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Effects of different diets on growth and survival of *Betta bellica* (Sauvage, 1884)

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Abstract: *Betta bellica* (Sauvage, 1884) is one of the ornamental fish species native to Malaysia that have potential to be commercialised. The aim of this study was therefore to investigate the effects of different diets on the growth and survival rate of *B. bellica*. Four different types of diets were investigated in the study: Micropellets (A), live *Moina* (B), mixed feed containing micropellets and live *Moina* in a 1:1 ratio (C) and freeze-dried *Moina* (D). The daily feeding corresponded to 10% of the body weight of the fish (w/w), with a weekly water change of 50%. After four-week of experimental period, the survival rate and percentage body weight gain of *B. bellica* were determined. The results of the study showed that *B. bellica* had a survival rate of 91.1%, 96.7%, 98.9% and 74.4% when fed micropellets, *live Moina*, mixed feed and *freeze*-dried *Moina*, respectively. Although the percentage survival of *B. bellica* fed freeze-dried *Moina* was lower compared to the other diets, there was no significant difference (P > 0.05) between all diets in terms of survival rate. The results showed that body weight gain of *B. bellica* fed with mixed diet (383.1%) was significantly higher (P < 0.05) than freeze-dried *Moina* (196.6%), but there was no significant difference (P > 0.05) when compared to *micropellets* (295.6%) and live *Moina* (341.9%). This result suggests that a mixed diet of micropellets and live *Moina* in a 1:1 ratio is promising for the growth of *B. bellica*. Further research into other potential diets is crucial as they would provide additional better options for feeding and rearing of *B. bellica*.

Keywords: Diet, *Betta bellica*, growth, survival

Abstrak: *Betta bellica* (Sauvage, 1884) merupakan salah satu spesies ikan asli di Malaysia yang berpotensi untuk dipasarkan dalam industri hiasan. Objektif kajian ini adalah untuk mengkaji kesan diet yang berbeza terhadap kadar pertumbuhan dan kemandirian *B. bellica*. Empat jenis diet berbeza telah dikaji dalam kajian ini: mikropelet (A), *Moina* hidup. (B), diet campuran mikropelet dan *Moina* hidup pada nisbah 1:1 (C), dan *Moina* kering beku (D). Pemakanan harian diberi pada kadar 10% (w/w) berat badan ikan, dan air ditukar pada kadar 50% setiap minggu. Selepas empat minggu tempoh kajian, kadar kemandirian dan peratus penambahan berat badan *B. bellica* ditentukan. Hasil kajian menunjukkan bahawa kadar kemandirian *B. bellica* apabila diberi diet mikropellet, *Moina* hidup, makanan campuran dan *Moina* kering beku masing-masing ialah 91.1%, 96.7%, 98.9% dan 74.4%. Walaupun peratus kemandirian *B. bellica* diberi makan *Moina* kering beku adalah lebih rendah berbanding *B. bellica* diberi diet lain, tetapi tidak terdapat perbezaan yang signifikan (P > 0.05) antara semua diet dari segi kemandirian. Keputusan menunjukkan bahawa peratusan penambahan berat badan *B. bellica* yang diberi diet campuran (383.1%) adalah signifikan lebih tinggi (P < 0.05) berbanding *Moina* kering beku (196.6%), tetapi tiada perbezaan yang signifikan (P > 0.05) berbanding mikropelet (295.6%) dan *Moina* hidup (341.9%). Keputusan ini menunjukkan bahawa diet campuran mikropelet dan *Moina* hidup pada nisbah 1:1 berkesan untuk meningkatkan pertumbuhan *B. bellica*. Penyelidikan lanjut terhadap diet-diet lain yang berpotensi adalah penting bagi memberi pilihan tambahan untuk ternakan *B. bellica*.

Introduction

Betta bellica (Sauvage, 1884), commonly known as the slender betta, is a freshwater species belonging to the Betta genus in the family Osphronemidae. The name *bellica* is derived from the Latin word *bellicus*, meaning warlike, and is due to the green coloration of this species. *B. bellica* is also locally known as "Betta Manja", as its natural, friendly behaviour makes it ideal for keeping as an ornamental fish (Zaini, 2019). In the ornamental fish trade, adult *B. bellica* are sold for around RM 100 per pair.

B. bellica (Figure 1) is widely distributed throughout Peninsular Malaysia (including Terengganu, Pahang, Johor, Perak, Selangor), Indonesia and Thailand (Tan & Ng, 1996; Tan & Ng, 2005a; Tan & Ng, 2005b; Panijpan et al., 2014; Sule et al., 2016; Panijpan et al., 2020). *B. bellica* was first discovered in a swamp forest in Perak, typically found in blackwater streams or peat swamp forests with acidic pH values between 4.0 and 6.0 (Ng et al., 1992; Tan & Ng, 2005a). This species is classified as least concern in the IUCN Red List (IUCN, 2022). However, this species is still threatened by habitat destruction due to land clearing for development. Oil palm or rubber plantations and agricultural activities have expanded in recent decades, destroying about two-thirds of the peat swamp forests in Peninsular Malaysia, Sumatra and Borneo (Stibig et al., 2014; Lampela et al., 2017; Cooper et al., 2020).

B. bellica is the largest fish in the Betta bubble nester group and can grow up to 10 cm long (Zaini, 2019). This species is carnivorous (Monvises et al., 2009). According to a study by Chung et al. (1994), numerous odonate nymphs were found in the guts of wild specimens of *B. bellica*, suggesting that this species eats insects and their larvae at the water surface. Although *B. bellica* is a potential ornamental fish for keeping in community aquaria, little is known about the most suitable diet for rearing *B. bellica* juveniles in captivity. Live foods such as *Moina* sp. and *Artemia* were commonly used for rearing Betta species. *Moina macrocopa* is one of the common live feeds for freshwater species (Suhaimi et al., 2022). Today, commercial pellets are preferred for rearing modern Betta fish. Nevertheless, no studies have been conducted to determine if commercial pellets are suitable for rearing wild Betta, such as *B. bellica*. Knowledge of the nutritional requirements of *B. bellica* is crucial for successful captive rearing. Therefore, the aim of this study was to determine the most suitable diet for the growth and survival of *B. bellica* juveniles. This study may provide information on suitable diets for *B. bellica* juveniles and contribute to the development of better management plans for rearing of this species.

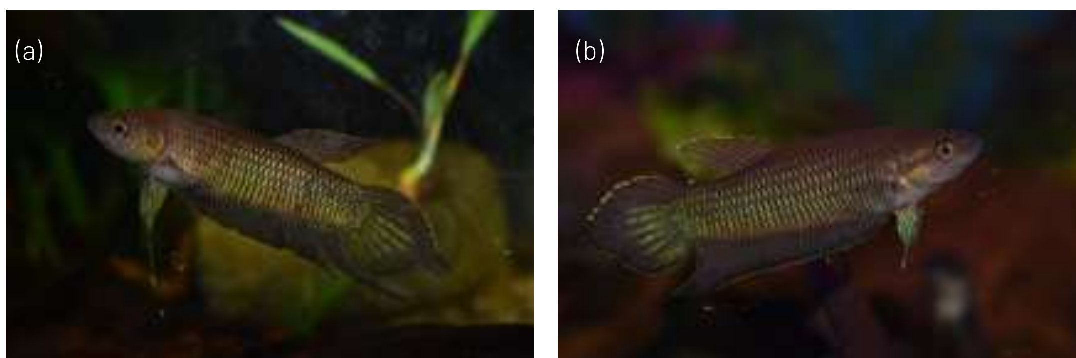

Figure 1. Adult (a) male and (b) female *Betta bellica*

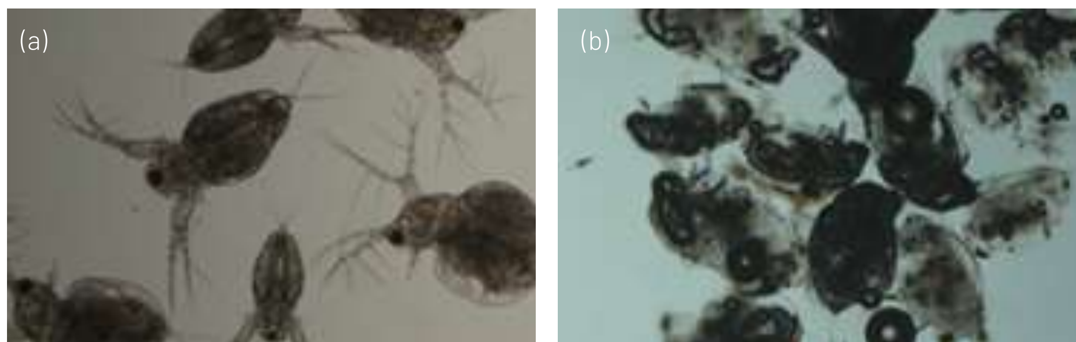

Figure 2. Microscopic view of (a) live *Moina* and (b) freeze-dried *Moina* at 40x magnification.

Materials and methods

This study was conducted for four weeks in the Ornamental Fish Unit, Fisheries Research Institute Glami Lemi (FRIGL), Jelebu, Negeri Sembilan, Malaysia, using five-week-old juveniles of *B. bellica* (F1) produced from domesticated wild stock. One week prior the experiment, the black peat granules (Sera North America, Inc.) were soaked in 30:70 dechlorinated tap water to create amber water that was slightly acidic (pH 5.5–6.5) and ideal for *B. bellica*.

Dietary preparations

Four different diets were used in this study, consisting of micropellets (A), live *Moina* (B), a mixture of micropellets and live *Moina* at 1:1 ratio (C) and freeze-dried *Moina* (D) (Figure 3). The commercially available Hikari® Tropical Micropellets (Kyorin Food Industries, Ltd, Japan) were used as control feed. Live *Moina* and freeze-dried *Moina* were obtained from the Fish Nutrition Unit, FRIGL. Live *Moina* were harvested daily. Freeze-dried *Moina* were prepared by freeze-drying for 24 h and stored at 3 - 5°C. Prior to the feeding trial, *B. bellica* juveniles were fed commercial micropellets as a control diet for one week to acclimatize the fish.

Figure 3. Different feeds used in this study. (a) micropellet, (b) live *Moina*, (c) freeze-dried *Moina*.

Feeding trials

A total of 360 *B. bellica* juveniles with an initial body weight between 0.040 g and 0.085 g were used for the study. Fish were randomly selected, weighed and divided into twelve groups of 30 fish each, which were then placed in the experimental tanks measuring 37 cm (L) x 20 cm (W) x 27 cm (H) and filled with 10 L of water. The experimental tanks were separated into four groups (1, 2, 3, 4) and were fed diets A, B, C, and D at 10% of body weight twice daily for four weeks. Group 1 were fed with diet A, group 2 with diet B, group 3 with diet C and group 4 with diet D. In the mixed

diet of micropellets and live *Moina* (treatment C), each diet was fed at 5% of body weight daily. The experiment was performed in triplicates and conducted at an ambient temperature of 25-28°C throughout the experimental period. The total weight of the fish in each experimental tank was measured weekly. The proportion of food for each treatment was adjusted weekly based on the data from the fish weight samples. A 50% water change was carried out each week. Water quality was tested for pH, ammonia and nitrite before initiation of the experiment and at the end of the experiment.

Data Collection and Statistical analysis

At the end of the experiment, the total number of surviving fish from each tank was counted, and all surviving fish from each tank were weighed in bulk. Growth performances were evaluated by total and percentage of weight gain and specific growth rate (SGR). The growth performance and survival rate of *B. bellica* were calculated using the following formula:

Total weight gain (g) = final body weight, W1 (g) - initial body weight, W0 (g)

$$\text{Percent weight gain (\%)} = \frac{\textit{Total body weight gain (g)}}{\textit{Initial body weight (g)}} \times 100$$

$$\text{Specific growth rate (SGR)(\%/day)} = \frac{\textit{Ln (Final body weight)} - \textit{Ln (Initial body weight)}}{\textit{Number of days}} \times 100$$

$$\text{Survival rate} = \frac{\textit{(Final number of fish - Initial number of fish)}}{\textit{(Initial number of fish)}} \times 100.$$

Three water quality parameters, namely ammonia (NH3), nitrite (NO2) and pH were determined and expressed in mean ± SD. All statistical analyses were performed with statistic version 8.0. One-way analysis of variance (ANOVA) was used to investigate whether there were significant differences in the parameters of growth performance, survival rate and water quality between the four treatments. Tukey's pairwise comparison test was performed to determine the significant differences between the means of the treatment groups. The difference with a P-value of less than 0.05 ($P < 0.05$) was considered significant.

Results and Discussion

Growth performance

The combination treatment of micropellets and live *Moina* at 1:1 ratio (treatment C) gave the highest percentage of body weight gain (383.1%), followed by live *Moina* (treatment B) with 341.9%, micropellets (treatment A) with 295.6% and freeze-dried *Moina* (treatment D) with 196.6% (Table 1). The total body weight gain of fish fed live *Moina* was significantly higher ($P < 0.05$) than that of fish fed freeze-dried *Moina*. There were no significant differences ($P > 0.05$) in the body weight gain between the micropellets (A), live *Moina* (B) and mixed diet of micropellets and live *Moina* (C) (Figure 4). In addition, there were also no significant differences ($P > 0.05$) in the percentage body weight gain between the micropellet (A), live *Moina* (B) and freeze-dried *Moina* (D) treatments (Figure 4).

Table 1. Growth performance and survival of *B. bellica* juveniles fed different diets for four weeks.

Treatments	Average weight (g)		Total weight gain (g)	Percent weight gain (%)	SGR (%)	Survival rate (%)
	Initial	Final				
A	0.06 ± 0.02	0.23 ± 0.04	0.17 ± 0.02	295.6 ± 70.2[ab]	5.1 ± 0.6[ab]	91.1 ± 1.9[a]
B	0.05 ± 0.01	0.23 ± 0.01	0.18 ± 0.01	341.9 ± 69.9[ab]	5.5 ± 0.6[ab]	96.7 ± 3.3[a]
C	0.05 ± 0.01	0.26 ± 0.03	0.20 ± 0.02	383.1 ± 42.2[a]	5.8 ± 0.3[a]	98.9 ± 1.9[a]
D	0.05 ± 0.01	0.15 ± 0.00	0.10 ± 0.01	196.6 ± 70.5[b]	4.0 ± 0.8[b]	74.4 ± 20.4[a]

Values are given as means of three replicate treatment groups ± SD. Values in the same column with different superscripts are significantly different (Tukey's test, $P < 0.05$). SGR - Specific growth rate. A: micropellets, B: live *Moina*, C: mixture of micropellets and *Moina* at 1:1 ratio, D: freeze-dried *Moina*.

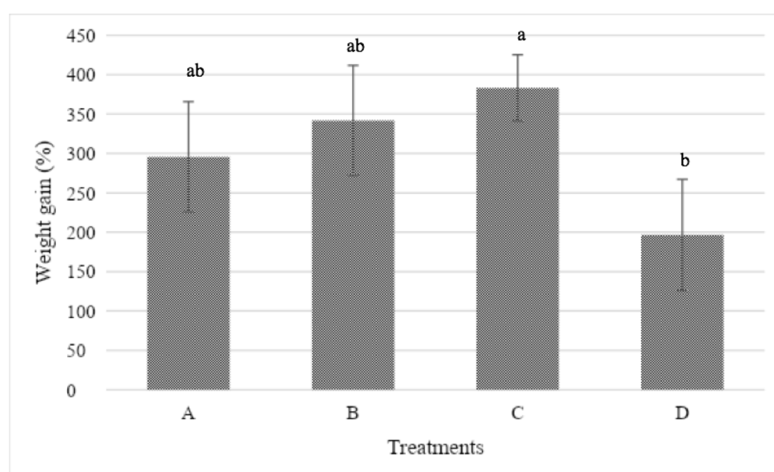

Figure 4. Average percentage body weight gain of *B. bellica* fed different diets for four weeks. The bars represent the standard deviation. Groups with different superscript letters were significantly different (Tukey's HSD test for pairwise comparison, $P < 0.05$). A: micropellets, B: live *Moina*, C: mixture of micropellets and *Moina* at 1:1 ratio, D: freeze-dried *Moina*.

In this study, growth was highest in the mixed diet group (C), suggesting that a combination of micropellets and live *Moina* in a 1:1 ratio is most suitable for rearing *B. bellica* juveniles. Compared to micropellets, which contained 43.0% crude protein and 7.0% crude fat, *Moina* had a higher crude protein and crude fat content of 62.8% and 18.3%, respectively (Hanan et al., 2020). In contrast, the ash composition was higher in micropellets (17.0%) than in *Moina* (10.2%). In this study, the proximate composition value of live *Moina*, freeze-dried *Moina* and micropellet is measured based on dry basis. Consequently, the mixed diet treatment was the ideal treatment that provided high protein, fat and ash content to *B. bellica* juveniles. The amount of ash corresponds to the mineral content of the feed, which often contains elements such as calcium, phosphorus, potassium and magnesium (Chapman & Miles, 2020). As the growth performance of *B. bellica* juveniles fed micropellets (A) and live *Moina* (B) was not significantly different ($P > 0.05$) from that of the mixed diet group (C), this suggests that these diets were similarly acceptable to *B. bellica* juveniles. Commercially available micropellets contain all essential nutrients for rearing *B. bellica*. Therefore, in the absence of live *Moina*, micropellets offer a practical alternative as a nutritious food and can be used in place of live *Moina*.

There was no significant difference in the growth performance and survival rate of the micropellet control treatment compared to the other treatments of live *Moina*, mixed diet and freeze-dried *Moina*. This result is consistent with a study on *Heterobranchus longifilis*, which found that the growth rate of larvae fed commercial trout diet was not significantly different from that of live *Moina* (Kerdchuen & Legendre, 1994). Similar studies also show that there was no significant difference in the percentage weight gain of fry fed live *Moina* and a mixture of live and artificial feed (Ovie & Ovie, 2002; Cheikyula & Ofojekwu, 2003). In another study, fish larvae fed on microparticulate feed showed poorer growth and survival rates, which could be attributed to the low nutrient content of the feed, a poorly developed digestive system or the low ingestion rate of the larvae (Holt, 2011).

