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Editor's Note

Although slightly delayed, for us, it is better late than never to make restitution. As we all know, the 11th Diseases in Asian Aquaculture (DAA11) Symposium, Kuching Sarawak was successfully organized in August 2022. A total of 141 abstract submissions from 23 countries which cover a wide range of aquatic animal health aspects, including biosecurity in aquaculture, epidemiology, detection, prevention, and control of diseases in finfish, crustacean and shellfish and trends in fish and shrimp health management was received and presented orally and as posters. Some of the papers presented were already published in MFJ Volume 22, 2022, which serves as a special edition devoted to the DAA11 and launched during the opening ceremony. Since there are still several papers collected for this purpose, we decided to issue the second special edition volume of DAA11 (Volume 24, 2024). This special edition seeks to promote young researchers' works to get published and disseminate their findings. It is also hoped that the papers will provide the reader with current information on the animal health aspects, especially within the local settings.

Environmentally Friendly Alternatives to Chemicals in Aquatic Animal Health Management: Malaysian Experiences

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Abstract: Aquaculture expansion is typically correlated with culture intensification and can result in overcrowding and poor water quality, which promotes pathogen propagation and increases disease outbreaks and mortality. In aquaculture, chemicals and drugs lead to several significant issues, including antimicrobial resistance development and dissemination, health risk to farmers and consumers, environmental pollution, and extra costs to farmers. In Malaysia, alternative medicine research and development (R&D) have been conducted and evaluated since 2005. The first attempt involved developing an oral vaccine against streptococcosis in tilapia. Subsequently, environmentally friendly alternatives to chemicals were attempted to manipulate pathogen lifecycles and incorporate plant-based antimicrobial solutions into fish diets. A recent innovation named Break and Protect (BP) aimed to trap and remove marine leeches from infested grouper by disrupting the leech lifecycle was developed and upgraded to BP2, which could remove leeches from 14 farmed fish species. Developed in 2017, KRIPeK is a portable kit for the on-site treatment of fish swim bladder disorders. Additionally, several plant-based antimicrobial solutions were developed to prevent and treat bacterial diseases in fish and stimulate and promote weight gain when administered to fish culture. Furthermore, commercial essential oils, such as cinnamon oil, prevented and treated marine ectoparasites. These locally produced, environmentally friendly alternatives have good potential to be applied in aquaculture for alternative treatment and intervention of disease outbreaks.

Keywords: Local plant, commercial herbs, prevention, treatment, early detection

Abstrak: Perkembangan pesat dalam akuakultur biasanya berkorelasi dengan intensifikasi ternakan, dan ini mampu mengakibatkan kualiti air yang buruk, mendorong penyebaran pathogen, peningkatan penyakit serta kematian ternakan. Penggunaan bahan kimia dan ubat-ubatan dalam akuakultur membawa kepada beberapa isu penting termasuk pengembangan dan penyebaran mikrob rintang antibotik, risiko kesihatan kepada petani dan pengguna, pencemaran terhadap alam sekitar dan penambahan kos operasi. Di Malaysia, R&D mengenai ubatan alternatif telah dijalankan sejak tahun 2005. Kajian pertama melibatkan pembangunan vaksin oral terhadap jangkitan *Streptococcus* sp. pada ikan tilapia. Kemudian, rawatan alternatif mesra alam berasaskan manipulasi kitaran hidup patogen dan pengembangan diet ikan. Antaranya inovasi yang dinamakan "Break and Protect (BP)" yang merupakan alat untuk memerangkap dan mengeluarkan lintah laut dari ikan kerapu terak di

sangkar dengan memutuskan kitaran hidup lintah laut. Inovasi ini ditambahbaik dan dinamakan BP2 dan mampu menyingkiran lintah laut dalam 14 spesies ikan ternak. Produk lain yang dicipta pada tahun 2017 adalah KRIPeK, iaitu kit mudah alih untuk rawatan masalah pundi renang ikan. Di samping itu, telah dilakukan pembangunan beberapa antimikrob berasaskan tumbuhan seperti SirehMAX™, GARLEX, dan SitroPro untuk mencegah dan merawat penyakit bakteria pada ikan di samping merangsang dan menggalakkan penambahan berat badan apabila diberikan kepada ternakan. Penggunaan minyak pati komersial seperti minyak kulit kayu manis juga terbukti bermanfaat dalam mencegah dan merawat ektoparasit marin. Alternatif mesra alam yang dihasilkan oleh penyelidik tempatan yang dibentangkan dalam kertas kerja ini mempunyai potensi yang baik untuk diaplikasikan dalam akuakultur bagi rawatan alternatif dan intervensi wabak penyakit.

Introduction

Global fish production was approximately 178 million tonnes in 2020, with an estimated total first sale value of USD 406 billion. Of this production, 88 million tonnes (approximately 49%; USD 265 billion) were from aquaculture production (Food and Agriculture Organization of the United Nations [FAO], 2022). Aquaculture has expanded rapidly, and sustainable aquaculture development remains essential to meet the rising demand for seafood. The specific nature of aquaculture practices predisposes farmed aquatic organisms to pathogens and potential disease outbreaks through the translocation and introduction of new species of aquaculture stocks that can lead to pathogen co-introduction (Shinn et al., 2023).

The rising issues such as co-infection and recurring disease stem from new pathogens and/or re-emerging infectious diseases. The FAO estimated that the economic effect of disease on 81 global aquaculture species was USD 6 billion annually (Shinn et al., 2015). Almost all aquaculture operations, especially the highly intensive and super-intensive farms, rely on medicants and control agents. As the aquaculture industry has expanded, many aquaculturists use chemicals as their first choice in the agriculture sector to treat pests, control algae and unwanted vegetation, and enhance growth and production. When properly applied, pesticides, algaecides, herbicides, and fungicides control pests and noxious vegetation. However, their improper use and runoff from agriculture can affect water and sediment quality, alter microbial communities and biodiversity, kill non-target animals and plants, and affect farm workers' health. Additionally, uncontrolled antibiotics or control agent use in aquaculture might lead to the emergence of antimicrobial-resistant (AMR) organisms. Thus, aquaculture operators are currently applying "best practices" and "know-how" when using such chemicals.

Human health and environmental concerns regarding chemical use in aquaculture are reflected in the FAO Code of Conduct for Responsible Fisheries (FAO, 1995) and the Guidelines for the Use of Chemicals in Aquaculture and Measures to Eliminate the Use of Harmful Chemicals (2013). In Malaysia, chemicals or chemo-therapeutants are used to treat fish and shrimps (Mohamed et al., 2000; Choo, 2000; Ibrahim, 2003). The chemicals used in Malaysian aquaculture are represented by four major groups: topical disinfectants, antimicrobials, probiotics, and anaesthetics (Mohamed et al., 2000). Topical disinfectants are used mainly to eliminate external opportunistic bacteria, fungi, and protozoans, while antimicrobials are antibacterial drugs. Probiotics or bacterial concentrates

enhance microbial degradation of organic accretions in the pond and can reduce the biochemical oxygen demand and increase the possibility of anaerobiosis. Anaesthetics includes substances used to sedate fish during transport and handling.

The increasing use of chemicals and antimicrobials in humans and food-producing animals is driving AMR (Laxminarayan et al., 2013), which is amongst the defining global health challenges of this era. Similarly, production intensification and the increasing incidence of infectious disease outbreaks associated with aquatic animal pathogens are driving antimicrobial use (Cabello et al., 2013) and AMR (Miller and Harbottle, 2018). Antimicrobial application in aquaculture is more harmful than in terrestrial food animal production as it leads to wider environmental exposure pathways for drug distribution through water, with important ecosystem health implications.

Many nations have banned the use of antimicrobials, particularly antibiotics, in the aquaculture sector due to the risks they present to consumers, the environment, and fish. Emphasising aquaculture disease, the search for alternative, ecologically friendly disease treatment methods has expanded over the past 10 years. A recent review reported that vitamins, prebiotics, probiotics, post-biotics, and parabiotics can be used as alternative treatment strategies in aquaculture (Thora et al., 2020). The two approaches are as follows: i) treatment of aquaculture water and the pathogen to reduce the overall load, and ii) treatment of fish stocks to render them less susceptible to infections by increasing general stress resistance or the stimulation of an immune response to protect against pathogens. There are also several disease managements approaches. The development of protective vaccines, immunoglobulin Y (IgY) as a feed additive, nano-bubbles to improve water quality and allow fish to thrive, environmentally friendly alternatives to synthetic chemicals, and good aquaculture practices are important approaches to overcoming chemical dependency. Since the Ninth Malaysia Plan (2006–2010), the National Fish Health Research Centre (NaFisH), a centre under the Fisheries Research Institute (FRI), Department of Fisheries, embarked on research, development, and innovation (R&D&I) of alternative treatments of fish to reduce the overall volume of chemicals used in aquaculture. The specific R&D&I objectives are to: i) avoid using hormones, antimicrobials, chemicals, and synthetic drugs in aquaculture; ii) overcome AMR; iii) reduce operational costs (no withdrawal period); and iv) ensure no hazardous chemical residues in fish tissues.

This article reviews the R&D&I on alternatives to chemicals in aquatic animal health management in Malaysia, especially FRI initiatives and outputs. The issues and challenges in delivering the innovations and technologies to end-users are briefly discussed.

R&D&I of environmentally friendly alternative chemicals

Vaccine development

Vaccination was clearly the potential solution for controlling and preventing infectious diseases since the 1980s, as demonstrated by the Norwegians in controlling vibriosis and furunculosis (Sommerset et al., 2005). Vaccines are a preferred alternative to chemicals, particularly antibiotics. In Malaysia, fish vaccine R&D began approximately 20 years ago, with most efforts concentrating on

vaccines against bacterial infections, most notably streptococcosis, vibriosis, and motile aeromonad septicaemia (MAS) (Ridzuan et al., 2022). There is a dire need for new locally produced vaccines, especially from local isolates as only three aquatic vaccines are approved for use in Malaysia (Department of Veterinary Services Malaysia, 2022).

Streptococcosis is a key disease of global tilapia aquaculture production and is prevalent in Malaysian tilapia production (Siti-Zahrah et al., 2004, 2008). Infection takes place through two species: *Streptococcus agalactiae* and *S. iniae* (Ali et al., 2020; Liao et al., 2020). The first reported case of streptococcosis in Malaysia was in the late 1990s in Sungai Pahang, Pahang, and affected cultured red hybrid tilapia (300–400 g), resulting in 60% mortality. In 2000, a series of outbreaks were recorded in Tasik Kenyir, Terengganu, and Tasik Pergau, Kelantan, causing approximately 50% mortality of cultured tilapia (Amal et al., 2008; Siti-Zahrah et al., 2008). Subsequently, mass mortality of cage-cultured red hybrid tilapia was reported in Tasik Kenyir (Najiah et al., 2012) and Temerloh, Pahang (Laith et al., 2017).

MAS is also common in Malaysia and most frequently linked to *Aeromonas hydrophila* and other *Aeromonas* species (*A. sobria*, *A. veronii*, and *A. caviae*). For example, *A. hydrophila* cases have been reported in *Clarias* spp. (Laith et al., 2013), *Pangasius* spp. (Mahmood et al., 2019), and tilapia (Pauzi et al., 2020) in Malaysia. Vibriosis has also been a Malaysian marine aquaculture issue since the 1970s (Leong et al., 1997), when marine finfish culture was established. However, it was not until the 1990s that seabass, grouper, and snapper were frequently infected. Fish are susceptible to vibriosis throughout the hatchery and grow-out phases. Several *Vibrio* spp. have been identified as etiological agents, including *V. alginolyticus*, *V. vulnificus*, and *V. harveyi.* Vibriosis outbreaks are not limited to Peninsular Malaysia and are also reported from across Borneo and East Malaysia (Ransangan and Mustafa, 2009).

Table 1 presents the main R&D on fish vaccines in Malaysia. A streptococcosis vaccine is one of the most investigated prospects due to the aggressive series of outbreaks affecting Malaysian tilapia cultures (Table 1). Furthermore, most studies focused on developing an oral vaccine rather than an injectable or immersion vaccine. The oral route is preferred as 80% of local tilapia farmers are small-scale operators. Injectable or immersion routes require additional infrastructure, labour, and effort, which increases the farmers' burden. Oral vaccines are generally prepared by infusing the antigen into feed and are more suitable for mass vaccination of fish of various sizes and ages against *Streptococcus*.

Many studies on vaccine development against major diseases in Malaysia were limited to laboratory-based investigations (Table 1). Most of these studies based their research on inactivated vaccines, while only a few used live attenuated vaccines (LAV). This is unsurprising as the whole-cell inactivated vaccine is the traditional vaccine preparation commonly used to control several piscine bacterial diseases in Southeast Asia (Kayansamruaj et al., 2020). Inactivated vaccines are prepared by physical and chemical treatments, such as heat inactivation, UV, irradiation, or formalin inactivation. However, LAV are used principally to control infectious diseases caused by known and novel identified pathogenic organisms. LAV are uncommon in aquaculture probably due to safety issues after their administration (Kayansamruaj et al., 2020).

Recombinant subunit vaccines (DNA or recombinant protein vaccines) can be used to reduce the possibility of regaining virulence. The first recombinant oral vaccine produced and used to combat disease in tilapia in Malaysia was reported by Nur-Nazifah et al. (2014). The vaccine was the result of joint R&D by NaFisH, FRI, Universiti Putra Malaysia (UPM), and Agro-Biotechnology Institute Malaysia (NIBM) experts. In July 2015, a patent for the was filed with Malaysia Intellectual Property Organization (MyIPO) and granted in June 2022 [Oral Vaccine Against Streptococcosis of Fish (MY-191398-A)].

Only a few R&D studies reached the field-testing stage (Table 1), and none were up-scaled or reached the pre-commercialisation or commercialisation stages. This limitation occurs in Malaysia and Southeast Asia. Only four types of fish vaccine have been registered in Southeast Asia to be used in tilapia, striped catfish, and Asian seabass, which are significantly fewer than those registered for salmonids in the USA, Europe, and Latin America (Kayansamruaj et al., 2020).

Table 1: R&D on fish vaccine development in Malaysia (extracted and modified from Ridzuan et al. (2022)).

Pathogens	Type/route	Targeted fish species	Stage of trial	References
S. agalactiae	Inactivated/oral	Red tilapia hybrid (*Oreochromis* sp.)	Lab	Firdaus-Nawi et al. (2013)
	Inactivated/spray immersion		Lab	Noraini et al. (2013)
	Recombinant/oral		Lab	Nur-Nazifah et al. (2014)
	Recombinant/oral		Field	Abu (2015)
	Inactivated/Oral		Lab	Ismail et al. (2016)
	Inactivated/Oral		Field	Ismail et al. (2016)
	Inactivated/Oral		Lab	Sa'aidatun et al. (2018)
	Inactivated/biofilm		Lab	Kahieshesfandiari et al. (2019)
	Live attenuated/oral		Lab	Laith et al. (2017)
S. iniae	Inactivated/oral	Red tilapia hybrid (*Oreochromis* sp.)	Lab	Hayat et al. (2020) Hayat et al. (2021)
A. hydrophilia	Recombinant/ intraperitoneal (injection)	African catfish (*Clarias gariepinus*)	Lab	Matusin (2015)
S. iniae; *A. hydrophila;*	Inactivated; bivalent/oral	Red tilapia hybrid (*Oreochromis* sp.)	Lab	Monir et al. (2020)
V. harveyi; *S. agalactiae*; *A. hydrophila*	Inactivated; polyvalent/oral	Asian seabass (*Lates calcarifer*)	Lab	Mohamad et al. (2021)
V. harveyi	Live attenuated/ intraperitoneal	Tiger grouper (*Epinephelus fuscoguttatus*)	Lab	Mohd-Aris et al. (2019)
	Inactivated/intraperitoneal	Marine red tilapia (*Oreochromis* sp.)	Lab	Mohd-Aris et al. (2019)
	Live attenuated/ intraperitoneal	Asian seabass (*L. calcarifer*)	Lab	Chin et al. (2020)
V. alginolyticus	Recombinant/ intraperitoneal (injection)	Tiger grouper (*E. fuscoguttatus*)	Lab	Nehlah et al. (2016), Nehlah et al. (2017)
V. alginolyticus; *V. harveyi*	Recombinant bivalent/ intraperitoneal	Asian seabass (*L. calcarifer*)	Lab	Silvaraj et al. (2020)

Plant-based extracts for treating fish diseases

Carefully selected plant-based extracts have been traditionally used to treat and prevent fish diseases for centuries. This approach is significant especially in addressing consumer concerns about the harmful and adverse effects of synthetic chemicals. Plant-derived chemicals are a wide group of chemical compounds found naturally in plants. The extensive existence of these compounds has demonstrated advantages in terms of their antioxidant, antibacterial, and antifungal activities (Khameneh et al., 2019).

The R&D in this area began during the Ninth Malaysia Plan (2006-2010) and continues under the current Twelfth Malaysia Plan (2021–2025). The objectives of this R&D are to: i) recommend the use of natural substances; ii) avoid the use of antimicrobials; iii) reduce AMR; iv) reduce operational costs; and v) eliminate the risk of chemical residues in fish tissues. The outputs from the R&D activities are described in the following sections.

SirehMAX®

SirehMAX® is one of the earliest R&D products by researchers from the FRI Tanjung Demong, Besut, Terengganu; NaFisH; and FRI Glami Lemi, Negeri Sembilan. SirehMAX® is a *Piper betle* (locally known as sireh) leaf extract that was initially developed to overcome bacterial infections in marine fish but later was demonstrated to be effective for treating infections in freshwater fish (Ahmad-Baihaqi et al., 2015). The SirehMAX® R&D began in 2009, and a patent was registered in 2015 for "Compositions containing *P. betle* Extract for Prevention and Treatment of Bacterial Diseases in Aquatic Animals" (MY-176273-A). Subsequently, the product was registered as national trademark (SirehMAX®, Class 5, Journal Number 50/2016, 6 Jun 2016) for pharmaceutical purposes for fish; additives to and supplements for foods for fish; a natural, antibacterial remedy for aquarium and pond fish; veterinary preparations for treating fish; a preparation to reduce or kill bacteria and/or parasites on fish; a phytotherapeutic, natural remedy for fish and a fish immune stimulant. These approaches were related to *P. betle* leaf extract use in the aquamarine and aquaculture industry.

