EFFECTS OF DIETARY SUPPLEMENTATION OF LOCAL MEDICINAL HERBS ON HYBRID GROUPER JUVENILE (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*)

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DECLARATION

Hereby, I declare that all the material in my thesis are of my own except for quotations, excerpts, equations, summaries and references which have been duly acknowledged.

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ABSTRACT

In treating bacterial diseases in aquaculture, non-organic substances such as antibiotics have been commonly used. However, researchers have discovered that with its continuous usage, the bacteria became resistant to antibiotic. Thus, the usage of herbs in treating aquaculture diseases has gained interest worldwide. Present study was conducted to screen the antioxidant and antimicrobial properties of some local medicinal herbs (Experiment 1) and to determine the growth, feed utilization, apparent digestibility coefficient, blood analysis and survival after challenged with *Vibrio harveyi* VHJR7 of the hybrid grouper juvenile fed with diets supplemented with herbs through a feeding trial (Experiment 2). In experiment 1, methanolic extracts from four fresh local medicinal herbs; *Piper betle* (betel), *Curcuma longa* (turmeric), *Etlingera coccinea* ("tuhau") and *Clinacanthus nutans* (snakegrass) were tested. The antioxidant activity was tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity. Meanwhile, the antibacterial activity was tested using the disc-diffusion method using four types of marine pathogens; *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi* and *Vibrio parahaemolyticus*. Concentrations of 0, 20, 50 and 100 mg/mL were tested for the antibacterial activity. A feeding trial was then conducted with the supplementation of the three best performing herbs in the diets; which were betel (D-betel), turmeric (D-turmeric) and tuhau (D-tuhau) at 0.5% of supplementation in the isoproteic (50%) and isolipid (12%) diets. Diet without herb supplementation was used as the control. Hybrid grouper, *Epinephelus fuscoguttatus* x *E. lanceolatus* juveniles with initial body weight of 8.91 ± 0.02g were fed with the experimental diets for 8 weeks. During the trial, triplicate groups of 20 fish were cultured in 100L fiberglass tanks for each experimental diet with a flow-through seawater system. The fish were fed with experimental diets until apparent satiation level twice a day. Results from the screening (Experiment 1) showed that the highest antioxidant activity was found in betel extract (more than 50% of activity), followed by turmeric (20 – 40%), tuhau and snakegrass (20 – 30%). Betel extract also showed strong antibacterial activity (>14.0 mm), followed by a moderate activity of tuhau (10 – 14mm), a weak activity of turmeric (<10mm) and no activity at all from snakegrass (0mm). Screening of these herbs *in-vitro* showed that among all the herbs tested, betel leaves extract gave the best results for the antioxidant and antibacterial activity. For the feeding trial (Experiment 2), highest growth was seen in fish fed D-turmeric, followed by D-tuhau while fish fed D-betel showed the lowest growth. However, the growth of fish fed D-turmeric and D-tuhau showed no significant difference than the control diet ($p > 0.05$). Better feed utilization was also observed in fish fed D-turmeric and D-tuhau compared to D-betel. Apparent digestibility coefficient (ADC) of protein and lipid of fish fed D-turmeric and D-tuhau were significantly higher than the control ($p < 0.05$). The herb supplementation did not affect the blood analysis except for the white cell count where lower values were obtained and higher platelet count was observed. When challenged with *Vibrio harveyi* VHJR7, fish fed D-betel showed highest survival amongst all and the superoxide dismutase activity in blood increased significantly after the challenge test. In conclusion, the herbs turmeric and tuhau improved the growth and feed efficiency of the hybrid grouper juvenile while betel provided better results in the challenge test.

ABSTRAK

KESAN PENGGUNAAN HERBA PERUBATAN TEMPATAN SEBAGAI MAKANAN TAMBAHAN UNTUK JUVENIL KERAPU HIBRID (Epinephelus fuscoguttatus x Epinephelus lanceolatus)