Moina is one of the most common live feeds for freshwater fish (Ingram, 2009; Poynton et al., 2013; Ramesh et al. 2014). In this study, juvenile *B. bellica* fed with live *Moina* showed higher growth compared to control treatment A and treatment D due to the high protein content. Live *Moina* may also be preferrable because its jerky, whimsical movements stimulated the predatory response of the fish (Mayer & Wahl, 1997; Gogoi et al., 2016). In addition, *Moina* contains digestive enzymes such as proteinases, lipases, amylases and peptidases, which act as exoenzymes in the stomach of fish larvae (Miah et al., 2013). Studies have shown that fish larvae fed with live feed grew and survived better than those fed only with formulated feed (Sales & Janssens, 2003; Finn & Kapoor, 2008; Uribe et al., 2018). Due to the nutritional variability of *Moina* with an average crude protein content of 50%, it is therefore important to enrich it with nutrient-rich feed before using it as fish feed (Rottmann et al., 2018; Nugroho et al., 2021; Suhaimi et al., 2022).

The study's results demonstrated that total body weight gain was highest in treatment C (combination of micropellets and live *Moina*), followed by treatments A (micropellets), B (live *Moina*), and D (freeze-dried *Moina*). Eventhough live *Moina* has a higher protein content than micropellets, *B. bellica* juveniles fed micropellets has a higher body weight gain than those fed live *Moina*. Protein content and utilization are significant factors influencing fish growth rates. Studies involving *B. splendens* and juvenile red snapper (*Lutjanus argentimaculatus*) have shown that protein is more efficiently utilized in formulated feed compared to live feed (Abbas et al., 2005; Mandal, 2010). Additionally, the quality of the feed, including its composition, also plays a crucial role. High protein efficiency in feed, combined with optimal levels of protein, fat, and ash, promotes fish growth (Shearer et al., 1992; Abbas et al., 2005; Yong et al., 2015).

As shown in the present study, a combination of micropellets and live *Moina* in a 1:1 ratio showed the best growth performance among the four treatments tested. This result is also consistent with several studies that have shown that feeding fish larvae with a combination of live and formula feed in their early stages can lead to better growth and survival (James & Sampath, 2004; Akbary et al., 2010). Studies have shown that fish larvae fed only formulated feed had poorer growth and lower survival rates than those fed only live food (Sales & Janssens, 2003; Finn & Kapoor, 2008; Uribe et al., 2018). This was also reported in the study by Akbary et al. (2010), in which it was observed that larvae of rainbow trout (*Oncorhynchus mykiss*) fed a combination of live and artificial feed grew faster than fish fed only *Artemia* or commercial feeds. Meanwhile, a mixed diet of live *Artemia*, earthworms, beef liver and pellet feed were found to result in high food intake and good growth performance in green swordtails (*Xiphophorus helleri*) (James & Sampath, 2004).

In this study, the growth of *B. bellica* was significantly ($P < 0.05$) lower when fed with treatment D (freeze-dried *Moina*) than with treatment C (mixed diet of micropellets and live *Moina* in a 1:1 ratio). This could be due to nutrient losses and lower digestibility of freeze-dried

Moina compared to live *Moina*. Live *Moina* retain their nutritional value better than freeze-dried *Moina*, as essential nutrients are preserved in the living state. Studies have shown that freeze-drying does not significantly reduce the nutritional value of *Moina* or other zooplankton, including proteases and other important enzymes. However, when freeze-dried zooplankton is placed in water for ten minutes, enzyme activity and most amino acids are lost (Grabner, 1981; Rottman et al., 2018). Several studies have shown that live plankton is important for rearing of certain fish larvae, as they rely on the digestive enzymes of the plankton and require an exogenous enzyme to increase the digestive kinetics of the fish (Dabrowski & Glogowski, 1977; Kolkovski, 2001; Maas et al., 2021). A study on juveniles of another carnivorous Betta species, *B. splendens*, has shown that they can only digest a few carbohydrates due to their short gut and lack of carbohydrate-digesting enzymes (Thongprajukaew et al., 2011). This also applies to the *B. bellica* in the present study, which is a carnivorous species. Therefore, feeding live food may increase the digestive activity and thus the ability of juvenile fish to digest formulated food (Kolkovski et al., 1997; Rønnestad et al., 2013). Apart from that, the palatability of freeze-dried *Moina* is less enticing for a carnivorous fish like *B. bellica* than live *Moina*. For various fish, live food is actually more preferable than artificial feeds (Das et al., 2007).

Survivability

There were no significant differences ($P > 0.05$) in survival rates between all treatment groups (Table 1). Nevertheless, the survival rate was highest for mixed diet (treatment C) at 98.9%, while the freeze-dried *Moina* (treatment D) had the lowest survival rate at 74.4%. At the beginning of this experiment the pH, Total ammonia (NH_3-N) and nitrite (NO_2) values were 6.50 ± 0.00, 2.74 ± 0.01 ppm and 0.00 ± 0.00 ppm respectively.

At the end of the experiment, 25.6% of the *B. bellica* juveniles in the freeze-dried *Moina*-fed treatment died. Therefore, water quality was tested to verify the cause of the mortality. The pH values of the water in the fish tanks with treatments A, B, C and D were 6.12 ± 0.06, 5.92 ± 0.14, 6.21 ± 0.05 and 5.87 ± 0.20, respectively. The pH values in this experiment were within an acceptable range of 4.0 – 7.0 for all treatment groups. In nature, *Betta* sp. can tolerate relatively low pH values. A study on the species composition of peat swamp environments found that *B. bellica* occurs in extremely acidic water with a pH of 3 (Ahmad & Samat, 2015). The concentrations of Total ammonia (NH_3-N) in treatments A, B, C and D were measured as 2.90 ± 0.31 ppm, 3.41 ± 0.77 ppm, 3.20 ± 0.59 ppm and 3.97 ± 1.01 ppm, respectively. The nitrite (NO_2) content for A, B, C and D was 0.05 ± 0.01, 0.05 ± 0.01, 0.05 ± 0.00 and 0.52 ± 0.81, respectively. The freeze-dried *Moina* treatment showed very poor water quality for total ammonia and nitrite levels on day 28 compared to the other treatments. The mass mortality of *B. bellica* juveniles in the freeze-dried *Moina*-fed treatment was likely due to nitrite toxicity from elevated nitrite levels. Observations revealed that feeding with freeze-dried *Moina* caused the water to turn cloudy, potentially affecting water quality. Therefore, freeze-dried *Moina* was not suitable for *B. bellica* juveniles as it is not only inhibiting the growth but also impaired the water quality, contributing to fish mortality. Currently, there is limited information in the scientific literature on the toxicity limits of ammonia and nitrite for bettas. However, even low levels of these compounds can be harmful to ornamental fish (Pleeging & Moons, 2017).

Freeze-dried *Moina* and micropellets offer useful advantages in feed management in terms of handling, storage and feeding convenience. Their extended shelf life and ease of storage make them a convenient feed option for aquaculture operations, as they eliminate the need for continuous culture maintenance required for live *Moina* (Rottman et al., 2018, Devi et al., 2019). However, the cost of maintaining live feed cultures is low compared to artificial feeds. Live *Moina* provides a cost-effective option. However, during the rainy season when live *Moina*

is limited, micropellets serve as an alternative. In this study, micropellet, live *Moina* and mixed diet were better treatments for water quality management as it showed better water quality in terms of total ammonia and nitrite levels compared to freeze dried *Moina*. Apart from this, live feed obtained from polluted environmental sources such as sewage treatment plants that are exposed to pathogenic bacteria increases the risk of disease transmission to the farmed fish (Czeczuga et al., 2008; Hanan et al., 2020; Joshua et al., 2022). Cultivation of hygienic *Moina* in semi-open and open outdoor system offers an advantage to reduce risk of disease transmission caused by dirty and polluted water (Amatul-Samahah et al., 2023).

Conclusion

The mixed feeding of live *Moina* and micropellets in a 1:1 ratio was optimal for the growth and survival of *B. bellica* juveniles. Although the combination of feeding live *Moina* and micropellets to *B. bellica* juveniles showed the highest growth and survival rate, either live *Moina* or micropellets also gave good results and can be used as diet for Betta. Given the importance of coloration in ornamental fish, future studies may look into the influence of *Moina* on the color enhancement of *B. bellica*.

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Antibacterial activity of selected plant extracts against fish pathogens

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Abstract: Medicinal plants are increasingly preferred in aquaculture as alternative sources for disease control due to their diverse bioactive compounds, including tannins, alkaloids, terpenoids, saponins, phenolics, steroids, and flavonoids. These compounds exhibit varying biochemical properties and solubility profiles across different solvents. Hence, this study aims to assess the antibacterial activity of six types of plants in four distinct solvents against fish pathogens. Six leaf extracts were included in this study: senduduk (*Melastoma malabathricum*), semambu (*Azadirachta indica*), mahkota dewa (*Phaleria macrocarpa*), munggai (*Moringa oleifera*), mengkudu (*Morinda citrifolia*), and gelenggang (*Senna alata*), underwent antimicrobial testing against common fish pathogenic bacteria, including Gram-positive (*Streptococcus agalactiae*) and Gram-negative species (*Vibrio alginolyticus*, *Vibrio vulnificus*, *Aeromonas hydrophila*, and *Edwardsiella tarda*). Antimicrobial activity was evaluated via the disc diffusion method, measuring the size of Zone of Inhibition (ZOI). The result indicated that most of the extracts using methanol and ethanol as solvent exhibited significant antimicrobial properties compared to n-hexane and ethyl acetate. The highest potential was observed in the extract of *Senna alata* against *E. tarda* (PCA17) with ZOI of 19 mm, surpassing the effectiveness of the commercial antibiotic oxytetracycline with ZOI of 10 mm. The experiment proved the effectiveness of some selected plant extracts as natural antimicrobials and suggested the possibility of using them in medications to treat infections caused by the test bacteria.

Keywords: Antibacterial activity, extract, solvents, methanol.

Abstrak: Tumbuhan semakin menjadi pilihan utama dalam bidang akuakultur sebagai sumber alternatif bagi mengawal penyakit kerana kepelbagaian sebatian bioaktifnya seperti tanin, alkaloid, terpenoid, saponin, fenolik, steroid, dan flavonoid. Sebatian-sebatian ini mempunyai sifat bio-kimia yang berbeza dan profil keterlarutan yang berbeza dalam pelarut yang berbeza. Oleh itu, kajian ini bertujuan untuk menilai aktiviti antibakteria enam jenis tumbuhan menggunakan empat pelarut berbeza terhadap patogen ikan. Enam ekstrak daun iaitu senduduk (*Melastoma malabathricum*), semambu (*Azadirachta indica*), mahkota dewa (*Phaleria macrocarpa*), munggai (*Moringa oleifera*), mengkudu (*Morinda citrifolia*), dan gelenggang (*Senna alata*), telah diuji untuk aktiviti antimikrob terhadap bakteria patogenik umum ikan, termasuk bakteria Gram-positif (*Streptococcus agalactiae*) dan spesies Gram-negatif (*Vibrio alginolyticus*, *Vibrio vulnificus*, *Aeromonas hydrophila*, dan *Edwardsiella tarda*). Aktiviti antimikrob dinilai melalui kaedah difusi agar, dengan mengukur saiz Zon Perencatan (ZOI). Keputusan menunjukkan bahawa kebanyakan ekstrak menggunakan metanol dan etanol sebagai pelarut menunjukkan sifat antimikrob yang signifikan berbanding dengan n-heksana dan etil asetat. Potensi tertinggi diperhatikan dalam ekstrak *Senna alata* terhadap *E. tarda* (PCA17) dengan ZOI sebanyak 19 mm, melebihi keberkesanan antibiotik komersial oxytetracycline

iaitu ZOI sebanyak 10 mm. Eksperimen membuktikan keberkesanan sesetengah ekstrak tumbuhan yang dipilih sebagai antimikrob semula jadi dan mencadangkan kemungkinan untuk menggunakannya dalam ubat-ubatan untuk merawat jangkitan yang disebabkan oleh bakteria tersebut.

Introduction

In the specific context of the aquaculture sector—the farming of fish and other aquatic organisms—disease prevention and control have emerged as significant challenges. Various pathogens, including those responsible for infectious diseases such as vibriosis, aeromoniasis, streptococcosis, and edwardsiellosis, pose substantial threats to aquatic organisms. These pathogens can proliferate rapidly within aquaculture systems, leading to detrimental effects on fish and other aquatic species (Hanson, Hemstreet, & Hawke, 2019; Amaro et al., 2020; Maulu et al., 2021 ; Loch et al., 2017). Consequently, effective strategies for disease management are essential to mitigate the impact of these pathogens on aquaculture production.

In addressing the challenges of disease management in aquaculture, research has increasingly focused on the antibacterial efficacy of medicinal plant extracts against bacteria affecting fish. As a result, the current research development demonstrates the growing interest of medicinal plants as effective treatments in the challenging matter of disease management in aquatic organisms (Zhu, 2020; Mariappan, Kaliyamurthi, & Binesh, 2023). There are many medicinal plants studied for their purpose of treatment and prevention against fish pathogens. However, comparing the results of published articles on the antimicrobial effect of these plants was often difficult due to the utilization of non-standard methodology in plant extraction techniques, solvent used, plant origin, bacterial strain and endpoint identification (Nawaz et al., 2020; Veranita et al., 2020). For example, methanol leaf extract senduduk (*Melastoma malabathricum*) has been reported to exhibit antibacterial activity against *Escherichia E. coli*, commonly bacteria encountered in aquaculture environments (Das et al., 2021). Then, semambu (*Azadirachta indica*), commonly known as neem, displays that methanol extract has the strongest growth inhibitory effect on both standard and clinical isolated strains of *Pseudomonas aeruginosa* (Maleki et al., 2017).

Then, extract from mahkota dewa (*Phaleria macrocarpa*) using ultrasonic bath extraction has been recognized for its antimicrobial activity against motile aeromonas septicemia (MAS) disease caused by the bacterium *A. hydrophila* (Sarendah & Kusdarwati, 2020). Ethanolic extracts from munggai (*Moringa oleifera*) have shown antibacterial effects against fish pathogens, with highest inhibition diameter against *E. tarda* was 12.95 mm at a concentration of 375 mg/L after 24 hours (Riyadi et al., 2021). mengkudu (*Morinda citrifolia*) macerated extract has been investigated for its antimicrobial properties, with extracts displaying inhibitory effects against fish pathogens such as *A. hydrophila* in koi fish (*Cyprinus carpio*) (Mulyani, 2022). In addition, Soxhlet-ethanol gelenggang extract has demonstrated antibacterial activity against various aquatic pathogens (*Staphylococcus aureus*, *Escherichia E. coli* and *Pseudomonas aeruginosa*), suggesting its potential application in aquaculture settings (Ekpiken, Nfongeh, & Bassey,2021).

Hence, the aim of this study was to assess the antimicrobial efficacy of six different plants against five bacterial strains commonly encountered in aquaculture environments, known for their pathogenicity. This targeted approach allows us to evaluate the potential of the plant extracts in combating these prevalent bacterial pathogens in aquaculture, contributing

to the development of effective disease management strategies in the industry.

Material and Methods

Plant sample

The plant materials selected for this study consist of six different plants: senduduk (*Melastoma malabathricum*), semambu (*Azadirachta indica*), mahkota dewa (*Phaleria macrocarpa*), munggai (*Moringa oleifera*), mengkudu (*Morinda citrifolia*), and gelenggang (*Senna alata*). These plants were procured from the supplier Secret Barn Sdn. Bhd, in pharmaceutical-grade powdered form. The collection of these plants, specifically matured leaves, is located at Kedah and Perak, Malaysia. The leaves were dried under controlled conditions at temperatures below 35°C in a dark environment. Following the drying process, the leaves were finely ground into powder before being packaged and delivered.

Bacterial culture

A total five fish pathogenic bacterial strains were used in this study: Gram-positive (*Streptococcus agalactiae SA2*) and *Gram-negative* (*V. alginolyticus STS1, V. vunificus KBM1, A. hydrophila PS1*, and *E. tarda PCA17*). These strains were obtained from the bacterial bank at National Fish Health Research Centre (NaFisH) located in Batu Maung, Pulau Pinang, Malaysia which already confirmed through growth on appropriate agar media, followed by a comprehensive assessment involving morphological, physiological, standard biochemical analyses and molecular identification (specific Polymerase Chain Reaction, PCR).

Preparation of plant extracts

The maceration extraction procedure was outlined by Othman et al. (2018) with slight modification. The sample of leaves powder was divided into four portions: A, B, C, and D (Table 1). The ratio of leaf powder to solvent for each portion was maintained at 1:10 (g/mL) to ensure efficient extraction. Portion A, consisting of 50 g of leaf powder, was immersed in 500 mL of absolute methanol (99.5%; HmbG® Chemicals, Germany), while B, C and D underwent a similar process with 500 mL of ethanol (95%; HmbG® Chemicals, Germany), ethyl acetate (99.5%; HmbG® Chemicals, Germany) and n-hexane (98%; Bendosen) All mixtures were immersed for 72 hours with shaking at 150 rpm (Cole-Parmer Orbital Shaker). After 72 hours, each mixture underwent filtration and evaporation (LabTech EV311 Plus Rotary Evaporator) to yield the crude extract.

Portion	Solvent	Chemical Structure	Polarity	Ratio (g/mL)
A	Methanol (99.5% Purity)	CH_3OH	Most Polar	1:10
B	Ethanol (95% Purity)	CH_3CH_2OH	Polar	1:10
C	Ethyl acetate (98 % Purity)	$CH_3COOCH_2CH_3$	Intermediate polarity	1:10
D	n-Hexane (99.5% Purity)	$CH_3(CH_2)_4CH_3$	Nonpolar	1:10

Table 1. Solvents used for plant extraction.

Preparation of stock solution

All the plant crude extracts were initially prepared by creating a standard stock solution. Following the removal of the solvent from the concentrate, 95% ethanol is chosen as the diluent to facilitate standardization and comparability across different extracts and

experimental conditions, ensuring uniformity in extract preparation, dilution protocols, and assay procedures. The selection of ethanol as the diluent for the plant extract concentrate aligns with considerations of solubility, safety, stability, and practicality, thereby optimizing the formulation process for subsequent analysis and application (González et al., 2018). The dilution was conducted using a concentrate-to-liquid ratio of 1:10 (w/v), as established by Othman et al. (2018), yielding a final concentration of 100 mg/ml.

In vitro antibacterial activity test

Antimicrobial susceptibility test was performed for all bacterial strains by modified Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guideline. Sterile Antibiotic Assay Discs (Whatman®) with 6 mm diameter were used with 20 μL of prepared plant extracts (100 mg/ml) pipetted onto the disc with 10 μL on each side. The discs were allowed to dry for 15 mins before use.

