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Brown Banana Leaf (*Musa x paradisiaca*) Improve *Betta splendens* Hatching, Larvae, Survival and Growth Performance While Affecting its Sex Ratio

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ABSTRACT

The effectiveness of brown banana leaf (BBL) *Musa x paradisiaca* as a culture media for rearing *Betta splendens* larvae was investigated. Larvae (n=630, 3 days old, 6 replicates) were reared in 7 rearing medium; C1 (no leaf, control), C2 (0.7 g brown catappa leaf, positive control), T1 (0.7 g; Treatment 1), T2 (0.8 g; Treatment 2), T3 (0.9 g, Treatment 3), T4 (1.0 g, Treatment 4) and T5 (1.1 g, Treatment 5) of BBL per litre of rearing water respectively. Throughout the 80 days trial period, larvae were first fed microworms twice daily for two weeks, then Artemia for another two weeks, followed by ad libitum feeding of commercial feed twice daily. Water quality was monitored every two days, and water was changed every two weeks. The hatching rate, survival, growth parameters and sex ratio were determined after 80 days. There were significant differences ($P<0.05$) in all the parameters observed. The highest hatching and survival rates were found in treatment C2 (95.86%; 95.56%) and T5 (95.63%; 95.56%). For treatment T1, T2, T3 and T4, the hatching (HR) and survival rate (SR) was 85.75±7.31% (HR), 68.89±13.11% (SR); 75.54±2.04% (HR),76.67±15.64% (SR); 89.76±0.587% (HR), 74.44±22.08% (SR) and 92.63±2.23% (HR), 61.11±6.56% (SR) respectively. The lowest percentage of hatching rate was shown by C1 at 68.08±1.089% while the lowest survival rate was shown by T4. The highest specific growth rate (SGR) was T3 (6.00±0.34% day^{-1}) (weight) and T5 (3.45±0.10% day^{-1}) (length). The SGR for C2, TI, T2 and T4 was recorded at 5.837±0.34, 5.857±0.5, 5.81±0.429 and 5.612±0.243 % day^{-1} respectively. The sex ratios were skewed in 2 treatments; T1 (12 male: 19 female) and T5 (19 male: 9 female) with the rest showing no significant difference to the 50:50 (M:F) ratio. Hatching rate, SGR and sex ratio was not recorded for C1 as all larvae reached 100% mortalities after two weeks of the rearing trials. Based on the result, it is safe to conclude that BBL is a good and safe alternative/replacement to catappa leaf for usage in the aquaculture industry.

Keywords: Larvae culture, Ornamental fish, Phytochemicals

INTRODUCTION

Ornamental fish industry is a global multimillion dollar business (dos Anjos *et al*., 2009) worth USD15 to USD30 billion per year (Evers *et al*., 2019). The Siamese fighting fish (*Betta splendens* Regan, 1910) is one of the popular ornamental species among aquarists and hobbyists especially in Southeast Asia. Based on a simple search across online forums and marketplaces, the price of a single *B. splendens* can reach up to USD 30, depending on the variety. There is no reliable source of information on the exact pricing, as it is highly subjective and market driven. However, a study by Rahmatulloh *et al.* (2023) reported that in Palembang, Indonesia, the highest recorded price for *B. splendens* could reach approximately USD 2,000. In Malaysia, it was reported that the trade value of this species reached RM9.4 million in 2023 (BERNAMA, 2024). Despite its long history in scientific study and commercial culture, *B. splendens* still lacks standardized and effective husbandry and larval nutritional protocols, with only limited peer-reviewed literature available on these aspects (Lichak *et al*., 2022 and Murray *et al*., 2024).

The larvae and juveniles of *B. splendens* are known to be very sensitive and fragile. High mortalities can occur when the rearing environment fails to meet the species' water quality requirements (Kajimura *et al*., 2023). Nutrition, pathogen, water quality, and stress are aspect that contributes to larval survival (Herath & Atapaththu, 2012). One of the key factors influencing *B. splendens* culture is water quality, as this species prefers slightly acidic conditions (Watson *et al.*, 2019). Farmers commonly use catappa leaves (*Terminalia catappa*) in the rearing medium as they are considered eco-friendly due to their high content of phenolic compounds and tannins (Annegowda *et al.*, 2010), which lower water pH and create a calmer environment resembling the fish's natural habitat (Ashraf & Bengtson, 2007). In addition, catappa leaves are known for their antimicrobial properties, which help protect fish from pathogens (Chitmanat *et al.*, 2003; Neelavathi *et al.*, 2013). However, since the plant grows mainly in coastal areas (Mallik *et al.*, 2013), farmers and breeders especially those where their farm location are far from coastal areas used brown banana leaf (*Musa spp*.), a much more accessible plant that can be found almost everywhere as an alternative to Catappa leaf in *B. splendens* cultivation.