Dalam merawat peyakit berpunca dari bakteria dalam akuakultur, bahan tidak organik seperti antibiotik sering digunakan. Namun begitu, dengan penggunaan antibiotik yang berterusan, bakteria dapat membina ketahanan terhadap antibiotik. Justeru, penggunaan herba dalam menangani penyakit akuakultur telah menarik minat untuk dikaji. Kajian ini telah dilakukan untuk menyaring ciri-ciri antioksidan dan antimikrobial yang terdapat dalam herba perubatan tempatan yang dipilih (Eksperimen 1) dan juga untuk menentukan pertumbuhan, penggunaan makanan, pekali pencernaan nyata (ADC), analisis darah dan kemandirian setelah dicabar dengan Vibrio harveyi VHJR7 bagi kerapu hybrid juvenil yang diberi pemakanan yang ditambah dengan herba melalui percubaan makanan (Eksperimen 2). Dalam Eksperimen 1, ekstrak methanolik dari empat herba perubatan tempatan segar; sirih (Piper betle), kunyi (Curcuma longa), tuhau (Etlingera coccinea) dan belalai gajah (Clinacanthus nutans) telah diuji. Aktiviti antioksidan juga diuji menggunakan asai hapus-sisa radikal bebas 1,1-difenil-2-pikrilhidrazil (DPPH). Manakala, aktiviti antibakteria telah diuji meggunakan kaedah penyebaran-cakera dengan empat jenis patogen marin; Vibrio alginolyticus, V. anguillarum, V. harveyi dan V. parahaemolyticus. Kepekatan 0, 20, 50, dan 100mg/mL telah digunakan bagi menguji aktiviti antibakteria. Kemudian, satu percubaan makanan telah dijalankan dengan penambahan tiga herba terbaik ke dalam makanan; iaitu sirih (betel), kunyit (turmeric) dan tuhau (tuhau) pada kadar 0.5% penambahan dalam pemakanan isoprotik (50%) dan isolipid (12%). Makanan tanpa herba tambahan telah digunakan sebagai makanan kawalan. Kerapu hybrid juvenil, E. fuscoguttatus x E. lanceolatus dengan berat awal 8.91 ± 0.02g telah diberi makanan eksperimental tersebut selama 8 minggu. Tiga kumpulan ikan dengan 20 ekor ikan setiap kumpulan telah dikultur di dalam tangka gentian kaca dengan sistem air masin mengalir berterusan. Ikan tersebut diberi makanan sebanyak 2 kali sehari sehingga kesemua ikan mempamerkan reaksi kenyang. Hasil daripada saringan herba (Eksperimen 1) menunjukkan bahawa aktiviti antioksidan yang tertinggi didapati dalam ekstrak sirih (lebih daripada 50% daripada aktiviti), diikuti oleh kunyit (20 – 40%) serta tuhau dan belalai gajah (20 – 30%). Ekstrak sirih juga menunjukkan aktiviti antibakteria yang kuat (>14mm), diikuti dengan aktiviti antibakteria yang sederhana oleh tuhau (10 -14mm), aktiviti lemah oleh kunyit (<10mm) dan tiada langsung aktiviti daripada belalai gajah (0mm). Saringan herba secara in-vitro ini menunjukkan bahawa antara semua herba yang telah dikaji, ekstrak daun sirih memberikan hasil yang terbaik untuk aktiviti antioksidan dan antibakteria. Untuk percubaan makanan (Eksperimen 2) pula, pertumbuhan tertinggi yang dilihat dalam ikan yang diberi makan turmeric diikuti oleh tuhau manakala ikan yang diberi makan betel menunjukkan pertumbuhan yang paling rendah. Walau bagaimanapun, pertumbuhan ikan yang diberi makan turmeric dan tuhau tidak menunjukkan perbezaan ketara daripada makanan kawalan ($p>0.05$). Penggunaan makanan yang baik juga dilihat dalam ikan yang diberi makan turmeric dan tuhau berbanding betel. Pekali pencernaan nyata (ADC) protin dan lipid dalam ikan yang diberi makan turmeric dan tuhau berbeza dengan ketara daripada diet kawalan ($p<0.05$). Sementara itu, nilai indeks hepatosomatik (HSI) pada ikan *yang diberi makan betel menunjukkan perbezaan yang ketara ($p<0.05$) berbanding rawatan lain. Penggunaan herba sebagai makanan tambahan tidak mempengaruhi analisis darah kecuali pada ikan yang diberi makan betel dimana kiraan sel putih dimana nilai yang lebih rendah diperolehi manakala kiraan platelet pula tinggi. Apabila dicabar dengan Vibrio harveyi VHJR7, ikan yang diberi makan betel menunjukkan kemandirian yang lebih tinggi antara semua dan aktiviti enzim superoksida dismutase (SOD) dalam darah meningkat dengan ketara selepas dicabar. Kesimpulannya, herba kunyit dan tuhau mampu meningkatkan pertumbuhan dan juga kecekapan pengambilan makanan kerapu hybrid juvenil manakala sirih memberikan hasil yang lebih baik dalam ujian cabaran bakteria.*