The advantages of SirehMAX® include broad-spectrum antimicrobial properties against gram-negative and gram-positive bacteria (*Vibrio* sp., *Streptococcus* sp., *A. hydrophila*, and *Nocardia* sp.) (Nik-Haiha et al., 2011). SirehMAX® was effective in treating infections in *Lates calcarifer* (Ahmad-Baihaqi, 2018; Othman et al., 2018), grouper (Azila et al., 2018), *Pangasius* sp. (Rimatulhana et al., 2018), and *Tor* sp. (Hanan et al., 2018) of all ages and in all environments (freshwater, brackish, and saltwater). The product is easily biodegradable in each environment and can be readily applied with feed (orally) or given in a bath treatment. SirehMAX® can be used for treatment and prophylaxis, and farmers do not have to implement a withdrawal period.

SitroPro®

SitroPro is a combination of plant extracts, notably lemongrass (*Cymbopogon citratus*), and represents one of the earliest R&D investigations into alternative treatments based on natural substances

by the FRI, particularly FRI Gelang Patah, Johor. SitroPro® was very efficient for treating protozoan parasites and ectoparasites, especially the marine leech (*Zeylanicobdella arugamensis* (Fadzillah et al., 2017; Fadzilah et al., 2019). Initial observations revealed that SitroPro was an immune-stimulant in *L. calcarifer* (Fadzilah and Azmi, 2018; Fadzilah et al., 2019). SitroPro® application is mainly oral (mixed with feed) and through bath treatment. In 2016, SitroPro® was registered under a national trademark (Class 31, Journal Number 30/2017, 29 Jun 2016).

GARLEX

R&D on GARLEX, a garlic extract used for treating fish diseases, was initiated within the Eleventh Malaysia Plan (2016–2020). Garlic was used due to its widely known antimicrobial properties. Additionally, the product increases the appetite of the fish. Initial findings demonstrated that GARLEX inhibited almost 80% of bacterial growth in the main organs of infected fish after one month of being mixed in the diet (Nik Haiha et al., 2017; Shaharah et al., 2022). Other investigations on GARLEX included: antimicrobial susceptibility against several fish pathogens; toxicity testing to juvenile grouper (*Ephinephelus* sp.); palatability studies; effect of various GARLEX concentrations on lysozyme activity in juvenile hybrid grouper; and the growth rate of grouper fed a GARLEX-containing diet. GARLEX R&D is ongoing, and the promising results have enabled the registration of the extraction and application methods under Malaysian copyright (Notification Number: LY00028224) in January 2021.

In addition to SirehMAX, SitroPro, and GARLEX, another related R&D project is Leech Guard, which began at the end of the Eleventh Malaysia Plan (2016–2020). Leech Guard is used to treat marine leeches on fish. Leech Guard is an extract prepared from noni (*Morinda citrifolia*) leaves. Noni was selected based on the beneficial phytochemicals in the plant, which demonstrate antibacterial, antiviral, antifungal, antitumor, anthelminthic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects (Assi et al., 2017). Almost 200 phytochemicals have been identified and isolated from different parts of *M. citrifolia* (Singh, 2012). This product is still under R&D and is undergoing field-testing.

Plant extracts exhibit antimicrobial activities but it is occasionally difficult to obtain them bulk and obtain a standardised quantities of active compounds from the extracts. Thus, mass-producing the plant extracts is complicated and costly. Accordingly, using commercial plant essential oils is much more practical. For example, NaFisH demonstrated that incorporating cinnamon essential oil in shrimp feed was effective in preventing marine ectoparasite infestation (Kua et al., 2022).

Simple diagnostic and treatment field kits

The FRI has developed simple field treatment and diagnostic kits to be used by farmers to enable the early detection of disease conditions and avoid larger-scale potential issues and losses. Among the first field kits developed were a swim bladder disorder treatment kit for use in tiger grouper (KRIPek) and the marine leech trap (Break and Protect, BP).

KRIPeK

Swim bladder disorders in farm-raised fish are an acute, severe condition that can have a considerable economic effect. The swim bladder must be properly inflated for effective buoyancy control, swimming prowess, and feeding success. Malaysian farmers reported swim bladder disorders in groupers (*Epinephelus* spp.) that resulted in mortality three days after the onset of positive buoyancy, which could increase up to 100% mortality within 15 days (Rimatulhana et al., 2021). Affected fish include marketable-sized fish or broodstock that exhibit positive buoyancy disorder and eventually die of starvation, overexposure to direct sunlight, or secondary bacterial infection. High mortality rates in marine fish have been reported in Taiwan, the Philippines, and Malaysia (Chang et al., 2016; Nagasawa and Cruz-Lacierda, 2004; Rimatulhana et al., 2021). The existing treatment methods (air removal using a syringe, life jacket on the fish body, and antibiotics) are not practical in farmed fish and are subject to persistent issues, such as recurrence after air removal, long recovery period, AMR, and mortality. The KRIPek (Kit Rawatan Ikan Perut Kembung) was designed specifically to treat marine fish with clinical signs of a bloated swim bladder. The KRIPeK consists of three main parts: i) pictorial manual; iii) waterproof folding container; and iii) a two-step protocol, which are packed in a box that weighs < 3 kg for easy handling and transportation. The technical services cover two stages: i) air removal from the swim bladder using a needle, followed by ii) force-feeding using a functional diet through a tube. Laboratory trials reported that KRIPeK resulted in a significantly improved recovery time (after 24 h) and higher survival rate (60%–80%) in treated fish compared with the 30%–58% in untreated fish. Field trials by farmers in Sarawak and Penang yielded higher survival rates of 70%–100%. KRIPek was registered in October 2021as the intellectual property of FRI with MyIPO under Utility Innovation (filing number UI202100576).

Break and Protect (BP)

BP is another FRI product to be used on-site. BP was based on the issue of the marine leech *Z. arugamensis* in farmed marine fish, particularly those in cage systems. Mortality due to *Z. arugamensis* infestations on marine fish have been reported in the Philippines, Singapore, and Malaysia (Cruz-Lacierda et al., 2000; Murwantoko et al., 2017; Mahardika et al., 2018; Kua et al., 2019; Azmey et al., 2020). Beside mortality, heavy infestation of this parasite can cause serious injury and secondary infection. Parasitic infestation and secondary infections are the main contributors associated with losses in farmed fish (Xu et al., 2007; Zhang et al., 2015; Kotob, 2017). *Z. arugamensis* is also a vector for bacteria, such as *V. alginolyticus* (Kua, 2008; Kua et al., 2009). With both characteristics, marine leech infestations are commonly associated with secondary infections such as by bacteria or viruses. The marine leech infestation must be addressed first to reduce the secondary infection. If preventive measures are implemented in a timely manner, then infestations can spread and cause mortality, leading to greater economic loss to the farmer.

Leech infestations in Penang, Malaysia, alone caused losses of RM 6 million in 2014 and RM 1.1 million in 2016. The magnitude of losses caused by marine leeches has resulted in this parasite being placed on the Malaysian notifiable disease list. Until 2014, 14 marine fish species were identified as potentially infected by marine leeches with a prevalence of 40%–100%. Marine

leeches are parasites that attach to fish and suck their blood as their main food, stressing the fish and typically resulting in secondary infections that can be fatal in caged fish. Traditional treatment involves removing the leeches manually by hand, brush, or towel, which is stressful. Leech removal using chemicals (formalin, acriflavine, or antibiotics) is not environmentally friendly.

BP is novel as it utilises knowledge of the marine leech life cycle and the natural behaviour of fish that can minimise secondary infection or disease occurrence that leads to mortality (Kua, 2008; Kua et al., 2010). The device provides an area for adult marine leeches to lay their cocoons. Placement of the inert device in the floating cages attracts the fish to hide inside the device, carrying their leeches with them. Within the device, the leeches detach from the host and lay their cocoons if the environment is conducive to do so. After a few days, the device is removed, along with both adult leeches and their cocoons. BP2 can be reused after cleaning. Continuously removing and redeploying the device over a fixed period aids in reducing marine leech prevalence.

The device was first designed in 2010 for trapping and removing marine leeches and reduced leech prevalence from 70% to 20% (Kua, 2014; Kua, 2015). The device uses no chemicals and removed adult leeches and cocoons from fish cages. The initial design was termed BP and was registered as a patent in 2009 but was later withdrawn due to improvements in the BP design for more efficient leech trapping. In 2017, another registration for the leech remover was made (PI No. 2017703059) and termed BP2. Trials at fish culture sites demonstrated that the continuous use of BP2 in marine fish cages reduced the prevalence of marine leech infestation from 80%–100% to 20%–28%.

BP2 was developed using a Ministry of Science and Innovation (MOSTI) grant for the initial stage and later supported by a Department of Fisheries development grant within the Eleventh Malaysia Plan. BP2 has subsequently undergone several improvements in design, size, type, and colour of substrates to accommodate uses in all marine fish species. BP2 was directed towards fish farmers in Southeast Asian countries facing marine leech infestations. BP2 has been undergoing field trials at three main culture areas where polyculture is practiced and has been implemented at six main culture areas throughout Peninsular Malaysia. The main culture area is in the Kingdom of Brunei. BP2 has won local and international awards, including the Commonwealth Secretary-General's Innovation for Sustainable Development Award (Commonwealth Innovation Awards 2021 Government category winner) in 2021 and is produced commercially by a local company.

Early detection methods at culture sites

Huge economic losses due to the incidence of viral, bacterial, and parasitic agents in shrimp farms have been reported globally (Shinn et al., 2018). The gross national losses in Malaysia due to the emerging shrimp disease acute hepatopancreatic necrosis disease (AHPND) was valued at USD 1.3 billion in 2011–2013 (Kua, 2018). Commonly encountered diseases [white spot disease virus (WSD), infectious hypodermal and haematopoietic necrosis virus (IHHNV), and vibriosis] and new emerging diseases [AHPND, infectious myonecrosis virus (IMNV), *Enterocytozoon hepatopenaei*, decapod iridescent virus 1, and white faeces syndrome] will continue to be major constraints in the development of the Malaysian shrimp industry.

The current methods for detecting shrimp disease are commercial rapid detection kits, PCR, real-time PCR, RT-PCR, bacteria isolation, and histopathology. Some of these methods require skilled labour and are tedious, expensive, and time-consuming. In a disease outbreak, uncertainty about the disease cause and absence of established emergency protocols might hinder farmers' capacities to respond swiftly and appropriately. This hindrance potentially leads to significant economic losses. Shrimp affected by disease tend to be smaller and exhibit slow growth. Delayed infection detection and management will lead to rising operational costs from high feed conversion ratios (FCR) and daily production losses. Mortality in farms can reach 40%–100%; thus, the industry needs rapid detection methods.

Shrimp Health On-site Spotter (SHOS-Spotter) is an on-site early detection method used to determine the health status of farmed shrimp within 1–3 h. SHOS-Spotter fulfils rapid detection of early signs of disease, thus immediate mitigation actions can be taken to minimise losses due to a disease outbreak. The shrimp health status can be determined by assessing the condition of the intestines. The SHOS-Spotter involves examining the gut contents using a portable microscope to determine the presence or absence of sloughed epithelial cells from the hepatopancreas (HP). Scores of 0–2 (0 indicates healthy shrimp, and scores of 1 and 2 indicate unhealthy shrimp) are assigned based on microscopic observations considering the HP cell density or number in the gut lumen. SHOS-Spotter allows farmers to independently determine whether their shrimp have sick or healthy intestines. SHOS-Spotter was developed based on shrimp farmers' issues due to acute, high rates of shrimp mortality due to disease, toxin, or a critical change in the environment. Field trials of SHOS-Spotter demonstrated that gut score readings in shrimp aligned with AHPND-positive cases, a correlation validated by PCR. Therefore, SHOS-Spotter can be a reliable indicator of shrimp health. This product was registered in October 2021 as a national trademark under Class 11 (filing number TM2021029905).

Issues and challenges

Although there has been significant R&D&I exploring alternatives to chemical use in aquaculture; unfortunately, there have been very few commercial outputs. There are several barriers to the commercialisation of FRI technology, and the relevant issues and challenges are discussed below.

More studies are required

The extraction, isolation, and characterisation of active ingredients in botanicals and herbal preparations should be established. Major challenges, such as translating *in vitro* studies to *in vivo* experiments and finally to fish clinical trials, should be addressed. More studies are needed to better define the factors that could affect extraction efficacy and the extracts. For example, the extraction factors (solvent type and amount, temperature, and extraction time) should be evaluated. Furthermore, information on the efficiency of the plant extracts (degree of tissue penetration, optimum dose/concentration, active compounds, and quantity) should be considered. Moreover, ongoing studies are needed to gain a better understanding of the exact mechanisms and pharmacodynamic and pharmacokinetic properties of the molecules.

Lack of commercialisation funding

Commercialisation is a long and complex process that involves initial investment in R&D, intellectual property filing, securing pre-seed and seed funding, market funding, and prototype funding. The lack of funding and incentives to support private-sector research and commercialisation is a major impediment to the rate at which licenced products are released on the market. The lack of pre-seed finance beyond the proof-of-concept stage precludes the smooth translation of potential research findings into tangible commercial outputs.

Management of Intellectual Property (IP) and commercialisation

Maintaining IPs requires significant funding, which is also one of the main hindrances. FRI R&D product and outcome commercialisation was initiated only a few years ago. Thus, the researchers and officers handling commercialisation lack knowledge and experience regarding business planning and other aspects of commercialisation. Additionally, limited personnel handle these responsibilities.

Conclusion

Some significant studies have been conducted in Malaysia to mitigate the use of chemicals in aquaculture, especially in managing and treating aquatic disease. The research efforts include vaccination with locally produced vaccines; plant-based extracts; and using essential oils, field treatment kits, and an early warning disease system. Policymakers, researchers, and the private sector should collaborate to address the issues of commercialising innovations and R&D technologies to benefit farmers. Additionally, more research is needed, especially in the up-scaling, field evaluation, and field verification of R&D products and technologies. For example, the selected phytocompounds detailed in this article are potent antimicrobial agents against fish pathogens including bacteria, viruses, and parasites. Furthermore, it is important to use extraction methods suitable for specific compounds as the amount of product compounds can vary. Most importantly, phytocompounds are only produced in small volumes restricted to research purposes only. This issue should be addressed as further research is needed to synthesise the compounds for industry use. Further research on natural products should be conducted to maximise the use of medicinal plants and capitalise on the safety aspects offered by phytocompounds, which might outweigh synthetically or semi-synthetically derived agents. The current disease detection technologies and effective treatment, prevention, and control methods are integral to contain or impede infectious disease spread in cultured fish.

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Blue-Green Algae and Assessment of Microcystin in Shrimp Aquaculture Farms in Sarawak

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***Abstract*:** Blue-green algal or cyanobacterial blooms occur frequently in aquaculture ponds and can cause severe water quality deterioration, including scum formation and toxin production. In this study, 20 small-scale shrimp farms were assessed from February 2021 to November 2021 to determine blue-green algae abundance and microcystin levels in the tissue of shrimps using enzyme-linked immunosorbent assay. There was a high cell count of *Microcystis* sp. (6.77×10^8 cells/L) in Muara Tebas, *Anabaena* sp. (4.99×10^7 cells/L) in Telaga Air, and *Pseudanabaena* sp. (1.69×10^8 cells/L) in Kuala Baram. Low microcystin levels were detected in the shrimp samples throughout the study. The results demonstrated that blue-green algae monitoring in shrimp aquaculture farms is necessary.

Keywords: Blue-green algae, microcystin, ELISA, shrimps, aquaculture

Abstrak: Alga biru-hijau atau ketumbuhan sianobakteria kerap berlaku di kolam akuakultur. Ini boleh menyebabkan penurunan kualiti air yang teruk termasuk pembentukan scum dan penghasilan toksin. Sebanyak 20 ladang udang skala kecil dinilai dari Februari 2021 hingga November 2021 untuk kelimpahan alga biru-hijau dan tahap mikrosistin dalam tisu udang menggunakan ujian imunoassain enzim (ELISA). Terdapat jumlah sel yang tinggi bagi *Microcystis* sp. pada 6.77×10^8 sel/L di Muara Tebas, *Anabaena* sp. pada 4.99×10^7 sel/L di Telaga Air dan *Pseudanabaena* sp. pada 1.69×10^8 sel/L di Kuala Baram. Mikrosistin dikesan tetapi pada tahap rendah dalam sampel udang yang dikumpulkan dari ladang udang di Sarawak sepanjang kajian. Kajian ini menunjukkan bahawa pemantauan alga biru-hijau dalam ladang akuakultur udang sangat diperlukan.

Introduction

Blue-green algae blooms can cause severe water quality deterioration, including scum formation, toxin production, hypoxia, and foul odours and tastes (Guzman-Guillen et al., 2013). Blue-green algae negatively affect ecosystems as they colonise a wide range of niches in aquatic and terrestrial environments (Codd et al., 2005). Furthermore, human exposure to toxic blue-green algae or their toxins results in mild to life-threatening health implications (Buratt et al., 2017; Badar et al., 2017; Lundqvist et al., 2017; Massey et al., 2018).

Excessive blue-green algae growth is a common concern in shrimp aquaculture farms that depletes oxygen levels in tiger shrimp aquaculture farms, leading to mortality. Blue-green algae blooms can cause serious economic losses in the aquaculture industry (Qin et al., 2013). Thus, bloom-forming blue-green algae are undesirable in aquaculture ponds (Hans and Craig, 2007).

