the Reed and Muench method (Lei et al., 2021).

1. The percentage of wells infected for each dilution was calculated.

2. The proportional distance was calculated , $PD =$

$$\frac{(\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - 50\%)}{\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - \%of\ wells\ infected\ at\ dilution\ below\ 50\%}$$

3. To calculate TCID50, the proportional distance, index was applied to the dilution that produced the percentage infected immediately above 50%

Table 2. TCID50 of FPV

Dilution inoculum	No. of wells infected	No. of wells not infected	Cumulative infected	Cumulative not infected	Percentage infected
10^{-3}	6	0	51	0	100%
10^{-4}	6	0	42	0	100%
10^{-5}	6	0	33	0	100%
10^{-6}	6	0	24	0	100%
10^{-7}	5	1	15	1	90%
10^{-8}	2	4	4	5	44%
10^{-9}	2	4	2	9	18%

4.1.3 Feline Parvovirus confirmation by PCR

For quality control purposes, FPV confirmation via PCR was conducted prior to virus propagation. The small intestine sample from a cat previously confirmed to be infected with FPV tested positive for FPV, showing a 538 bp band, as illustrated in Figure 4.

Figure 4. Agarose gel electrophoresis of the amplification product from the small intestine of an FPV-infected cat. Lane 1: negative control, Lane 2: 100 bp, Lane 3: positive control and Lane 4: small intestine sample of cat previously infected with FPV.

4.2 Cytotoxicity of different concentration of *Kyllinga Brevifolia*

CellTiter-Blue assays estimate the viability of CRFK cell lines by quantifying cellular metabolic activity based on reduction of resazurin dye to resorufin. Following incubation with the CellTiter-Blue reagent, the fluorescence signals are measured using the ELISA plate reader at wavelength 570 nm and 600 nm. The intensity of this fluorescence is proportional to the metabolic activity of the cells.

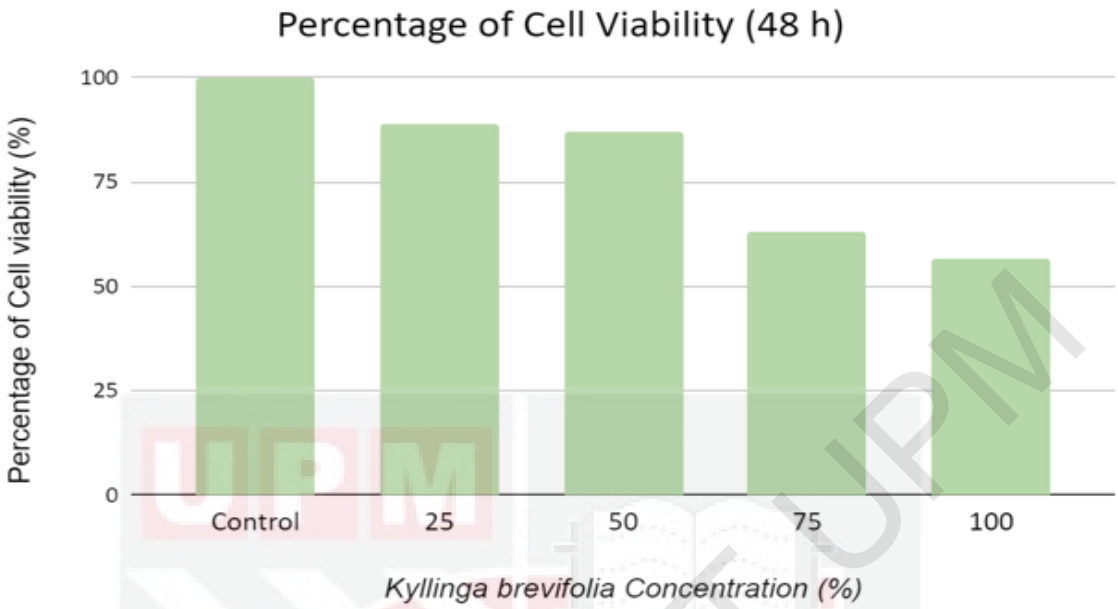

Figure 5. Cell viability in different concentrations of KB. Cell viability exhibits a concentration-dependent response

The viability of CRFK cell lines, as determined by the CellTiter-Blue Cell Viability Assay, exhibits a concentration-dependent response to KB (Figure 5). After incubation, the viabilities and confluences of CRFK observed are as depicted in Figure 6. 100% KB resulted in 56.44%, 75% KB yielded 62.98%, 50% KB resulted in 86.95%, and 25% KB led to 88.69% cell viability. 25% and 50% KB difference in cells viability was no significant ($P>0.05$), however both concentrations shown a significant difference in cells viability compared to 75% and 100% KB ($P<0.05$).

