



UNIVERSITI PUTRA MALAYSIA

**EFFECTS OF SELECTED HERBAL EXTRACTS ON GROWTH, DISEASE
RESISTANCE AND IMMUNE RESPONSE IN ASIAN SEABASS, *Lates
calcarifer* (BLOCH, 1790)**

RASHIDAH BINTI ABDUL RAZAK

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By

RASHIDAH BINTI ABDUL RAZAK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in
Fulfilment of Requirements for the Degree of Doctor of Philosophy

November 2018

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DEDICATION

WITH LOVE AND APPRECIATION TO:

MY PARENTS: ABDUL RAZAK JUSOH AND SAMSIAH ABDULLAH

MY HUSBAND: MUHAMMAD FARIZAL ABDUL HALIM

MY DAUGHTER: MALIKHA ALEESYA



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Prof Dato' Mohamed Shariff Mohamed Din, PhD
Faculty : Veterinary Medicine

Asian seabass, *Lates calcarifer* is among the main marine fish species cultured in Malaysia with the highest production. Nevertheless, disease outbreaks during culture periods have become a major constraint that contributes to severe economic losses. The frequent use of antibiotics for disease treatment has caused a negative impact with the development of resistant bacterial strains. Thus, the use of herbal treatments is now receiving greater attention as an alternative to overcome disease problems. As herbal medicine is effective in healing and curing human diseases it is also being applied as chemotherapeutics and feed additives in fish. Therefore, the general objective of this study was to determine the effect of herbal supplementation on the growth performance, immune response and disease resistance in Asian seabass. Extracts of *Clinacanthus nutans*, *Syzygium polyanthum*, *Vitex negundo*, *Polygonum chinense*, *Alpinia conchigera*, *Premna foetida*, *Brucea javanica* and *Pimenta dioica* were screened for their antimicrobial activities against the common marine fish pathogens, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Aeromonas hydrophila* using disk diffusion method. The results showed that the methanolic extracts of *P. chinense*, *P. dioica* and *P. foetida*, and aqueous extracts of *S. polyanthum* and *P. dioica* have moderate to strong antimicrobial activities against all tested pathogens at concentration of 300 mg/mL. These five crude extracts were further fractionated using methanol, dichloromethane and ethyl acetate whereby ethyl acetate fractions gave moderate, strong to very strong antimicrobial activities against all tested bacteria except for *P. foetida* extract against *V. parahaemolyticus*. Brine shrimp cytotoxicity study on the potential five crude extracts showed that only the methanolic extract of *P. foetida* and *P. chinense* have low toxicity while the others were moderately toxic. Therefore, extract of *P. foetida* (PFA) and *P. chinense* (PCE) were chosen for feed supplementation at concentrations of 2, 5 and 10 g/kg for 60 days, followed by challenged with *V. harveyi* for 14 days to investigate their potential as growth and disease resistance promoters in Asian seabass. The results showed that PCE at 5 g/kg (PCE-5) diet was the best fish growth promoter with 47.24 g/fish of body weight gain, 35.8 of feed efficiency, 1.14 specific growth rate and 98.8% survival. Fish fed with PCE-5 diet have also obtained significantly higher (p

< 0.05) albumin, albumin: globulin ratio, phagocytic activity and serum total immunoglobulin compared with other diets on day 60. After two weeks of challenging study, all treatment diets showed significantly ($p < 0.05$) higher survival than the control. Fish fed with PCE-10 diet had the highest survival (90%), followed by PCE-5 and PCE-2 diets with 86.7% survival respectively. PCE-10 diet supplementation also showed significantly ($p < 0.05$) increases in some of the haemato-biochemical parameters, lysozyme and respiratory burst activities, and total immunoglobulin compared to the control. The histological study also revealed that fish fed with both plant extracts showed milder lesions compared to the control upon exposure to *V. harveyi*. Therefore, this study revealed that *P. chinense* supplementation in fish diet has a beneficial effect on the growth, survival, biochemical, immunological and disease resistance in Asian seabass.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**KESAN-KESAN EKSTRAK HERBA YANG TERPILIH KE ATAS
PERTUMBUHAN, KETAHANAN PENYAKIT DAN REAKSI IMUN DALAM
SIAKAP, *Lates calcarifer* (BLOCH, 1790)**