Microcystins are among the most common and dangerous blue-green algae toxins (Singh et al., 2012; Merel et al., 2013; Rastogi et al., 2014; Huisman et al., 2018) and are produced by the genera *Microcystis*, *Anabaena*, and *Planktothrix* (DeFigueiredo et al., 2004; Bouaïcha et al., 2019). Microcystins accumulate in shrimp tissues and pose a health risk to humans when consumed (Peng, 2010; Gutiérrez-Praena et al., 2013; Massey et al., 2018; Nielsen and Jiang, 2020). Most microcystin-producing blooms are dominated by *Microcystis* (Dai et al., 2008; Xu et al., 2008; Bouaïcha et al., 2019) and are hepatotoxins and neurotoxins that cause liver failure and even death (Carmichael et al., 2001) and chronic effects in humans even with low-level exposure (Chorus et al., 2001). Microcystin production in blue-green algae have lethal and sub-lethal effects in humans and tiger shrimps (Chen et al., 2014). The issue of toxin production stems from the formation of toxic algal blooms, or scums or mats, and the subsequent human and animal health risks are considered a subject of concern globally, especially when considering the massively increasing anthropogenic activities and global warming issues (Reichwaldt et al., 2012; El-Shehawy et al., 2012; Paerl et al., 2016; Testai et al., 2016; Wang et al., 2021). This issue is due to progressive eutrophication that induces algal excessive growth and enhances toxin formation (Rastogi et al., 2015). Additionally, global warming is expected to increase bloom frequency and duration. Therefore, toxic blue-green algal blooms are a global emerging environmental threat.

Blue-green algae blooms are becoming more frequent worldwide. The recent incidence frequency, severity, and duration have increased globally (Chorus and Bartram, 1999). The succession of toxic blue-green algal species and biomass fluctuations, which are influenced by seasonal changes in environmental factors (nutrients, grazing, light, and temperature), is believed to affect the microcystin concentrations. Hence, research on water quality dynamics and practical management of water quality issues in aquaculture ponds is very important and has tangibly benefited tiger shrimp producers (Tucker et al., 2008). Blue-green algae have been implicated in fish kills, and shrimp are routinely farmed in waters that contain blue-green algae, which likely affect productivity. Shrimps that ingest these toxins might not die but are weakened, increasing their susceptibility to pathogens. The algae can also affect growth rates and feed conversion ratios. Blue-green algae are widely viewed as pollution indicators and their presence in aquaculture production systems at high numbers indicates improper system management.

In Malaysia, there is very little monitoring and assessment of these blooms in the shrimp aquaculture industry. Therefore, this study was conducted to determine the blue-green algae abundance and assess microcystin levels in shrimp aquaculture farms in Sarawak.

Materials and Methods

Aquaculture farm location

This study involved 11 locations in Sarawak, Malaysia: Bandar Baru Semariang, Santubong, Selabat, Muara Tebas, Rambungan, Telaga Air, Selumit, Mukah, Oya, Kuala Baram, and Sempadi (Figure 1). The shrimp aquaculture farms were all earth ponds filled with river water.

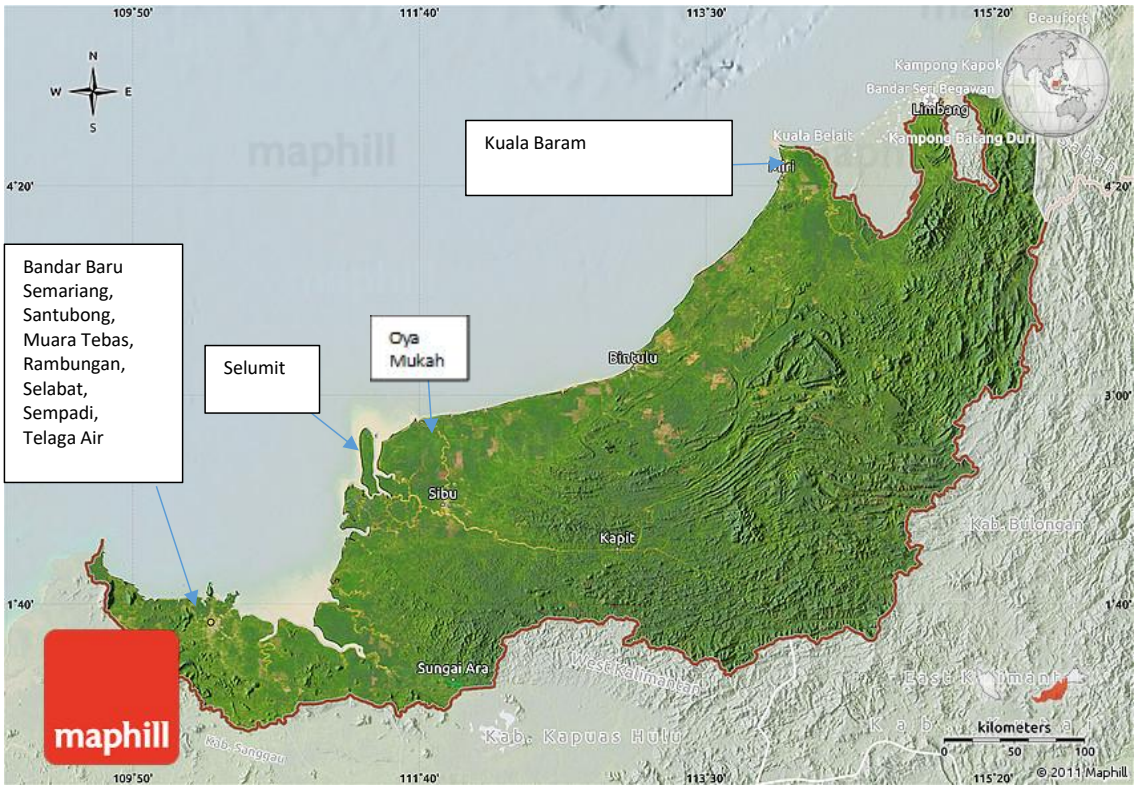

Figure 1. Map of the 11 locations in Sarawak.

Physical water parameters

Sampling was conducted at each shrimp aquaculture farm from February 2021 to November 2021. Three areas of the pond were sampled per farm. Water temperature, salinity, turbidity, and pH were measured on-site with a portable probe (Horiba) at 0.5 m from the water surface. Water samples were then obtained 0.15 m below the water surface. Approximately 100 mL of the water sample was placed and stored immediately in a high-density polyethylene (HDPE) bottle. The sample bottles were placed in a cooler container containing ice to maintain the freshness of the sample and to protect it from sunlight, and transported to the laboratory for analysis.

Nutrient analysis

Nutrients were analysed within two days after sample collection (Jackson, 2000). Four nutrients were quantified: nitrate, phosphate, nitrite and ammonia. The water samples were pre-filtered through a 0.45-µm membrane filter before analysis. Ultra-pure water (Sartorius Stedim Biotech) was used throughout the study, ion analysis was conducted according to Jackson (2000), and the results were compared against ion standards. The phosphate, nitrate, nitrite, and ammonia concentrations were determined using a Hach 2800 spectrophotometer. Phosphate, nitrate, nitrite, and ammonia in the water were measured by filtering the samples through 0.45-µm pore size filters (Millipore, New Bedford, MA, USA).

Microcystin analysis

The water samples were collected in glass containers and tested within 24 h. The samples were stored in a refrigerator if they had to be held for longer periods (i.e. up to five days). The reagents and samples were allowed to reach ambient temperature before use. The number of microtiter plate strips required were counted and removed from the resealable pouch. The remaining strips were kept in the pouch and stored tightly with the desiccant. The standards, control, sample diluent, enzyme conjugate, antibody, substrate, and stop solutions were ready to be used and did not require dilution. The 5 × Wash Solution concentrate was diluted with deionised or distilled water at a 1:5 ratio (100 mL concentrate was added to 400 mL deionised or distilled water). The stop solution contained diluted sulphuric acid (H_2SO_4) and was handled with care.

Microcystin was analysed by collecting the extracts on GF/F filters (pore size, 0.7 mm; Whatman). The microcystin was analysed in duplicate according to the methods and procedures of the Abraxis enzyme-linked immunosorbent assay (ELISA) kit. The standard solutions, control, or sample (50 µL) was added to the wells of the test strips according to the working scheme. The antibody solution (50 µL) was added to the individual wells successively using a multi-channel pipette. Then, the wells were covered with parafilm and the contents were mixed by moving the strip holder in a circular motion on the benchtop for 30 s. The strips were incubated for 90 min at room temperature, then the cover was removed, the contents of the wells were decanted into a sink, and the inverted plate was blotted on a stack of paper towels. The strips were washed three times using 250 µL wash buffer (1×) per well and in each washing step. The inverted plate was blotted after each wash step on a stack of paper towels.

The enzyme conjugate solution (100 µL) was added to the individual wells successively. Then, the wells were covered with parafilm and the contents were mixed as described above. The strips were incubated for 30 min at room temperature, then processed as described above, and washed as described above. Next, 100 µL substrate (colour) solution was added to the individual wells. Then, the wells were covered with parafilm and the contents were mixed as described above before the strips were incubated for 20–30 min at room temperature protected from sunlight. Subsequently, 50 µL stop solution was added to the wells in the same sequence as for the substrate solution. The absorbance was read at 450 nm using a microplate ELISA reader within 15 min after the addition of the stop solution.

Only positive results > 1 ppb (minimum permissible level [MPL]: 1 ppb) were confirmed using high-performance liquid chromatography (HPLC) (Hitachi, Japan). The extract was filtered through Whatman No. 1 filter paper and ammonium sulphate was added to the filtrate to 40% saturation (0.246 g/mL). The suspension was left to settle for 1 h at 4 °C and filtered through a Whatman No. 1 filter paper, followed by 0.45- and 0.22-µm filters. The extract was divided into 50-mL aliquots and stored in the dark at 4°C until used.

A C_{18} gravity column (Aldrich Cat. No. 37,763-5, 10 g) was wetted with 100 mL HPLC-grade methanol and washed with 50 mL ultrapure water. A 50-mL aliquot of crude microcystin extract was added to the column and allowed to adsorb. The column was washed with 100 mL Milli-Q water to remove water-soluble compounds, then with 50 mL 30% methanol to remove less polar material. A 70% methanol solution was added to the column and 2.0-mL fractions collected. Microcystin was confined to the sixth fraction and no further concentration was required. The fractions were analysed using a 20-min linear gradient of 15%–35% acetonitrile in 8 mM ammonium acetate, with a 10-min isocratic elution at 35% acetonitrile (8 mM ammonium acetate). Peak detection was achieved using a GBC LC 1250 fast-scanning UV/V is detector at 238 nm. The eluting peaks were scanned between 200 nm and 300 nm at 1-nm intervals to determine the maximum and minimum absorbance.

Total blue-green algae abundance

Qualitative blue-green algae abundance

The water samples (100 mL each) were poured into a measuring cylinder then allowed to sediment for 48 h. The top layer of the water samples was subsequently removed, leaving the bottom 30 mL in the measuring cylinder, which was allowed to sediment for another 24 h. Then, the top 10 mL was removed and the bottom 20 mL was stored in a small glass universal bottle. The samples were stored in the dark or continued with screening.

Total cyanobacteria concentration

The samples (1 mL each) were placed on glass slides and covered with a glass coverslip and viewed under a microscope (Olympus, Japan IX-70, ×1000 magnification). All the cells in the sample were speciated and the blue-green algal species were counted. The total blue-green algae in each sample were determined by direct microscopical count using an inverted light microscope (Olympus, Japan IX70) and a Sedgewick-Rafter chamber, which was specially designed and presented to the Fisheries Research Institute Bintawa by a Japanese scientist. Blue-green algal morphotypes produce structures visible to the naked eye, such as pinhead or larger, spherical or irregular colonies (*Microcystis* sp.) and bundles of filaments (*Anabaena* sp.) that appear as sawdust in shape and length. Species were identified using the taxonomic keys described by Omura et al. (2012).

Results

Physical water parameters

The physical parameters of the water samples varied between the locations and farms (Figures 2 and 3). The temperature was 25.4–31.2°C, and most ponds were <31°C. A water temperature between 25°C and 32°C is optimum for shrimp species growth (Marine Water Quality Criteria and Standards, MWQS-DOE). The water pH during sampling was 6.62–10.21. Aquaculture water with pH 7–9 is ideal for fish and crustacean growth while pH 9–11 can cause slow development in aquatic species (MWQS-DOE). The sample turbidity was 5–391 mg/L, and the salinity was 5.2–30.1 ppt.

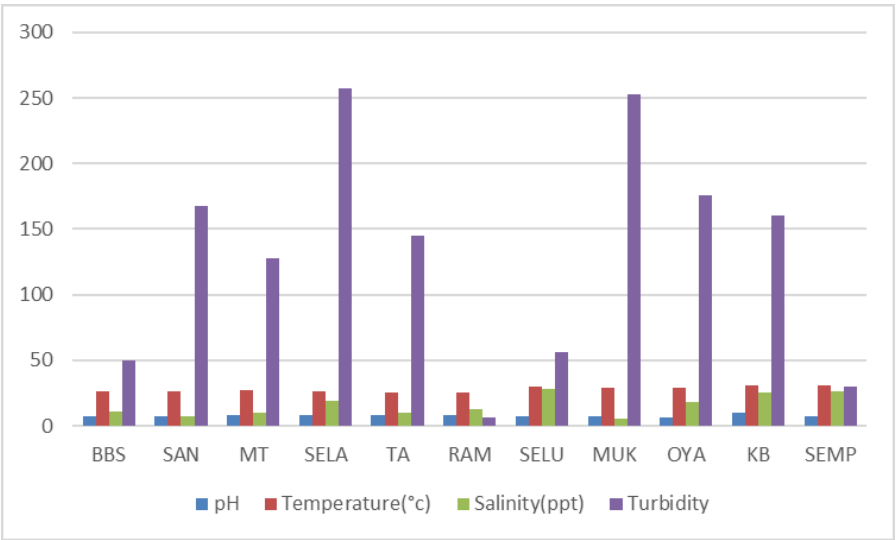

Figure 2. Physical water parameters of the 11 locations in Sarawak.

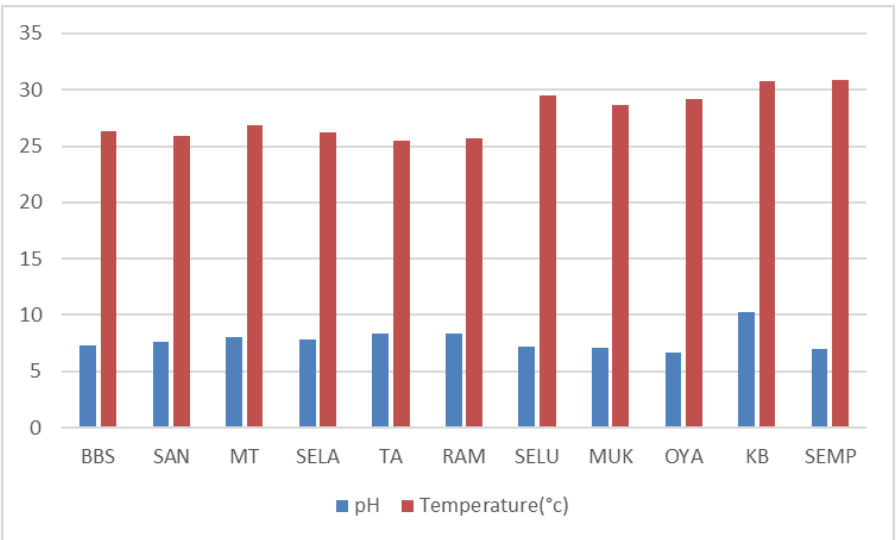

Figure 3. Physical water parameters (pH and temperature) of the 11 locations in Sarawak.

Nutrient analysis

Among the dissolved inorganic nutrients in the water samples, the nitrate concentration was 0.3–3.5 mg/L, which was within the desired concentrations (0.2–10 mg/L) (MWQS-DOE). The nitrite concentration was 0.003–0.532 mg/L (preferred concentration: <1 mg/L). The concentrations of unionised ammonia (0–3.6 mg/L) and phosphate (0.05–1.44 mg/L) were below and within the acceptable ranges, respectively (Figure 4). The concentrations of nitrate and unionised ammonia were higher than the desired concentration of <1.28 mg/L and <0.10 mg/, respectively [Department of Fisheries-recommended water quality for business proposal for commercial farming of black tiger shrimp (*Penaeus monodon*)].

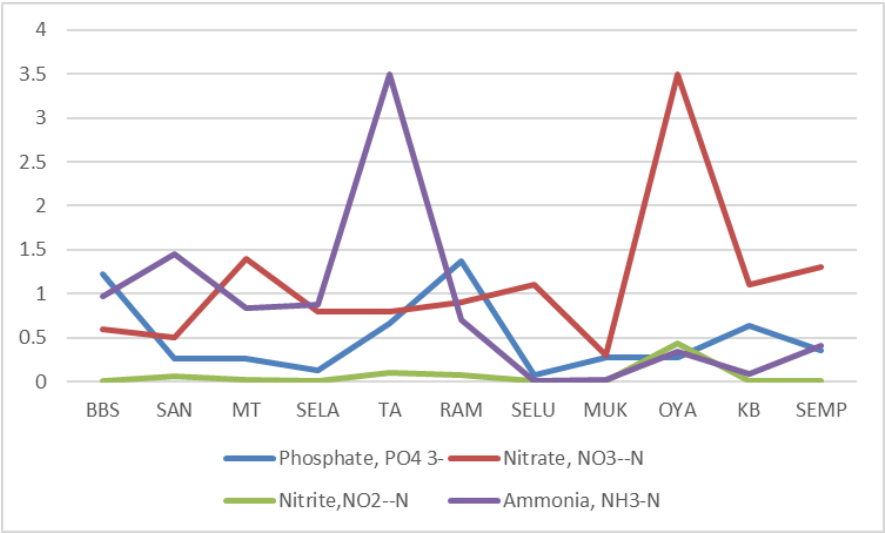

Figure 4. Water nutrient analysis chart of the 11 locations in Sarawak

Microcystin analysis

Microcystin was detected in the tissue of shrimps at the Muara Tebas farm (1/20 farms, 5%), and a level of 0.448 ppb was detected.

Total blue-green algae abundance

Blue-green algae were observed in the aquaculture farms during the very hot period of the year. The blue-green algae were dominated by *Microcystis* sp., *Anabaena* sp. and *Pseudanabaena* sp. Nineteen farms (95%) experienced blue-green algae blooms as the cell counts were >0.3 × 10^3 cells/L. Only three farms (15%) were positive for the toxic blue green algae *Microcystis* sp. The highest concentration was detected in Muara Tebas (6.0 × 10^8 cells/L), followed by Santubong (4.49 × 10^7 cells/L). The lowest *Microcystis* sp. biomass was observed in Sempadi (4.5 × 10^6 cells/L).