The bacterial cultures were adjusted to 0.5 McFarland's turbidity standard and inoculated onto Tryptone Soya Agar, Oxoid™ (dehydrated) (*A. hydrophila, S. agalactiae, E. tarda*) and Tryptic Soy Agar containing 1.5% sodium chloride (*V. alginolyticus, V. vulnificus*) to mimic the salinity conditions suitable for the growth. To assess the inhibitory effects of the plant extracts, the plant extract-impregnated discs were placed on the agar. Oxytetracycline (30 μg) acted as positive control while an ethanol-impregnated disc was used as a negative control (Fig. 1). This control will ensure that any observed antimicrobial effects are attributed to the bioactive compounds in the plant extracts, not the solvent itself.

The agar plates were subsequently incubated for 24 hours at 28°C - 30°C. Following incubation, the results for the zone of inhibition (ZOI) were measured and recorded to indicate the antibacterial activity. The zone of inhibition was determined by measuring the diameter of the clear zone surrounding the discs to the nearest millimeter, including the 6 mm diameter of the antibiotic disc, using a ruler. The experiments were conducted in triplicate.

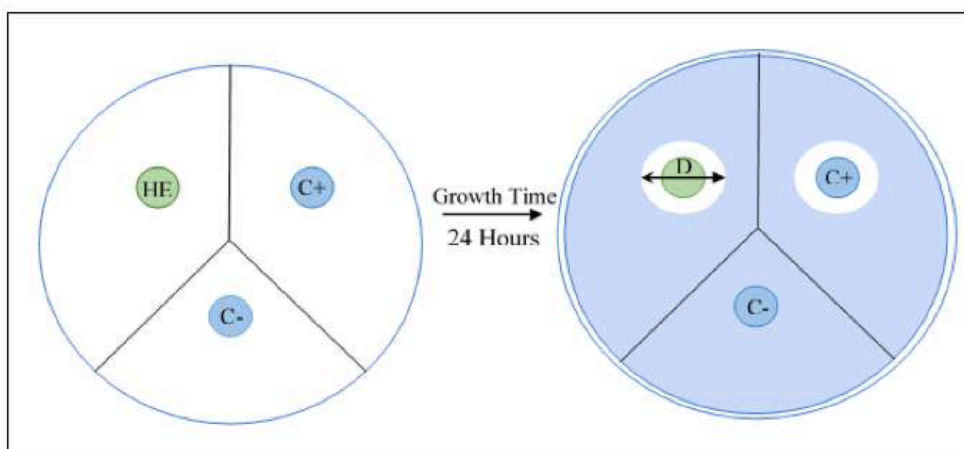

Figure 1. Agar-Disc Diffusion Method. HE = Herbal extract, C+ = Positive Control, C- = Negative Control, D = Diameter Zone of Inhibition.

Results and Discussion

In this study, the findings show the important role of solvent selection in extraction processes conducted under the same extraction method. It reveals that the use of different

solvents influences the difference of antibacterial effects observed. This variability is attributed to variations in the polarity of solvents, resulting in significant fluctuations in the concentration of bioactive compounds within the extracts. This finding is supported by an earlier study which found that the optimal solvent for extraction depends on the specific plant materials and the particular chemicals targeted for separation. This is because plant materials consist of a range of bioactive compounds with differing solubility properties in various solvents (Farahmandfar et al., 2019, Nawaz et al., 2020).

In Fig. 2, the methanolic extracts from senduduk, gelenggang, Mahkota dewa, and semambu leaves exhibited the most significant inhibition zones compared to extracts from munggai and mengkudu. The graph indicates that the methanol extracts of senduduk and gelenggang showed the most significant effect against all five bacterial strains tested, with diameter zone of inhibition (ZOI) ranging from 7 mm to 19 mm. The largest ZOI measured is at 19 mm against *E. tarda* (PCA17), surpassing the diameter ZOI of Oxytetracycline (OTC), which was 10 mm. The superior antibacterial activity of the gelenggang methanolic extract against *E. tarda*, compared to OTC, may be attributed to several factors specific to this bacterial strain and the chemical composition of the plant extract. One possible explanation is that the gelenggang extract contains bioactive compounds that may inhibit the growth of *E. tarda*, as reported in a recent study against other bacteria (Fatmawati, Purnomo, & Bakar, 2020). These compounds such as tannin may interact with specific molecular targets or pathways within the bacterial cell, leading to alteration of cell wall composition (Ehiowemwenguan, Inetianbor, & Yakubu, 2014).

In addition, an important finding of this study is that the ZOI for senduduk extract (15 mm), semambu extract (13 mm), and mahkota dewa extract (15 mm) also exceeds the ZOI of OTC when tested against *S. agalactiae* (SA2) (ZOI = 0 mm) and *E. tarda* (PCA17) (ZOI = 10 mm). This suggests that these extracts may possess higher susceptibility against the tested bacterial strains compared to OTC. Then, for antimicrobial efficacy of methanolic mengkudu and munggai extracts, it was observed that these extracts exhibited minimal to no zone of inhibition when tested against various bacterial strains. This outcome suggests a lack of significant antibacterial activity associated with these extracts.

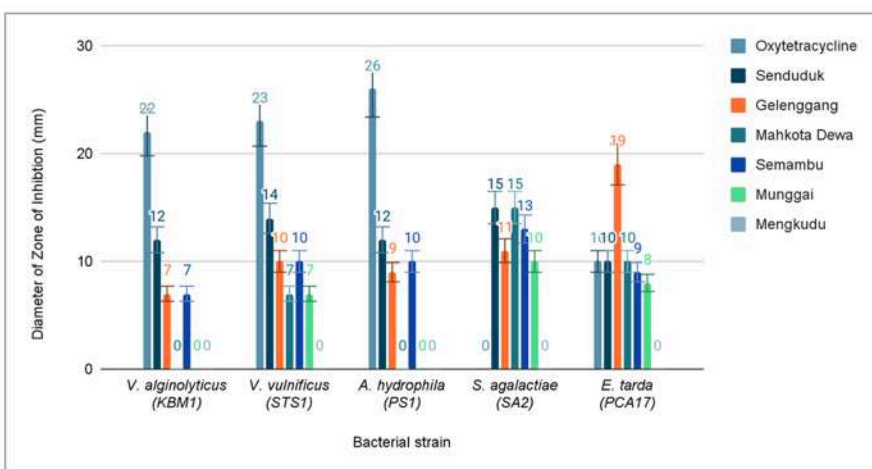

Figure 2. Graph of the inhibition zones in the disc diffusion method against fish pathogen bacteria using methanol as solvents for extraction.

Next, the results in Fig. 3 showed that only the ethanolic extracts of senduduk and gelenggang exhibited a ZOI compared to the other plant extracts. Specifically, the senduduk ethanolic extract demonstrated the largest inhibitory zone against *E. tarda* (PCA17) at 19 mm and against *S. agalactiae* (SA2) at 18 mm. This ZOI exceeded that of oxytetracycline (OTC), which measured 10 mm and 0 mm against the respective bacterial strains, indicating the superior effectiveness of the plant extract compared to the commercial antibiotic. In contrast, the gelenggang ethanolic extract displayed relatively low inhibitory effects across all tested bacteria, with zone diameters of only 7 mm and 8 mm.

The significant antibacterial activity of the senduduk (*Melastoma malabathricum*) ethanol extract against both *E. tarda* (PCA17) and *S. agalactiae* (SA2), compared to oxytetracycline, may be attributed to various factors specific to these bacterial strains and the chemical composition of the plant extract. senduduk, is known to contain secondary metabolites such as phenolic compounds, flavonoids, alkaloids, and terpenoids (Ismail et al., 2022). These compounds have been extensively studied for their antimicrobial properties and their ability to disrupt bacterial cell structures or interfere with essential cellular processes (Khameneh et al., 2019).

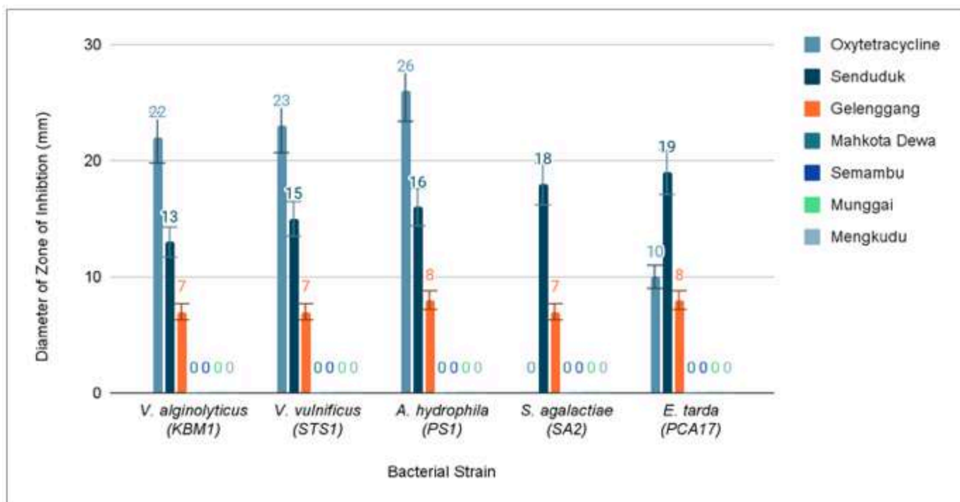

Figure 3. Graph of the inhibition zones in the disc diffusion method against fish pathogen bacteria using ethanol as solvents for extraction.

Fig. 4 & 5 indicate an absence of ZOI in the ethyl acetate and n-hexane plant extracts compared to those obtained through other solvent extraction methods. Ethyl acetate and n-hexane were identified as the least efficient extractants, failing to exhibit any discernible antibacterial activity against the tested microorganisms. This observation is similar to previous research on *N. speciosa* fruit extract, where the plant was less effective when extracted by low polar solvents such as hexane (Naufalin & Herastuti, 2017). This study suggests the importance of selecting the appropriate solvent to optimize the extraction of bioactive compounds.

Screening of the antibacterial properties of the selected plant revealed differences in the antibacterial activities of the extracts. These could be due to the differences in their chemical composition as well as in the mechanism of action of their bioactive constituents (Dubale et al., 2023). Despite the abundance of secondary metabolites in all extracts, their

presence alone does not dictate antibacterial activity. The antibacterial activity is also influenced by the concentration of secondary metabolites and potential interactions with other components (Lelario et al., 2018). Subsequently, the concentration of secondary metabolites is influenced by factors such as solvent type, extraction method, plant species, habitat conditions, and other relevant variables like temperature and pH (Karimi et al., 2020).

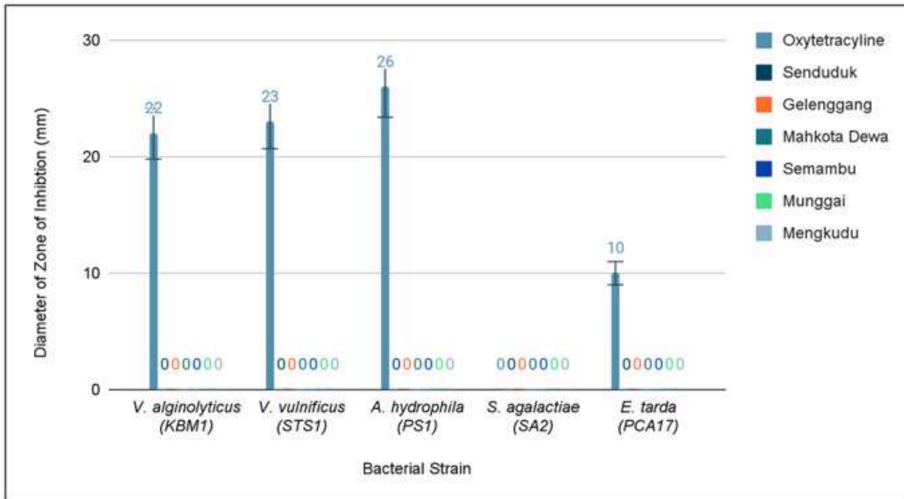

Figure 4. The inhibition zones in the disc diffusion method against fish pathogen bacteria using n-hexane as solvents for extraction.

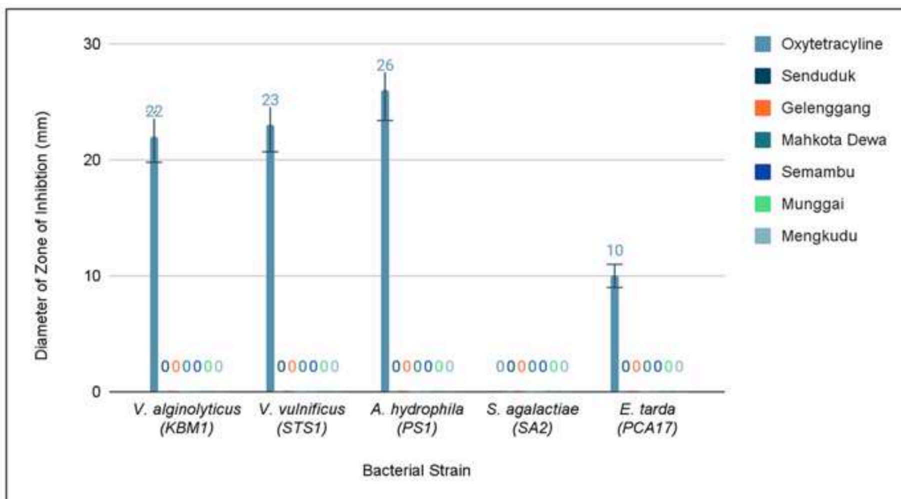

Figure 5. The inhibition zones in the disc diffusion method against fish pathogen bacteria using ethyl acetate as solvents for extraction.

The results of this study emphasize the significant correlation between the demonstrated antibacterial activity and the choice of solvent employed for extraction. By conducting a series of consecutive extractions utilizing solvents with varying polarities, it was revealed that both methanol and ethanol are effective solvents for extracting antimicrobial compounds from diverse plant (Figure 2 and 3). The presence of discernible antibacterial activity in the methanolic and ethanolic extracts indicates that solvents with higher polarity

possess greater efficiency in extracting bioactive compounds with antimicrobial properties from the targeted plant materials.

This discovery is similar to previous research findings that highlight the superior efficacy of polar solvents in extracting phytochemicals with notable antimicrobial activity from plant sources (Rafińska et al, 2019 ; Rezaei & Ghasemi Pirbalouti, 2019). The remarkable effectiveness of polar solvent extraction can be attributed to several factors grounded in the physicochemical properties of the solvent. Polar solvents exhibit the capacity to dissolve polar compounds inherent in plant material. This solvent capability is facilitated by the presence of hydroxyl (-OH) functional groups in both methanol and ethanol, enabling these solvents to engage in hydrogen bonding interactions with solute molecules (Potts et al., 2021). For instance, phenolic compounds, flavonoids, alkaloids, and terpenoids, recognized for their antibacterial properties, are among the bioactive compounds that can be effectively extracted using polar solvents (Mehmood et al., 2022). The selective extraction of these compounds ensures the concentration of antibacterial compounds, thereby enhancing the therapeutic potential of the obtained plant extracts.

In the comparative analysis between ethanol and methanol as solvents for extracting antibacterial compounds from plants, the findings indicate that methanol exhibited greater efficacy. This difference can be attributed to the varying polarities of the two solvents. Methanol, possessing greater polarity, demonstrates enhanced solubilization of antibacterial bioactive compounds present in plant materials. However, both efficacy and safety considerations were needed taken into account while selecting the methanol as extraction solvent. Despite the known toxicity of methanol, it was initially employed for extraction purposes only. Following the extraction process, hazardous methanol was effectively removed using a rotary evaporator. Consequently, the substitution of methanol with ethanol as a diluent is proposed, aiming to provide a safer alternative for subsequent testing procedures (González et al., 2018). This approach ensured the preservation of bioactive compounds while mitigating health risks associated with methanol exposure.

For low polar solvents, the inability of n-hexane and ethyl acetate to effectively extract antibacterial compounds from the tested plants is apparent due to the lower concentration or abundance of antibacterial compounds extracted. This inefficacy is consistent with previous research findings and is further supported by the results of inhibitory zone categorization. For instance, the crude n-hexane extract of *P. aurata* exhibited minimal inhibitory activity against the tested microorganisms, *V. parahaemolyticus* and *V. harveyi* (Lestari, Massinai, & Haris, 2022). This is because n-hexane is more proficient in extracting oils from seeds and leaves, which contrasts with its limited capacity to extract bioactive compounds with antibacterial properties from certain plant materials (Tesfaye & Tefera, 2017).

In addition, the study also highlights the comparative efficacy of oxytetracycline and plant extracts against fish pathogens. Extracts from three plants—senduduk, gelenggang, and Mahkota dewa—utilizing ethanol and methanol as solvents, demonstrated superior activity against two out of five tested bacteria, specifically both Gram-positive (*S. agalactiae*) and Gram-negative bacteria (*E. tarda*). Concurrently, oxytetracycline has been reported ineffective against *E. tarda* due to the emergence of antibiotic resistance. This loss of effectiveness can be attributed to the development of resistance mechanisms in *E. tarda* strains to tetracycline antibiotics (Manzoor et al., 2023). Additionally, the *S. agalactiae* strain also experiences similar issues and was recently reported to be resistant to erythromycin, levofloxacin, tetracycline, and chloramphenicol (Liu et al., 2023).

Consequently, these findings hold significant implications as they suggest the potential utility of plant-derived alternatives for medication. Plant-derived antimicrobials offer inherent advantages, including natural biodegradability, which facilitates the reduction of antimicrobial residues in the environment (Li et al, 2023). This characteristic encourages sustainable behaviors and helps minimize any ecological consequences. Moreover, the utilization of plant-derived antimicrobials presents a promising alternative way for combating antibiotic resistance, particularly in cases where bacterial strains have developed resistance to conventional antibiotics (Yang et al., 2018). Overall, screening natural sources like plants for antimicrobial drugs emphasizes the importance of conducting further research in this field to expand the range of alternative treatments available for combating diseases with resistance, particularly in the context of aquaculture practices.

Conclusions

In conclusion, this study has provided valuable insights into the antibacterial activity of six medicinal plant species against various fish pathogens. The results highlight the significant impact of the solvent used for plant extraction on the effectiveness of the extracts. Specifically, methanol and ethanol extracts demonstrated significant antibacterial activity. The methanolic extracts of senduduk, gelenggang, and Mahkota Dewa leaves exhibited notable inhibition zones against *S. agalactiae* and *E. tarda*. However, the efficacy of these extracts was limited to only a subset of the tested fish pathogens. Ethanolic extracts were effective against some bacteria but showed limited activity against others. Similarly, methanolic extracts were potent against specific pathogens but not all.

