STUDIES ON THE EFFECT OF CINNAMALDEHYDE ON INNATE IMMUNE RESPONSE AGAINST NERVOUS NECROSIS VIRUS IN MEDAKA OLHE-131

THESIS

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肉桂醛對稻田魚 OLHE-131 細胞之先天性免疫反應與抵抗神經壞死病毒之研究

Studies on the Effect of Cinnamaldehyde on Innate Immune Response Against Nervous Necrosis Virus in Medaka OLHE-131

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摘要

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論文摘要內容：

神經壞死病毒(NNV)為含有兩條正向單股 RNA 分子之病毒，其造成許多種石斑魚種的神經壞死疾病。本研究以稻田魚肝臟細胞株 OLHE-131 與稻田魚(medaka)成魚為實驗動物模式，研究肉桂醛對 NNV 感染的抗病毒功能。細胞毒性結果顯示 5 μg/ml 與 10 μg/ml 肉桂醛為適合處理 OLHE-131 細胞之濃度，且處理 OLHE-131 後之免疫相關基因 IFN-α、IFN-d、MX、ISG15、ISG56、GIG1-α之mRNA 表現量均顯著增加。依據細胞形態分析，結果顯示以 5 μg/ml 肉桂醛處理 OLHE-131 可以防止 NNV 感染造成之細胞 CPE 現象，此結果說明肉桂醛具有抵抗 NNV 感染之抗病毒功效。以含有 60 mg/kg 肉桂醛之飼料餵食稻田魚一個月後，結果顯示相較於控制組，餵食肉桂醛之稻田魚的抗病毒免疫相關基因如 IFN-α、IFN-d、MX、ISG15、ISG56、GIG1-α 的表現量均顯著增加。研究結果說明肉桂醛具有免疫調節功效，於水產養殖上具有抵抗 NNV 感染的應用潛力。

關鍵詞：肉桂醛、稻田魚、NNV（神經壞死病毒）、免疫相關基因表達

Abstract

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The contents of abstract in this Thesis:

Nervous necrosis virus (NNV) is a bipartite single-stranded positive-sense RNA molecule virus, which caused severe nervous necrosis disease in many kinds of grouper species. In the present study medaka liver cell line OLHE-131 and medaka fish were used as models to study the effect of cinnamaldehyde on the antiviral activity against RGNNV infection. Cytotoxicity assay showed that 5 μg/mL and 10 μg/mL were the appropriate concentration for treating OLHE-131, and the mRNA expressions level of immune-related genes such as IFN-α, IFN-d, MX, ISG15, ISG56, GIG1-α were increased in the presence of 5 μg/mL and 10 μg/mL of cinnamaldehyde. Based on the morphology assay, 5 μg/mL of cinnamaldehyde can prevent NNV-induced CPE suggesting cinnamaldehyde potential exhibit antiviral activity against NNV infection. Dietary supplementation with cinnamaldehyde (60 mg/kg) in adult medaka for one month revealed the immune-related genes such

as IFN-α, IFN-d, MX, ISG15, ISG56, GIG1-α were significantly increased suggesting cinnamaldehyde possess immunomodulatory functions and potential is an effective immunostimulatory against NNV virus in aquaculture.

Keywords: Cinnamaldehyde, Medaka, NNV (Necrosis Nervous Virus), Immune-related genes

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July, 24th 2017
Asthervina Widyastami P.

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I. INTRODUCTION

1.1 Background

Fish is well-recognized as a major nutritional of protein, essential amino-acids and minerals need from an animal source (Easterling, 2007; FAO, 2009, 2010; Rice and Garcia, 2011). Fish and seafood represented around 16.6% of animal protein intake and will be increasing globally (FAO, 2012). As global fisheries demand is considered by ecosystems productivity and management effectiveness, per capita fish consumption able to be maintained or increased by aquaculture system as an increasing contribution to sustainable of global fish supplies (Merino *et al*., 2012).

Aquaculture is controlled culturing of aquatic animals. Cultivation of fish has a significant contribution in social, environmental, and economic for the global market (FAO, 2014) and aquaculture is the most increasing fish trade was entering in the global market (Tveteras *et al*., 2012). However, the outbreak of disease affecting as a big problem in aquaculture environment, it's causing huge economic losses in recent years (Wang *et al*., 2010).

Nervous necrosis virus (NNV) is one of the aquatic pathogens that have been reported infection for many kinds of marine teleost species and affecting a massive mortality not only juvenile but also adult fish, mainly in grouper cultivation (Munday *et al*., 2002). This virus belongs to *Betanodavirus* and *Nodaviridae* family which is a non-enveloped icosahedral virus with 25-30 nm diameter (Lai *et al*., 2002). The genome of NNV is containing two single-stranded, positive-sense RNA molecules (RNA1 and RNA2). RNA1 segment encoding an RNA-dependent RNA polymerase, while the smaller RNA2 encoding the coat protein (CP) and protected among different NNV strains (Shieh and Chi, 2005). NNV are classified into five groups, those are striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus

(BFNNV), tiger puffer nervous necrosis virus (TPNNV) and redspotted grouper nervous necrosis virus (RGNNV) and the fifth betanodavirus isolated from turbot (*Scophthalamus maximus*) (Johansen *et al*.,2004). NNV attack brain and retina which showed by necrosis of the central nervous system and perform with clinical signs such as erratic swimming pattern and darkening of the fish (Bovo, 1999). Antibiotics have been used to overcome many problems in aquaculture in the past but should be controlled at a minimum usage. Moreover, antibiotics are considered may give adverse effect for the animal (fish) and human health (Gudding, 1999 and Ramesh, 2016). Recently, natural product usage as a controller of pathogens is considered as the eco-friendly alternative method.

Cinnamaldehyde is a yellow liquid oily with strong cinnamon odor and sweet taste that was extracted from bark, leaves, and roots of *Cinnamomum* spp. (Paranagama *et al*., 2010). Cinnamaldehyde is a bioactive compound that has been identified to insecticidal (Huang and Ho., 1998), antimicrobial (Faikoh *et al*., 2014; Ravishankar *et al*., 2010; Demo *et al*., 2001; Kwon *et al*., 2003), antifungal (Bang *et al*., 2000 and Xie *et al*., 2004), anti-inflammatory (Liao *et al*., 2008), immunomodulatory (Koh *et al*., 1998), anticancer (Imai *et al*., 2002), and anti-angiogenic activities (Kwon *et al*., 1997). Thus, also induces apoptotic cell death (Fang *et al*., 2004; Wu *et al*., 2005) and inhibits tumor growth (Christopher *et al*., 2009). But less information about the antiviral activity of cinnamaldehyde, especially in fish application.

At present, medaka (*Oryzias latipes*) (Furusawa *et al*., 2006) and zebrafish (*Danio rerio*) (Lu *et al*., 2008) are fish model proved infected by *Betanodavirus* under experimental conditions. Medaka has several experimental surpluses compare to other fish and higher vertebrates. Medaka is small, cost-effective, easy to breed in large number, has a short life cycle and the whole medaka genomic sequences are available and many animal experimental techniques for gene function analysis can be applied to medaka (Kinoshita *et al*., 2009; Taniguchi *et al*., 2006). It has been proven successfully

according to Adachi et.al (2010) research about the examination of susceptibilities of some medaka cell lines derived from different strains and organs infect by RGNNV. Based on this background, the aim of this study is to investigate the effect of **cinnamaldehyde** on the antivirus activity against NNV infection in adult medaka and medaka liver (OLHE-131) cell line.

II. LITERATURE REVIEW

2.1 Medaka (*Oryzias latipes*)

Medaka is known as the vertebrate model used in many kinds of studies of development, genetics, environmental research and human disease (Ishikawa, 2000; Wittbrodt *et al*., 2002). Medaka includes one of ornamental fish and uses aquarium maintaining set for laboratory experiments that the most popular in many countries (Ayyappan, 2011). In the laboratory, an illumination cycle of 14-h light and 10-h dark is generally recommended. For successful daily breeding in the laboratory, temperatures ranging from 25 to 28 ^{o}C are most suitable. In nature, medaka eats animal and vegetable foods such as phytoplanktons and zooplanktons. In the laboratory, synthetic diets (e.g. Tetra Min) and nauplia of the brine shrimp are given several times a day (Shima and Hiroshi, 2004).

2.1.1 Taxonomy, Morphology and Life History

Japanese medaka, *Oryzias latipes* is eurythmy, a small size (3-4 cm), egg-laying freshwater fish, omnivorous, its eggs are fertilized and develop externally. Japanese medaka was first described under the scientific name of *Poecilia latipes* in 1850 by Philip Franz von Siebold (Temminck and Schlegel, 1850), and renamed to *Oryzias latipes* (Jordan and Snyder, 1906) based on the preferred habitat of medaka in the rice (*Oryzias sativa*) fields. Japanese named means a tiny fish with big eyes and mostly used by people who live in Japan (Wittbrodt *et al*., 2002).

Medaka has many colour body (Karakawa and Shibata, 1980). The physiology, embryology, and genetics of medaka have been extensively used for study for the past 100 years (Yamamoto, 1975). A native distribution for *Oryzias latipes*, Medaka is in East Asian countries, especially in Japan, Korea,

China, and Taiwan (Wittbrodt *et al*., 2002). *Oryzias latipes* is an orange-red variety of the medaka fish (Sakamoto *et al*., 2001) that has characteristics like a small size, easy to breed, short generation time, high fertilization and transparent eggs, so it's suitable for embryogenesis studies such us transgenic experiments (Inoue and Takei, 2002; Chen *et al*., 2009; Sasado *et al*., 2010) following that characteristic, the medaka morphology can be shown in **Appendix.1**

Appendix 1. Morphology of Japanese Medaka. (Wittbrodt *et al*., 2002).

The scientific classification of binomial name *Oryzias latipes* is,

Kingdom	:	Animalia	
Superphylum	:	Deuterostomia	
Phylum	:	Chordata	
Subphylum	:	Vertebrata	
Infraphylum	:	Gnathostomata	
Superclass	:	Gnathostomata	
Class	:	Actinopterygii	
Subclass	:	Actinopterygii	
Infraclass	:	Actinopteri	
Superorder	:	Atherinomorphae	
Order	:	Beloniformes	
Suborder	:	Adrianichthyidae	
Family	:	Adrianichthyidae	
Genus	:	Oryzias	
Series	:	Smegmamorpharia	
Species	:	*O. latipes*	(ZipcodeZoo, 2017)

2.2 Nervous *Necrosis* Virus (NNV)

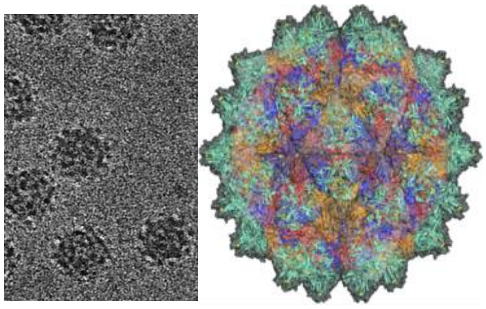
Nervous necrosis virus (NNV) or Betanodavirus was first discovered on an aquaculture farm of Japanese parrotfish (*Calotomus japonicus*) in Nagasaki, Japan during 1986. In this case, many fish fries died which 6-25 mm length during summer season (June-July). It causes fish fry lost their balance and developed necrosis in the brain and retina then viral particles were identified as the cause and leading to the name of nervous necrosis virus (NNV) (Yoshikoshi and Inoue, 1990). NNV is one of aquatic pathogen type of virus that can infect more than 30 species including the grouper, which is valuable fish in Taiwan. NNV was affecting up to 90-100% mortality in aquaculture industry (Kuo *et al*., 2016).