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LIST OF ABBREVIATIONS

ADC	-	Apparent digestibility coefficient
ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Chemists
ATCC	-	American Type Culture Collection
BC	-	Before Christ
BHT	-	Butylated hydroxyl toluene
BW	-	Body weight
BWG	-	Body weight gain
Conc.	-	Concentration
CF	-	Condition factor
DPPH	-	1,1-diphenyl-2-picrylhydrazyl
EDTA	-	Ethylenediaminetetraacetic acid
FCR	-	Feed conversion ratio
FI	-	Feed intake
GCMS	-	Gas Chromatography-Mass Spectrometry
HSI	-	Hepatosomatic index
Ig	-	Immunoglobulin
IPF	-	Intraperitoneal fat
MHA	-	Mueller-Hinton Agar
MIC	-	Minimum inhibition concentration
NPU	-	Net protein utilization
PCV	-	Packed cell volume
PBS	-	Phosphate Buffer Saline
PER	-	Protein efficiency ratio
RCC	-	Red cell count
S.D	-	Standard deviation
SGR	-	Specific growth rate
SOD	-	Superoxide dismutase activity
sp.	-	Species
SPSS	-	Statistical Package of Social Sciences

TFI	-	Total feed intake
TSB	-	Tryptic Soy Broth
UV-Vis	-	Ultraviolet-visible
VSI	-	Viscerosomatic index
WCC	-	White cell count
WG	-	Weight gain

LIST OF SYMBOLS

°C	-	Degree Celsius
%	-	Percentage
US$	-	United States of America Dollar
RM	-	Ringgit Malaysia
et al.	-	And others
G	-	Gauge
±	-	Plus minus sign
cm	-	Centimeter
mm	-	Millimeter
g	-	Gram
kg	-	Kilogram
mg	-	Milligram
µg	-	Microgram
mg/mL	-	Milligram per milliliter
mL	-	Milliliter
L	-	Liter
mmol/L	-	Millimoles per liter
unit/mL	-	Unit per milliliter
pH	-	Potential hydrogen
h	-	Hour(s)
c.f.u g^{-1}	-	Colony forming unit per gram
rpm	-	Revolutions per minute
M	-	Molar

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Aquaculture

Aquaculture is a mushrooming industry with an annual production of 80 million tonnes (Kolkovski and Kolkovski, 2011). Over the decades, the significant increase of fish production led to the necessity of intensive fish culture. Culturing fishes in enclosed spaces such as ponds, net cages and tanks are the norm to enhance productivity per unit space (Harikrishnan *et al.*, 2011b). With its growing industry, aquaculture has become a key component of the animal health industry (Kolkovski and Kolkovski, 2011). Stressors such as transporting, overcrowding, grading, poor water quality and handling deteriorates the health of cultured fish (Li *et al.*, 2004). When the health of fish deteriorates, it results in poor physiological environment and suppression of immune system which increases the chances of the fish to be susceptible to infectious agents and causing disease outbreaks. Events like this are risks as they lead to financial losses however they are common in aquaculture industry (Harikrishnan *et al.*, 2011a).

Several preventive measures were done to overcome the problem but even with these partially successful preventions including the use of antibiotics, the estimated annual economic loss were still huge for the last two decades. It was estimated that China lost over US$ 400 million in 1993, India forfeited US$ 17.6

million in 1994 while Thailand, over US$ 500 million in 1996 (www.agriculture.de/acms1/conf6ws9fish.htm). It is also well understood that harmful microbes, nutritional disorders and bad water quality or environmental disorders are causes of diseases and major obstacle to the fish culture worldwide (Kumar and Aanatharaja, 2007). Antibiotics is defined as drugs which are of natural or synthetic source and has the ability to kill or inhibit the growth of micro-organisms. In aquaculture, usage of antibiotics causes deliberate issues due to its adverse effects for example antibiotic accretion in the tissue and immunosuppression (Gudding *et al.*, 1999). These antibiotics and chemotherapeutants are mainly administered in the culture practices through medicated feeds or food materials and could enter the environment via leaching from uneaten feeds and also from the unabsorbed parts in excretion (Robinson *et al.*, 2007). The residual leaching into the surrounding environment also affects higher animals and humans such as poisoning (Vaseeharan and Thaya, 2014). However, the countless attempts to control and prevent these outbreaks using antibiotics and other chemical chemotherapeutants have been unsuccessful (Jadhav *et al.*, 2006). Due to the uncontrolled and repetitive uses of these antibiotics, antibiotic-resistant pathogens are developed and making future treatments and preventions even more challenging (Flores *et al.*, 2003).

