UNIVERSITI PUTRA MALAYSIA

INVESTIGATION OF THE POTENTIAL ANTIVIRAL EFFECT OF TENDER COCONUT WATER (COCOS NUCIFERA L.) ON FELINE PARVOVIRUS (FPV) INFECTION: AN IN VITRO STUDY

RAFFIDAH BINTI MOHD ISA

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A project paper submitted to the
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CERTIFICATION

It is hereby certified that I have read this project paper entitled “Investigation of The Potential Antiviral Effect of Tender Coconut Water (*Cocos Nucifera L.*) on Feline Parvovirus (FPV) Infection: An *In Vitro* Study”, by Raffidah binti Mohd Isa and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

In the name of Allah, the Most Gracious, the Most Merciful,

I dedicate this thesis to the Almighty Allah, the Source of all knowledge and wisdom. May His blessings guide and illuminate my path. I express gratitude to my family, friends, and supervisor for their unwavering support throughout this academic journey. May this humble effort be pleasing to Allah and contribute positively to the betterment of knowledge and understanding.

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And to all those whose names may not be written here but whose contributions, no matter how small, have played a part in the completion of this thesis.

Thank you for being a part of this journey.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BHK	Baby Hamster Kidney cells21/
bp	Base pairs
CBC	Complete Blood Count
CRFK	Crandell-Rees Feline Kidney Cell
CO_2	Carbon dioxide
CPE	Cytopathic effect
°C	Degree Celsius
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELISA	enzyme-linked immunoassay
FBS	Fetal bovine serum
FPV	Feline Parvovirus
g	Gravitational force
H_0	Null hypothesis
H_a	Alternative hypothesis
HCC38	Human Cancer Cell-38
hpi	hours post-infection
HSD	Honest Significant Difference
MCF7	Michigan Cancer Foundation-7
MCW	Mature Coconut Water

Min	Minute
mL	Milliliter
MLV	Modified live virus
nm	nanometer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p value	Probability value
RPM	revolutions per minute
SPSS	Statistical Package for Social Sciences
TCW	Tender Coconut Water
$TCID_{50}$	50% tissue culture infectious dose
µL	Microliter
µm	Micron
USA	United States of America
VCO	Virgin coconut oil
%	Percent
2D	Two-Dimension
3D	Three-Dimension
40X	40 times

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek Ilmiah Tahun Akhir.

PENYELIDIKAN KEBERKESANAN ANTIVIRAL POTENSI AIR KELAPA MUDA (*COCOS NUCIFERA L.*) KE ATAS JANGKITAN FELINE PARVOVIRUS (FPV): SATU KAJIAN DALAM KAWALAN *IN VITRO*

Oleh

Raffidah Binti Mohd Isa

2023

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Penyelia Bersama: Dr. Norfitriah Mohamed Sohaimi & Dr. Nur Fazila Saulol Hamid

Air kelapa muda (AKM) atau *Cocos nucifera L.* adalah cecair biologi dan steril yang mengandungi pelbagai elektrolit, gula, dan asid amino. Baru-baru ini, dikatakan bahawa ia berkesan dalam merawat panleukopenia kucing yang disebabkan oleh parvovirus kucing (FPV), penyakit yang sangat berjangkit dan mempunyai kematian tinggi pada kucing. Tanda-tanda klinikal penyakit ini termasuk kehilangan selera makan, muntah, demam, cirit-birit, lesu, dehidrasi yang teruk, dan dalam kebanyakan kes, leukopenia yang ketara. Walaupun ramai pemilik haiwan peliharaan

berkongsi pengalaman mereka di media sosial dan laman web bahawa kucing mereka sembuh dari FPV selepas diberi air kelapa muda, setakat ini tiada kajian yang membuktikan kesan antivirusnya. Oleh itu, kajian ini bertujuan untuk menentukan potensi kesan antiviral TCW selepas jangkitan FPV *in-vitro.* Sebelum kajian antiviral dilakukan, kesitotoksian sel ginjal kucing Crandell-Rees (CRFK) selepas menambah steril TCW dalam empat kepekatan berbeza (25%, 50%, 75%, dan 100%) dinilai dengan menggunakan ujian kelansungan Cell Titer-Blue®. Sel CRFK dijangkiti dengan $1x10^5$ $TCID_{50}$/ mL FPV dan dirawat dengan 75% TCW kemudian diinkubasi selama 48 jam pada 37 °C dalam 5% CO_2 dalam plat 96-mikrowell. Sel CRFK yang dijangkiti FPV dan sel CRFK yang diinkubasi dengan PBS masing-masing berfungsi sebagai kawalan positif dan negatif. Kesan sitopatik (CPE) dinilai dalam sel CRFK yang dirawat FPV selepas 48 jam inkubasi dan titer virus yang berjangkit diukur dengan ujian $TCID_{50}$. Hasilnya, kepekatan 75% TCW memberikan peratus kelansungan sel tertinggi berbanding kawalan dan kepekatan lain. CPE diperhatikan dalam kedua-dua kumpulan, sel CRFK yang dirawat TCW dan kawalan positif yang dicirikan oleh sel yang menyusut, memanjang, bulat, dan sel tertanggal. Tambahan pula, $TCID_{50}$ sel CRFK yang dirawat TCW tetap pada $1x10^5$ $TCID_{50}$/ mL. Kesimpulannya, kajian *in-vitro* ini menunjukkan bahawa TCW tidak menyebabkan penurunan titer virus FPV dan tidak mempunyai kesan antiviral seperti yang diperakui oleh kebanyakan pemilik haiwan peliharaan. Walau bagaimanapun, kajian ini memerlukan pengesahan lanjut daripada kajian *in-vivo* dan faktor lain seperti usia, status imun hos dan perjalanan penyakit.

Kata kunci: Kesan antiviral; *in-vitro*; Panleukopenia kucing; Feline parvovirus (FPV); Air kelapa muda steril (TCW)

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 - Final Year Project.

INVESTIGATION OF THE POTENTIAL ANTIVIRAL EFFECT OF TENDER COCONUT WATER (*COCOS NUCIFERA L.*) ON FELINE PARVOVIRUS (FPV) INFECTION: AN *IN VITRO* STUDY

By

Raffidah Binti Mohd Isa

2023

Supervisor: Associate Professor Dr. Nor Yasmin Abd Rahaman

Co-supervisor: Dr. Norfitriah Mohamed Sohaimi & Dr. Nur Fazila Saulol Hamid

Tender coconut water (TCW) or *Cocos nucifera L.* is a biological and sterile liquid that contains a variety of electrolytes, sugars, and amino acids. Recently, it is said to be effective in treating feline panleukopenia caused by feline parvovirus (FPV), a highly infectious disease and high mortality in cats. Clinical signs of this disease include anorexia, vomiting, fever, diarrhea, lethargy, severe dehydration and in most cases, profound leukopenia. Although many pet owners shared their experience on the social media and websites that their cats survived FPV after giving tender coconut water, thus far there is no study proving its antiviral effect.