Nervous necrosis virus spreads in various kinds of geographic distribution for example BFNNV exist in cold-water fish species in North America, Norway and Japan like sea bass (*Dicentrarchus labrax*) in France (Grotmol *et al*., 2000; Johnson *et al*., 2002; Nishizawa *et al*., 1997; Nylund *et al*., 2008; Thiéry *et al*., 2004). TPNNV has been reported only found in Japan from Tiger puffer (*Takifugu rubripes*) and Japanese flounder (*Paralichthys olivaceus*) (Nishizawa *et al*., 1997). SJNNV is endemic to Japan waters then was detected in Spain and Portugal fish reared (Cutrín *et al*., 2007; García-Rosado *et al*., 2007; Thiéry *et al*., 2004) and RGNNV found in various kinds of warm-water fin fish and widest geographical range involving Asia, Australia, USA, and the Mediterranean (Munday *et al*., 2002; Chérif *et al*., 2009; Ciulli *et al*., 2006, 2007; Dalla Valle *et al*., 2001; Skliris *et al*., 2001; Thiéry *et al*., 2004). Beside of they have been known infecting over 40 marine fish species worldwide, including the population in the South Pacific, Africa, Australia, Asia, South Pacific and North America (Walker and Winton, 2010). They also were reported to infect freshwater fish in both natural and experimental populations (Yanong, 2010).

2.2.1 Characterization of Nervous *Necrosis* Virus

Nervous necrosis virus (NNV) is a non-enveloped spherical virus, containing bipartite RNA genomes. Both RNA molecules (RNA1, 3.1 kb; RNA2, 1.4 kb) are positive-sense single-stranded RNA (+ssRNA) without a poly (A) tail at their 3'-ends. The virus encodes two structural proteins, viral RNA-dependent RNA polymerase (RdRp) by RNA1 and coat protein (CP) by RNA2. During virus replication, a subgenomic RNA3 (0.4 kb) derived from RNA1 is also produced and encodes two nonstructural proteins B1 and B2, respectively have functions connected to apoptosis and RNA interfering (Shetty *et al*., 2012; Tan *et al*., 2001; Chen *et al*., 2009; Fenner *et al*., 2006). NNV is termed as viral encephalopathy and retinopathy (VER) that was highly emerging disease can cause serious damage typical of extensive vacuolation to the piscine central nervous system (Glazebrook *et al*., 1990; Yoshikoshi and Inoue, 1990).

Betanodavirus is a member of the family *Nodaviridae* define the different features from other viruses. These features are a small, non-enveloped and icosahedral-shaped capsid that covers genetic material made up of single-stranded, positive-sense RNA. The "non-enveloped" means that this virus lack an outermost layer made up of lipids and protein, and the "capsid" is the protein "shell" that surrounds the genetic material of the virus. "Single-stranded, positive-sense RNA" just the type of genetic material number (Yanong, 2010). Based on those characteristics, the nervous necrosis virus morphology can be shown at **Appendix.2**



Appendix 2. Nervous Necrosis Virus Morphology (National Synchrotron Radiation Research Center, 2015).

2.2.2 Nervous *Necrosis* Virus Classification

Recently, Bentanodavirus are classified based on phylogenetic analysis of NNV genomic RNA 2 sequences, Those are Barfin flounder nervous necrosis virus (BFNNV), Tigger puffer nervous necrosis virus (TPNNV), Striped jack nervous necrosis virus, (SJNNV) Spotted grouper nervous necrosis virus (RGNNV) (Adachi *et al*., 2010) and currently the fifth genotype was suggested from turbot fish (*Scophthalmus maximus*) (Johansen *et al*., 2004). Thus, allowed identification of four major different genotypes and have different optimal incubation temperature. Thus, 15-20^{o}C for barfin flounder nervous necrosis virus (BFNNV) genotype, 20^{o} C for the tigger puffer nervous necrosis virus (TPNNV) genotype, 20-25^{o} C for the striped jack nervous necrosis virus (SJNNV) genotype and 25-30^{o} C for red-spotted grouper nervous necrosis virus (RGNNV) genotype (Iwamoto *et al*., 2000).

2.2.3 Nervous *Necrosis* Virus Spread

Viruses appear into the environment from clinically ill or carrier hosts, anyway they do not replicate outside the host like living animals or people but they were maintained and transmitted to a susceptible host (Pirtle and Beran, 1991). Viruses can survive for only a limited time outside the cell protection or host. Hence, living hosts are necessary to the virus survival in nature, because the virus is possible only by repeating or replication infectious cycles in appropriate hosts. The disease will outbreak in hosts of other species to which the virus is transmitted from some link of the perpetuation chain (Matumoto, 1969). Betanodaviruses are highly resistant in the aquatic environment and survive for a long time in seawater at low temperature (Frerichs *et al*., 2000) then at 25^{o}C or higher, the survival rate significantly occurs. Clinically ill in the aquatic environment was directly proportional to the appearance of disease outbreak is likely to persist during long periods and represent a source of infection for wild susceptible species. In frozen fish, the virus might stay for

long periods and it might be a potential risk if raw fish is used for feeding (OIE, 2016 and Mori *et al*., 2005).

Nervous necrosis virus was transmitted to the host through horizontal and vertical transmission (Peducasse *et al*., 1999). Horizontal spread causes by environmental factors. In the laboratory studies had demonstrated that betanodavirus can spread horizontally (from infected to uninfected fish in the same water place) or by exposure to contaminated water (water that contains a virus). Betanodaviruses might be transmitted by feeding of contaminated live foods, including Artemia (brine shrimp), *Tigriopus japonicus* (copepod), and *Acetesinte medius* (shrimp), or through raw contaminated fish (Gomez *et al*., 2010). Vertically transmission has been shown from broodstock to offspring (Breuil *et al*., 2002; Kai *et al*., 2010), that was potentially inside or outside of fertilized eggs.

2.2.4 Nervous *Necrosis* Virus Diagnosis

Betanodaviruses or NNVs mostly affected mortality in larval and juvenile stages. In the fact, the clinical signs are targeted the nervous system that causes mortalities up to 100%. Infected larvae and juvenile stages often show some clinical signs such as abnormal swimming behaviour, including spinning and vertical positioning, curving of the body; and muscle tremors. Nervous necrosis virus also causes hyperinflation of the swim bladder, so ill fish are found at the surface primarily. Infected fish might have traumatic lesions due to uncontrolled swimming or spinning and the most clinical sign in adult fish is abnormal swimming (Bovo and Florio, 2008) then skin pigmentation, either darkening or lightening depend on the species may also be seen (Yanong, 2010).

Fish infection by NNV might be found in a number of different reservoirs although in wild or cultured condition. In Nguyen *et al*., (1997) study explained that subclinical striped jack (*Pseudocaranx dentex*) broodstock (as carriers) were infected with betanodavirus in multiple organs including gonads and

intestines, but not within the central nervous system. It means that those fish no clinical disease signs, might spread the virus through their gametes (eggs or sperm) or feces and betanodavirus (RGNNV) has been demonstrated in the tissue using PCR of several species of wild marine invertebrates (Gomez *et al*., 2008).

Diagnostic laboratory can be used to determine whether or not about betanodavirus infection, those are immunohistochemistry (Le Breton *et al*., 1997); virus isolation which requires seeding in the cell culture (Chi *et al*., 1999); electron microscopy; PCR diagnostic or DNA probes (pieces of genetic material that will bind specifically to betanodaviruses) to evidence of viral DNA (Starkey *et al*., 2004); real-time PCR (Dalla Valle *et al*., 2005). According to histopathology assay, the most specifically finding is the presence of vacuolation "holes" and necrosis (presence of dead tissue) whereas in normal fish would not be appearance (Yanong, 2010).

2.2.5 Nervous *Necrosis* Virus Handling

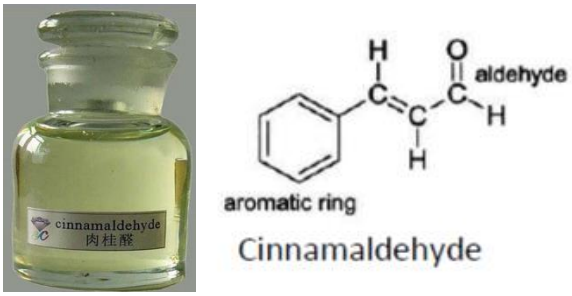
Betanodavirus infections have been associated with mortality in large populations, sometimes achieve 100% mortality. Good husbandry and biosecurity are important. Some researchers study about betanodavirus handling, those are vaccine for bath immunization in grouper larvae (*Epinephelus coioides*) (Kai and Chi, 2008); single intramuscular vaccination with formalin-inactivated (Pakingking *et al*., 2010); oral chitosan-DNA vaccine (Valero *et al*., 2016); and ozone disinfection (Battaglene and Morehead, 2006). International vaccine studies that vaccination of larvae and adult fish can reduce the disease incidence, and that vaccination of broodstock can reduce potential transmission of virus to offspring (Lin *et al*., 2007; Kai *et al*., 2010; Pakingking *et al*., 2010) but still no commercial vaccines are available in the USA.

2.3 Cinnamaldehyde

Cinnamaldehyde is a major compound of Cinnamon from the genus *Cinnamomum* spp. Cinnamon is a genus of the Lauraceae family and differs to two main varieties, those are, the Ceylon or true cinnamon (*C. zeylanicum* Blume) which grows in Sri Lanka and Southern India and cassia (*C. aromaticum* Ness) which grows in China, Indonesia and Vietnam (Hamidpour, 2015; Shan *et al*., 2007; Ranasinghe *et al*., 2012). The essential oil of *Cinnamon cassia* is containing 80-90% cinnamaldehyde with little or no eugenol, which is different between *C. zeylanicum* bark is containing 60-80% cinnamaldehyde with approximately 2% eugenol, then the essential oils from its leaves were found rich in eugenol (70-75%) (Shan *et al*., 2007).

Cinnamaldehyde has a molecular mass less than 500 Da, pKa value of −4.44, log P (partition coefficient) of 1.98, polar surface area (2D) of 17.07 Å2, molecular surface area (3D) of 194.04 Å3, molecular polarizability of 15.78, zero hydrogen bond donors, one hydrogen bond acceptor and molar refractivity of 42.13. In the medicinal application, cinnamaldehyde may act as an orally active drug and can be developed as an interesting template for drug design rationales (Sheraz *et al*., 2016).