Only four farms (20%) had *Anabaena* sp. concentrations > 0.3 × 10^3 cells/L (Table 1). *Anabaena* sp. abundance was highest in Telaga Air (4.99 × 10^7 cells/L), whereas it was lowest in

Santubong (1.50×10^3 cells/L). *Anabaena* sp. was not detected in Bandar Baru Semariang, Muara Tebas, Selabat, Rambungan, Selumit, Oya, and Sempadi. *Pseudanabaena* sp. was detected in 18 farms (90%). The highest cell count was in Kuala Baram (1.69×10^8 cells/L), while the lowest was in Oya (0.302×10^3 cells/L). *Pseudanabaena* sp. was not detected in Rambungan and Sempadi.

Table 1. Table of cyanobacterial biomass in the 11 locations in Sarawak.

Cyanobacteria / Location	*Anabaena* sp. (cells/L)	*Pseudanabaena* sp. (cells/L)	*Microcystis* sp. (cells/L)
Bandar Baru Semariang	0	5.52×10^6	0
Santubong	1.50×10^3	7.86×10^5	4.49×10^7
Muara Tebas	0	4.07×10^7	0
Selabat	0	3.32×10^7	6.77×10^8
Telaga Air	4.99×10^7	5.35×10^7	0
Rambungan	0	0	0
Selumit	0	2.8×10^5	0
Mukah	8.05×10^3	2.533×10^3	0
Oya	0	0.302×10^3	0
Kuala Baram	6.63×10^6	1.69×10^8	0
Sempadi	0	0	4.52×10^8

Relationship between physical parameters of water with blue-green algae

Temperature and pH were significantly correlated ($p < 0.05$) with blue-green algae biomass (Table 2). However, the nutrients and blue-green algae intensity in the water column were not related. Furthermore, the water sample contained low phosphate, nitrate, nitrite, and ammonia levels.

Table 2. Relationship between physical and nutrient parameters with blue-green algae biomass in shrimp farms in Sarawak

Parameter	Blue-green algae biomass
Temperature	0.419*
pH	0.426*
Salinity	-0.112
Turbidity	-0.145
Nutrients	
Nitrate	-0.239
Phosphate	-0.138
Nitrite	-0.126
Ammonia	-0.118

Note: *p < 0.05; - indicates no correlation.

Discussion

Blue-green algae thrive under conducive temperatures, light, and nutrient status and therefore their abundance increases. In this study, all sampled farms had relatively high-water temperatures, where most were ~30°C during sampling. In this study, the highest recorded temperature was 31.2 °C in Selabat, which also had high levels of *Pseudanabaena* sp. (3.32×10^7 cells/L) and *Microcystis* sp. (6.77×10^4 cells/L). Most blue-green algae species attain an optimal growth rate at 25–35 °C (Kumar et al., 2011; Lurling et al., 2012). Other studies reported that some blue-green algae species, such as *Microcystis* sp., can out-compete other phytoplankton species at ≥30 °C (Fujimoto et al., 1997). This result suggests that blue-green algae can thrive at very high temperatures in aquaculture farms. A similar finding was reported in Penang, where the blue-green algal cell density was significantly correlated with temperature when the temperature was 27.1–32.3 °C (Mohd. Nasarudin and Ruhana, 2007). Similar results were reported in a study on *Tor tambroides* ponds in Serian, Sarawak (Mohd. Nasarudin and Ruhana, 2011).

In the present study, water pH was positively correlated with blue-green algal biomass. Kuala Baram had the highest pH (10.21) and the highest *Pseudanabaena* sp. levels (1.69×10^8 cell/L). The water pH influences blue-green algae activity and intensity in the water column (You *et al.*, 2007). Photosynthetic activities increase and carbon dioxide is released during intense increases in blue-green algae populations, increasing the pH value that favours cyanobacteria dominance (Dokulil and Teubner, 2000).

Many scientific studies have stated that cyanobacterial proliferation is closely related to the nutrient concentration of the water body (Chorus and Bartram, 1999). This phenomenon was also reported in aquaculture ponds (Kankaanpää et al., 2005). Many studies have also reported that multiple environmental factors and nutrients affect blue-green algal abundance in aquaculture ponds. In the present study, the four water nutrients and physical parameters (temperature and pH) enabled blue-green algae growth and proliferation in aquaculture ponds leading to toxic levels. All four nutrients analysed were within their permitted levels in the water samples. The nitrite concentration was very low and below the acceptable range of 1 mg/L. Similar results were obtained and compared with the water analysis of freshwater fish (*T. tambroides*) ponds in Sarawak, where the nitrite levels were 0.001–0.007 mg/L and were not significantly related to the blue-green algae cell density in aquaculture water body (Mohd. Nasarudin and Ruhana, 2007).

To gain a better understanding of the potential blue-green algae producing toxin, the blue-green algae population was assessed using ELISA. Microcystin was detected at <1 ppb [maximum acceptable value [MAV]: 1 ppb as per the Malaysian Ministry of Health (MOH)] in shrimp tissue from one site. Toxin accumulation in shrimp is a potential threat to human food safety. In humans, microcystins were identified for the first time in the serum (mean: 0.228 ng/mL) of a chronically exposed human population (fishermen at Lake Chaohu, China), with an indication of hepatocellular damage (Chen et al., 2009).

There have been public health concerns regarding microcystin occurrence in aquaculture farms in many countries. Our results provided an important baseline for the status of the presence

of blue-green algae species and microcystin assessment in 11 locations of Sarawak. It is important to educate farmers and aquaculturists on the risks and consequences associated with microcystin in shrimp.

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Assessment of Antibacterial Activity of Fresh Garlic Juice Extract against *Vibrio* spp. Isolated from Hybrid Grouper

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***Abstract*:** Antimicrobial resistance has gained worldwide attention. Investigations of the antimicrobial properties of plants and botanical substances, particularly garlic (*Allium sativum*) have attracted significant attention due to their promising abilities in this field. This study evaluated the antibacterial activity of fresh garlic juice extract against *Vibrio* spp. isolated from hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*). The antibacterial activity of different concentrations of garlic juice extract was evaluated using the Kirby-Bauer disc diffusion susceptibility test. The positive and negative controls were commercial antibiotic and ethanol, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract against *Vibrio alginolyticus*, *V. vulnificus*, and *V. parahaemolyticus* were determined using broth dilution. The *Vibrio* spp. isolates exhibited varying sensitivity levels to the garlic juice extract concentrations. The inhibitory zone diameter varied with the *Vibrio* species used but remained constant with regardless of the amount and concentration of fresh garlic juice extract used. *V. vulnificus* was the least sensitive to 100% fresh garlic juice extract, followed by *V. parahaemolyticus* and *V. alginolyticus*. The inhibition zone diameter increased together with the increased concentrations of garlic juice extract. The garlic juice extract MIC and MBC against *Vibrio* spp. were <1%. The results demonstrated that a small amount of fresh garlic juice extract (<1%) was sufficient to inhibit the bacterial growth. Thus, garlic juice extract was effective against the test bacteria and can be utilised to treat vibriosis in hybrid grouper.

Keywords: *Epinephelus* spp., Garlic juice extract, *Vibrio* spp., antimicrobial activity

***Abstrak*:** Isu rintangan antimikrobial telah mendapat perhatian pada peringkat global. Kajian tentang keupayaan antibakteria oleh tumbuhan dan herba ini telah menarik perhatian ramai pihak. Bawang putih, atau *Allium sativum*, menunjukkan keputusan yang meyakinkan dalam menangani isu ini. Matlamat kajian ini adalah untuk menilai aktiviti antibakteria oleh ekstrak jus bawang putih

segar. Kajian berkenaan aktiviti antibakteria oleh ekstrak jus bawang putih segar terhadap *Vibrio* spp. yang disampel daripada ikan kerapu hibrid (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*) telah berjaya dilaksanakan. Ujian resapan cakera Kirby-Bauer telah digunakan untuk menilai aktiviti antibakteria bagi ekstrak jus bawang putih pada kepekatan berbeza. Antibiotik komersil dan etanol telah masing-masing digunakan sebagai kawalan positif dan negatif. Kaedah pencairan *broth* telah digunakan bagi menentukan kepekatan perencatan minimum (MIC) dan kepekatan bakteria minimum (MBC) ekstrak bawang putih terhadap *Vibrio alginolyticus*, *Vibrio vulnificus*, dan *Vibrio parahaemolyticus*. Keputusan menunjukkan bahawa *Vibrio* spp. menunjukkan tahap sensitiviti yang berbeza terhadap ekstrak jus bawang putih yang berbeza kepekatan. Bergantung pada jenis *Vibrio* yang digunakan dalam eksperimen, diameter zon perencatan berbeza-beza, tetapi jumlah ekstrak jus bawang putih segar yang digunakan dan kepekatannya kekal sama. *V. vulnificus* ialah spesies yang paling kurang sensitif terhadap 100% ekstrak jus bawang putih segar, diikuti oleh *V. parahaemolyticus,* dan kemudian *V. alginolyticus* Diameter zon perencatan pertumbuhan bakteria meningkat dengan peningkatan kepekatan ekstrak jus bawang putih. MIC dan MBC ekstrak jus bawang putih terhadap *Vibrio* spp. mula bertindak balas di bawah kepekatan 1%. Keputusan menunjukkan bahawa ekstrak jus bawang putih berkesan terhadap bakteria yang diuji dan boleh digunakan untuk merawat penyakit vibriosis dalam ikan kerapu.

Introduction

The hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) was developed at the Borneo Marine Research Institute (BMRI), University Malaysia Sabah, Malaysia (Ch'ng and Senoo, 2018). The unique characteristics of hybrid grouper, such as ease of handling and management, high tolerance of low water salinity (Liang et al., 2013) and low water pH (Mustafa et al., 2013), and rapid growth (De et al., 2014), led to it being in high demand and highly popular for mariculture in Southeast Asia (Mustafa, 2012). A total of 3,636.69 tonnes of hybrid grouper were produced in 2020, representing 1.2% of all mariculture production in Malaysia. Due to its advantages, the hybrid grouper is the most cultivated of all groupers. In 2020, Pulau Pinang recorded the highest production at 1,218.46 tonnes, followed by Perak with <200 tonnes (DOFM, 2021).

Fish vibriosis is a systemic condition that affects marine, estuarine, and some freshwater fish (Ross et al., 1968; Ghittino et al., 1972). Vibriosis cases were first noted in the 1500s along the Italian coast (Bullock, 1977). *Vibrio* spp. is a gram-negative bacterium that invades fish, shrimp, and other aquatic animals and leads to disease outbreaks, causing severe economic loss (Austin, 2010). The rapid development of mariculture has resulted in vibriosis becoming a major cause of fish loss and a production-limiting factor. *Vibrio* spp. can kill >50% of cultured fish during an outbreak (Reed and Francis-Floyd, 1996). In Malaysia, the first outbreaks of vibriosis among marine fish (snapper, grouper, and seabass) were reported in 1990 and lead to a RM 30.72 million loss (Shariff, 1995). Grouper with vibriosis exhibits clinical abnormalities such as lethargy, loss of appetite, and swimming on the surface. Skin, fin, and mouth ulcers, corneal opacity, pop-eye, and the loss of one eye are among the external vibriosis lesions. Affected fish in the advanced stages display skin discoloration and haemorrhages (Amalina et al., 2019).

Many farms and hatcheries use vaccines, chemotherapeutic agents, and antibiotics to enhance fish immunity and prevent disease outbreaks. Antibiotics are the most frequently applied approach in global aquaculture (Mariappan et al., 2023) to kill and inhibit pathogen growth and to promote fish growth (Chowdhury et al., 2009). Lulijwa et al. (2020) reported that 67 different antibiotic compounds were used in 11 major countries producing aquaculture products from 2008 to 2018, where oxytetracycline (OTC), florfenicol, and sulphadiazine were the most commonly used antibiotics. Despite the benefits of antibiotics, these chemical drugs have a negative effect on the environment and humans. Antibiotics can accumulate in sediments and farmed animal tissues (Deb, 1998; Lulijwa et al., 2020; Subasinghe et al., 2000). The antibiotic residue in farmed aquatic food can affect food safety (Chen et al., 2018). Furthermore, consuming antibiotic residue might lead to adverse drug reactions (ADR) and antibiotic resistance to beneficial bacteria (Liu et al., 2017), placing consumers at risk following exposure (Alderman and Hastings,1998). Therefore, natural plant extracts have been considered an effective alternative to control disease outbreaks to reduce or avoid dependence on antibiotics.

Developing safe and effective alternatives is necessary to prevent harmful effects. Herbal medicine is an excellent replacement for commonly used drugs (or chemicals). Herbal medicines are non-allergenic to the host and non-toxic, with selective toxicity. Furthermore, they are economical and chemically stable (Fatima et al., 2011). Cristea et al. (2012) reported that herbal medicine is antimicrobial, antiparasitic, anticarcinogenic, an appetite enhancer, growth promoter, antioxidant, digestive system stimulator, and insecticidal. Hence, screening medicinal plants is important for therapeutic use. Garlic (*Allium sativum*) has been cultured for centuries for its medicinal properties and is used as flavouring in cuisine (Lewis and Elvin-Lewis, 2003). Ankri and Mirelman (1999) reported that garlic has broad antibacterial, antifungal, antiparasitic, and antiviral activity. Corzo-Martinez et al. (2007) reported that garlic promoted aquatic organism health by regulating pathogens, particularly bacteria and fungi.

There have been many studies on the antibacterial activity of garlic juice extract, but studies on the antibacterial activity of garlic juice extract on *Vibrio* spp. are scarce. This study investigated the *in vitro* antimicrobial activity of fresh garlic juice extract against *Vibrio* spp. bacteria in juvenile hybrid grouper.

Materials and Methods

Preparation of garlic juice extract

Fresh garlic was obtained from the local market in Besut, Terengganu, Malaysia. The garlic juice extract was obtained by grinding peeled garlic cloves in a juice maker (Panasonic, Japan). The stock extract was filtered through muslin cloth, then stored in a sealed bottle at 4 °C before use. The filtrate was considered a 100% stock solution.

Bacterial culture

Vibrio alginolyticus, *V. vulnificus*, and *V. parahaemolyticus* were isolated from a vibriosis outbreak among hybrid grouper at the Fisheries Research Institute Tanjung Demong (FRI TD). Infected hybrid grouper exhibited lethargy, loss of appetite, and skin, mouth, and fin ulceration. The bacteria were identified using an Analytical Profile Index (API) 20NE identification system (BioMerieux, Marcy l'Etoile, France). The isolates were sub-cultured onto tryptic soy agar (TSA, Merck, Germany) containing 1.5% sodium chloride for 24 h at 30 °C. Five colonies per isolate were cultured in 250 mL tryptone soy broth containing 1.5% sodium chloride for 24 h at 30 °C with continuous shaking at 150 rpm. Then, 1 mL suspension was collected and serially diluted 10 times in 9 mL phosphate-buffered saline (PBS, Merck) before 0.1 mL per serial dilution was plated onto TSA plates containing 1.5% sodium chloride. The colony-forming unit/mL was determined after 24-h incubation at 30 °C.

Kirby-Bauer disc diffusion susceptibility test

The performance of the fresh garlic juice as an antibacterial was assessed by adding 40 µL fresh garlic juice extract (20%, 40%, 60%, 80%, and 100%) to 6-mm filter paper discs, then allowing them to dry for 10 min. The discs were then placed on TSA plates containing 1.5% sodium chloride and seeded with the respective test microbes alongside a 30 µg OTC disc (Oxoid™, United Kingdom) (positive control) and another disc to which absolute ethanol had been added (negative control). The plates were then incubated for 16–18 h at 35 °C; thereafter, the inhibition zone around each disc was measured and recorded. Each concentration was prepared in triplicate.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The absolute garlic juice extract was serially diluted using PBS to obtain concentrations of 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.20%, 0.09%, 0.045%, 0.023%, and 0.011%. Subsequently, 1 mL bacterial suspension was added to every 10 mL dilution of fresh garlic juice extract. The blank control was PBS without fresh garlic juice extract. The mixtures were plated on TSA plates containing 1.5% sodium chloride and incubated for 24 h at 30 °C. The lowest concentration that did not demonstrate any bacterial growth was used as the minimum bactericidal concentration (MBC), while the lowest concentration that prevented bacterial growth was considered the minimum inhibitory concentration (MIC) (Palavesam *et al.*, 2006).

Statistical analysis

The data were analysed using SPSS 23 (SPSS Inc., Chicago, IL, USA) and are expressed as the means ± SD. Significant differences between the control and exposed groups were analysed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences with $p < 0.05$ were considered significant.

Results

Bacteriological examination

All *Vibrio* isolates were confirmed to have good identification (>90%) before use as test bacteria in this study. Table 1 depicts the API 20NE profiling results.

Table 1. API profiles of the *Vibrio* isolates

Biochemical test	*V. alginolyticus*	*V. vulnificus*	*V. parahaemolyticus*
NO3	+	+	+
TRP	+	+	+
GLU	+	+	+
ADH	-	-	-
URE	-	-	-
ESC	+	+	-
GEL	-	+	+
PNG	-	+	+
GLU	+	-	+
ARA	+	-	-
MNE	+	-	+
MAN	+	-	+
NAG	-	-	+
MAL	-	-	+
GNT	+	-	-
CAP	-	-	-
ADI	-	-	-
MLT	+	-	+
CIT	-	-	-
PAC	+	-	-

Antimicrobial susceptibility testing

The bacterial growth inhibition by the fresh garlic juice extract was tested using the Kirby-Bauer disc diffusion method against *V. alginolyticus*, *V. vulnificus*, and *V. parahaemolyticus* (Table 2). The inhibition zone diameter varied according to the *Vibrio* species tested although the concentration and volume of fresh garlic juice extract given were similar throughout the experiment. *V. vulnificus* was the most sensitive to the 100% fresh garlic juice extract, followed by *V. parahaemolyticus* and *V. alginolyticus*.