Therefore, this study aimed to determine the potential antiviral effect of TCW following FPV infection *in-vitro*. Before antiviral study was carried out, the toxicity of Crandell-Rees Feline Kidney Cell (CRFK) after adding sterile TCW in four different concentrations (25%, 50%, 75% and 100%) was assessed by using Cell Titer-Blue® Cell Viability Assay. CRFK cells were infected with $1x10^5$ $TCID_{50}$ of FPV and treated with 75% tender coconut water and then incubated for 48 hours at 37 °C in 5% CO_2 in 96-microwell plates. FPV-infected CRFK cells and CRFK cells incubated with PBS served as positive and negative controls, respectively. Cytopathic effect (CPE) was assessed in FPV-treated CRFK cells after 48 hours of incubation and infectious viral titer was measured by $TCID_{50}$ assays. As a result, the concentration of 75% TCW yielded the highest percentage of cell viability as compared to control and other concentrations. CPEs were observed in both groups, TCW-treated CRFK cells and positive control as characterized by shrunken, elongated, rounded, and detached cells. Additionally, $TCID_{50}$ of TCW-treated CRFK remained at $1x10^5$ $TCID_{50}$/ mL . In conclusion, this *in vitro* study demonstrated that TCW is not causing reduction in FPV virus titers and does not possess any antiviral effects as claimed by most pet owners. Nevertheless, this study requires further confirmations from *in vivo* study and other factors such as age, immune status of the host and course of the disease.

Keywords: Antiviral effect; *in-vitro*; Feline panleukopenia; Feline parvovirus (FPV); Sterile Tender coconut water (TCW)

CHAPTER 1

1.0 INTRODUCTION

Feline parvovirus (FPV) causes feline panleukopenia, which is a highly infectious disease and high mortality in cats. Clinical signs include anorexia, vomiting, fever, diarrhea, lethargy, severe dehydration and in most cases, profound leukopenia (Squires, 2023). Unfortunately, sometimes no obvious signs are present, and a cat will die without apparent warning. FPV also can cause cerebellar hypoplasia in utero or early neonates. The virus is mainly transmitted through fecal–oral route, and the virus is resilient as they are remarkably stable in the environment, remaining infectious for up to a year, depending on the conditions (Janke et al., 2021). Supportive care and vaccination are mainly methods of treatment and prevention for FPV. In acute cases of feline panleukopenia requires supportive care nursing in an isolation unit and prompt fluid therapy together with antibiotics as primary treatments. Intravenous (IV) fluid replacement and maintenance aim is to balance electrolytes in the blood, but it does not cure the disease (Tello & Perez-Freytes, 2017).

Survival rates of 20%–51% have been reported in cats that received supportive treatment for feline panleukopenia while mortality rate of this disease is over 90% in kittens if not treated (Kabir et al., 2023). A study conducted at Shakalaka Vet Care and Surgery in Sabah, Malaysia, 91 out of 1570 cats were diagnosed with feline panleukopenia, resulting in an overall prevalence of 5.8%. The analysis also highlighted that a significant number of cases involved cats aged 1-6 months who were both

unvaccinated and non-neutered (Pacini et al., 2023).

There is limited treatment efficacy, however, vaccination is generally very effective in preventing the disease, even though it does still occur in some high-risk situations. Inactivated and modified-live virus vaccine is used as it provides long-lasting immunity for protection of feline panleukopenia (Rehme et al., 2022). Recently, tender coconut water (TCW) (*Cocos nucifera L.*) is being said to be effective as supportive treatment in treating FPV (Rukmini et al., 2017), leading to a declining rate of cat owners choosing vaccination. Several studies have indicated that TCW is recognized as a natural remedy abundant in nutrients, encompassing vitamins, essential amino acids, palmitic and oleic acids, and dietary minerals (Tuyekar et al., 2021). It is used as a dehydrating agent in cholera, diarrhea, and treatment of cancer, and as a hair nutrient in alopecia (Verma et al., 2012). While numerous pet owners have shared their experiences on social media and websites claiming that their cats recovered from feline panleukopenia after receiving tender coconut water, there is currently no scientific study confirming its antiviral efficacy.

Therefore, the objective of this study was:

1. To determine the potential antiviral effect of tender coconut water in feline parvovirus infection.

Hypothesis of this study was:

Null hypothesis (H_0): Tender coconut water is not effective to treat feline parvovirus.

Alternative hypothesis (H_a): Tender coconut water is effective to treat feline parvovirus.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Feline Parvovirus

Feline parvovirus (FPV) was first discovered in 1928 by Verge and Christoforoni in France (Sykes & Parrish, 2021). FPV is classified as the feline parvovirus subgroup of the genus Parvovirus, within the family *Parvoviridae* (Siegl et al., 1985). FPV is a small, non-enveloped and single-stranded DNA virus which makes it stable in the environment (Figure 1). It can survive at freezing temperatures and room temperatures, and it can also survive the use of certain disinfectants, including iodine and alcohol (Grzyb, 2023). FPV is the main causative agent for feline panleukopenia. FPV can infect not only domestic cats, but also other species such as raccoons, foxes, and minks (Stuetzer & Hartmann, 2014).

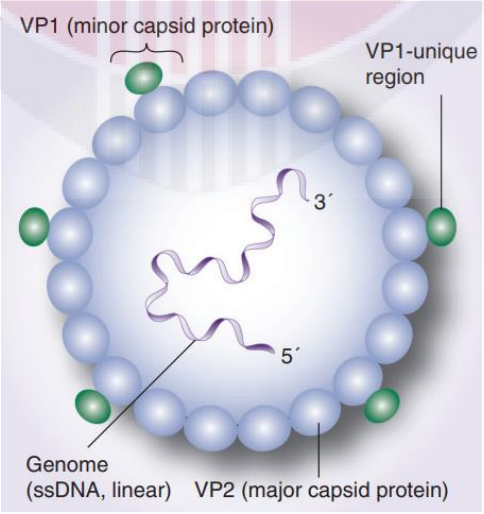


Figure 1: Schematic structure of a parvovirus particle (Plentz & Modrow, 2011).

2.1.1 Transmission of Feline Parvovirus

Feline parvovirus (FPV),also known as feline panleukopenia virus is a highly contagious and potentially deadly virus that affects cats. The virus is primarily transmitted through direct contact with an infected cat or its bodily fluids, as well as through contact with contaminated environments. The virus is shed in high concentrations in the feces, urine, saliva, and nasal secretions of infected cats (Pacini et al., 2023). Direct contact with an infected cat, especially through grooming, biting, or close social interaction, can facilitate the transmission of the virus (Hwang et al., 2018). Feline parvovirus is highly resistant and can survive in the environment for up to a year (Squires, 2023). Contaminated objects, such as food and water bowls, litter boxes, bedding, toys, and even the hands and clothing of caretakers, can be the source of infection to the cat (Mehta, 2021).

2.1.2 Pathogenesis of Feline Parvovirus

The pathogenesis of feline panleukopenia caused by FPV involves a complex series of events that affect the hematopoietic system and other rapidly dividing cells. FPV enters the host primarily through the oral route, often via ingestion of contaminated feces, urine, or fomites (Sykes, 2014). The virus targets rapidly dividing cells, with a predilection for cells in the bone marrow, lymphoid tissues, and the intestinal epithelium. The viral genome is a single-stranded DNA, and its replication is closely linked to host cell replication in nucleus (Caliaro et al., 2019). One hallmark of feline panleukopenia is severe leukopenia, resulting from the destruction of rapidly dividing cells in the bone marrow. This leads to a decrease in white

blood cells, particularly lymphocytes. The lymphoid tissues, including the thymus and lymph nodes, also experience significant damage, contributing to immunosuppression (Url et al., 2003). Intestinal epithelium is a major target of FPV, leading to the characteristic gastrointestinal signs of the disease. The virus causes necrosis of the crypt epithelial cells, resulting in villous atrophy and malabsorption. Diarrhea, vomiting, and dehydration are common clinical manifestations. FPV can also affect the cerebellum and lead to neurological signs, such as ataxia and tremors (Marks, 2016). This neurotropic potential adds another layer to the complex pathogenesis of feline panleukopenia. The severe depletion of white blood cells, damage to the gastrointestinal tract and dehydration can lead to organ failure which contributes to the high mortality rate associated with feline parvovirus (Truyen et al., 2009).