Banana plant is a species that has many cultivars (Calberto *et al*., 2015), are traded and consumed worldwide. According to Kema *et al.* (2021), in Southeast Asia, banana plants are abundant and being grown in backyards or home gardens for domestic consumption. Aside from its major use as food (banana fruit) for humans and animal farming, several literatures have reported that other portions of this plant have the potential to be utilized in aquaculture as a natural treatment to assist/increase productivity. In Vietnam, farmers used banana leaf from various cultivars as feed for fish cultured in ponds (Dongmeza *et al*., 2009). In Rohu (*Labeo rohita*) aquaculture, banana peels and banana peel flour from *M. acuminata* were given as feed and have showed a positive impact on growth performance, increment in survival rate, and increase immunity against *Aeromonas hydrophila* (Giri *et al*., 2016). There is also a report on the application of banana leaf as substrate in breeding *B. splendens* where the percentage of fry survival is higher compared to rigifoam and polythene (Rajapakshe *et al*., 2020). Till date, no study has been systematically done to explore and optimize the potential of brown banana leaf and ensure the safety and efficiency of its usage in ornamental fish culture despite being used by local fish farmers and breeders for quite some time. Previous studies are mostly focused on the benefits as an ingredient in fish feed but not its usage as a rearing medium especially in *B. splendens* larvae culture. Therefore, this study was carried out to determine the effects of brown banana leaf rearing medium on survivability, growth performance, and sex ratio of *B. splendens* larvae thus confirming whether banana leaf can be a good, safe, and sustainable alternative to Catappa leaf.

MATERIALS & METHODS

Location and Ethics Statement

These experiments were conducted at the Quality and General Analysis Laboratory and the Ornamental Fish Breeding Unit Hatchery of the Faculty of Fisheries and Aquaculture Sciences (FSPA), Universiti Malaysia Terengganu and at a private hatchery in Kampung Sungai Deraka, Kuantan, Pahang. Moral and ethical aspect of the research such as animal handling and minimum number of fish needed for valid statistical analysis complied with the Research Ethics Guidelines of Universiti Malaysia Terengganu (2014).

Sample Preparation

Samples of brown banana leaf (BBL) (*Musa x paradisiaca*) were collected from nearby locations at Kuala Nerus (5.3679° N, 103.0472° E), Terengganu (2 km radius from the university campus). The collected samples were transported to the laboratory for taxonomic verification. The leaves were washed with distilled water to remove dirt and other contaminants before being used. Fertilized eggs and larvae used in this experiment were obtained from routine breeding of FSPA hatchery broodstocks.

Experimental Design

A total of five treatments and two controls (6 replicates each) were set in this study. They were: C1 (no leaf), C2 (0.7 g brown catappa leaf (BCL)), T1 (0.7 g BBL), T2 (0.8 g BBL), T3 (0.9

g BBL), T4 (1.0 g BBL) and T5 (1.1 g BBL) per 1 L of rearing water. The concentrations were determined based on the acute lethal toxicity levels of *Musa* × *paradisiaca* and *T. catappa* extracts on fish, as reported by Yunus *et al.* (2019) and Aswadi (2023). Stock rearing water were prepared by immersing BCL and BBL in leaf form according to the dose of each treatment in freshwater (unchlorinated municipal tap water) for one day prior to usage. This rearing water was then used for the rearing of broodstock and larvae.

To measure the hatching rate in each treatment, 9 pairs of *B. splendens* broodstocks obtained from the hatchery stock were reared in each rearing medium treatment (Total n: 63 pairs, male (3.55±0.06 g in weight; 4.02±0.04 cm in total length) and female (2.10±0.08 g in weight; 3.58±0.06 cm in total length)) using a 2 L plastic aquarium. The aquarium was arranged randomly, and a plastic board were used as a partition to avoid the pairs from seeing other breeding pairs which might influence their breeding behaviour. Observation on the hatching rate was made 48 hours after fertilization of each pairs occurred. Hatching rate (%) was calculated by using the formula: the number of larvae hatched/ number of eggs laid multiplied with 100. Photos of the bubble nest containing eggs was captured and the number of eggs was counted using ImageJ software.

For survival, growth, and sex ratio observation, 630 larvae aged three days after hatch (from the breeding trials) were used in this rearing trial. They were randomly selected and distributed evenly (15 larvae per treatment) into 3 litres plastic tanks each containing the specified amount of BBL and BCL.