Table 2. Mean inhibition zone (mm) of garlic juice extract against the tested bacteria

Bacteria tested	Control	20% GE	40% GE	60% GE	80% GE	100% GE	OTC (30 μg)
V. alginolyticus	0.0 ± 0.00^{a}	10.3 ± 0.07^{b}	17.1 ± 0.14^{c}	19.1 ± 0.14^{d}	20.1 ± 0.07^{e}	20.2 ± 0.00^{e}	19.2 ± 0.07^{d}
V. vulnificus	0.0 ± 0.00^{a}	15.2 ± 0.07^{b}	21.3 ± 0.14^{c}	22.0 ± 0.28^{d}	25.1 ± 0.14^{e}	26.3 ± 0.21^{f}	22.0 ± 0.70^{d}
V.parahaemolyticus	0.0 ± 0.00^{a}	9.90 ± 0.14^{b}	14.3 ± 0.14^{c}	20.1 ± 0.07^{d}	20.2 ± 0.07^{d}	23.0 ± 0.07^{e}	23.2 ± 0.14^{e}

GE = Fresh garlic juice extract, OTC =Oxytetracycline
Values are the mean ± SD (n = 2). Analysis was performed using one-way ANOVA, followed by the Tukey test with *post hoc* multiple comparisons. The negative control exhibited no antibacterial activity

Inhibition zone of V. alginolyticus

Table 2 summarises the inhibition zone diameter of *V. alginolyticus* as tested by 100%, 80%, 60%, 40%, and 20% fresh garlic juice extracts, ethanol (negative control), and commercial antibiotic (OTC, positive control). The widest inhibition zone diameter was observed for the discs impregnated with 100% (20.2 ± 0.00 mm) and 80% (20.1 ± 0.49 mm) fresh garlic juice extract, followed by OTC (19.2 ± 0.07 mm), 60% (19.1 ± 0.14 mm), 40% (17.1 ± 0.14 mm), and 20% fresh garlic juice extract (10.3 ± 0.07 mm), while no inhibition (0 ± 0.00 mm) was observed for the negative control disc. The data indicated a positive correlation between the fresh garlic juice extract concentration and the corresponding increase in the *V. alginolyticus* inhibition zone.

Inhibition zone of V. vulnificus

Table 2 summarises the inhibition zone diameter of *V. vulnificus* as tested by 100%, 80%, 60%, 40%, and 20% fresh garlic juice extracts, ethanol (negative control), and commercial antibiotic (OTC, positive control). The widest inhibition zone diameter was observed for the discs impregnated with 100% (26.3 ± 0.21 mm) and 80% (25.1 ± 0.14 mm) fresh garlic juice extract, followed by 60% fresh garlic juice extract (22.2 ± 0.28 mm), OTC (21.7 ± 0.7 mm), 40% fresh garlic juice extract (21.3 ± 0.14 mm), and 20% fresh garlic juice extract (15.2 ± 0.07 mm), while no inhibition (0 ± 0.00 mm) was observed for the negative control disc. The data indicated a positive correlation between the fresh garlic juice extract concentration and the corresponding increase in the *V. vulnificus* inhibition zone.

Inhibition zone of V. parahaemolyticus

Table 2 summarises the inhibition zone diameter of *V. parahaemolyticus* as tested by 100%, 80%, 60%, 40%, and 20% fresh garlic juice extracts, ethanol (negative control), and commercial antibiotic (OTC, positive control). The widest inhibition zone diameter was observed in the discs impregnated with OTC (23.2 ± 0.14 mm) and 100% fresh garlic juice extract (23.0 ± 0.07 mm), followed by 80% (20.2 ± 0.21 mm), 60% (20.1 ± 0.07 mm), 40% (14.3 ± 0.14 mm), and 20% fresh garlic juice extract (9.9 ± 0.14 mm), while no inhibition (0 ± 0.00 mm) was observed for the negative control disc. The data indicated a positive correlation between the fresh garlic juice extract concentration and the corresponding increase in the *V. parahaemolyticus* inhibition zone.

MIC and MBC

Table 3 summarises the MIC and MBC of the fresh garlic juice extract against the tested bacteria. The lowest MBC was against *V. alginolyticus* (0.023%), followed by that against *V. vulnificus* (0.045%) and *V. parahaemolyticus* (0.09%). Similarly, the MIC was lowest against *V. alginolyticus* (0.011%) followed by that against *V. vulnificus* (0.023%) and *V. parahaemolyticus* (0.045%). The results indicated that a low concentration of fresh garlic juice extract (<1%) was sufficient to inhibit the growth of the three *Vibrio* species (Table 3).

Table 3. The MIC and MBC of fresh garlic juice extract against tested bacteria

Bacterial isolates	MBC	MIC
V. alginolyticus	0.023%	0.011%
V. vulnificus	0.045%	0.023%
V. parahaemolyticus	0.09%	0.045%

MIC = Minimum inhibitory concentration; MBC = minimum bactericidal concentration.

Discussion

Vibrio spp. are commonly encountered in coastal and estuarine environments (Baker-Austin et al., 2018) and can be responsible for bacterial infections in farmed aquatic species. Antibiotics are commonly applied to address infections, but their usage has resulted in more negative effects than positive ones **(**Alderman and Hastings,1998).

Our results demonstrated that every concentration of fresh garlic juice extract exerted some degree of inhibition against all three pathogenic *Vibrio* species. The sensitivity of the bacteria towards fresh garlic juice extract aligned with the concentration of the extract, where the inhibition zones increased gradually with the concentration of garlic juice used. The positive control OTC also inhibited the bacterial growth, yielding results that were equal to 60%–100% fresh garlic juice. Tetracycline was thought to be effective against *Vibrio* spp., which is consistent with previous evidence (Amalina et al., 2012).

Fresh garlic juice extract (100% and 80%) demonstrated significantly better antibacterial activity against *V. alginolyticus* and *V. vulnificus* compared to OTC ($p < 0.05$). Furthermore, 60% fresh garlic juice extract demonstrated equal antibacterial activity to OTC. *V. parahaemolyticus* was more resistant to the extract, where 100% extract produced similar, non-significant antibacterial activity to OTC ($p > 0.05$). Amalina et al. (2019) indicated that most *V. parahaemolyticus* isolates from seafood in Malaysia were highly resistant to at least one antibiotic, including tetracycline. The extensive and unregulated use of antibiotics in aquaculture for disease prevention instead of treatment and the rapid disease spread are the main factors in the wide emergence of multi-drug resistance (MDR) in *V. parahaemolyticus*. Furthermore, the lack of control and misuse of antibiotics in aquaculture contribute to the emergence and spread of antibiotic-resistant bacteria and the dissemination of antibiotic-resistant genes (Tan et al., 2020). Genes that confer antibiotic resistance can be transferred between bacteria, even across different species. This horizontal gene transfer can occur naturally, but can be accelerated

by antibiotics overuse and misuse. This development is a significant concern for human and animal health (Miranda et al., 2018). Hence, the low MIC and MBC of the fresh garlic juice extract suggested the potential of the extract to replace commercial antibiotics to control vibriosis in grouper.

Natasya-Ain et al. (2018) reported that garlic extract demonstrated antibacterial activity against four marine pathogens: *Aeromonas hydrophila*, *V. harveyi*, *V. anguillarum*, and *V. alginolyticus*. Furthermore, Yadhav et al. (2015) proved that garlic was effective against five bacterial pathogens: *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Escherichia coli*. Li et al. (2007) discovered that the organosulfur compound in garlic contributed the most biological function. Garlic organosulfur exerts its antibacterial effect when the free sulfhydryl groups of proteins, including those in enzymes, can form disulfide bonds with the sulfur in the highly reactive organosulfur compounds. The creation of disulfide bonds inactivates the enzymes, thus killing the bacteria.

Another mechanism is the bacterial cell membrane interaction with the organosulfur molecule. This contact weakens the bacterial cell membrane, causing cell content leakage and death. Additionally, it is believed that the garlic organosulfur chemicals affect gene expression, DNA replication, and protein synthesis (Bhatwalkar et al., 2021). Khashan (2014) demonstrated that garlic was effective against *S. aureus* activity due to the presence of the allicin compound in garlic extract. Allicin also has antibiotic, antidiabetic, and antihypertensive properties (Shinkafi, 2013). Apart from allicin, ajoene and other aliphatic sulfur compounds are the primary antibacterial organosulfur compounds in garlic (Bhatwalkar et al., 2021). Our results demonstrated that fresh garlic juice extract has abilities equivalent to the commercial antibiotic OTC in fighting *Vibrio* spp. infection.

Conclusions

Fresh garlic juice extract has fulfilled the important criteria needed as an antibacterial agent. A higher concentration of garlic juice extract might produce greater inhibition of bacterial activity. Furthermore, the MIC and MBC of the fresh garlic juice extract were very low. However, further research is needed to determine the potential of the extract for therapeutic application in fish. The next step is to conduct challenge trials using the three *Vibrio* species examined here and examine the potential of fresh garlic juice extract to control or manage infections. Therefore, fresh garlic juice extract should be verified as a potential herbal medicine that might replace the antibiotics commonly used in aquaculture to treat vibriosis.

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Pathogenicity of Betanodavirus Strains in Asian Seabass (*Lates Calcarifer*) Under Temperature Fluctuation Stress

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***Abstract*:** Viral nervous necrosis (VNN) caused by betanodavirus is responsible for mass mortality in various marine cultured fish species. VNN can be transmitted horizontally and vertically. In this study, three betanodavirus strains (BE2006, AVA, and KL0022) were obtained from National Fish Health Research Centre (NaFisH) and cultured in the E-11 cell line. The median tissue culture infective dose ($TCID_{50}$) for all isolates was determined before being used in *in vivo* experiments. The $TCID_{50}$ of KL0022, BE2006, and AVA were 10^4 $TCID_{50}$/mL, 10^7 $TCID_{50}$/mL, and 10^6 $TCID_{50}$/mL, respectively. The KL0022 strain had the lowest $TCID_{50}$, followed by that of AVA and BE2006. Subsequently, 360 healthy juvenile Asian seabass (5 ± 2 g) were divided equally into eight tanks to represent four treatment groups with duplicates: Group 1 (AVA), Group 2 (BE2006), Group 3 (KL0022), and Group 4 (negative control). The fish were observed daily for clinical signs or mortality. On the appearance of clinical signs or mortality, the fish were killed immediately and underwent a reverse transcriptase–PCR (RT-PCR) and virus cell culture. The experiment was terminated after 30 days. The clinical signs observed among the sick and dead fish were darker body colouration and lethargy. The highest mortality was recorded in the KL0022 group, indicating that the strain had the highest virulence, followed by the AVA and BE2006 groups. All dead and sick fish from the treatment groups were positive for betanodavirus by RT-PCR, while the dead fish from the negative control group tested negative.

Keywords: VNN, betanodavirus, temperature, pathogenicity, Asian seabass, $TCID_{50}$

Abstrak: Penyakit Viral Nervous Necrosis (VNN) yang disebabkan oleh jangkitan betanodavirus bertanggungjawab ke atas kejadian kematian besar-besaran dalam pelbagai spesis ikan marin yang dikultur. Kajian terdahulu telah melaporkan penyakit ini boleh disebarkan melalui dua arah, iaitu secara mendatar dan menegak. Tiga strain betanodavirus (BE2006, AVA dan KL0022) diperoleh daripada Pusat Penyelidikan Kesihatan Ikan Kebangsaan (NaFisH dan dibiakkan dalam sel-sel E-11. Pada permulaan, lima puluh peratus dos jangkitan kultur tisu (TCID 50) untuk semua isolat ditentukan terlebih dahulu sebelum digunakan dalam eksperimen in vivo. TCID 50 untuk semua strain yang diuji ialah KL0022 10 4 TCID 50 /ml, BE2006 = 107 TCID 50 /ml dan AVA = 106 TCID

50 /ml. Strain KL 0022 didapati mempunyai TCID 50 paling rendah diikuti oleh AVA dan seterusnya BE 2006. Kemudian, tiga ratus enam puluh (360) ekor anak siakap Asia yang sihat seberat 5 ± 2g dibahagikan sama rata ke dalam lapan biji tangki untuk mewakili empat kumpulan rawatan dengan pendua; Kumpulan 1 (strain AVA), Kumpulan 2 (strain BE2006), Kumpulan 3 (strainKL0022) dan Kumpulan 4 (kawalan negatif). Semua anak-anak ikan tersebut dipantau setiap hari untuk sebarang tanda klinikal atau kematian. Pada kemunculan tanda-tanda klinikal atau kematian, ikan-ikan itu dimatikan serta-merta dan kehadiran virus dikesan melalui kaedah reverse transcriptase-polymerase chain reaction (RT-PCR) dan kultur sel virus. Eksperimen tersebut kemudiannya ditamatkan selepas tempoh tiga puluh (30) hari. Tanda-tanda klinikal yang diperhatikan di kalangan ikan yang sakit dan mati adalah seperti warna badan yang lebih gelap dan lesu. Kematian tertinggi telah direkodkan dalam kumpulan yang dijangkiti strain KL0022 yang menunjukkan virulensi tertinggi diikuti kumpulan AVA dan BE2006 dengan virulensi terendah. Kesemua ikan mati dan sakit daripada kumpulan rawatan telah diuji positif terhadap betanodavirus melaui kaedah RT-PCR, manakala ikan yang mati daripada kumpulan kawalan negatif telah diuji negatif terhadap betanodavirus.

Introduction

Aquaculture in Malaysia began in the 1920s with the extensive polyculture of Chinese carp species, such as bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*), and grass carp (*Ctenopharyngodon idellus*), which were brought directly from China in former mining pools. Recently, the aquaculture sector has grown rapidly and become a very profitable industry, contributing 11% to the national agriculture gross domestic product (GDP) and 0.8% of the national GDP. In 2021, the Malaysian aquaculture industry recorded a total production of 417,187 metric tonnes (mt), accounting for 24% of national fisheries production and involving 21,241 people (DOF, 2023). Mariculture is the main contributor to the aquaculture industry, with a total production of 311,284 mt and a value of RM 2.52 billion. In 2021, Asian seabass (*Lates calcarifer*) was the principal marine species cultured in Malaysia, with a total production of 34,187 mt and a wholesale value of RM 480,159 (DOF, 2023).

The aquaculture industry faces many obstacles and challenges that can impede its growth, and diseases are one of the major threats (Murray et al., 2005). Viral nervous necrosis (VNN) due to betanodavirus infection is a common disease in cultured marine fish and is considered the biggest issue in Asian seabass cultivation (Nallala et al., 2021). Fish can be infected by betanodavirus by horizontal or vertical transmission (Atirah et al., 2019). Horizontal transmission might originate from the culture environment, while vertical transmission occurs through infected broodstock (Antonovics et al., 2017). A shortage of marine fish seed has resulted the importation of seed from neighbouring countries, such as Thailand and Indonesia, which are the main sources of the betanodavirus infection introduced into the Malaysian mariculture system (Knibb et al., 2017).

The source of betanodavirus infection in fish remains unclear, although recent studies indicated that it can be vertically transmitted from broodstock to offspring, while other studies speculated that stress or primary infection by the marine bacterium *Vibrio alginolyticus* leads to secondary infection by betanodavirus (Atirah et al., 2019). Observations and recent reports from farmers suggested that temperature fluctuations might be the main predisposing factor resulting in betanodavirus infection in

Asian seabass. Thus, this study was conducted to determine the effect of temperature as a predisposing factor towards the pathogenicity of betanodavirus strains in Asian seabass.

Materials and Methods

Viral isolates and cell culture techniques

Local betanodavirus isolates were obtained from the National Fish Health Research Centre (NaFisH), Penang, and cultured in an E-11 cell line. Isolates of the betanodavirus strains BE2006, KL0022, and AVA were used. BE2006 was isolated from a diseased golden pompano (*Trachinotus blochii*) in Langkawi Island, Kedah, Malaysia (Rangsangen et al., 2011), while KL0022 was isolated from fingerling hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*) during a NaFisH epidemiology study in Langkawi Island (Ariff et al., 2019). AVA was obtained from the Agri-Food and Veterinary Authority of Singapore.

The E-11 cells were maintained at 25 °C. The cultures were seeded in a 25 cm^2 culturing flask (Corning™) containing 0.7×10^6 cells and filled with Leibovitz's L-15 medium (Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco) and 0.05 mg/mL gentamicin reagent (Gibco). The seeding densities were calculated using an automated cell counter (Countess® II automated cell counter, Thermo Fisher Scientific). The cell culture was sub-passaged in a laminar flow cabinet using aseptic reagents and equipment.

The E-11 monolayer almost reached confluency and was washed twice with HBSS (Invitrogen™), then 500 **µ**L of each betanodavirus isolate stock culture supernatant was inoculated onto the cells. Thereafter, the flask was incubated at 25 °C for 1 h before it was supplemented with 5 mL L-15 medium with 2% FBS and 0.05 mg/mL gentamicin reagent (Iwamoto et al., 2000; Nishi et al., 2016).

When the cytopathic effect (CPE) of the infected cells was observed in 80% of the cells, the infected culture was harvested using four freeze-thaw cycles (Souto et al., 2018). Then, the infected culture supernatant was centrifuged for 15 min at 5000 rpm and filtered using a 0.45-**µm** pore size syringe filter (Minisart® Sartorius AG, Germany). The virus supernatant was stored at -80 °C for further use.

RT-PCR confirmation of virus

Total RNA was extracted from the harvested virus culture using Easy-Blue™ according to the manufacturer's protocol (iNtRON Biotechnology, Inc). The extracted RNA underwent PCR assay using the protocol adapted from the OIE Manual (OIE, 2018). The PCR involved two reactions: an initial RT-PCR using primers F2 and R3, followed by a second PCR using primers NFRG and NRGG (Table 1). The expected result was a 258-bp product indicating the presence of betanodavirus. The PCR detection was validated against a positive control of betanodavirus using an isolate obtained from the Agri-Food and Veterinary Authority of Singapore that was also maintained by NaFisH.