Trans-cinnamaldehyde is suspected have two conformers, s-*cis* and s-*trans*, which are different from each other in the orientation of the aldehyde group (Egawa *et al*., 2008). Cinnamaldehyde molecular formula is C_9H_8O that has many kinds of synonyms which are 3-phenylprop-2-enaldehyde; beta-phenylacrolein; cinnamic aldehyde; cinnamic aldehyde, (E)-isomer; supercinnamaldehyde; trans-3-phenylprop-2-enaldehyde (Pubchem, 2017). Cinnamaldehyde structure can be shown in **Appendix. 3**



Appendix 3. Cinnamaldehyde (GoogleImage, 2017).

2.3.1 Cinnamaldehyde Application

Cinnamaldehyde either obtained from natural extracts or synthesized in the laboratory; demonstrates excellent biological activities. Cinnamaldehyde inhibits the activity of NF-kB and the production of tumour necrosis factor alpha (TNF-α) induced interleukin-8 (IL-8) (Reddy *et al*., 2004; Cabello *et al*., 2009). It also reduces IL-1β-induced cyclooxygenase-2 activity (Guo *et al*., 2006). The effects of cinnamaldehyde extracted from *C. zeylanicum* on blood glucose levels of streptozotocin-induced diabetic rats have been studied by Babu *et al*. (2007) The oral administration of cinnamaldehyde restored the altered plasma enzymes such as aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase and acid phosphatase levels to near normal. They also found that cinnamaldehyde isolated from *C. zeylanicum* significantly reduced serum total cholesterol and triglyceride levels in streptozotocin-induced diabetic rats (Babu *et al*., 2007).

In addition, oral administration of cinnamaldehyde isolated from *C. zeylanicum* significantly improved altered enzyme activity of pyruvate kinase and phosphoenolpyruvate carboxykinase in experimental diabetic rats (Anand *et al*., 2010). The oral administration of cinnamaldehyde significantly improved the hepatic and muscle glycogen content. Fang *et al*. (2004) reported the anticancer effect of trans-cinnamaldehyde from *C.osmophloeum*. trans-Cinnamaldehyde displayed potential effects in restraining tumour cell growth and enhancing tumour cell apoptosis.

A recent study reported the potential effects of cinnamic aldehyde isolated from *C. cassia* against myocardial ischemia (Song *et al*., 2013). One of the major constituents of essential oil extracted from *C. zeylanicum* named (E)-cinnamaldehyde has been reported to show potential antityrosinase activity (Marongiu *et al*., 2007). This compound has also been reported to exert chemopreventive activity against several types of cancer cells. The results indicated that cinnamaldehyde induced apoptosis involved disruption of mitochondrial potential enhanced ROS generation, and the mitochondrial release of cytochrome c and Smac/DIABLO into the cytosol, which in turn promoted caspase-3 to its active form and the subsequent cleavage of PARP. The results obtained in this study showed that cinnamaldehyde is an apoptotic inducer that acts on the mitochondrial death pathway in PLC/PRF/5 cells and its effect could be blocked by CsA and z-VAD-fmk (Lin *et al*., 2013).

2.4 The Immune System in Fish

Fish immunology is allowed attention because fish assume as a greater importance models in environmental toxicology and as alternative models in biomedical research (Lin *et al.*, 2016). The immune system of fish is nearby vertebrates. They have a mechanism to against many kinds of a microorganism to keep their health. The healthy fish is maintained by the immune system to protect from a variety of stressors in the aquaculture environment (Uribe *et al*., 2011).

The immune system of fish and other vertebrates is divided into non-specific (innate) immunity and specific (adaptive) immunity. The innate immune system is the first line of defence that provides an immediate response to an invading pathogen and is composed of both humoral and cellular factor. The adaptive immune system is the second line of defence that responds to an invading pathogen and reacts in an appropriate manner to eliminate the specific invading organism, they also composed of both cellular and humoral factor. (Alcorn *et al*., 2008)

2.5 Innate Immune-Related Genes

2.5.1 Type I Interferon

Type I IFN is one of the most powerful innate immune responses that are mediated to against viruses infection. Interferon (IFN) is one of the cytokines which involved in the antiviral process (Zhang and Gui, 2012). In mammals, the mechanism of type I interferon (IFN) response had been characterized. When the virus attack, viral proteins, and their nucleic acid are detected by host pattern recognition receptors (PRRs) including retinoic acid-inducible gene I (RIG-I), a retinoic acid-inducible gene I-like receptors (RLRs) and Toll-like receptors (TLRs) (Beutler *et al*., 2007). Interferon is secreted from the cell and binds to its cognate receptor (IFNAR), which binding triggers the Janus kinase signal transduction and activator of transcription (JAK-STAT) signaling pathway then will lead to the formation of interferon-stimulated gene (ISG)-factor 3 (ISGF3) that binds to IFN-stimulated regulatory element (ISRE) of various ISGs (Schneider *et al*., 2014). ISGs are the workhorses of the innate antiviral response; thus are the effector molecules to work together for limiting every step of a virus's replication (Poynter and Stephanie, 2016).

Type I interferon also termed with "host-antiviral state" in both autocrine and paracrine fashion by inducing the expression of hundreds of interferon-stimulated genes (ISG) (Sadler and Williams,2008; Schoggins and Rice, 2011). IFNs exert the antiviral effect with upregulation of the downstream ISGs likely through Janus kinase (Jak)-signal transducer and activator of the transcription (Stat) pathway (Shi *et al*., 2012; Skjesol *et al*., 2010; Yu *et al*., 2010).

2.5.2 Mx

Mx proteins are dynamin-like GTPases with antiviral activity. Mx1 induces a broad antiviral state by forming oligomers around viral nucleocapsids, ultimately targeting them for degradation (Schneider *et al*., 2014). Fish Mx proteins have a different mechanism of action with mammals.

In grouper, Mx was shown to limit GNNV replication by direct interactions, primarily it was seen that coat protein of the virus binds to the effector domain of Mx then do blocking function (example binding nucleoprotein) and it was occurred in nucleus or cytoplasm (Chen *et al*., 2008; Haller *et al*., 2007; Kochs and Haller, 1999; Engelhardt *et al*., 2004).

Mx1 is an IFN induced protein expressed in cells like macrophages and hepatocytes. It was best known for its antiviral activity against orthomyxoviruses. Mx1 was the first member of a small gene family and high ability as antiviral. Most species have one to three Mx protein isoforms with a different antiviral function depending on their intracellular localization (Lee and Vidal, 2002). Mx2 differs from Mx1 in that Mx2 localizes to the nucleus and its antiviral activity reveals on a nuclear localization signal (Kane *et al*., 2013).

The mechanism of Mx proteins is involved in endocytosis, intracellular vesicle transport, and mitochondria distribution. Mx proteins mainly contain three conserved domains: an N-terminal GTPase Domain (GTP-binding), a middle domain Responsible for interaction with the GTPase effector domain (GED), and the C-Terminus GED domain, which recognizes the virus. Two amphipatic a-helices form leucine zippers in the C-terminus. And then, Mx proteins able to be self-assemble into higher ordering-like structures to form a helical stack (Lee and Vidal, 2002). The higher order structures may represent a storage form whereas the monomers are likely the active form of MxA (Janzen *et al*., 2000). Activation of Mx by IFN involves the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the formation of an IFN-stimulated gene factor 3 (ISGF3) multi-meric complex, which in turn migrates into the nucleus and binds an IFN-stimulated response element (ISRE) upstream of the Mx gene (Darnel *et al*., 1994; von Wussow *et al*., 1990). It is not yet clear how viral infection activates Mx but it seems to be independent of ISGF3, involving STAT1 instead and possibly the IFN-

regulatory factors 1 and 3 (IRF1and IRF3) (Bandyopadhyay *et al*., 1995; Pine, 1992; Grant *et al*., 1995).

2.5.3 ISG15

Interferon-stimulated gene 15 (ISG15) also termed with vig-3 is a ubiquitin-like modifier induced by type I interferon that can be found in an unconjugated form both intracellular and released from interferon stimulated cells into the extracellular environment that covalently binds to its target protein in a process called as ISGylation. ISG 15 has been described attentionally as a potential regulator immune response and has been shown to mediate protection in a number of different viral invasion types (Morales and Deborah, 2013; O'Farrell *et al*., 2002; Schneider *et al*., 2014).

ISG15 has been identified in many fish species that was induced by IFN (Røkenes *et al*., 2007). The overexpression of orange-spotted grouper ISG15 in grouper spleen cells inhibiting grouper nervous necrosis virus (GNNV) and overexpression of zebrafish ISG15 in *Epithelioma papulosum cyprinid* (EPC) cells inhibited VHSV and IHNV; and after knockdown of ISG15 by RNAi led to an increase in megalocytivirus viral load in tongue sole head kidney lymphocytes (HKL) (Huang *et al*., 2013; Langevin *et al*., 2013; Wang *et al*., 2012). However, ISG15 does not protect against all fish viruses, such as in orange-spotted grouper (*Epinephelus coioides*) ISG15 can work to against Grouper nervous necrosis virus (GNNV) but it was not effective to against Singapore grouper iridovirus (SGIV) viral transcripts (Huang *et al*., 2013).

ISG15 mechanism in some fish such as *Danio rerio; Cynoglosus semilaevis*; *Epinephelus coioides* use ISGylation of viral and host proteins located in nucleus/cytoplasm against Birnaviridae; Iridoviridae; Nodaviridae; Rhabdoviridae; and Iridoviridae (Langevin *et al*., 2013; Huang *et al*., 2013; Furnes *et al*., 2009; Wang *et al*., 2012).

2.5.4 ISG 56/IFIT1

Fish ISG56 proteins contain tetratricopeptide repeat domains and transcription is upregulated in response to viral infection (Long and Sun, 2014; O'Farrell *et al*., 2002; Zhou *et al*., 2013). Although fish ISG56 has been known to have antiviral effects, its mechanism of action is poorly understood. The ISG56/IFIT1 protein contains 490 amino acids which have a molecular weight of 58kDa and highly inducible by interferon alpha, thus implicating play an important role for IFIT3 in interferon-mediated antiviral action. Member of the ISG56/IFIT1 gene family has been known in inhibition of translation and synthesis of protein through interaction with eukaryotic initiation factor (eIF3), inhibition of interferon-beta production, inhibition of cell migration, and promotion of antiproliferative and antiviral effects (Schmeisser *et al*., 2010).

ISG56 (also named as IFIT1) is a member of ISGs, and encode multifunctional proteins with tetratricopeptide repeats (Fensterl and Sen, 2011). ISG56 is strongly upregulated by viral infection. ISG56 interact with various proteins and RNAs and exert a wide variety of biological functions including regulation of translation, inhibition of viral replication, cell migration, cell proliferation and cell death (Fensterland Sen, 2011; Reich, 2013). Antiviral mechanism of ISG56 is interference with viral transcription through binding to eukaryotic initiation factor (eIF)3 or binding/sequestering viral 50 PPP-RNA or non-20-O methylated viral RNA (Zhou *et al*., 2013; Diamond, 2014). Fish also express members of the IFIT family, including ISG56 (O'Farrell *et al*., 2002).