For the first two weeks of the experiment, larvae were given microworms that were cultured in the hatchery (twice daily), followed by *Artemia* (Bio-Marine, USA) (twice daily) for the next two weeks. Ad libitum feeding using commercial feed (Sanyu Betta, 30% crude protein) was done twice daily as soon as the larvae reach one month old. Water quality (DO, pH, total ammonia and temperature) were measured every two days and 70% water change was done every two weeks throughout the 80 days rearing trial. The water quality parameters throughout this experiment were maintained as follows: temperature (28 ± 2°C); dissolved oxygen (6.5 ± 0.2 mg/L); pH (7.25 ± 0.30) and total ammonia (0.12 ± 0.1 mg/L) (Norazmi-Lokman *et al*., 2020).

Survival and Growth Rate

Total numbers of mortality in treatments were counted and recorded every day throughout the 80 days of the experiment. The survival rate was calculated by dividing the total number of fish at the end of the experiment with the total number of fish at the start of the experiment and was them multiplied with 100. Growth rate criteria such as total length and total weight were measured during the initial (day 0) and final day (day 80) of the experiment. The fish were starved overnight and anesthetized before weight and length measurements were taken. Length of the fish was measured using standard ruler meanwhile growth was measured using Savio Mini Digital Scale. Then, mean total length, mean weight gain, body weight gain and specific growth rate were calculated using the following formulas (Mandal *et al*., 2010):

a) Mean total length gain (mm)
= Final total length-Initial total length
b) Mean weight gain (g)
= Final body weight- Initial body weight
c) Body weight gain (%)
= [(Final body weight – Initial body weight)/ Initial body weight] x 100
d) Specific growth rate (SGR) length (mm %/day)
= [(Ln (Final length) – Ln (Initial length))/ Number of days] x 100
e) Specific growth rate (SGR) weight (g %/day)
= [(Ln (Final weight) – Ln (Initial weight))/ Number of days] x 100

Sex Ratio

Identification of sex in *B. splendens* for each treatment was carried out by observing commonly known secondary sex characteristics of the species such as their body size, body coloration and the size of fins at the end of the experiment (Day 80) (Table 1).

Table 1. Secondary characteristics in differentiation of male and female

Characteristics	Male	Female
Colour	Brightly coloured	Subdued in colour
Fins	Long and flowing	Short
Body shape	Slim and narrow	Short and wider
Opercular membrane	Large and visible without flaring	Small and only visible during flaring
Ovipositor spot	Do not have	Have egg spot between ventral and anal fins

Data Analysis

Statistical analysis was performed using SPSS software version 25. The results were expressed as mean ± standard deviation (SD). All data taken were subjected to normality test using a Shapiro-Wilk's test and for equality of variance using a Levene's test. Kruskal Wallis H test leading to median post hoc test were run to assess the survival rate meanwhile hatching rate and growth performances were conducted using One Way Analysis of Variance (ANOVA) and followed by Tukey post hoc test. In order to determine the sex ratio significant for each treatment, Chi-Square tests were used. All P values less than 0.05 were considered statistically significant (Laerd, 2022).

RESULTS

Total Hatching Rate

Hatching rates of eggs was significantly different (P<0.05) among treatments at 48 hours after observation (Figure 1). The highest hatching rate was observed in those of C2 (0.7 g/L BCL) (95.86±0.66%) and T5 (1.1 g/L BBL) (95.63±0.74%) while C1 (0.8 g BBL) had the lowest percentage of hatching rate (68.08±1.089%). For treatment T1, T2, T3 and T4, the hatching rate was 85.75±7.31%, 75.54±2.04%, 89.76 ± 0.587% and 92.63 ± 2.23% respectively. The statistical analysis also showed that all treatments containing leaf (C2, T1, T2, T3, T4 and T5) had significantly higher hatching rates compared to treatment without leaf (C1).