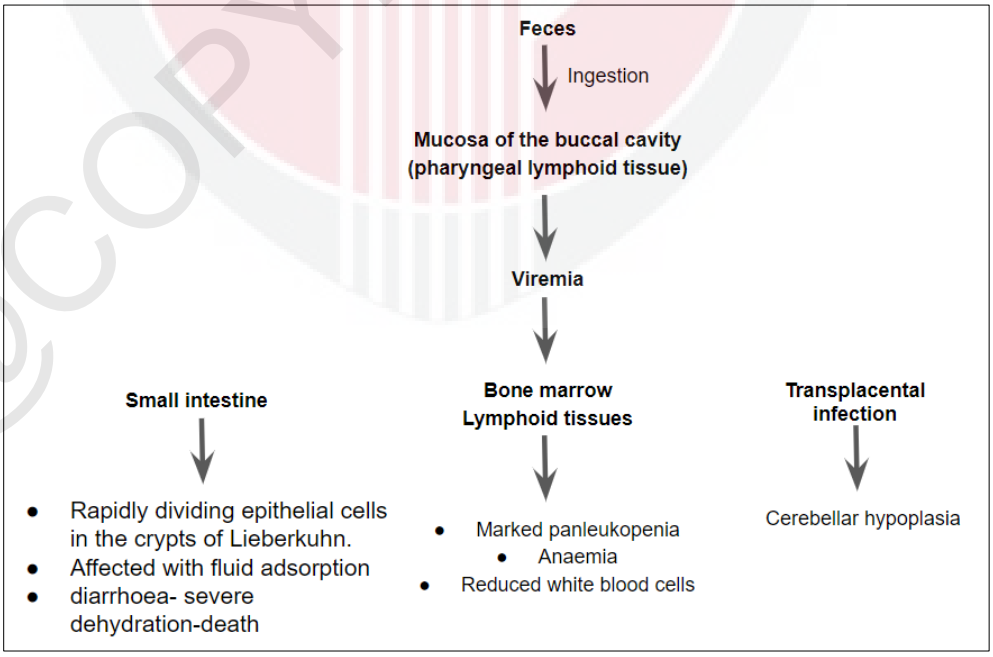


Figure 2: Pathogenesis of Feline Parvovirus (Parrish, 1995)

2.1.3 Clinical signs of Feline Panleukopenia

The majority of feline panleukopenia infections exhibit subclinical manifestations in unvaccinated and healthy cats (Grzyb, 2023). Those felines that present clinical signs are typically less than one year old. Per acute cases may experience sudden and unexpected death, often observed in fading kittens (Munnich , 2022). Acute cases are characterized by a fever ranging from 40 °C to 41.7 °C, accompanied by depression and anorexia following an incubation period of two to seven days. Vomiting, typically bilious and inappetence, tends to emerge one to two days after the onset of fever. Some cases may include hypersalivation, associated with nausea or abdominal pain. While diarrhea may lag vomiting, it is not consistently present, and only 3%–15% of cases involve hemorrhagic diarrhea (Squires, 2023). Rapid onset of extreme dehydration is common, and affected cats may spend extended periods near their water bowls without consuming substantial amounts of water. Terminal cases may experience hypothermia and are at risk of developing septic shock and disseminated intravascular coagulation. The duration of this self-limiting illness rarely exceeds five to seven days, with the highest mortality rates observed in kittens under 5 months old.

2.1.4 Diagnosis of Feline Panleukopenia

Various diagnostic methods, including commercial rapid test kit, complete blood count (CBC), PCR, and virus isolation, are available to confirm feline panleukopenia. Despite these options, a presumptive diagnosis is effectively established through post-mortem and histological examination

(Jacobson et al., 2021). Commercial rapid test kits like SensPERT® Feline Parvovirus Test Kit and IDEXX SNAP Parvo test kit are commonly used for FPV diagnosis, detecting specific antigens in fecal samples with a sensitivity and specificity of 95.8% and 99.7% (Mosalanezhad et al., 2009). CBC parameters reflect panleukopenia, evidenced by decreased white blood cells, neutropenia, and lymphopenia, highlighting the impact of FPV on rapidly dividing cells in the bone marrow and lymphoid tissues (Squires, 2023). PCR is a valuable tool for the rapid, accurate, and sensitive detection of feline parvovirus in cats, identifying subclinical shedding and quantifying viral load, especially post-vaccination with modified live virus (Decaro et al., 2005).

In a specific case study, a presumptive diagnosis of FPV was based on post-mortem findings, revealing abnormalities like thymus shrinkage, intestinal wall thickening, distension, color changes, and bleeding on the outer surface (Sykes, 2014). Additionally, observations included enlarged mesenteric lymph nodes, potential blood-tinged liquid in the intestine, and mucosal lining bleeding. Histopathological analysis showed transmural hemorrhages, widespread fibrin, and platelet accumulation in small gastrointestinal vessels of the stomach, ileum, and colon (Pacini et al., 2023). In the small intestine, inflammatory infiltration of the lamina propria of the villi, crypt dilation, and necrosis were observed, occasionally featuring intranuclear bodies. Virus isolation, a specialized and infrequently used procedure for FPV diagnosis, involves obtaining clinical samples like feces, blood, or tissue, followed by examination for CPE indicating viral replication

(Yang et al., 2022). Additionally, typical parvovirus CPE were observed in virus isolation, where cells appeared shrunken, elongated, rounded, and detached (Pacini et al., 2023). Confirmation of FPV presence is achieved through additional diagnostic methods, including immunofluorescence, PCR, or other molecular techniques, ensuring accurate virus identification (Lyi et al., 2014).

2.1.5 Treatment and Prevention

Dehydration frequently occurs in cats with feline panleukopenia due to intense diarrhea and vomiting. Providing intravenous fluids is a key aspect of supportive care to address dehydration and electrolyte imbalances (Squires, 2023). Malnutrition is a concern in affected cats, and nutritional support, often administered through syringe feeding or specialized diets, helps sustain energy levels and bolster the weakened immune system. It is crucial to control vomiting to maintain hydration and proper nutritional intake, and antiemetic medications can be used for this purpose. While there isn't a specific antiviral drug approved for treating feline panleukopenia, some studies have explored the use of medications like famciclovir and interferon (Ferri et al., 2020). These studies, however, have limitations, highlighting the need for further research to establish the effectiveness of antiviral drugs in treating FPV infection. Vaccination stands out as the primary method for preventing FPV infection. Modified live virus (MLV) vaccines are widely used and have demonstrated high efficacy in providing immunity (Jakel et al., 2012). Given the highly contagious nature of FPV, it's crucial to implement effective hygiene and biosecurity measures

to prevent the virus's spread. This involves proper disinfection of contaminated environments and the isolation of infected individuals (Peng et al., 2023).