2.5.5 GIG1

The innate immune system is known to be key of important in primary fish defence (Sun *et al*., 2011). Fish may also have some unique innate immune factors (e.g., Gig), or antiviral mechanism causes its aquatic environment (Yang *et al*., 2011). Gig family members are ISGs specific to fish that do not exist to have human homologs and Crucian carp Gig 1 as a typical ISG with

antiviral function by the Jak-Stat pathway. Gig 1 was found in cytoplasm upon viral invasion and limited GCHV replication (Sun *et al*., 2014). Overexpression of fish Gig proteins inhibits viral replication and induces a protective antiviral state; Gig1 and Gig2 have protective activity against GCHV, Gig2 has demonstrated effectiveness against spring viremia of carp virus (SVCV) and *Rana grylio* virus (RGV) (Sun *et al*., 2013, 2014; Li *et al*., 2012).

Gig family members are ISGs specific to fish that do not appear to have human homologs (Sun *et al*., 2014). Gig1 and Gig2 expression are upregulated in response to virus infection, and both promoters contain ISREs (Sun *et al*., 2013, 2014; Li *et al*., 2012). however, recombinant IFN induced Gig2 in zebrafish and Gig1 in crucian carp (Jiang *et al*., 2009; Li *et al*., 2012; Sun *et al*., 2013; Zhang and Gui, 2004). Gig 1 was found in cytoplasm upon viral infection and limited GCHV replication (Sun *et al*., 2014). The antiviral mechanisms of these ISGs are still unknown. Overexpression of fish Gig proteins limits viral replication and induces a protective antiviral state; Gig1 and Gig2 have protective activity to against GCHV, Gig2 has demonstrated effectiveness against spring viremia of carp virus (SVCV) and rana grylio virus (RGV) (Li *et al*., 2012; Sun *et al*., 2013, 2014). Again, further research is necessary to understand the induction and expression patterns for Gig1 and Gig2 as well as its mechanism of action.

III. MATERIALS AND METHODS

3.1 Cell line and Virus

The medaka cell line OLHE-131 was purchased from Riken cell bank. The medaka cell line OLHE-131 was cultured at 28ºC in Leibovitz's L-15 medium (L-15) (Gibco, USA) containing 15% fetal bovine serum (FBS). GF-1 cells were cultured in L-15 medium supplemented with 5% FBS. Betanodavirus used in this study was RGNNV. The virus was prepared from inoculated GF-1 cells. Viral titers were determined by $TCID_{50}$ (Reed and Muench, 1938) using GF-1 cells. GF-1 cell is suitable cell line to produce RGNNV.

3.2 Nervous *Necrosis* Virus (NNV) Preparation

Betanodavirus strain Red Grouper Nervous Necrosis Virus (RGNNV) was obtained from was used in this study. The virus was prepared for in vitro and in vivo experiments as the slight modification method described by Maekawa *et al* (2016) and Adachi *et al* (2010). In brief, RGNNV was seeded on GF-1 cells L-15 medium containing 5% FBS then incubated at 28ºC for 72 h. For stocking, the supernatants were taken and were kept into -80ºC until further use. For viral titer was determined by $TCID_{50}$.

3.3 $TCID_{50}$

To determine the viral titer, $TCID_{50}$ assay was needed to check the concentration of virus in the undiluted suspension and end point of this virus. The $TCID_{50}$ method followed by Reed and Muench (1938). GF-1 cells were seeded in 96-well microplates (1×10^4 cell well $^{-1}$ in L-15 medium with 5% FBS) as much as 90 µL well^{-1} and was incubated for 24 h at 28ºC. The virus was prepared to serially dilute from 10^{-1} to 10^{-10}. For 10^{-1} was prepared 10 µL virus stock and was mixed with 90µL L-15 medium with 5% FBS then dilution series to 10^{-10} and no added virus for negative control. In this case, $TCID_{50}$ was checked from 10^{-5} to 10^{-10} then was incubated for 24 h at 28ºC for 96 h. After

incubation, the supernatants were removed and the cells were washed to remove uninfected viral particles by PBS 1x once then were observed under a microscope to CPE checked. The $TCID_{50}$ always checked in every use.

3.4 Fish

Adult medaka fish were acclimatized in several 10-L transparent plastic tanks with water circulating system (28^{o}C), under condition 14-h light and 10-h dark cycle and fed daily. In this study, fish had 3 months old and weighing 150-300 mg $fish^{-1}$ was used. For diet treatment separated into 4 groups (Control, A group, B group and C group) and daily feed 2% BW once or twice a day. For NNV challenge by Takami's IM injection at 10^6 $TCID_{50}$ 10 μl^{-1} $fish^{-1}$ (Maekawa *et al*., 2016).

3.5 Diet Formulation and Preparation

All medaka's diets were formulated in **(Table 1).** Medaka's diets divided into 4 groups. The control diet was formulated containing 100% basal diet, A group, B group and C group were formulated containing 0,03%, 0,06%, 0,09% Cinnamaldehyde from total 100% basal diet respectively. Cinnamaldehyde was purchased from Aldrich company.

Dry ingredients were mixed together using an RT-N04 mixer and add wet ingredients then mixed together and RO water was added to obtain great moisture level. Diets were passed a grinder with a 0,5-cm die to form "mini ball-like', then air-dried overnight at 60^{o}C to avoid destruction of nutrition and long-term storage. After drying, diets were ground into small pellets of appropriate size using a grinder. Diets were sieved with mesh no. 100 to separate big pellet, powder, and small pellet. The small pellet was kept at 4^{o}C in plastic containers until use.

Table 1. Fish feed ingredients formulation

Ingredients	Experimental diets (g/kg)		
	A	B	C
Fish meal	100	100	100
Soybean meal	470	470	470
Gluten	180	180	180
Corn starch	54	54	54
Carboxymethyl cellulose	10	10	10
α – Starch	100	100	100
Canola oil (Omega-3) **(liquid)**	56.97	56.94	56.91
Vitamin mix	10	10	10
Mineral mix	19	19	19
Cinnamaldehyde (CA)	0.03	0.06	0.09
Total	1000	1000	1000

Control is 1 kg basal diet; A group is 30 mg of CA kg^{-1} basal diet; B group is 60 mg of CA kg^{-1} basal diet; C group is 90 mg of CA kg^{-1} basal diet

3.6 RNA Extraction

3.6.1 OLHE-131 Cell Line

Total RNA of the OLHE-131 cell line was extracted from 6 cm^2 disk about 3000 µL $well^{-1}$ then discard the supernatant and wash with 1 mL once with PBS 1x after that add 1 mL Trypsin for removed the cell from disk and added 500 µL L-15 15% FBS then mixed thoroughly to move in 1.5 mL sterile tube. Centrifuged 2000 rpm at 23ºC for 5 min.And then discard the supernatant and collect the pellet. Furthermore, added 1 mL Tripure Isolation Reagent (Roche, USA) for isolation of RNA, DNA and Protein in a single-step liquid-phase separation from the same sample then incubated around 5 minutes at room temperature. Added 200 µL of chloroform and mixed vigorously for 15 seconds then incubated at room temperature around 2 to 3 minutes. Centrifuged

the samples 12000 rpm for 15 min at 4ºC. The mixture will be separated into 3 parts, the lower red phenol-chloroform phase was protein, an interphase was DNA and a colourless upper aqueous phase was RNA. Transferred only the aqueous phase carefully to the new 1.5 mL sterile tube. Then added 500 µL of isopropyl alcohol per 1 mL of Tripure Isolation Reagent and mixed slowly to precipitate the RNA from the aqueous phase. Centrifuged 12000 rpm for 10 min at 4ºC. A gel-like pellet on the side and bottom of the tube was formed. Removed the supernatant completely and wash the RNA pellet once with 75% ethanol, added 1 mL 75% ethanol per 1 mL of Tripure Isolation Reagent and no need to mix then centrifuged 7500 rpm for 5 minutes at 4ºC. Removed the supernatant then drying the RNA pellet for 5 to 10 minutes until dry then dissolved by RNA water (DEPC-treated water) around 20 µL to 40 µL depended on pellet amounts. Checked the purity of RNA samples by OD at 260 nm and 280 nm.

3.6.2 Adult Medaka

Total RNA was extracted from the whole fish body. Prepared the section set and the homogenous stick was soaked by H_2O_2 (9 %) overnight. Kill the fish used ice and then cut the whole fish body then put inside the 15 mL falcon. Added 4 mL Tripure Isolation Reagent (Roche, USA) and mixed using homogenizer (IKA® T10 basic) and then added 800µL chloroform, centrifuged 4000 rpm for 15-45 minutes at 8ºC. The mixture will be separated into 3 parts, the lower red phenol-chloroform phase was protein, an interphase was DNA and a colourless upper aqueous phase was RNA. Transferred only the aqueous phase carefully to the new 1.5 mL sterile tube. Then added 500 µL of isopropyl alcohol per 1 mL of Tripure Isolation Reagent and mixed slowly to precipitate the RNA from the aqueous phase. Centrifuged 12000 rpm for 10 min at 4ºC. A gel-like pellet on the side and bottom of the tube was formed. Removed the supernatant completely and wash the RNA pellet once with 75% ethanol, added 1 mL 75% ethanol per 1 mL of Tripure Isolation Reagent and no need to mix then centrifuged 7500 rpm for 5 minutes at 4ºC. Removed the supernatant then

drying the RNA pellet for 5 to 10 minutes until dry then dissolved by RNA water (DEPC-treated water) around 20 µL to 40 µL depended on pellet amounts. Checked the purity of RNA samples by OD at 260 nm and 280 nm.

3.7 Quantitative Real-Time Reverse Transcriptional PCR (qRT-PCR) Analysis

Quantitative real-time reverse transcriptional PCR (qRT-PCR) was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA). Gene expressions level were normalized to that elongation factor 1α (EF1α). The mean threshold cycle (low CT) was used to determine relative expressions level. Sequences of primers used for the qRT-PCR study (Maekawa *et al*., 2016) are listed in **Table 2.**

Table 2. Primers used in this study for quantitative real-time RT-PCR.

Usage	Gene		Sequence (5'- 3')
real-time PCR	EF1a	Forward	5'- ATTTGCGGGGTTTGCAC - 3'
		Reverse	5'- TGGGAGCTTTTATACGGACTGG - 3'
	IFNa	Forward	5'- GAGCTGAACAGCTGCCTGAA - 3'
		Reverse	5'- TTTCTTGCCCAGTTTTCTGC - 3'
	IFNd	Forward	5'- TGCTGGAGAAGATGGTAAATAACG - 3'
		Reverse	5'- CCACCTCGATGTCTTCTGTAGC - 3'
	GIG1a	Forward	5'- CCTGGAGCTCTCATGGGTTC - 3'
		Reverse	5'- TCCTCACCATCCCACAGTTG - 3'
	ISG15	Forward	5'- GACCCAGCGGTTGGTCTATG - 3'
		Reverse	5'- AACTGCAGAGACCGGGAGTC - 3'
	ISG56	Forward	5'- CTTCAGAAAGGCCACAGAAACC - 3'
		Reverse	5'- AGCCAGGGACCCTCATCTTC - 3'
	Mx	Forward	5'- CTATCATGAAACTGAAGGACATTGG - 3'
		Reverse	5'- AAGCTTGCTTGGGCCAGTAG - 3'
	NNV	Forward	5'- GACGCG CTTCAAGCAACTC - 3'

	Reverse	5’- CGAACACTCCAGCGACACAGTA - 3’

3.8 In Vitro

In vitro test used OLHE-131 cell line that was derived from medaka liver. Medaka liver represent as Medaka fish for in vitro study because for in vivo study this study used Medaka as model fish, and the other hand Adachi *et al*., (2010) explained that OLHE-131 cell line has been proven can be infected by NNV.