Table 1. List of PCR primers used in the study

Reaction	Primer	Primer sequence	Process	Amplicon size	Reference
First RT-PCR	F2	5′-CGTGTCAGTCATGTGTCGCT-3′	RT-PCR	420 bp	Nishizawa *et al.* (1994)
	R3	5′-CGAGTCAACACGGGTGAAGA-3′			
Second PCR	NFRG	5′-CSGCGAAACCAGCCTGCAGG-3′	Nested PCR	258 bp	de la Pena *et al.* (2008)
	NRGG	5′-ACCTGAGGAGACTACCGCTG-3′			

TCID50 calculations

The $TCID_{50}$ was determined using the following protocol. One day before titration, the microliter plate (96 wells, flat-bottom) was seeded with E-11 cells. After 24 h, 10-fold serial dilutions from the different betanodavirus cell lines were made in the maintenance medium; 3–9 wells were used for the dilutions of 10^{-1} to 10^{-9}, while three wells were used for the controls. After 1-h adsorption, all wells were carefully emptied, and 100 µL maintenance medium was added to the test and control wells. The plates were then incubated at 25 °C. Plates were examined daily, and observations were made as CPE developed. The $TCID_{50}$ was calculated using the method by Reed and Muench (1938) and compared with the method of Muthannan Andavar Ramakrishnan (2016).

Animal ethics

The fish were handled following the guidelines for the care and use of animals in scientific research. The protocols were justified and approved by the International Islamic University of Malaysia (IIUM) Institutional Animal Care and Use Committee (IACUC) and were certified in IACUC-2020-015.

Fish and rearing conditions

Three hundred and sixty healthy VNN-negative seabass fry (5 ± 2 g), 2–3 inches in length were obtained from FRI Tanjung Demong, Terengganu, Malaysia and randomly assigned in equal numbers to eight tanks representing four groups with duplicates: Group 1 (AVA), Group 2 (BE2006), Group 3 (KL0022), and Group 4 (negative controls). The fish were stocked at a density of 40 fry per 30 L water. All experimental fish were acclimatised for at least seven days before the experiment began.

During acclimatisation, the light cycle was held at a constant 12 h of lighting per day. Feeding was *ad libitum* with commercial pellet while water was completely renewed daily and continuously aerated. Before the experiment began, five fish per group were killed and swab samples were obtained from the organs for bacterial and parasitic examination to ensure that the fish were healthy and disease-free. The water quality was determined daily using a handheld HQ40d Meter (Hach, Loveland, CO, USA) and maintained at an optimum level.

Experimental design

The experiment was conducted at the FRI Tanjung Demong experimental facility. For the experimental challenge, the 40 fry were transferred to a tank containing 5 L aerated seawater. Then, 50 mL virus inoculum was added to each group. After 2-h immersion, the fish were transferred to 30 L clean seawater. Unscheduled water temperature fluctuation was imposed on the fish as the stress factor to depress their immune systems. All fish were observed daily and clinical changes or mortality were recorded. Upon the appearance of clinical signs or mortality, the fish were killed and processed for PCR and virus cell culture. The experiment was terminated 30 days post-betanodavirus infection, and RT-PCR and viral cell culture were conducted.

Results and Discussion

The $TCID_{50}$ of KL0022, BE2006, and AVA were calculated using the methods of Reed and Muench (1938) and Muthannan Andavar Ramakrishnan (2016) and were 10^4 $TCID_{50}$/mL, 10^7 $TCID_{50}$/mL, and 10^6 $TCID_{50}$/mL, respectively. All strains were from the widely distributed red-spotted grouper nervous necrosis virus (RGNNV) species (Nishizawa *et al.*, 1997). The $TCID_{50}$ of the other reported RGNNV strains were $10^{5.80}$ $TCID_{50}$/mL and $10^{5.05}$ $TCID_{50}$/mL (Biasini et al., 2022).

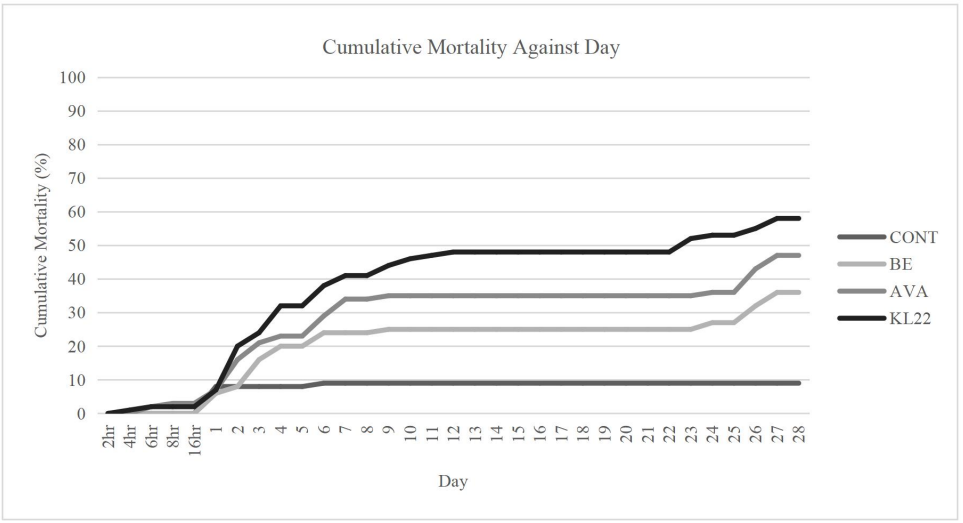

Figure 1. The cumulative mortality of Asian seabass following experimental challenge with three betanodavirus strains

The percentage of cumulative mortality was directly proportional to the days of infection (Figure 1). The highest mortality was recorded in the KL0022 group, indicating that the strain had the highest virulence, followed by the AVA and BE2006 groups (Table 2). The first mortality observed in the infected groups was within 24 h. However, other studies reported VNN outbreaks after 20 days in seven-band grouper (*E. septemfasciatus*) (Tanaka et al., 1998) and after 30 days in guppies, zebrafish, oscars, and goldfish (Zorriehzahra et al., 2013). The early mortality observed in this study might have been due to the stress of exposure to the unscheduled temperature fluctuation. The percentage of cumulative mortality did not increase significantly from day 10 to 21, suggesting that the virus was

in the dormant stage during that time. From day 22 until the end of the study, mortality was observed again but in lower numbers. In agreement with this result, Chi *et al.* (1999), Le Breton (1999), and Maltese and Bovo (2007) also reported that water temperature plays a significant role in the degree of infections by betanodavirus and mortality in fish.

Table 2. Total percentage of fish mortality throughout the experiment

Tank	Fish (no.)	Cumulative mortality	Percentage cumulative mortality (%)	Sick fish
Control	80	9	11.25	0
BE2006	80	36	45	14
AVA	80	47	46.25	9
KL0022	80	58	72.5	13

The first clinical sign observed in this experiment was darker body colouration, followed by lethargy before mortality occurred. The clinical signs observed in this study were the typical clinical signs of betanodavirus infection in Asian seabass (Banerjee et al., 2014; Azad et al., 2006). The darker pigmentation due to the high expression of melanin indicated that the fish were under stress conditions (Choi et al., 2020). Lethargic or "tired" fish syndrome occurs due to many reasons, but betanodavirus-infected fish become inactive due to the loss of appetite.

All sick and dead fish from the treatment groups tested positive by RT-PCR, confirming that the clinical signs and mortalities were attributable to betanodavirus infection (Figure 3). The dead fish from the negative control group tested negative for betanodavirus, indicating that these mortalities were due to natural causes. T1–T5 were the target regions for detecting the RNA2 coat protein gene to identify betanodavirus (Nishizawa et al., 1994). However, the histopathological observation did not detect vacuolation in the fish tissue samples from the treatment groups, suggesting that acute infection had occurred. Multiple vacuolation in the cytoplasm and detachment of the infected cells are the typical CPE in chronic betanodavirus infection (Low et al., 2017).

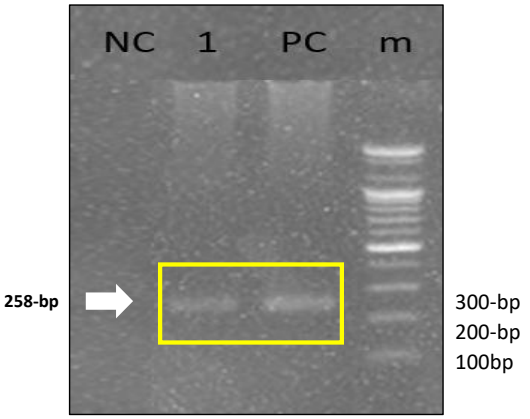

Figure 3. Agarose gel electrophoresis analysis of nested PCR amplification of betanodavirus from the infected group. NC, Negative control; 1, infected dead fish sample; PC, positive control; m, 100-bp marker.

Conclusion

Temperature fluctuation was confirmed as a main predisposing factor during acute betanodavirus infection. The local isolate KL0022 was the most virulent strain in Asian seabass. Given that Asian seabass is a popularly cultured and favoured aquaculture species, VNN control and prevention should be explored further to maintain sustainable fry production and prevent the rapid spread of VNN, notably within Malaysia.

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The Effect of Commercial Cinnamon Essential Oil on Bacterial Prevalence in Farmed Red Snapper (*Lutjanus argentimaculatus*)

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***Abstract*:** Bacterial infections cause high mortality and substantial losses to fish farmers. The threat of bacterial infection in aquaculture has raised interest in finding new treatments. Using plant essential oils as antibacterial agents is a new trend in preventing and treating bacterial disease. Thus, this study aimed to evaluate the effect of cinnamon essential oil (EOCIN) on bacterial species pathogenic to red snapper (*Lutjanus argentimaculatus*). The study duration was 43 days, beginning on 23 November, 2021, and was conducted on Jerejak Island, Pulau Pinang, cage cultures. Fish (mean size: 18.64 ± 3.31 g) were divided into control and treatment groups. The control fishes were fed normal pellets while the treatment fishes were fed for 14 consecutive days with pellets sprayed with 1.5% (v/w) EOCIN. After treatment completion, the fishes were sampled on days 15, 29, and 43. Twenty control fishes and 20 treatment fishes were sampled for each sampling session. The field trial results revealed that 25% of the treatment fish appeared clinically healthier than the control fish. The internal organs of the treatment fish had a low prevalence of *Vibrio* spp. compared to the control: *V. vulnificus* and *V. parahaemolyticus* were prevalent in 5% and 5% of the treatment group compared with 25% and 10% in the control group, respectively. The fish health assessment at 14 and 28 days after EOCIN feed regimen completion demonstrated a similar prevalence of *Vibrio* sp. isolated from the two sampling days: *V. vulnificus*, 5% and *V. parahaemolyticus*, 6%. However, there was a significant increase in the prevalence of *Photobacterium damselae*, which was prevalent in 50% of the treatment group compared to only 39% in the control group. This preliminary study demonstrated that feeding an EOCIN-medicated diet to farmed red snapper reduced the prevalence of certain bacterial species in their internal organs. However, the prevalence of bacteria once the feeding regimen was halted. Thus, the antibacterial effect of EOCIN was effective against several pathogenic *Vibrio* sp. However, EOCIN did not prevent *P. damselae* colonisation in the fish after a 14-day treatment. This, further studies are needed to define the optimal dosing regimen for managing *Vibrio* infections in fish.

Keywords: Cinnamon essential oil, red snapper, bacterial prevalence

Abstrak: Jangkitan bakteria diketahui menyebabkan kematian yang tinggi dan kerugian besar kepada penternak. Ancaman jangkitan bakteria dalam akuakultur telah menimbulkan minat untuk mencari rawatan baharu. Terdapat trend baharu menggunakan minyak pati tumbuhan sebagai agen antibakteria dalam rawatan langkah pencegahan terhadap penyakit bakteria. Oleh itu, objektif kajian ini adalah untuk menilai kesan minyak pati kayu manis (EOCIN) terhadap spesies bakteria patogen ikan lazim dalam ikan merah (*Lutjanus argentimaculatus*). Keputusan percubaan lapangan mendedahkan

bahawa 25% daripada ikan yang menerima 14 hari EOCIN dalam makanan kelihatan sihat secara klinikal berbanding ikan yang menerima pelet biasa. Prevalens bakteria yang rendah didapati dalam organ dalaman ikan kumpulan EOCIN berbanding kawalan; *Vibrio vulnificus* (5% EOCIN berbanding dengan 25% kawalan), dan *Vibrio parahaemolyticus* (5% EOCIN berbanding dengan 10% kawalan). Pemerhatian lanjut selepas 14 dan 28 hari pemberian EOCIN, corak peningkatan prevalens boleh dikesan dalam dua kumpulan bakteria; *V. vulnificus* dan *V. parahaemolyticus* (5% EOCIN berbanding dengan 6% kawalan). Peningkatan ketara dalam prevalens boleh dikesan dalam *Photobacterium damselae* dengan prevalens 50% dalam EOCIN berbanding dengan 39% dalam kawalan. Kajian awal ini menunjukkan bahawa pemberian EOCIN kepada ikan merah ternak mengurangkan prevalens bakteria dalam organ dalaman ikan. Walau bagaimanapun, prevalens bakteria meningkat selepas pemberhentian rawatan EOCIN dalam diet mereka. Oleh itu, kesan antibakteria EOCIN didapati mengurangkan prevalens beberapa *Vibrio* sp. yang patogenik dan *P. damselae* daripada menjangkiti ikan selepas 14 hari rawatan EOCIN. Kajian lanjut diperlukan untuk mengesahkan keberkesanan dos dan rejim rawatan untuk pemberian EOCIN sebagai bahan tambahan dalam makanan terhadap keadaan kesihatan ikan secara keseluruhan terutamanya mengenai prevalens bakteria dan kejadian penyakit**.**

Introduction

The persistent high demand for fish protein, valued as an affordable protein source, continues to propel the growth of the aquaculture sector and its multispecies industries (FAO, 2016; DOF, 2019). Sustainable aquaculture requires good management practices in maintaining the health of fish (Mzula et al., 2021). One of the greatest challenges in sustainable aquaculture is the management of infectious diseases caused by parasites, bacteria, viruses, and fungi.

Bacterial infection cause high mortality and substantial losses to fish farmers. Treating bacterial infections with antibiotics is common, where antibiotics act as therapeutic or prophylactic agents (Thiang et al., 2020). Asia-Pacific countries have the largest antibiotic usage percentage, accounting for 93.8% of global antibiotic use, where 57.9% of usage is from China (Schar et al., 2020). The choice of antibiotic depends on the type of bacteria as each antibiotic has its own mechanism of action (Patel et al., 2023). For example, vancomycin targets gram-positive bacteria and is not efficient against gram-negative bacteria as it cannot cross the gram-negative bacteria cell wall (van Duijkeren et al., 2018).

There is a recent growing trend of using plant essential oils (EOs) as antibacterial agents. The application of EOs as antibacterial agents was effective against a range of bacterial infections, for example, *Aeromonas salmonicida*, *V. harveyi*, and *Streptococcus agalactiae* (Dawood et al., 2021). Cinnamon belongs to the genus *Cinnamomum* and has antibacterial properties (Nabavi *et al.,* 2015). Additionally, cinnamon has anti-inflammation, immunostimulant, growth-promoting, antineoplastic, and antioxidative effects (Charles, 2012; Habiba et al., 2021). These properties stem from the phenolic compounds of cinnamon. Given its benefits, dietary cinnamon is suggested for finfish species (Habiba et al., 2021).

The commonly used antibiotics in aquaculture are tetracycline, sulfonamides, oxolinic acid,

erythromycin, nitrofurans, chloramphenicol, and virginiamycin (ASEAN, 2013; Wan Norhana et al., 2020). Despite the abundance of antibiotics in the market, the lack of awareness of proper antibiotic use for treating bacterial diseases has resulted in antibiotic resistance in aquaculture (Tangcharoensathien et al., 2021). The development of antimicrobial-resistant (AMR) pathogens in fish and the aquatic environment resulting from improper antimicrobial agent use in farmed fish has become a major concern (Miller and Harbottle, 2018). In 2021, the World Health Organization (WHO) declared that AMR is one of the top global public health threats. Therefore, new and alternative treatments are required to prevent AMR development. This approach has led to an increase in trials exploring the utility of natural-based compounds from herbal and plant products or extracts, such as the EOs of cinnamon, clove oil, and garlic, as alternative antibacterial agents.

Human medicine has used medicinal plants and herbs for treating disease for thousands of years, and many are still used as modern treatments. Recently, a rising trend in the use of plant-based products, such as EOs, has gained scientific interest as EOs have beneficial properties, such as antimicrobials (Menanteau-Ledouble et al., 2015; Morales-Covarrubias et al., 2016), anti-inflammatory (Na-Phatthalung et al., 2017), antiparasitic (Zhang et al., 2013), and immunostimulants (Van Hai et al., 2015). Perez-Sanchez et al. (2018) reported that plant EOs were effective against bacterial infections such as *Escherichia coli* and *Staphylococcus aureus*, probably due to their main compounds: thymol and carvacrol. High antimicrobial activities of commercial cinnamon EO (EOCIN) were reported from a Kirby-Bauer disc diffusion assay (mean inhibition zone diameter [DIZ]: 17.4 ± 3.9) against 10 isolates of *V. parahaemolyticus* from *Penaeus vannamei* with acute hepatopancreatic necrosis disease (AHPND), where the minimum inhibitory concentration (MIC) was 0.39–0.78 mg/L (Padilah et al., 2019).

Disc diffusion testing demonstrated that the EOs of *Cinnamomum micranthum* leaves and twigs were effective against *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *Lactococcus garvieae*, *Debaryomyces hansenii*, *Photobacterium damselae*, *Streptococcus* sp., and *A. hydrophila* (Yeh et al., 2009). The varied inhibitory activity might have been due to the difference of antibacterial constituents in the different parts of the plant. The antibacterial screening of 3.12% EOCIN from *C. bejolghota* determined that it strongly inhibited all *Streptococcus mutans* isolates with MIC of 12.8–51.2 and 64–256 mg/mL, respectively (Fani and Kohanteb, 2011). In another study, *C. camphora* EOCIN demonstrated important antibacterial potency against *E. coli*, with an MIC and minimum bactericidal concentration (MBC) of 200 µg/L (Wu et al., 2019). Considering the benefits of EOCIN, this study evaluated the effect of EOCIN against bacterial infections in farmed red snapper (*Lutjanus argentimaculatus*).