1.2 Health Management in Aquaculture

1.2.1 Antibiotics

Substances that hold the capability to either terminate or inhibit growth of microorganisms are called antimicrobial agents. Post-discovery in 1928 by Fleming, antibiotics had become important in maintaining humans and animal's health and well-being. Antibiotics could be derived from natural sources or synthetic origins and since they were claimed to be safe (non-toxic) to the host, this enables them to be used as chemotherapeutic agents for bacterial infectious diseases treatment. Besides being used by humans especially in medicine field, antimicrobials were also used as supplements in animal's food and aquaculture feed. Usage of antibiotics could be categorised as therapeutic, prophylactic or metaphylactic (Saga and Yamaguchi, 2009).

Antibiotics were usually delivered to fishes via inclusion into formulated feed. As fish does not metabolises antibiotics naturally, most of them flows back into the environment through faeces where it was estimated that 75% of the antibiotics fed had been discharged back into the water (Burridge *et. al.*, 2010). Issues concerned with treatment of antibiotics in aquaculture were potential impacts on the aquatic environment (marine and freshwater) and also antimicrobial resistance developed by fish pathogens such as *Aeromonas salmonicida, A. hydrophila, Edwardsiella tarda, Yersinia ruckeri, Photobacterium damselae* and *Vibrio anguillarum* due to antimicrobial exposure. Resistance towards antimicrobials had been documented widely in medicinal field, both humans and veterinary.

1.2.2 Chemicals

Chemotherapy is infectious diseases treatment that involves usage of drugs or chemicals. It is regarded as the last resort in disease control. There are three known methods for application of chemical treatment to fish; external, systemic and parental treatment. Nevertheless, there are disadvantages of chemotherapy such as causing adverse effects or destroying nitrification process in biofilters, possessing adverse effects on natural foods, have potential to leave harmful residues in the host, ineffective bath immersions, potential for resistant strains to develop and immunosuppressive effects of certain drugs (Cruz-Lacierda and Erazo-Pagador, 2001).

However, these treatments have their disadvantages. Among them is that they are expensive to be used extensively in fish farming and some of the treatments are only effective towards one pathogen type (Harikrishnan *et al.*, 2011b; Pasnik *et al.*, 2005; Sakai, 1999). Harmless, preventive and lasting methods of health management should be focused more especially when human health and environment are concerned. Due to these concerns, natural products such as medicinal herbs or probiotics are some of the proposed solutions in aquaculture (Citarasu, 2010; Lee *et al.*, 2009; Makkar *et al.*, 2007; Mohapatra *et al.*, 2013; Panigrahi and Azad, 2007).

1.3 Alternatives of Health Management in Aquaculture

1.3.1 Probiotics and Prebiotics

Probiotics were described as microbial dietary adjuvants that profits the host physiology via mucosal modulating and systemic immunity besides enhancing the nutritional and microbial balance in the intestinal tract (Villamil *et al.*, 2002). Up to date, most of probiotics studies conducted in fish were taken from strains isolated and selected from the aquatic environment. A wide range of microalgae, yeast, gram-positive bacteria and gram-negative bacteria had been classified as probiotics (Gatesoupe, 1999). Moreover, there are several studies that validates the probiotic action modes in the aquatic environment where feed conversion ratio and feed utilization improves, adhesion capacity to the intestinal mucosa that hindered the adherence of pathogenic bacteria revealed, production of extra-cellular antibiotic like products or iron binding agents (siderophores) that prevent the growth of some pathogenic flora, improves water quality in presence of red tide planktons problems and enhance fish immune response (Kesarcodi-Watson *et al.*, 2008).

Meanwhile prebiotics were defined as non-digestible food ingredients by the host which possess benefits via selective intestinal tract metabolism (Gibson *et al.*, 2004). Oligosaccharides (fructooligosaccharide (FOS), mannanoligosaccharide (MOS)), polysaccharides, protein hydrolysates and polyols were some examples of prebiotics. They came from a vast source and were stable compounds that emits no residue and no induced resistance besides possess the ability to selectively proliferate intestinal bacteria, enhances immunity and demonstrates anti-viral activity (Gibson *et al.*, 2004).