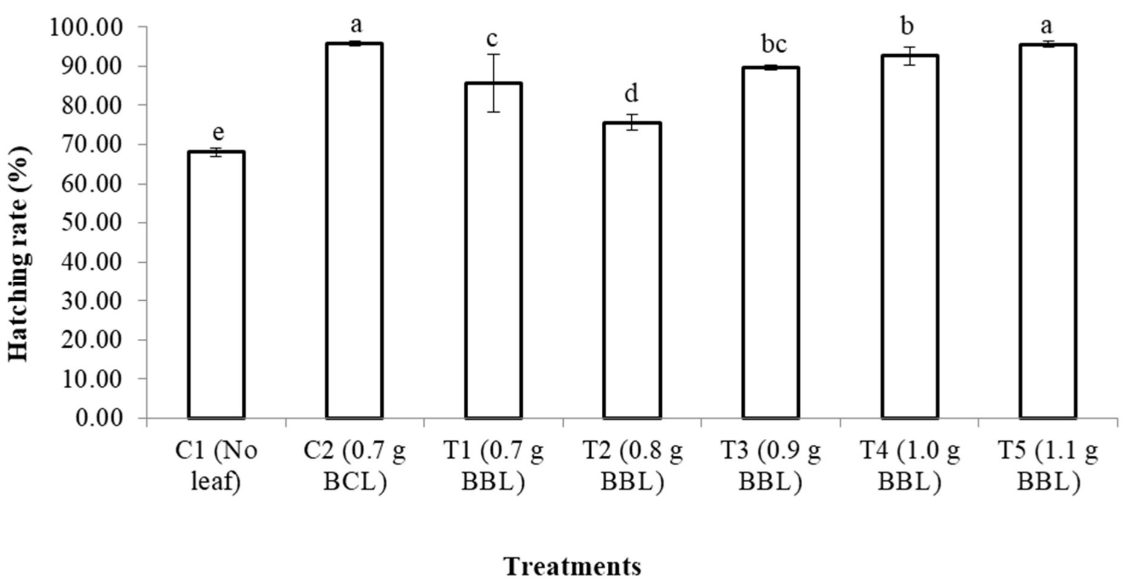

Figure 1. Hatching rates of *Betta splendens* after 48 hours in C1 (control, no leaf), C2 (0.7 g brown Catappa leaf, BCL) as positive control, T1: 0.7 g, T2: 0.8 g, T3: 0.9 g, T4: 1.0 g and T5: 1.1 g of brown banana leaf (BBL). Data are presented as mean ± SD (n= 9). Bars with different letters are significantly different (P < 0.05).

Survival rate and growth performance

The survival rate, SGR (weight and length) of larvae reared in 0.7 g BCL and different weight of BBL after 80 days of experiment is presented in Table 2. After 80 days of the experiment, survival rate and SGR were significantly different ($P<0.05$) among the treatments. However, no result was recorded for C1 (control, no leaf) as all larvae reached 100% mortalities after two weeks of the rearing trials. The results showed that C2 (0.7 g BCL) (95.56%) and T5 (1.1 g BBL) (95.56%) had the highest larval survival. Meanwhile, T4 (1.0 g BBL) (61.11%) had the lowest survival rate compared to other BBL treatments. The obtained result indicated SGR (weight) was significantly lower in larvae reared in T4 (1.0 g of BBL) compared to other treatments. This is in contrast with SGR length where the result showed T4 (1.0 g BBL) and T5 (1.1 g BBL) were the highest meanwhile C2 (0.7 g BCL) was the lowest among treatments.

Table 2. The survival rate and SGR (weight and length) of *Betta splendens* in C2 (0.7 g brown catappa leaf, BCL) as positive control, T1: 0.7 g, T2: 0.8 g, T3; 0.9 g, T4: 1.0 g and T5: 1.1 g of brown banana leaf (BBL) after 80 days of experimental period. Data are expressed as the mean ± SD (n=30). Values with different superscript are significantly different ($P<0.05$).

Treatments	Survival rate (%)	SGR weight (g %/day)	SGR length (mm %/day)
C2 (0.7 g BCL)	95.56 ± 3.44^{a}	5.837 ± 0.341^{a}	3.260 ± 0.232^{a}
T1 (0.7 g BBL)	68.89 ± 13.11^{b}	5.857 ± 0.500^{a}	3.383 ± 0.150^{bc}
T2 (0.8 g BBL)	76.67 ± 15.64^{b}	5.810 ± 0.429^{a}	3.392 ± 0.194^{bc}
T3 (0.9 g BBL)	74.44 ± 22.08^{b}	$5. 997 \pm 0.342^{a}$	3.334 ± 0.151^{ab}
T4 (1.0 g BBL)	61.11 ± 6.56^{b}	5.612 ± 0.243^{b}	3.428 ± 0.102^{c}
T5 (1.1 g BBL)	95.56 ± 3.44^{a}	5.929 ± 0.188^{a}	3.451 ± 0.104^{c}

Sex ratio

The sex ratios in each treatment after 80 days of the rearing trials are as shown in Table 3. In C2 (0.7 g BCL) (positive control), the sex ratio was 50% male and 50% female while a slightly skewed sex ratio was observed in T1 (0.7 g BBL) toward female population with 61.3% female and T5 (1.1 g BBL) toward male population with 67.9% male. For treatments T2, T3, and T4, the proportions of males were 46.4%, 55.2%, and 55.0%, respectively. Nevertheless, chi-square analysis indicated that the sex ratios did not deviate significantly from the expected 1:1 ratio observed in the control group (T2).

Table 3. Sex ratio of *Betta splendens* in 0.7 g brown Catappa leaf (BCL) and different weight of brown banana leaf (BBL) after 80 days of experimental period.

