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Romi Novriadi
Balai Perikanan Budidaya Laut Batam, Directorate General of Aquaculture. PO BOX 60 Sekupang Batam - 29422

K.B Haw
Product Engineer at AquaCultural Fresh and Marine Exploratory (S) Pte.Ltd 15; Seletar West Farmway (1), 798123. Singapore

Correspondence:
Romi Novriadi
Balai Perikanan Budidaya Laut Batam, Directorate General of Aquaculture. PO BOX 60 Sekupang Batam - 29422

Preliminary study: The use of herbal extracts against iridovirus in tiger grouper *Epinephelus Fuscoguttatus* culture

Romi Novriadi, K.B Haw

Abstract
In tiger grouper *Epinephelus fuscoguttatus* production, disease outbreaks caused by systemic viral infection are being increasingly reported as an important limiting factor for the production sustainability. The infection of viral disease leads to great economic loss and become a significant challenge to the expansion of tiger grouper production. Here we demonstrated that conditioning tiger grouper with combination of medicinal plant extracts enhanced the survival in comparison to non-treated group. However, the virus was still detected in clinically normal tiger grouper 8 days after experimental inoculation and herbal bio conditioning treatment, indicating that some may be asymptomatic carriers within the aquaculture system. These results indicated that the administration of herbal extracts derived from combination of herbal biomedicines plants generate protective immunity in the tiger grouper against iridovirus infection.

Keywords: *Herbal extracts, Tiger grouper, Percentage survival, Iridovirus*

1. Introduction
Systemic iridoviral disease has been previously reported in many freshwater and marine fish species and cause severe economic losses within the aquaculture system (Inouye *et al*., 1992; Matsuoka *et al*., 1996; Miyata *et al*., 1997; Hyatt *et al*., 2000; Iwamoto *et al*., 2002) [12, 20, 22, 11, and 13]. One of these infectious diseases is Grouper iridovirus disease that has been recorded in orange-spotted grouper *Epinephelus coioides*, yellow grouper *Epinephelus awoara*, giant grouper *Epinephelus lanceolatus* (Sung *et al*., 2010) [30] and tiger grouper *Epinephelus fuscogutattus* (Novriadi *et al*., 2014a) [23]. Grouper iridovirus, belong to *Iridoviridae* family, are large icosahedral cytoplasmic deoxyriboviruses with viral particle sizes ranging from 120 to 350 nm in diameter (Sung *et al*., 2010) [30] and occurred not only in fry and juvenile grouper but also in 1–2-year-old and market-sized grouper. Unfortunately, although some injectable methods are applicable, individual immunization of thousands of fish is very labor, time and cost intensive (Shin *et al*., 2013).
The concepts of using medicinal herbs have received much attention as an alternative strategy for producing immunomodulating compounds because of their relatively low capital cost and they are risk-free to the environmental contamination. Herbs have been used as medicine and immune booster mainly due to their capability to induce the activation of specific and non-specific immune mechanisms of aquatic organisms (Yin *et al*., 2008) [32]. The herbs contain with many immunologically active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils, which can enhance immune functions against pathogenic microorganisms (Yin *et al*., 2008; Dhayanithi *et al*., 2013, Novriadi *et al*., 2014b,c) [32, 6, 24, 25]. Interestingly, the application of mangrove as a medicinal herbs are being used in a wide range of applications including in the control of bacterial, fungal and viral diseases (Dhayanithi *et al*., 2013) [6].
To observe the possibility of medicinal herbs to overcome the Grouper iridovirus (GIV) infection in tiger grouper *Epinephelus fuscoguttatus*, we developed a non-invasive experimental infection technique through cohabitation protocol for horizontal transmission of *Iridovirus* (GIV). Cohabitation trials consisted of placing healthy tiger grouper in the same tank as tiger grouper that had first been experimentally infected or mock infected by injection with virulent Iridovirus. The dynamics of Iridovirus within the internal organ of individual test animals was followed, in parallel with a percentage survival observation and expression study by the use of PCR techniques