However, according to the European Commission (2003) regulation, prebiotics are not authorised as feed additives due to several drawbacks from its products. This is due to several reasons such as prebiotics could neither kill or inhibit pathogens which does not provide any preventions against bacterial infections besides causing adverse reactions due to fermentation in the gastrointestinal tract such as bloating and diarrhoea (De Vrese and Schrezenmeir, 2008). Moreover, the exorbitant cost to produce prebiotics makes it cost ineffective hence restraints their usage in animal husbandry and aquaculture industry (Badia *et al.*, 2013).

1.3.2 Medicinal herbs

Medicinal herbs had been known to possess immunostimulant characteristics for centuries. Application of medicinal herbs as natural and harmless compounds exhibits potential in aquaculture as an alternative to immunoprophylactics and antibiotics (van Hai, 2015). This had caused escalation of interests in medicinal herbs across the globe as these plants are cheap, utilise minimal preparations and leaving minimal negative effects on animals and the environment. There is an array of medicinal herbs comprised of herbs, spices, seaweeds, herbal medicines, herbal extracted compounds and commercial plant-derived products had been used for studies in various aquatic animals. Utilization of medicinal plants does not limit to the parts only such as roots, leaves, stem and flowers but the whole plants could also be used or just the extracted compounds of the plants. In addition, it could be applied either in singles or combination of several varieties, and even fused with other immunostimulants which could be done either through water treatments, feed additives and enrichments. However, different types and parts of medicinal plants bear different dosages and period of administration which the optimal levels had not yet being considered and despite showing prospects to boosts growth and immunity (act as antibacterial and antiviral agent), the mechanisms were not fully understood (van Hai, 2015).

Increment of intensive aquaculture causes disease outbreak to occur frequently. Hence, medicinal herbs had gained interests in aquaculture in order to provide eco-friendly and safe compounds besides being an alternative to antibiotics and chemicals compounds while enhance fish immunity and control diseases in the same time. Medicinal herbs such as herbs, seeds and spices in various forms (crude, extracts, mixed and active compounds) has the potential to be used as immunostimulants in order to boost fish immune system and prevention from microbial diseases (Awad and Awaad, 2017).

The medicinal herbs act by stimulating the cellular and humoral immune response which could be observed via elevation in the fish immune parameters (Abdel-Tawwab *et al.*, 2010). Various concentrations of medicinal herbs supplemented either through injection, immersion or oral administration exhibit various levels of immune stimulation. Besides boosting immunity system and prevent diseases, medicinal herbs also could act as growth promoter and immunomodulator (Acar *et al.*, 2015). There are practices that uses several medicinal herbs to substitute protein in fishmeal as a cheaper alternative and had been efficiently proven (Awad and Awaad, 2017).

Rural fish farmers have been using medicinal herbs long ago (Caruso *et al.*, 2013). They introduced the medicinal herbs directly into rearing water to increase water quality, boost fish resistance to pathogens, minimise fish stress and combating fish diseases (Caruso *et al.*, 2013). Studies that had been done on medicinal herbs also reported that fish that were treated with it enhanced the immune parameters (Dügenci *et al.*, 2003; Yuan *et al.*, 2007).

1.4 Problem Statement

Hybrid grouper of tiger grouper (*Epinephelus fuscoguttatus*) and giant grouper (*Epinephelus lanceolatus*) was first produced in Borneo Marine Research Institute hatchery (Ch'ng and Senoo, 2008). Compared to their parental species, the hybrid grouper possesses better flesh quality and able to grow faster (Luin *et. al.*, 2013). Since then, hybrid grouper had become one of the main marine species cultured and commercialised especially in South East Asia region and China.

Hybrid grouper are more resistant towards most diseases and the production in the current market is stable. Nevertheless, in intensive culture of hybrid grouper, it is important to take note on the health management in order to ensure a sustainable production. One of the ways to promote better health management is by administering local medicinal herbs to the hybrid groupers.

Malaysia has a variety of local medicinal herbs that had been used by the locals for a long time, mainly as food consumption. Despite the abundance of local medicinal herbs in Malaysia, there are less studies done on incorporating local medicinal herbs into aquaculture especially for hybrid groupers. Hence, it is required to understand which local medicinal herbs that could boost the immunity of the hybrid grouper without jeopardizing its growth and survivability.

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