Oleh

RASHIDAH BINTI ABDUL RAZAK

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Ikan siakap, *Lates calcarifer* merupakan antara spesis ikan marin utama yang diternak di Malaysia dengan pengeluaran hasilnya yang tertinggi. Walaubagaimanapun, penularan penyakit semasa tempoh ternakannya, telah menjadi satu kekangan yang menyumbang kepada kerugian ekonomi yang teruk. Penggunaan antibiotik yang kerap untuk rawatan penyakit telah menyebabkan satu kesan negatif dengan penghasilan strain bakteria berdaya rintangan. Oleh itu, rawatan menggunakan herba kini menerima banyak perhatian sebagai satu alternatif bagi mengatasi masalah penyakit ikan. Oleh kerana ubat herba telah terbukti berkesan dalam memulihkan pelbagai penyakit manusia, jadi ia juga telah digunakan sebagai bahan kemoteraputik dan makanan tambahan kepada ikan. Jadi, matlamat utama kajian ini adalah untuk menentukan kesan penambahan herba ke atas prestasi tumbesaran, reaksi imun dan ketahanan penyakit pada ikan siakap. Ekstrak *Clinacanthus nutans*, *Syzygium polyanthum*, *Vitex negundo*, *Polygonum chinense*, *Alpinia conchigera*, *Premna foetida*, *Brucea javanica* dan *Pimenta dioica* telah ditapis untuk aktiviti antimikrob terhadap patogen ikan laut iaitu *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* dan *Aeromonas hydrophila* menggunakan kaedah penyebaran cakera. Keputusan menunjukkan bahawa ekstrak metanolik *P. chinense*, *P. dioica* dan *P. foetida*, dan ekstrak akueus *S. polyanthum* dan *P. dioica* mempunyai kesan aktiviti antimikrob sederhana hingga kuat terhadap semua pathogen yang diuji pada kepekatan 300 mg/mL. Lima ekstrak mentah ini seterusnya dipecah-asingkan menggunakan metanol, diklorometana dan etil asetat di mana pecahan-pecahan etil asetat mempunyai aktiviti antimikrob yang sederhana, kuat dan sangat kuat terhadap semua bakteria yang diuji kecuali ekstrak *P. foetida* terhadap *V. parahaemolyticus*. Kajian ketoksisan udang air masin ke atas lima ekstrak mentah telah menunjukkan hanya ekstrak metanolik *P. foetida* dan *P. chinense* mempunyai ketoksisan rendah manakala yang lain adalah sederhana toksik. Oleh itu, ekstrak *P. foetida* (PDA) dan *P. chinense* (PCE) telah dipilih sebagai penambah makanan pada kepekatan 2, 5 dan 10 g/kg selama 60 hari, diikuti dengan ujian cabaran terhadap *V. harveyi* selama 14 hari untuk menyiasat potensinya sebagai penggalak pertumbuhan dan ketahanan penyakit pada ikan siakap. Keputusan menunjukkan diet PCE-5 adalah penggalak pertumbuhan terbaik dengan

47.24 g/pertambahan berat badan ikan, 35.8 kecekapan makanan, 1.14 kadar pertumbuhan spesifik dan 98.8% kadar kemandirian. Ikan diberi makan dengan diet PCE-5 juga dengan ketara ($p < 0.05$) memiliki tahap albumin, nisbah albumin: globulin, aktiviti fagosit dan jumlah immunoglobulin serum yang lebih tinggi berbanding diet lain pada hari 60. Selepas 2 minggu kajian cabaran, kesemua diet rawatan menunjukkan kelangsungan hidup lebih tinggi yang ketara ($p < 0.05$) berbanding kawalan. Ikan diberi makan dengan diet PCE-10 mempunyai kadar kemandirian tertinggi (90%), diikuti diet PCE-5 dan PCE-2 dengan masing-masing memiliki 86.7% kadar kemandirian. Makanan tambahan PCE-10 juga menunjukkan peningkatan ketara ($p < 0.05$) dalam beberapa parameter hemato-biokimia, aktiviti lisozim, letusan respirasi dan jumlah immunoglobulin berbanding kawalan. Kajian histologi juga menunjukkan ikan diberi makan dengan kedua-dua ekstrak tumbuhan menunjukkan lesi yang lebih ringan berbanding kawalan selepas pendedahan kepada *V. harveyi*. Oleh itu, kajian ini mendedahkan bahawa penambahan *P. chinense* dalam diet ikan mempunyai kesan bermanfaat terhadap tumbesaran, kadar kemandirian, biokimia, immunologi dan ketahanan penyakit pada ikan siakap.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