2. Material and Methods

2.1. Tiger grouper

7 - 8 cm of Tiger grouper (*Epinephelus fuscoguttatus*) were collected from Batam Mariculture Development Center (Batam, Kepulauan Riau, Indonesia) and maintained for two weeks in an indoor infrastructure. The percentage survival of tiger grouper and water quality was monitored daily during the cohabitation, herbal bioconditioners immersion and post immersion. In order to limit the impact of environmental parameters on immune health status of tiger grouper, similar environmental conditions as their natural environment and filtered seawater was used during the rearing period. These tiger grouper were presumed to be healthy because none died during two weeks of observation period. Since Parasitic diseases, *Viral Nervous Necrosis* and Vibriosis have been identified as the main infectious agents associated with *E. fuscoguttatus* mortality in Batam (Novriadi, 2014a) [23]. These groupers were tested for the presence of other pathogenic microorganisms. Contaminated groupers were not considered for further analysis and fish must be collected again. These groupers were also tested and must found to be free from *Vibriosis* and parasite at the initial period of experiment (Time zero), using conventional identification and microscopic observation, respectively.

2.2. Virus stock

Sleepy Grouper Iridovirus (SGIV) was obtained from a spleen homogenate of SGIV-infected tiger grouper, and propagated in Grunt fin (GF) cells (Clem *et al*., 1961) [3] as previously described (Lua *et al.*, 2005) [18]. The virus titer was determined using the 50% tissue culture infective dose (TCID50) method (Reed and Muench, 1938) [27]. The viral stock was stored in 1 ml aliquots at -80^0 C until further use.

2.3. Experimental infection by intramuscular injection followed by cohabitation

Groupers were anaesthetized for one hour at 20 °C. Good aeration system was provided in order to improve the anaesthetic efficiency (personal observations). Then, 150 µl of the SGIV inoculum (5.0 X 10^5 TCID 50/ml), were injected into the abdomen of groupers. After injection, groupers were transferred to tanks filled with 1 µm-filtered seawater at 30 ‰ salinity, maintained under static conditions at 28-30 °C with aeration. Cohabitation was designated as "virulent Iridovirus". Under both conditions, a ratio of five healthy groupers to one injected was used and total initial density was 1 individual per litre. After 96 hours of cohabitation, all injected groupers were transferred to the new tanks for herbal immersion study and the seawater was replaced with new filtered seawater at 30 ‰ salinity. All experiments were performed under static water conditions at 28-30 °C with aeration and semi sinking pellet. During the post-cohabitation periods, groups of groupers were sampled from the main cohabitation tanks and used for Iridovirus assessments study.

2.4. Experimental treatment by herbal bioconditioners

Groupers that have been infected by Iridovirus were immersed in 20 mg/L of herbal solution (AquaHerb©) for 72 hours and maintain in water salinity 30‰, water temperature 28 - 30^0 C, dissolved oxygen > 5 mg/l and pH ranged from 7.8 – 8,2. After 48 hours of conditioning period, the seawater in the rearing tank was replaced with fresh-filtered seawater for the next 96 hours. During the conditioning and post-conditioning, groupers were fed with semi sinking pellets and water quality was observed daily. The experiment was performed with four replicates per treatment and survival was determined 8 days after each herbal immersion treatments. At the end of study, experimental fish were randomly selected for Iridovirus assessments study.

2.5 Survival (%) of Tiger grouper

The survival (%) of tiger grouper were determined according to the procedures described by Marques *et al.* (2004). For this purpose, the number of live tiger grouper was registered before priming, feeding or challenge with bacteria by counting with the naked eye under laminar flow to maintain the gnotobiotic condition. At the end of experiment, the number of swimming grouper was scored and survival (%) was calculated according to the following equation :

Survival (%) = Final number of surviving grouper / initial number of grouper x 100%

2.6 Clinical and Gross Examination

The clinical and gross examination of unhealthy fish was performed by a clinical doctor of veterinary medicine and fish disease specialist during the post cohabitation and herbal conditioning period. The mortality rate and the examination results during this period was noted daily.

2.7 Histological analysis

Post conditioning tiger grouper were dissected directly in the field at the end of the experiment. Target organs (liver, kidney and intestine) were quickly removed and fixed in Bouin's solution for 24 h in room temperature. The tissues of target organs were dehydrated and routinely processed for paraffin wax embedding. According to Bucke (1994), 5-7 µm thick sections were cut by a rotary microtome and stained with hematoxylin and eosin. Tissues were screened for a variety of histopathological features and lesions under 400X magnifications