Further research is needed to optimize extraction methods and formulation techniques to maximize the therapeutic potential of these plant-derived compounds. Then, conducting Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) studies will provide more detailed insights into the antibacterial activity of these extracts. Additionally, it is important to explore other medicinal plant species that may have potent antimicrobial properties against a broader spectrum of fish pathogens. This research will contribute to the development of effective alternatives to conventional antibiotics, thereby reducing the risks associated with antimicrobial resistance and environmental contamination in aquaculture settings.

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Effect of stocking density on growth performance of tiger prawn *Penaeus monodon* from Madagascar in ponds

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Abstract: Tiger prawn *(Penaeus monodon)* is among aquaculture species that contribute to food security in Malaysia. To maximise its aquaculture production, the effect of stocking density on the survival rate of *P. monodon* originating from Madagascar was studied. The size of the ponds used for this study ranged from 1,936 to 2,170m^2. *P. monodon* was cultured at three stocking densities (40, 60 and 80 ind/m^2), in triplicates. Throughout the culture period, 10-20% of water replacement was done weekly with chlorine-treated brackish water, from day 20 until the end of the production cycle. The feeding regime was five (5) times per day from the second day of culture until day 60 and four (4) times per day from day 61 onwards. The survival rate (SR), average body weight (ABW) and feed conversion ratio (FCR) of the prawns were recorded at the end of the cycles, which took up to five (5) months for each culture. The SR (%), ABW (g) and FCR for stocking densities /m^2) of 40, 60 and 80 were 83.47±4.51, 88.30±8.26, 81.50±6.18; 27.33±2.35, 26.81±1.55, 24.72±1.15 and 1.74±0.04, 1.83±0.02, 1.87±0.28 respectively. One-way ANOVA indicated, there was no significant differences (P>0.1) were observed in the SR, ABW and FCR between the stocking densities. Meanwhile, the total harvest (kg) that was recorded at 2,077+179, 2,415+917, 3,381+348 respectively showed a significant difference (P<0.1) between the stocking densities. This suggests that the survival rate, average body weight and feed conversion ratio were not affected by the stocking density while high stocking density (80 ind./m2) in ponds will be an effective technique to maximize the production of tiger prawns in Malaysia.

Keywords: tiger prawn, *Penaeus monodon*, stocking density, survival, average body weight,

Abstrak: Udang harimau *(Penaeus monodon)* adalah antara spesies akuakultur yang dapat menyumbang kepada keterjaminan makanan di Malaysia. Untuk memaksimumkan pengeluaran *P. monodon*, kesan kepadatan stok terhadap kadar hidup *P. monodon* yang berasal dari Madagaskar telah dikaji. Saiz kolam yang digunakan untuk kajian ini adalah di antara 1,936 hingga 2,170m^2. *P. monodon* diternak pada tiga kepadatan stok (40, 60 dan 80 ind./m^2), dengan tiga replikat. Semasa ternakan, 10-20% air ditukar menggunakan air payau terawat menggunakan klorin setiap minggu bermula dari hari ke-20 sehingga akhir pusingan ternakan udang. Bermula dari hari kedua ternakan sehingga hari ternakan ke-60, udang diberi makan lima (5) kali sehari dan mulai hari ke-61 ternakan dan seterusnya, udang diberi makan empat (4) kali sehari. Kadar hidup (SR), purata berat badan (ABW) dan nisbah penukaran makanan (FCR) udang dicatatkan pada akhir ternakan yang mengambil masa sehingga lima (5) bulan untuk setiap pusingan. SR (%), ABW (g) dan FCR untuk kepadatan stok (/m2) 40, 60 dan 80 masing-masing ialah 83.47±4.51, 88.30±8.26, 81.50±6.18; 27.33±2.35, 26.81±1.55, 24.72±1.15 dan 1.74±0.04, 1.83±0.02, 1.87±0.28. Statistik satu-hala ANOVA menunjukkan tiada perbezaan yang signifikan (P>0.1) diperhatikan untuk SR, ABW dan FCR antara kepadatan stok yang berlainan. Sementara itu, jumlah tuaian (kg) yang dicatatkan pada kepadatan stok 40, 60, 80 (/m^2) adalah 2,077+179, 2,415+917, 3,381+348

menunjukkan perbezaan yang signifikan (P<0.1) di antara kadar tebaran yang berlainan. Ini menunjukkan bahawa kadar hidup, purata berat badan dan nisbah penukaran makanan tidak dipengaruhi oleh kadar tebaran manakala kadar tebaran yang tinggi di kolam (80 ind./m^2) boleh menjadi faktor yang berkesan untuk memaksimumkan pengeluaran udang harimau di Malaysia.

Introduction

Production of tiger prawn *(Penaeus monodon)* and white shrimp *(Penaeus vannamei)* are among aquaculture species that contribute to the food security in Malaysia. In 2010, the production of *P. monodon* was 18,118.00 mt and increased to 140,530.00 mt in 2014 (Jabatan Perikanan Malaysia, 2010, 2014). However, the production dropped to 4,286.00 mt in 2015 due to early mortality syndrome (EMS) (Jabatan Perikanan Malaysia, 2010; 2014). Then, the production showed an increasing trend from 5,654.73 mt to 10,132.92 mt in 2016 and 2017 (Jabatan Perikanan Malaysia, 2016; 2017). This increment in production of *P. monodon* was most probably contributed by Early Mortality Syndrome (EMS) crisis that hit the local white shrimp *(P. vannamei)* industry which causing the Malaysian farmers to opt for the tiger prawn as the primary species for prawn culture (FAO, 2018).

The *P. monodon* has a natural distribution range from East Africa to the South Pacific Islands (Salote et al., 2011). The species from Madagascar, Africa of Specific Pathogen Free (SPF) status, was domesticated in 2001 and produced over 30,000mt of superior quality head-on prawn for European markets (Groumellec et al., 2011). Taking advantage of the SPF status, this study used the domesticated *P. monodon* from Madagascar as the subject species of this study.

Traditionally, intensive culture system with stocking density ranges from 20 – 60 ind./m^2 for *P. monodon* in small (0.1 to 1.0 ha) ponds with square or rectangular shape (FAO, 2005) is commonly practiced in Thailand, Malaysia, and Australia. This method is favourable since industry players claimed it is more beneficial in terms of profit per culture cycle. To maximize the production of *P. monodon*, the effect of stocking density on the survival rate of *P. monodon* was studied. Three stocking densities (40, 60 and 80 ind./m^2), in triplicates were applied in this study.

Materials and Methods

Experimental design

P. monodon were cultured in three (3) treatments of stocking density (SD); 40, 60, 80 ind./m^2 for five (5) months, each in triplicate. The differences in survival rate (SR), average body weight (ABW) and feed conversion ratio (FCR) of the *P. monodon* culture were subjected to one-way analysis of variance (ANOVA). These data were computed using SPSS (version 7.5) of the Statistical Package for the Social Sciences (IBM SPSS) Statistics 23 software.

Research ponds

Nine (9) earthen ponds located in Fisheries Research Institute Gelang Patah, Johor, Malaysia were used in this study. The ponds size used for this study ranged from 1,936 to 2,170 m^2 with a depth of approximately 1.5 m. Each pond was equipped with 4 paddle-wheel aerators (1 horsepower each) to maintain the water currents and aeration.

Pond preparation

Firstly, the ponds were alkalised with calcium carbonate, ranging between 100-300 parts per million (ppm). After approximately 24 hours, the ponds were filled with chlorine-treated brackish water at approximately of 1.3 m depth. The pH of the brackish water was optimised with dolomites of 50 ppm for 14 days where the application was conducted twice a week. Similarly, the probiotics (Bio solution, CP, Indonesia) were added at 20 ppm twice per week for 14 days, prior to stocking the ponds with post-larvae tiger prawns. Meanwhile, the application of lime or other chemicals was done when necessary.

Sources of tiger prawns (P. monodon)

The post-larvae (PL-10) of the *P. monodon* originated from the Madagascar were obtained from a local hatchery in Perak, Malaysia. The post-larvae were transported in plastic bags and upon arriving, they were acclimatised in the culture pond for 1 hour. Post-larvae from three (3) bags were sampled randomly for counting prior to be released into the ponds accordingly with the rest of the post-larvae stocks. The experiments were conducted in 9 ponds to accommodate triplicate of each stocking density of 40, 60 and 80 ind./m2.

Feeding and water management

The tiger prawns were fed five (5) times a day, starting from the second day of culture (DOC) until DOC 60 and scheduled at 7:30 am, 10:30 am, 2:30 pm, 6:00 pm and 9:30 pm. The feeding frequency on DOC61 and onwards was reduced from 5 times to 4 times per day. The feeds were supplemented with Azomite of 3,000 ppm. Azomite which is a naturally occurring volcanic mineral and was applied as feed additive to enrich the feed. The feed was also coated with diluted molasses that act as additional feed additive. During the entire culture period, probiotics of Bio Solution CP brand were administered directly into the pond at a concentration of 20 ppm three times a week for day 14 onwards.

After DOC 25, weekly water exchange was executed of approximately 10 - 20% using pump of the chlorine-treated water source from the reservoir. This regular water exchange was based on established aquacultural practices aimed at maintaining optimal water quality, particularly by managing the levels of dissolved oxygen and removing waste products such as uneaten feed and excreta, which could not be fully mitigated by probiotics alone. Regular water exchanges help prevent the build-up of harmful substances that can deteriorate water quality, potentially leading to health issues in prawns. Although adding probiotics three times weekly at a concentration of 20 ppm contributes to a healthy microbial balance (Amiin et al., 2023) and enhances water quality, it does not entirely eliminate the need for physical water exchanges. These exchanges are crucial for maintaining stable water parameters, thereby supporting the probiotics' effectiveness and ensuring the environmental stability necessary for the prawns' optimal growth and health. Thus, the combined approach of using probiotics and conducting regular water exchanges is not redundant but rather synergistic, each component playing a vital role in sustainable aquaculture practices. The water exchange routine was implemented based on findings from a similar study (Shakir et al., 2014), which highlighted that key water parameters, particularly dissolved oxygen (DO) and pH, significantly influence yield in high stocking density ponds. This study maintained the DO level at 4 mg L^{-1} or ppm, adhering to aquaculture standard practices, especially for marine species, to ensure that the physiological needs of the prawns are met, thus preventing stress and health issues that could negatively impact their growth and survival. Regular monitoring and adjustment of water quality, including DO levels, is integral to the management strategy, supporting optimal conditions in high-density aquaculture environments. The water parameters were recorded twice daily in the morning (7.30 am) and evening (5.30 pm) throughout the culture period. Water parameters were maintained

at recommended levels according to several references such as Thai Agricultural Standard (TAS7401, 2009), Boyd and Fast (1992), Hansell (1993), Chen and Chen (1992) and Boyd and Thunjai (2003), on aquaculture and *P. monodon* culture.

Partial harvest

Practically, *P. monodon* farming with a high stocking density (> 60 ind./m^2) is harvested partially to optimize the size of the cultured prawns at the end of the cycle period. Partial harvesting was applied to all triplicate culture ponds with stocking density of 40, 60 and 80 ind./m^2. Partial harvest started after the *P. monodon* reached an average individual weight of 20.00 g, and the harvesting was continued approximately every two weeks.

Results and Discussion

Survival rate (SR)

In this study, the survival rates (SR) for stocking densities (SD) of 80, 60, and 40 were observed as 81.50±6.18%, 88.30±8.26%, and 83.47±4.51%, respectively (Fig. 1). The analysis revealed no statistically significant differences in survival rates among the various stocking densities, with a p-value of 0.470. This finding indicates that survival rates are not adversely affected by changes in stocking densities within the tested ranges, and surprisingly, no distinct trends were observed that suggest higher or lower stocking densities result in correspondingly higher or lower survival rates.

These findings align with earlier research conducted by Chakraborty et al. (1996), where varying stocking densities of 40, 41, and 55 ind./m2 resulted in survival rates of 70.00%, 60.23%, and 68.63%, respectively. Similarly, Suresh & Shailender (2013) and Alokesh et al. (2013) reported a decrease in survival rate as stocking densities increased. Thus, the relationship between stocking density and survival rate appears to vary based on specific experimental conditions.

Nonetheless, the statistical analysis using one-way ANOVA in this study demonstrated no significant differences (P > 0.1) in the survival rates across varying stocking densities (Table 1). On an individual mortality perspective, cannibalism among prawns, often exacerbated by limited food availability, emerged as a significant factor. This heightened competition for food led to increased instances of contact among individuals during feeding, consequently triggering cannibalistic responses (Abdussamad & Thampy, 1994). Additionally, the vulnerability during moulting phases, where a prawn's body becomes weak and soft, further amplified the occurrences of cannibalism (Abdussamad & Thampy, 1994). It remains crucial to recognize and consider various other factors that could potentially influence survival rates, including water quality and pond ecology, as highlighted by Alokesh et al. (2013). These multifaceted factors collectively contribute to the intricate dynamics that impact survival rates in aquaculture settings.

Average body weight (ABW)

In this research, we conducted a comprehensive examination of the influence of different stocking densities on the Average Body Weight (ABW) of the cultured prawn. Notably, the results revealed a remarkable similarity in ABW among the various stocking densities employed. Specifically, the ABW values were found to be 27.33±2.35 g, 26.81±1.55 g, and 24.72±1.15 g for stocking densities (SD) of 40, 60, and 80 individuals per square meter (Fig. 1), respectively. It's noteworthy that the statistical analysis further confirmed these observations, indicating no statistically significant difference (P> 0.1) in ABW (Table 1) across the various stocking densities.

Furthermore, the study's management strategy incorporated partial harvesting, which commenced when the average individual prawn weight reached approximately 20.00 g. This approach was diligently executed, with subsequent harvesting events occurring at approximately two-week intervals starting from 120-day until 180-day culture period. The consistent ABW values and the absence of significant differences between stocking densities underscore the robustness and uniformity of the culture system employed, contributing valuable insights to the optimization of prawn aquaculture practices.

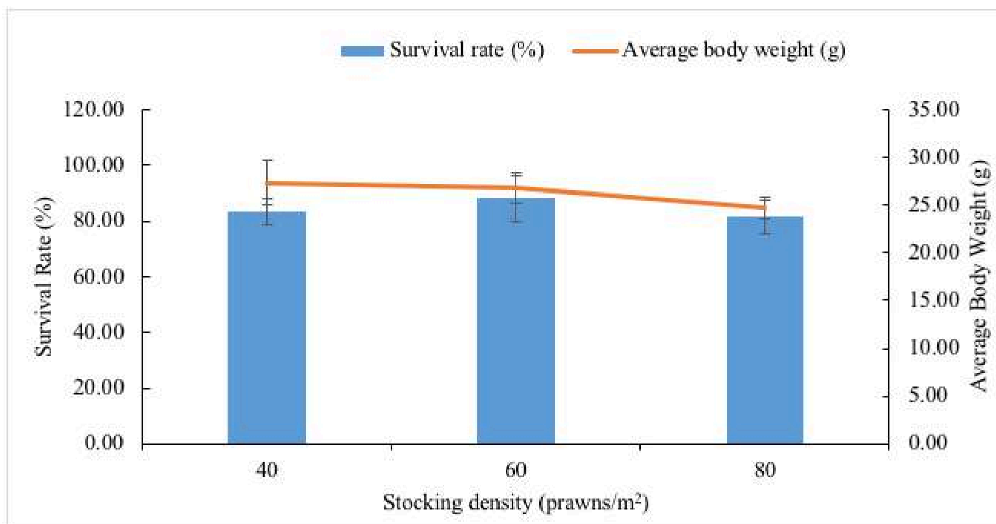

Figure 1. Survival rate (%) and average body weight (g) per stocking density of *P. monodon*.

Table 1. One-Way Analysis of Variance (ANOVA) of Survival Rate (SR), Average Body Weight (ABW), Feed Conversion Ratio (FCR) and Total Production (kg) of *P. monodon*

Measure	Stocking density (individual/ m^2)	N	Mean	SD	95% CI for Mean	F	DF	p-value
Survival rate (SR%)	40	3	83.47	4.51	72.26 to 94.67	0.860	2, 6	.470
	60	3	88.30	8.26	67.78 to 108.82			
	80	3	81.55	6.18	66.20 to 96.89			
Average body weight (ABW%)	40	3	27.33	2.35	21.49 to 33.18	1.863	2, 6	.235
	60	3	26.81	1.55	22.98 to 30.65			
	80	3	24.72	1.15	21.87 to 27.57			
Feed conversion ratio (FCR)	40	3	1.74	0.04	1.63 to 1.85	0.538	2, 6	.610
	60	3	1.83	0.02	1.78 to 1.89			
	80	3	1.87	0.28	1.19 to 2.56			
Total production (kg)	40	3	2077.00	179.39	1631.38 to 2522.62	4.144	2, 6	.074
	60	3	2415.33	916.94	137.54 to 4693.13			
	80	3	3380.55	347.57	2517.14 to 4243.96			

Note: CI = Confidence Interval; df = degrees of freedom; SD = Standard Deviation; F and p-values are from ANOVA tests examining differences between groups.

Feed conversion ratio (FCR)

The Feed Conversion Ratio (FCR) is a crucial metric in aquaculture, representing the efficiency with which the feed provided to the prawns is converted into body weight. This study meticulously investigated the FCR of tiger prawns across three distinct stocking densities (SD) - 40, 60, and 80. The feed conversion ratio (FCR) did not significantly differ across the stocking densities studied, with a p-value of 0.610. This result indicates that feed efficiency is maintained regardless of the density at which prawns are stocked. The findings, illustrated in Fig. 2, indicated FCR values of 1.74±0.04, 1.83±0.02, and 1.87±0.28 for SD 40, 60, and 80, respectively. This observed trend aligns with established research, including but not limited to Jannatul et al. (2019), Zaki et al. (2004), and Chakraborty et al. (1996), consistently highlighting the inverse correlation between stocking density and FCR. It is well-documented, supported by Sandifer et al. (1987), that escalating stocking densities tend to reduce feed conversion efficiency due to heightened intraspecific competition for available food resources.