2.2 Coconut water

2.2.1 Difference between Tender Coconut and Mature Coconut

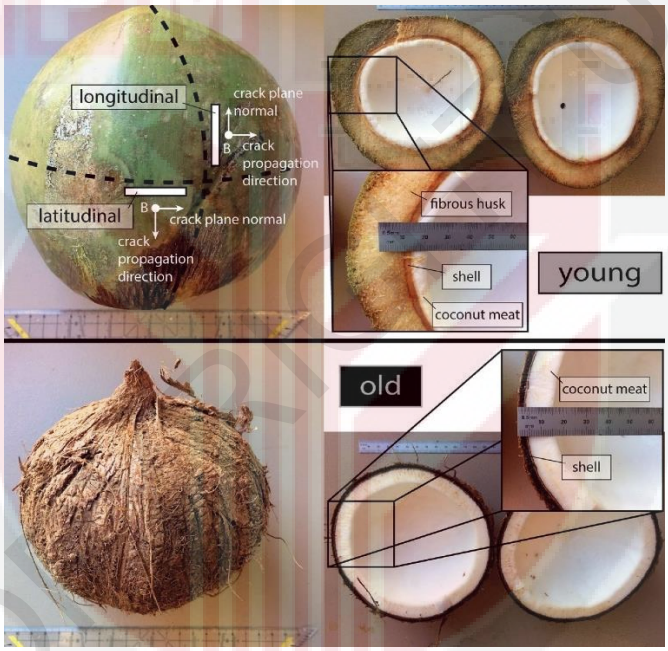


Figure 3: Difference between tender coconut and mature coconut (Gludovatz et al., 2017)

Comparing tender and mature coconuts involves looking at their nutritional content, physical traits, and potential uses. Figure 3 illustrates the distinctions between tender coconut and mature coconut. Tender coconuts, often called green coconuts, are harvested from young palms, and are known for high water content, soft white meat, and a sweet taste. In

contrast, mature coconuts come from older palms, having thicker and harder shells with firmer, mature meat (Leung et al., 1972). Nutritionally, tender coconuts are valued for electrolyte-rich water, making them a popular natural hydrating beverage. The soft meat is rich in vitamins, minerals, and antioxidants, as demonstrated in a study where 100% tender coconut water (TCW) increased dried periodontal ligament cell viability to 2.58% compared to untreated cells at 1.06% (Ali et al., 2011). On the other hand, mature coconuts have denser meat with higher fat levels, and the extracted oil is a significant source of saturated fats. Physical differences extend to the husk, with tender coconuts having a thinner, more flexible shell than the thick, woody husk of mature coconuts (Pandiselvam et al., 2019). These distinctions influence extraction, processing, and utilization methods in various industries.

2.2.2 Phytochemical Constituents of Tender Coconut Water

Studies on tender coconut water (TCW) phytochemical composition have gained interest because of their possible influence on cellular health. TCW contains various bioactive compounds such as caffeic acid, epicatechin and catechin that may contribute to health benefits such as antioxidant, anti-infalmmatory and anti-cancer (Halim et al., 2023). Studies have identified phytochemicals like cytokinin, auxins, and gibberellins in TCW, known for essential roles in cell growth and development (Yong et al., 2009). These phytohormones might influence cellular processes and have potential applications in cell culture systems. Vitamins such as vitamin B1, vitamin B2 and vitamin C to prevent thiamine deficiency in cats (Truyen et al., 2009).

Additionally, TCW includes antioxidants such as ascorbic acid (vitamin C) and flavonoids, associated with anti-inflammatory and antioxidant properties (Lima et al., 2015). A study found that TCW exhibited a higher anti-inflammatory effect (42.52%) during the second phase of edema in rat paw compared to mature coconut water (MCW) (25.94%) (Rao et al., 2016). These compounds may contribute to cellular protection against oxidative like potassium and magnesium, crucial for cellular function (Appaiah et al., 2014). The presence of these minerals and electrolytes makes coconut water a valuable option for hydration and replenishing essential nutrients. Thus, it can overcome electrolyte disturbances such as hypokalemia, hypoglycemia, hypoproteinemia, and anemia in affected cats. The phytochemicals in TCW may have potential cytotoxic effects on certain cancer cells, suggesting a possible role in cancer therapy or prevention (Rudzińska et al., 2023). Coconut water vinegar delayed 4T1 breast cancer progression in mice by inducing apoptosis and delaying metastasis (Mohamad et al., 2019). However, more research is needed to clarify specific mechanisms and potential applications in clinical settings.

2.2.3 Sterilization Method of Tender Coconut Water

The sterilization of TCW is a crucial step to ensure safety, prolong shelf life, and meet quality standards for cell culture. Various methods are used to sterilize TCW, with a focus on their impact on microbiological safety, nutritional content, and overall quality. Autoclaving is one method explored in some studies, involving subjecting the water to high pressure and temperature (1.5 bars and 121°C) for 20 minutes to effectively eliminate

microbial contaminants. However, autoclaving results in nutrient loss, reducing the internal nutrient concentration to 23.5% (Awua et al., 2011). Another common method is membrane filtration, specifically using a 0.22-micron membrane, to remove bacteria and larger microorganisms, ensuring a microbiologically safe product (Bobbitt & Betts, 1992). This process employs porous membranes to physically separate particles based on size, enhancing the throughput of coconut water while maintaining membrane integrity and consistent microbial inactivation compared to other sterilization methods. In conclusion, the literature supports 0.22-micron membrane filtration as a viable and effective method for sterilizing coconut water, offering advantages in terms of microbial safety and preserving sensory and nutritional qualities (Hu & Wu, 2023). Ongoing research aims to refine and optimize this method for broader industry adoption.

2.3 Cell Titer-Blue® Cell Viability Assay

The Cell Titer-Blue Viability Assay is a widely utilized method for evaluating cell viability and proliferation, playing a crucial role in various fields such as cell biology, drug discovery, and toxicology (Riss, 2016). This assay relies on the reduction of the redox dye resazurin to its fluorescent product resorufin by metabolically active cells, offering a quantitative measure of cell viability (Van Den Driessche et al., 2014). Researchers have extensively used the Cell Titer-Blue assay to assess the cytotoxic effects of pharmaceutical compounds, environmental agents, and other substances on various cell lines (Aslanturk, 2018). Its versatility makes it valuable for high-throughput screening, allowing for a swift and reliable evaluation of cell

viability in diverse experimental conditions. The assay's sensitivity and broad applicability extend to both 2D and 3D cell cultures, enhancing its suitability for more accurate modeling of physiological conditions. The Cell Titer-Blue assay has been applied to identify potential confounders in a 2D in vitro drug response screening assay affecting data replicability, reproducibility, and cell viability of MCF7 and HCC38 breast cancer cell lines (Larsson et al., 2020). Comparative studies have explored its reliability and accuracy in comparison to other viability assays, demonstrating its effectiveness and reproducibility. Researchers appreciate the non-destructive nature of the assay, allowing for longitudinal studies on the same cell population. However, it is noteworthy that cell viability above 100% is not uncommon, particularly in the Cell Titer-Blue assay, as random experimental fluctuations within +/- 10% occur due to metabolic activities among cells. Additionally, treatment stimulation may support cell growth and promote cell proliferation, leading to increased metabolism up to 200% of baseline activity (Heinrich, 2012).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Crandell-Rees Feline Kidney (CRFK) Cell Cultivation

The cell lines used in this study were Crandell-Rees Feline Kidney (CRFK). CRFK was cultured in T25 flask (BKMAM, China) and Dulbecco's modified eagle medium (DMEM) (Gibco™, New York) was used which was supplemented with 10% fetal bovine serum (FBS) (Biowest, USA), penicillin (HiMedia, USA), streptomycin (Biowest, USA) and sodium bicarbonate (Sigma-Aldrich, Malaysia) incubated in incubator (BINDER, Germany) at 37°C in 5% CO_2. The CRFK cell lines were normally confluent at 80%-90% in 2 to 3 days. CRFK cell growth and confluency were observed under an inverted microscope (Olympus, Malaysia).