Treatments	Sexual maturity (%)		χ^2	df	P
	Male	Female			
C2 (0.7 g BCL)	50	50			
T1 (0.7 g BBL)	38.7	61.3			
T2 (0.8 g BBL)	46.4	53.6			
T3 (0.9 g BBL)	55.2	44.8			
T4 (1.0 g BBL)	55.9	44.1			
T5 (1.1 g BBL)	67.9	32.1	14.385	5	0.013

DISCUSSION

Evidently it can be seen in this study, the absence of any brown leaf in the rearing water of *B. splendens* larvae leads to the collapse of the culture. After two weeks, all the fish in C1 treatment dies while treatments containing Catappa and banana leaf showed a significant and positive result in terms of hatching, survival and growth rates. A unique observation on the skewness of the sex ratio of fishes reared in water containing BBL was discovered.

The highest hatching rates were observed in C2 (0.7 g BCL, positive control) and T5 (1.1 g BBL). Previous studies have reported that fish egg hatchability increases when the fertilized eggs were treated with plants. For instance, when fennel (*Foeniculum vulgara)* was administered at a dosage of 150mg/kg to convict cichlid (*Cichlasoma nigrofasciatum*), it led to a hatching rate of 92.33±1.63% (Sotoudeh & Yeganeh, 2017). Similarly, the use of ginseng root (2 g/kg) in rainbow trout (*Oncorhynchus mykiss*) resulted in hatching rates of 85.2±7.01 (Kadak *et al*., 2019).

Previous studies of phytochemical in plant showed that hydrolysable tannin had a positive impact in stimulating rohu (*Labeo rohita*) immunological parameters (Prusty *et al*., 2007) and boosted carbohydrate accumulation and digestion in grass carp (*Ctenopharyngodon idellus)* (Yao *et al*., 2019). In striped bass (*Morone saxatilis*) larvae, tannic acid was added into feed which led to increases in growth rates and survival rates (Ashraf & Bengtson, 2007). According to Shirmohammadli *et al*, (2018), the hatching and survival percentage in eggs treated with tannic acid (hydrolysable tannin) was much higher compared to clay suspension treatment and the eggs also showed lower fungal contamination. As phytochemicals (tannins, flavonoids and saponins) are known to be present in the culture water of this study (Aswadi, 2023; Norazmi-Lokman & Aswadi, 2023), it can be said that this was the contributing factor to the excellent performance of survival and hatching rate observed in this study. Nevertheless, further investigation is needed to determine the mechanism of tannin on fertilized eggs and how it contributes to the favourable hatching rate.

Environmental conditions are known to have significant impact on reproduction particularly during the embryonic and larval development stages (Baumgartner *et al,* 2008). Within the realm of environmental factors, pH is a parameter that is routinely observed and assessed, particularly when it comes to the development of eggs and the performance of fish larvae (dos Santos *et al*., 2020). The ideal pH values for the embryonic and larval development stages vary depending on the species. For instance, in Pacu (*Piaractus mesopotamicus*), common carp, (*Cyripinus carpio*) (Sapkale *et al*., 2011), Chum salmon (*Oncorhynchus keta*) (Bell *et al*., 1969) and rainbow trout, (*Oncorhynchus mykiss*) (Hagenmaier, 1974), the optimal pH levels were found to be 9, 7.5, 7.5-8.0, and 6.5 respectively.

The variation in the ideal pH levels is associated with the activity of hatching enzymes, specifically chorionase. This activity is primarily influenced by both the fish species and the water's pH (Bell *et al*., 1969; Hagenmaier, 1974). Fertilized fish eggs possess a chorion and plasma membrane that together create a protective and resilient barrier (egg hardening). This barrier serves to safeguard the developing embryo from external environmental conditions (Marimuthu *et al*., 2019). Prior to hatching, fish embryos develop specialized hatching glands that subsequently produce hatching enzymes. These enzymes play a crucial role in breaking down the eggshell during the hatching process (Jezierska *et al*., 2009). However, if fertilized eggs exposed to highly acidic or alkaline pH conditions, it could lead to damage by causing the eggshells to become excessively hard. This in turn, hinders the functioning of hatching enzymes and the embryo, resulting in unsuccessfully embryo hatching and the mortality of larvae (Peterson & Martin-Robichaud, 1983; Kareem *et al*., 2017). According to Marimuthu *et al,* (2019), fertilized eggs and early life of African catfish, *C. gariepinus* were low under extreme pH at both acidic and alkaline concentrations.

The complete dataset of water quality parameters, including pH, recorded throughout this study is available in Norazmi-Lokman and Aswadi (2023). In the current study, the pH of the rearing water of the treatments with highest hatching rate C2, and T5 was 6.60±0.13 and 6.59±0.16 respectively which is also the optimum pH for *B. splendens*. There was a difference in the pH value of each treatment where the pH value decreases with the increment of BBL usage. These conditions were foreseen because the presence of tannins in BBL can lead to reduction in the pH of the water in the culture. It is therefore suggested that the rearing water needs to be slightly acidic (6.5 to 6.65) to obtain hatching rates of more than 90% in *B. splendens* production.