2.8 Polymerase chain reaction detection

Polymerase chain reaction (PCR) was performed and forward and reverse primers were 1-F: 5'-CTC AAA CAC TCT GGC TCA TC-3' (20 µM) and 1-R: 5'-GCA CCA ACA CAT CTC CTA TC-3' (20 µM), respectively (Wang *et al*., 2003). Amplification reactions were performed in a total volume of 25 µL containing 17.375 µL DEPC water, 0.5 µL dNTP mix (2.5 µM), 2.5 µL 10× buffer, 0.125 µL Taq DNA polymerase, 1.5 µl $MgCl_2$, 2 µL DNA template obtained and quantified from SGIV-affected gill tissue, and 10 µM of each primer (0.5 µL). The DNA was quantified before it was used in the PCR reaction. Wild type SGIV isolated from grouper was served as positive control. DNA sequences were amplified in a thermal cycler (Gene Amp PCR System 9700; Applied Biosystems, Foster City, CA, USA) using the following program: 94 °C for 5 min, followed by 30 cycles consisting of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 10 min, then a final extension period at 4 °C. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (2 µL) on 2% agarose gel with healthy nucleic acid staining in 1× TAE buffer. PCR products were documented using the UV gel documentation.

2.9 Preparation of Agarose Gel for Electrophoresis

Approximately 1.8 g of Agarose were dissolved in 100 mL of 1X TAE (Tris acetate ethylene diamine tetra acetate) buffer solution using a hot plate until the agarose melt. To the melted agarose solution, 4 µl of ethidium bromide was added and mixed gently. The solution was poured into the gel moulder with the channels and it was allowed to solidify. The amplified products mixed with the required amount of gel loading dye

slowly loaded into the channels. Five to Six channels contained the amplified samples. Other wells contained a 100bp molecular marker, positive control and negative control, respectively. The samples were run in an electrophoresis unit at 115 volts for 35 minutes. The gel was documented using gel documentation system and photographed.

3. Results

Tiger grouper *Epinephelus fuscoguttatus* is recognized as economically-important marine fish species and abundantly cultured in Indonesia for domestic consumption and overseas export. However, due to the (super) intensive culture, various diseases, especially Grouper Iridovirus infection occur in grouper marine culture and frequently create serious problems. Recently, this viral infection had cause up to 50% mortality of humpback grouper *Cromileptes altivelis* in Teluk Mandeh – West Sumatera (INFHEM, 2013) and affected farmed groupers, 100-200 g and 2-4 kg in size, during the sampling campaign in Singapore and Malaysia (Lio-Po and de la Pena, 2004) [17]. The most promising method in prevention of Iridovirus infection is the enhancement of resistance of the fish, which can be achieved by the application of vaccines and immunomodulating compounds. However, vaccines is effectively only against one kind of pathogens and the application of immunostimulants only last for short period (Ardo, 2013) [1]. Therefore, it is very interesting to observe the effect of herbal bioconditioners that can act as immunostimulants as an alternative strategy to overcome the diseases problem, especially for Iridovirus infection.

During the cohabitation, the daily monitoring of a biotic factor in the experimental tanks did not show any variations. However, during the herbal bioconditioners, several parameters such as: phosphate, nitrite, nitrate and ammonia showed significant variation in their concentration. The daily pH ranged from 7.82 – 8.17, temperature ranged from 28.9 – 30.1 and salinity ranged from 30 – 31 ‰. The ammonia and nitrite levels during post conditioning ranged from 0.04 – 0.21 mg/l and 0.001 – 0.026 mg/l. Turbidity varied from 2.85 to 3.77 ppm. Total bacteria in the rearing water ranged from 1.7 – 1.9 x 10^2 CFU/ml and Total *Vibrio* ranged from 1.05 – 1.28 x 10^2 CFU/ml.

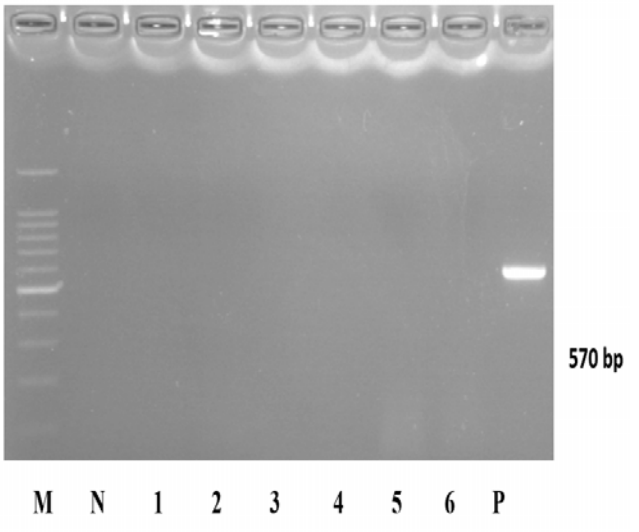

Fig 1: Agarose gel of the PCR product of Tiger grouper *Epinephelus fuscoguttatus* prior to experimental infection with Iridovirus. LANE M: DNA molecular weight marker (); LANE 1: Sample 1; LANE 2: Sample 2; LANE 3: Sample 3; LANE 4: Sample 4; LANE 5: Sample 5; LANE P: Positive control; LANE N: Negative Control.