3.2 Feline Parvovirus Stock Preparation

1 g of sterile sand was added into 1 g of cat intestine tissue previously positive with FPV. 1 mL of PBS (Sigma-Aldrich, Malaysia) was added, and tissue was ground using mortar and pestle. 9 mL of PBS was added to make 10% suspension and centrifuged at 1008 *g* (3000 rpm) for 10 min (Benchmark Scientific, China) to separates cells from the supernatant. Supernatant was filtered using a membrane filter 0.22 µm (IT-Tech, Malaysia) and a syringe (IT-Tech, Malaysia) to remove debris and bacteria. Once FPV stock was prepared, virus confirmation was done by PCR before proceeding to virus propagation. Virus propagation was done by adding 200 µL of FPV stock in 4 mL DMEM media into T25 flasks containing CRFK

cells. CRFK cells were observed after 48 hours of incubation at 37°C in 5% CO_2. Virus titration for FPV stock was done by $TCID_{50}$ method, that is applicable to viruses inducing CPE. The titration was performed by placing the virus on an established cell monolayer and the procedure was as follows where CRFK cells were seeded at 10,000 cells/well/100 µL and serial dilution of FPV was done until dilution of 10^{-11}. CPE was observed until viral dilution 10^{-9} after 48 hpi. Once the CPE has stopped progressing, the viral titer was estimated by the Reed and Muench method (Lei et al., 2020). This method evaluates an endpoint where 50% of the cell cultures are infected.

3.3 Collection of Tender Coconut Water

A good quality of TCW was used in this study. Under sterile conditions, the TCW was cut with a large sterile knife until a relatively soft flesh was exposed. A sterile number 15 scalpel blade (Hi-Tech Instruments, Malaysia) was used to cut a triangular piece through the exposed coconut flesh. TCW was filtered by using Corning® 500 mL bottle top vacuum filter (Corning®, China), 0.22 µm to remove debris and bacteria for cell culture.

3.4 *In vitro* Cell Viability Assessment by Cell Titer-Blue® Cell Viability Assay

Four different concentrations of TCW (25%, 50%, 75% & 100) were prepared with DMEM media. 50 µL of TCW for each concentration were added into 96-well plates (Corning®, China) containing 10,000 CRFK cells and incubated for 48 hours as shown in Figure 4. DMEM media was

changed and 20 µL resazurin reagent (Promega, USA) was added. CRFK cells with four different concentrations were incubated for four hours. CRFK cells viability was measured at wavelength 570 nm and 600 nm by Spectramax M3 multi-mode microplate reader (Thermo Fisher Scientific, USA). Cytotoxicity effects of CRFK among four different concentrations of TCW was analyzed by using SPSS software version 29 with One-Way analysis of variance (ANOVA) followed by multiple comparison test using Turkey's Honest Significant Difference (HSD). (See Appendix 1)

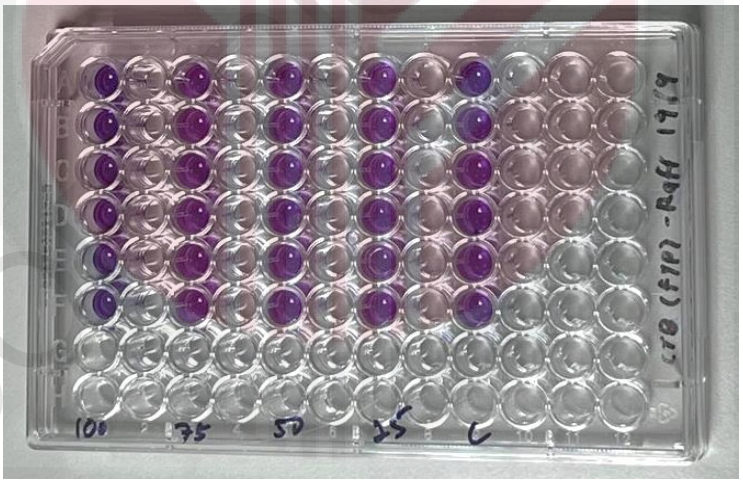


Figure 4: The 96-well plate for cell viability assay of four concentrations of TCW.

3.5 Determination Antiviral Properties in 75% of Tender Coconut Water

The FPV viral stock, with an initial titer of $10^{8.9}$, was diluted in DMEM media to reach a titer of 10^5 when infecting CRFK cells. Dilution was done by

adding 100 µL of virus stock in 900 µL DMEM media, 10 µL diluent was then added to 990 µL media. CRFK cells were infected with viral titer 10^5 $TCID_{50}$/ mL which acts as positive control and CRFK added with 75% TCW acts as negative control. Infected CRFK was treated with 75% of TCW in a 96-microwell plate and incubated for 48 hours at 37 °C in 5% CO_2. CPE was assessed in TCW-treated CRFK cells and infectious viral titers were measured by $TCID_{50}$ assays. FPV viral titer after treated with 75% TCW was compared to the initial FPV viral titer before treated and the reduction was assessed based on Table 1 according to Ruppach (2014).

Table 1: Log_{10} reduction factors in viral clearance studies

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

CHAPTER 4

4.0 RESULT

4.1 Characterisation of Feline Parvovirus Stock

Feline Parvovirus Confirmation by PCR

Figure 5 revealed the PCR result of infected cat's small intestine. Small intestine of cat that previously infected with FPV was confirmed by PCR before virus propagation was done. The PCR result for FPV was positive with 583 bp.

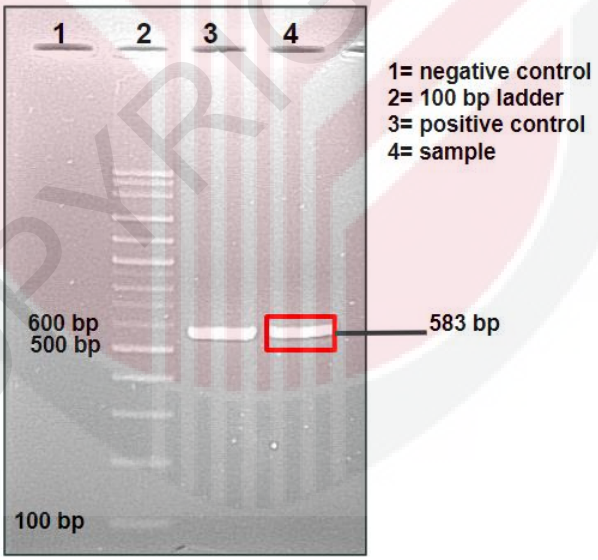


Figure 5: Agarose gel electrophoresis of amplification of feline parvovirus- infected cat. a) Lane 1: negative control, Lane 2: 100 bp, Lane 3: positive control and Lane 4: small intestine sample of cat previously infected with FPV.

$TCID_{50}$ of FPV Stock

Before evaluating the $TCID_{50}$ of FPV following the addition of 75% TCW, $TCID_{50}$ was initially conducted to assess viral infectivity and establish the viral titer of the FPV stock. As indicated in Table 2, CPE was observed until viral dilution 10^{-9} at 48 hpi, and the viral titer was subsequently calculated using the Reed and Muench method (Lei et al., 2020). The calculation involved the formula presented below, resulting in a calculated viral titer of FPV stock at $1X10^{8.9}$ $TCID_{50}$/ mL.

Table 2: Determination $TCID_{50}$ of FPV stock using the Reed–Muench method**.**

Dilution Inoculum	No. of wells infected	No. of wells not infected	Cumulative positive (A)	Cumulative negative (B)	Infected rate (%) A/(A+B)
10^{-3}	6	0	33	0	100
10^{-4}	6	0	27	0	100
10^{-5}	6	0	21	0	100
10^{-6}	6	0	15	0	100
10^{-7}	5	1	9	1	90
10^{-8}	2	4	4	5	44
10^{-9}	2	4	2	9	18
10^{-10}	0	6	0	15	0

$$I = \left[\frac{(\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - 50\%)}{(\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - \%\ of\ wells\ infected\ at\ dilution\ below\ 50\%)}\right]$$

$$= \frac{90\% - 50\%}{90\% - 44\%}$$

$$= 0.9$$

The index is then applied to the dilution that produced the percentage infected immediately above 50%.