3.8.1 Cytotoxicity

Cytotoxicity measure was followed by Roche manufacturer's protocol. The OLHE-131 cell line was seeded in 96-well microplates (1 x 10^4 cell well $^{-1}$ in L-15 medium with 15% FBS) as much as 90 µL well^{-1} and was incubated for 24 h at 28^{o}C. Add 10 µL of Cinnamaldehyde followed by 100 µL at concentrations 50 µg mL $^{-1}$, 40 µg mL $^{-1}$, 30 µg mL $^{-1}$, 20 µg mL^{-1}, 15 µg mL^{-1}, 10 µg mL^{-1}, 5 µg mL^{-1} and DMSO was diluted in L-15 medium, 15% FBS was the control (0.1% DMSO) then were incubated at 30^{o}C for 24 h to get the optimal concentration and incubation of Cinnamaldehyde. Each treatment was performed in triplicate followed Federer (2014). After incubation, each well was added with 10 µL WST-1 **(need dark condition)** then incubation for 1.5 h to 2 h at 28^{o}C and read to ELISA reader with wavelength 440 nm for measuring the absorbance of the WST-1 product.

3.8.2 Immune-Related Genes Expression

The OLHE-131 cell line was seeded in 6 cm^2 disk (8 x 10^5 cell well $^{-1}$ in L-15 medium with 15% FBS) as much as 2700 µL well^{-1} and was incubated for 24 h at 28^{o}C. Add 300 µL of Cinnamaldehyde followed by 3 mL at an optimal concentration of Cinnamaldehyde 10 µg mL $^{-1}$, below an optimal concentration of Cinnamaldehyde 15 µg mL $^{-1}$, and then DMSO was diluted in L-15 medium, 15% FBS was the control and then were incubated at 28^{o}C for 0

h, 12 h, and 24 h. After incubation, appropriate time was harvested for the immune-related gene expressions assay. Each treatment was performed in nine replicate followed Federer (2014).

Total RNA of cell treated by Cinnamaldehyde was extracted and further purified using TRIZOL RNA Isolation Reagent (Invitrogen) and followed by TRIZOL RNA Isolation Protocol. First-strand complementary (c) DNA synthesis in reverse transcription-PCR (RT-PCR) was achieved from BIO-RAD iScript cDNA Synthesis Kit's protocol. Messenger (m) RNA expression of immune-related genes including IFN-α, IFN-d, MX, ISG56, ISG15, GIG1a and EF1α (internal control) were tested the expression fold using KAPA SYBR FAST qPCR Kit Master Mix (2x) ABI Prism in Quantitative real-time reverse transcriptional PCR (qRT-PCR). After amplification, data acquisition and analysis were performed by Sequence Detection Software (SDS vers 2.2 Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was chosen as the calculation method (Livak and Schmittgen, 2001). The difference in the cycle threshold (C_T) value of individual immune gene and its housekeeping gene (EF1-α), called ΔC_T, was calculated. $\Delta\Delta C_T$ = (ΔC_T of optimal concentration of Cinnamaldehyde or control for each immune-related gene) - (ΔC_T of its housekeeping gene).

3.8.3 In Vitro Challenge Test

The OLHE-131 cell line was seeded in 24 well (1×10^5 cell well $^{-1}$ in L-15 medium with 15% FBS) as much as 900 µL well^{-1} and was incubated for 24 h at 28ºC. Add 100 µL of Cinnamaldehyde and/or NNV followed by 1 mL at an optimal concentration of Cinnamaldehyde 10 µg mL $^{-1}$ and 5 µg mL $^{-1}$. DMSO was diluted in L-15 medium, 15% FBS was the control (0.1% DMSO) and then were incubated at 30ºC for 5 days with daily observation to obtain the CPE. The NNV titer number was $2{,}5 \times 10^8$ TCID50 mL^{-1} following the higher virus titer of stock. Each treatment was performed in the three replicate followed Federer (2014).

3.9 In Vivo

In vivo test used medaka adult fish. Medaka represents as experimental model fish for in vivo study and the other hand Furusawa *et al*., (2006) and Maekawa *et al*., (2016) proved that Medaka can be infected by NNV.

3.9.1 Preliminary Test

3.9.1.1 Feed Trial and Cytotoxicity for Medaka Adult Fish

Adult medaka fish was 3 months old and weighing 150-300 mg fish $^{-1}$ was used preparing in 3-L tanks with a water circulating system (28^{o}C) under condition 14-h light and 10-h dark cycle and fed daily. For the preliminary test, tanks were prepared about 4 tanks (Control, A group, B group and C group). Each tank contains 10 fish and acclimatizes around 1 weeks. After acclimatization fish were fed 2% BW by a dietary supplemented appropriate group a day for a month. Passed a month, sacrifice the fish through each tank was taken 5 fish to get the Total RNA from the whole body and evaluate the immune-related genes expressions as described above. For cytotoxicity, the survival rate (%) of adult medaka fish was daily observation for 1 month each 4 days accumulation.

3.9.1.2 NNV Susceptibility For Medaka Adult Fish

Adult medaka fish was 3 months old and weighing 150-300 mg fish $^{-1}$ was used preparing in 3-L tanks with a water circulating system (28^{o}C) under condition 14-h light and 10-h dark cycle and fed daily. For the preliminary test, tanks were prepared about 3 tanks to be performed in triplicate followed Federer (2014). Each tank contains 15 fish and acclimatizes around 1 weeks. After acclimatization fish were injected by Intraperitoneal method with 10 µL RGNNV suspension 1 x 10^{8} $TCID_{50}$ mL^{-1} and observed daily for 2 months then count the RPS (Relative Percent Survival) each week accumulation.

3.10 Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze the data. When ANOVA identified differences among groups, Tukey test, and Dunnet test were conducted to examine significant difference among treatment groups using SigmaPlot 12.5 software and GraphPad Prism 7 software. Data were shown as the mean ± Standard Error of the Mean (SEM). Statistical significant differences required that $p < 0.05$.

IV. RESULT

4.1 In Vitro Cytotoxicity

Cytotoxicity assay is intended to obtain the optimal concentration of the cinnamaldehyde in the treatment of OLHE-131 cells by using WST-1 assay, where the optimal concentrations known to be non-toxic to the cells. Based on **Figure 1.** has been known that at a concentration of 20 µg mL^{-1} was an optimal concentration of cinnamaldehyde, it was proven by the absence of a significant difference in the number of viable cells when compared to control, while the cinnamaldehyde at concentrations above 20 µg mL^{-1} there were significant differences when compared to controls. it was suspected that concentrations above 20 µg mL^{-1} were the toxic concentration of the cells.

4.2 In Vitro Immune-Related Genes Expression Assay

In order to prove whether the optimal concentration can enhance the selected immune-related genes expression in medaka liver (OLHE-131) cell line, the quantitative real-time PCR (qPCR) analysis was performed at the different time points treatment. In this case, 10 µg mL^{-1} and 5 µg mL^{-1} have been chosen for selected concentration. Actually, based on cytotoxicity assay 20 µg mL^{-1} was the optimal concentration for cinnamaldehyde treatment, but for morphology, a figure appeared that 20 µg mL^{-1} and 15 µg mL^{-1} can occur the damaged cell. In brief, 10 µg mL^{-1} and 5 µg mL^{-1} suspected as safe concentration to genes analyzed such as IFNa, IFNd, Mx, ISG15, ISG56, and GIG1a.

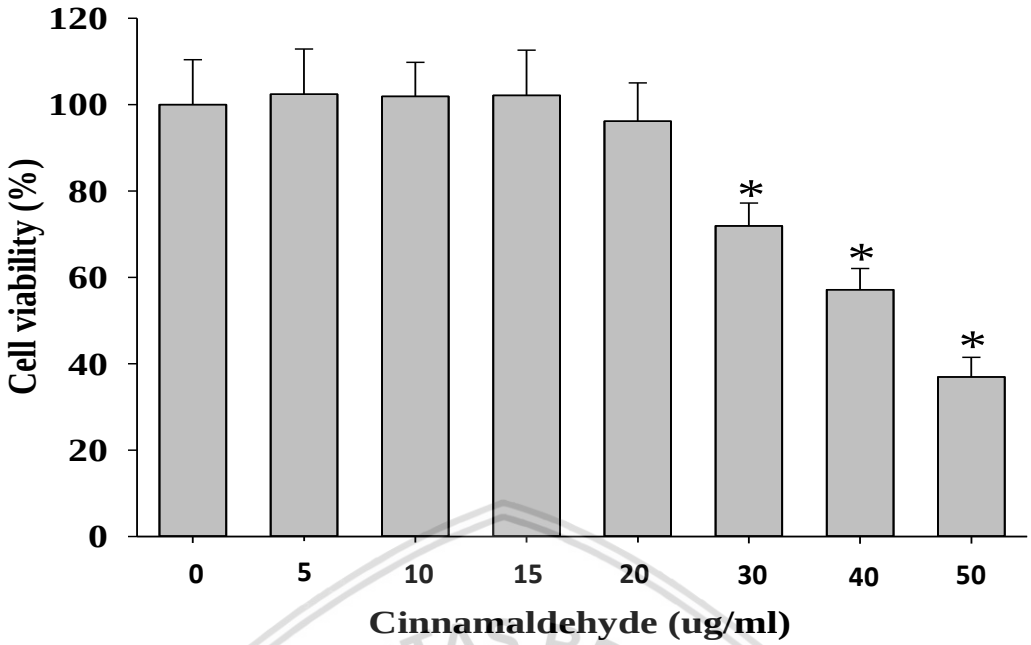


Figure 1. WST-1 analysis of the cinnamaldehyde cytotoxicity after 24 hours post-treatment with different concentrations (µg mL^{-1}).