Materials and methods

Study area and design

The study duration was 43 days, beginning on 23 November, 2021, at a cage culture site on Jerejak Island, Pulau Pinang, Malaysia. The red snappers (mean body weight: 18.64 ± 3.31 g) were cultured in 5 m × 5 m cages stocked at 400 fish/m^2. The fish were divided into a control group and an EOCIN-fed group. The EOCIN treatment was incorporated into pelleted feed given to red snapper.

The trial was conducted using duplicate cages for each condition.

Diet preparation and sampling procedures

The control group was fed a normal pelleted diet, while the treatment group received a diet top-dressed with EOCIN. Both groups were fed for 14 days. EOCIN (3 ppm) was prepared by mixing 3 mL EOCIN with 30 mL distilled water, and spraying the mixture onto 30 kg pellets. The sprayed EOCIN was mixed well with the food pellets using a fish meal mixer and dried completely before use. After the 14-day feeding regimen, the fish were switched to a normal pellet diet and sampled on day 15, 29, and 43 (20 control fish and 20 EOCIN-fed fish per sample point).

Water quality sampling and analysis

Water quality sampling and analysis were performed following the methods by Mutea et al. (2021). The water quality parameters analysed were: 1) physical parameters [pH, temperature, salinity, and dissolved oxygen (DO)]; and 2) chemical parameters (ammonia, sulphate, nitrate, and iron). The physical parameters were measured *in situ* using a YSI multiparameter Pro Plus instrument (YSI Corporation, NY, USA). For the chemical parameters, water samples were collected at the surface and at 1- and 3-m depth using a Wildco® water sampler (Yulee, FL, USA) and kept in 1-L screw-capped polyethylene bottles. The bottles were immediately placed in a cool box at 4 °C to ensure proper preservation of the samples. The chemical parameters were analysed in the laboratory using a DR2800 spectrophotometer (Hach, Loveland, CO, USA).

Observation of clinical signs and collection of fish samples

The weight and total length of the fish were measured. The behaviour and clinical signs and abnormalities observed during the physical examination of each fish were recorded prior to opening of the body cavity following decontamination of the ventrolateral surface of the fish by swabbing with 70% alcohol. A portion of the samples from the internal organs (spleen, liver, and kidney) were obtained aseptically from individual fish and cultured on tryptic soy agar (TSA, Millipore, Switzerland) with 1.5% sodium chloride for bacteria isolation. The plates were examined for bacteria growth at 30^{o}C after 24–48-h incubation. The colony morphology on the plates was recorded. Distinct colonies were subcultured to obtain pure colonies for further testing.

Identification of bacteria

After the pure colonies were obtained, the bacterial isolates were identified using conventional biochemical tests following Austin and Austin (2007) and Bergey's Manual of Determinative Bacteriology. The bacteria identification involved Gram staining and determining the colony morphology (colour, shape, and size) and phenotypic characteristics (haemolysis characteristic on blood agar), growth on MacConkey agar and thiosulphate citrate bile salt (TCBS) agar, and catalase, oxidase, motility, and positive oxidative-fermentative sugar tests. The isolate was confirmed using

commercially available API 20E and API 20E kits (Bacteria Identification System, BioMerieux, Marcy-l'Etoile, France).

Statistical analysis

The clinical signs data were analysed using an independent t-test with a 95% confidence interval to evaluate the significant difference of means between the control and treatment groups.

Results and discussion

The initial screening recorded a mean sample weight of 18.64 ± 3.31 g. The mean fish body weight increased and was 32.82 ± 9.16 g (control) and 36.0 ± 6.07 g (treatment group) on the last day of experiment (day 43). Notably, the EOCIN-fed fish demonstrated superior body weight gain compared to those in the control group (Figure 1). The mean fish weight increased by approximately 93% in the treatment group compared to 76% in the control (p = 0.203) over the 43-day experiment.

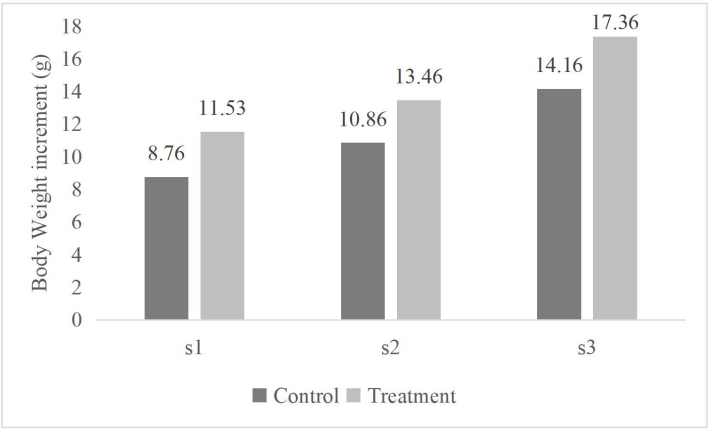

Figure 1. The fish body weight gain was assessed at three sampling points (n = 20 fish per group per sampling). s1 = first sampling, s2 = second sampling, s3 = third sampling

The health status of the fish was examined before the feeding trial by performing a necropsy examination and bacterial isolation from the internal organs. Preliminary screening before the EOCIN treatment revealed that the fish had external lesions, such as fin and tail rot (13.33%), skin ulcers (10.00%), and scale drop (3.33%) (Table 1).

The scoring of the clinical signs between the control and treatment groups was significantly different. Approximately 75% of the fish in the control group had external lesions of the tail and fin rot compared to 50% in the treatment group. The control fish also had lesions/pathological changes, such as scale drop and skin ulcers, with 5% and 15% prevalence, respectively, whereas these changes were not observed in the treatment group (Table 1).

However, the evaluation of the clinical signs on days 29 and 43 revealed the opposite to that

was observed on day 15. Once the EOCIN treatment stopped, the subsequent percentage of external lesions began to increase in the treatment group compared to the control group. On day 29 of sampling (14 days after cessation of EOCIN treatment), the percentage of external lesions of tail rot was higher (90%) in the treatment group compared to 72.22% in the control group.

The day 43 samples had similar patterns, with the fish in the treatment group exhibiting a higher percentage of scale drop (35%) compared to the control (5%). The treatment group also had a higher percentage of tail and fin rot (95%) than the control (85%).

Table 1. Clinical signs observed in farmed *l. argentimaculatus* during gross examination (%)

Clinical signs	Pre-experiment fish health assessment	Gross observation (%)					
		14 days with EOCIN		14 days without EOCIN		28 days without EOCIN	
		Control	EOCIN	Control	EOCIN	Control	EOCIN
Tail and fin rot	13.33	75.00	50.00	72.22	90.00	85.00	95.00
Scale drop	3.33	5.00	0.00	44.40	40.00	5.00	35.00
Ulcer	10.00	15.00	0.00	6.00	0.00	10.00	15.00

It is also important to consider water quality as a part of good on-site management practices. Table 2 lists the physical parameters of the water, while Table 3 presents the chemical parameters of the culture environment. Comparing the results with the appropriate water quality criteria for aquaculture livestock, the DO at the study site was within the acceptable range (>5 mg/L). The pH for all three sampling days was also in the appropriate range (pH 6.5–8.5).

However, the readings obtained for sulphide were slightly higher than the recommended range of <10 mg/mL. Table 3 demonstrates that the sulphide concentration was 11 mg/mL for samplings 1 and 3 for both groups. the sulphate concentration was higher during sampling 2, where it was 13 and 14 mg/mL for the control and treatment groups, respectively. Sulphide is released into the environment by sulphur-containing organic waste. In the presence of oxygen, sulphide is quickly oxidised to sulphate. Conversely, in the absence/lack of oxygen, sulphate is converted to sulphide (Boyd, 2007). The concentration of unionised ammonia was appropriate for all three sampling days for both the control and treatment groups, i.e. <0.02 mg/mL.

Table 2. Water quality of the culture environment at each sampling point

	14 days with EOCIN	14 days without EOCIN	28 days without EOCIN
Temperature (°C)	28.59	28.29	28.79
DO (mg/L)	6.03	5.56	6.46
Salinity (ppt)	30.21	29.90	30.04
pH	7.68	7.76	7.78

Table 3. The chemical parameters of the culture environment

Regimen	14 days with EOCIN		14 days without EOCIN		28 days without EOCIN	
Parameters (mg/L)	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Unionised ammonia (NH_3)	0.01	0.01	0.01	0.02	0.01	0.02
Sulphide (S^{2-})	11.00	11.00	0.013	14.00	11.00	11.00
Nitrite (NO^{2-})	0.01	0.01	0.01	0.02	0.01	0.01
Iron (Fe)	0.02	0.03	0.02	0.03	0.02	0.02

In addition to water quality, other factors should be emphasised in fish farming. The reliance on antibiotics to combat bacterial diseases in the rapidly growing aquaculture industry is unavoidable (Cabello et al., 2013). Antibiotics use in the aquaculture industry is mainly for disease treatment, control, and prevention and for promoting the growth of cultured species (Bush et al., 2011, Romero et al., 2012). The misuse and improper usage of antibiotics in aquaculture drove the emergence of antibiotic-resistant bacteria (ARB) or AMR pathogens (Patil et al., 2016; Paulson et al., 2016).

There are a few reported cases due to resistant pathogens in aquaculture. A previous study reported 100% mortality in goldfishes caused by *Edwardsiella tarda* infection, where *E. tarda* promoted AMR spread through horizontal gene transfer. The discovery of resistance genes on transferable plasmids and integrons among the AMR-associated genera *Aeromonas*, *Yersinia*, *Photobacterium*, *Edwardsiella*, and *Vibrio* have been reported (Miller and Harbottle, 2018). As AMR becomes a major public health issue, scientists worldwide have shifted to alternatives to antimicrobials. One alternative is the application of natural plants to treat infectious disease. Medicinal plants contain antimicrobial compounds and have drawn much attention. Many of these compounds, which include various polyphenols, alkaloids, terpenoids, polypeptides, lectins, and polyacetylenes, have been approved as a GRAS (Generally Recognised as Safe) material for food consumption and have negligible side effects (Simoes et al., 2009).

In this study, we evaluated the effect of EOCIN dietary supplement on farmed red snapper. On day 15, the prevalence of *Vibrio* spp. in the treatment group was reduced compared to the control: *V. vulnificus*, 5% vs. 25%; *V. parahaemolyticus*, 5% vs. 10% (Table 3). These findings demonstrated the effect of dietary cinnamon in enhancing antibacterial capacity in fish. Our result was consistent with the study by Habiba *et al.* (2021), where dietary cinnamon enhanced the antibacterial capacity in European seabass (*Dicentrarchus labrax*) and significantly decreased the number of *Vibrio* spp. in the fish treated with cinnamon compared to those fed a cinnamon-free diet.

Table 3. Pathogenic bacteria prevalence (%) in *L. argentimaculatus*

Sampling day	Day 15		Day 29		Day 43	
Prevalence/group	**14 days with EOCIN**		**14 days without EOCIN**		**28 days without EOCIN**	
	Control	**EOCIN**	**Control**	**EOCIN**	**Control**	**EOCIN**
V. alginolyticus	5	15	17	15	15	15
V. vulnificus	25	5	6	5	20	25
V. parahaemolyticus	10	5	6	5	5	0
P. damselae	25	25	39	50	50	40

The antibacterial mechanism of action of EOCIN has not been elucidated. However, EOCIN caused cell deformities and organelle dysfunction by penetrating hydrophobic compounds into bacterial and parasitic cells (Dawood et al., 2021). EOCIN can also inhibit the adhesion of harmful pathogens to epithelial cells by improving the intestinal mucus secretion of host cells (Jamroz et al., 2006). This result might explain the lower prevalence of pathogenic bacteria in the EOCIN-fed fish compared to those fed the control diet in the present study.

However, when the EOCIN treatment was ended after 14 days, the treatment group had an increasing pattern of bacterial prevalence compared to the control: *V. vulnificus* and *V. parahaemolyticus*, 5% in EOCIN vs. 6% in control; *P. damselae*, 50% in EOCIN vs. 39% in control). These results suggested that including EOCIN in the diet enhanced fish immunity. However, ending the EOCIN treatment rapidly halted the antimicrobial benefits conferred by EOCIN.

Conclusion

Plant EOs have great potential for use in aquaculture as they contain diverse properties that can improve animal health and growth. This preliminary study demonstrated that administrating EOCIN to farmed red snapper reduced the prevalence of bacteria in the internal organs of the fish, reducing the probability of bacterial infection in the cultured fish. Cinnamaldehyde is a major compound related to the EOCIN antibacterial activity. This study reported the antibacterial activity against the prevalence of *Vibrio* spp., namely *V. vulnificus* and *V. parahaemolyticus*, in red snapper. Oral application is the preferred method and is most suitable for minimal handling and to reduce stress to administer EOCIN as dietary supplements with antioxidants, representing an alternative to prevent or treat oxidative stress in fish. The 14-day dietary supplementation of EOCIN at 1.5% volume per kg diet increased the weight gain of the fish compared to the control, proving the growth-promoting activity of EOCIN. With the growing concerns regarding AMR, EOCIN might be an alternative to commercial antibiotics.

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Unveiling the Potential of Medicinal Plants in Combating Shrimp Acute Hepatopancreatic Necrosis Disease (AHPND)

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Abstract: Acute hepatopancreas necrosis disease (AHPND) represents a substantial threat to the global shrimp-farming sector, with *Vibrio parahaemolyticus* recognized as the primary etiological agent. This study responds to the critical imperative for efficient control against AHPND by investigating the therapeutic potential of medicinal plant extracts. Screening of 85 common medicinal plants was systematically undertaken to discern extracts capable of attenuating *V. parahaemolyticus* pathogenicity. Among these, aqueous extracts of two plant species, denoted as CTM 27 and CTM 50, exhibited discernible inhibition of *V. parahaemolyticus* proliferation, as corroborated by zone inhibition assays. Notably, extracts sourced from an additional two plant varieties, CTM 15 and CTM 44, manifested a remarkable capacity to counteract *V. parahaemolyticus* antagonistic behaviour. This phenomenon, characteristic of *V. parahaemolyticus* and its kin, involves the secretion of effector proteins aimed at impeding the growth of competitive bacterial strains within shared ecosystems. Subsequent investigations unveiled the efficacy of CTM 15 and CTM 44 extracts in reversing *V. parahaemolyticus* antagonistic effects against *Escherichia coli*, thereby intimating their prospective utility in ameliorating interspecies competition within shrimp farming habitats. These findings underscore the auspicious therapeutic potential inherent in select medicinal plant extracts for combating AHPND and ameliorating shrimp production outcomes. The identified extracts proffer a host of advantages, including thermal stability and cost-effectiveness, rendering them appealing candidates for development into disease management modalities. Nonetheless, further inquiry is requisite to elucidate the underlying mechanisms of action and optimize formulations for practical deployment in shrimp farming contexts.

Keywords: *AHPND,* antagonism, medicinal plant, shrimp

Abstrak: Penyakit nekrosis hepatopankreas akut (AHPND) merupakan ancaman yang besar kepada sektor ternakan udang global, dengan *Vibrio parahaemolyticus* (*V. parahaemolyticus*) dikenal pasti sebagai agen etiologi utama. Kajian ini bertindak balas terhadap keperluan penting untuk strategi kawalan yang berkesan terhadap AHPND dengan menyiasat potensi terapeutik ekstrak tumbuhan perubatan. Penyaringan pelbagai jenis meliputi 85 tumbuhan perubatan lazim telah dijalankan secara sistematik untuk mengenal pasti ekstrak yang mampu mengurangkan patogenisiti *V. parahaemolyticus*. Antara ini, ekstrak akuas daripada dua spesies tumbuhan, yang ditanda sebagai CTM 27 dan CTM 50, menunjukkan penindasan yang ketara terhadap proliferasi *V. parahaemolyticus*, seperti yang disahkan oleh ujian zon penindasan. Perlu diperhatikan, ekstrak yang diperoleh dari dua varieti tumbuhan

tambahan, CTM 15 dan CTM 44, menunjukkan keupayaan yang luar biasa untuk menentang tingkah laku antagonistik *V. parahaemolyticus*. Fenomena ini, yang merupakan ciri *V. parahaemolyticus* dan keluarganya, melibatkan pengeluaran protein efektor yang bertujuan untuk menghalang pertumbuhan strain bakteria yang bersaing dalam ekosistem yang berkongsi. Penyiasatan seterusnya menemui keberkesanan ekstrak CTM 15 dan CTM 44 dalam membalikkan kesan antagonistik *V. parahaemolyticus* terhadap Escherichia coli, dengan demikian mengisyaratkan kegunaan prospektif mereka dalam memperbaiki pertandingan antara spesies dalam habitat ternakan udang. Penemuan ini menekankan potensi terapeutik yang menjanjikan yang terkandung dalam ekstrak tumbuhan perubatan tertentu untuk memerangi AHPND dan memperbaiki hasil pengeluaran udang. Ekstrak yang dikenal pasti menawarkan beberapa kelebihan, termasuk kestabilan terma dan kos yang berpatutan, menjadikan mereka calon yang menarik untuk pembangunan menjadi modal pengurusan penyakit. Walau bagaimanapun, penyelidikan lanjut diperlukan untuk menjelaskan mekanisme tindakan yang mendasari dan mengoptimumkan formulasi untuk penggunaan praktikal dalam konteks ternakan udang. Kesimpulannya, kajian ini menawarkan paradigma yang pionir untuk pengekalan AHPND, menekankan potensi ekstrak tumbuhan perubatan sebagai alternatif yang berkesan dan lestari kepada intervensi antibakteria konvensional.

Introduction

Acute hepatopancreas necrosis disease (AHPND) poses a significant threat to the global shrimp-farming industry, resulting in substantial economic losses and production declines. AHPND is primarily caused by *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a pathogen with escalating prevalence since its emergence in South Asian countries in late 2013 (Kumar et al., 2021). This disease manifests with severe mortality rates, reaching up to 100% within a short period after stocking shrimp, thereby profoundly impacting shrimp production in affected regions. The economic repercussions are staggering, with collective losses exceeding $43 billion across Asia (Boyd et al., 2022).