Stressful condition encountered by fish in captivity such as water replacement might result in high mortality of fish larvae. *Betta splendens* is a species adapted to blackwater environments with a preferred pH range of 5.6 to 6.8. Providing appropriate water conditions for rearing is crucial to ensure the sustainable production of this species. Cultivating blackwater fishes in captivity using clear water has been associated with the occurrence of larval deformities and increased mortality in fish larvae, as observed in studies by Sung & Abol-Munafi (2019) and Ramos *et al.* (2020).

This current study shows that the use of BBL reduces mortality of *B. splendens* larvae. The highest survival rate for cultured *B. splendens* larvae was recorded at the end of the experiment in two groups: C2 (0.7 g BCL, positive control) and T5 (1.1 g BBL). It is well known that *T. catappa* promotes higher survival rate of *B. splendens.* Previously, it has been demonstrated that rearing *B. splendens* in *T. catappa* leaves leads to an increase survival rate, as supported by research conducted by Chansue & Assawongkasem, (2008) and Nugroho *et al*. (2016). The findings from this study, which indicate that the survival rates of larvae reared in BBL and BCL are similar, strongly suggest that BBL can serve as a suitable substitute for BCL in the rearing of *B. splendens* larvae and juvenile. This observation aligns with an acute-lethal toxicity assessment where similar BBL concentrations were found to be non-toxic and shows no adverse impact on *B. splendens* survival rates (Aswadi, 2023). However, a higher amount of BBL is needed to get the same result as those observed in BCL treatment.

Generally, it is established that using plant extracts enhances the survival rate of fish in comparison to control treatments (without plant extract). It shows that survival rate was higher in all treatments with leaf extract compared to in control treatment where 100% mortality occurred after two weeks of experiment. In immersion studies involving pandanus leaf (*Pandanus amaryllifolius*), lemongrass (*Cymbopogon citratus*) and horse radish (*Moringa oleifera*) with Nile tilapia (*O. niloticus*) and African catfish (*C. gariepinus*), it was found that they exhibited a greater survival rate in comparison to control treatment (Sopiah *et al*., 2018; Doctolero *et al*., 2021). Besides that, oral administration in hybrid catfish (*Clarias microcephalus x C. gariepinus*), African catfish (*C. gariepinus*), tilapia (*O. mossambicus*) and goldfish (*Carasius auratus*) fed with asthma weed (*Euphorbia hirta*), mulberry leaf meal (*Morus alba*), Bermuda grass (*Cynodon dactylon*), beal *(Aegle marmelos),* winter cherry (*Withania somnifera*), ginger *(Zingiber officinale)* and neem (*Azadirachta indica*) also showed higher survival rates than in control treatment (Immanuel *et al*., 2009; Kumar *et al*., 2013; Olaniyi *et al*., 2016; Panase *et al*., 2018). The authors suggested that the outcomes observed might be attributed to the presence of phytochemicals in these plants, which could have potentially assisted the fish in the treatment groups in coping with stressful conditions and improving their overall well-being. However, the mode of action of these plants on the survival of fish is not yet understood and needs further study.

Phytochemicals in plants are acknowledged as immunostimulatory agents due to their ability to stimulate immunes system in fish (Makled *et al*., 2020; Rosidah & Pratiwy, 2022). The immune system in fish comprises both innate immunity and adaptive immunity which work together in synergy to provide protection against pathogens and these phytochemicals have been shown to affect both immune pathways (Secombes & Wang, 2012; Ahmadifar *et al*., 2021). Immunostimulators enhance innate immunity by increasing the activity of phagocytic cell, lysozyme, the complement systems, and lymphocyte (Sakai, 1999). Jatropha (*Jatropha vernicosa*), Sage (*Salvia officinalis*) and garlic (*Allium sativum*) which are rich in flavonoids, saponins, coumarin and essential oils, have been documented to possess the capability to defend against bacterial infection by enhancing phagocytosis and lysozyme activity in yellowtail fish (*Seriola rivoliana*), Nile tilapia (*O. niloticus*) and European sea bass (*Dicentrarchus labrax*) (Reverter *et al*., 2014; Abdellatief *et al*., 2018; Serradell *et al*., 2020; Silva-Jara *et al*., 2020).

Phytochemicals might additionally strengthen innate immunity by obstructing virus transcription and diminishing its replication within host cells, potentially leading to higher survivability (Citarasu, 2010). This has been reported in grass carp (*Ctenopharyngodon idella*) and Asian sea bass (*Lates calcarifer*), where phytochemicals have been suggested to function as inhibitors against reovirus and nervous necrosis virus (Chen *et al*., 2017; Islam *et al*., 2021).