The overexpression of IFNa that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-immersion of cinnamaldehyde, 10 µg mL^{-1} shown the significant difference compared to the control, it means the IFNa gene was activated enough by 10 µg mL^{-1} of cinnamaldehyde but it does not affect to 5 µg mL^{-1} of cinnamaldehyde when compared to control. For 12 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to the control and each treatment, it means the IFNa gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when they were compared. For 24 h post-immersion of cinnamaldehyde, 10 µg mL^{-1} shown the significant difference compared to the control and 5 µg mL^{-1}, it means the IFNa gene was increased dramatically by 10 µg mL^{-1} of cinnamaldehyde when they were compared including control **(Figure 2.).**

The overexpression of IFNd that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to the control and each treatment, it means the IFNd gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when they were compared. For 12 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to each treatment including control, it means the IFNa gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when compared to each other. For 24 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} concentrations shown the decrement overexpression value when compared to the previous time points, but 5 µg mL^{-1} appeared significantly decrement in comparison control. It means the IFNa gene was weak dramatically by 5 µg mL^{-1} of cinnamaldehyde when compared to control **(Figure 2.).**

The overexpression of Mx that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant

difference to another treatment and control, it means the Mx gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when compared to each treatment including control. For 12 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the decrement value than 0 h time point but the huge significant differences appeared in 10 µg mL^{-1} when compared to the control, it means the Mx gene was activated enough by 10 µg mL^{-1} of cinnamaldehyde than control and 5 µg mL^{-1} has slight significant difference compared to the control and huge significant difference compared to another treatment. For 24 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} concentrations shown the decrement overexpression value when compared to the previous time points, but 5 µg mL^{-1} appeared significantly decrement in comparison control. It means the IFNa gene was weak dramatically by 5 µg mL^{-1} of cinnamaldehyde when compared to control **(Figure 2.).**

The overexpression of ISG15 that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-immersion of cinnamaldehyde, 10 µg mL^{-1} shown the significant difference to the control and each treatment, it means the ISG15 gene was activated enough by 10 µg mL^{-1} of cinnamaldehyde when compared to the control and 5 µg mL^{-1}. For 12 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant different appeared in 10 µg mL^{-1} as the highest expression when they were compared, it means the ISG15 gene was activated stable by 10 µg mL^{-1} of cinnamaldehyde than control and 5 µg mL^{-1}. For 24 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} concentrations shown the decrement overexpression value when compared to the previous time points, but 5 µg mL^{-1} appeared significantly decrement in comparison control. It means the ISG15 gene was weak dramatically by 5 µg mL^{-1} of cinnamaldehyde when compared to control **(Figure 2.).**

The overexpression of ISG56 that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-

immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to the control, it means the ISG56 gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when compared to each treatment including control. For 12 h post-immersion of cinnamaldehyde, 10 µg mL^{-1} shown the decrement value than previous time points but still shown the significant difference compared to the control and another treatment. And for 5 µg mL^{-1} has up-regulated expression and shown the significant difference compared to the control, it means the ISG56 gene was activated enough by 5 µg mL^{-1} of cinnamaldehyde especially compared to the control. For 24 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} concentration shown the decrement overexpression value when compared to the previous time points, but 5 µg mL^{-1} and 10 µg mL^{-1} still appeared the significantly different in comparison to each treatment including control. It means the ISG56 gene was strong dramatically by 5 µg mL^{-1} of cinnamaldehyde then 10 µg mL^{-1} when compared to control **(Figure 2.).**

The overexpression of GIG1a that was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde has a different result at each time points. For 0 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to the control and each treatment, it means the GIG1a gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde when compared to control and another treatment. For 12 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} shown the significant difference compared to the control and each treatment, it means the GIG1a gene was activated enough by 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde especially compared to control. For 24 h post-immersion of cinnamaldehyde, 5 µg mL^{-1} and 10 µg mL^{-1} and 5 µg mL^{-1} concentrations shown the decrement overexpression value when compared to the previous time points, but 10 µg mL^{-1} appeared significantly decrement in comparison to each treatment including control. It means the GIG1a gene was strong dramatically by both concentrations of cinnamaldehyde, especially when compared to control **(Figure 2.).**

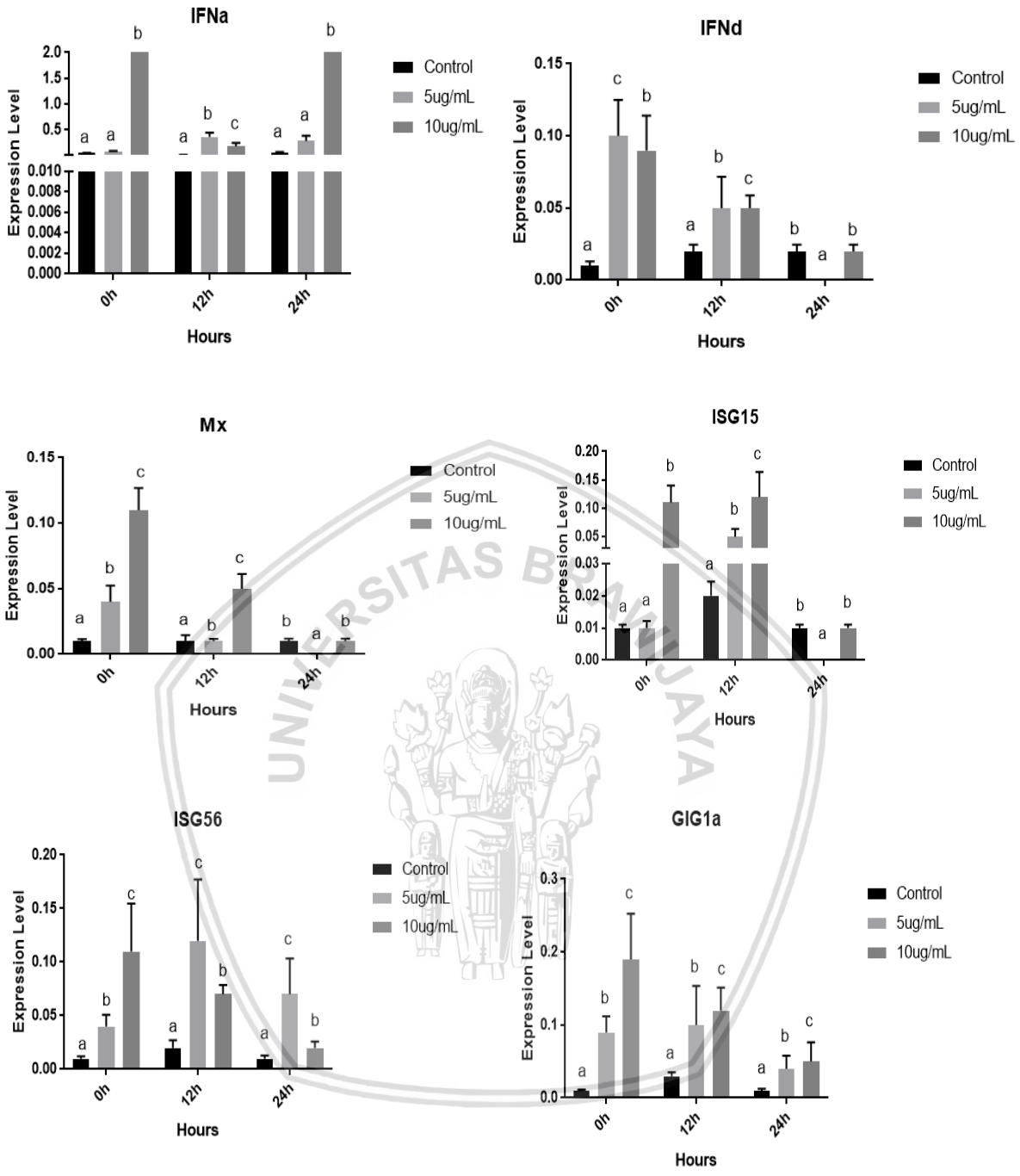


Figure 2. Relative gene expression level of OLHE-131 cell line was treated with 5 µg mL^{-1} and 10 µg mL^{-1} of cinnamaldehyde at different time points (0h, 12h, and 24h) compared to the control. The expression level was determined by qRT-PCR.

4.3 In Vitro Challenge

Firstly, infectivity of cinnamaldehyde against RGNNV on medaka liver cell line (OLHE-131) by detecting CPE from 1 to 5 days post-inoculation (dpi). Red Grouper Necrosis Nervous Virus with titer number 2.5 x 10^8 TCID50 mL^{-1} was added into each well either had or no cinnamaldehyde inside. **Figure 3.** shown that in this study, the OLHE-131 cells were grouped into 6 groups those are control group as negative control, NNV group as positive control, 5 µg mL^{-1}, NNV + 5 µg mL^{-1} group, 10 µg mL^{-1}, and NNV + 10 µg mL^{-1} group. For control group that has added no virus or cinnamaldehyde, there was no CPE found. For NNV group the CPE exerted since 1 dpi and got worse at 4 dpi. At 1 dpi the cells just occur the morphology change from normal shape became tiny shape and at 4 dpi the cell starts to be dead and the worst condition occurs at 5 dpi. Meanwhile, more cell death were found as like became round. For 5 µg mL^{-1} group, the cell condition was much cell be to survive and a little bit damaged cell from 1 dpi to 5 dpi and for NNV + 5 µg mL^{-1} group also found the survival cell although some of the cells occur the CPE characteristic. For 10 µg mL^{-1} group the damaged cells were found since 1 dpi and got worst for the next day but for NNV + 10 µg mL^{-1} group was found the survival cell although it just small part and gave the worst effect in the end.

4.4 Feed Trial and Cytotoxicity for Medaka Adult Fish

In vivo study, fish that has been fed daily a month with dietary supplementation of cinnamaldehyde with appropriate groups were checked the immune-related genes were those are IFN-α, IFN-d, MX, ISG56, ISG15, and GIG1a.

The overexpression of IFNa that was treated with 30 mg kg^{-1},60 mg kg^{-1} and 90 mg kg^{-1} of cinnamaldehyde has different significance level compare to control. For in this gene, IFNa was shown the significant different expression level at 60 mg kg^{-1} and 90 mg kg^{-1}. It means in those concentrations, the IFNa

was activated enough in comparison to the control. Hence, 30 mg kg^{-1} of cinnamaldehyde was not strong enough to activate the IFNa gene **(Figure 4.).**

The overexpression of IFNd that was treated with 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg $kg^{-1}$of cinnamaldehyde has different significance level compare to control. For in this gene, IFNd was shown the significant different expression level at 60 mg kg^{-1} and 90 mg kg^{-1}. It means in those concentrations, the IFNd was activated enough in comparison to the control. Hence, 30 mg kg^{-1} of cinnamaldehyde was not strong enough to activate the IFNd gene **(Figure 4.).**

The overexpression of Mx that was treated with 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} of cinnamaldehyde has different significance level compare to control. For in this gene, Mx was shown the significant different expression level at 60 mg kg^{-1} and 90 mg kg^{-1}. It means in those concentrations, the Mx was activated enough in comparison to the control. Hence, 30 mg kg^{-1} of cinnamaldehyde was not strong enough to activate the Mx gene **(Figure 4.).**

The overexpression of ISG15 that was treated with 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} of cinnamaldehyde has different significance level compare to control. For in this gene, ISG15 was shown the significant different expression level at 60 mg kg^{-1} and 90 mg kg^{-1}. It means in those concentrations, the ISG15 was activated enough in comparison to the control. Hence, 30 mg kg^{-1} of cinnamaldehyde was not strong enough to activate the ISG15 gene **(Figure 4.).**

The overexpression of ISG56 that was treated with 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} of cinnamaldehyde has different significance level compare to control. For in this gene, ISG56 was shown the significant different expression level at 30 mg kg^{-1}, 60 mg $kg^{-1,}$ and 90 mg kg^{-1}. It means in those concentrations, the ISG56 was activated enough in comparison to the control. **(Figure 4.).**