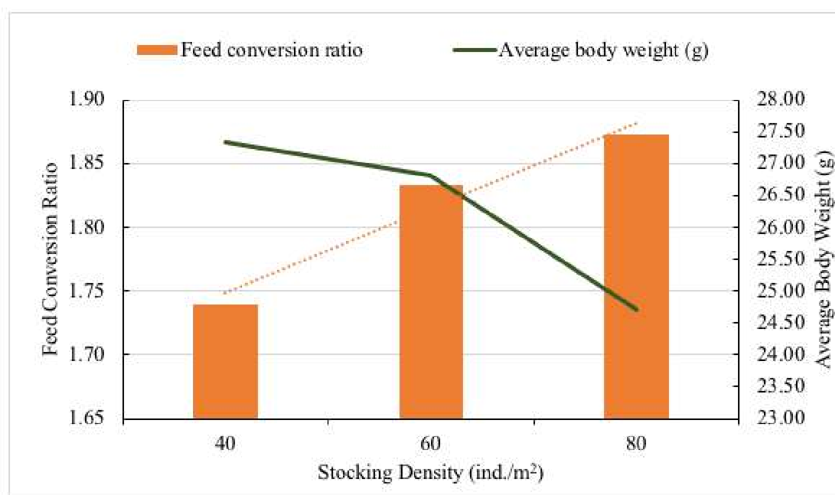

Figure 2. Feed conversion ratio and average body weight per stocking density of *P. monodon*

Contrary to the anticipated trend, meticulous statistical analysis employing one-way ANOVA revealed no statistically significant differences (P>0.1) in the FCR of tiger prawns across the varying stocking densities (Table 1). This intriguing uniformity in FCR across all treatments (SD 40, 60, and 80) suggests a level of stability and control in the feeding regimen adopted, a facet thoroughly elucidated in the methodology. During the initial two months of the culture period (Day of Culture 2 to Day of Culture 60), a high feeding frequency of five times daily was rigorously implemented. This approach, in line with the findings of Hasan et al. (2012), is recognized to optimise production and profitability while minimising feed wastage, potentially equalising the FCR. Subsequently, from Day of Culture 61 onwards, the feeding strategy was dynamically adapted, with feed proportions determined by body weight and specific days of culture, ensuring a consistent and efficient FCR.

These results underscore the significant role of a well-structured feeding regimen in influencing the FCR and, consequently, the overall efficiency and productivity of tiger prawn aquaculture. The findings furnish invaluable insights for aquaculture practitioners to refine and tailor their feeding strategies, ultimately contributing to sustainable and economically viable prawn farming practices. Future research may delve into the intricate interplay of feeding regimes, stocking densities, and growth parameters to optimise FCR, thus fostering sustainable aquaculture practices.

Total production

The comprehensive production analysis of tiger prawns within distinct stocking densities - 40, 60, and 80 - yielded noteworthy outcomes. The cumulative production for each stocking density was meticulously recorded, manifesting as 2,077±179 kg, 2,415±917 kg, and 3,381±348 kg, respectively. Employing a rigorous one-way ANOVA statistical analysis, a significant variance in total production was observed across the three stocking densities (SD 40, SD 60, and SD 80), with a p-value of 0.074 (Table 1). This suggests a trend where higher densities might lead to increased total production, potentially enhancing yield per unit area and indicating that higher stocking densities can effectively boost production outputs in aquaculture environments. This pivotal discovery underscores the undeniable influence of stocking densities on the productivity of *P. monodon* culture, positioning this variable as a pivotal lever within the aquaculture domain.

Intriguingly, this finding holds promising implications for the aquaculture industry, providing a substantive basis to endorse higher stocking densities for the cultivation of tiger prawns. Elevated stocking densities, as evidenced in SD 80, elucidate the potential for bolstered production, thus presenting an opportunity to optimise operational efficiency and overall output. This empirical insight enriches our understanding of the delicate balance between stocking density and yield, nurturing a trajectory of enhanced sustainability and productivity within the realm of aquaculture.

Water quality

Frequent water exchange, typically conducted on a weekly basis, has emerged as a pivotal factor contributing to the remarkable survival rates exceeding 80% observed in *P. monodon* culture. Conversely, inadequate water management practices often result in deteriorating water quality, subsequently impeding growth rates and overall survival (Liu et al., 2017; Aguilar et al., 2012). Notably, rigorous monitoring of vital water parameters, including pH (measured both in the morning and evening), total ammonia nitrogen (TAN), nitrite ($NO2^{-}$)), alkalinity, calcium, and magnesium, was diligently carried out throughout the culture period. This meticulous monitoring regimen significantly contributed to a comprehensive evaluation of the aquacultural milieu, providing valuable insights into the environmental factors influencing prawn growth and well-being.

The pH levels, fundamental in aquaculture, were subject to meticulous monitoring throughout the culture period. The recorded pH exhibited a fluctuating pattern, oscillating within the range of 8.3 to 9.1. Notably, these values slightly surpassed the recommended threshold of 8.3 as stipulated by the Thai Agricultural Standard (TAS 7401, 2009), and also exceeded the optimal range for prawn and prawn culture suggested by Boyd & Fast (1992), which spans from 7 to 9. It is imperative to maintain an appropriate pH level as excessively high pH, exceeding 10.6, as cautioned by Tsai (1990), could have lethal implications, particularly for penaeid prawns.

In the context of alkalinity, a critical water parameter, the recorded values ranged from 108.8 to 118.8 ppm. These observations conformed to the recommended limit set by Hansell (1993), denoting alkalinity exceeding 80 ppm, which is regarded as favourable for aquaculture environments. This is especially crucial for *P. monodon*, as elucidated by Chanratchakool (2003), where alkalinity levels above 150 ppm, in conjunction with a pH exceeding 8.3, facilitate mineral deposition on the exoskeleton of the prawn. However, it is prudent to avoid excessive alkalinity, as it could disrupt essential physiological processes, underscoring the delicate balance required in maintaining optimal water quality parameters for successful aquaculture.

The analysis of Total Ammonia Nitrogen (TAN) levels revealed concentrations ranging from 0.4 to 0.6 ppm (Fig. 3), slightly surpassing the recommended level of 0.4 ppm outlined in the Thai Agricultural Standard (TAS 7401-2009). Intriguingly, Chen et al. (2016) presented a compelling finding suggesting a higher tolerance of *P. monodon* to ammonia nitrogen than conventionally understood. Through an exposure experiment at 67.65 ppm, a group of *P. monodon* exhibited a remarkable survival rate of 88.67 ± 9.81% throughout the research period, challenging established assumptions regarding the species' ammonia nitrogen tolerance.

Conversely, the levels of nitrite (NO2-) observed ranged from 0.4 to 1.0 ppm (Fig. 3), contributing to the optimal conditions for *P. monodon* culture. This observation corroborates the findings of Chen & Chen (1992), elucidating that an excess of nitrite in tiger prawn culture induces significant growth deformation. Adverse growth effects, including reductions in weight and length and an imbalanced ratio of carapace length to total length, manifested even at relatively low levels of nitrite exposure, as low as 2 ppm (Chen & Chen, 1992).

The elemental composition of the aquaculture environment, particularly calcium and magnesium, holds significant importance, revealing concentrations within the range of 325 to 335 ppm for calcium and 500 to 525 ppm for magnesium (Fig. 3). Calcium, a crucial element, plays a vital role in the formation of the prawn's exoskeleton, a process particularly pronounced during moulting. Inadequate calcium levels, falling below 30 ppm, have been associated with moulting abnormalities observed in prawn ponds, underlining the criticality of adequate calcium concentrations for healthy moulting processes (Boyd & Thunjai, 2003).

Furthermore, magnesium, an essential cofactor, intricately interlinked with various metabolic processes, including carbohydrate, fat, and protein metabolism (Truong et al., 2020), stands out as a significant factor in the aquaculture environment. Given its multifaceted involvement in metabolic pathways, the presence of optimal magnesium levels may indicate a favourable environment conducive to the high survival rate and the observed nearly uniform Average Body Weight (ABW) across the culture. These findings shed light on the potential influence of these elemental constituents in shaping prawn growth and overall well-being, offering valuable insights for aquaculture management and water quality optimization.

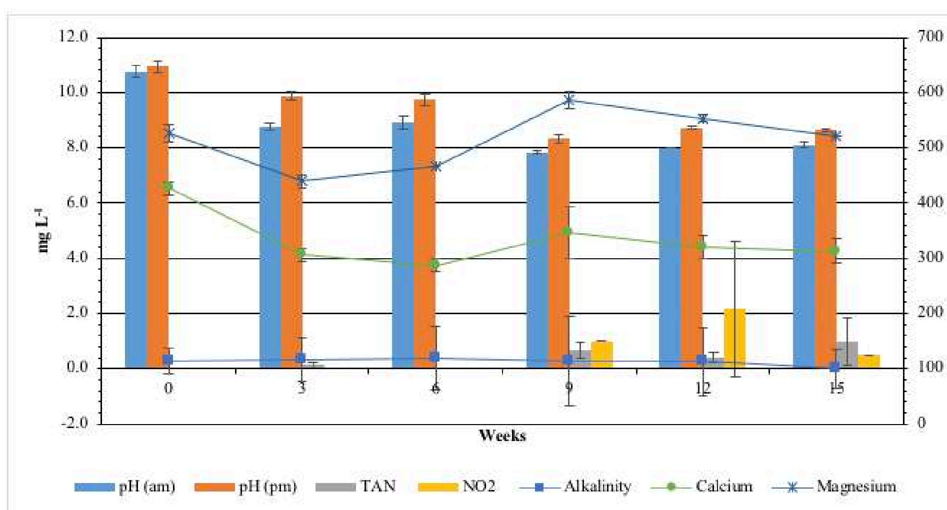

Figure 3. Water quality of *P. monodon* culture

Strategic and consistent administration of probiotics, occurring two times per week on the first 14 days of culture and three times per week after 14 days onwards at a concentration of 20 ppm, was meticulously employed in this study to optimise the growth of the tiger prawn, *P. monodon*. Probiotics, acting as carriers of non-pathogenic bacteria, significantly enhance the organism's disease resistance mechanisms. This enhancement is achieved through competitive exclusion, where probiotics outcompete pathogenic bacteria, thereby substantially reducing the likelihood of disease occurrence within the culture system (Fanzafar, 2006). The judicious use of probiotics, thus, stands as a pivotal strategy in ensuring the maintenance of optimal water quality, complementing regular water exchange practises fundamental in aquaculture management.

Furthermore, probiotics exert a profound influence on organic matter breakdown, culminating in a substantial reduction of sludge and slime formation within the culture environment. This degradation process significantly contributes to improved water quality by minimising disease incidences attributed to pathogens such as *Vibrio* sp., *Aeromonas* sp., and viruses. Moreover, probiotics serve to augment zooplankton populations, ameliorate odour issues, and ultimately enhance overall aquaculture production (Sahu et al., 2008). Notably, a study focusing on the impact of probiotics on the growth of the tiger prawn, *P. monodon*, echoed the efficacy of probiotics in aquaculture. The study, conducted by Hasan et al. (2012), emphasised the routine application of probiotics weekly, showcasing its potential in bolstering water quality management and harvest outcomes, encompassing crucial parameters such as survival, body weight, feed conversion, and production.

Conclusion

The findings of this current study unequivocally demonstrate that various stocking densities applied to *P. monodon* cultures do not exert a statistically significant influence on growth performance, a result confirmed through rigorous statistical analysis with a significance threshold of $P>0.1$. This conclusion is strongly supported by the consistently high survival rates exceeding 80% across all treatments (SD40, 60, and 80), coupled with the closely comparable values of Average Body Weight (ABW) and Feed Conversion Ratio (FCR) observed within these treatments. Thus, within the experimental conditions, the evidence overwhelmingly suggests that high stocking density (80 ind./m^2) in aquaculture ponds is a pragmatic and viable strategy for tiger prawn farming, demonstrating its potential to significantly augment production, ultimately leading to higher yields. Nevertheless, it is imperative to underscore that the adoption of higher stocking densities necessitates meticulous attention to the maintenance of crucial water parameters and quality. Specifically, regular water exchange at a rate of 10 - 20% of the total volume emerges as a crucial practice to ensure optimal conditions for growth. Additionally, continuous monitoring of water quality parameters, complemented by interval application of probiotics, becomes instrumental in maintaining a balanced and favourable culture environment. The successful implementation of this stocking strategy, combined with rigorous water management practices, strongly advocates for further explorations into even higher stocking densities, thereby paving the way for prospective advancements and optimizations in tiger prawn aquaculture.

Acknowledgement

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Life cycle characteristic of brackish water cladoceran *Diaphanosoma celebensis*

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Abstract: Due to Artemia's high cost and sporadic supply, alternate zooplankton species have to be identified to reduce or completely replace its use. The objective of this study is to explore alternative zooplankton species like *Diaphanosoma* spp as potential replacements for *Artemia*. *Diaphanosoma spp*. is promising live food organism as alternative species for *Artemia* due to its high reproduction rate. This study examines the life cycle development of cladoceran *Diaphanosoma celebensis* by observing embryo in brood pouch of the mother. In the present experiment, amictic females and devoid of neonates were used and conducted using one (1) Litre conical flask with 15 ppt filtered sea water and Nannochloropsis as feed. The changes in embryonic development were observed every 15 minutes. The growth of the embryos began after the cleavage of egg mass. The time taken for egg mass to develop to first juvenile instar was 50 ± 0.8 h and then to adult female in 96 ± 0.8 h. The final result indicated that *D. celebensis* undergoes parthenogenesis (development into an embryo without being fertilized) since they could produce eggs individually. The length of juvenile was 0.87 ± 0.05mm while mature adult was 1.38 ± 0.05mm. This life cycle study indicated that size of *D. celebensis* is suitable and can be used as live feed for marine fish larvae.

Keywords: Marine live feed, Zooplankton, Cladoceran, Growth rate, *Diaphanosoma celebensis*

Abstrak: Akibat dari kos *Artemia* yang tinggi dan bekalan yang semakin berkurangan, spesies zooplankton alternatif perlu dikenal pasti untuk pengunaan Artemia. Diaphanosoma adalah organisma makanan hidup yang boleh digunakan sebagai spesies alternatif kepada *Artemia* kerana kadar pembiakannya yang tinggi. Kajian ini mengkaji kitaran hidup cladoceran, *Diaphanosoma celebensis* dengan pemerhatian kepada embrio dalam kantung induk. Di dalam kajian ini, betina amiktik tanpa neonat telah digunakan menggunakan kelalang kon satu (1) Liter dengan 15 ppt air laut ditapis dan Nannochloropsis sebagai makanan. Perubahan dalam perkembangan embrio diperhatikan setiap 15 minit. Pertumbuhan embrio bermula selepas pembelahan jisim telur. Masa yang diambil untuk jisim telur berkembang menjadi instar juvana pertama ialah 50 ± 0.8 jam dan kemudian kepada betina dewasa dalam 96 ± 0.8 jam. Keputusan akhir menunjukkan bahawa *D. celebensis* mengalami parthenogenesis (perkembangan menjadi embrio tanpa disenyawakan) kerana *D. celebensis* boleh menghasilkan telur secara individu. Diadapati panjang juvenil ialah 0.87 ± 0.05 mm manakala dewasa matang ialah 1.38 ± 0.05 mm. Kajian kitaran hayat ini menunjukkan bahawa saiz *D. celebensis* adalah sesuai dan boleh digunakan sebagai makanan hidup larva ikan marin.

Introduction

Live feed has been considered to be one of the crucial factors in the commercial culture of larvae of many fishes and crustacean species (Kovalenko et al., 2002). It also has a significant impact on the total cost of production. Due to the fierce competition in the fish market, it is essential to develop more effective methods of cultivating fish larvae

of the highest calibre. Cladocerans were chosen in the present experiment, as they are a dominant group in aquatic food chain (Dodson and Frey 2001), In addition, Cladocerans are the natural dietary item for many marine and freshwater fish and crustaceans (de la Pena, 2001). According to Sipau´ba- Tavares and Bachion (2002), the species is resistant to handling involved in the culture system and can be utilized as live feed for fish. Mostly, prawn and fish depend on zooplankton at some stage during their lifespan and some feed exclusively on zooplankton during their entire life cycle (Sumitha 2006).

Cladocera or commonly called "water fleas" (Mittman B. et al., 2014) are quite small ranging from 0.5 mm to 3 mm. Cladocera are an excellent natural food source is for aquatic animals especially fish larvae and extensively used as live food in hatcheries (Budhin et al., 2016). Cladocera fulfill the aspects such as nutritionals value, resistance to any water conditions, population growth rate and suitable body size to produce adequate of live feed for fish feeding in larva cultur ure (He et al., 2001).

Diaphanosoma celebensis is an example of a cladoceran that is suitable for marine larval culture (Segawa et al., 1988). *D. celebensis* undergoes a process called parthenogenesis, wherein sexual reproduction periodically added to sexual reproduction (Nandini & Sarma, 2003). Assuming conditions such as food, water and temperature are appropriate, Cladoceran culture can rapidly generate a large number of individuals. *Diaphanosoma spp*. can reproduce parthenogenetically and has a high tolerance to a wide range of water salinities (Sipauba-Tavares and Bachion, 2002; Hagiwara et al. 2016).

In normal practice, marine fish larvae rearing rely on main live feed such as rotifers and *Artemia* (Marcus and Murray, 2001). In spite of the fact that rotifers and *Artemia* have been proven successful as animal protein source in rearing the larvae of many species, several problems remain. Problems involve variable nutrient composition maybe because of different batches and strains (Watanabe et al., 1980) and irregular supply and availability (Fermin, 1991), high cost (De la Pena et al., 1998), potential introduction of pathogens into the culture system and cultures are prone to crashes for rotifer (Kovalenko et al. 2002). (Lavens and Sorgeloos, 2000) Furthermore, mention that due to low yields, the quantity of imported *Artemia* cysts is insufficient to meet the demands of the aquaculture industry. Hatcheries frequently generate marine fish larvae with uneven sizes and low survival rates because of these issues (Madkour, K et al. 2023).

High cost for *artemia*, also one of crucial factors since lowest possible cost is essential for the successful development of fish culturing since artificial diets studied so far appear not to be fully adequate and the use of live feed yields better results (Watanabe and Kiron 1994). Occasionally, the size of live feed is crucial since the sort of feed that can be ingested depend on the size of the fish's mouth (Russo et al. 2009; Rønnestad, I et al. 2013). One of the example marine fish, seabass can ingest organism which are 80% their mouth size (Duray and Kohno, 1988). De la Pena et al., (1998), have cited the suitability of replacement *Artemia* with *D. celebensis*.

The purpose of this study is to get a thorough understanding of embryogenesis in brackish Cladoceran, *D. celebensis*. Studies about its reproduction, growth, and prospective use as live feed in aquaculture for marine fish larvae are lacking. To determine the association between age and overall length of *D. celebensis*, growth rate was also investigated. Understanding the selectivity of fish larvae may be crucial for optimising feeding in rearing systems. In order to demonstrate the suitability of this Cladoceran as live feed to replace *Artemia*, the size of

the neonate and *D. celebensis* under laboratory circumstances were evaluated in detail.