= $10^{7.9}$ $TCID_{50}$/0.1 mL

= $10^{8.9}$ $TCID_{50}$ / mL

Feline Parvovirus Cytopathic Effects

FPV stock with viral titer 1X$10^{8.9}$ $TCID_{50}$/ mL was used to infect CRFK cells. Figure 6 showed the formation of CPE, characterized by shrunken, elongated, rounded, and detached cells after 48 hpi under 40X magnification.

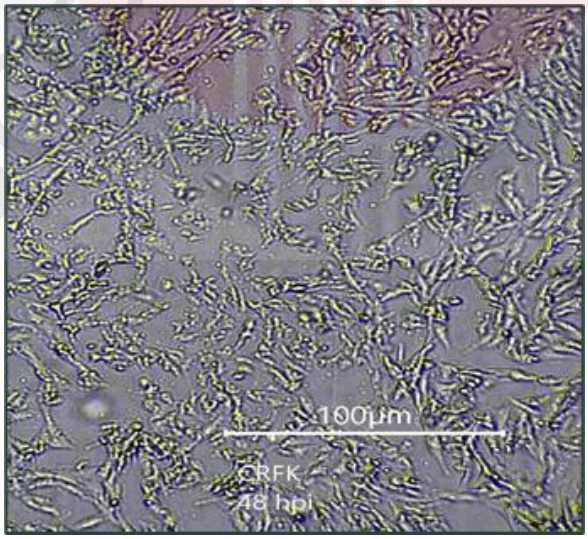


Figure 6: Cytopathic Effects of FPV in CRFK, 48 hpi. 40X magnification.

4.2 Percentage of CRFK Cells Viability in Four Different Concentrations of TCW (25%, 50%, 75% & 100%)

The Cell Titer-Blue assay assessed the viability of CRFK cells by quantifying cellular metabolic activity through the reduction of resazurin dye to resorufin. CRFK cells were measured at wavelengths of 570 nm and 600 nm. CRFK cells viability was calculated following this calculation based on Table 3. The result was presented in Figure 7.

$$Percentage\ of\ cell\ viability = \frac{Average\ absorbance\ ratio\ of\ each\ concentrations}{Average\ absorbance\ ratio\ of\ control} \times 100$$

Table 3: Datasets of CRFK cells viability measured at wavelength 570 nm and 600 nm by Spectramax M3 multi-mode microplate reader.

570 nm	**100%**	**75%**	**50%**	**25%**	**control**
A	1.4803	1.4173	1.3985	1.3938	1.3908
B	1.4737	1.4279	1.4085	1.3445	1.3852
Average	1.477	1.4226	1.4035	1.3938	1.388
600 nm	**100%**	**75%**	**50%**	**25%**	**control**
A	0.7781	0.7213	0.6534	0.6949	0.7264
B	0.9327	0.6981	0.8204	0.8621	0.7405
Average	0.8554	0.7097	0.7369	0.7785	0.73345
Absorbance ratio %	**100%**	**75%**	**50%**	**25%**	**control**
A	190.2454697	196.4924442	214.0342822	200.5756224	191.4647577
B	158.0036453	204.5408967	171.6845441	155.9563856	187.0627954
Average absorbance	**174.1245575**	**200.5166705**	**192.8594132**	**178.266004**	**189.2637766**
Std	22.79841267	5.691115356	29.94578699	31.55056493	3.112657393
% Viability	**92.00099496**	**105.9456142**	**101.8998018**	**94.18918254**	**100**

Based on Figure 7 showed the percentage of CRFK cell viability across four distinct concentrations of TCW (25%, 50%, 75%, and 100%). The results obtained through the Cell Titer-Blue® cell viability assay is outlined as follows. Although there is no significant different ($p>0.05$) for all TCW concentrations (25%, 50%, 75% and 100%), 75% TCW demonstrated the highest CRFK cell viability, reaching 106% when compared to the control which is 100%. The other concentrations, specifically 25%, 50%, and 100%, displayed viabilities of 94%, 101%, and 92%, respectively. Notably, CRFK cells in 100% TCW demonstrated the lowest CRFK cells viability.

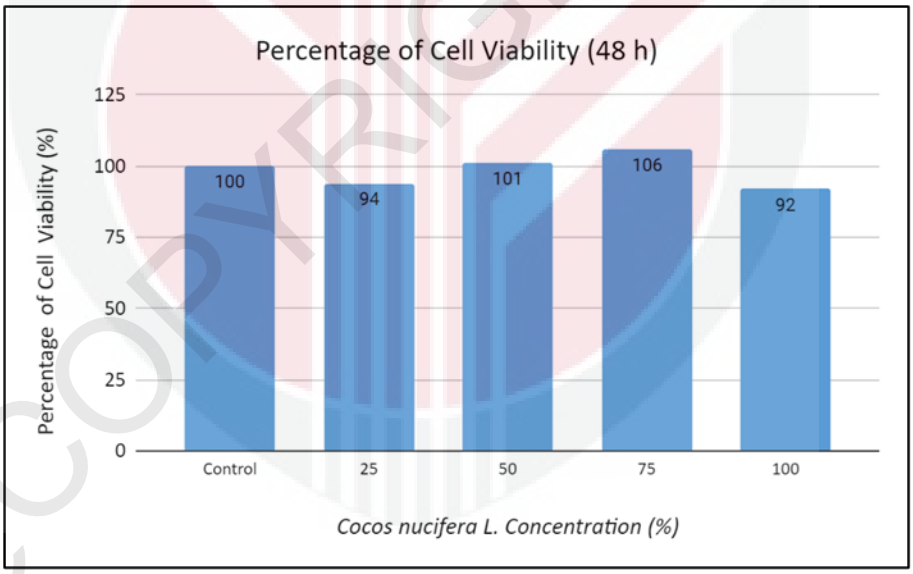


Figure 7: Percentage of CRFK Cells Viability in Four Different Concentration of *Cocos nucifera L.* (25%, 50%, 75% & 100%).

The following outcome illustrates the cytotoxic effects of four different concentrations of TCW following a 48-hour incubation period. According to Figure 8, 75% TCW exhibited the highest confluency of CRFK cells

compared to other concentrations, with 100% TCW resulting in the lowest cell confluency.