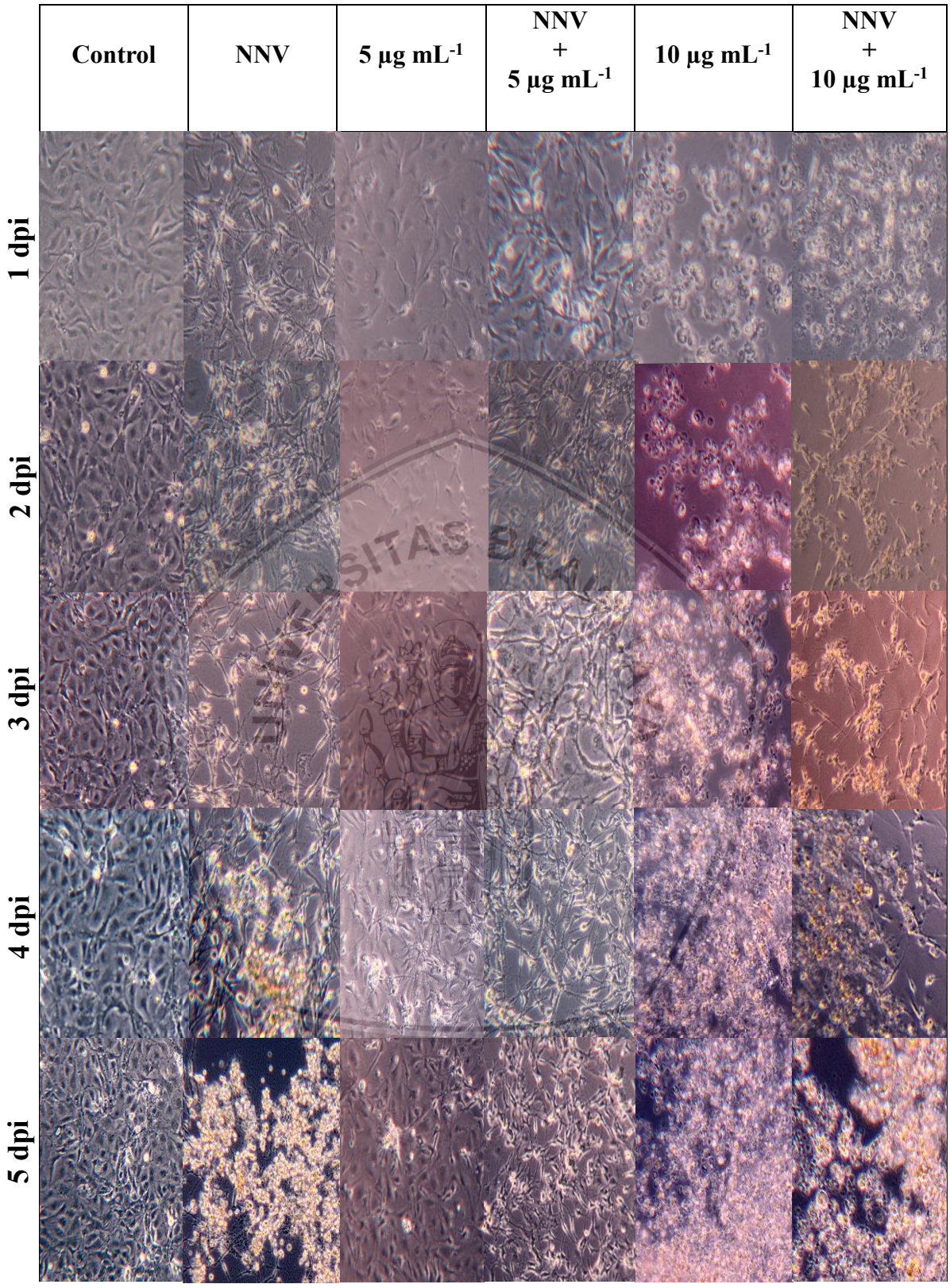


Figure 3. Infectivity of RGNNV against Cinnamaldehyde on OLHE-131 cell line. Each cell line (1 x 10^5 cell well $^{-1}$) was inoculated with RGNNV titer number 2.5 x 10^8 TCID50 mL^{-1} and incubated at 30^{o}C.

The overexpression of GIG1a that was treated with 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} of cinnamaldehyde has different significance level compare to control. For in this gene, GIG1a was shown the significant different expression level at 60 mg kg^{-1} and 90 mg kg^{-1}. It means in those concentrations, the GIG1a was activated enough in comparison to the control. Hence, 30 mg kg^{-1} of cinnamaldehyde was not strong enough to activate the GIG1a gene **(Figure 4.).**

In this study, all immune-related genes significantly increased after treatment using dietary supplementation with cinnamaldehyde in 60 mg kg^{-1} and 90 mg kg^{-1} compared to control. It means in 60 mg kg^{-1} and 90 mg kg^{-1} the dietary supplementation with cinnamaldehyde affected to induce the immune-related genes in comparison to control.

The cytotoxicity assay shown that the evaluation of survival rate on adult medaka fish were 100% for control, 100% for A group (30 mg kg^{-1}), 80% for B group (60 mg kg^{-1}), 80% for C group (90 mg kg^{-1}) at the end of observation **(Figure 5.).**

4.5 Cumulative Survival of Adult Medaka NNV Assay Via IP Injection

To determine the survival rate of adult medaka post infection by RGNNV around 2 months which observed daily then calculated each week to obtain the survival rate percentage. The result showed that the evaluation of cumulative survival rate decreases dramatically after 7 to 21 days post-injection (dpi) and dead continually to 56 days post-injection (dpi) **(Figure 6.).**

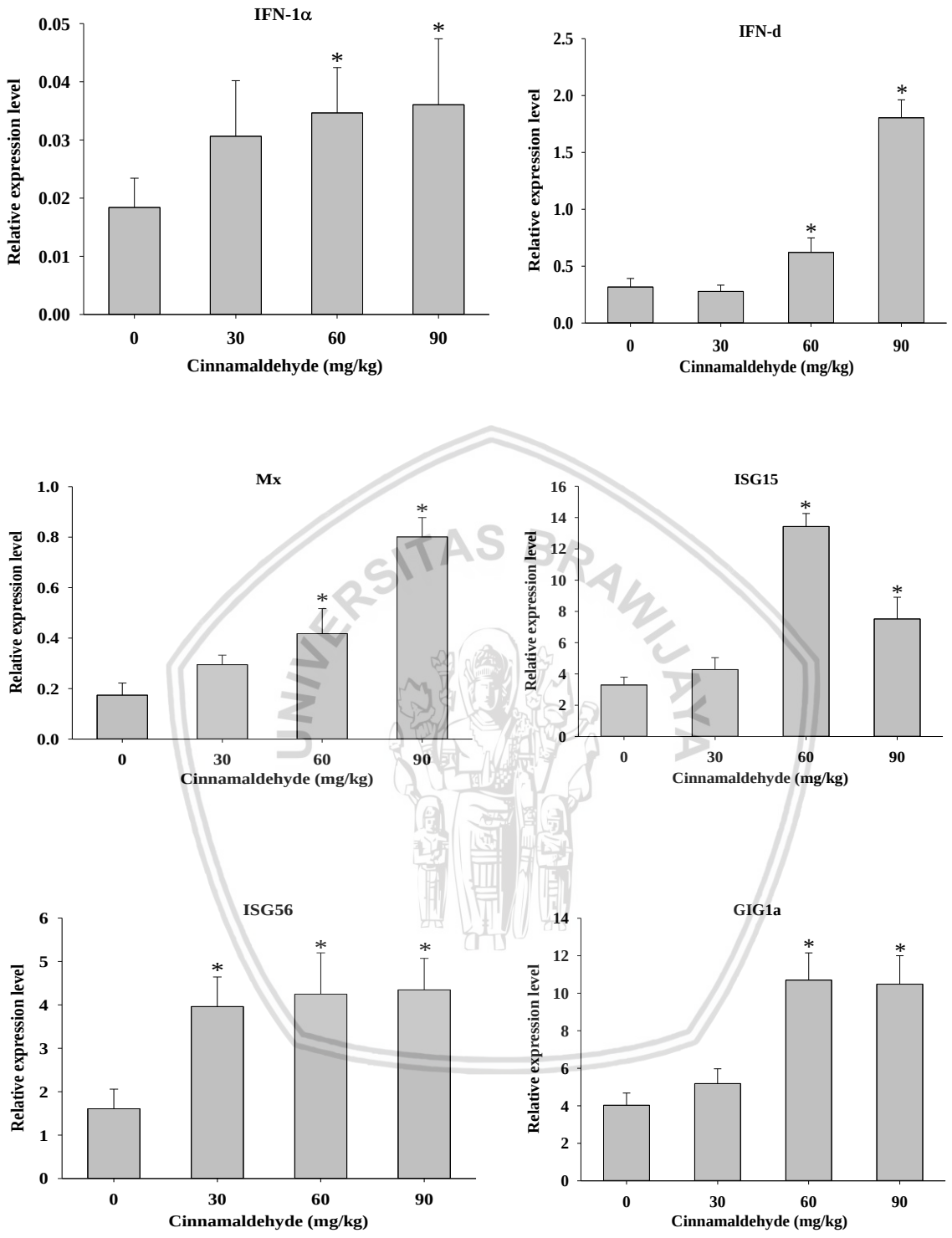
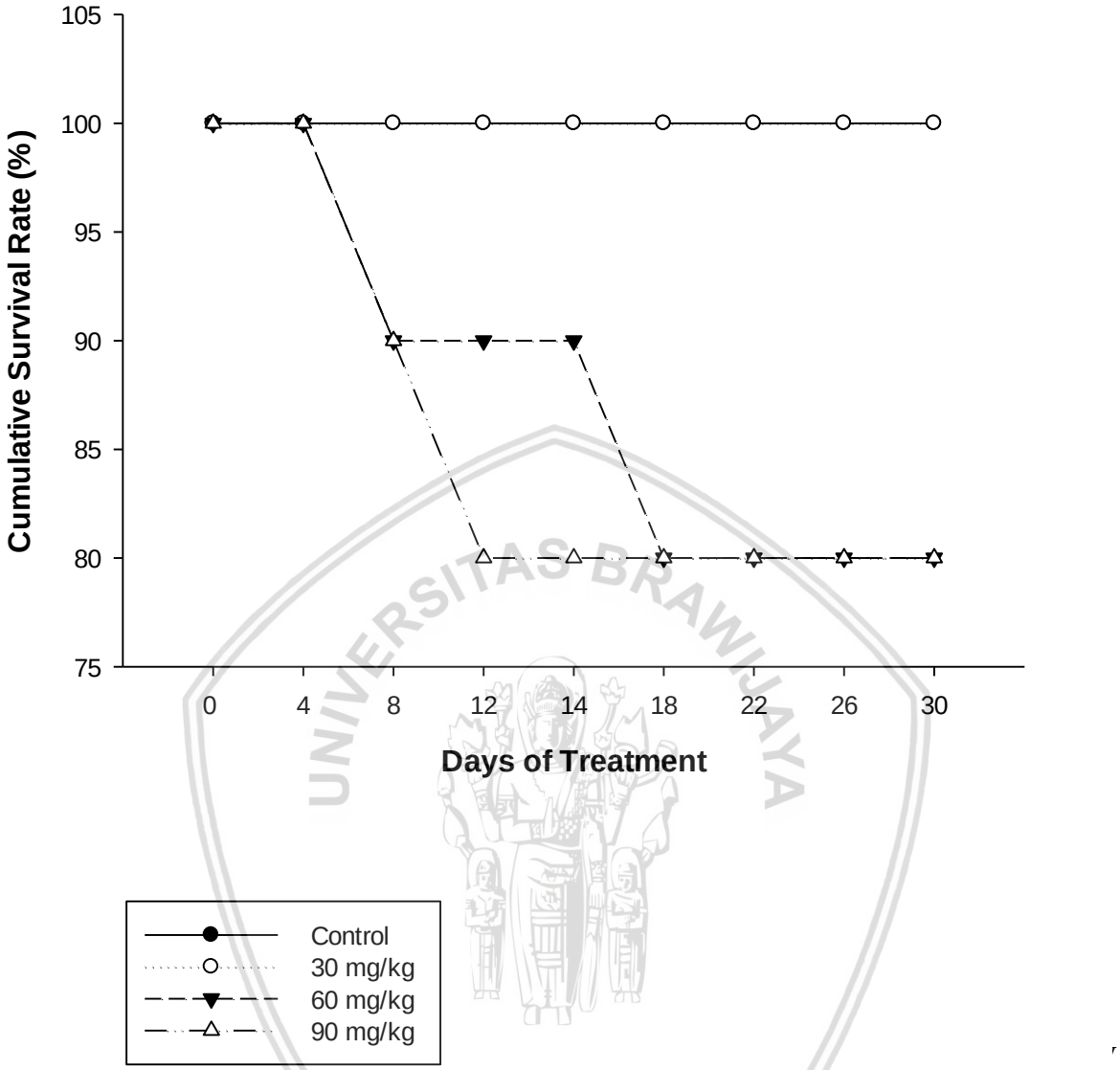