The decision to use 25% concentration of KB for treatment was driven by the cellular morphology of CRFK after introducing different concentrations of KB. Higher KB concentrations resulted in decreased cell confluency and reduced viability.

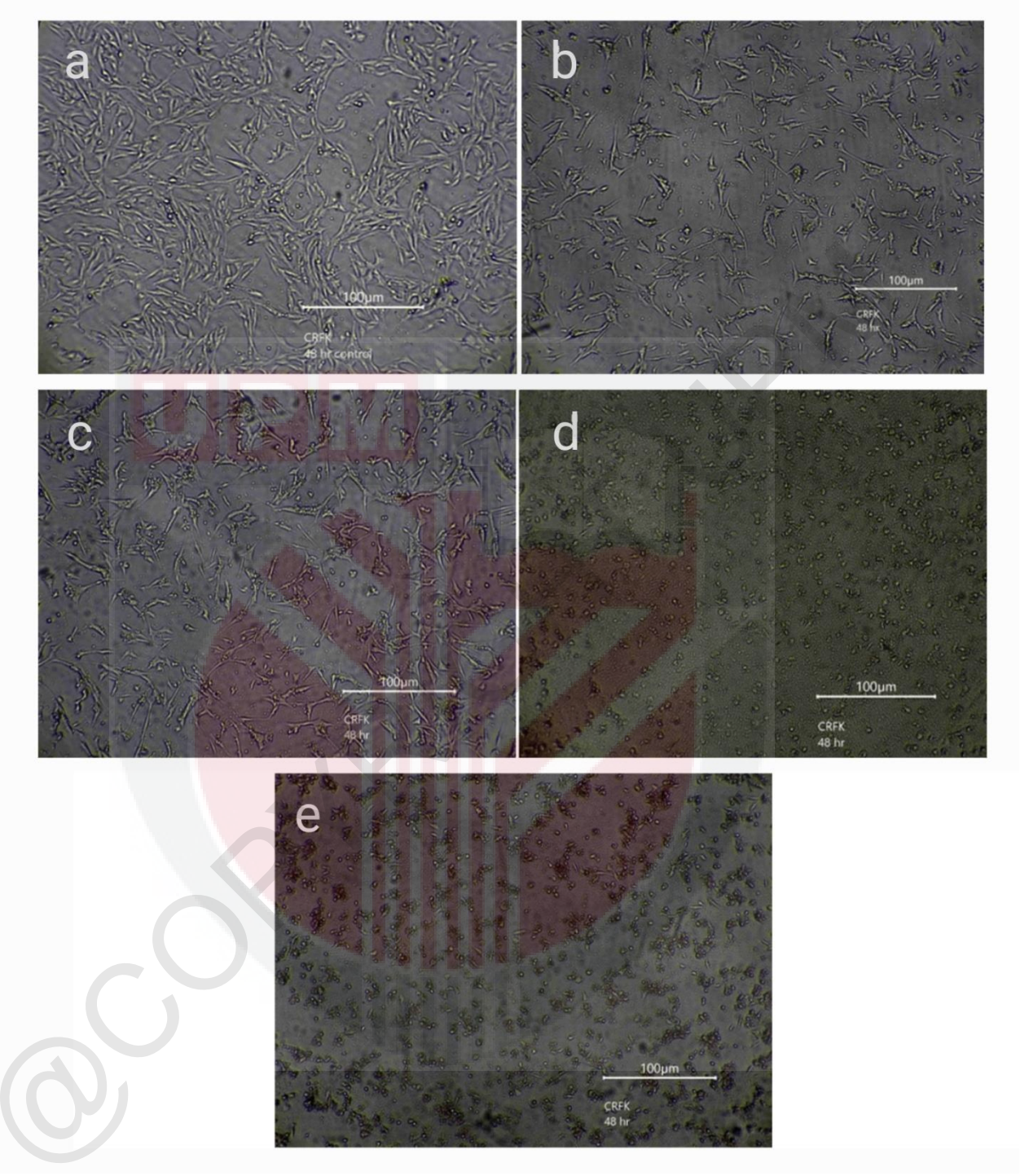

Figure 6. CRFK after 48 hours treatment with KB. Control (a), 25% KB (b), 50% KB (c), 75% KB (d), 100% KB (e) An increase in KB percentage is associated with a decrease in CRFK cell confluency.

4.2 Determining the antiviral properties of *Kyllinga Brevifolia*

Cytopathic effects (CPE) were observed 48 hours post-infection in both the positive control group (CRFK + FPV) and the treatment group (CRFK + FPV + 25% KB). CPE is characterized by shrunken, elongated, rounded and detached CRFK cells. FPV stock was confirmed by PCR with best pair 538 bp. However, less CPE formation was observed in KB-treated cells. The TCID50 value for FPV prior to treatment was $10^{8.9}$.Table 2 shows the $TCID_{50}$ of FPV following treatment with KB. Meanwhile, Figure 7. illustrates the CPE formation in KB treated CRFK cells and non-treated CRFK cells 48 hpi, together with uninfected CRFK cells for comparison.

:

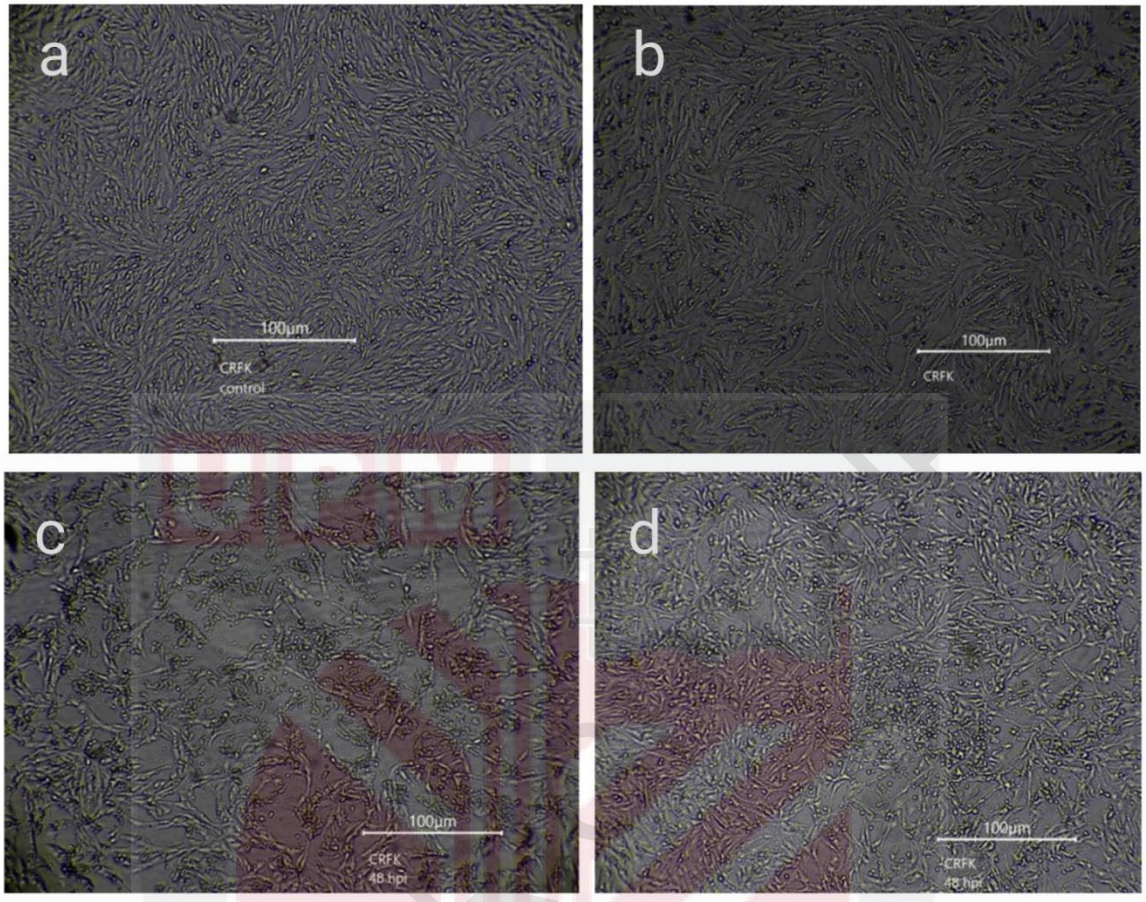

Figure 7. CRFK.KB treated CRFK cells 48 hpi with controls. CRFK (a), CRFK + 25% KB (b), CRFK + FPV 48 hpi (c), CRFK + FPV + 25% KB 48 hpi (d). Less CPE was observed in KB treated cell compared to positive control 48 hpi.

Table 3. TCID50 of FPV after treatment with KB

Dilution inoculum	No. of wells infected	No. of wells not infected	Cumulative infected	Cumulative not infected	Percentage infected
10^{-1}	5	0	10	0	100%
10^{-2}	3	2	5	2	71%
10^{-3}	2	3	2	5	28%

In KB-treated cells, the Feline Parvovirus titer was reduced from 1×10^{5} to $1\times10^{3.5}$ $TCID_{50}$. Infected CRFK treated with 25% KB also showed less CPE formation 48 hpi.

CHAPTER 5

DISCUSSION, CONCLUSIONS AND RECOMMENDATION

5.1 DISCUSSION

Based on this *in vitro* study, KB causes reduced CPE and significant FPV viral titer reduction from 1×10^5 to $1\times10^{3.5}$ $TCID_{50}$ in KB-treated infected CRFK cells. The reduction in FPV titers was contributable according to Ruppach (2014), signifying that the viral titer reduction following treatment with KB was significant. The significant viral titer reduction might indicate that KB might possess potential antiviral properties against FPV infection. These findings might be related to the active compound of KB, which includes flavonoid glycosides and quercetin triglycoside (Apers et al., 2002).

The active compound found in KB, quercetin, has demonstrated antiviral properties against various viruses, as evidenced by several studies (Agarwal et al., 2020; Wu et al., 2015). Notably, quercetin has shown effectiveness against the coronavirus responsible for the onset of COVID-19, a disease