| | |
|--------------------|--|
| °C | Degree Celsius |
| 16S rRNA | 16S ribosomal RNA |
| AAHU | Aquatic Animal Health Unit |
| ABW | Average body weight |
| A: G | Albumin: globulin ratio |
| AWG | Average weight gain |
| Alb | Albumin |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| ANOVA | Analysis of variance |
| AST | Aspartate transaminase |
| ATCC | American type culture collection |
| BHA | Butylated hydroxyanisole |
| BWG | Body weight gain |
| CF | Condition factor |
| CFU | Colony formation unit |
| CLSI | Clinical and Laboratory Standards Institute |
| cm | centimetre |
| CO ₂ | Carbon dioxide |
| DCM | dichloromethane |
| ddH ₂ O | Double distilled water |
| DHA | Docosahexaenoic acid |
| DMF | N, N-dimethyl formamide |
| DMSO | Dimethyl sulfoxide |
| DO | Dissolved oxygen |
| DoF | Malaysian Department of Fisheries |
| DPPH | 2,2-diphenyl-1-picryhydrazyl |
| EA | Ethyl acetate |
| EAF | Ethyl acetate fraction |
| EFA | Essential fatty acids |
| EJF | Environmental Justice Foundation |
| Eos | Eosinophils |
| EPA | Eicosapentaenoic acid |
| EUS | Epizootic ulcerative syndrome |
| FAO | Food and Agriculture Organization of the United Nation |
| FCR | Feed conversion ratio |
| FDA | US Food and Drug Administration |
| FE | Feed efficiency |
| FER | Feed efficiency ratio |
| FRAP | Ferric Reducing Antioxidant Power |
| g | gram |
| GAE | Gallic acid equivalent |
| GC-MS | Gas chromatography–mass spectrometry |
| Glo | Globulin |
| Glu | Glucose |
| h | hour |
| H and E | Haematoxylin and eosin |
| Hb | Haemoglobin |
| HPV | Human papillomavirus |

| | |
|------------------|--|
| Ht | Haematocrit |
| HUFA | Unsaturated fatty acids |
| HSV | Herpes simplex virus |
| IHNV | Infectious hematopoietic necrosis virus |
| kg | kilogram |
| L | litre |
| LA | Lysozyme activity |
| LC ₅₀ | 50 percent lethal concentration |
| LD ₅₀ | 50 percent lethal dose |
| Lym | Lymphocytes |
| µg | microgram |
| µL | microlitre |
| MCHC | Mean corpuscular haemoglobin concentration |
| MCV | Mean corpuscular volume |
| MeOH | Methanol |
| mg | milligram |
| MHA | Mueller Hinton agar |
| MHB | Mueller Hinton broth |
| MIC | Minimum inhibition concentration |
| min | minute |
| mL | millilitre |
| mm | millimetre |
| MMC | Melanomacrophage center |
| mmt | Million metric tonnes |
| Mon | Monocytes |
| mt | Metric tonnes |
| NaCl | Sodium chloride |
| NBT | Nitroblue tetrazolium |
| Neu | Neutrophils |
| OD | Optical density |
| OMV | Oncorhynchus masou virus |
| PA | Phagocytic activity |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PCV | Packed cell volume |
| PEG | Polyethylene glycol |
| PER | Protein efficiency ratio |
| PMS | Premenstrual syndrome |
| PWG | Percentage weight gain |
| RBA | Respiratory burst activity |
| RBC | Red blood cells |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RPS | Relative percent survival |
| RT-PCR | Real-time polymerase chain reaction |
| SGR | Specific growth rate |
| SOFIA | The State of World Fisheries and Aquaculture |
| SSA | Southern Shrimp Alliance |
| TCBS | Thiosulfate citrate bile salts sucrose |
| TGC | Thermal growth coefficient |
| TI | Total immunoglobulin |