In adaptive immunity, a complex network of cells, genes, proteins, and cytokines are involved in the reactions, which encourage the host to react to antibodies and antigens (Uribe *et al*., 2011; Rosales & Uribe-Querol, 2017). The identification of antigen (proteins located on the pathogen's surface) in fish relies on the major histocompatibility complex (MHC) molecules, which are glycoprotein receptors encoded by genes within the MHC (Abbas *et al*., 2019). Subsequent to this recognition, T lymphocyte (T cells) release cytokines which later stimulate B lymphocytes (B cells) to generate antibodies for the purpose of neutralizing and eliminating the pathogens (Salinas *et al*., 2011). Research conducted on Bangkal (*Nauclea subdita*), Stinging neetle (*Urtica dioica*) and fennel (*Radix bupleuri*) which contain flavonoids, sterols, tannins, alkaloids, and phenolics, has shown that they contributed to the up-regulation of immune gene expression levels such as major histocompatibility complex class II (MHC2), IkappaB kinase (IKK α), cytokines interleukin (IL)-8, IL-1β and IL-6 associated with higher survival rate in rainbow trout (*Oncorhynus mykiss*) and hybrid grouper (*Epinephelus lanceolatus x E. fuscoguttatus*) (Reverter *et al*., 2014; Zou *et al*., 2019; Mehrabi *et al*., 2020). Hence, it is proposed that the phytochemicals found in BBL in the current study may exhibit a similar interaction, potentially contributing to the enhanced survival rate observed in *B. splendens* larvae.

In the present study, 80 days of BBL immersion enhanced growth performance specifically specific growth rate (SGR) with better values found in fish with inclusion level of T5 (1.1 g of BBL). Growth performance in current study may be related to the phytochemicals in the plant extracts that affecting growth-related genes such as growth hormone (GH) and insulin-like growth factor, (IGF) in the aquatic animals (Ahmadifar *et al*., 2021). Once the pituitary gland releases growth hormone, its role is to activate the liver by attaching to the growth hormone receptor (GHR). This activation prompts the liver to produce and secrete IGFs such as insulin-like growth factor, 1 (IGF-1) and insulin-like growth factor, 2 (IGF-2) (Carnevali *et al*., 2005). After that, IGFs act on target cells to induce proliferation and differentiation and eventually promotes growth in fish body (Guo *et al*., 2018). In studies of beluga sturgeon (*Huso huso*), zebrafish (*Danio rerio*), rohu (*Labeo rohita*) and tilapia (*O. mossambicus*) showed that expression levels of GH, IGF-1 and IGF-2 increases (Kamboh & Zhu, 2013; Midhun *et al*., 2016; Safari *et al*., 2020; Ahmadifar *et al*., 2022). The authors suggested that flavonoids and tannins are responsible in raises the concentration of IGF-1 by up regulating the binding of GH and liver GHR and thus enhancing the growth performances. It is suggested that tannin and flavonoids in the BBL may enhanced the growth rate by upregulating these genes of *B. splendens* in the present study. These phytochemicals may improve fish growth performances by activating a series of function with up-regulating/stimulates the growth-related genes.

Sex determination is one of significant parts in the maintenance of fish populations. In ornamental fishes, when one sexual category displays dazzling colouration or higher growth rate, the phenotypic sex manipulation becomes essential (Uguz *et al*., 2003; Cogliati *et al*., 2010). This is the same condition occurring in male *B. splendens* where they are more desired which lead to high market demand meanwhile female commonly demand for breeding purposes. Commonly, phenotypic sex manipulation uses steroid which give rise to the public concerns (Katare *et al*., 2015) as they cause environmental and public health problems (Pattiasina *et al*., 2021). Thus, it is required to strive to get adequate male so that it is economically cost-effective and environmentally friendly.

In the present study, the sex ratio of *B. splendens* was skewed from the expected 1:1 expected ratio. The highest concentration treatment T5 (1.1 g BBL) produces the highest number of male (19 male: 9 female), meanwhile lowest treatment T1 (0.7 g/L BBL) produces the highest number of female (12 male: 19 female) after 80 days of exposure. It seems that higher concentration in immersion of plant extract seems to influence the total of male population. In the present study, the highest concentration (T5, 1.1 g/L BBL) produces the highest number of males. Similar result was found in study of African catfish (*C. gariepinus*) and guppy (*Poecilia reticulata*) showed increases in male percentage in the highest dose (9 g/30 L) and (0.15 g/L) respectively after immersion in the puncture vine, *Tribulus terrestris* extract (Cek *et al*., 2007; Turan & Cek, 2007). The authors suggested that this may be due to the phytochemical released by the plant extract where the higher the concentration the higher the amount of phytochemical released.