Materials and Methods

Diaphanosoma celebensis monoculture preparation

The experiments were conducted at the Plankton laboratory, Institut Penyelidikan Perikanan Tanjung Demong, Besut, Terengganu (IPPTD). *D. celebensis* was obtained from the hatchery ponds using 150-micron plankton net. The sample were then cultured in 300 litre indoor tank. During the culture period, the culture was harvested every 2 days to get rid of excess debris and foreign microscopic organism such as copepod and rotifer. One individual of *celebensis* were selected and cultured in 1-litre conical flask containing 400 ml 15 ppt filtered sea water without external stresses like aeration. *Nannochloropsis oculata*, were used as food for *D. celebensis* was cultured under continuous light with gentle aeration throughout the monoculture process.

Female adult selection

Adult females were isolated and collected using a 1 ml pipette over the course of one week. Adult *D. celebensis* were arranged individually in a petri dish with a diameter of 5.0 cm filled with filtered sea water (35 ppt). For the experiment, females without an embryo or without neonates were used. Every 15 minutes, females were placed on a glass slide and checked with an inverted microscope (Leica DM1000, 40 x magnification) to observe if there were any eggs in the brood pouch. The presence of eggs was a sign of the embryo's early stages of development.

Observation on embryonic development

The timing was recorded as 0 hours beginning with the appearance of egg mass. After a few minutes of the embryo's emergence from the brood pouch, the final molt signalled the end of development. Embryos were successfully developed in the brood pouches with temperatures fluctuating between 18˚C to 22˚C. The timing of embryogenesis of *D. celebensis* were 50 h. All embryos were observed using Leica Inverted Microscope DM1000 with magnification 40 x while pictures were captured using Leica Application Suite (LAS) EZ Software.

Life cycle of Diaphanosoma celebensis

First juvenile instar (after final molt) was placed in another petri dish contain filtered seawater and N. oculata with density of 10 to 20 x 10^4 cells ml were given as food. At the same magnification, juvenile lengths were periodically measured for triplicate. Timing were recorded until the presence of egg mass in brood pouch. This indicates the adult stage of *D. celebensis*. This experiment, which involved monitoring a single female *D. celebensis* in a petri dish, was carried out multiple times since, when kept in petri dishes for an extended period, involve sudden deaths.

Results

Embryogenesis of Diaphanosoma celebensis

Embryo were observed every 15 minutes to see the change in development. The moment the egg mass was present in brood pouch (Figure 1) (a): time were recorded as 0 ±0.8 h. The egg is ellipsoidal in shape and filled with granules of yolk. At the two poles, there is narrow zone of transparent peripheral plasma. The cleavage had begun even though there were not clear to be seen due to its opacity. At 16 ± 0.8 h, the formation of limb rudiments appears (Figure 2)(b).The commencement of the process coincides with the casting off of the membrane. By the 19 ± 0.8 h, the gnathobases of all limbs are separated by contraction and form a row of hexagons (Figure 1)(c). The rudiment that will shape as antennae will grow out at the second pair of thoracic limbs.

Next, rudiments at retina reshaped into eye capsule at 34 ± 0.8 h. Then, eye pigment appears as two small red-brown dots (Figure 1)(d). The eyes are small but noticeable due to the pigmentation. The embryos already have first heartbeat and the movement of the organ and gut peristalsis can be seen clearly as they response actively to any movement of the mother. By this time, the embryos already resemble the mother with almost complete post abdomen and gut (Figure 2)(a).

At 50 ± 0.8 h, the embryo (neonate) leaves the brood pouch (Figure 2(b)). At this point, post abdominal claw are extended. The carapace had covered all the limb and post abdominal. Swimming antenna also extended upwards (Figure 2)(c). The first juvenile is invisible to the naked eye since the gut have no food.

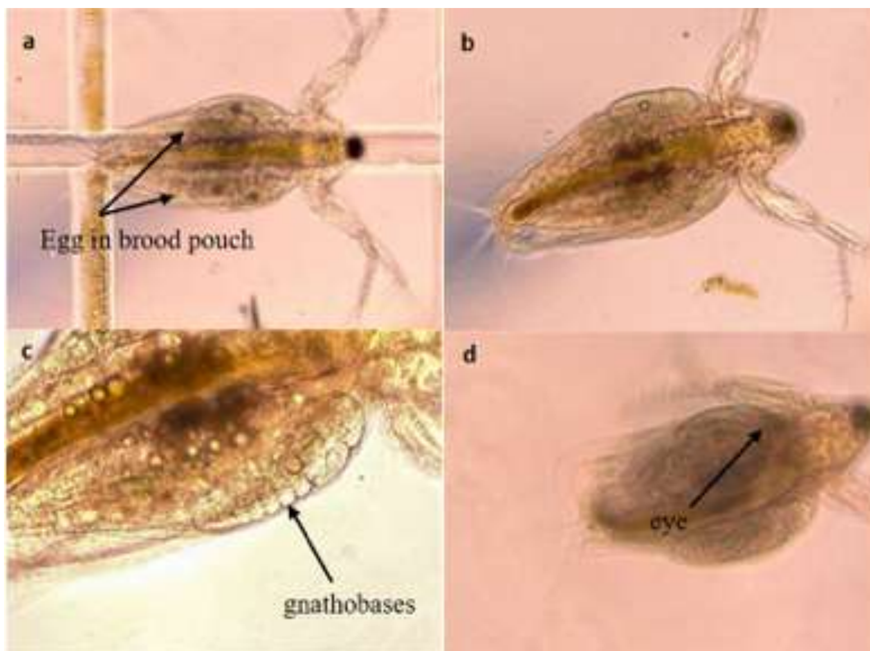

Figure 1. a) At 0 ± 0.8 h, the presence of ellipsoidal shape egg mass in brood pouch filled with granules of yolk. Magnification of 40x. **b)** Formation of limbs rudiments at 16 ± 0.8 hr with magnification of 40x. **c)** At 19 ± 0.8 hr, presence of gnathobases of limbs as well as

the formation of rudiment at thoracic limbs. Magnification of 40x. **d)** At 34 ± 0.8, small but noticeable eye pigment appears. Magnification of 40x.

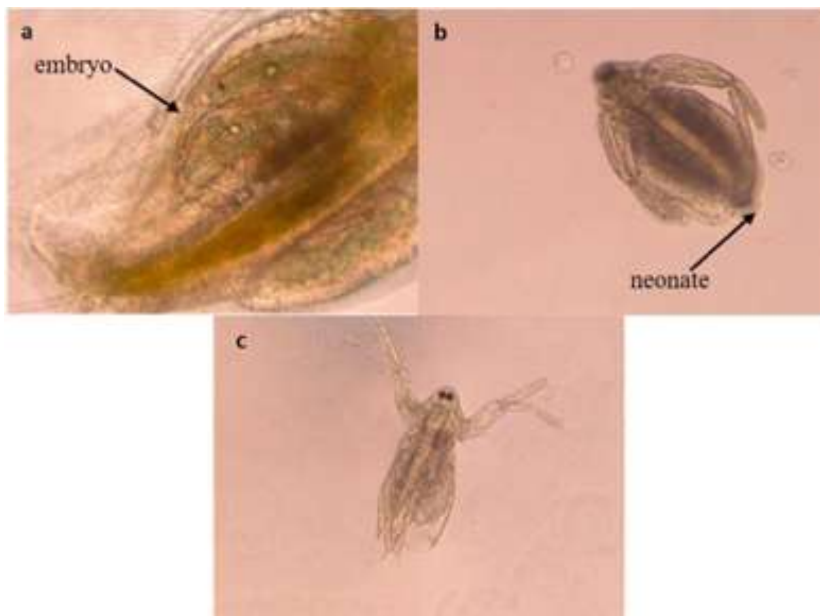

Figure 2. a) Embryo of *Diaphanosoma celebensis*. The embryos already resemble the mother with almost complete post abdomen and gut. Magnification of 40x. b) At 50 ± 0.8 h, release neonate from the brood pouch. c) First juvenile instar with extended antenna are invisible with naked eye. Magnification of 40x.

Life cycle of Diaphanosoma celebensis

Three replicates of first juveniles were observed and their lengths were measured. The mean length of the first juvenile was 0.868 ± 0.047 mm at 1 h. At 24 h, the mean body length of samples was 1.036 ± 0.049 mm. There was slightly increment in length from about 24 h to 30 h after release. The detailed life cycle is shown in Figure 3 and mean age and body length of adult *D. celebensis* are given in Table 1.

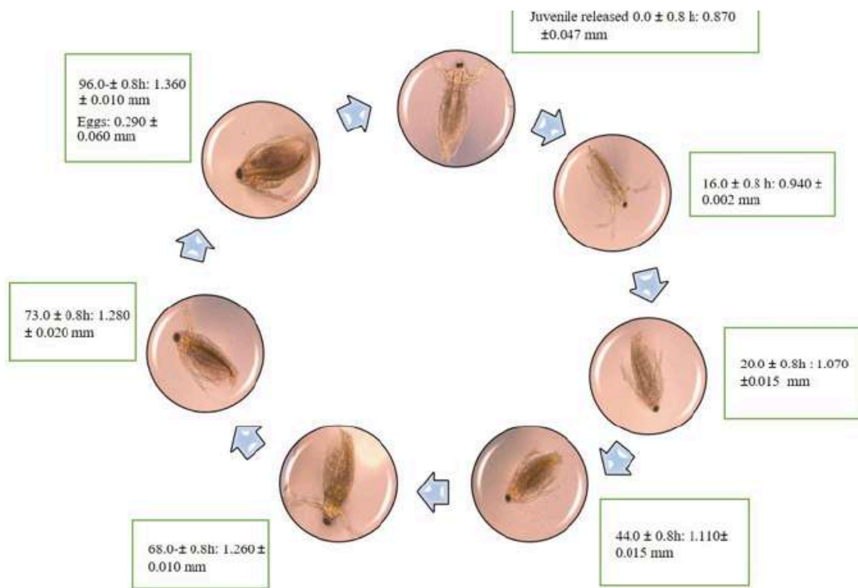

Figure 3. Life cycle of *Diaphanosoma celebensis* observe from 0 to 96 hour. Magnification of 40x

Table 1. The average length of *Diaphanosoma celebensis* correspond with age of the instar in hours.

AGE (hour)	LENGTH (mm)	RANGE
1	0.868 ± 0.047	0.773 - 0.934
3	0.955 ± 0.019	0.936 - 0.973
6	0.917 ± 0.056	0.805 - 0.983
9	0.986 ± 0.009	0.977 - 0.995
24	1.036 ± 0.049	0.937 - 1.09
26	1.085 ± 0.005	1.08 - 1.09
30	1.065 ± 0.050	0.964 - 1.12
48	1.227 ± 0.056	1.12 - 1.31
70	1.383 ±0.043	1.31 - 1.46

Discussion

Embryo development

First juvenile released is invisible to naked eyes maybe because of no food in gut. However, Herzig (1984) stated that the gut of *Diaphanosoma neonates* is filled with yolk. On terms of final molt of neonates after released, some previous studies did not observe this including Parejko, 1992 and these cases are also supported by Pavlova (1959) which he did not observe this molt. However, compared with studies from Kotov and Boikova (1998), since the authors found the molt occur after some minutes right after released from brood pouch, they proposed a completely new scheme of embryogenesis of Diaphanosoma based on recognition of instar, separated by molt as it accepted for Cladocerans. In their study, they used two types of Cladocerans: *D. brachyurum* and *Sida crystallina*. Both molts do not vary in time that can be compared with *D. celebensis*. The authors also proposed the entire process of embryological development can be classified into several staged.

The first instar was from the presence of the egg mass into the brood pouch to shedding of the outer egg membrane, with two phases, early embryogenesis and the beginning of later embryogenesis. The instar continues for 15 h in *D. celebensis*. The second instar started when the formation short rudiments of second antennae extending to the first and the entire body closely surrounded by rudiment of carapace. During this stage, there were many processes occur like the formation of the row of hexagon, carapace extends to thoracic limb, rudiments of claws and post abdominal setae became more visible. These phases typically take 18 h. Third instar where the second antennae are free and movable include heartbeat and gut peristalsis, while the thoracic limbs and post abdomen are enclosed in carapace. The fourth instar where more developed embryos have longer second antennae and larger eyes. Instar duration is 16 h in *D. celebensis*.

In this study, timing for embryonic development of *D. celebensis* was 50 h compared to *D. brachyurum* which in same family (Kotov and Boikova, 1998) is quite similar to their timing was 52 h from presence of egg mass to juvenile released. They used in – vitro method which living embryos were removed from the female brood pouch. In this study, removal of the embryos was done several times during early embryogenesis but often lead to death so the study focuses on the observing of embryo from brood pouch.

Life cycle and growth rate

In his 1994 study, Pandian explored the parthenogenetic reproduction of numerous marine Cladocera species. *D. celebensis* and *D. brachyurum* are both members of the Sididae family, and their morphologies are comparable. The Cladocerans were characterised by Kotov and Boikova (1998) as having post abdominal claws that are elongated and six pairs of leaf-like trunk limbs contained within a carapace. Moreover, *D. celebensis* has a carapace that protects six pairs of appendages. Seawater with a salinity filter that was used throughout the experiment ranged from 15 to 18 ppt (Park, J. C., & Park, H. G. 2010). The impact of salinity on parthenogenetic reproduction or survival rate has been the subject of numerous investigations. According to one of the researches, done by Achuthankutty et al. in 2000, females raised in lower salinity environments had longer life spans, more and larger offspring, and higher neonatal weights. Salinity was the only variable component in the study, and as a result, it affected both growth and the birth of neonates. This may help to explain why, in contrast to this experiment, the size of the first juvenile instar was considerably greater (0.87 mm by the first hour).

Furthermore, maturation of neonates into parthenogenetic females is probably an indication of the availability of an optimum quantity of food, because parthenogenetic reproduction in tropical cladocerans is normally triggered by the availability of food (Vijayalakshmi and Venugopal, 1972). This statement also proven by Provasoli et al., 1970, that stated about rate of neonate's production influenced by the type of alga and cell concentration. Throughout the experiment, *D. celebensis* was still being fed with *Nannochloropsis oculata* so the females still can produce neonates. Thus, in order to manage D. celebensis reproduction, food quality and quantity are crucial parameters. Microalgae quality was also cited by Sipau'ba-Tavares and Pereira (2008) as a key determinant of zooplankton development, growth, and reproduction. Further research on the benefits of enriching *D. celebensis* with the proper microalgae is advised.

Future study

Researching *Diaphanosoma* sp. as a substitute for *Artemia* in aquaculture is a promising avenue. *Artemia*, though widely used, can be expensive and environmentally unsustainable due to the high demand for its cysts. Potential future works such as:

1. **Nutritional Comparison:** Evaluation of *Diaphanosoma* sp. and *Artemia* in terms of protein content, lipid composition, vitamins, and minerals. Understanding the nutritional profile will determine its suitability as a feed substitute.
2. **Growth Performance:** By contrasting growth rates, survival rates, and feed conversion ratios with those fed diets containing *Artemia*.
3. **Digestibility:** Analysing the target species ability to absorb nutrients and energy from *Diaphanosoma* spp.
4. **Immunostimulatory Effects:** Determination if aquatic species are subjected to any immunostimulatory effects by *Diaphanosoma* sp. Assess factors like stress tolerance, illness resistance, and immunological response.
5. **Economic Viability:** Ascertain whether employing *Diaphanosoma* sp. in place of *Artemia* is economically feasible, perform a cost-benefit analysis, elements including market demand, production costs, and the accessibility of live feed will also be considered.
6. **Environmental Impact:** Variables such as the utilisation of land and water, energy usage, the production of waste, and greenhouse gas emissions will be considered
7. **Optimization of Culture Techniques:** Improving culture methods to produce *Diaphanosoma* sp. in large quantities. Considering factors such as water quality management, feeding schedule, stocking density, temperature, salinity, and pH.
8. **Feeding Behavior Studies:** Examination of how aquatic animals feeding behaviour when given diets containing *Diaphanosoma* sp., to guarantee acceptability and palatability.
9. **Toxicological Studies:** Making sure that *Diaphanosoma* sp. is free of any dangerous materials or pollutants that might have an unfavourable effect on aquatic life or humans.
10. **Consumer Acceptance:** Evaluation on how well aquatic items made from animals given diets based on *Diaphanosoma* sp. are received by consumers.

Investigating these possibilities will undoubtedly yield important information about *Diaphanosoma* sp. potential as an affordable and sustainable replacement for *Artemia* in live feeds for aquaculture.

Conclusion

Given its quick regeneration and lower juvenile and adult sizes than *Artemia*, *D. celebensis* has a strong chance of becoming a substitute or supplement of the natural live feed that is currently utilised for hatcheries' larval rearing of fish. *D. celebensis* is a species that has the potential to greatly improve the quantity of sea bass produced in hatcheries since its size range is consistent with the amount of zooplankton required by larvae that grow quickly.

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First Report on Tetrodotoxin in Puffer Fish (*Lagocephalus sp.*) Fillet from Fish Landing Jetty, Penang, Malaysia

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Abstract: For decades, puffer fish consumption has been attributed to food poisoning occurrences in Malaysia. In Malaysian waters, puffer fish (*Lagocephalus sp.*) are commonly found, and some species are known to contain the neurotoxin or tetrodotoxin (TTX). The selling of puffer fish fillets in Pulau Pinang, dubbed as crystal fish, was highlighted by local media, and raised concern from the State Health Department, Pulau Pinang as they could pose risks to consumers. This study was carried out to detect TTX in 60 puffer fish fillets (*Lagocephalus sp.*) landed at Batu Maung, Penang fish jetty and filleted by a skilled operator. TTX levels in the puffer fish fillets were carried out using Liquid Chromatography-Mass Spectrophotometry/Mass Spectrophotometry (LC-MS/MS). TTX was detected in 40% of the fillets tested, ranging from 0.14 to 2.14 µg/g. Although the level of TTX is still consider safe to consumed but the finding from this study called for public awareness and education on the potential dangers of consuming this product. Mislabeling of puffer fish fillets as crystal fish should also be prohibited, so that the consumer is not deceived. The jetty authorities should monitor the puffer fish landings closely and ensure accurate identification of the species especially the potentially toxic species of puffer fish. Additionally, puffer fish fillets from *Lagocephalus* sp. should be regularly tested for toxicity.