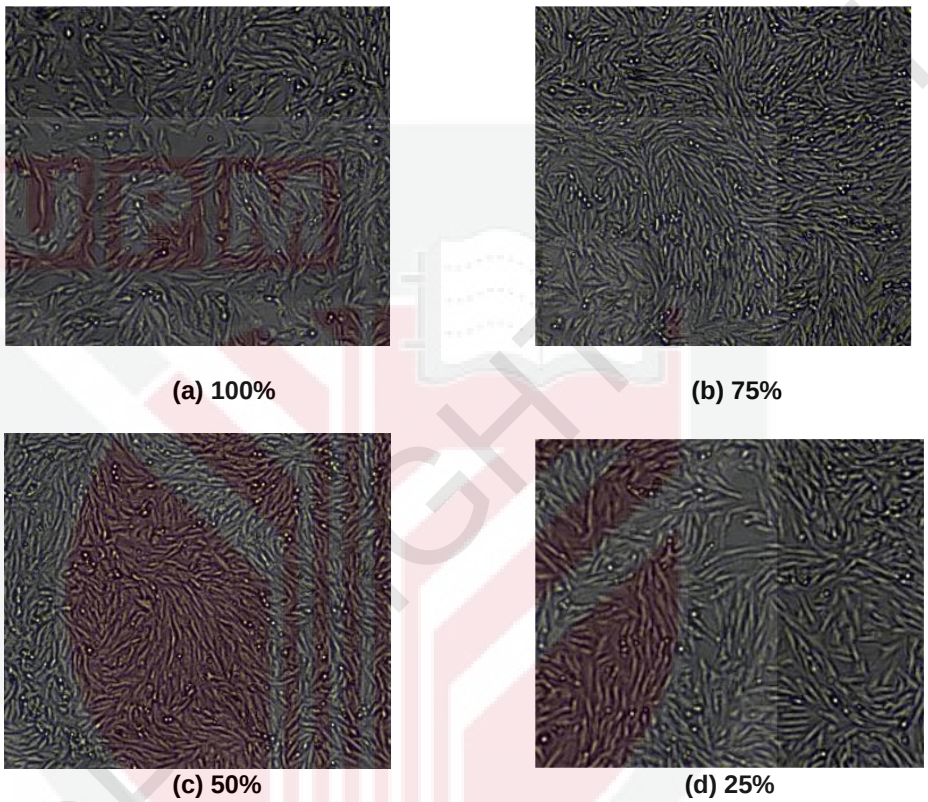


Figure 8: Confluency of CRFK Cells at Four Distinct Concentration of TCW (a) 100% (b), 75% and (c) 25%. 40X magnification.

4.3 $TCID_{50}$ of FPV after adding 75% of Tender Coconut Water

The FPV stock with viral titer $1X10^{8.9}$ was diluted to a viral titer of $1X10^{5}$ for the infection of CRFK cells, and the $TCID_{50}$ of FPV after the addition of 75% TCW was compared to the diluted FPV based on Table 5. $TCID_{50}$ of FPV treated with 75% TCW after 48 hpi was calculated based on Table 4 by

using the Reed and Muench method and the viral titer was $1X10^{4.8}$ $TCID_{50}$/ mL.

Table 4: Determination $TCID_{50}$ of TCW- treated CRFK cells using the Reed–Muench method.

Dilution Inoculum	No. of wells infected	No. of wells not infected	Cumulative positive (A)	Cumulative negative (B)	Infected rate (%) A/(A+B)
10^{-1}	4	0	13	0	100
10^{-2}	4	0	9	0	100
10^{-3}	3	1	5	1	83
10^{-4}	2	2	2	3	40

$$I = \left[\frac{(\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - 50\%)}{(\%\ of\ wells\ infected\ at\ dilution\ above\ 50\% - \%\ of\ wells\ infected\ at\ dilution\ below\ 50\%)}\right]$$

$$= \frac{83\% - 50\%}{83\% - 40\%}$$

$$= 0.8$$

The index is then applied to the dilution that produced the percentage infected immediately above 50%

= $10^{3.8}$ $TCID_{50}$/0.1 mL

= $10^{4.8}$ $TCID_{50}$/ mL

As depicted in Table 5, the contrast in the viral titer of FPV before and after the introduction of 75% TCW is evident. The data reveals a decrease in the $TCID_{50}$ of FPV, reaching $1X10^{4.8}$ $TCID_{50}$/ mL following the incorporation of

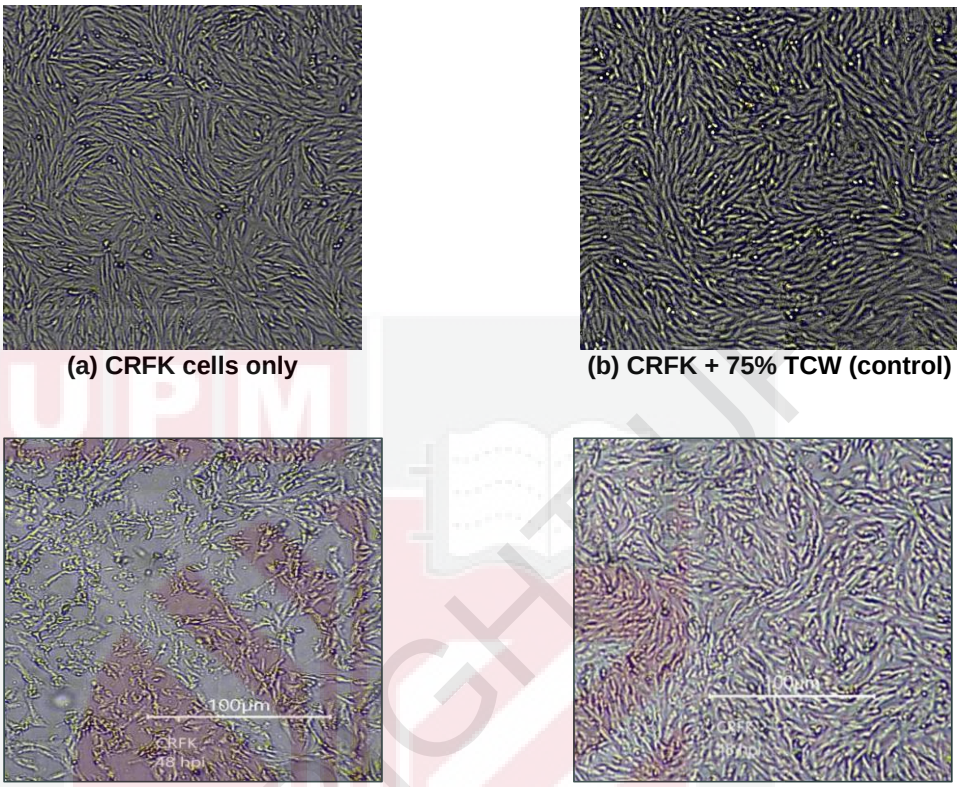
75% TCW. Conversely, the $TCID_{50}$ of diluted FPV used to infect CRFK cells before treatment with 75% TCW was noted at $1X10^5$ $TCID_{50}$/ mL.

Table 5: Viral titer reduction difference before and after added 75% TCW.

Feline parvovirus	**Viral Titer ($TCID_{50}$/ mL)**
Before added with 75% TCW	10^5
After added with 75% TCW	$10^{4.8}$
Viral Titer reduction difference	**0.2**

4.4 CRFK Cell Morphology and CPE Observation

In Figure 9 microscopic analysis reveals the morphology of CRFK cells with 75% TCW exhibit a more favorable appearance when compared to CRFK cells only where the cells appeared more elongated, rounded shape and increased in cell confluency. Microscopic analysis reveals distinct changes in CPE formation and cell morphology following exposure to the experimental compound which was 75% of TCW. Under 40X magnification TCW-treated CRFK cells and positive control groups both have CPE formation in which cells appear shrunken, elongated, rounded, and detached after 48 hpi.



(a) CRFK cells only

(b) CRFK + 75% TCW (control)

(c) CRFK + FPV, 48 hpi (Positive group)

(d) CRFK + FPV + 75% TCW, 48 hpi (TCW-treated CRFK cells group)

Figure 9: Morphology of CRFK cells following the exposure of 75% TCW - (a) CRFK cells only, (b) CRFK cells added with 75% of TCW (negative control), (c) CRFK infected with FPV (positive control) and (d) CRFK + FPV + 75% TCW (TCW-treated CRFK cells group) after 48 hours of incubation. 40X magnification.

CHAPTER 5

5.0 DISCUSSION

Coconut water has been discovered to encompass diverse bioactive constituents, including lauric acid, nicotinic acid, L-lysine, and L-Arginine, which contribute to its antibacterial, anticancer, and anti-inflammatory attributes (Lima et al., 2015). Coconut water has been claimed to possess antiviral properties, as numerous pet owners have found their cats surviving parvovirus when giving coconut water. However, scientific studies on the antiviral properties of TCW remain insufficient to substantiate these claims. Consequently, this study aimed to investigate the potential antiviral effects of coconut water.