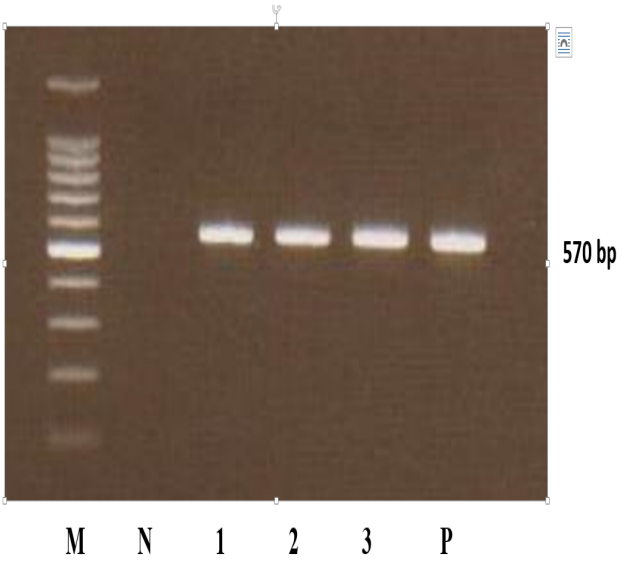

Fig 2: Agarose gel of the PCR product of Tiger grouper *Epinephelus fuscoguttatus* after experimental infection with Iridovirus. LANE M: DNA molecular weight marker (); LANE 1: Sample 1; LANE 2: Sample 2; LANE 3: Sample 3; LANE P: Positive control; LANE N: Negative Control.

The experimental tiger groupers were tested for the presence of Iridovirus by using *Polymerase Chain Reaction* method prior to cohabitation. The result presented in Figure 1 showed that all experimental groupers are free from iridovirus based on the absence of band within the samples. At the end of cohabitation period, the influence of Iridovirus infection on tiger groupers was evaluated. Based on their clinical signs, challenged groupers start to develop a clinical signs suspected of iridovirus, such as: low appetite, rapid opecular movements and remain at the bottom. Our results also showed that all experimental fish has been infected by iridovirus based on the appearance of DNA at 570 bp (Figure 2**).** These infections are typically observed from 4 days post challenge and onwards, but with some differences between subtypes. In order to understand the dynamics of infection, we studied the interaction between Iridovirus and host responses through the histological analysis in their primary target organs (intestine, kidney and liver). The result presented in Figure 3 indicated that fish infected by iridovirus due to severe degenerative and necrotic changes in the intestinal mucosa, kidney and liver.

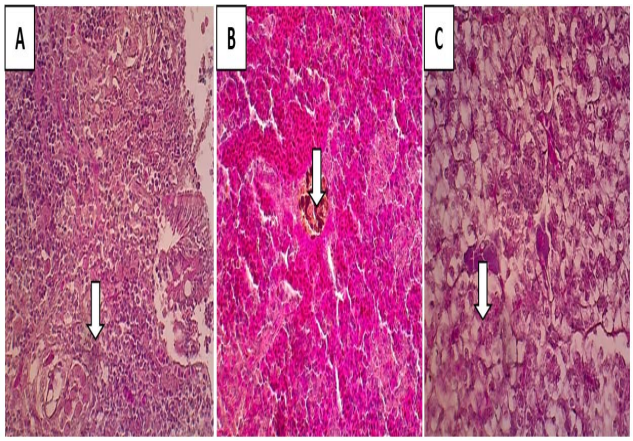

Fig 3: Histological analysis in different tissue of Tiger grouper *Epinephelus fuscoguttatus* following herbal bioconditioners to overcome the experimental infection with Grouper Iridovirus showing a necrosis in (a) intestine ; (b) kidney and (c) liver