Keywords: Awareness, LC-MS/MS, Neurotoxin, Skilled person, Tetraodontidae, Toxic species

Abstrak: Sejak beberapa dekad, penggunaan ikan buntal telah dikaitkan dengan kejadian keracunan makanan di Malaysia. Di perairan Malaysia, ikan buntal (*Lagocephalus* sp.) biasa ditemui, dan beberapa spesies diketahui mengandungi neurotoksin atau tetrodotoxin (TTX). Penjualan filet ikan buntal di Pulau Pinang, digelar sebagai ikan kristal, diketengahkan oleh media tempatan, dan menimbulkan kebimbangan Jabatan Kesihatan Negeri Pulau Pinang kerana ia boleh mendatangkan risiko kepada pengguna. Kajian ini dijalankan untuk mengesan TTX dalam 60 filet ikan buntal (*Lagocephalus* sp.) yang didaratkan di jeti ikan Batu Maung, Pulau Pinang dan disiang oleh pekerja yang mahir. Tahap TTX dalam filet ikan buntal telah ditentukan menggunakan Kromatografi Cecair-Spektrofotometri Jisim/Spektrofotometri Jisim (LC-MS/MS). TTX dikesan dalam 40% daripada fillet yang diuji, dalam julat antara 0.14 hingga 2.14 µg/g. Walaupun paras TTX yang dikesan masih dianggap selamat untuk dimakan, namun penemuan daripada kajian ini dapat memberi kesedaran dan pendidikan kepada orang ramai tentang potensi bahaya pengambilan produk ini. Salah pelabelan filet ikan buntal sebagai ikan kristal juga harus dilarang, supaya pengguna tidak tertipu. Pihak berkuasa jeti perlu memantau pendaratan ikan buntal dengan teliti dan memastikan pengecaman tepat spesies tersebut terutamanya spesies ikan buntal yang berpotensi toksik. Selain itu, filet ikan buntal daripada *Lagocephalus* sp. harus kerap diuji ketoksikannya.

Introduction

Tetrodotoxin (TTX) is a strong and powerful marine toxin (Hwang and Noguchi, 2007), as well as TTX analogues commonly associated with puffer fish or known as ikan buntal in Malaysia. Puffer fish is also known by different names throughout different countries, such as *fugu* (Japan), *bok* (Korea), *buntek* (Indonesia), *ca noc* (Vietnam), *trey kampot* (Cambodia), *pa pao* (Lao PDR) and *pla pakpao* (Thailand) (Barlow, 2004; Cohen et al., 2009; Lorenzetti, 2015). TTX is a potent sodium channel inhibitor and it affects the transmission of signals from nerves to muscles, causes paralysis and could be fatal (Hwang and Noguchi, 2007). TTX is heat resistant and hence cannot be eliminated by freezing, cooking, or washing (Noguchi and Ebesu, 2001). There is also no known antidote for TTX and management of intoxication relies upon appropriate supportive care, including respiratory support measures (Bucciarelli et al., 2021; Bane *et al*., 2014; Noguchi and Ebesu, 2001). Hence, puffer fish consumption may lead to fatalities, as regularly reported in Japan, Thailand and China, where they are commonly consumed (Noguchi and Ebesu, 2001). Puffer fish could be found in rivers, mangroves and coastal waters of tropical and subtropical regions of Atlantic, Indian and Pacific oceans. There are approximately 196 species and 27 genera of puffer fish in the Tetraodontidae family (Froese and Pauly, 2018). With reference to Froese and Pauly (2018) list, approximately 37 of the species were recorded in Malaysian Waters (Ambak et al., 2010) and the exact number at present could be higher than that.

According to fisheries data, the total landings of puffer fish in Malaysia in 2020 were approximately 1337 tonnes, with the highest landings occurring in Perak (804 tonnes), followed by Sarawak (228 tonnes) and Sabah (192 tonnes) (Department of Fisheries (DOF), 2020). Generally, puffer fish are captured as bycatch from trawls or gill nets. *Lagocephalus lunaris* (Bloch & Schneider, 1801) (Green rough-backed puffer, buntal-pisang kasar), *L. spadiceus* (Richardson, 1845)(Half-smooth golden pufferfish, buntal-pisang muda), *L. sceleratus* (Gmelin, 1789)(Silverside blaasop, buntal-pisang kerisi) and *L. wheeleri* (Abe, Tabeta and Kitahama, 1984) (pufferfish, buntal-pisang emas) are the most common species caught. Another significant species, *Xenopterus naritus* (Richardson, 1848)(Yellow puffer fish, buntal kuning), is exclusive to the estuary of Sarawak, where it is traditionally consumed and regarded as a delicacy. It has good protein resources. The puffer fish muscle contains 79.97% moisture (wet weight basis), 88.22% protein, 5.08% ash, 0.25% fibre and 0.47% fat (dry weight basis) (Mohd Nor Azman et al., 2018). Various pufferfish products were available for consumers especially in Sarawak. Most of them are whole fresh fish, cooked (ready-to-eat), dried (whole), salted (eggs), smoked (whole) and also fermented (whole) (Mohd Nor Azman, A. 2017).

TTX poisoning is not common but occasionally reported in Malaysia. Various incidences of intoxication related to the consumption of puffer fish contaminated with TTX have been reported in Malaysia since 1985. Puffer fish poisoning cases have been reported in Terengganu, Johor, Sabah and Sarawak from 1985 to 2022 but no case was reported between 2009 and 2020, and some of them resulted in fatalities (Table 1). The toxic species involved were mostly *Lagocephalus* sp. and *X. naritus* (Chua and Chew, 2006; Zolkepli, 2008; Murali, 2009). Most of the cases, the victims or their family members caught and prepared the fish implicated without knowledge or experience to handle pufferfish. In addition, they also consumed inedible parts such as liver, ovary and skin. Several poisoning cases were also reported due to the consumption of the flesh.

Table 1. Cases of puffer fish poisoning in Malaysia (1985-2022).

Year	Cases (Fatalities)	Location	Reference
1985	4 cases (1 death)	Sabah	Lyn (1985)
1987	18 cases (9 death)	Sabah	Kan *et al*. (1987)
1997	1 case	Terengganu	Loke and Tan (1997)
2008	34 cases	Johor	Chua and Chew (2009)
2009	5 cases (1 death)	Terengganu	Muraili (2009)
	3 cases (1 death)	Sarawak	Man *et al*. (2009)
	6 cases (1 death)	Sabah	
2021	2 cases	Selangor	Pers comm.
2022	2 cases	Johor	Pers comm.

In 2017, the Penang State Health Department is concerned about the possibility of puffer fish fillets being sold under a different name and is alerted to this possibility. This concern is supported by statistics from the Department of Fisheries (DOF) that indicate consistent, albeit insignificant, puffer fish landings in Penang. In 2015, approximately 45 tonnes of puffer were landed, followed by 218, 111, 183, 82 and 108 tonnes in 2016, 2017, 2018, 2019 and 2020, respectively (DOF, 2015; 2016; 2017; 2018; 2019; 2020). A trained fisherman with more than ten years of experience fillets the fish and sells it as "crystal fish" to seafood restaurants and wet markets in Penang. If the puffer fish contains a significant amount of TTX, it may pose a public health threat. Since little is known about TTX in Peninsular Malaysia puffer fish, this brief study was conducted to detect TTX in fillets of puffer fish (*Lagocephalus* sp.) from the Batu Maung fish jetty.

Materials and Methods

Specimen collection

This study utilized 60 pieces of fillets from 30 freshly landed specimens of puffer fish (*Lagocephalus* sp.) captured in May 2018 from Penang waters and landed at Batu Maung, Penang fish jetty. A health inspector from the Penang State Health Department Office collected the samples. The samples were weighed and their standard length was measured. The samples were identified based on their morphological characteristics prior to their filleting by a trained individual (Lim and Gambang, 2009; Froese and Pauly, 2018). Sixty fillets were produced, and the samples were transported to the Fisheries Research Institute on ice for further analysis.

Tetrodotoxin (TTX) standard

The TTX standard (1 mg, > 98% purity) was obtained from Groupe Biomedix, Malaysia (Ascent Scientific, Japan; Batch number APN09032-1-1). The stock solutions of TTX were diluted with 0.03 M acetic acid to produce standard solutions (0.2-1000.0 ng/mL or ppb)(Fisher Scientific, UK) and stored at -20 °C. Reference material for 4-epiTTX and 4,9-anhydroTTX is not commercially available, so the concentration of TTX analogues was determined using the TTX calibration equation. Using the conventional linear TTX within the range of 0.2-1000 ng/mL, a calibration curve was generated for monitoring TTX at 320 Da in the total ion current (TIC) mode ($y = 484.152x$, $r2 = 0.9963$) (Fig. 1). The limit of detection (LOD) and the limit of quantification (LOQ) in this study were estimated based on the signal-to-noise ratio (S/N) of reference solution (Table 2.).

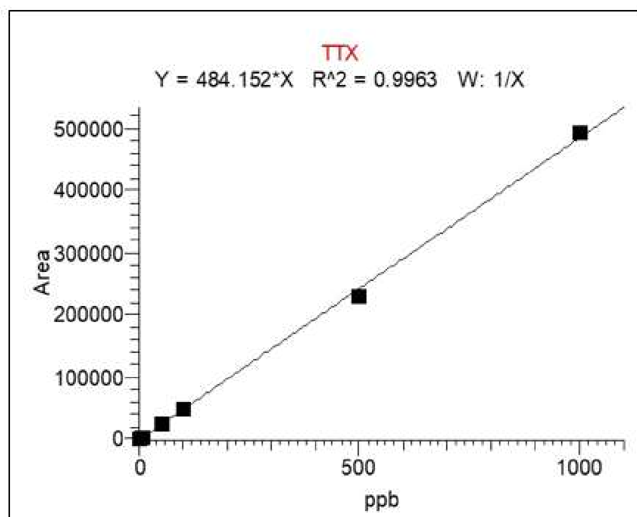


Figure 1. Calibration curve of TTX.

Table 2. Retention time, LOD (S/N > 3) and LOQ (S/N > 10) for TTX analysed by LC-MS/MS.

Toxin	m/z > m/z	Retention time (min)	LOD (ng on column)	LOQ (ng on column)
TTX	320 > 162	3.99	0.05	0.25

Sample preparation and extraction

A small portion (2.0±0.1 g) of each fillet was extracted with an equal volume of 0.1% acetic acid in triplicate. The samples were then homogenised for three minutes using an ultrasonic probe (OMNI-Ruptor 4000, Georgia, United States). TTX was extracted by heating in a boiling water bath (BUCHI B-480, Germany) for 10 min in accordance with the official guidelines of the Japan Food Hygiene Association (2005), with minor modifications (Mohd Nor Azman et al., 2015). After being cooled in slurry ice, the mixture was centrifuged for 15 min at 5,000 rpm (Eppendorf 5430, Hamburg, Germany). Prior to analysis with liquid chromatography, mass spectrophotometry/mass spectrophotometry (LC-MS/MS), the supernatant was filtered with a 0.2 µm nylon membrane filter (Thermo Electron, USA).

LC-MS/MS analysis

For mass spectrometric detection with a TSQ Quantum Discovery MAX (Thermo Electron, USA), an MS Surveyor pump with an autosampler was connected to a mass spectrometer with an electrospray ionisation (ESI) probe that operated in positive mode. LC and MS conditions were carried out according to Diener et al. (2007) and Mohd Nor Azman et al. (2015). The Xcalibur 2.1.0 software was used to perform peak identification, data collecting, and calibration graph plot. TTX was separated using a ZIC-HILIC column (SeQuant, Haltern, Germany) (150 mm × 2.1 mm, 5 µm) with a guard column (20 mm × 2.1 mm, 5 µm) (SeQuant, Haltern, Germany). In water, mobile phase A contained 10 mM ammonium formate (NH_4COOH) and 10 mM formic acid (CH_2O_2), while in acetonitrile/water (ACN/H_2O) (8: 20, v/v), mobile phase B contained 5 mM NH_4COOH and 2 mM CH_2O_2. The flow rate for gradient elution was 250 µL/min. The gradient programme began at 100% B and fell linearly to 65% B in 0.1 min. It remained at 65% B for 7.0 min before returning to 100% B after 3.0 min. Prior to the second injection, there was a 20-min equilibration period (Mohd Nor Azman et al., 2015).

Statistical analysis

SPSS (Statistical Package for the Social Sciences) version 16.0 for Windows was used to analyse the data. The data were reported as the mean and standard deviation in triplicates.

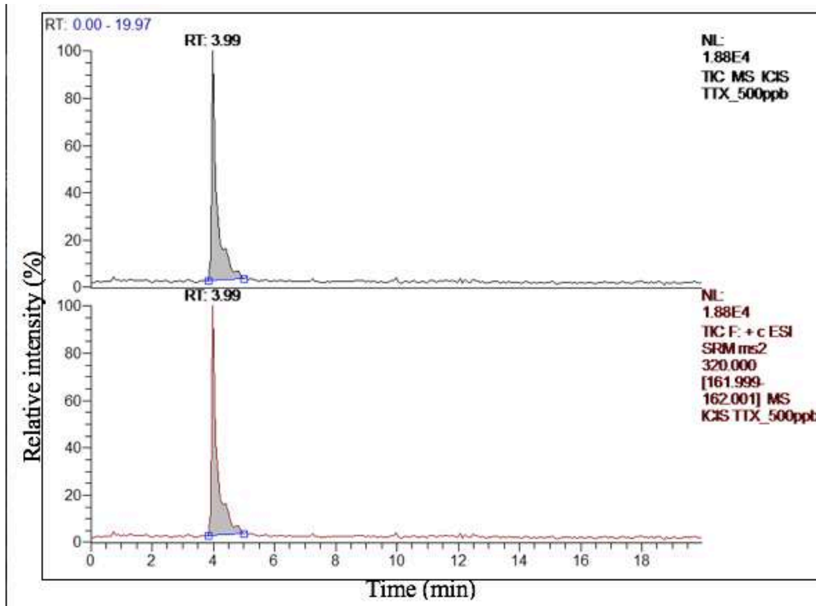
Results and Discussion

A total of 30 specimens with a mean total length of 16.2±2.2 cm and a mean body weight of 122.3±52.1 g was used in this study. The specimens were identified as *L. lunaris* (Green rough-back puffer) or locally known as buntal pisang kasar based on the distribution patterns of small spines on their dorsal bodies. (Fig.e 2). This species has an oval form that spreads to the dorsal fin ray base (Ngy et al., 2008).

Figure 2. Elliptical shape of small spines on the dorsal body (red oval line) of puffer fish *L. lunaris*.

Fig. 3 depicts the results of the TTX concentration detection in *L. lunaris* fillets. The mass chromatogram for the TTX standard solution was scanned at m/z 320 and a retention time of 3.99 min was recorded. The *L. lunaris* fillet extracts examined exhibited a peak at 3.99 min retention time, consistent with the standard TTX concentration (Figure 3a). 40% of the fillets contained TTX concentrations between 0.14 μg/g (0.64 MU/g) and 2.14 μg/g (9.73 MU/g). However, the concentrations were below the Japanese regulatory limit (Japanese Food Hygiene Association, 2005) recommended safety limit (2.20 μg/g or 10 MU/g) and were classified as non-toxic. The toxicity score was classified as 10-100 MU/g (2.20-22.0 μg/g) for weak toxicity, 100-1000 MU/g (22.0-220 μg/g) for moderate toxicity, and over 1,000 MU/g (220 μg/g) for high toxicity. (Noguchi and Arakawa, 2008). Only one sample recorded 2.14 μg/g (9.73 MU/g), which is extremely close to the recommended safety limit (Figure 4).

(a) TTX standard



(b) Fillet of *Lagocephalus lunaris*

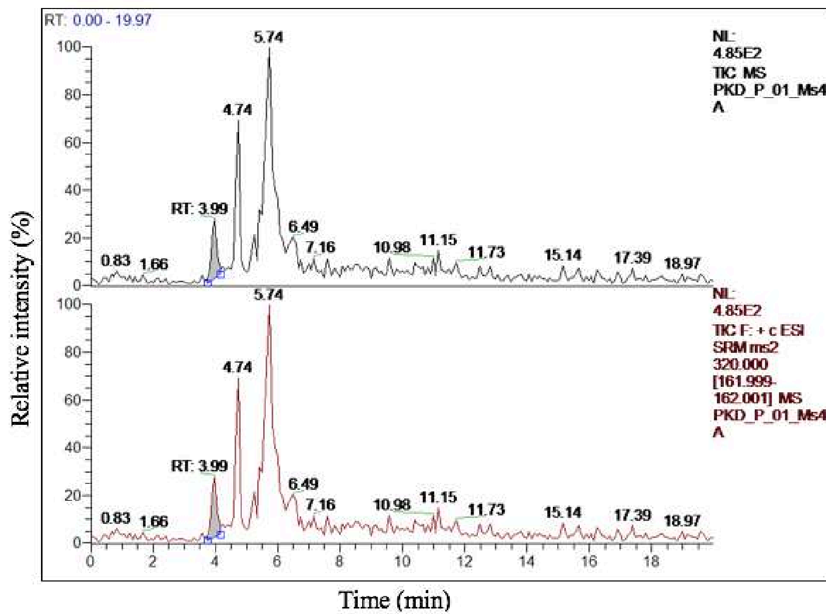


Figure 3. Full scan Total Ion Current (TIC) and Selected Reaction Monitoring (SRM) chromatography of TTX analysed by LC-MS/MS in (a) TTX standard and (b) fillet of *L. lunaris*.

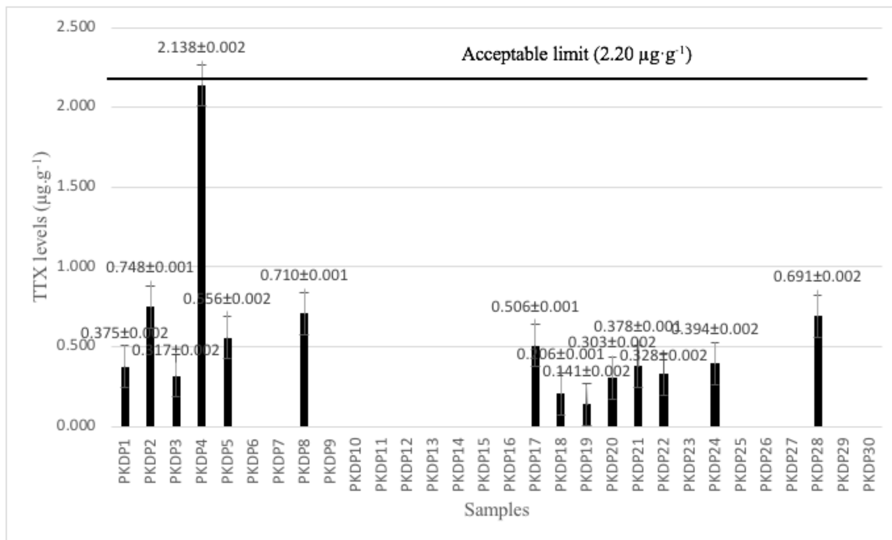


Figure 4. Mean±SD TTX concentration (µg/g) in the fillets of *L. lunaris*. The black line indicates the level of TTX at 2.2 µg/g (10 MU/g), the limit of toxicity in the fillet.