Based on the results of this study indicate that TCW-treated CRFK cells led to a reduction in the FPV viral titer by 0.2 which the FPV viral titer reduced from 10^5 to $10^{4.8}$ $TCID_{50}$/ mL. However, according to Ruppach (2014), it is evident that a reduction of 0.2 in the viral titer is not deemed statistically significant. In other words, the observed decrease in the FPV viral titer after the introduction of 75% TCW does not reach a level that is considered. Thus, 75% TCW does not demonstrate a notable antiviral effect against FPV and does not possess antiviral properties. However, lack of a significant reduction does not necessarily rule out the potential antiviral properties of TCW (Gómez-García et al., 2021). Parvoviruses replicate in the nucleoplasm, undergoing complex intracellular trafficking before their genome enters the cell nucleus for replication. This complexity suggests

that antiviral substance efficacy may depend on the specific viral lifecycle and intracellular processes (Caliaro et al., 2019). Hence, according to Liu & Du (2012), assessing antiviral properties should consider intricate interactions between the virus and host cell, along with the specific mechanisms of action of tested compounds.

According to Adan et al. (2016), Cell Titer-Blue assay is valuable for assessing the potential impact of natural products on cell viability and cytotoxicity, providing insights into their effects on living cells. Thus, performing cell viability assays on TCW is crucial to understand its influence on cell cultures. Based on the CRFK cell viability result, the optimal concentration for CRFK cell growth is identified as 75% which the percentage of cell viability was 106% higher than control. According to Erikstein et al. (2010) stated that there is a significant increase in the reduction of the redox dye resazurin to its fluorescent product resorufin by metabolically active cells. It is essential to acknowledge that cell viability exceeding 100% is not unusual, particularly in the Cell Titer-Blue assay, where random experimental fluctuations within +/- 10% may arise due to metabolic activities among cells (Heinrich, 2012). Furthermore, the stimulation from treatment may facilitate cell growth and encourage cell proliferation, resulting in an elevated metabolism of up to 200% compared to baseline activity (Riss, 2016). While the assessment of cell viability percentages indicated that 75% TCW represents the optimal concentration, leading to a notable increase in cell viability, the examination of cytotoxicity effects on CRFK cells across four distinct TCW concentrations using the

Turkey HSD comparison test by ANOVA revealed no statistically significant differences ($p>0.05$) among the concentrations. Consequently, the cytotoxic effects appear to be quite similar when compared to the control. Nevertheless, the selection of 75% TCW as the treatment for this study is supported by an analysis of CRFK cell morphology after a 48-hour exposure to TCW at various concentrations. The results indicate that CRFK cells treated with 75% TCW exhibited the most favorable characteristics of morphology compared to cells exposed to other concentrations. Therefore, the findings indicate that a 75% concentration is considered safe and non-toxic for CRFK cells, with fewer cell deaths observed within 48 hours of exposure to TCW, and it promotes optimal cell confluency. This concentration can be suggested to pet owners for their cats, and future in vivo studies may explore concentrations that are even more favorable and not harmful to feline cells. The morphological examination of CRFK cells reveals a more favorable appearance when treated with 75% TCW, showing an elongated, rounded shape and enhanced cell confluency compared to untreated cells. According to Sekar et al. (2013), TCW contains essential components such as sugars, fats, calcium, phosphorus, iron, amino acids, mineral salts, vitamin B complex, vitamin C, and cytokines, fostering cell growth and maintaining a conducive environment for cellular health. This nutrient-rich composition establishes TCW as a suitable medium for cell growth and development. Previous studies, including those on BHK 21/C13 and 4T1 cell lines, demonstrated growth rates in TCW comparable to higher than traditional growth media (Bhatt et al., 2014). Thus, despite widespread belief among pet owners that coconut water

possesses antiviral properties, these findings suggest that TCW primarily aids in cell maintenance, ensuring electrolyte balance, nutrient supply, and anti-inflammatory properties that protect cells from damage which can treat the clinical signs of FPV infection.

Slower formation of CPE in treated CRFK cells with 75% TCW compared to the positive control after 48 hpi suggests a potential impact of TCW on the viral replication and associated CPE. According Agol (2012), CPE refers to the cell changes or alterations resulting from a viral infection, which can cause negative structural, metabolic, or functional modifications in the infected cell. The delayed CPE formation in TCW-treated CRFK cells indicates that TCW may have a mitigating effect on the viral-induced cellular changes, potentially slowing down the progression of viral replication and associated damage. A study revealed that coconut water vinegar effectively slowed down the progression of 4T1 breast cancer in mice by promoting apoptosis and delaying metastasis (Mohamad et al., 2019). In addition, Lakshmanan et al. (2017) stated that delayed CPE in TCW-treated CRFK cells is due to TCW having anti-inflammatory properties that can help protect cells from damage and reduce inflammation. A study has shown that TCW exhibited a higher anti-inflammatory effect of 42.52% during the second phase of edema in rat paw, in comparison to mature coconut water (MCW), which showed 25.94% inhibition. (Rao et al., 2016). Therefore, the results indicate that TCW may provide a potential protective effect against virus-induced cellular changes, thereby reducing viral infection and associated damage.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, TCW showed no significant antiviral impact on FPV in treated cell cultures as it does not cause viral titer reduction and does not possess antiviral properties, despite claims by pet owners. While it supports cell health in terms of electrolyte balance, nutrient supply, and anti-inflammatory properties.

To enhance the effectiveness of future investigations into the potential antiviral treatments for FPV, it is recommended that further research focus on comparing time-point strategies for virus infection treatment. Additionally, exploring the antiviral properties of other coconut products, such as virgin coconut oil (VCO) containing lauric acid and monolaurin as having antiviral properties (Su et al., 2023). Therefore, it can be considered for future research on FPV treatment.

CHAPTER 7

7.0 REFERENCES

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APPENDIX

APPENDIX 1

One-Way ANOVA Test for Cytotoxicity Effects of CRFK Cells among Four Different Concentrations of TCW

(I) TCW Percentage (%)	(J) TCW Percentage (%)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
25	50	-14.5934	22.1541	.957	-103.4649	74.2780
	75	-22.2506	22.1541	.844	-111.1221	66.6208
	100	4.14144	22.1541	1.000	-84.7300	93.0129
	Control	-10.9977	22.1541	.984	-99.8692	77.8737
50	25	14.5934	22.1541	.957	-74.2780	103.4649
	75	-7.6572	22.1541	.996	-96.5287	81.2142
	100	18.7348	22.1541	.905	-70.1366	107.6063
	Control	3.5956	22.1541	1.000	-85.2758	92.4671
75	25	22.2506	22.1541	.844	-66.6208	111.1221
	50	7.6572	22.1541	.996	-81.2142	96.5287
	100	26.3921	22.1541	.758	-62.4793	115.2636
	Control	11.2528	22.1541	.983	-77.6186	100.1244
100	25	-4.1414	22.1541	1.000	-93.0129	84.7300
	50	-18.7348	22.1541	.905	-107.6063	70.1366
	75	-26.3921	22.1541	.758	-115.2636	62.4793
	Control	-15.1392	22.1541	.952	-104.0107	73.7322
Control	25	10.9977	22.1541	.984	-77.8737	99.8692
	50	-3.5956	22.1541	1.000	-92.4671	85.2758
	75	-11.2528	22.1541	.983	-100.1244	77.6186
	100	15.1392	22.1541	.952	-73.7322	104.0107