V. parahaemolyticus strains associated with AHPND harbor a unique extrachromosomal plasmid encoding two toxin genes, PirA and PirB, which are crucial for the pathogenesis of the disease. The presence of these toxins distinguishes AHPND-causing *V. parahaemolyticus* (*V. parahaemolyticus* AHPND) from non-AHPND strains, highlighting the virulence mechanisms underlying AHPND pathogenicity (Lim et al., 2020). Despite extensive efforts to mitigate the spread of AHPND, current control measures, including pond management practices and antibiotic treatments, have shown limited efficacy and are often associated with undesirable outcomes such as antibiotic resistance (Ghosh et al., 2021).

Given the limitations of existing control strategies, there is a pressing need to explore alternative approaches for managing AHPND in shrimp farming. Immunostimulants and other bioactive compounds derived from natural sources have garnered interest due to their potential to enhance shrimp immunity and disease resistance without the drawbacks associated with conventional treatments (Mohan et al., 2019). Among these alternatives, medicinal plant extracts have emerged as promising candidates for controlling AHPND. However, despite growing interest in plant-based therapies, there remains a significant gap in our understanding of their efficacy and mechanisms of action in mitigating AHPND.

This study seeks to address this gap by investigating the therapeutic potential of medicinal plant extracts against AHPND-causing *V. parahaemolyticus* strains. Through a systematic screening approach, we aim to identify plant extracts capable of attenuating *V. parahaemolyticus* pathogenicity and enhancing shrimp health and survival. By elucidating the bioactive components and mechanisms underlying the observed effects, we seek to contribute to the development of sustainable and effective strategies for AHPND management in shrimp farming.

In this context, this study aims to advance our understanding of the role of medicinal plants in combating AHPND and provide insights into their potential application as alternative treatments in shrimp aquaculture. By bridging the gap between traditional knowledge of medicinal plants and modern scientific approaches, we strive to offer innovative solutions to address the challenges posed by AHPND and safeguard the sustainability of the shrimp-farming industry.

Materials & Methods

Isolation, Culture Preparation and Experimental Control of VPAHPND Strain

The *VP*AHPND strain, sourced from Universiti Putra Malaysia, was isolated from *Penaeus vannamei* exhibiting symptoms of AHPND. Isolation techniques involved streaking bacterial samples onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and subsequent subculture in Tryptic Soy Broth (TSB) medium supplemented with 3% NaCl. Purity of the isolated colonies was confirmed through morphological and biochemical characterization. The strain was maintained long-term at -80°C in glycerol stocks. A single colony of *VP*AHPND was inoculated into 10 ml of TSB medium supplemented with 3% NaCl and incubated overnight at 30°C with agitation at 220 rpm. After 16 hours of incubation, bacterial growth was assessed by measuring the optical density (OD) at 600 nm using a spectrophotometer. The culture density was adjusted to a final concentration of 10^7 colony-forming units (CFU)/ml based on OD measurements and confirmed by viable plate counts. Sterile TSB medium served as negative controls to detect any contamination during culturing procedures. Positive controls, using known concentrations of *VP*AHPND, were included to validate inoculum density calculations. Additional validation methods, such as viable plate counts on selective agar plates and viability staining assays, were performed to corroborate turbidity measurements and assess bacterial viability.

CTM 27 & CTM 50 Herbal Material Selection, Extraction and Preparation

A total of 85 types of powdered herbal extracts were selected for this study. The powdered herbs, with a mesh size of 80, were obtained from Sun Ten Pharmaceutical Manufacturing (M) Sdn. Bhd. To ensure consistency, the powdered herbs were stored in a controlled environment at ambient room temperature (28°C) until further use. Each type of herbal extract was derived from various parts of the plant, including the whole plant, root bark, stem, leaf, root, flower, fruit, fruit skin, and seed, to capture a comprehensive range of bioactive compounds. To prevent degradation of bioactive compounds, the herbal stock solutions were prepared at a concentration of 100 mg/ml and stored in a chiller maintained at 4°C. This controlled storage temperature ensured the stability and integrity of the herbal extracts throughout the study duration. Additionally, the powdered herbs were

stored under ambient room temperature conditions to prevent moisture absorption and preserve their physicochemical properties.

Antibacterial activity of CTM 27 & CTM 50 against V. parahaemolyticus

Overnight cultures of *V. parahaemolyticus* were prepared by inoculating a single colony from glycerol stock into 5 ml of Luria broth supplemented with 3% NaCl. The cultures were then incubated on a shaking incubator at 30°C with 250 rpm for 18-24 hours to achieve optimal growth. To prepare the bacterial lawn, 500 µl of the overnight *V. parahaemolyticus* culture was spread evenly onto Tryptone Soy Agar (TSA) plates using a sterilized L-Shaped Cell Spreader. The plates were then air-dried in a laminar flow hood for 5 minutes with the lid partially opened to ensure thorough drying of the bacterial lawn. Eight paper discs, each with a diameter of 0.5 cm, were saturated with different concentrations of CTM 27 & CTM 50 herbal extracts. Additionally, one paper disc saturated with distilled water served as a negative control. These paper discs were then placed on the surface of the TSA plates inoculated with *V. parahaemolyticus* using sterilized forceps. Following the placement of paper discs, the TSA plates were sealed with parafilm and incubated in an inverted position at 30°C for 18-24 hours. After the incubation period, the microbial growth inhibition zones around each paper disc were measured using a calliper or ruler. The diameter of the clear zones devoid of bacterial growth was recorded in millimetres. The recorded inhibition zone measurements were analyzed to assess the antibacterial activity of CTM 27 & CTM 50 against *V. parahaemolyticus*. Statistical analysis, such as comparing the inhibition zone sizes between the herbal extracts and negative control, was performed to determine the significance of the observed antibacterial effects.

Reverse antagonistic mechanism of V. parahaemolyticus AHPND

The conventional disc diffusion method was employed with modifications to investigate the inhibitory effects of herbal extracts against *VP*AHPND antagonism. Overnight cultures of shrimp gut bacteria isolates and *VP*AHPND were prepared to achieve adjusted turbidity of 0.8 ± 0.1 at OD600 nm prior to experimentation. The inoculum was then used to prepare agar lawns of bacteria isolates using the pour-plate method. Agar plates, previously cultured overnight, were divided into eight segments to facilitate the inoculation of the assay. Live-cell cultures of *VP*AHPND were selected for the treatment assay as they exhibited visible outgrowth zones from the inoculated paper discs on agar lawns of bacteria isolates. Sterile filter paper discs (~6 mm) were impregnated with herb extract solutions prepared in a 96-well plate. The paper discs were then inoculated onto agar plates and incubated overnight in a controlled environment at 30 ± 1 °C. Subsequently, the inhibition zones (mm) formed around the paper discs were measured and recorded.

Statistical Analysis

A comprehensive statistical analysis was conducted using One Way Analysis of Variance (ANOVA) to assess the significance of differences between multiple groups. The results were expressed as mean ± standard deviation to provide a measure of central tendency and dispersion. The analysis was performed using IBM SPSS Statistics 20 software, a widely recognized tool for statistical analysis in research settings.

Result & Discussion

In this study, 85 common medicinal plants were screened to evaluate their potential for mitigating the pathogenicity of *Vibrio parahaemolyticus* (*V. parahaemolyticus*). Zone inhibition studies were conducted using aqueous extracts of the selected plants, with particular focus on two plants identified as CTM 27 and CTM 50. The results revealed that these two herbal extracts exhibited specific inhibition of *V. parahaemolyticus* growth, as evidenced by the formation of inhibition zones on agar plates.

Table 3.1 presents the inhibition zones observed for each herbal extract tested against *V. parahaemolyticus*. Notably, CTM 27 derived from *Glycyrrhiza uralensis Fisch* (Stem) demonstrated a significant inhibition zone of 30±2.52 mm, indicating potent antibacterial activity against *V. parahaemolyticus*. Similarly, CTM 50 obtained from *Ligustrum lucidum* (Fruit) exhibited an inhibition zone of 20±3.61 mm, further highlighting its efficacy in inhibiting *V. parahaemolyticus* growth.

Table 3.1. Inhibition zone of direct inhibition of 1-day overnight culture medium.

CTM	Herb name	Part	Inhibition Zone (mm)
27	*Glycyrrhiza uralensis* Fisch	Stem	30±2.52
50	*Ligustrum lucidum*	Fruit	20±3.61

In contrast, the remaining herbal samples tested did not exhibit significant antibacterial effects against *V. parahaemolyticus*, as evidenced by the absence of inhibition zones. This observation suggests that CTM 27 and CTM 50 possess unique antibacterial properties specifically targeting *V. parahaemolyticus*, distinguishing them from the other herbal extracts screened in this study.

Our results emphasize the potential of CTM 27 and CTM 50 as promising candidates for further investigation as natural antibacterial agents against *V. parahaemolyticus*. These findings contribute to the growing body of research aimed at exploring alternative therapeutic approaches for combating *V. parahaemolyticus*-associated infections, with implications for public health and disease management strategies. Further studies are warranted to elucidate the underlying mechanisms of action and evaluate the efficacy of CTM 27 and CTM 50 in vivo, paving the way for their potential application in clinical settings.

Figure 1 and 2 illustrate the inhibition zones observed for CTM 27 and CTM 50, derived from the stem of *Glycyrrhiza uralensis Fisch* and the fruit of *Ligustrum lucidum*, respectively, against *Vibrio parahaemolyticus* (*V. parahaemolyticus*). These findings underscore the antibacterial properties exhibited by both herbal extracts, suggesting their potential in combating *V. parahaemolyticus* bacterial strains. Notably, *Glycyrrhiza uralensis Fisch* holds significant historical and therapeutic value within Chinese traditional medicine, having been utilized for centuries in mainland China for diverse ailments such as metastasis, cancer, and tumorigenesis (Auyeung & Ko, 2017). Belonging to the Family *Fabaceae* (*Leguminosae*), *Glycyrrhiza uralensis Fisch* is widely distributed across regions including Russia, Europe, Asia, and Turkey. Conversely, CTM 50, derived from the fruit of *Ligustrum lucidum*, represents a native Chinese plant categorized under the family Oleaceae (Pang et al*.,* 2015).

Historically, this herb has been employed for the prevention of conditions such as diabetes, coronary heart disease, and osteoporosis (Chen et al., 2017).

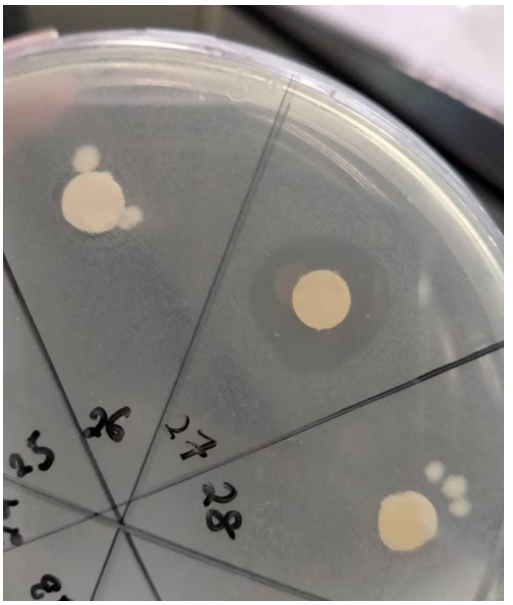

Figure 1. Inhibition zone of direct inhibition method using CTM 27 on *V. parahaemolyticus* lawn

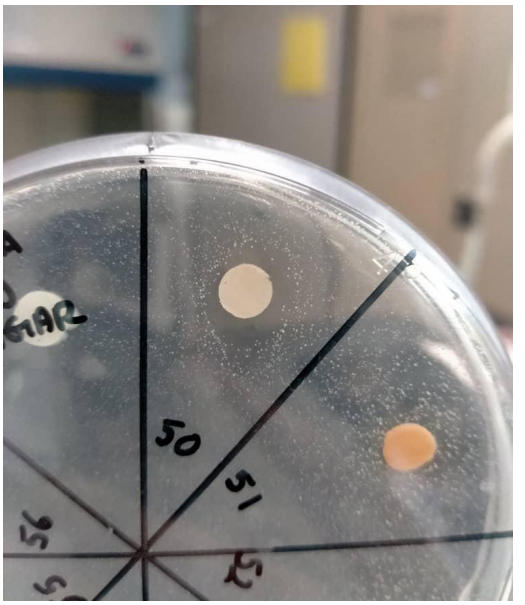

Figure 2. Inhibition zone of direct inhibition method using herb number 50 on *V. parahaemolyticus* lawn.

Recent research, notably by Ayeka et al. (2016), has highlighted the significant potential of *Glycyrrhiza uralensis Fisch* as an anticancer agent and immune booster, both *in vitro* and *in vivo* contexts. This herb has garnered substantial scientific interest over the years, with numerous studies investigating its chemical and biological properties. Notably, a chemical analysis conducted by He et al. (2006) identified over 100 phenolic compounds in *Glycyrrhiza uralensis Fisch*, a considerable portion of which are isoprenoid-substituted phenols. Among these compounds, isoflavones stand out, with oxygen substitutions typically occurring at the C-5 position. The herb also contains various flavonoids, chalcones, flavonones, arylcoumarins, and isoflavenes, many of which exhibit inhibitory effects against bacterial growth. Additionally, *Glycyrrhiza* species are rich in phenolic compounds like 3-aryl-coumarins, flavonoids, benzofurans, and coumestans (Fan et al., 2015), further contributing to its therapeutic potential.

Further studies have demonstrated the antitumor activity of *Glycyrrhiza uralensis Fisch*, particularly evident in its polysaccharides' direct inhibition of tumor cells such as CT-26 cells, even at low concentrations (Ayeka et al., 2016). Recent investigations have also unveiled the potent antibacterial activity of ethanolic extracts from *Glycyrrhiza uralensis Fisch* roots against *Streptococcus* mutans, reaffirming its medicinal importance as an antimicrobial and antioxidant agent (Visavadiya et al., 2009).

Similarly, attention has been directed towards the fruit of *Ligustrum lucidum*, known as "*Ligustri Lucidi Fructus*" in herbal medicine and "Lu-Zhen-Zi" in Chinese medicine. This fruit is

renowned for its tonic properties, particularly in revitalizing kidney and liver functions. It exhibits a range of biological activities, including antiviral, anti-inflammatory, immunomodulatory, antioxidant, anti-tumor, and hepatoprotective effects (Che & Wong, 2015). Notably, compounds such as ursolic acids and oleanolic acids found in Ligustrum lucidum play crucial roles in controlling inflammation, hyperlipidemia, hepatotoxicity, and pain, while phenolics, triterpenes, and secoiridoid glycosides further contribute to its pharmacological profile (Paula et al., 2019).

In light of these findings, it is evident that both *Glycyrrhiza uralensis Fisch* and *Ligustrum lucidum* fruit exhibit antibacterial effects against *V. parahaemolyticus*, suggesting the presence of phytochemicals with potential bactericidal properties. Notably, phenolic compounds, a major class of secondary metabolites in plants, particularly polyphenols and phenolics, are likely contributors to these observed antibacterial effects (Swallah et al., 2020).

Moreover, in addressing the antagonistic effects of *VP*AHPND on shrimp gut bacteria growth, the study explored the phyto-therapeutic potential of *Ziziphus jujuba* fruit (CTM 15) and *Chrysanthemum indicum* flower (CTM 44). By employing a revised disc-diffusion model integrating herbal extracts with *VP*AHPND inoculation, the study aimed to evaluate the inhibitory effects of these herbs on *VP*AHPND antagonism. Results indicated that both methanolic and aqueous extracts exerted significant inhibitory effects on *VP*AHPND growth, with specific herb treatments demonstrating pronounced effects on microbial outgrowth, particularly observed with *Chrysanthemum indicum* flower extracts (M44+*V. parahaemolyticus*). Notably, the herbs extracts exhibited specificity in attenuating *VP*AHPND activity without disrupting shrimp gut microbiota, highlighting their potential as targeted therapeutic agents against this pathogen. Subsequent characterization through minimum inhibitory concentration (MIC) determination further underscored the antimicrobial efficacy of these herbal extracts against *V. parahaemolyticus*AHPND, with promising implications for future antimicrobial therapy.

Conclusion

In this comprehensive study, we rigorously evaluated 85 different herbal extracts to identify those capable of inhibiting the antibacterial activity of *Vibrio parahaemolyticus* (*V. parahaemolyticus*), employing a screening technique designed to minimize disruptions to non-target microbiota. The primary aim was twofold: firstly, to identify herbs with the potential to counteract the antibacterial effects of *V. parahaemolyticus*, and secondly, to determine the Minimum Inhibitory Concentration (MIC) required for effective antibacterial activity. Recognizing the limitations and potential side effects associated with chemical approaches to disease control, particularly in aquaculture settings, our focus on herbal remedies aligns with the imperative to mitigate stress on aquatic ecosystems and minimize the risk of future infections, thus promoting biological and eco-friendly interventions.

In our direct inhibition tests, notable antibacterial effects were observed with CTM 27, stemming from *Glycyrrhiza uralensis* Fisch, and CTM 50, derived from the fruit of *Ligustrum lucidum*, as evidenced by the discernible inhibition zones observed on *V. parahaemolyticus* cultures. These findings suggest the presence of specific phytochemicals within these herbs that possess the capability to target and neutralize *V. parahaemolyticus* cells.

Furthermore, our investigation revealed significant antimicrobial activity in extracts obtained from various medicinal plant sources, including stems, fruits, or flowers. Particularly noteworthy was the apparent effectiveness of extracts from *Glycyrrhiza uralensis* Fisch and *Ligustrum lucidum* in inhibiting *V. parahaemolyticus*'s antibacterial effects, underscoring the potential of these herbal remedies in combating aquatic diseases. Previous research has highlighted the immunomodulatory and microbicidal properties associated with flavonoids and phenolic compounds present in fruits and plants, attributes which likely contribute to the observed antimicrobial effects. Although the precise antibacterial mechanisms of these herbal extracts against *V. parahaemolyticus* remain incompletely understood, our findings suggest a promising avenue for the discovery of novel antibacterial agents derived from plant sources for the treatment of AHPND.

In summary, the herbal extracts investigated in this study represent a promising reservoir of bioactive compounds with potential applications in aquaculture disease management. Further elucidation of their antibacterial mechanisms and exploration of their therapeutic potential could yield valuable insights and novel strategies for combating AHPND and other aquatic diseases, ultimately enhancing the sustainability and resilience of aquaculture systems.

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