| | |
|------|--|
| TP | Total protein |
| TPU | University Agriculture Park |
| TSA | Tryptone soy agar |
| TSB | Tryptone soy broth |
| U | Unit |
| UPM | Universiti Putra Malaysia |
| UN | United Nations |
| VHSV | Viral haemorrhagic septicaemia rhabdovirus |
| VNN | Viral nervous necrosis |
| WBC | White blood cells |
| WHO | World Health Organization |
| WG | Weight gain |
| WOR | World Ocean Review |
| WSSV | White spot syndrome virus |
| w/v | Weight per volume |
| w/w | Weight per weight |
| YRV | Yellow-head rhabdovirus |



CHAPTER 1

INTRODUCTION

1.1 Background of study

The aquaculture industry is growing rapidly as one of the major sources of food production worldwide (FAO, 2017a). Global production of aquaculture has increased almost twofold from 61.6 million metric tonnes (mmt) to 110.2 mmt in quantity and threefold in value from USD 81.6 million to 243.5 million between 2006 to 2016 (FAO, 2017a). The increase of aquaculture production is to contribute to the world fish supplies as the fisheries resources are now being overfished (SOFIA, 2014). According to SOFIA (2014), the proportion of overfished stocks increased tremendously from 10% in 1974 to 28.8% in 2011. Furthermore, world marine capture production has also increased from 84.5 mmt to 92.0 mmt over the past 15 years (FAO, 2017b). These high amounts of global capture fishery production can contribute to the depletion of the marine fish abundance (Hutching and Reynolds, 2004). According to Worm *et al.* (2006), if the current practices in the marine capture continue, the total fish stocks will collapse 100% by 2048. Therefore, in order to overcome the serious threat of overfishing and limitation on wild capture, aquaculture has become one of the solutions for seafood production and marketing globally (Klinger and Naylor, 2012).

The aquaculture industry has grown tremendously over the past years in response to the increasing market demand for food. The global food demand is estimated to increase by 50% between 2012 to 2050 (FAO, 2017d). One of the vital sources of food for humans is fish (WOR, 2013) as it is high in protein, micronutrients and fatty acids that are essential for human brain development (Tacon and Metian, 2013).

In order to meet the higher demand for fish as food, farmers are intensively culturing fish. For example, in Malaysia, the total number of ponds, cages, pools and tanks have increased almost twofold, rising from 151 798 to 276 369 over the past 10 years from 2006 to 2016 (DoF, 2007; DoF, 2017). However, the rapid expansion of intensive aquaculture has caused a severe environmental problem (Ottinger *et al.*, 2016). Intensive aquaculture involves high stocking densities and high feed input, produces effluent discharges loaded with high nutrients (Piedrahita, 2003; Read and Fernandes, 2003). EJF (2003) reported that the effluent discharges from the culture ponds were channelled into the environment have polluted the soil and water. The unsustainable practice of discharging untreated effluent will affect the water quality until a level where it is toxic to the aquatic animals (Naylor *et al.*, 2001).

Poor water quality from the toxic unionised ammonia that is above optimum level will increase the fish susceptibility to non-infectious diseases (Ngueku, 2014). Moreover, the poor water quality also caused stress in fish which suppressed the immune functions, making the fish more susceptible to infectious diseases (Boyd, 2017). These non-infectious and infectious aquatic animal diseases have become a major limiting factor in

aquaculture. Currently, the aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens (Bondad-Reantaso *et al.*, 2005). Bacterial diseases have become a major constraint to the aquaculture, thus far at least 13 genera of bacterial species have been reported to be pathogenic to aquatic animals (Klesius and Pridgeon, 2011). With intensification and expansion of aquaculture activities, disease problems have also increased. The annual loss of revenue due to diseases is estimated to reach up to USD 6 billion (Assefa and Abunna, 2018). For example, in Brazil, diseases have caused an annual loss of about 15% of fish production, which valued about USD 84 million (Tavares-Dias and Martins, 2017). China which is the leading country for aquaculture also experienced a loss of 15% of their total fish production to diseases (Leung and Bates, 2013). A total loss of about USD 1 billion was also reported in India due to diseases in shrimp (Mishra *et al.*, 2017). High mortality and high economic losses in the aquaculture industry have resulted in several farmers not making a profit, and some were declared bankrupt (Lafferty *et al.*, 2015). To overcome disease problems, farmers use antibiotics and chemicals in controlling the disease outbreak in aquaculture.