Figure 4. Relative gene expression level of feed trial with dietary supplementation of cinnamaldehyde after 1 month-treatment by different concentrations of cinnamaldehyde such as 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} in comparison to control.



supplementation of cinnamaldehyde after 1 month-treatment by different concentrations of cinnamaldehyde such as 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} in comparison to control.

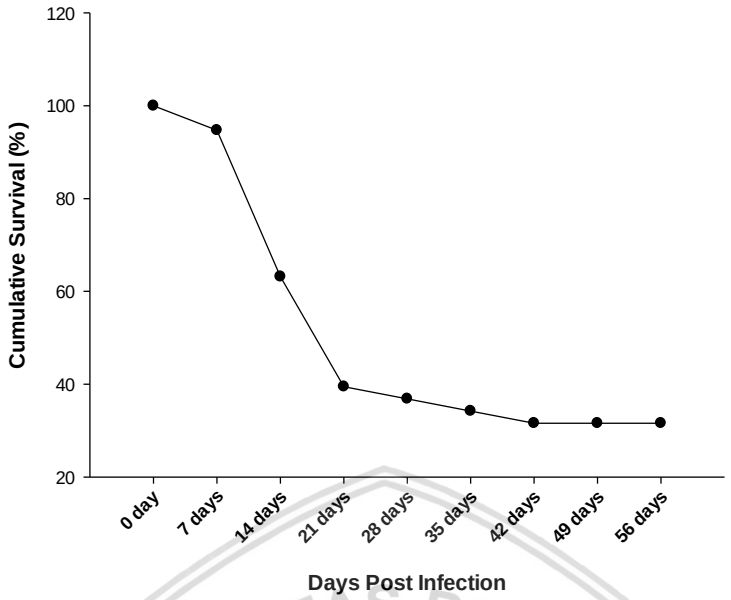


Figure 6. The cumulative survival rate of Adult Medaka with IP Injection of RGNNV (1 x 10^8 $TCID_{50}$ mL^{-1}).

V. DISCUSSION

5.1 In Vitro Cytotoxicity

The optimal concentration was determined about 20 µg mL^{-1}. It has been proved a no significant difference the viable cell compared to control. It means the cell can survive even has added by 20 µg mL^{-1} although occur the damaged cell but not to kill the cell and this concentration was suspected as tolerance concentrate. The non-significant difference means no different the number of viable cells between 20 µg mL^{-1} of cinnamaldehyde and control. Hence, 20 µg mL^{-1} of cinnamaldehyde was determined as non-toxic concentration. Otherwise, the concentration above 20 µg mL^{-1} was toxic concentration, there was a significant difference of viable cell compared to control. It was indicated that the concentration 20 µg mL^{-1} above were able to kill the cell or be a toxicity to the cell.

5.2 In Vitro Immune-Related Genes Expression Assay

The expression of all selected immune-related genes in OLHE-131 cell line was analyzed by quantitative real-time PCR (qPCR) performed both concentrations (10 µg mL^{-1} and 5 µg mL^{-1}) were significantly different at different time points compared to the control. For IFNa expression value was increased at each time points. Hence, others genes expression value decreased as time goes by such as IFNd, Mx, ISG15, ISG56, and GIG1a. Even though, the decrement value does not effects for the significantly different in comparison to control. It was assumed might be no virus added inside this treatment, because all selected genes were including antiviral activity genes. So, in this assay, all immune-related genes assumed that these cinnamaldehyde concentrations can be potential to be an antivirus agent as immunomodulators. Faikoh (2014), reported that cinnamaldehyde had increased some of the endogenous genes and one of them is an interferon (INF) γ as anti-microbial treated in zebrafish. In the other hand Lin *et al*., (2016), explained

that Immunomodulators are substances that modify the immune system response to treat it. They led and potentiate the weapons of the immune system and make it highly prepared state for any invade it might encounter.

5.3 In Vitro Challenge Experiment

In present assay explained that RGNNV can infected the OLHE -131 cell line at 30^{o}C that was related with Adachi *et al* (2010) explained that OLHE-131 apparent the CPE in 4-5 days after inoculated with 10^3 $TCID_{50}$ mL^{-1} and cinnamaldehyde has play an important role to against NNV infection because although the viral titer that used in this study was higher than Adachi *et al* (2010) study but the OLHE-131 cell exhibiting a few CPE characteristic and morphology change on cell but still can survive at 5 µg mL^{-1} of cinnamaldehyde compare to the negative control and positive control (NNV group) but for 10 µg mL^{-1} of cinnamaldehyde shown an interesting phenomena, because the cell was treated only 10 µg mL^{-1} of cinnamaldehyde worst result than NNV + 10 µg mL^{-1} of cinnamaldehyde. This phenomenon might be assumed that cinnamaldehyde can against the NNV infection through the extracellular pathway. This assumes supported by Liu *et al* (2009), explained that CPE on HeLa cells exerted by the control group; cells developed a vacuole and swell and became round; they fused together, shown the typical “bunches of grapes” change and finally became necrotic and exfoliate. In the cinnamaldehyde-treated groups with 0.319 mg mL^{-1}, the cells appeared normal, with only a few cells exhibiting the CPE characteristics; atrophy-like; string-like, overlapping, and exfoliation.

5.4 In Vivo Cytotoxicity

For in vivo cytotoxicity result all the survival rate (%) of treatment groups such as 30 mg kg^{-1}, 60 mg kg^{-1} and 90 mg kg^{-1} dietary supplementation of cinnamaldehyde include control shown that more than 50% of survival fish. It means all of these concentrations can be accepted or non-toxic substances. It was supported on CCOHS (2017), that Lethal $Dose_{50}$ (LD_{50}) is a number of

substances and given all at once which causes the death of 50% of a group of test animals. LD_{50} can be found in many ways of entry or administration but dermal (applied for skin) and oral (given by mouth) administration methods are the most general.

5.5 Immune-Related Genes for Feed Trial of Cinnamaldehyde Supplementation

In present study describes immune-related genes expressions level of IFN-α, IFN-d, MX, ISG56, ISG15, and GIG1a. All immune-related genes play role in innate immune response to antiviral activity like as Goodbourn *et al* (2000) reported that viruses must replicate in the face of powerful immune defence mechanisms including those induced by interferon (IFN), the effectiveness of the IFN response has led to many viruses developing specific mechanisms that antagonize the production or action of IFNs and added by Poynter and DeWitte-Orr (2016), immunity response to a viral infection, IFN signaling results in the expression of a diverse group of genes known as interferon-stimulated genes (ISGs) in fish fewer ISGs have been identified and while there is evidence they limit viral infection, almost nothing is known of their respective antiviral mechanisms. Through medaka as a model fish that was treated by different concentration dietary-supplemented of cinnamaldehyde shown increasing of IFNs and ISGs (Interferon-stimulated genes) level. It means the dietary-supplemented by cinnamaldehyde affected to induce the IFNs and ISGs. It will be a primary result that cinnamaldehyde can be potential to face virus invasion.

5.6 Cumulative Survival of Adult Medaka NNV Assay Via IP Injection

The susceptibility of adult medaka to NNV which was injected by 1 x 10^8 $TCID_{50}$ mL^{-1} of RGNNV shown the fish dead found in day by day around 2 months observation. Therefore, the evaluation of cumulative survival rate decreases dramatically after 7 to 21 days post-injection (dpi) and dead continually to 56 days post-injection (dpi). Munday *et al*., (2002), studied that

NNV infection is an acute infectious disease and death occur within one week of symptom onset. During the experimental period, the infected medaka died without display erratic swimming except for sudden death. Some of them show the physical damage like swelling of swim bladder and pigmentation. It was related to Munday *et al*., (2002) and Chen *et al*., (2014) explained that an infected fish a spiral swimming pattern, dashes under water and floats back to the surface, darkened body colour, less appetite, and clusters near side of the pool. In this assay, prove that adult medaka can be infected by RGNNV.

VI. CONCLUSIONS

According to the results, it might conclude that cinnamaldehyde can be potential as antiviral agent either immunomodulator or therapeutic agent; 5 µg mL^{-1} was an optimal concentration to against RGNNV infection on In vitro study and medaka liver (OLHE-131) cell line can be potential as a model cell line for RGNNV infection. Furthermore, 60 mg kg^{-1} feed-supplemented of cinnamaldehyde was an optimal concentration to enhances the innate immune response on In vivo study and also medaka fish can be potential as a model fish for RGNNV infection because of their effectiveness usage in this study.

Although for In vitro study 10 µg mL^{-1} of cinnamaldehyde treatment was increasing significant different for the immune-related gene expressions as like IFNa, IFNd, Mx, ISG15, ISG56, GIG1a and EF1-α as internal control which compared to the control but for challenge experiment the OLHE-131 cell line was challenged by 2.5 x 10^8 $TCID_{50}$ mL^{-1} of RGNNV the cell morphology got worst then the cell was treated by RGNNV + 5 µg mL^{-1} which were shown the survival cell until 5 days post inoculation compared to all pairwise at each time point.

For In vivo study describes that the expression of the immune-related gene as like IFNa, IFNd, Mx, ISG15, ISG56, GIG1a and EF1-α as internal control were significant different increased at 60 mg kg^{-1} feed-supplemented of cinnamaldehyde compare to the control. This result might be suspected that 60 mg kg^{-1} feed-supplemented of cinnamaldehyde was the best concentration and can be used as an immunomodulator for the fish application.

The survival rate of adult medaka was injected by 1 x 10^8 $TCID_{50}$ mL^{-1} RGNNV exhibited dramatically decrement after 7 days post-injection. This result suspected that the adult medaka can be potential as RGNNV infection model fish.

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