The situation in which puffer fish were sold as fillets in Penang is not unique. Hwang and Noguchi (2007) also reported incidents in which large quantities of puffer fish from the genus *Lagocephalus* were landed, processed into fillets, and sold on the local market. This is interpreted as the fishermen's desire to maximise profits by selling all of their catch. In addition, puffer fish have a high protein content and a favourable fatty acid profile (Aydin et al., 2013). However, not all puffer fish is poisonous. *L. spadiceus*, for instance, is a non-toxic species (Berry and Hassan, 1973; Kungsuwan, 1994; Brillantes et al., 2003; Man et al., 2010; Mohd Din and Mohamad, 2011) and was used as raw material for fish balls in Thailand's processing factories (Brillantes et al., 2003). In Taiwan, the non-toxic puffer fish *L. gloveri* is utilised as a food source (Chen et al., 2002).

In this study, distinguishing between *Lagocephalus* species is quite difficult. This statement was also mentioned by Ngy et al. (2008) when differentiating *L. lunaris* from other *Lagocephalus* species (*L. spadiceus*, *L. wheeleri*) because their external morphology is very similar and they are frequently captured together. Nevertheless, accurate identification of *L. lunaris*, *L. wheeleri* and *L. spadiceus* is essential for public health purposes (Ngy et al., 2008). In Japan and Taiwan, over ten cases of poisoning have been caused by consuming *L. lunaris* misidentified as a non-toxic *L. wheeleri* or *L. spadiceus* have been reported, with one fatality (Noguchi and Ebesu, 2001; Hwang et al., 2002; How et al., 2003).

In the present study, TTX was detected in 40% of the fillets between 0.14 µg/g (0.64 MU/g) and 2.14 µg/g (9.73 MU/g). Only one sample recorded 2.14 g/g, which is extremely close to the recommended level (2.20 µg/g) (10 MU/g). Why is detection present in only 40 % of samples? As previously reported, puffer fish acquire TTX from TTX-bearing organisms (i.e., their prey), which include bacteria, starfish, gastropods, crustaceans, flatworms, and ribbon worms (Noguchi et al., 2006a). The various levels of TTX detected in puffer fish may be attributable to the diverse diets consumed in various regions or habitats (Mohd Nor Azman et al., 2014). Furthermore, it has been demonstrated that the toxicity of puffer fish varies significantly between individuals, types of tissue, sex, maturity stage, regions, and seasons

in which they were caught (Kungsuwan, 1994; Nagashima et al., 2001; Yu and Yu, 2002; El-Sayed et al., 2003; Noguchi et al., 2006b; Rodriguez et al., 2012; Christidis et al., 2021). The findings of this study have shown several possibilities. First and foremost, species caught in Penang waters during the summer (May) have low TTX concentrations. Secondly, the individual who handled the puffer fish was well-trained, as there was minimal TTX contamination (if any) from the skin, liver, intestines, and gonads into the flesh. In addition, previous studies demonstrated a low concentration of TTX in the flesh of puffer fish prepared by an expert at two locations in Sarawak (Mohd Nor Azman et al., 2015). Thirdly, our observation is consistent with that of other researchers who found the highest concentrations of TTX in the puffer fish's gonads, liver, intestines, and skin and not in its flesh or muscle (El-Sayed et al., 2003; Noguchi et al., 2006a; Chua and Chew, 2009). Despite the fact that numerous studies report high TTX levels in puffer fish muscle (El-Sayed et al., 2003; Noguchi et al., 2006a; Helbig and Luckas, 2010), the high levels of TTX in the flesh were migrated from the highly toxic skin (Oshiro et al., 2021). In addition, the species examined in this study (*L. lunaris*) was found to have the lowest TTX concentration (4.92 g/g) when compared to other *Lagocephalus* species, such as *L. sceleratus* (18.40 g/g) and *L. inermis* (62.70 g/g) (Mohd Nor Azman et al., 2014).

Although the results of this study indicated that the fillets were classified as non-toxic based on the Japanese regulatory limit, they are still dangerous to consume, particularly if more than 100 g of the puffer fish fillet is consumed. This is because the severity of TTX poisoning is dependent on the amount taken. The least fatal oral dose and minimum acute dose of TTX for 50 kg individuals are approximately 2 mg (10,000 MU) and 0.2 mg (1,000 MU), respectively (Katikou et al., 2009). However, it can differ depending on age, health, and toxin susceptibility (Cohen et al., 2009). Since the reference material for 4-epiTTX and 4,9-anhydroTTX was not determined in this study, some peaks detected in the samples might be the TTX analogues. These TTX analogues could contribute to the toxicity of the sample.

TTX-containing puffer fish may pose a concern to public health. However, this fish is consumed in many Asian (China, Japan, Taiwan, Thailand, Malaysia) and Mediterranean (Turkey, Egypt, Israel, and Lebanon) countries (El-Sayed et al., 2003; Chamandi et al., 2009; Aydin, 2011) countries as well as other regions of the world. As TTX is predominantly deposited in the liver, ovaries, intestines, and skin of puffer fish (Kao, 1966; Noguchi et al., 2006a), the Japanese Ministry of Health, Labour, and Welfare has banned the consumption of puffer fish liver, ovary, intestines, and skin (Arakawa et al., 2010). However, the flesh of numerous toxic species is edible (Mahmud et al., 2001). It is safe for human consumption if all toxic parts have been eliminated. Therefore, only trained chefs are permitted to cook it. The leading causes of TTX poisoning in Japan have been identified as unlicensed chefs and unqualified persons (Isbister and Kiernan, 2005). Preparing puffer fish necessitates skills and competencies to assure its safety for consumption. However, food poisoning due to eating of puffer fish still occur regularly. In Japan, only licensed and certified chefs are permitted by law to cook or serve puffer fish to consumers (Cohen et al., 2009). In addition, the Japanese Ministry of Health, Labor, and Welfare published recommendations regarding the consumption of puffer fish. In Egypt, puffer fish are prohibited from commercial sale. However, locals consume them due to their abundance along the Red Sea and Gulf of Suez. Due of their low cost, they can still be seen in fish markets. *L. sceleratus* is considered among the best delicious seafood in Suez. Additionally, it has therapeutic qualities, especially as a back pain reliever (Sabrah et al., 2006). The restrictions regarding the sale and consumption of puffer fish in a variety of nations are presented in Table 3.

Table 3. Regulations regarding sale and consumption of puffer fish in several countries (modified from Anon, 2014).

Country	Regulation	References
European Union	Fishery products derived from poisonous fish, belonging to the Tetraodontidae, Molidae, Diondontidae and Canthigasteridae families, may not be placed on the market.	European Commission Directives 853/2004 (EC, 2004)
Turkey	Prohibited the fishing and the sale of puffer fish (Tetraodontidae)	Aydin et al. (2013)
Japan	• Specifies which parts of 21 species of puffer fish can be ingested. • Specific harvesting guidelines for certain pufferfish. • Specific training on handling puffer fish, registration requirements for chefs, requirements for restaurants and regulation on farming of puffer fish.	Noguchi and Arakawa (2008)
China	China's FDA forbade the food industry from processing puffer fish, despite the fact that puffer fish is produced for export.	CHP (2015)
Taiwan	Fishermen should not capture or farm puffer fish to supply food processing and catering as ingredients.	CHP (2015)
Canada	Importation of puffer fish of the family Tetraodontidae is strictly prohibited	Hwang et al. (2002)
New Zealand	The presence of tetrodotoxin is assessed in puffer fish, which is a "prescribed food." (i.e. presence of tetrodotoxin would result in a failed import and would not be permitted to import into New Zealand).	MPI (2012)
Singapore	• The import of live puffer fish is no longer allowed • All puffer fish products from Japan (muscle meat from wild and farmed puffer fish and farmed puffer fish fins, skin and milt) can only be imported from Singapore Food Agency-accredited sources.	SFA (2022)
United States	• When necessary, precautions are taken to ensure that the fish captured are free of toxins, or when they are treated to remove the toxins, puffer fish can be ingested safely. • The Food and Drug Administration (FDA) advises restaurants and fish markets that serve or sell puffer fish (also known as puffer, fugu, bok, blowfish, globefish, swellfish, balloonfish, or sea squab) not to purchase or sell this product unless it originates from a proven safe source.	USFDA (2007a) USFDA (2007b)

Currently, no specific license or permit is required for the sale, preparation or handling of puffer fish by the authority in Malaysia. In the Malaysia Food Act (1983), Act 281, and Fisheries Development Act (1971), Act 49, however, a general clause prohibits the sale and preparation of any food that is poisonous, harmful, or injurious to health. There are no written regulations in Malaysia regarding how to cook, store, or consume puffer fish. However, Sarawak residents frequently consume fresh and dried puffer fish and eggs (Parvaneh et al., 2012).

Conclusion

Our investigation revealed TTX was detected in 40% of the puffer fish fillet samples obtained from Batu Maung, Penang fish jetty, however, the levels are below the recommended safety limit. Even then, the toxicity is subjected to many factors such as types of species, tissues, sex, maturity stage, regions, and seasons. Therefore, the health risk is potentially

there. Since there is no specific regulation on this, education and awareness are vital to alert the consumers on the potential harms. Mislabelling of puffer fish fillet as crystal fish should be reprimanded, so that consumers are not deceived. On the other hand, the landing port authorities need to monitor the puffer fish landings closely, ensuring accurate identification of the species, especially the potentially toxic species. Samples of fillets ought to be sent for toxicity determination from time to time. Since the toxic and non-toxic puffer fish are challenging to differentiate, it would be good if the trained personnel that prepare the fish could also learn to identify the toxic and non-toxic fish so as to minimise the risk.

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INSTRUCTION TO AUTHORS

MALAYSIAN FISHERIES JOURNAL

Aim and Scope

The journal seeks to provide a forum for dissemination of research findings in all aspects of fisheries science. Manuscripts describing research work relevant to local communities are most welcome to aid in the advancement of sustainable fisheries. The standardized format set below is an adaption from some international journal.

Submission of Papers

A paper is considered for publication on the understanding that:

- It reports original unpublished work
- It is approved by all named authors
- It does not contain tables and figures that have been published elsewhere
- All acceptable manuscripts will be reviewed by the Publication Committee
- Acknowledgement and action on each point raised by the reviewer will be requested from the author if the manuscripts to be accepted

Different type of Submissions

1. Full length paper

These should describe new and carefully confirmed findings and experimental procedures that should be given in sufficient detail for others to verify work. The length of a full paper should be the minimum required to describe and interpret the work clearly. The paper should comprise the following sections: (a) Abstract; (b) Introduction; (c) Materials and Methods; (d) Results; (e) Discussion; (f) Acknowledgement; (g) References; (h) Tables; (i) Legends to figures; (j) Figures. The results and discussion section may be combined.

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A Short Communication is suitable for recording the results of complete small investigation or giving details of new methods, techniques or apparatus, not more than 3000 words. The style of main sections need not conform to that of full-length papers. Progress reports are not acceptable.

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Short Notes are one to two printed pages in length. They are suitable for reports of simple findings such as properties of an already well-described enzyme or of observations not requiring elaboration. They should be written with a short summary, with no main sub-division, may contain one table or figure, or two if the text is brief and no more than three references.

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These are reports of processes or procedures which may be published as an annex to a full length of paper or on their own provided that the work is of sufficient interests to other workers in the field.

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These should be centered on current issues that are of interests to all. The length of the paper is between 6000 – 10000 words. The references must be more than 30.

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ii. Up to six keywords or phrases

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It should include key references to appropriate works and up-to-date primary literature. The rationale of the research undertaken should be explained. The introduction should clearly state the aims and objectives of the paper.

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Materials and Methods should be described in sufficient detail to allow the work to be repeated. Specify and describe the study site and test animals where appropriate. Sub headings are used to itemize the main parts. Materials and methods should be written in the past tense either in active of passive voice. In this section, study dates, number of subjects, groups, evaluation criteria, exclusion criteria and statistical methods should be described sequentially. The origin of materials and/or suppliers of equipment should be named if necessary.

Results and Discussion

The sections may be separated, though authors may find it's easier to combine them. Use tables or graphs as appropriate but do not repeat information in the text. The reproducibility of the findings must be clearly stated, the number of times the experiment was conducted, the number of replicate samples, etc., should be stated. Statistical analysis of results must specify the procedure being used with a reference given. If results are given as a percentage of a control value, the 100% should be given.

Discussion should provide the explanation and interpretation of results or findings by comparing with the prior studies. It should bring out those essential points of the work, the implications and practical significance of the findings, their limitation and relevance to previous studies. It should not be a recapitulation of the results.

References

References in text shoul be cited as: Smith (1993) or (Smith, 1993). Two authors as: Smith and Brown (1993) or (Smith and Brown, 1993). Three or more authors as: Smith et al. (1993) or (Smith et al., 1993). A series of references should be appearing in chronological order, e.g., White and Black 1991; Black and White 1992. References to papers by the same authors in the same year are distinguished by letter a, b, etc. (e.g., 1989a, or 1991a, b). Publications having no obvious authors are cited as Anon. (1990) in the text and bibliography. References with 4 and more authors should be written as: Abbasi, A. A., Paparidis, Z., Malik, S., Goode, D. K., et al. (2007). References to grey literature such as in-house reports, contract reports and non-referred papers are not appropriate and should be avoided. At the end of papers, References are listed in alphabetical order by the first word in the reference (usually the author's last name). References with three or more authors should be placed in chronological order after considering of the names of the first and second authors. The author must ensure that references cited in the text agree with those listed in the bibliography. Some sample reference styles follow:

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   Example: Saha, B. C. and/& Zeikus, J. G. (1989). Improve method for preparing high maltose conversion syrup. *Biotechnology and Bioengineering*, **34**, 229-303.

   Example: Debnath, P. P., Delamare-Deboutteville, J., Jansen, M. D., Phiwsaiya, K., et.al (2020). Two-year surveillance of tilapia lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *J Fish Dis*. **43**(11),1381-1389.

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Most edited books state on the cover or title page that they are edited by an author or multiple authors. The format is the same as a print book, except the editor's name is in the author's position. Include a parenthesis afterwards with the abbreviation (Ed.) for an edited book by one author or (Eds.) for an edited book with two or more authors.

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Example:

a) Primrose, G.B. (Ed.). (1987). *Modern Biotechnology*. Blackwell Scientific, Oxford.

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Some edited books contain chapters written by various authors. Use the format below to cite an author's individual chapter in an edited book.

Chapter author's Last name, F. M. (Year published). Title of chapter. In F. M. Last name of Editor (Ed.), Title of book (p. x or pp. x-x). Publisher.

The title of the chapter is not italicized, while the title of the book is. The chapter author's name is reversed at the beginning of the reference, but the editor's name is written in standard order.

Example:

a) Longacre, W. A., and Ayres, J. E. (1968). Archeological lessons from an Apache wickiup. In S. R. Binford and L. R. Binford (Eds.), Archeology in cultural systems (pp. 151-160). Blackwell, Oxford, UK.

In the above example, Longacre and Ayers are the authors of the individual chapter and Binford and Binford are the editors of the entire book.

b) Gudding, R. (2014). Vaccination as a preventive measure. In R. Gudding, , A. Lillehaug, and O. Evensen, (Eds.), Fish Vaccination (pp. 12-21). John Wiley & Sons Ltd, West Sussex, UK.

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Conference proceedings published as a whole book follow the same reference format as whole: i) journal, ii) edited books or iii) book chapter

Example:

i. Duckworth, A. L., Quirk, A., Gallop, R., Hoyle, R. H., Kelly, D. R. (2019). Cognitive and noncognitive predictors of success. Proceedings of the National Academy of Sciences, USA, 116(47), 23499–23504. https://doi.org/10.1073/pnas.1910510116
ii. Kushilevitz, E., and Malkin, T. (Eds.). (2016). Lecture notes in computer science: Vol. 9562. Theory of cryptography. Springer. https://doi.org/10.1007/978-3-662-49096-9
iii. Benedel, A. L., Jourdan, L. and Biernacki, C. (2019). Probability estimation by an

adapted genetic algorithm in web insurance. In R. Battiti, M. Brunato, I. Kotsireas & P. Pardalos (Eds.), Lecture notes in computer science: Vol. 11353. Learning and intelligent optimization (pp. 225-240), Springer. https://doi.org/10.1007/978-3-030-05348-2 21

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Brief of appreciation to whom it is due.

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Use only recommended SI Units. Use superscripts presentation (mg mL-1). Below are few examples of abbreviations of the most commonly used SI units:

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Time	Second	s
Time	Minute	min
Electric current	Ampere	A
Area	square meter	m^2
Volume	cubic meter	m^3
Frequency	Hertz	Hz

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Use only recommended SI Units. Use superscripts presentation (e.g: mg mL-1) and common abbreviations such as 'm' for meter, 'kg' for kilogram, 'min' for minute and so on.

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Results should be clear and concise. The discussion should explore the significance of the results of the work. Avoid extensive citations and discussion of published literature. If appropriate, Results can be written in a separate section from Discussion. This especially if the Discussion is extensive and includes all the Results of the study.

Table

Please submit tables as **editable text** and not as images. Tables use double space and 12pt Times New Roman fonts.

Table 1. Use Times New Roman 12 font

Component	Content (%, w/w)
Protien	44.9 ± 0.37
Carbohydrate	22.3 ± 0.94
Water content	13.7 ± 0.02
Ash	6.1 ± 0.19

Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

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Figure should be on the left margin. **Embed the figures in the text** with minimum resolution of 300 dpi. Separate **figure files in JPEG or PNG formats should be supplied together with the article**. Ensure that each figure has a caption. A caption should comprise a brief title (not on the figure itself) and a description of the figure. Keep text in the figure themselves to a minimum but explain all symbols and abbreviations used.

Figure 1. Left: Trap one funnel (1F)

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Graphs must be supplied in figure formats. The fonts of the graph must be clear and readable. Black and white graphs are preferred.

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Reference list

References should be arranged first alphabetically and then further sorted chronologically. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of Results and Discussion section.

Author contribution (Optional)

Please list the contribution of each author here, e.g.: M.I. designed the research and supervised all the process, L.A. collected and analyzed the data and wrote the manuscript.

Acknowledgments

List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proofreading the article, etc.).

Conflict of Interest (Optional)

Please state any conflict of interest regarding the research or the research funding.

References

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