that led to a global pandemic in 2019. In the context of coronaviruses, quercetin exhibits antiviral characteristics by impeding virus replication and infection through the inhibition of viral enzymes, as demonstrated by Agarwal et al. (2020). In addition, based on *in vitro* study on MDCK cells by Wu et al. (2015), Quercetin demonstrates antiviral effects against the Influenza A virus by inhibiting the virus's entry into cells, thereby preventing infection.

Nevertheless, there is a lack of studies demonstrating the antiviral properties of the active compounds in KB against FPV. Notably, in *in vitro* study conducted by Saraiva et al. (2002), it was observed that quercetin demonstrated antiviral effects against Canine Parvovirus (CPV). Given that CPV belongs to the same family and genus as FPV and shares close relations with FPV, this finding is particularly noteworthy. The antiviral mechanism, as

highlighted by Kang (2019), involves the inhibition of virus adsorption and penetration into cells. While these observations shed light on the potential antiviral properties of certain compounds, further research is essential to establish the specific efficacy of KB's active compounds against FPV.

KB has the capability to reduce the viral titer of FPV, suggesting its potential as a treatment for FPV infection. Numerous pet owners in Malaysia have been offering KB as treats, and cats have been consuming these grass voluntarily while roaming outdoors. Despite this voluntary ingestion, uncertainties persist regarding the absorption of KB and the precise quantity of active compounds within it that may possess antiviral properties against FPV.