1.2 Statement of problem

Antibiotics such as oxytetracycline, florfenicol, saraflloxacin and potentiated sulphonamides have been used to control diseases in the aquaculture industry in some countries in Europe (Armstrong *et al.*, 2005) and Asia (Serrano, 2005). Although these antibiotics are authorised for use in aquaculture (Serrano, 2005) with high potential in controlling some bacterial diseases, but there are many problems associated with their usage. Antibiotics cannot be recommended in the aquaculture because of their side effects and potential dangerous residue to the consumer in spite of the positive effects of these chemicals/antibiotics on the fish (Citarasu, 2010). Moreover, some countries have banned the use of antibiotics as growth promoters in animal feed, and the Republic of Korea is the first Asian country to implement it (FAO, 2018). In addition, some of the side effects of the antibiotics usage are residue problems such as persistence in sediment and water column (Armstrong *et al.*, 2005), and the development of antibiotic resistance (Serrano, 2005). Therefore, these emerging antibiotics resistance is a public health concern in animal medicine and human worldwide (Landers *et al.*, 2012). Increase awareness and concern about this problem has led to the suggestion of alternative method such as the use of the medicinal plant (Caruso, 2016).

Medical plants have been globally known and used for thousands of years as traditional medicine in humans (Bulfon *et al.*, 2015) and has more recently attracted the attention of scientists and researchers as an alternative in preventing diseases occurrence in aquaculture. Consequently, medical plants have been tested in fish and shellfish, and successfully being proven as a growth promoter, immunostimulant, agent for antibacterial, antiviral, antifungal and anti-stress, appetite stimulators and aphrodisiac (Citarasu, 2010).

Local medicinal plants have been shown to possess many potential bio-activities such as antimicrobial activity (Chung *et al.*, 2004; Philip *et al.*, 2009; Zaidan *et al.*, 2005) that can be used for replacing antibiotics and chemical drugs (Savoia, 2012). Medicinal plants

also contain various nutrients that can promote growth and used as immunostimulants for enhancement of the immune system of fish to prevent and control microbial diseases (Vaseeharan and Thaya, 2014). Therefore, the present study was undertaken to elucidate the effect of local medicinal plants as antibacterial, growth promoter, immunostimulant and disease resistance in Asian seabass, *Lates calcarifer*.

1.3 Hypotheses

H_0 : The extracts of the selected local medicinal plants do not possess antimicrobial activity against fish pathogens

H_1 : The extracts of the selected local medicinal plants possess antimicrobial activity against fish pathogens

H_0 : Diet supplemented with plant extracts showing optimum antibacterial activity and low toxicity do not improve the growth, disease resistance and immune response in Asian seabass, *Lates calcarifer*

H_1 : Diet supplemented with plant extracts showing optimum antibacterial activity and low toxicity improve the growth, disease resistance and immune response in Asian seabass, *Lates calcarifer*

1.4 Objectives

The aim of this study was to evaluate the potential of medicinal plants as an antibacterial agent in Asian seabass.

The specific objectives of the present study were:

- 1) To investigate the antimicrobial activity of crude extracts of the selected medicinal plants and their fractions of against bacterial pathogens
- 2) To determine the toxicity level of the selected plant extracts showing optimum antibacterial activity
- 3) To analyse the effect of dietary enhancement on growth, survival, haemato-biochemical parameters and immune response of Asian seabass fed plant extracts showing optimum antibacterial activity and low toxicity
- 4) To investigate the disease resistance in Asian seabass challenged with *Vibrio* infection after feeding with the selected plant extracts.

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