Herbal bioconditioners were used after cohabitation for 72 hours without any water replacement and equipped with good aeration system. The results presented in Figure 4 indicate that for the groupers immersed with 20 mg/L AquaHerb was able to significantly induce higher survival 94±1, 47% at 8 days post AquaHerb immersion in comparison to 25±1, 36% in the group of groupers that did not received AquaHerb immersion. Surprisingly, at the end of post immersion, only 50% of the survival fish in the group of AquaHerb immersion that able to eliminate the iridovirus infection based on the absence of bands (570 bp) in their electrophoresis result (Figure 5)

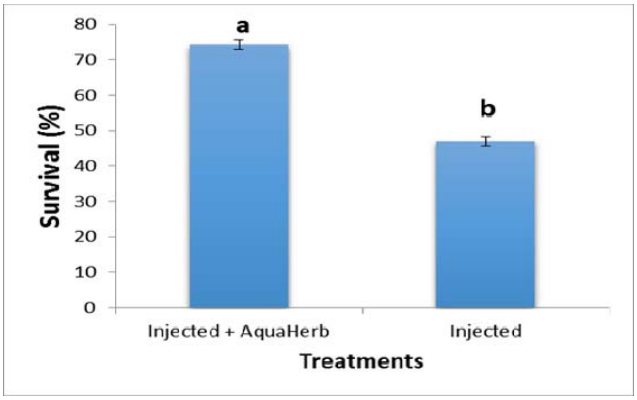

Fig 4. Histogram of the mean survival (%) of groupers injected with Iridovirus followed by immersion with AquaHerb (herbal bioconditioners). Survival was counted after 8 days post AquaHerb immersion. Control groupers were not immersed with AquaHerb during the experiment.

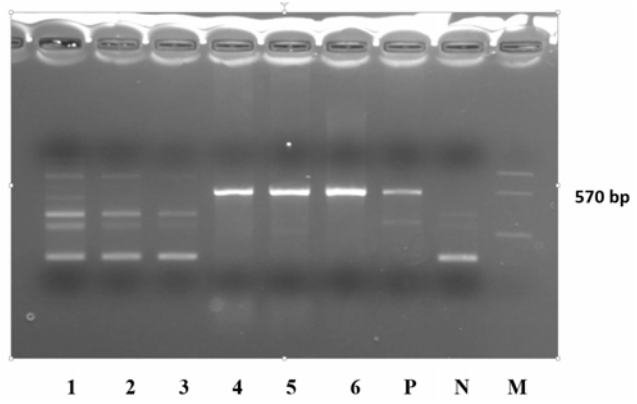

Fig 5. Agarose gel of the PCR product of Tiger grouper *Epinephelus fuscoguttatus* after herbal bioconditioners. Analysis was performed 96 hours post cohabitation. LANE M: DNA molecular weight marker (); LANE 1: Sample 1; LANE 2: Sample 2; LANE 3: Sample 3; LANE 4: Sample 4; LANE 5: Sample 5; LANE 6: Sample 6; LANE P: Positive control; LANE N: Negative Control

4. Discussion

Diseases caused by Iridovirus are known as one of the most widespread in marine fish culture. This infection has caused severe mortality and become one of the main inhibition factor for the sustainability of tiger grouper production. Ultimately, the entire stock that infected by this virus was destroyed. Several studies have reported that herbal medicines can be used to enhance resistance of marine fish against pathogen, including in the control of bacterial, fungal and viral diseases (*Dhayanithi et al.,* 2013) [6]. In this study, extracts of commercial herbal medicines AquaHerb were chosen because of their recorded ability to enhance the activity of the immune system (Novriadi *et al*, 2014a, b) [23, 24].

The results from this study showed that commercially available herbal bioconditioners (AquaHerb) produced from a specially-selected mangrove extract *Rhizophora mucronata*, Pinaceau Extracts *Pinus armandii* and Melaleuca Extracts *Melaleuca alternifolia* was able to modulate the immune system and significantly improved the Tiger grouper *Epinephelus fuscoguttatus* against iridovirus infection in comparison to control (p<0,05*)*. Corroboration for our results comes from the work of (Direkbusarakom *et al*. 1996a) [7]. Who have reported that herbs have been found to have antiviral activity against fish viruses in tissue culture. Moreover, (Direkbusarakom *et al*. 1996b) [8]. stated that the ethanol extract complexed with polyvinylpyrrolidone from *Clinacanthus nutans* fed to the black tiger shrimp (*Penaeus monodon*) were able to provide a protection and induce the survival of herbal-treated shrimp up to 94±1,47% in comparison to control that only survived 25±1,36% after 8 days of challenged. This observation further substanties the probability of herbal biomedicines as antiviral agents mainly due to the ability of herbal active compounds to inhibit and reduce the replication of virus in the host cells and enhance the immune system of fish (Citarasu, T, 2010) [5, 28].