Phytochemicals might affect sex ratio by interacting with the endogenous hormone. Phytochemicals might instigate biological responses in fish such as estrogenic effects and disruption in aromatase expression/activity (CYP19) and are considered as endocrine disrupting chemicals (EDCs) (Ng *et al.*, 2006; Cheshenko *et al*., 2008). Ribeiro *et al*, (2012) stated that phytochemical compounds achieve their effects by blocking and binding or deactivating oestrogen receptor sites which affecting the sex hormone bioavailability by promoting the production of hormone-binding globulin. This has been reported in African catfish (*C. gariepinus*), Nile tilapia (*O. niloticus*) and zebrafish (*Danio rerio*), (Mukherjee *et al*., 2015; Gabriel *et al*., 2017; Nian *et al*., 2017; Syarifuddin *et al*., 2019; Gharaei *et al*., 2020; Wokeh & Orose, 2020; Aziz *et al*., 2022).

There were two possible scenarios suggested by Sirotkin & Harrath (2014) on the expression of phytochemicals in endocrine modulation. Firstly, the phytochemicals in plants are capable of controlling endocrine systems (estrogenic and androgenic activity) due to their molecular structure that is similar with estradiol (17- β-estradiol) (Younes & Honma, 2011; Rearick *et al*., 2014). In addition, the action of estrogens is activated by binding to the estrogen receptors (Matthews *et al*., 2000). Since the molecular structure of saponins and flavonoids is similar to androgens and estrogens (D' Arrigo *et al*., 2021), they have the capacity to compete, mimic and replace endogenous estrogens at binding sites on estrogen receptor sites (Pilsakova *et al*., 2010). This was reported in a study where puncture vine (*Tribulus terrestris*) extract raises testosterone level in convict cichlid (*Cichlasoma nigrofasciatum*) due to the presence of estradiol glycosides (saponins) which acted as inductors (Yeganeh *et al*., 2017). Besides that, saponins from plant extract also acted as mediators in which they facilitated the production of androgens from estradiol thereby raising testosterone levels (Tadayon *et al*., 2018).

Another possible scenario is where saponins and flavonoids inhibit the aromatase activity by blocking the biosynthesis and action of estrogen (Berrino *et al*., 2001; Pilsakova *et al*., 2010; Ribeiro *et al*., 2012). Khumpirapang *et al*. (2021) reported that flavonoids caused sex reversal of *B. splendens* by producing 95% male larvae. In *B. splendens*, sex determination and gonad differentiation occurred in the early stages of life cycle. However, the possibility of sex reversal is present because ovarian differentiation begins earlier than formation of testicular tissues (Omeje, 2016). When cholesterol is converted into estradiol-17-β through the enzymatic action of aromatase, sexual differentiation into ovarian cells occurs (Nakamura *et al*., 1998; Nagahama 2002). Thus, when phytochemicals block the aromatase activity, this leads to a reduction of estrogen biosynthesis in cells which resulting to testicular development instead of the ovary development (Dabrowski *et al*., 2005). This scenario was supported by Khumpirapang *et al*, (2021) where flavonoids had significant effect on sex reversal of *B. splendens* by producing 95% of male larvae. It can be concluded that these scenarios may took place and influence the male population in our study.

CONCLUSION

In conclusion, the main objective of this study was to demonstrate the effect of (BBL) in *B. splendens* aquaculture was successfully achieved. The findings of this study reveal that BBL has the same effects as BCL where these two treatments have the highest hatching and survival rate. In *B. splendens* larvae culture, BBL treated fish has better growth performance compared to BCL where the highest SGR weight was in T3 (0.9 g BBL) and SGR length in T5 (1.1 g BBL). Based on observation of hatching rate, survival rate and growth performances, the findings of this work indicated that culture water with 1.1 g/L of BBL is the most optimum for *B. splendens*. Furthermore, the immersion of BBL has presented that it is effective in earning the preferred gender population. Specifically, higher male population of *B. splendens* can be obtained by T5 treatment (1.1 g BBL) meanwhile a higher female population in T1 treatment (0.7 g BBL). It is safe to conclude that BBL is a good and safe alternative/ replacement to Catappa leaf. Other than that, the water quality does not give a negative impact during the immersion of BBL in any treatments. The knowledge and methods developed in this study will succour further enhancement in *B. splendens* breeding and larvae culture. However, further studies are required to determine the mechanism underlying influences of BBL in physiology and reproductive behaviour in *B. splendens*. Additional study is also required to provide conclusive evidence regarding BBL efficacy to be used as sex reversal agent in *B. splendens* culture. Nevertheless, this study has opened a path toward a BBL application in this species.

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