An *in vitro* study conducted on CRFK indicates that high concentrations of KB can potentially exert toxicity on cells, with 25% KB demonstrating the highest cell viability. However, further *in vivo* investigations are essential to establish the optimal non-toxic concentration of KB for treating feline panleukopenia in cats. It is important to determine the optimal non-toxic concentration to provide guidance for cat owners on how to prepare KB for their cats. An *in* vivo study by Helliön-Ibarrola et al. (1999), reported that the intraperitoneal LD_{50} of crude rhizome extract from KB is 575 mg/kg.Compared to this *in vitro* investigation, it was observed that the KB extract exhibited a concentration-dependent increase in toxicity, with no significant difference in cell viability between 25% and 50% ($P>0.05$). However, both 25% and 50% concentrations displayed a significant variance in cell viability when compared to 75% and 100% concentrations of KB ($P<0.05$). It is noteworthy that the study conducted by Helliön-Ibarrola et al. (1999) also utilized an *in vivo* approach, employing crude rhizome extract with KB concentration measured in mg/kg units to assess toxicity, while this study employed an *in vitro* methodology, utilizing an aqueous extract of KB at different percentage concentrations to evaluate toxicity

In addition, *in vivo* studies are needed to confirm the antiviral properties of KB against FPV infection as *in vivo* studies are only suggestive, and not conclusive. The intricate interplay between the active compounds in KB and the virus within a living organism warrants a more holistic investigation to validate and expand upon the potential therapeutic benefits of KB in the context of FPV infection in felines.

KB might possess a potential to be used as a preventive and treatment for FPV infection. However, it is essential to consider the epidemiological triad, which involves virus, host, and environment factors. For virus factor, KB can be used to prevent disease development as it causes significant FPV viral titer reduction. However, the host factor, emphasizing immunization through complete vaccination is more crucial to prevent serious infection. Maintaining a virus-free environment is also crucial, necessitating the use of appropriate disinfectants, particularly in high-risk areas like cat shelters. This comprehensive investigation is vital to enhance our understanding of KB's potential therapeutic benefits and to establish more informed guidelines for its use in feline health management.

5.2 CONCLUSION

In conclusion, based on the *In vitro* study, *Kyllinga Brevifolia* causes a reduced cytopathic effect (CPE) and a decline in viral titer from $10^{5.0}$ to $10^{3.5}$ on CRFK cell lines after infection with FPV. These findings suggest potential antiviral properties against FPV. However, it's important to note that the antiviral efficacy of *Kyllinga Brevifolia* against FPV remains inconclusive and requires further confirmation through further *in vivo* studies.

5.3 RECOMMENDATIONS

In this *in vitro* study, active compound that caused the reduction of viral titers was not identified. Currently there are no studies that provide a thorough analysis of the active compounds in KB, therefore, there are other possibilities a different active compound,

besides quercetin, could be accountable for the reduction in viral titer. Therefore, for further study, it is recommended to identify the active compound that possess the antiviral effects can be identified with high performance liquid chromatography (HPLC) analysis. Additionally, it is recommended to determine the plant part (roots, stems, leaves, flowers) that contains the highest concentration of these active compounds. Lastly, to confirm the antiviral properties of *Kyllinga Brevifolia* against FPV*,* further *in vivo s*tudies are required.

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APPENDIX

Appendix A

One-Way ANOVA Test Results for Cytotoxicity Effects of *Kyllinga Brevifolia* on CRFK at Four Different Concentrations

(I) Kyllinga Brevifolia (%)	(J) Kyllinga Brevifolia (%)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
25	50	16.1966667	5.8743855	.054	-.245364	32.638697
	75	61.2640667*	5.8743855	.000	44.822036	77.706097
	100	73.5497500*	5.8743855	.000	57.107720	89.991780
50	25	-16.1966667	5.8743855	.054	-32.638697	.245364
	75	45.0674000*	5.8743855	.000	28.625370	61.509430
	100	57.3530833*	5.8743855	.000	40.911053	73.795114
75	25	-61.2640667*	5.8743855	.000	-77.706097	-44.822036
	50	-45.0674000*	5.8743855	.000	-61.509430	-28.625370
	100	12.2856833	5.8743855	.190	-4.156347	28.727714
100	25	-73.5497500*	5.8743855	.000	-89.991780	-57.107720
	50	-57.3530833*	5.8743855	.000	-73.795114	-40.911053
	75	-12.2856833	5.8743855	.190	-28.727714	4.156347

*. The mean difference is significant at the 0.05 level.