Several studies have indicated that mangroves and plant extract are being used in a wide range of applications for centuries (Kathiresan, 2000) [15] including in the control of viral diseases (Dhayanithi *et al*., 2013) [6]. Molecules derived from the combination of several plant extracts have had an excellent record of providing novel chemical structures for the development of new therapeutic agents (Dhayanithi *et al*., 2013) [6]. Based on our laboratory examinations, AquaHerb contain with several active principle components, such as alkaloids, flavonoids, terpenoids and steroids which have been reported as antistress, antimicrobial properties, appetite stimulation, tonic and immunostimulation in finfish and shrimp culture (Citarasu *et al*., 2002; Sivaram *et al*., 2004) [4, 28]. The combination of several herbal extracts may have a greater accuracy than chemotherapeutic agents (Maqsood *et al*., 2011) [19] and offer viable solution, especially in preventing or controlling infectious microbes (Citarasu, 2010) [5, 28].

The application of several medicinal herbs as a treatment significantly induce higher survival in tiger grouper against iridovirus infection. This in line with the study from Ardo *et al*., (2008) [32] who stated that the administration of two Chinese herbs *Astragalus membranaceus* and *Lonicera japonica* significantly enhanced phagocytic, respiratory burst activity and had a moderate effect on the highest level of immunoglobulin. Moreover, rainbow trout fed with diets contained with several plant extracts, namely: extracts of mistletoe *Viscum album,* nettle *Urtica dioica* and ginger *Zingiber officinale*, showed an enhanced extracellular respiratory burst activity *(p<0,001)* compared to the control group (Dugenci *et al*., 2003) [9]. Thus, the administration of herbal extracts or their products enhance the innate and adaptive immune response of against bacterial, viral and parasitic disease.

In this preliminary study, the infected fish showed various symptoms characteristic to the same pathogens used in the experiment, including low appetite, rapid opercular movements and remain at the bottom. To confirm the occurrence of Iridovirus, further analysis was carried out by the use of *Polymerase Chain Reaction* (PCR) method. PCR method has a wide range of applications as a fundamental molecular biological tool for virus detection, especially Iridovirus in fish tissue (Chinchar and Mao, 2000; Jeong *et al*., 2006) [2, 14]. The assay involves amplification of a portion of the small units of the virus isolated from kidney and spleen

(Oshima *et al*., 1998) [26]. The results showed that all experimental fish infected by iridovirus based on the appearance of bands (570 bp) in the electrophoresis result.

The administration of herbal bioconditioners were able to eliminate the iridovirus from fish tissue. However, approximately only 50% of the fish are truly free from infection and the remaining fish still have iridovirus DNA inside their body tissue despite they survived from infection. This phenomenon has received considerable attention in our mariculture center mainly due to the ability of herbal treatments to enhance the survival of tiger grouper against iridovirus injection. Study from (Harikrishnan *et al*., 2010) [10]. stated that under *in vitro* conditions, Dasyscyphin C ($C_{28}H_{40}O_8$) extract of leaves of *Eclipta prostrata* have been shown antiviral activity against fish nodavirus. In addition, study from (Khanna *et al*., 2011) [16]. Showed similar results and stated that the administration of Gymnemagenol at 20 µg mL−1 was able to inhibit the proliferation of grouper nervous necrosis virus (GNNV) until 53% at the end of the 6th days by inhibiting the proliferation of GNNV infected SIGE cells.

The application of medicinal herbs and plant extracts have been proven useful against a broad spectrum of pathogens, have strong antiviral activity (Sivasankar *et al*., 2015) [29] and synergistic effects without develop any herbal-resistance toward pathogens. In addition, the use of herbal are inexpensive, locally available and biodegradable with no adverse effects to the environment (Syahidah *et al*., 2015) [31]. However, the capability of herbs to clinically reduce the viral infection seems to be more specific. Study from (McCutcheon *et al*., 1995) [21]. Revealed that the antiviral properties of plants demonstrates that plants may inhibit one type of virus, but have no activity against most others. Therefore, these results suggest that the administration of several herbal or their combination have been found to have more non-specific immuno-stimulating effects against viral infection.

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